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MECANISMOS NEURO-HUMORAIS E ENDOTELIAIS NA INSUFICIÊNCIA CARDÍACA – IMPLICAÇÕES FISIOPATOLÓGICAS E TERAPÊUTICAS

NEUROHUMORAL AND ENDOTHELIAL MECHANISMS IN HEART FAILURE IMPLICATIONS IN PATHOPHYSIOLOGY AND TREATMENT

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Aos meus pais, por tudo o que me deram

"Success is going from one failure to another without loss of enthusiasm"
S. J. Withrow

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CAPÍTULO I

INTRODUÇÃO GERAL E OBJECTIVOS

"Com o saber cresce a dúvida"

Johann Wolfgang von Goethe (1749-1832)

INSUFICIÊNCIA CARDÍACA

A insuficiência cardíaca (IC) é uma síndrome clínica complexa resultante de um processo de disfunção ventricular agudo ou crónico. Trata-se de uma condição debilitante com elevada morbilidade e mortalidade, tanto em Portugal (Ceia e col., 2002) como nos restantes países industrializados, prevendo-se que a sua incidência continue a aumentar pelo menos até ao final do primeiro quartel do século XXI (Levy e col., 2002). De forma similar, a IC também representa uma importante causa de morbilidade e mortalidade em medicina veterinária (Gordon e col., 2006; Atkins e col., 2007; Besche e col., 2007). Pelo exposto, torna-se premente a optimização do diagnóstico e do tratamento da IC, constituindo, deste modo, um dos mais importantes desafios e prioridades na investigação cardiovascular.

INSUFICIÊNCIA CARDÍACA DIASTÓLICA E SISTÓLICA

Na prática clínica, a IC pode dividir-se em IC diastólica e IC sistólica. Esta classificação foi reconhecida há cerca de 70 anos por Fishberg, tal como descrito recentemente por Katz e Zile (Katz e Zile, 2006). Ao longo do tempo foram propostas várias definições de IC diastólica. Assim, em 1993, a IC diastólica foi definida como uma "condição resultante de um aumento da resistência ao enchimento de um ou de ambos os ventrículos e que conduz a sintomas de congestão devido a um inadequado desvio para cima da relação pressão-volume telediastólica" (Brutsaert e col., 1993). Aproximadamente

dez anos depois foi sugerida outra definição que a caracteriza como uma "condição em que a câmara ventricular é incapaz de acomodar um volume adequado de sangue durante a diástole a pressões diastólicas normais de forma a manter um volume de ejecção apropriado (Zile e Brutsaert, 2002a). Sendo estas definições de natureza puramente funcional, foi proposta a seguinte definição clínica "síndrome clínica caracterizada por sinais e sintomas de IC, com fracção de ejecção preservada e disfunção diastólica" (Zile e Brutsaert, 2002a), que tem sido utilizada posteriormente por outros investigadores (Baicu e col., 2005; Aurigemma e col., 2006). Existem, no entanto, outras definições para a IC diastólica tais como, "IC com função sistólica preservada" ou "IC com fracção de ejecção do ventrículo esquerdo normal". Na IC diastólica, a principal alteração funcional é o compromisso do relaxamento do ventrículo esquerdo associado ao aumento da rigidez passiva (Zile e col., 2004).

No que concerne à IC sistólica, em 1933, Sir Thomas Lewis definiu-a como "uma condição em que o coração não é capaz de ejectar adequadamente o seu conteúdo" (Chatterjee e Massie, 2007). Em 1980, Braunwald descreveu a IC como "um estado fisiopatológico em que uma alteração na função cardíaca é responsável pela incapacidade do coração bombear adequadamente o sangue de modo a responder às necessidades metabólicas dos tecidos" (Braunwald, 1980). Neste caso, a principal alteração funcional é o compromisso da função sistólica, principal mecanismo responsável pela diminuição da fracção de ejecção, com ou sem disfunção diastólica concomitante (Konstam, 2003; Aurigemma e col., 2006). Esta forma de IC é igualmente denominada por "IC com fracção de ejecção reduzida".

Porém, alguns investigadores defendem a hipótese de que a IC representa uma entidade fisiopatológica única, em que o quadro clínico inicial se caracteriza por disfunção diastólica predominante, evoluindo no sentido de um agravamento progressivo da função

cardíaca sistólica (De Keulenaer e Brutsaert, 2007). Pelo contrário, os defensores da distinção entre IC diastólica e IC sistólica apontam argumentos que não se limitam ao tipo de disfunção cardíaca, sustentando que entre as duas entidades existem diferenças estruturais, funcionais e moleculares que poderão condicionar uma abordagem terapêutica distinta (Paulus e col., 2007). Quando comparadas, estas duas síndromes diferem em diversos aspectos a nível ultra-estrutural, incluindo, por exemplo, o diâmetro e a tensão passiva dos cardiomiócitos, a densidade dos miofilamentos e a expressão de isoformas de proteínas do citoesqueleto, particularmente da titina (Makarenko e col., 2004; Nagueh e col., 2004; Borbely e col., 2005; van Heerebeek e col., 2006).

Estudos epidemiológicos levados a cabo recentemente têm demonstrado um aumento progressivo da prevalência da IC diastólica, sendo que em alguns deles esta é responsável por mais de metade dos casos de IC (Owan e Redfield, 2005; Bhatia e col., 2006; Owan e col., 2006). Para a crescente importância desta entidade clínica muito tem contribuído a sua estreita associação ao envelhecimento, à hipertensão arterial, à diabetes *mellitus* e à doença coronária (Paulus e col., 1998; Gandhi e col., 2001; Gaasch e Zile, 2004; Owan e Redfield, 2005).

Embora tenha havido avanços significativos no tratamento da IC sistólica, escassos progressos foram alcançados na IC diastólica. A melhoria do prognóstico da IC sistólica deve-se principalmente às descobertas terapêuticas que mostraram atenuar a remodelagem cardíaca e melhorar as alterações hemodinâmicas. Aqui, os moduladores neuro-humorais, tais como os antagonistas do sistema renina-angiotensina-aldosterona ou do sistema adrenérgico, desempenham um papel importante ao melhorarem significativamente os sintomas e a qualidade de vida e ao diminuirem a mortalidade (Chatterjee e Massie, 2007). Em relação à IC diastólica, não existe até ao momento nenhum tratamento que se reflicta em termos de melhoria de prognóstico. Sabe-se, porém, que os bloqueadores dos

receptores da angiotensina diminuem a morbilidade mas não a mortalidade destes doentes (Yusuf e col., 2003).

(DIS)FUNÇÃO CARDÍACA DIASTÓLICA

Tal como referido anteriormente, a disfunção diastólica ocorre na IC diastólica, podendo também existir na IC sistólica. Esta disfunção relaciona-se com alterações na complacência (distensibilidade), no enchimento ou relaxamento do ventrículo esquerdo, independentemente da fracção de ejecção ser normal ou não e do doente ser sintomático ou assintomático (Aurigemma e col., 2006). Esta disfunção pode ser devida a um espessamento (hipertrofia) da parede ventricular, a cardiomiopatias restritivas ou infiltrativas e/ou taquicardia. Como resultado, a relação pressão-volume telediastólica do ventrículo esquerdo desloca-se para cima e para a esquerda, a complacência da câmara diminui (aumento da rigidez), o padrão de enchimento altera-se e as pressões de enchimento ventricular aumentam (Glantz e Parmley, 1978; Kitzman e col., 2002; Zile e Brutsaert, 2002a; Zile e Brutsaert, 2002b; Angeja e Grossman, 2003; Zile e col., 2004).

A disfunção diastólica ventricular é também comum em medicina veterinária, particularmente no Gato com doença miocárdica (Fox e col., 1995; Bright e col., 1999; Gavaghan e col., 1999; Maass e Leinwand, 2000; Fuentes, 2003), conquanto também ocorra na cardiomiopatia dilatada canina (Borgarelli e col., 2001; O'Sullivan e col., 2007).

Conceptualmente, os mecanismos responsáveis pela disfunção diastólica podem ser divididos em factores miocárdicos e extra-miocárdicos. A função diastólica é determinada pelas propriedades passivas da parede ventricular e da sua interacção com o processo activo de relaxamento micárdico. Outros determinantes incluem as estruturas que envolvem o ventrículo, a aurícula esquerda, as veias pulmonares, a válvula mitral e a frequência cardíaca (Leite-Moreira, 2006). No entanto, com excepção da frequência

cardíaca, estes últimos determinantes são extrínsecos ao ventrículo e normalmente não são considerados como uma verdadeira causa de disfunção ou insuficiência diastólica. Além disso, o diagnóstico de IC diastólica implica a exclusão destes determinantes como causa de alterações do enchimento ventricular (Gaasch e Zile, 2004).

As propriedades passivas da parede ventricular são influenciadas pela rigidez miocárdica (complacência), espessura da parede e remodelagem geométrica da câmara ventricular (tamanho ou volume). Não obstante a importância dos dois últimos determinantes, a rigidez miocárdica e os mecanismos envolvidos na sua modulação têm merecido particular atenção no seio da comunidade científica. Deste modo, a disfunção diastólica por compromisso da rigidez miocárdica pode resultar de alterações de mecanismos intrínsecos ao próprio cardiomiócito (citosqueleto) e à matriz extracelular, resultando neste caso de alterações na rede de colagénio extra-miocárdico (Leite-Moreira, 2006).

A disfunção diastólica tem um papel predominante na instalação da IC congestiva, já que o aumento do volume e da pressão telediastólicos do ventrículo esquerdo podem conduzir ao aumento das pressões venosas pulmonares e a edema pulmonar. O aumento da pressão de enchimento do ventrículo esquerdo correlaciona-se com os sinais de congestão e a tolerância ao exercício em humanos, independentemente da gravidade da disfunção sistólica (Packer, 1990; Nishimura e Tajik, 1997). Actualmente, a avaliação da função diastólica é uma componente importante no diagnóstico e monitorização da IC.

A proposta recentemente publicada das associações de IC e Ecocardiografia da Sociedade Europeia de Cardiologia preconiza que a disfunção diastólica pode ser determinada por métodos invasivos (cateterismo) e não-invasivos (ecocardiografia) (Paulus e col., 2007). No que respeita à avaliação ecocardiográfica, a função diastólica pode ser avaliada recorrendo a diversos parâmetros. Dos parâmetros mais utilizados, citam-se os

que se prendem com a análise por Doppler espectral do fluxo mitral e fluxo das veias pulmonares e mais recentemente por Doppler tecidular (DT) do anel mitral (Oyama, 2004).

O padrão de enchimento mitral é actualmente uma ferramenta não-invasiva de inegável valor na quantificação da função diastólica em doentes com suspeita de IC com função sistólica preservada (Galderisi, 2005). A avaliação do fluxo transmitral reflecte as diferenças instantâneas de pressão entre a aurícula e o ventrículo esquerdo, que por sua vez estão relacionadas com a velocidade de relaxamento e com a complacência miocárdica das duas câmaras (Appleton e col., 2000). O fluxo mitral obtido por Doppler espectral engloba as fases de enchimento ventricular, traduzidas na onda precoce E e onda tardia A. Num indivíduo normal o pico da onda E é superior ao pico da onda A, resultando numa relação E/A>1. Os padrões anormais do fluxo mitral, embora não específicos de qualquer doença, permitem distinguir vários estadios de disfunção diastólica e modificam-se à medida que a doença miocárdica progride. Na disfunção diastólica precoce, o relaxamento do ventrículo esquerdo está comprometido, levando a uma menor contribuição do enchimento precoce (diminuição do pico da onda E) e aumento de dependência da contracção auricular (aumento do pico da onda A). Esta alteração no relaxamento resulta numa relação E/A<1 e num prolongamento do tempo de relaxamento isovolumétrico e do tempo de desaceleração da onda E. À medida que a doença progride, a complacência do ventrículo esquerdo diminui e, consequentemente, aumentam as pressões de enchimento de ambas as câmaras cardíacas esquerdas, promovendo uma pseudonormalização do fluxo mitral (relação E/A normal e tempos de relaxamento isovolumétrico e de desaceleração da onda E normais a diminuídos). Em situações de disfunção diastólica avançada, a complacência do ventrículo esquerdo é reduzida, com consequente aumento da sua pressão de enchimento e frequente disfunção sistólica auricular, resultando num padrão restritivo de fluxo mitral (aumento do pico da onda E, diminuição do pico da onda A; relação E/A ≥2 e tempos de relaxamento

isovolumétrico e de desaceleração da onda E diminuídos) (Nishimura e Tajik, 1997; Oyama, 2004).

Contrariamente ao Doppler convencional que avalia o fluxo sanguíneo, o DT pulsado examina o movimento do tecido cardíaco, sobretudo a velocidade do anel mitral (Sutherland e col., 1999). Com base no descrito anteriormente, no padrão de enchimento pseudonormal pode considerar-se erroneamente a existência de uma função diastólica normal. Devido a esta limitação inerente à análise das velocidades do fluxo mitral, o DT do anel mitral veio obviar esta e outras limitações. Basicamente, o padrão de normalidade do DT, ao nível do anel mitral, sobrepõe-se ao padrão do fluxo mitral (E>A e E'>A'). No entanto, em situações de compromisso da função diastólica, e devido ao facto do DT não ser tão dependente da carga, vai permitir diferenciar o padrão pseudonormal do padrão normal do fluxo mitral, com uma velocidade precoce (E') patologicamente inferior a A', possibilitando identificar rapidamente alterações da função diastólica (Sohn e col., 1997). Por outro lado, a relação entre o pico da onda E do fluxo mitral e a velocidade de relaxamento diastólico precoce (E'), obtida por DT ao nível do anel mitral, correlaciona-se de forma significativa com as pressões de enchimento do ventrículo esquerdo (Ommen e col., 2000).

MECANISMOS NEURO-HUMORAIS

A IC envolve a activação de múltiplas vias celulares, metabólicas e neuro-humorais perante uma agressão miocárdica (Baker e col., 1989; Ganguly e col., 1989; Ferrari e col., 1996; Kjaer e Hesse, 2001). Um elevado número de agentes neuro-humorais têm sido implicados na progressão para a IC, em parte devido ao facto dos seus níveis plasmáticos estarem elevados nesta síndrome (quadro 1).

Quadro 1: Activação Neuro-humoral na Insuficiência Cardíaca (Francis e col., 2004).

- Sistema nervoso simpático (aumento de norepinefrina e epinefrina)
- Endotelina
- Arginina vasopressina
- Renina e Angiotensina II
- Aldosterona
- Neuropeptídeo Y
- Peptídeo natriurético auricular e peptídeo natriurético do tipo B
- Insulina, cortisol, hormona de crescimento, factor de necrose tumoral α, interleucina-6, peptídeo intestinal vasoactivo, adrenomedulina, urodilantina, urotensina-II, cardiotrofina-I
- Dopamina
- Prostaglandinas (PGI_2, PGE_2)
- Peptídeos vasodilatadores (bradicinina)

Esta resposta neuro-humoral subjacente ao desenvolvimento de IC está bem documentada em humanos e diversos estudos suportam a hipótese de que respostas semelhantes ocorram também no Cão e Gato com IC secundária a doenças cardíacas espontâneas (Asano e col., 1999; Prosek e col., 2004a; Prosek e col., 2004b; Baumwart e Meurs, 2005; Boswood e col., 2007; DeFrancesco e col., 2007).

Os mediadores neuro-humorais libertados, actuando de forma endócrina, parácrina ou autócrina, promovem um espectro de efeitos que, embora possam ser considerados

inicialmente compensadores, rapidamente se tornam deletérios, contribuindo para o ciclo vicioso de auto-agravamento que caracteriza esta síndrome (Katz, 2000). A reforçar a importância dos mecanismos neuro-humorais está ainda o facto do seu bloqueio representar um dos avanços mais significativos da terapêutica farmacológica da IC, com reflexos directos no prognóstico da doença e, como tal, na sobrevida dos doentes (Jessup e Brozena, 2003).

À medida que a disfunção ventricular progride, ocorre a activação de diversos sistemas neuroendócrinos, incluindo o sistema nervoso simpático e o sistema renina-angiotensina. Estes, embora fisiologicamente promovam o aumento da contractilidade e da frequência cardíaca e preservem o equilíbrio hidro-salino, contribuem para a remodelagem cardíaca, vasoconstrição periférica, retenção de sódio e cardiomegalia progressiva (Schrier e Abraham, 1999). Para além destes, são também activados outros sistemas vasoconstritores, como o sistema da arginina-vasopressina e da endotelina-1 (ET-1) (Schrier e Abraham, 1999; Attina e col., 2005). Em oposição a estes, ocorre a activação de outros mecanismos neuro-humorais (peptídeos natriuréticos, prostaglandinas vasodilatadoras e provavelmente o sistema dopaminérgico), predominantemente vasodilatadores, natriuréticos e anti-proliferativos (Gomes e col., 2004).

Actualmente, está bem documentado o papel crucial que os mecanismos neuro-humorais desempenham na IC sistólica, de tal forma que o avanço mais significativo na sua terapêutica farmacológica decorreu, em grande parte, da introdução, nos anos 80, do conceito de desregulação desses mecanismos (Cohn e col., 1981; Dzau, 1987). Por outro lado, o papel destes mecanismos não está totalmente estabelecido na IC diastólica. De facto, apesar da importância crescente que a IC diastólica tem vindo a assumir, a sua fisiopatologia e tratamento são ainda largamente desconhecidos (Gaasch e Zile, 2004; Kass e col., 2004; Leite-Moreira, 2006).

Durante muito tempo considerava-se que os mediadores neuro-humorais apenas seriam capazes de alterar cronicamente as propriedades diastólicas do miocárdio mediante a indução de fibrose e hipertrofia (Gaasch e Zile, 2004). Contudo, a literatura sugere que a rigidez diastólica pode ser modulada de forma aguda por alguns destes mediadores, caso do óxido nítrico (NO) (Grocott-Mason e col., 1994; Heymes e col., 1999), da ET-1 (Leite-Moreira e col., 2003) e da angiotensina II (AngII) (Leite-Moreira e col., 2006).

O SISTEMA DA ENDOTELINA

O sistema da ET consiste em três isopeptídeos (ET-1, ET-2 e ET-3) compostos por 21 aminoácidos, várias isoformas de peptidases activadoras e dois receptores acoplados a proteínas G, ET_A e ET_B. A ET-1 é um peptídeo vasoconstritor produzido pelo endotélio vascular, isolado há 20 anos. É a isoforma predominante no sistema cardiovascular humano, possuindo um terminal C hidrofóbico e duas pontes cisteína no terminal N (Yanagisawa e col., 1988).

A ET-1 resulta de um processo de clivagem enzimática que engloba várias etapas. O gene da ET origina a pré-pró-ET, peptídeo com cerca de 200 aminoácidos, que por sua vez sofre clivagem por acção de uma protease semelhante à furina e se transforma em *big* ET, de 38 aminoácidos. Esta é processada em ET pelas suas enzimas de conversão (ECE-1, ECE-2 e ECE-3) (Davenport e Maguire, 2006).

No sistema cardiovascular, os componentes da família da endotelina são expressos em vários tecidos, designadamente no endotélio vascular e endocárdico, nas células musculares lisas, nos cardiomiócitos e em diferentes células do sistema imune (Brunner e col., 2006). A nível cardiovascular, a ET-1 pode ser sintetizada não só pelo endotélio (vascular e endocárdico), mas também por células miocárdicas (Mebazaa e col., 1993; Suzuki e col., 1993; Tonnessen e col., 1995).

A ET-1 é um regulador local (autócrino e parácrino), uma vez que a sua libertação é predominantemente abluminal (Wagner e col., 1992) e a sua semivida plasmática é curta. A semivida da ET-1 deve-se principalmente à depuração pulmonar, onde os receptores ET_B desempenham um papel importante na sua remoção da circulação (Fukuroda e col., 1994), embora a depuração renal, hepática e cardíaca também estejam descritas (Dupuis e col., 1996; Dupuis e col., 1999; Johnstrom e col., 2005).

Nos mamíferos, os efeitos da ET-1 são mediados por dois tipos de receptores acoplados a proteínas G, ET_A e ET_B, que se distinguem não só pela sua afinidade de ligação, mas também pela sua distribuição nos tecidos e células e pelos seus efeitos fisiológicos (Arai e col., 1990; Sakurai e col., 1990). Os receptores ET_A, o subtipo mais abundante no tecido cardíaco, promovem vasoconstrição, aumento do inotropismo e mitogénese (Brunner e col., 2006). Além disso, como recentemente demonstrado, a activação deste tipo de receptores conduz a um aumento da distensibilidade miocárdica em situações de sobrecarga, sendo este efeito dependente da activação do trocador sódio/hidrogénio (Na^+/H^+) (Leite-Moreira e col., 2003) e da presença de um endotélio endocárdico (EE) intacto (Brás-Silva e Leite-Moreira, 2006). Por seu lado, os receptores ET_B induzem vasodilatação, mediada pela libertação de NO (Tsukahara e col., 1994) e de prostaciclinas (de Nucci e col., 1988), e efeitos inibitórios do crescimento associados a apoptose (Mallat e col., 1995; Okazawa e col., 1998). A nível vascular é possível ainda subdividir os receptores ET_A, de localização muscular, em ET_{A1} e ET_{A2} dependendo se são ou não sensíveis ao antagonista BQ-123, respectivamente (Sudjarwo e col., 1994). Por seu turno, a nível cardíaco (Leite-Moreira e Brás-Silva, 2004) e vascular (Sudjarwo e col., 1994) é também possível subdividir os receptores ET_B, em ET_{B1}, de localização endotelial com efeitos inotrópicos negativos e vasodilatadores, e ET_{B2}, de localização muscular com efeitos inotrópicos positivos e vasoconstritores.

A ET-1 tem um importante papel fisiológico na regulação da homeostasia cardiovascular. A sua geração excessiva e desregulada tem, contudo, sido associada às principais doenças cardiovasculares. Na IC, os níveis plasmáticos, salivares e tecidulares cardíacos de ET-1 encontram-se aumentados, correlacionando-se positivamente com a gravidade da doença e negativamente com o prognóstico (Attina e col., 2005). A sobreactivação do sistema da ET-1 está também descrita em modelos animais espontâneos de IC (Vollmar e col., 1995; Prosek e col., 2004a; Prosek e col., 2004b). O mecanismo subjacente à sua elevação nesta síndrome, não está ainda completamente definido. Sabe-se contudo que a produção de ET-1 na IC é disseminada, envolvendo todos os órgãos sujeitos tanto a uma baixa perfusão, como a uma diminuição do *shear* stresse (Sakai e col., 1996; Brunner e col., 2006). Não obstante, alguns autores têm defendido que os elevados níveis de ET-1 poderão contribuir para manter a função do coração insuficiente (Sakai e col., 1996). Além disso, a ET-1 parece também modular a eficácia da contracção miocárdica, o que poderá ter particular relevância na IC, onde o trabalho do coração está aumentado devido à elevação da pré-carga (Winegrad, 1997). Deste modo, o aumento dos níveis de ET-1 parece ser benéfico a curto prazo, uma vez que constitui um suporte inotrópico para o miocárdio insuficiente. No entanto, a longo prazo, parece ser prejudicial devido aos efeitos da ET-1 na redução do fluxo coronário, aumento da pós-carga e indução de hipertrofia e remodelagem.

O SISTEMA β-ADRENÉRGICO

A estimulação β-adrenérgica é um importante mecanismo regulador da função cardíaca em situações de maior exigência circulatória. Por outro lado, alterações nos mecanismos de transdução de sinal associados aos receptores β-adrenérgicos são determinantes na progressão para a IC.

Até ao momento foram identificados três subtipos de receptores β-adrenérgicos, β_1, β_2 e β_3, havendo dúvidas relativamente à existência do subtipo β_4 (Brodde e col., 2006). O miocárdio humano expressa receptores adrenérgicos β_1 e β_2, com um claro predomínio dos primeiros, sendo que a relação β_1/β_2 é cerca de 70%/30% nas aurículas e 80%/20% nos ventrículos (Brodde, 1993; Brodde e Michel, 1999).

Nos cardiomiócitos, a estimulação dos receptores β-adrenérgicos (β_1 e β_2) activa a proteína cinase A (PKA) dependente de monofosfato de adenosina cíclico (AMPc), via proteína G_s. A PKA fosforila diversas proteínas essenciais na função cardíaca envolvidas quer na homeostasia do cálcio (Ca^{2+}) intracelular (receptores rianodínicos, fosfolambam e canais de Ca^{2+} do tipo L) (Bers e Guo, 2005; Bers, 2006), quer na regulação das interacções actina-miosina (troponina I cardíaca e proteína ligada à miosina) (Sumandea e col., 2004; Cazorla e col., 2006). As alterações rápidas na homeostasia do Ca^{2+} parecem ser as responsáveis pelo efeito inotrópico positivo subjacente à estimulação β-adrenérgica. Contudo, modificações pós-translacionais das componentes dos filamentos finos e grossos também podem contribuir para este efeito, dado que a fosforilação da troponina I cardíaca acelera o ciclo das pontes cruzadas e reduz a sensibilidade dos miofilamentos ao Ca^{2+}, promovendo os efeitos inotrópicos e lusitrópicos positivos secundários à estimulação β-adrenérgica.

Adicionalmente, a PKA fosforila também uma outra proteína muitas vezes designada como terceiro filamento do sarcómero, a titina (Yamasaki e col., 2002). Estudos recentes demonstraram que a fosforilação da titina pela PKA diminui a tensão passiva tanto em cardiomiócitos de Rato como de Vaca (Yamasaki e col., 2002; Fukuda e col., 2005). Por outro lado, estudos adicionais verificaram que o aumento da actividade da PKA promove uma diminuição da tensão passiva em cardiomiócitos isolados de doentes com IC (Borbely e col., 2005; van Heerebeek e col., 2006), efeito também atribuído à fosforilação da titina (Kruger e Linke, 2006). Esta diminuição da rigidez passiva mediada pela PKA é

interessante sob o ponto de vista terapêutico, dado que o aumento da actividade desta cinase, secundário à estimulação β-adrenérgica, poderá melhorar a função diastólica dos doentes com IC diastólica (van Heerebeek e col., 2006).

Relativamente aos receptores β_3, apesar da sua expressão estar essencialmente limitada ao tecido adiposo (Krief e col., 1993), vários grupos demonstraram efeitos cardíacos secundários à activação destes receptores, bem como a presença do seu ARNm no coração e cardiomiócitos humanos e de outras espécies (Gauthier e col., 2000; Kitamura e col., 2000; Cheng e col., 2001). Estes receptores estão acoplados a uma proteína G inibitória e parecem mediar um efeito inotrópico negativo dependente da via do NO (Gauthier e col., 2000), embora a sua importância continue por determinar (Heubach e col., 2002).

NOVOS MODULADORES NEURO-HUMORAIS

Como vimos anteriormente, o tratamento farmacológico da IC consiste principalmente na modulação do estado neuro-humoral, mediante a inibição de sistemas adversos vasopressores/promotores de retenção de volume. A utilização de inibidores da enzima de conversão da angiotensina, tanto em medicina humana (CONSENSUS, 1987; SOLVD, 1991) como em medicina veterinária (COVE, 1995; IMPROVE, 1995; Ettinger e col., 1998), e de bloqueadores β-adrenérgicos (Packer e col., 1996; Korczyk e Doughty, 2006; Fauchier e col., 2007) são um exemplo e o seu benefício terapêutico está provado clinicamente (Sharpe, 2001). No entanto, apesar do evidente impacto clínico desta abordagem terapêutica, a IC continua a estar associada a um mau prognóstico, para além da sua incidência e prevalência estarem a aumentar (Stewart, 2003). Neste contexto é necessário investigar vias alternativas ou adicionais que modulem as respostas neuro-humorais na disfunção cardíaca.

Para além dos clássicos, existem novos mediadores neuro-humorais que desempenham também um papel importante na fisiopatologia da IC. Entre estes incluem-se, por exemplo, a adrenomedulina (AM) e a urotensina-II (U-II), os quais poderão proporcionar novas oportunidades terapêuticas nesta síndrome. Pelo facto dos seus efeitos miocárdicos, sistólicos e diastólicos, não estarem ainda bem esclarecidos, optámos por realizar uma revisão bibliográfica mais detalhada sobre estes dois sistemas neuro-humorais.

O SISTEMA DA ADRENOMEDULINA

CONSIDERAÇÕES GERAIS

A AM é um peptídeo vasodilatador potente identificado e isolado inicialmente do feocromocitoma humano (Kitamura e col., 1993a). Contudo, estudos subsequentes demonstraram a sua expressão em diversos tecidos normais, incluindo a glândula supra-renal, o coração, o rim e os vasos sanguíneos (Kitamura e col., 1993b), bem como níveis plasmáticos relativamente elevados deste peptídeo (Kitamura e col., 1994a). Adicionalmente, vários estudos constataram níveis plasmáticos elevados de AM no contexto da hipertensão, da IC, da insuficiência renal e da sépsis (Ishimitsu e col., 1994b; Nishikimi e col., 1995; Hirata e col., 1996; Nishio e col., 1997). Estas observações levaram a especulações por alguns investigadores sobre um papel fisiopatológico potencialmente relevante da AM no desenvolvimento das doenças cardiovasculares.

O número anual de publicações científicas relacionadas com a AM tem vindo a aumentar consideravelmente desde a sua descoberta em 1993, o que denota a importância deste peptídeo na progressão de diversas doenças.

ESTRUTURA DA ADRENOMEDULINA

A isoforma humana da AM (hAM) é constituída por 52 aminoácidos sendo estruturalmente semelhante ao peptídeo relacionado com o gene da calcitonina (PRGC) (Kitamura e col., 1993a). Os resíduos de cisteína, localizados nas posições 16 e 21 junto do terminal N, estão ligados por pontes dissulfureto, formando uma estrutura em anel constituída por 6 aminoácidos (Kitamura e col., 1993a).

A AM pertence a uma superfamília de peptídeos que inclui o PRGC, um vasodilatador potente, a amilina e mais recentemente a AM-2 ou intermedina (Roh e col., 2004; Takei e col., 2004).

A hAM foi também sequenciada no Porco (Kitamura e col., 1994b), no Cão (Ono e col., 1998), nos Bovinos (Kitamura e col., 2001), no Rato (Sakata e col., 1993) e no Ratinho (Okazaki e col., 1996). A sequência de aminoácidos da isoforma humana difere das isoformas porcina, canina e bovina em apenas 1, 2 e 4 aminoácidos, respectivamente, enquanto as isoformas do Rato e do Ratinho são mais curtas, contendo somente 50 aminoácidos (Sakata e col., 1993) (figura 1).

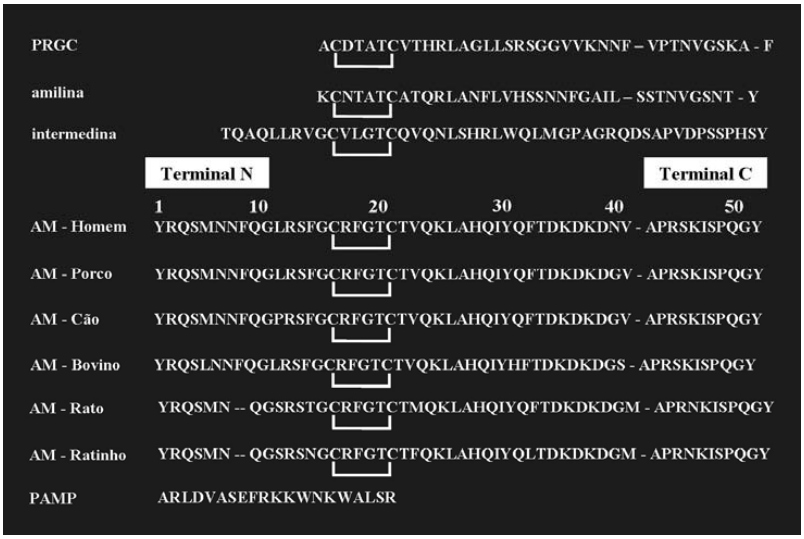

Figura 1: Isoformas da adrenomedulina e peptídeos relacionados em diversas espécies. AM, adrenomedulina; PRGC, peptídeo relacionado com o gene da calcitonina; PAMP, peptídeo do terminal N da pró-AM (20 aminoácidos). A ligação entre os resíduos de cisteína representa uma ponte dissulfureto. O terminal C da AM, do PRGC e da amilina é amidado (Ishimitsu e col., 2006).

SÍNTESE, SECREÇÃO E METABOLISMO DA ADRENOMEDULINA

O gene da hAM localiza-se num único locus do cromossoma 11 (p15.1-3) e possui 4 exões e 3 intrões (Ishimitsu e col., 1994a). O ARNm codifica uma molécula precursora de grande tamanho denominada pré-pró-AM, constituída por 185 aminoácidos. Junto ao

seu terminal N existe um peptídeo sinalizador de 21 aminoácidos. Inicialmente ocorre o corte e a separação do peptídeo sinalizador da pré-pró-AM, originando uma pró-hormona de 164 aminoácidos (pró-AM). Posteriormente, mediante a acção sequencial de endopeptidases, exopeptidases e finalmente enzimas amidadas (Lopez e Martinez, 2002), geram-se dois peptídeos amidados biologicamente activos: a AM (52 aminoácidos), situada junto ao terminal C da molécula precursora, e o peptídeo do terminal N da pró-AM (PAMP), com 20 aminoácidos (figura 2). Ambos os peptídeos resultam da mesma molécula precursora, mas a relação PAMP/AM não é equivalente e depende do órgão ou tecido em questão.

Durante o processamento, a AM é secretada sob a forma imatura com um terminal C glicado, sendo constituída por 53 aminoácidos (Sakata e col., 1993). Só após a sua amidação enzimática é que se origina a forma madura da AM, biologicamente activa e quimicamente menos estável (figura2) (Kitamura e col., 1998). Assim, a AM circulante inclui as duas formas, amidada (madura) e não amidada (glicada ou intermédia), representando esta última 85% da AM total presente no plasma (Cao e col., 2003). Em situações fisiológicas normais as concentrações plasmáticas da AM situam-se na ordem dos picomolares (2-10 pM) (Ichiki e col., 1994; Kitamura e col., 1994a; Lewis e col., 1998). Em estados patológicos, as concentrações plasmáticas das fracções total e madura de AM estão aumentadas, sugerindo que a AM glicada funciona como reservatório.

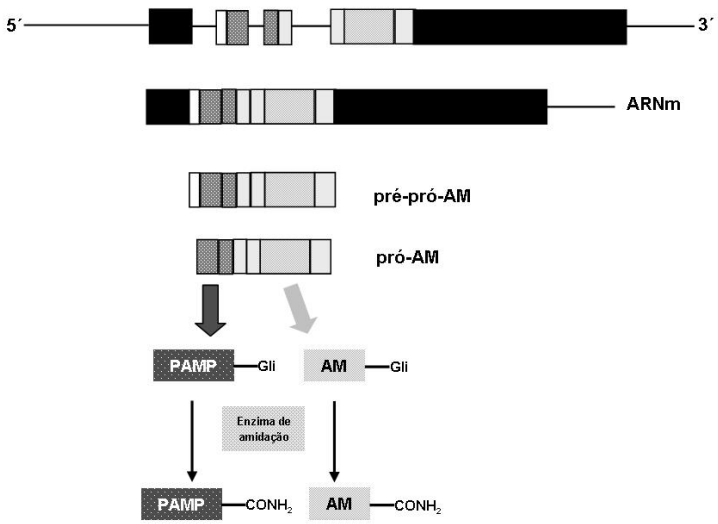

Figura 2: Síntese da adrenomedulina. A transcrição do gene da AM origina um ARNm que contém os 4 exões que compõem o gene. A tradução deste transcrito dá lugar à pré-pró-AM de 185 aminoácidos que, após a eliminação do peptídeo sinalizador, se converte em pró-AM. Este peptídeo origina dois peptídeos bioactivos, o PAMP e a AM. AM, adrenomedulina; PAMP, peptídeo do terminal N da pró-AM (Samson, 1999).

A AM tem uma semivida aproximada de 20 min (Meeran e col., 1997). Os pulmões parecem ser o principal local de depuração sistémica da AM madura (Nishikimi e col., 1994; Dupuis e col., 2005), mediante um processo enzimático que envolve duas fases, primariamente por metaloproteases e posteriormente seguido da acção de aminopeptidases (Lewis e col., 1997).

A AM é produzida por diversos tecidos (rim, pulmão e coração) (Kitamura e col., 1993b). Contrariamente ao peptídeo natriurético auricular e ao peptídeo natriurético tipo B, também produzidos no coração e que modulam efeitos cardiovasculares semelhantes aos da AM, esta está expressa de forma ubiquitária em vários tecidos não-cardiovasculares (Hinson e col., 2000). A AM está expressa e é secretada através de uma via constitutiva por diversos tipos de células do sistema cardiovascular, incluindo fibroblastos, cardiomiócitos, células inflamatórias e células endoteliais e musculares lisas vasculares

(Minamino e col., 2002). De qualquer modo, as células vasculares são a principal fonte de AM circulante.

A síntese/secreção de AM, especialmente no sistema cardiovascular, é regulada por diversos factores, tais como factores mecânicos (*shear* stresse), citocinas inflamatórias (interleucinas, factor de necrose tumoral e lipolissacarídeos), hormonas (AngII, ET-1 e aldosterona) e factores metabólicos (hipóxia, isquemia, stresse oxidativo e hiperglicemia) (Eto e col., 2003; Beltowski e Jamroz, 2004).

RECEPTORES DA ADRENOMEDULINA E MECANISMOS DE TRANSDUÇÃO DE SINAL

A distribuição dos locais de ligação da AM foi extensivamente estudada no Rato (Owji e col., 1995; Juaneda e col., 2003). A expressão dos receptores da AM é elevada no coração e pulmões, embora estes receptores também estejam expressos na supra-renal, no rim e no sistema nervoso central. Em humanos, o endotélio vascular e as células imunes gastrointestinais também exibem receptores específicos da AM (Hagner e col., 2002a; Hagner e col., 2002b).

Está descrita a existência de receptores que se ligam com elevada afinidade tanto à AM como ao PRGC (Zimmermann e col., 1995). Diferentes estudos anteriores demonstraram que o receptor do tipo do receptor da calcitonina (RTRC) pode actuar como receptor da AM ou do PRGC, dependendo da expressão de diferentes membros de uma nova família de proteínas com um único domínio transmembranar, denominadas proteínas modificadoras da actividade do receptor (PMAR) (McLatchie e col., 1998; Udawela e col., 2004). Até ao momento sabe-se que a família das PMAR é constituída por três isoformas: PMAR1, PMAR2 e PMAR3 (McLatchie e col., 1998; Sexton e col., 2001; Hay e col., 2003). A combinação do RTRC com a PMAR2 resulta no receptor do tipo 1 da AM (AM_1), enquanto a co-expressão do RTRC com a PMAR3 origina um receptor do tipo 2 da

AM (AM_2) (Buhlmann e col., 1999; Hay e col., 2004). Os papéis diferenciais destes dois receptores não estão convenientemente esclarecidos. Pensa-se que os receptores AM_1 e AM_2 estão sujeitos a diferentes mecanismos de regulação em termos de vias de sinalização intracelular que por sua vez modulam a actividade do receptor (Bomberger e col., 2005a; Bomberger e col., 2005b).

O AMPc foi inicialmente sugerido como o principal mensageiro secundário que modula a acção da AM. Contudo, a AM também aumenta a síntese de NO e, consequentemente, os níveis de monofosfato de guanosina cíclico (GMPc) (Hirata e col., 1995; Hayakawa e col., 1999). Possíveis explicações para o aumento da síntase do NO incluem a PKA activada pelo AMPc e a via dependente da cínase do fosfatidil-inositol 3 (PI3K)/Akt também activada pela AM (Nishimatsu e col., 2001) (figura 3). Outro estudo demonstrou que a AM estimula as cínases reguladas por sinais extracelulares (ERK) via activação das cínases de tirosina, podendo modular o estado mitogénico da célula (Iwasaki e col., 1998).

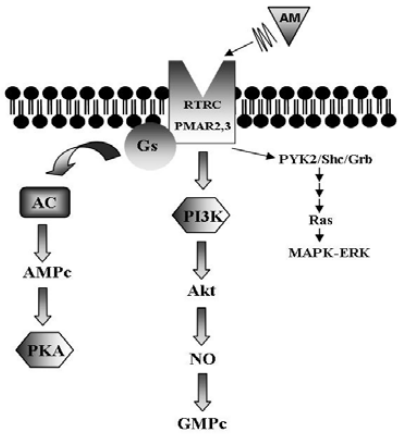

Figura 3: Receptores de membrana e vias sub-celulares que condicionam os efeitos da adrenomedulina.

EFEITOS CARDIOVASCULARES DA ADRENOMEDULINA

Os efeitos cardiovasculares da AM foram os primeiros a ser descobertos e a associação estreita entre este peptídeo e diferentes doenças cardiovasculares tem sido extensivamente estudada tal como se pode comprovar pela literatura actualmente existente (Nishikimi e Matsuoka, 2005; Shimosawa e Fujita, 2005).

Dado que a AM é secretada em grande quantidade pelo tecido vascular, nomeadamente pelas células endotelias e musculares lisas vasculares, a sua principal acção consiste no controlo parácrino da função vascular (Minamino e col., 2002). A AM produz vasodilatação comparável à induzida pelo PRGC. Em ratos anestesiados, a administração intravenosa de *bolus* de AM promove uma diminuição da resistência periférica total, resultando na redução da pressão arterial que persiste durante mais de 10 minutos (Kitamura e col., 1993a). A AM também induz vasodilatação e um aumento do fluxo sanguíneo em diversos leitos vasculares, incluindo a circulação cerebral, coronária, pulmonar e renal (Lippton e col., 1994; Hirata e col., 1995; Lang e col., 1997; Yoshimoto e col., 1998). De forma semelhante, em humanos, a AM quando administrada sob a forma de infusão intravenosa também provoca redução da pressão arterial dependente da concentração, acompanhada de redução da resistência periférica total (Lainchbury e col., 1997; Lainchbury e col., 2000b; Nagaya e col., 2000). Este efeito hipotensor ocorre com concentrações plasmáticas fisiológicas de AM e causa activação reflexa mínima do sistema nervoso simpático e do sistema renina-angiotensina aldosterona, sugerindo que a AM pode directamente inibir estes sistemas neuroendócrinos (Lainchbury e col., 1997).

O efeito vasodilatador da AM é principalmente mediado pela produção de AMPc. Contudo, o NO poderá ser, alternativamente, o mecanismo primário da vasodilatação, dado que a AM também estimula a sua produção. A corroborar esta hipótese está o facto do

efeito vasodilatador da AM poder ser atenuado após a remoção do endotélio ou após a administração de um inibidor da síntase do NO (Feng e col., 1994; Hirata e col., 1995).

No que concerne aos efeitos cardíacos, existe uma grande heterogeneidade de resultados entre os estudos relativamente ao verdadeiro efeito inotrópico da AM. Diferentes estudos descreveram um efeito inotrópico positivo da AM por mecanismos dependentes e independentes do AMPc (Szokodi e col., 1998; Ihara e col., 2000; Bisping e col., 2007). Porém, outros autores advogam um efeito inotrópico negativo da AM em cardiomiócitos isolados de Coelho associado a um aumento de NO e GMPc (Ikenouchi e col., 1997). Noutro estudo em que foram utilizaradas trabéculas miocárdicas humanas não foi observado qualquer efeito inotrópico (Saetrum Opgaard e col., 2000a). Estes resultados poderão estar relacionados com diferenças inerentes à própria espécie animal e à preparação experimental utilizada.

No Homem, a administração sistémica de AM aumenta o débito cardíaco em indivíduos saudáveis e com IC, secundariamente à diminuição da pressão sanguínea sistémica (pós-carga cardíaca) e à dilatação coronária com aumento do fluxo neste território (Parkes, 1995; Lainchbury e col., 2000b; Nagaya e col., 2000).

A AM tem um papel protector sobre o coração ao reduzir a remodelagem ventricular (Nakamura e col., 2002), sendo um potencial factor supressor da hipertrofia miocitária e da proliferação de fibroblastos (Tsuruda e col., 1998; Tsuruda e col., 1999). Por outro lado, a AM tem um efeito antiapoptótico nos cardiomiócitos mediado pela via dependente da PI3K/Akt (Okumura e col., 2004), a mesma que modula o efeito antiapoptótico nas células endoteliais vasculares e a angiogénese induzida pela AM (Kim e col., 2002; Tokunaga e col., 2004).

ADRENOMEDULINA NA INSUFICIÊNCIA CARDÍACA

No Homem, as concentrações plasmáticas de AM estão elevadas no âmbito da hipertensão (Ishimitsu e col., 1994b; Kohno e col., 1996; Sumimoto e col., 1997), do enfarte do miocárdio (Kobayashi e col., 1996) e da IC congestiva (Kato e col., 1996). O aumento das suas concentrações promove diminuição da pressão sanguínea e redução do volume de ejecção, actuando como um mecanismo protector.

Na IC, as concentrações plasmáticas aumentadas de AM correlacionam-se com as classes funcionais da *New York Heart Association* (NYHA) (Nishikimi e col., 1995; Randa Abdel Kader e col., 2007), e representam um possível indicador de prognóstico (Pousset e col., 2000). No Homem, relacionam-se com a gravidade da disfunção cardíaca, correlacionando-se negativamente com a fracção de ejecção do ventrículo esquerdo e positivamente com a pressão diastólica, a pressão de encravamento capilar e da artéria pulmonar e os níveis circulantes de peptídeos natriuréticos e de renina (Jougasaki e col., 1996; Kato e col., 1996; Yu e col., 2001). A AM e os seus receptores são sobreexpressos no miocárdio de modelos animais de IC induzida por sobrecarga de volume ou de pressão (Nishikimi e col., 1997; Totsune e col., 2000; Yoshihara e col., 2000; Cueille e col., 2002; Nishikimi e col., 2003a), bem como de doentes com IC quando comparados com indivíduos normais (Jougasaki e col., 1995). Adicionalmente, na IC o miocárdio é uma fonte importante de AM, contribuindo de forma significativa para o aumento dos níveis circulantes da mesma (Jougasaki e col., 1996).

A administração intravenosa de AM aumenta o débito cardíaco e reduz a pressão de encravamento pulmonar, tendo pouco efeito na frequência cardíaca e na pressão arterial, resultando assim num aumento do volume urinário e da excreção urinária de sódio (Nagaya e col., 2000). Estes efeitos benéficos podem ser mediados pela diminuição da pós-carga devida à vasodilatação periférica e ao possível efeito inotrópico da AM. Em modelos

animais, a administração crónica de AM atenua a progressão da disfunção cardíaca e melhora o prognóstico em ratos com IC crónica (Nishikimi e col., 2003b).

Em suma, a administração de AM ou de agonistas do seu receptor, bem como o bloqueio farmacológico do seu catabolismo representam uma potencial estratégia terapêutica na IC. Recentemente foi demonstrado em estudos experimentais o benefício do tratamento combinado de AM com omapatrilato, um inibidor das vasopeptidases, devido à elevação das concentrações plasmáticas de AM e, como tal, à potenciação dos seus efeitos cardioprotectores (Nishikimi e col., 2006; Rabkin e Klassen, 2007).

O SISTEMA DA UROTENSINA II

CARACTERÍSTICAS GERAIS

A U-II é um peptídeo vasoactivo potente, originalmente isolado da urofísis, órgão terminal do sistema neurosecretor caudal existente nos peixes teleósteos (Pearson e col., 1980). Trata-se de um peptídeo cíclico que partilha uma sequência de aminoácidos semelhante à somatostatina (Bern e col., 1985). Posteriormente a U-II foi clonada em várias espécies de mamíferos, embora o interesse à sua volta se tenha intensificado só mais tarde, quando esta foi clonada no Homem (Coulouarn e col., 1998) e identificada como o ligando endógeno do receptor UT (Ames e col., 1999).

Todas as isoformas conhecidas da U-II nos mamíferos, anfíbios e peixes partilham a mesma estrutura cíclica do terminal C (Cis^{5}-Cis^{10}) que confere grande parte da actividade biológica. Pelo contrário, o comprimento e a sequência do terminal N dependem da espécie (Coulouarn e col., 1998; Coulouarn e col., 1999; Douglas e Ohlstein, 2000; Elshourbagy e col., 2002).

Em 2003, Sugo e colaboradores identificaram um novo peptídeo, o peptídeo relacionado com a U-II, a partir do cérebro de Rato (Sugo e col., 2003). Este peptídeo conserva a mesma estrutura cíclica da U-II e liga-se de igual forma ao receptor UT, sendo que os seus efeitos biológicos parecem ser semelhantes aos induzidos pela U-II (Sugo e col., 2003; Prosser e col., 2006).

À semelhança de outros sistemas vasoconstritores neuro-humorais (sistema renina-angiotensina, sistema da ET) que são importantes na patogénese e na progressão das doenças cardiovasculares, dados emergentes sugerem que a U-II representa também um sistema potencialmente importante nestas doenças. Todavia, apesar dos diversos estudos

efectuados nesta área, vários aspectos do papel da U-II na fisiologia e na fisiopatologia do sistema cardiovascular permanecem por esclarecer.

SÍNTESE DA UROTENSINA-II E RECEPTORES

A isoforma humana da U-II (hU-II) é constituída por 11 aminoácidos e, à semelhança de outros peptídeos, a forma madura bioactiva deriva de formas pré-pró, mediante um processo de clivagem proteolítica. No Homem foram identificadas duas formas de pré-pró-U-II com 124 e 139 aminoácidos (Coulouarn e col., 1998; Ames e col., 1999). Contrariamente ao que sucede com a forma bioactiva, a sequência de aminoácidos da pré-pró-U-II apresenta pouca homologia entre espécies, sendo que a hU-II é homóloga à do peixe e à do sapo somente em 16% e 25%, respectivamente.

A via que conduz à produção de U-II não é totalmente conhecida. Até à data não foi identificada de forma definitiva nenhuma enzima de conversão da U-II (Tolle e van der Giet, 2007). O ARNm da pré-pró-U-II é expresso em diferentes células de mamíferos, incluindo as células musculares lisas vasculares (Douglas e col., 2002), as células endoteliais (Douglas e col., 2002; Totsune e col., 2003), as células endoteliais endocárdicas (Douglas e col., 2002), os motoneurónios (Coulouarn e col., 1998) e os fibroblastos cardíacos de Rato (Tzanidis e col., 2003). Contudo, não foi determinado se a pró-hormona é processada localmente nestas células ou é secretada e processada num local diferente. A circulação poderá constituir uma alternativa para a actividade da enzima de conversão da U-II, embora tenha sido sugerida a sua existência em células mesoteliais epicárdicas humanas, desempenhando uma actividade semelhante à da furina (Russell e col., 2004). Dados recentes, baseados em gradientes arterio-venosos, apontam o coração, o fígado e o rim como possíveis locais de síntese de U-II (Charles e col., 2005).

A U-II actua mediante a ligação a um receptor transmembranar associado a proteínas G, o receptor UT (Ames e col., 1999; Mori e col., 1999). Em 1995, Marchese e colaboradores identificaram o gene GPR14 que codificava um receptor órfão associado a proteínas G relacionado com a somatostatina (Marchese e col., 1995). A U-II foi reconhecida como o ligando endógeno desse receptor no Rato, tendo sido inicialmente denominado de receptor GPR14 (Tal e col., 1995). Entretanto, o receptor homólogo a este foi clonado no Homem com a designação de receptor UT.

O receptor UT, composto por 389 aminoácidos, é codificado por um gene localizado no cromossoma 17q25.3 (Ames e col., 1999). O receptor UT contém sete domínios transmembranares e pertence à classe A da família dos receptores transmembranares associados a proteínas G, homólogos ao receptor da rodopsina (Proulx e col., 2007). Este receptor partilha cerca de 27% de identidade com a sequência proteica dos receptores da somatostatina e dos opióides (Ames e col., 1999; Liu e col., 1999; Mori e col., 1999; Nothacker e col., 1999). O receptor UT do Rato e do Ratinho são homólogos em 92% e o do Homem é homólogo ao do Macaco em 95%. Por outro lado, as isoformas do Rato e do Macaco só partilham 74% de homologia (Elshourbagy e col., 2002; Proulx e col., 2007). O receptor UT do Gato também já foi clonado, sendo que apresenta 74% e 77% de homologia com o do Macaco e do Rato, respectivamente (Aiyar e col., 2005).

A principal via de sinalização intracelular associada ao receptor UT é a ligação e a activação do subtipo $G_{\alpha q/11}$ da proteína G heterotrimérica (Tzanidis e col., 2003). A sua activação leva ao aumento de trifosfato de inositol (IP_3) (Saetrum Opgaard e col., 2000b) e à mobilização do Ca^{2+} intracelular (Ames e col., 1999).

Embora sejam principalmente expressos no sistema nervoso central, tanto a U-II como o seu receptor são expressos de forma abundante no sistema cardiovascular. A este nível, o sistema da U-II é expresso nos cardiomiócitos, nas células musculares lisas e

endoteliais vasculares e nos fibroblastos (Ames e col., 1999; Maguire e col., 2000; Totsune e col., 2001; Douglas e col., 2002).

EFEITOS CARDIOVASCULARES DA UROTENSINA II

O estudo desenvolvido por Ames e colaboradores constituiu um marco importante na história da U-II. Neste estudo, a U-II revelou ser o vasoconstritor mais potente identificado até à data em mamíferos, com uma potência superior à ET-1. Este estudo demonstrou que a sua administração em primatas promoveu uma resposta vascular bifásica, dependente da concentração de U-II. Esta resposta caracterizou-se por uma redução inicial da pressão arterial, à qual se seguiu uma fase hipertensiva que culminou em depressão miocárdica e colapso circulatório fatal, consistente com um efeito dilatador e constritor (Ames e col., 1999).

De facto, a U-II induz uma vasoconstrição potente e prolongada de diferentes vasos arteriais em diferentes espécies, sendo esta irreversível durante várias horas, mesmo após lavagem (Camarda e col., 2002; Behm e col., 2004). No entanto, os efeitos da U-II dependem da espécie animal, do leito vascular e do segmento do leito vascular (Douglas e col., 2000). Por exemplo, no Rato, a U-II contrai a aorta torácica (Ames e col., 1999), não exercendo qualquer efeito no segmento abdominal deste vaso, dependendo o seu efeito da expressão do receptor ao longo do vaso.

Contudo, a U-II também pode actuar como um vasodilatador dependente do endotélio ao aumentar a libertação endotelial de NO (Bottrill e col., 2000; MacLean e col., 2000), pelo menos em alguns leitos vasculares do Rato (Bottrill e col., 2000; Katano e col., 2000). Para além do envolvimento da via do NO na vasodilatação mediada pelo receptor UT (Lacza e Busija, 2006), foi sugerido que as prostaciclinas e a PGE_2 também desempenham um papel importante neste efeito (Ishihata e col., 2005).

No caso particular da vasculatura humana, o papel exacto da U-II continua a representar um tema de grande controvérsia. Alguns investigadores não observaram qualquer efeito vasoactivo da U-II, tanto *in vivo* como *in vitro* (Hillier e col., 2001; Camarda e col., 2002; Wilkinson e col., 2002), enquanto outros documentaram resultados diferentes que incluiram vasoconstrição significativa *in vivo* (Bohm e Pernow, 2002) e *in vitro* (Maguire e col., 2000), bem como efeitos vasodilatadores (Stirrat e col., 2001).

Pelo exposto torna-se evidente que a bioactividade vascular da U-II é variável, dependendo da espécie animal, do leito vascular e também das preparações experimentais utilizadas. O mecanismo pelo qual a U-II promove a vasoconstrição é complexo. A interacção da U-II com o receptor UT induz a activação da fosfolípase C (PLC) e a libertação de IP_3 que, por sua vez, promove a mobilização do Ca^{2+} intracelular (Ames e col., 1999). Porém, existem outras vias de sinalização intracelular que também estão envolvidas na vasoconstrição induzida pela activação do receptor UT, tais como: os canais de Ca^{2+}, as cínases de tirosina, as cínases de proteínas activadas por mitogénios (MAPKs) (subfamília p38 e as $ERK_{1/2}$) e as vias da RhoA/cínase da Rho e da proteína cínase C (PKC) (Sauzeau e col., 2001; Rossowski e col., 2002; Russell e Molenaar, 2004; Tasaki e col., 2004).

Para além dos efeitos vasculares, a U-II exerce efeitos directos sobre o miocárdio. No entanto, pouco se sabe acerca do seu efeito sobre a contractilidade miocárdica, visto estar descrito que a U-II pode ter um efeito inotrópico positivo ou negativo, podendo também não induzir qualquer efeito. Em diversos estudos *in vivo*, a administração sistémica de U-II induziu uma diminuição da contractilidade ventricular esquerda com concomitante diminuição da pressão sanguínea, tanto no Macaco (Ames e col., 1999; Zhu e col., 2004) como no Rato (Gardiner e col., 2001; Hassan e col., 2003). Porém, este efeito sobre a contractilidade poderá ser explicado pelo facto da U-II afectar o tono vascular e a

pressão sanguínea, nomeadamente através da vasoconstrição coronária. Deste modo, numa tentativa de melhor esclarecer o efeito inotrópico directo da U-II, foram realizados estudos *in vitro* recorrendo a tecido miocárdico isolado. Em miócitos isolados de ventrículo esquerdo de Cão, a U-II (10 nM-10 μM) deprimiu a contractilidade miocárdica (Morimoto e col., 2002). Não obstante, em trabéculas humanas isoladas da aurícula e do ventrículo direito (Russell e col., 2001) e em músculos papilares de Rato a U-II promoveu um efeito inotrópico positivo (Gong e col., 2004), possivelmente por um mecanismo dependente da PKC (Russell e Molenaar, 2004). A U-II participa ainda na regulação miocárdica mediante a modulação central dos eixos simpático-supra-renal e pituitário-supra-renal (Lin e col., 2003; Watson e col., 2003).

Outra função importante da U-II é o seu papel na hipertrofia e remodelagem cardíaca. Ela é capaz de induzir hipertrofia de cardiomiócitos neonatais em cultura e promover a deposição de colagénio por fibroblastos neonatais em cultura (Tzanidis e col., 2003; Bousette e col., 2006b). Zhang e colaboradores documentaram *in vivo* o papel da U-II na modulação da hipertrofia cardíaca. Estes investigadores demonstraram que num modelo animal de Rato sujeito a hipóxia, em que existe hipertrofia ventricular direita, os níveis de U-II e de receptores UT eram superiores no ventrículo direito quando comparados com o esquerdo (Zhang e col., 2002). Em cardiomiócitos em cultura de Rato, a U-II é capaz de estimular a libertação de certos peptídeos, tais como o peptídeo natriurético auricular, o peptídeo natriurético tipo B (Zou e col., 2001), e citocinas como a interleucina-6 (Johns e col., 2004). Daqui ressalta o facto de factores neuro-humorais libertados após a estimulação com a U-II poderem modular os efeitos cardíacos da mesma (Zhu e col., 2006).

Resumindo, no sistema cardiovascular, a U-II pode modular o tono vascular, a contracção miocárdica, a frequência cardíaca e o crescimento e a proliferação celulares (quadro 2) (Russell, 2004).

RESPOSTA VASCULAR		
Contracção	Resposta potente e variável	(Maguire e col., 2000)
Relaxamento	Dependente do endotélio	(Katano e col., 2000; Stirrat e col., 2001)
Hiperpermeabilidade vascular	Aorta torácica (Rato): avaliada pelo extravasamento de plasma	(Gendron e col., 2004)
Proliferação das células musculares lisas vasculares	Células musculares lisas da aorta (Coelho)	(Watanabe e col., 2001)
Organização do citoesqueleto da actina	Células musculares lisas da aorta (Rato): aumento da F-actina, diminuição da G-actina	(Sauzeau e col., 2001)
RESPOSTA CARDÍACA		
Inotropismo positivo	Ventrículo e aurícula direita (Homem)	(Russell e col., 2001)
Inotropismo negativo	Ventrículo esquerdo e miócitos de ventrículo esquerdo (Cão)	(Morimoto e col., 2002)
Depressão miocárdica	Possivelmente secundária à vasoconstrição (Macaco)	(Ames e col., 1999)
Arritmias	Resposta inferior quando comparada com a ET-1 (átrio humano)	(Russell e col., 2001)
Taquicardia	Central: hipertensão e taquicardia Periférica: hipotensão e taquicardia reflexa	(Lu e col., 2002; Lin e col., 2003; Watson e col., 2003)
Bradicardia	Rato, central (área cerebral A_1): hipotensão e bradicardia	(Lu e col., 2002)
Hipertrofia dos cardiomiócitos	Crescimento de cardiomiócitos neonatais (Rato)	(Zou e col., 2001; Tzanidis e col., 2003)
Organização do sarcómero	Cardiomiócitos neonatais (Rato)	(Zou e col., 2001)
Produção de matrix extracelular	Fibroblastos cardíacos neonatais (Rato): aumento da síntese de colagénio	(Tzanidis e col., 2003)

Quadro 2: Efeitos cardiovasculares da urotensina II.

UROTENSINA II E INSUFICIÊNCIA CARDÍACA

Vários estudos em doentes com IC têm descrito elevadas concentrações plasmáticas de U-II na IC (Ng e col., 2002; Richards e col., 2002; Russell e col., 2003; Simpson e col., 2006), embora nem todos observem estes resultados (Dschietzig e col., 2002). Na literatura está documentada a associação entre as concentrações plasmáticas de hU-II e a disfunção diastólica na doença cardíaca isquémica (Heringlake e col., 2004). Dos estudos realizados

foram poucos os que demonstraram existir correlações entre as concentrações plasmáticas de U-II e as classes funcionais da NYHA (Lapp e col., 2004; Gruson e col., 2006).

No estudo levado a cabo por Russell e colaboradores foi sugerido que na IC a U-II é produzida no coração, dado que as suas concentrações plasmáticas na raiz aórtica eram superiores às da artéria pulmonar, sendo esta parcialmente removida da microcirculação (Russell e col., 2003).

O sistema tecidular cardíaco da U-II está também alterado no âmbito da IC experimental e clínica. Assim, foram vários os estudos que demonstraram sobrexpressão cardíaca da U-II e do seu receptor em modelos animais (Johns e col., 2004; Bousette e col., 2006a). Também no Homem, está documentada sobreexpressão do sistema da U-II na IC crónica secundária a cardiomiopatia isquémica ou dilatada (Douglas e col., 2002), e a sua correlação positiva e negativa com as dimensões telediastólicas do ventrículo esquerdo e com a fracção de ejecção, respectivamente.

Considerando os potenciais efeitos deletérios da U-II na IC, foram realizados estudos em modelos animais para avaliar os efeitos do bloqueio da sua actividade. Num modelo experimental de IC isquémica, após a oclusão da artéria coronária no Rato, o tratamento crónico com o bloqueador selectivo do receptor UT, SB-611812, melhorou *in vivo* a função cardíaca, ao reduzir significativamente a dilatação e a hipertrofia ventricular bem como a taxa de mortalidade (Bousette e col., 2006a). *In vitro* reduziu a disfunção diastólica, mediante a diminuição de fibrose miocárdica, e inibiu a proliferação de fibroblastos induzida pela U-II (Bousette e col., 2006b). Embora estes estudos apontem para a possibilidade do bloqueio da actividade da U-II representar um potencial alvo terapêutico na IC, estudos subsequentes num modelo animal de isquemia-reperfusão e no Homem com enfarte miocárdico agudo sugeriram que a U-II pode desempenhar um papel cardioprotector (Khan e col., 2007; Prosser e col., 2007).

De qualquer forma, a U-II e o seu receptor continuam a ser potenciais alvos terapêuticos, tendo sido já desenvolvidos vários antagonistas não peptídicos dos receptores com o intuito de minimizar os efeitos deletérios da sobreactivação do receptor UT (Lescot e col., 2007). Em 2006 foi publicado o único estudo clínico realizado no Homem para avaliar a eficácia de um antagonista do receptor UT, o palosuran (Sidharta e col., 2006). Apesar dos resultados promissores, este antagonista só foi avaliado no contexto do tratamento da nefropatia diabética.

OBJECTIVOS

O trabalho experimental que integra a presente dissertação de doutoramento teve como objectivo geral o estudo de mecanismos neuro-humorais e endoteliais envolvidos na IC. Tendo sido usado o Coelho como modelo experimental, o nosso objectivo inicial consistiu na validação do exame ecocardiográfico em animais saudáveis desta espécie. Posteriormente, foi nossa intenção caracterizar os efeitos miocárdicos intrínsecos a diferentes sistemas neuro-humorais, tanto em corações saudáveis como na presença de IC induzida experimentalmente. Deste modo, os objectivos específicos consistiram em:

1. Avaliação ecocardiográfica no Coelho

A ecocardiografia é actualmente o meio de diagnóstico de eleição na avaliação morfológica e funcional do coração e grandes vasos. O Coelho doméstico, *Oryctolagus cuniculus*, é largamente utilizado pelo Homem na investigação biomédica. Por outro lado, tem-se assistido a um aumento da sua popularidade enquanto animal de companhia. Neste contexto, em primeiro lugar propusemo-nos caracterizar os valores de referência para alguns parâmetros ecocardiográficos convencionais e outros parâmetros obtidos por Doppler tecidular, recorrendo a dois diferentes protocolos anestésicos. Dispusemo-nos ainda avaliar a concordância do valor do índice de Tei obtido segundo diferentes técnicas ecocardiográficas (modo-M, Doppler pulsado e Doppler tecidular pulsado).

2. Estudo da modulação da função cardíaca por mediadores neuro-humorais clássicos: a endotelina-1 e o sistema β-adrenérgico

a. Estudo dos efeitos miocárdicos da estimulação dos receptores ET_B na insuficiência cardíaca

Este estudo surgiu na sequência de um estudo previamente realizado pelo nosso grupo, com o objectivo de tentar perceber se os efeitos miocárdicos mediados pelos receptores ET_B da ET-1 estavam preservados em músculos papilares de corações insuficientes. Nesse primeiro estudo, constatou-se em corações saudáveis que os efeitos da ET-1 mediados pelos receptores ET_B são opostos na presença e ausência de um endotélio endocárdico funcionante. Desta forma, pareceu-nos importante analisar os efeitos da estimulação selectiva deste subtipo de receptores como possível marcador funcional de disfunção endotelial endocárdica no modelo experimental de IC induzida pela doxorrubicina.

b. Estudo do papel do óxido nítrico e das prostaglandinas na modulação dos efeitos diastólicos da ET-1

Dando continuidade aos estudos levados a cabo no nosso laboratório que avaliaram os efeitos agudos deste peptídeo na função diastólica, mais concretamente nas propriedades diastólicas do miocárdio e a sua modulação pelo endotélio endocárdico em músculos papilares isolados do ventrículo direito de Coelho, procurou-se esclarecer o papel de dois importantes mediadores endoteliais, o NO e as prostaglandinas, na modulação destes efeitos. Tendo em conta a importância do endotélio cardíaco, endocárdico e vascular, na modulação dos efeitos miocárdicos da ET-1 e o facto de na insuficência cardíaca poder

ocorrer disfunção endotelial, investigámos também se o efeito da ET-1 sobre as propriedades diastólicas se encontra preservado num modelo animal de IC.

c. Estudo dos efeitos do sistema β-adrenérgico sobre as propriedades diastólicas do miocárdio

Estudos recentes demonstraram que a fosforilação da titina pela PKA promove uma diminuição da rigidez miocárdica. Sendo a estimulação β-adrenérgica um dos estímulos mais potentes para a activação intracelular desta cínase, propusemo-nos avaliar os seus efeitos sobre as propriedades diastólicas do miocárdio em músculos papilares isolados do ventrículo esquerdo de Coelho. Ao mesmo tempo, e com a intenção de melhor perceber estes efeitos, foi também nosso objectivo averiguar os mecanismos que lhes estão subjacentes em termos de receptores e de vias de transdução do sinal envolvidas.

3. Estudo dos efeitos miocárdicos de novos sistemas neuro-humorais: a adrenomedulina e a urotensina-II

Como vimos atrás, evidências crescentes apontam para a importância de determinados sistemas neuro-humorais na modulação aguda das propriedades diastólicas do miocárdio, entre eles a ET-1, o NO e a AngII. Deste modo, e na sequência dos estudos anteriores, investigámos, em músculos papilares isolados do ventrículo direito de Coelho, os efeitos miocárdicos de dois novos importantes mediadores neuro-humorais do sistema cardiovascular, a AM e a U-II, dando particular ênfase aos efeitos sobre as propriedades diastólicas. Além disso, e com intuito de melhor perceber estes efeitos, foi também nosso objectivo averiguar que mecanismos lhes estão subjacentes em termos de receptores e de vias de transdução do sinal envolvidas.

O último trabalho que integra esta dissertação surgiu na sequência dos resultados obtidos no estudo da U-II, tendo sido nosso propósito investigar o envolvimento dos sistemas da AngII e da ET-1 nos efeitos miocárdicos da U-II.

Do exposto na introdução desta dissertação torna-se evidente que apesar de todo o conhecimento que já existe sobre os sistemas neuro-humorais e destes serem objecto de investigação intensa, os seus efeitos na progressão da IC, a discriminação dos efeitos miocárdicos mediados por cada um dos tipos de receptores dos vários agentes, bem como a caracterização dos mecanismos celulares e moleculares que lhes estão subjacentes não estão ainda completamente esclarecidos. A investigação e esclarecimento de alguns aspectos darão seguramente uma contribuição para a melhor compreensão da fisiologia cardiovascular e da fisiopatologia da IC e poderá ajudar a delinear novas estratégias terapêuticas.

CAPÍTULO II

AVALIAÇÃO ECOCARDIOGRÁFICA NO COELHO

PARTE A: PARÂMETROS ECOCARDIOGRÁFICOS DE REFERÊNCIA POR MODO-M E DOPPLER CONVENCIONAL

M-mode and Doppler echocardiographic reference values for male New Zealand white rabbits

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Objective—To determine M-mode and Doppler echocardiographic reference values in healthy New Zealand white rabbits.

Animals—52 healthy male rabbits.

Procedures—The rabbits were anesthetized and M-mode measurements of the left ventricle, left atrium, and aorta and Doppler measurements of pulmonary and aortic outflow and mitral inflow were recorded.

Results—Mean ± SD heart rate during echocardiographic examination was 155 ± 29 beats/min. Mean ± SD measurements in diastole and systole for the interventricular septum thickness, left ventricular internal diameter, and left ventricular free wall thickness were 2.03 ± 0.37 mm and 3.05 ± 0.45 mm; 14.37 ± 1.49 mm and 10.25 ± 1.22mm; and 2.16 ± 0.25 and 3.48 ± 0.55 mm, respectively. Mean ± SD left atrial-to-aortic diameter ratio was 1.17 ± 0.14, and mean ± SD mitral valve E-point–to–septal separation interval was 1.71 ± 0.29 mm. Mean ± SD for fractional shortening and ejection fraction were 30.13 ± 2.98% and 61.29 ± 4.66%, respectively. Mean ± SD maximal aortic and pulmonary artery outflow velocities were 0.85 ± 0.11 m/s and 0.59 ± 0.10 m/s, respectively, and the peak E-to-peak A wave velocity ratio of the mitral valve was 2.19 ± 0.46.

Conclusions and Clinical Relevance—Results provide echocardiographic reference values for young adult male New Zealand white rabbits anesthetized with ketamine and medetomidine. Values obtained from unanesthetized rabbits, rabbits sedated with other agents, or rabbits of different size may differ from those reported here. (*Am J Vet Res* 2006;67:1725–1729)

The domestic rabbit (*Oryctolagus cuniculus*) is becoming increasingly popular as a companion animal. Cardiac disease has been described in pet rabbits,[1,2] and the species has been widely used in cardiovascular research.[3–5]

Echocardiography is a useful technique for diagnosing cardiovascular disease in small animals.[6] It has been used for cardiac imaging and evaluation in experimental and clinical settings and has become an indispensable tool in the specialty of veterinary cardiology because it enables noninvasive measurement of cardiac structures. Knowledge of findings in healthy animals is essential for interpretation of results in clinical patients. Values obtained from clinically normal animals by use of 2-dimensional and M-mode echocardiography have been reported in a variety of animals, including dogs,[7–13] cats,[14–17] ferrets,[18,19] chinchillas,[20] guinea pigs,[21] hamsters,[22] and birds,[23] but to the authors' knowledge, reference values for rabbits have not been published.

The purpose of this study was to determine reference values for echocardiographic M-mode and Doppler measurements in clinically healthy rabbits that were lightly anesthetized with ketamine and medetomidine.

Abbreviations

IVS	Interventricular septum
LVID	Left ventricular internal diameter
LVFW	Left ventricular free wall
FS	Fractional shortening

Materials and Methods

The study was performed according to the Portuguese Law for Animal Welfare. The anesthetic and testing methods conformed to the Guide for the Care and Use of Laboratory Animals published by the National Academy Press. Fifty-two healthy male New Zealand white rabbits, 16 to 18 weeks of age and weighing 2.2 to 3.2 kg, were used. Rabbits were free of signs of cardiovascular or respiratory tract disease and were determined to be clinically normal on the basis of a physical examination that included careful thoracic auscultation. The rabbits were housed in stainless steel cages in a controlled environment, at temperatures of 20° to 25°C with 12 hours of light and 12 hours of dark/day. A commercial pellet diet and water were supplied ad libitum. Feed was withheld for a maximum of 4 hours before rabbits underwent echocardiographic examination to reduce abdominal distension from intestinal fill, which can mechanically compress the diaphragm and lungs, particularly when the abdomen is compressed during the segment of echocardiographic examination in which images are obtained via the subcostal approach. The weight of each rabbit was recorded prior to anesthesia.

Ketamine hydrochloride[a] (2 mg/kg) and medetomidine hydrochloride[b] (0.15 mg/kg) were administered IM to each rabbit to minimize defensive movements and facilitate complete echocardiographic examination. Rabbits were typically completely immobilized within 2 minutes of injection. For the right parasternal views, rabbits were placed in right lateral recumbency over a gap in the tabletop through which the ultrasound probe was brought from below and placed on a shaved area on the cranial aspect of the lower portion of the right thoracic wall. The hair was clipped in the subcostal portion of the abdominal wall for the subcostal apical 4-chamber

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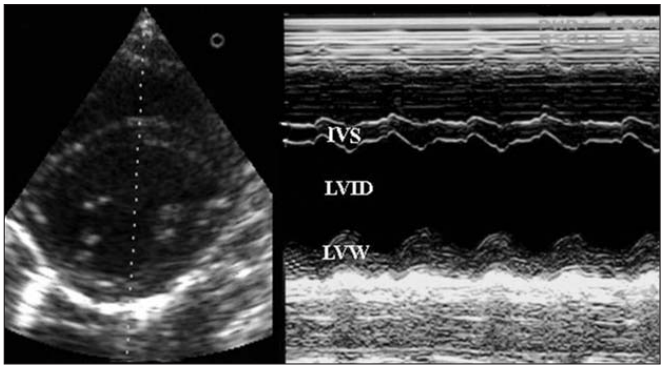

Figure 1—Right parasternal short-axis echocardiographic view (left panel) with 2-dimensional guided M-mode tracing (right panel) of the left ventricle of a healthy male New Zealand white rabbit. View was obtained just below the level of the mitral valve. LVW = Left ventricular (free) wall.

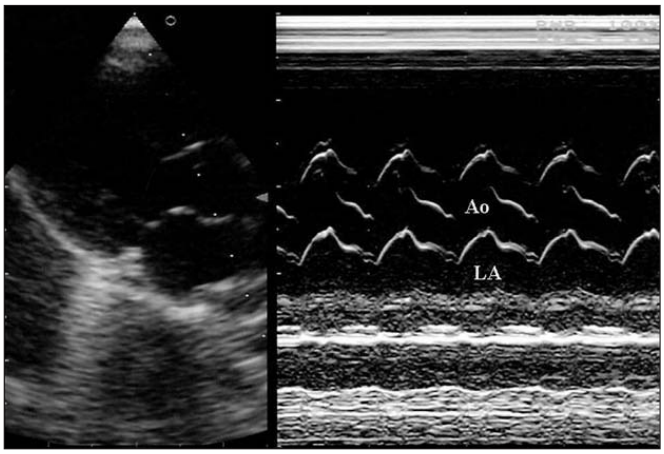

Figure 2—Right parasternal long-axis echocardiographic view (left panel) with 2-dimensional guided M-mode tracing (right panel) at the level of the aortic valve of a healthy male New Zealand white rabbit. Ao = Aorta. LA = Left atrial appendage.

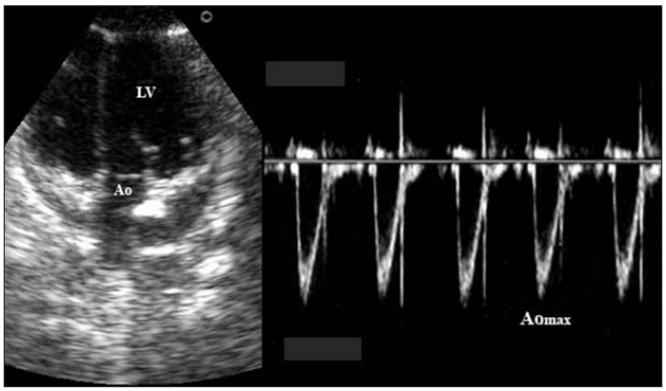

Figure 3—Five-chambered apical echocardiographic view (left panel) and pulsed-wave spectral Doppler recording (right panel) of the aortic outflow tract of a healthy male New Zealand white rabbit. LV = Left ventricle. Ao_{max} = Maximal aortic outflow velocity. *See* Figure 2 for remainder of key.

view, which was obtained with rabbits positioned in dorsal recumbency. Echocardiographic measurements were obtained from standard views.[24] Doppler imaging of aortic and mitral valve blood flow was optimized by use of the subcostal apical view. Transthoracic 2-dimensional and M-mode echocardiography and Doppler imaging were performed with a system[c] that included color Doppler capabilities with a 5-MHz transducer. Calipers were used to measure structures to the nearest millimeter by means of a leading-edge–to–leading-edge technique according to accepted echocardiographic standards for dogs.[24–26]

From the right parasternal short-axis view, 2-dimensional guided M-mode tracings were made just below the mitral valve at the level of the papillary muscles for measurements of the IVS, LVID, and LVFW in diastole and systole (**Figure 1**). The right parasternal long-axis view with 2-dimensional guided M-mode was used for the measurements of the E-point–to–septal separation interval in the plane of mitral valves, and the aortic and left atrial appendage diameters were evaluated at the level of the aortic valve (**Figure 2**). These measurements were made from the leading edge of the first endocardial surface to the leading edge of the second endocardial surface. The E-point–to–septal separation interval was measured from the point of maximal opening of the mitral valve (E-point) to the IVS. Fractional shortening was calculated from measurements for the LVID in systole and diastole by use of the following formula:

$$FS\ (\%) = [(LVIDd - LVIDs)/LVIDd] \times 100$$

where d is diastole and s is systole. Left ventricular ejection fraction was calculated by use of the cube method according to this formula:

$$\text{Ejection fraction} = [(LVIDd^3 - LVIDs^3)/LVIDd^3] \times 100$$

Doppler examinations were performed according to protocols established for dogs and cats.[27–29] Heart rate was calculated directly from the pulsed Doppler tracings. Pulmonary flow velocities were determined by use of pulsed Doppler (n = 52 rabbits) from the right parasternal short-axis view. Aortic flow (n = 35 rabbits) and mitral E- and A-wave velocities (35 rabbits), with the A-wave corresponding to atrial contraction during late diastole, were recorded via pulsed Doppler from the subcostal apical 5- and 4-chamber views[28] (**Figures 3 and 4**). In the great vessels, the sample volume was positioned in the center of the vessel, just beyond the valve leaflets. In the mitral valve, the sample volume was placed in the visual center of the inflow tract, on the ventricular side of the valve at the tips of the mitral valve leaflets when they were opened. Alignment was maximized in the 2-dimensional view, and no angle of correction was used. Variables recorded for each rabbit included maximal pulmonary and aortic out-

flow velocity (ie, PA_{max} and Ao_{max}-to- m/s) and maximal E- and A-wave velocities, and an E wave-to-A wave ratio was calculated. Velocities were recorded as the maximal value on the outer edge of the peak velocity spectrum.[28]

Recording was typically completed approximately 30 minutes after administration of ketamine and medetomidine. Anesthesia was reversed with atipamezole[d] (0.15 mg/kg) administered IM, and all rabbits recovered fully almost immediately after atipamezole administration.[30] All data were collected by use of a trackball-driven cursor and the ultrasound system software. The measured beats were selected on the basis of quality of the recording and presence of a regular cardiac rhythm. Three representative cardiac cycles were analyzed, and a mean value was calculated for each measurement. From these means, the overall mean, SD, and range for all variables measured in all rabbits were calculated. Multiple linear regression analyses and Pearson correlation coefficients were used to compare rabbit body weights with their respective mean M-mode and Doppler echocardiographic measurements. The minimal α value for statistical significance was $P < 0.05$.

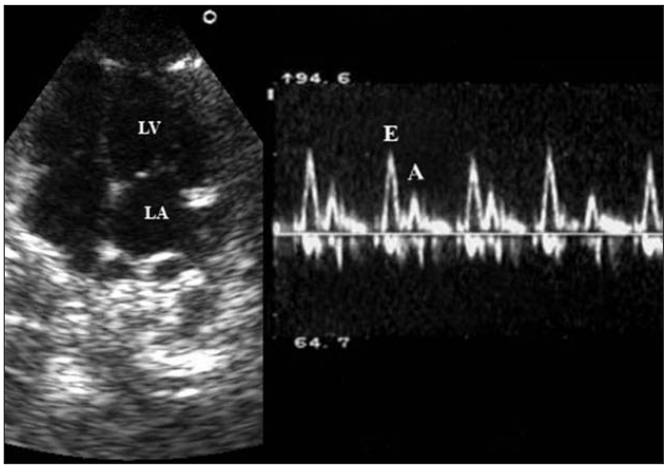

Figure 4—Four-chambered apical echocardiographic view (left panel) and pulsed-wave spectral Doppler recording (right panel) of mitral valve inflow in a healthy male New Zealand white rabbit. E = Point of maximal mitral valve E-wave velocity. A = Point of maximal mitral valve A-wave velocity. *See* Figures 2 and 3 for remainder of key.

Results

Values obtained with 2-dimensional, M-mode, and Doppler echocardiography in all 52 rabbits were summarized (**Table 1**). Mitral valve A- and E-wave velocities were only obtained in the last 35 of the 52 rabbits. No significant linear relationship or correlation was found between body weight and any of the M-mode or Doppler values obtained in this study. No echocardiographic abnormalities were observed in any of the rabbits.

Table 1—Values for 2-dimensional, M-mode, and Doppler echocardiographic variables in male New Zealand white rabbits anesthetized with a combination of ketamine and medetomidine administered IM.

Variable	No. of rabbits	Mean (SD)	Range
BW (kg)	52	2.59 (0.25)	2.2–3.2
IVSd (mm)	52	2.03 (0.37)	1.43–3.10
IVSs (mm)	52	3.05 (0.45)	2.17–4.03
LVIDd (mm)	52	14.37 (1.49)	11.87–19.06
LVIDs (mm)	52	10.05 (1.22)	7.83–13.53
LVFWd (mm)	52	2.16 (0.25)	1.60–2.80
LVFWs (mm)	52	3.48 (0.55)	2.43–4.55
FS (%)	52	30.13 (2.98)	22.60–36.83
EF (%)	52	61.29 (4.66)	49.07–70.0
Ao (mm)	52	8.26 (0.76)	6.73–9.80
LA (mm)	52	9.66 (1,14)	7.53–12.0
LA:Ao	52	1.17 (0.14)	0.94–1.54
EPSS (mm)	52	1.71 (0.29)	1.20–2.33
Doppler HR (bpm)	52	155 (29)	115–234
Ao_{max} (m/s)	35	0.85 (0.11)	0.56–1.06
PA_{max} (m/s)	52	0.59 (0.10)	0.34–0.84
Mitral E (m/s)	35	0.59 (0.10)	0.41–0.83
Mitral A (m/s)	35	0.28 (0.07)	0.19–0.44
Mitral E:A	35	2.19 (0.46)	1.34–3.55

BW = Body weight. IVSd = Thickness of the IVS in diastole. IVSs = Thickness of the IVS in systole. LVIDd = LVID in diastole. LVIDs = LVID in systole. LVFWd = Thickness of the LVFW in diastole. LVFWs = Thickness of the LVFW in systole. EF = Ejection fraction. Ao = Aortic diameter. LA = Left atrial appendage diameter. EPSS = Mitral valve E-point–to–septal separation interval. HR = Heart rate. bpm = Beats per minute. Ao_{max} = Maximal aortic outflow velocity. PA_{max} = Maximal pulmonary artery outflow velocity. Mitral E = Maximal mitral E-wave velocity. Mitral A = Maximal mitral A-wave velocity.

Discussion

Male New Zealand white rabbits are a good model for cardiovascular research because their size makes surgical manipulation of the heart more feasible than in smaller animals, they are less expensive to procure and maintain than dogs, and the composition of rabbit myosin and the kinetics of calcium in the rabbit are similar to those in human myocardium.[31] Pet rabbits can develop cardiovascular disease, and radiography, electrocardiography, and echocardiography are useful noninvasive diagnostic procedures that can be used in evaluation of cardiac disease to provide highly specific assessment of cardiac size, dysrhythmias, and internal structure and function (dynamics), respectively.[6,32]

Measurements of LVID in diastole and systole, IVS in diastole and systole, thickness of the LVFW in diastole and systole, and the left atrial appendage and aortic diameters are widely used for evaluation of cardiac morphology. The echocardiographic values calculated for FS, ejection fraction, and maximal aortic and pulmonary outflow velocities (Ao_{max}, and PA_{max}) are used to assess systolic function, whereas the mitral valve E wave-to-A wave ratio is used to assess diastolic function.

Pet rabbits may be manually restrained safely for many procedures, but research animals and any rabbits less accustomed to handling may require sedation or anesthesia to permit examination procedures, including the prolonged echocardiographic examination that may be required to obtain the necessary measurements. Inhaled isoflurane in oxygen provides rapid induction and recovery with minimal cardiovascular effects.[33]

However, when anesthesia in rabbits is induced by inhalation of isoflurane, especially by face mask, rabbits may react to the smell by holding their breath for extended periods and struggling, which result in hypoxemia and hypercapnia.[34–36] For this reason, rabbits in the present study were anesthetized with IM injection of a combination of ketamine and medetomidine. This anesthetic combination yielded good immobilization and allowed the ultrasonographer to obtain adequate 2-dimensional and M-mode images for measurements and to position the Doppler cursor so as to derive reliable measurements for the outflow velocities, and mitral valve E- and A-wave velocities. Use of this anesthetic combination resulted in short recovery times because of the ability to reverse the effects of medetomidine with atipamezole.[37–39]

The mean ± SD heart rate of the rabbits in our study was 155 ± 29 beats/min, a range that was lower than the range of mean heart rates (180 to 250 beats/min) previously reported for conscious rabbits.[40] Previous work in rabbits revealed that administration of ketamine-medetomidine combinations induces moderate bradycardia, but the effects on mean arterial pressure were minimal, and higher doses were used in those studies[41–44] than were used in the present study. It is known that administration of a combination of ketamine and xylazine alters cardiac function in mice, manifested by decreases in heart rate, FS, and ejection fraction and increases in LVID and IVS in diastole.[45,46] Considering that medetomidine and xylazine are both α_2-adrenergic receptor agonists, it is possible that our results would differ from those obtained in conscious rabbits.

Echocardiographic M-mode measurements reported for dogs vary proportionally with body size (weight), which varies by breed.[47] The M-mode measurements reported for cats,[14,48] ponies and horses,[49] ferrets,[19] and chinchillas,[20] however, do not vary with body size, presumably because different breeds of these species are similar in size. Although the body weight of rabbits in the present study varied from 2.2 to 3.2 kg, no significant associations between body weight and echocardiographic measurements were detected. Because there were no significant changes in echocardiographic values within this range of body weights, the authors speculate that cardiac measurements do not increase with increased weight after rabbits reach maturity. However, rabbits and breeds of rabbits do vary considerably in size, and the values obtained in these young adult male New Zealand white rabbits may be different than those that would be obtained in larger rabbits or in smaller breeds of rabbits.

Rabbits did not undergo testing with thoracic radiography, electrocardiography, CBC, serum biochemical analyses, or urinalyses to rule out the possibility of subclinical or underlying cardiac or pulmonary disease. However, all 52 rabbits used in the study were considered to be clinically normal on the basis of thorough physical examinations prior to being anesthetized, and all rabbits recovered from anesthesia without incident. Values for the echocardiographic variables in the study rabbits should represent reference values for use in echocardiographic examination of young adult male New Zealand white rabbits when obtained during ketamine-medetomidine anesthesia.

a. Imalgene 1000, Merial Portuguesa—Saúde Animal, Rio de Mouro, Portugal.
b. Domitor, Pfizer Saúde Animal, Seixal, Portugal.
c. Aloka Color Doppler SSD-2200, Aloka Co, Tokyo, Japan.
d. Antisedan, Pfizer Saúde Animal, Seixal, Portugal.

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CAPÍTULO II

AVALIAÇÃO ECOCARDIOGRÁFICA NO COELHO

PARTE B: AVALIAÇÃO ECOCARDIOGRÁFICA COM DOPPLER TECIDULAR

Accepted in *The Veterinary Journal*

Echocardiographic evaluation including tissue Doppler imaging in New Zealand white rabbits sedated with ketamine and midazolam

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Abstract

Limited data are available on the use of more recent echocardiographic parameters in the rabbit. Echocardiographic examination, including conventional echocardiography and tissue Doppler imaging (TDI), was performed on 26 male New Zealand white rabbits under ketamine-midazolam sedation. An important emphasis was given to more recent systolic and diastolic parameters, such as myocardial performance index (Tei index), and mitral annular motion (from septal and lateral sides of the left ventricle) obtained with pulsed TDI.

Parameters that assess systolic and diastolic function (fractional shortening, Tei index, and maximal mitral E- and A-wave velocities) were comparable to those reported in the literature for rabbits in the awake state. This less cardiodepressive anaesthetic protocol could be a good alternative in performing echocardiographic evaluation whenever such caution is necessary. TDI is feasible in healthy rabbits and potentially suitable for the investigation of left ventricle systolic and diastolic function.

Keywords: Doppler echocardiography; Tissue Doppler Imaging; Reference value; Rabbit; Anaesthesia

Introduction

Cardiac disease has been described in pet rabbits (Martin et al., 1987) and the species is widely used in cardiovascular research (Bras-Silva et al., 2006; Lange et al., 2006; Barraud et al., 2007). Echocardiography is a useful non-invasive method for the in vivo evaluation of ventricular dimensions and performance in experimental and clinical settings.

Doppler echocardiography provides additional useful information on cardiac conditions in humans and small animals. Parameters obtained by tissue Doppler imaging (TDI) have been shown to be more independent of pre- and after-load than classic haemodynamic Doppler measurements and can be used to quantify regional myocardial function accurately and more objectively (Sohn et al., 1997; Firstenberg et al., 2001; Nagueh et al., 2001). Pulsed TDI of the mitral annulus and myocardial wall has been suggested as a means to assess systolic and diastolic left ventricular (LV) function, both in human and veterinary medicine (Oki et al., 1999; Chetboul et al., 2005; Teshima et al., 2005; Chetboul et al., 2006; O'Sullivan et al., 2007).

The Tei-index, a new parameter to assess myocardial performance, has been proposed for the assessment of global cardiac performance (systolic and diastolic function) in a wide variety of congenital and acquired cardiac abnormalities (Dujardin et al., 1998; Bruch et al., 2002; Haque et al., 2002; Harjai et al., 2002; Gaibazzi et al., 2005; Dyer et al., 2006).

Reference values for various M-mode, flow Doppler and tissue Doppler echocardiographic parameters have been reported in rabbits in the conscious state as well as during different anaesthetic combinations (Fontes-Sousa et al., 2006; Stypmann et al., 2007). Nevertheless, examination of the awake rabbit is more difficult, more time-consuming, and needs special training, especially with research animals or animals less

accustomed to handling. Anaesthesia is an alternative, although this might affect cardiac function, and the extent will depend on the type of anaesthesia (Schaefer et al., 2005). It is therefore important to know the effect of standardised sedation protocols on echocardiographic parameters. Recent studies used ketamine-alpha-2 agonist combinations to perform echocardiography in rabbits (Fontes-Sousa et al., 2006; Stypmann et al., 2007) but the major obstacle with this combination is its potential for cardiac and respiratory depression (Sanford and Colby, 1980). As an alternative, ketamine in combination with midazolam, a short-acting benzodiazepine, has been described for chemical restraint in rabbits associated with minimal cardiorespiratory depression (Dupras et al., 2001).

The purpose of the present study was to determine reference values for echocardiographic M-mode, Doppler, and pulsed TDI measurements in clinically healthy New Zealand White rabbits sedated with ketamine and midazolam.

Materials and Methods

The study was performed according to the Portuguese Law for Animal Welfare. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Twenty-six young adult healthy male New Zealand white rabbits (16-20 weeks old and weighting 2.3±0.4 kg) were studied. Rabbits were healthy and free of signs of cardiovascular or respiratory tract disease on the basis of a physical examination that included careful thoracic auscultation and were considered normal on the basis of their echocardiogram. The animals were housed in stainless steel cages in a controlled environment, at temperatures of 20 to 25 ºC, with a 12:12 h light dark cycle, and were fed

with a standard pellet diet and water ad libitum. The weight of each rabbit was recorded prior to anaesthesia.

A combination of ketamine-hydrochloride (20 mg/kg; Imalgene 1000, Merial) and midazolam (2 mg/kg; Midazolam APS, Farma–APS) was administered SC to each rabbit to minimise defensive movements and facilitate complete echocardiographic examination. Typically, the rabbits were completely immobilised within 5-10 min.

Echocardiography was carried out under light anaesthesia and spontaneous respiration, using a GE Vivid 7 system (GE VingMed) equipped with tissue Doppler technology. The standard phased-array, variable-frequency (3.5-6.9 MHz) transducer was used for two-dimensional, Doppler, and TDI. Recordings were made under continuous ECG monitoring (lead II) by fixing the electrodes on the limbs at a sweep speed of 100 and 200 mm/s for off-line analysis. All echocardiographic acquisitions were made in sinus rhythm.

Rabbits were placed in right or left lateral recumbency to obtain right and left parasternal views, respectively, over a gap in the tabletop through which the ultrasound probe was brought from below and placed on a shaved area on the anterior aspect of the lower portion of the thoracic wall. Echocardiographic measurements were obtained from standard views (Thomas et al., 1993).

From the right parasternal short-axis view, two-dimensional guided M-mode tracings were made just below the mitral valve at the level of the papillary muscles for measurements of the interventricular septum (IVS), left ventricular internal diameter (LVID), and left ventricular free wall (LVFW) in diastole and systole. The right parasternal long-axis view with two-dimensional guided M-mode was used for the measurements of the E-point–to–septal separation interval in the plane of mitral valves. In this same view, the aortic and left atrial diameters were evaluated at the level of the aortic

valve. These measurements were obtained applying the leading-edge method of the American Society of Echocardiography (Sahn et al., 1978). Left ventricular ejection fraction was calculated by use of the cube method according to the formula: ejection fraction = $[(LVIDd^3 – LVIDs^3)/LVIDd^3] \times 100$.

Doppler examinations were performed according to protocols established for dogs and cats (Gaber, 1991). Heart rate was calculated directly from the pulsed Doppler tracings. Variables recorded for each rabbit included maximal pulmonary artery and aortic outflow velocity, namely the maximal pulmonary outflow velocity (PA_{max}) and maximal aortic outflow velocity (Ao_{max}), maximal E- and A-wave velocities, E:A ratio, isovolumetric relaxation time (IVRT), isovolumetric contraction time (IVCT), left ventricle ejection time (LVET) and Tei index.

The velocities were recorded as the maximal value on the outer edge of the peak velocity spectrum. Pulmonary artery flow velocity was determined by use of pulsed Doppler from the right parasternal short-axis view. The velocities of aortic flow and mitral flow- peak early diastolic wave (E) and peak atrial contraction wave (A) were recorded via pulsed Doppler from the left parasternal apical 5- and 4-chamber views. Mitral inflow velocity pattern was recorded with the sample volume between the tips of the leaflets. In the great vessels, the sample volume was positioned in the center of the vessel, just beyond the valve leaflets, and colour Doppler was used to help align the cursor parallel to blood flow. Alignment was maximised in the 2-dimensional view and no angle of correction was used.

IVRT was measured as the time interval between end of aortic outflow and onset of the mitral inflow by pulsed Doppler. IVCT was measured as the time interval between the end of mitral inflow and onset of aortic outflow by pulsed Doppler. The Tei index was calculated as described, Tei = (IVCT + IVRT)/LVET (Tei et al., 1995).

TDI was performed from the left parasternal apical 4-chamber view as previously described (Nagueh et al., 2001; Gan et al., 2004). In brief, the mitral annular motion was measured from the septal and lateral (free wall) side with pulsed TDI. Colour TDI was used to aid in sample volume placement, and the cursor was aligned as parallel as possible to the longitudinal axis of LV wall motion. Gain and filter settings were adjusted to eliminate background noise and to allow the recording of clear tissue signals. Measurements included peak early diastolic (E_m), late diastolic (A_m) and systolic (S_m) mitral annular velocities (Fig. 1), with calculation of E_m:A_m and E:E_m ratios.

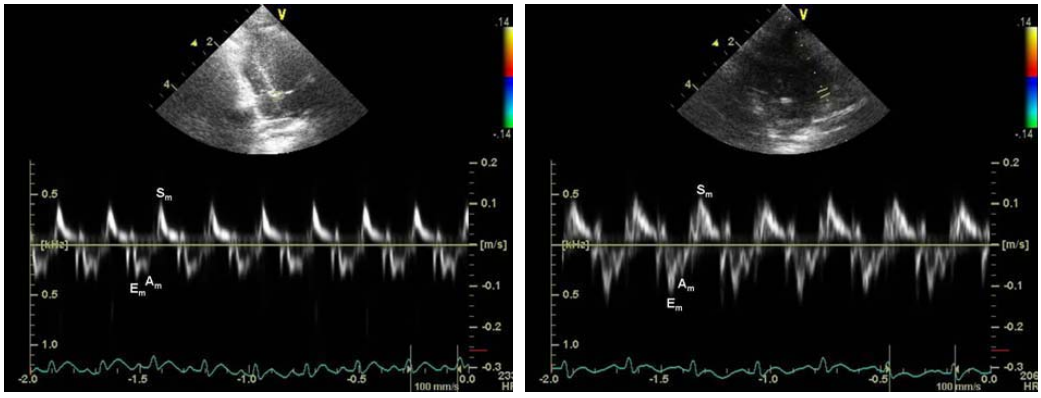

Fig. 1. The velocity profiles (E_m, A_m, S_m) obtained from pulsed tissue Doppler imaging of septal (left) and lateral (right) mitral annulus in rabbits. The values of E_m peak velocities are significantly different between septal (lower values) and lateral side (higher values) of the mitral annulus. E_m: the peak early diastolic velocity. A_m: the peak atrial diastolic velocity. S_m: the peak systolic velocity.

All data were collected by use of a trackball-driven cursor and the ultrasound system software. The measured beats were selected on the basis of quality of the recording and presence of a regular cardiac rhythm. For each parameter the mean of three representative cardiac cycles was recorded. From these means, the overall mean, standard deviation (SD), and range for all variables measured in all rabbits were calculated. All images were stored digitally on optical discs and analysed retrospectively. The measurements were performed offline using dedicate software (EchoPAC 7).

Statistical analysis

The statistical analysis was performed using the software SPSS for Windows, 15.0. Mean values, SD, maximum and minimum values (range) and percentiles for the echocardiographic parameters were computed. Pearson correlation coefficients (r) were used to study the association between rabbit body weights and heart rates and their respective mean M-mode and Doppler echocardiographic measurements. The P-level for statistical significance was set at 0.05.

Results

All 32 echocardiographic measurements (M-mode, 2D, Doppler echocardiography and TDI) were easily recorded in all rabbits in order to obtain reference values for the breed when sedated with ketamine-midazolam. No animal died during or after the examination. Mean bodyweight was 2.2 kg (SD=0.4, range 1.9-3.5 kg). Heart rate was 262±37 bpm (mean±SD) and was stable during the whole examination. Recording was typically completed approximately 20 min after administration of ketamine and midazolam. A wave was usually superimposed to E wave due to the elevated heart rate, but it was possible to distinguish between the peak velocity of E and A wave with normal or higher frame rates (Fig. 2).

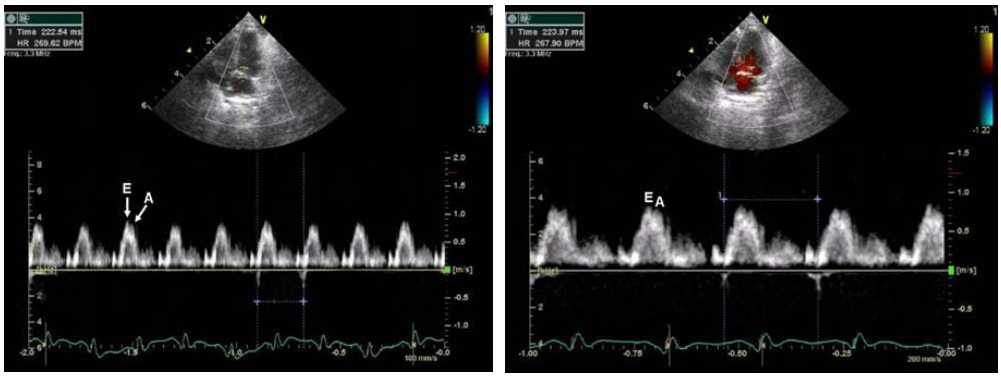

Fig. 2. Pulsed Doppler recordings of left ventricle inflow depicting the peak velocity of E and A waves, obtained at normal (left) and higher (right) sweep speeds from a rabbit with a heart rate above 250 bpm.

Tables 1 and 2 summarise the results of the 2-dimensional and M-mode measurements, Doppler echocardiography including conventional Doppler and TDI. Heart rate correlated with few echocardiographic parameters. A weak significant negative correlation was found between heart rate and LVIDs (r=0.50, P=0.01). Weak positive correlations were found between heart rate and E_m LW (r=0.42, P=0.03) and E_m:A_m LW (r=0.50, P=0.01). The bodyweight correlated weakly positive with Ao (r=0.52, P<0.01) and weakly negative with E_m:A_m septal (r=0.44, P= 0.03).

Table 1 - Values for 2-dimensional and M-mode echocardiographic variables in 26 male New Zealand white rabbits sedated with a combination of ketamine and midazolam.

Parameter	Mean ± SD	Per 5th	Per 95th
IVS_d (mm)	2.65 ± 0.31	2.23	3.20
IVS_s (mm)	3.63 ± 0.34	2.97	4.13
$LVID_d$ (mm)	13.51 ± 1.05	11.97	15.23
$LVID_s$ (mm)	8.64 ± 0.82	7.37	10.00
$LVFW_d$ (mm)	2.25 ± 0.29	1.90	2.77
$LVFW_s$ (mm)	3.15 ± 0.38	2.60	3.93
FS (%)	36.01 ± 4.31	31.18	42.83
EF (%)	69.58 ± 5.33	62.99	77.73
Ao (mm)	6.57 ± 0.46	5.87	7.43
LA (mm)	7.49 ± 1.14	5.90	9.50
LA:Ao	1.15 ± 0.19	0.82	1.43
EPSS (mm)	1.41 ± 0.25	1.13	1.83

SD, standard deviation; IVS_d and IVS_s, thickness of the interventricular septum in diastole and systole, respectively; $LVID_d$ and $LVID_s$, left ventricular internal diameter in diastole and systole, respectively; $LVFW_d$ and $LVFW_s$, thickness of the left ventricular free wall in diastole and systole; FS, fractional shortening; EF, ejection fraction; Ao, aorta diameter; LA, left atrial diameter; and EPSS, E-point to septal separation.

Table 2 – Doppler echocardiographic measurements including tissue Doppler imaging and calculated indices in 26 male New Zealand white rabbits sedated with a combination of ketamine and midazolam.

Parameter	Mean ± SD	Per 5th	Per 95th
Doppler HR (bpm)	262.77 ± 37.17	213.01	329.03
Ao_{max} (m/s)	0.86 ± 0.12	0.67	1.08
PA_{max} (m/s)	0.78 ± 0.12	0.61	0.98
Mitral E (m/s)	0.78 ± 0.15	0.60	1.05
Mitral A (m/s)	0.55 ± 0.11	0.42	0.76
Mitral E:A	1.44 ± 0.16	1.26	1.65
IVRT (ms)	31.42 ± 6.19	23.77	39.94
IVCT (ms)	25.00 ± 3.68	19.02	30.43
LVET (ms)	95.72 ± 10.21	79.89	112.22
Tei index	0.60 ± 0.10	0.48	0.76
S_m LW (m/s)	0.11 ± 0.02	0.08	0.14
E_m LW (m/s)	0.16 ± 0.05	0.09	0.25
A_m LW (m/s)	0.09 ± 0.03	0.06	0.13
E_m:A_m LW	1.83 ± 0.43	1.34	2.61
E:E_m LW	5.24 ± 1.55	3.43	8.03
S_m septal (m/s)	0.10 ± 0.02	0.07	0.12
E_m septal (m/s)	0.11 ± 0.04	0.07	0.19
A_m septal (m/s)	0.08 ± 0.02	0.04	0.12
E_m:A_m septal	1.55 ± 0.44	0.65	2.22
E:E_m septal	7.75 ± 2.69	4.17	12.24

SD, standard deviation; HR, heart rate; Ao_{max}, maximal aortic outflow velocity; PA_{max}, maximal pulmonary outflow velocity; Mitral E, maximal mitral E wave velocity; Mitral A, maximal mitral A wave velocity; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; LVET, left ventricle ejection time; S_m LW, peak systolic mitral annular velocity from left wall; E_m LW, peak early diastolic mitral annular velocity from left wall; A_m LW, late early diastolic mitral annular velocity from left wall; S_m septal, peak systolic velocity of septal mitral annulus; E_m septal, peak early diastolic velocity of septal mitral annulus; and A_m septal, late early diastolic velocity of septal mitral annulus.

Discussion

Rabbits are an important model for cardiovascular research, mainly as they are small and relatively inexpensive but large enough to allow physiological experiments (Muders and Elsner, 2000). There are also various similarities between human and rabbit myocardium including a predomination of the β-myosin heavy-chain isoform, a positive force-frequency relationship and excitation-contraction coupling processes (Kavinsky et al., 1984; Ezzaher et al., 1992; Hasenfuss, 1998).

A complete Doppler-echocardiographic examination including TDI evaluation was obtained in rabbits anaesthetised with ketamine and midazolam. This anaesthetic combination yielded good immobilization and allowed the ultrasonographer to obtain adequate 2D, M-mode, flow Doppler and TDI images for quantitative measurements. The combination is relatively common in research as well as in veterinary practice. In one study using this anaesthesia, some physiological and blood parameters were reduced (mean arterial pressure, CO_2 arterial pressure) or not affected (O_2 arterial pressure) relative to ketamine-midazolam-xylazine and tiletamine-zolazepam-xylazine anaesthesia (Dupras et al., 2001).

The mean±SD heart rate observed in the present study was slightly higher than the range of mean heart rates previously reported for conscious rabbits (180-250 bpm) (Marano et al., 1996; Gil et al., 2004), and considerably higher when compared with rabbits anaesthetised with ketamine-α_2 agonists (medetomidine or xylazine) (Fontes-Sousa et al., 2006; Stypmann et al., 2007). Our results are in accordance with a previous study using rabbits, which found that ketamine-midazolam promoted the highest heart rate when compared with other anaesthetic combinations (Dupras et al., 2001). In fact, although these agents cause minimal cardiorespiratory depression, it has also been reported in humans that they may increase heart rate (Marlow et al., 1991).

Some echocardiographic parameters are particularly sensitive to high heart rates, e.g. mitral E- and A-wave that fuse and may not be distinguishable. Nevertheless, the use of high-speed tracing (100-200 m/s) allowed the assessment of E- and A- wave peak flow velocities, even at elevated heart rates, but deceleration time of the E-wave could not be measured.

In recent years, TDI has emerged as a new modality that is less affected by loading conditions and so provides a strong complementary role in the assessment of diastolic function (Leite-Moreira, 2006). In the present study, mitral annulus velocity obtained from the septal and lateral (free wall) side with pulsed TDI was markedly higher than the values observed in awake rabbits or those anaesthetised with ketamine-xylazine (Stypmann et al., 2007). This higher mitral annulus velocity could be explained by sympathetic stimulation induced by ketamine (positive chronotropic and inotropic effects) and the minimal cardiovascular effects associated with midazolam anaesthesia (Dupras et al., 2001).

The ratio obtained between transmitral E velocity and annular E_m, $E:E_m$ ratio, has been reported to be an accurate index of the level of filling pressure of the assessed ventricular chamber. In previous studies carried out in humans this ratio had a strong correlation with pulmonary capillary wedge pressure (PCWP) and LV diastolic pressure (LVDP) (Nagueh et al., 1999; Ommen et al., 2000). In small animal medicine, it has been reported that an $E:E_m$ value >9.1 indicated a mean left atrial pressure >20 mmHg in dogs with experimentally induced acute mitral regurgitation (Oyama et al., 2004).

Some potential limitations of the current study deserve attention, since we only used healthy anaesthetised rabbits. First, the work partially allowed us to assess the influence of the specific sedation used on the various echocardiographic parameters, since the same rabbits had not been examined in the conscious state. Nevertheless, some of the systolic and diastolic parameters were similar to those reported previously in the conscious

state (fractional shortening, Tei index, and maximal mitral E- and A-wave velocities) (Stypmann et al., 2007).

Second, the work does not allow to asses efficacy of the newer echocardiographic parameters, such as Tei-index and TDI, or superiority above conventional parameters for detecting myocardial disease. This will have to be demonstrated in future studies in rabbits with induced or spontaneous cardiomyopathies.

Finally, we did not evaluate intra-operator variability. Poor repeatability has been reported in the acquisition of the velocities from the long-axis posterior wall and interventricular septum using pulsed TDI analysis (Simpson et al., 2007). Another study showed that the intra-examination variability was better under anaesthetised conditions (Chetboul et al., 2004), which was attributed to perfect immobility of the animal that improved repeatability of TDI measurements.

Conclusions

Echocardiographic reference values for New Zealand white rabbits anaesthetised with ketamine-midazolam are presented providing reference values for future studies. Emphasis was given to more recent indices that simultaneously reflect systolic and diastolic cardiac function, such as the Tei index derived from pulsed Doppler echocardiography and the pulsed TDI of the mitral annulus. Most of the results presented are comparable to those found in non-anaesthetised rabbits, and thus ketamine-midazolam anaesthesia may offer a good alternative when sedation is necessary.

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CAPÍTULO II

AVALIAÇÃO ECOCARDIOGRÁFICA NO COELHO

PARTE C: AVALIAÇÃO DO ÍNDICE DE TEI DO VENTRÍCULO ESQUERDO POR DIFERENTES TÉCNICAS ECOCARDIOGRÁFICAS

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Left ventricular Tei Index in rabbit: agreement between echocardiography techniques

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Abstract

Objective: To report the normal values and examine the agreement for the left ventricular Tei index (LVTI) measured by tissue Doppler imaging (TDI), pulsed wave Doppler (PWD) and M-mode echocardiography (MME), in healthy New Zealand white rabbits.

Animals: Were included 26 clinically healthy male rabbits.

Procedures: Echocardiographic examination including TDI, PWD and MME was performed. The animals were sedated with a subcutaneous combination of ketamine and midazolam. Intraclass correlation coefficients (ICC) were used to measure absolute agreement between the three echocardiography methods. ICC were computed for the parameters *a* and *b* and for LVTI. Two methods were considered to have good agreement if the ICC was higher than 0.75.

Results: For the *a* value the Pearson correlation coefficients between the techniques were all high (≥0.7) and statistically significant. However, only the septal TDI and the lateral TDI had a good agreement (ICC=0.86). For the *b* value the correlations were generally low with exception of the one between the septal and the lateral TDI. Similarly to the parameter *a*, the TDI techniques were the only ones having a good agreement (ICC=0.77). For the LVTI only the TDI techniques presented a significantly positive correlation. All the other correlations were close to zero with a paradoxal negative significant correlation between the LVTI-PWD and the LVTI-lateral TDI.

Conclusions and Clinical Relevance: For the LVTI the absolute agreement was poor for all the techniques.

Keywords

Tei index, echocardiography, tissue Doppler imaging, left ventricle, rabbit model.

Introduction

Traditional echocardiographic assessment of left ventricle (LV) diastolic function relied on Doppler patterns of mitral inflow. Transmitral velocities are directly related to left atrial pressure (preload) and independently and inversely related to ventricular relaxation. The use of mitral valve inflow patterns to assess diastolic function remains limited, since mitral inflow patterns are highly sensitive to preload and can change dramatically with progression of diastolic dysfunction [1].

Pulsed tissue Doppler imaging (TDI) derived from Doppler echocardiography can quantify the velocity of myocardial wall and/ or valve annulus motion. Parameters obtained by TDI have been shown to be more independent of pre and afterload than classic hemodynamic Doppler measurements [2-4]. In human, pulsed TDI of the myocardial wall immediately adjacent to mitral annulus has been demonstrated to reflect systolic and diastolic left ventricular function in normal subjects and in a wide number of cardiac diseases [5-8].

The Tei index (TI) has become a widely used echocardiographic parameter for the assessment of global systolic and diastolic function in human with congenital and acquired cardiac disease. The major advantage of this index is that it is not age or heart rate dependent and does not depend on any geometric assumption [9-11]. The TI is calculated according to the equation $(a\text{-}b)/b$.

In the literature there are, especially in human, several reports regarding left ventricular Tei index (LVTI) data using the more conventional echocardiography methods, expressed in mean and standard deviation [12-18]. Other statistical analysis that established more accurately the relations and agreement between echocardiography techniques both in healthy subjects and in several diseases are not so well documented.

The purpose of this study was to report the normal values and examine the agreement for the LVTI measured by TDI, pulsed wave Doppler (PWD) and M-mode echocardiography (MME), in healthy New Zealand white rabbits. To our knowledge such comparison of the various methods for assessing LVTI was not previously reported.

Materials and Methods

The study was performed according to the Portuguese Law for Animal Welfare. The anaesthetic and testing methods conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The examinations were performed in 26 clinically healthy male New Zealand white rabbits (*Oryctolagus cuniculus*), 16 to 18 weeks of age and weighing 1.7 to 3.5 kg. Rabbits were free of signs of cardiovascular or respiratory tract disease and were determined to be clinically normal on the basis of a physical examination that included careful thoracic auscultation. The rabbits were housed in adequate cages in a controlled environment, at temperatures of 20 to 25ºC with 12 hours of light and 12 hours of dark per day. A commercial pellet diet and water were supplied ad libitum. The weight of each rabbit was recorded prior to anaesthesia.

A subcutaneous combination of ketamine-hydrochloride[a] (20 mg/kg, SC) and midazolam[b] (2 mg/kg, SC) were administered to each rabbit to minimize defensive movements and facilitate complete echocardiography examination, under regular conditions. The anaesthetic combination allowed them to breathe spontaneously. Recording was typically completed approximately 30 minutes after administration of ketamine and midazolam.

Echocardiography Studies

Transthoracic standard two-dimensional, MME, PWD and color Doppler echocardiography examinations were performed. The apical 4 chamber TDI of the mitral annulus was also included in the echocardiography evaluation. PWD of the mitral inflow and LV outflow and MME of the mitral and aortic valves were also acquired in all animals. The exam was performed from right and left parasternal locations, using an ultrasound unit[c] equipped with a 7-MHZ phased-array transducer. All measurements were recorded with simultaneous electrocardiography at a sweep speed of 100 and 200 mm/sec for off-line analysis. Three representative cycles were measured and averaged for each rabbit. All images were stored in the system for off-line analysis. Care was taken to maintain adequate contact while avoiding excessive pressure on the chest of the rabbit. For the right and left parasternal views, rabbits were placed in right and left lateral recumbency over a gap in the tabletop through which the ultrasound probe was brought from below and placed on a shaved area on the anterior aspect of the lower portion of the right and left thoracic wall. Echocardiography measurements were obtained from standard views [19]. Callipers were used to measure structures to the nearest millimetre by means of a leading-edge–to–leading-edge technique according to accepted echocardiography standards for dogs [19-21].

The right parasternal long-axis view with two-dimensional guided MME was used for evaluate the mitral valve motion with its several points and the aortic and left atrial appendage diameters were evaluated at the level of the aortic valve. These measurements were made from the leading edge of the first endocardial surface to the leading edge of the second endocardial surface. Doppler examinations were performed according to protocols established for dogs and cats [22-24]. Heart rate was calculated directly from the PWD tracings. Aortic flow and mitral E- and A-wave velocities were recorded via PWD from

left parasternal apical 5-chamber view. The sample volume was positioned between the aortic and mitral valves to alow simultaneous acquisition of the PWD tracings of the LV inflow and outflow tracts. Alignment was maximized in the 2-dimensional view and no angle of correction was used. The velocities were recorded as the maximal value on the outer edge of the peak velocity spectrum [23].

Longitudinal velocities within the myocardium were recorded with TDI from the apical window with the pulsed mode. The sample volume (2mm) was placed within a myocardial segment and the spectral recording of velocities within the segment obtained. For optimal recording of tissue velocity, both gain and filter settings were set low. As recommended, the sample volume was placed at the junction of the left ventricle wall and the mitral annulus [25]. Recordings were made in the septum and in the left ventricle lateral wall.

Tei Index Evaluation

The LVTI was calculated as previously described [11,14,16-18,25-27]. For evaluation of the LVTI-TDI, LVTI-PWD and LVTI-MME we used a measurement technique previously described in human [15]. For the 3 methods the *a* component equals the sum of isovolumic contraction time (ICT) plus ejection (ET) time plus isovolumic relaxation time (IRT). The *b* component is equal to the left ventricular ejection time. The LVTI is calculated according to the equation (*a*-*b*)/*b*. LVTI-MME *a* component was measured from mitral valve closure to the subsequent mitral valve opening on the mitral valve MME tracing. The *b* component of LVTI-MME was measured from aortic valve opening to aortic valve closure on the aortic valve MME tracing (figure 1). MME recording was obtained from the standard paraesternal long axis view.

LVTI-PWD *a* component measurement was made from the trailing edge of the PWD late mitral A wave to the leading edge of the subsequent PWD early mitral E wave. The *b* component for the LVTI-PWD was measured from the leading edge to the trailing edge of the left ventricular outflow tract PWD tracing (figure 2).

The LVTI-TDI *a* component was measured from the trailing edge of the mitral annular A` wave to the leading edge of the subsequent TDI mitral annular early diastolic (E`) wave. The LVTI-TDI *b* component was measured from the leading edge to the trailing edge of the TDI mitral annular systolic (S) wave (figure 3). Both TDI and PWD recordings were made from the standard apical view, and PWD acquisitions were made in the same cardiac cycle (i.e., five chamber view).

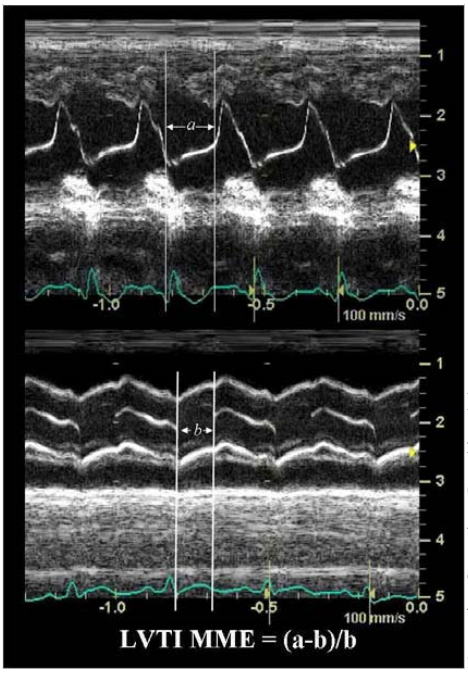

Figure 1: Left ventricular Tei index (LVTI) measurement using M-mode echocardiography (MME). The *a* value is measured from mitral valve closure to the following mitral valve opening. The *b* value corresponds to the interval between aortic valve opening and closure. LVTI is calculated as: (*a-b)/b*.

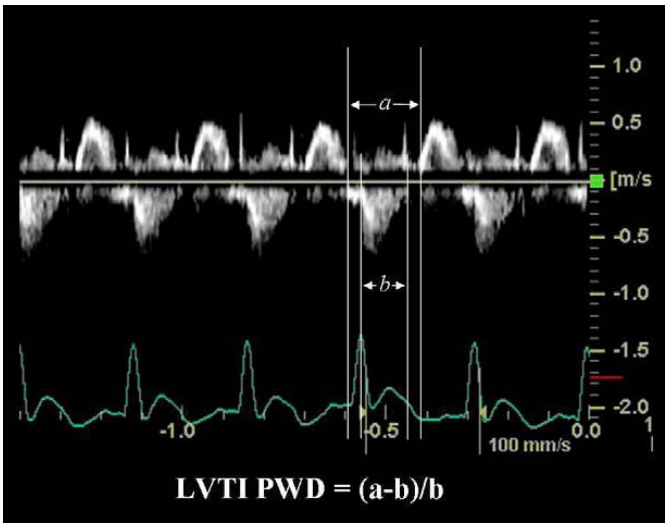

Figure 2: Left ventricular Tei index (LVTI) measurement using pulsed wave Doppler echocardiography (PWD). The *a* component is measured from the trailing edge of late diastolic transmitral PWD flow A wave to leading edge of subsequent early diastolic transmitral PWD flow E wave. The *b* component is obtained by measuring the time interval between the leading and trailing edges of LV outflow systolic PWD tracing. LVTI is calculated as: (*a-b)/b*.

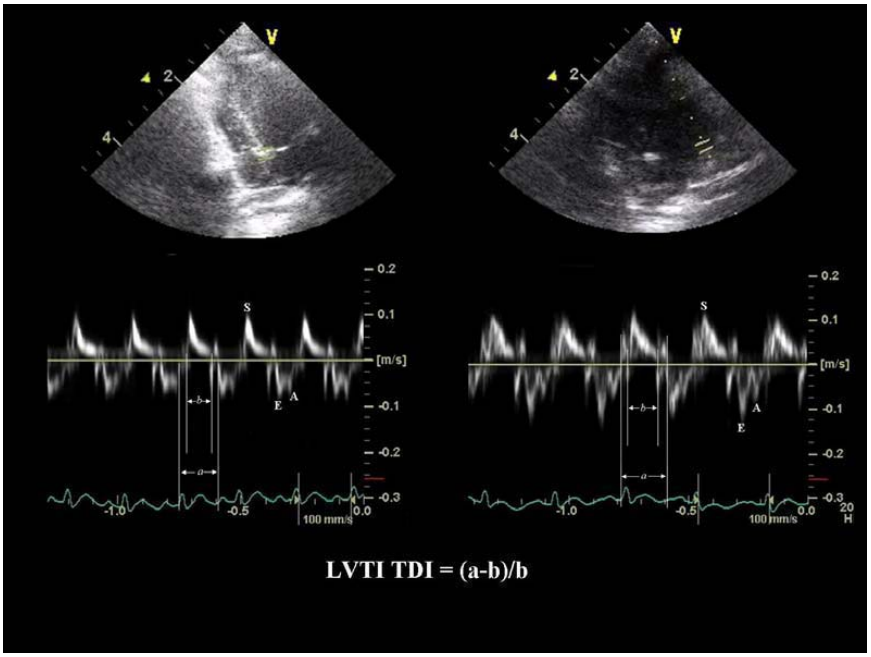

Figure 3: Left ventricular Tei index (LVTI) evaluation using tissue Doppler imaging (TDI), in the interventricular septum (left) and LV lateral wall (right). The *a* component is measured from the trailing of late diastolic TDI mitral annular A wave to leading edge of subsequent early diastolic TDI mitral annular E wave. The *b* component corresponds to the time interval between the leading and trailing edges of the systolic TDI mitral annular S wave. LVTI is calculated as: (*a-b)/b*.

Statistical Analysis

All data were collected by use of a trackball-driven cursor and the ultrasound system software. The measured beats were selected on the basis of quality of the echocardiography recording, quality of the electrocardiogram recording and presence of a regular cardiac rhythm. Data were expressed as mean ± SD and range for the body weight, heart rate and LVTI acquisitions using three echocardiography methods. Intraclass correlation coefficients (ICC) were used to measure absolute agreement between the three echocardiography methods. ICC were computed for the parameters *a* and *b* and for LVTI. Two methods were considered to have good agreement if the ICC was higher than 0.75.

Because some readers might be more familiar with the usual Pearson's correlation, this coefficient was also included in the results. However, one must realise that the Pearson correlation only measures linear association and not necessarily agreement. The analysis was performed using SPSS® ver. 15.0 and the ICC model chosen was the two-way mixed. Results were considered to be statistical significant for *p*-values less than 0.05.

Results

For the body weight the mean ± SD was 2.25 ± 0.41 Kg. The heart rate ranged between 191 and 330 bpm and the mean ± SD was 263 ± 37. LVTI normal values for MME, PWD and LV septal and lateral wall TDI echocardiography methods expressed as mean ± SD, were respectively 0.27 ± 0.15, 0.59 ± 0.10, 0.67 ± 0.23 and 0.64 ± 0.14.

All echocardiography acquisitions were made in sinnus rhythm. Figure 4 shows the comparison of the *a* value obtained by the three different echocardiography techniques: MME, PWD and TDI (LV septal and lateral acquisitions). The Pearson correlation coefficients between the techniques were all high (≥0.7) and statistically significant.

However, only the septal TDI and the lateral TDI had a good agreement (ICC=0.86). Graphically it is possible to see that although the measures of the *a* parameter are strongly associated, as indicated by the Pearson correlation, the measurements obtained by TDI (both septal and lateral) tended to be higher than the MME and PWD techniques.

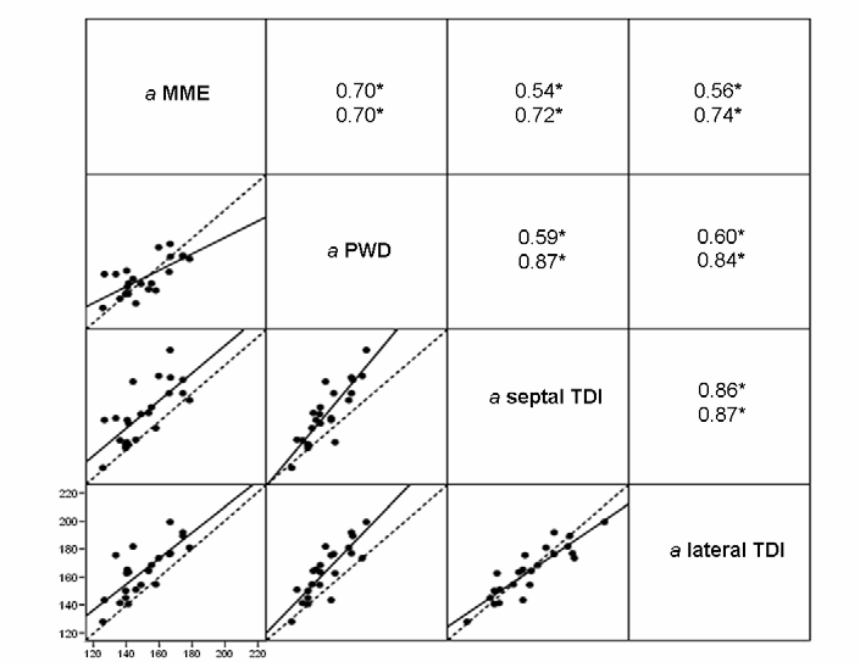

Figure 4: Comparison of the *a* value obtained by three different echocardiography methods: M-mode (MME), Pulsed wave Doppler (PWD) and tissue Doppler imaging (TDI) – left ventricle septal and lateral wall. Results include the 26 animals of the study. **Lower diagonal of the figure**: scatter plots representing the relation between the *a* component obtained with each technique. Each dot corresponds to a pair of *a* values for one animal. The dashed line represents the absolute agreement between the techniques. The solid line represents the fitted linear relationship (linear regression line) between each pair of techniques. The scale presented in the lower corner scatter plot is maintained throughout the other scatter plots. **Upper diagonal of the figure**: Intraclass correlation coefficient (top) and Pearson's correlation coefficients (below) for each pair of techniques. *$p<0.05$.

The comparison of the *b* value obtained by three echocardiography methods is presented in figure 5. The correlations were generally low with exception of the one between the septal and the lateral TDI. Similarly to the parameter *a*, the TDI techniques were the only ones having a good agreement between each other (ICC=0.77). The values for the *b* parameter measured by MME tended to be higher than the other techniques.

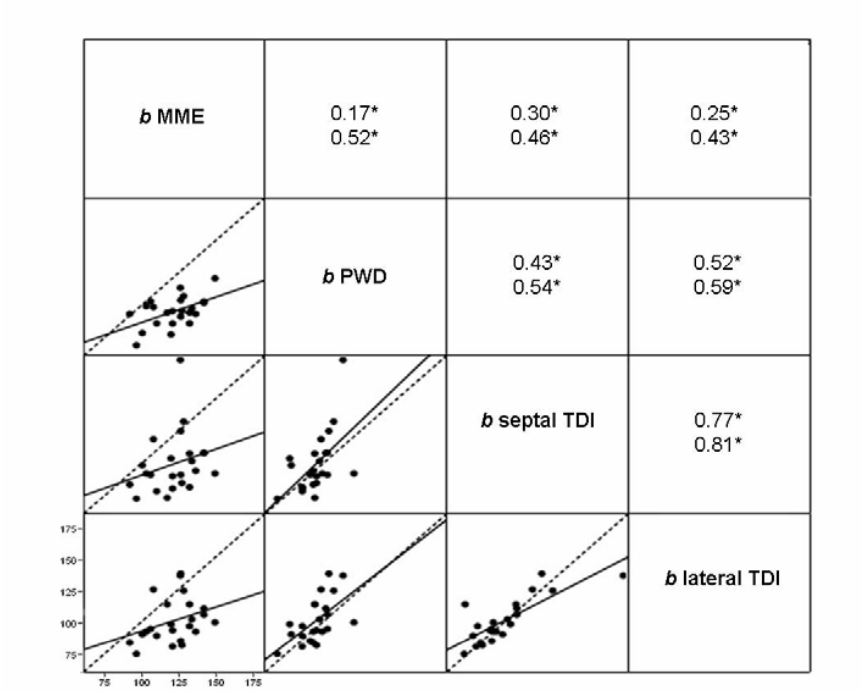

Figure 5: Comparison of the *b* value obtained by three different echocardiography methods: M-mode (MME), Pulsed wave Doppler (PWD) and tissue Doppler imaging (TDI) – left ventricle septal and lateral wall. Results of the 26 animals measured included in the study. **Lower diagonal of the figure**: scatter plots representing the relation between the *b* component obtained. Each dot corresponds to a pair of *b* value for one animal. The dashed line represents the absolute agreement between the techniques. The solid line represents the fitted linear relationship (linear regression line) between each pair of techniques. The scale presented in the lower corner scatter plot is maintained throughout the other scatter plots. **Upper diagonal of the figure**: Intraclass correlation coefficient (top) and Pearson's correlation coefficients (below) for each pair of techniques. *p<0.05.

The comparison of the LVTI data using MME, PWD and TDI methods is shown in figure 6. For the LVTI only the TDI techniques presented a significantly positive correlation. All the other correlations were close to zero with a paradoxal negative significant correlation between the LVTI-PWD and the LVTI-lateral TDI. For the LVTI the absolute agreement was poor for all the techniques. The higher ICC was obtained between the TDI techniques (ICC=0.60) but lower than 0.75.

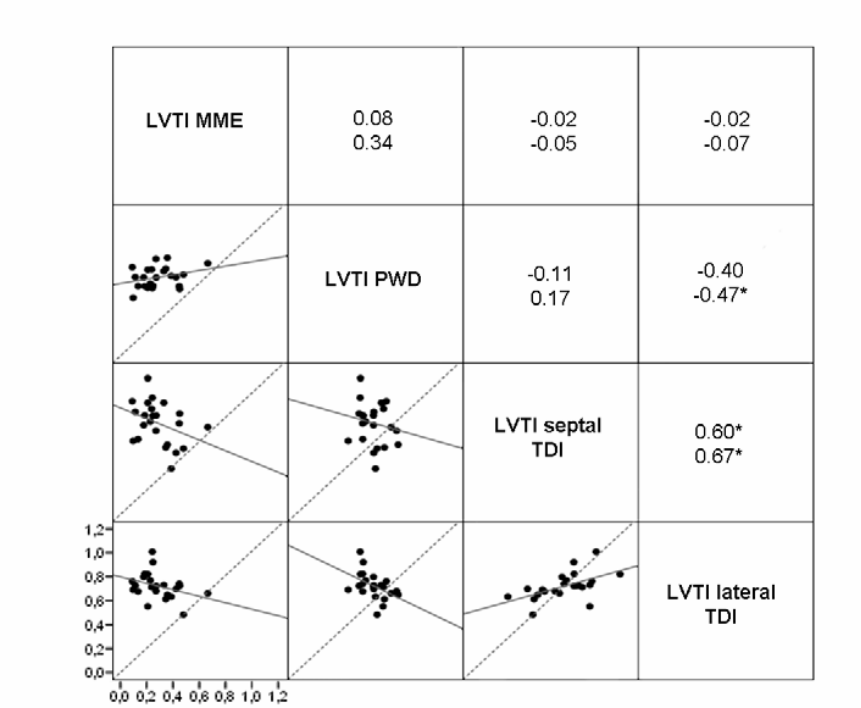

Figure 6: Comparison of the left ventricle Tei index (LVTI) value obtained by three different echocardiography methods: M-mode (MME), Pulsed wave Doppler (PWD) and tissue Doppler imaging (TDI) – left ventricle septal and lateral wall. Results of the 26 animals measured included in the study. **Lower diagonal of the figure**: scatter plots representing the relation between the TEI obtained. Each dot corresponds to a pair of TEI scores for one animal. The dashed line represents the absolute agreement between the techniques. The solid line represents the fitted linear relationship (linear regression line) between each pair of techniques. The scale presented in the lower corner scatter plot is maintained throughout the other scatter plots. **Upper diagonal of the figure**: Intraclass correlation coefficient (top) and Pearson's correlation coefficients (below) for each pair of techniques. *p<0.05.

Discussion

The present study reported normal values and examined the agreement for the LVTI measured by TDI, PWD and MME echocardiography, in healthy New Zealand white rabbits, having shown that for the LVTI the absolute agreement was poor for all the techniques.

The TI may be obtained by different echocardiographic techniques, according to the formula (*a*-*b*)/*b*. In all echocardiographic methods the *a* value equals the sum of isovolumic contraction time (ICT) plus ejection (ET) time plus isovolumic relaxation time (IRT). The *b* value is equal to the left ventricular ejection time.

The TI, as originally described by Tei, has two important limitations. One is that the time interval between the end and the onset of mitral inflow and ejection time is measured sequentially (i.e., not in the same cardiac cycle) [28]. The other limitation is that by using the formula (*a*-*b*)/*b* without measuring the individual isovolumetric intervals one cannot determine whether the altered global function in mainly due to systolic, diastolic or combined dysfunction [29]. There are critical discrepancies in LVTI values obtained using the three echocardiography methods because they measure different time intervals for the *a* and *b* components of the LVTI. Cui et al. [15] study demonstrated that the MME *a* value begins at the same time as the TDI *a* value but ends before both the TDI *a* or PWD *a* values ends. The TDI *a* component begins after the PWD *a* component begins and ends after the PWD *a* component ends. Therefore, the *a* measurements are similar for both TDI and PWD, but shorter for MME. The TDI *b* component begins slightly before the PWD *b* begins and the TDI *b* ends slightly before the PWD *b* ends. The MME *b* measurement begins at the same time as the TDI *b* but ends after both the TDI *b* and PWD *b*, resulting in a longer MME *b* versus both TDI *b* and PWD *b*. These acquisition differences, innate to

each echocardiography technique, cause the LVTI-MME to be lower than the LVTI-PWD or LVTI-TDI.

LVTI-PWD measurements may require the use of different cardiac cycles to measure more accurately the *a* and *b* components and define precisely the beginning and ending points. In these cases even slight changes in heart rate between the time of evaluation of *a* and *b* waves may be a source of error [15]. In order to overcome this problem, in our work, we performed the LVTI-PWD acquisitions in the same cardiac cycle, as previously reported [25,30]. In theory, measuring time intervals using myocardial velocities is not equivalent to the measurement of blood flow time intervals. Gaibazzi et al. [14] found mild agreement between the LVTI PWD and TDI techniques when used in a single healthy subject, what is not in total accordance with our results. When evaluated using only the mean and SD the results were very similar to ours. However, some methodological differences can, at least in part, explain these apparent discrepancies. In the present study the *a* and *b* values for the LVTI-PWD calculation were obtained in the same cardiac cycle (five chamber view), and the electrocardiogram monitoring was used systematically to overcome some difficulties in the acquisition of the correct intervals.

The use of the LVTI formula raises another problem because very small variations in the *a* and *b* components acquire much higher magnitude in the final value of LVTI.

Left ventricular dysfunction results in both prolongation of isovolumetric contraction time and isovolumetric relaxation time with ejection time shortening. As a result, the LVTI is increased in patients with LV dysfunction and is well documented in several diseases [31,32].

In human LVTI-MME mean ± SD was consistently and significantly less than LVTI-TDI and LVTI-PWD [15,17]. Spencer et al. [27] demonstrated some age-dependent changes of LVTI in a population of adult patients. In fact, previous studies demonstrated

that LV ejection time may change with age [33,34]. Other authors have already documented significant prolongation of IVRT with age in the normal heart [35-38]. Cui et al. [15] found no significant association between LVTI by any of the 3 methods and age or heart rate after controlling for the effect of body surface area.

Our results regarding the *a* and *b* value of the LVTI formula are similar to the previous studies reported in the literature. The mean and SD of the LVTI are also in agreement with the literature. The differences among the various techniques in the LVTI data observed in the present study are presumably due to the several potential error factors discussed above and can therefore explain the poor absolute agreement between techniques for LVTI. Comparison of LVTI values obtained by different echocardiographic techniques must therefore be interpreted with caution. In this setting LVTI application should probably be limited to the follow-up of a group of patients using the same echocardiographic technique in order to monitor the progression of cardiac (dys)function. Of the various echocardiography techniques, LVTI-TDI is probably the most precise one, as the *a* and *b* components can be measured in the same cardiac cycle.

In conclusion, despite the discrepancies between the LVTI measurements using different echocardiography techniques, this myocardial performance index remains a potentially useful tool for serial evaluation of systolic and diastolic global ventricular function, if its drawbacks and limitations are taken in account.

Footnotes:

a. Imalgene 1000, Merial Portuguesa, Rio de Mouro, Portugal
b. Midazolam APS, Farma – APS, Produtos Farmacêuticos, S.A., Lisboa, Portugal
c. GE Vivid 7 system – GE VingMed, GE, Portugal

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CAPÍTULO III

MEDIADORES NEURO-HUMORAIS CLÁSSICOS

PARTE A: EFEITOS MIOCÁRDICOS DA ESTIMULAÇÃO DOS RECEPTORES ET_B NA INSUFICIÊNCIA CARDÍACA

Impaired Response to ET_B Receptor Stimulation in Heart Failure: Functional Evidence of Endocardial Endothelial Dysfunction?

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Inotropic effects of selective ET_B receptor stimulation depend on the functional integrity of the endocardial endothelium (EE), which is negative when it is intact and positive when it is damaged. These results have been attributed to the existence of two subtypes of ET_B receptors in the heart: (i) ET_{B1}, located on the EE, decreases inotropy; (ii) ET_{B2}, located on myocardial cells, increases inotropy. In the present study we investigated the functional integrity of the EE in a heart failure (HF) model (doxorubicin-induced cardiomyopathy) by evaluating the contractile response to ET_{B1} receptor stimulation. New Zealand White rabbits were treated with doxorubicin (DOX-HF, 1 mg/kg, iv, twice weekly for 8 weeks) or with saline. Contractile effects of increasing doses of a selective agonist of endothelial ET_B receptors, IRL-1620 (10^{-9} to 10^{-6} *M*), were studied in papillary muscles (Krebs-Ringer: 1.8 m*M* $CaCl_2$, 35°C) from control ($n = 10$) and DOX-HF rabbits ($n = 7$). Isotonic and isometric twitches were recorded and analyzed. Reported parameters included active tension (AT) and maximum velocities of tension rise (dT/dt_{max}) and decline (dT/dt_{min}). On echocardiography, DOX-HF rabbits had increased left ventricular (LV) end-diastolic and end-systolic diameters and reduced ejection fraction (52% ± 2% vs. 61% ± 1%). Contrary to control papillary muscles, DOX-HF muscles showed a steady decrease in contractility between 1 and 4 Hz. In the control group, IRL-1620 induced dose-dependent negative inotropic and lusitropic effects that decreased at 10^{-6} *M*: 26% ± 3%, AT; 17% ± 3%, dT/dt_{max}; and 16% ± 5%, dT/dt_{min}. In the DOX-HF group, these effects were significantly reduced. At the same concentration, IRL-1620 decreased AT (8% ± 3%) and dT/dt_{max} (8% ± 3%), without significantly affecting dT/dt_{min}. This study showed an impaired response to endothelial ET_B receptor stimulation, providing for the first time strong evidence of the occurrence of EE dysfunction in the failing heart and further highlighting the potential use of ET_B receptor stimulation as a marker of EE function. Exp Biol Med 231:893–898, 2006

Key words: heart; endothelin; endothelial function; ET_B receptors; contractile function; heart failure

Introduction

The discovery in 1988 of endothelin-1 (ET-1), one of the most potent endogenous vasoconstrictor peptides, by Yanagisawa and colleagues (1) represented a landmark in the field of cardiovascular research. Since its discovery, a great deal of effort has been made toward gaining a better understanding of the key roles (developmental, physiological, and pathological) played by this peptide, particularly with regard to the cardiovascular system.

ET-1 exerts its actions mainly through two types of receptors, the so-called type A (ET_A) and type B (ET_B) receptors. Both are G protein–coupled transmembrane proteins, with different molecular and pharmacologic characteristics and functions based on their location (2–4).

ET_A receptor stimulation elicits vasoconstriction (5) and mitogenesis (6) and increases inotropism (7, 8) and myocardial distensibility in conditions of cardiac overload (9). ET_B receptor activation promotes vasodilatation mediated by nitric oxide and prostacyclin (10) release and has growth-inhibitory effects (11) associated with apoptosis (12). In addition, ET_B receptors play a determinant role in the clearance of circulating ET-1 (13).

There is increasing experimental and clinical evidence in support of an important role of ET-1 in the pathophysiology of heart failure (HF) (14). The endothelin system is activated in patients with chronic HF. Plasma big ET-1 and ET-1 concentrations have been correlated with clinical and hemodynamic measures of severity in patients with HF and inversely with prognosis (14, 15).

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Doxorubicin is a commonly used chemotherapeutic agent that is associated with the development of dose-dependent cardiomyopathy and irreversible and progressive HF characterized by bilateral enlargement, thinning of the ventricular wall, and reduction of the ejection fraction. Doxorubicin-induced HF (DOX-HF) has been used in different animal species to study the pathophysiologic mechanisms and to evaluate different treatment modalities for HF (16).

It was recently shown that the inotropic effect of selective ET_B receptor stimulation depends on the functional integrity of the endocardial endothelium (EE), which is negative when it is intact and positive when it is damaged. These results have been attributed to the existence of two subtypes of ET_B receptors in the heart: ET_{B1}, which is located on the EE and decreases inotropy, and ET_{B2}, which is located on myocardial cells and increases inotropy (17). The differential effects of ET_B stimulation in the presence and absence of an intact EE indicate that the analysis of such effects might be used as an experimental tool to test the functional integrity of the EE (17). In this context, the main goal of the present study was to investigate the functional integrity of the EE in an HF model (DOX-HF) by evaluating the contractile response to ET_{B1} stimulation.

Materials and Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, Revised 1996).

HF Model. A well-documented regimen was used for the induction of HF due to doxorubicin toxicity (DOX-HF) (18). Adult male New Zealand White rabbits (*Oryctolagus cuniculus*; 2.0–3.0 kg) received doxorubicin *via* a marginal ear vein by bolus injection (1 mg/kg) twice weekly for 8 weeks. Control rabbits received the vehicle (0.9% saline) in equivolumetric doses over the same period. The progression of cardiac dysfunction was monitored echocardiographically to estimate morphological and functional alterations during the development of HF.

Echocardiographic Evaluation. All animals were evaluated by echocardiography at the beginning of the study and then every 2 weeks during the study. Echocardiographic examination was performed with the rabbits lightly anesthetized with an intramuscular combination of ketamine (15 mg/kg) and medetomidine (0.15 mg/kg), and rabbits were allowed to breathe spontaneously. The animal was placed prone on a table with an area removed so that the ultrasound probe could be brought from below and placed on a shaved area of the anterior chest wall. The echocardiograms were obtained using a 5-MHz transducer (Aloka Color Doppler SSD-2200 echocardiograph; Aloka S.A., Tokyo, Japan), and the exam was performed from the right paraesternal position. Three representative cycles were measured and averaged for each rabbit. Parameters analyzed were heart rate, anterior and posterior end-diastolic and end-systolic wall thickness, left ventricular end-systolic and end-diastolic diameters (ESD and EDD, respectively), fractional shortening (FS; FS= [EDD − ESD]/EDD), and ejection fraction.

Papillary Muscle Studies. *Experimental Preparation.* The study was performed in isolated right papillary muscles ($n = 31$) from the control and DOX-HF groups 1 week after the last drug or saline administration. Rabbits were anesthetized with intravenous pentobarbital sodium salt (25 mg/kg). A left thoracotomy was performed, and beating hearts were quickly excised and immersed in modified Krebs-Ringer (KR) solution (composition in m*M*: NaCl, 98; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 2.4; KH_2PO_4, 1.2; glucose, 4.5; $CaCl_2 \cdot 2H_2O$, 1.8; $NaHCO_3$, 17; $C_3H_3NaO_3$, 15; CH_3COONa, 5; atenolol, 0.02) at 35°C with cardioplegic 2,3-butanedione monoxime (BDM; 3%) and 5% of newborn calf serum and gassed with 95% O_2/5% CO_2, to obtain a pH between 7.38 and 7.42.

After dissection, papillary muscles (length: 4.2 ± 0.3 mm; weight: 2.9 ± 0.3 mg; preload: 4.3 ± 0.3 mN) were mounted vertically in a 10-ml Plexiglas organ bath containing the above-described KR solution and were attached to an electromagnetic length-tension transducer (University of Antwerp, Belgium). Preload was estimated according to muscle dimensions, and the electrical stimulus (0.6 Hz) was set at 10% above threshold. Twenty minutes later, bathing solutions were replaced by corresponding KR solutions without BDM. During the next 2 hrs, muscles were stabilized. Bathing solutions were then replaced by corresponding KR solutions without calf serum, and L_{max} was calculated. Protocols were initiated after obtaining two similar isotonic and isometric control twitches separated by a 10-min interval.

Experimental Protocols. Effects of increasing doses of a selective agonist of endothelial ET_B receptors, IRL-1620 (10^{-9} to 10^{-6} *M*), were studied in papillary muscles from the control ($n = 10$) and DOX-HF ($n = 7$) groups.

In another set of papillary muscles from control ($n = 7$) and DOX-HF ($n = 7$) groups, isometric contractility-frequency relationships were obtained by plotting the maximum velocity of tension rise against the frequency of contraction. In summary, after an initial period of contraction at 0.6 Hz, the frequency of stimulation was stepped up at 3-min intervals to 1 Hz, 2 Hz, 3 Hz, and 4 Hz. Drugs were obtained from Sigma Chemical Company (St. Louis, MO).

Data Analysis. Isotonic and isometric twitches were recorded and analyzed. Selected parameters included active tension (AT, mN/mm^2); maximum velocity of tension rise (dT/dt_{max}, $mN/mm^2/sec$); maximum velocity of tension decline (dT/dt_{min}, $mN/mm^2/sec$); peak isotonic shortening (PS, $\%L_{max}$); maximum velocity of shortening (dL/dt_{max}, L_{max}/sec), maximum velocity of lengthening (dL/dt_{min}, L_{max}/sec); and time to half relaxation (tHR, msec).

Only data obtained from isometric twitches will be

Table 1. Mean Values of the Contractile Parameters in Papillary Muscles from the Control and Doxorubicin-Induced Heart Failure (DOX-HF) Groups[a]

Contractile parameter	Control group ($n = 17$)	DOX-HF group ($n = 14$)
AT (mN/mm^2)	25.3 ± 3.0	25.8 ± 2.4
dT/dt_{max} ($mN/mm^2/sec$)	175.4 ± 18.5	173.2 ± 15.7
dT/dt_{min} ($mN/mm^2/sec$)	−137.0 ± 15.9	−132.6 ± 13.2
PS ($\%L_{max}$)	12.0 ± 0.1	12.0 ± 0.1
dL/dt_{max} (L_{max}/sec)	0.9 ± 0.01	0.8 ± 0.07
dL/dt_{min} (L_{max}/sec)	−3.0 ± 0.4	−2.7 ± 0.4
tHR (msec)	377.0 ± 14.6	407.1 ± 21.1

[a] Values are means ± SEM. EE, endocardial endothelium; AT, active tension; dT/dt_{max}, maximum velocity of tension rise; dT/dt_{min}, maximum velocity of tension decline; PS, peak isotonic shortening; dL/dt_{max}, maximum velocity of shortening; dL/dt_{min}, maximum velocity of lengthening; tHR, time to half relaxation.

described, as the analysis of isotonic twitches yielded globally similar results. In the various protocols, results are given as percent changes from baseline. For the parameters that are expressed as negative values (e.g., dT/dt_{min}), such percent change refers to the absolute values.

Statistical Methods. Values are means ± SEM. Echocardiographic data of doxorubicin-treated animals at the beginning and at the end of the study were compared with a paired *t* test. Baseline performance of papillary muscles from control and doxorubicin-treated rabbits was compared with an unpaired *t* test. Effects of increasing concentrations of IRL-1620 and of increasing stimulation frequencies of papillary muscles from control and doxorubicin-treated rabbits were analyzed with a repeated-measures two-way analysis of variance. When significant differences were detected, the Tukey's *post hoc* test was selected to perform multiple comparisons; $P < 0.05$ was accepted as significant.

Results

Mean values of the contractile parameters in papillary muscles from the control group ($n = 17$) and from the DOX-HF group ($n = 14$) are shown in Table 1. Although baseline performance of rabbit papillary muscles was similar in all experimental protocols, contractility of papillary muscles from the control group did not significantly decline with increasing frequency (between 1 Hz and 4 Hz), whereas the papillary muscles from the DOX-HF rabbits showed a decrease in contractility with increasing frequency, indicative of contractile dysfunction and a reduced contractile reserve (Fig. 1). Additionally, in the DOX-HF group, the echocardiographic evaluation demonstrated a progressive increase of end-diastolic (from 14.3 ± 0.8 mm to 15.6 ± 0.4 mm) and end-systolic (from 10.4 ± 0.3 mm to 11.7 ± 0.4 mm) short-axis diameters and a reduction in fractional shortening (from 30% ± 1% to 24% ± 1%) and ejection fraction (from 61% ± 1% to 52% ± 2%) of the left ventricle, consistent with the presence of dilated cardiomyopathy and HF.

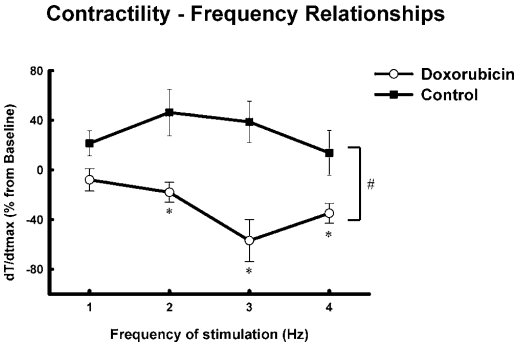

Figure 1. Contractile response of rabbit papillary muscles from the control group ($n = 7$) and from the doxorubicin-induced heart failure (DOX-HF) group ($n = 7$) to steady increases in stimulation frequency. Contractility-frequency relationships were then obtained by plotting maximum velocity of tension rise against frequency of contraction. Control muscles showed a steady increase in contractility between 1 Hz and 4 Hz, whereas in the DOX-HF group, muscles responded in the opposite way. $P < 0.05$: *, versus baseline; #, versus control.

Myocardial Effects of Selective ET_B Receptor Stimulation by IRL-1620. Figures 2 and 3 illustrate the effects of selective stimulation of the endothelial ET_B receptor with the agonist IRL-1620 in the various experimental conditions. In the control group, IRL-1620 induced dose-dependent negative inotropic and lusitropic effects. At 10^{-6} *M*, it significantly decreased AT (26% ± 3%), dT/dt_{max} (17% ± 3%), dT/dt_{min} (16% ± 5%), and tHR (11% ± 2%). In the DOX-HF group, these effects were significantly reduced. At the same concentration IRL-1620 decreased AT (8% ± 3%) and dT/dt_{max} (8% ± 4%), without significantly affecting dT/dt_{min} or tHR (Figs. 2 and 3).

Discussion

The present study showed that in the presence of HF induced by doxorubicin (DOX-HF), the myocardial response to selective endothelial ET_B receptor stimulation is impaired. Thus, in healthy animals (control group), IRL-1620 induced significant negative inotropic and lusitropic effects that were clearly reduced in papillary muscles from the failing hearts.

The progression of cardiac dysfunction was monitored echocardiographically to estimate morphologic and functional alterations during the development of HF. In addition, as contractile dysfunction in papillary muscles is most often not evident from changes in baseline performance of muscles that are contracting at low stimulating frequencies, but rather is evident based on an impaired response to increased frequencies (19), contractility-frequency relationships were performed. We found that although baseline

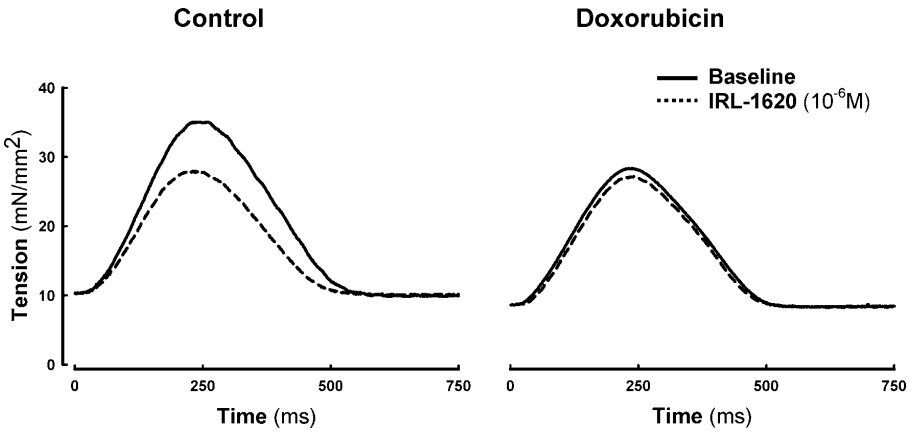

Figure 2. Representative isometric twitches performed in rabbit papillary muscles from the control group and from the doxorubicin-induced heart failure (DOX-HF) group, showing the effects of selective endothelial ET_B receptor stimulation by IRL-1620 (10^{-6} *M*). IRL-1620 induced negative inotropic and lusitropic effects in the representative twitch from the control group, effects that were clearly reduced in the example from the DOX-HF group.

performance of normal and DOX-HF muscles was similar, contrary to the former, the latter showed decreased contractility to increased frequencies, indicating contractile dysfunction and reduced contractile reserve.

The role of cardiac endothelium (EE and myocardial capillary endothelium) in HF has only recently been addressed. Typical morphologic EE cellular lesions have now been described in conditions of ventricular volume (20) or pressure (21, 22) overload. Experimental *in vitro* studies have demonstrated selective damage of the EE after exposure to high concentrations of a number of neurohormones and stressors known to be pathogenic risk factors *in vivo*, such as high plasma levels of catecholamines, angiotensin, atrial natriuretic peptide, serotonin, vasopressin, ox–low-density lipoproteins, homocysteine, cholic acid, and eosinophils. These lesions were accompanied by profound changes in the mechanical performance of the subjacent myocardium. Most cardiovascular risk factors known to be pathogenic for other vascular endothelial cells appear to also affect EE as an early target, contributing to the etiology and progression of cardiac failure (23). The association of such EE lesions with these conditions indicates that they might contribute causally to cardiac failure, but experimental evidence that they do so has been missing.

Until recently, a major limitation for the evaluation of EE dysfunction was the nonexistence of a functional marker, like acetylcholine for the vascular endothelium. We have recently gathered evidence that the response to selective ET_B receptor stimulation might be used as such a marker. In fact, similar to acetylcholine in the vasculature, myocardial effects of ET_B receptor stimulation depend on the presence or absence of a functional EE. When the EE is intact, endothelial ET_B receptor stimulation promotes negative inotropic and lusitropic effects that are mediated by nitric oxide and prostaglandins. On the contrary, when the EE is damaged, myocardial ET_B receptor stimulation induces positive inotropic and lusitropic effects (17). Therefore, if we use a selective endothelial ET_B receptor stimulator, we shall obtain negative inotropic and lusitropic effects when the EE is intact and no significant effects when the EE is damaged, as was previously shown (17). In this setting, the present study, having shown that papillary muscles from failing hearts had a blunted response to selective endothelial ET_B receptor stimulation, provides strong evidence in favor of the presence of EE dysfunction in the HF model used. Thus, as is the case with vascular endothelial dysfunction, it seems that cardiac endothelial dysfunction is present and/or may contribute to HF progression.

Although some concern can be raised with regard to the selectivity of IRL-1620 at higher concentrations, especially at 10^{-6} *M*, the results of this and other studies (17, 24) are not in favor of such a possibility. In fact, if this was the case, IRL-1620 (10^{-6} *M*) should increase contractility of papillary muscles devoid of an intact EE. We showed, however, that in these circumstances, IRL-1620 does not have any significant effects on muscular performance (17, 24).

Doxorubicin is an antineoplastic antibiotic widely used in the treatment of a variety of cancers, and its clinical use is limited as a result of a severe, dose-dependent cardiotoxicity (16, 18). In this context, our findings might also be relevant to better understand the pathophysiology of DOX-induced cardiomyopathy so that we can develop efficient protective and/or therapeutic strategies in patients treated with this chemotherapeutic agent.

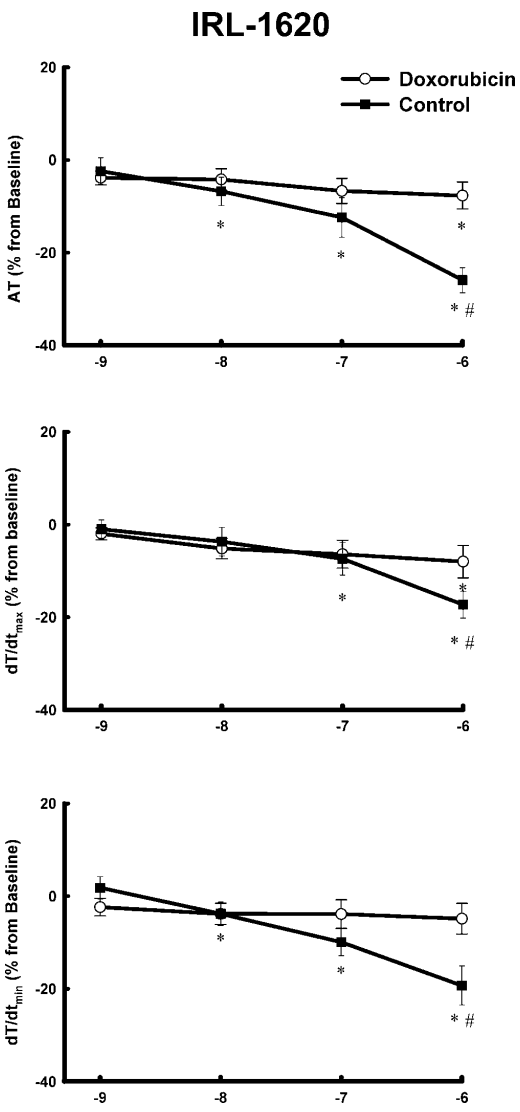

Figure 3. Concentration-response curves for the effect of selective endothelial ET_B receptor stimulation by IRL-1620 on contractile parameters in the various experimental conditions: control group (full squares, $n = 10$) or doxorubicin-induced heart failure (DOX-HF) group (open circles, $n = 7$). AT, active tension, top panel; dT/dt_{max}, maximum velocity of tension rise, middle panel; and dT/dt_{min}, maximum velocity of tension decline, bottom panel. Mean ± SEM; % baseline. $P < 0.05$: *, versus baseline; #, versus control.

This study showed an impaired response to endothelial ET_B receptor stimulation, indicating the presence of EE dysfunction in the experimental model of HF induced by doxorubicin and reinforcing the importance of ET_{B1} receptors as functional markers of endothelial integrity. Additionally, these results might be relevant for a better understanding of the role of EE in the pathophysiology of HF.

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CAPÍTULO III

MEDIADORES NEURO-HUMORAIS CLÁSSICOS

PARTE B: PAPEL DO ÓXIDO NÍTRICO E DAS PROSTAGLANDINAS NA MODULAÇÃO DOS EFEITOS DIASTÓLICOS DA ENDOTELINA-1

Physiol. Res. 57: 000-000, 2008

Nitric Oxide and Prostaglandins – Important Players in Endothelin-1 Induced Myocardial Distensibility

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Summary

This study investigated whether endothelin (ET)-1-induced increase in myocardial distensibility is preserved in heart failure (HF) and whether it is modulated by nitric oxide (NO) and prostaglandins. New Zealand white rabbits were treated with doxorubicin (1 mg/kg, intravenously twice a week for 8 weeks, DOX-HF group) or saline (control group). Effects of ET-1 (0.1, 1, 10 nM) were tested in papillary muscles from the DOX-HF group and a control group in the presence of: i) intact endocardial endothelium (EE); ii) damaged EE; iii) N^G-nitro-L-arginine (L-NNA; NO synthase inhibitor), and iv) indomethacin (INDO; cyclooxygenase inhibitor). In the presence of an intact EE, ET-1 promoted concentration-dependent positive inotropic and lusitropic effects that were maintained after damaging the EE, in the presence of L-NNA or INDO and in the DOX-HF Group. ET-1 reduced resting tension at the end of the isometric twitch (increased diastolic distensibility) by 3.2±1.3 %, 6.0±1.6 % and 8.8±2.7 % (at 0.1, 1 and 10 nM, respectively), in muscles with intact EE, effect that was completely abolished after damaging EE, in the presence of L-NNA or INDO or in the DOX-HF Group. This study demonstrated that the increase in myocardial distensibility induced by ET-1 is absent in HF and is dependent of NO and prostaglandin release.

Key words

Endothelin • Endothelium • Heart failure • Diastolic properties • Myocardial distensibility

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Introduction

The discovery of endothelin (ET)-1, one of the most potent endogenous vasoconstrictor peptides, by Yanagisawa *et al.* (1988) represented a landmark in the field of cardiovascular research. Since its discovery, a great deal of effort has been exerted in gaining a better understanding of the key roles (developmental, physiological, and pathological) played by this peptide, particularly with regard to the cardiovascular system, where the components of the endothelin system are widely expressed, namely in vascular and endocardial endothelium, smooth muscle cells and cardiomyocytes (Brunner *et al*. 2006). ET-1 acts in two main subtypes of G-protein coupled receptors (ET_A and ET_B) and has mainly local autocrine and paracrine actions, since it is released abluminally and has a short half-life. In heart failure (HF), the plasma, salivary and tissue levels of ET-1 are increased and are positively related to the stage of the disease and negatively to its prognosis (Attina *et al*. 2005). ET_A receptors mediate vasoconstriction, mitogenesis and positive inotropism. ET_B receptor activation promotes mainly vasodilatation and has growth inhibitory effects associated with apoptosis. These receptors also mediate the pulmonary clearance of circulating ET-1 and the reuptake of ET-1 by endothelial cells. In the heart (Leite-Moreira and Bras-Silva 2004) and in the vasculature (Endoh *et al*. 1998), it is possible to further subclassify the ET_B receptors into ET_{B1} receptors, located on the vascular and endocardial endothelium and responsible for vasodilatation and negative inotropism, and ET_{B2} receptors, located on

vascular muscular and myocardial cells and responsible for vasoconstriction and positive inotropism, respectively.

Unlike the well-known role of chronically elevated ET-1 levels in progression of cardiac fibrosis and ventricular remodeling, the acute diastolic effects of ET-1 in the failing myocardium remain less explored. We have previously reported, in healthy animals that ET-1 acutely decreases myocardial stiffness under the conditions of cardiac overload (Leite-Moreira *et al*. 2003). Although mediated by ET_A receptor stimulation (Leite-Moreira *et al*. 2003), this effect requires an intact endocardial endothelium (EE) and active endothelial ET_{B1} receptors (Bras-Silva and Leite-Moreira 2006). This is in agreement with the growing experimental evidence for a paracrine regulation of cardiac systolic and diastolic performance by endocardial endothelial cells that is analogous to vascular endothelial control of vascular tone (Brutsaert 2003). Until recently, a major limitation for the evaluation of EE dysfunction was the non-existence of a functional marker, such as acetylcholine for the vascular endothelium. We have recently gathered evidence that the response to selective ET_B receptor stimulation might be used as such a marker. Using this approach we documented endocardial endothelial dysfunction in an experimental model of HF, the so-called doxorubicin-induced HF (Bras-Silva *et al.* 2006).

In this context, the present study was conducted in order to investigate whether the diastolic effects of ET-1 were preserved in HF, and whether they are dependent on two of the most important endothelial mediators, nitric oxide and prostaglandins.

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication Nº 85-23, Revised 1996). The study was performed on New-Zealand White rabbits (*Oryctolagus cuniculus*; n=37; 1.8-3.0 kg).

Heart failure model

A well documented regimen was used for the induction of HF due to doxorubicin toxicity (DOX-HF) (Arnolda *et al.* 1985). Adult male New Zealand White rabbits received doxorubicin (DOX) *via* a marginal ear vein by bolus injection (1 mg/kg) twice weekly for 8 weeks (n=16) followed by a washout period of 1 week. Control rabbits (n=21) received the vehicle (0.9 % saline) in equivolumetric doses over the same period. Echocardiographic evaluation of all the animals was used to monitor left ventricular dilatation and dysfunction during the development of HF. In two subgroups of control and DOX-HF hemodynamic evaluation was also performed. The experimental protocols were carried out in an isolated papillary muscle model.

Echocardiographic evaluation

All animals were evaluated by echocardiography at the beginning and every two weeks during the administration of DOX or vehicle. Echocardiographic examination was performed as previously described (Fontes-Sousa *et al.* 2006). Briefly, the rabbits were lightly anesthetized with an intramuscular combination of ketamine hydrochloride (2 mg/kg) and medetomidine hydrochloride (0.15 mg/kg), being allowed to breath spontaneously. The animals were placed prone on a table with an area removed so that the ultrasound probe could be brought from below and placed on a shaved area of the anterior chest wall. The echocardiograms were obtained using a 7.5 MHz transducer (Vivid 3 General Electrics echocardiograph, Portugal) and the examination was performed from the right parasternal short-axis view. Two-dimensional guided M-mode tracings were made just below the mitral valve at the level of the papillary muscles for measurements of the left ventricular internal diameter and the left ventricular wall was free in diastole and systole. Three representative cycles were measured and averaged for each rabbit at each time point. Analyzed parameters were: heart rate, anterior and posterior end-diastolic and end-systolic wall thickness, left ventricular end-systolic and end-diastolic diameters (ESD and EDD, respectively), fractional shortening (FS) [FS=(EDD-ESD)/EDD].

Hemodynamic assessment

The instrumentation of the animals for hemodynamic studies was performed one week after the last administration of the vehicle (n=6) or DOX (n=9), respectively, as previously described (Leite-Moreira *et al*. 1999, Leite-Moreira and Correia-Pinto 2001). In summary, animals were premedicated with ketamine hydrochloride (50 mg/kg *i.m.*) and xylazine hydrochloride (5 mg/kg *i.m.*). An auricular vein was cannulated, and a prewarmed solution containing 20 mM KCl and 40 mM $NaHCO_3$ in 500 ml of 0.9 % NaCl was administered to compensate for perioperative fluid losses.

A tracheostomy was performed, and mechanical ventilation was initiated (Harvard Small Animal Ventilator, model 683), delivering oxygen-enriched air. Respiratory rate and tidal volume were adjusted to keep arterial blood gases and pH within physiological limits. Anesthesia was maintained with ketamine hydrochloride (33 ml/kg/h *i.m.*) and pentobarbital sodium (12.5 mg/kg *i.v.* before opening the chest, and then 2.5 mg/kg *i.v.* as needed). A 20-gauge catheter was inserted into the right femoral artery and connected to a pressure transducer to monitor heart rate and arterial pressure, and to obtain samples for blood gas analysis. The heart was exposed by a median sternotomy, and the pericardium was widely opened. Transient aortic constrictions were performed by abruptly occluding the aorta with a silk suture placed around the ascending aorta during the diastole separating two heartbeats. This was achieved by pushing a plastic tube against the aorta with one hand while pulling the silk suture with the other hand. Aortic constriction was quickly released to avoid neurohumoral reflex changes in cardiac function (3, 4). **Please, indicate the respective references by first author names** Peak systolic pressure of isovolumetric heartbeats, which can be obtained with aortic occlusions, is a sensitive index of left ventricular contractility. A 3-F high-fidelity micro-manometer (SPR-524, Millar Instruments, Houston, TX, U.S.A.) was inserted through an apical puncture wound into the left ventricular (LV) cavity, positioned at the midventricular level, and secured in place with a purse-string suture to measure LV pressure. The manometer was calibrated against a mercury column and zeroed after stabilization for 30 min in a water bath at body temperature. A limb electrocardiogram (DII) was recorded throughout.

After complete instrumentation, we allowed the animal preparation to stabilize for 30 min before the beginning of the experimental protocol. Recordings were made with respiration suspended at the end of expiration.

Parameters were converted on-line to digital data with a sampling frequency of 500 Hz. LV pressures were measured at end diastole and peak systole. Peak rates of LV pressure rise (dP/dt_{max}) and pressure fall (dP/dt_{min}) were also measured. The relaxation rate was estimated with the time constant τ by fitting the isovolumetric pressure fall to a monoexponential function.

Anesthetics ketamine hydrochloride (Imalgene 1000®), medetomidine hydrochloride (Domitor®) and xylazine hydrochloride (Rompum®) were obtained from Merial Portuguesa – Saúde Animal, Pfizer Saúde Animal, and Bayer, Portugal, respectively.

Papillary muscle studies

The study was performed in isolated right papillary muscles from the control (n=41) and DOX-HF (n=15) groups one week after the last drug or saline administration. Rabbits were anesthetized with intravenous pentobarbital sodium (25 mg/kg). A left thoracotomy was performed and beating hearts were quickly excised and immersed in modified Krebs-Ringer (KR) solution (composition in mmol/l: NaCl 98; KCl 4.7; $MgSO_4$ 2.4; KH_2PO_4 1.2; glucose 4.5; $CaCl_2$ 1.8; $NaHCO_3$ 17; $C_3H_3NaO_3$ 15; CH_3COONa 5; atenolol 0.02) at 35 ºC with cardioplegic 2,3-butanedione monoxime (BDM; 3 %) and 5 % of newborn calf serum and gassed with 95 % O_2/5 % CO_2, to obtain pH between 7.38-7.42.

After dissection, papillary muscles (length: 4.2±0.3 mm; weight: 2.9±0.3 mg; preload: 4.3±0.3 mN) were mounted vertically in a 10 ml plexi glass organ bath containing the above-described KR solution and attached to an electromagnetic length-tension transducer (University of Antwerp, Belgium). Preload was estimated according to muscle dimensions and the electrical stimulus (0.6 Hz) was set at 10 % above threshold. Twenty minutes later, bathing solutions were replaced by corresponding KR solutions without BDM. During the next two hours, muscles were stabilized. Bathing solutions were then replaced by corresponding KR solutions without calf serum and maximum physiological length (L_{max}) was calculated. Protocols were initiated after obtaining two similar isotonic and isometric control twitches separated by a 10 min interval.

Experimental protocols

In a set of papillary muscles from control (n=8) and DOX-HF (n=7) groups, isometric contractility-frequency relationships were obtained by plotting maximum velocity of tension rise against frequency of contraction. In summary, after an initial period of contraction at 0.6 Hz, the frequency of stimulation was stepped up at 3-min intervals to 1 Hz, 2 Hz, 3 Hz and 4 Hz.

Myocardial effects of increasing concentrations of ET-1 (0.1, 1, and 10 nM) were studied in rabbit papillary muscles from: i) Control Group with intact endocardial endothelium (EE) (n=9); ii) Control Group with damaged EE (n=9); iii) Control Group in the presence N^G-nitro-L-arginine (L-NNA; nitric oxide synthase inhibitor, 1 μM, n=8); iv) Control Group in the presence of indomethacin (INDO; cyclooxygenase inhibitor, 1 μM, n=7) and (v) DOX-HF Group (n=8).

The concentrations of ET-1 were selected on the basis of several studies showing that its physiological effects on contraction and distensibility of myocardial tissue preparations or whole heart preparations are exerted by concentrations in the nanomolar range (Shah *et al*. 1989, Firth *et al*. 1990, Leite-Moreira *et al*. 2003, Bras-Silva and Leite-Moreira 2006).

EE was damaged by briefly (1 s) exposing the isolated papillary muscle to a weak solution (0.5 %) of the detergent Triton X-100 (Brutsaert *et al.* 1988, Leite-Moreira and Bras-Silva 2004).

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Data analysis

Isotonic and isometric twitches were recorded and analyzed. Selected parameters include: resting tension (RT) at the beginning (RT_{beg}, mN/mm^2) and at the end (RT_{end}, mN/mm^2) of the twitch; active tension (AT, mN/mm^2); maximum velocity of tension rise (dT/dt_{max}, $mN/mm^2/s$); maximum velocity of tension decline (dT/dt_{min}, $mN/mm^2/s$); peak isotonic shortening (PS, $\%L_{max}$); maximum velocity of shortening (dL/dt_{max}, L_{max}/s), maximum velocity of lengthening (dL/dt_{min}, L_{max}/s) and time to half relaxation (tHR, ms).

When a pharmacological inhibitor (L-NNA or INDO) was used, the term baseline refers to the condition in the presence of those inhibitors before the addition of ET-1.

In the various protocols, results are given as the percentage change from baseline. For the parameters that are expressed as negative values (e.g. dT/dt_{min}) such percentage change refers to the absolute values.

Statistical methods

Values are means ± S.E.M. Baseline performance of papillary muscles from control and DOX-treated rabbits were compared with an unpaired t-test. Effects of increasing concentrations of ET-1 and of increasing stimulation frequencies on papillary muscles from control and DOX-treated rabbits were analyzed with a repeated-measures two-way ANOVA. Echocardiographic data of DOX-treated animals at the beginning and at the end of the study were compared with a paired t test. Hemodynamic measurements at baseline and after treatment with DOX or saline were analyzed with a repeated-measures two-way ANOVA. When significant differences were detected, the Student-Newman-Keuls test was selected to perform multiple comparisons. Differences were considered to be significant when $P<0.05$.

Results

Cardiac hemodynamics and echocardiography

The hemodynamic features of the experimental groups are summarized in Table 1. In comparison with the control group, the DOX-HF group presented a lower systolic pressure, dP/dt_{max} and peak systolic isovolumetric pressure. The left ventricular filling pressure, as estimated by left ventricular end-diastolic pressure, was increased in DOX-HF, whereas the dP/dt_{min} was decreased and the relaxation time constant τ was increased in the DOX-HF (Table 1).

Table 1. Hemodynamic data of rabbits from the control and doxorubicin-induced heart failure (DOX-HF) groups.

	Control group (n=6)	DOX-HF group (n=9)
LVSP, mmHg	64.8±4.7	47.7±9.7*
LVEDP, mmHg	1.2±0.3	2.28±0.34*
dP/dt_{max}, mmHg/s	3026.0±244.0	1274±266.0*
dP/dt_{min}, mmHg/s	-2004.0±378.0	-992.0±171.0*
LVP_{ISO}, mmHg	148.9±9.2	84.6±13.5*
τ, ms	36.6±7.7	68.9±7.1*

Values are mean ± S.E.M. LVEDP and LVSP, left ventricular end-diastolic and systolic pressures, respectively; dP/dt_{max} and dP/dt_{min}, peak rates of ventricular pressure rise and fall, respectively; LVP_{ISO}, peak systolic isovolumetric pressure; τ, time constant of isovolumetric relaxation. *$P<0.05$ *vs.* Control group.

Furthermore, the echocardiographic evaluation in the DOX-HF group demonstrated a progressive increase of the end-diastolic (from 14.2±0.3 to 15.2±0.3 mm) and end-systolic (from 9.9±0.2 to 11.1±0.3 mm) short-axis diameters and a reduction in fractional shortening (from 32±1 to 26±1 %) and ejection fraction (from 64±1 to 56±2 %) of the left ventricle. This was consistent with the presence of dilated cardiomyopathy and HF. None of the other parameters measured changed significantly after doxorubicin treatment, namely heart rate (158±6 vs. 144±7 bpm), left wall thickness in diastole (2.26±0.07 vs. 2.20±0.05 mm) and left wall thickness in systole (3.40±0.13 vs. 3.42±0.11 mm).

Table 2. Mean values of the baseline contractile parameters in papillary muscle from the control and doxorubicin-induced heart failure (DOX-HF) groups.

Contractile parameter	Control group		DOX-HF group (n=15)
	With EE (n=32)	Without EE (n=9)	
AT (mN/mm^2)	23.3 ±2.7	17.4 ±1.9*	26.3±4.3
dT/dt_{max} ($mN/mm^2/s$)	163.5±17.1	112.5±11.6*	164.5±21.3
dT/dt_{min} ($mN/mm^2/s$)	–133.1±15.3	–95.2±9.6*	–137.8±22.2
PS (% of L_{max})	12.0±0.1	9.0±0.1*	11.0±0.1
dL/dt_{max} (L_{max}/s)	0.89±0.1	0.61±0.06*	0.71±0.05
dL/dt_{min} (L_{max}/s)	–3.20±0.40	–2.01±0.2*	–2.43±0.2

Values are means ± S.E.M. EE, endocardial endothelium; AT, active tension; dT/dt_{max}, maximum velocity of tension rise; dT/dt_{min}, maximum velocity of tension decline; PS, peak isotonic shortening; dL/dt_{max}, maximum velocity of shortening; dL/dt_{min}, maximum velocity of lengthening. * P<0.05 *vs.* control group with intact EE.

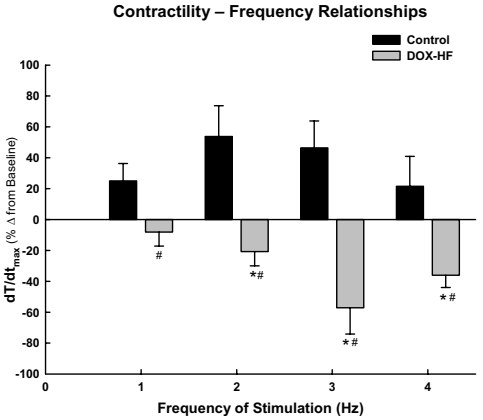

Fig. 1. Contractile response of rabbit papillary muscles from the control group (n=8) and from the doxorubicin-induced heart failure (DOX-HF) group (n=7) to steady increases in stimulation frequency. Contractility-frequency relationships were then obtained by plotting maximum velocity of tension rise against frequency of contraction. Control muscles showed a steady increase in contractility between 1 Hz and 4 Hz, whereas in the DOX-HF group, muscles responded in the opposite way. P<0.05: *, *vs.* baseline; #, *vs.* control.

Papillary muscle studies

Mean values of the baseline contractile parameters in papillary muscles from the control group with intact EE (n=32) and from the DOX-HF group (n=15) are shown in Table 2. Removal of the EE (n=9) resulted in a negative inotropic effect. Although baseline performance of rabbit papillary muscles was similar in the control group with intact EE and in the DOX-HF group, the contractility of papillary muscles from the control group did not significantly decline with increasing stimulation frequency, between 1 Hz and 4 Hz, while the papillary muscles from the DOX-HF rabbits showed a significant decrease in contractility over the same range of stimulation frequencies, indicating contractile dysfunction and reduced contractile reserve (Fig. 1). In the presence of an intact EE, ET-1 promoted concentration-dependent positive inotropic and lusitropic effects: AT increased by 15.3±5.4 %, 47.2±9.8 % and 88.6±18.3 %; dT/dt_{max}, 15.4±5.9 %, 47.1±12.3 % and 103.7±21.5 %; and dT/dt_{min}, 13.3±4.9 %, 42.4±6.8 % and 85.6±16.9 % (at 0.1, 1 and 10 nM, respectively). These effects were maintained after damaging the EE, in the presence of L-NNA or INDO and in the DOX-HF Group (Fig. 2).

Concerning the effects of ET-1 on myocardial distensibility, we found that RT significantly decreased after an isometric twitch in the presence of ET-1. Such a decrease was not significant at baseline and became progressively larger with increasing doses of ET-1 in muscles with intact EE. In fact, compared with its value at the beginning of the twitch (RT_{beg}), RT at the end of an isometric twitch (RT_{end}) decreased 3.2±1.3, 6.0±1.6 and 8.8±2.7 % in the presence of 0.1, 1 and 10 nM of ET-1, respectively (Fig. 3). Such a decrease in RT reflects an increase in myocardial distensibility, because restoring the value of RT to its initial value results in an increase in the resting length of the muscle. However, no significant differences between RT_{end} and RT_{beg} were found, when ET-1 was given after damaging the EE or in papillary muscles from the DOX-HF group. Similarly, ET-1 did not significantly alter myocardial distensibility after blocking release of NO or prostaglandins by L-NNA or INDO, respectively.

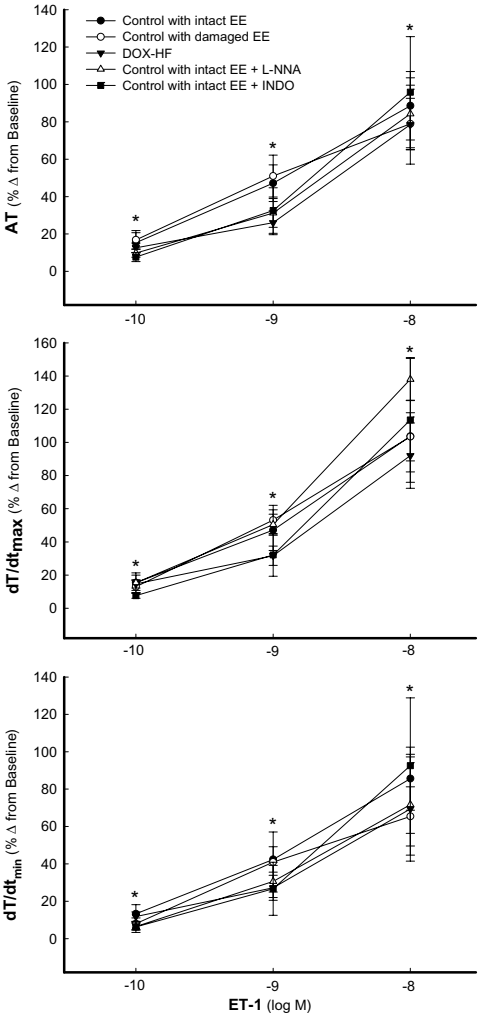

Fig. 2. Concentration-response curves for the effect of ET-1 on the contractile parameters of rabbit papillary muscles in various experimental conditions: Control group with intact endocardial endothelium (EE; full circles, n=9); Control group with damaged EE (open circles, n=9); Control group with intact EE and in the presence of N^G-nitro-L-arginine (L-NNA, open triangles, n=8) ; Control group with intact EE and in the presence of Indomethacin (INDO, full squares, n=7) and doxorubicin-induced heart failure group (DOX-HF; full triangles, n=8). AT, active tension; dT/dt_{max}, maximum velocity of tension rise; dT/dt_{min}, maximum velocity of tension decline. Mean ±S.E.M.; percentage of baseline. * P<0.05 *vs.* baseline.

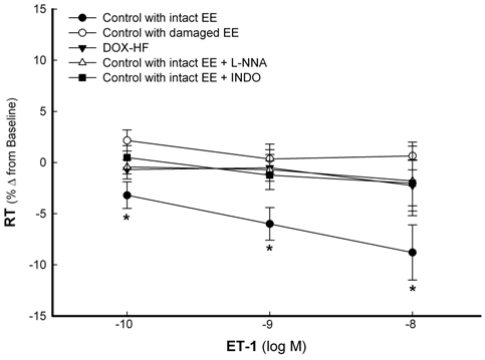

Fig. 3. Concentration-response curves for the effect of ET-1 on resting tension (RT) of rabbit papillary muscles in various experimental conditions: Control group with intact endocardial endothelium (EE; full circles, n=9); Control group with damaged EE (open circles, n=9); Control group with intact EE and in the presence of N^G-nitro-L-arginine (L-NNA, open triangles, n=8) Control group with intact EE and in the presence of indomethacin (INDO, full squares, n=7) and doxorubicin-induced heart failure group (DOX-HF; full triangles, n=8). Mean ±S.E.M.; percentage of baseline. * P<0.05 *vs.* baseline.

Discussion

The present study showed that the increase in myocardial distensibility induced by ET-1 is absent in DOX-HF and is dependent on NO and prostaglandin release.

The progression of cardiac dysfunction was monitored echocardiographically to estimate morphological and functional alterations during the development of HF. Hemodynamic studies performed one week after the last administration of DOX also showed the presence of systolic and diastolic dysfunction in DOX-HF animals. In addition, as contractile dysfunction in papillary muscles is most often not evident from changes in baseline performance of muscles that are contracting at low stimulating frequencies, but rather on an impaired response to increased frequencies (Endoh 2004), contractility-frequency relationships were studied. We found that although baseline performance of normal and DOX-HF muscles was similar, contrary to the former, the latter showed decreased contractility to increased frequencies, indicating contractile dysfunction and a reduced contractile reserve.

Positive inotropic and lusitropic effects of ET-1 have been previously described by several authors in various experimental preparations, although the magnitude of the effects varied among distinct animal

species (Endoh *et al*. 1998). Rabbits belong to the most sensitive animals to ET-1, which was one of the reasons for carrying out the experiments in this species. The magnitude of positive inotropic and lusitropic effects obtained in the present study is in agreement with previously published data on rabbit papillary muscles (Li *et al.* 1991, Leite-Moreira *et al.* 2003, Leite-Moreira and Bras-Silva 2004, Bras-Silva and Leite-Moreira 2006). These inotropic and lusitropic effects of ET-1 were maintained after damaging EE, blocking NO and prostaglandins release and in the DOX-HF group. Previous studies *in vivo* and *in vitro* showed that the contractile effects of ET-1 were increased (Li and Rouleau 1996), attenuated (Möllmann *et al.* 2006), maintained (Bras-Silva and Leite-Moreira 2006) or even reversed (Kelso *et al.* 1996, Thomas *et al.* 1996, MacCarthy *et al.* 2000) in the presence of HF. This difference could be explained by the different methodological approaches, different animal species and various experimental models of HF.

In the present study, we therefore observed that despite the occurrence of baseline contractile dysfunction in failing hearts, baseline performance of papillary muscles was similar in control and doxorubicin-treated animals. Furthermore, these muscles exhibited the same inotropic and lusitropic response to ET-1, but a distinct inotropic response to increasing stimulation rates, closer to the physiological range. The negative force-frequency relationship is a well-known feature of the failing myocardium that can be at least partially attributed to disturbed calcium homeostasis and energy imbalance (Endoh 2004). On the other hand, the contractile response to ET-1 involves distinct cellular mechanisms, which might explain its similar effects in the normal and failing myocardium. Furthermore, ET-1 has the ability to increase cardiac contractile efficiency by lowering ATPase activity (McClellan *et al*. 1996) and oxygen consumption (Takeuchi *et al.* 2001) and was considered essential for the contractile efficiency of the failing myocardium (Sakai *et al*. 1996).

With regard to the effects of ET-1 on the diastolic properties of the myocardium, we found that the decrease in resting tension (increase in myocardial distensibility) observed after an afterloaded twitch in the presence of ET-1 was not present in the failing myocardium. We also confirmed that damaging the EE also blocked this effect confirming our previous observations (Bras-Silva and Leite-Moreira 2006). In previous studies we have also shown that this effect of ET-1 on myocardial distensibility was mediated by ET_A receptor stimulation (Leite-Moreira *et al.* 2003), and dependent on endothelial ET_{B1} receptor activity, even if the direct stimulation of either endothelial ET_{B1} or myocardial ET_{B2} receptors did not elicit any effect on this parameter (Bras-Silva and Leite-Moreira 2006). If we take into account that endocardial endothelium is dysfunctional in the DOX-HF model (Bras-Silva *et al.* 2006) and that the acute effects of ET-1 on myocardial distensibility are blocked when the EE is damaged, it seems plausible that the blunted effects of ET-1 on myocardial distensibility in the failing myocardium could be explained by EE dysfunction.

Once NO and prostaglandins are two of the most important endothelial mediators and they are known to be released by the endothelium in response to ET_{B1} receptor stimulation (de Nucci *et al.* 1988, Thiemermann *et al.* 1989, Filep *et al.* 1991, Hirata *et al.* 1993 Leite-Moreira and Bras-Silva 2004), which also influences ET-1 effect on myocardial distensibility (Bras-Silva and Leite-Moreira 2006), we investigated how these two agents modulate the ET-1 effects. We found that similarly to what happened after damaging EE, after blocking of NO or prostaglandin release the ET-1-induced decrease in resting tension (increase in distensibility) was not observed.

NO has been shown to increase diastolic distensibility (Paulus and Shah 1999, Paulus *et al.* 1994). This effect seems to be mediated by reduction of myofilamentary calcium sensitivity because of phosphorylation of troponin I by cGMP-dependent protein kinase (Shah and MacCarthy 2000). Direct myocardial actions of prostaglandins are still not clear. With regard to inotropy both negative (Schor and Hohlfeld 1992) and positive (Mohan *et al.* 1995) effects were shown in isolated papillary muscles. Regarding lusitropy prostaglandins were recently shown to preserve early active diastolic relaxation (Kisch-Wedel *et al.* 2005) and to blunt the premature onset of tension decline promoted by ghrelin (Soares *et al.* 2006). These two agents, NO and prostaglandins, have also been implicated in the negative inotropic effects resulting from selective ET_{B1} receptor stimulation (Leite-Moreira and Bras-Silva 2004). It seems that independently of the direct actions of each of these endothelial agents, they are able to regulate both systolic and diastolic effects of ET-1.

Concerning the pathophysiological relevance of our findings, we must point out that a lower resting tension of the cardiac muscle indicates the ventricle can

reach higher filling volumes at lower filling pressures, which is undoubtedly quite a powerful adaptation mechanism. These acute beneficial ET-1 effects on diastolic function seem to be overcome by its role in progression to cardiac fibrosis and ventricular remodeling when its levels remain chronically elevated (Brunner *et al.* 2006). Additionally, the results of the present study emphasize that humoral influences on diastolic cardiac function are modulated by the interaction with endocardial endothelial mediators, such as NO and prostaglandins, which being altered in the failing heart might provide new elements for the comprehension of the pathophysiology of HF. Finally, doxorubicin is an antineoplastic antibiotic widely used in the treatment of a variety of cancers, and its clinical use is limited as the result of a severe, dose-dependent cardiotoxicity (Monnet and Chachques 2005). In this context, our findings might also be relevant to a better understanding of the pathophysiology of DOX-induced cardiomyopathy, so that efficient protective and/or therapeutic strategies can be developed in patients treated with this chemotherapeutic agent.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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CAPÍTULO III

MEDIADORES NEURO-HUMORAIS CLÁSSICOS

PARTE C: EFEITOS DA ESTIMULAÇÃO β-ADRENÉRGICA SOBRE A FUNÇÃO DIASTÓLICA

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β-adrenergic stimulation acutely decreases myocardial stiffness: a novel β_1-adrenoceptor, PKA and PKC mediated effect

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ABSTRACT

Myocardial effects of isoprenaline (10^{-10} to 10^{-5}M), a non-selective β-adrenergic agonist, were tested in rabbit papillary muscles either alone (n=8) or after pre-treatment with nadolol (non-selective β-adrenoceptor antagonist, 10^{-5} M; n=7), atenolol (β_1-adrenoceptor antagonist, 2.10^{-5}M; n=8), KT5720 (PKA inhibitor, 10^{-6} M; n=6), chelerythrine (PKC inhibitor, 10^{-5}M; n=6), or 5-(*N*-methyl-*N*-isobutyl)-amiloride (Na^+/H^+ exchanger inhibitor, 10^{-6}M; n=8). Passive length-tension relations were constructed before and after adding 10^{-5}M of isoprenaline (10^{-5}M, n=6).

Isoprenaline concentration dependently increased inotropy, lusitropy and resting muscle length (L/L_{max}). At 10^{-5} M, isoprenaline increased: 110.2±14.8% active tension, 310.1±35.8% maximal velocity of tension rise, 189.8±25.4% maximal velocity of tension decline and 1.024±0.01% of L/L_{max}. Correcting resting muscle length to its initial value resulted in a 29.6±3.4% decrease of resting tension, indicating decreased muscle stiffness, as confirmed by the right and downward shift of the passive length-tension relation induced by isoprenaline.

Selective β_1-adrenoceptor blockade and PKA and PKC inhibition attenuated the effects of isoprenaline on myocardial stiffness. In conclusion, β-adrenergic stimulation decreases myocardial stiffness, an effect that represents a novel mechanism of acute neurohumoral modulation of diastolic function. These findings suggest that this system could be a powerful regulator of cardiac filling, which might be involved in the pathophysiology of diastolic dysfunction.

Keywords: β-adrenergic stimulation, diastolic function, distensibility, myocardial stiffness, heart

1. INTRODUCTION

For many years, the evaluation of myocardial function has focused mainly in the chronotropic and inotropic state of the heart. However, cardiac relaxation (lusitropy) has emerged as an important feature, as it contributes to a proper pump function, allowing adequate time for ventricular diastolic filling (Katz and Smith, 1984; Smith and Katz, 1984). In fact, the clinical importance of assessing the lusitropic state induced by pharmacological interventions is presently recognized.

β-adrenergic stimulation is an important physiological mechanism to enhance cardiac performance during increased circulatory demands. Activation of these receptors on cardiac myocytes initiates signaling pathways that increase contractility and accelerate relaxation. Presently, three β-adrenoceptor subtypes have been identified, β_1-, β_2-, and β_3-adrenoceptor. Mammalian cardiac myocytes express mainly β_1-adrenoceptor, ranging from 60-80% depending on the species, and β_2-adrenoceptor. These receptors differently modulate systolic and diastolic functions (Brodde *et al.*, 2006).

The effects of β-adrenergic stimulation are partially mediated by cAMP-dependent protein kinase A (PKA), which phosphorylates a host of intracellular substrates, including several membrane channels and accessory proteins on the thin (actin) and thick (myosin) filaments. Fast changes in intracellular Ca^{2+}-handling are thought to be largely responsible for the positive inotropy. Concerning its positive lusitropy, it is primarily dependent on some kinases such as PKA that in turn phosphorylates several downstream proteins responsible for its effects. Some examples are the phosphorylation of: 1) phospholamban, enhancing Ca^{2+} reuptake into the sarcoplasmatic reticulum (Bers, 2006; Bers and Guo, 2005); 2) troponin I (TnI), decreasing myocardial calcium (Ca^{2+}) sensitivity on the thin filaments by increasing the rate at which Ca^{2+} dissociates from troponin C (TnC) (Fentzke *et al.*, 1999; Garvey *et al.*, 1988; Johns *et al.*, 1997; Robertson *et al.*, 1982;

Wattanapermpool *et al.*, 1995; Zhang *et al.*, 1995) and 3) myosin binding protein-C (MyBP-C), accelerating crossbridge cycle and increasing myofibrillar ATPase activity (Gruen *et al.*, 1999; Kunst *et al.*, 2000). All these mechanisms may lead to a faster rate of myofibrillar relaxation, thereby shortening twitch duration.

In addition to the thin and thick filaments, striated muscles also contain a third filament system composed by the giant protein titin. Similarly to TnI, TnC and MyBP-C, titin is also phosphorylated by PKA in response to β-adrenergic stimulation (Yamasaki *et al.*, 2002). Several studies in different myocardial preparations observed that this PKA-mediated phosphorylation acutely decreases passive tension (PT), an effect ascribed to phosphorylation of the stiff N2B titin isoform (Fukuda et al., 2005; Kruger and Linke, 2006; Yamasaki et al., 2002).

We have previously demonstrated acute changes of the myocardial passive properties after exposure to several neurohumoral agents like endothelin-1 (Leite-Moreira *et al.*, 2003), angiotensin II (Leite-Moreira *et al.*, 2006) and urotensin II (Fontes-Sousa et al., 2007). Similarly, nitric oxide (NO) has also been shown to increase myocardial distensibility (Paulus et al., 1994; Shah and MacCarthy, 2000). Furthermore, diastolic dysfunction induced by excessive afterload was attenuated by β-adrenergic stimulation, highlighting the lusitropic effects of this neurohumoral system (Leite-Moreira *et al.*, 2001).

In this context, considering that titin phosphorylation by PKA induces an increase of myocardial distensibility, as previously outlined, and β-adrenergic stimulation is one of the most important stimuli for PKA activation, the aim of the present study was to investigate the effects β-adrenergic stimulation on myocardial distensibility, as well as the underlying mechanisms.

2. MATERIALS AND METHODS

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication Nº 85-23, Revised 1996).

2.1. Experimental preparation

Isometric and isotonic contractions were analyzed in papillary muscles isolated from the right ventricle of rabbits. Male New Zealand White rabbits (*Oryctolagus cuniculus*; 2.1±0.1 kg; *n*=31) were anesthetized with intravenous sodium pentobarbital (25mg.kg^{-1}). A left thoracotomy was performed, beating hearts were quickly excised and immersed in a modified Krebs-Ringer solution (composition in mM: 98 NaCl, 4.7 KCl, 2.4 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 4.5 glucose, 1.8 $CaCl_2.2H_2O$, 17 $NaHCO_3$, 15 sodium pyruvate, 5 sodium acetate) at 35ºC with cardioplegic 2,3-butanedione monoxime (BDM; 3%) and 5% Newborn Calf Serum. The solutions were in equilibrium with 95% O_2 and 5% CO_2, to obtain a pH between 7.38-7.42.

The time from thoracotomy to dissection was ~3 min. The right ventricle was opened and papillary muscles were isolated by first dividing the chordae tendinae at the muscle tip and then freeing the muscle base and a small amount of surrounding myocardium from the ventricular wall. Only long, thin, uniformly cylindrical muscles were used.

After dissection, papillary muscles (*n*=49; length: 4.3±0.2mm; weight: 3.4±0.2mg; preload: 3.9±0.2mN) were mounted vertically in a 10ml plexi glass organ bath containing the aforementioned Krebs-Ringer solution. The lower muscular end was fixed in a phosphorbronze clip, and the upper tendinous end was attached to an electromagnetic length–tension transducer (University of Antwerp, Belgium).

Preload was initially set between 3 and 4 mN according to muscle dimensions. The preparations were stimulated at 0.6 Hz with a voltage of 10% above threshold (typically 3-6 mV) by rectangular pulses of 5 ms duration through two platinum electrodes arranged longitudinally alongside the entire muscle. After 20 min, bathing solutions were replaced by corresponding Krebs-Ringer solutions without BDM and the muscle started to contract. One hour later, bathing solution was replaced by corresponding serum-free Krebs-Ringer solution. During the next 2 hours, the muscles were stabilized. Finally, the muscles were stretched to a muscle length at which active force development was maximal (L_{max}). During the experiment, changes in diastolic muscle length and muscle shortening were measured by the isotonic transducer. Protocols were initiated after obtaining two similar isotonic and isometric control twitches separated by a 10 min interval. At the end of the experiment the muscles were removed, lightly blotted and then weighed. Muscle cross-sectional area was calculated by dividing the weight of the muscle by its length at L_{max}. A cylindrical shape and a specific density of 1.0 were assumed. Muscle tension was then expressed as force normalized per cross-sectional area ($mN.mm^{-2}$).

2.2. Experimental protocols

To evaluate the effects of β-adrenergic stimulation on contraction, relaxation and diastolic properties of the myocardium, increasing concentrations of isoprenaline (10^{-10} to 10^{-5} M)**,** a non-selective β-adrenergic agonist, were studied in rabbit papillary muscles in **A.** control muscles (n=8), and **B.** in the presence of: (i) nadolol (10^{-5} M; n=7), a non-selective β-adrenoceptor antagonist; (ii) atenolol (2.10^{-5} M; n=8), a selective β_1-adrenoceptor antagonist; (iii) KT5720 (KT, 10^{-6} M; n=6), an inhibitor of PKA; (iv) chelerythrine (10^{-5} M; n=6), an inhibitor of protein kinase C (PKC); and (v) 5-(*N*-methyl-*N*-isobutyl)-amiloride (10^{-6} M; n=8), an inhibitor of Na^+/H^+ exchanger (NHE).

These substances were dissolved in the bath Krebs-Ringer solution before the addition of isoprenaline, and muscle twitches were recorded after a stable response was obtained, typically 20 minutes later. The exceptions, atenolol and nadolol, were included in the initial Krebs-Ringer solution. After that, isoprenaline was added cumulatively without any washout between, with a maximal effect occurring approximately 4-5 min later. Also, passive length-tension relations were constructed before and after a single concentration of isoprenaline (10^{-5}M, n=6). Notably in each experimental protocol, all papillary muscles were obtained from different animals. All chemicals were obtained from Sigma Chemical Co, St Louis, Mo. Most of the stock solutions were dissolved in distilled water and stored at -20 ºC until use. KT5720 was dissolved in DMSO with a final concentration in the bath less than 0.1%.

2.3. Data analysis

Isotonic and isometric twitches were recorded and analyzed with dedicated software (University of Antwerp, Belgium). Selected parameters included: resting tension (RT; $mNmm^{-2}$), active tension (AT; $mNmm^{-2}$); maximal velocities of tension rise (dT/dt_{max}; $mNmm^{-2}s^{-1}$) and decline (dT/dt_{min}; $mNmm^{-2}s^{-1}$); peak isotonic shortening (PS; $\%L_{max}$); maximal velocities of shortening (dL/dt_{max}; $L_{max}s^{-1}$) and lengthening (dL/dt_{min}; $L_{max}s^{-1}$); time to half-relaxation (tHR, ms); and time to active tension (tAT; ms).

In the various protocols, results are given as percent change from baseline. For the parameters that are expressed as negative values (e.g. dT/dt_{min}) such percent change refers to the absolute values. When a pharmacological inhibitor was used, the term baseline refers to the performance in the presence of those inhibitors, before the addition of isoprenaline.

2.4. Statistical methods

Values are means ± S.E.M. and *n* represents the number of experiments. Effects of increasing concentrations of isoprenaline alone on the different experimental parameters were analyzed by one-way repeated-measures ANOVA. Effects of increasing concentrations of isoprenaline under various experimental conditions were analyzed with a repeated-measures two-way ANOVA. Effects on the various parameters of a single concentration of the antagonists were analyzed with a paired t-test. When significant differences were detected with any of the ANOVA tests, the Student-Newman-Keuls test was selected to perform pairwise multiple comparisons. $P < 0.05$ was accepted as significant.

3. RESULTS

Morphometric characteristics and baseline performance of papillary muscles did not vary significantly between the different experimental groups (Table 1). Concentration-response curves to isoprenaline in the various experimental conditions are illustrated in Figs. 1-6.

Table 1 – Morphologic and contractile characterization of papillary muscles (n = 49)

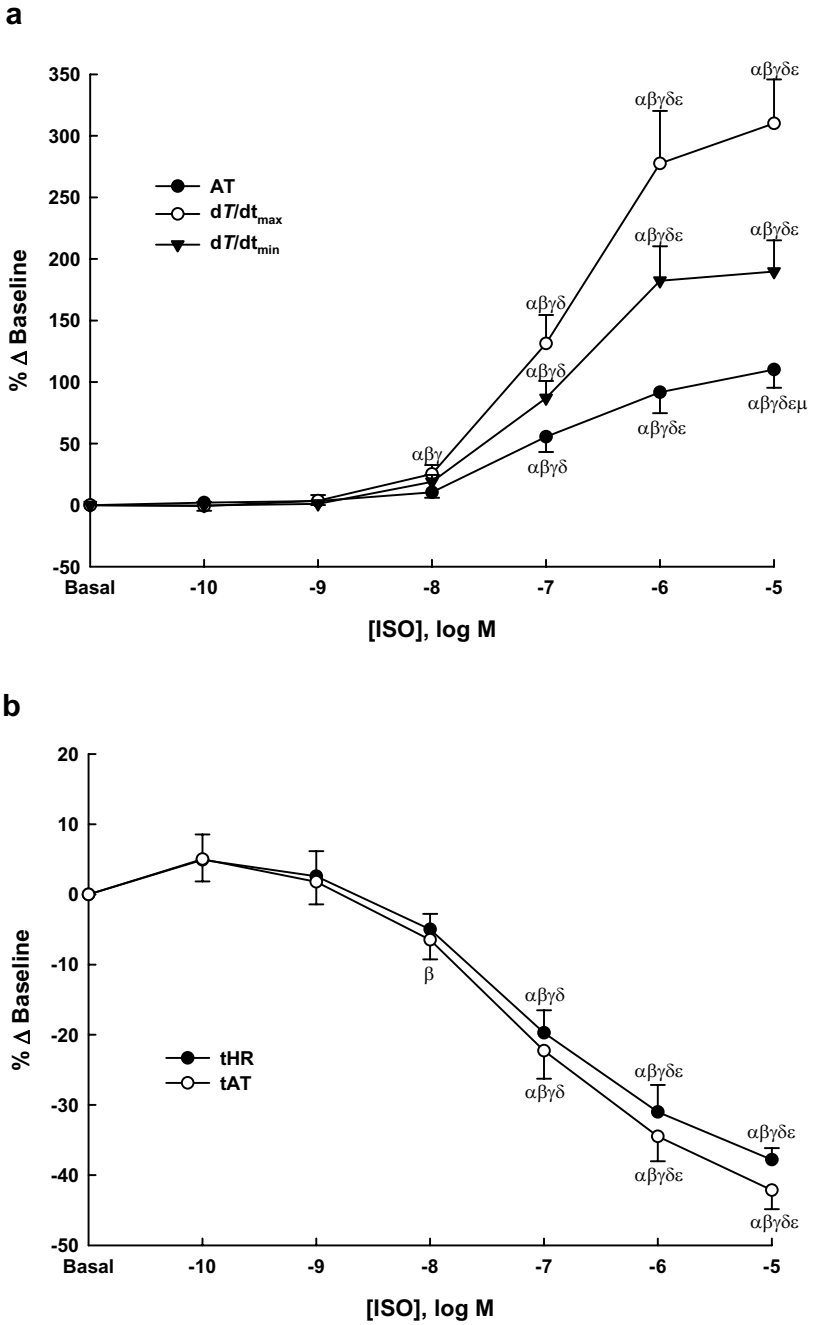
Parameter	Value
Lenght (mm)	4.3 ± 0.2
Weight (mg)	3.4 ± 0.2
Preload (mN)	3.9 ± 0.2
AT (mN/mm^2)	27.4 ± 2.6
dT/dt_{max} ($mN/mm^2/s$)	185.6 ± 16.1
dT/dt_{min} ($mN/mm^2/s$)	- 134.0 ± 10.8
tHR (ms)	401.7 ± 12.3
tAT (ms)	246.3 ± 7.3
PS (%L_{max})	12.4 ± 0.8
dL/dt_{max} (L_{max}/s)	0.9 ± 0.1
dL/dt_{min} (L_{max}/s)	-3.3 ± 0.3

AT: active tension; dT/dt_{max}, dT/dt_{min}: maximum velocity of tension rise and decline, respectively; tHR: time for half relaxation; tAT: time to active tension; PS: peak isotonic shortening; dL/dt_{max}, dL/dt_{min}: maximum velocity of shortening and lengthening, respectively. Values are means ± S.E.M.

Increasing concentrations of isoprenaline enhanced both contractility (AT, dT/dt_{max}, PS and dL/dt_{max}) and lusitropy (dT/dt_{min}, tAT, tHR and dL/dt_{min}) (Fig. 1). The highest concentration of isoprenaline (10^{-5}M) increased 110.2±14.8% AT, 310.1±35.8% dT/dt_{max}, 189.8±25.4% dT/dt_{min} and decreased 42.2±2.7% tAT and 37.8±1.7% tHR ($P < 0.05$). Additionally, at this concentration, isoprenaline increased 73.3±11.0% PS, 199.9±15.2% dL/dt_{max} and 210.9±42.5% dL/dt_{min}.

Concerning the diastolic properties of the myocardium, in addition to increasing relaxation rate (dT/dt_{min}), decreasing time to half relaxation (tHR) and promoting an earlier onset of relaxation (tAT), we observed that isoprenaline progressively increased resting muscle length at a constant resting tension, up to 1.024±0.01% of L/L_{max} (Fig. 2). Correcting muscle length, at the end of the experiment, to its initial value resulted in a 29.6±3.4% decrease of resting tension (RT), without altering other contractile parameters. All these results indicate an increase in muscle distensibility or, on the other hand, a decrease in muscle stiffness.

This aspect is further explored in Fig. 3 where passive length-tension relations at baseline and in the presence of isoprenaline (10^{-5}M) are presented. This relation is right and downward shifted by isoprenaline. In other words, at each resting tension, muscle length was always significantly higher in the presence of isoprenaline, indicating that this agent acutely increases distensibility and lowers stiffness of the myocardium.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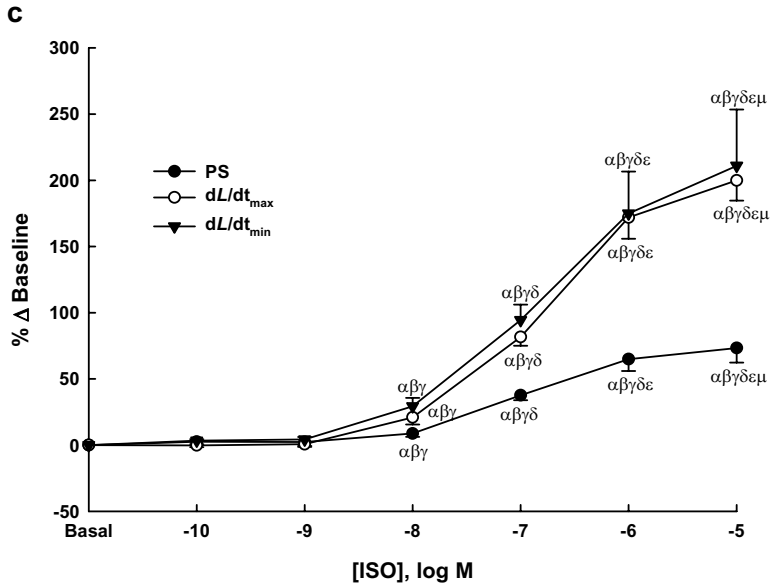


Fig. 1 - Effect of increasing concentrations of isoprenaline (ISO, 10^{-10} to 10^{-5}M; *n*=8) on **a** active tension (AT), peak rates of tension rise and decline (d*T*/dt_{max} and d*T*/dt_{min}, respectively); on **b** time to half relaxation (tHR) and time to active tension (tAT) and on **c** peak isotonic shortening (PS), maximal velocities of shortening and lengthening (d*L*/dt_{max} and d*L*/dt_{min}, respectively). Data are means ± S.E.M., expressed as percent variation from baseline. *P*< 0.05: α *vs* baseline, β *vs* 10^{-10}M ISO, γ *vs* 10^{-9}M ISO, δ *vs* 10^{-8}M ISO, ε *vs* 10^{-7}M ISO, μ *vs* 10^{-6}M ISO.

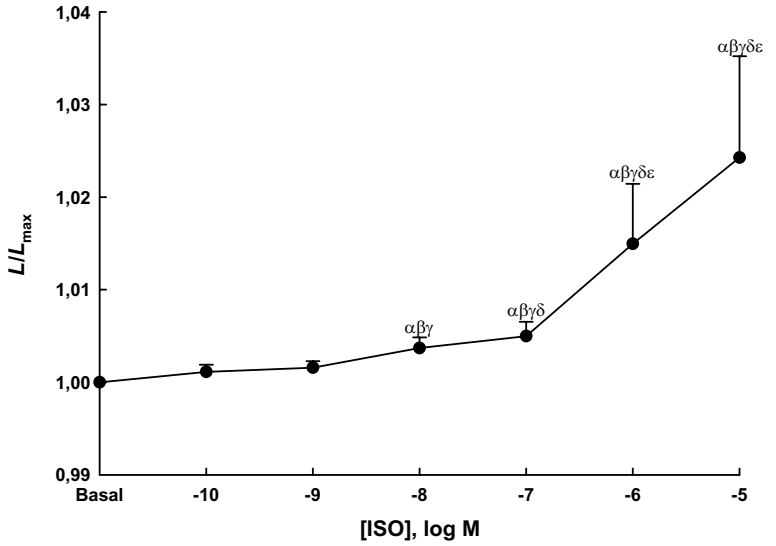


Fig. 2 - Effect of increasing concentrations of isoprenaline (ISO, 10^{-10} to 10^{-5}M; *n*=8) on resting muscle length (*L*/L_{max}). Data are means ± S.E.M., expressed as percent variation from baseline. *P*< 0.05: α *vs* baseline, β *vs* 10^{-10}M ISO, γ *vs* 10^{-9}M ISO, δ *vs* 10^{-8}M ISO, ε *vs* 10^{-7}M ISO, μ *vs* 10^{-6}M ISO.

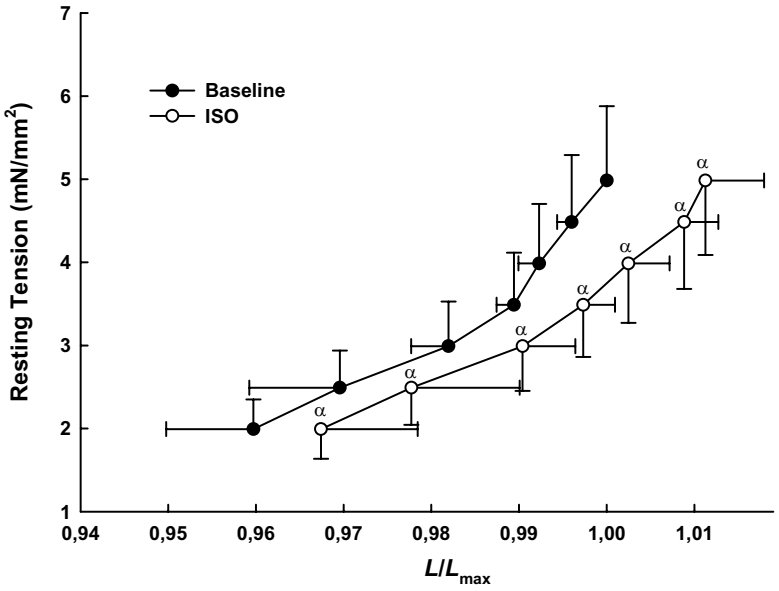
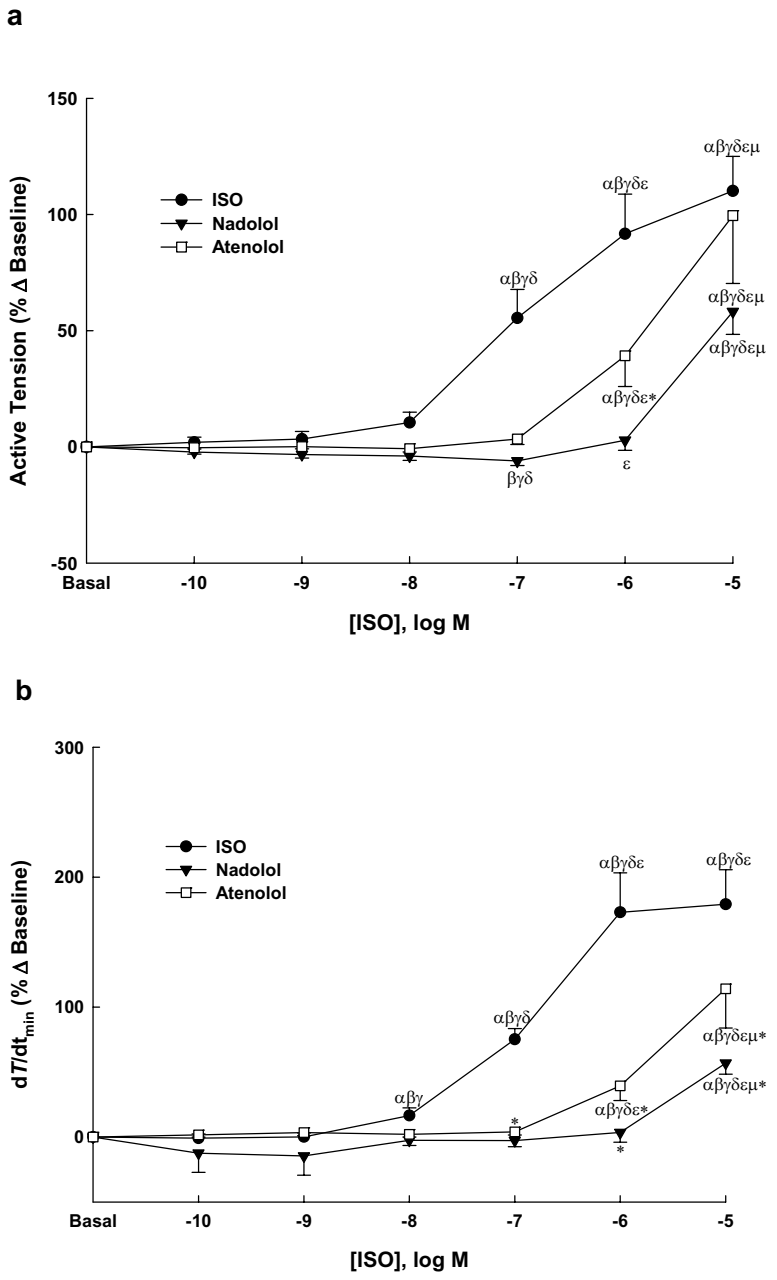


Fig. 3 - Passive length-tension relations at baseline and in the presence of isoprenaline (ISO, 10^{-5}M, *n*=6). Data are mean ± S.E.M. *P*< 0.05: α ISO *vs* baseline.

Effects of isoprenaline in the presence of non-selective β-adrenoceptor (nadolol) or selective β_1-adrenoceptor (atenolol) antagonists or after inhibition of PKA (KT5720), PKC (chelerythrine), NHE [5-(*N*-methyl-*N*-isobutyl)-amiloride] are illustrated in Fig. 4, 5 and 6. The majority of these agents did not significantly modify *per se* any of the analyzed contractile parameters. The exception was the presence of chelerythrine, which decreased 58.1±5.6% AT (*P*<0.01).

The inotropic effects of isoprenaline were not significantly affected by any of the evaluated agents (Fig. 4a and 5a). However, in the presence of the non-selective β-adrenoceptor antagonist, nadolol, or the selective β_1-adrenoceptor antagonist, atenolol, there was a tendency to an attenuation of the inotropic effect of isoprenaline (Fig. 4a). Concerning myocardial relaxation, both agents significantly attenuated the effects of isoprenaline on dT/dt_{min} and tAT (Fig. 4b and 4c).

The effect of isoprenaline on resting muscle length was significantly decreased by PKC or PKA inhibition and in the presence of the non-selective inhibitor of β-adrenoceptor (nadolol) or the selective inhibitor of β_1-adrenoceptor (Fig. 4d, 5b and 6).



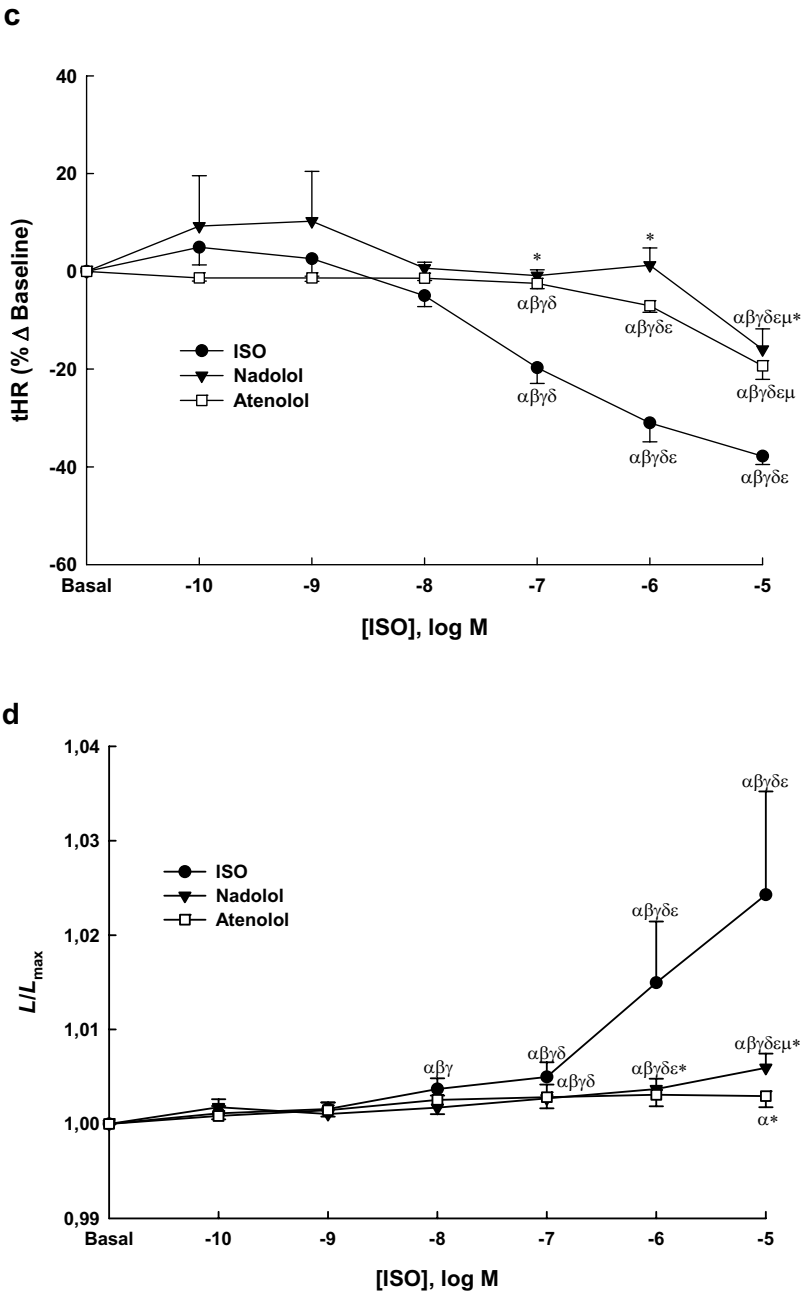


Fig. 4 - Effect of increasing concentrations of isoprenaline (ISO, 10^{-10} to 10^{-5}M; *n*=8) on **a** active tension (AT), on **b** peak rates of tension decline (dT/dt_{min}), on **c** time to active tension (tAT) and **d** passive muscle length (L/L_{max}) in the absence (*n*=8) or presence of a non-selective β-adrenoceptor antagonist (nadolol) (10^{-5}M; *n*=7) or a β_1-adrenoceptor antagonist (atenolol) (2.10^{-5}M; *n*=8). Data are mean ± S.E.M., expressed as percent variation from baseline. *P*< 0.05: α *vs* baseline, β *vs* 10^{-10}M ISO, γ *vs* 10^{-9}M ISO, δ *vs* 10^{-8}M ISO, ε *vs* 10^{-7}M ISO, μ *vs* 10^{-6}M ISO and * *vs* ISO alone.

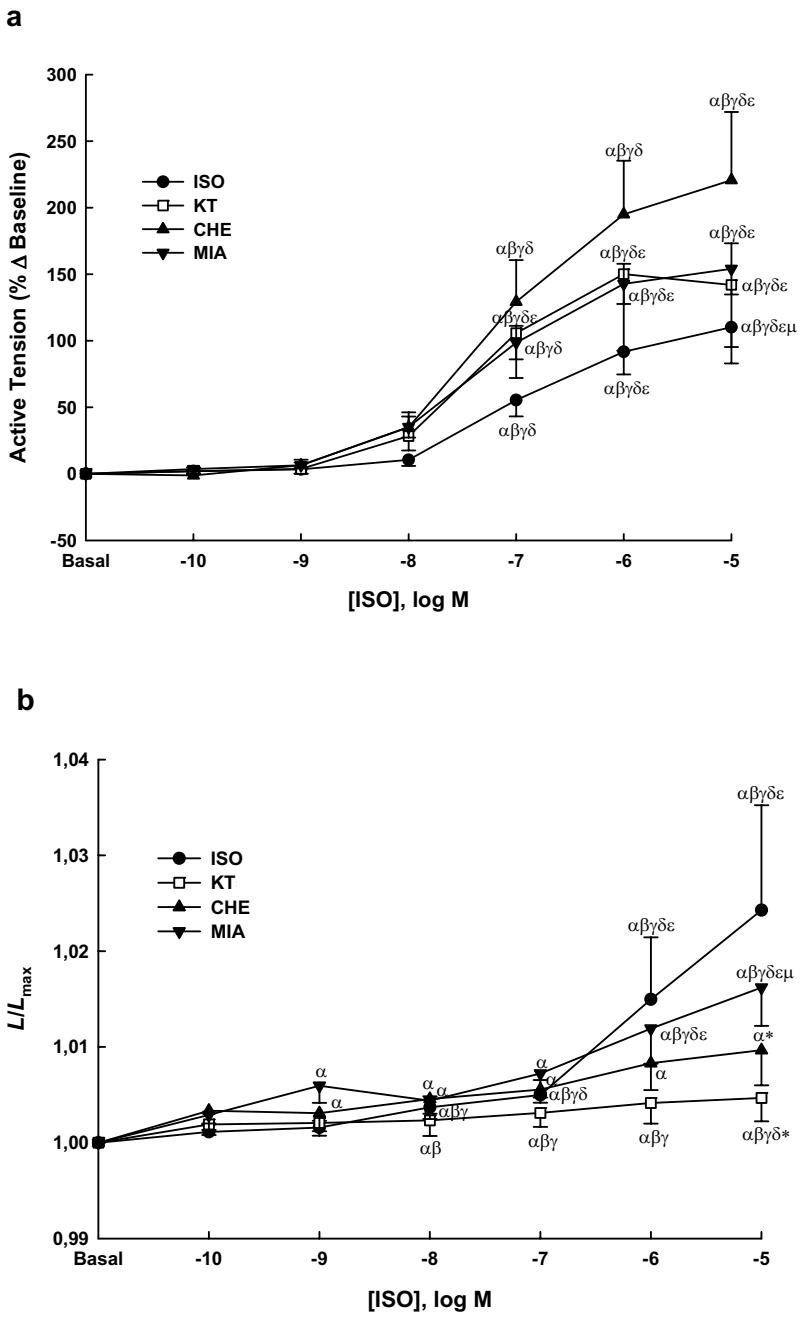


Fig. 5 - Effect of increasing concentrations of isoprenaline (ISO, 10^{-10} to 10^{-5}M; *n*=8) on **a** active tension (AT) and **b** passive muscle length (L/L_{max}) in the absence (*n*=8) or presence of inhibitors of PKA (KT, 10^{-6} M, *n*=6), PKC (CHE, 10^{-5}M; *n*=6) or NHE (MIA, 10^{-6}M; *n*=8). Data are mean ± S.E.M., expressed as percent variation from baseline. $P< 0.05$: α *vs* baseline, β *vs* 10^{-10}M ISO, γ *vs* 10^{-9}M ISO, δ *vs* 10^{-8}M ISO, ε *vs* 10^{-7}M ISO, μ *vs* 10^{-6}M ISO and * *vs* ISO alone.

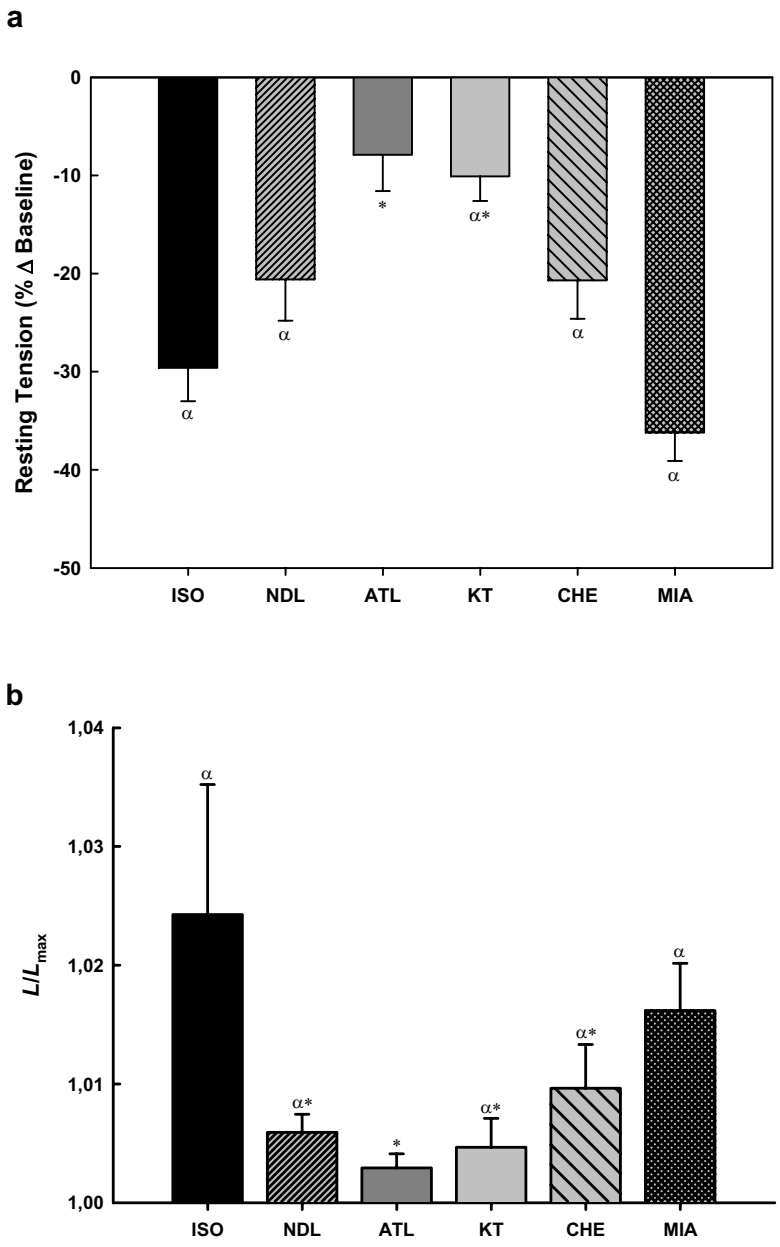


Fig. 6 - Effects of isoprenaline (ISO, 10^{-5}M) on **a** resting tension and **b** resting muscle length (L/L_{max}) in the absence (n=8) or presence of a non-selective β-adrenoceptor antagonist (NDL, 10^{-5}M; n=7), a β_1-adrenoceptor antagonist (ATL, 2.10^{-5}M; n=8), a PKA inhibitor (KT, 10^{-6} M, n=6), a PKC inhibitor (CHE, 10^{-5}M; n=6) or a NHE inhibitor (MIA, 10^{-6}M; n=8). Data are means ± S.E.M., expressed as percent variation from baseline. $P< 0.05$: α *vs* baseline, * *vs* ISO alone.

4. DISCUSSION

The present study addresses the changes in the passive properties of the myocardium of rabbit papillary muscles in response to β-adrenoceptor activation by isoprenaline. This study clearly demonstrates that β-adrenergic stimulation induces, besides the well-documented positive inotropic and lusitropic effects (Bers, 2002), a significant concentration-dependent acute increase in myocardial distensibility dependent on the activation of β_1-adrenoceptor, PKA and PKC.

The stimulation of β-adrenoceptor by the sympathetic nervous system plays a pivotal role in regulating myocardial function and morphology in the normal and failing heart. Several studies focusing the lusitropic effects of β-adrenergic stimulation support that crossbridge cycle and several other phosphorilatable events are the major determinants of the intrinsic rate of myocardial relaxation (Bronzwaer and Paulus, 2005). However, changes in passive proprieties of myocardium induced by β-adrenergic stimulation remain to be clarified. In fact, together with myocardial relaxation, passive properties of the ventricular wall, such as myocardial stiffness, wall thickness and chamber geometry (size or volume) are the major determinants of diastolic function.

The traditional view on cardiac β-adrenoceptor signal transduction is that, under physiological conditions, catecholamines induce positive inotropic, lusitropic, and chronotropic responses through the β_1-adrenoceptor-mediated activation of the Gs-adenylate cyclase-cAMP-PKA pathway. However, besides β_1-adrenoceptor, two other genetically and pharmacologically distinct β-adrenoceptor subtypes, β_2-adrenoceptor and β_3-adrenoceptor are also identified in various types of cells. Both β_1- and β_2-adrenoceptors are coupled to the Gs-adenylyl cyclase-cAMP-PKA pathway, but the β_2-adrenoceptor is also responsible for the activation of pertussis toxin-sensitive Gi proteins (Kilts *et al.*, 2000; Xiao *et al.*, 1999), and each type exhibits significantly different signal transduction

mechanisms as previously outlined (Steinberg, 1999; Xiao, 2001). Both types of β-adrenoceptors are present in rabbit ventricular myocytes (Marian, 2006), and the failing rabbit heart exhibits molecular changes in β-adrenergic signaling similar to those observed in human heart failure (Maurice *et al.*, 1999), which makes it a suitable experimental model to study β-adrenergic stimulation.

In the current study, we first evaluated if the classical pathways (β-adrenoceptor and PKA) were involved in the modulation of myocardial stiffness. To determine whether the activation of PKA is required for isoprenaline-induced increase of myocardial distensibility, we examined the effect of KT5720, a highly selective inhibitor of PKA (Bishopric *et al.*, 1992; Haikala *et al.*, 1997; Iwai-Kanai *et al.*, 1999; Kiehn *et al.*, 1998). This inhibitor was not able to alter the inotropic and lusitropic effects of isoprenaline. In line with our results, a previous study in guinea pig cardiac muscles reported that the same agent did not block the inotropism induced by β-adrenergic stimulation (Gotoh, 1995). Another study suggested that β-adrenoceptor stimulation might increase the peak L-type Ca^{2+} current via PKA-independent activation of Ca^{2+} channels (Yatani *et al.*, 1999). Furthermore, Curran and collaborators demonstrated that β-adrenergic stimulation increases calcium leak from sarcoplasmatic reticulum via calcium/calmodulin-dependent protein kinase (Curran *et al.*, 2007). Actually, the effects of sustained β_1-adrenoceptor stimulation (inotropy, cell growth and cell death) are primarily due to this pathway, rather than PKA signaling (Wang *et al.*, 2004; Zhu *et al.*, 2003). So, under certain physiological and pathological circumstances, this "non-classical" signaling pathway becomes more relevant (Singh *et al.*, 2001; Xiao, 2001). All these results corroborate the hypothesis that inotropic and lusitropic effects induced by β-adrenoceptor stimulation cannot be explained exclusively by cAMP-dependent mechanisms.

The present study demonstrates that β_1-adrenoceptor activation modulates distensibility via PKA activation. Additionally, it was already shown that increased PKA activity lowers passive stiffness also in engineered rat heart tissue (Zimmermann *et al.*, 2002) and in failing human cardiac cells (Borbely *et al.*, 2005; van Heerebeek *et al.*, 2006). This PKA-mediated decrease in passive myocardial stiffness is potentially relevant from a pathophysiologic point of view, as impairment of β-adrenergic signalling in heart failure may contribute to diastolic dysfunction in this syndrome.

In previous studies acute modulation of myocardial stiffness by angiotensin II and endothelin-1 was shown to be mediated by PKC and NHE activation (Leite-Moreira *et al.*, 2003; Leite-Moreira *et al.*, 2006). We therefore investigated their role on the effects of β-adrenergic stimulation on myocardial distensibility and found that indeed they are modulated by PKC.

Myocardial stiffness is determined by cytoskeleton of cardiomyocytes and the extracellular matrix (Kass *et al.*, 2004). Most of the elastic force of the cardiomyocytes is now thought to reside in the cytoskeletal protein, titin (Kass *et al.*, 2004). Changes in its isoform composition and phosphorylation status have been shown to alter diastolic function and myocardial passive properties (Kruger and Linke, 2006; LeWinter et al., 2007). Based on these evidences, we are tempted to propose that the acute increase of distensibility induced by isoprenaline could be attributed to changes in titin phosphorylation status since the other mentioned mechanisms can only be modulated chronically. Nevertheless, this aspect needs further investigation.

Finally, concerning the pathophysiologic relevance of our findings, we must point out that a decrease of 30% in passive tension of the isolated muscle indicates that β-adrenergic stimulation might allow the ventricle to reach high filling volumes at almost one third lower filling pressures, which is undoubtedly a quite powerful adaptation

mechanism. These acute beneficial effects of β-adrenergic stimulation on diastolic function are overcome by its role in progression to cardiac fibrosis and ventricular remodeling when its levels remain chronically elevated (Benjamin *et al.*, 1989; Ponicke *et al.*, 2003).

In conclusion, besides its well-known effects in myocardial contractility, the present study highlights the new effect of β-adrenergic stimulation on myocardial stiffness decrease. This effect requires the activation of β_1-adrenoceptor, and is mediated by PKA and PKC. This novel effect of β-adrenoceptor stimulation broadens our concepts with regard to the acute neurohumoral modulation of diastolic function and represents a potentially powerful regulator of cardiac filling.

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CAPÍTULO IV

NOVOS MEDIADORES NEURO-HUMORAIS

PARTE A: ADRENOMEDULINA COMO UM NOVO REGULADOR DA RIGIDEZ MIOCÁRDICA

Submitted to *Peptides*

Adrenomedullin as a novel regulatory peptide of myocardial stiffness: contribution of endocardial endothelium and nitric oxide

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Running Title: Adrenomedullin decreases myocardial stiffness

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Abstract

Adrenomedullin (AM) effects were studied in rabbit papillary muscles by adding increasing concentrations (10^{-10} to 10^{-6}M) either alone or after pre-treatment with L-NNA, indomethacin, AM22-52 (AM receptor antagonist), KT5720 (PKA inhibitor), as well as after endocardial endothelium (EE) removal. Passive length-tension relations were constructed before and after a single concentration of AM (10^{-6}M).

AM concentration-dependently induced negative inotropic and lusitropic effects, and increased resting muscle length (RL). At 10^{-6}M, AT, dT/dt_{max} and dT/dt_{min} decreased 20.9±4.9%, 18.3±7.3% and 16.7±7.8%, respectively, and RL increased to 1.010±0.004 L/L_{max}. Correcting RL to its initial value resulted in a 26.6±6.4% decrease of resting tension, indicating decreased muscle stiffness, also patent in the down and rightward shift of the passive length-tension relation. The negative inotropic effect of AM was dependent on its receptor, PKA, the EE and NO, while the effects of AM on myocardial stiffness were abolished by EE damage and NO inhibition. This latter effect represents a novel mechanism of acute neurohumoral modulation of diastolic function, suggesting that AM is an important regulator of cardiac filling.

Keywords: Adrenomedullin; inotropism; myocardial stiffness; endocardial endothelium; NO

1. Introduction

Adrenomedullin (AM) is a peptide identified and isolated from human pheochromocytoma [22], and initially annotated as a vasodilator peptide. AM acts as a circulating hormone, which elicits various biological activities in a paracrine or autocrine manner.

Human AM (hAM) is a 52 amino acid peptide with structural homology to calcitonin gene–related peptide (CGRP) [22]. AM is produced in several tissues (kidney, lung, and heart) [23], and its production is upregulated by several factors such as oxidative stress, pro-inflammatory cytokines, angiotensin II, hypoxia, hyperglycemia, infusion of natriuretic peptide, and aldosterone, among other factors [1].

There is increasing experimental and clinical evidence in support of an important role of AM in the pathophysiology of a variety of cardiovascular diseases. In spite of its relatively low plasmatic levels [22], various clinical studies have shown that they correlate with the severity of diseases, such as heart failure (HF), acute myocardial infarction, and hypertension [18, 19, 31, 35, 37].

At the cardiovascular level, AM can be synthesized and secreted from various cells, including vascular endothelial cells, vascular smooth muscle cells, cardiomyocytes and fibroblasts [1, 10]. Furthermore, AM and its receptors are expressed in the normal and failing myocardium [39, 41].

In normal animals [42] and in an ovine model of pacing-induced HF [48], AM was shown to reduce peripheral resistance and to increase cardiac output. These data have led investigators to suggest that AM may be involved in the control of cardiac function and that AM is activated in HF to modulate the opposing effects of the vasoconstricting and sodium-retaining factors ET-1 and angiotensin II.

The direct myocardial effects of AM remain, however, largely unknown. With regard to contractility, positive [16, 55], negative [15, 17, 30, 33, 47], and no significant [36, 49, 50, 54] inotropic effects have been reported. On the other hand, its effects on the diastolic properties of the myocardium were not yet investigated. Recent evidences have shown that these properties and more specifically myocardial stiffness can be acutely modulated by nitric oxide (NO) [52], ET-1 [26], angiotensin II [27] and urotensin II [11].

Diastolic HF has emerged over the last two decades as a separate clinical entity. Approximately half of the patients presenting with symptoms of congestive HF exhibit a near normal left ventricular systolic function at rest, which is thought to be caused by a predominant abnormality in diastolic function [44]. Determinants of diastolic function include myocardial relaxation and passive properties of the ventricular wall, such as myocardial stiffness, wall thickness and chamber geometry (size or volume). Other determinants include the structures surrounding the ventricle, the left atrium, pulmonary veins and mitral valve, and heart rate [25].

So, the present study was conducted to characterize the systolic and diastolic myocardial effects of AM and to clarify the intracellular pathways that underlie them.

2. Materials and methods

2.1 Animals and tissue preparation

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication Nº 85-23, Revised 1996).

2.2 Myocardial effects of adrenomedullin

2.2.1 Experimental preparation

Isometric and isotonic contractions were measured in papillary muscles isolated from the right ventricle of rabbits. Male New Zealand White rabbits (*Oryctolagus cuniculus*; 1.3–2.6kg; *n*=34) were anesthetized with intravenous sodium pentobarbital (25mgkg^{-1}). A left thoracotomy was performed, and beating hearts were quickly excised and immersed in a modified Krebs-Ringer (KR) solution (composition in mM: 98 NaCl, 4.7 KCl, 2.4 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 4.5 glucose, 1.8 $CaCl_2.2H_2O$, 17 $NaHCO_3$, 15 sodium pyruvate, 5 sodium acetate, 0.02 atenolol) at 35ºC with cardioplegic 2,3-butanedione monoxime (BDM; 3%) and 5% Newborn Calf Serum. Atenolol was used to prevent β-adrenergic mediated effects. The solutions were in equilibrium with 95% O_2 and 5% CO_2, to obtain a pH between 7.38-7.42.

The time from thoracotomy to dissection was ~3 min. The right ventricle was opened and papillary muscles were isolated by first dividing the chordae tendinae at the muscle tip and then freeing the muscle base and a small amount of surrounding myocardium from the ventricular wall. Only long, thin, uniformly cylindrical muscles were used. After dissection, papillary muscles (*n*=55; length: 4.8±0.2mm; weight: 3.7±0.2mg; preload: 3.4±0.1mN) were mounted vertically in a 10ml plexi glass organ bath containing the aforementioned KR solution. The lower muscular end was fixed in a phosphorbronze clip, and the upper tendinous end was attached to an electromagnetic length–tension transducer (University of Antwerp, Belgium).

Preload was initially set between 3 and 4 mN according to muscle dimensions. The preparations were stimulated at 0.6 Hz with a voltage of 10% above threshold (typically 3-6 mV) by rectangular pulses of 5 ms duration through two platinum electrodes arranged longitudinally alongside the entire muscle. After 20 min later, bathing solutions were

replaced by corresponding KR solutions without BDM and the muscle started to contract. One hour later, bathing solution was replaced by corresponding serum-free KR solution. During the next 2 hours, the muscles were stabilized. Finally, the muscles were stretched to a muscle length at which active force development was maximal. At this point, this length (mm) known as maximum physiological length (L_{max}) was measured with a microruler. During the experiment, changes in diastolic muscle length and muscle shortening were measured by the isotonic transducer. Protocols were initiated after obtaining two similar isotonic and isometric control twitches separated by a 10 min interval.

At the end of the experiment the muscles were removed, lightly blotted and then weighed. Muscle cross-sectional area was calculated by dividing the weight of the muscle by its length at L_{max}. A cylindrical shape and a specific gravity of 1.0 were assumed [11]. Muscle tension was then expressed as force normalized per cross-sectional area ($mNmm^{-2}$).

2.2.2 Experimental protocols

Effects of increasing concentrations of human AM-(1-52) ($C_{264}H_{406}N_{80}O_{77}S_3$) (AM; 10^{-10} to 10^{-6} M) on contraction, relaxation, and diastolic properties of the myocardium were studied in rabbit papillary muscles in the following conditions: **A.** control muscles with intact endocardial endothelium (EE), **B.** after selective removal of EE by a brief (1 s) immersion of the papillary muscle in a weak solution (0.5%) of the detergent Triton X-100 [4, 5], followed by abundant wash with Triton-free KR solution, and **C.** in muscles with intact EE in the presence of: (i) N^G-Nitro-L-Arginine (L-NNA; 10^{-5} M), a NO synthase inhibitor; (ii) indomethacin (Indo; 10^{-5} M), a cyclooxygenase inhibitor; (iii) human AM-(22-52) ($C_{159}H_{252}N_{46}O_{48}$) (AM22-52; 10^{-6} M), an antagonist of AM receptor; and (iv) KT5720 (KT, 10^{-6} M), an inhibitor of PKA. These substances were dissolved in the KR solution before the addition of AM, and muscle twitches were recorded after a stable

response was obtained, typically 15-20 min later. After that, AM was added cumulatively without any washout between. Finally, in another subset of muscles, passive length-tension relations were constructed in the absence and in the presence of the highest concentration of AM. Of note, that in each experimental protocol, all papillary muscles were obtained from different animals.

2.2.3 Data acquisition and analysis

Isotonic and isometric twitches were recorded and analyzed with dedicated software (University of Antwerp, Belgium). Selected parameters included: resting tension (RT; $mNmm^{-2}$), active tension (AT; $mNmm^{-2}$); maximal velocities of tension rise (dT/dt_{max}; $mNmm^{-2}s^{-1}$) and decline (dT/dt_{min}; $mNmm^{-2}s^{-1}$); peak isotonic shortening (PS; $\%L_{max}$); maximal velocities of shortening (dL/dt_{max}; $L_{max}s^{-1}$) and lengthening (dL/dt_{min}; $L_{max}s^{-1}$); time to half-relaxation (tHR, ms); and time to active tension (tAT; ms).

In the various protocols, results are given as percent change from baseline. For the parameters that are expressed as negative values (e.g. dT/dt_{min}) such percent change refers to the absolute values. When a pharmacological inhibitor was used or the EE damaged, the term baseline refers to the performance in the presence of those inhibitors or after damage of EE, before the addition of AM.

2.3 Drugs and materials

Drugs were obtained from the following sources: human AM-(1-52) and human AM-(22-52): Bachem (Bubendorf, Switzerland); all other chemicals: Sigma Chemical Co (St Louis, MO, USA). Stock solutions of all chemicals were dissolved in distilled water and stored at -20 ºC until use.

2.4 Statistical methods

Values are presented as means ± standard error of mean (S.E.M.) and *n* represents the number of experiments. Effects of increasing concentrations of AM alone on the different experimental parameters were analyzed by one-way repeated-measures ANOVA. Effects of increasing concentrations of AM under various experimental conditions were analyzed with a repeated-measures two-way ANOVA. Effects on the various parameters of a single concentration of the antagonists were analyzed with a paired t-test. When significant differences were detected with any of the ANOVA tests, the Student-Newman-Keuls test was selected to perform pairwise multiple comparisons. $P < 0.05$ was accepted as significant.

3. Results

Morphometric characteristics and baseline performance of papillary muscles did not vary significantly between the different experimental groups (means ± S.E.M. presented in Table 1). Concentration-response curves to AM in the various experimental conditions are illustrated in Figs. 1-6.

Table 1 – Morphologic and contractile characterization of papillary muscles (n = 55)

Parameter	Value
Length (mm)	4.8 ± 0.2
Weight (mg)	3.7 ± 0.2
Preload (mN)	3.4 ± 0.1
AT (mN/mm^2)	23.0 ± 1.6
dT/dt_{max} ($mN/mm^2/s$)	147.2 ± 11.2
dT/dt_{min} ($mN/mm^2/s$)	- 124.8 ± 8.2
tHR (ms)	413.3 ± 10.2
tAT (ms)	263.3 ± 6.6

AT: active tension; dT/dt_{max}, dT/dt_{min}: maximum velocity of tension rise and decline, respectively; tHR: time for half relaxation; tAT: time to active tension. Values are means ± S.E.M.

Effects of increasing concentrations of AM on myocardial contractility (inotropy) and relaxation (lusitropy) are illustrated in Fig. 1, where it can be seen that these concentrations decreased both contractility (AT and dT/dt_{max}) and lusitropy (dT/dt_{min}). The highest concentration of AM (10^{-6}M) decreased 20.9±4.9% AT, 18.3±7.3% dT/dt_{max}, 16.7±7.8% dT/dt_{min}, 11.9±3.8% PS, 13.7±4.8% dl/dt_{max}, 10.9±5.3% dl/dt_{min} ($P < 0.05$). Effects on tHR and tAT (onset of relaxation) were not statistically significant.

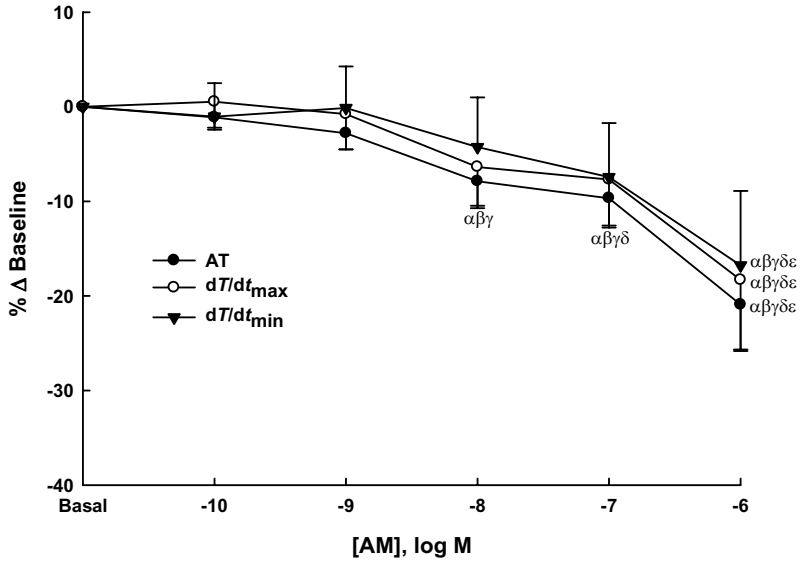

Fig. 1 - Effect of increasing concentrations of adrenomedullin (AM, 10^{-10} to 10^{-6} M, $n = 9$) on active tension (AT) and peak rates of tension rise and decline (dT/dt_{max} and dT/dt_{min}, respectively). $P< 0.05$: α vs. baseline, β vs. 10^{-10} M AM, γ vs. 10^{-9} M AM, δ vs. 10^{-8} M AM, ε vs. 10^{-7} M AM.

With regard to the diastolic properties of the myocardium, we observed that AM progressively increased resting muscle length (Fig. 2) at a constant resting tension. Correcting muscle length, at the end of the experiment, to its initial value resulted in a 26.6 ± 6.4% decrease of resting tension, without altering the other contractile parameters. These

results indicate an increase in muscle distensibility, or on the other hand, a decrease in muscle stiffness. This aspect is further explored in Fig. 3 where passive length–tension relations at baseline and in the presence of AM (10^{-6}M) are depicted. In this figure, it can be seen that this relation is right and downward shifted by AM. In other words, at each resting tension, muscle length was always significantly greater in the presence of AM, indicating that this peptide acutely increases distensibility and lowers stiffness of the myocardium.

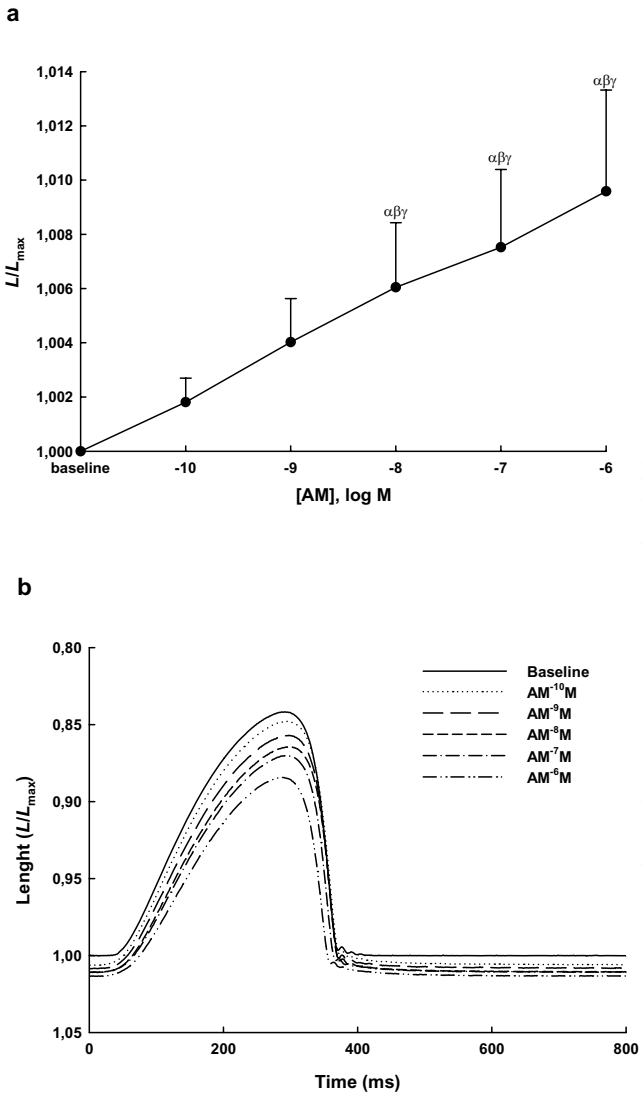

Fig. 2 - Effect of increasing concentrations of adrenomedullin (AM, 10^{-10} to 10^{-6} M, $n = 9$) on **a** resting muscle length (L/L_{max}). Data are mean ± S.E.M., expressed as percent variation from baseline. $P < 0.05$: α vs. baseline, β vs. 10^{-10} M AM, γ vs. 10^{-9} M AM, δ vs. 10^{-8} M AM, ε vs. 10^{-7} M AM. Panel **b** shows a representative example of isotonic twitches at baseline and in the presence of increasing concentrations of AM.

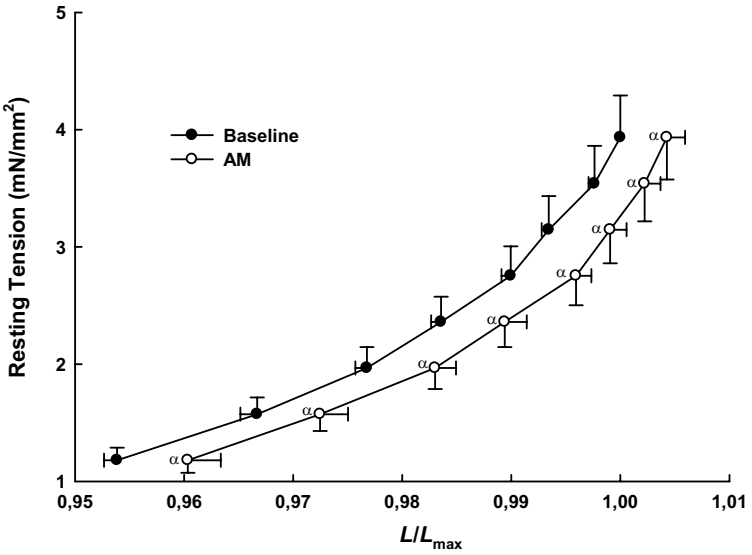

Fig. 3 - Passive length-tension relations at baseline and in the presence of adrenomedullin (AM, 10^{-6}M, *n*=6). Data are mean ± S.E.M. $P< 0.05$: α vs. baseline.

Effects of AM after damaging the EE, in presence of a selective AM receptor antagonist (AM22-52), or after inhibition of cyclooxygenase (Indo), NO synthase (L-NNA), or PKA (KT) are illustrated in Fig. 4, 5 and 6. While AM22-52, Indo and KT did not significantly modify *per se* any of the analyzed contractile parameters, selective destruction of the EE or the presence of L-NNA resulted in a significant decrease of AT by 33.1±5.6% and 5.8±2.4%, dT/dt_{max} by 31.5±6.4 and 4.6±3.1% and dT/dt_{min} by 27.0±6.8 and 6.5±3.4%, respectively.

The myocardial effects of AM were also significantly altered by these agents. For instance, the negative inotropic effect of AM was abolished when the EE was damaged or in the presence of L-NNA (Fig. 4a). Furthermore, in the latter condition the effects of AM on passive muscle length were no more statistically significant, having been totally abolished when the EE was damaged (Figs. 4b and 6). On the other hand, both AM22-52 and KT blunted the negative inotropic effect of AM (Fig. 5a), but did not alter the effect of

AM on resting length and tension (Figs. 5b and 6). Finally, none of the effects of AM altered by Indo (Figs. 4a, 4b and 6).

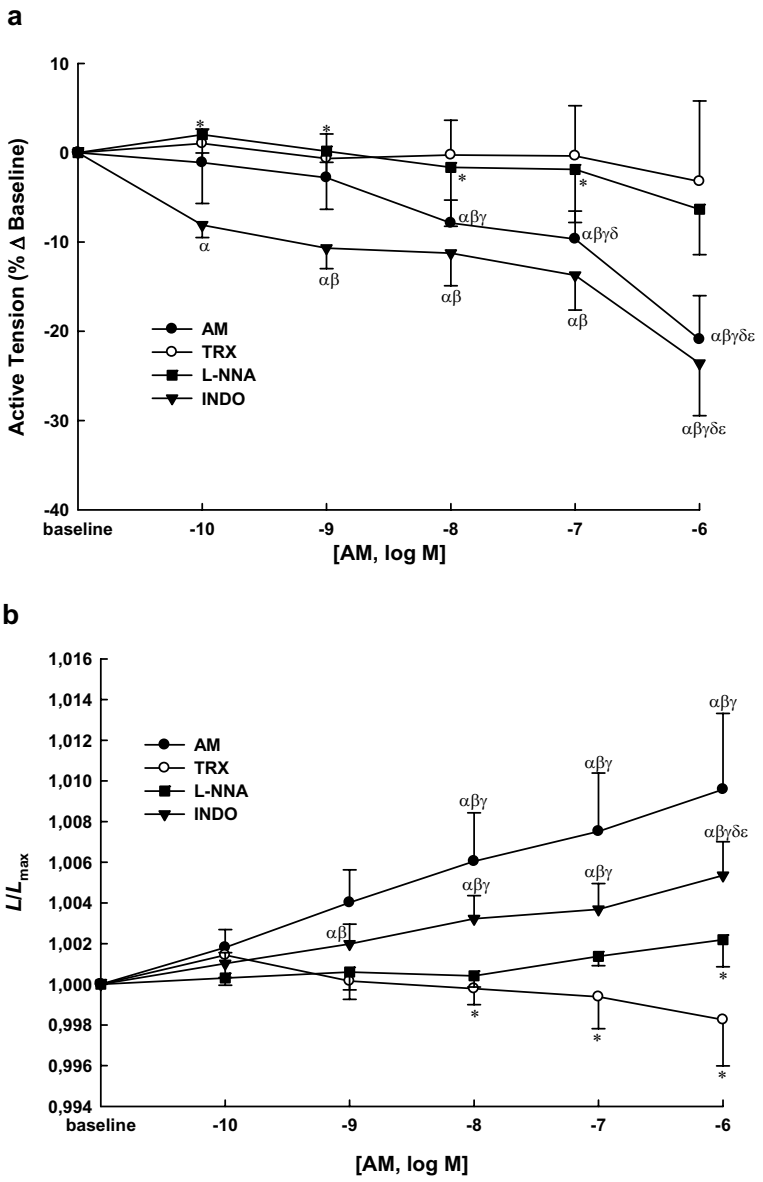

Fig. 4 - Effect of increasing concentrations of adrenomedullin (AM, 10^{-10} to 10^{-6} M) on **a** active tension and **b** passive muscle length (L/L_{max}) in the absence (n=9) or presence of damaged endocardial endothelium (TRX, n=9), NO synthase inhibition (L-NNA, 10^{-5} M, n=7), cyclooxygenase inhibition (INDO, 10^{-5} M, n=9). Data are mean ± S.E.M., expressed as percent variation from baseline. $P< 0.05$: α vs. baseline, β vs. 10^{-10} M AM, γ vs. 10^{-9} M AM, δ vs. 10^{-8} M AM, ε vs. 10^{-7} M AM, * vs. AM alone.

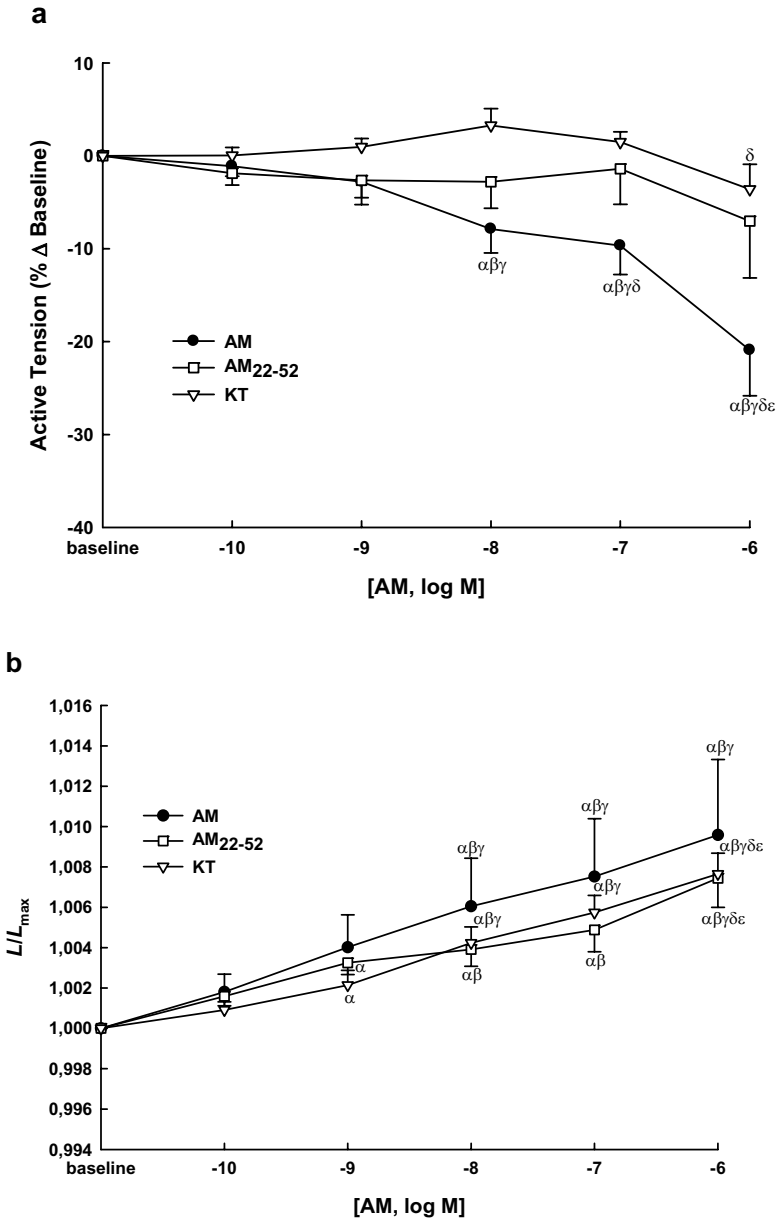

Fig. 5 - Effect of increasing concentrations of adrenomedullin (AM, 10^{-10} to 10^{-6} M) on **a** active tension and **b** passive muscle length (L/L_{max}) in the absence (n=9) or presence of selective AM receptor antagonist (human AM-(22-52)) (AM22-52, 10^{-6} M, n=8) or PKA inhibitor (KT5720) (KT, 10^{-6} M, n=7). Data are mean ± S.E.M., expressed as percent variation from baseline. $P< 0.05$: α vs. baseline, β vs. 10^{-10} M AM, γ vs. 10^{-9} M AM, δ vs. 10^{-8} M AM, ε vs. 10^{-7} M AM, * vs. AM alone.

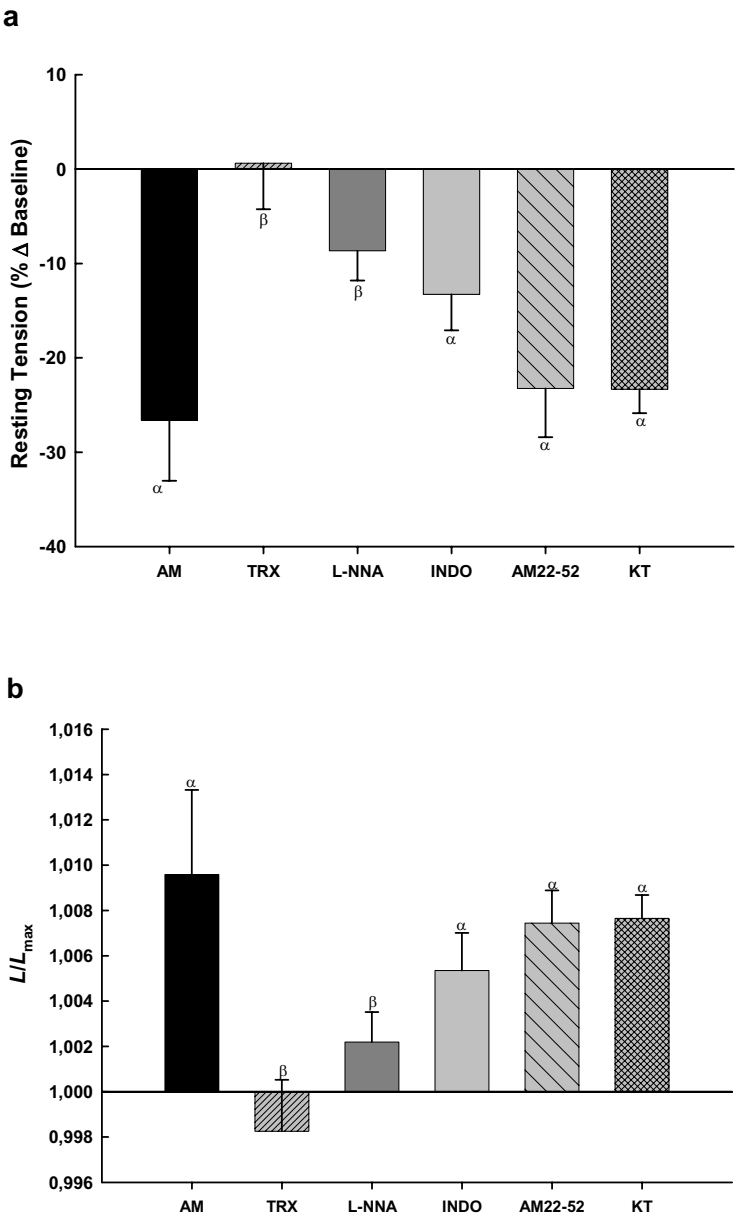

Fig. 6 - Effects of adrenomedullin (AM, 10^{-10} to 10^{-6} M) on **a** resting tension and **b** resting muscle length (L/L_{max}) in the absence (n=9) or presence of damaged endocardial endothelium (TRX, n=9), NO synthase inhibition (L-NNA, 10^{-5} M, n=7), cyclooxygenase inhibition (INDO, 10^{-5} M, n=9), selective AM receptor antagonist (human AM-(22-52)) (AM22-52, 10^{-6} M, n=8), or PKA inhibitor KT5720 (KT, 10^{-6} M, n=7). Data are means ± S.E.M., expressed as percent variation from baseline. $P< 0.05$: α vs. baseline, β vs. AM alone.

4. Discussion

The present study shows that AM induces significant concentration-dependent negative inotropic and lusitropic effects, and an acute increase in myocardial distensibility. The former effects are completely abolished by AM receptor blockade, PKA inhibition, EE removal or NO synthase inhibition. In contrast, the effect of AM on myocardial distensibility was no more observed when the EE was damaged or NO synthase inhibited. These observations suggest that this novel effect of AM requires an intact EE and is dependent of NO release.

A negative inotropic effect of AM was previously found in vitro [17, 33, 47], which is in line with our results. Nevertheless, this effect is apparently in disagreement with data from some other in vitro [16, 55] and in vivo [34, 40] studies, in which acute AM infusion increased cardiac index and stroke volume index. In vivo, this increase in cardiac output has been primarily attributed to a fall in cardiac afterload as a result of decreasing mean arterial pressure. A lack of inotropic and lusitropic effects of AM has also been reported in normal and heart-failure dogs [24]. Reasons for these discrepancies between studies presumably include species differences and distinct experimental models.

Though the major signal transduction pathway activated by AM appears to be Gs-mediated adenylate cyclase/cAMP/PKA system [16], not all effects of AM can be explained by this pathway [14]. A previous study suggested a contribution of NO to the negative inotropic effect promoted by AM in adult rabbit cardiac ventricular myocytes, which decreased intracellular Ca^{2+} concentration through a cGMP-dependent mechanism [17]. In the present study, besides NO and PKA, the negative inotropic effect of AM was also modulated both by its receptor and by the endocardial endothelium. Although the activation of the adenylate cyclase-cAMP system is one of the major pathways for the stimulation of cardiac contractility in the mammalian hearts [32], a recently published study observed a switch from G_s coupling to PKA-dependent G_i coupling with AM. This

resulted in a shift from positive inotropy to negative inotropy, which was time dependent and dose dependent [29] and is consonant with our results.

Likewise other neurohumoral agents, such as NO [45], ET-1 [26], angiotensin II [27] and urotensin II [11], we observed that AM acutely modulates myocardial stiffness, which is an important determinant of ventricular filling and, therefore, of diastolic function [25]. This effect was significantly blunted by EE removal and by inhibition of NO. The EE has also been involved in the effect on distensibility of some of these neurohumoral agents [3, 7, 46]. Similarly to vascular endothelial dysfunction [8], it seems that cardiac endothelial dysfunction is present and/or may contribute to HF progression [2]. So, considering that cardiac endothelium, both vascular and endocardial, regulates performance of underlying cardiac muscle, the results of the present study could help to better understand the physiopathology of HF.

Since NO is one of the most important endothelial mediators and AM activates endothelial nitric oxide synthase (eNOS) activity [38, 53], we investigated how this agent modulates AM effects. We found that after blocking NO release, AM-induced increase in resting muscle length (enhanced myocardial distensibility) was no more observed. In fact, it has been previously suggested that NO has an important role not only in the regulation of cardiac contractility [21], but also in the increase of diastolic distensibility [43, 45].

Specific AM receptors coupled to stimulation of adenylyl cyclase have been reported in myocardial tissue [20]. In addition, there is evidence for receptor sites that bind both AM and CGRP with fairly high affinity [57]. It was recently shown that the calcitonin receptor–like receptor (CRLR) can function either as an AM receptor or as a CGRP receptor, depending on the expression of different members of a novel family of single-transmembrane-domain proteins called receptor-activity–modifying proteins (RAMPs) [28, 56]. So far, the RAMP family has been shown to consist of three isoforms: RAMP1,

RAMP2 and RAMP3 [13, 28, 51]. Thus, the combination of CRLR plus RAMP2 results in an AM receptor 1 (AM_1), whereas CRLR co-expression with RAMP3 results in an AM receptor 2 (AM_2) [6, 12].

The AM peptide fragment AM22-52 has been described as an antagonist of both AM_1 and AM_2 receptors [9]. In the present study, AM was observed to promote a negative inotropic effect and an increase of myocardial distensibility, through the activation of AM22–52 sensitive and insensitive receptors, respectively. Since AM22-52 is a more selective antagonist at the AM_1 (CRLR/RAMP2) than at the AM_2 (CRLR/RAMP3) receptor [13], we hypothesize that the increase in myocardial distensibility induced by AM is possibly modulated by the AM_2 rather than by the AM_1 receptor. In contrast, the negative inotropic effect is most likely the result of AM_1 receptor activation, although further studies are needed to clarify these issues.

Finally, concerning the pathophysiologic relevance of our findings, we must point out that a decrease of 27% in passive tension of the isolated muscle indicate that AM might allow the ventricle to reach the same diastolic volume with almost 30% lower filling pressures, which is undoubtedly a potentially important adaptation mechanism. As the acute effects of AM on diastolic function were determined in an in *vitro* model, it allows determining the effects of AM on intrinsic myocardial diastolic properties, excluding those resulting from load and coronary tonus changes. However, the effects of AM in *vivo*, where other important adaptation mechanisms also affect diastolic filling pressures, may differ from those reported here.

On the other hand, the results of the present study emphasize that humoral influences on diastolic cardiac function are modulated by the interaction with endocardial endothelial and its mediators, such as NO, which being altered in the failing heart might provide new elements for the comprehension of the pathophysiology of HF.

5. Conclusions

Since its discovery, there has been great interest in AM as a promising endogenous peptide for the treatment of cardiovascular diseases. The present study provided new insights into the direct cardiac actions of AM. It described, for the first time, the modulation of diastolic function by AM, which represents a potentially powerful regulator of cardiac filling. These findings might improve our understanding about the role of AM, namely on diastolic function, which has been greatly overlooked in most studies.

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CAPÍTULO IV

NOVOS MEDIADORES NEURO-HUMORAIS

PARTE B: EFEITOS MIOCÁRDICOS DA UROTENSINA-II

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ORIGINAL ARTICLE

Urotensin II acutely increases myocardial length and distensibility: potential implications for diastolic function and ventricular remodeling

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Abstract Urotensin II (U-II) is a cyclic peptide that may be involved in cardiovascular dysfunction. In the present study, the acute effects of U-II on diastolic properties of the myocardium were investigated. Increasing concentrations of U-II (10^{-8} to 10^{-6} M) were added to rabbit papillary muscles in the absence (n=15) or presence of: (1) damaged endocardial endothelium (EE; n=9); (2) U-II receptor antagonist, urantide (10^{-5} M; n=7); (3) nitric oxide (NO) synthase inhibitor, N^G-Nitro-L-Arginine (10^{-5} M; n=9); (4) cyclooxygenase inhibitor, indomethacin (10^{-5} M; n=8); (5) NO synthase and cyclooxygenase inhibitors, N^G-Nitro-L-Arginine (10^{-5} M) and indomethacin (10^{-5} M), respectively, (n=8); or (6) protein kinase C (PKC) inhibitor, chelerythrine (10^{-5} M; n=9). Passive length–tension relations were constructed before and after a single concentration of U-II (10^{-6} M; n=3). U-II concentration dependently decreased inotropy and increased resting muscle length (RL). At 10^{-6} M, active tension decreased 13.8±5.4%, and RL increased to 1.007±0.001 L/L_{max}. Correcting RL to its initial value resulted in an 18.1±3.0% decrease in resting tension, indicating decreased muscle stiffness, which was also suggested by the down and rightward shift of the passive length–tension relation. This effect remained unaffected by EE damage and PKC inhibition. In contrast, the presence of urantide and NO inhibition abolished the effects of U-II on myocardial stiffness, while cyclooxygenase inhibition significantly attenuated them. U-II decreases myocardial stiffness, an effect that is mediated by the urotensin-II receptor, NO, and prostaglandins. This represents a novel mechanism of acute neurohumoral modulation of diastolic function, suggesting that U-II is an important regulator of cardiac filling.

Keywords Urotensin II · Diastolic function · Myocardial distensibility · Myocardial stiffness · NO · Prostaglandins · UT receptor

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Introduction

Urotensin II (U-II) is a vasoactive peptide, first isolated from the urophysis of teleost fish (Bern et al. 1985), and recently cloned in several mammalian species, including humans (Conlon et al. 1996; Coulouarn et al. 1998, 1999; Douglas et al. 2000). U-II acts by binding to G-protein-coupled receptors that were first identified in the rat (GPR14; Marchese et al. 1995; Tal et al. 1995) and later in humans [urotensin-II (UT) receptor; Ames et al. 1999]. The G-protein associated with the UT receptor belongs to the Gq class (Opgaard et al. 2000), which is the same class of G-proteins that bind to AT1, ET_A, and α-adrenergic receptors (Wheeler-Jones 2005).

U-II has been shown to have potent vasoactive properties depending on the vascular bed and the species tested (Bohm and Pernow 2002; Bottrill et al. 2000; Camarda et al. 2002; Douglas et al. 2000; Gardiner et al. 2001; Russell and Molenaar 2004; Stirrat et al. 2001). Additionally, U-II (Douglas et al. 2002; Matsushita et al. 2001), as well as its receptor (Ames et al. 1999), is highly expressed in the heart (cardiomyocytes) and blood vessels. Taking into consider-

ation these facts, several experimental and clinical studies evaluated whether this peptide plays a role in cardiovascular regulation and the pathophysiology of heart failure (Douglas et al. 2002; Dschietzig et al. 2002; Gong et al. 2004; Johns et al. 2004; Russell et al. 2001, 2003; Tzanidis et al. 2003). However, the role of U-II within the myocardium remains poorly understood, particularly in the setting of disease.

Furthermore, U-II was reported to affect the process of cell growth in the heart. This peptide exerted mitogenic effects on smooth muscle cells (Sauzeau et al. 2001; Watanabe et al. 2001), induced collagen and fibronectin synthesis by cardiac fibroblasts, and caused cardiac hypertrophy (Tzanidis et al. 2003), thereby contributing to ventricular remodeling and deterioration of systolic and diastolic function, similarly to what has been described for other vasoconstrictor peptides such as angiotensin II (Ang II) and endothelin-1 (ET-1; Weber et al. 1994). These chronic effects have classically been considered the main mechanisms through which neurohumoral agents may influence the diastolic properties of the myocardium. However, some of these agents have been, over recent years, shown to acutely modulate myocardial stiffness. These include nitric oxide (NO; Heymes et al. 1999; Ito et al. 1997; Shah et al. 1994), ET-1 (Leite-Moreira et al. 2003), and Ang II (Leite-Moreira et al. 2006) but not ghrelin (Soares et al. 2006). In isolated cardiomyocytes, an increase in diastolic cell length is observed after exposure to a cGMP analogue or a NO donor, and in intact hearts, NO shifts downward the diastolic pressure–volume loop during filling, both indicating increased myocardial distensibility.

To further clarify this issue, we conducted the present study in rabbit papillary muscle with the aim of characterizing the diastolic effects of U-II and some of their underlying mechanisms. A preliminary report has recently appeared (Fontes-Sousa et al. 2006).

Material and methods

Animals and tissue preparation

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication number 85-23, Revised 1996).

Functional experiments

Experimental preparation

Isometric and isotonic contractions were measured in papillary muscles isolated from the right ventricle of rabbits. Male New Zealand white rabbits (*Oryctolagus cuniculus*; 1.4–2.7 kg; n=53) were anesthetized with intravenous sodium pentobarbital (25 mg kg^{-1}). A left thoracotomy was performed, and beating hearts were quickly excised and immersed in a modified Krebs–Ringer (KR) solution (composition in millimolar, 98 NaCl, 4.7 KCl, 2.4 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 4.5 glucose, 1.8 $CaCl_2 \cdot 2H_2O$, 17 $NaHCO_3$, 15 sodium pyruvate, 5 sodium acetate, and 0.02 atenolol) at 35°C with cardioplegic 2,3-butanedione monoxime (BDM; 3%) and 5% Newborn Calf Serum. Atenolol was used to prevent β-adrenergic mediated effects. The solutions were in equilibrium with 95% O_2 and 5% CO_2, to obtain a pH between 7.38 and 7.42.

The time from thoracotomy to dissection was ~3 min. The right ventricle was opened, and papillary muscles were isolated by first dividing the chordae tendinae at the muscle tip and then freeing the muscle base and a small amount of surrounding myocardium from the ventricular wall. Only long, thin, uniformly cylindrical muscles were used.

After dissection, papillary muscles (n=73; length, 4.3± 0.2 mm; weight, 3.4±0.2 mg; preload, 3.4±0.1 mN) were mounted vertically in a 10-ml plexiglass organ bath containing the aforementioned KR solution. The lower muscular end was fixed in a phosphorbronze clip, and the upper tendinous end was attached to an electromagnetic length–tension transducer (University of Antwerp, Belgium).

Preload was initially estimated according to muscle dimensions. After 10 min, muscles were stimulated at interstimulus interval of 1,670 ms and voltage of 10% above threshold by rectangular pulses of 5 ms duration through two platinum electrodes. Twenty minutes later, bathing solutions were replaced by corresponding KR solutions without BDM, and the muscle started to contract. One hour later, bathing solution was replaced by corresponding serum-free KR solution. During the next 2 h, the muscles were stabilized. Finally, the muscles were stretched to a muscle length at which active force development was maximal. At this point, this length (millimeter) known as maximum physiological length (L_{max}), was measured with a microruler. During the experiment, changes in diastolic muscle length and muscle shortening were measured by the isotonic transducer. Protocols were initiated after obtaining two similar isotonic and isometric control twitches separated by a 10-min interval.

At the end of the experiment, the muscles were removed, lightly blotted, and then weighed. Muscle cross-sectional area was calculated by dividing the weight of the muscle by its length at L_{max}. A cylindrical shape and a specific gravity of 1.0 were assumed (Leite-Moreira et al. 2006). Muscle tension was then expressed as force normalized per cross-sectional area (mN mm^{-2}).

Experimental protocol

Effects of increasing concentrations of human U-II (hU-II; 10^{-8} to 10^{-6} M) on contraction, relaxation, and diastolic

properties of the myocardium were studied in rabbit papillary muscles in control muscles with intact endocardial endothelium (EE), after selective removal of EE by a brief (1 s) immersion of the papillary muscle in a weak solution (0.5%) of the detergent Triton X-100 (Brutsaert et al. 1988, 1996), followed by abundant wash with Triton-free KR solution, and in the presence of: (1) urantide ($C_{51}H_{66}N_{10}O_{12}S_2$; URT; 10^{-5} M), an antagonist of U-II receptor; (2) N^G-Nitro L-Arginine (L-NNA; 10^{-5} M), a NO synthase inhibitor; (3) indomethacin (Indo; 10^{-5} M), a cyclooxygenase inhibitor; (4) N^G-Nitro-L-Arginine plus Indo and (5) chelerythrine (CHE, 10^{-5} M), an inhibitor of protein kinase C (PKC). In a small subset of muscles (n=5), the effects of U-II were tested in a KR solution containing nadolol (10^{-5} M) instead of atenolol. These substances were dissolved in the KR solution before the addition of U-II, and muscle twitches were recorded after a stable response was obtained, typically 15–20 min later. After that, U-II was added cumulatively without any washout between. Finally, in another small subset of muscles, passive length–tension relations were constructed in the absence and in the presence of the highest concentration of U-II. Of note, in each experimental protocol, all papillary muscles were obtained from different animals.

Data acquisition and analysis

Isotonic and isometric twitches were recorded and analyzed with dedicated software (University of Antwerp, Belgium). Selected parameters included: resting tension (RT; mN mm^{-2}), active tension (AT; mN mm^{-2}); maximal velocities of tension rise (dT/dt_{max}; mN mm^{-2} s^{-1}) and decline (dT/dt_{min}; mN mm^{-2} s^{-1}); peak isotonic shortening (PS; $\%L_{max}$); maximal velocities of shortening (dL/dt_{max}; $L_{max}s^{-1}$) and lengthening (dL/dt_{min}; $L_{max}s^{-1}$); time to half-relaxation (tHR, ms); and time to active tension (tAT; ms).

In the various protocols, results are given as percent change from baseline. For the parameters that are expressed as negative values (e.g. dT/dt_{min}), such percent change refers to the absolute values. When a pharmacological inhibitor was used or the EE damaged, the term baseline refers to the performance in the presence of those inhibitors or after damage of EE, before the addition of U-II.

Drugs and materials

Drugs were obtained from the following sources: hU-II, Bachem (Bubendorf, Switzerland); urantide, Peptides International (Louisville, Kentucky, USA); all other chemicals, Sigma Chemical (St Louis, MO, USA). Stock solutions of all chemicals were dissolved in distilled water and prepared in aliquots at 100 times the final bath concentration, except for hU-II which stock concentration was 5.10^{-5} M. All stock solutions were stored at −20°C until use.

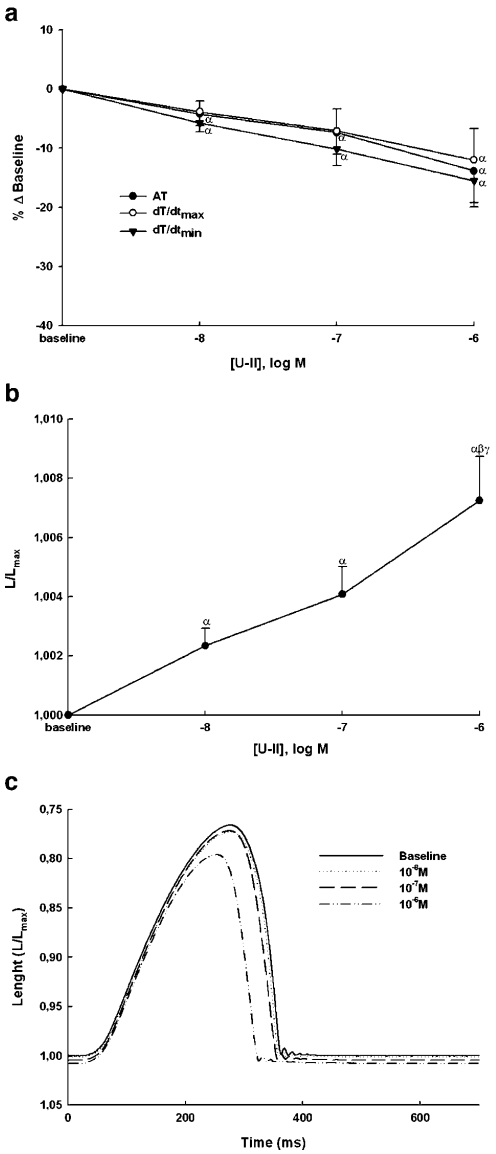

Fig. 1 Effect of increasing concentrations of urotensin II (U-II, 10^{-8} to 10^{-6} M, n=15) on **a** active tension (AT), peak rates of tension rise and decline (dT/dt_{max} and dT/dt_{min}, respectively) and **b** resting muscle length (L/L_{max}). Data are mean±SE, expressed as percent variation from baseline. $P<0.05$: α vs baseline, β vs 10^{-8} M U-II, γ vs 10^{-7} M U-II. **c** Representative example of isotonic twitches at baseline and in the presence of increasing concentrations of U-II

Statistical analysis

All values are given as mean±standard error of mean (SE), and *n* represents the number of experiments. Effects of increasing concentrations of U-II alone on the different experimental parameters were analyzed by one-way repeated-measures analysis of variance (ANOVA). Effects of increasing concentrations of U-II under various experimental conditions were analyzed with a repeated-measures two-way ANOVA. Effects on the various parameters of a single concentration of the antagonists were analyzed with a paired *t* test. When significant differences were detected with any of the ANOVA tests, the Student–Newman–Keuls test was selected to perform pairwise multiple comparisons. A P value less than 0.05 was considered to be significant.

Results

Baseline performance of rabbit papillary muscles was similar in all experimental protocols. Mean values of the contractile parameters from the 73 papillary muscles were as follows: AT, 19.8±1.3 mN mm^{-2}; dT/dt_{max}, 135.2±8.3 mN mm^{-2} s^{-1}; dT/dt_{min}, −113.1±6.7 mN mm^{-2} s^{-1}; PS, 13.0±0.7% of L_{max}; dL/dt_{max}, 1.0±0.1 L_{max}s^{-1}; dL/dt_{min}, −3.3±0.2 L_{max}s^{-1}; tAT, 243.1±5.5 ms; tHR, 382.6±8.8 ms.

Effects of increasing concentrations of U-II (10^{-8}, 10^{-7}, and 10^{-6} M) on papillary muscle function are summarized and illustrated in Fig. 1, where it can be seen that U-II induced concentration-dependent negative inotropic (AT, dT/dt_{max}) and lusitropic (dT/dt_{min}) effects. When the papillary muscle was stimulated with the two lowest concentrations of U-II (10^{-8} M and 10^{-7} M), muscle tension gradually decreased to reach a maximal decrease within 15 min for each one (Fig. 2). When the papillary muscle was stimulated with the higher concentration of U-II (10^{-6} M), muscle tension reached the maximal decrease within 30 min (Fig. 2). The highest concentration (10^{-6} M) of U-II decreased 13.8±5.4% AT (Fig. 1a), 12.0±5.3% dT/dt_{max} (Fig. 1a), 15.5±4.4% dT/dt_{min} (Fig. 1a), 11.2±3.8% PS, 9.7±3.3% dl/dt_{2max}, 13.4±3.5% dl/dt_{min}, 3.7±1.3% tHR, and 3.8±1.6% tAT (onset of relaxation).

With regard to the diastolic properties of the myocardium, we observed that U-II progressively increased resting muscle length (Fig. 1b) at a constant RT. Correcting, at the end of the experiment, muscle length to its initial value resulted in an 18.1±3.0% decrease in RT, without altering the other contractile parameters. This indicates an increase in muscle distensibility or, on the other hand, a decrease in muscle stiffness. Figure 3 illustrates mean length–tension relations in the absence and presence of the highest concentration of U-II, where it can be seen that the increase in muscle distensibility is observed over the entire range of muscle lengths studied.

The effect of U-II was not significantly different in the muscles in which atenolol was replaced by nadolol in the KR solution.

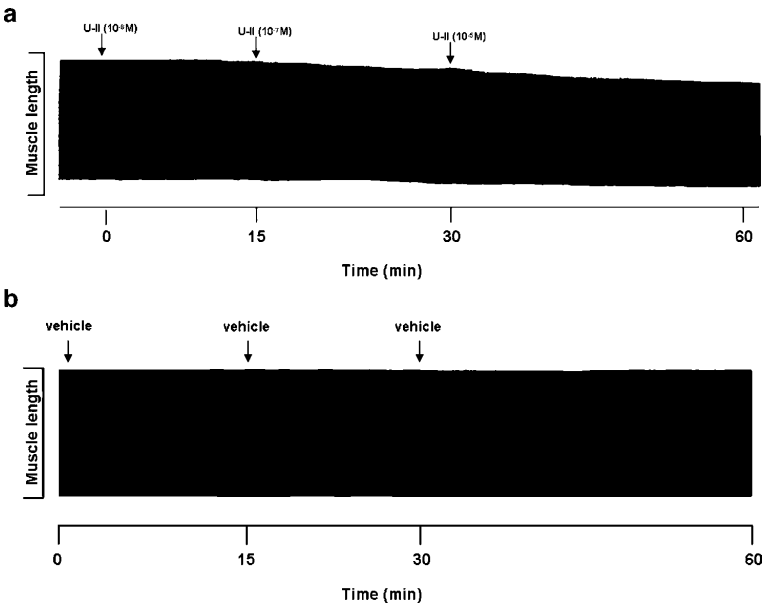

Fig. 2 Representative recording of the myocardial response of a rabbit papillary muscle to **a** urotensin II (U-II) and **b** vehicle (control muscle). *Arrows* in **a** cumulatively increasing concentrations of 10^{-8} to 10^{-6} M U-II and **b** equal volumes of the vehicle

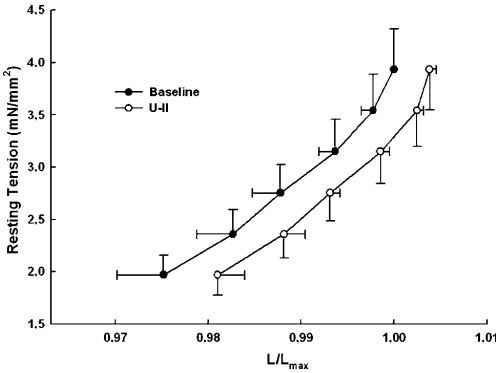

Fig. 3 Passive length–tension relations at baseline and in the presence of urotensin II (U-II, 10^{-6} M, n=3). Data are mean±SE

Effects of U-II after damaging the EE, in presence of a selective UT receptor antagonist (URT), or after inhibition of cyclooxygenase (Indo), NO synthase (L-NNA), or PKC (CHE) are illustrated in Figs. 4, 5 and 6.

Selective destruction of the EE or the presence of CHE resulted in a significant decrease in AT by 45.4±5.7% and 44.7±4.3%, respectively. The other inhibitors did not significantly modify per se any of the analyzed contractile parameters.

None of the agents significantly altered the effects of U-II on myocardial contractility (AT, dT/dt_{max}, PS, dL/dt_{max}), relaxation (dT/dt_{min}, dL/dt_{min}) or muscle twitch duration (tAT, tHR). Effects on AT, dT/dt_{max}, and dT/dt_{min} are illustrated in Figs. 4a and 5a. On the contrary, URT, L-NNA, and Indo significantly attenuated the effects of U-II on myocardial distensibility, although these effects were not affected by the presence of CHE or EE removal (Figs. 4b and 5b). In the presence of Indo, the effect of U-II on muscle length was markedly reduced, leading to a decrease in passive tension of only 11.6±3.1% (Fig. 6). On the other hand, in presence of URT and L-NNA, the effects of U-II on passive muscle length and RT were no more statistically significant, having been totally abolished when L-NNA and Indo were simultaneously present in the bath (Fig. 6).

Discussion

This study clearly demonstrates that U-II induces a significant concentration-dependent acute increase in myocardial distensibility. This effect is attenuated by cyclooxygenase inhibition and completely abolished by U-II receptor blockade or NO synthase inhibition. This suggests that such effect is mediated by UT receptor stimulation and dependent of NO and prostaglandins release.

U-II binds to a 389-amino acid G-protein-coupled receptor termed UT (Ames et al. 1999). The UT receptor is coupled to the $G\alpha_{q/11}$ signal transduction pathway, the same of AT1, ET_A, and α-adrenoceptors, which are linked to phospholipase C activation and the consequent increase in inositol trisphosphate and diacylglycerol, with mobilization of intracellular Ca^{2+} (Ames et al. 1999; Opgaard et al. 2000; Tzanidis et al. 2003). In the isolated rabbit aorta, the vasoconstrictor effect of U-II is mediated by a phospholipase C-dependent increase in inositol phosphates, probably mediated by a G_q-protein-coupled receptor (Opgaard et al. 2000). On the other hand, in the rat aorta, the contraction induced by U-II is mediated by a Ca^{2+}/calmodulin/myosin light chain (MLC) kinase system and modulated by the Ca^{2+} sensitization mechanisms to increase MLC phosphorylation (Tasaki et al. 2004).

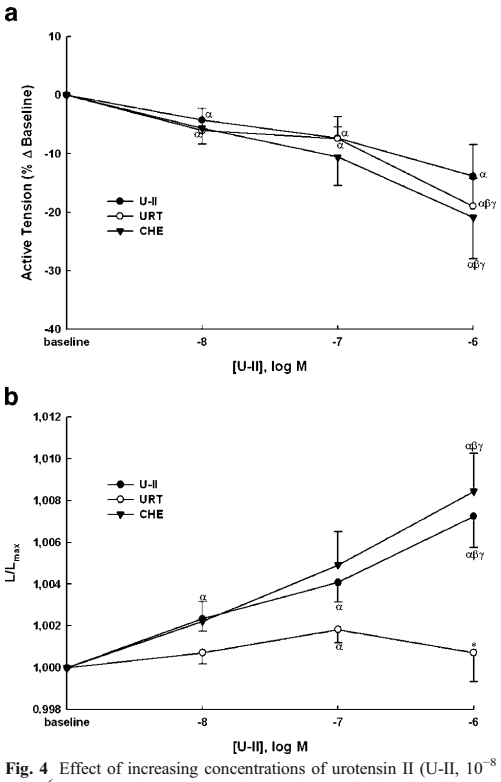

Fig. 4 Effect of increasing concentrations of urotensin II (U-II, 10^{-8} to 10^{-6} M) on **a** active tension and **b** passive muscle length (L/L_{max}) in the absence (n=15) or presence of selective UT receptor antagonist (urantide; URT, 10^{-5} M, n=7) or PKC inhibitor chelerythrine (CHE, 10^{-5} M, n=9). Data are mean±SE, expressed as percent variation from baseline. P<0.05: α vs baseline, β vs 10^{-8} M U-II, γ vs 10^{-7} M U-II, * vs U-II alone

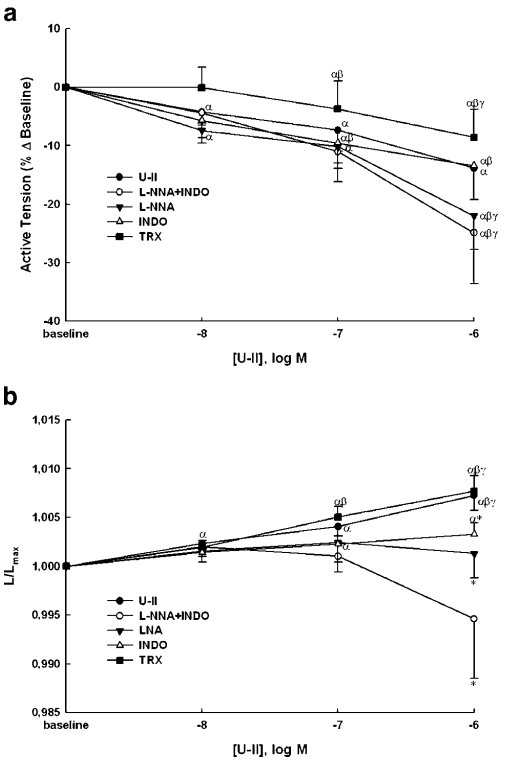

Fig. 5 Effect of increasing concentrations of urotensin II (U-II; 10^{-8} to 10^{-6} M) on **a** active tension and **b** passive muscle length (L/L_{max}) in the absence (n=15) or presence of NO synthase and cyclooxygenase inhibition (L-NNA, 10^{-5} M and Indo, 10^{-5} M, respectively, n=8), NO synthase inhibition (L-NNA, 10^{-5} M, n=9), cyclooxygenase inhibition (Indo, 10^{-5} M, n=8) or damaged endocardial endothelium (TRX, n=9). Data are mean±SE, expressed as percent variation from baseline. $P<0.05$: α vs baseline, β vs 10^{-8} M U-II, γ vs 10^{-7} M U-II, * vs U-II alone

In the present study, we found a mild concentration-dependent negative inotropic effect that was not altered either by EE removal or any of the used inhibitors. A similar effect was previously described in isolated canine cardiomyocytes (Morimoto et al. 2002), while a more pronounced one was reported in vivo first in nonhuman primates (Ames et al. 1999) and later in rats (Hassan et al. 2003), in response to systemic infusion of U-II, which was attributed to coronary vasoconstriction. On the contrary, in human isolated right atrial trabeculae (Russell et al. 2001) and in rat isolated left ventricular myocardium (Gong et al. 2004), a slight positive inotropic effect via a PKC-dependent mechanism (Russell and Molenaar 2004) was described. These discrepancies may be due to differences in the experimental preparation or the animal species used. Overall, however, the inotropic effects of U-II in vitro described in the literature are mild and of much smaller magnitude than those of for instance ET-1 and β-adrenergic stimulation (Russell 2004).

Myocardial stiffness is an important determinant of ventricular filling and, therefore, of diastolic function (Leite-Moreira 2006). As outlined in the introduction, classically, it was considered that neurohumoral agents only could influence the diastolic properties of the myocardium through chronic changes, as those induced by fibrosis and hypertrophy (Kass et al. 2004). More recent studies, however, have shown that diastolic stiffness may be acutely modulated by NO (Heymes et al. 1999; Shah et al. 1994), ET-1 (Leite-Moreira et al. 2003), Ang II (Leite-Moreira et al. 2006), and β-adrenoceptor stimulation or protein kinase A (PKA) activation (Borbely et al. 2005; Fukuda et al. 2005; van

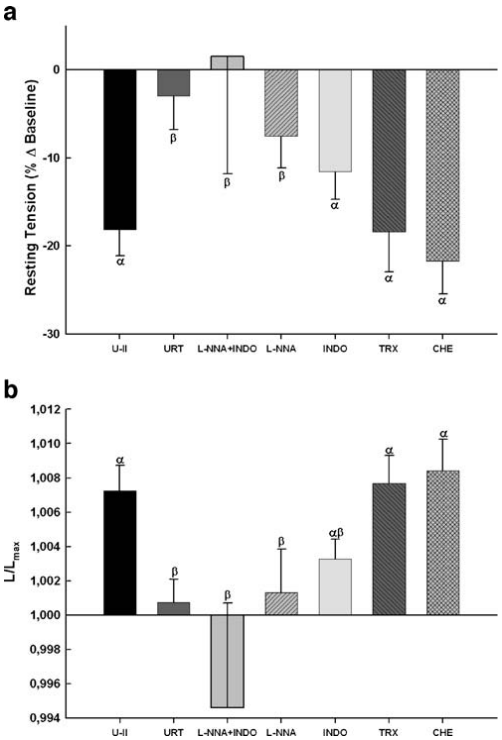

Fig. 6 Effects of urotensin II (U-II; 10^{-6} M) on **a** resting tension and **b** resting muscle length (L/L_{max}) in the absence (n=15) or presence of selective UT receptor antagonist (urantide; URT, 10^{-5} M, n=7), NO synthase and cyclooxygenase inhibition (L-NNA, 10^{-5} M and Indo, 10^{-5} M, respectively, n=8), NO synthase inhibition (L-NNA, 10^{-5} M, n=9), cyclooxygenase inhibition (Indo, 10^{-5} M, n=8), damaged endocardial endothelium (TRX, n=9) or PKC inhibitor chelerythrine (CHE, 10^{-5} M, n=9). Data are mean±SE, expressed as percent variation from baseline. $P<0.05$: α vs baseline, β vs U-II alone

Heerebeek et al. 2006; Yamasaki et al. 2002), while the present study demonstrates that the same is true for U-II.

Several actions of NO on myocardial contractile function have been reported, including changes in relaxation and diastolic properties of the myocardium. NO production and release have been detected in the sequence of endothelial UT receptor stimulation and seems to modulate the U-II-induced vasoconstriction in some experimental preparations (Ishihata et al. 2006). NO has been previously shown to increase myocardial distensibility, presumably as a result of protein kinase G (PKG)-mediated phosphorylation of myofilaments (Prendergast et al. 1997; Shah et al. 1994), which could explain the effects observed in the present study of U-II on this property.

UT receptor shares some subcellular pathways and interacts with ET_A and AT1 receptors (Li et al. 2005; Wang et al. 2007). With regard to diastolic function, we have recently shown, in the same animal species, that both ET_A (Leite-Moreira et al. 2003) and AT1 (Leite-Moreira et al. 2006) stimulation increase myocardial distensibility through PKC and Na^+/H^+ exchanger-mediated effects. It is also important to underline that while the effect of ET-1 on myocardial distensibility was only observed in acutely afterloaded twitches, in the case of Ang II it was present even in isotonic contractions. With regard to U-II, the results of the present study indicate that its effects on myocardial distensibility are not mediated by PKC but instead dependent on UT receptor stimulation and NO and prostaglandins release. Interestingly, however, even if these agents are released by the endothelium, EE removal did not alter the effects of U-II on myocardial distensibility. This apparent discrepancy can be easily explained if we take into account that the microvascular coronary endothelium, another important source of NO and prostaglandins (Brutsaert 2003), remained intact even after removal of the EE. Note that NO can also be released by the cardiomyocytes themselves (Massion et al. 2003). Data related with the expression of the UT receptor in the heart support this hypothesis. In fact, expression of this receptor was shown in cardiomyocytes and vascular endothelial cells but not yet in the EE (Russell 2004).

Finally, concerning the pathophysiologic relevance of our findings, we must point out that decreases of 18% in passive tension of the isolated muscle indicate that U-II might allow the ventricle to reach the same diastolic volume with almost 20% lower filling pressures, which is undoubtedly a potentially important adaptation mechanism. As the acute effects of U-II on diastolic function were determined in an in vitro model, this excludes systemic and humoral effects of U-II; consequently, the effects of U-II in vivo, where other important adaptation mechanisms also affect diastolic filling pressures, may differ from those reported in this paper.

These acute beneficial effects of U-II on diastolic function may become deleterious on the long term due to its role in the promotion of cardiac fibrosis and hypertrophy, when its levels remain chronically elevated (Bousette et al. 2006; Yamamoto et al. 2002), and by its effects on coronary arteries by accelerating the development of atherosclerosis, thereby leading to coronary artery disease (Watanabe et al. 2006). Furthermore, we have to consider that a sustained increase in myocardial length, as the one promoted by U-II, might contribute to ventricular dilatation, which is another important feature of ventricular remodeling.

In conclusion, this study describes, for the first time, the modulation of diastolic function by U-II, which increases myocardial distensibility, an effect that requires the activation of UT receptor and is mediated by NO and prostaglandins release. This novel effect of U-II broadens our concepts with regard to the acute neurohumoral modulation of diastolic function and represents a potentially powerful regulator of cardiac filling. In addition, taking into account that U-II and its receptor exhibits increased expression in cardiac tissue and plasma in human heart failure, these results might help to better understand the pathophysiology of this syndrome.

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CAPÍTULO IV

NOVOS MEDIADORES NEURO-HUMORAIS

PARTE C: INTERACÇÃO DO SISTEMA DA UROTENSINA II COM OS SISTEMAS DA ANGIOTENSINA II E ENDOTELINA-1

Submitted to the *Physiological Research*

Urotensin II-induced increase in myocardial distensibility is modulated by angiotensin II and endothelin-1

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Running Title: Urotensin II and myocardial distensibility

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Summary

In cardiovascular disorders several endogenous regulators, including angiotensin II (AngII), endothelin-1 (ET-1) and urotensin-II (U-II), are released from various types of cells. Because its plasma levels are elevated, it seems likely that cardiac function might be regulated by crosstalk among these peptides. So, we aimed to study if the myocardial effects of U-II depend on the interaction with AngII and ET-1 systems.
Effects of U-II (10^{-8}, 10^{-7}, 10^{-6}M) were tested in rabbit papillary muscles in the absence and in the presence of losartan (a selective AT1 receptor competitive antagonist) and PD-145065 (a nonselective antagonist of ET-1 receptors).
U-II promoted concentration-dependent negative inotropic and lusitropic effects that were abolished in the presence of both antagonists. Also, U-II increased resting muscle length (increased distensibility), up to 1.008±0.002 L/L_{max}. Correcting it to its initial value resulted in a 19.5±3.5% decrease of resting tension, indicating decreased muscle stiffness. This later effect was completely abolished in the presence of losartan and significantly attenuated by PD-145065, leading in the later condition to a decrease in passive tension of only 11.6±2.7%.
This study demonstrated an interaction of the U-II system with the AngII and ET-1 systems in terms of regulation of systolic and diastolic function.

Key words

Urotensin II • Angiotensin II • Endothelin-1 • Cardiac function • Myocardial distensibility

Introduction

Urotensin II (U-II) is a vasoactive cyclic peptide that was originally isolated from fish urophysis, and has been cloned from humans since 1998 (Coulouarn *et al.* 1998). UII has been identified as the endogenous ligand for the orphan G protein-coupled receptor, GPR14 (U-II receptor, UT) (Ames *et al.* 1999; Douglas *et al.* 2002). Both U-II and its receptor are expressed in the mammalian cardiovascular system namely in the myocardium, vascular smooth muscle cells and endothelial cells (Johns *et al.* 2004; Egginger *et al.* 2006). Human U-II (hU-II) effectively constricts isolated arteries from non-human primates. The potency of vasoconstriction is of a greater magnitude than that of endothelin 1 (ET-1), making U-II the most potent mammalian vasoconstrictor (Ames *et al.* 1999).

Furthermore, U-II was reported to affect the process of cell growth in the heart. This peptide exerted mitogenic effects on vascular smooth muscle cells (Sauzeau *et al.* 2001; Watanabe *et al.* 2001a;b) and human endothelial cells (Shi *et al.* 2006), induced collagen and fibronectin synthesis by cardiac fibroblasts, and caused cardiomyocyte hypertrophy (Tzanidis *et al.* 2003; Johns *et al.* 2004; Russell 2004). Thereby, U-II contributes to ventricular remodeling and deterioration of systolic and diastolic function, similarly to what has been described for other vasoconstrictor peptides such as angiotensin-II (AngII) and ET-1 (Weber *et al.* 1994; Ito 1997).

Moreover, elevation of U-II in the plasma and hearts of patients with congestive heart failure has been observed, and these circulating levels were related to the functional class of the disease and correlated negatively with left ventricular ejection fraction (Douglas *et al.* 2002; Russell *et al.* 2003; Russell 2004; Gruson *et al.* 2006). Also, U-II correlated significantly with big-ET-1 and brain natriuretic peptide, suggesting that U-II

could play a role in worsening the course of congestive heart failure and is associated with established markers of cardiovascular dysfunction (Gruson *et al.* 2006).

Unlike the well-known role of chronically elevated U-II levels in progression to cardiac fibrosis and ventricular remodeling, the acute diastolic effects of U-II remain less explored. We previously found that AngII (Leite-Moreira *et al.* 2006), ET-1 (Leite-Moreira *et al.* 2003) and U-II acutely increase myocardial distensibility. In the case of U-II this effect is mediated by UT receptor, NO and prostaglandins (Fontes-Sousa *et al.* 2007). The intracellular signaling of U-II and its interaction with other vasoconstrictors such as AngII and ET-1 are poorly understood, although is has been established that U-II shares some subcellular pathways and interacts with these vasoactive systems (Tasaki *et al.* 2004; Li *et al.* 2005; Wang *et al.* 2007). Regulation of myocardial distensibility induced by crosstalk between U-II and AngII or ET-1 has not been studied yet.

In this context, we aimed to study if the myocardial effects of U-II depend on the interaction of this system with other autocrine/paracrine mediators, like ET-1 and AngII. Specifically, our main goal was to investigate if the recently described effects of U-II on myocardial distensibility are dependent on the activation of these two classical systems.

Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication Nº 85-23, Revised 1996).

Experimental preparation

Isometric and isotonic contractions were measured in papillary muscles isolated from the right ventricle of rabbits. Male New Zealand White rabbits (*Oryctolagus cuniculus*; 1.2–2.7 kg; *n*=19) were anesthetized with intravenous sodium pentobarbital (25mgkg^{-1}). A left thoracotomy was performed, and beating hearts were quickly excised and immersed in a modified Krebs-Ringer (KR) solution (composition in mM: 98 NaCl, 4.7 KCl, 2.4 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 4.5 glucose, 1.8 $CaCl_2.2H_2O$, 17 $NaHCO_3$, 15 sodium pyruvate, 5 sodium acetate, 0.02 atenolol) at 35ºC with cardioplegic 2,3-butanedione monoxime (BDM; 3%) and 5% Newborn Calf Serum. Atenolol was used to prevent β-adrenergic mediated effects. The solutions were in equilibrium with 95% O_2 and 5% CO_2, to obtain a pH between 7.38-7.42.

The time from thoracotomy to dissection was ~3 min. The right ventricle was opened and papillary muscles were isolated by first dividing the chordae tendinae at the muscle tip and then freeing the muscle base and a small amount of surrounding myocardium from the ventricular wall. Only long, thin, uniformly cylindrical muscles were used.

After dissection, papillary muscles (*n*=27; length: 4.4±0.2mm; weight: 3.4±0.4mg; preload: 3.5±0.2mN) were mounted vertically in a 10ml plexi glass organ bath containing the aforementioned KR solution. The lower muscular end was fixed in a phosphorbronze clip, and the upper tendinous end was attached to an electromagnetic length–tension transducer (University of Antwerp, Belgium).

Preload was initially estimated according to muscle dimensions. After 10 min, muscles were stimulated at interstimulus interval of 1670 ms and voltage of 10% above threshold by rectangular pulses of 5 ms duration through two platinum electrodes. Twenty minutes later, bathing solutions were replaced by corresponding KR solutions without

BDM and the muscle started to contract. One hour later, bathing solution was replaced by corresponding serum-free KR solution. During the next 2 hours, the muscles were stabilized. Finally, the muscles were stretched to a muscle length at which active force development was maximal. This length (mm) is known as maximum physiological length (L_{max}). Protocols were initiated after obtaining two similar isotonic and isometric control twitches separated by a 10 min interval.

At the end of the experiment the muscles were lightly blotted and then weighed. Muscle cross-sectional area was calculated by dividing the weight of the muscle by its length at L_{max}. A cylindrical shape and a specific gravity of 1.0 were assumed. Muscle tension was then expressed as force normalized per cross-sectional area ($mNmm^{-2}$).

Experimental protocols

The effects of increasing concentrations of hU-II (10^{-8}, 10^{-7} and 10^{-6}M) on contraction, relaxation, and diastolic properties of the myocardium were studied in rabbit papillary muscles in the absence (n=12) or in the presence of (i) losartan (10^{-6}M; n=8), a selective AT1 receptor competitive antagonist, or (ii) PD-145065 ($C_{52}H_{65}N_7O_{10}$; 10^{-6}M; n=7), a nonselective antagonist of ET-1 receptors. These substances were dissolved in the KR solution before the addition of U-II, and muscle twitches were recorded after a stable response was obtained, typically 15-20min later. After that, U-II was added cumulatively without any washout between.

Of note, that in each experimental protocol all papillary muscles were obtained from different animals. hU-II was obtained from Bachem (Bubendorf, Switzerland). Losartan and PD-145065 were obtained from Cayman Chemical Company Europe and Sigma Chemical Co (St Louis, MO, USA), respectively. Peptides were prepared in aliquots and stored at -20 ºC.

Data acquisition and analysis

Isotonic and isometric twitches were recorded and analyzed with dedicated software (University of Antwerp, Belgium). Selected parameters included: resting tension (RT; $mNmm^{-2}$), active tension (AT; $mNmm^{-2}$); maximal velocities of tension rise (dT/dt_{max}; $mNmm^{-2}s^{-1}$) and decline (dT/dt_{min}; $mNmm^{-2}s^{-1}$); peak isotonic shortening (PS; $\%L_{max}$); maximal velocities of shortening (dL/dt_{max}; $L_{max}s^{-1}$) and lengthening (dL/dt_{min}; $L_{max}s^{-}$); time to half-relaxation (tHR, ms); and time to active tension (tAT; ms).

In the various protocols, results are given as percent change from baseline. For the parameters that are expressed as negative values (e.g. dT/dt_{min}) such percent change refers to the absolute values. When the pharmacological inhibitors were used, the term baseline refers to the performance in the presence of those inhibitors, before the addition of U-II.

Statistical methods

Values are presented as means ± standard error of the mean (SEM) of *n* experiments. Effects of increasing concentrations of U-II alone on the different experimental parameters were analyzed by one-way repeated-measures ANOVA. Effects of increasing concentrations of U-II under various experimental conditions were analyzed with a repeated-measures two-way ANOVA. Effects on the various parameters of a single concentration of the antagonists were analyzed with a paired t-test. When significant differences were detected with any of the ANOVA tests, the Student-Newman-Keuls test was selected to perform pairwise multiple comparisons. $P<0.05$ was accepted as significant.

Results

Baseline performance of rabbit papillary muscles was similar in all experimental protocols. Mean values of the contractile parameters from the 30 papillary muscles were: active tension 26.7±3.0 mN/mm^2; dT/dt_{max} 181.6±21.1 mN/mm^2·s; dT/dt_{min} -135.2±11.3 mN/mm^2·s; peak shortening 15.9±1.3% of L_{max}; dL/dt_{max} 1.17±0.09 $L_{max}.s^{-1}$; dL/dt_{min} -4.72±0.44 $L_{max}.s^{-1}$; time to half relaxation 397.0±17.7 ms. The presence of losartan or PD-145065 did not per se significantly change muscle performance.

U-II induced concentration-dependent negative inotropic (AT, dT/dt_{max}) and lusitropic (dT/dt_{min}) effects (Fig. 1). The highest concentration (10^{-6}M) of U-II decreased 15.8±5.6% AT, 13.5±5.4% dT/dt_{max}, and 18.1±4.5% dT/dt_{min}. With regard to the diastolic properties of the myocardium, we observed that U-II progressively increased resting muscle length (Fig. 1) at a constant resting tension. Correcting, at the end of the experiment, muscle length to its initial value resulted in a 19.5±3.5% decrease of resting tension, without altering the other contractile parameters. These effects indicate an increase in muscle distensibility, or on the other hand, a decrease in muscle stiffness.

In the presence of a nonselective endothelin ET_A/ET_B receptor antagonist (PD-145065), the negative inotropic (Fig. 2) and lusitropic (Fig. 2) effects of U-II were abolished. Similarly, losartan, a selective competitive AT1 receptor antagonist, completely abolished the negative inotropic and (Fig. 2) and lusitropic (Fig. 2) effects of U-II.

The effects of U-II on myocardial distensibility were significantly attenuated by PD-145065 (Fig. 2), leading to a decrease in passive tension of only 11.6±2.7%. On the other hand, in the presence of losartan the effects of U-II on passive muscle length and RT were no more statistically significant (Fig. 2).

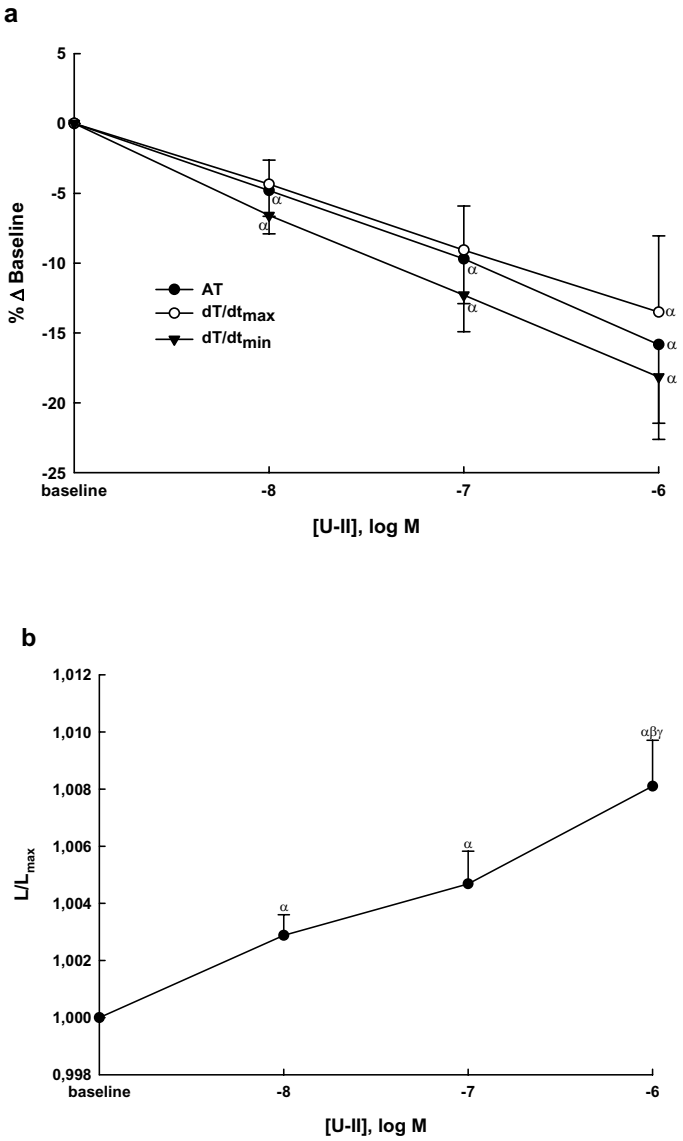

Figure 1. Effect of increasing concentrations of urotensin II (U-II; 10^{-8} to 10^{-6}M) on active tension (AT), peak rates of tension rise and decline (dT/dt_{max} and dT/dt_{min}, respectively) (top) and muscle length (L/L_{max}, bottom). Data are means ± SEM, expressed as percent variation from baseline. $P<0.05$: α *vs.* baseline, β *vs.* 10^{-8}M U-II, γ *vs.* 10^{-7}M U-II.

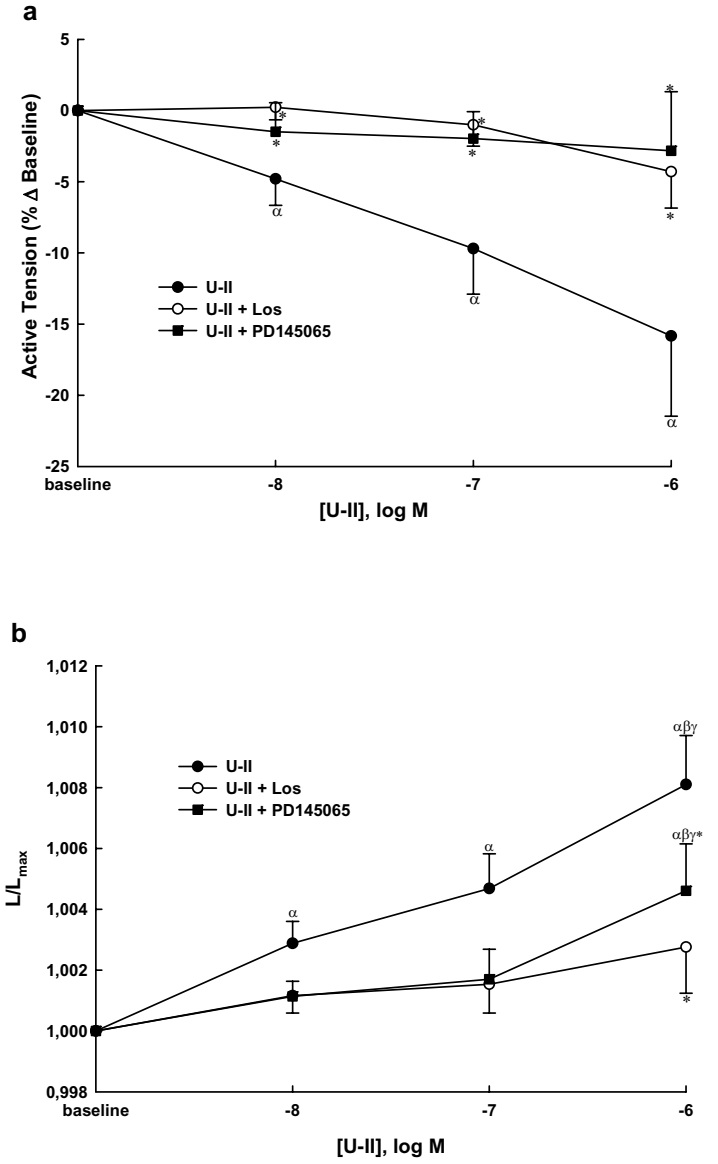

Figure 2. Effect of increasing concentrations of urotensin II (U-II; 10^{-8} to 10^{-6}M) on active tension (top) and muscle length (bottom, L/L_{max}) in the absence or presence of a selective AT1 receptor antagonist (losartan) (Los, 10^{-6}M) or a nonselective antagonist of ET-1 receptors (PD145065, 10^{-6}M). Data are means ± SEM, expressed as percent variation from baseline. $P< 0.05$: α vs baseline, β *vs.* 10^{-8}M U-II, γ *vs.* 10^{-7}M U-II, * *vs.* U-II alone.

Discussion

The role of U-II in cardiovascular physiology and diseases remains largely uncertain. Recent experimental and clinical studies have revealed increased expression of U-II and UT receptor in animals with experimentally induced heart failure and myocardial infarction and in patients with heart failure, hypertension, atherosclerosis, and diabetic nephropathy, suggesting a potential role of U-II in both cardiovascular and renal diseases (Zhu *et al.* 2006). On the other hand, in cardiovascular diseases, the expression of numerous neurohumoral factors such as AngII (Pfeffer and Braunwald 1990), ET-1 (Best and Lerman 2000), catecholamines (Ueyama *et al.* 2003), thromboxane A2 (Miyahara *et al.* 1997), and serotonin (Levy 2006) has been shown to be up-regulated. These studies give rise to the hypothesis that the interaction between U-II and other vasoactive substances may be crucial in modulating the cardiovascular effects of U-II under a certain disease status. Cross talk of intracellular signalling pathways is probably the underlying mechanism of the interaction between U-II and other vasoactive substances (Zhu *et al.* 2006).

Both ET-1 and AngII receptor systems are coupled to phospholipase C-Gαq protein signaling pathways, resulting in activation of protein kinase C isoforms and inositol phosphates, and both systems induce pathological hypertrophy accompanied by contractile dysfunction and poor clinical outcomes (Braunwald and Bristow 2000). U-II shares similar biological activities and signaling pathways with these hypertrophic Gq-coupled receptor ligands, since it has been also observed the coupling of its receptor to activated protein kinase C-dependent pathways (Saetrum Opgaard *et al.* 2000; Russell and Molenaar 2004).

The decrease of passive tension as the one promoted by U-II represents a potentially important adaptation mechanism, since it demonstrates that U-II might allow the ventricle to reach the same diastolic volume with almost 20% lower filling pressures

(Fontes-Sousa *et al.* 2007). However, we must consider that a sustained increase in myocardial length, as the one induced by U-II, might contribute to ventricular dilatation, which is another important feature of ventricular remodeling. Also, the acute beneficial effects of U-II on diastolic function may be also overcome by its role in the promotion of cardiac fibrosis and hypertrophy (Bousette *et al.* 2006b).

The present study showed that the increase of myocardial distensibility induced by U-II is dependent on Ang II and ET-1 systems. The development of inhibitors of these neurohumoral systems has proven to be favourable in treating many cardiac diseases by inhibiting or reversing cardiovascular remodeling. Drugs like angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and aldosterone antagonists have been demonstrated to reduce mortality and morbidity in patients (Sleight 2002; Dimopoulos *et al.* 2004). Additionally, recent studies demonstrated, in a rat model of coronary artery ligation, that SB-611812, a specific UT receptor antagonist, significantly improved cardiac dysfunction (Bousette *et al.* 2006a) and promoted a reduction of cardiac remodeling (Bousette *et al.* 2006b).

It is therefore reasonable to hypothesize that some cardiovascular effects could result from the interaction between different neurohumoral systems. From a physiopathological and clinical point of view, these results are potentially relevant, since the inhibition of a given neurohumoral system might also modulate the effects resulting from the activation of other systems. However, from the data presented, we can not deduce the specific signaling pathways that underlie these results. Further investigations are needed to clarify this issue.

In conclusion, in this animal species the acute decrease of myocardial stiffness induced by U-II is mediated by AngII and ET-1 systems. These results may contribute to a more complete understanding of the role of U-II in the acute modulation of myocardial

function. They also show that neurohumoral systems might have potential points of interaction. Furthermore, this might add to our understanding of the pharmacologic effects of the receptor antagonists of these peptides.

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CAPÍTULO V

DISCUSSÃO GLOBAL E CONCLUSÕES

Os objectivos desta dissertação consistiram, por um lado, na caracterização ecocardiográfica do Coelho saudável e por outro na avaliação dos efeitos intrínsecos de diferentes sistemas neuro-humorais na regulação da função miocárdica em corações saudáveis e na presença de IC. Dentre os sistemas neuro-humorais clássicos avaliámos o sistema da ET-1 e o sistema β-adrenérgico, enquanto a avaliação de novos sistemas neuro-humorais recaiu sobre os sistemas da AM e da U-II. Os estudos que encetámos visaram assim contribuir para o estudo da avaliação ecocardiográfica no Coelho e para o esclarecimento do efeito miocárdico de diferentes sistemas neuro-humorais e de suas implicações na fisiopatologia e tratamento da IC.

Tendo em conta as discussões parcelares elaboradas nas publicações incluídas na dissertação, as considerações finais deter-se-ão sobre os aspectos referentes ao conjunto de resultados reunidos neste trabalho no que respeita (i) à avaliação ecocardiográfica no Coelho e (ii) à modulação neuro-humoral da função cardíaca, com particular atenção sobre a função diastólica.

AVALIAÇÃO ECOCARDIOGRÁFICA NO COELHO

Actualmente, o Coelho doméstico (*Oryctolagus cuniculus*) é um importante animal de companhia (Graham, 2006; Mullan e Main, 2007), sendo também um modelo experimental de grande importância na investigação cardiovascular (Muders e Elsner, 2000). A ecocardiografia é uma técnica essencial na avaliação não invasiva da função cardíaca global e, como tal, no diagnóstico de doenças cardiovasculares. Estudos prévios reportaram os valores ecocardiográficos de referência de diversas espécies, incluindo, entre outros, o Cão (Lombard, 1984; Crippa e col., 1992; Kayar e col., 2006), o Gato (Fox e col.,

1985; Dummel e col., 1996), o Furão (Stepien e col., 2000; Vastenburg e col., 2004) e o Rato (Watson e col., 2004; Weytjens e col., 2006). No entanto, à data em que iniciámos os trabalhos desta dissertação, a literatura não havia estabelecido os valores ecocardiográficos de referência no Coelho saudável. Como tal, propusemo-nos caracterizar nesta espécie os valores normais de alguns paramêtros ecocardiográficos recorrendo a dois protocolos anestésicos diferentes, cetamina-medetomidina (Fontes-Sousa e col., 2006) e cetamina-midazolam (Fontes-Sousa e col., 2008b; Moura e col., 2008).

Nos dois primeiros trabalhos desta dissertação (estudos nº1 e nº2) caracterizámos alguns parâmetros ecocardiográficos obtidos por ecocardiografia em modo-M (EMM), Doppler convencional e DT. Em termos morfológicos realizámos as medições em diástole e em sístole do diâmetro interno do ventrículo esquerdo, da espessura do septo interventricular e da parede posterior do ventrículo esquerdo, bem como as medições da aurícula esquerda e da aorta. Para a avaliação da função sistólica procedemos ao cálculo da fracção de encurtamento, da fracção de ejecção e das velocidades máximas dos fluxos aórtico e pulmonar, enquanto a relação E:A da válvula mitral permitiu estimar a função diastólica (Fontes-Sousa e col., 2006; Fontes-Sousa e col., 2008b). Adicionalmente foram também caracterizados outros parâmetros de avaliação da função cardíaca global, sistólica e diastólica, o índice de Tei (IT) por Doppler pulsado (DP) e as velocidades do anel mitral por DT com as suas componentes S', E' e A' (Fontes-Sousa e col., 2008b).

A ecocardiografia por DT é uma técnica relativamente recente que visa complementar o estudo convencional em áreas sensíveis da patologia cardiovascular. Estudos prévios demonstraram que os parâmetros obtidos por DT são mais independentes da pré-carga e da pós-carga quando comparados com as avaliações hemodinâmicas Doppler clássicas (Sohn e col., 1997; Firstenberg e col., 2001; Nagueh e col., 2001). O DT pulsado, em particular, permite quantificar a velocidade da parede miocárdica e/ou o

movimento do anel mitral. Em estudos clínicos realizados em medicina humana e em medicina veterinária, a avaliação do DT pulsado da parede miocárdica imediatamente adjacente ao anel mitral reflecte a função sistólica e diastólica do ventrículo esquerdo em condições normais e na presença de diversas doenças cardíacas (Oki e col., 1999; Chetboul e col., 2005; Teshima e col., 2005; Chetboul e col., 2006).

No estudo nº2 recorremos à combinação anestésica cetamina-midazolam (Fontes-Sousa e col., 2008b), menos cardiodepressora do que a utilizada no primeiro estudo (Dupras e col., 2001), sendo que alguns dos resultados obtidos foram comparáveis aos obtidos em outros estudos levados a cabo em animais acordados (Stypmann e col., 2007). Considerando que a realização da avaliação ecocardiográfica no Coelho acordado poderá ser mais difícil, especialmente no âmbito da experimentação animal, o recurso à combinação cetamina-midazolam poderá representar uma potencial alternativa nestes casos.

O IT é um parâmetro ecocardiográfico largamente utilizado na avaliação da função sistólica e diastólica (Tei, 1995). O IT pode ser obtido por diferentes técnicas ecocardiográficas de acordo com a equação $(a\text{-}b)/b$. Em todos os métodos, o valor de *a* representa o somatório da fase de contracção isovolumétrica com a fase de ejecção e a fase de relaxamento isovolumétrico. O valor de *b* traduz a fase de ejecção do ventrículo esquerdo (figura 4). A principal vantagem deste índice reside no facto de não depender da idade ou da frequência cardíaca, assim como da geometria ventricular (Tei e col., 1995; Eidem e col., 1998; Poulsen e col., 2000). Diferentes autores reportaram em humanos valores de IT, expressos sob a forma de média e desvio padrão, usando diferentes métodos ecocardiográficos (Tham e Silverman, 2004; Abd El Rahman e col., 2005; Gaibazzi e col., 2005; Cui e Roberson, 2006). Em medicina veterinária e particularmente no Coelho são escassos os estudos que abordam o IT (Baumwart e col., 2005; Teshima e col., 2006;

Serres e col., 2007; Stypmann e col., 2007; Teshima e col., 2007). À data de início deste projecto não existiam dados em medicina veterinária relativos à avaliação do IT obtido por diferentes técnicas ecocardiográficas.

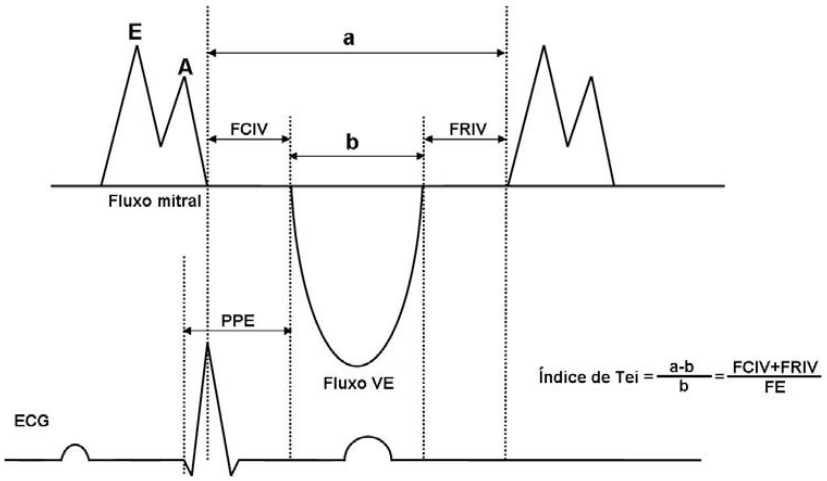

Figura 4: Cálculo do índice de Tei, tal como descrito originalmente. FCIV, fase de contracção isovolumétrica; FE, fase de ejecção; FRIV, fase de relaxamento isovolumétrico; PPE, período de pré-ejecção; VE, ventrículo esquerdo (Tei e col., 1995).

Assim, no último trabalho deste capítulo (estudo nº3), reportámos no Coelho os valores de referência do IT do ventrículo esquerdo (ITVE) obtido por EMM, DP e DT (septal e lateral). Por outro lado, dada a ausência de informação relativa a uma análise estatística que estabelecesse de uma forma mais precisa as relações e a concordância entre técnicas ecocardiográficas, avaliámos também a concordância entre as diferentes técnicas (Moura e col., 2008).

Verificámos que os valores do parâmetro *a* obtidos pelas diferentes técnicas ecocardiográficas apresentaram uma forte associação, tal como indicado pela correlação de Pearson, embora as medições obtidas por DT (tanto septal como lateral) tenham denotado uma tendência para ser mais elevadas do que as obtidas pelas outras duas técnicas. Contudo, somente com o DT septal e o DT lateral se obteve uma boa concordância. No

respeitante ao valor do parâmetro *b*, as correlações foram geralmente baixas com excepção da correlação entre o DT septal e DT lateral. De forma semelhante ao observado para o parâmetro *a*, as técnicas de DT foram as únicas que apresentaram uma boa concordância entre si. Houve uma tendência para os valores de *b* medidos por EMM serem mais elevados do que os obtidos pelas outras técnicas. Para o ITVE somente as técnicas de DT (septal *vs* lateral) apresentaram uma correlação positiva significativa, sendo que a concordância absoluta foi fraca para todas as técnicas.

Efectivamente, existem discrepâncias importantes relativamente aos valores obtidos do ITVE usando as três técnicas ecocardiográficas utilizadas no nosso estudo, dado que cada uma mede diferentes intervalos de tempo para as componentes *a* e *b* do ITVE. Um estudo recente demonstrou que o início da componente *a* obtida por EMM se sobrepõe ao início da componente *a* por DT, mas termina antes do fim da componente *a* obtida por DT e DP (Cui e Roberson, 2006). Adicionalmente, a componente *a* do DT inicia-se após o início da componente *a* do DP e termina após o final desta. Deste modo, as medições de *a* são similares tanto para o DT como para o DP, sendo inferiores na EMM. Por outro lado, o início e o final da componente *b* do DT ocorrem ligeiramente antes do início e do final da componente *b* do DP, respectivamente. O início da componente *b* da EMM ocorre ao mesmo tempo da componente *b* do DT mas termina após a componente *b* do DT e do DP, resultando num valor de *b* obtido por EMM mais longo quando comparado com o *b* obtido por DP e DT. Estas diferenças nas aquisições, intrinsecamente inerentes a cada técnica ecocardiográfica, resultam num ITVE-EMM inferior ao ITVE-DP e ao ITVE-DT. A corroborar estas observações, diferentes autores demonstraram em humanos que a média ± desvio-padrão do ITVE-EMM é consistentemente e de forma significativa inferior ao ITVE obtido por DP e DT (Tham e Silverman, 2004; Cui e Roberson, 2006), tendo sido observado o mesmo no nosso estudo (Moura e col., 2008).

O IT, tal como descrito originalmente por Tei, apresenta duas limitações importantes. Por um lado, o intervalo de tempo entre o final e o início do fluxo mitral e a fase de ejecção não são avaliados no mesmo ciclo cardíaco (Harada e col., 2002). Por outro lado, ao usar a fórmula (*a*-*b*)/*b* sem medir cada um dos intervalos isovolumétricos, não é possível determinar até que ponto a alteração da função cardíaca se deve a uma disfunção sistólica, diastólica ou sisto-diastólica (Dujardin e col., 1998).

A avaliação do ITVE-DP pode implicar o uso de diferentes ciclos cardíacos para medir mais acuradamente as componentes *a* e *b* e definir de forma precisa o início e o fim de cada componente. Neste caso, mesmo pequenas variações da frequência cardíaca entre o momento de medição de *a* e *b* podem representar uma fonte de erro (Cui e Roberson, 2006). Numa tentativa de ultrapassar esta limitação, realizámos as aquisições do ITVE-DP no mesmo ciclo cardíaco, tal como já tinha sido descrito previamente (Quinones e col., 2002). Todavia, de todas as técnicas ecocardiográficas, o cálculo do ITVE por DT será provavelmente o mais preciso, uma vez que as componentes *a* e *b* são sempre passíveis de ser medidas no mesmo ciclo cardíaco.

Podemos concluir que o ITVE pode ser obtido no Coelho pelas três técnicas ecocardiográficas avaliadas, embora a comparação dos seus valores deva ser interpretada de forma cautelosa. Por outro lado, na avaliação do ITVE deve recorrer-se à mesma técnica ecocardiográfica com vista a monitorizar a progressão da (dis)função cardíaca. O IT, enquanto índice de desempenho miocárdico, continua a ser uma ferramenta potencialmente valiosa na avaliação seriada da função ventricular global, sistólica e diastólica, desde que se tenha em consideração as suas limitações e desvantagens.

MODULAÇÃO NEURO-HUMORAL DA FUNÇÃO CARDÍACA

Como foi abordado na introdução desta dissertação, é do consenso geral a importância da componente diastólica na função cardíaca global, bem como as consequências clínicas resultantes do seu compromisso. Nesta perspectiva, é importante conhecer os mecanismos fisiopatológicos envolvidos na sua modulação para que sejam implementadas estratégias preventivas e terapêuticas que impeçam a progressão para um fenótipo disfuncional de IC. Desta forma, uma das linhas de investigação do nosso grupo tem sido descrever novos mecanismos de modulação aguda da função diastólica, nomeadamente a dimuição da rigidez miocárdica por diferentes agentes neuro-humorais.

Nesta série de trabalhos recorremos ao modelo de músculo papilar isolado do ventrículo direito de Coelhos brancos neozelandeses. Este modelo é ideal para a realização de estudos funcionais e farmacológicos, pois permite controlar de forma rigorosa a carga e deste modo avaliar a acção de determinado agente nas propriedades intrínsecas do miocárdio. Por outro lado, exclui factores extrínsecos pertubadores tais como a activação neuro-humoral sistémica, a perfusão coronária, o acoplamento ventrículo-arterial e a interacção ventricular. O Coelho foi seleccionado como modelo experimental pelo facto do miocárdio desta espécie apresentar mais similitudes com o miocárdio humano quando comparado com o de outras espécies como o Rato e o Ratinho, nomeadamente em termos de isoformas das cadeias pesadas de miosina e de homeostasia do Ca^{2+} (Hasenfuss, 1998; Bers, 2002).

Efeitos Miocárdicos da Estimulação Selectiva dos Receptores ET_B na Insuficiência Cardíaca

A importância do EE na modulação da função miocárdica é evidente (Brutsaert, 2003), embora não esteja ainda bem esclarecido o seu papel na fisiopatologia da IC. Com efeito foi documentada a existência de lesões morfológicas das células do EE em condições de sobrecarga ventricular de volume (Masuda e col., 1989) ou de pressão (Chu e col., 1995; Smiley e Tyagi, 1999). Por outro lado, estudos *in vitro* demonstraram a ocorrência de lesões do EE acompanhadas por alterações profundas na função mecânica do miocárdio subjacente após a exposição a elevadas concentrações de diversas neuro-hormonas e outros factores considerados como factores de risco *in vivo* (Brutsaert, 2003).

Como já referido na introdução desta dissertação, a ET-1 actua através da ligação a dois tipos de receptores, ET_A e ET_B. Um estudo prévio descreveu que o efeito inotrópico da estimulação selectiva dos receptores ET_B é dependente do estado funcional do EE, sendo negativo quando este está intacto e positivo quando está danificado (Leite-Moreira e Brás-Silva, 2004). Estes resultados foram atribuídos à existência de dois subtipos de receptores ET_B no coração, tal como descrito previamente a nível vascular (de Nucci e col., 1988; Sudjarwo e col., 1994): ET_{B1}, de localização endotelial e promotores de inotropismo negativo, e ET_{B2}, de localização miocárdica e promotores de inotropismo positivo. Com base nestes resultados, propôs-se que a avaliação destes efeitos pode constituir uma ferramenta experimental na avaliação da integridade funcional do EE, à semelhança da acetilcolina para o endotélio vascular.

Nesse sentido investigámos a integridade funcional do EE num modelo de IC (cardiomiopatia tóxica induzida pela doxorrubicina) (estudo nº4), avaliando a resposta contráctil à estimulação dos receptores ET_{B1} mediante a utilização de um agonista selectivo dos receptores ET_B endoteliais, IRL1620.

O modelo de IC induzida pela doxorrubicina tem sido usado em diferentes espécies animais no estudo dos mecanismos fisiopatológicos e na avaliação de diferentes estratégias terapêuticas na IC (Monnet e Chachques, 2005). Na monitorização da progressão da disfunção cardíaca recorreu-se à ecocardiografia, tendo sido observado um aumento progressivo dos diâmetros telessistólicos e telediastólicos, bem como uma diminuição das fracções de encurtamento e de ejecção do ventrículo esquerdo, alterações consistentes com a literatura (Monnet e Chachques, 2005). Adicionalmente, elaborámos relações contractilidade-frequência dado que é mais fácil identificar disfunção contráctil em músculos papilares a contrair a frequências mais elevadas (Endoh, 2004). Constatámos que, apesar da *performance* basal dos músculos dos corações saudáveis e insuficientes ser semelhante, estes últimos manifestaram uma diminuição da contractilidade quando sujeitos a elevações da frequência cardíaca, denotando disfunção miocárdica e redução da reserva contráctil (Brás-Silva e col., 2006; Brás-Silva e col., 2007).

O estudo evidenciou que neste modelo de IC se verifica a existência de disfunção endotelial endocárdica com base na resposta alterada à estimulação dos receptores ET_B (Brás-Silva e col., 2006), reforçando o papel destes como potenciais marcadores da integridade funcional do EE. Outros estudos apoiam a hipótese de ocorrência de disfunção do EE na IC, nomedamente o facto dos efeitos da ET-1 sobre a distensibilidade miocárdica serem dependentes do EE intacto (Brás-Silva e Leite-Moreira, 2006) e se encontrarem significativamente atenuados nos músculos provenientes de corações insuficientes (estudo nº5) (Brás-Silva e col., 2007).

Papel do Óxido Nítrico e das Prostaglandinas na Modulação dos Efeitos Diastólicos da Endotelina-1

O segundo estudo deste conjunto de trabalhos (estudo nº5) surgiu no seguimento de um outro efectuado pelo nosso grupo, no qual a ET-1 revelou ser um importante modulador das propriedades diastólicas do músculo papilar de Coelho. As propriedades diastólicas do miocárdio podem ser inferidas a partir da tensão passiva (TP), isto é, da tensão do músculo quando este não está a contrair, mantendo o seu comprimento constante. Na presença de ET-1, o abalo muscular condicionava, para além do aumento da tensão activa, uma redução da TP no final da contracção, isto é, a TP no final da contracção era menor do que no início da mesma (Leite-Moreira e col., 2003). Este efeito representa um aumento da distensibilidade miocárdica e é dependente da estimulação dos receptores ET_A, da activação do trocador Na^+/H^+, da integridade do EE e da actividade dos receptores ET_{B1} endoteliais (Leite-Moreira e col., 2003; Brás-Silva e Leite-Moreira, 2006). Considerando que o NO e as prostaglandinas são dois dos mais importantes mediadores endoteliais e que a sua libertação pelo endotélio é modulada pelos receptores ET_{B1}, averiguámos o seu potencial papel no efeito promovido pela ET-1. Na presença de inibidores da síntese de NO ou de prostaglandinas, a ET-1 não promoveu qualquer alteração da TP, denotando que estes mediadores regulam os efeitos da ET-1 sobre a função diastólica, mais concretamente sobre as propriedades passivas do miocárdio (Brás-Silva e col., 2007).

Também o aumento da distensibilidade miocárdica despertado pela AM (Fontes-Sousa e col., 2007b) e pela U-II (Fontes-Sousa e col., 2007a) é modulado pela libertação de NO. O papel do NO no aumento da distensibilidade miocárdica já tinha sido descrito anteriormente (Paulus e Shah, 1999), tendo sido atribuído à diminuição da sensibilidade dos miofilamentos ao Ca^{2+}, por fosforilação da troponina I pela proteína cínase dependente

do GMPc (Shah e MacCarthy, 2000). Relativamente às prostaglandinas, já lhe foram atribuídos alguns efeitos sobre a função diastólica, em termos de propriedades passivas (Fontes-Sousa e col., 2007a) e relaxamento (Kisch-Wedel e col., 2005; Soares e col., 2006).

Verificámos ainda neste estudo que, apesar dos efeitos inotrópicos da ET-1 nos músculos papilares de animais saudáveis com EE intacto não terem sido significativamente diferentes dos observados nos músculos papilares de animais saudáveis com EE danificado e de animais com IC, o aumento da distensibilidade não foi estatisticamente significativo nestas duas últimas condições (Brás-Silva e col., 2007). A explicação para a ausência desta resposta no miocárdio insuficiente poderá estar relacionada com a disfunção do EE presente no modelo de IC induzida pela doxorrubicina (Brás-Silva e col., 2006).

Efeitos da Estimulação β-Adrenérgica sobre a Função Diastólica

O sistema β-adrenérgico desempenha um papel importante como regulador da função cardíaca. Para além do efeito inotrópico positivo, o sistema β-adrenérgico modula o relaxamento miocárdico ao desencadear um efeito lusitrópico positivo. Este efeito sobre o relaxamento tem sido atribuído à fosforilação de diversas proteínas pela PKA, tais como o fosfolambam, a troponina I e a proteína C ligada à miosina (Lohse e col., 2003). Para além destas, a PKA também fosforila a titina (Yamasaki e col., 2002) e estudos recentes demonstraram que esta fosforilação diminui agudamente a TP a nível do músculo cardíaco (Fukuda e col., 2005; Kruger e Linke, 2006). Ainda relativamente ao seu efeito sobre o relaxamento cardíaco, um estudo prévio do nosso grupo verificou que a estimulação β-adrenérgica atenua a disfunção diastólica induzida pela pós-carga (Leite-Moreira e col., 2001).

Nesta perspectiva desenvolvemos um estudo para avaliar o efeito da estimulação β-adrenérgica pela isoprenalina sobre as propriedades diastólicas do miocárdio (estudo nº6) em músculos papilares isolados de Coelho. Para além das semelhanças entre o coração do Homem e do Coelho referidas anteriormente, o coração insuficiente desta espécie exibe também alterações moleculares do sistema β-adrenérgico semelhantes às observadas na IC no Homem (Maurice e col., 1999), enfatizando uma vez mais a selecção desta espécie como modelo experimental.

A isoprenalina promoveu um efeito inotrópico e lusitrópico positivo, efeitos já descritos anteriormente (Lohse e col., 2003). Porém, o resultado inovador deste estudo prende-se com o aumento da distensibilidade miocárdica após a incubação com a isoprenalina. Este agente aumentou de forma significativa o comprimento passivo do músculo que, quando corrigido para o seu valor basal inicial, representou uma considerável diminuição da TP. Este achado foi também reforçado pelo desvio para a direita e para baixo da relação TP-comprimento. Desta forma, para cada TP o comprimento do músculo foi sempre maior na presença de isoprenalina (Fontes-Sousa e col., 2008a).

Tanto o bloqueio não selectivo dos receptores β-adrenérgicos como o bloqueio selectivo dos receptores β_1-adrenérgicos mostraram uma tendência para atenuar os efeitos da isoprenalina sobre a contractilidade, tendo diminuído de forma significativa os seus efeitos sobre o relaxamento. Adicionalmente, bloquearam os efeitos da isoprenalina sobre o comprimento passivo e consequentemente sobre a TP. Estes resultados sugerem, assim, que a modulação das propriedades diastólicas pelo sistema β-adrenérgico é mediada pelos receptores β_1-adrenérgicos. Por seu turno, a PKA e a PKC modulam o efeito da estimulação β-adrenérgca sobre a distensibilidade miocárdica, visto que a inibição da PKA ou da PKC diminuíram de forma significativa este efeito (Fontes-Sousa e col., 2008a). Efectivamente, estudos anteriores já tinham demonstrado que o aumento da actividade da

PKA diminui a TP no músculo cardíaco (Borbely e col., 2005; Kruger e Linke, 2006). Também a via dependente da PKC está envolvida no aumento da distensibilidade miocárdica promovido pela ET-1 (Leite-Moreira e col., 2003) e pela AngII (Leite-Moreira e col., 2006).

Adrenomedulina como um Novo Regulador da Rigidez Miocárdica

No estudo nº7 avaliámos os efeitos miocárdicos da AM. Constatámos em músculos papilares de ventrículo direito de Coelho que este peptídeo promove um efeito inotrópico e lusitrópico negativo, bem como uma diminuição da rigidez miocárdica ou, por outras palavras, um aumento da distensibilidade miocárdica. O desvio para a direita e para baixo da relação TP-comprimento veio corroborar este efeito. O bloqueio do receptor da AM, a inibição da PKA, a remoção do EE e a inibição da síntese de NO aboliram os efeitos sobre a contractilidade e o relaxamento. Por outro lado, o efeito da AM sobre a distensibilidade requer a presença de um EE intacto e depende da libertação de NO.

À semelhança dos nossos resultados, diversos estudos demonstraram igualmente um efeito inotrópico negativo da AM (Perret e col., 1993; Ikenouchi e col., 1997; Mukherjee e col., 2002). Todavia, estes resultados estão aparentemente em desacordo com os de outros estudos, nos quais foi reportado uma ausência de efeito inotrópico (Lainchbury e col., 2000a) ou, em oposição, a ocorrência de um efeito inotrópico positivo (Szokodi e col., 1998; Ihara e col., 2000). Possíveis justificações para estas discrepâncias poderão dever-se a diferenças relacionadas com a espécie animal ou a com preparação experimental.

Dado que a AM activa a síntase endotelial do NO (Shimekake e col., 1995; Nishimatsu e col., 2001) fomos averiguar até que ponto este estaria envolvido nos efeitos da AM. Ikenouchi e colaboradores (1997) observaram em cardiomiócitos de Coelho em

cultura que o NO contribuía para o efeito inotrópico negativo induzido pela AM, tal como constatado presentemente (Fontes-Sousa e col., 2007b).

Para além do NO e do EE, a PKA também modula o efeito inotrópico negativo da AM. Embora a activação do sistema adenilato cíclase-AMPc seja um dos principais mecanismos responsáveis pela estimulação da contractilidade cardíaca em corações de mamíferos (Morgan, 1991), um estudo mais recente observou que a AM induz um efeito inotrópico positivo ou negativo mediante a activação do sistema PKA dependente, respectivamente, de proteínas G estimulatórias ou inibitórias (Mittra e col., 2004).

O fragmento peptídico AM22-52 é considerado um antagonista dos receptores AM_1 e AM_2 (Eguchi e col., 1994), embora estudos mais recentes apontem para uma maior selectividade para com os primeiros receptores (Hay e col., 2003). O efeito inotrópico negativo da AM ocorreu através da activação dos receptores sensíveis à AM22-52. Contrariamente, o efeito da AM sobre a distensibilidade aparentemente não depende dos receptores sensíveis à AM22-52, levando-nos a supôr que este efeito da AM será provavelmente dependente da activação dos receptores AM_2, embora sejam necessários estudos futuros para esclarecer esta hipótese.

Efeitos Miocárdicos da Urotensina II e sua Interacção com os Sistemas da Angiotensina II e Endotelina-1

Finalmente, nos dois últimos trabalhos desta dissertação investigámos os efeitos despertados por um novo sistema neuro-humoral, o sistema da U-II (estudos nº8 e 9). Verificámos pela primeira vez que a U-II induz um aumento da distensibilidade miocárdica dependente da concentração (Fontes-Sousa e col., 2007a; Fontes-Sousa e col., 2008c). Este efeito é atenuado pela inibição da síntese de prostaglandinas e completamente abolido pelo bloqueio do receptor UT ou pela inibição da síntese de NO. Deste modo, o efeito da U-II

sobre a distensibilidade miocárdica é mediado pelo receptor UT e depende da libertação de NO e de prostaglandinas (Fontes-Sousa e col., 2007a). Curiosamente, considerando que estes dois agentes são libertados pelo endotélio, a remoção do EE não alterou os efeitos da U-II sobre a distensibilidade. Esta aparente discrepância poderá ser explicada pelo facto do endotélio coronário microvascular, outra fonte importante de NO e prostaglandinas, se manter intacto após a remoção do EE (Brutsaert, 2003). De notar ainda que o NO também pode ser libertado pelos próprios cardiomiócitos (Massion e col., 2003).

Constatámos também que a U-II induziu um efeito inotrópico negativo que não foi alterado em nenhum dos protocolos experimentais levados a cabo. Embora, estudos *in vitro* e *in vivo* tenham demonstrado também o mesmo efeito inotrópico, estudos realizados em trabéculas humanas e em músculos papilares de Rato observaram um efeito inotrópico positivo por um mecanismo dependente da PKC. Tal como sucedeu com a AM, estas diferenças poderão estar relacionadas com a espécie em causa, bem como com a preparação experimental utilizada.

O receptor UT encontra-se ligado à proteína $G\alpha_{q/11}$, a qual activa a fosfolipase C com consequente acumulação de IP_3 e de diacilglicerol, com mobilização do Ca^{2+} intracelular (Ames e col., 1999; Saetrum Opgaard e col., 2000b; Tzanidis e col., 2003). Desta maneira, o receptor UT partilha algumas vias subcelulares e interage com os sistemas de receptores da angiotensina e da endotelina por intermédio dos receptores AT1 e ET_A (Li e col., 2005; Wang e col., 2007). Adicionalmente, e tendo em consideração que tanto a AngII (Leite-Moreira e col., 2006) como a ET-1 (Leite-Moreira e col., 2003) também aumentam a distensibilidade miocárdica, a possível interacção da U-II com estes dois sistemas vasoactivos surgiu como uma hipótese possível. O último estudo desta tese (estudo nº9) veio comprovar esta hipótese, tendo demonstrado que o antagonismo competitivo dos receptores AT1 e o bloqueio não selectivo dos receptores ET_A/ET_B

aboliram e atenuaram, respectivamente, o aumento da distensibilidade miocárdica induzida pela U-II (Fontes-Sousa e col., 2008c). Porém, permanecem por apurar as vias de sinalização específicas subjacentes a estes efeitos.

Implicações fisiopatológicas

A rigidez miocárdica é um importante determinante do enchimento ventricular e, consequentemente, da função diastólica (Leite-Moreira, 2006). Durante muito tempo considerou-se que os mediadores neuro-humorais apenas seriam capazes de alterar cronicamente as propriedades diastólicas do miocárdio através da modificação de factores como a fibrose e a hipertrofia (Gaasch e Zile, 2004). Contudo, estudos anteriores demonstraram que alguns agentes neuro-humorais modulam de forma aguda a rigidez diastólica, tais como o NO (Grocott-Mason e col., 1994; Heymes e col., 1999), a ET-1 (Leite-Moreira e col., 2003) e a AngII (Leite-Moreira e col., 2006). Nesta dissertação confirmámos o efeito da ET-1 e contribuímos para o esclarecimento das vias de transdução do sinal envolvidas. Verificámos ainda que, para além destes agentes, a activação do sistema β-adrenérgico (Fontes-Sousa e col., 2008a), a AM (Fontes-Sousa e col., 2007b) e a U-II (Fontes-Sousa e col., 2007a; Fontes-Sousa e col., 2008c) também desempenham um papel importante na modulação da distensibilidade miocárdica. Embora, curiosamente, se saiba que alguns deles, como a ET-1 e a U-II, quando activados cronicamente são importantes promotores de fibrose e hipertrofia, e portanto de uma diminuição da distensibilidade ventricular (Bousette e col., 2006a; Brunner e col., 2006), o presente trabalho demonstra que pelo contrário, quando activados agudamente, aumentam significativamente essa distensibilidade.

A redução da TP promovida pelos diferentes agentes neuro-humorais significa que o ventrículo consegue atingir o mesmo volume telediastólico com pressões de enchimento

menores, o que traduz um poderoso mecanismo de adaptação fisiológica que se encontra comprometido na IC e na presença de disfunção endotelial. O facto de termos utilizado um modelo *in vitro* permite avaliar os efeitos dos diferentes agentes neuro-humorais nas propriedades intrínsecas diastólicas do miocárdio. Todavia, importa sublinhar que o aumento do comprimento muscular, como o observado no conjunto dos resultados obtidos nesta tese, poderá levar a dilatação ventricular contribuindo, assim, para a remodelagem cardíaca.

O EE e os seus mediadores estão envolvidos na modulação do efeito da ET-1 e da AngII sobre a distensibilidade miocárdica (Brás-Silva e Leite-Moreira, 2006; Brás-Silva e col., 2007; Castro-Chaves e col., 2007). Esta dissertação permitiu confirmar que o mesmo ocorre com a AM (Fontes-Sousa e col., 2007b). De forma semelhante à disfunção endotelial vascular (Drexler e col., 1992), parece que a disfunção endotelial cardíaca está presente e/ou contribui para a progressão para a IC (Brás-Silva e col., 2006). Deste modo, considerando que o endotélio cardíaco, vascular e endocárdico, e os seus mediadores regulam funcionalmente o miocárdio subjacente, os resultados da presente dissertação poderão contribuir para um melhor esclarecimento da fisiopatologia da IC.

Por outro lado, torna-se razoável especular que alguns efeitos cardiovasculares possam resultar da interacção entre diferentes sistemas neuro-humorais. Do ponto de vista fisiopatológico e clínico, estes resultados são potencialmente relevantes, dado que a inibição de um dado sistema neuro-humoral pode condicionar os efeitos resultantes da activação de outros sistemas.

Apesar dos inúmeros avanços no tratamento da IC, esta síndrome continua a representar um grave problema de saúde pública. Assim, os resultados agora reunidos reforçam a ideia relativamente à modulação neuro-humoral da função diastólica, o que poderá contribuir para o esclarecimento da fisiopatologia da IC.

CONCLUSÕES

No seu conjunto, os resultados obtidos permitem concluir o seguinte:

1. O exame ecocardiográfico no Coelho branco neozelandês permitiu:
    a. Descrever os valores ecocardiográficos de referência de alguns parâmetros ecocardiográficos obtidos por modo-M, DP e DT, recorrendo a dois protocolos anestésicos diferentes;
    b. Demonstrar que a concordância absoluta para o ITVE é fraca para todas as técnicas ecocardiográficas avaliadas (modo-M, DP e DT).

2. No modelo de IC induzida pela doxorrubicina, a resposta à estimulação selectiva dos receptores ET_{B1} está significativamente atenuada, indicando a existência de disfunção endotelial endocárdica neste modelo experimental. Por outro lado, reforça a importância dos receptores ET_{B1} como marcadores da integridade funcional do EE.

3. A ET-1 aumenta de forma aguda a distensibilidade diastólica em condições de sobrecarga no miocárdio ventricular do Coelho. Este efeito depende da libertação de NO e prostaglandinas e está ausente na IC que cursa com disfunção do EE.

4. A estimulação β-adrenérgica, para além de promover um aumento da contractilidade e do relaxamento no miocárdio ventricular de Coelho, induz uma diminuição da rigidez miocárdica, sendo que este efeito requer a activação dos receptores β_1-adrenérgicos e é mediado pelas vias da PKA e da PKC.

5. Os efeitos agudos da AM no miocárdico ventricular de Coelho incluem:
    a. Efeitos inotrópico e lusitrópico negativos dependentes da activação do seu receptor, da PKA, da integridade do EE e da libertação de NO;
    b. O aumento do comprimento passivo muscular, denotando uma diminuição da rigidez miocárdica. Este efeito depende da integridade do EE e da libertação do NO.

6. No miocárdico ventricular de Coelho, a U-II promove:
    a. Efeitos inotrópico e lusitrópico negativos e um aumento da distensibilidade miocárdica;
    b. O aumento da distensibilidade miocárdica induzida pela U-II é mediado pelo receptor UT e depende da libertação de NO e de prostaglandinas. Adicionalmente, o aumento da distensibilidade miocárdica induzida pela U-II depende da activação dos sistemas da AngII e da ET-1.

CAPÍTULO VI

SUMMARY AND CONCLUSIONS

Heart failure (HF) is a complex clinical disorder resulting from any structural insult causing cardiac dysfunction. Diastolic heart failure (DHF) and systolic heart failure (SHF) are two clinical subsets of the syndrome of HF that are most frequently encountered in clinical practice. Epidemiological studies have clearly shown that DHF is a common cause of chronic HF and causes a significant increase in cardiovascular morbidity and mortality.

Diastolic dysfunction is well recognized and common in patients with HF. Whether DHF shares most pathogenic mechanisms with SHF remains to be established. The neurohumonal hypothesis has become central to our understanding of SHF. In DHF its role is not fully established. So, the main goal of the present thesis was to elucidate the role of different neurohumoral mechanisms on intrinsic myocardial properties in healthy hearts and in the progression to HF, with a particular emphasis on the diastolic properties. Experimental work was performed in papillary muscles isolated from the right ventricle of New Zealand White rabbits (healthy and with doxorubicin-induced HF). Evaluated neurohumoral mechanism included endothelin-1 (ET-1), sympathetic nervous system, adrenomedullin (AM) and urotensin II (U-II). On the other hand, considering that echocardiography is an essential diagnostic tool for the evaluation of global cardiac function and the fact that rabbit is an important animal model, we also characterize some echocardiographic parameters in this species.

The results allowed us to formulate the following conclusions:

1. Echocardiography evaluation in anaesthetised New Zealand white rabbits allowed us to:
    a. Report normal values of some echocardiographic parameters obtained from M-mode, Doppler echocardiography and Tissue Doppler Imaging (TDI);
    b. Demonstrate that the absolute agreement for left ventricular Tei index (LVTI) is poor for all the techniques evaluated (M-mode, pulsed wave Doppler and TDI);

2. In the doxorubicin-induced HF, the response to endothelial ET_{B1} selective stimulation is impared, indicating the presence of endocardial endothelial (EE) dysfunction in this experimental model and reinforcing the importance of ET_{B1} receptors as functional markers of EE integrity.

3. ET-1 acutely increases diastolic distensibility in conditions of cardiac overload. This effect is dependent on NO and prostaglandins release being absent in HF with EE dysfunction.

4. Besides its well-known effects on myocardial contractility and relaxation, β-adrenergic stimulation decreases myocardial stiffness, an effect that requires the activation of β_1-adrenoceptors and is mediated by PKA and PKC.

5. Acute effects of AM on rabbit myocardium include:
    a. Negative inotropic and lusitropic effects dependent on the receptor of AM, PKA, EE integrity and NO release.
    b. An increase of passive muscle length, denoting a decrease of myocardial stiffness. This effect depends on EE integrity and NO release.

6. Acute effects of U-II on rabbit myocardium include:
    a. Negative inotropic and lusitropic effects;
    b. An increase of passive muscle length, denoting an increase of myocardial distensibility;
    c. The increase of myocardial distensibility induced by U-II depends on UT receptor and NO and prostaglandins release; additionally, this increase on myocardial distensibility is also dependent on AngII and ET-1 systems.

CAPÍTULO VII

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CAPÍTULO VIII

PUBLICAÇÕES

Ao abrigo do Decreto-Lei nº 216/92 fazem parte integrante desta dissertação os seguintes trabalhos já publicados ou em publicação:

I. **Fontes-Sousa AP**, Brás-Silva C, Moura C, Areias JC, Leite-Moreira AF. M-mode and Doppler echocardiographic reference values in healthy New Zealand white male rabbits. *American Journal of Veterinary Research* 2006; 67(10): 1725-1729.

II. **Fontes-Sousa AP**, Moura C, Carneiro CS, Teixeira-Pinto A, Areias JC, Leite-Moreira AF. Echocardiographic evaluation including tissue Doppler imaging in New Zealand white rabbits sedated with ketamine and midazolam. *The Veterinary Journal* 2008 (em publicação).

III. Moura C, **Fontes-Sousa AP**, Teixeira-Pinto A, Areias JC, Leite-Moreira AF. Left ventricular Tei Index in rabbit: agreement between echocardiography techniques. *American Journal of Veterinary Research* (em revisão).

IV. Brás-Silva C, Monteiro-Sousa D, Duarte AJ, Guerra MS, **Fontes-Sousa AP**, Moura C, Areias JC, Leite-Moreira AF. Nitric oxide and prostaglandins – important players in endothelin-1 induced myocardial distensibility. *Physiological Research* 2007 (em publicação).

V. Brás-Silva C, **Fontes-Sousa AP**, Moura C, Areias J, Leite-Moreira AF. Impaired response to ETB receptor stimulation in heart failure. Functional evidence of endocardial endothelial dysfunction?. *Experimental Biology and Medicine* 2006; 231(6): 893-898.

VI. **Fontes-Sousa AP**, Falcão-Pires I, Brás-Silva C, Leite-Moreira AF. β-adrenergic stimulation acutely decreases myocardial stiffness: a novel β_1- adrenoceptor, PKA and PKC mediated effect (enviado para publicação).

VII. **Fontes-Sousa AP**, Santos-Carneiro C, Pires AL, Leite-Moreira AF. Adrenomedullin as a novel regulatory peptide of myocardial stiffness: contribution of endocardial endothelium and nitric oxide. *Peptides* (em revisão).

VIII. **Fontes-Sousa AP**, Brás-Silva C, Pires AL, Monteiro-Sousa D, Leite-Moreira AF. Urotensin II acutely increases myocardial length and distensibility: Potential implications for diastolic function and ventricular remodeling. *Naunyn-Schmiedeberg's Archives of Pharmacology* 2007; 376(1-2):107-115.

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