ALESSANDRA LINARDI

ATIVIDADES E EXPRESSÃO DE PEPTIDASES EM RATOS TRATADOS CRONICAMENTE COM L-NAME, UM INIBIDOR DA BIOSSÍNTESE DE ÓXIDO NÍTRICO, E EM RATOS ESPONTANEAMENTE HIPERTENSOS

Este exemplar corresponde à versão final da Tese de Doutorado, apresentada à Pós-Graduação da Faculdade de Ciências Médicas - UNICAMP, para obtenção do título de Doutor em Farmacologia da Farmacêutica – Alessandra Linardi.

Campinas, 12 de agosto de 2004.

Prof. Dr. Stephen Hyslop
- Orientador -

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"O homem se torna muitas vezes o que ele próprio acredita que é. Se eu insisto em repetir para mim mesmo que não posso fazer uma determinada coisa, é possível que eu acabe me tornando realmente incapaz de fazê-la. Ao contrário, se tenho a convicção de que posso fazê-la, certamente adquirirei a capacidade de realizá-la, mesmo que não a tenha no começo."

(Gandhi)

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SUMÁRIO

ANP - peptídeo natriurético atrial

APA - aminopeptidase A

APM - aminopeptidase M

APP - aminopeptidase P

CCVD - canais de Ca^{2+} voltagem dependentes do tipo L

cFP - (N-[1-(R,S)-carboxi-3-fenilpropil]-Ala-Ala-Phe-*p*-aminobenzoato

cGMP - guanosina-3',5'-monofosfato cíclica

CGRP - peptídeo relacionado ao gene da calcitonina

CS-866 - antagonista de receptores AT_1 de angiotensina II

DOCA - acetato de deoxicorticosterona

DPP IV - dipeptidil peptidase IV

DTT - ditiotreitol

ECA - enzima conversora de angiotensina

ECE - enzima conversora de endotelina

EC33 - (S)-3-amino-4-mercaptobutil ácido sulfônico

ERO - espécies intermediárias reativas de oxigênio

ICC - insuficiência cardíaca congestiva

JA2 - *N*-[1-(R,S)-carboxi-3-fenilpropil]-Ala-Aib-Tyr-*p*-aminobenzoato

2K-1C - 2 kidney -1 clip

L-NA - L-nitro arginina

L-NAME - N^{ω}-nitro-L-arginina metil éster

L-NIO - *N*(5)-(1-iminoetil)-L-ornitina

L-NMMA - N^{ω}-monometil-L-arginina

L-NNA - L^{ω}-nitro-L-arginina

MEP 24.15 - metaloendopeptidase 24.15

NAH - núcleo anterior do hipotálamo

NEP 24.11 - endopeptidase neutra 24.11

NO - óxido nítrico

NOS - óxido nítrico sintase

NPY - neuropeptídeo Y

Lista de abreviaturas

NTS -	núcleo do trato solitário
PC18 -	2-amino-4-metilsulfonil butano tiol
SHR -	ratos espontaneamente hipertensos
SHR-SP -	ratos espontaneamente hipertensos propensos a derrame
SIN-1 -	3-morfolinosidnonimina
SNS -	sistema nervoso simpático
SRA -	sistema renina angiotensina
VIP -	polipeptídeo vasoativo intestinal
WYK -	ratos Wistar Kyoto
Y-27632 -	inibidor específico para Rho-quinase

Resumo

As peptidases desempenham um papel importante na modulação da atividade de peptídeos endógenos em situações normais e patológicas. Neste trabalho, investigamos a atividade e expressão de quatro peptidases (aminopeptidase M – APM, dipeptidil peptidase IV – DPP IV, endopeptidase neutra – NEP 24.11 e metaloendopeptidase 24.15 – MEP 24.15) em tecidos (aorta, cérebro, coração, fígado, pulmão e rim) de ratos em dois modelos de hipertensão: a hipertensão induzida pelo tratamento crônico de ratos com N^{ω}-nitro-L-arginina metil éster (L-NAME), um inibidor da biossíntese de óxido nítrico (NO), e em ratos espontaneamente hipertensos (SHR). A atividade enzimática foi avaliada por ensaios enzimáticos fluorométricos ou colorimétricos enquanto a expressão foi avaliada por western blotting. Em ratos tratados com L-NAME (80 mg kg^{-1} day^{-1}, p.o., durante 4 semanas), não houve alteração na atividade das quatro peptidases estudas em cérebro, coração, fígado, pulmão e rim (a NEP 24.11 foi detectada apenas no pulmão e rim). Entretanto, na aorta torácica, a atividade da APM mostrou um pequeno decréscimo enquanto as atividades da DPP IV e MEP 24.15 foram significativamente aumentadas. A expressão de DPP IV e MEP 24.15 também foi aumentada pelo tratamento com L-NAME. As atividades da MEP 24.15 recombinante ou DPP IV renal não foram afetadas pela incubação com L-NAME (1-100 μM) ou nitroprussiato de sódio e 3-morfolinosidnonimina (1-100 μM cada), ambos doadores de NO. A administração de N-[1-(R,S)-carboxi-3-fenilpropil]-Ala-Aib-Tyr-*p*-aminobenzoato (JA2), um inibidor de MEP 24.15/MEP 24.16, em ratos anestesiados com pentobarbital sódico aumentou a resposta hipotensora da bradicinina. No modelo SHR, a atividade da DPP IV foi aumentada no cérebro, enquanto que as atividades da MEP 24.15 e NEP 24.11 foram aumentadas no pulmão (MEP 24.15 e NEP 24.11) e rim (NEP 24.11). Por outro lado, as atividades da DPP IV e MEP 24.15 foram reduzidas na aorta torácica; a APM foi inalterada e a NEP 24.11 não foi detectada neste tecido. Western blotting para DPP IV e MEP 24.15 mostrou redução correspondente na expressão de ambas as enzimas na aorta e um aumento para DPP IV no cérebro; não houve alteração na expressão da MEP 24.15 no pulmão e nem da NEP 24.11 no pulmão ou rim. A administração

do JA2 em SHR não alterou as respostas à bradicinina. Estes resultados indicam que há alterações na atividade e expressão das peptidases estudadas (exceto APM) nos dois modelos de hipertensão. Entretanto, a variabilidade nestas alterações entre os dois modelos sugere que a hipertensão *per se* não é a causa principal destas diferenças, mas que provavelmente resultam de alterações bioquímicas associadas a cada modelo e seletivas para cada peptidase.

Abstract

Peptidases play an important role in modulating the activity of endogenous peptides in normal and pathological situations. In this work, we investigated the activity and expression of four peptidases (aminopeptidase M - APM, dipeptidyl peptidase IV - DPP IV, metalloendopeptidase 24.15 - MEP 24.15 and neutral endopeptidase 24.11 - NEP 24.11) in rat tissues (aorta, brain, heart, kidney, liver and lung) in two models of hypertension, namely, chronic treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME), a nitric oxide (NO) synthase inhibitor (80 mg kg^{-1} day^{-1}, p.o. for 4 weeks), and spontaneously hypertensive rats (SHR). Enzymatic activity was assayed fluorometrically or colorimetrically and protein expression was assessed by western blotting. Treatment with L-NAME did not significantly alter the activities of the four peptidases in brain, heart, kidney, liver and lung (NEP was detected only in kidney and lung). In contrast, in thoracic aorta, the activity of APM was slightly but significantly reduced whereas those of DPP IV and MEP 24.15 were markedly enhanced. Immunoblotting for DPP IV and MEP 24.15 showed increased expression in aortic tissue. Neither L-NAME (1-100 μM) nor the NO donors sodium nitroprusside and 3-morpholinosydnonimine (1-100 μM each) had any consistent effect on the activity of recombinant MEP 24.15 or renal DPP IV. Administration of the MEP 24.15/MEP 24.16 inhibitor N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate (JA2) in pentobartibal-anesthetized rats significantly potentiated the hypotensive response to bradykinin. In SHR, the activity of DPP IV was significantly increased in brain, whereas MEP 24.15 and NEP 24.11 activities were markedly enhanced in lung (MEP 24.15 and NEP 24.11) and kidney (NEP 24.11). In contrast, the activities of DPP IV and MEP 24.15 were markedly decreased in aortic tissue; APM was unaltered and NEP 24.11 was not detected in this tissue. Immunoblotting for DPP IV and MEP 24.15 showed decreased expression in aortic tissue and increased expression of DPP IV in the brain; there was no alteration in the expression of MEP 24.15 in the lung or of NEP 24.11 in kidney and lung. The administration of JA2 to SHR did not alter the responses to bradykinin. These results indicate that there were alterations in the activity and expression of the peptidases studied (except for APM) in both models

of hypertension. However, the different patterns of alterations seen between the two models suggests that the changes were not caused by the hypertension *per se*, but rather were probably the result of biochemical alterations associated with each model and selective for each peptidase.

1. INTRODUÇÃO

A literatura tem demonstrado cada vez mais o envolvimento de fatores estruturais e funcionais na elevação da resistência periférica, característica principal dos modelos de hipertensão experimental e humana. Desde 1940, quando foi demonstrado que a renina atuava no angiotensinogênio, liberando angiotensina I (BRAUN-MENÉNDEZ *et al.*, 1940; PAGE e HELMER, 1940) e, em seguida na década de 50, quando foram reconhecidas duas formas de angiotensina, formadas por clivagem através da enzima conversora de angiotensina (ECA) (ver SKEGGS, 1956), até a identificação do fator de relaxamento do endotélio (FURCHGOTT e ZAWADSKI, 1980) como sendo o óxido nítrico (NO) (IGNARRO *et al.*, 1987; PALMER *et al.*, 1987; MONCADA *et al.*, 1988), e a descoberta da endotelina (YANAGISAWA *et al.*, 1988), pesquisas têm explorado de forma intensa os mecanismos envolvidos na fisiopatologia da hipertensão. Neste contexto, torna-se imprescindível estudar a ação de peptídeos endógenos, assim como sua liberação e degradação pelas peptidases para compreensão da patologia da hipertensão e para o desenvolvimento de novas terapias para o tratamento da mesma. Assim, desde o surgimento do primeiro modelo de hipertensão experimental desenvolvido por GOLDBLAT *et al.* (1934), o qual observou que ao introduzir um clipe na artéria renal de cachorros, os mesmos desenvolviam uma forma de hipertensão secundária, o uso de modelos animais de hipertensão arterial tem-se mostrado bastante promissor para o estudo de sistemas peptidérgicos e sua influência na regulação da pressão arterial.

1.1. MODELOS DE HIPERTENSÃO

1.1.1. HIPERTENSÃO INDUZIDA PELA INIBIÇÃO CRÔNICA DA BIOSSÍNTESE DE ÓXIDO NÍTRICO

1.1.1.1. Óxido nítrico (NO)

O óxido nítrico (NO) é um mediador biológico multifuncional, o qual serve como molécula chave em muitos processos fisiológicos, como a comunicação neuronal e a regulação do tônus vascular (MONCADA *et al.*, 1991; GROSS e WOLIN, 1995). Sua síntese ocorre através da L-arginina, tendo como produto intermediário a N^G-hidroxi-L-arginina, a qual é convertida em L-citrulina, com

conseqüente liberação de NO (PALMER e MONCADA, 1988). A reação é catalisada por um grupo de enzimas denominadas de NOS (HEMMENS e MAYER, 1998). Há três isoformas de NOS: a NOS endotelial (eNOS), a NOS neuronal (nNOS) e a NOS induzível (iNOS). A eNOS e nNOS são isoformas constitutivas, as quais dependem do complexo Ca^{2+}/calmodulina para produzirem NO, liberando o mesmo em baixas concentrações (nM) e em condições fisiológicas. Já, a iNOS é uma isoforma induzível, a qual produz NO em condições patológicas e em altas concentrações (μM), por longos períodos. Embora a iNOS esteja presente constitutivamente em alguns tecidos como epitélio pulmonar e túbulos distais do rim, é principalmente expressa em células inflamatórias após a indução por citocinas e outros mediadores inflamatórios. Sua atividade é independente de Ca^{2+} (ANDREW e MAYER, 1999; HECKER *et al.*, 1999).

O NO exerce amplo espectro de atividades biológicas *in vivo*, os quais são em parte dependentes da guanosina-3',5'-monofosfato cíclica (cGMP), produzida após a estimulação da enzima guanilato ciclase solúvel pelo mesmo (FÖRSTERMANN *et al.*, 1986; MONCADA *et al*, 1991). Nas células musculares lisas dos vasos, esta ativação resulta em relaxamento. O NO também pode atuar diretamente em canais de potássio dependentes de cálcio, levando a uma hiperpolarização dependente do endotélio nos vasos, resultando em vasodilatação (BOLOTINA *et al.*, 1994). Além de regular o tônus vascular, o NO modula a adesão leucocitária no endotélio (KUBES *et al.*, 1991), inibe a proliferação celular no músculo liso (GARG e HASSID, 1989; SCOTT-BURDEN *et al.*, 1992) e a agregação plaquetária (RADOMSKI *et al.*, 1987), e regula a formação de massa óssea (ARMOUR, *et al.*, 2001), o consumo de oxigênio renal (LAYCOCK *et al.*, 1998) e a angiogênese (LEE *et al.*, 1999; ZICHE e MORBIDELLI, 2000).

Além dos efeitos do NO mediados pela formação de cGMP, como o controle do tônus vascular (MONCADA e HIGGS, 1993), o NO também exerce diversas outras funções independentes da formação deste nucleotídeo cíclico (para revisão ver PFEILSCHIFTER *et al.*, 2001). Tais funções incluem a modulação da síntese protéica (CURRAN *et al.*, 1991), controle de atividade enzimática (TROY *et al.*, 1996) e a regulação da expressão (ativação ou

repressão) de genes para α-actina (KAWADA *et al.,* 1996), citocinas (REMICK e VILLARETE, 1996), fatores de crescimento (TSURUMI *et al.,* 1997; GUO *et al.,* 1998), moléculas de adesão (BIFFL *et al.,* 1996; LEFER e LEFER, 1996; MUROHARA *et al.,* 1996; KUPATT *et al.,* 1997), proteínas envolvidas em apoptose (BRÜNE *et al.,* 1998a,b), receptores (KEH *et al.,* 1996; MICHELSON *et al.,* 1996; REDMOND *et al.,* 1996; WALLACE e BOOZE, 1997; ICHIKI *et al.,* 1998; USUI *et al.,* 1998), e enzimas, tais como a ciclooxigenase–2 (COX-2) (AMIN *et al.,* 1997), citocromo P-450 (KHATSENKO, 1998), metaloprotease de matriz (LALA e ORUCEVIC, 1998), superóxido dismutase (BRADY *et al.,* 1997) e a própria NOS (CHEN e MEHTA, 1997).

Alterações atribuídas à atividade ou expressão das NOS e conseqüentemente na biodisponibilidade de NO têm sido relacionadas com processos patológicos como a hipertensão (PANZA, 1997), hipercolesterolemia e arteroesclerose (BLAIR *et al.,* 1999; KUHLENCORDT *et al.,* 2001), diabetes (OYADOMARI *et al.,* 2001), insuficiência cardíaca (WATANABE *et al.,* 2000) e cicatrização prejudicada (LEE *et al.,* 1999). Estudos em algumas patologias humanas têm avaliado a expressão da NOS e os efeitos da administração de doadores de NO. Em modelos experimentais, o papel da NOS é estudado pela manipulação de sua atividade usando inibidores da enzima como o L-NAME (N^{ω}-nitro-L-arginina metil éster), N^{ω}-monometil-L-arginina (L-NMMA) ou a *N*(5)-(1-iminoetil)-L-ornitina (L-NIO). Com relação ao L-NAME, um análogo da L-arginina, o mesmo é hidrolisado em L-nitroarginina, a qual inibe a atividade da NOS (PFEIFFER *et al.,* 1996). Através do uso de L-NAME para inibir a NOS, foi possível demonstrar que o NO regula o tônus vascular em humanos, uma vez que esta inibição resultou no aumento da pressão sangüínea (SANDER *et al.,* 1999). Em outros sistemas vasculares, como o pulmão, a queda de atividade da NOS pode prejudicar a regulação do tônus vascular, resultando em hipertensão pulmonar (KANNO *et al.,* 2001; LEE *et al.,* 2001; PEARSON *et al.,* 2001).

A administração crônica de L-NAME em ratos até oito semanas resulta em hipertensão prolongada (ARNAL *et al.,* 1992; BAYLIS *et al.,* 1992; RIBEIRO *et al.,* 1992). Neste modelo de hipertensão, ocorrem alterações hemodinâmicas e

morfológicas, principalmente no tecido cardíaco (MORENO Jr. *et al.*, 1995, 1997) e renal (para revisões, ver ZATZ e BAYLIS, 1998, e JOVER e MIMRAN, 2001). O infarto do miocárdio observado em ratos após a administração de L-NAME indica um papel do NO na regulação da resposta arterial sistêmica (GABALLA *et al.*, 1999). A administração de L-NAME também prejudica a função endotelial e aumenta o dano isquêmico no modelo cardíaco de arterioesclerose em camundongos (TOKUNO *et al.*, 2001). No rim, o L-NAME aumenta a pressão sangüínea sistêmica, e causa mudança na hemodinâmica renal e função tubular, incluindo consumo de oxigênio, fibrose e glomeruloesclerose (BAYLIS *et al.*, 1992; ADLER *et al.*, 2001).

Além das alterações hemodinâmicas e renais, o tratamento de ratos com L-NAME durante duas semanas leva a um aumento na expressão de cininogênio e calicreína (CHAO *et al.*, 1996) em diversos tecidos (cérebro, coração, fígado, e rim). Similarmente, o tratamento crônico de cães com L^{ω}-nitro-L-arginina (L-NNA) leva a um aumento na expressão da enzima ciclooxigenase-1 (COX-1) e, conseqüentemente, a uma maior produção de prostaglandinas (BEVERELLI *et al.*, 1997). Quanto a receptores, USUI *et al.* (1998) relataram que a inibição da síntese de NO pelo L-NAME em ratos, aumentou a expressão de RNAm para receptores do tipo I (AT_{1A}, AT_{1B}) de angiotensina II e, conseqüentemente, elevou o número de receptores expressos na glândula adrenal, sendo que este efeito não era resultado da hipertensão que acompanhava a inibição de NOS. Em adição, ICHIKI *et al.* (1998) relataram que o NO reduz a expressão de receptores AT_1 em cultura de células do músculo liso vascular por uma via independente de cGMP. Já, KATOH *et al.* (1998) descreveram um aumento na expressão de RNAm que foi acompanhado por um aumento no número de receptores AT_1 em tecido cardíaco de animais tratados com L-NAME, no entanto esses resultados foram observados apenas na primeira semana de tratamento, sendo que após quatro semanas os níveis retornaram aos dos animais controle.

1.1.1.2. Sistema renina-angiotensina

Vários trabalhos têm demonstrado o envolvimento do sistema renina angiotensina (SRA) na resposta pressórica após o tratamento crônico com L-NAME. RIBEIRO *et al.* (1992) demonstraram que a administração crônica concomitante de um antagonista de receptores AT_1 (losartan) com L-NAME preveniu a hipertensão neste modelo. Trabalhos posteriores envolvendo a administração de antagonistas de receptores AT_1 ou inibidores da ECA confirmaram esses resultados (POLLOCK *et al.,* 1993; NAVARRO-CID *et al.,* 1996; TAKASE *et al.,* 1996; TAKEMOTO *et al.,* 1997). Estudos *in vitro* (THARAUX *et al.,* 1997), inclusive em rim isolado (GARDES *et al.,* 1994), sugerem que o NO estimula a síntese ou secreção de renina. Além disso, vários autores relatam um decréscimo de renina circulante em animais com inibição crônica de NOS (RIBEIRO *et al.,* 1992; YAMADA *et al.,* 1996; KNOBLICH *et al.,* 1996). TAKEMOTO *et al.* (1997) observaram um aumento na atividade da ECA em aorta e coração, mas não em fígado, pulmão, rim e plasma, de ratos tratados cronicamente com L-NAME, e GONZALEZ *et al.* (2000) também descreveram um aumento na atividade da ECA em aorta de ratos tratados com L-NAME durante oito semanas.

1.1.1.3. Sistema nervoso simpático

Além do SRA, a literatura tem demonstrado o envolvimento do sistema nervoso simpático (SNS) na regulação do tônus vascular no modelo crônico de L-NAME. Assim, CUNHA *et al.* (1993) relataram uma queda da pressão arterial em ratos tratados cronicamente com L-NAME, após bloqueio ganglionar. No mesmo trabalho, observou-se que o bloqueio de receptores β-adrenérgicos produziu queda na pressão arterial e freqüência cardíaca nos ratos tratados com L-NAME, quando comparados ao controle. Similarmente, um aumento da atividade do SNS após tratamento crônico com L-NAME tem sido descrito (MATSUOKA *et al.,* 1994; ZANCHI *et al.,* 1995). Mais recentemente, foi demonstrado que a administração de um antagonista de receptores AT_1 (candesartan) no NTS produziu queda na pressão arterial e freqüência cardíaca em ratos tratados com L-NAME, quando

comparados aos controles (ESHIMA *et al.*, 2000). PECHÁNOVÁ *et al.* (2004) avaliaram o balanço de sistemas vasoativos no modelo de tratamento crônico com L-NAME e observaram que o SNS tem um papel fundamental na manutenção de pressão arterial nesse modelo, apesar do bloqueio do SRA normalizar a hipertensão, como citado anteriormente. Estes autores sugeriram que a interação entre o sistema nervoso central (SNC), NO e angiotensina II neste modelo de hipertensão resulta em um elevado tônus simpático na vasculatura, a qual é normalizada com a inibição do SRA. Esses resultados sugerem que o aumento da atividade do SNS contribui para a hipertensão causada pela inibição crônica de NOS, e que a ação do SRA no NTS via receptores AT_1 está envolvida neste aumento da ativação do SNS.

1.1.1.4. Sistema natriurético atrial

Os peptídeos natriuréticos, dos quais os principais são ANP, BNP e CNP, constituem uma família de peptídeos cardíacos e vasculares, com papel fundamental na regulação da pressão arterial e volume sangüíneo (para revisão ver D´SOUZA *et al.*, 2004). Os efeitos biológicos desses peptídeos são mediados por dois subtipos de receptores de guanilato ciclase associados à membrana: NPR-A e NPR-B (CHINKERS *et al.*, 1989; CHANG *et al.*, 1989) e um terceiro subtipo, o NPR-C, que atua como um receptor que inativa as ações dos peptídeos natriuréticos (MAACK *et al.*, 1987, 1993).

Vários trabalhos têm relatado a ação modulatória do NO na síntese e liberação de ANP (LEW *et al.*, 1989; SANCHEZ-FERRER *et al.*, 1990; LESKINEN *et al.*, 1995). Assim, SANCHEZ-FERRER *et al.* (1990) e LESKINEN *et al.* (1995) demonstraram que o NO liberado do endocárdio inibe a liberação de ANP de miócitos cardíacos. Em adição, os níveis plasmáticos de ANP estão elevados em ratos tratados cronicamente com L-NAME (LESKINEN *et al.*, 1995; LEE *et al.*, 2000). CARNIO *et al.* (2004) também demonstraram que a administração aguda de L-NNA aumentou os níveis plasmáticos de ANP e que permaneceram elevados até quatro dias após o tratamento. Estes autores observaram que o tratamento crônico com L-NNA induziu a liberação de ANP do SNC e neurohipófise,

sugerindo um controle neuroendócrino que possivelmente envolve baroreceptores. Já, LEE *et al.* (2000) demonstraram que mesmo após a administração de losartan, a expressão de ANP no átrio continuou aumentada. Entretanto, em ratos que receberam L-arginina juntamente com L-NAME, a expressão de ANP permaneceu inalterada, sugerindo um possível envolvimento do NO na modulação de ANP que independe de alterações hemodinâmicas. Em outro trabalho, LEE *et al.* (2002) demonstraram aumento nas concentrações plasmáticas de ANP em três modelos de hipertensão: ratos 2K-1C (2 kidney-1 clip), DOCA (acetato de deoxicorticosterona)-sal e inibição crônica com L-NAME. Na aorta torácica, os receptores NPR-A e NPR-C foram aumentados no grupo DOCA–sal, mas estavam diminuídos nos ratos tratados com L-NAME e 2K-1C. Similarmente, YOSHIMOTO *et al.* (1995) observaram aumento na expressão de receptores NPR-A em grupos DOCA-sal e com inibição crônica de NO. LEE *et al.* (2002) também demonstraram que a expressão de receptores AT_1 assim como de ECA foram significativamente aumentadas nos grupos 2K-1C e L-NAME, mas nos ratos DOCA-sal estava diminuída, sugerindo uma regulação recíproca pelo SRA local e ANP.

ARNAL *et al.* (1992) relataram que os níveis basais de cGMP em aorta dependem principalmente da atividade da guanilato ciclase solúvel e em menor escala da atividade da guanilato ciclase particulada em ratos tratados cronicamente com L-NAME. Posteriormente, LEE *et al.* (2002) observaram um aumento da atividade da guanilato ciclase particulada no grupo DOCA-sal e uma queda da atividade em grupos 2K-1C e L-NAME. Estas alterações observadas não eram devido a um aumento da pressão arterial ou a mudanças nos níveis plasmáticos de ANP, mas há uma interação entre o SRA e os peptídeos natriuréticos, já que em ratos DOCA-sal a atividade do SRA está diminuída (para revisão ver SCHENK e MCNEILL, 1992; LEE *et al.*, 2002) e nos grupos 2K-1C (HASHIMOTO *et al.*, 1983; LEE *et al.*, 2002) e L-NAME (RIBEIRO *et al.*, 1992; TAKEMOTO *et al.*, 1997; GONZALEZ *et al.*, 2000) há um aumento da atividade do SRA.

1.1.1.5. Endotelinas

As endotelinas, potentes peptídeos vasoconstritores de 21 aminoácidos, atuam através de dois subtipos de receptores, ET_A e ET_B (ARAI *et al.,* 1990; SAKURAI *et al.,* 1990). No músculo liso, a ativação dos receptores leva à contração (DE NUCCI *et al.,* 1988), já no endotélio, os receptores ET_B, quando estimulados, induzem a liberação de NO e prostaciclina causando vasodilatação (SCHIFFRIN, 1995b). No coração, a endotelina é produzida em células endoteliais, músculo liso, fibroblastos e cardiomiócitos, sendo que há um aumento na expressão do peptídeo em resposta à isquemia (ITO *et al.,* 1996) ou à ação da angiotensina II (ITO *et al.,* 1993). No rim, os receptores ET_A modulam a vasoconstrição e retenção de sódio (CLAVELL *et al.,* 1995; WILKINS *et al.,* 1995), enquanto os receptores ET_B exercem um efeito natriurético (WEBB *et al.,* 1998). THORIN *et al.* (1999) descreveram que antagonistas ET_A ou ET_A/ET_B causam aumento no diâmetro externo de artérias coronárias em cachorro, após a inibição de prostaciclina, entretanto a resistência do tônus vascular não foi alterada. O bloqueio de receptores ET_B não altera o diâmetro das artérias coronárias após a inibição da prostaciclina. Já, a inibição de NO causa uma redução do diâmetro das artérias coronárias, enquanto que o bloqueio adicional com antagonistas ET_A ou ET_A/ET_B restaura o diâmetro das artérias para níveis observados antes da inibição de NO. O mesmo trabalho relata que *in vitro*, em artérias coronárias pré-contraídas com L-NA (L-nitro-arginina) ou prostaglandina $F_{2\alpha}$, o bloqueio do receptor ET_A causa relaxamento, mas o bloqueio de receptores ET_B não tem efeito.

O papel da endotelina na fisiopatologia da hipertensão tem sido bastante controverso (para revisão ver SPIEKER *et al.,* 2001; SAVOIA e SCHIFFRIN, 2004; TSURUDA *et al.,* 2004). Alguns estudos têm demonstrado o envolvimento das endotelinas em diferentes formas de hipertensão genética e experimental. Assim, ratos DOCA-sal (LARIVIÈRE *et al.,* 1993a, b; LI *et al.,* 1994), ratos espontaneamente hipertensos (SHR)-DOCA-sal (SCHIFFRIN *et al.,* 1995a), e ratos Dahl sensíveis ao sal (DOUCET *et al.,* 1996) são formas de hipertensão experimental nos quais a ativação do sistema endotelina parece estar envolvida.

Esses modelos têm uma expressão aumentada de endotelina-1 no endotélio vascular e ao se administrar um antagonista ET_A/ET_B (bosentan) ou um antagonista seletivo ET_A ocorre uma queda da pressão arterial (LI *et al.*, 1994; SCHIFFRIN *et al.*, 1997b; DOUCET *et al.*, 1996). Entretanto, no modelo SHR os níveis vasculares de endotelina não estão alterados (LARIVIÈRE *et al.*, 1993b; SCHIFFRIN *et al.*, 1995a), assim como não há alterações na pressão arterial com a administração de antagonistas para ET_A/ET_B (SCHIFFRIN *et al.*, 1995b). De modo semelhante, não há nenhuma alteração na expressão de endotelina em animais tratados cronicamente com L-NAME ou alterações na pressão arterial após a administração de antagonistas dos receptores de endotelina (SCHIFFRIN *et al.*, 1997a), apesar de ter sido observada proteção renal após a administração dos antagonistas (VERHAGEN *et al.*, 1998). De modo geral, estes resultados demonstram que, a importância das endotelinas e, suas ações nos receptores ET_A ou ET_B, variam de acordo com o modelo estudado de hipertensão. Em modelos de hipertensão onde a expressão de endotelina está aumentada, a administração de antagonistas dos receptores destes peptídeos não somente reduz a pressão arterial, como inibe o remodelamento hipertrófico de pequenas artérias (para revisão ver SCHIFFRIN, 2000).

1.1.1.6. Estresse oxidativo

A importância do estresse oxidativo na fisiopatologia do tônus vascular e, conseqüentemente, no quadro de hipertensão, tem sido cada vez mais investigada. USUI *et al.* (1999) relataram que a inibição crônica de NO aumentou a formação de radicais de ânion superóxido (O_2^-) e a atividade da ECA endotelial. Posteriormente, KITAMOTO *et al.* (2000) demonstraram que a administração de um antagonista AT_1 de angiotensina II (CS-866) previniu a produção de O_2^- na aorta de ratos tratados cronicamente com L-NAME. O mesmo trabalho também relatou um decréscimo da atividade dos fatores de transcrição sensíveis ao sistema redox (NF-κB e AP-1), na aorta de ratos tratados com L-NAME após a administração do CS-866. Assim, a interação de NO com espécies intermediárias reativas de oxigênio (ERO) pode ter um papel importante na modulação de

respostas vasculares neste modelo de hipertensão (ATTIA, *et al.,* 2001 BAUERSACHS *et al.,* 1998; KITAMOTO *et al.*, 2000).

1.1.1.7. A via Rho/Rho quinase

A via Rho/Rho-quinase e seu envolvimento na fisiopatologia da hipertensão também vem sendo amplamente explorados na literatura. A via Rho/Rho-quinase regula a fosforilação da cadeia leve de miosina e, conseqüentemente, contribui para a contração do músculo liso (KUREISHI *et al.,* 1997). UEHATA *et al.* (1997) demonstraram que um inibidor específico para Rho-quinase (Y-27632) reduziu significativamente a pressão arterial em ratos hipertensos. Além disso, a atividade da Rho-quinase está aumentada em vasos sangüíneos no quadro de hipertensão (MUKAI *et al.,* 2001), e sua inibição induz vasodilatação preferencial no antebraço de pacientes hipertensos, sendo que o mesmo não é observado em normotensos (MASUMOTO *et al.,* 2001). A RhoA e Rho-quinase estão distribuídas no sistema nervoso central (OLENIK *et al.*, 1997; HASHIMOTO *et al.*, 1999) e, a ativação da Rho/Rho-quinase no núcleo do trato solitário (NTS) contribui para manutenção da pressão sangüínea basal via o SNS no modelo SHR (ITO *et al.*, 2003). Em trabalho recente, ITO *et al.* (2004) demonstraram que a microinjeção de Y-27632 no NTS reduziu significativamente a pressão arterial, freqüência cardíaca e a atividade nervosa simpática renal em ratos tratados cronicamente com L-NAME, quando comparados aos controles. A infusão do inibidor intracisternalmente também suprimiu a hipertensão causada pela inibição crônica de NO. Além disso, os autores observaram que a translocação membranal de RhoA e a fosforilação das ERM (ezrina, radixina, moesina; proteínas alvo da Rho-quinase) foram aumentadas no NTS de ratos tratados com L-NAME. Estes resultados sugerem que a via Rho/Rho-quinase está ativada no NTS de ratos tratados cronicamente com L-NAME, contribuindo, dessa forma, para o mecanismo da hipertensão neste modelo.

1.1.1.8. Canais de Ca^{2+}

Alguns trabalhos têm focalizado atenção especial aos canais de Ca^{2+} voltagem dependentes do tipo L (CCVD), já que há evidências que o aumento de influxo de Ca^{2+} tem um papel importante na patogênese da hipertensão (para revisão ver ZICHA e KUNES, 1999). A administração sistêmica de um antagonista de canais de Ca^{2+} (nifedipina) em ratos tratados com L-NAME aboliu a hipertensão desse modelo, assim como em ratos Dahl-sensíveis ao sal e SHR (KUNES *et al.*, 2003), mas não causou alterações na pressão arterial de animais normotensos. Embora esses resultados tenham sido interpretados como conseqüência do bloqueio do influxo de Ca^{2+} no músculo liso das arteríolas, a ação dos antagonistas de canais de Ca^{2+} é mais complexa visto que os mesmos podem também bloquear a entrada de Ca^{2+} nas células endoteliais (para revisão ver KUNES *et al.*, 2004), estímulo essencial para formação de NO pela NOS constitutiva. Antagonistas como a nifedipina podem exercer efeitos contrastantes envolvendo a vasodilatação devido ao bloqueio da entrada de Ca^{2+} através dos CCVD na célula muscular lisa dos vasos, e/ou vasoconstrição devido à queda na entrada de Ca^{2+} nas células endoteliais. Dessa forma, a reposta destes antagonistas na pressão arterial dependerá da razão entre os mecanismos constritores e relaxantes que estarão atuando antes da administração dos mesmos, assim como da concentração de Ca^{2+} no endotélio e músculo liso dos vasos, tecidos diretamente relacionados com o controle do tônus vascular (ZICHA e KUNES, 1999).

Os trabalhos discutidos acima indicam que há uma multiplicidade de mecanismos envolvidos na hipertensão induzida pela inibição crônica da biossíntese de NO, especialmente no que diz respeito ao envolvimento de peptídeos como a angiotensina II, bradicinina e ANP. O envolvimento de peptídeos neste modelo implica também as peptidases, já que são responsáveis pela ativação ou inativação dos mesmos. Porém, com exceção da ECA, pouco se sabe do envolvimento de outras peptidases neste modelo de hipertensão com L-NAME e sua regulação pelo NO.

1.1.2. RATOS ESPONTANEAMENTE HIPERTENSOS (SPONTANEOUSLY HYPERTENSIVE RATS - SHR)

O modelo SHR foi introduzido por OKAMOTO e AOKI em 1963. É um modelo que se assemelha bastante à hipertensão primária no homem e, em função disso, tem sido bastante explorado para se entender a fisiopatologia da hipertensão humana. No desenvolvimento da hipertensão no modelo SHR, o primeiro fator envolvido parece ser o controle neural, no qual os nervos agiriam estimulando o metabolismo de proteínas, o que levaria a mudanças estruturais nos vasos sangüíneos (LOMBARD *et al.,* 1984; para revisão ver RUSKOAHO, 1984; RUSSEL, 2002). A literatura tem demonstrado que o desenvolvimento da hipertensão em vários modelos animais, incluindo o SHR, DOCA-sal e Dahl-sensíveis ao sal, está relacionado com aumento na atividade do SNS, sendo que esse aumento poderia elevar a pressão arterial via vasoconstrição direta e também aumentando a força e freqüência cardíaca (TAKEDA e BUNAG, 1980; TAKEDA *et al.,* 1991; CABASSI *et al.,* 2002; LEENEN *et al.,* 2002). Em adição, o aumento da atividade nervosa simpática renal causa secreção de renina e ativa o SRA, liberando angiotensina II que promove vasoconstrição e inibe a natriurese. Da mesma forma, um aumento da atividade simpática leva a efeitos tróficos no músculo liso vascular o que aumenta a resistência vascular e a resposta vasoconstritora (para revisão ver VEERASINGHAN e RAIZADA, 2003). O segundo fator envolvido no desenvolvimento da hipertensão em SHR estaria relacionado com alterações intrínsecas nos vasos sangüíneos. Assim, YAMORI *et al.* (1981) demonstraram que culturas de células de músculo liso de aortas de SHR cresciam mais rapidamente quando comparada às de animais normotensos, mesmo em condições livres da influência de pressão arterial e fatores neuro-humorais.

1.1.2.1. Sistema nervoso simpático

No modelo SHR, a inervação simpática renal é mais densa (HEAD, 1989) e desenvolve-se mais rápido (GATTONE *et al.,* 1990) durante as duas primeiras semanas após o nascimento, quando comparado aos ratos Wistar Kyoto (WKY). Além disso, a concentração de norepinefrina renal é duas vezes maior no neonato

e aproximadamente três vezes mais alta no SHR adulto, comparado ao WKY (CAPLEA *et al.*, 2002). Uma vez que o fator de crescimento neuronal é elevado no SHR neonato, comparado com WKY (FALCKH *et al.*, 1992) ou ratos normotensos Donryu (KAPUSCINSKI *et al.*, 1996), tem sido sugerido que este fator deve contribuir para hiperinervação simpática renal em SHR (CHARCHAR *et al.*, 1998). Em adição, um estudo recente demonstrou que o aumento na formação de radicais de oxigênio também deve contribuir para a elevada atividade simpática renal em SHR (SHOKOJI *et al.*, 2003). Os autores relataram que a administração intravenosa de tempol (inibidor da superóxido dismutase) reduz a pressão arterial em SHR, sendo que essa redução é acompanhada de um decréscimo na atividade simpática renal.

Há um aumento nas concentrações plasmáticas de norepinefrina em SHR quando comparados com WKY (HOWE *et al.*, 1979; ZUGCK *et al.*, 2003). YAMADA *et al.* (1989) relataram que uma redução na densidade de receptores α_2 adrenérgicos promove um aumento na ativação central do tônus simpático em SHR. Os receptores α_2 adrenérgicos pré-sinápticos, quando ativados, exercem efeitos inibitórios na liberação de norepinefrina cardíaca dos terminais simpáticos (STARKE *et al.*, 1989; LANGER, 1997; MILLER, 1998). ZUGCK *et al.* (2003) demonstraram uma redução na expressão dos receptores pré-sinápticos adrenérgicos do subtipo α_{2A} em SHR, o que poderia contribuir para um aumento na liberação de norepinefrina nesses animais. Além disso, os autores relataram que a inibição dos receptores α_2 adrenérgicos pré-sinápticos pela ioimbina, resultou em um aumento significativo da liberação de norepinefrina no coração de WKY. Dessa forma, uma redução na densidade desses receptores, assim como, prejuízos funcionais nos mesmos devem causar um aumento nas concentrações de norepinefrina em SHR.

1.1.2.2. Neuropeptídeos

Em relação ao papel dos neuropeptídeos em ratos SHR, WATSON *et al.* (2002) demonstraram que quando os mesmos eram tratados com um fator de crescimento neuronal, o qual estimula a síntese do peptídeo relacionado ao gene

da calcitonina (CGRP), ocorria uma queda da pressão arterial, a qual voltou a níveis elevados novamente após a administração do antagonista $CGRP_{8-37}$. Já, o neuropeptídeo Y (NPY) é liberado de terminações simpáticas e da medula adrenal (para revisão ver ZUKOWASKA *et al.*, 2003), sendo que seus níveis plasmáticos estão aumentados em algumas condições patológicas como hiperatividade simpática, hipertensão, insuficiência cardíaca congestiva e insuficiência renal (ZUKOWSKA-GROJEC e WAHLESTEDT, 1993; ZUKOWSKA-GROJEC, 1995).

O NPY-(1-36), quando administrado centralmente, reduz a pressão arterial e promove bradicardia em ratos anestesiados e acordados (FUXE *et al.*, 1983; HÄRFSTRAND *et al.*, 1984; FUXE *et al.*, 1987). No entanto, o fragmento C-terminal NPY-(13-36), quando administrado no NTS ou ventrículo lateral, induz vasoconstrição (AGUIRRE *et al.*, 1990a; NARVÁEZ *et al.*, 1992, 1993). O NPY-(13-36) também pode ter efeitos opostos aos do NPY-(1-36) quando injetado intraventricularmente (AGUIRRE *et al.*, 1990b) ou no NTS (YANG *et al.*, 1993). O NPY-(1-36) atua via receptores Y_1 e Y_2 (SHEIKH *et al.*, 1989), entretanto tem sido descrito que na regulação cardiovascular central, a ativação dos receptores Y_1 pelo NPY-(1-36) exerce ações vasodepressoras, enquanto a ativação dos receptores Y_2 pelo fragmento NPY-(13-36) produz vasoconstrição (FUXE *et al.*, 1990). No entanto, perifericamente, a ativação do receptor Y_1 promove vasoconstrição (ZUKOWSKA-GROJEC, 1997; FRANCO-CERECEDA e LISKA, 1998) e proliferação de células do músculo liso vascular (KIM *et al.*, 1998; ZUKOWSKA-GROJEC *et al.*, 1998), sendo que essa resposta é aumentada pela pré-ativação de receptores β-adrenérgicos (ZUKOWSKA-GROJEC *et al.*, 1996; ZUKOWSKA-GROJEC, 1997).

Em SHR a administração central de NPY-(1-36) tem pouca influência na pressão arterial (HÄRFSTRAND *et al.*, 1984, 1988), o que estaria de acordo com a demonstração por MACCARONE e JARROT (1985) de que os níveis do NPY-(1-36) no leito cerebral estão reduzidos nos SHR. A administração intracerebroventricular do fragmento NPY-(13-36) não afeta a pressão arterial em WKY, mas a mesma dose exerce uma reposta vasopressora significativa e de longa duração em SHR (AGUIRRE *et al.*, 1990a). Subseqüentemente, AGUIRRE

et al. (1995) mostraram que o aumento da pressão arterial induzido pelo NPY-(13-36) em SHR estava relacionado a uma maior expressão de receptores Y_2 nesses animais.

1.1.2.3. Sistema renina angiotensina

Além da importância dos neuropeptídeos, vários trabalhos têm demonstrado o envolvimento do SRA local no quadro hipertensivo de SHR. Um aumento na atividade cardíaca da ECA e no número de receptores de angiotensina na hipertrofia ventricular esquerda de SHR já foram observados, mesmo quando a atividade plasmática da ECA estava reduzida (SUZUKI *et al.*, 1993; CAMPBELL *et al.*, 1995; DIEZ *et al.*, 1997). Além disso, VARAGIC *et al.* (2001) relataram que doses mínimas de inibidores de ECA foram efetivas em reduzir a hipertrofia ventricular esquerda em SHR, embora não tenham alterado a hemodinâmica desses animais. Um aumento na expressão e atividade de componentes do SRA cerebral tem sido observado em vários modelos de hipertensão, incluindo o SHR (BERECEK *et al.*, 1987), assim como uma elevação na expressão do angiotensinogênio cerebral precede o desenvolvimento da hipertensão nesse modelo (TAMURA *et al.*, 1996). Já, microinjeções de losartan no hipotálamo de SHR resulta em um decréscimo da pressão arterial (YANG *et al.*, 1992), sendo que o efeito depressor parece ser devido também à uma redução da atividade simpática (BERECEK *et al.*, 1987; HUANG e LEENEN, 1998). Dessa forma, no cérebro a angiotensina II contribui para regulação cardiovascular via ação no hipotálamo e medula (aumentando a atividade simpática), reduzindo a sensibilidade dos baroreceptores e estimulando a secreção de vasopressina (AVERILL e DIZ, 2000; MCKINLEY *et al.*, 2001; DAMPNEY *et al.*, 2002).

Comparada a angiotensina II, o papel da angiotensina III no controle da pressão arterial ainda não está muito esclarecido, embora tem sido sugerido que a mesma é responsável pelos níveis centrais da angiotensina II mediante ação da aminopeptidase A (para revisão ver REAUX *et al.*, 2001). Assim, a ação da angiotensina II nos baroreceptores é atenuada pela inibição da conversão de angiotensina II em III (LUOH e CHAN, 1998; REAUX *et al.*, 1999b), e a

administração intracerebroventricular de angiotensina II ou III causa um aumento da pressão arterial em SHR e WKY (ABHOLD *et al.*, 1987; WRIGHT *et al.*, 1985, 1989b), sendo que essa resposta envolve aumento da atividade simpática, inibição baroreflexa no NTS e liberação de vasopressina (PHILLIPS, 1987). Entretanto, enquanto a angiotensina II atua via receptores AT_1, a angiotensina III parece atuar em receptores AT_1 e AT_2 para deprimir a resposta baroreflexa (LUOH e CHAN, 1998; REAUX *et al.*, 2001).

Durante a administração de inibidores de ECA ou antagonistas AT_1 ocorre um aumento nos níveis plasmáticos de angiotensina-(1-7) (IYER *et al.*, 1998; YAMADA *et al.*, 1999; CHAPPELL *et al.*, 2000). A angiotensina-(1-7) é um peptídeo do SRA bastante atuante, e exerce uma variedade de efeitos fisiológicos, incluindo a estimulação da biossíntese de NO (HEITSCH *et al.*, 2001) e do ânion superóxido das células endoteliais, potencialização das ações vasodilatadoras da bradicinina na microvasculatura de SHR (FERNANDES *et al.*, 2001), estimulação da liberação de prostaglandinas e vasopressina, diurese, e natriurese (FERRARIO 1998; TOM *et al.*, 2003). BENTER *et al.* (1995) e KOST *et al.* (1998) relataram que a infusão intravenosa de angiotensina-(1-7) reduz a pressão arterial em SHR, mas o mesmo não ocorre em WKY.

STEGBAUER *et al.* (2004) observaram que a angiotensina-(1-7) aumentou a liberação de norepinefrina em rim isolado de SHR propenso a derrame (SHR-SP; Stroke Prone), mas não teve efeito em WKY, assim como a angiotensina-(1-7) não induziu vasodilatação ou vasconstrição nos rins de ambos os grupos. No entanto, os mesmos autores também relataram que, a angiotensina-(1-7) inibe o aumento da atividade simpática renal promovida pela angiotensina I e II em rins isolados de SHR-SP e WKY. Em baixas doses, a angiotensina-(1-7) inibe a vasoconstrição renal induzida pela angiotensina I e II em SHR-SP, sendo que esse resultado é observado em WKY, mas em doses maiores. Assim, concluiu-se que a angiotenisna-(1-7) tem efeitos pré- e pós-sinápticos diferentes em SHR-SP, por facilitar a liberação pré-sináptica de norepinefrina, bloquear as ações da angiotensina I e II e não ter efeitos vasculares diretos (STEGBAUER *et al.*, 2004). Em adição, as ações da angiotensina-(1-7) no NTS parecem contribuir para

modulação baroreflexa, promovendo bradicardia e atuando no tônus vascular (CHAVES *et al.*, 2000). Em SHR, a sensibilidade para angiotensina-(1-7) no NTS está reduzida, o que deve contribuir para a redução no controle baroreflexo desse modelo (CHAVES *et al.*, 2000).

CAMPBELL *et al.* (1995) observaram que os níveis de angiotensina I, II e angiotensina-(1-7) variaram de acordo com o tecido e idade dos ratos Donryu e SHR. No entanto, a angiotensina-(1-7) e a angiotensina I não foram detectadas no cérebro desses animais, apesar de ter sido observado um aumento da angiotensina II em SHR jovens (seis semanas). Já, nos rins os níveis de angiotensina I, II e angiotensina-(1-7) foram menores em SHR quando comparados com ratos Donryu. No coração, os autores observaram redução apenas nos níveis de angiotensina-(1-7) e angiotensina II em SHR. No pulmão, os níveis de angiotensina I e II, assim como a razão de angiotensina II-I, estavam reduzidos nos SHR, sendo que a angiotensina-(1-7) não foi detectada. Da mesma forma, em frações membranais de pulmão de ratos Sprague-Dawley, a angiotensina I é rapidamente convertida em angiotensina II, mas ao se administrar inibidores de ECA, não há formação significativa de angiotensina-(1-7) (ALLERED *et al.*, 2000). Por outro lado, KOHARA *et al.* (1993) descrevem que SHR apresentam níveis plasmáticos de angiotensina I e II similares aos WKY, no entanto os níveis plasmáticos de angiotensina-(1-7) foram mais altos em SHR.

Assim, a literatura tem demonstrado que além do SRA sistêmico, o SRA tecidual exerce ações importantes na manutenção da pressão arterial. Em paralelos, as vias alternativas de degradação da angiotensina I têm sido cada vez mais exploradas. Nesse contexto, GUO *et al.* (2001) relataram a importância da enzima quimase (EC 3.4.21.39) como uma via alternativa de conversão de angiotensina I para angiotensina II em ratos SHR. A quimase é uma serina protease secretada de mastócitos, e que está envolvida na síntese de angiotensina II. A angiotensina II liberada por essa via parece não estar relacionada com a regulação da pressão arterial, no entanto atua diretamente no remodelamento estrutural observado em doenças do sistema cardiovascular. Além da liberação de angiotensina II, a quimase atua degradando matrix extracelular,

ativando fatores de crescimento e promovendo a síntese de endotelinas, ações que em conjunto devem contribuir para alterações na reposta vascular (para revisão ver DOGGRELL e WANSTALL, 2004). GUO *et al.* (2001) demonstraram um aumento na atividade e expressão da quimase em aortas de SHR *versus* WKY, e que em células da musculatura lisa vascular a conversão de angiotensina I para angiotensina II foi reduzida pela quimostatina, um inibidor de quimase, sugerindo um possível papel da quimase na fisiopatologia da hipertensão nesse modelo.

1.1.2.4. Óxido nítrico

Vários autores têm demonstrado que a produção central de NO participa na regulação do tônus simpático periférico. Assim, a atividade simpática é aumentada com o bloqueio central da produção de NO (GEROVA *et al.*, 1995; TSENG *et al.*, 1996) ou diminuída pela administração de doadores de NO no cérebro (HORN *et al.*, 1994; LEWIS *et al.*, 1991), indicando que o NO deve afetar a atividade simpática em múltiplas regiões do cérebro.

Em um trabalho de QADRI *et al.* (1999) foi demonstrado que o bloqueio da NOS neuronal no cérebro pelo 7-nitro-indazoli aumentou a pressão arterial em SHR, mas não em WKY. Já, PLOCHOCKA-ZULINSKA e KRUKOFF (1997) relataram um aumento na expressão da NOS neuronal no hipotálamo e tronco cerebral de SHR. Mais recentemente, QADRI *et al.* (2003) demonstraram um decréscimo significativo na atividade da NOS neuronal no córtex cerebral e tronco cerebral em SHR jovens (3 a 4 semanas). Por outro lado, os autores observaram um aumento da atividade da enzima em regiões cerebrais cruciais para a manutenção da pressão arterial, como o hipotálamo e tronco cerebral em SHR adultos (12 a 13 semanas). O mesmo trabalho também demonstrou que o tratamento de SHR adultos com enalapril ou losartan reduziu a atividade da NOS no hipotálamo, mas não afetou a enzima no tronco cerebral. No entanto, a hidralazina, um vasodilatador que não age no SRA, não influenciou a atividade da NOS no hipotálamo ou tronco cerebral. Os autores concluíram que os efeitos anti-hipertensivos de inibidores de ECA ou bloqueadores de AT_1 são mediados pelo

NO hipotalâmico, sendo que apenas a queda da pressão arterial não influencia a atividade da NOS no cérebro.

A inibição crônica de NO promove danos estruturais renais severos e progressivos em ratos Wistar, como isquemia glomerular, glomeruloesclerose e expansão intersticial (BAYLIS *et al.,* 1992; RIBEIRO *et al.,* 1992; ARCOS *et al.,* 2000; PEREIRA e MANDARIN-DE-LACERDA, 2001). Além disso, a inibição crônica de NO pode antecipar a hipertensão, causar proteínuria e aumentar a mortalidade em SHR, quando comparados com WKY. A existência de um SRA renal tem sido sugerida devido a altas concentrações de angiotensina II no filtrado glomerular e túbulo proximal (SEIKALY *et al.,* 1990). A administração de inibidores da ECA previne alterações estruturais e funcionais causadas pela inibição de NO, sugerindo que a hipertensão induzida pela deficiência de NO está associada a um aumento da reatividade vascular e estimulação do SRA (CHARPIE *et al.,* 1997). PEREIRA *et al.* (2004) demonstraram que o enalapril previne danos renais em ratos Wistar e SHR tratados cronicamente com inibidor de NO (L-NAME), e também relataram que o enalapril beneficiou a microvasculatura do córtex renal tanto de ratos Wistar e SHR tratados com L-NAME, quanto de ratos normotensos, sugerindo que a microcirculação cortical é regulada por outros mecanismos além da síntese de NO.

1.1.2.5. Sistema natriurético atrial

Tem sido bem documentado que o ANP exerce um efeito importante no controle baroreflexo da freqüência cardíaca em humanos (EBERT e COWLEY, 1988; VOLPE, 1992) e animais (THORÉN *et al.,* 1986; VOLPE *et al.,* 1987; FERRARI *et al.,* 1990). Na hipertensão humana e em modelos animais, o controle baroreflexo está prejudicado (MANCIA *et al.,* 1986; KORNER *et al.,* 1995), sendo que no modelo SHR a sensibilidade baroreflexa está reduzida (HEAD e ADAMS, 1992). THOMAS *et al.* (1997) observaram que o ANP aumentava a bradicardia reflexa em WKY conscientes, quando baroreceptores cardiopulmonares ou quimioreceptores cardíacos eram ativados, mas o reflexo baroreceptor arterial não foi alterado. No entanto, em SHR a infusão de ANP não causou nenhum dos

efeitos observados no WKY, sugerindo que esses animais são resistentes às ações do ANP e que o mesmo atua em vias vagais aferentes não arteriais. MATSUBURA *et al.* (1990) demonstraram uma relação entre o aumento na expressão e concentração plasmática de ANP e a hipertrofia cardíaca na hipertensão renovascular, e sugeriram que o ANP poderia estar envolvido em um mecanismo compensatório. Da mesma forma, THOMAS *et al.* (1998) relataram que em SHR onde a hipertrofia ventricular esquerda foi prevenida por um inibidor de ECA, as ações do ANP na resposta baroreflexa cardíaca foram restauradas. No entanto, esse resultado parece não ter relação com a pressão arterial, já que apenas a normalização da mesma não altera a falta de sensibilidade de SHR para o ANP (THOMAS *et al.*, 1998). Esta insensibilidade ao ANP deve contribuir para o quadro hipertensivo nesses animais. Por outro lado, as concentrações de ANP estão elevadas em SHR (IMADA, *et al.*, 1985; GUTKOWSKA *et al.*, 1986), sendo que a expressão cardíaca de ANP (BOLUYT *et al.*, 1994) é maior em SHR quando comparada ao WKY. É provável que as concentrações elevadas de ANP nos SHR levem à redução no número dos receptores para o mesmo, o que poderia explicar a insensibilidade às ações do peptídeo nesse modelo.

Uma dieta rica em NaCl leva a uma redução na liberação de sódio no núcleo anterior do hipotálamo (NAH) em SHR, o que não acontece em WKY (CHEN *et al.*, 1991; OPARIL *et al.*, 1996). Já que o NAH exerce ação inibitória na atividade simpática e pressão arterial, a redução do sódio nessa região poderia exarcebar o quadro hipertensivo (para revisão ver CARISON *et al.*, 2001). JIN *et al.* (1991) observaram um aumento do ANP no NAH em SHR, sendo que o ANP inibe a liberação de sódio no cérebro e reduz a atividade dos neurônios do NAH (YANG *et al.*, 1990, ZHU *et al.*, 1996). As concentrações de ANP no NTS estão reduzidas em SHR com dieta rica em NaCl (JIN *et al.*, 1991), sendo que a microinjeção do mesmo no NTS reduz a sensibilidade baroflexora cardíaca em SHR mas não em WKY (JIN *et al.*, 1992). Estes resultados sugerem que o ANP atuaria no NTS para inibir o controle baroreflexo e a atividade simpática.

O ANP exerce ações renais, tais como aumento da razão de filtração glomerular (CHEN *et al.*, 2001), inibição da reabsorção de sódio e água,

supressão da renina, e inibição das ações da vasopressina (ZEILD, 1990; INOUE *et al.*, 2001). KATO *et al.* (1987) observaram uma redução no número de sítios ligantes de ANP com alta afinidade para o peptídeo ANP-(1-28) no glomérulo de SHR comparado com ratos WKY. WOODARD *et al.* (2002) relataram alterações funcionais nos receptores NPR-A (ver item 1.1.1.4.) em SHR quando comparados aos WKY. Estes autores observaram um decréscimo no número de receptores NPR-A com alta afinidade para o ANP-(1-28) nas membranas papilares e glomerulares de SHR, no entanto o receptor NPR-A mostrou grande afinidade pelo ligante ANP-(1-28) em SHR.

TREMBLAY *et al.* (1993) relataram um aumento na produção de cGMP em SHR. WOODWARD *et al.* (2002) observaram que a produção de cGMP nos receptores NPR-A foi mais eficiente no glomérulo intacto de SHR, assim como houve um aumento na atividade da guanilato ciclase particulada na membrana glomerular de SHR quando comparado com WKY, sugerindo alterações funcionais do receptor NPR-A em SHR. Em estudo mais recente, WOODARD *et al.* (2004) realizaram cruzamentos entre linhagens SHR e WKY (SHR machos *vs.* WKY fêmeas ou o inverso) obtendo uma linhagem F1 que foi cruzada entre si produzindo uma segunda linhagem F2. Os autores observaram que o aumento na produção de cGMP dos receptores NPR-A é uma característica mantida na geração F2. Entretanto, também observaram que o aumento na produção de cGMP induzido pelo ANP-(1-28) não foi reprodutível em ratos DOCA-sal quando comparado aos SHR, o que sugeriu que a alteração na produção de cGMP vista nos SHR estava geneticamente associada à elevação da pressão arterial.

1.1.2.6. Estresse oxidativo

A produção exacerbada de ânion superóxido (O_2^-) pelos vasos tem sido observada em vários modelos de hipertensão, incluindo o SHR (DOHI *et al.*, 1995; SUZUKI *et al.*, 1995). Em cultura de células do músculo liso vascular (GRIENDLING *et al.*, 1994) e em aortas intactas de ratos (RAJAGOPLAN *et al.*, 1996) a administração de angiotensina II estimula a geração de O_2^- pelo aumento da atividade da enzima NADPH oxidase. A NOS endotelial (COSENTINO *et al.*,

1998; KERR *et al.,* 1999) e a xantina oxidase (MIYAMOTO *et al.,* 1991) também estão envolvidas na produção de superóxido. Um aumento na produção de O_2^- e sua interação com NO é considerado fator importante na disfunção endotelial observada em SHR (GRUNFELD *et al.,* 1995; TSCHUDI *et al.,* 1996; SCHNACKENBERG *et al.,* 1998). O O_2^- liberado do endotélio associa-se ao NO formando peroxinitrito (ONOO-), um potente mediador citotóxico e pró-inflamatório (SALVEMINI *et al.,* 1996, 1998, 1999, CUZZOCREA *et al.,* 2001). Alguns mecanismos têm sido propostos para ações deletérias do peroxinitrito. Assim, o peroxinitrito poderia atuar no ácido araquidônico formando F_2-isoprostanas, que exercem potentes efeitos vasoconstritores e antinatriuréticos (CARLOS e RECKLCHOFF, 1999). Além disso, o peroxinitrito também poderia ligar-se a grupos tióis da glutationa e produzir *S*-nitroglutationa (BALZY *et al.,* 1998), que prejudica a produção de NO e a vasodilatação. Já, em cultura de células endoteliais bovinas, a exposição ao peroxinitrito reduz a mobilização de cálcio induzida por agonistas vasodilatadores (KOOY e ROYALL, 1994), sendo que esse efeito parece ocorrer devido à inativação das bombas de cálcio pelo peroxinitrito (GROVER *et al.,* 2003). Em um trabalho recente, CUZZOCREA *et al.* (2004) demonstraram que a administração de M40403, o qual remove superóxido seletivamente sem interferir com outras moléculas como o NO, reduz a pressão arterial em SHR. No entanto, ao se administrar L-NAME, o M40403 perde seu efeito nesses animais. Além disso, os autores observaram um aumento do relaxamento endotélio-dependente em aorta isolada, após a administração de M40403, o qual foi maior em SHR quando comparados aos WKY. Assim, a ação anti-hipertensiva do M40403 é devido à eliminação do O_2^- e conseqüente aumento da meia-vida do NO (GRYGLEWSKI *et al.,* 1986; RUBANYI e VANHOUTTE, 1986). Na ausência de O_2^-, haveria menos formação de peroxinitrito e, conseqüentemente, menos liberação de endoperoxidases vasoconstritoras (LANDINO *et al.,* 1996).

1.1.2.7. Endotelinas

Os níveis vasculares de endotelina não estão alterados no modelo SHR (LARIVIÈRE *et al.,* 1993b, 1995; SCHIFFRIN *et al.,* 1995a), assim como não há alterações na pressão arterial com a administração de antagonistas para ET_A/ET_B (SCHIFFRIN *et al.,* 1995b). No entanto, em SHR-DOCA-sal ocorre um aumento na expressão de endotelina-1 no endotélio vascular (SCHIFFRIN *et al.,* 1995a) e, ao se administrar um antagonista ET_A/ET_B (bosentan) ou um antagonista seletivo ET_A, ocorre uma queda da pressão arterial (SCHIFFRIN *et al.,* 1997a). ROSSI *et al.* (2003) relataram um aumento dos receptores ET_A em SHR-SP quando comparado com SHR. Por outro lado, ocorre um aumento de receptores ET_A em arteríolas cerebrais de SHR com dieta rica em sal. Já, o receptor ET_B não está alterado em SHR-SP ou SHR. BLEZER *et al.* (1999) demonstraram que a inibição de receptores ET_A atenua o inicio do derrame em SHR-SP, quando o tratamento é aplicado no inicio dos experimentos, mas os benefícios desaparecem com a interrupção da administração do inibidor. Esses resultados sugerem que o aumento de receptores ET_A deve estar relacionado com o inicio do processo que desencadeia o derrame em SHR-SP, e a inibição dos mesmos protege os animais do derrame.

Por outro lado, SVENTEK *et al.* (1996) demonstraram que SHR tratados com L-NAME desenvolveram hipertensão maligna, sendo que um aumento na expressão de endotelina-1 foi detectado nos grandes vasos. Mas, em um trabalho posterior, LI *et al.* (1996) relataram que SHR tratados cronicamente com L-NAME e bosentan (inibidor ET_A/ET_B) não demonstraram queda da pressão arterial. Modelos de hipertensão que tem uma expressão elevada de endotelina como ratos DOCA-sal, (DENG e SCHIFFRIN, 1992a) e SHR-DOCA-sal (SCHIFFRIN *et al.,* 1995a) apresentam uma hipertrofia vascular severa, enquanto que modelos que não têm a expressão aumentada de endotelina-1, como o SHR (DENG e SCHIFFRIN, 1992b) e 2K-1C (DENG e SCHIFFRIN, 1991) têm uma hipertrofia vascular menos severa. LI *et al.* (1996) observaram um aumento da expressão de endotelina-1 apenas nos grandes vasos de SHR-L-NAME, os quais exibiram hipertrofia vascular quando comparado com SHR. Os autores sugeriram que a

perda do efeito do bosentan na pressão arterial de SHR-L-NAME era devido à ausência de endotelina ou da hipertrofia endotelina-dependente nas pequenas artérias. A administração intracerebroventricular de L-NAME em SHR promove uma resposta hipertensora bifásica composta por um aumento transitório da pressão arterial que retorna a níveis basais após cinco minutos aproximadamente, seguida de uma elevação da pressão arterial prolongada (resposta tardia) (SALAS *et al.*, 2003). Nesse trabalho, a administração de PD 145065 (bloqueador de receptores centrais ET_A/ET_B) atenuou a primeira fase da hipertensão, mas não afetou a resposta tardia indicando que o NO modula a ação central da endotelina. Sabe-se que além da síntese no endotélio vascular e células musculares lisas (YANAGISAWA *et al.*, 1988), a endotelina é produzida no sistema nervoso central e influência a neurotransmissão (KUROKAWA, *et al.*, 1997). Em SHR, a concentração de endotelina-1 no SNC é menor que no WKY (IYER *et al.*, 1995) e os seus receptores estão reduzidos no hipotálamo e medula ventrolateral (GULATI e REBELLO, 1992). Já, a administração de L-754,142 (bloqueador periférico de receptores ET_A/ET_B) atenua ambas as respostas promovidas pelo L-NAME em SHR (SALAS *et al.*, 2003). No mesmo trabalho, observou-se que a administração de indometacina aboliu ambas as repostas promovidas pelo L-NAME em SHR. Baseado nos resultados de STANIMIROVIC *et al.* (1993), que relataram que a endotelina estimula a produção de prostaglandinas em células endoteliais de capilares cerebrais, SALAS *et al.* (2003) sugeriram que o NO inibe a atividade da endotelina, sendo que a deficiência do mesmo, promovida pelo L-NAME, levaria a uma maior atuação da endotelina na liberação de prostaglandinas. Da mesma forma, SALAS *et al.* (2003) também demonstraram que a resposta tardia promovida pelo L-NAME em SHR foi prevenida pela clorisondamina (bloqueador ganglionar de receptores nicotínicos), mostrando que a inibição do NO ativa o SNS na resposta tardia em SHR.

1.1.2.8. A via Rho/Rho quinase

SEASHOLTZ *et al.* (2001) observaram um aumento na atividade e expressão de RhoA (uma pequena proteína G) em aortas de ratos SHR quando

comparados a ratos Kyoto e em aortas de ratos Kyoto tratados com L-NAME cronicamente. Como citado anteriormente (ver item 1.1.1.7.), a via de sinalização que envolve a RhoA e seu efetor Rho quinase parece estar associada a vasoconstrição, já que a administração de um inibidor da Rho quinase (Y-27632) reduziu a pressão arterial em ratos hipertensos (UEHATA *et al.*, 1997). ITO *et al.* (2003) relataram que microinjeções do inibidor Y-27632, no NTS de SHR e WKY, reduziu a pressão arterial, freqüência cardíaca e a atividade simpática renal em ambos os grupos, mas o decréscimo observado em SHR foi significativamente maior que no WKY. Estes autores também observaram que após a transfecção de um vetor de adenovirus que expressa negativamente a Rho-quinase (AdDNRhoK), no NTS de SHR e WKY, a excreção urinária de norepinefrina e a atividade simpática renal diminuíram em ambos os grupos, mas essa redução foi mais proeminente em SHR. No entanto, antes da transfecção, a excreção urinária de norepinefrina já era significativamente mais alta em SHR, sugerindo uma atividade simpática exacerbada nesse modelo. Além disso, ITO *et al.* (2003) observaram que os níveis de expressão da RhoA no NTS foram significativamente maiores em SHR do que WKY. Embora, trabalhos demonstrem que não há alterações na expressão de Rho quinase em ratos hipertensos (SEASHOLTZ *et al.*, 2001; SEKO *et al.*, 2003), a inibição da mesma claramente exerce efeitos depressores e inibitórios na atividade simpática.

1.2. PEPTÍDEOS

Os peptídeos com atividade biológica representam uma classe importante de mediadores endógenos. Este grupo heterogêneo é composto de moléculas que variam em tamanho de aproximadamente 5 a 50 aminoácidos e inclui substâncias como a angiotensina (REGOLI *et al.*, 1974; VALLOTON, 1987; HANNAN e WIDDOP, 2004), cininas (bradicinina e calidina) (ROCHA e SILVA *et al.*, 1949; REGOLI e BARABÉ, 1980; BHOOLA *et al.*, 1992), encefalinas (JANECKA *et al.*, 2004), endotelinas (ET-1, ET-2 e ET-3) (YANAGISAWA *et al.*, 1988; MASAKI, 2004), peptídeo natriurético atrial (ANP) e peptídeos correlatos (GENEST e CANTIN, 1988; D´SOUZA *et al.*, 2004), neurotensina (DAVIS *et al.*, 1992; DAVIS e

KONINGS, 1993), CGRP (AMARA *et al.*, 1982; ROSENFELD *et al.*, 1983; HOLZER, 1992; DUMONT *et al.*, 2004; SHIN e HONG, 2004), peptídeos do trato gastrointestinal como o glucagon e o polipeptídeo vasoativo intestinal (VIP) (HENNING e SAWMILLER, 2004), taquicininas (substância P e neurocininas A e B) (GEPPETTI *et al.*, 1988; HOLZER, 1988; MAGGI, 1995) e vasopressina (HUANG e TANG, 2004), entre outros. Todos estes peptídeos atuam através de receptores específicos localizados na superfície de células endoteliais, epiteliais, neuronais, músculo liso e esquelético, e células de defesa (leucócitos, linfócitos, macrófagos) onde ativam uma variedade de respostas intracelulares. As atividades exercidas por estas moléculas são diversas, e incluem controle do tônus e permeabilidade vascular, algesia, neurotransmissão, crescimento celular, e modulação da resposta inflamatória (Fig. 1), podendo atuar de forma autócrina, endócrina ou parácrina.

Embora tenham suas ações específicas, quase todos os peptídeos endógenos mostram interações (inibição, estimulação, potenciação, sinergismo) entre os seus efeitos (KUSSEROW e UNGER, 2004) (Fig. 2), e isso é refletido no desenvolvimento de medicamentos para atingir mais de um sistema peptidérgico, tais como os sistemas natriurético + renina-angiotensina (para revisões ver NAWARSKAS *et al.*, 2001; TABRIZCHI, 2003; WORTHLEY *et al.*, 2004) e renina-angiotensina + endotelina (JENG *et al.*, 2002a; KOWALA *et al.*, 2004), no tratamento de hipertensão e outras patologias.

Assim, peptídeos tais como as cininas (especialmente a bradicinina) (para revisão ver SHARMA *et al.*, 2003) e a família dos peptídeos natriuréticos (ANP – peptídeo natriurético atrial, BNP – peptídeo natriurético do cérebro, CNP – peptídeo natriurético tipo C) (para revisões ver WORTHLEY *et al.*, 2004 e D´SOUZA *et al.*, 2004), cuja ação vascular principal é a vasodilatação, antagonizam a vascoconstrição promovida por peptídeos constritores como a angiotensina e as endotelinas. No caso do ANP, a infusão deste peptídeo reduz a pressão arterial, aumenta o fluxo urinário e a excreção de sódio e o cGMP, e inibe a secreção de renina e aldosterona (JANSSEN *et al.*, 1989).

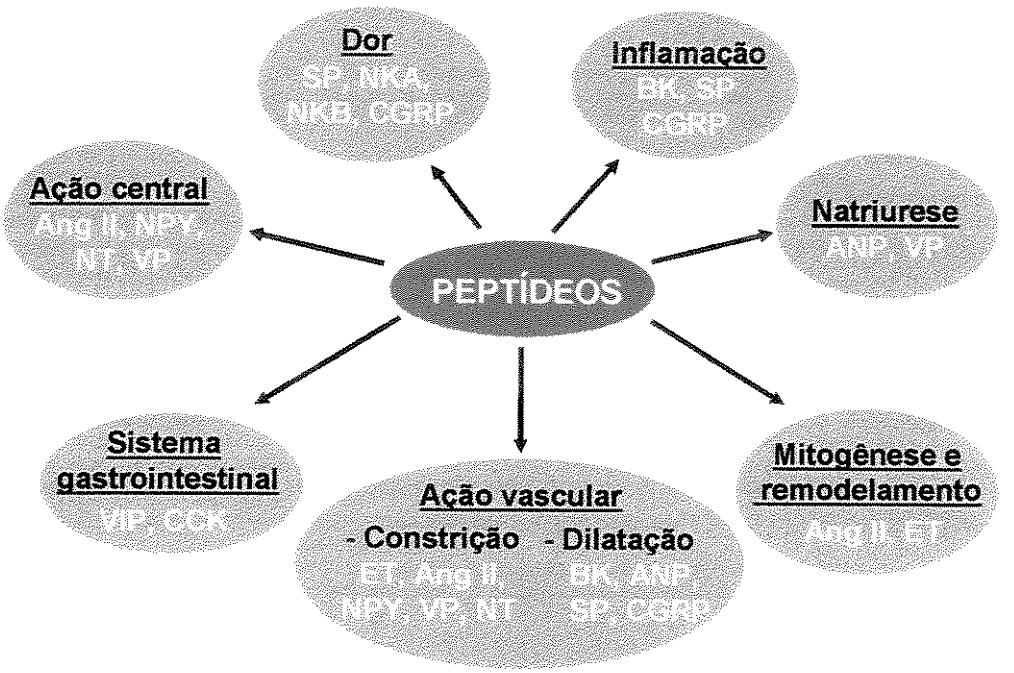

Figura 1: Ações fisiológicas de alguns dos principais peptídeos em mamíferos. **Ang II** – angiotensina II, **ANP** – peptídeo atrionatriurético, **BK** – bradicinina, **CCK** – colicistocinina, **CGRP** – peptídeo relacionado ao gene de calcitonina, **ET** – endotelina, **NKA** e **NKB** – neurocininas A e B, **NPY** – neuropeptídeo Y, **NT** – neurotensina, **SP** – substância P, **VIP** – peptídeo vasoativo intestinal, e **VP** – vasopressina.

Além disso, o ANP inibe a produção de endotelina, a proliferação de células do músculo liso vascular e hipertrofia cardíaca (JOHNSTON *et al.*, 1989; DAVIDSON *et al.*, 1996). Por outro lado, as endotelinas, vasopressina e catecolaminas podem estimular diretamente a secreção de ANP (RUSKOAHO, 1992; LEVIN, 1998).

As angiotensinas exercem uma variedade de papéis importantes no sistema cardiovascular. A angiotensina II, produzida pela ECA a partir da angiotensina I, é conhecida como potente vasoconstritor, mas também estimula o SNS, liberando noradrenalina e inibindo a recaptação da mesma (JACKSON *et al.*, 1985; ESHIMA *et al.*, 2000), além de atuar na neuro-hipófise aumentando a liberação de vasopressina (GANONG, 1984) (caminho não mostrado na Fig. 2).

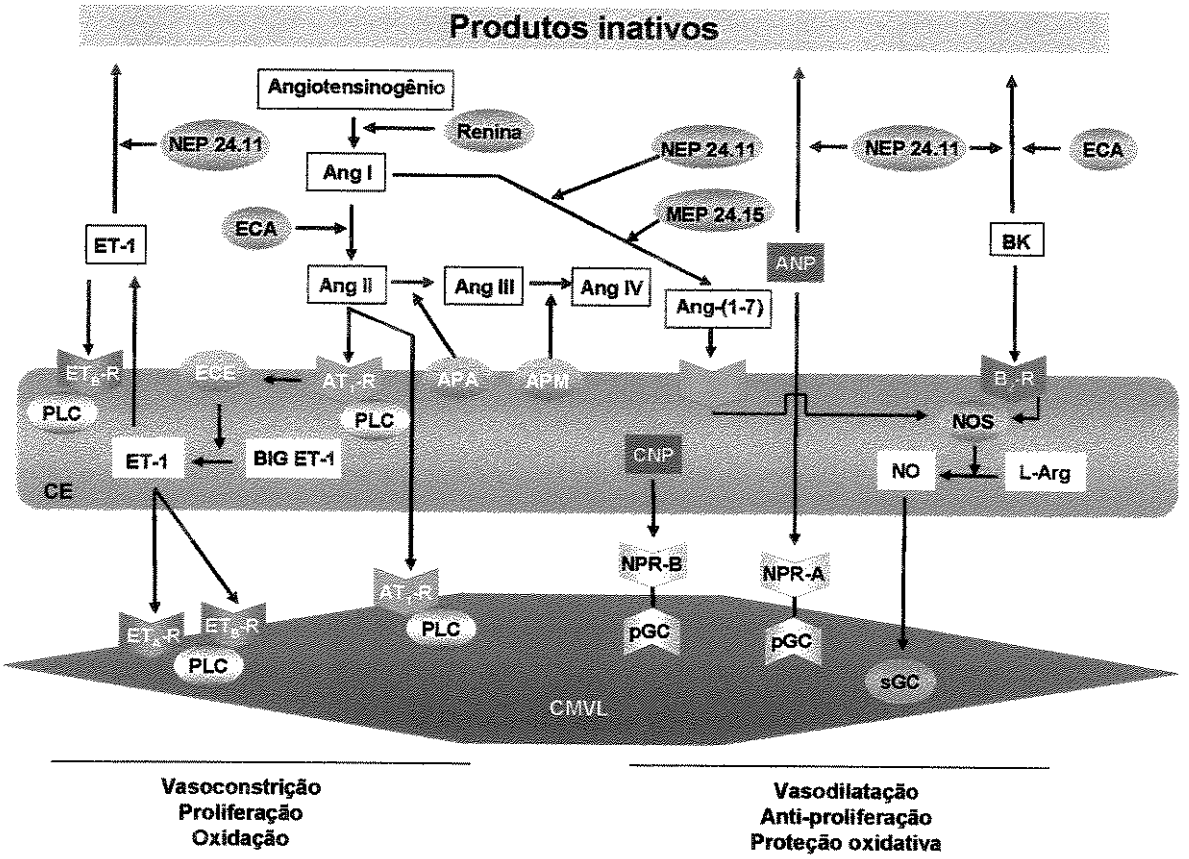

Figura 2. Interações entre alguns sistemas peptidérgicos a nível vascular.

APA – aminopeptidase A, **ANP** – peptídeo atrionatriurético, **APM** – aminopeptidase M, **AT_1** – receptor do tipo 1 para angiotensina, **B_2** – receptor do tipo 2 para bradicinina, **CE** – célula endotelial, **CMVL** – célula de músculo vascular liso, **CNP** – peptídeo atrionatriurético do tipo C, **ECA** – enzima conversora da angiotensina, **ECE** – enzima conversora da entodelina, **ET-1** – endotelina 1, **ET_A/ET_B** – receptores dos tipos A e B para endotelina, **pGC/sGC** – guanilil ciclase particulada e solúvel, **MEP 24.15** – timet oligopeptidase, **NEP 24.11** – neprilisina, **NO** – óxido nítrico, **NOS** – óxido nítrico sintase, **PLC** – fosfolipase C (modificada de QUASCHINING *et al.*, 2003).

A angiotensina III, produzida pela ação de aminopeptidase A sobre angiotensina II, estimula a liberação de vasopressina (para revisão ver REAUX *et al.*, 2001). A administração intracerebroventricular de angiotensina III aumenta a atividade da vasopressina e induz sua liberação na corrente sangüínea (HOHLE *et al.* 1995; ZINI *et al.*, 1996). Em WKY ou SHR, a administração intracerebroventricular da angiotensina III aumenta a pressão arterial, sendo que esta resposta envolve um aumento na atividade do SNS, inibição sináptica de baroreceptores no núcleo do trato solitário (NTS) e liberação de vasopressina (WRIGHT *et al.*, 1985; PHILLIPS, 1987).

A angiotensina IV é produzida pela ação de aminopeptidase M sobre a angiotensina III. Alguns trabalhos descrevem que a angiotensina IV atua como um vasodilatador e aumenta o fluxo sangüíneo no rim e cérebro (WRIGHT *et al.*, 1995; KRAMAR *et al.*, 1997). Mas, na circulação pulmonar sua administração parece causar vasoconstrição (NOSSAMAN *et al.*, 1995). Em culturas de células endotelias de pulmão, a angiotensina IV ativa a eNOS, sendo essa ativação dependente do receptor AT_4 (PATEL *et al.*, 1998).

A angiotensina-(1-7) atua promovendo vasodilatação (BENTER *et al.*, 1993, 1995), estimula a liberação de vasopressina (SCHIAVONE *et al.*, 1988), causa natriurese (DELLIPIZZI *et al.*, 1994; HILCHEY e BELL-QUILLEY, 1995; HANDA *et al.*, 1996), promove a liberação de prostaglandinas (JAISWAL *et al.*, 1992, 1993), aumenta a ação vasodilatadora de bradicinina (PAULA *et al.*, 1995; ABBAS *et al.*, 1997; LIMA *et al.*, 1997), estimula a liberação de NO (PORSTI *et al.*, 1994; BROSNIHAN *et al.*, 1996; LI *et al.*, 1997), têm ações antiproliferativas no músculo vascular liso (FREEMAN *et al.*, 1996), e promove um aumento na expressão de endotelina-1 (ITO *et al.*, 1993), que pode estimular os receptores ET_B em células endoteliais (Fig. 2), levando à liberação de NO e de prostaciclina (SCHIFFRIN, 1995b).

1.3. PEPTIDASES

O controle dos níveis circulantes de peptídeos é efetuado principalmente pela ação enzimática de peptidases. Tal regulação ocorre por duas vias principais. A primeira via envolve a endocitose do complexo peptídeo-receptor que posteriormente é desfeito em endossomas, organelas intracelulares cujo meio interno é de pH ácido e, subseqüentemente, o peptídeo é transportado para os lissosomos onde ocorre a degradação por peptidases. A segunda via envolve a degradação de peptídeos circulantes através da ação de peptidases ancoradas na membrana plasmática da célula e orientadas para o meio extracelular, ou de peptidases livres na circulação. Devido sua importância no controle de níveis circulantes de peptídeos, qualquer alteração na quantidade ou atividade de determinada peptidase poderá resultar em mudanças significativas na

concentração local (ou sistêmica) do(s) peptídeo(s) metabolizado(s) pela mesma e, conseqüentemente, nas ações farmacológicas destes.

A formação de peptídeos bioativos a partir de precursores inativos envolve uma série de eventos mediados por peptidases específicas. Esses eventos podem ocorrer dentro da célula, na superfície celular, e no meio extracelular (circulação). De modo geral, a produção de peptídeos endógenos segue o caminho intracelular clássico de biossíntese e secreção onde a formação de RNAm leva à síntese de uma proteína precursora na forma de um pré-pró-peptídeo (CULLINAN *et al.*, 1991). A clivagem seqüencial das formas pré- e pró-peptídeo leva à geração do peptídeo ativo que é secretado da célula por exocitose. Este caminho é típico, por exemplo, das endotelinas, onde o pró-peptídeo é metabolizado para o peptídeo ativo na própria célula através de uma enzima conversora de endotelina, localizada nas vesículas transportadoras envolvidas na exocitose (MASAKI, 2004). Em outros casos, o peptídeo ativo pode ser derivado de um precursor inativo presente na circulação, como é o caso da bradicinina, produzida a partir da ação de calicreínas sobre o cininogênio de alta massa molecular (para revisão ver CAMPBELL, 2000; SKIDGEL *et al.*, 2003), e das angiotensinas produzidas a partir da angiotensinogênio pela ação da renina e, subseqüentemente, ECA (para revisões, ver HANNAN e WIDDOP, 2004 e XIAO *et al.*, 2004).

Após sua síntese e liberação para a circulação geral, muitos peptídeos sofrem a ação de várias peptidases, as quais podem ativá-los ou inativa-los (Fig. 2). Assim, a ECA converte a angiotensina I em angiotensina II (SKEGGS *et al.*, 1956; HANNAN e WIDDOP, 2004; XIAO *et al.*, 2004), e inativa a bradicinina (YANG *et al.*, 1970; para revisão ver CAMPBELL, 2000; SKIDGEL *et al.*, 2003). Já, a aminopeptidase A cliva a angiotensina II em angiotensina III, a qual é convertida em angiotensina IV pela aminopeptidase M (APM) em vários tecidos (WRIGHT *et al.*, 1995), enquanto a metaloendopeptidase 24.15 (MEP 24.15) e a endopeptidase neutra 24.11 (NEP 24.11) clivam a angiotensina I em angiotensina-(1-7) (para revisões ver FERRARIO *et al.*, 1998; SHRIMPTON *et al.*, 2002). A Figura 3 ilustra a ação de várias peptidases na degradação de angiotensina I.

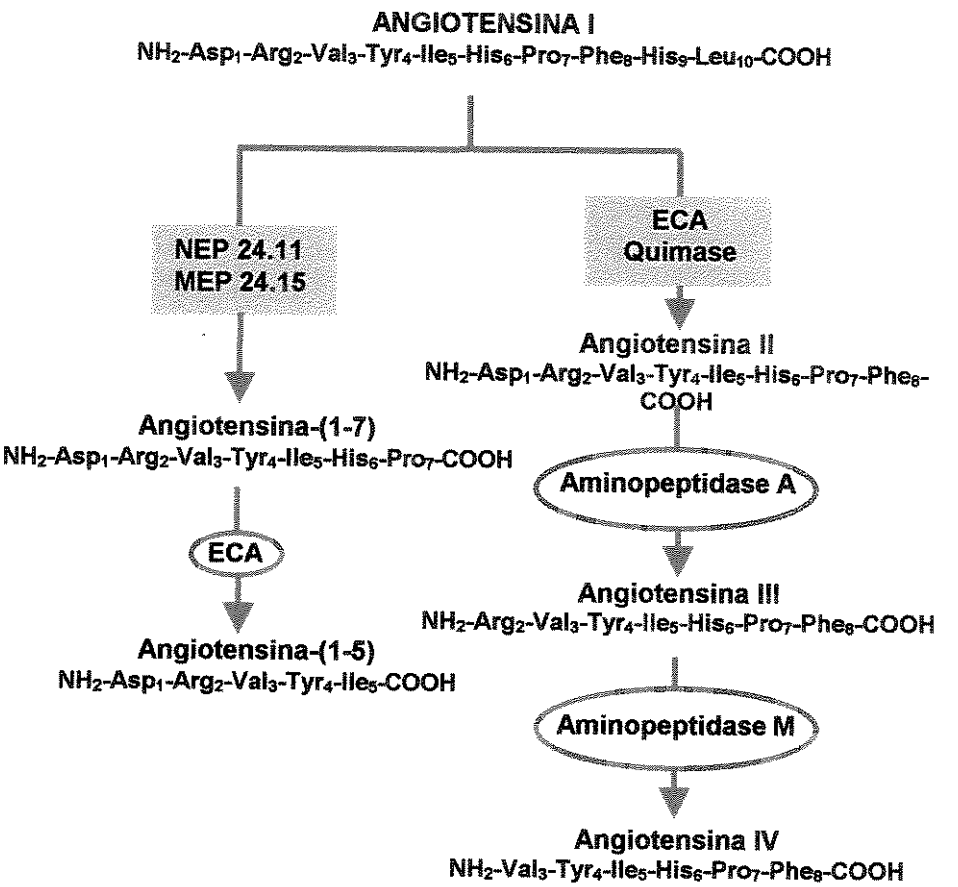

Figura 3. Vias alternativas de metabolização da angiotensina I em peptídeos biológicamente ativos.

ECA – enzima conversora da angiotensina, **NEP 24.11** – neprilisina, **MEP 24.15** – timet oligopeptidase (modificado de FERRARIO, 1998).

Atualmente, são reconhecidas pelo menos 13 famílias de peptidases contendo mais de 300 peptidases distribuídas intra- e extracelularmente em vertebrados, invertebrados, microorganismos e plantas. Apesar desta grande variedade, apenas um número reduzido tem sido demonstrado como tendo um papel importante no sistema cardiovascular de mamíferos. A Tabela 1 resume algumas das peptidases envolvidas na metabolização de peptídeos com ação vascular e a Figura 2 mostra os pontos de atuação de algumas destas peptidases na circulação e a nível endotelial.

PEPTIDASES	NOMENCLATURA	DISTRIBUIÇÃO	SUBSTRATOS
AMINOPEPTIDASES			
M	E.C. 3.4.11.2	Cérebro, coração, rim, pulmão, endotélio, músculo liso vascular	Ang III, Des(Asp1) Ang I, hepta(5-11) SP, hexa(6-11) SP, [Met]encefalina e Leu-encefalina
A	E.C. 3.4.11.7	Células epiteliais renais e intestinais, endotélio, cérebro	Ang II, colicistocinina 8
DIPEPTIDIL PEPTIDASES			
I	E.C. 3.4.14.1	Cérebro, fígado, pâncreas, pulmão, rim, linfócitos	Pró-catepsina G, pró-elastase, pró-quimase
IV	E.C. 3.4.14.5	Cérebro, coração, fígado, rim, pulmão, intestino, endotélio e linfócitos	SP, BK, colágeno, fibronectina, GLP-1, citocinas, NPY
PEPTIDIL DIPEPTIDASE			
ECA	E.C. 3.4.15.1	Cérebro, coração, fígado, pulmão, rim, endotélio, plasma	Ang I, BK
SERINA ENDOPEPTIDASE			
Quimase	E.C. 3.4.21.39	Artéria, coração, pulmão, mastócitos	Ang I, apolipoproteina B ou lipoproteinas de baixa densidade
METALOENDOPEPTIDASES			
NEP 24.11	E.C. 3.4.24.11	Cérebro, células do músculo liso vascular, endotélio, fibroblastos, miócitos, rim	ANP, Ang I, BK, SP, encefalinas,
MEP 24.15	E.C. 3.4.24.15	Cérebro, coração, fígado, pulmão, rim	BK, GnRH, NT, SP, dinorfina (1-8)
MEP 24.16	E.C. 3.4.24.16	Cérebro, coração, fígado, pulmão, rim	BK, GnRH, NT, SP, dinorfina (1-8)
ECE-1	E.C. 3.4.24.71	Endotélio, pulmão	Big-endotelina-1

Tabela 1. Exemplos de peptidases envolvidas no metabolismo de peptídeos no sistema cardiovascular.

O desenvolvimento de fármacos com ação inibitória sobre estas peptidases tem sido um caminho promissor para modulação do metabolismo de peptídeos e controle da pressão arterial. O foco principal destas pesquisas tem sido a ECA, para a qual já foram desenvolvidos vários inibidores a partir de estudos de FERREIRA (1965) e FERREIRA *et al.* (1970a, b) sobre a ação de peptídeos potencializadores de bradicinina presentes na peçonha da serpente *Bothrops jararaca*. Esta descoberta resultou no desenvolvimento do medicamento captopril, e derivados como enalapril, com potente ação inibitória sobre a ECA, e que são largamente usados no tratamento da hipertensão arterial (para revisões ver GAVRAS e BRUNNER, 2001)

Mais recentemente, outras peptidases também têm sido o objeto de investigações semelhantes. Assim, vários inibidores têm sido desenvolvidos para NEP 24.11 (para revisão ver CORTI *et al.*, 2001; WORTHLEY *et al.*, 2004), peptidase que tem grande importância fisiológica por estar envolvida na degradação de peptídeos vasodilatadores como ANP, BNP, CNP, substância P e bradicinina, mas também converte a big-endotelina-1 em endotelina-1, um potente peptídeo vasoconstritor (STEPHENSON e KENNY, 1987; KENNY e STEPHENSON, 1988; ERDÖS e SKIDGEL, 1989; LANG *et al.*, 1992; MURPHEY *et al.*, 1994). Outra peptidase cuja contribuição no controle cardiovascular tem sido investigado é a metaloendopeptidase 24.15 (MEP 24.15, timet oligopeptidase), para o qual já há inibidores que podem ser usados para investigar o papel desta enzima *in vivo* (SMITH *et al.*, 2000; NORMAN *et al.*, 2003a, b).

Uma abordagem recente no uso de inibidores de peptidases é o desenvolvimento de compostos com ação dupla, especificamente contra ECA e NEP 24.11 (para revisão ver CORTI *et al.*, 2001; QUASCHNING *et al.*, 2003; WORTHLEY *et al.*, 2004). Em ratos espontaneamente hipertensos (SHR) susceptíveis a derrame, o tratamento crônico com omapatrilato (inibidor de ECA/NEP 24.11) melhorou a vasodilatação endotélio-dependente (INTENGAN e SCHIFFRIN, 2000). Similarmente, em ratos Dahl sensíveis ao sal, o omapatrilato normalizou a vasodilatação endotélio-dependente e aumentou os níveis de cGMP (guanosina-3',5'-monofosfato cíclica) em artérias resistentes, quando comparado

com captopril (D'USCIO *et al.*, 2001). O omapatrilato também possue ações benéficas em pacientes com insuficiência cardíaca congestiva (ROULEAU, *et al.*, 2000). Os efeitos benéficos desta inibição simultânea de ECA e NEP 24.11 têm sido atribuídos a um acúmulo de peptídeos vasodilatadores (peptídeos natriuréticos e bradicinina) que teriam efeitos opostos à ação vasoconstritora da angiotensina e endotelina, principalmente através da sua capacidade de elevar os níveis intracelulares de cGMP e, conseqüentemente, causar relaxamento muscular (HOMAYOUN, *et al.*, 1989; VENEMA *et al.*, 1996). Mais recentemente, nos moldes dos estudos com inbidores duplos da ECA e NEP 24.11, tem se estudado a utilidade de inibidores mistos da enzima conversora de endotelina (ECE) e NEP 24.11 (JENG *et al.*, 2002b), de APM e NEP 24.11 (ROQUES e NOBLE, 1995; CHEN *et al.*, 2000; LE GUEN *et al.*, 2003), e de ACE, ECE e NEP 24.11 (tripla inibição) (INGUIMBERT *et al.*, 2002), além de antagonistas mistos dos receptores da angiotensina e endotelina (KOWOLA *et al.*, 2004).

Assim, apesar da reconhecida importância da ECA no controle cardiovascular, há outras peptidases com funções cardiovasculares importantes (Tabela 1), entre elas a aminopeptidase M, a dipeptidil peptidase IV, a metaloendopeptidase 24.15 e a endopeptidase neutra 24.11.

1.3.1. Aminopeptidase M

A aminopeptidase M (APM; EC 3.4.11.2), também conhecida como aminopeptidase N ou CD13, é uma metalo (Zn) glicoproteína transmembrana do tipo II (pertencente à superfamília das gluzincinas) (HOOPER, 1994). A APM é constituída de duas subunidades com o peso molecular aproximado de 160 kDa cada, tendo 10 sítios de N-glicolisação (aproximadamente 400 resíduos de carboidratos compondo em torno de 40% da massa molecular da proteína). Esta peptidase está ancorada à membrana plasmática através de uma região trans-membrana helicoidal próxima ao N-terminal. Uma pequena parte do N-terminal (8 a 10 aminoácidos) se estende para o citoplasma, enquanto o sítio catalítico é voltado para o meio extracelular (Fig. 4). Tem sido sugerido que a APM possui dois domínios para ligação do substrato, sendo um sítio no C-terminal e outro

centro ativo no domínio N-terminal, tendo atividade ótima em torno de pH 7,5 (CHECLER, 1993; para revisão ver RIEMANN *et al.*, 1999). É eficiente na remoção de aminoácidos da região N-terminal como a tirosina da [Met]encefalina e Leu-encefalina no córtex frontal, mas não degrada peptídeos contendo resíduos de prolina ou ácido piroglutâmico no N-terminal (DAVIS *et al.*, 1992).

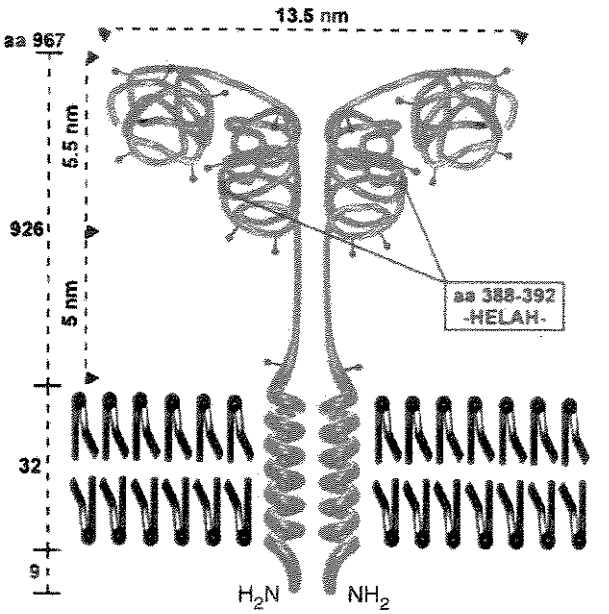

Figura 4. Estrutura esquemática da APM ou CD13 na superfície celular. Note que a forma dimérica de CD13 consiste de duas moléculas de APM unidas por ligações não covalentes. Os pontos de glicosilação estão indicados em vermelho. A região HELAH responsável pela ligação ao zinco no sítio ativo da enzima também está indicada (Riemann *et al.*, 1999).

A APM está amplamente distribuída no cérebro, microvasos cerebrais (HERSH *et al.*, 1987; DAUCH *et al.*, 1993), pulmão (JIANG *et al.*, 1992), fígado (OLSEN *et al.*, 1991), coração (BAWAB *et al.*, 1992; WOLFRUM *et al.*, 1999), rim (VLAHOVIC e STEFANOVIC, 1998) músculo liso vascular (PALMIERI *et al.*, 1985, 1989) e endotélio (PALMIERI *et al.*, 1985, 1989; FAVOLORO *et al.*, 1991; PAPAPETROPOULOS *et al.*, 1996). No endotélio e músculo liso vascular, a APM está envolvida na metabolização de peptídeos vasoativos, entre eles a des(Asp1)angiotensina I, angiotensina III, hepta(5-11)substância P e hexa(6-

11)substância P (PALMIERI *et al.*, 1985, 1989). HARDING *et al.* (1986) foram os primeiros a investigar a metabolização da angiotensina II e angiotensina III *in vivo*, e a relatar o envolvimento de aminopeptidases na degradação dos mesmos (para revisão ver REAUX *et al.*, 2001). Posteriormente, através de inibidores específicos para aminopeptidase A (APA) (EC33) (CHAUVEL *et al.*, 1994) e APM (EC27 e PC18) (FOURNIE-ZALUSKI *et al.*, 1992; CHAUVEL *et al.*, 1994) foi possível especificar quais enzimas estavam envolvidas no metabolismo das angiotensinas. Assim, ZINI *et al.* (1996) e REAUX *et al.* (1999a, b) demonstraram através da administração intracerebroventricular de angiotensinas, em camundongos, na ausência ou presença dos inibidores citados acima, o envolvimento da APA na degradação de angiotensina II para angiotensina III, e a ação da APM sobre a angiotensina III, degradando-a em angiotensina IV.

A literatura tem demonstrado o envolvimento de aminopeptidases na hipertensão. Assim, a administração intracerebroventricular de EC33 bloqueia a reposta hipertensora induzida pela angiotensina II em ratos normotensos e SHR anestesiados. Da mesma forma, o EC33 também promove uma resposta hipotensora quando administrado sozinho em SHR e ratos normotensos anestesiados e conscientes (REAUX *et al.*, 1999b). Em adição, a administração intracerebroventricular de PC18 em SHR e ratos normotensos induz uma elevação na pressão arterial (REAUX *et al.*, 2001). Estes resultados sugerem que o aumento da pressão arterial requer a conversão da angiotensina II em angiotensina III no cérebro. Já, RAMIREZ *et al.* (1997), ao compararem dois modelos diferentes de hipertensão, observaram reduções nas atividades enzimáticas da glutamil aminopeptidase (EC 3.4.11.7), arginil aminopeptidase (EC 3.4.11.6) e alanil aminopeptidase (EC 3.4.11.14) em frações membranais de rins de ratos 2K-1C. Por outro lado, esses mesmos autores mostraram que houve uma redução da atividade enzimática apenas da aspartil aminopeptidase (EC 3.4.11.-) nas frações membranais e solúveis de rins de animais com redução da massa renal, sem alterações das atividades das outras aminopeptidases.

HEALY e SONG (1999) observaram um aumento da atividade enzimática da APA no rim e plasma de SHR com quatro, seis e oito semanas de idade,

quando comparados aos respectivos WKY. No entanto, no cérebro e coração esse aumento foi observado apenas em SHR com dezesseis semanas de idade. Os autores também relataram que ao administrarem um inibidor de ECA, a atividade da APA decaiu no plasma e rim, sugerindo uma possível relação entre a formação de angiotensina II e o aumento da atividade da APA na manutenção da pressão arterial em SHR. WRIGHT *et al.* (1987) observaram reduções na atividade de aminopeptidases no cérebro de ratos SHR. Além disso, a administração intracerebroventricular de APM reduziu a pressão arterial em animais SHR e em menor intensidade em WKY (WRIGHT *et al.*, 1989a, 1990, 1991). Em trabalho posterior, WRIGHT *et al.* (1995) observaram que a resposta depressora promovida pela APM no cérebro de animais SHR foi atenuada pela administração intracerebroventricular de sartran (antagonista de receptores AT_1 e AT_2). Os autores também relataram que a administração intracerebroventricular de hexametônio (bloqueador ganglionar simpático) ou de um antagonista de vasopressina reduziu a reposta hipotensora da APM em SHR e WKY, sugerindo uma interação entre as angiotensinas, o SNS e vasopressina na resposta hipotensora da APM. PRIETO *et al.* (1998) observaram aumento da atividade enzimática de várias aminopeptidases, entre elas, a APM, na fração solúvel da neurohipófise e glândula adrenal em ratos com redução da massa renal e ingestão de salina. Porém, não houve alterações da atividade na fração membranal dos tecidos. Entretanto, em um trabalho mais recente, PRIETO *et al.* (2003) observaram um aumento nos níveis de APM no átrio direito de ratos 2K-1C, mas queda na atividade desta peptidase no pulmão. Estes estudos em conjunto, sugerem diferentes vias e propriedades para as aminopeptidases, de acordo com o modelo de hipertensão.

1.3.2. Dipeptidil peptidase IV

A dipeptidil peptidase IV (DPP IV; E.C. 3.4.14.5) é uma glicoproteína trans-membrana do tipo II (YARON e NAIDER, 1993), a qual está ancorada a membrana plasmática através de uma hélice hidrofóbica que se estende ao citoplasma com uma pequena região N-terminal (seis aminoácidos). Esta pequena

haste flexível liga a região ancorada a membrana com um domínio glicolisado altamente rico em cisteína. A dimerização da molécula parece ser um pré-requisito importante para a atividade enzimática (PÜSCHEL *et al.*, 1982), sendo que a enzima purificada é composta de duas subunidades idênticas de 110-130 kDa cada (para revisão ver AUGUSTYNS *et al.*, 1999). Entretanto em um trabalho recente, ENGEL *et al.* (2003) demonstraram que a DPP IV pode sofrer uma tetramerização, através da união de dímeros localizados na superfície de células diferentes, mediando o contato entre as mesmas (Fig. 5).

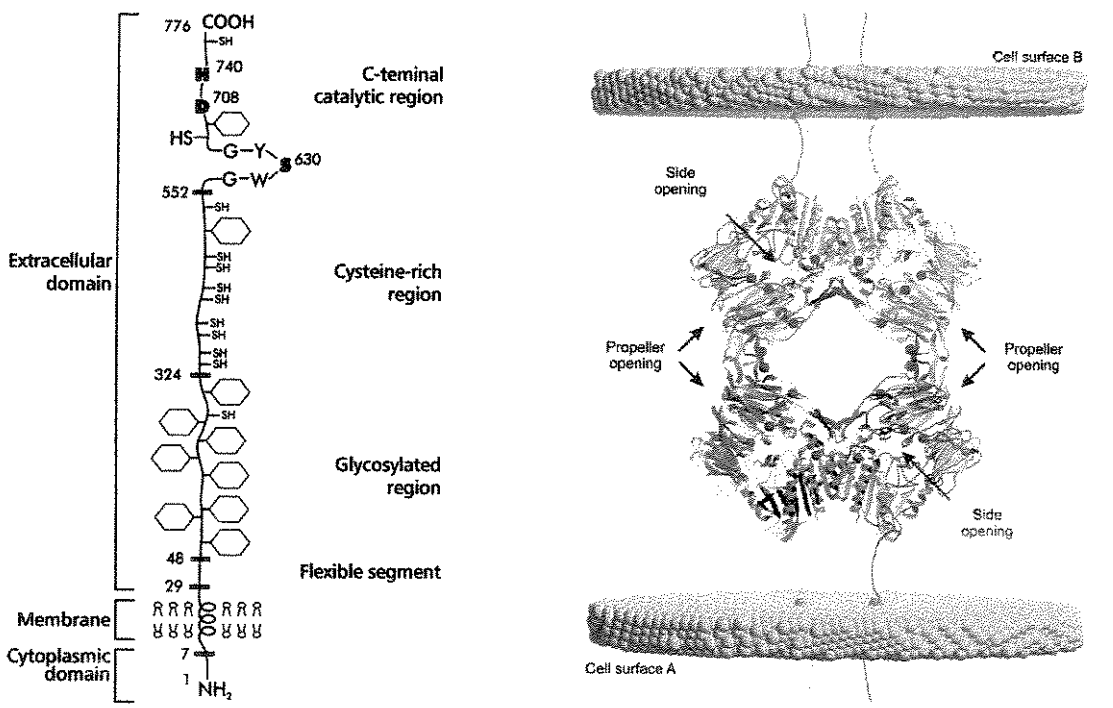

Figura 5. Estrutura esquemática da DPP IV ou CD26. Forma monomérica (à esquerda) e sua organização em forma de tetrâmero onde dois dímeros de células diferentes interagem (à direita). Note que a forma dimérica da CD26 consiste de duas moléculas de DPPIV (Mentlein, 1999; Engel *et al.*, 2003).

A DPP IV é uma serina protease pertencente à família prolil oligopeptidase (BARRETT e RAWLINGS, 1992), a qual cliva dipeptídeos na porção N-terminal de peptídeos onde o segundo aminoácido é, preferencialmente, a prolina (NH_2-X-Pro-Y), liberando X-Pro (MCDONALD e BARETT, 1986; MENTLEIN, 1988, 1999; NAUSCH *et al.*, 1990; MEDEIROS e TURNER, 1994; VANHOOF *et al.*, 1995),

embora possa também atuar (em grau menor) quando o segundo aminoácido é Ala (PÜSCHEL *et al.,* 1982; YARON e NAIDER, 1993). É encontrada em vários tecidos (baço, cérebro, coração, fígado, intestino, pulmão, rim e outros) e células (endoteliais, epiteliais e linfócitos) (HONG *et al.,* 1989; VLAHOVIC e STEFANOVIC, 1998; YARON e NAIDER, 1993; TRIBULOVÁ *et al.,* 2000).

Embora tenha um papel fundamental na imunologia devido ao fato de ser idêntica à molecula de adesão CD26 (= Ta1 e Tp103 de linfócitos) (YARON e NAIDER, 1993; ANSORGE *et al.,* 1997; MORIMOTO e SCHLOSSMAN, 1998; VON BONIN *et al.,* 1998; VAN DAMME *et al.,* 1999; OHTSUKI *et al.,* 2000), o papel biológico da DPP IV ainda não é bem estabelecido. Evidências mostram sua participação em vários processos bioquímicos como transporte renal, digestão intestinal de peptídeos contendo prolina, e ativação imunológica de células imunocompetentes.

Como acontece com muitas peptidases, a DPP IV parece estar envolvida tanto na ativação como na inativação de peptídeos. Assim, foi demonstrado que a DPP IV hidrolisa a substância P *in vivo* e *in vitro* em fragmentos (3-11) e (5-11) (HEYMANN e MENTLEIN 1978; AHMAD *et al.,* 1992). Alguns trabalhos demonstram que no sangue placentário há altos níveis de DPP IV, e devido ao mesmo ser uma fonte de substância P, é possível que a DPP IV esteja envolvida indiretamente na modulação da contratilidade placentária através da sua ação sobre a substância P (PÜSCHEL *et al.,* 1982; GRAF *et al.,* 1994). A DPP IV também parece ser responsável pela inativação do fator liberador do hormônio de crescimento, *in vitro* e *in vivo* (FROHMAN, *et al.* 1986, 1989), assim como foi demonstrado que a mesma atua inativando o polipeptídeo insulinotrópico dependente de glicose e o peptídeo-1 tipo-glucagon (MENTLEIN *et al.* 1993b; KIEFFER *et al.,* 1995, MENTLEIN, 1999). A β-casomorfina é outro peptídeo degradado em fragmentos inativos pela DPP IV (TIRUPPATHI *et al.,* 1993. A DPP IV também atua em membros da família dos polipeptídeos pancreáticos como o neuropeptídeo Y e o peptídeo YY, liberando seus respectivos fragmentos (3-36), com importantes ações biológicas (MENTLEIN *et al.,* 1993a; WRENGER *et al.* 1996).

MURPHEY *et al.* (2000) demonstraram que em humanos, a bradicinina é degradada em bradicinina (1-5), um metabólito mais estável e aparentemente inativo, tanto pela ECA como por outra via envolvendo aminopeptidase P (APP) e DPP IV. Neste caso, a bradicinina sofre uma hidrólise prévia pela APP na ligação Arg^1-Pro^2, seguida de outra hidrólise pela DPP IV na posição Pro^3-Gly^4. Além disso, LEFEBVRE *et al.* (2002) mostraram que no plasma de pacientes hipertensos com angioedema associado a inibidores de ECA, a atividade enzimática da DPP IV está reduzida. No entanto, não houve alteração na atividade de APP. Como a mesma degrada a bradicinina, os autores concluíram que o angioedema poderia estar associado à degradação de substância P, já que a mesma é hidrolizada pela DPP IV.

Ao investigar possíveis fatores envolvidos no remodelamento do miocárdio e hipertrofia cardíaca em ratos tratados com L-NAME (40 mg/kg; 4 semanas), TRIBULOVÁ *et al.* (2000) relataram que em vênulas não houve alteração na expressão de DPP IV baseado na histoquímica, mas observaram um aumento da enzima em locais de fibrose. MAGYAR *et al.* (2000) observaram um deslocamento da atividade da enzima para frações mais pesadas de rins em ratos Sprague-Dawley com hipertensão arterial induzida. Entretanto, não houve alterações da DPP IV em ratos SHR que tiveram um aumento adicional da pressão arterial, ou que tiveram a pressão arterial reduzida. Estes estudos indicam um possível envolvimento da DPP IV em mecanismos vasculares, inclusive em eventos patológicos como a hipertensão.

1.3.3. Endopeptidase neutra 24.11

A endopeptidase neutra 24.11 (NEP 24.11, neprilisina ou encefalinase; EC 3.4.24.11), uma Zn-metalopeptidase de 94 kDa ligada à membrana plasmática pela sua porção N-terminal, é o protótipo para as endopeptidases neutras de mamíferos do grupo M13 que incluem as enzimas conversoras da endotelina (ECE1 e ECE2), o KELL (antígeno de superfície de eritrócitos), e o produto do gene PEX. A estrutura destas enzimas consiste em uma região N-terminal citoplasmática curta, uma hélice transmembrana, e um domínio C-terminal

bastante grande que contém o sítio catalítico (Fig. 6). A NEP 24.11 encontra-se amplamente distribuída em vários tecidos, incluindo o endotélio vascular, células do músculo liso, miócitos cardíacos, fibroblastos, rins e cérebro (ERDÖS, 1990; ROQUES *et al.*, 1993). Entre seus substratos podemos citar o peptídeo natriurético, angiotensina I, angiotensina II, cininas, substância P, endotelina, peptídeos quimiotáticos e encefalinas (ERDÖS e SKIDGEL, 1989; RICHARDS *et al.*, 1992; YAMAMOTO *et al.*, 1992; ROQUES *et al.*, 1993).

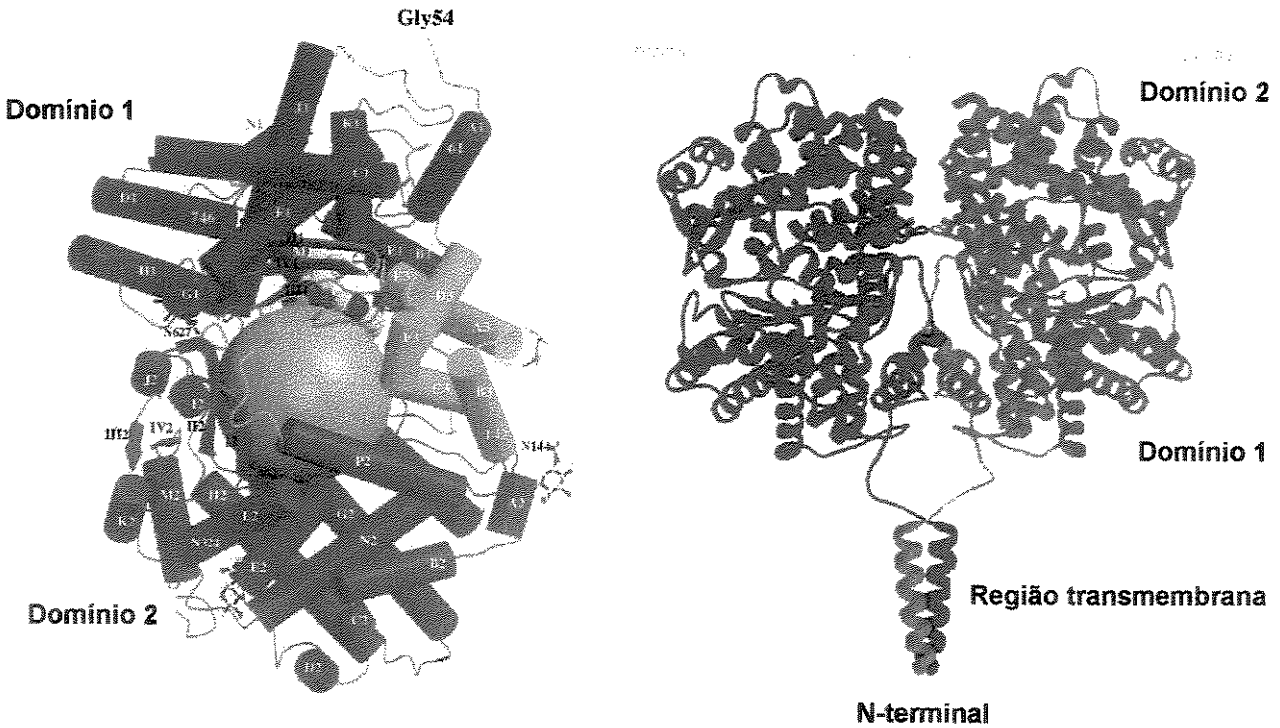

Figura 6. Estrutura esquemática da NEP 24.11. (A) Estrutura da região C-terminal (extracelular) de NEP 24.11 (neprilisina) composta de dois domínios contendo principalmente α-hélices. O domínio 1 (vermelho, e que vem logo após o segmento transmembrana a partir da Gly 54) contém o sítio ativo com o íon de zinco e resíduos envolvidos na catálise. O domínio 2 (azul escuro) é ligado ao domínio 1 por segmentos polipeptídicos (azul claro). A região onde encaixa o substrato é indicada pela esfera amarela. (B), Normalmente, a NEP existe como um monômero na membrana. Porém, uma mutação de E403 para C403 (na região que acopla os dois domínios) pode levar à dimerização da molécula (através de uma ligação sulfídrica), resultando em uma estrutura semelhante àquela indicada à direita, que é para ECE (que ocorre normalmente como um dímero). Figuras de OEFNER *et al.* (2000) e BUR *et al.* (2001).

A importância da NEP 24.11 na fisiologia cardiovascular têm sido relatada cada vez mais. Assim, ZHANG *et al.* (1998) demonstraram que em microvasos coronarianos isolados de cães, a produção de NO foi aumentada de uma maneira

dose-dependente em resposta ao fosforamidon e tiorfan, inibidores da NEP 24.11. Quando L-NAME ou HOE 140 (antagonista de receptores B_2) foi acrescentado à preparação, esse aumento de NO não foi observado. Esses dados sugerem que a inibição de NEP está relacionada à ativação de receptores B_2 e depende da atividade de NOS, e indicam também a interação da enzima com um sistema local de formação de cininas em microvasos coronarianos de cães.

No coração humano a NEP 24.11 é responsável por aproximadamente 50% da degradação de bradicinina (BLAIS *et al.*, 2000). Em estudos experimentais, a redução da isquemia e alterações na reperfusão, após a inibição da NEP 24.11, são mediados por cininas (ZHANG *et al.*, 1998). No entanto, na hipertensão, o candoxatril, inibidor de NEP 24.11, causa queda mínima na pressão arterial, mas a combinação do candoxatril com inibidor de ECA causa marcado decréscimo na pressão arterial (RICHARDS *et al.*, 1993). Dessa forma, os efeitos anti-hipertensivos dos inibidores de NEP 24.11 dependem da predominância de peptídeos vasodilatadores (bradicinina e peptídeos natriuréticos) ou vasoconstritores (endotelina). Já, na insuficiência cardíaca congestiva (ICC) a inibição da NEP 24.11, em curtos períodos de tempo, causa diurese dose-dependente, mas em longo prazo a diurese não é observada (GOOD *et al.*, 1995). Em cachorros com ICC, a inibição de NEP 24.11 em longo prazo melhora a excreção de sódio e aumenta a resposta renal para o ANP exógeno, levando à conclusão que a NEP 24.11 limita a resposta renal para o ANP na ICC.

A inibição da NEP 24.11 produz respostas variáveis na pressão sangüínea, que vão de decréscimo a aumento de pressão ou até mesmo nenhum efeito significativo em pacientes normotensos ou hipertensos. Os efeitos observados durante a inibição da NEP 24.11 se devem ao aumento nos níveis circulantes de vários peptídeos que são degradados por esta enzima, tais como cininas e peptídeos natriuréticos, os quais são vasodilatadores. A inibição desta peptidase também pode levar a aumentos nos níveis de angiotensina II e endotelina, potentes vasoconstritores, além de reduzir os níveis de angiotensina (1-7), peptídeo de ação vasodilatadora (NORTHRIDGE *et al.*, 1989; RICHARDS *et al.*, 1990; BEVAN *et al.*, 1992; FAVRAT *et al.*, 1995; ANDO *et al.*, 1995).

YAMAMOTO *et al.* (1992) demonstraram que a produção de angiotensina-(1-7) foi aumentada após a administração de angiotensina II em ratos SHR e que esse aumento foi mediado pela NEP 24.11, já que a administração de um inibidor da enzima reduziu a formação da angiotensina-(1-7). A administração de um anticorpo policlonal de angiotensina-(1-7) ou de um inibidor de NEP 24.11, parcialmente reverteu os efeitos anti-hipertensivos do tratamento com losartan e lisinopril em ratos SHR (IYER *et al.,* 1998). FERNANDES *et al.* (2001) também demonstraram que a angiotensina-(1-7) potencializa a vasodilatação induzida pela bradicinina no leito vascular mesentérico de ratos SHR. Já, FERRARIO *et al.* (2002) demonstraram que SHR tratados com omapatrilato, descrito anteriormente como um inibidor de ECA e NEP 24.11, tiveram um aumento nas concentrações plasmáticas de angiotensina I, II e angiotensina-(1-7), sugerindo vias alternativas de formação de angiotensinas (ver Fig. 3). No entanto, a razão entre angiotensina-(1-7)/angiotensina I, também foi reduzida nesses animais. Estes autores também observaram um aumento na excreção urinária de angiotensina-(1-7) nos animais que receberam ompatrilato.

Vários trabalhos têm demonstrado os efeitos da associação de inibidores de NEP 24.11 e ECA (para revisão ver CAMPBELL 2003 e WORTHLEY *et al.,* 2004). O raciocínio para esta abordagem é que diminuindo a produção de um vasoconstritor (angiotensina pela ECA) e diminuindo a degradação de vasodilatadores (ANP pela NEP 24.11 e bradicinina pela ECA e NEP 24.11) haveria uma melhora na pressão arterial de indivíduos hipertensivos. Um dos mais estudados destes duplos inibidores é o omapatrilato, que parece exercer efeitos hemodinâmicos benéficos em modelos experimentais de insuficiência cardíaca e hipertensão (RICHARDS *et al.,* 1993; TRIPPODO *et al.,* 1995; TIKKANEN *et al.,* 1998; QUASCHNING *et al.,* 2001). Assim, a administração oral do omopatrilato em sujeitos normotensos mostrou ser efetiva após 24 horas, como foi demonstrado pela inibição de ECA (avaliado pelos níveis séricos da mesma) e inibição da NEP 24.11 (avaliado pela concentração urinária de ANP), assim como pela redução dose-dependente da pressão arterial (LIAO *et al.,* 1997; VESTERQVIST *et al.,* 1997). Em adição, estudos clínicos demonstraram redução siginificativa da

pressão arterial em pacientes hipertensos, quando comparados à administração de enalapril (ver WORTHLEY *et al.*, 2004), assim como em pacientes com ICC, o omopatrilato exerce efeitos benéficos (de uma maneira dose-dependente) e melhora a função renal (MCCLEAN *et al.*, 2000).

Nem todos os trabalhos têm mostrado um efeito benéfico da inibição crônica de NEP 24.11. Assim, ANDO *et al.* (1995) observaram aumento da pressão sangüínea após a inibição de NEP 24.11 em voluntários normotensos, sendo que este efeito foi associado a um aumento nos níveis de endotelina. KENTSCH *et al.* (1996) relataram que a inibição de NEP 24.11 aumentou a resistência vascular periférica durante as primeiras horas após a administração do inibidor candoxatril, mas não causou nenhum efeito após 10 dias de terapia com o mesmo, em pacientes com insuficiência cardíaca. Vários estudos têm relatado que o omapatrilato exacerba o angioedema quando comparado ao tratamento com enalapril, em alguns casos havendo comprometimento das vias aéreas (WORTHLEY *et al.*, 2004). Esse efeito colateral tem sido atribuído a um aumento nas concentrações de bradicinina (NUSSBERGER *et al.*, 1998, MESSERLI e NUSSBERGER, 2000). Em outro estudo clínico de pacientes com ICC, o índice de mortalidade e hospitalização não foi diferente entre o grupo que recebeu omopatrilato ou enalapril (PACKER *et al.*, 2002). Assim, embora os resultados iniciais tenham sido promissores, o uso clínico do omopatrilato não tem sido recomendado devido aos efeitos colaterais causados pelo inibidor e pelo fato de que os resultados benéficos freqüentemente não foram diferentes do uso de inibidores de ECA como o enalapril.

1.3.4. Metaloendopeptidase 24.15

Nos últimos 10 anos, vários trabalhos têm mostrado um papel fisiológico importante para as metaloendopeptidases solúveis na regulação de peptídeos com ações centrais e periféricas. Neste contexto, a metaloendopeptidase 24.15 (MEP 24.15, timet oligopeptidase; EC 3.4.24.15) e a metaloendopeptidase 24.16 (MEP 24.16, neurolisina; EC 3.4.24.16) têm sido bastante estudadas. Ambas as enzimas estão amplamente distribuídas nos tecidos e células do corpo (TISLJAR e

BARRETT, 1990; MOLINEAUX *et al.*, 1991; DAUCH *et al.*, 1993; TISLJAR, 1993; CHECLER *et al.*, 1995). A MEP 24.15 é encontrada predominantemente na fração solúvel do citosol (DAHMS e MENTLEIN, 1992), embora CHU e ORLOWSKI (1985) demonstraram que até 20% da enzima estava associada à fração particulada (núcleo e membrana) em homogenatos de cérebro de ratos, enquanto a MEP 24.16 está associada à membrana (VINCENT *et al.*, 1996; SHRIMPTON e SMITH, 2000; SHRIMPTON *et al.*, 2002). Estas enzimas não possuem o peptídeo de sinal para secreção pela via convecional, o que poderia explicar sua localização predominantemente intracelular. Entretanto, pequenas quantidades delas são encontradas na supercífice celular e no meio extracelular (FERRO *et al.*, 1999). Para explicar esta presença extracelular, tem sido proposta a existência de uma via não-convencional de secreção para as duas peptidases (FERRO *et al.*, 2004).

Tanto a MEP 24.15 como a MEP 24.16 são metaloendopeptidases zinco-dependentes, com massa molecular de 78 kDa cada (BARRETT *et al.*, 1995; CHECLER *et al.*, 1995 SHRIMPTON *et al.*, 2002). As estruturas destas duas peptidases são muito parecidas, conforme demonstrado por análises estruturais (BROWN *et al.*, 2001; RAY *et al.*, 2002, 2004) (Fig. 7). A maioria dos aminoácidos que diferem entre as duas enzimas encontra-se na superfície das moléculas, com apenas 11% das diferenças sendo associadas ao sítio ativo (RAY *et al.*, 2002), o que poderia explicar a grande semelhança no perfil de substratos destas enzimas. Uma diferença importante entre as duas enzimas é que a MEP 24.16 é inativada por agentes redutores contendo tiol como o ditiotreitol (DTT), enquanto a MEP 24.15 é ativada por baixas concentrações de DTT (<10 mM). Além disso, a atividade e secreção da MEP 24.15 podem ser reguladas pela fosforilação (TULLAI *et al.*, 2000), o que não ocorre com MEP 24.16 devido à substituição do sítio de fosforilação (Ser^{644}) por leucina (RAY *et al.*, 2002). A atividade da MEP 24.15, e possívelmente a da MEP 24.16, também pode ser modulada pelo ATP por interação com o zinco presente no sítio ativo (PORTARO *et al.*, 2001).

A MEP 24.15 cliva preferencialmente peptídeos na posição carboxil de aminoácidos hidrofóbicos (ORLOWSKI *et al.*, 1983), enquanto que a MEP 24.16

exibe especificidade pela posição prolil (JIRACEK *et al.*, 1996). A distinção entre a MEP 24.15 e a 24.16 é bastante difícil já que ambas são muito semelhantes em termos de propriedades físico-químicas e especificidade para substratos. *In vitro,* as duas enzimas degradam o hormônio liberador de gonadotrofina, bradicinina, neurotensina, dinorfina$_{1-8}$ e a substância P. Entretanto a MEP 24.16 hidrolisa a neurotensina na posição Pro^{10}-Gly^{11}, enquanto a MEP 24.15 cliva especificamente na posição Arg^{8}-Arg^{9} (RIOLI *et al.*, 1998; para revisões ver SHRIMPTON e SMITH, 2000, e SHRIMPTON *et al.*, 2002).

Há vários estudos demonstrando um papel para a MEP 24.15 no controle da pressão arterial em mamíferos. *In vitro,* a MEP 24.15 cliva a bradicinina na posição Phe^{5}-Ser^{6} (ORLOWSKI *et al.*, 1983). A infusão intravenosa de cFP (N-[1-(R,S)-carboxi-3-fenilpropil]-Ala-Ala-Phe-*p*-aminobenzoato), um inibidor específico para MEP 24.15, em ratos normotensos produz uma queda marcante na pressão arterial, a qual é abolida pela pré-administração de um antagonista de receptores B_2 da bradicinina (GENDEN E MOLINEAUX, 1990). Entretanto, o mecanismo de ação deste inibidor não ocorre somente através da inibição da MEP 24.15, uma vez que a degradação do cFP *in vivo* leva à formação de um metabólito que inibe a ECA (CHAPPELL *et al.*, 1992; TELFORD *et al.*, 1995). Mais recentemente, SMITH *et al.* (2000) demonstraram que a administração intravenosa de um novo inibidor para MEP 24.15, o N-[1-(R,S)-carboxi-3-fenilpropil]-Ala-Aib-Tyr-p-aminobenzoato (JA2), potencializou o efeito hipotensor da bradicinina em coelhos, mas não afetou os efeitos hipertensivos da angiotensina I e II. No mesmo trabalho, estes autores mostraram que o JA2 não é degradado como o cFP, e portanto não interfere na atividade da ECA. NORMAN *et al.* (2001) relataram que em coelhos com hipertensão do tipo "renal wrap" a administração intravenosa de JA2 não causou alterações cardiovasculares.

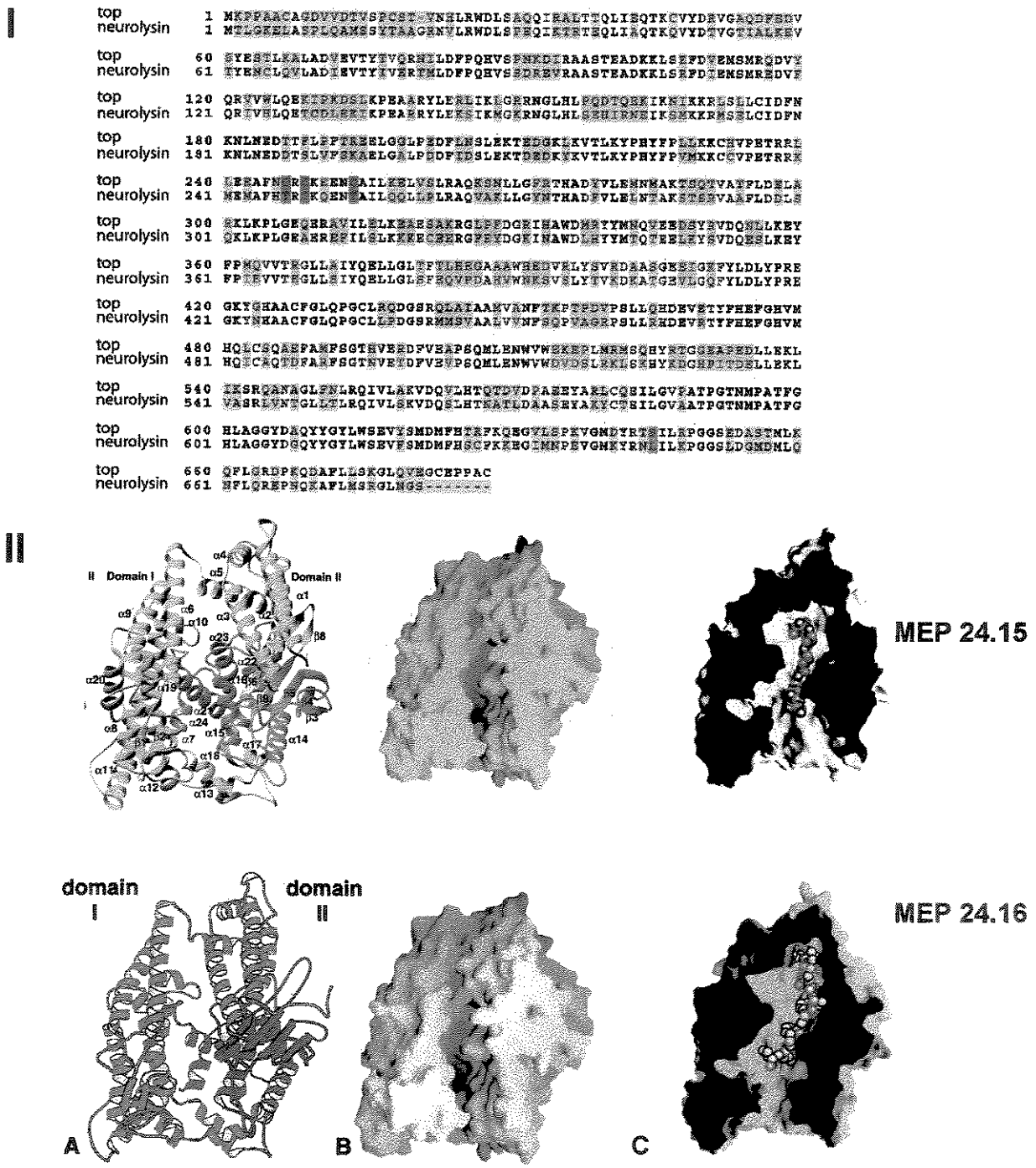

Figura 7. Comparação das estruturas de MEP 24.15 (timet oligopeptidase, TOP) e MEP 24.16 (neurolisina). Em **I**, a seqüência de aminoácidos, onde os resíduos diferentes estão em verde, as cisteínas envolvidas na multimerização da enzima estão em vermelho, e o sítio de fosforilação (Ser^{644}) está em azul. Em **II**, as estruturas em forma de fita (**A**), em modelo mostrando a superfície das moléculas (**B**), e em secção, mostrando o sítio ativo contendo a neurotensina como substrato (**C**). Em **IIA**, a esfera azul indica o íon de zinco no sítio ativo e a porção dourada (MEP 24.15) ou vermelha (MEP 24.16) indica a parte que é semelhante a outras metalopeptidases. Figuras de BROWN *et al.* (2001) e RAY *et al.* (2002, 2004).

Recentemente, NORMAN *et al.* (2003b) mostraram que a MEP 24.15 e 24.16 estão presentes em culturas de células endoteliais vasculares. Usando inibidores específicos para cada uma das enzimas ($PF^{PC}LP$-NH2 para MEP 24.16 e Z-F^{PC}ARF para MEP 24.15), estes autores demonstraram que em células endoteliais de aorta de ovelhas a degradação de bradicinina parece ser mais dependente da MEP 24.16 do que MEP 24.15. No entanto, em células endoteliais EA.hy926 ambas as peptidases contribuem para o metabolismo de bradicinina. Assim, células endoteliais de diferentes leitos vasculares podem expressar estas peptidases em diferentes proporções.

Além da possível degradação de bradicinina pela MEP 24.15, há evidências demonstrando o envolvimento da MEP 24.15, entre outras peptidases, em uma via alternativa de degradação da angiotensina I (FERRARIO *et al.,* 1998; SHRIMPTON *et al.,* 2002). Essa via paralela à da ECA leva à formação de angiotensina (1-7) que, como discutido anteriormente (ver item 1.2.), promove múltiplas ações no sistema cardiovascular.

2. OBJETIVOS

Baseado nos relatos de aumento da atividade da ECA no modelo L-NAME, e o envolvimento de peptídeos como a angiotensina II e o ANP, propomos, neste projeto:

1. Investigar se a inibição crônica na biossíntese de NO poderia afetar a atividade enzimática e expressão de outras peptidases implicadas na regulação cardiovascular. Foram estudadas as peptidases APM, DPP IV, MEP 24.15 e NEP 24.11 através de ensaios enzimáticos e western blotting.

2. Investigar se as alterações vistas no modelo de inibição crônica de L-NAME eram características deste modelo ou se aplicariam a outros modelos experimentais de hipertensão. Para isso, estudamos as mesmas peptidases nos mesmos tecidos de ratos espontaneamente hipertensos.

3. CAPÍTULOS

Artigo publicado

"Peptidase activities in rats treated chronically with N^{ω}-nitro-L-arginine methyl ester (L-NAME)"

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Peptidase activities in rats treated chronically with N^{ω}-nitro-L-arginine methyl ester (L-NAME)

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Abstract

The chronic treatment of rats with N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) biosynthesis, results in hypertension. This inhibition of NO production results in activation of the renin-angiotensin system, with increased activity of the carboxypeptidase angiotensin I-converting enzyme (ACE). Since chronic NO inhibition increases ACE activity, we hypothesized that this inhibition could also affect the activities of other peptidases involved in cardiovascular functions. To test this possibility, we examined the activities of aminopeptidase M (APM), dipeptidyl peptidase IV (DPP IV), metalloendopeptidase 24.15 (MEP 24.15) and neutral endopeptidase 24.11 (NEP 24.11) in rat brain, heart, kidney, liver, lung and thoracic aorta. Male Wistar rats were treated chronically with L-NAME (80 mg kg^{-1} per day) administered in the drinking water for 4 weeks and their organs then removed and processed for the determination of peptidase activities. Treatment with L-NAME did not significantly alter the activities of the four peptidases in brain, heart, kidney, liver and lung. In contrast, in aorta, the activity of APM was slightly but significantly reduced whereas those of DPP IV and MEP 24.15 were markedly enhanced; NEP 24.11 was not detected in this tissue. Immunoblotting for DPP IV and MEP 24.15 showed increased expression in aortic tissue. Neither L-NAME (1–100 μM) nor the NO donors sodium nitroprusside and 3-morpholinosydnonimine (SIN-1; 1–100 μM) had any consistent effect on the activity of recombinant MEP 24.15 or renal DPP IV. The importance of MEP 24.15 in peptide metabolism was confirmed in pentobartibal-anesthetized rats pretreated with the MEP 24.15 inhibitor *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate (JA2), which significantly potentiated the hypotensive response to bradykinin. The altered peptidase activities seen in aorta may contribute to modulating vascular responses in this model of hypertension.

Keywords: Angiotensin I-converting enzyme; Chronic inhibition; Hypertension; Nitric oxide; Peptidases; Renin-angiotensin system

1. Introduction

Nitric oxide (NO) plays a major role in modulating regional blood flow and arterial blood pressure in animals and humans. The chronic administration of NO synthase (NOS) inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) produces sustained arterial hypertension in rats [1] which may be accompanied by histopathological changes in cardiac and renal tissue [2]. L-NAME-induced hypertension is mediated to a large extent by increased formation of angiotensin II through increased activity of the renin-angiotensin system (RAS) since treating rats with angiotensin-converting enzyme (ACE) inhibitors such as enalapril [3], or with angiotensin II receptor antagonists such as losartan [1,2] restores blood pressure to near normal levels.

In addition to ACE, various other peptidases capable of metabolizing circulating peptides could potentially modulate blood pressure. These include membrane-bound

Abbreviations: ACE, angiotensin-converting enzyme; ANP, atrial natriuretic peptide; APM, aminopeptidase M; AT_1, angiotensin II type 1 receptor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; DPP IV, dipeptidyl peptidase IV; JA2, *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate; MEP 24.15, metalloendopeptidase 24.15; NEP 24.11, neutral endopeptidase 24.11; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; QFS, quenched fluorescent substrate; RAS, renin-angiotensin system; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; TBS, Tris-buffered saline

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enzymes such as aminopeptidase M (APM; EC 3.4.11.2) and neutral endopeptidase 24.11 (NEP 24.11; EC 3.4.24.11), which are capable of degrading peptides such as angiotensin, atrial natriuretic peptide (ANP), bradykinin, neurotensin and tachykinins [4–7]. Another important membrane-bound peptidase is dipeptidyl (amino)peptidase IV (DPP IV; EC 3.4.14.5) that cleaves peptides in which proline, and to a lesser extent, alanine or hydroxyproline, is the penultimate amino acid [8]. More recently, evidence has accumulated in support of a role for soluble neutral metallopeptidases 24.15 and 24.16 as regulators of endogenous peptide activity (reviewed in [9]). Metalloendopeptidase 24.15 (EC 3.4.24.15; MEP 24.15) is an important peptidase that degrades neurotensin, somatostatin, gonadotrophin releasing hormone, bradykinin, and various peptides with opioid activity [9].

Nitric oxide can modulate the expression and activity of a variety of peptides [10], receptors [11,12] and enzymes [13], including ACE, the activity and/or expression of which is increased in the tissues of rats treated chronically with L-NAME [14,15]. Based on the enhanced activity of ACE seen in this model, we hypothesized that the inhibition of NO biosynthesis could also affect the activities of other peptidases involved in regulating cardiovascular functions. To test this hypothesis, we examined the activities of four peptidases (APM, DPP IV, MEP 24.15 and NEP 24.11) in rats treated chronically with L-NAME.

2. Materials and methods

2.1. Reagents

Bovine serum albumin, *N*-dansyl-D-alanyl-glycyl, *N*-dansyl-D-alanyl-glycyl-*p*-nitrophenyl-alanylglycine, diprotin A, dithiothreitol (DTT), Hepes, leuhistin, L-Leu-*p*-nitroanilide, β-naphthylamine, *p*-nitroaniline, N^{ω}-nitro-L-arginine methyl ester, phosphoramidon and sodium nitroprusside were from Sigma. Acrylamide, ammonium persulfate, Coomassie brilliant blue R250, glycerol, Hybond-P PVDF membrane (0.45 μm), *N*,*N*′-methylene bis-acrylamide, sodium dodecyl sulphate (SDS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Temed), Tris base and donkey anti-rabbit IgG-peroxidase conjugate were from Amersham Biosciences. SIN-1 (3-morpholinosydnonimine) was from Cassella AG. Gly-Pro-β-naphthylamide was from Bachem. 7-Methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys-(2,4-dinitrophenyl) (QFS) was synthesized by Auspep and was a generous gift from Dr. A. Ian Smith (Baker Research Medical Institute, Australia), who also provided the MEP 24.15/24.16 inhibitor *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate (JA2). Purified human recombinant MEP 24.15 was prepared as described previously [16]. Rabbit anti-DPP IV polyclonal antibody was from Santa Cruz Technologies and antiserum specific for MEP 24.15 was produced in rabbits immunized with recombinant 24.15 as described [17]. Flat-bottomed 96-well plates were from Corning Inc., chemioluminescence kits (SuperSignal, West Pico) were obtained from Pierce and photographic film was from Kodak. Other reagents of analytical grade were obtained from Baker, Mallinkrodt or Merck.

2.2. Animals

Male Wistar rats (~150 g at the start of the experiment) were obtained from the Central Animal House Services (UNICAMP) and were housed at 23 ± 1 °C on a 12 h light/dark cycle with food and water ad libitum. The experiments described here were done in accordance with the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

2.3. Treatment with L-NAME and blood pressure measurements

Rats were treated with L-NAME (80 mg kg^{-1} per day) given in their drinking water for 4 weeks [1]. The amount of L-NAME ingested was calculated based on the water intake of the rats which was monitored daily. Control rats received tap water alone. Once a week, the rats were weighed and tail blood pressure was measured by a tail-cuff method [18].

2.4. NOS activity

Brain NOS activity was measured by the method of Förstermann et al. [19], as described by Faria et al. [20], using [^{3}H]-arginine as substrate. Enzyme activity was expressed as pmol of [^{3}H]L-citrulline formed/min/mg of protein.

2.5. Protein concentrations

Protein concentrations were determined by the method of Lowry et al. [21] using bovine serum albumin as standard.

2.6. Tissue preparation

After 4 weeks of treatment, the rats were anesthetized with sodium pentobarbital (>60 mg/kg, i.p.; Hypnol®) and perfused via the aorta with heparinized saline to wash out blood from the organs (brain, heart, kidney, liver, lung) which were then collected, snap frozen in liquid N_2, and stored at −80 °C until used. In the case of thoracic aorta, the rats were exsanguinated without perfusion in order to avoid damage to the endothelium and the vessels then rapidly removed and processed as described above. For measurement of enzymatic activities, tissues were homogenized (Ultraturrax, model T25, 24,000 rpm) at 4 °C in 5–10 volumes of 0.1 M Tris–0.32 M sucrose, pH 7.4. The homogenate was centrifuged at 3000 × *g* (20 min, 4 °C)

and the supernatant then collected and centrifuged at 20,000 × *g* (25 min, 4 °C). The resulting supernatant was used to measure MEP 24.15 activity and the precipitate was used for APM, DPP IV and NEP 24.11 activities. For aorta, preliminary experiments indicated that the most consistent results were obtained using only low speed centrifugation (3000 × *g*, 30 min, 4 °C) and all enzyme activities were assayed in the supernatant. Aliquots of these preparations were also used for immunoblotting.

2.7. Peptidase assays

2.7.1. Aminopeptidase M (APM)

APM activity was assayed using the chromogenic substrate L-Leu-*p*-nitroanilide [22]. The assay was done in 96-well plates containing 150–220 μl of 50 mM Tris–HCl, pH 7.4, 5–50 μl of sample, and 25 μl of substrate (final conc. 140 μM), to give a final volume of 250 μl/well. The increase in absorbance at 410 nm was followed for 20–30 min at 37 °C using a SpectraMax340 multiwell plate reader (Molecular Devices) and the amount of product formed was determined from a standard curve of *p*-nitroaniline. Enzyme activity was expressed as nmol of *p*-nitroaniline formed/min/mg of protein.

2.7.2. Dipeptidyl peptidase IV (DPP IV)

DPP IV activity was assayed using Gly-Pro-β-naphthylamide in an assay mixture containing 50–70 μl of 50 mM Tris–HCl, pH 8.0, 20 μl of substrate (final conc. 200 μM), and 10–30 μl of sample. After incubation for 15–30 min, the reaction was stopped by adding 900 μl of ammonium formate, pH 4.5 and the resulting fluorescence was measured (Hitachi F-2000 spectrofluorimeter) at 410 nm after excitation at 335 nm [23,24]. The amount of product formed was determined from a standard curve of β-naphthylamine and activity was expressed in nmol of β-naphthylamine formed/min/mg of protein. To examine the possibility of a direct effect of L-NAME and NO on DPP IV activity, renal DPP IV was assayed in the presence of L-NAME and the NO donors SNP and SIN-1 (1–100 μM). The activity was monitored as the increase in fluorescence over time.

2.7.3. Metalloendopeptidase 24.15 (MEP 24.15)

The activity of MEP 24.15 was determined fluorimetrically using the specific quenched fluorescent substrate (QFS) 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys-(2,4-dinitrophenyl), as described [25]. The assays were done under conditions of linearity (zero-order kinetics), with <10% of the total substrate being consumed during the assay. The reactions were run for 10–30 min in a final volume of 100 μl consisting of TBS (0.025 M Tris–HCl, pH 7.4, 0.125 M NaCl), 10 μM QFS, 0.5 mM DTT and 10–50 μl of sample. The activity was expressed as the increase in arbitrary fluorescence units (AFU)/min/mg of protein.

To examine the possibility of a direct effect of L-NAME and NO on MEP 24.15 activity, recombinant MEP 24.15 (16 ng/well) was assayed in TBS (without DTT), in the presence of L-NAME and the NO donors SNP and SIN-1 (1–100 μM). The activity was monitored as the increase in fluorescence over time.

2.7.4. Neutral metalloendopeptidase 24.11 (NEP 24.11)

NEP 24.11 activity was assayed using *N*-dansyl-D-alanyl-glycyl-*p*-nitrophenyl-alanylglycine as substrate. The reaction mixture consisted of 170–190 μl of 0.1 M Hepes, pH 6.4, 10–30 μl of sample and 50 μl of substrate (final concentration, 200 μM). After incubation at 37 °C for 10–20 min, the reaction was stopped by boiling the samples for 5 min. The resulting fluorescence was measured at 562 nm after excitation at 342 nm [26] and the amount of product (*N*-dansyl-D-alanyl-glycyl) formed was determined from a standard curve constructed in Hepes buffer. Enzyme activity was expressed as nmol of product formed/min/mg of protein.

2.7.5. Specificity of assays

The specificities of the activities assayed was confirmed by using leuhistin [27], diprotin A [28], *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate (JA2) [29] and phosphoramidon [30] to inhibit APM, DPP IV, MEP 24.15 and NEP 24.11, respectively. The specificity of the inhibition was tested in all of the organs investigated in this study and was >80% in all cases ($n = 5$ for each enzyme; results not shown). In the MEP 24.15 assay, 0.5 mM DTT was included to inhibit MEP 24.16 [16].

2.8. SDS–PAGE and immunoblotting

Aliquots (10 μg) of aortic extracts prepared as described above were electrophoresed (100 V constant) in 10% polyacrylamide gels in the presence of SDS [31] and the proteins then transferred to Hybond-P PVDF membranes [32]. Peptidases were detected by blotting with rabbit polyclonal IgG antibodies against DPP IV (diluted 1:800) or MEP 24.15 (1:5000) and then detected by chemioluminescence. The immunoreactive bands were evaluated by densitometry using the software Scion Image® and the peptidase levels were expressed as arbitrary densitometric units.

2.9. Role of MEP 24.15/24.16 in bradykinin-induced hypotension

Male Wistar rats (~200 g) were anesthetized as described above. The trachea was cannulated to facilitate breathing, and the right carotid artery and left femoral vein were cannulated with polyethylene tubing for the measurement of arterial blood pressure and drug administration, respectively. The arterial pressure was recorded continuously via a pressure transducer (Abbott) coupled to a

computer-controlled data acquisition system (Transonic Systems). Bradykinin (0.3 and 3 μg/kg, i.v.) was administered after allowing 15 min for stabilization of the preparation. In some experiments, JA2 (10 mg/kg, i.v.) was given 10–15 min before repeating the above doses of bradykinin. Since JA2 alone caused a transient hypotension at the dose used, the possible involvement of NO in this response was examined by testing this inhibitor in rats treated with L-NAME as described above.

2.10. Statistical analysis

The data are presented as the mean ± S.D. Statistical analyses were done using Student's *t*-test or analysis of variance (ANOVA) followed by the Bonferroni test. Values of $P < 0.05$ were considered as significant.

3. Results

3.1. The efficacy of chronic treatment with L-NAME

Systolic blood pressure increased significantly ($P < 0.05$) in rats treated with L-NAME (191 ± 3 mmHg after 4 weeks, $n = 10$) compared to control rats (133 ± 4 mmHg, $n = 10$). Treatment with L-NAME for 4 weeks also inhibited the activity of brain constitutive NOS by >90% ($P < 0.05$).

3.2. Effect of chronic treatment with L-NAME on peptidase activities

With the exception of NEP 24.11, which was consistently detected only in kidney and lung (greatest activity in

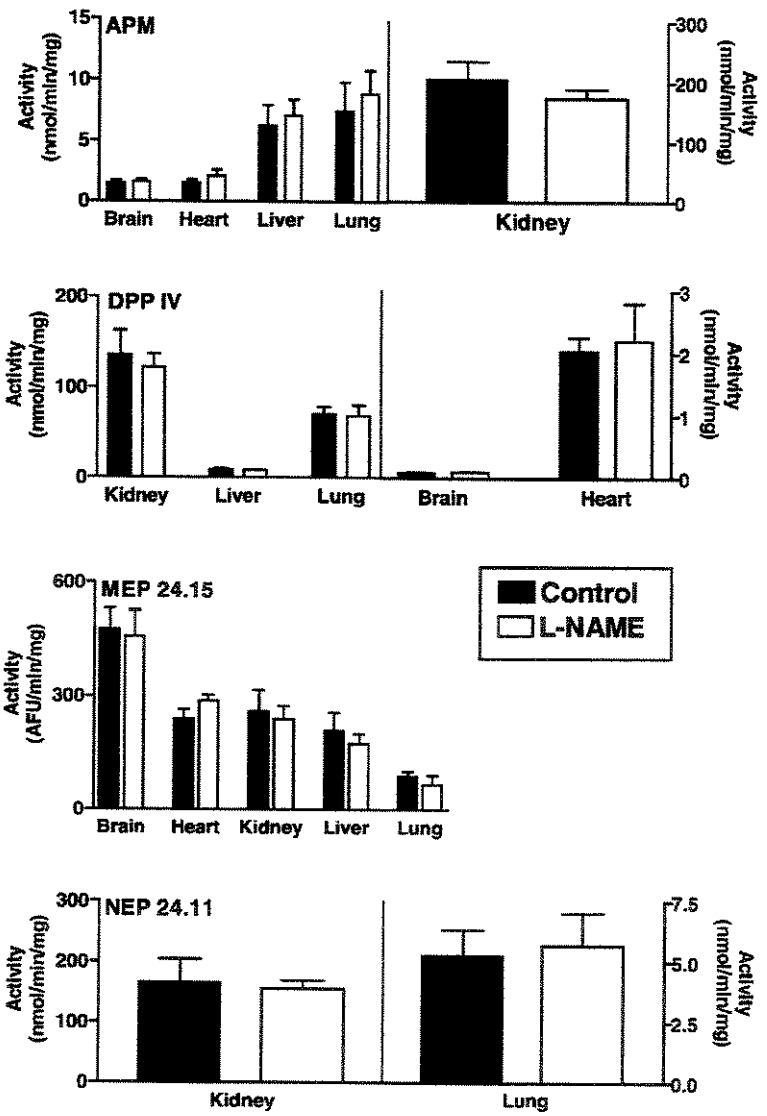

Fig. 1. APM, DPP IV, MEP 24.15 and NEP 24.11 activities in rat tissues after treatment with L-NAME for 4 weeks. The columns represent the mean ± S.D. of five rats each. There were no significant differences between control and L-NAME-treated rats. AFU, arbitrary fluorescence units.

the former), the other peptidases were detected in all of the tissues assayed. APM and DPP IV activities were highest in kidney and MEP 24.15 was highest in brain.

Fig. 1 shows that treating rats with L-NAME for 4 weeks did not significantly alter the enzyme activities of the four peptidases in any of the principal organs compared to the control rats. In contrast, the activity of aortic APM was slightly but significantly lower in L-NAME-treated rats whereas the activities of DPP IV and MEP 24.15 were markedly enhanced (Fig. 2). Since the levels of DPP IV and MEP 24.15 were increased in aortic tissue of L-NAME-treated rats, the expression of these enzymes was examined by western blotting. As illustrated in Fig. 3, immunoblots for DPP IV and MEP 24.15 showed increased expression of these enzymes in aortic tissue, which agreed with their enhanced enzymatic activity.

To further examine the influence of L-NAME and NO on the activity of DPP IV and MEP 24.15, renal membrane-

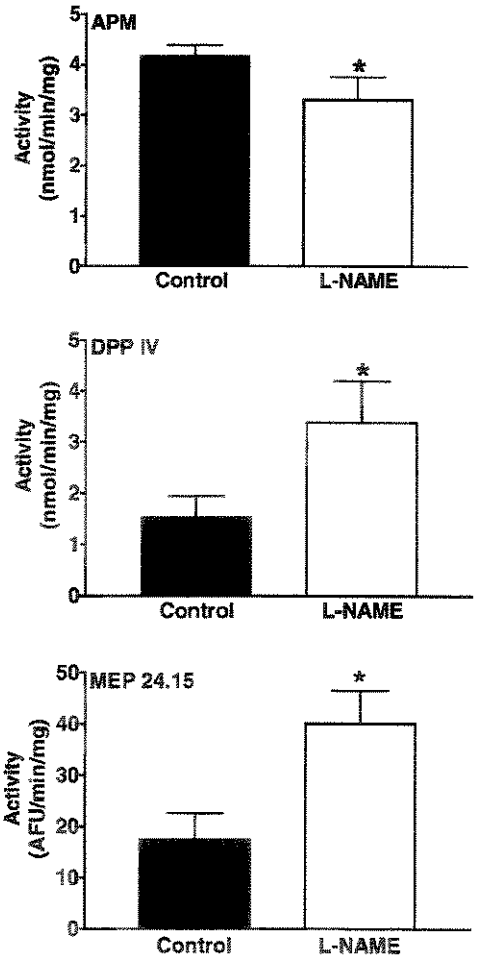

Fig. 2. APM, DPP IV and MEP 24.15 activities in rat aorta after treatment with L-NAME for 4 weeks. The columns represent the mean ± S.D. of five rats each. $^*P < 0.05$ compared to the corresponding control. AFU, arbitrary fluorescence units.

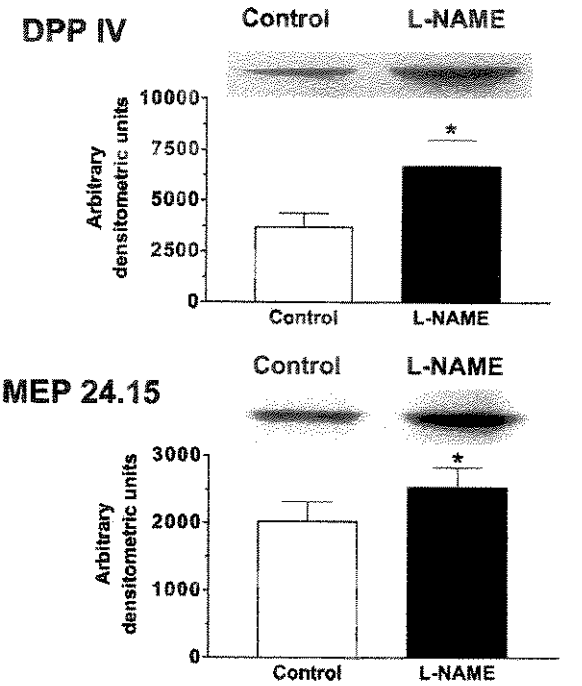

Fig. 3. Western blots for DPP IV (upper panel) and MEP 24.15 (lower panel) in rat aorta after treatment with L-NAME for 4 weeks. Representative blots and mean densitometric values for the expression of DPP IV and MEP 24.15 are shown for the control and L-NAME groups. Equal amounts of protein (10 μg) were applied to the gels in all cases. The columns represent the mean ± S.D. of four rats each. $^*P < 0.05$ compared to the corresponding control.

bound DPP IV and purified recombinant MEP 24.15 were incubated with various concentrations of L-NAME or the spontaneous NO donors SNP and SIN-1. As shown in Figs. 4 and 5, neither L-NAME nor the NO donors SNP and SIN-1 had any consistent, marked effect on the activity of DPP IV and MEP 24.15 at concentrations up to 100 μM.

3.3. Involvement of MEP 24.15/24.16 in bradykinin-induced hypotension

To examine the role of MEP 24.15/24.16 in the hypotension induced by bradykinin, pentobarbital-anesthetized rats were pretreated with the non-selective inhibitor JA2. Administration of the inhibitor (10 mg/kg, i.v., 15 min before bradykinin) caused a transient but significant decrease in blood pressure that returned to normal within 5 min (Fig. 6A); a lower dose of JA2 (5 mg/kg, i.v.) had no such effect, nor did the vehicle solution (10% 2-hydroxypropyl-β-cyclodextrin in 0.9% saline). This hypotensive response to JA2 was attenuated in rats treated with L-NAME for 4 weeks. In normotensive Wistar rats, JA2 significantly enhanced the hypotensive responses to bradykinin (Fig. 6B). In these experiments, bradykinin was administered after the blood pressure response to JA2 had returned to basal values.

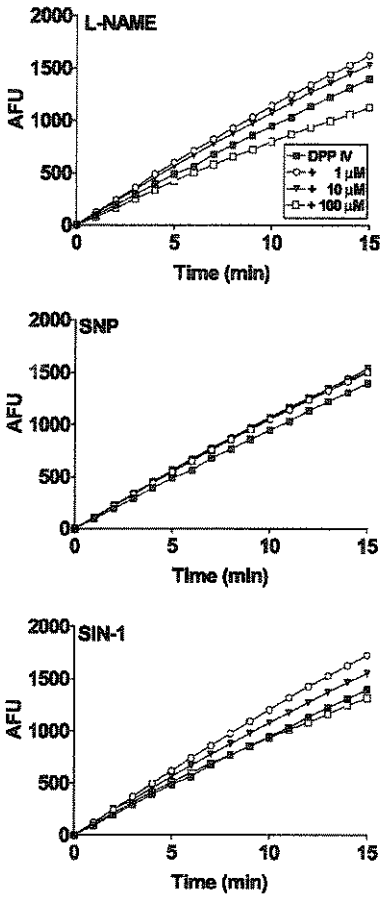

Fig. 4. DPP IV activity in the absence and presence of L-NAME, SNP and SIN-1. Peptidase activity using 5 μg of renal membrane preparation was assayed as described in Section 2. The points are the mean of two determinations, each in duplicate. AFU, arbitrary fluorescence units.

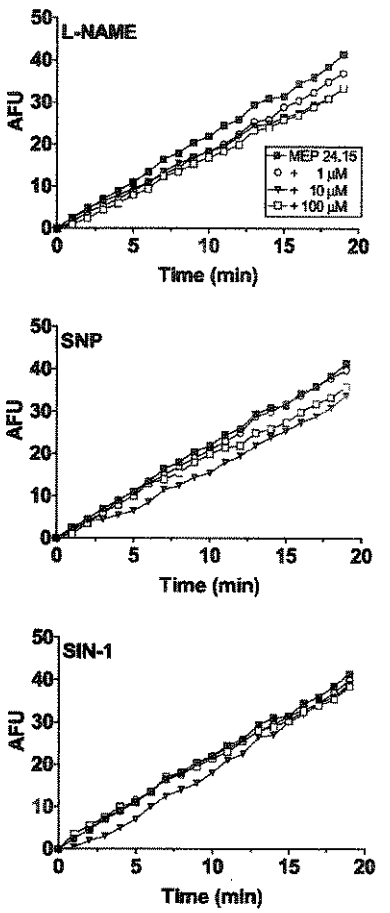

Fig. 5. MEP 24.15 activity in the absence and presence of L-NAME, SNP and SIN-1. Peptidase activity using 16 ng of purified enzyme was assayed as described in Section 2. The points are the mean of two determinations, each in duplicate. AFU, arbitrary fluorescence units.

4. Discussion

The chronic treatment of rats with L-NAME, an inhibitor of NO biosynthesis, leads to hypertension, with subsequent marked effects on the cardiovascular system (reviewed in [2]). To a large extent, the hypertension and vascular inflammatory responses observed in this model involve activation of the RAS since treating rats with ACE inhibitors or angiotensin II type 1 (AT_1) receptor antagonists reverts the above alterations [1,14,33,34]. In agreement with the involvement of the RAS, there is increased AT_1 receptor expression (mainly in the first week of treatment with L-NAME) [11] and increased ACE expression and activity in tissues, particularly heart and aorta [14,15,35]. The enhanced ACE activity contributes to vascular and myocardial damage associated with this model [36] and is mediated, at least partly, by oxidative stress, which is increased in L-NAME-treated rats [35]. In contrast to this role of ACE and angiotensin II in the hypertension resulting from chronic NO inhibition, little is known of the involvement of other peptidases in the cardiovascular responses in this model.

As shown here, treatment with L-NAME for 4 weeks did not significantly affect the activities of the four peptidases studied in brain, heart, kidney, liver and lung, but produced alterations in the levels of APM, DPP IV, and MEP 24.15 in aortic tissue. The altered activities seen in aorta agreed with the findings of Bouton et al. [37] for the serpin protease nexin-1 and of Takemoto et al. [14] and Gonzalez et al. [15] for ACE, all of whom reported increased activity of the corresponding enzyme in this vessel in this model. In addition, Takemoto et al. [14] demonstrated that, except in the heart, ACE activity was unaltered in other tissues examined (kidney, liver and lung). The finding of altered enzymatic activities in aorta, but not in other tissues, suggests that changes in peptidase activities in L-NAME-treated rats may be more associated with vascular than with non-vascular tissue or cell types. In this regard, it is possible that local changes in vascular peptidase activities within a given tissue may be masked when whole organ homogenates are used.

While there were no significant changes in APM activity in brain, heart, kidney, liver and lung after 4 weeks of treatment with L-NAME, a small but significant decrease

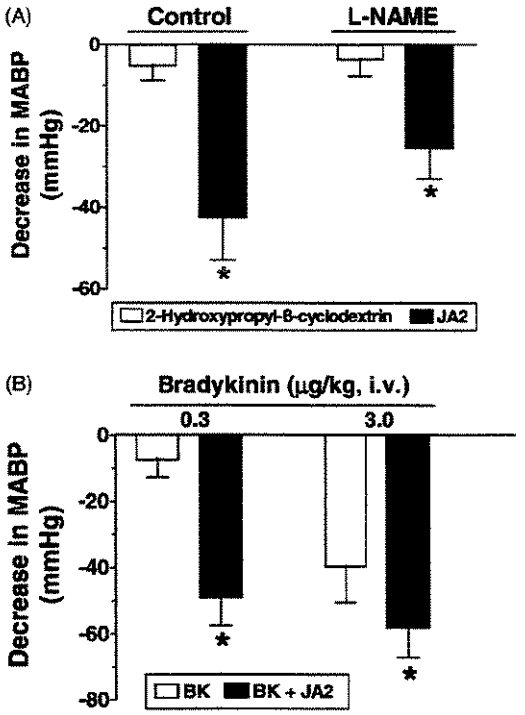

Fig. 6. Involvement of MEP 24.15/24.16 in the blood pressure response to bradykinin in pentobarbital-anesthetized male Wistar rats. (A) Effect of the dual MEP 24.15/24.16 inhibitor, JA2 (10 mg/kg, i.v.), on basal blood pressure in normotensive (control) rats and in rats treated with L-NAME for 4 weeks. JA2 alone produced transient hypotension in control and L-NAME-treated rats, with the response being attenuated in the latter. The vehicle solution alone (10% 2-hydroxypropyl-β-cyclodextrin in 0.9% saline) had minimal effect on the blood pressure. (B) Effect of MEP 24.15/24.16 inhibition by JA2 on the responses to bradykinin in anesthetized rats. Pre-treating the rats with JA2 potentiated the responses to bradykinin. Note that bradykinin was tested only after the blood pressure has returned to basal values following JA2 administration. The columns represent the mean ± S.D. of six rats each. $^{*}P < 0.05$ compared to the corresponding response seen with vehicle (2-hydroxypropyl-β-cyclodextrin) (A) or bradykinin (B) alone.

in this enzyme was seen in aorta. APM, or CD13, occurs in a variety of tissues, including aortic smooth muscle cells [38], heart and kidney. As shown here, the APM levels in kidney were consistently higher than in the other tissues in both control and L-NAME-treated rats. APM hydrolyzes the N-terminal of kallidin to form bradykinin [6,39] and can also degrade des(Asp^1)angiotensin I, angiotensin III and enkephalins [4,39]. Since angiotensin III shares many properties with angiotensin II, including vasoconstrictor activity [40], APM could have an important role in attenuating the actions of the former peptide. Thus, the decrease in aortic APM activity in L-NAME-treated rats could represent a mechanism for exacerbating the hypertension mediated by angiotensins II and III in this model since there would be less degradation of the latter peptide; this change in enzyme activity may be greater in other vessels and microcirculatory beds, some of which are rich in APM [41].

DPP IV is a high molecular mass (>110 kDa) membrane-bound, serine-type peptidase with a wide distribution in rat tissues. This peptidase can cleave a variety of peptides and proteins, the most important of which, in terms of vascular activity, are substance P, neuropeptide Y and peptide Y ([42], reviewed in [43]). Important actions of DPP IV, additionally known as CD26, are also associated with the immunological system, particularly the activation of immunocompetent cells, as well as in cell–cell interactions [43,44]. Since the chronic inhibition of NO biosynthesis results in a cellular response involving the infiltration of inflammatory cells as well as necrosis and fibrosis in cardiac, renal and vascular tissues [2,14,33,34], we investigated whether there were any alterations in the activity of this peptidase following treatment with L-NAME.

As shown here, the only tissue in which there was a significant change in DPP IV activity was aorta, and this increase in activity appeared to involve increased expression of the peptidase rather than a direct action of L-NAME or NO donors on the enzyme itself (Figs. 3 and 4). The enhanced activity and expression of DPP IV most probably occurred at the level of the endothelium since vascular smooth muscle cells are apparently devoid of this enzyme [38]. Although little is known about the changes in neuropeptide levels in chronic L-NAME-induced hypertension, it is possible that increased vascular activity of DPP IV could serve to degrade substance P, thereby abolishing its vasodilator action and enhancing angiotensin II-induced vasoconstriction. Conversely, however, enhanced DPP IV activity would abolish the vasoconstrictor action of neuropeptide Y. Since neuropeptide Y stimulates hypertrophy in rat ventricular cardiomyocytes [45,46], and since cardiomyocyte hypertrophy is seen in L-NAME-induced hypertension, locally increased levels of vessel DPP IV could provide some protection against this phenomenon if neuropeptide Y levels are elevated in this model. This hypothesis would agree with evidence implicating DPP IV in angiogenesis and vascular remodeling [47], and with the histochemical demonstration of enhanced DPP IV activity in areas of fibrosis in cardiac tissue of rats with L-NAME-induced hypertension [48].

NEP 24.11, a membrane-bound enzyme that is identical to common acute lymphoblastic leukemia antigen (CALLA), also known as CD 10, occurs in endothelial cells, vascular smooth muscle cells, cardiomyocytes, fibroblasts and other cell types [49]. In the present study, NEP 24.11 was detected only in kidney and lung. This distribution agrees with other reports showing that these two organs are the principal sites of NEP 24.11 activity in the rat; the levels in brain are approximately 7–10-fold lower than in these two tissues and are undetectable in heart, liver, and some other tissues ([50], reviewed in [51]).

Treatment with L-NAME did not alter NEP 24.11 activity in rat tissues. This lack of effect of chronic treatment with L-NAME on NEP 24.11 activity is particularly interesting in view of the ability of dual ACE-NEP inhibitors, such as omapatrilat, in improving vascular function in a variety of cardiovascular conditions, including hypertension of

various origins (reviewed in [7]). These dual inhibitors act primarily by preventing the degradation of ANP and related peptides (BNP and CNP) by NEP 24.11. ANP levels are elevated after chronic inhibition with L-NAME [10]. Whilst this elevation involves increased ANP mRNA expression, a reduced activity of NEP 24.11 could also contribute to the higher levels of this peptide, although this seems unlikely based on the unaltered activities seen here.

NEP 24.11 can also produce angiotensin-(1–7) from angiotensin I, and this heptapeptide may antagonize the vasoconstrictor actions of angiotensin II through its vasodilator action [52]. However, the reduced production of NO, an important mediator of angiotensin-(1–7) action [53], and the increased activity of ACE, an important enzyme in degrading angiotensin-(1–7) to angiotensin-(1–5) [54], seen in L-NAME-treated rats could be important factors in limiting the action of angiotensin-(1–7) in this model. In addition to its action on ANP and angiotensin-(1–7), NEP 24.11 can degrade bradykinin by cleaving the terminal Pro-Phe bond that is also cleaved by ACE [55]. However, since the RAS is highly activated in L-NAME-induced hypertension, the levels of bradykinin in the circulation would be expected to be lower than in non-treated rats. These observations suggest that the role of NEP 24.11 in this model of hypertension may be limited, although this peptidase may be important locally in countering angiotensin II-mediated vasoconstriction in L-NAME-induced hypertension, particularly in the microcirculation and in non-aortic vessels.

Apart from DPP IV, the only other peptidase showing increased activity in this study was MEP 24.15 in aortic tissue. MEP 24.15 is an emerging target for the development of therapeutic drugs since this peptidase is involved in the degradation of a variety of vasoactive peptides, including bradykinin, and the generation of angiotensin-(1–7) from angiotensin I [9]. Angiotensin-(1–7) exerts a variety of physiological effects, including stimulation of NO and superoxide anion release from endothelial cells [53], potentiation of the vasodilatory action of bradykinin, and stimulation of prostaglandin and vasopressin release, diuresis and natriuresis (reviewed in [52,56]). Since ACE activity is augmented during chronic inhibition of NO biosynthesis, pathways leading to the formation of angiotensin-(1–7) may be activated to compensate for the increased action of angiotensin II, and this could involve MEP 24.15, although, as noted above, enhanced ACE activity could rapidly degrade angiotensin-(1–7).

Several studies in vitro and in vivo have demonstrated that MEP 24.15/24.16 have an important role in modulating the responses to vasoactive peptides, particularly bradykinin [9,57,58]. Our results confirm these findings for this peptide in normotensive rats, as shown by the marked potentiation of the hypotensive response after pretreating rats with JA2. These observations thus indicate a role for MEP 24.15/24.16 in cardiovascular regulation in rats, in agreement with a recent investigation in rat cerebral microvasculature [57] and a study in rabbits [59]. Whereas the enzyme assays in vitro were done in the presence of 0.5 mM DTT which selectively inhibits MEP 24.16 activity [16], the dual inhibitory capacity of JA2 means that the response in vivo probably reflected the action of MEP 24.15 and/or 24.16. Recent work has indicated that MEP 24.16 may be more important than MEP 24.15 in peptide metabolism by cultured endothelial cells [58]. However, it is possible that the relative contribution of these two peptidases may vary depending on the model, the animal species, and the vascular bed studied. The transient hypotension seen in response to JA2 alone could reflect an immediate action of this inhibitor on MEP24.15/24.16 or could be caused by a direct, non-specific action of this compound on the vasculature, independent of an interaction with MEP24.15/24.16. The reduced response seen in L-NAME-treated rats suggests that this phenomenon is partially NO-dependent.

The mechanism responsible for the increase in the activities of aortic DPP IV and MEP 24.15 seen here is unclear. A direct effect of NO on these enzymes seems improbable because, unlike ACE [60], the activities of these peptidases were unaffected by NO-releasing compounds (SNP and SIN-1). The activities were also unaffected by incubation with L-NAME. A lack of a direct effect of NO-donors [*S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and DETA NONOate] and an NOS inhibitor (N^G-monomethyl-L-arginine, L-NMMA) on enzyme activity has also been reported for matrix metalloproteinase (MMP)-9 [61].

The enhanced expression of DPP IV and MEP 24.15 seen in western blotting suggested that the increase in enzymatic activities was mediated by events during biosynthesis of the peptidases, probably at the level of expression. This conclusion agreed with studies showing that NO regulates the expression of many proteins, including enzymes, by cGMP-dependent and independent mechanisms [13,62]. Such regulation could involve the modulation by NO of intracellular kinase signalling pathways [63] or the interaction of NO with specific regulatory regions in the genes concerned, e.g. heme oxygenase-1 [64]. The proportionally lower increase in MEP 24.15 expression seen here compared to that seen for activity may indicate that enhanced expression is only part of the explanation. Other factors, such as the intracellular levels of free ATP [65] and the degree of MEP 24.15 phosphorylation by protein kinase A at serine 644 [66] could perhaps be important in modulating MEP 24.15 activity in this model of hypertension (enzyme activity is inhibited by increased free ATP and phosphorylation).

In conclusion, the results of this study show that while the chronic treatment of rats with L-NAME does not significantly alter the levels of APM, DPP IV, MEP 24.15 and NEP 24.11 in various rat organs, there are significant changes in vascular tissue. The extent to which

these changes are characteristic of other large vessels and the microcirculation, as well as their contribution to modulating the hypertension in this model remain to be established. These findings also demonstrate that although the RAS is the principal peptidase pathway altered in this model of hypertension, other peptidases may also be affected. The tissue-selective changes in some of the peptidases studied here are reminiscent of those for ACE, the aortic activity of which is elevated after treatment with L-NAME [14,15] while there are no marked changes in serum or tissue (kidney, liver, lung) activities [14].

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Artigo submetido

"Peptidase activities and expression in spontaneously hypertensive rats"

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Peptidase activities and expression in spontaneously hypertensive rats

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Running title: Peptidases in spontaneously hypertensive rats

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Abbreviations: ACE, angiotensin-converting enzyme; ANP, atrial natriuretic peptide; APA, aminopeptidase A; APM, aminopeptidase M; AT_1, angiotensin II type 1 receptor; DPP IV, dipeptidyl peptidase IV; ECE, endothelin-converting enzyme; JA2, N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate; MEP 24.15, metalloendopeptidase 24.15; NEP 24.11, neutral endopeptidase 24.11; QFS, quenched fluorescent substrate; RAS, renin-angiotensin system; SHR, spontaneously hypertensive rats; TBS, Tris-buffered saline; WKY, Wistar Kyoto rats.

Abstract

The involvement of peptidases other than angiotensin-converting enzyme and neutral endopeptidase 24.11 (NEP 24.11) in cardiovascular regulation in spontaneously hypertensive rats (SHR) has not been extensively studied. In this investigation, we compared the activities and expression of aminopeptidase M (APM), dipeptidyl peptidase IV (DPP IV), metalloendopeptidase 24.15 (MEP 24.15) and NEP 24.11 in brain, heart, kidney, liver, lung and thoracic aorta from adult male Wistar-Kyoto (WKY) rats and SHR to assess the alterations in the latter model. Peptidase activities were determined using colorimetric or fluorometric substrates and protein expression levels were assessed by western blotting. APM, DPP IV and MEP 24.15 activities were detected in all of the tissues examined. There were no significant differences in APM activity between the two strains of rats. In contrast, in SHR, DPP IV was significantly increased in brain but was reduced (~75%) in aortic tissue, whereas MEP 24.15 was significantly increased in the lungs but decreased (~50%) in aorta compared to WKY rats ($p<0.05$ in all cases); there no significant differences in these activities in the other organs examined. NEP 24.11 was detected only in the kidneys and lungs and was significantly greater ($p<0.05$) in SHR than WKY rats. Western blotting revealed significant alterations in the expression of DPP IV that agreed with the changes in activity. A significant change in MEP 24.15 expression was seen in aorta but not in pulmonary tissue, whereas there were no changes in NEP 24.11 expression. Administration of the MEP 24.15/24.16 inhibitor JA2 had no effect on bradykinin-induced hypotension in SHR. These results indicate that the activities of several peptidases may be altered in SHR and could be important in modulating the hypertension in this model.

Keywords: Blood pressure; Cardiovascular; Hypertension; Peptidase; Peptides; Spontaneously hypertensive rat

The spontaneously hypertensive rat (SHR) was initially described by Okamoto and Aoki [1] and has since proven to be a valuable model of hypertension. Various investigations have implicated the renin-angiotensin system (RAS) in the pathogenesis of hypertension in SHR [2,3], with a central role for angiotensin-converting enzyme (kininase II or dipeptidyl carboxypeptidase, EC 2.4.15.1) which is responsible for the formation of the potent vasoconstrictor angiotensin II from angiotensin I. Neutral endopeptidase 24.11 (NEP 24.11, neprilysin, EC 3.4.24.11), which is related to endothelin-converting enzyme [4,5], has also been attributed a role in this model, particularly in view of its ability to degrade vasodilatory peptides of the atrial natriuretic peptide (ANP) family [6]. Indeed, the important roles of ACE and NEP 24.11 in modulating hypertension through their actions on angiotensin I and ANP, respectively, has led to the development of dual inhibitors for these peptidases [7-9]. Both ACE and NEP 24.11 are also involved in degrading the vasodilatory peptide angiotensin-(1-7) in SHR [10-12]. In addition, the peptidase chymase has been implicated in SHR because of its angiotensin-converting activity [13].

In addition to ACE, chymase and NEP 24.11, various other peptidases are capable of metabolizing circulating peptides, including membrane-bound aminopeptidase M (APM; EC 3.4.11.2) that can degrade angiotensin, ANP, bradykinin, neurotensin and tachykinins [14-16], dipeptidyl (amino)peptidase IV (DPP IV; EC 3.4.14.5), which cleaves peptides in which proline, and to a lesser extent, alanine or hydroxyproline, is the penultimate amino acid [17,18], and the soluble neutral metallopeptidases 24.15 and 24.16, that degrade neurotensin, somatostatin, gonadotrophin releasing hormone, bradykinin, and various peptides with opioid activity [19,20].

Despite the potential role for these peptidases in modulating the cardiovascular responses in SHR, there have been few comparative reports of their distribution and activities in this model. In this work, we examined the activities and expression of APM, DPP IV, MEP 24.15 and NEP 24.11

in various tissues of SHR rats and compared them with those of normotensive WKY rats to determine the patterns of variation in this model.

Materials and methods

Reagents. Bovine serum albumin, *N*-dansyl-D-alanyl-glycyl, dansyl-D-alanyl-glycyl-*p*-nitrophenyl-alanylglycine, diprotin A, dithiothreitol (DTT), Hepes, leuhistin, L-Leu-*p*-nitroanilide, β-naphthylamine, *p*-nitroaniline and phosphoramidon were from Sigma. Acrylamide, ammonium persulfate, Coomassie brilliant blue R250, glycerol, Hybond-P PVDF membrane (0.45 μm), N,N′-methylene bis-acrylamide, sodium dodecyl sulphate (SDS), N,N,N′,N′-tetramethylethylenediamine (Temed), Tris base and donkey anti-rabbit IgG-peroxidase conjugate were from Amersham Biosciences. Gly-Pro-β-naphthylamine was from Bachem. 7-Methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys-(2,4-dinitrophenyl) (QFS) and the MEP 24.15/24.16 inhibitor N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate (JA2) were synthesized by Auspep. Rabbit anti-DPP IV and anti-NEP 24.11 polyclonal antibodies were from Santa Cruz Technologies and antiserum specific for MEP 24.15 was produced in rabbits immunized with recombinant 24.15 as described [21]. Flat-bottomed 96-well plates were from Corning Inc., chemioluminescence kits (SuperSignal West Pico) were obtained from Pierce and photographic film was from Kodak. Other reagents of analytical grade were obtained from Baker, Mallinkrodt or Merck.

Animals. Male WKY and SHR (12 weeks old) were obtained from the Central Animal House Services (UNICAMP) and were housed at 23±1°C on a 12 h light/dark cycle with food and water ad libitum. The experiments described here were done in accordance with the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

Blood pressure measurements. The systolic arterial blood pressure of the rats was measured by a tail-cuff method [22] on the day before the experiments.

Protein concentrations. Protein concentrations were determined by the method of Lowry *et al.* [23] using bovine serum albumin as standard.

Peptidase assays. The processing of the tissues and the peptidase assays were done as described elsewhere [24]. The peptidase assays are summarized below.

Aminopeptidase M (APM) activity was assayed using the chromogenic substrate L-Leu-*p*-nitroanilide [25] in 50 mM Tris-HCl, pH 7.4. The increase in absorbance at 410 nm was followed for 20-30 min at 37°C using a SpectraMax340 multiwell plate reader (Molecular Devices) and the amount of product formed was determined from a standard curve of *p*-nitroaniline. Enzyme activity was expressed as nmol of *p*-nitroaniline formed/min/mg of protein.

Dipeptidyl peptidase IV (DPP IV) activity was assayed using Gly-Pro-β-naphthylamine in 50 mM Tris-HCl, pH 8.0. After stopping the reaction with ammonium formate, pH 4.5, the resulting fluorescence was measured (Hitachi F-2000 spectrofluorimeter) at 410 nm following excitation at 335 nm [26,27]. The activity was expressed in nmol of β-naphthylamine formed/min/mg of protein.

Metalloendopeptidase 24.15 (MEP 24.15) activity was determined fluorometrically (ex. 317 nm, em. 425 nm) using the specific quenched fluorescent substrate (QFS) 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys-(2,4-dinitrophenyl) in 0.025 M Tris-HCl, pH 7.4, containing 0.125 M NaCl and 0.5 mM DTT [28]. DTT was included in these assays to inhibit EMP 24.16 activity [21]. The assays were done under conditions of linearity (zero-order kinetics), with <10% of the total substrate being consumed during the assay. The activity was expressed as the increase in arbitrary fluorescence units (AFU)/min/mg of protein.

Neutral metalloendopeptidase 24.11 (NEP 24.11) activity was assayed using the substrate dansyl-D-alanyl-glycyl-*p*-nitrophenyl-alanylglycine (final concentration, 200 μM) in 0.1 M Hepes, pH 6.4, and measuring the fluorescence of the product (*N*-dansyl-D-alanyl-glycyl) formed at 562 nm after excitation at 342 nm [29]. Enzyme activity was expressed as nmol of product formed/min/mg of protein.

The specificities of the activities assayed were determined using selective inhibitors [24], and >80% inhibition was observed in all of the organs investigated (n=5 for each enzyme; results not shown).

SDS-PAGE and immunoblotting. Aliquots of aortic (10 μg), brain (20 μg), kidney (10 μg) or lung (20 μg or 50 μg) extracts [24] were electrophoresed (100 V constant) in 10% polyacrylamide gels in the presence of SDS [30] and the proteins then transferred to Hybond-P PVDF membranes [31]. Peptidases were detected by blotting with rabbit polyclonal IgG antibodies against DPP IV (diluted 1:500), MEP 24.15 (1:2000) or NEP 24.11 (1:500) and then detected by chemioluminescence. The immunoreactive bands were evaluated by densitometry using the software Scion Image® and the peptidase levels were expressed as arbitrary densitometric units.

Modulation of bradykinin-induced hypotension by MEP 24.15/24.16 in SHR. Male SHR were anesthetized with sodium pentobarbital (>60 mg/kg, i.p.; Cristália Indústria Farmacêutica, Itapira, SP, Brazil). The trachea was

cannulated to facilitate breathing, and the right carotid artery and left femoral vein were cannulated with polyethylene tubing for the measurement of arterial blood pressure and drug administration, respectively. The arterial pressure was recorded continuously via a pressure transducer (Abbott) coupled to a computer-controlled data acquisition system (Transonic Systems). Bradykinin (0.3 and 3 μg/kg, i.v.) was administered after allowing 15 min for stabilization of the preparation. In some experiments, JA2 (10 mg/kg, i.v.) was given 10-15 min before repeating the above doses of bradykinin.

Statistical analysis. The data are presented as the mean ± S.D. Statistical analyses were done using Student's t-test or analysis of variance (ANOVA) followed by the Bonferroni test. Values of $P<0.05$ were considered significant.

Results

Blood pressure levels in WKY and SHR rats

Systolic blood pressure was significantly ($p<0.05$) greater in SHR (173±16 mmHg, n=10) compared to WKY rats (123±9 mmHg, n=10) at the time of the experiments.

Peptidase activities and expression in WKY and SHR rats

With the exception of NEP 24.11, which was consistently detected only in kidney and lung (greatest activity in the latter), the other peptidases were detected in all of the tissues assayed. APM and DPP IV activities were highest in kidney and MEP 24.15 was highest in brain.

Figure 1 shows that the activity of APM was not significantly different in any of the principal organs of SHR compared to WKY rats. In contrast, the activity of DPP IV was significantly increased in brain, that of MEP 24.15 was greater in lung and NEP 24.11 was enhanced in kidney and lung. The activities of DPP IV and MEP 24.15 were significantly lower in thoracic aorta from SHR compared to WKY rats; there was no significant difference in the activity of APM (Fig. 2). Since the levels of DPP IV, MEP 24.15 and NEP 24.11 were altered in some tissues of SHR compared to WKY rats, the expression of these enzymes was examined by western blotting.

As shown in Figure 3, there was an increase in DPP IV expression in the brain and a decrease in aortic tissue, in agreement with the changes in enzymatic activity. The expression of MEP 24.15 was also lower in aorta but not significantly altered in lung (Fig. 4). In contrast, there was no difference in the protein levels NEP 24.11 in kidney and lung from SHR compared to WKY rats (Fig. 5).

Modulation of bradykinin-induced hypotension by MEP 24.15/24.16 in SHR

To examine whether MEP 24.15/24.16 was involved in the metabolism of bradykinin in SHR, pentobarbital-anesthetized SHR were injected with bradykinin (0.3 and 3 μg/kg, i.v.) before and after treatment with the specific but non-selective MEP 24.15/26.15 inhibitor JA2. Administration of the inhibitor (10 mg/kg, i.v., 15 min before bradykinin) did not affect the blood pressure of the rats nor did it affect the hypotension induced by bradykinin (Fig. 6).

Discussion

In this study, we examined the activities in SHR and WKY rats of four peptidases (APM, DPP IV, MEP 24.15 and NEP 24.11) that have been implicated in modulating the cardiovascular responses to a variety of peptides. The pattern of alterations for each peptidase varied among the major organs studied. Thus, whereas there were no changes in the activities of APM, DPP IV activity was elevated in brain but decreased in aorta, MEP 24.15 activity was increased in lung but decreased in aorta and NEP 24.11 was elevated in kidney and lung, the only two tissues in which this activity was detected. With the exception of NEP 24.11, these changes in activity were at least partly mediated by alterations in protein expression.

A variety of aminopeptidases (A, B, M, cystinyl and pyroglutamyl) have been implicated in experimental models of hypertension [32-34]. Aminopeptidase A (APA) is responsible for the conversion of angiotensin II to angiotensin III [35-37] and shows greater activity in SHR than in WKY rats [35], whereas aminopeptidases B and APM (=APN) produce angiotensin IV from angiotensin III; APM also degrades des(Asp^1)angiotensin I and enkephalins and hydrolyzes the N-terminal of kallidin to form bradykinin [14,15,38]. In SHR, aminopeptidases may have a greater importance in hypertension mediated by the central angiotensinergic system compared to the peripheral system [39]. In anesthetized SHR and WKY rats, the intracerebroventricular (i.c.v.) administration of APA results in an elevation in blood pressure [36,40] that is attenuated when this enzyme is inhibited [36]. In contrast, similar administrations of leucine aminopeptidase and APM cause a decrease in blood pressure [40-43]. Inhibition of aminopeptidase B activity enhances the central pressor response to angiotensin III [44-45], probably through is ability to prevent the conversion to angiotensin IV, with greater sensitivity in SHR compared to WKY rats.

As shown here, there were no significant differences in the activities of APM in the various tissues of SHR rats compared to WKY, despite its importance in reducing the levels of angiotensin III in the central nervous system [46]. This finding is similar to that of Dendorfer *et al.* [47] who reported no significant differences in the kininase activities (ACE, carboxypeptidase N and aminopeptidase P) of plasma from SHR and WKY rats. In contrast, Nakashima *et al.* [48] reported that renal APA was lower in SHR than in WKY rats, and that the placental activity of this peptidase was higher in hypertensive rats. On the other hand, Healy and Song [49] found markedly greater renal APA activity in 4-, 8- and 16-week-old SHR compared to WKY rats, with moderate increases in the levels of this peptidase also in adrenal gland, brain and heart. This variability in aminopeptidase activity in SHR is thus reminiscent of that seen with aminopeptidases in other experimental models of hypertension [32,34].

Dipeptidyl peptidase IV, a membrane-bound, serine-type peptidase, cleaves a variety of peptides and proteins, the most important of which, in terms of vascular activity, are substance P, neuropeptide Y and peptide Y [17,18,50]. Although DPP IV has important roles in immunological disorders and diseases such as diabetes [18,50,51], its importance in hypertension remains unclear. Pesquero *et al.* [52] suggested that DPP IV contributed to the enhanced pulmonary kininase activity in SHR following ACE inhibition, probably following removal of the N-terminal Arg of bradykinin by APP. DPP IV occurs in rat plasma [53,54] and vascular endothelium [55], where it can hydrolyze substance P, a potent vasodilatory neuropeptide. The reduced activity of DPP IV seen in aorta of SHR compared to WKY rats suggests that there may be less degradation of substance P, and this could serve to counter the hypertension in SHR by enhancing the reduced levels of substance P present in SHR [56].

DPP IV also degrades neuropeptide Y(1-36), a potent vasoconstrictor that acts through Y1 receptors, to produce NPY(3-36), that is no longer at Y1 receptors but activates Y2 and Y5 receptors in the peripheral and central nervous systems and in blood vessels, where they may be involved in angiogenesis [55,57]. Thus, peripherally, where there is decreased vascular DPP IV activity and possibly reduced NPY(1-36) degradation, vasoconstriction may be enhanced. Increased levels of circulating neuropeptide Y could also enhance the hypertrophy caused by this peptide in rat ventricular cardiomyocytes [58,59]. In cerebral arteries, NPY(1-36) produces vasoconstriction or vasodilation depending on whether it is applied extraluminally or luminally [57]. Since the enhanced DPP IV activity seen in the brain of SHR could lead to greater local degradation of NPY(1-36) in the vessel lumen, there could be less vasodilation in these vessels. Thus, the effect of changes in DPP IV will largely depend on the vasculature involved and on the circulating levels of substance P and neuropeptide Y in this model. Curiosly, although DPP IV is abundant in kidney [18,50,51],

there was no significant difference in the renal activity of this enzyme between the two strains of rats.

The increased activity of NEP 24.11 in SHR generally agrees with the role in cardiovascular regulation suggested for this peptidase based on studies with dual ACE/NEP inhibitors [7,9]. The augmented activity could lead to greater formation of the vasodilatory peptide angiotensin-(1-7), as well as enhanced degradation of ANP and bradykinin [11]. Since angiotensin-(1-7) enhances the release of vasodilator prostaglandins and nitric oxide, and potentiates the responses to bradykinin [61], the overall effect could be to counteract the hypertension [11], despite concomitant degradation of ANP and bradykinin. The lack of difference in the protein expression of NEP 24.11 between the two strains suggests that the increase in activity in SHR may be related to alterations in the enzymatic properties (Vmax) of the peptidase rather than to its expression.

The activity of another peptidase, MEP 24.15, which also forms angiotensin-(1-7) and degrades ANP and bradykinin, was increased in lung but not in renal tissue. In addition, the activity of MEP 24.15 in aorta was lower in SHR. Although MEP 24.15 activity was increased in pulmonary tissue, this did not reflect in increased protein expression. A similar observation has been reported by Healy and Song [49] for renal APA activity in 4-week-old SHR which was 41% greater than in WKY rats but with no significant change in protein expression; significant changes in expression were seen in 8- and 16-week-old SHR in which APA activities were 51% and 68% greater than in WKY rats, respectively.

MEP 24.15/24.16 have an important role in modulating the responses to vasoactive peptides, particularly the vasodilators bradykinin and angiotensin-(1-7) [19,20]. However, the relative contribution of MEP 24.15 and MEP 24.16 to peptide metabolism may vary among cell types [62], animal species, and the vascular bed studied. As shown here, the greatest difference in MEP 24.15 activity among tissues of the two strains of rats occurred in aorta, with a significant decrease in

SHR. This could reflect in less degradation of bradykinin and less formation of angiotensin-(1-7) by the vasculature of these rats, with opposite effects in pulmonary tissue. In contrast to studies indicating a role for MEP 24.15/24.16 in cardiovascular regulation in normotensive Wistar rats [24], in rat cerebral microvasculature [63] and in rabbits [64], these peptidases do not appear to have a major role in modulating the hypertension in responses to bradykinin in SHR, as shown here. This finding could be related to the reduced vascular activity of MEP 24.15 in these rats, and agrees with a previous study indicating that MEP 24.15/24.16 have little involvement in renal wrap hypertension in rabbits [65].

In conclusion, the results of this study indicate that the activity of several peptidases are altered in SHR compared to WKY rats, although the pattern of alterations differs for each enzyme. The overall effect of these changes on cardiovascular responses in vivo will therefore be the sum of a complex series of interactions. The extent to which these changes are attributable to hypertension *per se* or to metabolic and genetic factors associated with this model remains to be determined.

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Figure legends

Fig. 1. APM, DPP IV, MEP 24.15 and NEP 24.11 activities in SHR and WKY rat tissues. The columns represent the mean + 1 S.D. of five rats each. *$p<0.05$ compared to WKY rats. AFU, arbitrary fluorescence units.

Fig. 2. APM, DPP IV and MEP 24.15 activities in SHR and WKY rat aorta. The columns represent the mean + 1 S.D. of five rats each. *$p<0.05$ compared to WKY rats. AFU, arbitrary fluorescence units.

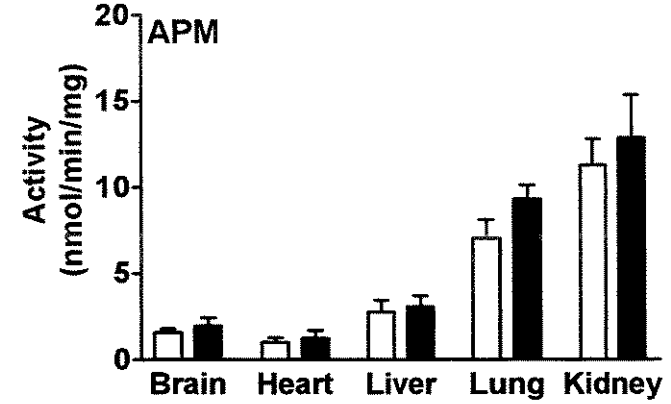
Fig. 3. Western blots for DPP IV in aorta (upper panel) and brain (lower panel) of SHR and WKY rats. Representative blots and mean densitometric values for the expression of DPP IV are shown. The columns represent the mean + 1 S.D. of five rats each. *$p<0.05$ compared to WKY rats.

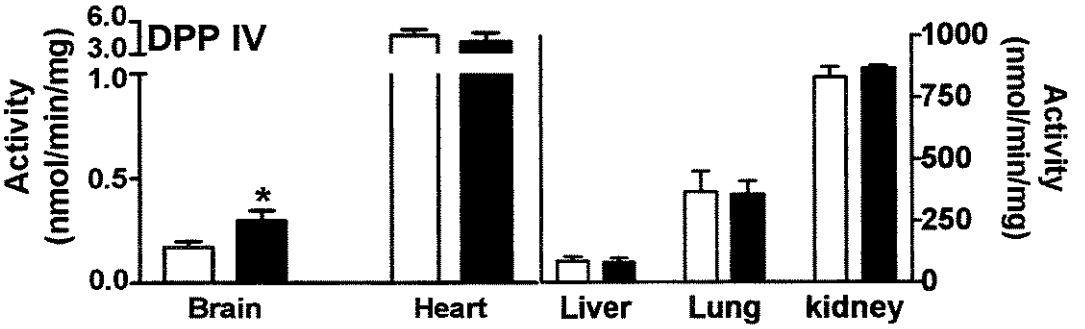
Fig. 4. Western blots for MEP 24.15 in aorta (upper panel) and brain (lower panel) of SHR and WKY rats. Representative blots and mean densitometric values for the expression of MEP 24.15 are shown. The columns represent the mean + 1 S.D. of five rats each. *$p<0.05$ compared to WKY rats.

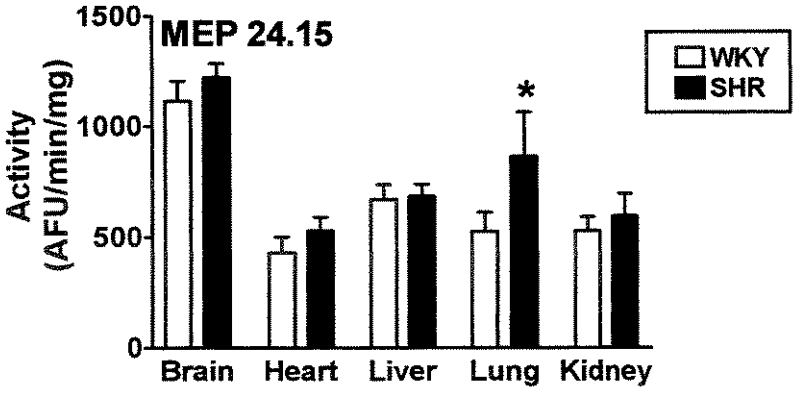
Fig. 5. Western blots for NEP 24.11 in kidney (upper panel) and lung (lower panel) of SHR and WKY rats. Representative blots and mean densitometric values for the expression of NEP 24.11 are shown. The columns represent the mean + 1 S.D. of five rats each.

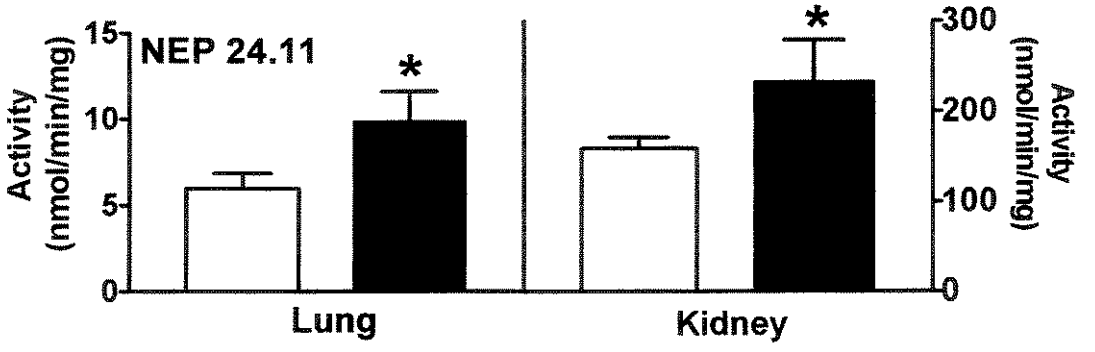
Fig. 6. Lack of involvement of MEP 24.15/24.16 in the hypotensive response to bradykinin in pentobarbital-anesthetized SHR. There were no significant differences in the responses to

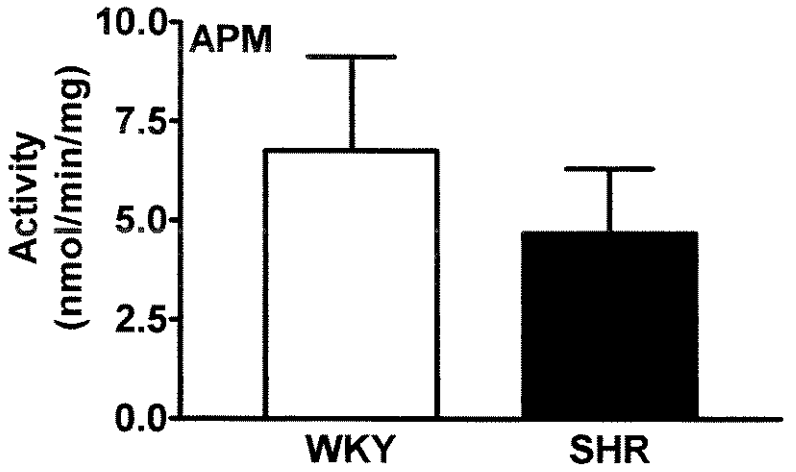
bradykinin before and after administration of the MEP 24.15/24.16 inhibitor JA2 (10 mg/kg, i.v.). Neither the vehicle solution alone (10% 2-hydroxypropyl-β-cyclodextrin in 0.9% saline) nor JA2 had a significant effect on the resting blood pressure. The columns represent the mean + 1 S.D. of six rats each.

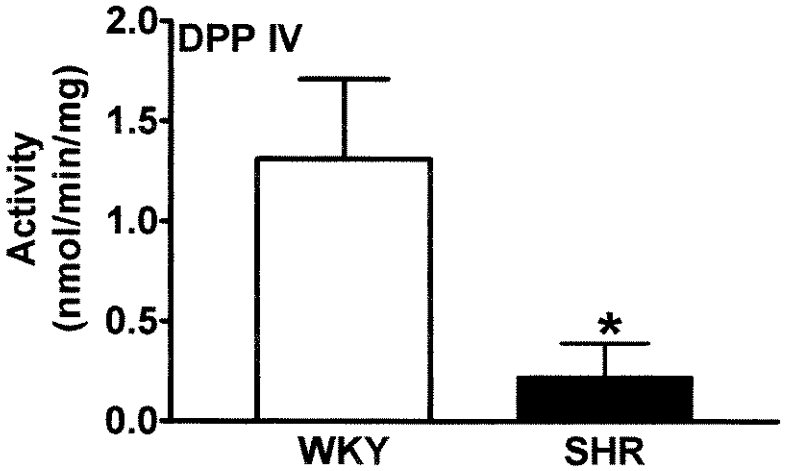

APM
Activity (nmol/min/mg)
20
15
10
5
0
Brain
Heart
Liver
Lung
Kidney

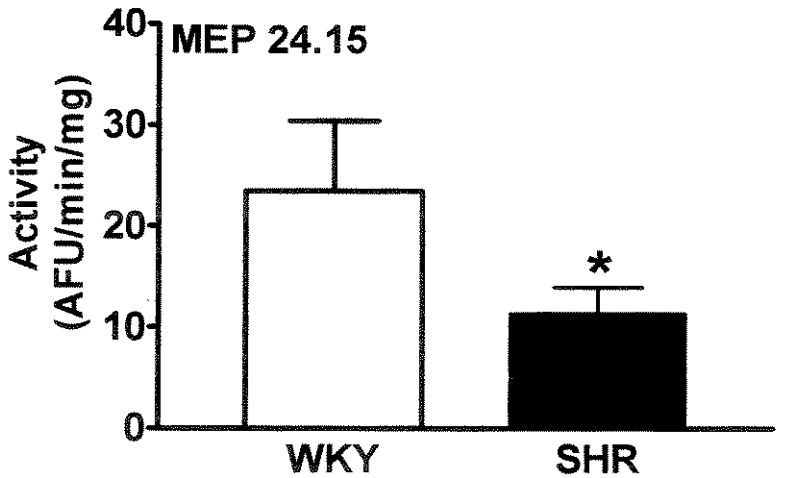

DPP IV
Activity (nmol/min/mg)
6.0
3.0
1.0
0.5
0.0
*
1000
750
500
250
0
Activity (nmol/min/mg)
Brain
Heart
Liver
Lung
kidney

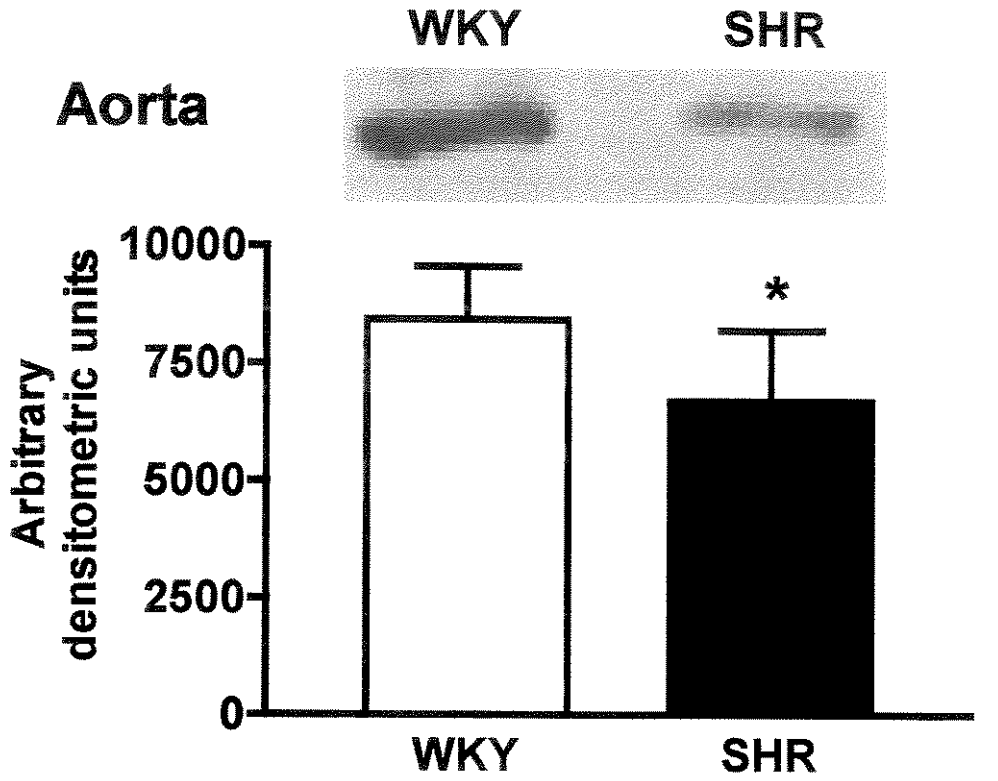

MEP 24.15
Activity (AFU/min/mg)
1500
1000
500
0
WKY
SHR
*
Brain
Heart
Liver
Lung
Kidney


NEP 24.11
Activity (nmol/min/mg)
15
10
5
0
*
*
300
200
100
0
Activity (nmol/min/mg)
Lung
Kidney


APM
Activity
(nmol/min/mg)
10.0
7.5
5.0
2.5
0.0
WKY
SHR


DPP IV
Activity
(nmol/min/mg)
2.0
1.5
1.0
0.5
0.0
*
WKY
SHR


MEP 24.15
Activity
(AFU/min/mg)
40
30
20
10
0
*
WKY
SHR


WKY
SHR
Aorta
Arbitrary densitometric units
10000
7500
5000
2500
0
*
WKY
SHR

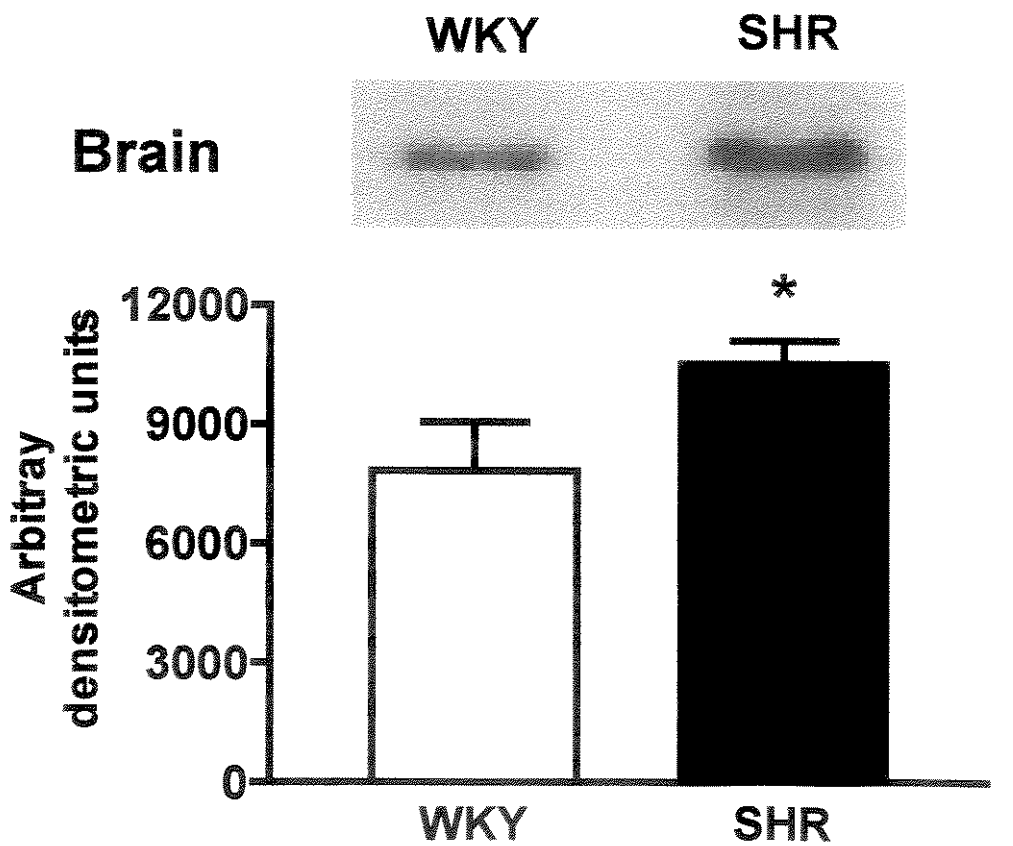

WKY
SHR
Brain
Arbitray densitometric units
12000
9000
6000
3000
0
*
WKY
SHR

Figure 3

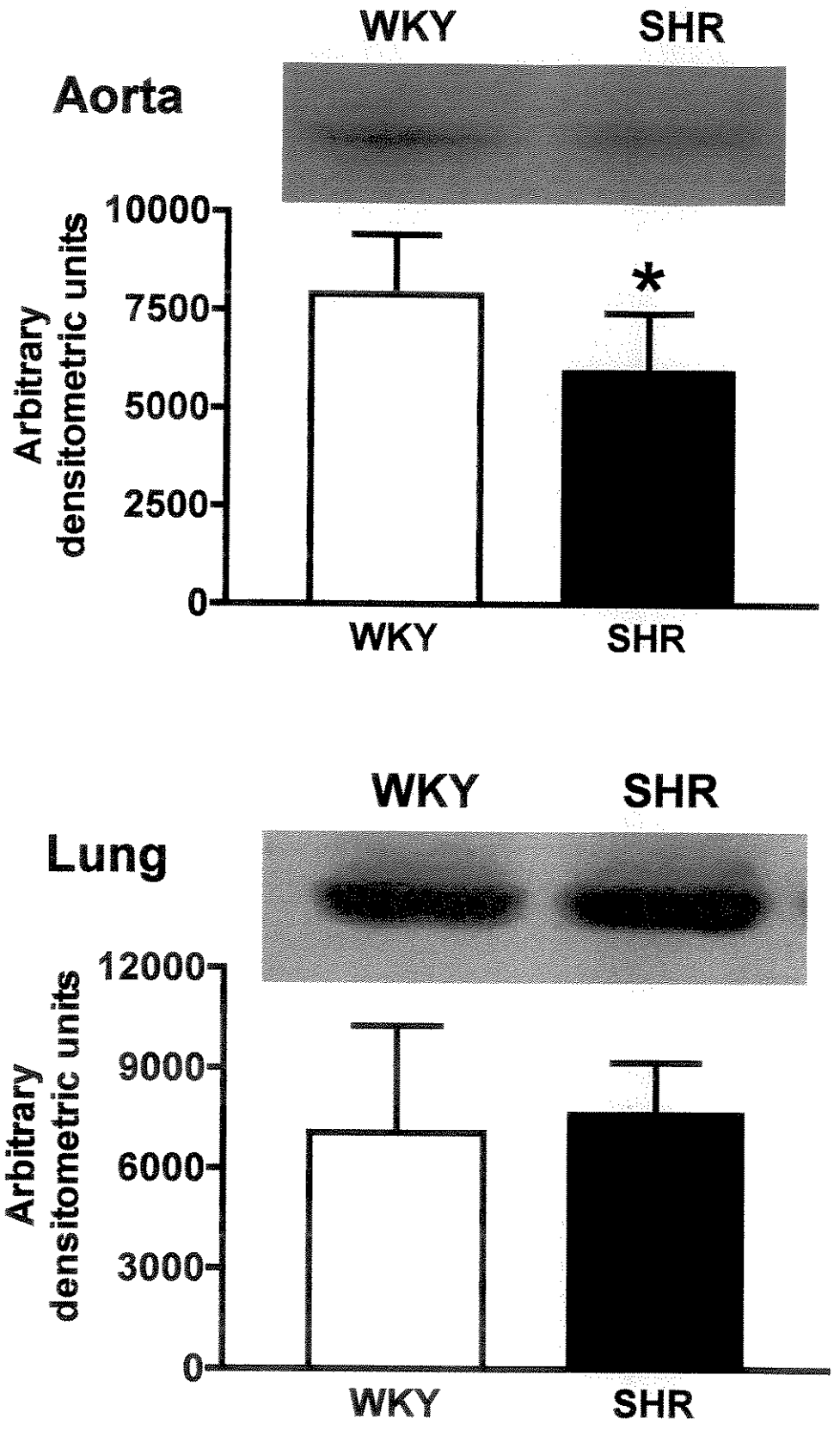
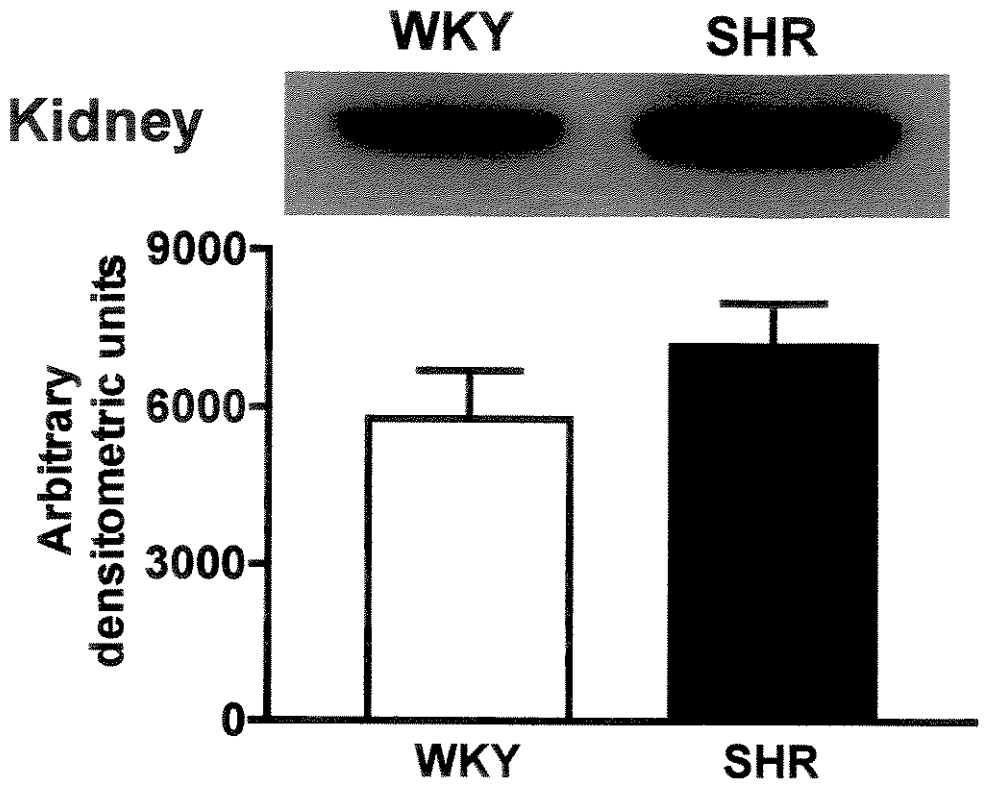

WKY
SHR
Aorta
10000
7500
5000
2500
0
Arbitrary densitometric units
*
WKY
SHR
WKY
SHR
Lung
12000
9000
6000
3000
0
Arbitrary densitometric units
WKY
SHR

Figure 4


WKY
SHR
Kidney
Arbitrary densitometric units
9000
6000
3000
0
WKY
SHR

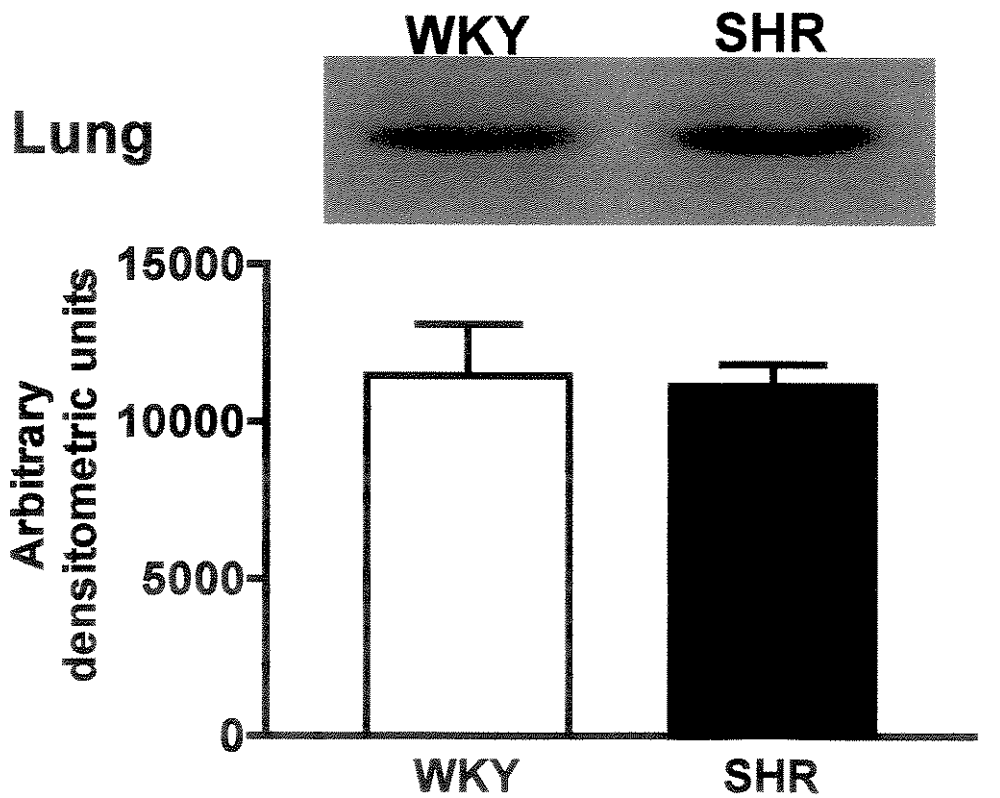

WKY
SHR
Lung
Arbitrary densitometric units
15000
10000
5000
0
WKY
SHR

Figure 5

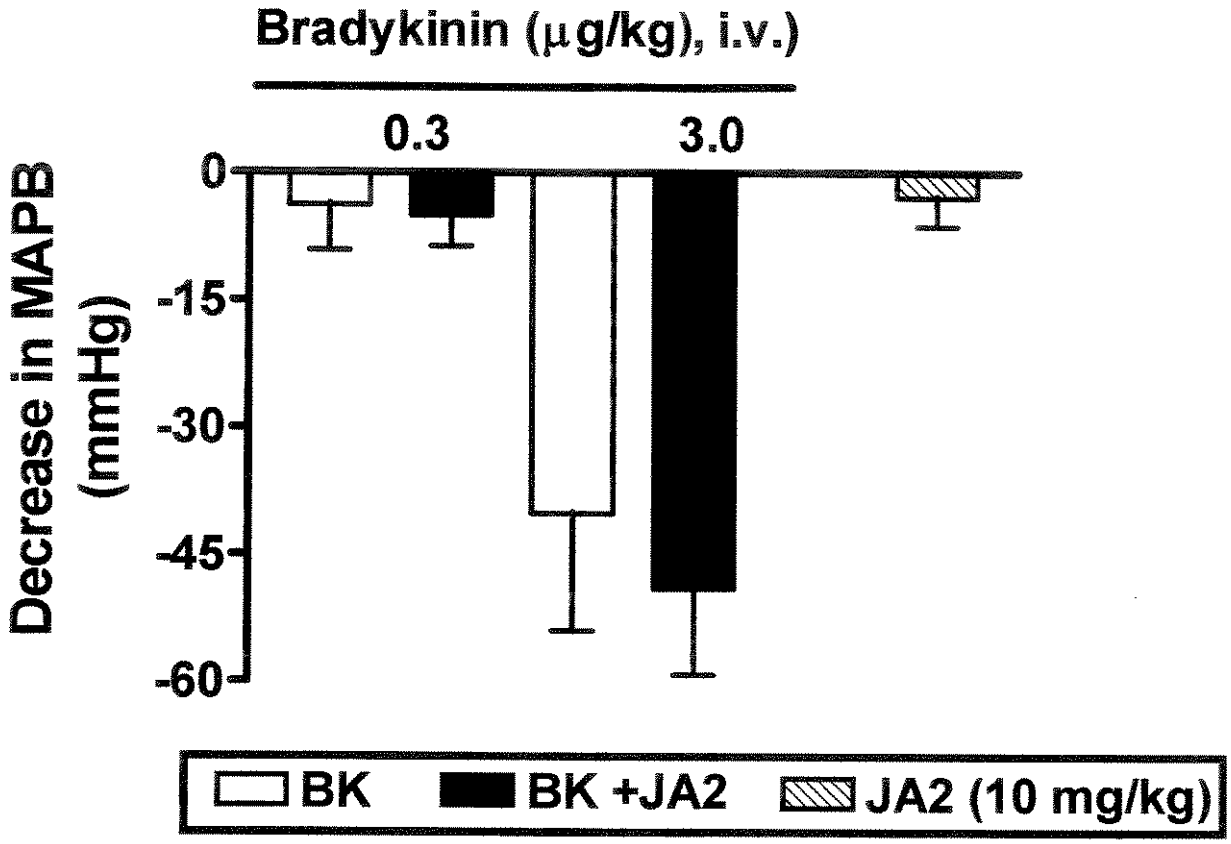

Bradykinin (μg/kg), i.v.
0.3
3.0
0
-15
-30
-45
-60
Decrease in MAPB (mmHg)
BK
BK +JA2
JA2 (10 mg/kg)

Figure 6

4. DISCUSSÃO GERAL

Neste trabalho, foram estudadas algumas peptidases envolvidas na regulação da pressão arterial em dois modelos experimentais de hipertensão: ratos tratados cronicamente com L-NAME e ratos espontaneamente hipertensos (SHR). O tratamento crônico com L-NAME promove efeitos marcantes no sistema cardiovascular, tanto a nível morfológico quanto a nível fisiológico (ZATZ e BAYLIS, 1998; JOVER e MIMRAN, 2001). Muitas das alterações observadas neste modelo resultam da ativação do SRA e da atividade da ECA, uma vez que parte delas, inclusive o aumento da resistência periférica, podem ser revertidos ou atenuados pela administração de inibidores de ECA ou antagonistas de receptores AT_1 de angiotensina (RIBEIRO *et al.,* 1992; TAKEMOTO *et al.,* 1997; USUI *et al.,* 2000; SANADA *et al.*, 2001). Já, o modelo SHR assemelha-se bastante à hipertensão humana e vem sendo amplamente empregado na pesquisa experimental. Da mesma forma que no modelo crônico de L-NAME, o SRA também exerce papel fundamental na fisiopatologia do modelo SHR, principalmente o SRA central (BERECEK, *et al.,* 1987; YANG *et al.,* 1992; TAMURA *et al.,* 1996).

Além da ECA, há outras peptidases envolvidas no controle das funções cardiovasculares. A endopeptidase neutra 24.11 (NEP 24.11) degrada peptídeos vasodilatadores como o ANP (ERDÖS e SKIDGEL, 1989) e ainda apresenta similaridades com a enzima conversora de endotelina (TURNER *et al.,* 2001; TURNER, 2003). Em vista disso, inibidores duplos de ECA e NEP 24.11 têm sido descritos na literatura (CAMPBELL, 2003; QUASCHNING *et al.,* 2003; WORTHLEY *et al.,* 2004). A aminopeptidase M (APM) degrada angiotensina III em angiotensina IV (ZINI *et al.*, 1996; REAUX *et al.,* 1999a, 2001), a dipeptidil peptidase IV (DPP IV) está envolvida na degradação da substância P, do neuropeptídeo Y e o peptídeo YY entre outros (AUGUSTYNS *et al.,* 1999), e a metaloendopeptidase 24.15 (MEP 24.15) cliva neurotensina, somatostatina, bradicinina, hormônio liberador de ganodotrofina e peptídeos opióides (SHRIMPTON e SMITH, 2000; SHRIMPTON *et al.,* 2002).

Nossos resultados demonstraram que o tratamento crônico com L-NAME por quatro semanas não afetou a atividade das quatro peptidases estudadas no

coração, cérebro, fígado, pulmão e rim, mas promoveu alterações nos níveis de APM, DPP IV e MEP 24.15 na aorta. Estes achados são semelhantes aos resultados de TAKEMOTO *et al.* (1997) e GONZALEZ *et al.* (2000), que relataram um aumento da atividade da ECA em aortas de animais tratados com L-NAME. Além disso, TAKEMOTO *et al.* (1997) demonstraram que, com exceção do coração, a atividade da ECA foi inalterada nos outros tecidos estudados (rim, fígado e pulmão). Estes resultados, em conjunto, indicam que neste modelo as alterações na atividade e expressão das peptidases variam de acordo com o tecido.

No modelo SHR o padrão de alteração para cada enzima também variou entre tecidos. Assim, não houve alterações na APM em nenhum dos tecidos estudados. Já a atividade da DPP IV foi elevada no cérebro, mas decaiu na aorta, enquanto a atividade da MEP 24.15 foi maior no pulmão, mas também decaiu na aorta. A atividade da NEP 24.11 foi elevada no pulmão e rim (a enzima foi detectada apenas nesses tecidos, assim como ocorreu no modelo L-NAME). Com exceção da NEP 24.11, as alterações na atividade destas peptidases foram acompanhadas por alterações na expressão protéica.

Há algumas possibilidades para explicar a ausência de alterações marcantes na atividade e expressão das peptidases na maioria dos tecidos estudados:

1) É possível que as principais alterações na atividade das peptidases ocorram na fase inicial do tratamento e desenvolvimento da hipertensão, sendo que após estabelecimento da mesma o organismo se adapta à nova situação e "normaliza" ou reajusta as funções celulares em um novo patamar ou nível. Esta é uma possibilidade apoiada pelo trabalho de KATOH *et al.* (1998) sobre receptores AT_1 da angiotensina II e por observações nossas sobre receptores B_2 da bradicinina (dados não publicados), que mostram aumento na expressão dos mesmos na primeira semana do tratamento, com normalização em intervalos maiores de tratamento ($\geq$2 semanas).

2) Outra possibilidade seria que, as alterações observadas estejam mais associadas a estruturas específicas de cada órgão, por exemplo vasos, em vez de

ser necessariamente uma resposta generalizada do órgão. Esta distribuição localizada poderia contribuir para a não-detecção de mudanças na atividade e expressão de peptidases nos tecidos quando se usa um homogenato total que poderia mascarar alterações locais.

3) É possível, também, que as alterações na atividade estejam relacionadas aos sítios/locais de lesões nos tecidos onde há necrose, fibrose e hipertrofia, e não á uma alteração disseminada de modo aleatória e generalizada nos órgãos. Isso estaria de acordo com os achados do TRIBULOVÁ *et al.* (2000) mostrando que ocorre um aumento na expressão da DPP IV em tecido cardíaco apenas em locais onde há lesões teciduais. Neste caso, de modo semelhante ao ítem (2), o uso de um homogenato total poderia mascarar estas alterações.

Observamos que em ratos Wistar normotensos a administração de JA2 potenciou a resposta hipotensora da bradicinina, o que está de acordo com os achados de SMITH *et al* (2000) demonstrando que a administração intravenosa de JA2 aumentou o efeito hipotensor da bradicinina em coelhos, mas não afetou a hipertensão promovida pela angiotensina I ou II. Já, nos SHR, onde a atividade de MEP 24.15 foi menor com relação aos Wistar normotensos não foi observado efeito significativo do JA2, indicando que nesse modelo, as enzimas MEP 24.15/MEP 24.16 provavelmente contribuem pouco para a modulação vascular. De modo semelhante, NORMAN *et al.* (2001) relataram que em coelhos com hipertensão do tipo "renal wrap" a adminstração intravenosa de JA2 não alterou a pressão dos animais. Estes achados indicam que o nível de atividade/expressão da MEP 24.15/MEP 24.16 e, portanto, sua relevância no controle cardiovascular, varia de acordo com o modelo e cepa de animal estudado.

A detecção de MEP 24.15 na aorta indica que a vasculatura é um sítio importante de ocorrência desta enzima. Esta conclusão está de acordo com a presença de MEP 24.15 e MEP 24.16 demonstrada em cultura de células endoteliais (NORMAN *et al.*, 2003b) e na microvasculatura cerebral (NORMAN *et al.*, 2003a). Estes dois estudos mostraram a metabolização da bradicinina por estas peptidases. Entretanto, NORMAN *et al.* (2003b) demonstraram que a contribuição relativa de MEP 24.15 e MEP 24.16 aparentemente varia de acordo

com a origem das células endoteliais usadas: a MEP 24.16 parece ser mais importante no metabolismo de bradicinina em cultura de células endoteliais de ovelha, enquanto que na linhagem endotelial EA.hy926 ambas as peptidases contribuem para o metabolismo deste peptídeo. Tal variação celular e tecidual na atividade destas peptidases poderia contribuir para diferenças nas respostas à bradicinina entre leitos vasculares. Nos ensaios enzimáticos realizados em nosso estudo, usamos 0,5 mM DTT no meio de reação, o qual seletivamente inibe a MEP 24.16 mas ativa a MEP 24.15 (RIOLI *et al.,* 1998). Assim, nossos resultados *in vitro* provavelmente refletem alterações na atividade de MEP 24.15. De modo semelhante, o antisoro usado no blotting foi específico para MEP 24.15. Na época em que os experimentos foram realizados, não havia disponível um antisoro específico para MEP 24.16 que pudesse ser usado para avaliar a expressão desta. Recentemente, conseguiu-se produzir um anti-soro anti-MEP 24.16 específico (informação do Dr. E. S. Ferro), mas ainda não foi usado para avaliar a expressão desta peptidase, nos modelos investigados aqui.

Não foi possível avaliar o papel da DPP IV, *in vivo*, nestes dois modelos devido à dificuldade encontrada em obter um inibidor estável desta peptidase para uso quando estes experimentos estavam sendo realizados.

De modo geral, as alterações na atividade peptidásica foram acompanhadas de mudanças na expressão, embora em alguns casos, isso não tenha ocorrido. Assim, a atividade da MEP 24.15 aumentou no tecido pulmonar, o mesmo ocorreu para NEP 24.11 tanto em tecido pulmonar como em renal, mas isso não refletiu em um aumento na expressão protéica no modelo SHR. Resultado similar foi observado por HEALY e SONG (1999) que relataram um aumento na atividade renal de APA que não foi acompanhado por alterações na expressão protéica em SHR com quatro semanas de idade. Por outro lado, observamos um decréscimo significativo na atividade e expressão de MEP 24.15 e DPP IV na aorta de SHR quando comparados aos WKY. Em relação a DPP IV, houve um aumento da atividade que também foi acompanhada por um aumento na expressão da enzima no cérebro de animais SHR. Já, no modelo L-NAME, houve um aumento da atividade e expressão de MEP 24.15 e DPP IV na aorta.

Pouco se sabe ainda dos mecanismos regulatórios da atividade enzimática e expressão gênica de algumas das peptidases investigadas aqui. É possível, por exemplo, que haja diferenças na sensibilidade ao NO, que é capaz de influenciar a atividade e expressão por mecanismos que dependem e independem de cGMP (PFEILSCHIFTER *et al.,* 2001). Esta variabilidade de respostas está de acordo com um estudo do ZHANG *et al.* (2003) mostrando repostas divergentes na expressão de RNAm para ANP e BNP em átrio de ratos tratados cronicamente com L-NAME durante 4 semanas. A estimulação por fatores como citocinas (REMICK e VILLARETE, 1996), a ativação de vias intracelulares de sinalização (ERK1/2, MAPK, NF-κB, p70S6K) (USUI *et al.,* 2000; SANADA *et al.,* 2001; MARTENS *et al.,* 2002; ZARAGOZA *et al.,* 2002; KNIPP *et al.,* 2004), a peroxidação de lipídeos (BAPAT *et al.,* 2002), a formação de radicais livres (KITAMOTO *et al.,* 2000), mudanças intracelulares associadas ao estresse oxidativo (HUSAIN e HAZELRIGG, 2002) e alterações na estabilidade do RNAm (EBERHARDT *et al.,* 2002; AKOOL *et al.,* 2003), bem como variações na estimulação da síntese protéica (MARTENS *et al.,* 2002) e a própria resposta hipertrófica (ZHANG *et al.,* 2003) poderiam contribuir para modular a atividade e expressão das peptidases. No caso da DPP IV e MEP 24.15, as alterações no modelo de inibição crônica de NO parecem não estar relacionadas diretamente a uma ação direta do NO ou do L-NAME sobre a atividade, o que difere, por exemplo da ECA, cuja atividade é modulada por NO (ACKERMAN *et al.,* 1998).

Em conclusão, a presente investigação demonstrou que há alterações na expressão e atividade de algumas peptidases em tecidos obtidos de ratos nos dois modelos de hipertensão estudados. Os resultados obtidos variam entre o modelo crônico de L-NAME e o modelo SHR, assim como o tecido estudado, o que sugere que as alterações observadas estejam relacionadas a diferenças bioquímicas e celulares associadas a cada modelo. Os mecanismos seletivos pelos quais algumas peptidases são afetadas e outras não, e a relação com a hipertensão *per se*, ainda precisam ser esclarecidos.

5. CONCLUSÕES

Os resultados deste trabalho permitiram concluir que:

1. O tratamento com L-NAME durante quatro semanas não afetou a atividade das peptidases estudadas em cérebro, coração, fígado, pulmão e rim. No entanto, o mesmo tratamento aumentou significativamente a atividade e expressão da DPP IV e MEP 24.15 em aortas. Estes achados sugerem que as alterações enzimáticas observadas no modelo crônico de L-NAME estão mais relacionadas ao tecido vascular, e que resultam em parte de uma maior expressão protéica.

2. A potencialização do efeito hipotensor da bradicinina por JA2 sugere o envolvimento de MEP 24.15/24.16 na regulação da pressão arterial em ratos Wistar normotensos.

3. Embora o SRA seja a principal via afetada no modelo crônico de L-NAME, outras peptidases também são influenciadas.

4. No modelo SHR houve alterações nas peptidases DPP IV, MEP 24.15 e NEP 24.11 na aorta, cérebro, pulmão e rim. Essas alterações variaram de acordo com o tecido e peptidase estudados, sendo, em alguns casos, acompanhados por alterações na expressão. Assim, as alterações nas atividades peptidásicas são mais generalizadas de que no modelo L-NAME.

5. A administração de JA2 não afetou a resposta à bradicinina nos ratos SHR. Este ausência de efeito pode estar relacionada à atividade reduzida de MEP 24.15 observada no tecido aórtico destes animais.

6. A atividade e expressão das peptidases investigadas variam de acordo com o modelo de hipertensão, o que sugere o envolvimento de mecanismos bioquímicos e celulares peculiares a cada modelo. Embora pareça que não há uma relação estreita entre estas alterações e a hipertensão *per se*, experimentos adicionais seriam necessários para esclarecer este ponto.

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7. APÊNDICE

Durante a vigência do doutorado foram desenvolvidos dois trabalhos paralelos em anexo:

1. O trabalho intitulado: "Novel natural peptide substrates for endopeptidase 24.15, neurolysin and angiotensin-converting enzyme", foi realizado em colaboração com o Dr. Emer S. Ferro do Departamento de Biologia Celular e Desenvolvimento, Instituto de Ciências Biomédicas, Universidade de São Paulo (USP).

2. Já, o outro trabalho intitulado: "Biological activities of a lectin from *Bothrops jararacussu* snake venom", foi desenvolvido em nosso laboratório.

Artigo publicado

"Novel natural peptide substrates for endopeptidase 24.15, neurolysin, and angiotensin-converting enzyme"

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Novel Natural Peptide Substrates for Endopeptidase 24.15, Neurolysin, and Angiotensin-converting Enzyme*

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Endopeptidase 24.15 (EC 3.4.24.15; ep24.15), neurolysin (EC 3.4.24.16; ep24.16), and angiotensin-converting enzyme (EC 3.4.15.1; ACE) are metallopeptidases involved in neuropeptide metabolism in vertebrates. Using catalytically inactive forms of ep24.15 and ep24.16, we have identified new peptide substrates for these enzymes. The enzymatic activity of ep24.15 and ep24.16 was inactivated by site-directed mutagenesis of amino acid residues within their conserved HEXXH motifs, without disturbing their secondary structure or peptide binding ability, as shown by circular dichroism and binding assays. Fifteen of the peptides isolated were sequenced by electrospray ionization tandem mass spectrometry and shared homology with fragments of intracellular proteins such as hemoglobin. Three of these peptides (PVNFKFLSH, VVYPWTQRY, and LVVYPWTQRY) were synthesized and shown to interact with ep24.15, ep24.16, and ACE, with K_i values ranging from 1.86 to 27.76 μM. The hemoglobin α-chain fragment PVNFKFLSH, which we have named *hemopressin*, produced dose-dependent hypotension in anesthetized rats, starting at 0.001 μg/kg. The hypotensive effect of the peptide was potentiated by enalapril only at the lowest peptide dose. These results suggest a role for hemopressin as a vasoactive substance *in vivo*. The identification of these putative intracellular substrates for ep24.15 and ep24.16 is an important step toward the elucidation of the role of these enzymes within cells.

Endopeptidase EC 3.4.24.15 (ep24.15; also referred to as thimet oligopeptidase) and endopeptidase EC 3.4.24.16 (ep24.16; also referred to as neurolysin) were initially detected in and purified from rat brain homogenates (1, 2). The cloned rat brain ep24.16 (3) showed 80% similarity and 63% identity with the previously cloned rat testis ep24.15 (4). Both peptidases share most of their natural substrates, including bradykinin, neurotensin, opioids, angiotensin I, and gonadotrophin-releasing hormone (5, 6). All of these natural substrates are hydrolyzed at the same peptide bond and at similar rates, except for neurotensin, which is hydrolyzed by ep24.15 and ep24.16 by cleavage of its Arg^8–Arg^9 and Pro^{10}–Tyr^{11} bonds, respectively (7).

Functional studies have suggested that ep24.15 and ep24.16 inactivate neuropeptides inside and outside the central nervous system. The central administration of *Z*-(Leu,Asp)Phe-$\psi(PO_2CH_2)$(Leu,Asp)Ala-Lys-Met, a fully specific endopeptidase ep24.15 inhibitor (8), prolongs the forepaw licking latency of mice in the hot plate test and following the injection of submaximally active doses of neurotensin (9). Likewise, the intracerebroventricular administration of Pro-Phe-$\psi(PO_2CH_2)$-Leu-Pro-NH_2, a selective ep24.16 inhibitor (10), significantly increases the neurotensin-induced antinociception of mice in the hot plate test (11).

Outside the central nervous system, *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate, an inhibitor of both ep24.15 and ep24.16, potentiates bradykinin-induced hypotension, which suggests that one or both of these peptidases participate in the metabolism of bradykinin (12). In macrophages, CFP-AAF-pAb, a mixed inhibitor of ep24.15 and ep24.16 (7), reduces antigen presentation through the major histocompatibility complex class I (MHC-I)[1] but not through MHC-II (12). Conversely, liposome-mediated introduction of ep24.15 into macrophages stimulates the antigen presentation of MHC-I, but not that of MHC-II. The observation that ep24.15 can degrade or bind to several MHC-I antigenic peptides (13, 14), which are 8–11 amino acid fragments generated in the cytoplasm by proteasomes (15, 16), raises the possibility that ep24.15 and ep24.16 participate in the intracellular metabolism of peptides. The nature of such peptides is unknown.

Angiotensin I-converting enzyme (ACE; peptidyldipeptidase

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[1] The abbreviations used are: MHC, major histocompatibility complex; ACE, angiotensin I-converting enzyme; HPLC, high performance liquid chromatography; QFS, quenched fluorescent substrate; BK, branykinin; Dyn, dynorphin A1–13; Aib, α-aminoisobutyric acid; NO, nitric oxide.

A) is a zinc metallopeptidase that cleaves the COOH-terminal dipeptide from angiotensin I to produce the potent vasopressor octapeptide angiotensin II (17) and inactivates bradykinin by the sequential removal of two COOH-terminal dipeptides (18). In addition to these two main physiological substrates, which are involved in blood pressure regulation and water and salt metabolism, ACE also cleaves COOH-terminal dipeptides from various oligopeptides with a free COOH terminus. ACE has also been implicated in a range of physiological processes unrelated to blood pressure regulation, such as immunity, reproduction, and neuropeptide regulation, based on its localization and/or the *in vitro* cleavage of a range of biologically active peptides. The role of ACE in blood pressure control and water and salt metabolism has been defined mainly by the use of highly specific ACE inhibitors (19). These inhibitors are effective in the treatment of hypertension, congestive heart failure, and diabetic nephropathy (20–22). Moreover, ACE has recently been implicated in the hydrolysis *in vivo* of the tetrapeptide Ac-Ser-Asp-Lys-Pro, which modulates hematopoietic stem cell proliferation by preventing their recruitment into the S phase (23). The acute administration of captopril, an ACE inhibitor, produces a 7-fold increase in the plasma concentration of Ac-Ser-Asp-Lys-Pro in normal volunteers, thus demonstrating the importance of ACE in the metabolism of this substrate (24).

In this study, we show that ep24.15 and ep24.16, when catalytically inactivated by site-directed mutagenesis of amino acid residues within their HEXXH motifs, can be used to identify new endogenous peptides present in crude peptide extracts prepared from rat tissues. The ep24.15 or ep24.16 enzyme-bound peptides were isolated and many of them fully sequenced by electrospray ionization tandem mass spectrometry. Based on these sequences, synthetic peptides were prepared and shown to interact strongly with ep24.15, ep24.16, and ACE. One of the peptides identified here (PVNFKFLSH), derived from the α_1 chain of hemoglobin, was among the best natural substrates identified so far for these enzymes, and caused dose-dependent hypotension in rats. This peptide, which we have named *hemopressin*, may have a role in blood pressure regulation and in cardiovascular disease.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Protein Expression—A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce a specific point mutation in the wild type ep24.15 or ep24.16 cDNA cloned into the expression vector pGEX4t-2 (7). Oligonucleotide primers were synthesized with mismatches coding for alanine, based on prokaryotic codon usage rules to obviate the use of rare codons that could hinder subsequent protein expression. Point mutations were specified as H473A, E474A, H477A, and E502A for ep24.15, and H474A, E475A, H478A, and E503A for ep24.16. PCR was done in a 50-μl mixture using 50 ng of template plasmid DNA, 14 pmol of each primer, 10 nmol of dNTPs, and 2.5 units of *turbo Pfu* DNA polymerase (Stratagene) in 0.5× *Pfu* polymerase reaction buffer. The thermocycler was programmed for an initial denaturation at 95 °C for 1 min followed by 16 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 15 min, with a final incubation at 72 °C for 10 min. One microliter (20 units) of *Dpn*I (New England Biolabs) was added to the sample (50 μl) and incubated at 37 °C for 16 h. The sample was then denatured at 65 °C for 30 min. Two microliters of the final sample were used to transform competent *Escherichia coli* XL1-blue cells by electroporation. Putative positive colonies were confirmed by double-stranded template dideoxy sequencing (25). Expression and purification of the wild type or mutant proteins for biochemical characterization were done as described (7), with all enzymes stored at −80 °C for subsequent analysis.

SDS-PAGE—The homogeneity of the recombinant enzyme preparations was assessed by electrophoresis under reducing conditions in 8% polyacrylamide gels containing SDS-PAGE, as described previously (26). Protein bands were detected by staining the gels with Coomassie Brilliant Blue R-250 (Bio-Rad).

DNA Sequencing—DNA was sequenced using a multicapillary MegaBace1000 sequencer, according to the protocol supplied with the DYEnamic ET dye terminator cycle sequencing kit (Amersham Biosciences).

Peptide Synthesis—Peptides were synthesized by the Resgen-Invitrogen Corporation using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry.

Peptide Extract from Rat Tissues—A crude peptide extract from rat brain or spleen was prepared as previously described (27). Briefly, male Wistar rats were killed and the brain and spleen were removed and rapidly frozen in liquid nitrogen prior to storage at −80 °C. Tissues from five rats were added to boiling 0.1 M acetic acid and homogenized (Polytron, Brinkmann). The tissue homogenates were boiled for 10 min, centrifuged at 50,000 × *g* for 30 min at 4 °C, and the supernatant was filtered through a Millipore centrifugal filter unit with a NMCO of 5,000. The filtrate was adjusted to pH 7.4 with 1 M Tris-HCl (pH 7.4) and then used in the experiments described below.

Enzyme-Peptide Binding Assay—Enzyme (1–5 nmol)-peptide complexes were produced by incubating a specific synthetic peptide or the peptide extract with catalytically inactive ep24.15 or ep24.16 in 200 μl of buffer (25 mM Tris-HCl, pH 7.5, containing 125 mM NaCl and 0.1% of bovine serum albumin) for 30 min at room temperature. At the end of this period, the reaction mixture was layered onto a dried Sephadex G-25 column (previously washed and equilibrated with Tris-buffered saline followed by centrifugation to remove the buffer) and centrifuged at 1000 × *g* for 2 min. The flow-through (~200 μl) was collected and the peptide content analyzed by high performance liquid chromatography (HPLC) using a Chromolith performance column (4.6 mm × 100 mm; Merck), with a linear gradient of 5–35% acetonitrile in 0.1% trifluoroacetic acid, for 20 min, and at a flow rate of 1 ml/min, as previously described (7). Control experiments were done by: (i) adding an excess of dynorphin A_{1-13} (30 μM) to the reaction mixture as a specific competitive inhibitor for ep24.15 and ep24.16, (ii) performing the assay in the presence of wild type active ep24.15 and ep24.16, and (iii) preforming the assay in the absence of ep24.15 and ep24.16 (reaction mixture containing only 0.1% of bovine serum albumin).

Peptide Sequencing by ESI-MS/MS—Peptides were sequenced by positive nano-electrospray ionization (nano-ESI+) using peptide-containing aliquots collected during HPLC. Typical conditions were a capillary voltage of 1 kV, a cone voltage of 30 V, and a desolvation gas temperature of 100 °C. The protonated peptides were subjected to collision-induced dissociation with argon in the 15–45 eV collision energy range. All of the mass spectrometry experiments were done with a Q-TOF mass spectrometer (Micromass, UK) in Qq-orthogonal time-of-flight configuration. Peptide sequences were determined manually from the ESI-MS/MS product ion mass spectra with the help of the PepSeq software (Micromass).

Determination of the Peptide Bonds Cleaved—The peptide bonds cleaved were identified by isolating the fragments by HPLC followed by ESI-MS/MS mass spectrometry sequencing, as described above.

Peptide Sequence Homologies—To identify the putative protein precursors of the peptides sequenced by ESI-MS/MS, a protein data base (www.ncbi.nlm.nih.gov/blast) was searched for short, nearly exact matches (rodentia origin), as previously described (28). When the perfect match for a given peptide was not found in a large protein sequence, more than one putative protein precursor containing part of the identified peptide was listed.

Enzyme Activity Assay and Determination of Kinetic Parameters—The enzymatic activity of wild type and mutant ep24.15 and ep24.16 was determined in duplicate in a continuous assay using the quenched fluorescent substrate (QFS) (7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-dLys-(2,4-dinitrophenyl), as previously described (29, 30). ACE (Sigma) enzymatic activity was measured similarly using the internally quenched fluorescent peptide Abz-FRK(2,4-dinitrophenol)P-OH (31). The relative inhibition constants (K_i) of the new synthesized peptides were determined in parallel with well known substrates or competitive inhibitors as a reference. The following equations were used to calculate the K_i values: $K_i = K_{i,app}/(1 + [S]/K_m)$, where [S] = molar concentration of the substrate, K_m = Michaelis-Menten constant, and $K_{i,app}$ = apparent inhibition constant, assuming [S] = K_m (10 μM) (26). The $K_{i,app}$ was calculated using the equation, $V_o/V_i = 1 + [I]/K_{i,app}$, where V_o = velocity of hydrolysis without the inhibitor, V_i = velocity of hydrolysis in the presence of the inhibitor, and [I] = molar inhibitor concentration. In a plot of $(V_o/V_i) - 1$ *versus* [I], the slope is $1/K_{i,app}$. To determine the K_i values, five solutions with synthetic peptide concentrations ranging from 0.1 to 100 μM were used to construct the graph $(V_o/V_i) - 1$ *versus* [I]. The relative hydrolysis ratio was determined using peptides at a concentration of 100 μM, under zero-order kinetics, with less than 10% of the substrate consumed by the end of the incubation period, which

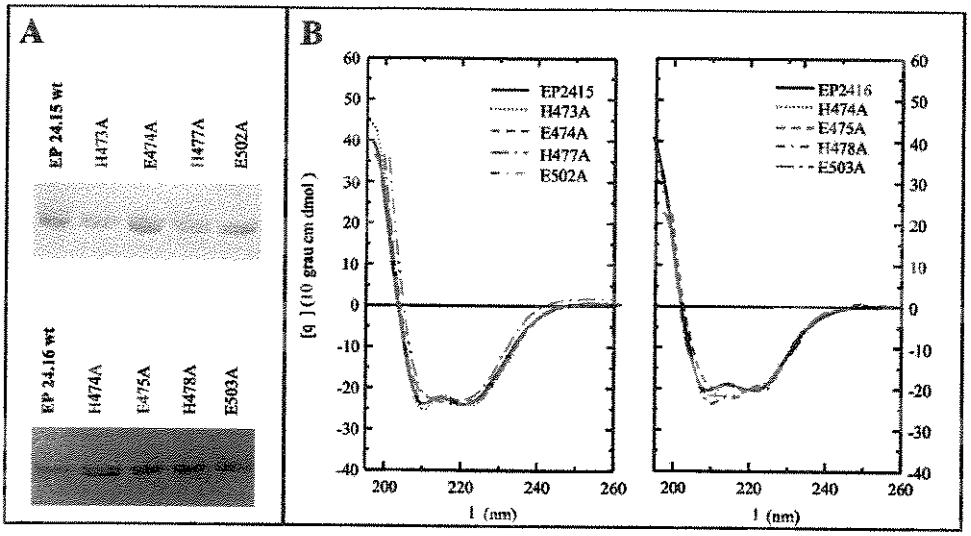

FIG. 1. **SDS-PAGE and circular dichroism spectra of wild type and mutated ep24.15 and ep24.16.** *A*, SDS-PAGE electrophoresis followed by Coomassie Blue staining showing the homogeneity of the wild type and mutated ep24.15 and ep24.16 enzymes (15 μg each) obtained after a single-step affinity chromatography on a Sepharose-glutathione *S*-transferase column. *B*, CD spectra were obtained using a Jasco model 720 spectrometer at 0.5-nm intervals in the wavelength range of 190 to 260 nm. The settings used were a resolution of 0.5 nm, a response time of 0.5 s, a scan speed of 10 or 20 nm/min (4 or 5 scans), a cell path length of 0.01 or 0.02 cm, and a temperature of 20–22 °C. The samples were prepared in 10 mM Tris-HCl (pH 7.4).

varied from 30 min to 2 h. The enzyme concentration varied from 5 to 50 ng/assay. All assays were done in triplicate.

Circular Dichroism (CD)—The secondary structure of selected mutants displaying a substantial decrease in catalytic and inhibitor binding capacity was examined by CD spectroscopy using a Jasco 720 spectropolarimeter. The instrument calibration was verified using an aqueous solution of d_{10}-camphorsulfonic acid, and the CD spectra were collected in the wavelength range of 190 to 260 nm at 0.5-nm intervals, with a resolution of 0.5 nm, a response time of 0.5 s, a scan speed of 10 or 20 nm/min for 4 or 5 scans, a cell path length of 0.01 or 0.02 cm, and a temperature of 20–22 °C. Samples were prepared in 10 mM Tris-HCl (pH 7.4). Secondary structure estimation of the proteins was done using the SELCON3 algorithm (27).

Protein Concentration—For the CD experiments, protein concentrations were determined as described by Gill and von Hippel (33). For all other purposes, protein concentrations were determined by the Bradford assay (34) using bovine serum albumin as standard.

Action of Selected Peptides on Blood Pressure in Anesthetized Rats—To examine the action of peptides PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL on blood pressure, male Wistar rats (~200 g) were anesthetized with sodium pentobarbital (>60 mg/kg, intraperitoneal; Hypnol™, Cristália, Itapira, SP, Brazil) and placed under a heating lamp to maintain body temperature. The trachea was cannulated to facilitate breathing, and the left carotid artery and left femoral vein were cannulated with polyethylene tubing for the measurement of arterial blood pressure and drug/peptide administration, respectively. The cannulas were kept patent with heparinized 0.9% (w/v) saline. The arterial pressure was recorded continuously via a pressure transducer (Abbott, Chicago, IL) coupled to a computer-controlled data acquisition system (Transonics Systems, Inc., Ithaca, NY). The experiments were initiated after at least 15 min for stabilization. Bradykinin (BK), angiotensin II, and peptides (PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL) were dissolved and administered in 0.9% saline. For PVNFKFLSH, the doses tested ranged from 0.001 to 10 μg/kg, whereas for LVVYPWTQRY and FDLTADWPL, only two doses (10 and 100 μg/kg) were examined. The order of dose administration was randomized in all experiments. The responsiveness of the preparations was assessed by administering a single dose of BK (3 μg/kg) and angiotensin II (3 μg/kg) before peptides PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL, and then at the end of the experiment to assess whether there was any alteration in the response to these two agonists. In separate experiments, enalapril (2 mg/kg, intravenously) was given 10–15 min before administration of the lowest doses (0.001, 0.01, and 0.1 μg/kg) of PVNFKFLSH to assess the influence of ACE inhibition on the action of this peptide. In all cases, bolus intravenous injections (100 μl) of peptides were washed in a further 100 μl of saline. The animal protocols and procedures described here were done in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the general principles for the care and use of animals established by the Brazilian College for Animal Experimentation (COBEA).

Statistical Analysis—The blood pressure changes were expressed as the mean ± S.E. of the peak changes in mean arterial blood pressure (in mm Hg) relative to the values recorded immediately prior to peptide administration. Differences between doses and treatments were compared using Student's *t* test or analysis of variance followed by the Tukey test, as appropriate. A value of $p < 0.05$ indicated significance.

RESULTS

Site-directed mutagenesis of the cDNA encoding rat testis ep24.15 and pig liver ep24.16 was used to prepare mutants in which the histidine and glutamic acid residues of the HEXXH motif conserved within an active site α-helix were genetically substituted. Two additional glutamate residues carboxyl to the HEXXH motif, Glu^{502} in ep24.15 and Glu^{503} in ep24.16, were also mutated. The wild-type ep24.15 and ep24.16 have previously been expressed in DH5α *E. coli* in a catalytically active form that resembles the proteins isolated from mammalian tissue (7). Isopropyl-1-thio-β-D-galactopyranoside induction of transformed DH5α *E. coli* triggers a time-dependent overexpression of specific proteins, the apparent molecular weight of which corresponds to the calculated mass of ep24.15 or ep24.16 fused with glutathione *S*-transferase; the maximal production of the fusion proteins similarly reached a plateau by 4 h (data not shown). Proteolytic removal of glutathione *S*-transferase and subsequent purification of the recombinant proteins allowed the recovery of apparently homogenous peptidases based on SDS-PAGE analysis (Fig. 1A). The production yield (~0.5 mg/liter of culture) was similar for all expressed proteins, suggesting that mutation of the above mentioned amino acid residues did not affect the relative levels of ep24.15 or ep24.16 expression in DH5α *E. coli*.

To ensure that the mutated ep24.15 and ep24.16 had not lost enzymatic activity as a result of gross structural alterations during mutagenesis and subsequent protein expression, the secondary structures of these enzymes were compared with those of the catalytically active wild-type proteins. The CD spectra suggested that both ep24.15 and ep24.16 had a typical α-helix secondary structure (Fig. 1*B*) that was not significantly modified by any of the mutations that inactivated the catalytic

TABLE I
Deconvolution of the CD spectra shown on Fig. 1B

Secondary structure estimation of the proteins was performed using data in the wavelength range of 190–260 nm using CONTIN algorithm (32). Protein was quantified according to the method described by Gill and von Hippel (33). The values are the mean ± S.E. of three determinations.

	Total α-helix	Total strand	Turns	Unordered
EP24.15 wt[a]	0.42 ± 0.05	0.16 ± 0.04	0.16 ± 0.03	0.27 ± 0.05
H473A	0.40 ± 0.05	0.20 ± 0.04	0.15 ± 0.03	0.26 ± 0.05
E474A	0.41 ± 0.05	0.20 ± 0.04	0.15 ± 0.03	0.26 ± 0.05
H477A	0.39 ± 0.05	0.16 ± 0.04	0.20 ± 0.03	0.26 ± 0.05
E502A	0.41 ± 0.05	0.16 ± 0.04	0.17 ± 0.03	0.25 ± 0.05
EP24.16 wt	0.47 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.26 ± 0.02
H474A	0.44 ± 0.02	0.14 ± 0.01	0.14 ± 0.01	0.27 ± 0.02
E475A	0.47 ± 0.02	0.12 ± 0.01	0.13 ± 0.01	0.28 ± 0.02
H478A	0.44 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.27 ± 0.02
E503A	0.44 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.28 ± 0.02
E510A	0.44 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.27 ± 0.02

[a] wt = wild type

TABLE II
Quantitative measurements of the peak area of dynorphin A_{1-13} (Dyn) and BK peptides separated by gel filtration, in the absence or presence of inactive ep24.15 (E474A) or ep24.16 (E475A)

The average of the two peaks areas obtained after gel filtration of the peptides in the absence of the inactive enzymes was taken as control (zero). Observe the proportional increment of the peptide peaks eluted in the presence of different concentrations of the inactive enzymes.

	Peak area		Average of first and second run	Resulting area relative to control experiments
	First run	Second run		
Dyn	20,605	19,657	20,131	Zero (control)
Dyn + E474A, 1 nM	51,399	48,460	49,929	29,798
Dyn + E474A, 5 nM	105,235	101,016	103,125	82,994
Dyn + E475A, 1 nM	59,854	59,880	59,867	39,736
Dyn + E475A, 5 nM	72,578	68,549	70,563	50,432
Bk	108,765	114,469	111,615	Zero (control)
Bk + E474A, 1 nM	132,327	137,971	135,149	23,534
Bk + E474A, 5 nM	148,450	157,923	153,186	41,571
Bk + E475A, 1 nM	150,060	153,752	151,906	40,291
Bk + E475A, 5 nM	164,417	161,976	163,196	51,581

activity (Table I). The effects of individual mutations on the catalytic activity of ep24.15 and ep24.16 were assessed using a QFS. As expected, complete ablation of enzymatic activity was observed when point mutations were made for H473A, E474A, H477A, and E502A in ep24.15, or H474A, E475A, H478A, and E503A in ep24.16.

Following the structural characterization of the wild type and mutant ep24.15 and ep24.16, we examined whether the inactive enzymes would bind to bioactive peptides such as dynorphin A_{1-13} and bradykinin. Initial binding assays done with ^{125}I-dynorphin A_{1-13} suggested a similar ability of all inactive enzymes to bind this peptide (data not shown). Therefore, the E474A and E475A mutants were selected for further experiments, as this specific glutamic acid is believed to be involved directly in substrate catalysis, whereas the other residues are involved in zinc ion coordination (19). To further characterize the ability of the E474A and E475A mutants to bind bioactive peptides, such as bradykinin and dynorphin A_{1-13}, enzyme-peptide complexes were allowed to form in solution and the excess of unbound peptide was removed by gel filtration. The resulting complexes were analyzed by HPLC. The results from these assays (Table II) supported the idea that catalytically inactive ep24.15 (E474A) and ep24.16 (E475A) retained the ability to bind bioactive peptides such as bradykinin and dynorphin A_{1-13}.

To assess the usefulness of these mutants for isolating new substrates for ep24.15 and ep24.16, the E474A and E475A mutants were incubated with a rat brain peptide extract and the enzyme-peptide complexes were separated from the excess of free peptides by gel filtration and then subjected to HPLC. The presence of either E474A or E475A in the incubation with peptide extract was critical for obtaining increasing amounts of specific peptide peaks, as shown in the HPLC chromatograms (Fig. 2). In control experiments without the inactive enzymes to complex and arrest the peptides, only small peaks were seen (Fig. 2). For the moment, it is unclear whether these smaller peaks represent lower amounts of peptides bound to the inactive enzymes. Equivalent experiments done with active ep24.15 or ep24.16 produced chromatograms without a significant increase in the peptide peaks, when compared with those obtained using the inactive enzymes (data not shown). Hence, catalytic inactivation before incubation with the crude peptide extracts was important to recover larger amounts of the putative natural substrates of these enzymes. To determine whether binding of the peptides by the inactive enzymes involved a specific interaction, dynorphin A_{1-13} (30 μM), a potent competitive inhibitor of both ep24.15 and ep24.16, was added to the peptide extracts. Dynorphin A_{1-13} clearly reduced the number of peptide peaks observed in the chromatograms (Fig. 2, *A* and *B*). Thus, it is reasonable to conclude that the above procedures were appropriate for identifying peptides that interact specifically with ep24.15 and ep24.16.

To identify the peptides that bound to inactive ep24.15 and ep24.16, the peptide peaks were collected manually during HPLC (Fig. 2*C*) and were further analyzed by nano-ESI-MS/MS. A representative deconvoluted ESI-MS/MS product-ion mass spectrum, which allowed the complete sequencing of peptide PVNFKFLSH, is shown in Fig. 3. The isotopic cluster separation of 0.5 mass/charge ratio (*m/z*) units of the precursor ion and its *m/z* reveals that doubly charged, doubly protonated peptide molecules of mass 1088.92 (M + H) were formed by ESI ionization and mass selected for MS/MS sequencing. The se-

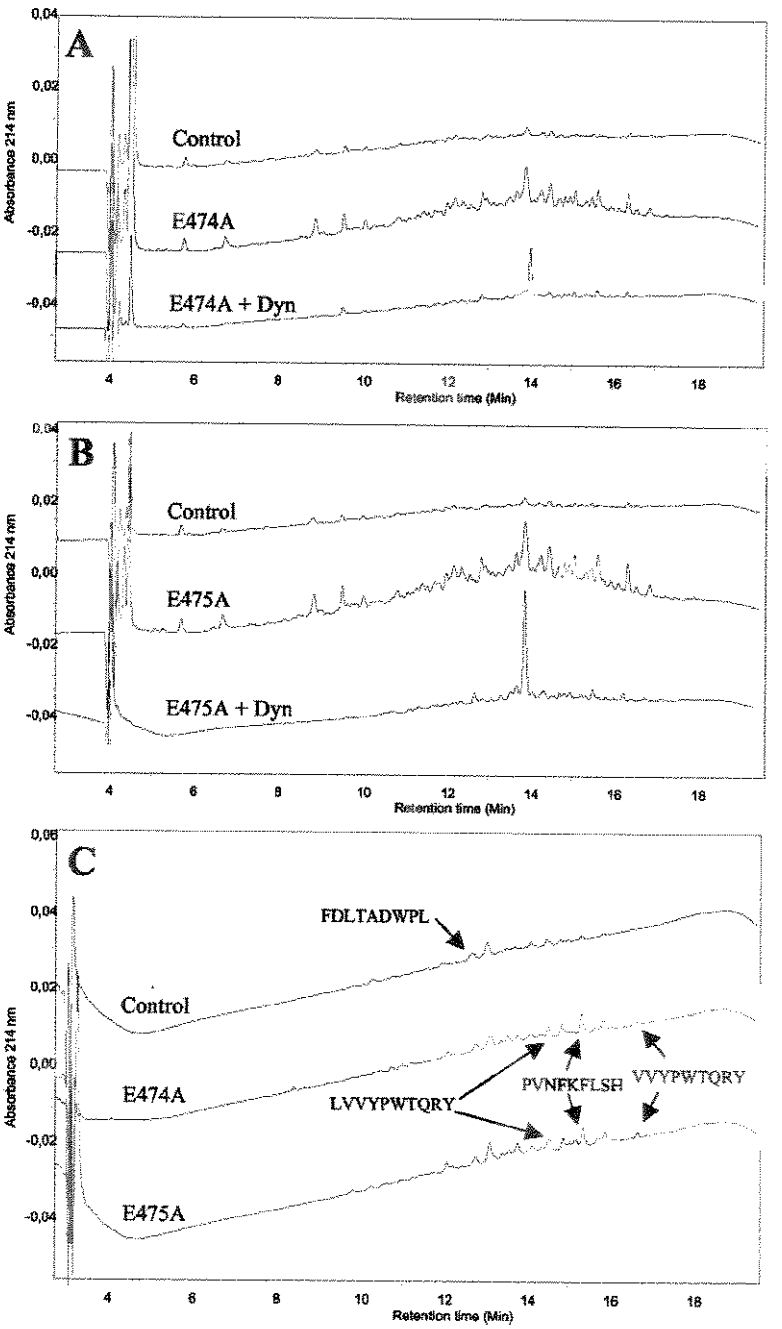

FIG. 2. **HPLC chromatograms showing the rat brain peptides arrested by inactive ep24.15 and ep24.16.** Following incubation of the rat brain peptide extract with inactive ep24.15 (E474A) or ep24.16 (E475A), the enzyme-peptide complexes were eluted through a Sephadex G-25 column and analyzed by HPLC, as detailed under "Experimental Procedures." Addition of the competitive inhibitor dynorphin A_{1-13} (*Dyn*; 30 μM) significantly reduced the amount of rat brain peptides arrested by inactive E474A (*A*) and E475A (*B*). The HPLC peptide peaks for E474A or E475A were collected manually and sequenced by ESI-MS/MS (*C*). Control experiments were done in the absence of inactive peptidases or in the presence of dynorphin A_{1-13} (30 μM) as a competitive peptide during incubation with the rat brain peptide extract (*A* and *B*).

quence of this peptide, and that of several other peptides sequenced from similar ESI-MS/MS spectra, was found to match specific sequences in various proteins (Table III).

To confirm whether the sequenced peptides were in fact ep24.15 and/or ep24.16 substrates, and to further validate this new method, four of the 15 peptides identified were chemically synthesized. One of these four peptides (FDLTADWPL) was selected for synthesis because it did not appear to be arrested by ep24.15 or ep24.16, as shown in the HPLC chromatograms (Fig. 2*C*). The other three peptides (LVVYPWTQRY, VVYPWTQRY, and PVNFKFLSH) were selected for synthesis because they were efficiently arrested by ep24.15 and ep24.16 (Fig. 2*C*). The constant of inhibition (K_i values) and relative hydrolyzes ratio for these peptides were determined in parallel with known bioactive peptides such as bradykinin, angiotensin I and II, and dynorphin A_{1-13} (Table IV).

The three peptides selected on the basis of a specific interaction with the inactive enzymes prevented the hydrolysis of QFS by ep24.15 or ep24.16 in a competitive enzyme assay, in contrast to the fourth peptide (FDLATDWPL), which did not inhibit the enzymes (Table IV). To estimate the affinity of these peptides for ep24.15 and ep24.16, the relative constant of inhibition (K_i) was determined. Peptide FDLTADWPL had a K_i above 100 μM, and was not efficiently degraded by these enzymes, even after prolonged incubations. On the other hand, peptides that specifically interacted with the inactive ep24.15 and ep24.16 enzymes had K_i values in the micromolar range (Table IV). Peptide PVNFKFLSH had the highest affinity for both enzymes, with a K_i eight times lower for ep24.16 (3.43 μM) than for ep24.15 (27.8 μM). Despite these differences, this peptide was a better substrate for ep24.15 and ep24.16 than bradykinin. Peptides LVVYPWTQRY and VVYPWTQRY, which

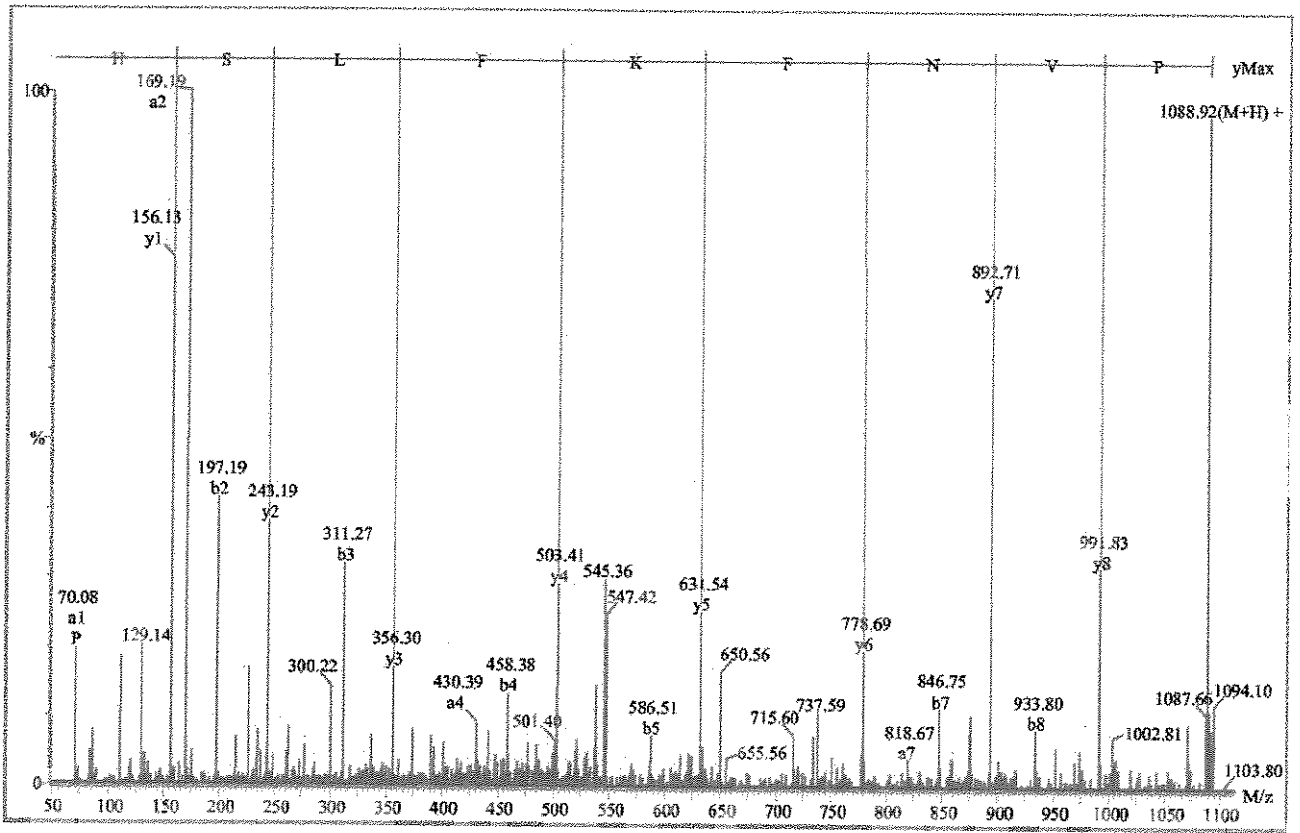

FIG. 3. **Representative peptide sequencing by ESI-MS/MS.** Product ion mass spectra for the mass-selected protonated peptide PVNFK-FLSH acquired using the high-resolution orthogonal time-of-flight mass analyzer after 26 eV collision-induced dissociation with argon. Note that all of the Y-type (Y1–Y8) ionic fragments were formed and clearly detected, which allowed complete sequencing of the peptide.

TABLE III

Analyses of the sequenced peptides by homology with proteins in the data base

The protein data base (www.ncbi.nlm.nih.gov/blast) was searched for short, nearly exact matches on *rodentia* organisms; (b) = brain; (s) = spleen.

Peptide sequenced	Putative precursor protein	Biological activity
VVYPWTQRY (b)	Hemoglobin β chain	Increased after ischemia (61)
LVVYPWTQRY (b)	Hemoglobin β chain	Specific binding to opioid receptor, guinea pig ileum contraction (61)
PVNFKFLSH (b)	Hemoglobin α chain	Unknown
FDLTADWPL (b)	Endogenous retrovirus HERV-K10 putative Protease; protein similar to F-box protein FBW7	Unknown
WSTVLTDVN (b)	Hypothetical protein XP_153553; olfactory receptor MOR13–2;	Unknown
VNMVPVGWASR (b)	Tripeptidyl-peptidase I precursor (TPP-I); zinc finger protein AT-BP2	Unknown
VYPWT (b)	Hemoglobin β chain	Unknown
HHPGDFTPAMHASLDK (s)	Hemoglobin α chain	Unknown
HPGDFTPAMHASLDK (s)	Hemoglobin α chain	Unknown
GDFTPAMHASLDK	Hemoglobin α chain	Unknown
NRTAE (s)	Protein-tyrosine phosphatase γ; SWI/SNF related, matrix-associated, actin-dependent regulator of chromatin, subfamily α-like 1 (*Homo sapiens*); cytokine-like nuclear factor n-pac (*H. sapiens*)	Unknown
KVNPDDVGGEALGRL (s)	Hemoglobin β II	Unknown
LNNPDDRWSKNA (s)	Hypothetical protein XP_070487 TRG γ-chain J-C (*H. sapiens*)	Unknown
TPGTDFWLHASLD (s)	Putative voltage-gated calcium channel γ-4 subunit (*H. sapiens*)	Unknown
QFWLHASL (s)	Oxidase (cytochrome *c*) assembly 1-like (*H. sapiens*)	Unknown

differed by a single NH_2-terminal amino acid, had similar K_i values for both ep24.15 and ep24.16. However, the relative hydrolysis ratio of VVYPWTQRY was five times higher for ep24.15 compared with ep24.16. In contrast, the peptide with the NH_2-terminal leucine, LVVYPWTQRY, was degraded at least five times faster by ep24.16 than by ep24.15 (Table IV), suggesting that a large nonpolar amino acid residue at the NH_2-terminal position could be of importance for defining specific substrates or inhibitors for ep24.15 or ep24.16.

The cleavage products of the peptides LVVYPWTQRY, VVYPWTQRY, and PVNFKFLSH, digested by either ep24.15 or ep24.16, were identified by ESI-MS/MS sequencing. Con-

TABLE IV

Side-by-side evaluation of the constant of inhibition (K_i values) and relative hydrolyses ratio of bradykinin and dynorphin A_{1-13} and the newly identified rat brain peptides for the ep24.15, ep24.16, and ACE

	K_i values (μM)			*Relative hydrolyze ratio (%)		
	EP24.15	EP24.16	ACE	EP24.15	EP24.16	ACE
Bradykinin	5.36	8.11	1.74	100*	100*	100*
Dynorphin A_{1-13}	0.04	0.22	>100	<0.01	<0.01	8.49
Angiotensin I	4.29	5.35	25.88	33.5	38.7	172.26
Angiotensin II	8.12	7.95	>100	6.99	20.88	<0.01
PVNFKFLSH	27.76	3.43	1.866	140	152.24	1146.19
VVYPWTQRY	10.02	7.04	10.63	10.62	1.9	138.89
LVVYPWTQRY	2.56	2.01	6.488	0.72	4.7	100.65
FDLTADWPL	>100	>100	>100	<0.01	<0.01	<0.01

TABLE V

Cleavage sites for ep24.15 and ep24.16 in selected peptides

The positions are hydrolyzed by EP24.15 (↑) and EP24.16 (↓).

Peptides	Cleavage sites
PVNFKFLSH	PVNF↑ ↓K↑ ↓F↑ ↓LSH
VVYPWTQRY	VVYPW↑ ↓T↑ ↓Q↑ ↓RY
LVVYPWTQRY	LVVYP↑ ↓W↑ ↓T↑ ↓Q↑ ↓RY

trary to previously described natural substrates for ep24.15 and ep24.16, hydrolysis of the peptides LVVYPWTQRY, VVYPWTQRY, and PVNFKFLSH involved at least three peptide bonds (Table V). These results agreed with data obtained using several synthetic peptides (36, 37), suggesting that these enzymes could also simultaneously hydrolyze more than one peptide bond in natural substrates.

Peptidases are not peptide-specific (38), and ep24.15 and ep24.16 share a series of substrates with ACE (7). Because the physiological function for ACE in the cardiovascular system is well known (20, 21), we examined the kinetic parameters of the peptides identified in this study. The ep24.15 and ep24.16 substrates LVVYPWTQRY, VVYPWTQRY, and PVNFKFLSH also interacted with ACE, with K_i values ranging from 1.7 μM up to 26 μM (Table IV). ACE hydrolyzed the peptide PVNFKFLSH more efficiently than it did angiotensin I or bradykinin (Table IV).

The effects of three of the peptides identified here (FDLTADWPL, LVVYPWTQRY, and PVNFKFLSH) were examined on the blood pressure of anesthetized rats. The intravenous injection of PVNFKFLSH produced immediate hypotension, the extent of which varied according to the dose (Fig. 4*A*). The rapid fall in blood pressure elicited by PVNFKFLSH was similar to that seen with bradykinin, but required a lower dose (0.01 *versus* 3 μg/kg for bradykinin). LVVYPWTQRY also produced hypotension at ≥10 μg/kg, whereas FDLTADWPL produced a slight effect only at 100 μg/kg. Based on this effect on blood pressure, PVNFKFLSH was named *hemopressin*. Whereas enalapril significantly enhanced the hypotensive response to bradykinin (decrease in mean arterial blood pressure: −14.9 + 4.2 mm Hg *versus* −28.2 + 2.4 mm Hg before and after enalapril, respectively; $n = 5$ each, $p < 0.05$), this ACE inhibitor had a significant effect only on the lowest dose of PVNFKFLSH. The responses to bradykinin were potentiated after the administration of PVNFKFLSH while those to angiotensin II were unaffected; LVVYPWTQRY had no such effect on the responses to these two agonists (Fig. 4*B*).

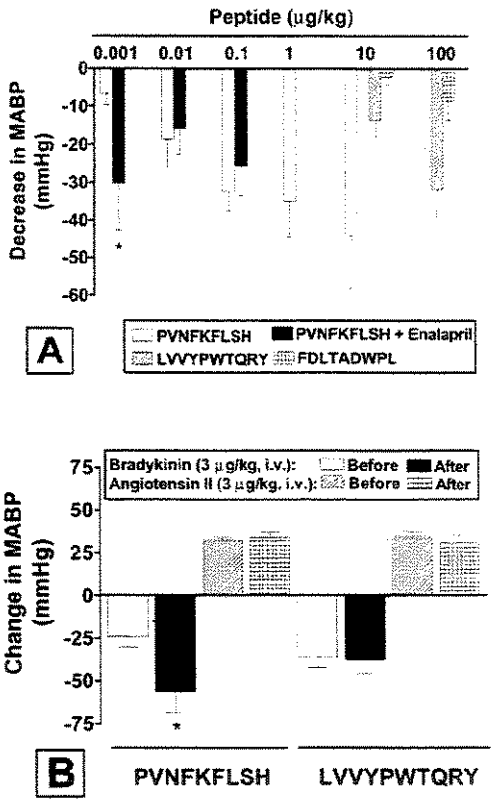

FIG. 4. **Hypotension produced by peptides PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL in pentobarbital-anesthetized male Wistar rats.** *Panel A* shows the responses to various doses of the three peptides. Note that peptide FDLTADWPL caused little hypotension at the doses tested. The responses to PVNFKFLSH in the presence of enalapril are also shown. *Panel B* shows the potentiation of the responses to BK, but not to angiotensin II, in rats treated with PVNFKFLSH. Note that LVVYPWTQRY produced no potentiation. The columns in *panels A* and *B* represent the mean ± S.E. of the change in mean arterial blood pressure in three to nine rats. The mean arterial blood pressure (*MABP*) of the three groups of rats after stabilization but before peptide administration were not significantly different (138 + 4, 133 + 5, and 142 + 4 mm Hg for rats given peptides PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL, respectively; analysis of variance followed by Tukey test). See "Experimental Procedures" for further experimental details. *, $p < 0.05$ compared with response without enalapril.

DISCUSSION

A major finding of the present study was the identification of a new peptide substrate for ep24.15, ep24.16, and ACE that causes hypotension. This peptide (named *hemopressin*) is a fragment of the α-chain of hemoglobin. This is the first report to identify intracellular protein fragments as natural substrates for endopeptidase 24.15 (ep24.15) and neurolysin (ep24.16). These findings suggest a role for ep24.15 and ep24.16 in intracellular peptide metabolism.

The substitution of amino acids His^{474}, Glu^{475}, His^{477}, or Glu^{502} abolishes the enzymatic activity of ep24.15 (39). The importance of the corresponding residues in ep24.16 for catalysis has not yet been experimentally demonstrated, but may be predicted from the recently determined tertiary structure (35). Indeed, substitution by alanine of the corresponding residues

on ep24.16 (H474A, E475A, H478A, and E503A) completely abolished the enzymatic activity. Because all of the proteins examined were expressed to an equivalent level, it is unlikely that the outcome observed with any of the substitutions resulted from improper protein folding. Analysis of the secondary structures by circular dichroism confirmed the similarity of the mutated proteins to the wild type, and indicated that inappropriate protein folding did not cause the loss of enzymatic activity. In addition, deconvolution of the circular dichroism data supported the previous assumption of secondary structure homology between these two oligopeptidases. In an attempt to identify new natural substrates for ep24.15 and ep24.16, catalytically inactive forms of these enzymes were used to identify peptides present in rat brain and spleen extracts.

All of the peptides isolated and sequenced here were within the size range previously reported for natural and synthetic substrates of ep24.15 and ep24.16 (36, 37, 53–55). Using a series of peptides structurally related to bradykinin, Oliveira *et al.* (53) showed that 5 amino acids was the minimum substrate size for ep24.15. Similar results were obtained for ep24.15 and ep24.16 using synthetic fluorescent substrates (37). The smallest peptide isolated here also contained 5 amino acids, which corroborated previous findings (37, 53). On the other hand, orphanin, a neuropeptide containing 17 amino acids, is the largest natural substrate described so far for ep24.15 (54). Using fluorescent substrates in a detailed, systematic analysis of the influence of substrate size on ep24.15 and ep24.16 catalysis, Oliveira *et al.* (37) confirmed that 17 amino acids was indeed the largest substrate size for both enzymes. The largest peptides identified here contained 16 amino acids, which also agrees with these earlier studies (37, 53–55).

There is increasing evidence that ep24.15 and ep24.16 may play a major role in the intracellular metabolism of peptides, probably at a stage beyond the proteasome (13–15, 40). As shown here, we have identified several putative intracellular substrates for ep24.15 and ep24.16. Because the substrates for ep24.15 and ep24.16 must be peptides containing 5–17 amino acids, there is a need for a proteolytic system able to generate such small peptides from larger proteins. The 20 S proteasome, a multicatalytic proteinase complex, is the main intracellular extralysosomal proteolytic system involved in ubiquitin-dependent and -independent intracellular proteolysis (41). In degrading cytosolic, mitochondrial, and nuclear proteins (42, 43), the proteasome generates peptides from 3 to 22 residues in size (16, 44, 45). The peptides generated by the proteasome are therefore within the optimum size range for substrates of ep24.15 and ep24.16 (14, 37). Of the new ep24.15 and ep24.16 substrates identified here, at least seven are hemoglobin fragments. Interestingly, ep24.15 is present in large amounts in human erythrocytes, where hemoglobin also occurs in large quantities (46). Short hemoglobin fragments have been shown to be generated directly by the proteolytic action of the proteasome (47, 48). Whereas additional studies will be necessary to clarify the putative enzymes involved in the generation of hemopressin *in vivo*, it seems reasonable to suggest that ep24.15 and ep24.16 may function in the later steps of intracellular protein degradation. The mechanisms whereby the peptides isolated here escaped degradation is unknown.

In addition to their well known receptor-mediated functions, some peptides also play a role in intracellular processes. For example, calmodulin-dependent protein kinase II is a multifunctional protein kinase with an important role in controlling a variety of cellular functions in the central nervous system (49). A 13-amino acid peptide (KKALRRQEAVDAL), known as autocamtide-2-related inhibitory peptide, is a highly specific inhibitor of calmodulin-dependent protein kinase II (50). In *Bacillus subtilis*, the RapA and RapB proteins are aspartylphosphate phosphatases that specifically dephosphorylate the Spo0F~P intermediate response regulator of the phosphorelay signal transduction system for sporulation (51). The RapA phosphatase activity on Spo0F~P is inhibited *in vivo* by a pentapeptide generated from the *phrA* gene, which displaces Spo0F~P from a preformed complex with RapA (51). The c-Jun NH_2-terminal kinase, a member of the stress-activated group of mitogen-activated protein kinases, is inhibited by a cell-permeable peptide that decreases intracellular c-Jun NH_2-terminal kinase signaling and confers long-term protection to pancreatic β-cells against interleukin-1β-induced apoptosis (52). Thus, by acting on the intracellular metabolism of peptides, ep24.15 and ep24.16 could contribute to the maintenance of cellular homeostasis.

Of the endogenous globin fragments identified in the present study, three (LVVYPWTQRY, VVYPWTQRY, and the fragment VYPWT) are apparently related to a family of peptides known a hemorphins, which are derived from the degradation of the β-chain of human hemoglobin and show morphine-like activity based on their ability to inhibit the contractions of electrically stimulated guinea pig ileum (Refs. 56 and 57 and references therein). LVVYPWTQRY and VVYPWTQRY are identical to human LVV-hemorphin-7 (57, 58) and VV-hemorphin-7 (59), respectively, except for their terminal amino acid residue, which is tyrosine instead of arginine and may reflect the rodent origin of our peptides.

Peptide PVNFKFLSH produced potent hypotension in anesthetized rats. This peptide is derived from the α_1-chain of rodent hemoglobin and shares no sequence identity with the hemorphins of the β-chain of human hemoglobin. Other peptides derived from the α-chain identified here included HHPGDFTPAMHASLDK and two truncated fragments of this peptide, but their effect on blood pressure was not examined. The mechanism by which PVNFKFLSH produces hypotension is still unclear but could involve a variety of pathways, including ion channel activation or blockade, the stimulation of nitric oxide (NO) formation through as yet unidentified receptors, the release of vasodilator peptides such as atrial natriuretic factor, or the inhibition of endogenous peptidase activity which could lead to an increase in circulating levels of hypotensive peptides.

In experiments not described here, we have observed that PVNFKFLSH does not contract or relax vascular (aorta) or nonvascular (guinea pig ileum) smooth muscle preparations. This finding is similar to the inability of hemorphins to contract isolated endothelium-denuded aortic strips from rats (60), and suggests that PVNFKFLSH probably does not have a direct action on vascular smooth muscle. The involvement of NO in the observed hypotension merits investigation, although Moisan *et al.* (57) observed that the blockade of NO production by L-N^{ω}-nitro-L-arginine methyl ester did not influence the hypertensive response to hemorphins.

Exogenous and endogenous peptides may be metabolized by a variety of peptidases, including the three enzymes studied here. To examine the influence of ACE on the hypotensive responses to PVNFKFLSH, rats were treated with enalapril to block this enzyme. Although the treatment was effective in potentiating BK-induced hypotension, it had little effect on the responses to PVNFKFLSH, except at the lowest dose of the peptide. This finding will have to be explored further in light of the role of ACE, and other peptidases such as the ep24.15 and ep24.16, in the metabolism of PVNFKFLSH. Experiments to address this aspect using specific inhibitors are in progress in our laboratory.

The ability of PVNFKFLSH to potentiate the hypotension to BK without affecting the hypertension to angiotensin II is

interesting, although it is still unclear whether this response is selective for BK or applies to vasodilatory peptides in general. This action of PVNFKFLSH could involve the sensitization of intracellular pathways to subsequent stimulation by BK or could involve the specific inhibition of a peptidase(s), possibly ACE, that degrades BK. The inhibition of ACE by PVNFKFLSH could influence the metabolism of other peptide substrates by this peptidase. The hypotensive action of PVNFKFLSH may involve therefore peptidase- and nonpeptidase-mediated pathways. Finally, the observation that FDLTADWPL, derived from a nonhemoglobin molecule(s), caused little hypotension compared with the varied effects observed for fragments from the α (PVNFKFLSH) and β (hemorphins and LVVYPWTQRY) chains of hemoglobin confirms data in the literature indicating that the degradation of hemoglobin is an important source of bioactive peptides, and could provide a lead for investigating the biological activities of the other peptides identified in this study.

In summary, we have demonstrated the feasibility of using catalytically inactive forms of ep24.15 and ep24.16 to identify new bioactive peptide substrates for these enzymes. However, the methodology should be applicable to other enzyme systems. One of the new substrates identified (*hemopressin*) is a fragment of the hemoglobin α-chain and reduces blood pressure in anesthetized rats. Further functional analyses will be necessary to evaluate the pharmacological and physiological relevance of hemopressin and of the other peptides identified in this study.

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Artigo submetido

"BIOLOGICAL ACTIVITIES OF A LECTIN FROM *BOTHROPS JARARACUSSU* SNAKE VENOM"

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Toxicon

BIOLOGICAL ACTIVITIES OF A LECTIN FROM *BOTHROPS JARARACUSSU* SNAKE VENOM

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Running title: Biological activities of snake venom lectin

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Abstract

Snake venoms contain saccharide-binding lectins. In this work, we examined the biological activities of a lectin (BjcL) purified from *Bothrops jararacussu* snake venom by chromatography on non-derivatized Sepharose 4B and Sephacryl S-200 HR. The protein, a homodimer with subunits of 14.5 kDa, gave a single immunoprecipitin line in immunoelectrophoresis and cross-reacted in ELISA with antivenoms raised against *Bothrops* spp. (lanceheads), *Micrurus* spp. (coral snakes), *Crotalus durissus terrificus* (South American rattlesnake), and arthropod (*Loxosceles gaucho, Phoneutria nigriventer* and *Tityus serrulatus*) venoms. BjcL agglutinated human formaldehyde-fixed erythrocytes at ≥100 ng/ml and was inhibited by lactose and EDTA (≥2 mM) and high concentrations (>100 mM) of glucose and sucrose, but not by N-acetylglucosamine. BjcL had no direct hemolytic activity and was devoid of esterase, PLA_2 and proteolytic activities. The lectin (up to 200 μg/ml) did not aggregate human platelet-rich plasma (PRP) or washed platelets (WP), nor did it alter the aggregation induced by ADP in PRP or by thrombin in WP. When injected into mouse hind paws, BjcL (10-100 μg/paw) caused edema and increased vascular permeability, with a maximum effect after 1 h that persisted for up to 6 h (edema) or gradually decreased after the peak interval (vascular permeability). No hemorrhage was observed in BjcL-injected paws. In anesthetized rats, *B. jararacussu* venom (200 μg/kg, i.v.) produced sustained hypotension (maximum decrease of ~60%) whereas a similar dose of BjcL decreased the blood pressure by <15%, with a rapid return to the resting level.

1. Introduction

Lectins are glycoproteins characterized by their ability to bind to specific sugars (Sharon and Lis, 1972). Since many membrane proteins facing the extracellular environment contain carbohydrate residues, the specific sugar-binding properties of lectins can be used to investigate the importance of sugar residues in cell-cell recognition and other biological responses (Lis and Sharon, 1986). Such binding usually requires two or more sugar sites on the membrane surface in order to permit the cross-linking of cells. Although initially isolated from plants, lectins have since been found to be widely distributed throughout the animal kingdom (Kocourek, 1986), including in snake (Ogilvie and Gartner, 1984) and arthropod (Liang and Pan, 1995; Khoang et al., 2001) venoms.

Since their initial description (Ogilvie and Gartner, 1984), snake venom lectins (SVL) have been isolated from a variety of species, including *Agkistrodon contortrix contortrix* (Gartner and Ogilvie, 1984), *Agkistrodon piscivorus leukostoma* (Gartner and Ogilvie, 1984), *Agkistrodon p. piscivorus* (Komori et al., 1999), *Bitis arietans* (Nikai et al., 1995), *Bothrops atrox* (Gartner et al., 1980), *Bothrops godmani* (Lomonte et al., 1990), *Bothrops jararaca* (Ozeki et al., 1994), *Bungarus fasciatus* and *Bungarus multicinctus* (Zha et al., 2001), *Crotalus atrox* (Gartner and Ogilvie, 1984), *Dendroaspis jamesonii* (Ogilvie et al., 1986), *Lachesis muta* (Gomes-Leiva and Aragon-Ortiz, 1986; Ogilvie et al., 1986; Aragón-Ortiz et al., 1989, 1990), *Trimeresurus okinavensis* (Nikai et al., 2000), and *Trimeresurus stejnegeri* (Liang and Wang, 1993). Several of these proteins have been partially or wholly sequenced (Hirabayashi et al., 1991; Aragón-Ortiz et al., 1996; Komori et al., 1999; Nikai et al., 2000; Carvalho et al., 2002) and, in some cases, their genes cloned (Xu et al., 1999; Zha et al., 2001).

SVL are C-type (Ca^{2+}-dependent), saccharide-binding [mostly galactose, but also mannose (Zha et al., 2001)] proteins that generally account for ≤1% of the dry venom weight, with most of them occurring as homodimers with a molecular mass of ~28 kDa (Lomonte et al., 1990; Hirabayashi et al., 1991; Ozeki et al., 1994). SVL characteristically cause hemagglutination, although other actions such as mitogenic activity in lymphocytes (Djaldetti et al., 1980; Hembold et al., 1985, 1986; Mastro et al., 1986), platelet aggregation (Gartner et al., 1980; Gomes-Leiva and Aragon-Ortiz, 1986; Ogilvie et al., 1986, 1989; Ozeki et al., 1994), induction of paw edema in mice (Lomonte et al., 1990), and the modulation of Ca^{2+} release from skeletal muscle sarcoplasmic reticulum (Ohkura et al., 1996; Hirata et al., 1999) have also been reported. In addition to true lectins, snake venoms also contain various proteins with C-type lectin-like motifs but which generally lack the ability to cause hemagglutination. Many of the latter proteins have important hemostatic actions, including the modulation of platelet aggregation (Clemetson et al., 1998, 2001; Markland, 1998; Andrews and Berndt, 2000; Braud et al., 2000; Wisner et al., 2002).

The venom of the South American pitviper, *Bothrops jararacussu* (jararacuçu) contains a lectin (BjcL) (Carvalho et al., 1998, 2002). Studies with cultured cells have shown that BjcL can inhibit the proliferation of various tumor cell lines (Pereira-Bittencourt et al., 1999; Carvalho et al., 2001). However, little is known of the immunological properties and other biological activities of this protein. In this report, we describe some additional properties of BjcL.

2. Material and methods

2.1. Materials

N-Acetylglucosamine, acrylamide, agarose, ammonium persulfate, bromophenol blue, 4-chloro-1-naphthol, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), lactose, β-

mercaptoethanol, N,N'-methylene-bis-acrylamide, molecular weight markers for polyacrylamide gel electrophoresis, rabbit anti-horse IgG-peroxidase conjugate, sodium dodecyl sulfate (SDS), N,N,N′,N′-tetramethylethylenediamine (TEMED), Tris, trypsin and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sepharose 4B, Sephacryl S-200 HR and chromatographic columns were from AmershamBiosciences (Piscataway, NJ, USA). Iloprost was from Schering. Sodium pentobarbital (Hypnol®) was from Cristália (Espírito Santo do Pinhal, SP, Brazil). All other reagents were of the highest grade available. Multi-well plates (high protein binding) for ELISA were from Corning (Corning, NY, USA) and microtiter V-well plates for hemagglutination were from Greiner.

2.2. Venom and antivenoms

Bothrops jararacussu venom obtained from adult snakes of both sexes was from the Instituto Butantan (São Paulo, SP, Brazil) or private suppliers. Commercial equine antivenoms raised against *Bothrops* species (*B. alternatus, B. jararaca, B. jararacussu, B. moojeni*, and *B. neuwiedi*), against *Micrurus* (coral snakes) species (*M. frontalis* and *M. corallinus*), against *Crotalus durissus terrificus* (South American rattlesnake), and against arachnids (spiders – *Loxosceles gaucho* and *Phoneutria nigriventer* and scorpion – *Tityus serrulatus*) (Cardoso et al., 2003) were obtained from the Instituto Butantan.

2.3. Animals

Male Wistar rats (200-250 g) and male Swiss white mice (25-30 g) were obtained from the university's central breeding colony and housed on a 12 h light/dark cycle at 22°C with free access

to water and standard rodent chow (Purina®). The experiments described here were done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

2.4. Purification of B. jararacussu venom lectin (BjcL)

Bothrops jararacussu venom (100 mg batches) was dissolved in 5 ml of Tyrode solution (composition, in mM: NaCl 137, Na_2PO_4 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, $MgCl_2$ 0.49 and KCl 2.7) and centrifuged (2000 *g*, 10 min, 25°C) to remove insoluble material. The resulting supernatant was applied to a column (1 cm x 10 cm) of Sepharose 4B equilibrated with Tyrode solution at 20°C and the column then washed with the same solution at a flow rate of 1 ml/min until the absorbance at 280 nm had returned to baseline. The lectin bound to the column was eluted with Tyrode solution containing 200 mM lactose and fractions of 1 ml were collected. The elution profile was monitored by reading the absorbances of the fractions in a Uvikon 810 spectrophotometer (Kontron Instruments, Milan, Italy). The lectin obtained in the preceding step was dialyzed against distilled water, lyophilized, and resuspended in 0.1 M Tris-HCl, pH 7.5, before being applied to a column (1 cm x 30 cm) of Sephacryl S-200 HR equilibrated with this same buffer. The protein was eluted at a flow rate of 0.5 ml/min using the same buffer and 1 ml fractions were collected. The elution profile was monitored as described above.

2.5. Protein concentration

The protein concentrations of venom and purified BjcL solutions were estimated by the absorbance at 280 nm, assuming that an A_{280} nm of 1.0 = 1.0 mg of protein/ml.

2.6. Electrophoresis

SDS-PAGE (Laemmli, 1970) was done in mini-gels (8 cm x 10 cm, 10% acrylamide) using a Mighty Small II SE260 apparatus (Hoefer-Pharmacia). *Bothrops jararacussu* venom and BjcL were diluted in 0.063 M Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% glycerol and 0.001% bromophenol blue and then boiled for 4 min before electrophoresis at 100 V (constant). In some experiments, β-mercaptoethanol (5 mM) was included in the sample preparation. At the end of the run, the gels were silver stained, dried and documented. Molecular mass markers were included in the runs.

2.7. Immunoelectrophoresis

Samples of *B. jararacussu* venom and BjcL were run in 1% agarose gels in 0.1 M Tris-HCl, pH 8.0, at room temperature and a fixed voltage (120 V) for 90 min. The gels were prepared on microscope slides. After electrophoresis, commercial bothropic antivenom was added to a trough cut in the agarose and the slides then incubated in a humidified chamber for 48 h. After this period, the slides were washed in 0.15 M NaCl for 12 h, then dried at 37°C for 36 h and stained with 0.4% amido black in 10% acetic acid for 10 min. After destaining in 5% acetic acid, the slides were dried and documented.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The reactivity of *B. jararacussu* venom and purified BjcL with commercial equine antiserum raised against a pool of venoms from *Bothrops* species (*B. alternatus, B. jararaca, B. jararacussu, B. moojeni* and *B. neuwiedi*) was assessed essentially as described by Valério et al. (2002). Briefly, 96-well plates were coated overnight at 4°C with 100 µl of bothropic antivenom

(diluted 1:1000 in 0.1 M sodium carbonate, pH 9.6) and then washed with 0.9% NaCl containing 0.05% Tween-20 followed by incubation with varying amounts of venom or BjcL diluted in incubation buffer (phosphate-buffered saline, PBS, containing 0.05% Tween-20 and 0.25% bovine casein) for 1 h at room temperature. The plates were subsequently washed with 0.9% NaCl containing 0.05% Tween 20 and incubated with an affinity (protein G-Sepharose)-purified IgG-peroxidase conjugate (1:1000, in incubation buffer) against *Bothrops* venom components. After further washes, the plates were incubated with substrate (100 µl of 0.2 mg of O-phenylenediamine/ml and 0.05% H_2O_2 in 0.15 M citrate buffer, pH 5.0) for up to 30 min in the dark at room temperature. The reactions were stopped by adding 50 µl of 5% H_2SO_4 and the final absorbances were read at 492 nm in a SpectraMax340 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

To examine the cross-reactivity of BjcL with commercial antivenoms, 96-well plates were coated overnight with BjcL (5 µg/well) diluted in sodium carbonate then washed and incubated for 1 h at room temperature with serial dilutions of antivenoms prepared in incubation buffer. After washing, the plates were incubated with a rabbit anti-horse IgG-peroxidase conjugate (diluted 1:1000 in incubation buffer) and then processed as described above.

2.9. Enzyme activities

Phospholipase A activity was assayed by the method of Holzer and Mackessy (1996) modified for 96-well plates (Beghini et al., 2000). Proteolytic and esterase activities were determined using casein (Delpierre, 1968) and TAME (Viljoen et al., 1979) as substrates, respectively.

2.10. Hemagglutination

Hemagglutinating activity was determined by the method of Nowak et al. (1976) using microtiter V-well plates and serial two-fold dilutions of *B. jararacussu* venom and BjcL. Each well contained 50 µl of a 10% suspension of human formaldehyde-fixed, trypsinized erythrocytes in phosphate-buffered saline (PBS, pH 7.4), and varying amounts of venom or BjcL in 100 µl of PBS. The negative control contained 50 µl of cell suspension and 100 µl of PBS. Following the addition of erythrocytes, the plates were shaken briefly and incubated at room temperature (~25°C) for 2 h. Unagglutinated erythrocytes formed a button at the bottom of the wells, whereas agglutinated erythrocytes formed a diffuse coat or mantle. To examine the requirement for Ca^{2+} and the inhibition by sugars, BjcL (10 µg/well) was incubated for 2 h at 25°C with different concentrations of sugars or EDTA diluted in PBS and the hemagglutinating activity then determined as described above.

2.11. Platelet aggregation

Blood from individuals who had not been on any medication for the previous 10 days was collected in 3.8% sodium citrate (9:1, v/v) and then centrifuged (200 *g*, 15 min, 25°C) to obtain platelet-rich plasma (PRP). The cell pellet was centrifuged again (2000 *g*, 15 min) and the resulting supernatant (platelet-poor plasma, PPP) was used to calibrate the aggregometer. Washed platelets (WP) were prepared as described by Radomski and Moncada (1983). For each test, 0.5 ml of platelet suspension was incubated with stirring for 3 min at 37°C in a Payton two-channel aggregometer. Subsequently, varying amounts of BjcL were added and the platelet response then monitored. The ability of BjcL to inhibit aggregation was examined by incubating platelets for 1-3 min with different quantities of BjcL prior to stimulation with ADP (0.5-10 µM, PRP) or thrombin

(50-100 IU/ml, WP). The aggregation was monitored for up to 5 min and the responses were compared with those obtained for these agonists in the absence of BjcL.

2.12. Paw edema and vascular permeability

Male Swiss mice were injected intravenously with Evans blue (50 µl of a 0.25% (w/v) solution/g of body weight) and 30 min later received an intraplantar injection (50 µl, in 0.9% saline) of venom (10, 30 or 100 µg/paw) or BjcL (2.5, 5, 10, 30 or 100 µg/paw) in the left hind paw. The contralateral paw was injected with saline (50 µl/paw) and served as the control. After 0.5, 1, 2, 4 and 6 h, the mice were killed with an overdose of anesthetic and the paw edema (Levy, 1969) and dye exudate (Gamsé *et al.*, 1980) then measured. For this, the paws were amputated at the tarsocrural joint and weighed on an analytical balance. The edema (expressed in mg) was calculated as the difference in weight between the left (treated) and right (untreated) paws. The paws were subsequently minced into small pieces, placed in a test tube with formamide (3 ml) and incubated in a water bath at 57°C for 24 h. At the end of this period, the absorbance of the supernatants was measured at 619 nm (Uvikon 810 spectrophotometer) and the amount of Evans blue present in the extracts was determined from a standard curve of the dye prepared in formamide. The results were expressed as µg of dye/mg paw weight.

2.13. Arterial blood pressure measurements

Male Wistar rats were anesthetized with sodium pentobarbital (>60 mg/kg, i.p.) and a tracheostomy was done to allow the animals to breath room air. The rats were cannulated for the measurement of arterial blood pressure (via a carotid artery) and the administration of anesthetic, venom or BjcL (via a femoral vein). Changes in blood pressure were recorded continuously via a

transducer (Abbott, Chicago, IL, USA) connected to a computer data acquisition system (Transonics Systems Inc., Ithaca, NY, USA). After allowing 15 min for stabilization, *B. jararacussu* venom or BjcL was injected and the changes in blood pressure were monitored for 20 min and expressed as the percent change relative to the values obtained immediately before venom or BjcL injection.

2.14. Statistical analysis

The results were expressed as the mean ± S.E.M. for the number of experiments or animals indicated. Statistical comparisons were done using Student's unpaired t-test or analysis of variance (ANOVA) followed by the Bonferroni test as appropriate. A value of $p<0.05$ indicated significance.

3. Results

3.1. Purification of BjcL

Figure 1A shows the elution profile of BjcL from a non-derivatized Sepharose 4B column in the presence of 200 mM lactose. Of the four lots of venom screened, one did not contain lectin; the remaining three lots gave the elution profile shown here. Chromatography of BjcL on Sephacryl S-200 HR yielded only one major peak that, in the absence of lactose, was retarded on the column and eluted with the void volume (24 ml) (Fig. 1B). The amount of protein recovered after the Sepharose 4B step accounted for <1% of the venom applied based on the absorbance at 280 nm. SDS-PAGE revealed a principal band of ~29 kDa which, in the presence of β-mercaptoethanol, reduced to a single band of ~14.5 kDa (Fig. 1C), indicating that BjcL occurred as a homodimer.

3.2. Immunological properties of BjcL

Immunoelectrophoresis of BjcL against commercial bothropic antivenom gave only one precipitin line compared to the venom (Fig. 2), and this finding was confirmed by immunodiffusion (results not shown). The ELISA reactivity of BjcL with bothropic antivenom compared to that of the venom is shown in Figure 3A. When tested against different antivenoms, BjcL reacted most with antivenom raised against the venoms of South American lanceheads (*Bothrops* spp.) followed by coral snakes (*Micrurus* spp.), South American rattlesnake (*Crotalus durissus terrificus*) and arachnids (spiders – *Loxosceles gaucho* and *Phoneutria nigriventer* and scorpion – *Tityus serrulatus*) (Fig. 3B).

3.3. Hemagglutination by BjcL

BjcL agglutinated human formaldehyde-fixed erythrocytes at concentrations ≥0.1 μg/ml (n=7) whereas *B. jararacussu* venom produced hemagglutination at ≥1.5 μg/ml (n=3). Both lactose and EDTA (≥2 mM; n=7 each) prevented hemagglutination by BjcL. At high concentrations (>100 mM), glucose and sucrose, but not N-acetylglucosamine (200 mM), inhibited BjcL-induced hemagglutination (n=3 each). BjcL had no direct hemolytic activity based on examination of the hemagglutination plates at the end of the assay.

3.4. Platelet aggregation

At concentrations up to 200 μg/ml, BjcL did not aggregate human platelets in PRP or in WP (n=3 each). Similarly, the lectin did not inhibit the aggregation induced by ADP (3 μM, n=3, PRP) or thrombin (100 IU/ml, n=3, WP) (Fig. 4).

3.5. Edema and vascular permeability

Bothrops jararacussu venom and BjcL produced edema and increased the vascular permeability in mouse hind paws, with both effects being maximal after 1 h (Fig. 5A,B). However, whereas the edema was generally maintained for up to 6 h after venom or lectin injection (Fig. 5A), vascular permeability tended to return to normal with time in both cases (~50% return to basal values after 6 h) (Fig. 5B). The edema and increase in vascular permeability caused by BjcL were dose-dependent when measured at the peak response (1 h) (Fig. 6A,B). In the case of *B. jararacussu* venom, no dose-dependence was seen for edema formation at the doses tested (maximal response was already observed at 10 μg/kg), whereas the increase in vascular permeability was greater with the highest dose (100 μg/kg) compared to the two lower doses (10 and 30 μg/kg). At all doses, the responses to BjcL were significantly smaller than those to venom (Fig. 6), and BjcL doses <10 μg/kg were without effect. No macroscopic hemorrhage was observed in mouse paws injected with BjcL, in contrast to the venom, which caused marked hemorrhage within minutes of injection, even at the lowest dose.

3.6. Arterial blood pressure

In anesthetized rats, the intravenous injection of *B. jararacussu* venom (200 μg/kg) produced immediate, sustained hypotension (~60% decrease in blood pressure) that lasted for 15 min followed by a gradual recovery after 20 min (Fig. 7). In constrast, a similar dose of BjcL (200 μg/kg) caused only a slight decrease (<15%) in blood pressure with a return to basal values after 5 min. A lower dose of BjcL (100 μg/kg) produced no significant change in blood pressure (data not shown).

4. Discussion

Various SVL have been isolated from venoms of the genus *Bothrops*, including *B. atrox* (Gartner et al., 1980), *B. godmani* (Lomonte et al., 1990), *B. jararaca* (Ozeki et al., 1994), and *B. jararacussu* (Carvalho et al., 1998). Although there has been no systematic analysis of the occurrence of SVL within a given genus or species, it is quite possible that more than one type of these lectins may occur within a given venom. In agreement with this, a survey of gene expression in the venom glands of *B. insularis* using expressed sequence tags (ESTs) identified gene clusters for at least two SVL – one related to puff adder (*B. arietans*) lectin (PAL) and another to *T. stegnejeri* lectin (TSL) (Junqueira-de-Azevedo and Ho, 2002).

Based on its properties (molecular mass of ~29 kDa with identical monomer subunits, ability to cause hemagglutination, inhibition by lactose and EDTA), the BjcL isolated here is apparently the same as that purified by chromatography of *B. jararacussu* venom on an immobilized D-galactose column (Carvalho et al., 1998). These properties were also very similar to those of Ca^{2+}-dependent (C-type) lectins isolated from the venoms of other species of *Bothrops* and *Crotalus*. The venom content of BjcL (<1%) was similar to that of several SVL (generally ≤1%) (Ogilvie et al., 1986; Ozeki et al., 1994; Nikai et al., 2000). The single band seen in SDS-PAGE and immunoelectrophoresis, and the lack of enzymatic activity (esterase, PLA_2 and protease) indicated a high degree of purity for BjcL.

Whereas the physico-chemical properties of SVL have been well studied, considerably less is known about their immunological characteristics. SVL are antigenic and generally show immunoprecipitin lines in immunodiffusion (Gartner and Ogilvie, 1984; Ogilvie et al., 1986; Lomonte et al., 1990; Hirata et al., 1999; Nikai et al., 2000). Intrageneric and intergeneric cross-reactivity with antisera to SVL has been observed in some cases. Gartner and Ogilvie (1984)

observed that antiserum to *A. c. leukostoma* lectin interacted with *L. muta* lectin, while a later studied showed that antiserum raised against *L. muta* lectin cross-reacted with lectins from *A. c. leukostoma, A. p. piscivorus, B. atrox* and *C. atrox* (Ogilvie et al., 1986). Lomonte et al. (1990) noted that a lectin from *B. goldmani* venom cross-reacted with commerical antivenom raised against a pool of venoms that included *Bothrops asper, Lachesis muta stenophrys* and *Crotalus durissus durissus*. In contrast to these findings, neither the antiserum to *L. muta* lectin nor that to *A. c. leukostoma* cross-reacted with a lectin from *Dendroaspis jamesoni* venom (Gartner and Ogilvie, 1984; Ogilvie et al., 1986). The lack of reactivity between these antisera to crotalid lectins and an elapid lectin may indicate an immunological divergence between lectins in the venoms of these two groups, although an insufficient number of elapid venom lectins has been studied to confirm this possibility. Nevertheless, these results indicate that, despite similar biological activities, there may be marked immunological differences among SVL. The immunological relatedness among most SVL does not necessarily extent to other C-type, lectin-like venom proteins that are not true lectins. Thus, Castro et al. (2003) observed that *B. jararaca* venom lectin showed little immunoreactivity with antibodies to the C-type, lectin-like protein bothrojaracin from this same venom.

In agreement with the above studies, ELISA and immunoelectrophoresis showed that BjcL was recognized by commercial antivenom raised against a pool of *Bothrops* venoms, which included *B. jararacussu* venom, thus indicating that this protein is antigenic. Cross-reactivity with various other antivenoms was also observed by ELISA, with the order of reactivity being bothropic > elapidic > crotalic > arthropod antivenom. The lower reactivity seen with crotalic antivenom compared to elapidic antivenom was unexpected since the genera *Bothrops* and *Crotalus* are phylogenetically, and thus taxonomically, more closely related to each other than to

Micrurus (coral snakes), and also because elapid species are generally considered to be poor in lectins (Ogilvie and Gartner, 1984). Although no SVL have yet been purified from *Micrurus* venoms, a recent report has identified saccharide-binding lectins in the venoms of the elapids *B. fasciatus* and *B. multicinctus* (Zha et al., 2001). The lower reactivity of BjcL with crotalic antivenom raised against *Crotalus durissus terrificus* venom agrees with the low hemagglutinating activity reported for venoms from this subspecies (Francischetti *et al.*, 2000) and may indicate that this venom has a lower content of true lectins (although it does contain convulxin, a potent platelet-aggregating C-type, lectin-like protein). Some of this variation in the immunoreactivity of BjcL could also reflect differences in the content of anti-SVL IgG in the antivenoms. The lowest reactivity for BjcL was observed with antiserum raised against arthropod (spider and scorpion) venoms. This may indicate that these venoms are either poor in true lectins, or that there is little immunological identity between their lectins and SVL. In support of the latter possibility, the lectins identified so far in arthropod venoms are smaller than SVL (<20 kDa) (Liang and Pan, 1995; Li and Liang, 1999; Lu et al., 1999; Khoang et al., 2001), and this could influence their antigenic properties and cross-reactivity with antisera to SVL.

As with *B. jararaca* venom lectin (Ozeki et al., 1994), BjcL did not aggregate human platelets. However, the ability to aggregate platelets apparently varies among SVL since Ogilvie et al. (1989) reported that lectins from the venoms of *A. p. leukostoma*, *C. atrox* and *L. muta* caused aggregation that was inhibitable by lactose, whereas a lectin from *B. atrox* venom caused only occasional platelet aggregation while *A. c. contortrix* venom lectin had no effect. The different abilities to aggregate platelets probably reflect the presence of structural variations among these proteins. This aggregation, which involves intracellular Ca^{2+} mobilization that is Na^{+}-independent (Wilson-Byl et al., 1991), is accompanied by the release of β-thrombomodulin, and is inhibitable

by agents (PGE_1 and PGI_2) that elevate intracellular cAMP levels, by monoclonal antibodies and a peptide (Arg-Gly-Asp-Ser) that block the GP IIb/IIIa receptor complexes on the platelet surface and, in the case of *L. muta* lectin, by antiserum to this protein (Ogilvie et al., 1989). Physiologically, SVL with platelet aggregating activity could enhance the action of other venom proteins, especially C-type lectin-like proteins that are active on platelets.

Bothrops venoms produce marked local effects such as edema, hemorrhage and necrosis (Gutiérrez and Lomonte, 2003) that are mediated principally by metalloproteinases (Gutiérrez and Rucavado, 2000) and myotoxic phospholipases A_2 (Gutiérrez and Lomonte, 1995). The contribution of SVL to these responses remains unclear. Lomonte et al. (1990) reported that a lectin from *B. goldmani* venom did not cause hemorrhage, myonecrosis, or lethality in mice, but did cause rapid (within 30 min) dose-dependent edema (12-50 μg/paw) that was sustained for up to 6 h. However, based on the low potency of this lectin and its low content in the venom, these authors concluded that this protein was probably not a major contributor to venom-induced edema. As shown here, BjcL also produced dose-dependent edema and increased vascular permeability, with the maximal responses occurring within 1 h and persisting for up to 6 h. Although the venom was more potent than BjcL at all doses, especially the lower ones, the time-dependent profiles for the edema and the changes in vascular permeability produced by BjcL paralleled those of the venom. The doses of BjcL required to produce edema were similar to those reported for various plant lectins in rat and mouse hind paws (Bento et al., 1993; Freire et al., 2003). Although we have not investigated the mechanism of BjcL-induced edema, it may well involve histamine and/or serotonin since Lomonte et al. (1990) observed that pretreating mice with cyproheptadine (a histamine and serotonin receptor antagonist) significantly reduced the

edema induced by lectin from *B. goldmani* venom. However, Aragon-Ortiz et al. (1990) observed that the lectin from *L. muta* venom did not cause histamine release from isolated mast cells.

The cardiovascular actions of SVL have not been extensively investigated. As shown here, the i.v. administration of BjcL to anesthetized rats caused only a small, transient hypotension at a dose of 200 μg/kg. In contrast, a similar dose of venom resulted in a marked and sustained decrease in blood pressure. This observation suggests that at this dose, BjcL probably did not contribute to the hypotension observed. Indeed, since BjcL accounts for <1% of the venom, a venom dose of 200 μg/kg would contain <2 μg of lectin/kg, a dose that has no effect on blood pressure (results not shown). Conversely, a BjcL dose of 200 μg/kg would correspond to a venom dose of ~20 mg/kg, sufficient to kill rats within 5 min. Although the average yield of venom from *B. jararacussu* is ~150-250 mg (Belluomini, 1963, 1968; Kaiser and Michl, 1971; Sanchez et al., 1992), which would correspond to <1.5-2.5 mg of BjcL, the amount of venom injected in a bite and the subsequent concentrations of circulating BjcL are unknown, so it is unclear what the real contribution of BjcL to blood pressure changes would be. It is possible that a higher dose of BjcL than that tested here may have had a greater effect on the cardiovascular system. This would agree with Aragón-Ortiz et al. (1989) who showed that *L. muta* venom lectin caused marked hypotension in rats at a dose of 1.5 mg/kg, with double this dose being lethal to the animals.

The mechanism by which SVL may affect the cardiovascular system is unclear, but could involve alterations in intracellular calcium levels and mobilization similar to those observed in skeletal muscle treated with *B. arietans* (Ohkura et al., 1996) or *T. okinavensis* (Hirata et al., 1999) venom lectin. Alterations in calcium fluxes and intracellular levels have also been implicated in the deleterious actions of plant lectins in cardiac tissue (Makino et al., 1988; Zhao et al., 1989; Ma et al., 1995). The hypotension induced by plant lectins involves an action on blood vessels

(Zhang et al., 1994; Christiansen et al., 1995), perhaps through decreased vasoconstrictor and increased vasodilator production. A study of the hypotension caused by a lectin from the edible muschroom *Tricholoma mongolicum* indicated that adenosine A_2 receptors and nitric oxide formation were involved, with no participation by the adrenergic, cholinergic, histaminergic or renin-angiotensin systems (Wang et al., 1996). Whether similar mechanisms are involved in the cardiovascular actions of SVL remains to be determined.

In conclusion, the findings of this study have extended the biological activities of BjcL and have shown that this lectin may be involved in the local effects (edema and increased vascular permeability) seen after envenoming by this species; the contribution of BjcL to changes in blood pressure is less clear. This protein apparently has no significant effect on platelet aggregation, at least in vitro. Together, these findings suggest that the actions of BjcL may be more local than systemic. The immunoreactivity of BjcL with commercial bothropic antivenom indicates that there may be neutralization of the biological activity of this protein during treatment with antivenom.

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FIGURE LEGENDS

Figure 1. Purification of BjcL on non-derivatized Sepharose 4B (A) and elution profile following gel filtration on Sephacryl S-200 HR (B). Panel C shows the SDS-PAGE profile of BjcL treated with β-mercaptoethanol; the non-reduced protein had a molecular mass of ~29 kDa (not shown). BjcL was purified from *B. jararacussu* venom as described in Methods. For electrophoresis, 7 μg were applied to a 10% gel which was run and the gels then silver-stained. The molecular mass markers (in kDa) were lysozyme (14.4), trypsin inhibitor (20.1), carbonic anhydrase (31), ovalbumin (45), BSA (66), and phosphorylase b (97).

Figure 2. Immunoelectrophoresis of *B. jararacussu* venom and BjcL. Forty micrograms of venom and BjcL were applied to wells cut in 1% agarose gels and electrophoresed at 120 V for 90 min. After electrophoresis, a central trough was cut in the gel and filled with commercial bothropic antiserum. The slide was then placed in a humidified chamber for 48 h to allow for diffusion to occur. Washing and staining were then done as described in Methods.

Figure 3. ELISA immunoreactivity of *B. jararacussu* venom and BjcL with commercial bothropic antivenom (A) and cross-reactivity of BjcL with various commercial antivenoms (B). The ELISAs were done as described in Methods.

Figure 4. Failure of BjcL (200 μg/ml) to induce platelet aggregation in PRP (upper traces) and washed platelets (lower traces) and lack of effect on aggregation induced by ADP (3 μM, in platelet-rich plasma, PRP) and thrombin (100 IU/ml, in washed platelets, WP). BjcL was

incubated with PRP or WP prior to stimulating the platelets with the respective antagonists. The tracings are representative of three experiments each.

Figure 5. Time-dependent changes in the edema (A) and vascular permeability (B) of mouse hind paws following the injection of *B. jararacussu* venom (30 μg/paw) and BjcL (30 μg/paw). The points represent the mean±S.E.M. of 4-5 mice.

Figure 6. Dose-dependence of the edema (A) and the increase in vascular permeability (B) produced by BjcL in mouse hind paws. Lower doses of BjcL (≤5 μg/kg) did not cause edema or alter vascular permeability. Note that at the doses tested, the changes observed with *B. jararacussu* venom showed little dose-dependence. The columns are the means±S.E.M.of 4-5 mice. *$p<0.05$ compared to venom. #$p<0.05$ compared to other venom doses. The responses to the three doses of BjcL in each panel were also significantly different ($p<0.05$) among themselves.

Figure 7. Blood pressure changes in anesthetized rats following the intravenous injection of *B. jararacussu* venom and BjcL (200 μg/kg each). The points are the mean±S.E.M. of 5 rats. The decrease in blood pressure from 0.5 to 2 min for BjcL and for all time points from 0.5 min onwards for venom was significantly different ($p<0.05$) from the pre-injection (resting) value indicated by the dashed line.

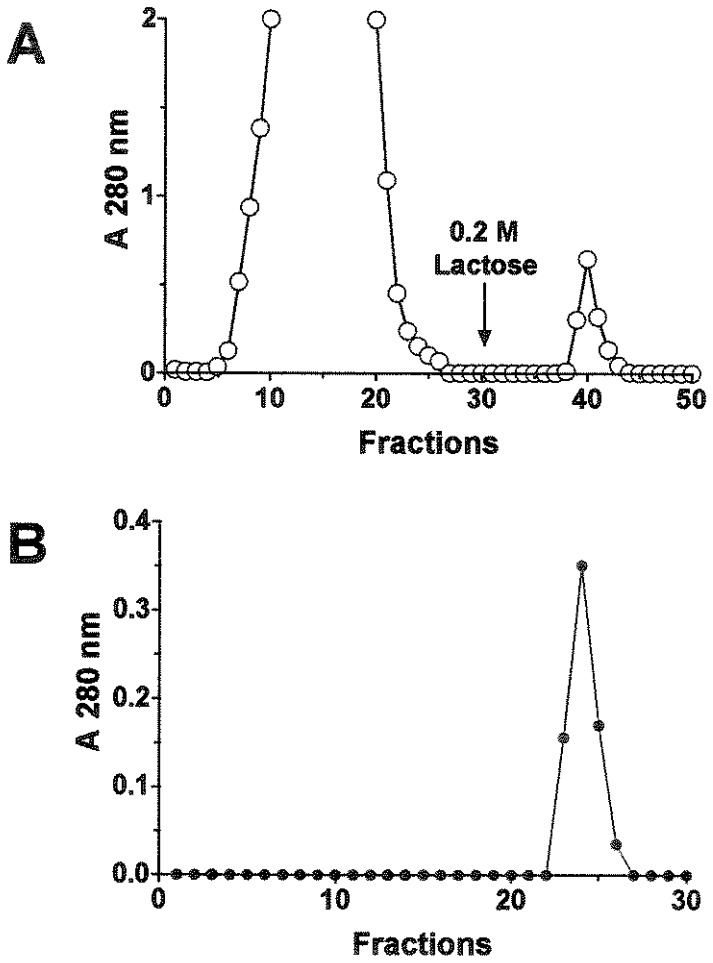
A
A 280 nm
2
1
0
0.2 M
Lactose
0
10
20
30
40
50
Fractions
B
A 280 nm
0.4
0.3
0.2
0.1
0.0
0
10
20
30
Fractions
C
kDa
M
V
L
97
66
45
31
20.1
14.4

FIGURE 1

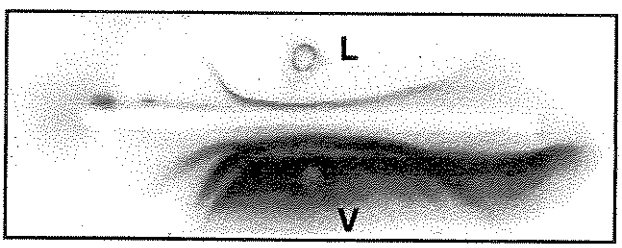

FIGURE 2

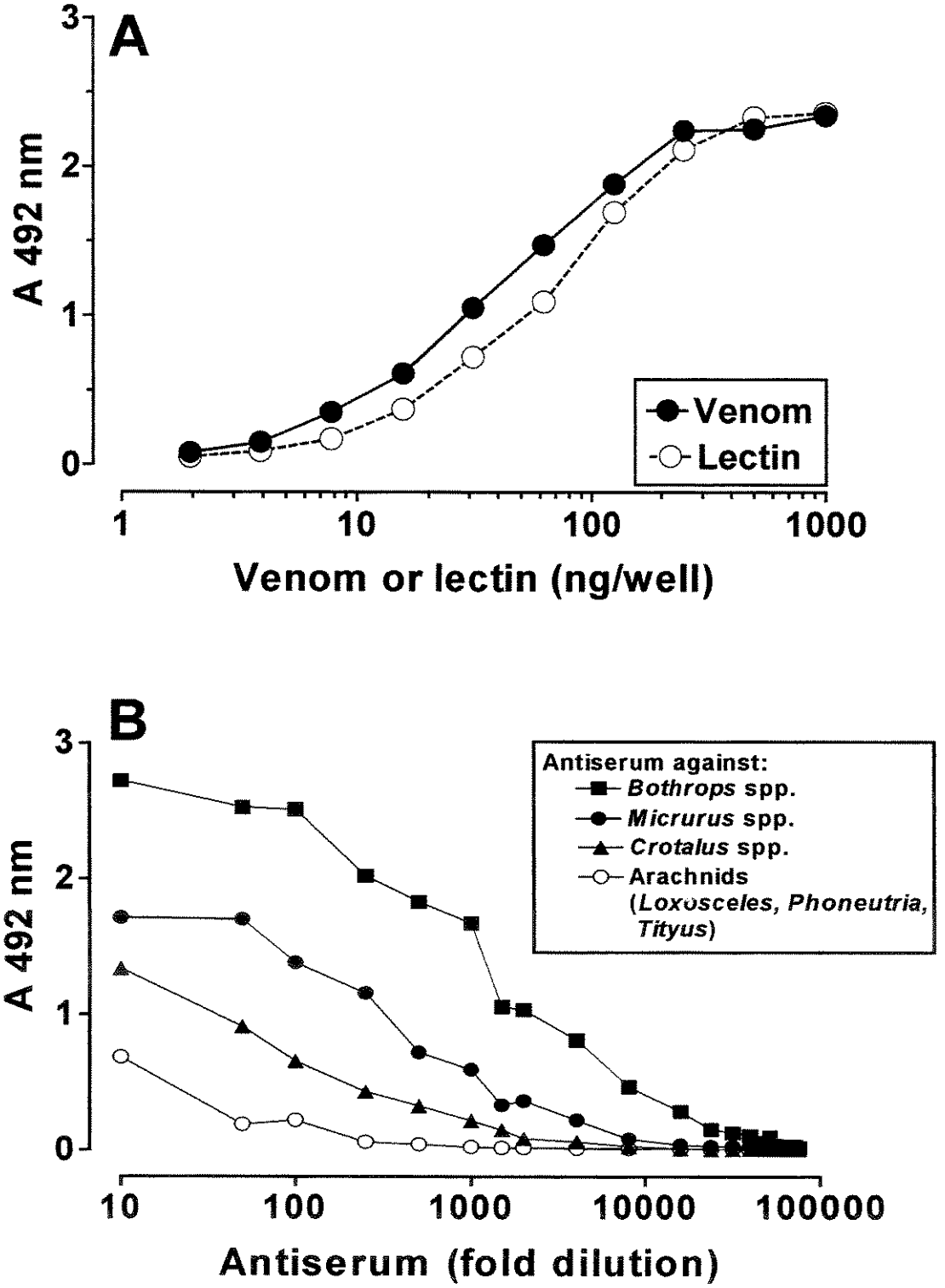
A
A 492 nm
3
2
1
0
Venom
Lectin
1
10
100
1000
Venom or lectin (ng/well)
B
A 492 nm
3
2
1
0
Antiserum against:
Bothrops spp.
Micrurus spp.
Crotalus spp.
Arachnids
(Loxosceles, Phoneutria, Tityus)
10
100
1000
10000
100000
Antiserum (fold dilution)

FIGURE 3

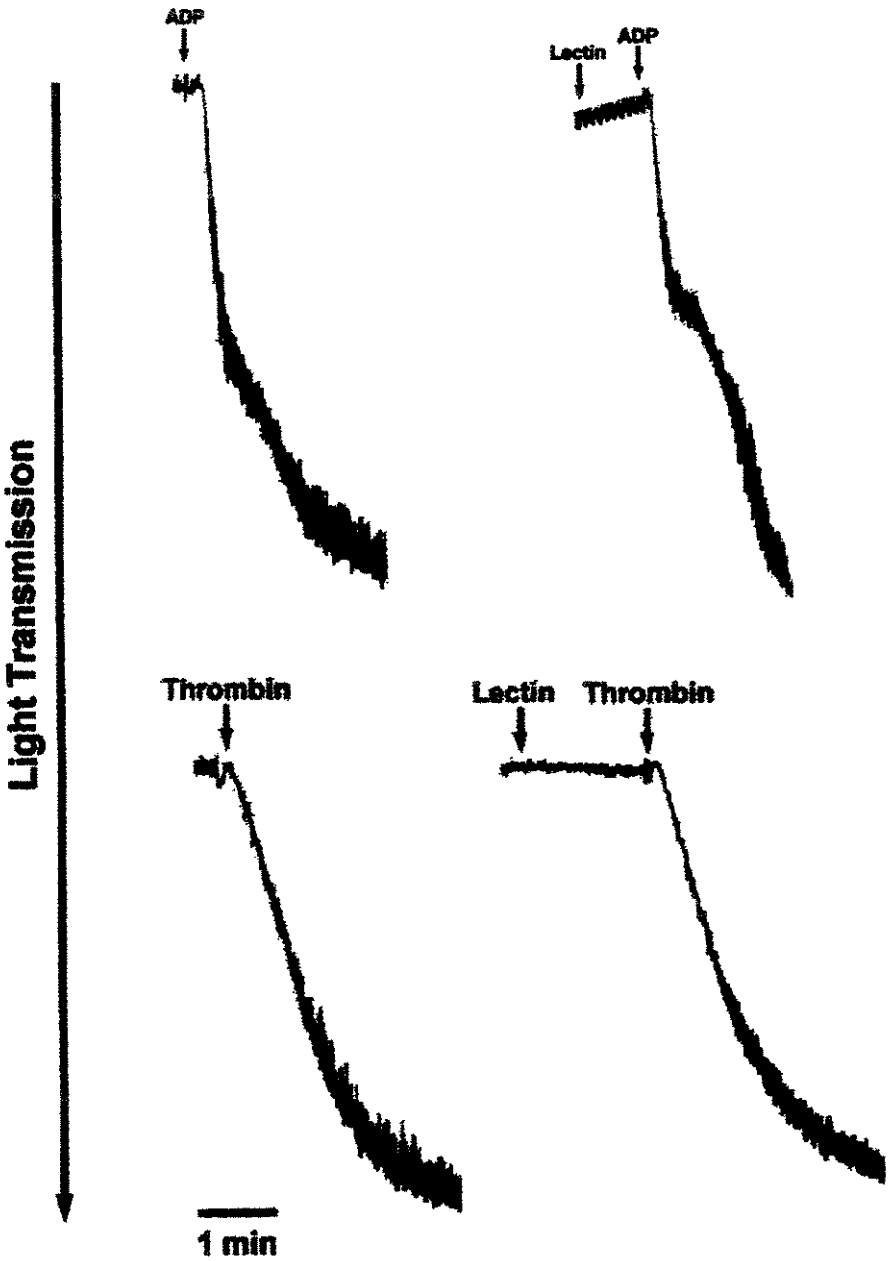
ADP
Lectin
ADP
Light Transmission
Thrombin
Lectin
Thrombin
1 min

FIGURE 4

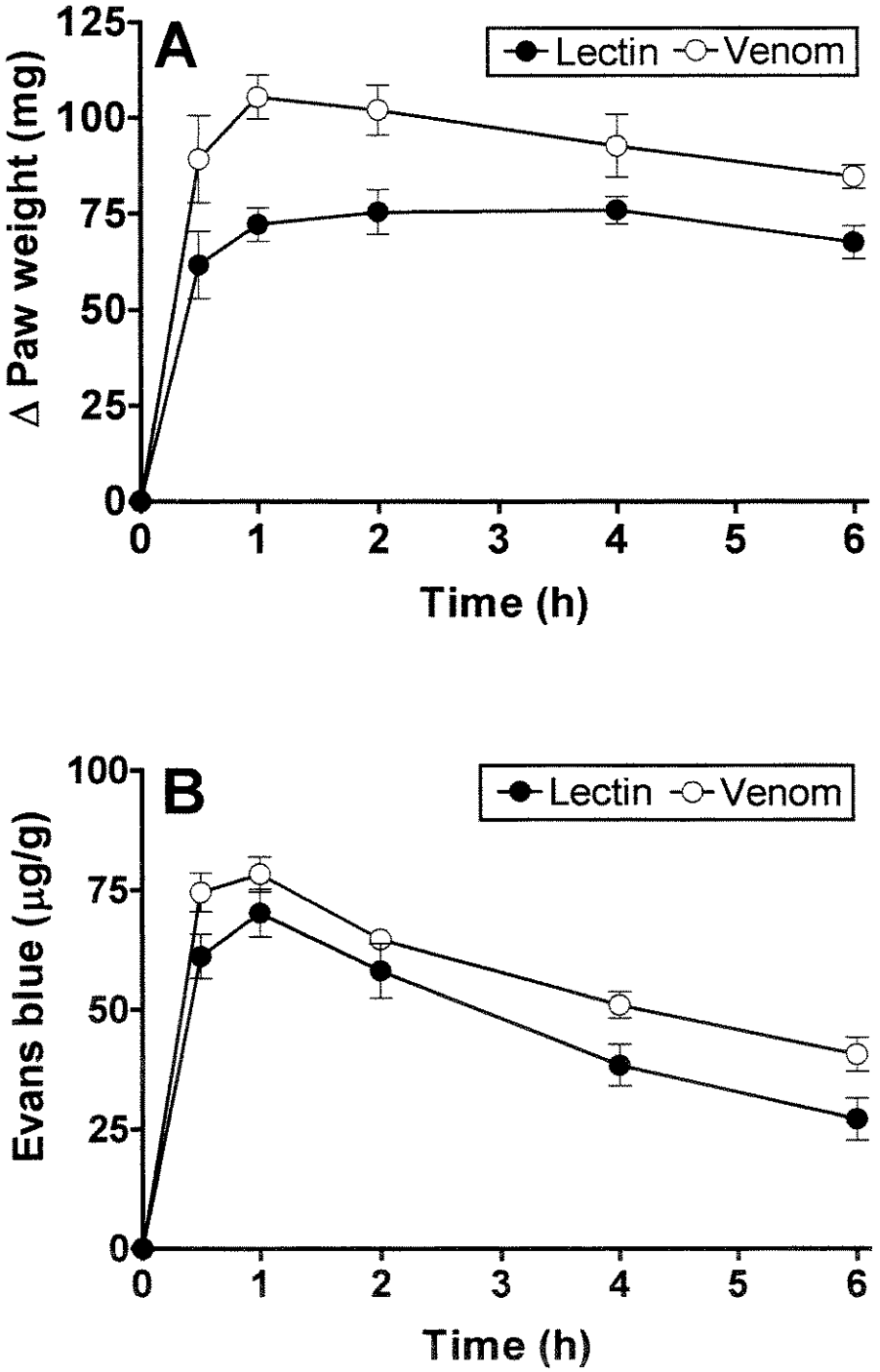
A
Lectin
Venom
Δ Paw weight (mg)
125
100
75
50
25
0
0
1
2
3
4
5
6
Time (h)
B
Lectin
Venom
Evans blue (µg/g)
100
75
50
25
0
0
1
2
3
4
5
6
Time (h)

FIGURE 5

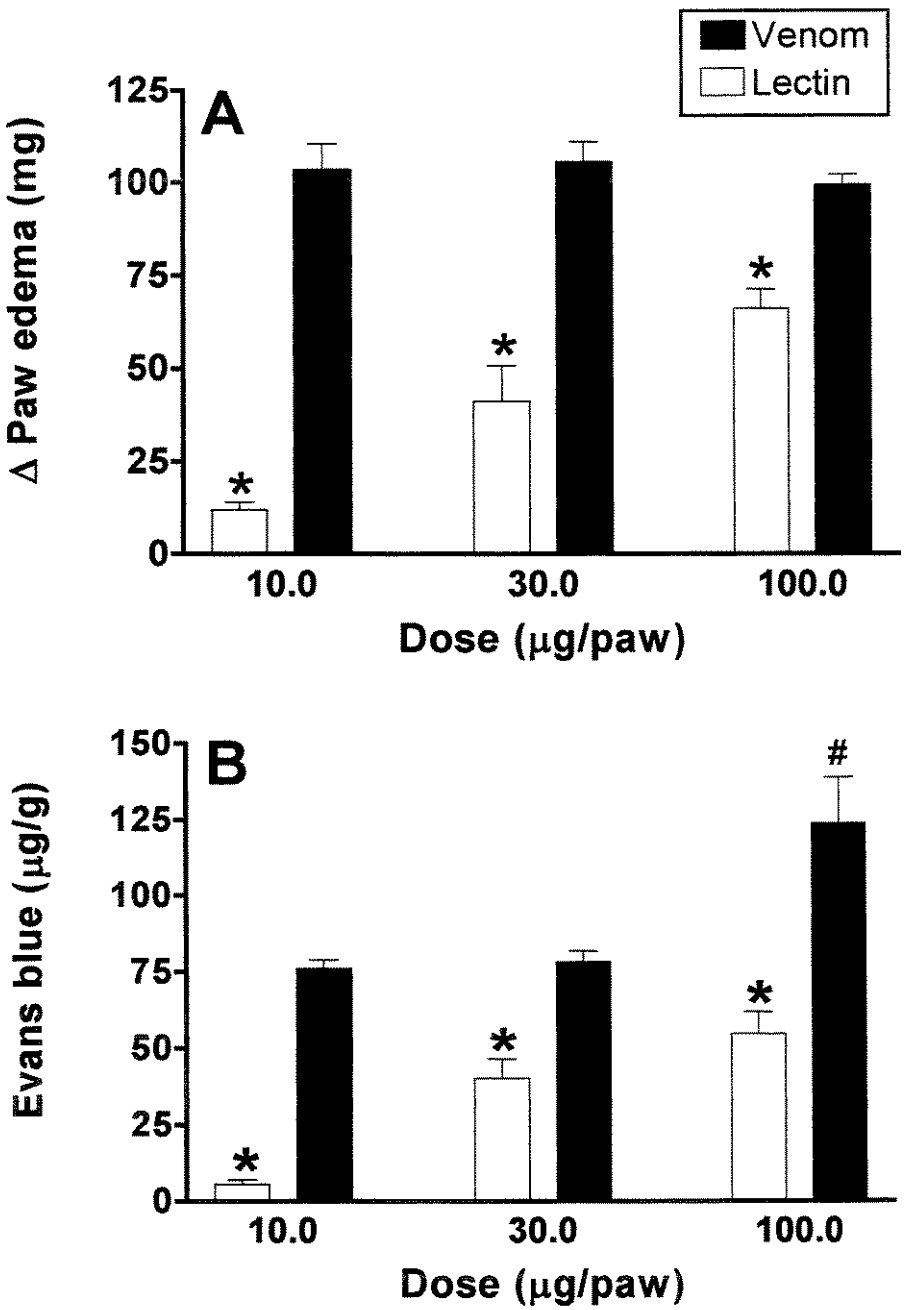
A
Venom
Lectin
Δ Paw edema (mg)
125
100
75
50
25
0
*
*
*
10.0
30.0
100.0
Dose (μg/paw)
B
Evans blue (μg/g)
150
125
100
75
50
25
0
*
*
*
#
10.0
30.0
100.0
Dose (μg/paw)

FIGURE 6

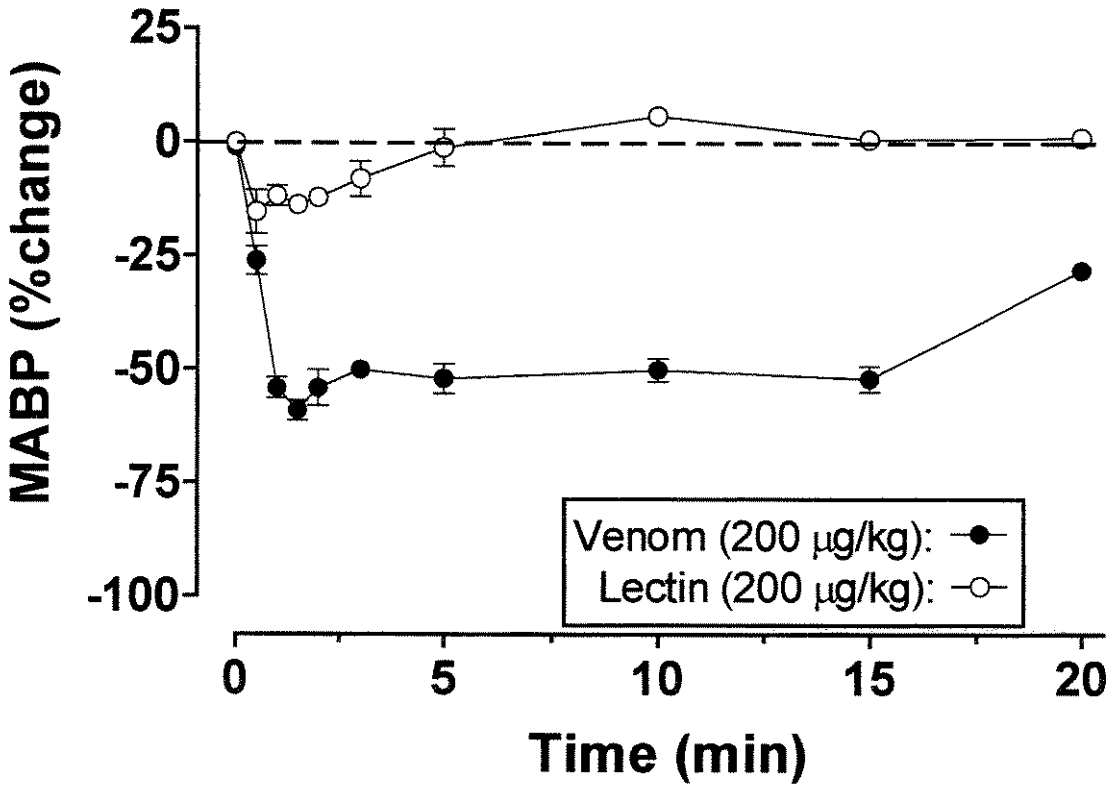
MABP (%change)
25
0
-25
-50
-75
-100
0
5
10
15
20
Time (min)
Venom (200 μg/kg):
Lectin (200 μg/kg):

FIGURE 7