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Kelly Fabiane Santos Ricardo

Prevenção das disfunções vasculares no início da aterogênese: Efeito da S-Nitroso-N-Acetilcisteína, SNAC, em camundongos "*knock out*" para o receptor da LDL.

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Kelly Fabiane Santos Ricardo

e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Biologia Funcional e Molecular. Área de concentração: Fisiologia

Orientadora: Prof Dra Marta Helena Krieger

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Dedico

À

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Aos meus pais Enedino e Iraci

Principalmente a minha mãe, Iraci Santos Ricardo.

Exemplo de coragem e força espiritual nos momentos mais difíceis...

E ainda, pelo imenso amor dedicado aos filhos!

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Ricardo e Thiago, hoje vocês estão na companhia do Pai.

Mas sempre serão motivos de orgulho para nós!

Saudades.....

Provações te buscaram.

Dificuldades te agitaram.

Tudo parece noite ao redor de teus passos.

Não te detenhas, no entanto, a fim de medir as sombras.

Prossegue trabalhando

E não te afastes da paciência.

Por nada te desesperes.

Dê tempo a Deus para que te acenda nova luz.

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RESUMO

Aterogênese e a hipercolesterolemia são patologias envolvidas principalmente com a disfunção vascular, a qual se correlaciona diretamente com o aumento no nível de estresse oxidativo no ambiente vascular. Alterações na biodisponibilidade da molécula de óxido nítrico (NO) e no sistema NO/NO sintase, constituem-se eventos chave no processo aterogênico vascular. Assim, as restaurações destas alterações podem ser promovidas por doadores de NO.

Recentemente, o reconhecimento de que os S-nitrosotióis atuam como fontes endógenas de NO têm se tornado muito evidente. Eles podem ocorrer naturalmente no plasma ou ainda podem ser sintetizados quimicamente. Dentre estes últimos, elegemos o SNAC (S-nitroso-N-acetilcisteína) para nossas investigações.

Em nosso primeiro estudo, caracterizamos o efeito hipotensor do SNAC em animais normotensos e hipertensos acordados. Os resultados mostraram que: i) O SNAC possui efeito hipotensor dose-dependente positivo, e parcialmente dependente do GMPc; ii) Seu efeito vasodilatador é mais potente e duradouro que o nitroprussiato de sódio (NPS); iii) A associação do NPS com N - acetil - L - cisteína (NAC) resultou em uma potencialização do efeito vasodilatador do NPS. Esses resultados indicam que o SNAC pode estar envolvido no mecanismo de vasodilatação promovido pelo NPS, atuando como um intermediário na doação de NO, por meio da reação de transnitrosação.

Em nosso segundo estudo, caracterizamos o efeito anti aterogênico do SNAC em camundongos knockout para o receptor da LDL, sob dieta hipercolesterolêmica (20% gordura, 1, 25 % colesterol e 0, 5 % de ácido cólico). Os camundongos foram divididos em 3 grupos: controle, HC (dieta hipercolesterolêmica), SNAC+HC (0,51 μmol/Kg, i.p

diariamente e dieta hipercolesterolêmica). Após duas semanas de tratamento, verificamos que camundongos sob dieta hipercolesterolêmica apresentaram as seguintes disfunções relacionadas ao início da aterogênese: i) alterações na motricidade vascular dependente e independente do endotélio, ii) aumento do estresse oxidativo, iii) desenvolvimento da área da lesão próxima a raiz da aorta, iv) aumento nos níveis de lípides plasmáticos e v) alterações no perfil lipídico, vi) aumento na expressão das NO sintases aórticas, vii) aumento na coloração para nitrotirosina.

Após duas semanas de tratamento com SNAC e dieta hipercolesterolêmica, verificou-se uma prevenção de 55 % na formação da placa e uma restauração nas alterações desencadeadas pela dieta hipercolesterolêmica. As disfunções na motricidade vascular foram normalizadas e acompanhadas de uma redução no estresse oxidativo e na superexpressão das NOS constitutivas. Finalmente, nossos resultados mostraram que no início do processo aterosclerótico há alterações no sistema NO/NO sintase e um aumento no estresse oxidativo. Essas alterações estão correlacionadas principalmente com o aumento no vasorelaxamento dependente do endotélio e com o aumento na expressão das NOS constitutivas. O SNAC mostrou ser uma droga promissora, pois pode reverter ou minimizar as alterações relacionadas com a fase inicial da aterosclerose.

ABSTRACT

Pathophysiology of the NO/NO synthase system and endothelium dysfunctional changes in early phases of the atherogenic process are incompletely understood. In hypercholesterolemic LDLr-/- mice, we addressed changes in endothelium-dependent relaxations, NO synthase expression and plaque burden in the absence or presence of the nitrosothiol NO donor S-nitroso-N-acetylcysteine (SNAC). Initially, we characterized the hypotensive effect of the S-nitroso-N-acetylcysteine (SNAC) in normotensive and hypertensive conscious rats. The results showed that i) SNAC reduced the medium arterial pressure in a dose–response manner in both normotensive and hypertensive animals; ii) At the same doses (EC_{50} of SNAC), SNAC showed a vasodilator effect in normotensive rats more potent and more prolonged than that of sodium nitroprusside (SNP); iii) SNAC acts by both cGMP-dependent and cGMP-independent pathways; iii) It was also shown that the thiol N - acetyl – L - cysteine (NAC) potentiates the action of SNP in hypertensive rats, pointing to the mediation of thiols in the vasodilator action of SNP in this condition. Such mediation may involve the formation of a more potent thiol complex with the nitroprusside anion or the transfer of ·NO to NAC, generating SNAC as a primary vasoactive species.

In the second study we characterized the SNAC effect on the endothelial dysfunction in hypercholesterolemic LDLr-/- mice for two weeks. Increase in plasma cholesterol/triglyceride levels was accompanied by aortic root lesions. Aortic vasorelaxation to acetylcholine was paradoxically increased, while endothelium-independent relaxations to sodium nitroprusside were decreased suggesting enhanced basal or stimulated NO release. This dysfunction was associated with enhanced aortic superoxide production and with increased levels of constitutive NOS isoform expression, particularly neuronal NOS. SNAC (0.51μmol/Kg i.p., per two weeks) treatment in HC mice (SNAC +

HC) decreased plaque extension by 55%, prevented the alterations in vasorelaxation and brought about a 20% decrease in cholesterol levels. In conclusion, the present study disclosed, in early stages of plaque development in LDLr-/- mice, particular changes in NO/NOS pathophysiology, characterized by increased endothelium-dependent vasorelaxation and increased constitutive NOS expression. Such changes were prevented by SNAC, which may therefore constitute a novel strategy to halt early plaque progression.

LISTA DE ABREVIATURAS

SNAC - S-nitroso-N-acetilcisteína

NPS - nitroprussiato de Sódio

NAC - N - acetil - L - cisteína

LDLr-/- - camundongos knockout para o receptor da LDL

NOS - óxido nítrico sintase

LDL - lipoproteína de baixa densidade

IDL - lipoproteína de densidade intermediária

VLDL - lipoproteína de muito baixa densidade

HDL - lipoproteína de alta densidade

$^{\cdot}NO$ - óxido nítrico

ApoE $LDLr^{-/-}$ - camundongos knockout para a ApoE a para o receptor da LDL

ERO - espécies reativas de oxigênio

$O_2^{\cdot -}$ - radical superóxido

$OH^{\cdot}$ – radical hidroxila

H_2O_2 - peróxido de hidrogênio

$ONOO^{-}$ - peroxinitrito

HOCl - ácido hipocloroso

RSH - tiol

Dieta HC - dieta hipercolesterolêmica (20 % de gordura, 1, 25 % de colesterol e 0, 5 % de ácido cólico)

SOD - superóxido dismutase

iNOS – enzima óxido nítrico sintase induzida

eNOS – enzima óxido nítrico sintase endotelial

nNOS - enzima óxido nítrico sintase neuronal

CML - célula muscular lisa

ACh-Acetilcolina

L-NAME - N^{G}-nitro-L-arginina

$h\nu$- Irradiação

SUMÁRIO

1 - INTRODUÇÃO

As doenças cardiovasculares são a maior causa da morbidade e da mortalidade no mundo ocidental (KEANEY, 2000). Dentre essas patologias, a principal é a aterosclerose, a qual se caracteriza por uma resposta inflamatória e fibroproliferativa, causada por agressões físicas e químicas à parede arterial (ROSS, 1999). As alterações vasculares que ocorrem na aterogênese estão relacionadas com um aumento no nível de estresse oxidativo promovido por alterações no ambiente redox vascular. Contudo, a elaboração de um modelo para explicar a regulação da função e da estrutura vasculares centrados em processos redox, constitui-se um dos maiores desafios na fisiologia vascular (LAURINDO, 2003).

As lesões ateroscleróticas se desenvolvem preferencialmente no arco aórtico devido a uma diferença na dinâmica do fluxo sanguíneo (LUSIS, 2000). Na fase inicial, essas lesões se caracterizam pelo aumento da concentração de LDL circulante, a qual se difunde passivamente através dos espaços intercelulares das células endoteliais. Isso, conseqüentemente, gera um acúmulo dessa lipoproteína na matriz subendotelial. Na parede do vaso a LDL pode sofrer alterações em sua estrutura, tais como: lipólise, proteólise e oxidação. Essas alterações aumentam a afinidade entre LDL e os macrófagos, originando as células espumosas (LUSIS, 2000).

A progressão das estrias gordurosas para lesões arteriais complexas requer a infiltração de células inflamatórias e também a proliferação de células musculares lisas, produtoras de matriz extracelular, as quais constituem o maior componente em volume no ateroma avançado (LUSIS, 2000; GENG; LIBBY, 2002). A identificação dos fatores envolvidos e a regulação das alterações funcionais e estruturais que ocorrem na fase inicial da aterogênese não estão totalmente esclarecidas. E, sobretudo, há enorme dificuldade em

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encontrar um modelo experimental adequado à resolução desse problema. Em decorrência disso, vários modelos animais são utilizados, tais como: coelhos, porcos, primatas e roedores. Atualmente, os animais geneticamente modificados têm sido muito utilizados em estudos relacionados com a aterogênese. Nesse grupo destacam-se os camundongos LDLr-/- (knockout para o receptor da lipoproteína de baixa densidade). Os camundongos dessa linhagem, quando alimentados com dieta rica em colesterol, desenvolvem em curto período de tempo, elevação nos níveis plasmáticos de colesterol e das frações de IDL, LDL, VLDL (ISHIBASHI et al., 1993; AJI et al., 1997; VÉNIANT et al., 2001). E, conseqüentemente, desenvolvem placa de ateroma, principalmente na aorta (TANGIRALA et al., 1995; MERAT et al., 1999). Essas alterações no perfil lipídico somadas ao incremento na permeabilidade endotelial às lipoproteínas e outros constituintes plasmáticos contribuem para o desencadeamento da disfunção endotelial (VERBEUSEN et al., 1986; FREIMAN et al., 1986).

Inúmeros estudos experimentais direcionados às alterações vasculares na aterosclerose documentam a utilização de estratégias terapêuticas antioxidantes na reversão da disfunção endotelial (LEVINI et al., 1996), na extensão do ateroma (NIGRIS et al., 2000) ou mesmo na infiltração vascular de macrófagos (GALIS et al., 1998). Assim, utilizamos no presente estudo camundongos LDLr-/- hipercolesterolêmicos, na fase inicial da aterosclerose, tratados com SNAC, um nitrosotiol doador de óxido nítrico (NO).

1.1. Endotélio e a reatividade vascular

As células endoteliais são capazes de detectar estímulos mecânicos, físicos e químicos respondendo adequadamente e processando as informações, liberando fatores que modificam o tônus vascular, a função plaquetária, a adesão de moléculas a sua superfície,

as funções metabólicas e a permeabilidade capilar (DA LUZ; FAVARATO, 2003; LAROIA et al., 2003). Dentre estas funções, o controle do tônus vascular se dá pela interação das células endoteliais e as células musculares lisas (CML), as quais compõem as camadas musculares dos vasos. Assim, o tônus vascular normal exercido pelas CMLs, é dependente de fatores mitogênicos, da inervação simpática, de metabólitos locais como potássio e adenosina, bem como de outros fatores vasodilatadores e vasoconstritores sintetizados pelas células endoteliais.

A molécula de ·NO desempenha a maior relevância dentre as substâncias vasodilatadoras secretadas pelo endotélio, sendo o fator preponderante na manutenção do tônus vascular. Sua biodisponibilidade é alterada em várias patologias como hipertensão, aterosclerose e hipercolesterolemia.

A função do ·NO não se restringe apenas ao controle do tônus da musculatura lisa do vaso, por meio da vasodilatação, mas também promove a inibição de diversos processos pró aterogênicos como, por exemplo, a oxidação da LDL, a adesão de monócitos, a agregação plaquetária, migração e a proliferação de células musculares lisas. Assim, a restauração da biodisponibilidade do ·NO pode prevenir ou restaurar as disfunções endoteliais, o que irá gerar uma melhora global da reatividade vascular (JIANG et al., 2000; NAPOLI; IGNARRO, 2001; VITA et al., 2002).

Nas condições fisiológicas, o conjunto de fatores citados acima promove a manutenção do tônus vascular basal e da fluidez do sangue e, ainda, inibe o processo inflamatório arterial, bem como a proliferação de células musculares lisas. Entretanto, na presença de fatores de risco para doenças cardiovasculares, o endotélio pode sofrer alterações funcionais e fenotípicas que contribuem para a instalação do processo

inflamatório, da trombose, da vasoconstrição e, finalmente da aterosclerose (VITA et al., 2002).

1.2. Disfunções vasculares promovidas pela aterogênese

A função vasomotora dependente do endotélio, mediada pelo ·NO, é uma indicação importante de sua integridade funcional, tanto *in vitro* como *in vivo*. Enquanto a uma resposta vasorelaxante independente do endotélio reflete a integridade funcional da CML. Tanto as disfunções dependentes como as independentes do endotélio podem indicar uma disfunção na biodisponibilidade do ·NO.

Alterações no vasorelaxamento dependente do endotélio foram caracterizadas em diversas patologias, tais como, hipertensão arterial, hiperlipidemia, aterosclerose entre outras. Vários autores descreveram que primatas, coelhos e camundongos duplo knockout para ApoE e para o receptor da LDL (ApoE LDLr-/-), alimentados com dieta hipercolesterolêmica (dieta HC) por 2, 4 ou 10 semanas, apresentaram uma redução na resposta vasorelaxante dependente do endotélio (FAGGIOTTO et al., 1984; KEANEY et al., 1995; LAPIMG et al., 1999). Contudo, uma análise minuciosa destes estudos evidencia que a patofisiologia das disfunções endoteliais que ocorrem no início da aterosclerose, não está totalmente esclarecida. E, além disso, a ligação entre hipercolesterolemia, alterações no sistema NO/NO sintase, aumento do estresse oxidativo e desacoplamento da NOS vascular apresenta uma série de questões em aberto (HARRISON et al., 2003; MÜGGE, 1998).

1.3. Espécies reativas de oxigênio (ERO)

Radical livre pode ser definido como qualquer espécie química que possui elétrons desemparelhados na última camada de valência e se constitui de um intermediário capaz de

existência independente (BETTERIDGE, 2000). Conseqüentemente, o radical livre pode retirar um elétron de uma outra substância para se estabilizar (ação oxidante), ou pode doar este elétron para uma outra substância para se estabilizar (ação redutora).

O termo espécies reativas de oxigênio (ERO) é usado para designar radicais livres e intermediários relacionados que, embora não sejam radicais livres *per se*, participam freqüentemente da produção dos mesmos.

O superóxido ($O_2^{\cdot -}$) e o radical hidroxila ($OH^{\cdot}$) são os principais radicais livres de importância biológica, formados a partir do oxigênio molecular. Outras espécies reativas de oxigênio, como o peróxido de hidrogênio (H_2O_2), peroxinitrito ($ONOO^-$) e o ácido hipocloroso (HOCl) não são radicais livres *per se*, mas possuem um forte efeito oxidante, e conseqüentemente, contribuem para o aumento do estresse oxidativo (KODJA; HARRISON, 1999; CAI; HARRISON, 2000).

O estresse oxidativo pode ser verificado nas patofisiologias vasculares através do aumento na geração das ERO. Este aumento ocorre quando mecanismos de defesa vasculares são esgotados por excesso de produção de radicais livres ou quando tais mecanismos não são acessíveis ou ainda não estão eficientes para tamponar o efeito dessas espécies (LAURINDO, 2003).

No sistema vascular, ambos o superóxido e o $^{\cdot}NO$, são radicais instáveis e muito reativos, sendo que a constante de reação está estimada em $7 \times 10^{9}\ M^{-1}\ s^{-1}$. Essa reação é três vezes mais rápida que a dismutação do superóxido pela superóxido dismutase. Assim, demonstrando que o aumento da geração de superóxido na parede vascular pode inibir as ações fisiológicas do $^{\cdot}NO$ (MÜGGE et al., 1998), gerando um aumento no nível de estresse oxidativo.

Em vários tipos celulares, particularmente em vasos, existe ampla evidência do envolvimento de ERO na sinalização, crescimento, proliferação, diferenciação, senescência e apoptose celular. Portanto, a disfunção endotelial, é considerada uma disfunção da sinalização redox (LAURINDO, 2003).

Em células de mamífero, as fontes enzimáticas de ERO incluem os seguintes sistemas: respiração mitocondrial, via do ácido araquidônico incluindo as enzimas lipoxigenase e ciclooxigenase, P450, xantina oxidase, NADH/NADPH oxidase, NO sintases, peroxidases e outras hemeproteínas. Mas no sistema cardiovascular a NADH/NADPH oxidase, a xantina oxidase e a NO sintase (ilustrado na Figura 1) têm sido preferencialmente estudados. (CAI; HARRISON, 2000).

Acredita-se que o estresse oxidativo pode ser prevenido ou reparado por meio de intervenções que bloqueiem as vias metabólicas de geração de ERO ou que simulem ou multipliquem os efeitos dos mecanismos de defesa fisiológicos (LAURINDO, 2003).

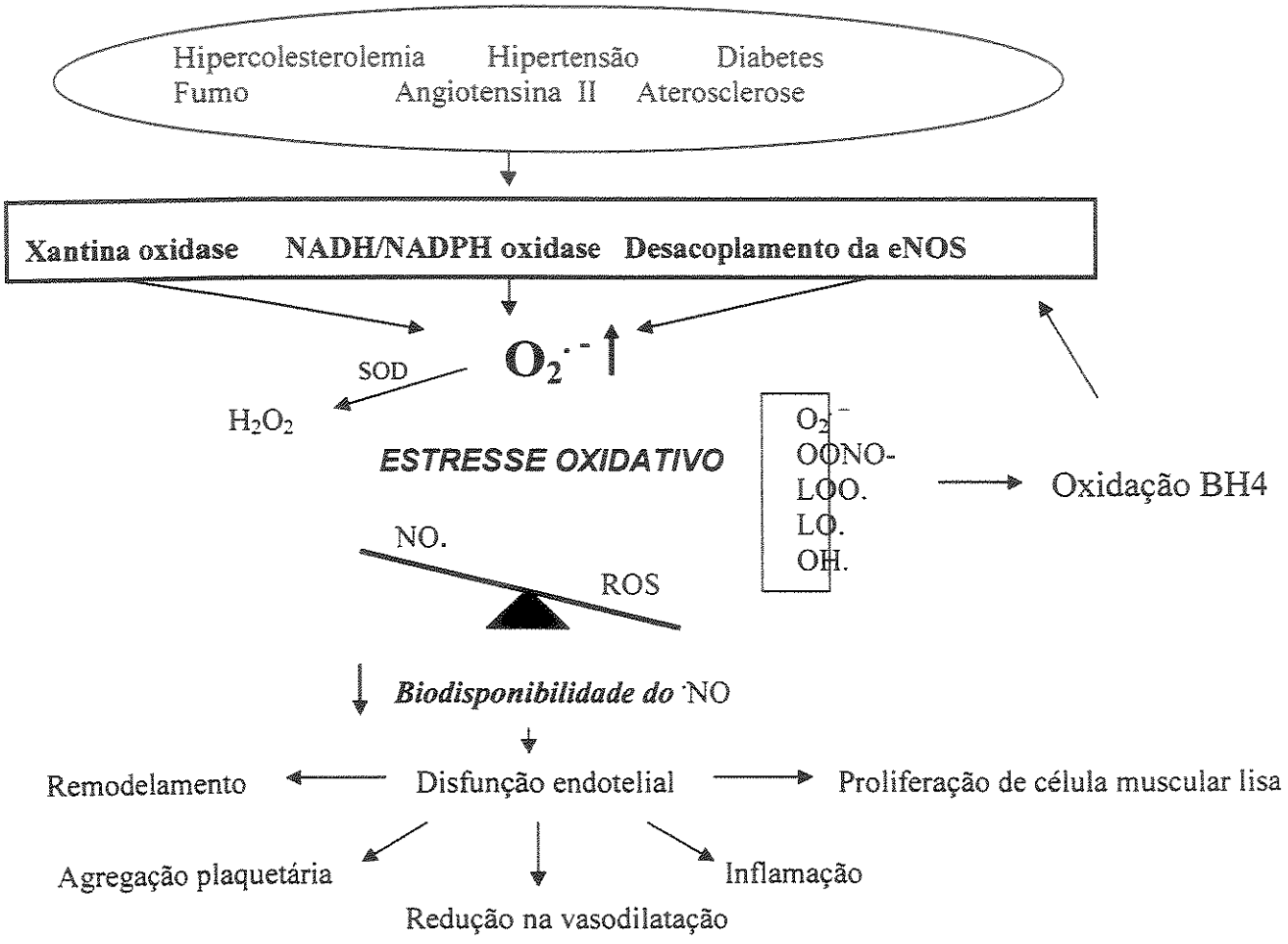

Figura 1: Fontes geradoras de superóxido com conseqüente redução da biodisponibilidade do oxido nítrico (Adaptado de CAI; HARRISON, 2000).

1.4. Biodisponibilidade de NO

O NO secretado pelas células é produzido por três isoformas bem caracterizadas das NO sintases, as quais oxidam a L-Arginina à L-Citrulina e são codificadas por genes distintos. As três isoformas das NOS têm uma estrutura molecular semelhante e requerem cofatores múltiplos, como as flavinas (FAD), NADPH e tetrahidrobiopterina (BH4), os quais são necessários para manter a dimerização da enzima e a produção de NO, conforme ilustrado na Figura 2.

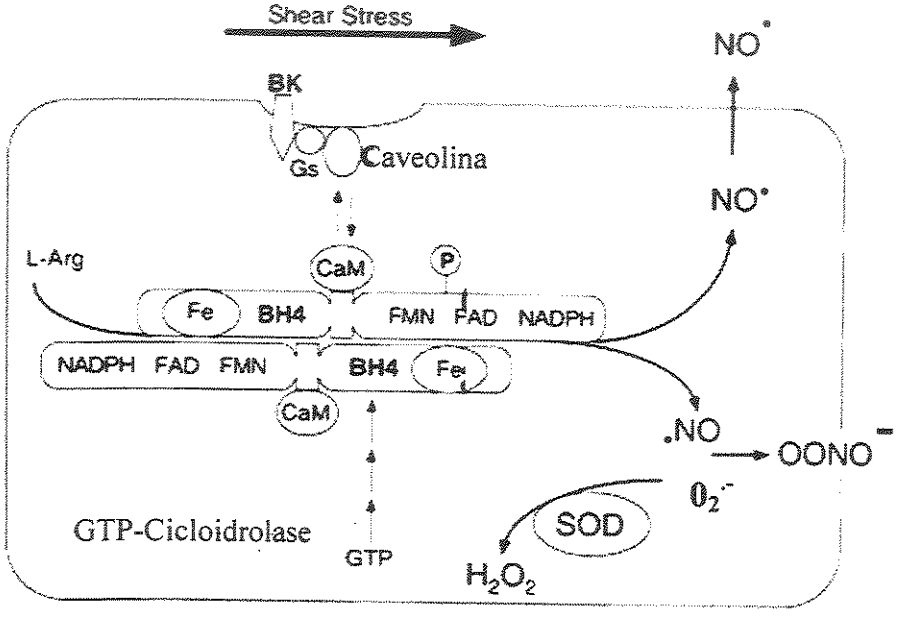

Figura 2: Regulação da NOS nas células endoteliais e sua interação com cofatores e superóxido (Reproduzido de CHANNON et al., 2000).

A isoforma neuronal (nNOS ou NOS 1) é expressa constitutivamente e sua atividade é regulada pelo complexo cálcio - calmodulina. Essa isoforma foi identificada inicialmente em neurônios, contudo hoje se sabe que também é expressa em células

musculares lisas da camada média e da adventícia dos vasos sanguíneos em condições normais e na aterosclerose (WILCOX et al., 1997; SCHWARZ et al., 1999). Recentemente, alguns autores correlacionaram a nNOS ao controle da pressão arterial (SULLIVAN et al., 2002; BOULANGER et al., 1998) e à aterogênese (WILCOX et al., 1997). A ação da nNOS pode ser alterada pela presença de formas "splice" alternativas conforme estudos relatados por Schwarz et al., (1999); Sullivan et al., (2002).

A eNOS é constitutivamente expressa em células endoteliais e sua atividade é regulada pelo complexo cálcio-calmodulina (CHANNON et al., 2000). Os mecanismos de ativação da eNOS têm se revelado os mais elaborados das 3 isoformas, talvez refletindo a complexidade do controle fisiológico dos diferentes leitos vasculares (MICHEL; FERON, 1997; GOVERS et al., 2001). Os mais conhecidos são: ativação via aumento da concentração citosólica de cálcio, "shear stress" e GMPc (HARRISON, 1997; LI; FÖRSTERMAN, 2000). Contudo, vários estudos descreveram uma relação entre o aumento na expressão da eNOS, a redução na biodisponibilidade do ·NO e um aumento de superóxido em vasos hipercolesterolêmicos e ateroscleróticos (MINOR et al., 1990; KANAZAWA et al., 1996; CHANNON et al., 2000; LAURSEN et al., 2001).

A iNOS foi identificada em vários tipos celulares, tais como: macrófagos, células endoteliais, células da musculatura lisa vascular e miócitos cardíacos. Essa isoforma é regulada primariamente ao nível de transcrição e é independente das concentrações de cálcio intracelular, podendo ser induzida por várias citosinas e endotoxinas pró - inflamatórias (GRIFFITH et al., 1995; KIBBE et al., 1999; VIARO et al., 2000). Vários autores descreveram uma relação entre o aumento na expressão da iNOS e lesões ateroscleróticas em aortas de camundongos ApoE (OZAKI et al., 2002). Esse aumento na expressão da iNOS está correlacionado principalmente ao aumento no estresse oxidativo,

por que além de gerar ·NO, esta enzima também gera superóxido por um fenômeno denominado desacoplamento (LAURINDO; LEITE, 2003).

Um outro mecanismo importante que influencia a atividade das NO sintases é a inibição alostérica pelo próprio ·NO, que é capaz de se ligar ao grupo heme da enzima e inibir o transporte de elétrons (GRYSCAVAGE et al., 1994; ESPEY et al., 2002).

Verifica-se que os mecanismos moleculares promovidos pelo sistema NO/NO sintase nas disfunções endoteliais, presentes na aterosclerose, não estão totalmente esclarecidos. Contudo, o aumento na expressão das três isoformas encontra-se diretamente ligado às disfunções vasculares, ao estresse oxidativo e às alterações no mecanismo de sinalização celular que ocorrem nas células endoteliais e musculares (WILCOX et al., 1997; WEVER et al., 1998; OZAKI et al., 2002).

1.5.Teoria oxidativa da aterogênese

A LDL pode sofrer oxidação em vários graus, os quais variam desde a peroxidação de fosfolípides específicos na superfície da partícula até uma extensa oxidação dos lípides internos da membrana (BERLINER et al., 1995). Em resumo, a LDL oxidada pode exercer os seguintes efeitos no ambiente vascular: i) sua retenção no espaço sub endotelial; ii) recrutamento, quimiotaxia e ativação de monócitos/macrófagos e células musculares lisas, as quais induzem a captação intracelular da LDL através dos receptores "scavenger"; iii) formação das células espumosas; iv) toxicidade para as células endoteliais; v) estímulo à produção excessiva de radicais superóxido por células endoteliais (LAURINDO et al., 1998).

Inúmeras evidências sugerem que a peroxidação de componentes da lipoproteína de baixa densidade (LDL) é um evento relevante na gênese do ateroma – denominado de "teoria oxidativa da aterogênese" (PALINSKI et al., 1989).

A teoria oxidativa da aterogênese está relacionada à fisiopatologia da disfunção endotelial. Uma vez, que vasos de animais hipercolesterolêmicos e mesmo de humanos expostos a fatores de risco para a aterosclerose, apresentam um relaxamento deficiente à acetilcolina (ACh) (HARRISON et al.,1997; VERGANI et al., 2000). Inicialmente foi hipotetizado que haveria uma redução na produção de ·NO, o que estaria relacionado a um aumento da resposta vasoconstritora. Entretanto, hoje se encontra demonstrado que não só a produção de óxido nítrico está aumentada na aterosclerose (LI; FORSTERMAN, 2000), mas também a de superóxido (MÜGGE et al., 1994).

Uma série de estudos foi conduzido e centrado na utilização de estratégias terapêuticas para reverter à disfunção endotelial relacionada à oxidação da LDL. O sucesso destas estratégias foi atribuído à redução do grau de oxidação da mesma, bem como, outras disfunções ligadas a aterogênese, tais como extensão da placa, produção aumentada de superóxido e infiltração de macrófagos.

1.6. Nitrosotióis (RSNO)

O ·NO, é um radical livre, instável e lipossolúvel, o qual pode ser estabilizado por moléculas carreadoras que prolonguem sua meia vida e preservem sua atividade biológica.

Sendo assim, os tióis (RSH) de baixo peso molecular são ideais para exercer esta função, pois além de serem abundantes no plasma humano reagem rapidamente com óxidos de nitrogênio, produzindo nitrosotióis (MATHEWS; KERR, 1993; OAE; SHINAHAMA, 1983; GASTON, 1999) os quais são biologicamente ativos e mais estáveis que o ·NO livre.

Adicionalmente, são vasodilatadores e possuem propriedade anti plaquetária, ambas mediadas pelo cGMP (IGNARRO et al., 1981). Contudo, estudos relatam que o vasorelaxamento produzido pelos tióis não é resultado apenas da liberação de ·NO, mas que o radical (R), o qual pode ser representado por uma molécula de cisteína ou glutationa, também pode influenciar a atividade biológica dos nitrosotióis (KOWALUK; FUNG, 1990).

Ainda, os S - nitrosotióis podem inibir a enzima conversora de angiotensina a qual desempenha um papel preponderante na homeostase cardiovascular, principalmente na manutenção do tônus constritor. A inibição da enzima conversora de angiotensina II determina redução na concentração de angiotensina II e a destruição de algumas cininas, principalmente a bradicinina. A menor concentração de angiotensina II e bradicinina vascular pode resultar na potencialização vasodilatadora. Este efeito pode, ainda, ser visto em decorrência do aumento na biodisponibilidade de óxido nítrico (PERSSON et al. 2000).

Os efeitos que os tióis exercem ocorrem via as três principais reações: liberação de óxido nítrico, transnitrosação e S-tiolação. Essas três vias de reações são ilustradas na figura 3.

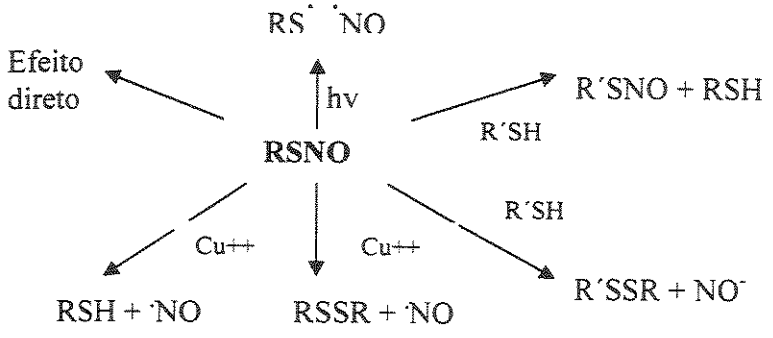

Figura 2: Reações biológicas dos RSNO (adaptado de HOOG, 2000).

Finalmente, os S-nitrosotióis são compostos endógenos que têm múltiplos efeitos farmacológicos, muitos dos quais possuem aplicações imediatas. Além disso, participam de várias vias de sinalização e das respostas ao estresse oxidativo.

2 -OBJETIVOS

A biodisponibilidade do 'NO e as vias de síntese do sistema NO/NO sintase no ambiente vascular, parecem ser fatores determinantes na manutenção funcional e no processo aterogênico. Assim, os objetivos do presente trabalho foram investigar:

1- O efeito hipotensor da SNAC (S-Nitroso-N-acetilcisteína) *in vivo* em ratos acordados normotensos e hipertensos.

2- O efeito da SNAC (S-Nitroso-N-acetilcisteína) nas disfunções vasculares promovidas pela aterogênese em camundongos LDLr-/- hipercolesterolêmicos.

3 - MÉTODOS, RESULTADOS E CONCLUSÃO

3.1.Characterization of the hypotensive effect of S-nitroso-N-acetylcysteine in normotensive and hypertensive conscious rats

Characterization of the hypotensive effect of S-nitroso-N-acetylcysteine in normotensive and hypertensive conscious rats

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[1] Abbreviations used: NO, nitric oxide/nitrogen monoxide; RSNO, S-nitrosothiol; NAC, N-acetyl-L-cysteine; GSH, glutathione; SNAC, S-nitroso-N-acetylcysteine; GSNO, S-nitrosoglutathione; SNP, sodium nitroprusside; MB, methylene blue; $O_2^{\bullet-}$, superoxide anion; MAP, mean arterial pressure; PBS, phosphate-buffered saline; L-NAME, N^G-nitro-L-arginine methyl ester; cGMP, cyclic guanosine 3, 5-monophosphate; CysH, -cysteine; CysNO, S-nitroso-L-cysteine; UV, ultraviolet; CT, charge transfer.

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Abstract

S-Nitrosothiols (RSNOs) are potent vasodilators found naturally in vivo. A variety of synthetic RSNOs have been considered as potential nitric oxide (NO) donors for biomedical applications. We have characterized the hypotensive effect of the RSNO S-nitroso-N-acetylcysteine (SNAC) in normotensive and hypertensive conscious rats. SNAC reduced the medium arterial pressure in a dose–response manner in both normotensive and hypertensive animals. At the same doses (EC_{50} of SNAC), SNAC showed a vasodilator effect in normotensive rats more potent and more prolonged than that of sodium nitroprusside (SNP). The hypotensive effect of SNAC was also more potent in methylene blue-treated rats, where the cGMP-dependent pathway had been blockaded. These data indicate that SNAC acts by both cGMP-dependent and cGMP-independent pathways. It was also shown that the thiol N-acetylcysteine (NAC) potentiates the action of SNP in hypertensive rats, pointing to the mediation of thiols in the vasodilator action of SNP in this condition. Such mediation may involve the formation of a more potent thiol complex with the nitroprusside anion or the transfer of NO to NAC, generating SNAC as a primary vasoactive species. The kinetic monitoring of the decomposition reactions of SNAC and SNP showed that both compounds are quite stable under the infusion conditions used. Therefore, their vasodilator action cannot be assigned to their breakdown with release of free NO in solution. As the two compounds are unlikely to cross the plasmalemma of smooth muscle cells, their actions are probably associated with the mediation of endogenous thiols in transnitrosation reactions.

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Introduction

S-Nitrosothiols (RSNOs)[1] have been recognized as novel nitric oxide (nitrogen monoxide) (NO) donor drugs. Several RSNOs have been shown to be potent smooth muscle relaxants and inhibitors of platelet aggregation [1-6]. Some of these compounds occur naturally in vivo, such as S-nitrosoalbumin, S-nitroso-L-cysteine (CysNO), and S-nitrosoglutathione (GSNO), and others have been synthesized chemically like S-nitroso-N-acetylpenicillamine, S-nitrosocaptopril, and S-nitrosomercaptoethylamine. Their synthesis can be achieved by the S-nitrosation reaction of the parent sulfhydryl-containing peptides, which are very reactive toward nitrosating species. L-Cysteine (CysH) and glutathione (GSH) (the most abundant nonprotein thiol found endogenously) are naturally occurring sulfhydryl-containing peptides found in nearly all cells. GSNO, formed in the S-nitrosation of GSH, has been considered to be an endogenous NO carrier involved in many biological functions like the ability to relax vascular smooth muscle cells and to prevent platelet aggregation [7,8]. CysH is the precursor of GSH and has been shown to be involved as an intermediate (CysNO) in various transnitrosation reactions with other NO carriers [9,10]. N-Acetyl-L-cysteine (NAC), an endogenous product of cysteine, is also an effective precursor and stimulator of GSH synthesis [11] and can be readily nitrosated yielding S-nitroso-N-acetylcysteine (SNAC). The S-nitrosation reaction of endogenous thiols is considered to be involved in the vascular vasorelaxing activity of nitrovasodilators like glyceril trinitrate, leading to the formation of RSNOs as intermediates [12,13].

RSNOs undergo decomposition in vitro, yielding free NO and a disulfide according to the following reaction:

$$2RSNO \rightleftharpoons RS\text{–}SR + 2NO^{\bullet} \quad (1)$$

This reaction is catalyzed by metal ions, notably Cu^{2+} ions [14,15], and is accelerated photochemically by irradiation with ultraviolet or visible light [16,17]. The thermal spontaneous release of NO from S-nitrosothiols was reported to be unrelated to the vascular relaxation and antiplatelet properties of these compounds, suggesting that direct extracellular effects of S-nitrosothiols may be responsible for their activity and that simple NO mimetic action cannot explain their mechanism of action [18-20]. Although it is conceivable that extracellularly generated free NO diffuses passively through the membrane of vascular smooth muscle cells, several recent observations [21-23], suggest that S-nitrosothiols may gain access to the intracellular milieu via a transnitrosation mechanism catalyzed by cell-surface thiols [24], whose equation can be written as

$$RSNO + R'SH \rightleftharpoons RSH + R'SNO \quad (2)$$

Several organic nitrates like nitroglycerin, widely used in the treatment of ischemic heart disease, are considered to be NO donor drugs. The same consideration applies to disodium pentacyanonitrosylferrate ($Na_2[Fe(CN)_5(NO)]$) (sodium nitroprusside, SNP), which is widely used in the treatment of hypertensive emergencies and severe cardiac failure [25-27]. As SNP is also frequently used as a reference vasodilator, we have compared its in vivo action with that of SNAC. Although SNP has been shown to be a very effective drug, it has some drawbacks: Aqueous SNP solutions decompose under illumination with room light, releasing the NO and cyanide (CN^-) ligands [28]. Both ligands are also released upon administration and in addition to the NO effects, toxic side

effects due to the accumulation of cyanide may lead to severe lactic acidosis, arrhythmia, and excessive hypotension [29]. RSNOs offer clear advantages over SNP and other nitrovasodilators, since they apparently do not lead to nitrate tolerance [30,31] and do not present any toxic side effect.

Despite the potential use of RSNOs as possible substitutes of SNP and other NO donor drugs, most studies on the evaluation of the vasorelaxant effects of RSNOs are based on in vitro experiments using vascular smooth muscle (isolated rat or rabbit aortic rings and/or mesenteric artery) and nonvascular smooth muscle (e.g., guinea pig trachea), which does not allow the interrelationships between the circulation and the vascular environment in the vasorelaxant response to be taken into account. For this reason, we have compared in this work the hypotensive effects of SNAC and SNP, using normotensive and hypertensive conscious rats as an animal model. To support our discussion about whether the different biological activities of these two compounds could be correlated with their spontaneous thermal NO release, we carried out kinetic experiments to characterize the spontaneous NO release from SNAC and SNP in vitro, under the same infusion conditions used in vivo. Our results have shown that SNAC has a vasodilator potency higher than that of SNP and a more prolonged action and that its action is partially cGMP independent. Kinetic monitoring of the NO release reactions of SNAC and SNP have shown that both compounds cannot have their biological actions assigned to the spontaneous release of NO in solution. Moreover, the previous combination of SNP and NAC at the same doses led to a potentiation of the vasodilator effect of SNP. Such results indicate that endogenous thiols may be involved as intermediates in the mechanisms of vasodilation of NO donor drugs (including SNP) through transnitrosation reactions.

Materials and methods

Chemicals

Gaseous NO was obtained from a gas cylinder (White Martins, Campinas, SP, Brazil). Synthetic air (N_2/O_2, 79/21 v/v, H_2O < 2 ppm, THC, $CO+CO_2$ < 0.3 ppm) was purchased from Air Liquide (Campinas, SP, Brazil). Anhydrous N-acetyl-L-cysteine and sodium nitroprusside dihydrate were purchased from Aldrich and used without further purification. Aqueous solutions were prepared in phosphate buffered saline (PBS), pH 7.4, using distilled–deionized water. N^G-Nitro--arginine methyl ester (L-NAME), PBS, and methylene blue (MB) were purchased from Sigma.

Synthesis of SNAC

S-Nitrosation of NAC was achieved by bubbling a mixture of NO/synthetic air through NAC solutions (61.2 mmol/L) in a quartz spectrophotometer cuvette (Aldrich). Gas flows were controlled by flowmeters (Aldrich) to allow mixtures of known NO/air ratios. A gas flow-through system with a glass mixer, polyethylene tubes, and Teflon connections was used to control the mixture and delivery of gases to the solution. Preadjusted ratios of $NO:O_2$ (5.6:5.6 mL/min) were used to allow for an excess of O_2 relative to NO. To follow the course of the S-nitrosation reaction, the gaseous mixture was bubbled through the solutions in short pulses, using a solenoid valve (Cole Parmer) controlled by an electronic circuit developed for this purpose. The reaction was followed spectroscopically at λ =545 nm, which corresponds to the maximum of the visible absorption band of SNAC. This band and the UV band at 336 nm were observed to increase continuously with the total time of bubbling until a maximum was reached (Fig. 1A). S-Nitrosation was carried out until the

achievement of this maximum, to ensure a complete reaction, avoiding an excess of the nitrosating reactant. Based on this procedure, the concentration of SNAC solution obtained was considered to be equal to the starting concentration of NAC solution (61.2 mmol/L).

In vitro stability of SNAC and SNP solutions

The stability of SNAC solutions was assessed by monitoring the SNAC concentration over time spectrophotometrically. Solutions were placed in quartz cuvettes and referenced against air, and changes in ground-state absorbances at 37 °C were recorded at 545 nm, under stirring, at time intervals of 1 h. A diode array spectrophotometer (Hewlett–Packard, Model 8453, Palo Alto, CA) with a temperature-controlled sample holder was used to monitor the spectral changes. Kinetic parameters were obtained by fitting the absorption vs time curves with the exponential equation for first-order reactions

$$A_\infty - At = (A_\infty - A_0)e^{-kt} \quad (3)$$

Where At and A_∞ are the absorbances at 545 (or 336) nm, at time t and at the end of the decomposition reaction (infinity value), respectively, and k is the first-order rate constant of the reaction. A Levenberg–Marquart nonlinear fitting program was used for the curve fittings. Each point in the kinetic curves presented is the average of two experiments with the error bars between the absorbance values expressed by their standard error of the mean (SEM).

Stability of aqueous SNP solutions relative to the aquation reaction with release of free NO was monitored by following the absorption band at 394 nm at intervals of 1 h.

Spectra of the SNP solutions were also obtained in 1-cm quartz cuvettes at 37 °C, under stirring.

Animal model and experimental design

Thirty-six adult male Wistar rats weighing 250–300 g were used for this study. The animals were supplied by "Centro de Bioterismo" CEMIB-UNICAMP. All procedures for animal experimentation were approved by the Institutional Animal Care and Use Committee and conducted according to the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, US National Academy of Sciences. The rats were housed in individual wire-mesh bottom cages (27x17x15 cm) under 12-h/12-h light/dark cycle and given free access to water and standard laboratory rat chow. For surgical procedures the rats were anesthetized with a combination of 40 mg/kg body wt ketamine hydrochloride (im, Ketalar; Parke Davis) and 6 mg/kg body wt xylazine (im, Rompun; Bayer). Both femoral arteries and veins of the animals were cannulated with polyethylene tubing (PE 10 connected to PE 50, filled with heparinized (50 U/mL) saline solution) for monitoring blood pressure and injecting drugs. The cannulas were exteriorized at the back of the rat's neck. At the end of the surgical procedures, the rats received a single dose of (40,000 U) penicillin G procaine. After vessels cannulation, the rats were kept in the individual cages for 24 h for recuperation before starting the administration of drugs. The arterial blood pressure was measured at a frequency of 1500 Hz in the conscious unrestrained animals using a pressure transducer (Gould-Strain Gauge) and a preamplifier (General Purpose Amplifier, Stemtech) attached to the intraarterial cannulae. The signals were integrated to obtain the mean arterial pressure (MAP). A mercury manometer connected to the transducer was used for calibrating the system. The systolic and diastolic

arterial pressures were calculated using the software DI 220 AT-CODAS (Data Q Instruments), from which the maximum differences between the mean arterial pressure in the basal condition and after drug administration (ΔMAP) were obtained. Before the administration of drugs the basal blood pressure was recorded for 20 min, in order to obtain a stabilized baseline reading. The total duration of the effects was calculated as the time elapsed between the onset of the decrease in the basal pressure and its full recovery. Freshly synthesized SNAC solutions and SNP, NAC, MB, and L-NAME solutions were prepared by dilution in PBS to obtain the desired infusion concentrations and kept in ice until administration. SNAC and SNP solutions were protected against room light by wrapping the flasks with aluminum foil. SNAC and SNP solutions were administered as a bolus through the venous cannulae in the conscious, unrestrained rats using injection volumes of 100 μL. The rats were randomly divided into three groups.

(i) Normotensive rats. Animals in this group were submitted to three separate studies. For dose–dependent studies, the rats were given 14 random doses of SNAC ranging from $2.8x10^{-2}$ to 854×10^{-2} μmol/kg body wt (n=6). For comparing the effect of SNAC with that of SNP, the animals were injected with SNP ($51x10^{-2}$ μmol/kg body wt, n=6). And, for evaluating the possible effect of NAC on the bioactivation of SNP, the animals were injected with a mixture of SNP and NAC, each one at a dose of ($51x10^{-2}$ μmol/kg body wt, n=6).

(ii) L-NAME-treated rats. Animals in this group were given L-NAME (3 mg/kg body wt, iv) in order to induce acute hypertension prior to drug administration. For dose-dependent

studies the acute hypertensive rats received seven random doses of SNAC ranging from $2.8x10^{-2}$ to $428x10^{-2}$ μmol/kg body wt (n=6). For evaluating the possible effect of NAC on the bioactivation of SNP in hypertensive animals, the animals were injected with a mixture of SNP and NAC, each one at a dose of ($51x10^{-2}$ μmol/kg body wt, n=6).

(iii) Methylene blue-treated rats (n=6). Animals in this group were given a continuous infusion of MB and PBS, through a pump at rate of 83 nmol/kg/min, followed by bolus administration of SNP ($51x10^{-2}$ μmol/kg body wt, iv). After recovery of basal MAP, the same animals received bolus injection of SNAC ($51x10^{-2}$ μmol/kg body wt). Control experiments with this group were conducted through the infusion of PBS, followed by the administration of SNP. After recovery of basal MAP, the same animals received an injection of SNAC under the same conditions described above (n=6).

Statistical analysis

The data were expressed as means ± standard error of the mean (SEM). Student's t test was used for comparisons between two groups. The value of $P<0.05$ was considered significant.

Results

Characterization of SNAC synthesis and decomposition in vitro

Fig. 1A shows the spectral changes obtained during the synthesis of SNAC. The visible band at 545 nm ($\varepsilon \approx 34\ M^{-1}\ cm^{-1}$) is accompanied by an UV band with a maximum at 336 nm, which has a much higher molar absorption coefficient ($\varepsilon \approx 778\ M^{-1}\ cm^{-1}$; Fig. 1A, inset). These absorption bands have already been characterized in other works as being due to S-nitrosothiol formation [17, 32,33] and were used to confirm the formation of SNAC in this work. The absorption band at 545 nm was used to monitor the synthesis of SNAC in aqueous PBS solution and its subsequent decomposition with NO release according to Eq. (1). Fig. 1B shows the kinetic curves corresponding to the synthesis and decomposition of SNAC. S-Nitrosation of NAC was conducted with accurate control of the frequency and duration of the gas pulses to avoid an excess of nitrosating reactant after the complete S-nitrosation of NAC (top of the synthesis curve). The monitoring of the decomposition reaction at 37 °C was started immediately after the completion of the synthesis and can be seen in the decomposition curve of Fig. 1B. This curve fitted well to a first-order exponential decay with a half-life of ≈1.7 h for a SNAC solution 61.2 mmol/L.

To evaluate the effect of SNAC concentration on the rate of NO release in buffered solutions (pH 7.4), the kinetic monitoring of a buffered dilute SNAC solution (0.24 mmol/L) was carried out also at 37 °C. It can be seen in curves ii and iii of Fig. 1C that the initial rate of NO release is highly reduced upon dilution. This dilution was used to reach the concentration of the solutions infused in the rats (0.24 mmol/L). Thus, curve ii in Fig. 1C corresponds to the actual rate of NO release from SNAC in aqueous buffered solution,

immediately before infusion. The half-life of SNAC in this condition was found to be ≈27 h at 37 °C.

Characterization of the thermal stability of SNP in the dark

The primary product of the decomposition of aqueous SNP solutions with NO release is the aquoferricyanide ion ($[Fe^{III}(CN)_5(H_2O)]^{2-}$) formed in the reaction

$$[Fe(CN)_5(NO)]^{2-}+H_2O \longrightarrow [Fe^{III}(CN)_5(H_2O)]^{2-}+NO \quad (4)$$

The appearance and growth of the two new absorption bands at 338 and 394 nm in the UV/vis spectrum of SNP can be assigned to the formation of the $[Fe^{III}(CN)_5(H_2O)]^{2-}$ species according to the equation above, as already demonstrated elsewhere [28]. Therefore, the absorptions at these wavelengths were used to monitor the stability of SNP relative to the release of NO.

Although SNP solutions are known to undergo photochemical decomposition under irradiation with UV/vis light with the release of the NO and CN ligands, in the dark aqueous SNP solutions have been shown to be stable over large periods of time [28]. No spectroscopic evidences of decomposition were found in the present study, after leaving aqueous-buffered 0.24 mmol/L SNP solutions in the dark at 37 °C for 8 h (Fig. 1C, curve i).

Hypotensive effect of SNAC in conscious normotensive rats

Random administration of 12 different doses of SNAC (from 2.8×10^{-2} to 854×10^{-2} μmol/kg body wt) in the normotensive rats led to a dose-dependent decrease in the MAP from -14±1 to -60±1.4 mm Hg, respectively (Fig. 2A). An EC_{50} of 51×10^{-2} μmol/kg body wt corresponding to a ΔMAP of 28 ±1.43 mm Hg was estimated from the data of Fig. 2A.

Hypotensive effect of SNAC in conscious L-NAME-treated rats

Acute hypertension induced through the administration of L-NAME (3 mg/kg, iv) lead to an increase of 28% in the basal MAP (105 ± 2.5 mm Hg) of the rats, which lasted for 1 h. Random administration of seven different doses of SNAC (from $2.8x10^{-2}$ to $428x10^{-2}$ μmol/kg) in the hypertensive rats also led to a dose-dependent decrease in the MAP from −12 ± 2.5 to −59 ± 0.5 mm Hg, respectively (Fig. 3B). An EC_{50} of $26x10^{-2}$ μmol/kg body wt, corresponding to a ΔMAP of 34±1.43 mm Hg, was estimated from the data of Fig. 2B.

Hypotensive effects of SNP and SNAC in normotensive and methylene blue-treated rats

Comparison between the hypotensive effect of SNP and SNAC at the same doses ($51x\ 10^{-2}$ μmol/kg body wt) has shown that SNAC produces a vasodilation more intense than that of SNP in normotensive rats under PBS infusion (Fig. 3A), leading to a ΔMAP 40% higher than that of SNP, on average. Similarly, SNAC was 43% more potent than SNP at the same doses in methylene blue-treated rats. However, while there is no significant difference between the effect of SNP in normotensive and methylene blue-treated rats, SNAC led to a ΔMAP 15% smaller in methylene blue-treated rats compared to that in normotensive rats. In addition to the higher potency of SNAC compared to SNP in normotensive rats, it was also observed that the duration of the hypotensive effect of SNAC in this case (9±2 min) was much greater than that obtained with SNP (1±0.04 min) at the same dose (Fig. 3B).

Effect of combined administration of SNP and NAC in normotensive and hypertensive rats

The combined administration of SNP and NAC in normotensive rats did not lead to a significant difference in the ΔMAP obtained, compared to the effect of SNP alone at the same dose ($51x10^{-2}$ μmol/kg body wt; Fig. 4). However, two significant differences were observed when SNP was infused together with NAC in acutely hypertensive rats, as can also be seen in Fig. 4. First, SNP alone had a more potent effect in the acutely hypertensive rats, leading to a ΔMAP 33% higher than that in normotensive rats. Second, NAC potentiated the effect of SNP in the hypertensive rats, leading to a ΔMAP 34% higher than that in normotensive rats. Interestingly, the magnitude of the hypotensive effect obtained with the combined administration of SNP and NAC in hypertensive rats (ΔMAP=36 ± 2 mm Hg) was very similar to the value obtained with the infusion of SNAC at the same dose, also in the hypertensive animals (ΔMAP=34 ± 2 mm Hg, Fig. 2B).

Discussion

The reactions involved in the synthesis of SNAC through the S-nitrosation of NAC by a mixture of NO and O_2 were already described elsewhere [17]. The overall stoichiometric reaction for a generic thiol (RSH) can be written as

$$4NO+O_2+4\ RSH \longrightarrow 4RSNO+2\ H_2O \qquad (5)$$

By monitoring the above reaction spectroscopically, it was possible to avoid an excess of the nitrosating reactant and to ensure the complete consumption of NAC. Therefore, it can be considered that the SNAC solutions used in the present study were free from excess

thiol, nitrosating species, and other ions, except those present in the PBS solution. The kinetic monitoring of the absorption bands of SNAC in aqueous-buffered solutions (pH 7.4) has shown that it undergoes thermal decomposition at 37 °C in the dark, as already reported in other works [34,35]. This monitoring also showed that the initial rate of spontaneous NO release from the solutions is strongly dependent on the starting concentration of SNAC. This result can be explained on the basis of a homolitic cleavage of the S–N bond, which leads to the production of free NO and a thiyl radical ($RS^{\bullet}$) according to

$$RS\text{–}NO \longrightarrow RS^{\bullet} + NO^{\bullet} \qquad (6)$$

The primary $RS^{\bullet}$ fragments formed in Eq. (6) also react with intact SNAC molecules forming the thermodynamically stable dimer RS–SR, with release of a further NO molecule, according to

$$RS^{\bullet} + RSNO \longrightarrow RS\text{–}SR + NO^{\bullet} \qquad (7)$$

In the concentrated SNAC solutions, the disappearance of the band at 545 nm is associated with the appearance of a new UV band as a shoulder at ca. 250 nm (not shown) that is consistent with the formation of RS–SR [35] (Eq. (7)). This self-catalytic decomposition reaction is expected to be more effective the more concentrated the solution. This fact is reflected in the great difference in the half-lives of concentrated (curve iii) and diluted (curve ii) solutions, obtained from the first-order fitting of the curves in Fig. 1C. Although the kinetics of SNAC decomposition in concentrated solutions follows a more

complex mechanism, due to the concerted occurrence of reactions (6) and (7), the good fitting of the kinetic curves of Fig. 1C to a single first-order equation can be used for estimating the half-lives of SNAC. The half-life of ≈ 27 h found for dilute aqueous-buffered SNAC solution indicates that at the infusion concentration used in the rats SNAC is quite stable and thus the spontaneous release of free NO cannot account for its potent vasodilator effect observed in vivo. Plasma RSNOs have been detected with concentrations in the low-nanomolar range for the low-molecular-weight nitrosothiols [36-40]. Therefore, it is also unlikely that reactions (6) and (7) are operative in the NO action of endogenous RSNOs.

Similarly, SNP solutions have shown to be stable at pH 7.4 and 37 °C over long periods in the dark [41]. SNP have bands assigned to two d → π*(NO) charge transfer (CT) transitions at *ca.* 394 nm ($d_{xy,yz} \rightarrow \pi^*(NO)$) and 497 nm ($d_{xy} \rightarrow \pi^*(NO)$) and two internal d–d transitions (shoulders) at *ca.* 265 nm ($d_{xy} \rightarrow d_{z2}$) and 330 nm ($d_{xy} \rightarrow d_{x2-y2}$). The absence of any detectable change in the UV/vis spectrum of aqueous SNP solution after prolonged periods in the dark can be taken as clear evidence of its thermal stability. Therefore, in the present study, neither SNAC nor SNP can have their biological actions assigned to the previous spontaneous release of free NO in solution.

The in vivo results obtained with SNAC in this animal model confirm that SNAC is a potent bioactive compound with NO-like effects. Our data also show that the in vivo vasodilator effect of SNAC presents a dose-dependent correlation in both normotensive and acute hypertensive rats. Moreover, the vasodilator effect obtained with SNAC in the hypertensive rats was greater than that in the normotensive rats ($EC_{50}=51\times10^{-2}$ μmol/kg body wt vs 28×10^{-2} μmol/kg wt, respectively).

The vasodilator response in acutely hypertensive subjects is known to have multiple central and local regulation mechanisms, which operate to recover the basal MAP [42]. An impaired bioavailability of NO is considered to occur in most animal models of hypertension, contributing to the increased peripheral resistance and possibly to the development of cardiovascular complications [43]. In some hypertension experimental models, the endothelium-dependent vascular relaxation was shown to be impaired, whereas the endothelium-independent vascular relaxation was not changed. Examples of such conditions can be observed in the acutely hypertensive model promoted by an infusion of angiotensin II and in the chronic model SHR [44]. In those two animal models of hypertension, the endothelium-dependent relaxation is considered to be impaired due to an increase in the superoxide ($O_2^{\bullet-}$) production which reacts with NO produced by the endothelium to form peroxynitrite ($ONOO^-$), leading to an imbalance between the relaxing and contracting factors [45,46]. In the present case, the acute hypertension induced by L-NAME, a nonselective inhibitor of NO synthase, is not assigned to an increase in the $O_2^{\bullet-}$. The peripheral vasoconstriction in such models is associated with a decrease in the endothelial NO production, which leads to a reduction in the accumulation of cGMP in the smooth muscle tissues [47].

To check if SNAC is active via cGMP-independent pathways, we carried out experiments with SNAC and SNP (as a control) in rats where the cGMP-dependent pathway of NO action had been blockaded with methylene blue. Our observation that the potency of SNAC in the methylene blue-treated rats is reduced but not suppressed (relative to the normotensive rats) shows that SNAC action is in fact partially cGMP independent. The same conclusion cannot be extended to SNP, as the potency of its effect is statistically

simil ar in normotensive and methylene blue-treated rats. Therefore, SNP seems to act mainly via a cGMP-independent pathway.

Our observation that the hypotensive effect obtained with SNAC in normotensive rats is more potent and more prolonged than the effect obtained with SNP at the same doses can be taken as an indication that the biological actions of SNAC and SNP may involve different main pathways. There have been two major hypotheses concerning the mechanisms of action of RSNOs in biological systems. The first involves the release of free NO through the homolitic cleavage of the S–N bond. This mechanism implies the subsequent reaction of NO with molecular dioxygen to yield dinitrogen trioxide, N_2O_3, which in turn may mediate the nitrosation of another thiol according to the equations

$$4\,^{\cdot}NO + O_2 \longrightarrow 2\,N_2O_3 \quad (8)$$

$$N_2O_3 + RSH \longrightarrow RSNO + NO_2^- + H^+ \quad (9)$$

Although there is some evidence that cells contain a cell-surface metalloprotein that is responsible for the catalysis of NO release from RSNOs, the transfer of NO^+ equivalents in blood and interstitial fluid through the mediation of N_2O_3 implies a low pH to allow an effective N_2O_3 concentration at the equilibrium, and such a condition is very far from the conditions at physiological pH. Our results based on the chemical stability of SNAC and SNP in vitro indicate that the primary release of free NO is not involved in the biological action of both compounds. On the other hand, it is unlikely that either SNAC or SNP will permeate the membranes of target cells readily to activate the intracellular signaling systems because they exhibit very small partition coefficients from aqueous solutions to nonpolar phases [48]. These small partition coefficients are consistent with the polar nature

of SNAC and with the charged form of the nitroprusside anion, under physiological conditions. The second hypothesis is that the major in vivo mechanism of RSNOs (and possibly of SNP, see below) is transnitrosation: the transfer of NO^+ equivalents to a protein thiol in blood. In the case of RSNOs, the nitroso group is reversibly transferred between two thiolates in the absence of the previous release of NO (Eq. (2)). In the case of SNAC, o

ne possibility is that it reacts with thiol-containing species on the cell membrane surface through transnitrosation reactions and that the S-nitrosated cell-surface thiols initiate the intracellular signal transduction process in the target cells. This mechanism implies a one-electron reduction of SNAC leading to the release of NO and to the regeneration of the parent thiol (NAC) by cell-surface electron transport chains [48, 49]. The first step in this transnitrosation mechanism can be represented for a generic RSNO as

$$\text{RS–NO+HS'–Plasma membrane} \longrightarrow \text{NO–S'–Plasma membrane} + \text{RSH} \quad (10)$$

Subsequent intracellular S-transnitrosation reactions can regenerate the cell-surface thiols and transport NO to the sGC, whose cysteine residues are considered to be the target sites of NO. A similar proposal has already been presented by Liu et al. [10] for explaining the biological activity of another S-nitrosothiol (SNO-4B) and much evidence from other works points to the same mechanism. For example, transnitrosation reactions have been demonstrated to occur naturally in vivo and to participate in physiological and pharmacological actions of S-nitrosothiols [50, 51]. In addition, membrane fractions of vascular smooth muscle cells have been shown to be capable of catalyzing the release of NO from S-nitrosothiols [18].

Although much has already been said about the possible mechanisms involved in the vasodilator action of SNP, the actual mechanism is not still clearly understood. It is generally considered that SNP requires bioactivation to release NO [52, 53] but the mechanism(s) of bioactivation is not firmly established as well [54, 55]. Once the nitrosyl ligand of SNP cannot be considered to be thermally labile under physiological conditions (as demonstrated by the kinetic monitoring of curve i; Fig. 1C), the mediation of endogenous NO carriers can also be evoked to explain the NO release from SNP. The observation that NAC potentiates the vasodilator effect of SNP in the hypertensive rats can be of great significance for the understanding of the mechanisms involved in this case. It is known from other works [26, 56, 57] that the nitroprusside anion reacts readily with thiolates generating S-nitrosothiol complexes in the reversible reaction

$$[Fe(CN)_5(NO)]^{2-} + RS^- \rightleftharpoons [Fe(CN)_5N(O)SR]^{3-} \qquad (11)$$

We have confirmed Reaction (11) between SNP and NAC by following the absorption band of the nitrosothiol complex formed at 522 nm in aqueous basic solution (data not shown). The nitrosothiol complex of nitroprusside with NAC is thermally unstable and decomposes yielding Fe (II) complexes and disulphide (Eq. (12)). The fate of NO in this process may involve its release as RSNO (Eq. (13)), although there are no evidences of this reaction in our or other studies so far.

$$[Fe^{II}(CN)_5N(O)SR]^{3-} \longrightarrow Fe^{II}(CN)_5(NO)]^{3-} + 1/2\ RSSR \qquad (12)$$

$$[Fe^{II}(CN)_5N(O)SR]^{3-} + H_2O \longrightarrow [Fe^{II}(CN)_5(H_2O)]^{3-} + RSNO \qquad (13)$$

The formation of a S-nitrosothiol complex with extracellular thiols (Eq. (11) is one of the possible pathways for the bioactivation of SNP after its infusion in the blood. The potentiation of its effect through the previous mixture of SNP with NAC is evidence that the complex or its decomposition products (possibly RSNO) are more potent than the free nitroprusside anion. Extracellular thiols might then facilitate the S-transnitrosation reactions and/or help to preserve the thiols on the cell surface, similar to the mechanism considered for the observed potentiation of SNO-4B-induced vasorelaxation by GSH [10]. The observation of a potentiation effect only in hypertensive animals is evidence that the acute hypertensive condition can be associated with an altered vascular redox and/or tonus state which reduces the capability of cell-surface thiols of reducing RSNOs to perform the transportation of NO to the interior of the cell. It has already been considered by Jourd'heuil et al. [22] that increasing the circulating low-molecular-weight thiols might result in the mobilization of NO pools from high-molecular-weight RSNOs that may not be accessible otherwise. It has also been shown by Scharfstein et al. [52] that the in vivo depressor effect of S-NO-albumin in the rabbit is increased by the intravenous administration of excess cysteine before S-NO-albumin. These results together with those obtained in the present work point to a common conclusion: that low-molecular-weight S-nitrosothiols like SNAC are efficient species for the delivery of NO to the vascular wall and can be formed as intermediates in the NO action of nitrovasodilators, including SNP.

In conclusion, we have shown that SNAC is a potent hypotensive NO donor drug with a more prolonged vasodilator effect than SNP in normotensive rats. The vasodilator action of SNAC in methylene blue-treated rats and its greater potency relative to SNP indicate that SNAC acts by both cGMP-dependent and cGMP-independent pathways. We have also demonstrated that the association of SNP and NAC potentiates the SNP action,

leading to an effect equivalent to that obtained with the same dose of SNAC. Such results point to a mediation of thiols in the vasodilator action of SNP in hypertensive states and may involve the formation of a more potent thiol complex with the nitroprusside anion or the formation of SNAC as a primary vasoactive species. The kinetic monitoring of the decomposition reactions of SNAC and SNP showed that both compounds are quite stable under the infusion conditions used. Therefore, their vasodilator action cannot be assigned to their breakdown with release of free NO in solution. Such results are in accordance with a mechanism that involves the mediation of endogenous thiols in transnitrosation reactions for the vasodilator action of exogenous NO donor drugs.

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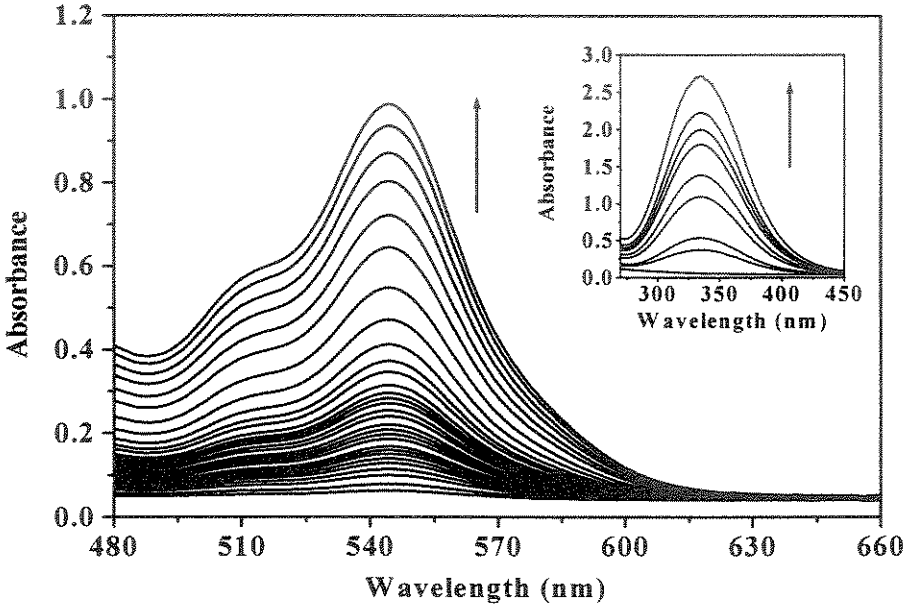
Figures Legends

Fig. 1. (A) Formation of SNAC by the S-nitrosation of NAC (61.2 mmol/L) shown by spectral changes in the UV/vis range after each gas (NO/air) pulse (for details see text). Spectra were recorded every 3–6 s. (B) Kinetic curves monitored at 545 nm for the synthesis of SNAC (left side) and for its thermal decomposition with NO release at 37 °C (right side). (C) Kinetic curves for monitoring the stability of SNP solution (i) (measured at 394 nm) and for monitoring the decomposition of SNAC solutions 0.24 mmol/L (ii) (measured at 336 nm) and 61.2 mmol/L (iii) (measured at 545 nm) at 37 °C. Each point in the curves represents the mean ± 1 SEM of two experiments.

Fig. 2. (A) Dose–responses of mean arterial pressure decrease (ΔMAP) to SNAC administered in bolus, iv, in normotensive conscious and unrestrained male Wistar rats. Each column represents the mean ± 1 SEM of six rats. (B) Dose responses of mean arterial pressure decrease (ΔMAP) to SNAC administered in bolus, iv, in L-NAME-treated acute hypertensive conscious and unrestrained male Wistar rats. Each column represents the mean ± 1 SEM of six rats.

Fig. 3. (A) Hypotensive effect of SNAC and SNP (both at 51×10^{-2} mol/kg body wt) administered in bolus, iv, in methylene blue + PBS-infused and PBS-infused rats. Each column represents mean ± 1 SEM of six rats. *$P<0.05$ compared to PBS infusion. (B) Time duration of hypotensive effects of SNAC and SNP (both at 51×10^{-2} μmol/kg body wt) administered in bolus, iv, in PBS-infused rats.

Fig. 4. Hypotensive effects of bolus, iv, administration of SNP and of a combination of SNP and NAC in normotensive and -NAME-treated acute hypertensive conscious and unrestrained male Wistar rats. Each column represents the mean ± 1 SEM of six rats. *P<0.05 compared to SNP-treated hypertensive rats.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Wavelength (nm)
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0.8
0.6
0.4
0.2
0.0
480
510
540
570
600
630
660
Absorbance
Wavelength (nm)
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2.5
2.0
1.5
1.0
0.5
0.0
300
350
400
450

Figure 1A

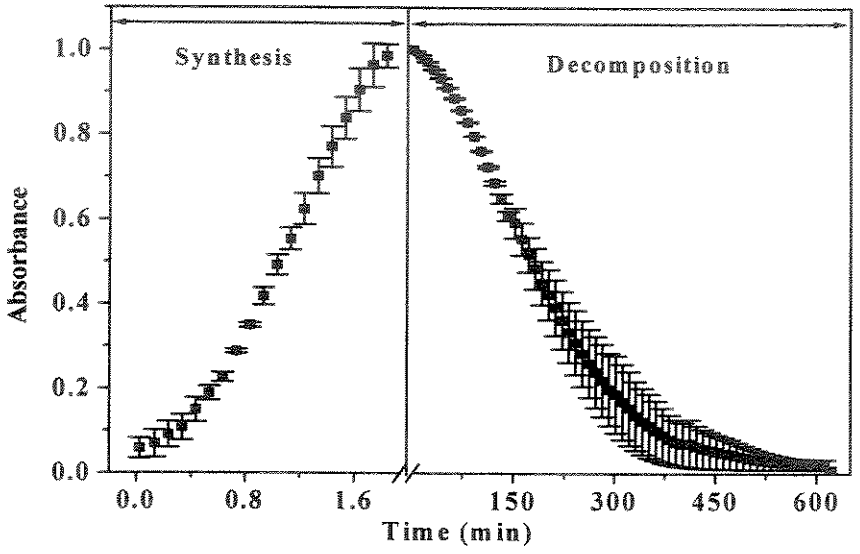
Synthesis
Decomposition
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.8
1.6
150
300
450
600
Time (min)

Figure 1B

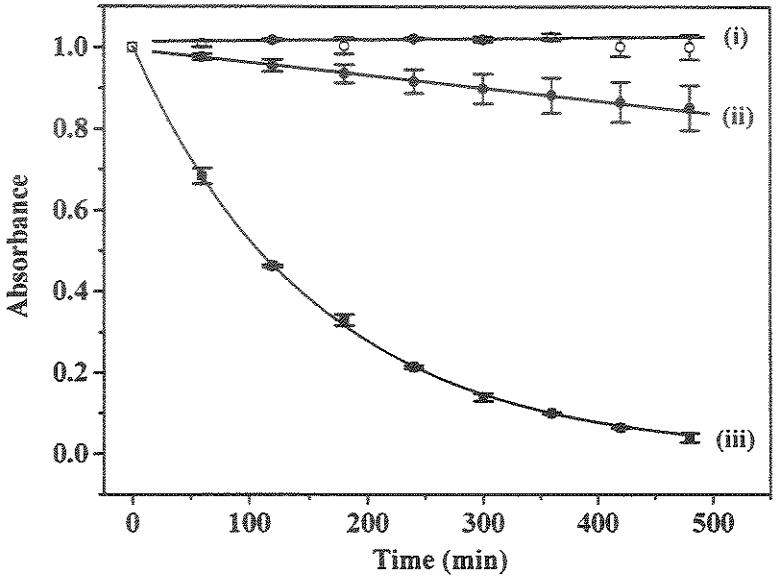

Figure 1C

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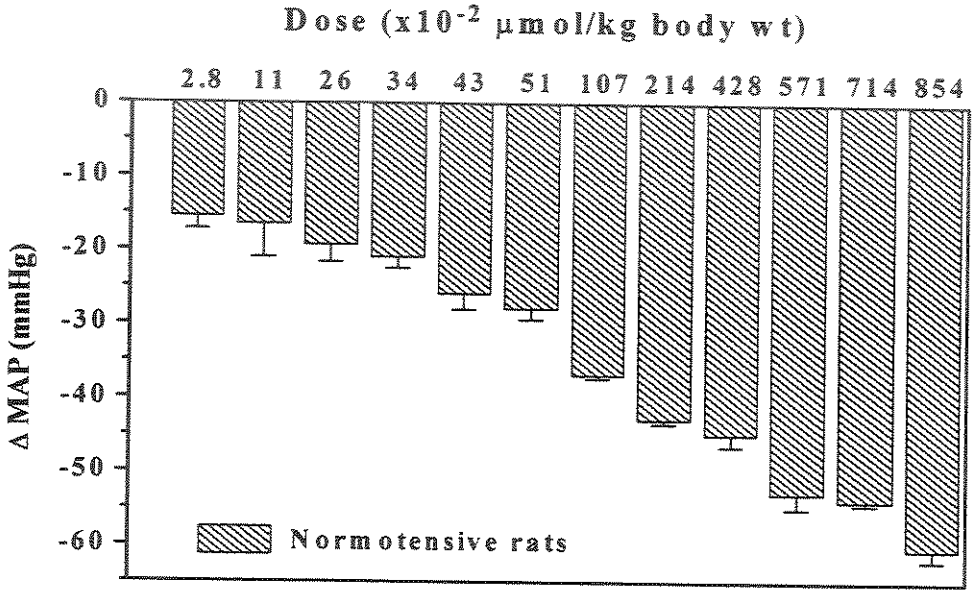
Dose (x10^{-2} µmol/kg body wt)
2.8 11 26 34 43 51 107 214 428 571 714 854
0
-10
-20
-30
-40
-50
-60
Δ MAP (mmHg)
Normotensive rats

Figure 2A

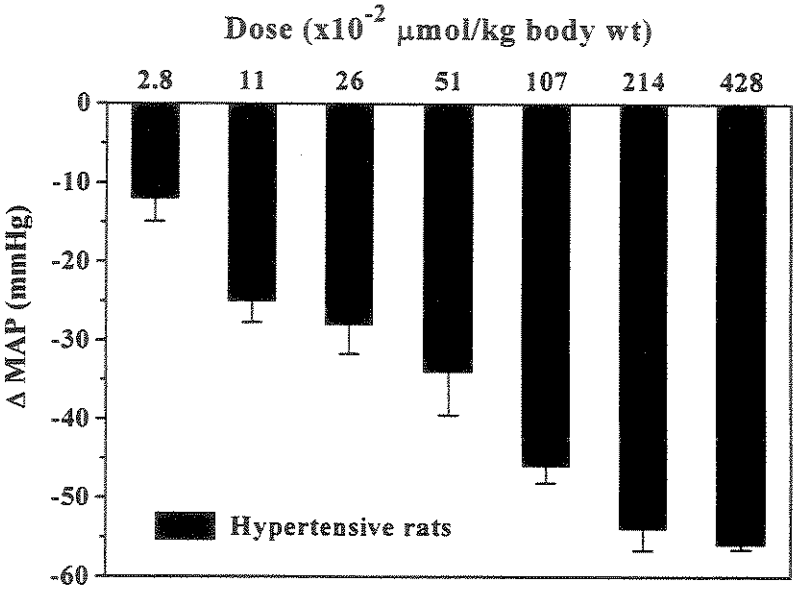
Dose (x10^{-2} µmol/kg body wt)
2.8
11
26
51
107
214
428
0
-10
-20
-30
-40
-50
-60
Δ MAP (mmHg)
Hypertensive rats

Figure 2B

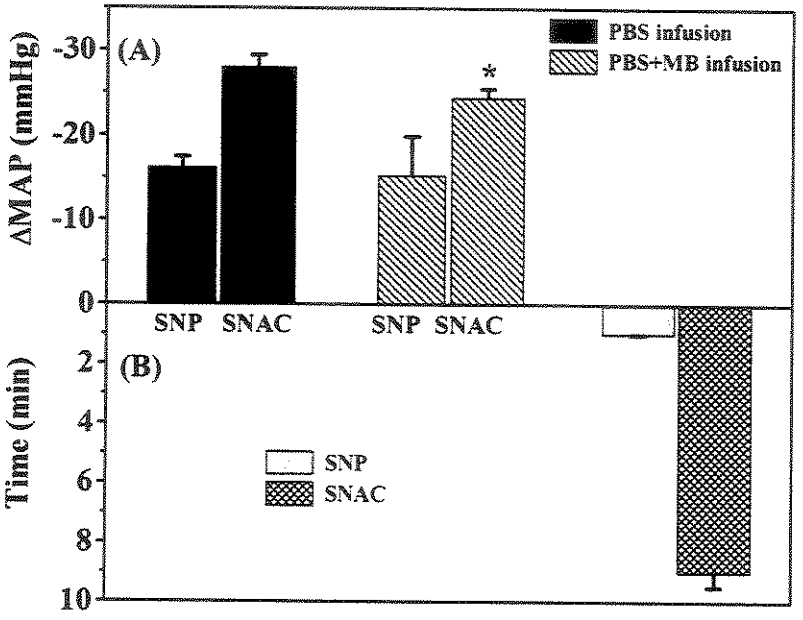
(A)
PBS infusion
PBS+MB infusion
ΔMAP (mmHg)
-30
-20
-10
0
*
SNP SNAC
SNP SNAC
(B)
Time (min)
2
4
6
8
10
SNP
SNAC

Figure 3

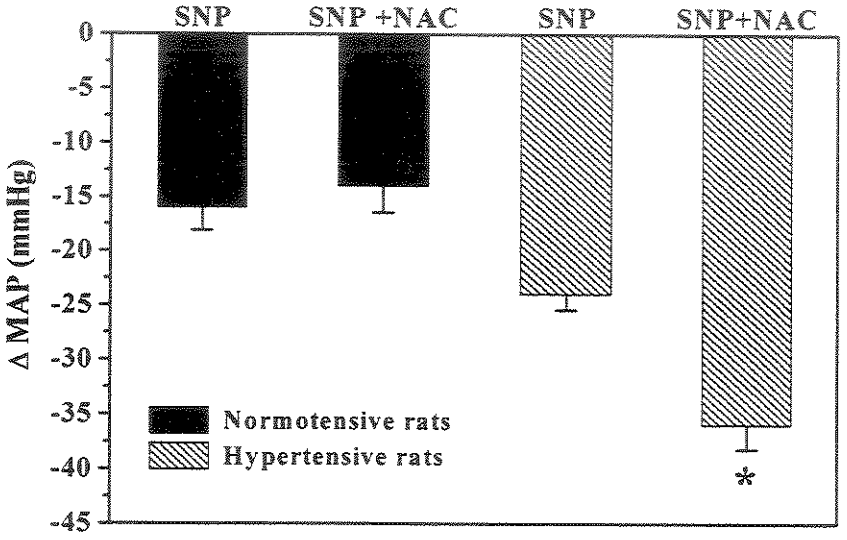
SNP
SNP +NAC
SNP
SNP+NAC
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
Δ MAP (mmHg)
Normotensive rats
Hypertensive rats
*

Figure 4

3.2. Antiatherogenic effects of S-nitroso-*N*-acetylcysteine in hypercholesterolemic LDL receptor knockout mice

Antiatherogenic effects of S-nitroso-*N*-acetylcysteine in hypercholesterolemic LDL receptor knockout mice

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Condensed abstract

This study assessed in LDLr-/- mice effects of the nitrosothiol S-nitroso-N-acetylcysteine (SNAC) in early prevention of plaque development and endothelium-dependent vasomotor changes. After 15 days of hypercholesterolemic diet , there was plaque accumulation and a paradoxical mild increase in acethylcholine-dependent vasorelaxation, together with increased expression of constitutive NOS isoforms. All such alterations were prevented by SNAC, which decreased plaque burden by 55%. These data underscore the antiatherogenic potential of S-nitrosothiols.

Abstract

Background: Pathophysiology of NO/NO synthase system and endothelium dysfunctional changes in early phases of the atherogenic process are incompletely understood. In hypercholesterolemic LDLr-/- mice, we addressed effects of the nitrosothiol NO donor S-nitroso-N-acetylcysteine (SNAC) in the early prevention of plaque development as well as changes in endothelium-dependent relaxation and NO synthase expression. **Methods and Results**: LDLr-/- mice were submitted to 1.25- % cholesterol-enriched diet for 15 days. Development of aortic root lesions accompanied increase in plasma cholesterol/triglyceride levels. Aortic vasorelaxation to acetylcholine was paradoxically increased, while endothelium-independent relaxation to sodium nitroprusside was decreased, suggesting enhanced basal or stimulated NO release. This dysfunction was associated with enhanced aortic superoxide production and with increased levels of constitutive NOS isoform expression, particularly neuronal NOS. SNAC administration (0.51 μmol/Kg/day i.p. for 15 days) decreased plaque extension by 55% in hypercholesterolemic mice. SNAC completely abrogated vasomotor changes and led to decreased constitutive NOS expression. SNAC induced only minor changes in plasma lipid profile. **Conclusion**: The present study disclosed, in early stages of plaque development in LDLr -/- mice, particular changes in NO/NOS pathophysiology, characterized by increased endothelium-dependent vasorelaxation and increased constitutive NOS expression. Such changes were prevented by SNAC, which may therefore constitute a novel strategy to halt early plaque progression.

Key Words: nitric oxide, atherosclerosis, nitric oxide synthases, endothelial dysfunction, oxidative stress, deficient receptor LDL knockout mice, nitrosothiol

INTRODUCTION

Endothelial cell dysfunction is most likely the initiating event in atherosclerosis[1]. Given the importance of redox signaling for endothelial function, established endothelial dysfunction sums up to decreased nitric oxide bioavailability. It is well known that acetylcholine-induced vasorelaxation is reduced in experimental models of hypercholesterolemia/atherosclerosis, although not in aortas from ApoE ko mice[2]. In addition, decreased endothelium-dependent dilation is uniformly observed in systemic vessels from patients with risk factors for atherosclerosis[3], as well as in diseased coronary arteries[4]. Such decreased NO effects can lead to LDL oxidation and inflammatory cell activation, which are well-known initial events of atherosclerosis. It is known that in these later stages, decrease in eNOS derived NO bioavailability is due to increased superoxide production by sources such as NAD(P)H oxidase[5], xanthine oxidase[6] or uncoupled NO synthase(s)[7]. However, the pathophysiology of early endothelial dysfunction changes in atherosclerosis remains incompletely understood. In particular, the natural evolution of vasoreactivity is unknown, and the link between hypercholesterolemia and increased NOS output/activity, enhanced oxidative stress and NOS uncoupling is still not elucidated[8]. Early endothelial dysfunction is associated with cell activation and oxidative stress[9], adhesion molecule expression[10], macrophage infiltration and uptake of modified LDL particles[11]. All such effects are likely to be counteracted by an increase in bioactive nitric oxide[12], while being worsened by reactive byproducts of the nitric oxide-superoxide reaction[13]. The opportunity to study such early events of atherogenic process and to test therapeutic interventions is a particular feature of the hypercholesterolemic LDLr-/- mouse model, considering that other models; such as the Apo E-/- mice, already exhibit significant lesions at the newborn stage, even in the absence of hypercholesterolemic diet.

Recently, the importance of S-nitrosothiols as potential NO reservoirs has become apparent, in line with the role of thiol nitrosylation as an effector mechanism of NO signaling[14]. Both naturally occurring and chemically synthesized nitrosothiols have been proposed as having potential clinical application[15]. Among the latter, SNAC showed hypotensive effects in normo or hypertensive conscious rats[16].

In the present study we sought to characterize changes in endothelial cell function as well as the pathophysiology of the NO/NO synthase system in an early phase of the atherogenic process in hypercholesterolemic LDLr -/- mice. A major focus of such observations was to examine the effect in these processes of the exogenous administration of the NO donor, SNAC.

MATERIALS AND METHODS

Protocols: LDLr-/- mice (LDL receptor knockout mice) were obtained from Jackson Laboratory (Bar Harbor, ME) by homologous recombination, as described by Ishibashi et al[17]. In this study, three-month-old male LDLr-/- mice weighing 25 ± 3 g were used. The animals were kept on cycles of 12 h of light and 12 h of darkness, at controlled temperature (22 ± 2 °C). Animals were handled under protocols (number 375-1) approved by the Institutional Committee for Ethics in Animal Research, (Campinas State University - UNICAMP, Brazil).

LDLr-/- mice were distributed into three groups that received diet and water ad libitum. 1) Control mice (C), which received commercial diet (Nuvital CR1); 2) hypercholesterolemic mice (HC) received a diet containing 20% fat, 1.25% cholesterol, 0.5% cholic acid (Dyets, Inc. Bethlehem); 3) Similarly handled hypercholesterolemic mice given SNAC (SNAC + HC;

daily dose of 0.51 μmol/Kg i.p.). After 15 days, mice were anesthetized with Ketamine (Bayer, 40 mg/Kg i.p) and Xylazine (Parke-Davis, 6 mg/Kg i.p.) for blood collection and subsequently killed by exanguination followed by tissue harvesting. SNAC was synthesized as described by Santos et al.[16]

Vascular reactivity: Mice were anesthetized and, after thoracotomy, the descending thoracic aortas were collected and dissected. Rings of ± 2 mm long were obtained from each aorta, were connected to a hook and kept in a organ bath containing Krebs-Henseleit buffer with the following composition (mM): NaCl 115.0; KCl 4.6; CaCl 2.5; KH_2PO_4 1.2; $MgSO_4$ 2.4; $NaHCO_3$ 25; glucose 11.0 and ascorbic acid 0.1), pH 7.4, 37 °C, aerated with 95% O_2 and 5% CO_2. The rings remained under 0.051 mN tension during 1 hour for stabilization. Tension cumulative was measured by an isometric transducer (F-60, Narco Biosystems) connected to a Narco Bio-System polygraph (Model DMP-4). Cumulative dose relaxation curves[18] to acetylcholine (Ach; 10^{-9} – 10^{-5} M; Sigma Chem. Co) or to sodium nitroprusside (SNP; 10^{-9} – 10^{-5} M; Sigma Chem. Co) were assessed in aortic segment after maximum contraction defined when three consecutive concentrations of phenylefrine (Phe;10^{-9} to 10^{-5} M, Sigma Chem. Co) did not increase developed tension. At this point a cumulative dose-relaxation curve were obtained. EC_{50} and EC_{75} were calculated as agonist concentration promoting 50% or 75%, respectively, of maximal individual effect[19]. Results are expressed for ≥ 5 experiments, each one with rings from different mice. Statistical calculations were performed with Software Graph Pad Prism (Graph Pad Software).

Superoxide production

Vascular superoxide production was assessed on homogenates of aortic segments from six mice/group with lucigenin (5 μM) chemiluminescence, as described[20]. Results were expressed in cpm/mg protein, quantified by Bradford method[21].

Lesion morphometry in ascending proximal aorta

Hearts and aortae were processed as previously described [22]. Since early lesion were localized in the aortic root and ascending aorta, this area was chosen for analysis using serial transversal sections, which were stained for lipids with Oil-Red O, as described by Rubin et al.[23] Image Pro Plus Software (version 3) for image analysis (Media Cybernetics) was used. Each aortic lesion area was expressed as the sum of lesion areas from 10 equally spaced sections/mouse. Results from six mice/group are expressed in μm^2.

Plasma lipoproteins

Plasma from C, HC or SNAC+ HC mice was obtained by centrifugation (12000 rpm, 15 min) of blood collected in heparinized tubes via retro orbital plexus. Total cholesterol and triglyceride were determined by enzymatic colorimetric kits (Wako Chemicals). Individual plasma samples (200 μl) were fractionated by FPLC using an HR10/30 Superose6 column (Amershan/Pharmacia Biotech), balanced with Tris-saline buffer pH7.4, as described by Jiao et al.[24] Total cholesterol was determined enzymatically in each FPLC fraction. Results (mg/dL) are from 3 mice/group.

Immunohistochemistry

Aortic fragments were collected; fixed overnight in 4% buffered formaldehyde, and processed for paraffin inclusion with standard methods. Rabbit polyclonal antibodies against nNOS, eNOS, iNOS and anti-nitrotyrosine were from Santa Cruz Biotechnology and used at 1:100 dilution[25]. Three slides from each group were performed. For 4 μm thick transversal sections were then depariffinized and endogenous peroxidase blocked with 0.03% H_2O_2 in 0.1 M PBS for 30 min. Next, the sections were pre-incubated with 3% BSA in 0.1 M PBS for 45 min to block the specific connections and then incubated. An adaptation was made in the technique described by Tambascia et al.[25], in order to analyze aortic iNOS by immunohistochemistry, as follows: after blocking the endogenous peroxidase with 0.03% H_2O_2, the slides were incubated with a citrate buffer pH 6, for 5 minutes in a microwave oven at maximum heat. The following procedures were identical to the protocol above described for immunohistochemistry analysis of eNOS and nNOS.

Western Analysis

Western analyses for NOS proteins were performed as described[25] with the same antibodies used for immunohistochemistry (1:1000 dilution), as well as secondary IgG anti-rabbit (1:300 dilution, Pharmacia). Equal amounts of protein (50 μg/lane) from three aortas from each group were processed. Densitometric analyses was performed with Scion Image software.

Statistical Analysis

Data are mean±SEM. Statistical analysis was performed by ANOVA and Tukey Test. Significant level was 0.05.

RESULTS

Paradoxical changes in endothelium-dependent and independent relaxation: prevention by SNAC

HC aortae presented increase in Phe-induced vasoconstriction, as compared with C mice (Figure 1A, 1D). Such supersensitivity to Phe was prevented by SNAC.

Endothelium-dependent relaxation exhibited a paradoxical result in HC mice, characterized by a mild increase rather than a decrease in the maximal response vs. C or SNAC+HC mice. A similar increase at the EC_{75} level was also observed in HC mice (Figure 1B, 1D), although not at the EC_{50} level (data not shown).

Endothelium- independent relaxation response was mildly decreased in HC mice versus C or SNAC+HC mice at the EC_{50} level (Figure 1C, 1D), whereas maximal response was not different.

Taken together, these data show that SNAC normalized the altered endothelium-dependent and independent vasorelaxation responses in HC mice (Figure 1B, 1C, 1D).

Decreased the oxidative stress by SNAC

Superoxide production was increased in aortas from HC mice versus C mice (13.3 ± 0.22 vs 7.31 ± 1.23 cpm x 10^6/mg protein, respectively), and was markedly decreased in SNAC + HC mice (1.4 ± 0,005 cpm x 10^6/mg protein, $p<0.05$, Figure 2.)

Decreased atherosclerotic lesion area by SNAC

As expected, C mice had no detectable atherosclerotic plaques, whereas HC mice developed significant lesions detected in proximal aorta, with average lesion area of 95.6 ± 2.0 μm^2 x 10^3. SNAC+HC mice showed a mean lesion area in the aorta approximately 55% smaller than HC mice (45.8 ± 4.9 vs 95.6 ± 2.0 μm^2 x 10^3, respectively), as shown in Figure 3.

Changes in plasma lipid profile by SNAC

HC mice showed significant increase in the total plasma cholesterol and triglyceride levels: 475% and 122%, respectively, vs. C mice (Table 1). Both the (LDL+VLDL) and the HDL fractions increased at least 5 fold, although the former exhibited greater relative increase (Table 1, Figure 4). After 15 days of SNAC treatment, there were minor changes in plasma lipid profile, with total plasma cholesterol and triglyceride levels decreased by ≤ 20% (Table 1, Figure 4).

Increased NOS expression in hypercholesterolemic mice is prevented by SNAC

Functional and structural vascular alterations in the HC mice were associated with increased aortic expression of NOS, particularly the constitutive isoforms. nNOS was identified mainly in macrophage-like cells localized in the subendotelial layer, as well as in the media and adventitia (Figure5 C). Such increased staining did not occur in SNAC + HC mice (Figure 5C). Increased nNOS expression was confirmed by Western analysis. With both techniques, enzyme expression was shown to be decreased by SNAC (Figure 6). The other constitutive isoform, eNOS, was identified in the intima of control arteries. In HC mice, immunoreactivity to eNOS increased also in the medial layer (Figure 5E). Such

results were confirmed by Western analysis (Figure 6). Overexpression of eNOS was normalized by SNAC (Figure 5F). A single 160-KDa band was observed in Western blots of the aortic homogenates stained with anti- nNOS antibody. In the Western Analysis, HC mice showed a 180% increase in nNOS expression in comparison with the C group, whereas in SNAC + HC, no increase in the expression of the nNOS was seen. The level of expression of nNOS protein after SNAC treatment in HC mice was very close to that determined in controls. A single 136-KDa band was observed in Western blots of the aortic homogenates stained with anti- eNOS antibody.A 165% of increase in the expression of the eNOS was detected in HC group. However, SNAC treatment (SNAC + HC) brought about only a 32.5% reduction in comparison with HC mice.

DISCUSSION

The present study focused on the antiatherogenic effect of SNAC in early stage of plaque development in LDLr-/- mice. Such stage was characterized by peculiar changes in endothelial function expressed as lack of the usually observed decrease in endothelium-dependent relaxation. Rather, there was a small, but significant increase acetylcholine-mediated vascular relaxation, together with decreased endothelium-independent relaxation to sodium nitroprusside. Experimental and clinical studies have consistently shown that endothelium-dependent relaxation is lowered in hypercholesterolemia and atherosclerosis in a way that correlates with other risk factors, and is likely to be predictive of clinical outcomes[3]. Experimentally, e.g., this type of classical endothelial dysfunction develops in primates, rabbits or in double apoE^{0}xLDLr^{0} mice after 2, 4 or 10 weeks, respectively, of hypercholesterolemic diet[26-28]. Therefore, the paradoxical vasomotor changes observed in our model are surprising, although by no means incompatible with the classical impairment of

vasodilator response. We attribute these findings to two possibilities. First, this may be an early stage of dysfunctional endothelium, which could be followed by installation of the more common profile of vasomotor changes. In fact, recent observations show that vascular reactivity is decreased at 6-8 weeks if hypercholesterolemia is present in this model (Sousa HP, MD, personal communication). Second, these findings might to some extent represent a particularity of the LDLr-/- model. In any case, the opportunity in this animal model to reveal such endothelial alterations, whether or not as a transient "time window", is a particularly important tool for exposing pathophysiological mechanisms potential relevant for early atherogenic endothelial dysfunction in humans[3].

The vasomotor alterations in the aortic ring of HC mice were concomitant with significant overexpression of constitutive NOS. Increased endothelial production of NO in the early stages of hypercholesterolemia and up-regulation of eNOS expression has been reported in several animal models of atherosclerosis[29,30]. In cultured endothelial cells, 4-day incubation with native LDL induces activation of NO synthase, although with increased production of both NO and superoxide[31]. The finding of increased constitutive NOS in atherosclerosis of LDLr -/- hyperlipidemic mice is compatible with earlier experiments showing that aortas of hypercholesterolemic rabbits release larger quantities of NO than normal vessels[30]. However, paradoxical increase in the extent of lesions has been described in mice overexpressing eNOS[32]. The significant degree to which nNOS was increased in our model was a particularly noteworthy finding of our study, not recognized previously in early vascular dysfunction during atherogenesis. Moreover, this increase was observed not in the endothelial layer, but in other cells scattered throughout the media, probably infiltrating macrophages and/or smooth muscle cells. In addition, the occurrence of increased acetylcholine-mediated vasorelaxation concomitant to increased nNOS

expression suggests that this isoform is able to replace dysfunctional endothelial NOS as an emergent source of NO. This compensatory role has been described previously in human atherosclerotic vessels[33]. In addition, nNOS overexpression in the aorta of SHR rats has been suggested to attenuate increased blood pressure[34]. Pharmacological evidence for functional nNOS in conductance and resistance arteries has been reported[35]. However, full structural characterization of a "vascular" or other extra-neural form of neuronal NOS is still incomplete. There is evidence of the localization of nNOS in a caveolar domain in smooth muscle cells[36], as well as for the existence of splice variant(s)[35,37]. How such non-endothelial cells are able to trigger off vasorelaxation upon acetylcholine challenge is still unclear. Smooth muscle cells exhibit muscarinic receptors, although at lower levels than do endothelial cells[38], and these receptors can be induced by interferon-gamma in monocytic cell lines[39]. Increased nNOS expression in cardiomyocytes were attributed to increase in cytosolic calcium concentration [40]. Finally, our data do not allow exclusion of an NO/NOS-independent mechanism as the cause of such enhanced relaxation in HC mice. For example, increased production of EDHF has been reported in hypercholesterolemia[41].

Administration of SNAC decreased the extent of plaques and reverted dysfunctional changes of endothelium and smooth muscle cells early in the course of atherosclerosis in hyperlipidemic LDLr -/- mice. Direct effects of NO and/or decreased superoxide levels due to its scavenging by NO are likely mechanisms underlying such effects. The minor SNAC effects in plasma lipid fractions are not sufficient to explain its effects. One such direct effect is the inhibition of lipid peroxidation. NO reacts with lipid peroxides at a constant diffusion-limited rate of $\sim 10^{10}\ M^{-1}.\ s^{-1}$, rendering this intermediate one of the most potent known biological antioxidants[42]. The adducts between nitric oxide and lipid peroxides may further act as NO reservoirs[43]. However, the *in vivo* half-life of SNAC is yet unclear,

considering that its *in vitro* effects last for 10 hours, while the *in vivo* hypotensive effect lasts only minutes[16]. Therefore, the importance, in this animal model, of direct SNAC effects due to sustained NO release is not unequivocal. The reversal by SNAC of the vasomotor alterations may thus have been due to an indirect sustained effect triggered by SNAC. In particular, SNAC treatment was associated with decreased expression of constitutive NOS, particularly nNOS. The lack of change in nitrotyrosine levels after SNAC suggest that pathways other than peroxynitrite generation were involved in nitrotyrosine formation e.g., a reaction between NO and oxygen or enzymatic reaction between nitrite and HOCl produced by myeloperoxidase from leukocytes[42].

The decreased sensitivity of hyperlipidemic aortas to nitroprusside is probably due to enhanced desensitization due to increased constitutive NOS-derived vessel wall NO levels. We attribute this to the inhibitory action of NO on NO synthase, possibly involving direct interaction between NO with enzyme-bound ferric heme. The reversal of this decreased sensitivity with SNAC may be due to a decrease toward normal constitutive NOS expression in the aorta. RSNOs are able to promote the S-nitrosation of thiol residue of proteins through trans-nitrosation reactions [15,44]. These reactions may be involved in the recovery of SMC sensitivity to SNP, observed after SNAC treatment, via modification of the enzymatic system involved in the redox equilibrium or signal transduction pathways.

In conclusion, our data show a novel antiatherogenic effect of the S-nitrosothiol SNAC, expressed as prevention of early dysfunctional endothelial changes in LDLr-/- mice. The mildly enhanced, rather than decreased, relaxation to acetylcholine associated with increased expression of constitutive NOS may provide an important clue to the subsequent changes that characterize established endothelial dysfunction in humans. In addition, the protective effects of a nitrosothiol compound in these processes may lead to

novel strategies to counteract such alterations and to minimize the development of atherosclerosis.

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Figure legends

Figure 1. Dose effect curves for phenylephrine (A), acetylcholine (B) and sodium nitroprusside (C) in aorta rings from LDLr -/- mice. In (B) and (C) , rings were precontracted with phenylephrine as described in the text. Table shows values for EC_{50} or EC_{75} for each agent, as defined in detail in the methods.

Figure 2. Vascular superoxide anion generation estimated by lucigenin - enhanced chemiluminescence in aortic segments from LDLr-/- mice (n= 6/group).*HC vs C, **HC vs SNAC +HC, ***C vs SNAC+HC. *,**,*** , $p < 0.05$. Data are means±SEM.

Figure 3: Quantitative analysis of atherosclerotic lesion area (μm^2) section in the proximal aorta from LDLr -/- mice. Total lesion area of treatment in 3 groups was quantified in oil-red O stained sections. Each symbol represents the average lesion size from individual mice, calculated from 10 sections/mice. Lower panels shows representative photomicrographs of Oil Red O – stained sections of aorta from C (A), HC (B) and SNAC + HC mice (C). Representative photographs. Magnification: 20x. *HC vs C, **HC vs SNAC +HC, ***C vs SNAC +HC. *, **,***, $p < 0.05$. Data are expressed as means±SEM, n=6/group.

Figure 4: Plasma FPLC lipoprotein profile in (σ) C, (O) HC and (□) SNAC + HC LDLr -/- mice. Each point represents the mean of plasma samples from triplicate measurements from 3/mice/group. Data are represented as an average distribution of total cholesterol. Fractions

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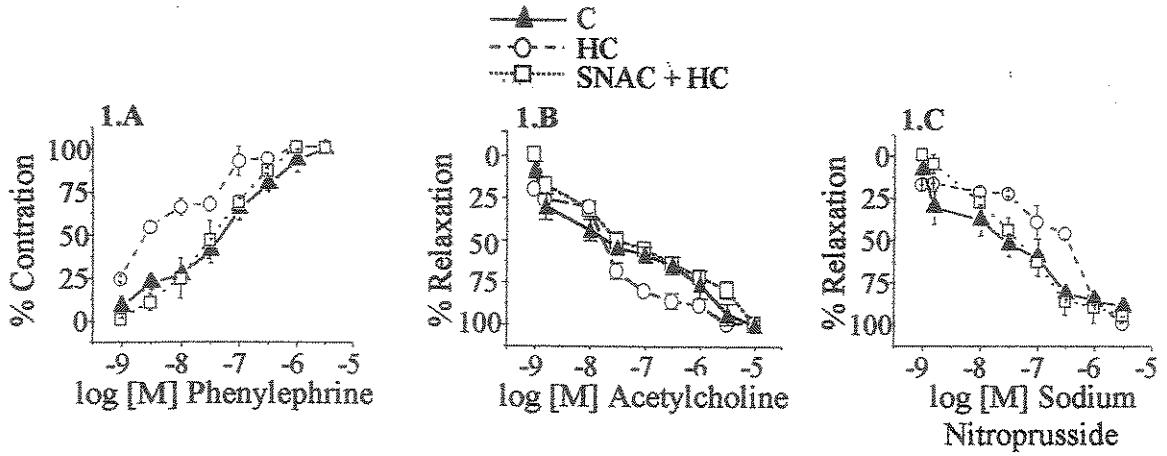
10 through 18 contain VLDL; fractions 19 through 25 contain LDL; and fractions 26 through 36 contain HDL. Fractions 37 through 40 are the non-lipoprotein associated proteins.

Figure 5: Immunohistochemical peroxidase staining of LDLr -/- mice aorta sections with anti-neuronal NOS (Panels A-C) and anti-endotelial NOS antibodies (Panels D-F). C mice (Panels A and D), HC (Panels B and E) and SNAC +HC mice (Panels C and F) Magnification:100x. Vascular lumen is positioned at the upper right corner.

Figure 6: Western analysis of neuronal (Panel A) and endothelial (Panel B) NOS protein expression in aorta of from C, HC and SNAC+HC LDLr-/- mice. Insert: Representative Western blot showing nNOS and eNOS expression, respectively. * HC vs C, **HC vs SNAC +HC, ***C vs SNAC +HC. *,**,***, $p < 0.05$. Data are mean±SEM.



1.D

	C	HC	SNAC + HC
-log EC_{50} Phe	7.31 ± 0.13	8.16 ± 0.17 *,**	7.35 ± 0.04
-log EC_{75} ACh	6.12 ± 0.17	7.74 ± 0.63 *,**	5.19 ± 0.12 ***
-log EC_{50} SNP	7.62 ± 0.26	6.54 ± 0.27 ***	7.41 ± 1

Mean ± SEM, n = 5. *, **, ***, $p < 0.05$. * HC vs C, ** HC vs SNAC + HC ,
*** C vs SNAC + HC.

Figure 1

Santos et al. Antiatherogenic effects of.

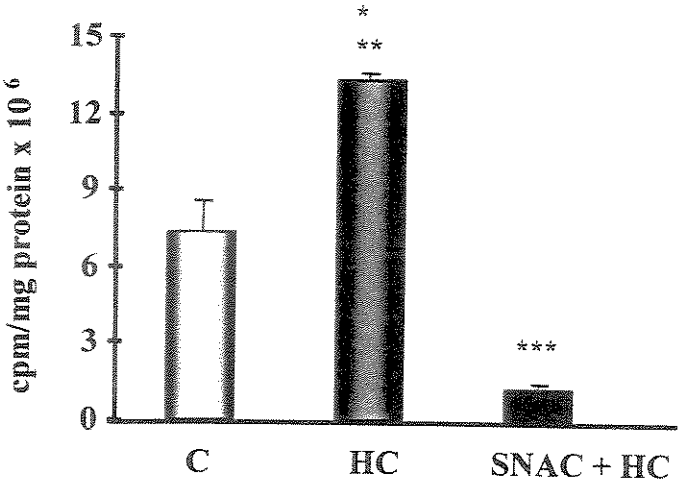


Figure 2

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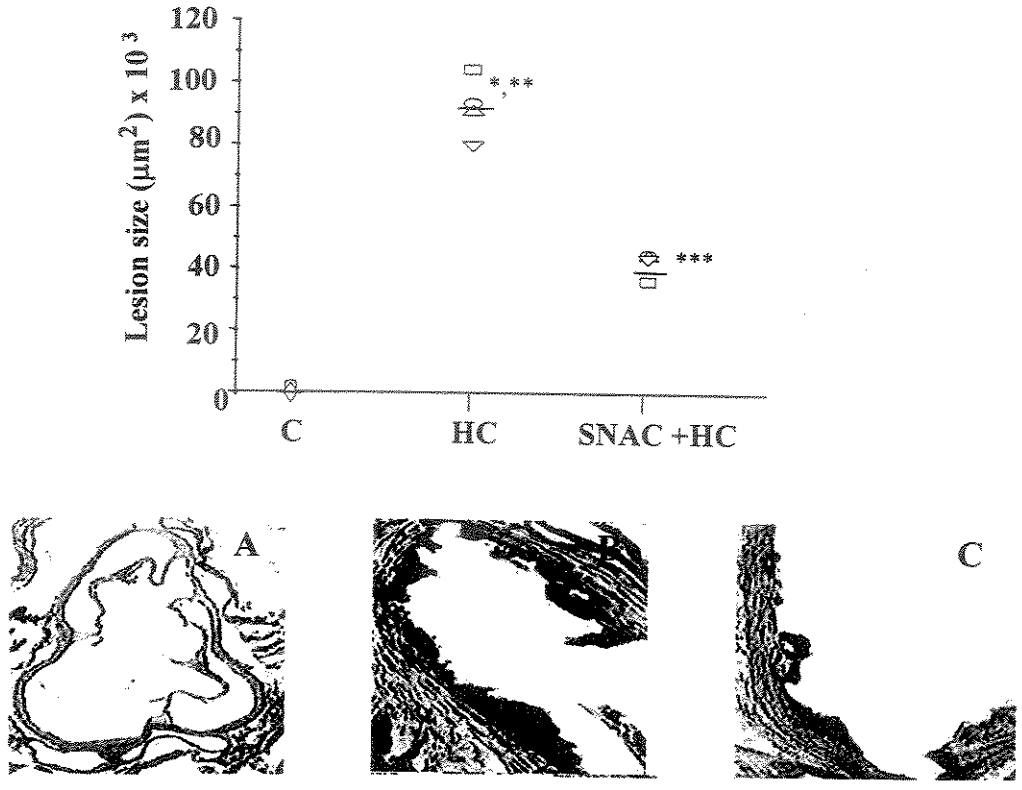

Figure 3

Santos et al. Antiatherogenic effects of.

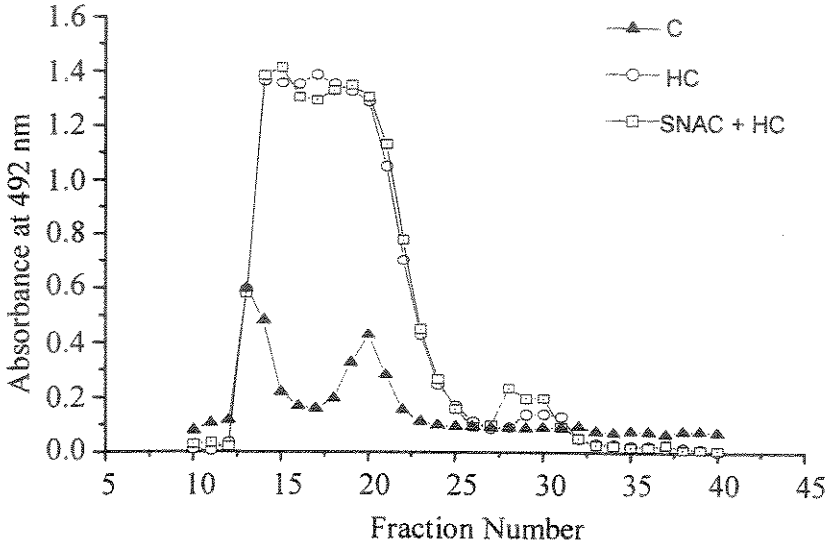

Figure 4

Santos et al. Antiatherogenic effects of.

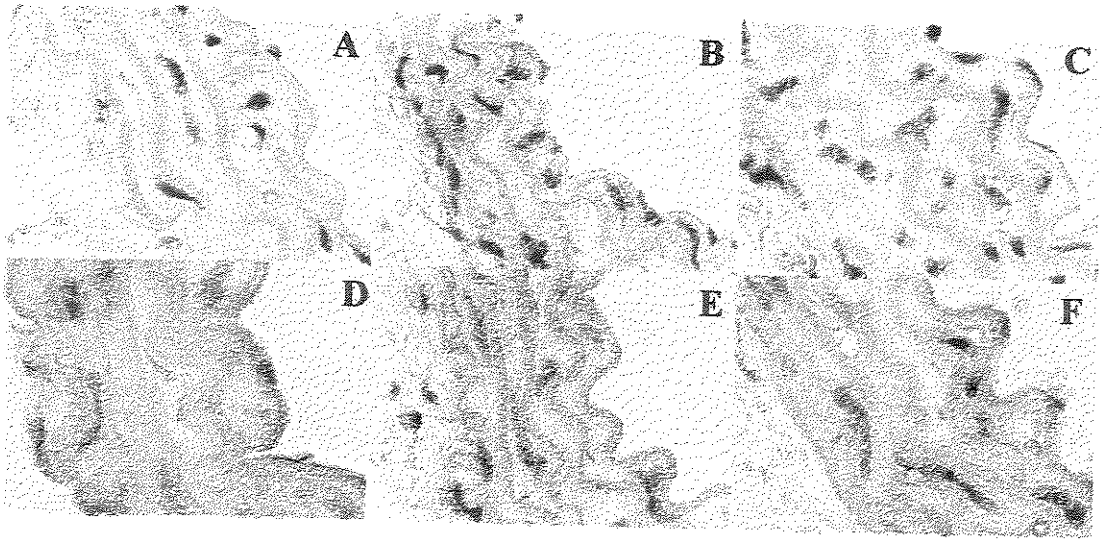

Figure 5

Santos et al. Antiatherogenic effects of.

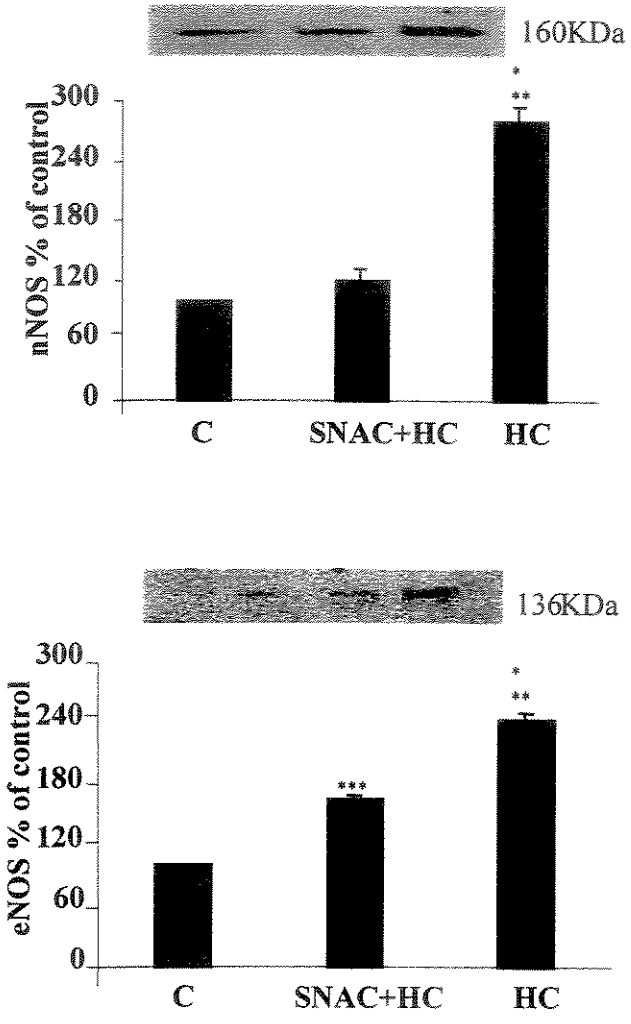

Figure 6

Santos et al. Antiatherogenic effects of.

4 - CONCLUSÕES

A caracterização do efeito hipotensor do SNAC em ratos acordados foi de suma importância para o conhecimento dos efeitos cardiovasculares deste nitrosotiol *in vivo*, do seu mecanismo de ação, bem como da sua correlação dose-efeito com outros doadores de NO amplamente utilizados. Os resultados deste estudo demonstraram que:

- O nitrosotiol SNAC mostrou ser uma droga com potente atividade hipotensora, sendo que esse efeito foi mais potente e prolongado que o nitroprussiato de sódio (NPS). Tais constatações justificam a sua utilização como hipotensor agudo e de fácil manuseio.
- O SNAC promoveu efeito hipotensor de maneira dose-dependente positiva, tanto em ratos acordados normotensos, como em hipertensos. E esse efeito foi parcialmente dependente do GMPc. O conhecimento deste mecanismo abre perspectivas para sua utilização em formulações com menor indução aos problemas de tolerância.
- O efeito sinérgico da administração de N-Acetil-L-cisteína (NAC) com nitroprussiato de sódio (NPS) potencializaram o efeito hipotensor promovido pelo NPS, produzindo um efeito hipotensor equivalente àquele obtido com a mesma dose de SNAC (EC_{50}). Esse incremento demonstrou a mediação que os tióis podem exercer na ação hipotensora promovida pelo NPS. Tal potencialização pode ter sido mediada pela formação de um complexo mais potente entre o tiol e o anion NPS, ou ainda, pode ser em decorrência da transferência do $^{\cdot}NO$ para a NAC, gerando SNAC como espécie vasodilatadora. Essas evidências encontram-se de acordo com o

mecanismo envolvido na reação de transnitrosação, mediada pelos tióis endógenos na ação hipotensora de drogas doadoras de óxido nítrico.

Os estudos que caracterizaram o efeito anti aterogênico promovido pela administração do SNAC em camundongos knockout para o receptor da LDL, sob dieta hipercolesterolêmica durante duas semanas, revelaram disfunções vasculares inéditas e importantes na fase inicial da aterogênese. Sobretudo, mostraram o potencial que este nitrosotiol exerceu na prevenção das alterações estruturais e funcionais da aterosclerose neste modelo experimental. Os resultados evidenciaram que:

- Camundongos sob dieta hipercolesterolêmica apresentaram as seguintes alterações vasculares relacionadas a aterogênese: i) desenvolvimento de área da lesão próxima a raiz da aorta, ii) aumento do estresse oxidativo, iii) disfunções de relaxamento vasculares dependente e independente do endotélio, iv) aumento nos níveis de lípides plasmáticos, v) alterações no perfil lipídico, vi) aumento na expressão das NOS sintases aórticas e vii) aumento na coloração para nitrotirosina aórtica. Dentre o conjunto de resultados, verificou-se que a instalação da placa de ateroma foi acompanhada pela supersensibilidade do relaxamento vascular endotélio-dependente, e pelo aumento de expressão das NOS constitutivas.
- Após duas semanas de tratamento com SNAC (0,51μmol/Kg i.p. diariamente) e dieta hipercolesterolêmica, foi verificado um efeito preventivo de 55 % na formação da placa e uma restauração das disfunções vasculares, desencadeadas pela dieta hipercolesterolêmica. As disfunções de motricidade vascular foram normalizadas

e acompanhadas da redução do estresse oxidativo e da superexpressão das NOS constitutivas. Tal constatação reforça a indicação da participação do sistema NO/NOS na progressão da placa aterosclerótica, e fundamentalmente, indica a possibilidade de manipulação deste sistema por meio do tratamento com o SNAC. Os resultados obtidos após o tratamento com SNAC indicam que este nitrosotiol pode ser uma droga promissora, pois se mostrou efetivo em prevenir ou minimizar importantes alterações estruturais e funcionais no período inicial desta patologia.

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