UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE FISIOLOGIA E FARMACOLOGIA

ESTUDO DOS EFEITOS CARDIOVASCULARES DO ÓLEO ESSENCIAL DO *Ocimum gratissimum* E DE SEU PRINCIPAL CONSTITUINTE, EUGENOL, EM RATOS HIPERTENSOS DOCA-SAL, ACORDADOS

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Estudos dos efeitos cardiovasculares do óleo essencial do *Ocimum gratissimum* e de seu principal constituinte, Eugenol, em ratos hipertensos DOCA – sal, acordado

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RESUMO

Os efeitos cardiovasculares do tratamento intravenoso (i.v.) com o óleo essencial do *Ocimum gratissimum* L. (Labiatae) (OEOG) e seu principal constituinte, eugenol (EUG) foram investigados no modelo experimental de ratos com hipertensão induzida por deoxicorticosterona (DOCA-sal). Em ratos hipertensos DOCA-sal e seus controles uninefrectomizados, injeções em bolus de OEOG (1-20 mg/kg) ou EUG (1-10 mg/kg) induziram hipotensão e bradicardia dose-dependentes. O tratamento com DOCA-sal aumentou significativamente as reduções máximas da pressão arterial induzidas por hexametônio (30 mg/kg, i.v.) bem como as respostas hipotensoras induzidas pelo OEOG ou pelo EUG sem afetar a bradicardia. Todavia, o aumento da hipotensão induzida pelo OEOG em ratos hipertensos, não foi afetado pelo pré-tratmento i.v. com hexametônio (30 mg/kg), propranolol (2 mg/kg) ou metilatropina (1 mg/kg). Estes resultados mostram que o tratamento i.v. com OEOG ou EUG reduz a pressão arterial de maneira dose-dependente, em ratos hipertensos DOCA-sal acordados e esta ação é aumentada quando comparada com os controles uninefrectomizados. O efeito hipotensor, parece estar mais relacionado ao relaxamento vascular ativo do que a redução do efluxo do tônus simpático para os vasos. Para corroborar esta hipótese, foram estudados os efeitos vasculares do OEOG e seu principal constituinte, EUG , e os possíveis mecanismos envolvidos nestes efeitos. Em preparações de aorta isolada, de ratos hipertensos DOCA-sal com endotélio, pré-contraídas com fenilefrina, o OEOG (1-1000 µg/mL) e EUG (0,006-6 mM) induziram relaxamento similar com valores de IC_{50} (média geométrica ± 95% intervalo de confiança) de 226,9 [147,8-348,3] µg/mL e 1,2 [0,6-2,1] mM, respectivamente. O efeito vasorrelaxante do OEOG foi significativamente reduzido pela remoção do endotélio (IC_{50} = 417,2 [349,5-497,8] µg/mL). Em meio livre de cálcio, as contrações induzidas por Ca^{2+} foram significativamente reduzidas e abolidas por concentrações de 300 e 1000 µg/mL de OEOG respectivamente, enquanto a concentração de 1000 µg/mL não teve efeito significativo sobre as contrações induzidas por cafeína. Resultados similares foram obtidos com o EUG (1,8 e 6 mM) tanto na contração induzida por $CaCl^2$ quanto naquela induzida por cafeína. Os dados sugerem que em ratos hipertensos DOCA-sal a resposta

hipotensora para o OEOG é principalmente devido a um relaxamento vascular ativo, o qual é parcialmente dependente da integridade do endotélio vascular e parece ser predominantemente mediado por uma inibição do influxo do Ca^{2+} plasmático do que por uma inibição da liberação do Ca^{2+} do retículo sarcoplasmático induzindo por Ca^{2+}. Seria de grande interesse estudar os efeitos cardiovasculares do OEOG e de seu principal constituinte EUG, em outro modelo de hipertensão como SHR.

Palavra-chave: óleo essencial, eugenol, aorta torácica isolada, hipertensão, efeito miorrelaxante, *Ocimum gratissimum*.

ABSTRACT

The cardiovascular effects of intravenous (i.v.) treatment with the essential oil of *Ocimum gratissimum* L. (Labiatae) (EOOG) and its main constituent, eugenol (EUG) were investigated in the experimental model of deoxycorticosterone-acetate (DOCA-salt) hypertensive rats. In both conscious DOCA-salt hypertensive rats and their uninephrectomized controls, i.v. bolus injections of EOOG (1-20 mg/kg) or Eug (1-10 mg/kg) induced dose-dependent hypotension and bradycardia. Treatment with DOCA-salt significantly enhanced the maximal decreases in MAP elicited by hexamethonium (30 mg/kg, i.v.) as well as the hypotensive responses to both EOOG and EUG without affecting the bradycardia. However, the enhancement of EOOG-induced hypotension in hypertensive rats remained unaffected by i.v. pretreatment with hexamethonium (30 mg/kg), propranolol (2 mg/kg) or methylatropine (1 mg/kg). These results show that i.v. treatment with EOOG or EUG dose-dependently decreased blood pressure in conscious DOCA-salt hypertensive rats, and this action is enhanced when compared with uninephrectomized controls. The hypotensive effect that seems related to an active vascular relaxation rather than withdrawal of sympathetic tone. To corroborate this hypothesis, we examined the vascular effects of EOOG and its main constituent, EUG and the putative mechanisms underlying these effects. In isolated aorta preparations with intact endothelium from DOCA-salt hypertensive rats, EOOG (1-1000 µg/mL) and EUG (0.006-6 mM) relaxed similarly the phenylephrine-induced contraction with IC_{50} (geometric mean ± 95% confidence interval) values of 226.9 [147.8-348.3] µg/mL and 1.2 [0.6-2.1] mM, respectively. Vasorelaxant effects of EOOG were significantly altered by removal of the vascular endothelium (IC_{50} = 417.2 [349.5-497.8] µg/mL). In calcium-free medium, the $CaCl_2$-induced contractions were significantly reduced and even abolished by EOOG at 300 and 1000 µg/mL, respectively, while EOOG (1000 µg/mL) was without significant effect on caffeine-induced contractions. Similar results were obtained with EUG (1.8 and 6 mM) on both $CaCl_2$- and caffeine-induced contractions. The data suggest that hypotensive responses to EOOG in DOCA-salt hypertensive rats are mainly due to an active vascular relaxation, which is partly dependent upon the integrity of the vascular endothelium and seems predominantly mediated through an inhibition of plasmalemmal Ca^{2+} influx rather than of Ca^{2+}-induced Ca^{2+} release from the

sarcoplasmic reticulum. It would be of interest to assess the cardiovascular effects of the EOOG, and its main constituent EUG, in another hypertensive model, such as SHR.

Keywords: essential oil, eugenol, isolated thoracic aorta, hypotension, myorelaxant effect, *Ocimum gratissimum*.

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LISTA DE ABREVIATURAS

ACh	acetilcolina
AC	adenilato ciclase
ANOVA	análise de variância
AMPc	3’5’-adenosina monofosfato cíclico
ATP	adenosina trifosfato
b.p.m.	batimentos por minuto
Ca^{2+}	íon cálcio
$CaCl_2$	cloreto de cálcio
Cl^-	íon cloro
CO_2	dióxido de carbono
$[Ca^{2+}]_i$	concentração de cálcio intracelular
DAG	diacilglicerol
DOCA-sal	modelo de hipertensão por sobrecarga de desoxicorticosterona e sal
EAZ	extrato de *Alpinia zerumbet*
ET	endotelina
EUG	eugenol
FC	freqüência cardíaca
FHDE	fator hiperpolarizante derivado do endotélio
FRDE	fator relaxante derivado do endotélio
g	grama
GC	guanilato ciclase
GDP	difosfato de guanosina
GMPc	3’5’-guanosina monofosfato cíclico
GTP	trifosfato de guanosina
HEX	hexametônio
i.p.	intraperitoneal
i.v.	intravenoso
IC_{50}	concentração efetiva para inibir 50% da resposta
IP_3	1,4,5-trifosfato de inositol

ISO	International Standard Organization
K^+	íon potássio
K_{ATP}	canais para potássio sensíveis ao ATP
K_{Ca}	canais para potássio sensíveis ao Ca^{+2}
L-NAME	N^G nitro-L-arginina-metil-ester
MA	metil-atropina
ME	metil-eugenol
mg	miligrama
mg/kg	miligrama por kilograma
min	minuto
ml	mililitro
MLCK	quinase da cadeia leve de miosina
mM	milimolar
mmHg	milímetros de mercúrio
Na^+	íon sódio
NCX	trocador Na^+/Ca^{2+}
nM	nanomolar
ONS	óxido nítrico síntase
OE	óleo essencial
OEAC	óleo essencial de *Aniba canellila*
OEAZ	óleo essencial de *Alpinia zerumbet*
OECN	óleo essencial de *Croton nepetaefolius*
OECZ	óleo essencial de *Croton zehntneri*
OEOG	óleo essencial do *Ocimum gratissimum*
OMS	organização mundial de saúde
ON	óxido nítrico
OP	óxido de piperitenona
PAD	pressão arterial diastólica
PAM	pressão arterial média
PAS	pressão arterial sistólica
FEN	fenilefrina
PIP_2	fosfatidilinositol 4,5-bifosfato
PKA	proteína quinase A
PKC	proteína quinase C

PM	peso molecular
ROCs	canais operados por receptor
RhoA	proteína GTPase
RhoGEF	fator de troca do nucleotídeo guanina
s	segundos
s.c.	sub-cutâneo
Trp-4-ol	terpinen-4-ol
UI	unidades internacionais
VOCs	canais operados por voltagem
α	alfa
β	beta
µg	micrograma
µL	microlitro
µm	micrometro
%	porcentagem

1. INTRODUÇÃO

1.1 Generalidades

O uso de plantas no tratamento e na cura de enfermidades é tão antigo quanto à civilização humana. Ainda hoje nas regiões mais pobres do Brasil e até mesmo nas grandes cidades, plantas medicinais são comercializadas em feiras livres, mercados populares e encontradas em quintais residenciais (MACIEL *et al.,* 2002). Durante a última década, o consumo de ervas tem aumentado consideravelmente em países como a América do Norte e Europa, particularmente na Alemanha e França (CAPASSO *et al.*, 2003). Estudos têm mostrado que dois terços das mulheres usam ervas para sintomas da pré-menopausa, 45% dos pais tratam as crianças com ervas e 45% das mulheres gestantes utilizam as ervas como remédio (ERNST, *et al.,* 2004). Mas, a maioria dos produtos utilizados não é licenciada e não possui uma demonstração de eficácia, segurança ou qualidade (DE SMET *et al.,* 2002). Embora, as ervas sejam freqüentemente promovidas como natural não são desprovidas de efeitos adversos (PALMER, *et al.,* 2003).

Para a Organização Mundial de Saúde (OMS), planta medicinal é qualquer planta que possua em um ou em vários de seus órgãos, substâncias usadas com finalidade terapêutica, ou como ponto de partida para a síntese de produtos químicos e farmacêuticos. A estas substâncias é dado o nome de princípios ativos, responsáveis pelo efeito terapêutico que a planta medicinal possui. As funções fisiológicas dos princípios ativos nas plantas ainda não estão completamente esclarecidas, mas a sua produção está associada à defesa da planta contra agentes externos como doenças, pragas, radiação solar, ou a resíduos do metabolismo vegetal. Estes princípios ativos possuem funções ecológicas importantes para a sobrevivência da espécie e são produzidos, em sua grande maioria, através do metabolismo secundário das plantas (MONTANARI, 2002).

Na Europa, plantas são largamente utilizadas na medicina tradicional, em particular para o tratamento da tosse, de problemas circulatórios, de dores musculares, problemas digestivos, gripe, insônia, ansiedade, e também para o tratamento de patologias do fígado, rins e bexiga. Algumas plantas também são

utilizadas na preparação de alimentos e como suplemento alimentar, além da utilização de substâncias derivadas destas para produção de cosméticos e perfumes (SILANO *et al. ,* 2004).

No Brasil, a utilização de plantas como meio curativo é uma prática bastante difundida e popular, às vezes, empregada de maneira equivocada e até mesmo malévola, visto que muitas plantas possuem princípios tóxicos e o seu uso indiscriminado pode causar sérios problemas (MATOS *et al.,* 2001; LORENZI & MATOS, 2002). Na região Nordeste, o uso de plantas medicinais e preparações caseiras assumem importância fundamental no tratamento das patologias que afetam as populações de baixa renda devido a deficiência de assistência médica (MATOS, 1989).

As plantas têm fornecido um grande número de agentes clinicamente úteis, possuindo um considerável potencial como fontes de novas drogas. Há estimativas de que existam aproximadamente 200.000 espécies de plantas no mundo, várias delas correndo o risco de extinção. Aproximadamente 20.000 espécies destas plantas são utilizadas na medicina tradicional, entretanto a maioria não foi avaliada do ponto de vista químico ou farmacológico. Postula-se que aproximadamente 25% de todos os medicamentos modernos sejam derivados das plantas de forma direta ou indireta (CALIXTO, 2000).

O mercado mundial de fitoterápicos movimenta cerca de US$ 22 bilhões por ano e vem seduzindo a cada ano mais adeptos nos países desenvolvidos. Em 2000, o setor faturou US$ 6,6 bilhões nos Estados Unidos da América e US$ 8,5 bilhões na Europa, sendo a Alemanha o maior mercado mundial de fitoterápicos (FERNANDES & ANTUNES *et al.,* 2000).

1.2. Óleos essenciais

Os óleos essenciais (OEs) são definidos como os produtos obtidos de partes de plantas, sendo que de forma geral são misturas complexas de substâncias voláteis, lipofílicas, geralmente odoríferas e líquidas. Do ponto de vista químico, os OEs das plantas são constituídos principalmente de uma mistura de lipídeos chamados terpenos. Os terpenos são hidrocarbonetos e aqueles que são oxigenados são denominados terpenóides. Os OEs são encontrados em várias plantas, mas são especialmente abundantes nas

mirtáceas, coníferas, labiadas, rutáceas, lauráceas e umbelíferas (MATOS & FERNANDES, 1975-1978; CRAVEIRO *et al.*, 1976a).

Os métodos de extração variam conforme a localização do óleo volátil na planta e com a proposta de utilização do mesmo. Os métodos mais comuns são: enfloração, destilação por arraste de vapor d'água, extração com solvente orgânico de forma contínua e descontínua, prensagem/espressão e extração por CO_2 supercrítico. O método mais empregado para a obtenção dos OEs é a destilação pelo vapor no qual o OE é evaporado e condensado por resfriamento (CRAVEIRO *et al.*, 1976b, 1981).

A ISO (International Standard Organization) define óleos voláteis como os produtos obtidos de partes de plantas através da destilação por arraste de vapor d'água, bem como os produtos obtidos por espressão / prensagem dos pericarpos de frutos cítricos (Rutaceae).

Dependendo da família, os óleos voláteis podem ocorrer em estruturas secretoras especializadas, tais como pêlos glandulares nas Lamiaceae, células parenquimáticas diferenciadas nas Lauraceae, Piperaceae e Peaceae, nos canais oleíferos nas Apiaceae ou em bolsas lisígenas ou esquizomógenas nas Pinaceae e Rutaceae. Os óleos têm a função de proteger as plantas de doenças e parasitas, além de atrair insetos que auxiliam na polinização. Adicionalmente, catalisam reações bioquímicas, agindo como hormônios ou reguladores, e dessa forma desempenham um importante papel na bioquímica da planta (LAVABRE, 1993).

A maioria dos OEs possui índice de refração e são opticamente ativos, sendo que essas propriedades são utilizadas na sua identificação e no controle de qualidade. Seus constituintes variam desde hidrocarbonetos terpênicos, álcoois simples e terpênicos, aldeídos, cetonas, fenóis, ésteres, éteres, óxidos, peróxidos, furanos, ácidos orgânicos, lactonas, cumarinas, até compostos sulfurados (MATOS & FERNADES, 1975-1978; LAVABRE, 1993).

Os terpenos representam uma classe de produtos naturais amplamente distribuídos no reino vegetal, sendo uma família de compostos quimicamente diversos (DI STASI *et al.,*, 1994). Possuem uma composição molecular típica, formada por duas ou mais unidades isoprênicas ($C_{10}H_{15}$) (NABETA *et al.,* 1995). Os terpenos representam a segunda classe com maior número de constituintes ativos obtidos de plantas, perdendo apenas para os flavonóides.

São classificados de acordo com as unidades de carbonos e as formas de ciclização, estando subdivididos em: monoterpenos (10 unidades carbono), sesquiterpenos (15 unidades carbono), diterpenos (20 unidades carbono), sesteterpenos (25 unidades carbono), triterpenos (30 unidades carbono) e tetraterpenos (40 unidades carbono) (DI STASI *et al.,*, 1994).

Os constituintes podem ser ativos ou inativos do ponto de vista farmacológico e as ações dos OEs provavelmente refletem uma ação combinada dos constituintes. Os constituintes ativos podem atuar de forma sinérgica ou não, ao passo que os constituintes inativos podem influenciar a farmacocinética dos ativos. Na mistura, esses compostos se apresentam em diferentes concentrações, e normalmente, um deles é o composto majoritário, sendo que outros estão em menores teores e alguns em baixíssimas quantidades. Além disso, a composição química dos OEs pode variar durante o dia e ao longo do ano e dependendo do clima , do solo, da variedade e da parte da planta de onde é extraído (folha, casca, caule, raiz). Devido a essa complexa composição química, postula-se que os OEs apresentam também uma diversidade de ações farmacológicas, sendo fontes potenciais para o desenvolvimento de novas drogas (CRAVEIRO *et al*., 1978, 1980; ALBUQUERQUE, 1982; MAGALHÃES, 1997).

Além dos óleos voláteis obtidos de plantas (fitogênicos), produtos sintéticos são encontrados no mercado, sendo que esses óleos sintéticos podem ser imitações dos naturais ou composições de fantasia. Para uso farmacêutico, somente os naturais são permitidos pelas farmacopéias, excetuando aqueles óleos que contêm somente uma substância como o óleo volátil de baunilha (que possui somente vanilina). Nesses casos, algumas farmacopéias permitem o uso do equivalente sintético.

As plantas aromáticas são empregados na medicina popular na forma de chás e infusatos no tratamento da rinite alérgica (BEZERRA, 1994), da cólica menstrual, da diarréia sanguinolenta, da amebíase e giardíase, e como sedativos, estomáquicos (FREISE, 1935; ITOKAWA *et al*., 1981; KIUCHI *et al*., 1992; BEZERRA, 1994), antimicrobianos, analgésicos, diuréticos (LUZ *et al.,* 1984; MENDONÇA, 1989; MENDONÇA *et al*., 1991), antimaláricos (KLAYMAN, 1985), anti-sifilíticos (MENDONÇA, 1989), hipotensores e /ou anti-hipertensivos (LUZ *et al*., 1984; MENDONÇA, 1989).

A indústria utiliza amplamente os OEs, como aromatizantes, para preparação de perfumes, sabões, desinfetantes e cosméticos, na preparação de alimentos como doces caseiros, licores, bebidas aromáticas, refrescos e aguardentes de cana (JACOBS, 1948; LE BOURHIS, 1968, 1970; LE MOAN, 1973; CRAVEIRO *et al*., 1977; ITOKAWA *et al*., 1981). Os OEs são empregados também para mascarar odores desagradáveis em ambientes de trabalho e instalações sanitárias, além de serem usados como insumo em diversos produtos das indústrias de plástico, tintas, borrachas, inseticidas e outras (CRAVEIRO *et al.*, 1981).

1.3. Aromaterapia

A aromaterapia pode ser definida como sendo o uso dos OEs (PRICE & PRICE, 1999). O termo "aromaterapia" foi criado por um químico francês, Maurice René de Gattefossé em 1937, que após ter queimado as mãos, colocou-as acidentalmente, em um tanque contendo OE de lavanda, pensando que fosse água. Para sua surpresa a dor passou e ocorreu cicatrização do ferimento sem infecção. A partir deste evento passou a pesquisar as atividades terapêuticas dos OEs, que eram usados com finalidade cosmética e como odorizante (HUDSON & DISTEL, 1999).

Nos últimos anos a aromoterapia vem conquistando uma posição ampla no mercado de trabalho, pois foi observado que sua utilização em empresas além de prevenir doenças relacionadas ao trabalho aumenta a produtividade dos trabalhadores (BARON, 1988). Outro efeito importante em perfumar os ambientes com OEs é seu efeito purificador, tanto bactericida, como antiviral e antifúngico, ajudando a reduzir as alergias e doenças respiratórias que podem ser causadas por ar condicionados. Eles também reduzem a possibilidade da infecção por doenças contagiosas que são propagadas em ambientes fechados, como escolas e escritórios (KNASKO, 1992).

1.4. Efeitos biológicos/farmacológicos e toxicológicos dos óleos essenciais

As substâncias odoríferas em plantas foram consideradas por muito tempo como "desperdício fisiológico", ou mesmo produtos de desintoxicação,

como eram vistos os produtos do metabolismo secundário (SIMÕES & SPITZER, 1999).

Existem trabalhos demonstrando que a toxicidade de alguns componentes dos óleos voláteis constitui uma proteção contra predadores e infestantes. Mentol e mentona são, por exemplo, inibidores do crescimento de vários tipos de larvas (SIMÕES & SPITZER, 1999). Existem também evidências de que alguns insetos utilizam óleos voláteis, seqüestrados de plantas, para se defenderem de seus predadores. Assim, os vapores de certas substâncias como o citronelal (utilizado para combater formigas) e α-pineno (utilizado para combater cupins) podem causar irritação suficiente em um predador para fazê-lo desistir de um ataque. Certos himenópteros, por exemplo, seqüestram (sem alteração química) α- e ß- pineno, entre outros componentes, do *Pinus sylvestris* L. (uma conífera européia). Dessa forma, as larvas desses insetos se defendem de predadores como as formigas (HARBORNE, 1993).

Algumas propriedades farmacológicas dos OEs estão relativamente bem estabelecidas: ação carminativa (alguns óleos produzem uma certa anestesia sobre a cárdia, permitindo seu relaxamento e conseqüente expulsão do ar do trato gastrintestinal como por exemplo o funcho, a erva-doce, a camomila e a menta); ação antiespasmódica (alguns óleos relaxam a musculatura lisa intestinal, diminuindo ou mesmo suprimindo espasmos, por exemplo a camomila, a macela, o alho, o funcho, a erva-doce, a sálvia); ação estimulante sobre secreções do aparelho digestivo, justificando a propriedade estimulante do apetite (cita-se como exemplo: o gengibre, a genciana, o zimbro); ação irritante tópica (alguns produtos, tais como a essência de terebintina, provocam um aumento da microcirculação local com conseqüente efeito rubefaciente e, em certos casos, uma ação anestésica local); ação secretolítica (a ação irritante tópica pode provocar a atividade secretora do epitélio respiratório, facilitando a fluidificação e a expulsão do muco, benéfica em alguns problemas respiratórios são exemplos, o eucalipto e anis-estrelado); ações sobre o sistema nervoso central: estimulante (óleos voláteis contendo cânfora), depressora (melissa, capim-limão) ou mesmo provocando convulsões em doses elevadas (losna, erva-de-santa-maria, sálvia, canela); ação anestésica local (óleo volátil do cravo-da-índia, pelo seu alto teor em eugenol (EUG)); ação

anti-séptica (uso externo) (alguns óleos voláteis incluindo o EUG inibem o crescimento de várias bactérias e fungos devido à presença de compostos fenólicos, aldeídos e álcoois) (SIMÕES & SPTIZER, 1999).

A toxicidade crônica dos óleos voláteis é pouco conhecida e ainda é necessário avaliar suas eventuais propriedades mutagênicas, teratogênicas e/ou carcinogênicas, enquanto a toxicidade aguda é mais conhecida causando: reações cutâneas, efeitos convulsivantes e efeitos psicotrópicos. Deve-se, também, atentar para a sensibilidade dos indivíduos aos inúmeros componentes químicos de um óleo volátil e a ingestão concomitante de certos medicamentos, pois todos esses fatores podem provocar o aparecimento de reações adversas e/ou tóxicas. Geralmente a toxicidade dos óleos voláteis é dose dependente; entretanto, existem situações, nas quais mesmo o uso de baixas doses pode provocar reações severas, principalmente nos casos de alergias de contato (sensibilização cutânea) e de fototoxicidade. O grau de toxicidade depende, também, da via de administração sendo a ingestão oral aquela que apresenta maiores riscos, especialmente se os óleos voláteis forem ingeridos concentrados. A maior parte dos dados relativos à toxicidade dos óleos voláteis diz respeito à administração oral (SIMÕES & SPTIZER,1999).

1.5. Efeitos cardiovasculares dos óleos essenciais e de seus principais constituintes

O Laboratório de Farmacologia Cardiovascular da Universidade Federal de Pernambuco tem investigado os efeitos farmacológicos de vários OEs, dos quais seis tiveram interesse especial. São os OEs provenientes de *Croton nepetaefolius* Baill. (Euphorbiaceae) (marmeleiro vermelho), *Mentha villosa* Huds. (Labiatae) (menta rasteira), *Alpinia zerumbet* ou *speciosa* K. Schum (Zingiberaceae) (colônia), *Croton zehntneri* Pax. (Euphorbiaceae) (canela de cunhã), *Aniba canelilla* Mez. (Lauraceae) (casca preciosa) e *Ocimum gratissimum* L. (Labiatae) (alfavacão), que são plantas medicinais aromáticas com grande uso na medicina popular e com efeito antiespasmódico.

O *O. gratissimum* é utilizado como calmante e a *A. zerumbet* como anti-hipertensivo. Fitoterápicos da *Mentha villosa* já são comercializados industrialmente no Nordeste para o tratamento de giardíase e amebíase.

Estudos realizados em roedores mostraram que os OEs dessas plantas, e seus principais constituintes, apresentam grande eficácia farmacológica em tecidos excitáveis. Em preparações de músculo liso possuem um efeito em comum: são depressores da motilidade (miorrelaxantes e antiespasmódicos) (MAGALHÃES, 1997; COELHO-DE-SOUZA, 1997; MAGALHÃES *et al.,* 1998a; COELHO-DE-SOUZA *et al.,* 1998; SOUSA, 1999; LEAL-CARDOSO & FONTELES, 1999; BEZERRA *et al.,* 2000; MAGALHÃES, 2002; MAGALHÃES *et al.,* 2003, 2004).

Um conjunto de evidencias sugere que esses óleos agem diretamente sobre a célula muscular lisa, atuando provavelmente ao nível intracelular e independentemente dos mecanismos situados na membrana plasmática (MAGALHÃES, 1997; COELHO-DE-SOUZA, 1997; COELHO-DE-SOUZA *et al.,* 1997, 1998; MAGALHÃES *et al.,* 1998a; SOUSA, 1999; MAGALHÃES, 2002, MAGALHÃES *et al.,* 2003, 2004). Os efeitos desses OEs sobre o músculo liso não são idênticos, possuindo inclusive aspectos que implicam mecanismos de ação diferentes. Entretanto, são poucos os dados na literatura que relatam os possíveis efeitos hipotensores e/ou anti-hipertensivos desses OEs (ou de seus respectivos constituintes), e menos ainda, os que analisam o mecanismo de ação no sistema cardiovascular. Portanto, estudos neste sentido podem vir a corroborar o uso destas plantas aromáticas, na medicina popular, para o tratamento da hipertensão arterial, cuja prevalência é alta em nossa sociedade.

Os estudos sobre os efeitos cardiovasculares de OEs das plantas supramencionadas são descritos resumidamente abaixo.

1.5.1. Croton nepetaefolius

O *C*. *nepetaefolius* é utilizado na medicina popular como antiespasmódico. O OE do *C*. *nepetaefolius* (OECN) possui como principais constituintes o 1,8-cineol e o metil-eugenol (ME). Foi mostrado que a administração intravenosa (i.v.) do OECN induz quedas dose-dependentes da pressão arterial média (PAM) e da freqüência cardíaca (FC) em ratos normotensos anestesiados ou acordados (LAHLOU *et al.,* 1996; LAHLOU *et al.,* 1999). A hipotensão e bradicardia induzidas pelo OECN são independentes. A bradicardia é de origem vagal, uma vez que foi bloqueada pelo pré-tratamento

com metil-atropina (MA) ou bivagotomia, e reduzida pelo pré-tratamento com hexametônio (HEX), enquanto a hipotensão parece não ser de origem pré-juncional, uma vez que ocorre o mesmo efeito quando a transmissão autonômica central para o sistema vascular está bloqueada pelo HEX (LAHLOU *et al.,* 1999). Portanto, foi postulado que a atividade hipotensora do OECN pode resultar de seus efeitos vasodilatadores diretos sobre a musculatura lisa vascular (LAHLOU *et al.,* 1999). A estimulação dos receptores muscarínicos vasculares que normalmente induz uma hipotensão, não está, provavelmente, envolvida neste processo, uma vez que o pré-tratamento com MA não afetou a hipotensão induzida pelo OECN.

A hipótese de uma ação direta do OECN sobre a musculatura lisa vascular para induzir o efeito hipotensor foi corroborada pelos achados em estudos realizados *in vivo* e *in vitro* utilizando ratos hipertensos (LAHLOU *et al.,* 2000). De fato, experimentos *in vivo* mostram que a administração i.v. do OECN em ratos hipertensos DOCA-sal, acordados, também induzem quedas dose-dependentes da PAM e da FC, mas os efeitos hipotensores foram significativamente amplificados, tanto na magnitude (absoluta ou relativa) quanto na duração, em relação aos ratos controles uninefrectomizados, normotensos (LAHLOU *et al.,* 1996, 2000). Em ratos hipertensos, o pré-tratamento i.v. com HEX reduziu a magnitude da bradicardia sem alterar a hipotensão. Experimentos *in vitro,* utilizando preparações de aorta isolada, mostram que os efeitos inibitórios do OECN na contração induzida pela fenilefrina foram significativamente maiores (a IC_{50} foi reduzida em 7 vezes) nas preparações, de ratos hipertensos DOCA-sal do que naquelas dos controles uninefrectomizados (MAGALHÃES *et al.,* 1998b; LAHLOU *et al.,* 2000; MAGALHÃES, 2002). O conjunto desses resultados sugere que a amplificação do efeito hipotensor induzido pelo OECN em ratos hipertensos DOCA-sal é devido principalmente ao aumento da resposta vascular ao óleo do que ao aumento da atividade do sistema nervoso simpático neste modelo de hipertensão (LAHLOU *et al.,* 2000).

Foi ainda demonstrado que a hipotensão induzida pelo ME (10 mg/kg) em ratos anestesiados ou acordados, foi associada a uma bradicardia significativa que pareceu ser de origem vagal, uma vez que foi reduzida significativamente pela bivagotomia ou pelo tratamento i.v. com MA (LAHLOU

et al., 2004a), como foi demonstrado para as respostas bradicardizantes ao OECN (LAHLOU *et al.*, 1999). Tais achados com a MA, apontam mecanismos independentes para a hipotensão e bradicardia induzida pelo ME e excluem a possibilidade de que a hipotensão seja devido a uma bradicardia concomitante. Nesse estudo, foi utilizada a combinação de experimentos *in vivo* e *in vitro*, mostrando pela primeira vez que o tratamento i.v. de ME em ratos anestesiados ou acordados, induz um efeito hipotensor devido mais a um relaxamento vascular ativo do que a retirada do tônus simpático (LAHLOU *et al.,* 2004a). Mecanismo de ação similar foi proposto para os efeitos cardiovasculares da administração i.v., em ratos acordados ou anestesiados, do principal constituinte do OECN, o 1,8-cineol (LAHLOU *et al.,* 2002a). Esses resultados podem sugerir que tanto o ME quanto o 1,8-cineol contribuem na mediação dos efeitos hipotensores de OE de algumas plantas aromáticas usadas, na medicina popular, para o tratamento da hipertensão.

1.5.2. Mentha x villosa

Mentha x villosa Huds. (Labiatae) é uma planta rasteira aromática, comumente encontrada em jardins de casas no nordeste do Brasil (MATOS, 1994). Ela é popularmente conhecida como menta-rasteira, hortelã-rasteira, hortelã-comum ou hortelã-da-folha-miúda (MATOS, 1994). Infusões e decocções das folhas de *Mentha x villosa* são geralmente usadas como estomáquico, ansiolítico, e também para o tratamento de cólicas menstruais e diarréia sanguinolenta (MATOS, 1994). O OE de *Mentha villosa* (OEMV), e o seu principal constituinte, o óxido de piperitenona (OP) foi reportado por possuir efeitos miorrelaxante intestinal e antiespasmódico em animais de laboratório (SOUSA *et al.,* 1997).

Foi relatado que o tratamento i.v. do OEMV, em ratos anestesiados, induz hipotensão e bradicardia que foram atribuídas às ações do principal constituinte desse óleo, o OP (LAHLOU *et al.,* 2001). Estes efeitos cardiovasculares também são independentes, uma vez que a bradicardia, mas não a hipotensão, induzida pelo OEMV parece necessitar da presença de um fluxo neural simpático funcional e operacional para o coração. Isto sugere que a atividade hipotensora do OEMV pode ser resultante de um efeito

vasodilatador direto sobre a musculatura lisa vascular (LAHLOU *et al.,* 2001). Esta hipótese foi corroborada por duas linhas de evidências. Primeira, o OEMV reduziu a contração induzida pelo potássio (60 mM) na preparação de aorta isolada de rato. Esta atividade relaxante da musculatura lisa foi significativamente reduzida pela incubação de anéis de aorta com endotélio intacto, na presença do N^G nitro-L-arginina-metil-ester (L-NAME), ou pela remoção do endotélio (LAHLOU *et al.,* 2002b). Isto sugere que o óxido nítrico (ON) liberado das células endoteliais vasculares parece estar envolvido parcialmente no relaxamento da aorta, induzido pelo OEMV, e conseqüentemente na mediação da hipotensão. Segunda linha de evidência mostrou-se que a resposta hipotensora para o OEMV i.v. é maior em ratos hipertensos DOCA-sal, acordados, do que nos ratos uninefrectomizados controles (LAHLOU *et al.,* 2002c). Como foi observado para o OECN (LAHLOU *et al.,* 2000), o aumento da hipotensão não foi afetado pelo pré-tratamento i.v. com o HEX. Isto sugere que pode estar relacionado principalmente ao aumento da resposta vascular para o OEMV do que ao aumento da atividade do sistema nervoso simpático, neste modelo de hipertensão (LAHLOU *et al.*, 2002c).

Os dados supramencionados estão de acordo com aqueles encontrados na literatura, que mostram que o efeito hipotensor do OEMV (GUEDES *et al.,* 2004a) assim como do OP (GUEDES *et al.,* 2002, 2004b), também é devido a uma ação vasodilatadora, atribuída a mecanismos que tanto são dependentes do endotélio (via ON e prostaciclina) quanto independentes deste (bloqueio dos canais de Ca^{2+}). Nas ações de bloqueio de canais de Ca^{2+}, o OP inibiu a contração induzida pela fenilefrina. O fato das mesmas concentrações do OP que inibem as contrações de fenilefrina não inibirem as contrações da cafeína sugere que o efeito inibitório do OP não pode ser atribuído à inibição direta da maquinaria contratil do músculo liso vascular. A liberação de Ca^{2+} induzida pela fenilefrina é atribuída à formação de IP_3 (1,4,5-trifosfato de inositol), enquanto a liberação de Ca^{2+} induzida pela cafeína é devido a um mecanismo de liberação de Ca^{2+} induzido pelo Ca^{2+} (KARAKI & WEISS, 1988). Assim, o OP pode inibir de uma maneira relativamente seletiva a liberação do Ca^{2+} mediada pelo IP_3 dos estoques intracelulares na preparação de aorta isolada de rato (GUEDES *et al.*, 2004a).

1.5.3. Alpinia zerumbet

A *Alpinia zerumbet***,** uma planta aromática originada no oeste da Ásia (MATOS, 2001), é abundante no nordeste do Brasil, onde é comumente conhecida como “colônia”. Infusões e decocções das folhas da *A. zerumbet* são comumente usadas por possuir propriedades diurética e anti-hipertensiva (MENDONÇA *et al.,* 1991; MATOS, 2001). Foi mostrado que a administração i.v. do OE da *Alpinia zerumbet* (OEAZ) em ratos acordados normotensos, induz uma hipotensão provavelmente atribuída à ação do seu principal constituinte, o terpinen-4-ol (Trp-4-ol) (LAHLOU *et al.,* 2002d). A hipotensão do OEAZ parece ser independente do sistema nervoso autônomo sugerindo que o OEAZ atua como um agente vasodilatador do músculo liso vascular.

Estudos em ratos hipertensos DOCA-sal mostram que a magnitude da resposta hipotensora para a administração i.v. de OEAZ, Trp-4-ol ou de HEX é maior em ratos hipertensos DOCA-sal do que em ratos normotensos uninefrectomizados, acordados (LAHLOU *et al.,* 2003). A amplificação da hipotensão induzida pelo OEAZ permaneceu inalterada pelo pré-tratamento i.v. com o bloqueador ganglionar HEX ou com MA. Este resultado corrobora com a hipótese prévia de que o OEAZ atua diretamente no vaso. O aumento da hipotensão induzida pelo OEAZ em ratos hipertensos DOCA-sal pode ser devido principalmente ao aumento da responsividade vascular para o OEAZ do que pelo aumento da atividade do sistema nervoso autônomo neste modelo de hipertensão (LAHLOU *et al.,* 2003).

MOURA e colaboradores., demonstraram que o extrato de *A. zerumbet* (EAZ) promoveu um efeito vasodilatador dependente do endotélio que não está envolvido com a secreção de prostaglandinas, receptores ativados por acetilcolina (Ach), histamina, epinefrina ou abertura de canais de K_{ATP} ou K_{Ca} . Parte desse efeito vasodilatador do EAZ pode ser modulado por receptores de bradicinina B_2 e provavelmente é dependente da ativação da via ON-GMPc (MOURA *et al.*, 2005).

1.5.4. Aniba canelilla

A *Aniba canelilla,* popularmente conhecida como “casca-preciosa”, é utilizada na medicina popular como antiespasmódica, estimulante digestivo e por possuir propriedades carminativa (MAIA, *et. al.,* 2001). A administração i.v. do OE de *A. canelilla* (OEAC) em ratos normotensos acordados ou anestesiados resultou em hipotensão e bradicardia dose-dependentes, dois efeitos que ocorrem independentemente. A reposta hipotensora do OEAC permaneceu inalterada pelo tratamento i.v. com o bloqueador ganglionar HEX, porém, foi reduzida parcialmente com o bloqueador muscarínico periférico a MA ou com o bloqueador da ON síntase (ONS), o L-NAME. O OEAC causou um relaxamento concentração-dependente em anéis de aorta pré-contraídos com potássio, um efeito que foi significativamente e similarmente inibido pela remoção do endotélio ou pela adição da atropina no meio de perfusão. Por outro lado, o OEAC aboliu a contração induzida pelo $CaCl_2$, mas não àquela induzida pela cafeína, em meio sem Ca^{2+}. Esses resultados sugerem que a hipotensão induzida pelo OEAC é devida a um relaxamento ativo do vaso. Este relaxamento parece ser parcialmente mediado pelo ON liberado após ativação dos receptores muscarínicos periféricos (relaxamento dependente do endotélio), e predominantemente através da inibição do influxo de Ca^{2+} para o interior da célula (relaxamento independente do endotélio). No entanto, a bradicardia induzida pelo OEAC parece ser principalmente dependente da presença do tônus parassimpático para o coração (LAHLOU *et al.,* 2005).

1.5.5. Croton zehntneri

O *Croton zehntneri* (Euphorbiaceae) é uma planta aromática abundante no nordeste do Brasil, onde é comumente conhecida como “canela de cunhã”. Na medicina popular, infusões e decocções das folhas do *C. zehntneri* são utilizadas geralmente no tratamento de ansiedade, anorexia e no tratamento de distúrbios gastrointestinais (BATATINHA *et al.,* 1995; CRAVEIRO *et al.,* 1977; LEAL-CARDOSO & FONTELES, 1999). Além disso, o *C. zehntneri* é caracterizado por um odor forte e agradável remanescente de anis e dos

cravos-da-índia. Os extratos de seu caule e as folhas são usados nos perfumes e como flavorizantes nos alimentos e bebidas (CRAVEIRO *et al.,* 1978, 1977).

Em ratos normotensos acordados, a administração i.v. do OE do *Croton zehntneri* (OECZ) induziu uma queda inicial da PAM seguida de uma resposta pressora, esses dois efeitos são atribuídos principalmente às ações do anetol e estragol, os principais constituintes do OECZ. A hipotensão inicial induzida pelo OECZ (fase I) foi mediada por mecanismos colinérgicos e pareceu decorrente da bradicardia concomitante de origem reflexa, enquanto a resposta pressora subseqüente (fase II) pareceu ser resultado de uma ação vasoconstrictora indireta do OECZ, provavelmente devido à inibição da produção do ON endotelial. Esta última hipótese foi suportada pelos achados "*in vitro*" mostrando que baixas concentrações de OECZ amplificaram as contrações da fenilefrina em anéis de aorta de rato. Efeito amplificador foi abolido pela retirada do endotélio ou pelo bloqueio da ONS com L-NAME. Estes achados *in vivo* e *in vitro* foram os primeiros relatos dos efeitos farmacológicos do OECZ sobre o sistema cardiovascular (de SIQUEIRA *et al.,* 2005). Em outro estudo, foi corroborado que o efeito hipotensor inicial (fase I) da administração i.v. de OECZ em ratos anestesiados é decorrente da bradicardia reflexa, mediada pela ativação dos receptores valinoides TPRV1 localizados nas terminações sensoriais vagais sensíveis a capsaicina (de SIQUEIRA *et al.,* 2006). Ademais, a administração intra-arterial do OECZ assim como seus principais constituintes, anetol e estragol, induziu uma resposta hipotensora reflexa de origem espinhal, como foi mostrado para a capsaicina (de SIQUEIRA *et al.,* 2006).

1.5.6. Gênero *Ocimum*

Dentre o gênero *Ocimum* encontramos várias espécies com efeitos já demonstrados na literatura como *Ocimum sanctum, Ocimum basilicum* e *Ocimum suave.* O *Ocimum sanctum* é encontrado em regiões da Índia e o sumo de suas folhas é usado para o tratamento de febres crônicas, hemorragias, disenteria e doenças da pele (AGRAWAL *et al.,* 1996; MAITY *et al.,* 2000). Estudos demonstram que o *Ocimum sanctum* apresenta atividade

antioxidante que foi atribuída à presença de compostos como os flavonóides, taninos, ácido ascórbico e carotenóides (GRANASOUNDARI *et al.,* 1997).

O *Ocimum basilicum* também conhecido como doce basil, é rico em OEs e vem sendo assunto de numerosos estudos químicos (GRAYER *et al.,* 1996). É utilizado pela população como planta medicinal, condimento e agente antimicrobiano (GRAYER *et al.,* 1996; HAMMER *et al.,* 1999).

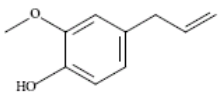
O *Ocimum suave* é encontrado na Ásia e no Oeste da África, onde é utilizado no tratamento de úlceras e como anticatártico (WATT *et al.,* 1962). Associado a outras plantas, o *Ocimum suave* é usado no tratamento de febre em crianças, problemas menstruais, dor estomacal e afecções brônquicas (BOUQUET, 1969).

O *Ocimum gratissimum* (Figura 1), popularmente conhecido como "alfavacão" pertence à família Labiatae e ao gênero *Ocimum*. A família das Lamiaceae inclui aproximadamente 220 gêneros (HEDGE, 1992) e 3.500 a 4.000 espécies (HARLEY, 1988; HEDGE, 1992). O gênero *Ocimum* compreende plantas ricas em OEs destinados às indústrias para produção de fármacos, perfumes e cosméticos (MORALES *et al.*, 1996). As folhas de *O. gratissimum,* contêm em OE 0,2% do peso da planta, cujos componentes principais são mono- e sesquiterpenos (PESSOA *et al.,* 2002).

O *O. gratissimum* é geralmente usado na culinária como condimento. Na medicina popular, infusões e decocções das folhas desta planta são geralmente usadas para o tratamento de problemas digestivos e como anti-séptico bucal (MATOS, 2001). No Oeste da África, extratos desta planta são usados como diaforético, laxante e também no tratamento de tosse, febre e conjuntivite (OLIVER *et al.,* 1960).

Figura 1: Ilustração do *Ocimum gratissimum.* Foto obtida de arquivo próprio.

O EUG (Figura 2) um dos constituintes presentes em OEs de várias plantas é usado na culinária como condimento e é comumente usado pelos dentistas para sedação de dores de dente, pulpite e hiperalgesia dental (LEAL-CARDOSO *et al.,* 1994). Entre os usos do EUG destaca-se o emprego na fabricação de dentifrícios, em perfumaria, saboaria e como clarificador em histologia. É também usado como matéria-prima para a obtenção de vanilina, empregada na aromatização de doces, chocolates, sorvetes e tabacos.



Eugenol

Figura 2: Estrutura química do eugenol.

1.5.6.1. Efeitos farmacológicos do óleo essencial de *Ocimum gratissimum* e de seu principal constituinte

O óleo essencial de *Ocimum gratissimum* (OEOG) foi estudado em vários sistemas fisiológicos, sendo mostrado que ele e o EUG possuem atividade antibactericida (NAKAMURA *et al.,* 1999). Ademais foi mostrado que são inibidores da eclosão dos ovos de *Haemonchus contortus,*o que sugere a possibilidade de sua utilização no tratamento de helmintoses gastrointestinais em pequenos ruminantes (PESSOA *et al.,* 2002). Madeira e colaboradores (2002), mostraram que o OEOG exerce um efeito relaxante na musculatura lisa

do intestino, justificando o uso desta planta na medicina popular para o tratamento de desordens gastrointestinais. Também foi mostrado que este possui propriedades anti-nociceptivas (AZIBA *et al.,*1999; RABELO *et al.,* 2003).

Foi relatado que o EUG induz efeitos no sistema nervoso central de mamíferos como hipotermia, diminuição da atividade motora espontânea e anticonvulsivante (DALLMEIER & CARLINI *et al.,* 1981). Além disso, o EUG induz efeitos miorrelaxantes e antiespasmódicos (LEAL-CARDOSO *et al.,* 1994), efeitos vasorrelaxantes em aorta torácica de ratos (DAMIANI *et al.,* 2003) e de coelhos (NISHIJIMA *et al.,* 1999), como também em leito mesentério vascular (CRIDDLE *et al.,* 2003).

Em nosso laboratório, estudos realizados em ratos normotensos acordados ou anestesiados mostram que a administração i.v. do OEOG induz efeitos hipotensores e bradicardizantes. Estes efeitos parecem ser atribuídos, ao menos em parte, as ações do EUG, o principal constituinte do OEOG. Estas respostas cardiovasculares ocorrem através de mecanismos distintos, pois somente a hipotensão induzida pelo OEOG é independente da presença do sistema nervoso autonômico, sugerindo que a hipotensão possa resultar de efeitos vasodilatadores diretos no músculo liso vascular (LAHLOU *et al.*, 2004b). Resultados obtidos com EUG mostram que sua administração i.v. em ratos normotensos anestesiados ou acordados, também induziu uma redução da PAM provavelmente devido mais a um relaxamento vascular ativo do que a retirada do tônus simpático. Inicialmente, este relaxamento não parece ser mediado pela via endotelial L-arginina/ON (LAHLOU *et al.*, 2004c). De fato, nas preparações de leito mesentérico de rato, pré-contraídas com potássio, o EUG induziu um efeito relaxante reversível e dependente da concentração. Este efeito não foi alterado pela adição de atropina no meio de perfusão (LAHLOU *et al.*, 2004c). A falta de dados na literatura em relação os efeitos cardiovasculares do OEOG e do EUG em ratos hipertensos nos levou a investigá-los.

1.6. Músculo liso vascular

O músculo liso encontrado na camada média dos vasos sanguíneos apresenta características do músculo liso unitário, o que significa que se comporta como um sincício funcional (GUYTON, 2006). As células do músculo liso vascular (MLV) são células que apresentam aspecto fusiforme com um comprimento de 30 a 60 μm e um diâmetro relativamente constante de 4 μm. O núcleo da célula se localiza no centro e tem forma elipisóide e compreende 20-30% do volume celular. O sarcolema no músculo liso apresenta em torno de 2 a 6% do volume celular e possui uma bicamada lipídica rica em entidades protéicas. O reticulo sarcoplasmático (RS) existe como uma estrutura tubular mas apresenta uma organização diferente daquela observada no músculo estriado. Não há sarcômeros bem definidos, porém apresenta estruturas chamadas “corpos densos” (análogos dos discos Z dos músculos estriados) que funcionam como meios de transmissão de forças mecânicas, de maneira a proporcionar um acoplamento entre os miofilamentos e o estroma do tecido conetivo (SOMLYO & SOMLYO, 1994).

As principais proteínas envolvidas no processo de contração do músculo liso são: miosina, actina, caldesmon, calmodulina e tropomiosina. A miosina do músculo liso é semelhante à encontrada nos músculos estriados e em outras células não musculares. É um hexâmero composto de duas cadeias pesadas (PM 200.000) e dois pares de cadeias leves (PM 20.000). A actina (PM 43.000) é uma proteína globular monomérica que em força iônica se encontra polimerizada na forma de um filamento helicoidal duplo. A calmodulina (PM em torno de 16.000) é uma proteína multifuncional ligante de cálcio, sendo um importante mediador de muitos efeitos regulatórios deste íon em vários sistemas, inclusive na contratilidade do músculo liso. O caldesmon (PM 140.000), apresenta uma estrutura dimérica que se encontra associada a actina na ausência de cálcio. Em presença deste íon, forma-se o complexo Ca^{2+}-calmodulina que se liga ao caldesmon deslocando-o da sua ligação com o filamento de actina, expondo assim os seus sítios de ligação com a miosina. A tropomiosina (PM 66.000) é uma proteína fibrosa, que junto com o caldesmon ocupa o sulco formado pelos dois filamentos de actina (PAIVA *et al.*, 2005).

As células endoteliais vasculares são continuamente expostas ao estresse provocado pela passagem do fluxo sanguíneo. O estresse de cisalhamento regula a estrutura e função das células endoteliais controlando a

expressão de genes mecanosensíveis e produzindo fatores vasoativos. Embora o estresse provocado pelo fluxo sanguíneo sobre as células endoteliais seja o principal mecanismo proposto para a regulação da enzima ONS, também são propostos outros mecanismos que envolvem a interação da ONS endotelial com proteínas, tais como, a calmodulina, a caveolina I e receptores da proteína G, a tirosina quinase e a tirosina fosfatase (KONE *et al.*, 2003).

1.6.1. Os íons cálcio e seus canais

A contração do MLV é regulada principalmente pela concentração de cálcio intracelular ($[Ca^{2+}]_i$) e pela sensibilidade dos elementos contráteis a este íon. No músculo liso vascular, a principal origem do Ca^{2+} mioplasmático é o meio extracelular, cuja entrada é mediada pelos canais de Ca^{2+} operados por voltagem (VOCs), pelos canais operados por receptor (ROCs), pelos canais para cátions não-seletivos e pelo trocador Na^+/Ca^{2+}. Além disso, o RS também contribui para o aumento do Ca^{2+} intracelular, cuja liberação pode ser mediada pelo IP_3 ou pelo próprio Ca^{2+} (WELLMAN & NELSON, 2003).

No MLV, a variação da $[Ca^{2+}]_i$ é bastante estreita, variando de 100 nM, quando as artérias estão completamente dilatadas a 350 nM em artérias contraídas (KNOT & NELSON, 1998; FARACI & SOBEY, 1996). A entrada de Ca^{2+} através dos VOCs é determinada pelo potencial de membrana (Em) celular que é modulado por vários tipos de canais iônicos, tais como os canais para K^+, Cl^- e outros cátions (WELLMAN & NELSON, 2003). Os ROCs são ativados pela ligação de hormônios, neurotransmissores ou fármacos a receptores específicos localizados na membrana das células musculares lisas, cuja contração acontece através de um mecanismo semelhante. Dentre estas substâncias pode-se citar a noradrenalina, a adrenalina, a serotonina, a ET-1, a angiotensina II, etc.

No músculo liso, o acoplamento excitação-contração se faz através de dois mecanismos principais: um eletromecânico e outro farmacomecânico (SOMLYO & SOMLYO, 1994). O primeiro tem início com a despolarização da membrana plasmática devido à entrada de cátions (por exemplo: Na^+ ou Ca^{2+}), alterando a diferença de potencial transmembrana e promovendo a abertura de VOCs (PAIVA *et al.,* 2005). Este evento permite a entrada de Ca^{2+} extracelular

para o interior da célula, obedecendo ao seu gradiente de concentração (NELSON *et al.*, 1990).

Dois tipos de canais para Ca^{2+} foram descritos no MLV: o canal do tipo L (*long lasting*) e o canal do tipo T (*transient*). A distribuição destes canais é variável, havendo, entretanto uma preponderância dos canais do tipo L, que por esta razão são os mais estudados. Os canais tipo L são considerados a principal via de entrada de Ca^{2+} do meio extracelular para o intracelular (CRIBBS, 2006). A ativação dos canais tipo L, passando pela transição do estado fechado para o aberto, ocorre após despolarização da membrana celular. A desativação ocorre quando a membrana celular é repolarizada ou hiperpolarizada. Estes canais são regulados pelo próprio Ca^{2+} intracelular que os inibe reversivelmente, como foi demonstrado em célula muscular lisa isolada de veia porta (OHYA *et al.*, 1988). O influxo sustentado de Ca^{2+}, por canais de cálcio tipo L, induz um nível tônico de vasoconstrição além de criar um modelo excitatório que auxilia na ação de substâncias vasoativas endógenas para melhor modular o diâmetro arterial (SONKUSARE *et al.,* 2006).

Os canais do tipo T são pouco estudados devido a ausência de drogas seletivas para a sua estimulação. Portanto, as suas funções são inferidas por correlação. São ativados em baixa voltagem e insensíveis aos bloqueadores de canais de Ca^{2+} mais comumente utilizados. O fato destes canais possuírem um potencial de ativação mais eletronegativo sugere que possam estar envolvidos na geração de correntes do tipo marca-passo, características da contração fásica dos músculos lisos (HOROWITZ *et al.*, 1996).

O acoplamento farmacomecânico envolve a interação de agonistas com os seus receptores na membrana das células musculares lisas, que, em sua grande maioria, são receptores formados por sete domínios transmembranais e acoplados a um complexo de proteínas G (α, β e γ) (MELDRUM *et al.*, 1991). No estado inativado, estas proteínas G estão acopladas entre si e a subunidade α está ligada ao difosfato de guanosina (GDP). Durante a ativação, ou seja, quando a droga se liga ao seu receptor, esse complexo protéico é ativado, e a subunidade G_α, então ativada, liga-se agora ao trifosfato de guanosina (GTP). A subunidade alfa-GTP se dissocia das demais e estimula a atividade da fosfolipase C. Esta enzima hidrolisa o fosfatidilinositol 4,5-bifosfato

(PIP_2), um fosfolipídio de membrana, em dois potentes segundos mensageiros: o IP_3 e o diacilglicerol (DAG) (Figura 3). O IP_3 atua no RS induzindo a liberação de Ca^{2+} e o DAG, junto com o Ca^{2+}, ativa a proteína quinase C (PKC), responsável pelo aumento da sensibilidade das proteínas contráteis ao Ca^{2+}, fosforilação da cadeia leve da miosina e pelo aumento da condutância iônica dos canais transmembranas (tipo L) (Figura 3). Os ésteres de forbol são utilizados para ativar a PKC, imitando as ações desenvolvidas pela DAG e causam a contração do músculo liso (WEBB, 2003).

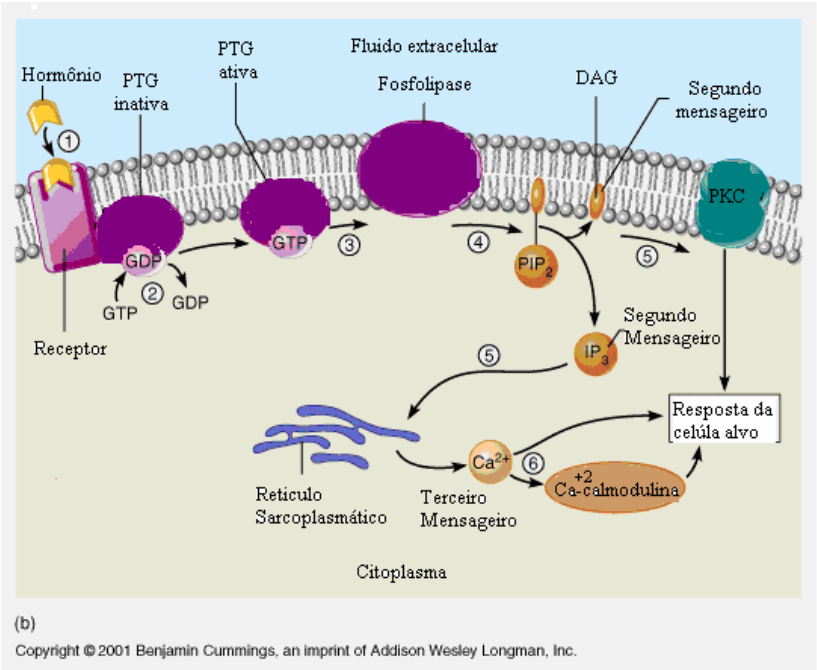


Figura 3. Mecanismo de ação de determinado agonista no acoplamento farmacomecânico (Adaptado Benjamin Cummings, 2001).

1.6.2. Contração Muscular

A contração desenvolvida pelas proteínas contráteis do MLV depende primariamente do aumento da concentração de Ca^{2+} ionizado no citoplasma (VOROTNIKOV *et al.*, 2002). Os canais iônicos são importantes na regulação da contração do músculo liso, pois atuam modulando o potencial da membrana plasmática e conseqüentemente a $[Ca^{2+}]_i$ (KEVIN & NELSON, 2005). O Ca^{2+} mioplasmático se liga a uma proteína citoplasmática chamada calmodulina, capaz de ligar até quatro íons Ca^{2+}, formando o complexo Ca^{2+}-calmodulina

(Figura 4). A ligação do Ca^{2+} altera a sua conformação, permitindo sua interação com a miosina quinase da cadeia leve (MLCK). A associação do complexo Ca^{2+}-calmodulina com a MLCK altera a conformação do complexo, deslocando a seqüência auto-inibitória desta, ativando a quinase e causando a fosforilação da miosina. A exposição dos sítios de ligação da miosina à actina é necessária para que haja a formação do complexo actino-miosina, que consiste na ligação cíclica da porção globular da miosina com a actina. A ligação é seguida pela mudança do ângulo de orientação do complexo actina-miosina, permitindo o deslizamento dos filamentos de miosina sobre a actina (HUXLEY & NIEDERGERKE, 1954). Por outro lado, no músculo em repouso os sítios de ligação da actina com a miosina estão encobertos pelo complexo tropomiosina-caldesmon, localizado ao longo dos filamentos finos. A mudança de conformação do caldesmon (induzida pelo complexo Ca^{2+}-calmodulina) libera o complexo tropomiosina-caldesmon, desbloqueando os sítios de ligação da actina e permitindo a formação do complexo actino-miosina. Portanto, o desenvolvimento de tensão do músculo liso depende de dois fatores: da ativação da miosina pela fosforilação direta e da ativação da actina pela desinibição causada pela saída do complexo tropomiosina-caldesmon. A energia destinada a este processo tem origem na molécula de ATP que é hidrolisada pela ATPase miosínica logo após a sua interação com a actina (HOROWITZ *et al.*, 1996).

Na maioria das vezes, a fosforilação da cadeia regulatória da miosina seria suficiente para determinar o desenvolvimento da contração do músculo liso, entretanto o caldesmon exerce efeito modulador neste processo (HOROWITZ *et al.*, 1996). No mesmo momento em que a PKC é ativada, ocorre a ativação do GTP ligado a proteína RhoA. O mecanismo preciso de ativação desta última pela proteína G ainda não está claro, mas envolve um fator de troca do nucleotídeo guanina (RhoGEF) e a migração do RhoA da membrana plasmática. Após a ativação, o aumento do Rho-quinase leva a inativação da miosina fosfatase (Figura 4) contraindo a célula (WEBB, 2003).

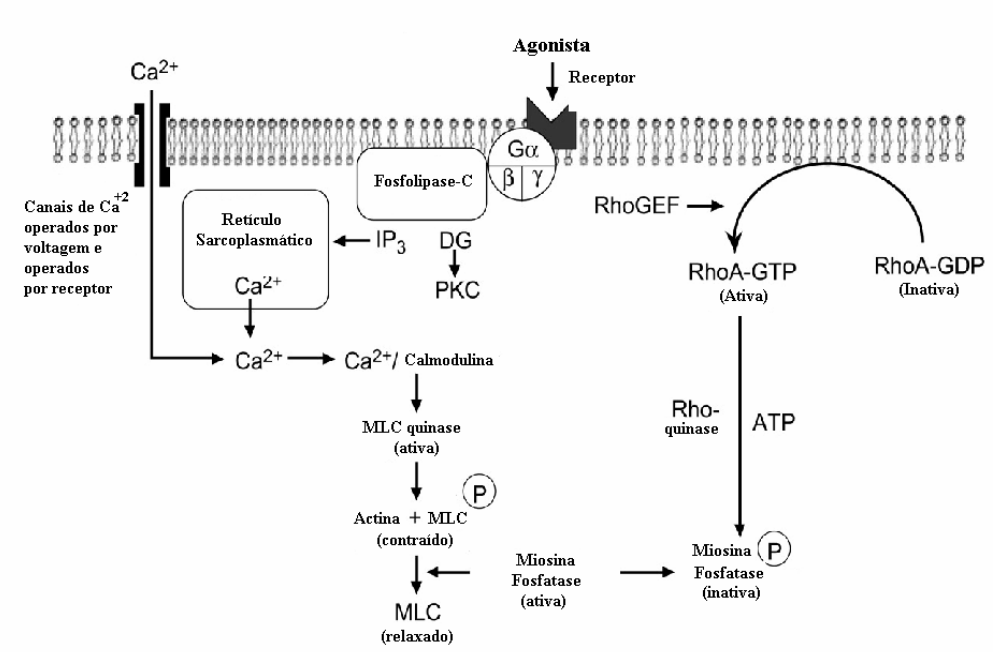


Figura 4: Regulação da contração do músculo liso (Adaptado de WEBB, 2003).

1.6.3. Relaxamento muscular

Estabelecida uma contração, o relaxamento do MLV pode ocorrer por vários tipos de processos que, na maioria dos casos, é devido à redução da $[Ca^{2+}]_i$. Esta redução que é ocasionada pelo seqüestro do Ca^{2+} através do RS ou pela extrusão pelas Ca^{2+} ATPases da membrana plasmática, leva à dissociação do complexo calmodulina-MLCK. A enzima fosfatase da cadeia leve da miosina desfosforila a miosina, reduzindo o número de pontes actina-miosina. Alguns dos complexos actomiosina restantes são lentamente dissociados, permitindo a manutenção de uma contração com reduzido gasto de energia (HAI & MURPHY, 1989).

Substâncias como o fator relaxante derivado do endotélio (FRDE) e o fator atrial natriurético aumentam a 3'5'-guanosina monofosfato cíclico (GMPc) e relaxam o MLV por atenuação do aumento da $[Ca^{2+}]_i$ em células isoladas e tecidos intactos (RAPAPORT *et al.*, 1985; KARAKI *et al.*, 1988). A redução na $[Ca^{2+}]_i$ mediado pelo GMPc pode envolver as seguintes vias:

a) abertura de canais de K^+ e conseqüente hiperpolarização, causando uma diminuição do influxo do Ca^{2+} (ROBERTSON *et al.*, 1993),

b) diminuição da entrada do Ca^{2+} independente da alteração do potencial de membrana, como o efeito do 8-bromo-GMPc em células musculares vasculares (ISHIKAWA *et al.*, 1993),

c) ativação da bomba Ca^{2+}-ATPase do RS e da membrana citoplasmática, dependente do GMPc com conseqüente diminuição do Ca^{2+} livre no citoplasma (POPESCU *et al.*, 1985; TWORT & VAN BREEMAN, 1988),

d) aumento da atividade trocadora Na^+/Ca^{2+} (NCX) como evidenciado em aorta isolada de rato (ITOH *et al*., 1983).

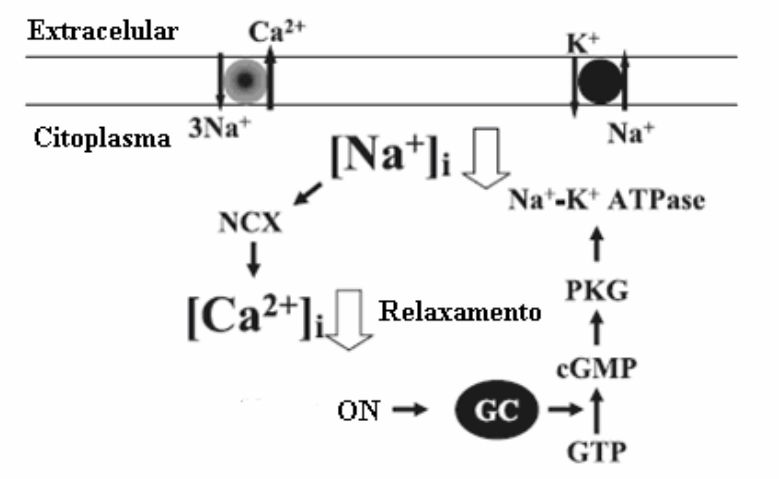


Figura 5: A ativação do trocador Na^+/Ca^{2+} (NCX) pelo GMPc. GC, guanilato ciclase; ON óxido nítrico; PKG, GMPc dependente (Adaptado de NISHIMURA, 2006).

NISHIMURA (2006), verificou que o ON induz um relaxamento no MLV principalmente através da ativação da guanilato ciclase solúvel (GC), que atua no GTP gerando o GMPc. O relaxamento mediado pelo GMPc envolve uma diminuição da $[Ca^{2+}]_i$ através da ativação da Ca^{2+}- ATPase presente tanto na membrana plasmática como no RS, como também através da Na^+/K^+ ATPase e os vários canais de K^+. O estímulo da bomba Na^+/K^+ ATPase encontrada na membrana plasmática é dado pela proteína quinase dependente de GMPc (PKG). Com a ativação da PKG e conseqüentemente da bomba, ocorre uma diminuição da concentração intracelular de Na^+, que estimula o trocador Na^+/Ca^{2+}, resultando na diminuição da $[Ca^{2+}]_i$ (Figura 5). Este autor ainda observou que o trocador Na^+/Ca^{2+}também poderia estar envolvido na diminuição da $[Ca^{2+}]_i$ mediada pelo AMPc. O isoproterenol (agonista β-adrenérgico) atua no receptor ativando a proteína G que estimula a adenilato

ciclase (AC). O aumento da atividade da AC pelo isoproterenol ou forskolin (um ativador da AC) leva a produção do AMPc através da hidrólise do ATP. O AMPc atua na PKA que estimula o trocador Na^+/Ca^{2+}, causando a saída de Ca^{2+} do interior celular e aumentando a entrada do Na^+. Conseqüentemente, ocorre uma redução da $[Ca^{2+}]_i$ o que leva ao relaxamento (Figura 6). O relaxamento do músculo liso também pode ocorrer devido ao aumento de AMPc cujo mecanismo principal é a fosforilação via PKA, da MLCK reduzindo à sua afinidade pela calmodulina (MCDANIEL *et al.*, 1991; SOMLYO & SOMLYO, 1994). Além disso, o AMPc pode reduzir a $[Ca^{2+}]_i$ por diminuir o influxo de Ca^{2+} no MLV e/ou por ativar canais de K^+ levando à hiperpolarização de membrana e redução do influxo de Ca^{2+} via VOCs (SADOSHIMA *et al.*, 1988; LINCOLN *et al.*, 1990). De acordo com estes últimos autores, durante o relaxamento do MLV induzido pelo AMPc pode ocorrer: a) hiperpolarização da membrana pela ativação de canais de K_{Ca} e inativação de canais de Ca^{2+} dependente de voltagem do tipo L; b) inibição da liberação de Ca^{2+} de estoques intracelulares; c) redução da fosforilação da cadeia leve da miosina; d) aumento do efluxo de Ca^{2+}.

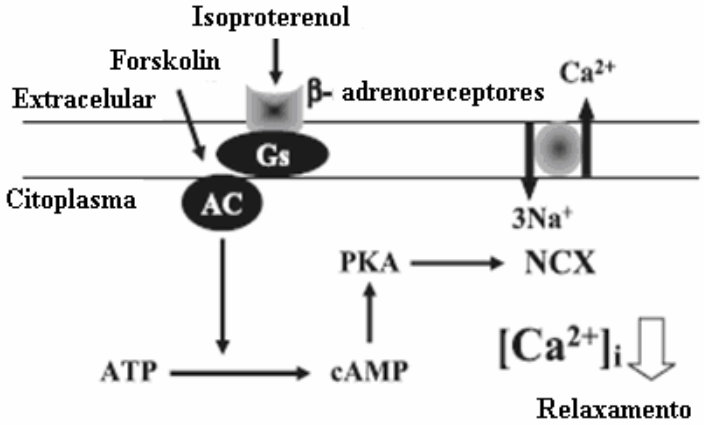


Figura 6: A ativação do trocador Na^+/Ca^{2+} (NCX) pelo AMPc. AC, adenilato ciclase; Gs, proteína G ligado ao GTP; PKA, proteína quinase dependente-AMPc (Adaptado de NISHIMURA, 2006).

O mecanismo primário para a diminuição do $[Ca^{2+}]_i$ induzido pelo aumento do AMPc é a ativação da PKG (LINCOLN *et al.*, 1990). Embora a PKG seja ativada pelo aumento de GMPc, altas concentrações de AMPc no meio intracelular também pode ativá-la (FRANCIS & CORBIN, 1994). Assim, o

relaxamento do MLV pode também ser devido a fosforilação via PKG e a ativação de proteínas que atuariam na homeostasia do Ca^{2+} intracelular, incluindo a Ca^{2+}-ATPase da membrana plasmática e o trocador Na^{+}/Ca^{2+} (LOHMANN *et al.*, 1997).

Na figura 7, pode-se observar que a diminuição da $[Ca^{2+}]_i$ é dada pela atuação da Ca,Mg-ATPase tanto no RS como na membrana plasmática, e também pelo trocador Na^{+}/Ca^{2+}, resultando na redução da $[Ca^{2+}]_i$. O complexo Ca^{2+}/calmodulina não será formado e conseqüentemente ocorrerá a inativação MLCK, desviando o balanço de atividade quinase para fosfatase, o que resultará em desfosforilação da cadeia leve de miosina e relaxamente muscular (WEBB, 2003). Durante o relaxamento, os canais de Ca^{2+} operados por voltagem ou por receptor presentes na membrana plasmática permanecem fechados resultando na diminuição da entrada de Ca^{2+} para a célula.

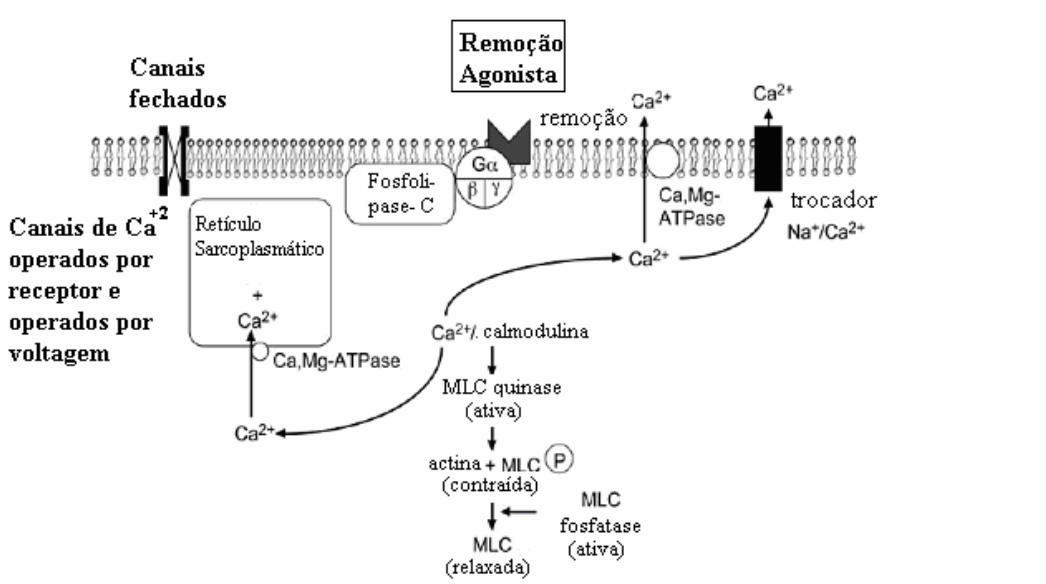


Figura 7: Relaxamento do músculo liso.

No MLV, o relaxamento produzido por algumas substâncias como ADP/ATP, adenosina, histamina, trombina e Ach são mediados pelo endotélio vascular através da liberação do ON ou do chamado fator hiperpolarizante derivado do endotélio (FHDE) (RAPAPORT *et al.*, 1985). Em 1980, Furchgott & Zawadzki foram os primeiros a identificar o ON, o qual é formado em células endoteliais a partir do aminoácido L-arginina, na presença da ONS

(VANHOUTTE, 2003). A ativação da ONS é dependente da $[Ca^{2+}]_i$ nas células endoteliais, sendo Ca^{2+}/calmodulina dependente. O ON se difunde das células endoteliais para o MLV, onde atua estimulando a enzima GC solúvel, que catalisa a produção de GMPc, causando a inibição do processo contrátil (Figura 8) (VANHOUTTE, 2003).

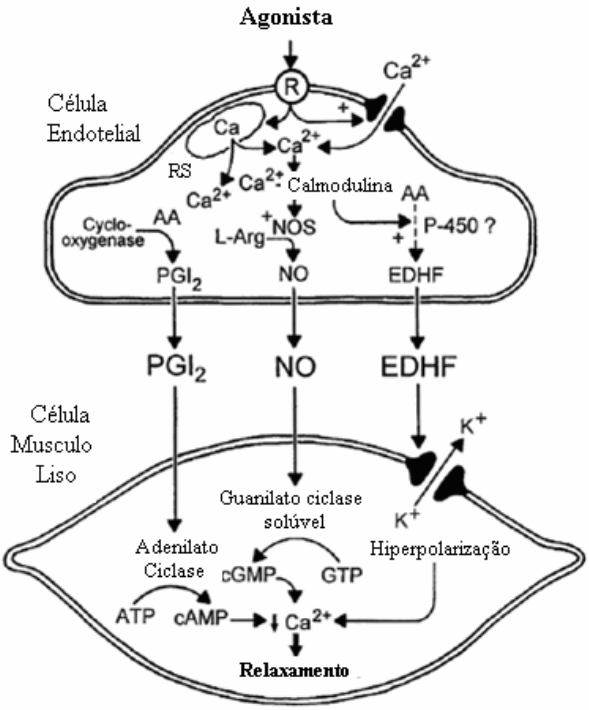


Figura 8: Substâncias secretadas pelo endotélio. A ativação de receptores endoteliais induz um influxo de Ca^{2+} no citoplasma da célula endotelial. Após a interação do Ca^{2+} com a calmodulina, ativa a ONS e as cicloxigenases, e leva a liberação de fatores hiperpolarizantes derivados do endotélio (FRDE). O ON causa relaxamento pela ativação e formação do GMPc a partir do GTP. O FRDE causa hiperpolarização e relaxamento pela abertura dos canais de K^+. As prostaciclinas (PGI_2) causam relaxamento pela ativação da AC que conduz a formação do AMPc. Quando os agonistas ativam as células endoteliais, o aumento IP_3 pode contribuir para o aumento de Ca^{2+} citoplasmático liberado pelo RS (Adaptado de VANHOUTTE, 2003).

Além de atuar no processo de relaxamento, o ON atua inibindo a proliferação celular, a agregação plaquetária, a adesão de leucócitos à parede vascular, além de ser um ativador do processo fibrinolítico (VANHOUTTE, 2003). Sendo, portanto, considerado o fator de origem endotelial mais

importante e quando a sua síntese e/ou liberação está reduzida compromete a homeostasia no endotélio vascular.

2. OBJETIVOS

2.1. Objetivo geral

- Estudar os efeitos cardiovasculares do OEOG e de seu principal constituinte, o EUG, em ratos hipertensos DOCA-sal através de uma abordagem *in vivo* e *in vitro*.

2.2. Objetivos específicos

- Estudar os efeitos do OEOG e do hexametônio sobre a PAM e a FC em ratos hipertensos DOCA-sal, acordados e em seus controles (ratos normotensos uninefrectomizados).

- Investigar se o EUG, o maior constituinte do OEOG, é o princípio ativo que medeia os efeitos cardiovasculares do OEOG.

- Investigar, em ratos hipertensos DOCA-sal acordados, o papel do sistema nervoso autônomo na mediação dos efeitos cardiovasculares do OEOG.

- Avaliar a ação vasorelaxante do OEOG e do EUG usando preparações de aorta torácica isolada de ratos hipertensos DOCA-sal.

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4. MANUSCRITOS

4. 1. MANUSCRITO 1

Enhanced Hypotensive Effects of the Essential Oil of *Ocimum gratissimum* Leaves and its Main Constituent, Eugenol, in DOCA-Salt Hypertensive Conscious Rats

Leylliane Fátima Leal Interaminense[1], José Henrique Leal-Cardoso[2], Pedro Jorge Caldas Magalhães[3], Gloria Pinto Duarte[1], Saad Lahlou[1]

Planta Med 71: 376-378, 2005.

Enhanced Hypotensive Effects of the Essential Oil of *Ocimum gratissimum* Leaves and its Main Constituent, Eugenol, in DOCA-Salt Hypertensive Conscious Rats

Leylliane Fátima Leal Interaminense[1], José Henrique Leal-Cardoso[2], Pedro Jorge Caldas Magalhães[3], Gloria Pinto Duarte[1], Saad Lahlou[1]

Abstract

The cardiovascular effects of intravenous (*i.v.*) treatment with the essential oil of *Ocimum gratissimum* (EOOG) and its main constituent, eugenol (Eug) were investigated in the experimental model of deoxycorticosterone acetate (DOCA-salt)-hypertensive rats. In both conscious DOCA-salt hypertensive rats and their uninephrectomized controls, *i.v.* bolus injections of EOOG (1–20 mg/kg) or Eug (1–10 mg/kg) induced dose-dependent hypotension and bradycardia. Treatment with DOCA-salt significantly enhanced the maximal decreases in mean aortic pressure (MAP) elicited by hexamethonium (30 mg/kg, *i.v.*) as well as the hypotensive responses to both EOOG and Eug without affecting the bradycardia. However, the enhancement of EOOG-induced hypotension in hypertensive rats remained unaffected by *i.v.* pretreatment with either hexamethonium (30 mg/kg) or methylatropine (1 mg/kg). These results show that *i.v.* treatment with EOOG or Eug dose-dependently decreased blood pressure in conscious DOCA-salt hypertensive rats, and this action is enhanced when compared with uninephrectomized controls. This enhancement appears related mainly to an increase in EOOG-induced vascular smooth relaxation rather than to enhanced sympathetic nervous system activity in this hypertensive model.

Ocimum gratissimum L. (Labiatae) is an aromatic plant abundant in north-eastern Brazil, where it is commonly known as "alfavaca". The plant is commonly used in cooking for flavouring. In folk medicine, infusions or decoctions of leaves from *O. gratissimum* are commonly used for the treatment of digestive problems and as a mouth antiseptic [1]. Leaves of *O. gratissimum* have an essential oil content of 0.2% of the plant dry weight, comprised principally of mono- and sesquiterpenes [2]. Recently, we showed that intravenous (*i.v.*) treatment of normotensive rats with EOOG and its main constituent, eugenol (Eug; 43.70%) dose-dependently decreased mean aortic pressure (MAP) [3], [4]. Such an effect remained unaffected by bilateral vagotomy or *i.v.* hexamethonium pretreatment, suggesting that it may result from a vasodilatory action of EOOG directly upon vascular smooth muscle rather than withdrawal of sympathetic tone [4]. Therefore, the present investigation was undertaken to gain further support for this hypothesis by assessing the influence of deoxycorticosterone-acetate (DOCA)-salt treatment, which increases basal sympathetic activity [5], on EOOG-induced hypotension.

Baseline MAP in DOCA-salt conscious rats was 166 ± 3 mmHg ($n = 31$ rats), and was significantly ($p < 0.001$) higher than that measured in their uninephrectomized controls (122 ± 2 mmHg, $n = 20$ rats). However, baseline heart rate (HR) values in hypertensive rats (375 ± 7 beats/min) were not statistically different from those in control rats (360 ± 7 beats/min). In both groups studied, *i.v.* injections of EOOG (1–20 mg/kg) or Eug (1–10 mg/kg) induced immediate and dose-dependent ($p < 0.001$) hypotension (Figs. **1A** and **2A**, respectively) and bradycardia (Figs. **1B** and **2B**, respectively), which peaked at the first 20–30 s after administration. Only the maximal percent decreases in MAP evoked by EOOG and Eug (Figs. **1** and **2**, respectively) were significantly ($p < 0.001$) enhanced by DOCA-salt hypertension. Furthermore, unlike normotensive rats, hypotensive responses to EOOG in DOCA-salt rats remained significantly ($p < 0.05$) reduced during a period of 1–5 min after the administration of the highest dose of EOOG (20 mg/kg). Maximal percent and absolute decreases in MAP elicited by hexamethonium (30 mg/kg, *i.v.*) in DOCA-salt hypertensive (−52 ± 6% and −92 ± 12 mmHg, respectively) were also significantly ($p < 0.001$) greater than those recorded in uninephrectomized controls (−32 ± 3% and −38 ± 4 mmHg, respectively). Pretreatment of DOCA-salt hypertensive rats with either methylatropine (1 mg/kg, *i.v.*) or hexamethonium (30 mg/kg, *i.v.*) significantly and similarly reduced the EOOG-induced bradycardia (Fig. **3B**, $p < 0.001$). However, neither pretreatment affected the EOOG-induced hypotension (Fig. **3A**), the magnitude of which remained statistically ($p < 0.001$) greater than in controls.

The current study shows that *i.v.* treatment of conscious DOCA-salt hypertensive rats and their uninephrectomized controls with EOOG or Eug dose-dependently decreased MAP and HR, as was reported in intact, normotensive rats [3], [4]. Treatment with DOCA-salt significantly enhanced the hypotensive responses to EOOG and Eug, an effect that could be related to the increased sympathetic activity in this hypertensive model, as evidenced by the significant increases in the hypotensive responses to hexamethonium in DOCA-salt hypertensive rats. However, this enhancement appears to be independent of the degree of vascular tone because it was unaffected after blockade of ganglionic transmission with hexamethonium.

Cardiovascular effects of EOOG in DOCA-salt rats appear to be partly attributable to the actions of its main constituent Eug, as previously discussed for normotensive rats [4]. This constituent has been reported to induce vasorelaxant effects on rat [11] and rabbit [12] thoracic aorta as well as on rat mesenteric vascular

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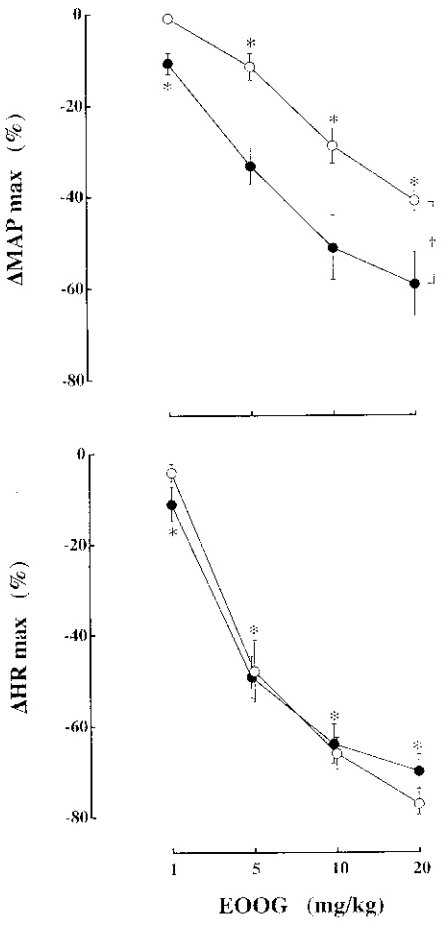

Fig. 1 Maximal decreases in mean aortic pressure (ΔMAP max; upper panel) and heart rate (ΔHR max; lower panel) elicited by the essential oil of *Ocimum gratissimum* (EOOG; 1–20 mg/kg, *i.v.*) in DOCA-salt hypertensive (solid circles) and uninephrectomized normotensive (open circles), conscious rats. Vertical bars indicate SEM (7 rats per group) and values represent means of changes expressed as a percentage of baseline. Baseline MAP (mmHg) and HR (beats/min) were 170 ± 6 and 370 ± 10, respectively, in DOCA-salt rats, and 122 ± 4 and 350 ± 9, respectively, in normotensive rats. † $p < 0.001$ by two-way ANOVA, * $p < 0.05$ by Dunnett's test.

unaffected by hexamethonium, suggesting that it is mainly related to increased vascular responsiveness to EOOG rather than to enhanced sympathetic nervous system activity in this hypertensive model. The present findings may add antihypertensive activity to the list of therapeutic uses of *O. gratissimum* in folk medicine.

Materials and Methods

EOOG was obtained as previously described [4], [6]. Analytical conditions, composition of EOOG and retention indices of its constituents have been previously described [4]. Eugenol (Sigma) and EOOG were dissolved in Tween (2%) and saline. All other drugs (Sigma) were dissolved in saline and administered in a volume of 1 mL/kg body weight.

Male Wistar rats (220–250 g) were cared for in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication 85–23, revised 1996). DOCA-salt and their uninephrectomized controls were obtained after 4-week period treatment with, respectively, DOCA and vehicle (olive oil), as previously described [7]. On the last day of treatment, rats of both groups were anesthetised with sodium pentobarbital (50 mg/kg, *i.p.*) and two catheters were implanted in the abdominal aorta (for the recording of MAP) and in the inferior vena cava (for drug administration), as previously described [3], [4], [7]. Twenty-fours hours later, baseline MAP and HR values were recorded on a Gilson model 5/6H (Medical Electronics Inc., USA), as previously described [3], [4], [7].

bed [3], [13]. In the current study, EOOG-induced hypotension was more potent on diastolic arterial blood pressure (data not shown). These *in vitro* data, together with the *in vivo* present findings, give further support to the hypothesis that the hypotensive response to EOOG is due to a decrease in peripheral vascular resistances. Vascular muscarinic receptors that normally mediate hypotension are probably not involved, since pretreatment of hypertensive rats with methylatropine did not affect the EOOG-induced hypotension. Thus, enhanced EOOG-induced hypotension in DOCA-salt hypertensive rats appear most likely related to an increased vascular smooth muscle relaxation induced by EOOG. Further investigations using isolated aortae from DOCA-salt hypertensive rats are needed to corroborate this hypothesis.

As was reported for intact normotensive rats [4], bradycardic effects of EOOG in DOCA-salt hypertensive rats appear to be dependent on an intact and operational parasympathetic nerve drive to the heart since they were significantly and similarly reduced by *i.v.* hexamethonium and methylatropine. The findings that DOCA-salt treatment enhanced EOOG-induced hypotension without affecting bradycardia, and that *i.v.* hexamethonium or methylaptropine reduced the EOOG-induced bradycardia without affecting hypotension, give further support to the concept of independent mechanisms for EOOG-induced hypotension and bradycardia [4].

The current study shows that DOCA-salt hypertension enhances the EOOG-induced hypotension. This enhancement remained

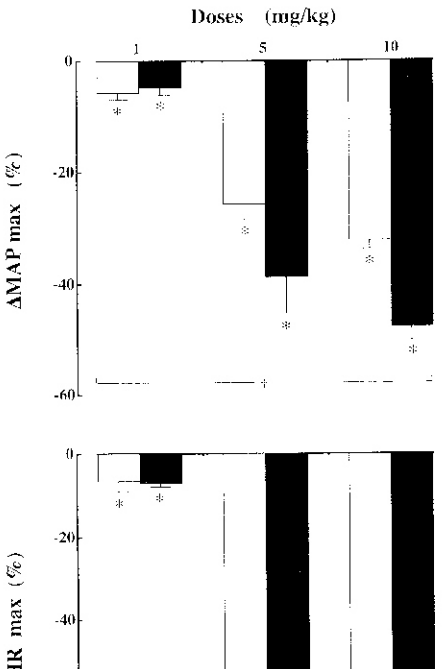

Fig. 2 Maximal decreases in mean aortic pressure (ΔMAP max; upper panel) and heart rate (ΔHR max; lower panel) elicited by eugenol (Eug; 1–10 mg/kg, *i.v.*) in DOCA-salt hypertensive (black columns) and uninephrectomized normotensive (white columns), conscious rats. Vertical bars indicate SEM (6–7 rats per group) and values represent means of changes expressed as a percentage of baseline. Baseline MAP (mmHg) and HR (beats/min) were 160 ± 6 and 351 ± 15, respectively, in DOCA-salt rats, and 123 ± 3 and 365 ± 11, respectively, in normotensive rats. † $p < 0.001$ by two-way ANOVA, * $p < 0.05$ by Dunnett's test.

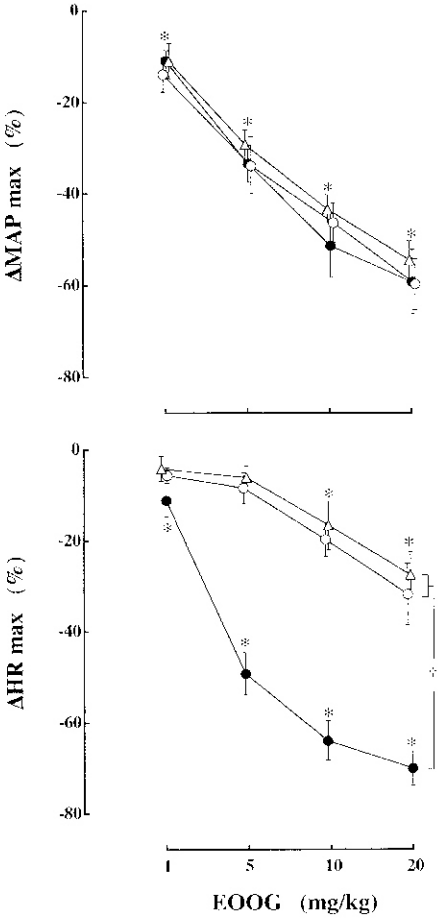

Fig. 3 Maximal decreases in mean aortic pressure (ΔMAP max, upper panel) and heart rate (ΔHR max; upper panel) elicited by the essential oil of *Ocimum gratissimum* (EOOG; 1 – 20 mg/kg, *i.v.*) in DOCA-salt hypertensive rats subjected to *i.v.* pretreatment with vehicle (solid circles), methylatropine (open triangles) or hexamethonium (open circles). Vertical bars indicate SEM (6 – 7 rats per group) and values represent means of changes expressed as a percentage of baseline. Baseline MAP (mmHg) and HR (beats/min) were 170 ± 6 and 370 ± 10, respectively, in vehicle-pretreated rats, 87 ± 9 and 366 ± 14, respectively, in hexamethonium-pretreated rats, and 173 ± 8 and 450 ± 14, respectively, in methylatropine-pretreated rats. † $p < 0.001$ by two-way ANOVA, *$p < 0.05$ by Dunnett's test.

Their maximal changes elicited by *i.v.* hexamethonium (30 mg/kg) or increasing bolus (100 μL) doses (1 – 20 mg/kg) of EOOG and Eug (1 – 10 mg/kg) were determined in both groups. Similar changes were also studied in DOCA-salt hypertensive rats pretreated 10 min before EOOG (1 – 20 mg/kg, *i.v.*) with vehicle (1 mL/kg), hexamethonium (30 mg/kg, *i.v.*) [8] or methylatropine (1 mg/kg, *i.v.*) [9]. In DOCA-salt hypertensive rats (n = 5), sodium nitroprusside (20 μg/kg) was used as a positive control [10] and induced a decrease in MAP of 35.0 ± 1.9% (mean ± SEM, n = 5).

Data are expressed as means ± the standard error of the mean. Significance ($p < 0.05$) of the results was assessed by means of unpaired Student's t-test, Mann-Whitney *U*-test and one-way or two-way analysis of variance (ANOVA), followed by Dunnett's test where appropriate.

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4. 2. MANUSCRITO 2

Pharmacological evidence of calcium channel blockade by essential oil of *Ocimum gratissimum* and its main constituent, eugenol, in isolated aorta rings from DOCA-salt hypertensive rats

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ORIGINAL ARTICLE

Pharmacological evidence of calcium-channel blockade by essential oil of *Ocimum gratissimum* and its main constituent, eugenol, in isolated aortic rings from DOCA-salt hypertensive rats

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Keywords
essential oil,
eugenol,
isolated thoracic aorta,
hypotension,
myorelaxant effect,
Ocimum gratissimum

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ABSTRACT

Intravenous (i.v.) treatment of conscious DOCA-salt hypertensive rats with the essential oil of *Ocimum gratissimum* L. (Labiatae) (EOOG) induced a hypotensive effect that seems related to an active vascular relaxation rather than withdrawal of sympathetic tone. To corroborate this hypothesis, the present study examined the vascular effects of EOOG and its main constituent, eugenol (EUG) and the putative mechanisms underlying these effects. Additionally, the role of the vascular β_2-adrenergic mechanism in the mediation of EOOG-induced hypotension has also been investigated. In conscious DOCA-salt hypertensive rats, the EOOG-induced hypotension was reversible and remained unchanged by i.v. pretreatment with propranolol (2 mg/kg). In isolated aorta preparations with intact endothelium from DOCA-salt hypertensive rats, EOOG (1–1000 μg/mL) and EUG (0.006–6 mM) relaxed the phenylephrine-induced contraction similarly with IC_{50} [geometric mean (95% confidence interval)] values of 226.9 (147.8–348.3) μg/mL and 1.2 (0.6–2.1) mM, respectively. Vasorelaxant effects of EOOG were significantly altered by removal of the vascular endothelium [IC_{50} = 417.2 (349.5–497.8) μg/mL]. In a calcium-free medium, the $CaCl_2$-induced contractions were significantly reduced and even abolished by EOOG at 300 and 1000 μg/mL, respectively, whereas EOOG (1000 μg/mL) did not have any significant effect on caffeine-induced contractions. Similar results were obtained with EUG (1.8 and 6 mM) on both $CaCl_2$- and caffeine-induced contractions, respectively. The data suggest that hypotensive responses to EOOG in DOCA-salt hypertensive rats are due to an active vascular relaxation, which is partly dependent upon the integrity of the vascular endothelium and seems predominantly mediated through an inhibition of plasmalemmal Ca^{2+} influx rather than Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum.

INTRODUCTION

Ocimum gratissimum L. (Labiatae) is an aromatic plant abundant in north-eastern Brazil, where it is commonly known as 'alfavaca cravo'. The plant is commonly used in cooking for flavouring. In folk medicine, infusions or decoctions of leaves of *O. gratissimum* are commonly used for the treatment of digestive problems and as a

mouth antiseptic [1]. In West Africa, extracts of this plant are used as diaphoretics, stomachics, laxatives, and also as a treatment for cough, fever and conjunctivitis [2]. Leaves of *O. gratissimum* have an essential oil content of 0.2% of the plant dry weight, comprised principally of monoterpenes and sesquiterpenes [3]. Notwithstanding, little is known about the biological actions of essential oil of *O. gratissimum* (EOOG) and its main constituent eugenol (EUG). It has been shown that EOOG exerts antispasmodic effects on guinea-pig isolated ileum, justifying the use of the plant in folk medicine for the treatment of gastrointestinal disorders [4,5]. Such a myorelaxant activity has also been demonstrated for EUG in rat isolated ileum, suggesting that the antispasmodic activity of EOOG could be attributed, in part, to the actions of this constituent [6]. EOOG has been reported to display an antinociceptive activity in two classical models of pain in mice, the writhing test and the formalin test [7]. This effect is most likely mediated by EUG, which is reported to have analgesic effects [8], and also by the second major compound present in EOOG, 1,8-cineole, which is reported to possess both anti-inflammatory and antinociceptive properties [9].

Previous studies in our laboratory showed that intravenous (i.v.) treatment of either normotensive [10,11] or deoxycorticosterone-acetate (DOCA-salt) hypertensive [12] rats with EOOG or its main constituent EUG (43.70% of total weight of the oil) elicited a dose-dependent decrease in mean arterial pressure (MAP) and heart rate (HR), two effects that occurred independently. The bradycardia appears dependent upon the presence on an intact and functional parasympathetic nerve drive to the heart while the hypotension is unrelated to withdrawal of sympathetic tone as it remained unaffected by i.v. pretreatment with hexamethonium. Hypotensive effects of EOOG and EUG are more potent on diastolic arterial pressure and also unrelated to cholinergic mechanisms as they were altered by neither bilateral vagotomy nor i.v. pretreatment with methylatropine [10–12]. Furthermore, in normotensive rat mesenteric bed preparations precontracted with potassium (60 mM), EUG was shown to induce a reversible and concentration-dependent vasodilator effect, which was partially dependent on the endothelium but remained unaffected by atropine [10,13]. It was therefore postulated that EOOG decreases blood pressure mainly through its vasodilatory action directly upon vascular smooth muscle rather than withdrawal of sympathetic tone. However, no information is available in the literature regarding the vascular effects of the EOOG. Therefore, the present investigation was undertaken to assess the potential vascular effects of the EOOG and EUG in isolated thoracic aorta with intact endothelium from DOCA-salt hypertensive rats, and to elucidate the mechanisms underlying this vascular activity. Additionally, experiments were performed in conscious DOCA-salt hypertensive rats to assess the role of vascular β_2-adrenergic mechanism in the mediation of EOOG-induced hypotension.

MATERIALS AND METHODS

Plant material

Aerial parts of *O. gratissimum* were collected between March and June 2002, at the experimental farm of the Federal University of Ceará, Fortaleza, State of Ceará, Brazil. The identification of the plants was confirmed by Dr F.J. Abreu Matos (Laboratory of Natural Products, Federal University of Ceará). A voucher specimen (no. 23929) is deposited in the herbarium of Prisco Viana at that University.

Extraction and chemical analysis

The EOOG was prepared from freshly chopped leaves by steam distillation and analysed chemically as previously described [14]. Analytical conditions were as follows: EOOG analysis was performed by gas chromatography and mass spectrometry (GC/MS; model 6971; Hewlett-Packard, Palo Alto, CA, USA). The column was a dimethylpolysiloxane DB-1 fused silica capillary column (20 m × 0.25 mm; 0.1 μm); the carrier gas helium (1 mL/min); the injector temperature was 250 °C; the detector temperature was 280 °C; and the column temperature was increased from 50 to 180 °C at 4 °C/min then from 180 to 250 °C at 20 °C/min. The mass spectra had an electronic impact at 70 eV. Composition of EOOG and the retention indices of their various constituents are presented in *Table I*. These compounds were identified using a mass spectral library search and ^{13}C-nuclear magnetic resonance spectroscopy [15].

Solutions and drugs

For in vivo experiments, EUG (Sigma Chemical Co., St Louis, MO, USA) and EOOG were dissolved in Tween 80 (2%), made up to the desired volume using sterile isotonic saline and sonicated just before use. Previous studies showed that this vehicle had no significant effects on either baseline MAP or HR over a period of 20 min [10,12,16,17]. Sodium pentobarbital (Sanofi, Libourne, France) and heparin (Laboratoires Léo SA,

Table I Chemical composition and retention indices of the constituents of the essential oil of *Ocimum gratissimum*.

Compounds	Composition (% of total weight)	Retention indices
α-Pinene	0.95	936
β-Pinene	3.02	976
β-Myrcene	0.70	992
1,8-Cineole	32.70	1035
Linalool	0.50	1106
α-Terpineol	0.60	1201
Eugenol	43.70	1389
β-Elemene	0.50	1402
trans-Caryophyllene	4.10	1428
α-Humulene	0.50	1458
Germacrene-D	1.30	1485
β-Selinene	4.00	1491
α-Selinene	1.30	1489
Total identified	100	

Source: Databank of the Department of Physics and Chemistry of the State University of Ceará, Brazil.

Montigny-le-Bretonneux, France) were used as commercially available injectable solutions. Propranolol hydrochloride was purchased from Sigma Chemical, dissolved in saline just before use and administered in volumes of 1 mL/kg body weight. Each i.v. injection was followed by a 60-μL (catheter volume) flush of physiological saline to ensure complete delivery of the dosage. For in vitro experiments, EOOG and EUG were first dissolved in Tween 80, made up with Tyrode's solution, and sonicated just before use. Phenylephrine (PHE) hydrochloride (Sigma), acetylcholine chloride (Sigma) and nifedipine hydrochloride (Sigma) were first dissolved in distilled water and were made up with Tyrode's solution. The perfusion medium used was a fresh modified Tyrode solution (pH 7.4) of the following composition (mM): NaCl 136, KCl 5, $MgCl_2$ 0.98, $CaCl_2$ 2, NaH_2PO_4 0.36, $NaHCO_3$ 11.9 and glucose 5.5.

DOCA-salt treatment

Male Wistar rats, weighing 200–220 g, were kept under conditions of constant temperature (22 ± 2 °C) with a standard light/dark cycle (12/12 h) and free access to food and water. All animals were cared for in compliance with the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996) and had prior approval from the local animal ethics committee. Under ether anaesthesia, all rats were subjected to unilateral nephrectomy. After a 1-week recovery, they were treated weekly with subcutaneous injections of DOCA (25 mg/kg) dissolved in olive oil (vehicle), and salt was administered by substitution of 1% NaCl solution for drinking water ad libitum. Circulatory and in vitro experiments were performed 4 weeks after the initiation of DOCA-salt treatment.

In vivo experiments

Rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and two catheters were implanted in the abdominal aorta (for the recording of arterial blood pressure) and in the inferior vena cava (for drug administration), as previously described [12]. Twenty-fours hours later, baseline MAP and HR were recorded on a Gilson model 5/6H polygraph (Medical Electronics Inc., Middletown, WI, USA), as previously described [12]. Their maximal changes elicited by i.v. injections of increasing bolus (100 μL) doses of EOOG (1–20 mg/kg) were determined in conscious rats which had been pretreated intravenously 10 min earlier with vehicle (1 mL/kg, $n = 9$) or propranolol (2 mg/kg, $n = 6$) [18–20]. When subsequent doses of EOOG were administered, MAP and HR were first allowed to return to their baseline levels, obtained before the first injection of the essential oil.

In vitro experiments

In another set of experiments, rats were stunned and then exsanguinated. Thoracic aortae were removed and immersed in perfusion medium at room temperature. After removing adhering fat and connective tissue, the aorta was cut into cylindrical strips (1 × 5 mm), which were suspended in a 5-mL organ baths containing perfusion medium continuously bubbled with air at 37 °C (pH 7.4). Strips were stretched with a passive tension of 1 g and tension was recorded using an isometric transducer (Grass Model FT03, Quincy, MA, USA) connected to a PC-based Dataq acquisition system (PM-100, CWE Inc., Akron, OH, USA). After an equilibration period of 60 min, control contractions were induced by adding 60 mM potassium chloride to the bath. When two successive control contractions showed similar amplitude, preparations were considered to be equilibrated. Four series of experiments were performed.

Series 1

In order to assess the effects of EOOG or EUG on PHE-induced contraction, aortic ring preparations with intact endothelium were exposed to increasing concentrations of EOOG (1–1000 μg/mL, $n = 6$) or EUG (0.006–6 mM corresponding to 1–1000 μg/mL, $n = 6$) during 5-min

period once a sustained contraction elicited by a submaximal concentration (3 μM) of PHE was established. Similar experiments ($n = 6$) have been performed with increasing concentrations (0.003–2.9 μM) of nifedipine, a well-known L-type Ca^{2+} channel blocker, used herein as a positive control. The effects of EOOG's vehicle at the same volume as that used for the different concentrations of the EOOG were also determined.

Series 2

In order to investigate whether EOOG-induced relaxation is dependent upon the integrity of the vascular endothelium, vascular responses to EOOG (1–1000 μg/mL, $n = 7$) were determined in endothelium-denuded rings precontracted by PHE (3 μM). The endothelium was removed immediately after dissection by gentle rubbing of the aortic lumen with a stainless steel wire. Each isolated aortic preparation with or without intact endothelium was challenged at the beginning of the experiment with 1 μM of acetylcholine. The absence of acetylcholine-induced vasorelaxant effects was taken as evidence that the preparation was effectively stripped of endothelium.

Series 3

This series of experiments was carried out to assess the effects of EOOG and EUG on K^+ (60 mM)-induced contraction in a Ca^{2+}-free medium. Calcium-free solutions were prepared by omitting $CaCl_2$ from normal Tyrode's solution. Ca^{2+} availability from extracellular Ca^{2+} entry was evaluated by recording the contraction induced by K^+ (60 mM) in Ca^{2+}-free medium (containing 2×10^{-5} M ethylene glycol bis(2-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA). After usual equilibration time (60 min) in normal Tyrode solution, the tissues were washed three times in Ca^{2+}-free medium, and then after 5 min they were challenged with 60 mM K^+. After 1 min, Ca^{2+} was cumulatively added still in the presence of high K^+ solution. The maximal contraction obtained with the control concentration–response curve for $CaCl_2$ (0.01–10 mM) was taken as 100%, and all contractions were calculated as a function of this value. EOOG (300 and 1000 μg/mL, $n = 7$) or EUG (1.8 and 6 mM, $n = 7$) was added to the preparations for 5 min, and then a second cumulative concentration–response curve for $CaCl_2$ was obtained.

Series 4

In this series of experiments, the effects of EOOG and EUG on caffeine-induced contractions in Ca^{2+}-free medium were determined as follows. After the usual stabilization time, the tissues were washed with Ca^{2+}-free solution for 6 min, with the temperature maintained in 25 °C. Caffeine (20 mM) was added, which produced a transient contraction. After washing the tissues with normal Tyrode solution, high potassium solution (K^+ 60 mM) was added for Ca^{2+} internal stores loading. The preparations were washed with Ca^{2+}-free solution followed again by the administration of caffeine, with EOOG (1000 μg/mL, $n = 7$) or EUG (6 mM, $n = 7$) added 5 min before the administration of caffeine.

Statistical analysis

All the results are expressed as mean ± standard error of the mean (SEM). Maximal changes (expressed as a percentage of baseline values) in MAP and HR after each dose of the EOOG were used to construct a dose–response curve. The IC_{50} value, defined as the EOOG or EUG concentration (μg/mL or μM, respectively) required to produce a half maximum reduction of PHE-induced contraction, was used to evaluate vascular sensitivity to EOOG or EUG. It was calculated by interpolation from semi-logarithmic plots, and expressed as geometric mean (95% confidence interval). Contractions data were expressed as a percentage of the PHE-induced contraction. The significance ($P < 0.05$) of the results was assessed by means of paired and unpaired Student's *t*-tests, and one- or two-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests, when appropriate.

RESULTS

In vivo experiments

In conscious DOCA-salt hypertensive rats, average baseline values of MAP and HR before any treatment were 168 ± 4 mmHg and 390 ± 13 beats/min, respectively (pooled data from 13 rats). These baseline values were of the same order of magnitude as those previously reported in the same preparation [12,21,22]. There was no significant change in either MAP or HR after the i.v. administration of EOOG's vehicle. However, increasing bolus doses of EOOG (1–20 mg/kg, i.v.) evoked dose-dependent decreases in MAP and HR ($P < 0.001$), which peaked at 20–40 s after administration (*Figure 1*) and became significant at the dose of 1 mg/kg (*Figure 2*). For all doses studied, pre-dose values of MAP were fully recovered within the first 1 min following EOOG treatment, but MAP remained significantly ($P < 0.05$) reduced 1–5 min after administration of the highest dose (20 mg/kg) (*Figure 1a*). As previously observed in

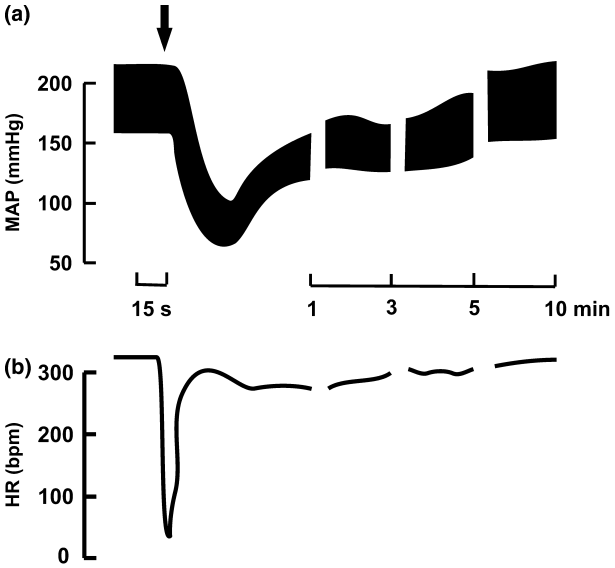

Figure 1 Representative recordings showing changes in (a) mean arterial pressure (MAP) and (b) heart rate (HR) induced by intravenous injection of the essential oil of *Ocimum gratissimum* (20 mg/kg) in DOCA-salt hypertensive, conscious rats. Arrow indicates the time of injection. bpm; beats per minute.

normotensive conscious rats [11], pre-dose values of HR were fully recovered within the first 1 min after the administration of 1 mg/kg EOOG, whereas HR remained significantly ($P < 0.05$) reduced 1–5 min after the administration of 5, 10 and 20 mg/kg EOOG (*Figure 1b*). Pretreatment with propranolol (2 mg/kg, i.v.) decreased significantly ($P < 0.05$) the baseline HR (347 ± 11 vs. 381 ± 16 beats/min) without affecting baseline MAP (180 ± 7 vs. 171 ± 7 mmHg). The EOOG-induced dose-dependent hypotension (*Figure 2a*) and bradycardia (*Figure 2b*) remained unaffected by propranolol pretreatment ($P > 0.05$, two-way ANOVA).

In vitro experiments

Effects of EOOG, EUG or nifedipine on contractions induced by phenylephrine (series 1)

In aorta rings with intact endothelium from DOCA-salt hypertensive rats, increasing concentrations of EOOG (1–1000 μg/mL) or EUG (0.006–6 mM), but not their vehicle (Tween in Tyrode's solution), inhibited the PHE-induced contractions in a concentration-dependent manner ($P < 0.001$, two-way ANOVA). The first inhibitory effect of EOOG and EUG became significant at a concentration of 30 μg/mL and 0.18 mM (corresponding to approximately 30 μg/mL), respectively (*Figure 3*). The IC_{50} [geometric mean (95% confidence interval)] values for EOOG- and EUG-induced vasore-

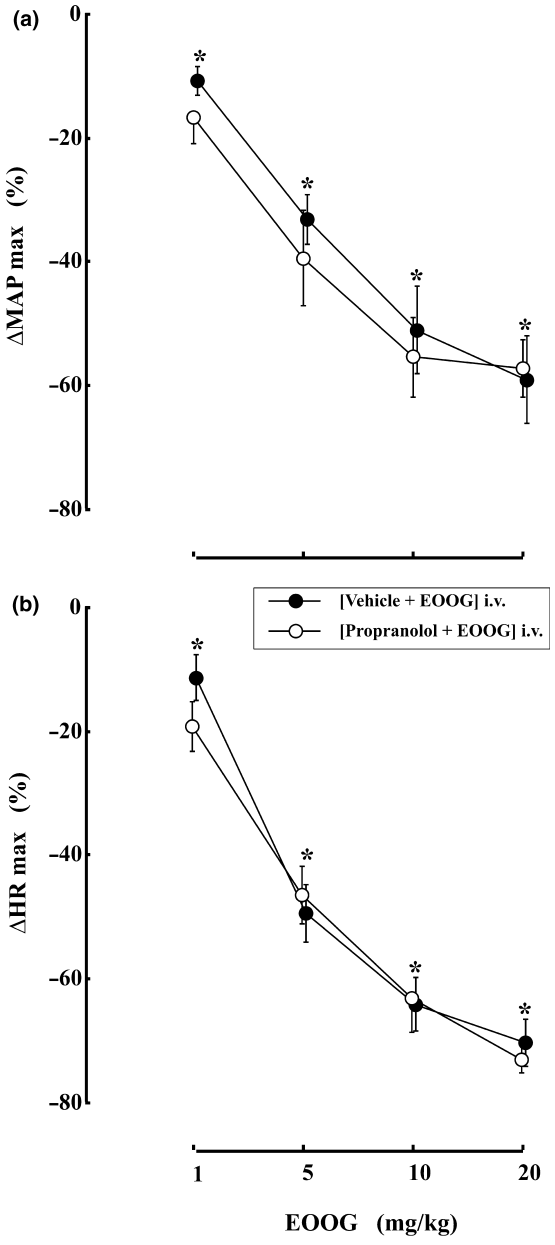

Figure 2 Maximal decreases in (a) mean arterial pressure (MAP) and (b) heart rate (HR) elicited by i.v. increasing bolus doses of the essential oil of *Ocimum gratissimum* (EOOG) in conscious DOCA-salt hypertensive rats subjected to i.v. pretreatment with vehicle (Tween 80 (2%) in isotonic saline; 1 mL/kg) or propranolol (2 mg/kg). Values are expressed as a percentage of baseline and vertical bars indicate SEM ($n = 6–9$ rats per group). Baseline MAP (mmHg) and HR (beats/min) were 168 ± 6 and 370 ± 10 in vehicle-pretreated rats, and, 180 ± 7 and 347 ± 11 in propranolol-pretreated rats, respectively. Propranolol pretreatment did not affect significantly the EOOG-induced cardiovascular responses. $P < 0.001$ among absolute values (one-way ANOVA) and $^*P < 0.05$ compared with corresponding baseline values (Dunnett's test).

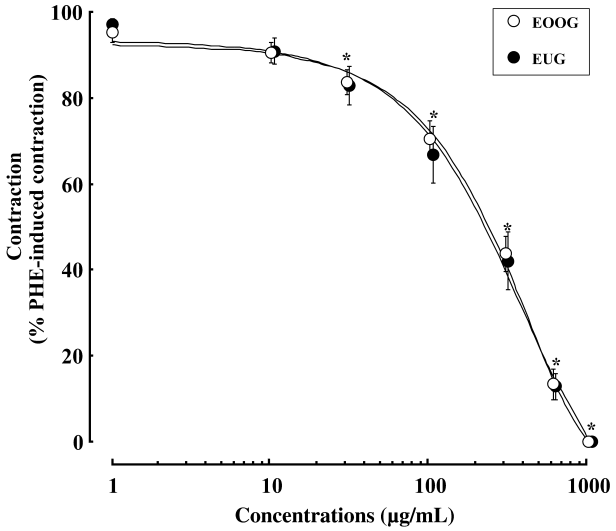

Figure 3 Effects of increasing concentrations of the essential oil of *Ocimum gratissimum* (EOOG; 1–1000 μg/mL) and its main constituent, eugenol (EUG; 1–1000 μg/mL corresponding to 0.006–6 mM), on the contraction induced by phenylephrine (PHE) in aortic rings with intact endothelium from DOCA-salt hypertensive rats. Vertical bars indicate SEM ($n = 6$ per group). EOOG and EUG elicited reversible and similar concentration-dependent vasodilator responses. $P < 0.001$ among absolute values (one-way ANOVA) and $^*P < 0.05$ compared with corresponding baseline values (Dunnett's test).

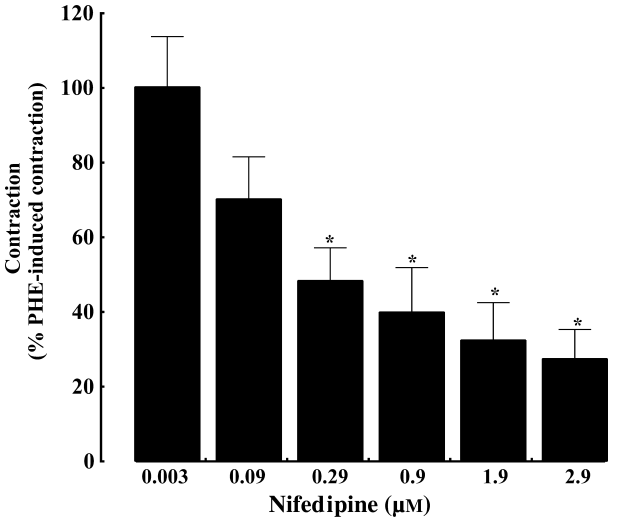

Figure 4 Effects of increasing concentrations (0.003–2.9 μM) of the positive reference drug nifedipine on the contraction induced by phenylephrine (PHE) in isolated aortic rings with intact endothelium from DOCA-salt hypertensive rats. Vertical bars indicate SEM ($n = 6$). Nifedipine inhibited the PHE-induced contraction in a concentration-dependent manner. $P < 0.001$ among absolute values (one-way ANOVA) and $^*P < 0.05$ compared with corresponding baseline values (Dunnett's test).

laxant effects were 226.9 (147.8–348.3) μg/mL and 1.2 (0.6–2.1) mM [corresponding to approximately 191.8 (106.3–346.3) μg/mL], respectively. There is no significant ($P > 0.05$) difference between the IC_{50} values for EOOG- and EUG-induced vasorelaxant effects. In these preparations, the known L-type Ca^{2+} channel blocker nifedipine (0.003–2.9 μM) inhibited the PHE-induced contraction in a concentration-dependent manner ($P < 0.001$; *Figure 4*). The first inhibitory effect of nifedipine became significant at a concentration of 0.29 μM ($P < 0.05$; *Figure 4*). The IC_{50} values for nifedipine-induced reduction of PHE-induced contractions were 0.2 (0.0057–6.6) μM [corresponding to approximately 0.07 (0.002–2.3) μg/mL].

Role of the vascular endothelium in EOOG-induced vasorelaxation (series 2)

In endothelium-denuded rings precontracted by PHE (3 μM), concentration–response curves for cumulative EOOG (1–1000 μg/mL) treatment showed no difference in the maximal response (*Figure 5*). However, the smooth muscle-relaxant activity of EOOG is partly dependent upon the integrity of the vascular endothelium as the mean geometric IC_{50} values for EOOG-induced vasorelaxant effects were significantly ($P < 0.05$) enhanced in endothelium-denuded preparations [417.2 (349.5–497.8) μg/mL] when compared with those in preparations with intact endothelium.

Effects of EOOG and EUG on Ca^{2+}-induced contractions in K^+-depolarized aorta (series 3)

In aorta preparations with intact endothelium incubated in Ca^{2+}-free medium in the presence of high KCl solution, increasing concentrations of $CaCl_2$ (0.01–10 mM) evoked the excepted concentration-dependent contractions ($P < 0.001$), an effect that became significant at a concentration of 0.1 mM ($P < 0.05$; *Figure 6*). This effect was significantly ($P < 0.05$; two-way ANOVA) reduced and abolished by EOOG at 300 and 1000 μg/mL, respectively (*Figure 6*). At the same range of concentration (corresponding to approximately 1.8 and 6 mM, respectively), EUG evoked similar effects as those elicited by EOOG (*Figure 7*). It is noteworthy that the maximal response to 10 mM of $CaCl_2$ in the presence of EOOG (29.0 ± 4.2%) was of the same order magnitude ($P > 0.05$) as that evoked in the presence of EUG (39.0 ± 6.1%), suggesting an equal potency of EOOG and EUG against $CaCl_2$-induced contractions.

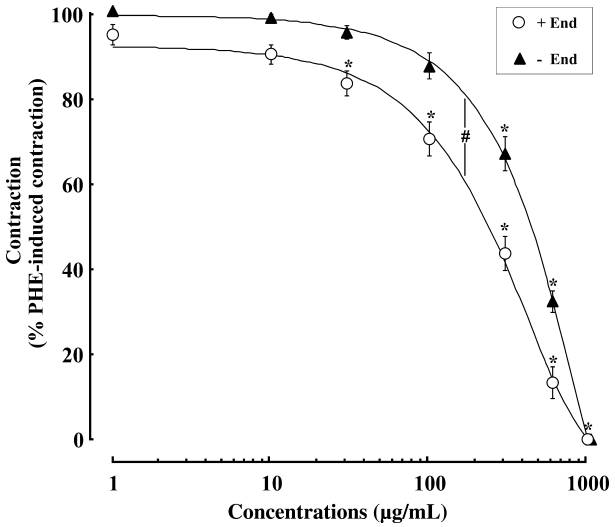

Figure 5 Effects of increasing concentrations (1–1000 µg/mL) of the essential oil of *Ocimum gratissimum* (EOOG) on the contraction induced by phenylephrine (PHE) in isolated aortic rings from DOCA-salt hypertensive rats with (+ End, $n = 6$) or without (– End) functional endothelium ($n = 7$). Vertical bars indicate SEM. The vasorelaxant effect of EOOG was significantly ($^{\#}P < 0.001$, two-way ANOVA) reduced by the mechanical removal of the vascular endothelium. $P < 0.001$ among absolute values (one-way ANOVA) and $^{*}P < 0.05$ compared with corresponding baseline values (Dunnett's test).

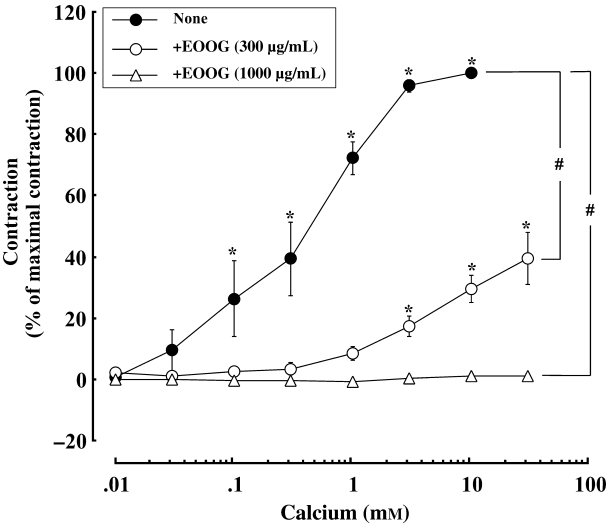

Figure 6 Effects of increasing concentrations (0.1–10 mM) of calcium (Ca^{2+}) on the contraction induced by potassium (60 mM) in isolated aortic rings with intact endothelium from DOCA-salt hypertensive rats in Ca^{2+}-free medium in the absence or in the presence of essential oil of *Ocimum gratissimum* (EOOG) at 300 or 1000 µg/mL. Vertical bars indicate SEM ($n = 7$ per group). The Ca^{2+}-induced concentration-dependent contractions were significantly ($^{\#}P < 0.001$, two-way ANOVA) reduced and abolished by EOOG at 300 and 1000 µg/mL, respectively. $P < 0.001$ among absolute values (one-way ANOVA) and $^{*}P < 0.05$ compared with corresponding baseline values (Dunnett's test).

Effects of EOOG and EUG on caffeine-induced contractions in Ca^{2+}-free medium (series 4)

In this series of experiments, caffeine was used as a pharmacological tool to investigate whether EOOG or EUG act through inhibition of Ca^{2+} release from the sarcoplasmic reticulum. In aortic rings with intact endothelium maintained in Ca^{2+}-free medium, caffeine-induced contractions remained significantly ($P > 0.05$, *Figure 8*) unchanged by the highest concentration of either EOOG (1000 µg/mL) or EUG (6 mM).

DISCUSSION

As discussed previously [11], it is unlikely that EOOG-induced cardiovascular changes could be related to a putative toxic effect of this essential oil. In fact, the oral acute toxicity LD_{50} values for EUG [23] and 1,8-cineole [24], the major constituents of EOOG, have been found to be greater than 2000 mg/kg [23,24]. Our previous hypothesis that EOOG-induced hypotension results mainly from a vasodilatory action of EOOG directly upon vascular smooth muscle [11] is corroborated by the present in vitro findings showing for the first time that, in aortic rings with intact endothelium from DOCA-salt hypertensive rats, EOOG and its main constituent, EUG, depressed the PHE-induced contractions in a concentration-dependent manner. Vascular β_2-adrenoreceptors do not seem to be involved in this effect as EOOG-induced hypotension in conscious DOCA-salt hypertensive remained unaltered by the non-selective β-adrenoceptor antagonist, propranolol. The present data suggest that the vasorelaxant activity of these agents is unrelated to a putative inhibition of the Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum. However, it seems partially dependent upon the integrity of the vascular endothelium and predominantly mediated through an inhibition of plasmalemmal Ca^{2+} inward current.

Previous reports suggested that endothelium-dependent relaxing factor (EDRF)/nitric oxide (NO) is involved in the inhibitory effects of EUG on isolated thoracic aortic preparations from normotensive rat [25] and rabbit [26]. However, EUG-induced concentration-dependent vasodilator responses in the rat mesenteric vascular beds were partially dependent on the endothelium, although apparently independent of EDRF/NO or prostacyclin

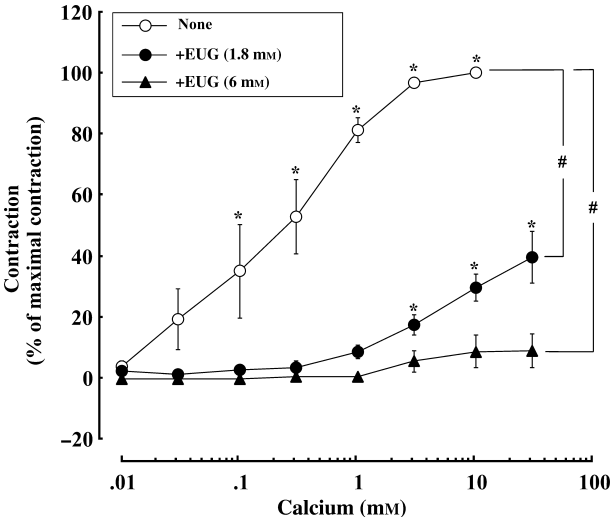

Figure 7 Effects of increasing concentrations (0.1–10 mM) of calcium (Ca^{2+}) on the contraction induced by potassium (60 mM) in isolated aortic rings with intact endothelium from DOCA-salt hypertensive rats in Ca^{2+}-free medium in the absence or in the presence of eugenol (EUG) at 1.8 and 6 mM. Vertical bars indicate SEM ($n = 7$ per group). The Ca^{2+}-induced concentration-dependent contractions were significantly ($^{\#}P < 0.001$, two-way ANOVA) reduced and abolished by EUG at 1.8 and 6 mM, respectively. $P < 0.001$ among absolute values (one-way ANOVA) and $^{*}P < 0.05$ compared with corresponding baseline values (Dunnett's test).

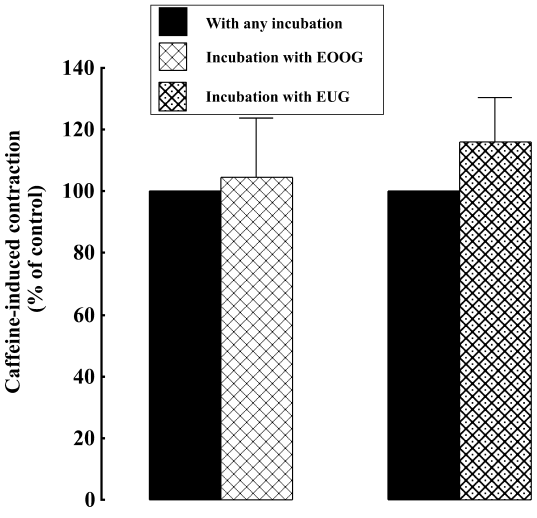

Figure 8 Effects of essential oil of *Ocimum gratissimum* (EOOG; 1000 μg/mL) and eugenol (EUG; 6 mM) on the contraction induced by caffeine (20 mM) in isolated aortic rings with intact endothelium from DOCA-salt hypertensive rats, maintained in Ca^{2+}-free medium. Vertical bars indicate SEM ($n = 7$ per group). Neither EOOG nor EUG affected the caffeine-induced transient contraction.

[13]. In the present study, putative participation of the vascular endothelium in mediation of EOOG-induced relaxation has been investigated. Our results show that the vasorelaxant effects of EOOG were also attenuated by mechanical removal of the endothelium, as evidenced by the significant increase in the IC_{50} of EOOG-induced reduction of PHE-induced contraction. This suggests that the vasorelaxation induced by EOOG is partly mediated by an endothelium-dependent mechanism involving EDRF/NO or prostacyclin release. Given the fact that such a mediation is minor (i.e. slight shift to the right of the concentration–relaxation response to EOOG without any change in the maximal response), we focused mainly on the mechanisms underlying the endothelium-independent relaxation of the EOOG, and its main constituent EUG.

In rat aorta, high K^+ induces membrane depolarization, which, in turn, opens the voltage-operated channels (VOCs), increases Ca^{2+} influx, and elicits sustained contraction. In contrast, contractions resulting from α_1-adrenoceptor stimulation by PHE are biphasic: an early phasic component due to intracellular Ca^{2+} release followed by a sustained component, which develops slowly and depends upon extracellular Ca^{2+} influx through receptor-operated channels (ROCs). The present study shows that both EOOG and EUG inhibited the PHE-induced contractions, an effect that could be mediated by EOOG- or EUG-induced decrease in Ca^{2+} entry through ROCs. However, it has been shown that one of the mechanisms of the tonic contraction following α_1-adrenoceptor activation with PHE is mediated by Ca^{2+} influx through nifedipine-sensitive, voltage-dependent L-type Ca^{2+} channels [27]. In a corollary to this, the current study shows that nifedipine, a well-known L-type Ca^{2+} channel blocker, inhibited the PHE-induced contractions, although its potency was about 5850-fold higher than that of EOOG or EUG. Thus, it is reasonable to suggest that vasorelaxant effects of EOOG and EUG related to decreased Ca^{2+} influx through ROCs, VOCs or both. To further corroborate the involvement of VOCs as a target for the EOOG- and EUG-induced vasodilatory effects, experiments were performed in aortic preparations depolarized with high KCl in Ca^{2+}-free medium. Under these conditions, both EOOG and EUG reduced and even fully abolished the contractions induced by $CaCl_2$, which are due exclusively to an increase in Ca^{2+} influx through VOCs. This suggests that the inhibitory effects of EOOG and EUG may also be attributed, at least in part, to blockade of Ca^{2+} influx through VOCs. Whether EOOG and EUG inhibit the transmembrane Ca^{2+} influx by

acting directly on voltage-dependent Ca^{2+} channels is a question that requires further investigation. Nevertheless, the Ca^{2+} channel antagonist profile of the EOOG and EUG is consistent with previous reports of other authors using EUG in aortic preparations from normotensive rats [25] and rabbits [26,28].

In rat aorta maintained in Ca^{2+}-free solution, caffeine induced only a transient contraction, which is attributable to the Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum [29]. As neither EOOG nor EUG altered the caffeine-induced transient contraction in Ca^{2+}-free medium, their vasorelaxant effect appears unrelated to a putative toxicity and seems restricted to an action upon the plasmalemmal Ca^{2+} channels rather than through inhibition of Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum. Furthermore, the inefficacy of EOOG or EUG in altering caffeine-induced contractions also rules out the possibility that participation of putative endothelium-independent intracellular sites of action, such as an inhibition of protein kinase C (PKC) or other enzymatic protein subsequent to activation of PKC related to the activation of contractile proteins like myosin light chain kinase (MLCK). However, as PHE was the contractile agent used herein, the possibility that inhibition of PHE-induced vasoconstriction by EOOG and EUG is partly attributable to blockade of Ca^{2+} release from sarcoplasmic reticulum upon activation of IP_3-sensitive Ca^{2+} channels [30] could not be completely discarded. Further experiments using aortic preparations precontracted by PHE in Ca^{2+}-free medium are required to examine the latter hypothesis.

The vasorelaxant activity of EOOG in aortic preparations from DOCA-salt hypertensive rats appear partly attributed to the actions of its main constituent, EUG. This constituent has been reported to induce vasorelaxant effects on isolated thoracic aortic preparations from normotensive rat [25] and rabbit [26,28] as well as on mesenteric vascular bed preparations from normotensive rats [10,13]. Interestingly, the present study shows that the inhibitory effect of EOOG on PHE-induced contractions was of the same potency as EUG. As only 43.7% of the EOOG was EUG, this observation indicates that EUG is not the only constituent that mediates the vasorelaxant activity of EOOG. Other constituents, such a 1,8-cineole (the second main constituent with 32.7% of total weight of the oil), may contribute to the vasorelaxant activity of EOOG as it was reported to inhibit significantly the KCl-induced contractions in aortic rings from normotensive rats [16]. The effects of EOOG on isolated aorta are not peculiar to that tissue alone because EOOG, as well as its major constituents (EUG and 1,8-cineole) have been reported to induce a concentration-dependent relaxation of guinea-pig isolated ileum segments precontracted with 60 mM potassium [4,5,6,31]. This myorelaxant activity explains the use of *O. gratissimum* in local folk medicine to treat gastrointestinal disturbances. These in vitro studies have suggested that EOOG and its main constituent act directly on the smooth muscle rather than indirectly on neurotransmitter release to induce their pharmacological effects [4,6,31].

In conclusion, the present results support the hypothesis that EOOG-induced hypotension in conscious DOCA-salt hypertensive rats is mainly due to an active vascular relaxation. The vasorelaxant activity of EOOG, which is partly attributable to the actions of EUG, seems partly dependent upon the integrity of the vascular endothelium (endothelium-independent relaxation) and predominantly mediated through an inhibition of plasmalemmal Ca^{2+} inward current (endothelium-independent relaxation) rather than inhibition of Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum. Further studies are necessary to assess the cardiovascular effects of the EOOG, and its main constituent EUG, in another hypertensive model, such as spontaneously hypertensive rats (SHR), and whether chronic oral treatment with EOOG is useful for the prevention and treatment of hypertension.

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5. ANEXO

5. 1. ANEXO 1

Cardiovascular effects of eugenol, a phenolic compound present in many plant essential oils, in normotensive rats.

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Cardiovascular Effects of Eugenol, a Phenolic Compound Present in Many Plant Essential Oils, in Normotensive Rats

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Abstract: Cardiovascular effects of intravenous (i.v.) treatment with eugenol (Eug), a natural pungent present in many plant essential oils, were investigated in normotensive rats. In either anesthetized or conscious rats, i.v. bolus injections of Eug (1 to 10 mg/kg) elicited immediate and dose-dependent hypotension and bradycardia. Magnitude of Eug-induced hypotension was similar in both groups. Pretreatment of anesthetized rats with bilateral vagotomy almost abolished the bradycardic responses to Eug without affecting the hypotension. Likewise, i.v. pretreatment of conscious rats with methylatropine (1 mg/kg) or hexamethonium (30 mg/kg) significantly reduced the Eug-induced bradycardia without affecting the hypotension. However, i.v. pretreatment with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl (L-NAME, 20 mg/kg), affected neither the hypotension nor the bradycardia elicited by Eug. In rat mesenteric bed preparations precontracted with potassium (60 mM), Eug (0.1–2 mM) induced a reversible and concentration-dependent vasodilator effect, which remained unaffected by atropine (1 μM). These results show that i.v. treatment of rats with Eug induces dose-dependent hypotension and bradycardia, which occurred independently. The bradycardia appears dependent upon the presence of an intact and functional parasympathetic nerve drive to the heart while the hypotension is due to an active vascular relaxation rather than withdrawal of sympathetic tone. Released nitric oxide from vascular endothelial cells seems to be not involved in the mediation of Eug-induced hypotension.

Key Words: autonomic nervous system, cardiovascular effects, eugenol, nitric oxide, mesenteric vascular bed, rat

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Eugenol (Eug), a natural pungent and the major constituent of oil of clove, is used as food flavor and fragrance agent, and is commonly used in dentistry for the sedation of toothache, pulpitis, and dental hyperalgesia.[1] Several biologic actions of Eug have previously been reported. For instance, this compound induces central nervous system effects in mammals such as hypothermia, decrease in spontaneous motor activity, anticonvulsant, and general anesthetic effects.[2] Eugenol is a potent depressant of peripheral nervous activity[3,4] and of excitation-contraction coupling in skeletal muscle.[1] This compound has also been documented to induce myorelaxant and antispasmodic effects[5] and vasorelaxant effects on either rat[6] or rabbit[7] thoracic aorta as well as on rat mesenteric vascular bed.[8] Eugenol was suggested to act, at least, as a Ca^{2+} channel antagonist either through voltage-dependent or through receptor-operated Ca^{2+} channels to induce its vasodilatory effects in the rat isolated aorta[6,7] but not in the small resistance vessels.[8] It was also reported that methyl-eugenol, an analogue of Eug, also possesses myorelaxant and antispasmodic effects in laboratory animals.[9]

Eugenol is also an important chemical constituent of the essential oils of many aromatic plants, such as *Eugenia caryophyllus* (Spr.) Merril et Harr, *Dicipelium cariophyllatum* Nees, *Pimenta dioica* (L) Merril, *Croton zehntneri* Pax et Hoffm, var. *eugenoliferum*, and *Ocimum gratissimum* L.[10–13] *Croton zehntneri*, and *O. gratissimum* have a rich essential oil content and are used in folk medicine of northeastern Brazil as stomachics, carminatives, and intestinal antispasmodics.[14] Previous studies from our laboratory showed that the essential oils of *C. zehntneri* and *C. nepetaefolius* (EOCN), which are rich in Eug analogues as well as in methyl-eugenol, possess myorelaxant and antispasmodic effects in laboratory animals.[9,15,16] It was also shown that intravenous (i.v.) treatment with EOCN induces dose-dependent decreases in mean aortic pressure (MAP) and heart rate (HR) in either anesthetized or conscious, normotensive rats.[17] Both in vivo and in vitro data suggested that the hypotensive response to EOCN[18] results from its vasodilatory action directly upon vascular smooth muscle rather than withdrawal of sympathetic tone. However, very few papers in the international literature have systematically studied the cardiovascular effects of Eug in rats.

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The present investigation was undertaken to address this issue and comprised 2 parts. The first part was performed in conscious, freely moving, or anesthetized rats to assess the cardiovascular effects of Eug as well as the role of autonomic nervous system and endothelial L-arginine/nitric oxide pathway in the mediation of these effects. The second one was performed in vitro using rat isolated mesenteric bed preparations, which represent a resistive network that contributes substantially to peripheral vascular resistance, to assess whether Eug-induced hypotension could result, at least in part, from its vasodilatory effects directly upon vascular smooth muscle.

METHODS

Animals

Male Wistar rats were kept under conditions of constant temperature (22 ± 2°C) with a standard light:dark cycle (12 hours light:12 hours dark) and free access to food and water. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996).

In Vivo Experiments

Rats (250–330 g) were anesthetized intraperitoneally (i.p.) with sodium pentobarbital (50 mg/kg), and catheters (PE-10 fused to PE-50) were implanted in the abdominal aorta (for the recording of arterial blood pressure) and in the inferior vena cava (for drug administration) through the left femoral artery and vein, respectively. These catheters, filled with heparin-saline solution (125 IU/ml), were exteriorized at the dorsal neck level. Postoperatively, the rats received an intramuscular injection of penicillin (24,000 IU), housed individually in plastic cages and allowed to recover for 48 hours before any circulatory experiments. At the time of experiment, the arterial catheter was connected to a blood pressure transducer (Statham P23 ID) coupled to a polygraph recorder; HR was obtained from a cardiotachometer triggered by the pressure pulses. Both signals were recorded on a Gilson model 5/6H (Medical Electronics Inc., Middletown, WI). The MAP was calculated as diastolic + [(systolic − diastolic)/3].

Before each experiment, blood pressure and HR were allowed to stabilize and were recorded during 10 to 15 minutes (according to the duration of effects) after i.v. treatment with Eug. When subsequent doses of Eug were administered, MAP and HR were first allowed to return to their baseline levels, obtained before the first injection of the compound. When the effects of an antagonist were tested, antagonist injection occurred 10 minutes before Eug administration. Doses of agonists or antagonists were chosen according to those recommended in the literature (references cited in text, series 1 and 2, below). Two series of experiments were performed as follows:

Series 1

This series of experiments was carried out in anesthetized rats to establish a dose-effect relationship. Rats were again anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Rectal temperature was kept close to 37°C by placing the animals on a thermostatically controlled table. Each animal received a series of increasing bolus (100 μL) doses (1, 3, 5, and 10 mg/kg) of Eug via the i.v. catheter, and time course of the changes in MAP and HR was recorded. These experiments were performed in both intact rats (n = 7), and in rats (n = 6) that had been subjected to a bilateral vagotomy performed at the cervical level 15 minutes earlier. In another set of intact rats (n = 6), time course of the decreases in MAP elicited by an i.v. injection of acetylcholine (ACh; 5 μg/kg), used herein as positive control[18,19] was determined and compared with that of Eug (10 mg/kg).

Series 2

This series of experiments was performed in conscious rats to establish a dose-effect relationship and to assess the role of the autonomic nervous system and the nitric oxide in the mediation of Eug-induced cardiovascular changes. Therefore, time course of the changes in MAP and HR elicited by i.v. injections of Eug (1, 3, 5, and 10 mg/kg) was determined in conscious rats that had been pretreated intravenously 10 minutes earlier with one of the following pretreatments: vehicle (1 mL/kg, n = 7), hexamethonium (30 mg/kg, n = 7), methylatropine (1 mg/kg, n = 6),[20] or L-NAME (20 mg/kg, n = 7). Each rat received increasing bolus doses as described previously. It should be noted that the dose (30 mg/kg) of hexamethonium chosen was sufficient to achieve complete ganglionic blockade.[21]

In Vitro Experiments

Rats (180–250 g) were stunned and then exsanguinated, and the superior mesenteric artery was cannulated. Thereafter, the mesenteric vascular bed was isolated and perfused at a constant hydrostatic pressure of 53 cm H_2O with a modified Tyrode solution continuously aerated. An equilibration period of 30 minutes was allowed to stabilize the mesenteric vascular bed, baseline perfusion flow. In control experiments, this was followed by a single concentration of potassium chloride (KCl, 60 mM) perfusion for 90 minutes, considering the adjustment for isotonicity of the buffer solution by equivalent reduction in NaCl. In the experimental group, after 20 minutes of the beginning of the perfusion with KCl, responses to increasing concentrations of Eug (0.1, 0.3, 1, 1.3, and 2.0 mM) alone (n = 6) or in association with atropine (1 μM, n = 6) were performed in a cumulative manner. A washout was then allowed until the baseline flow rates were achieved. Concentration-response curves to Eug were determined only once in each mesenteric vascular bed preparation and the Eug was directly added to the buffer solution. It is noteworthy that atropine at 1 μM was able to prevent the vasodilator responses to ACh (0.3 μM).

Drugs and Solutions

Sodium pentobarbital (Sanofi, Libourne, France) and heparin (Laboratoires Léo S.A., Montigny-le-Bretonneux, France) were used as commercially available injectable solutions. Hexamethonium bromide, methylatropine bromide, Eug, and ACh chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin G benzathine salt was purchased from Lafepe (Recife, PE, Brazil). For in vivo experiments, Eug was dissolved in Tween 80 (2%), brought to the chosen volume with sterile isotonic saline and sonicated just before use. Previous studies showed that this vehicle had no effects on either baseline MAP or HR during a period of 20 minutes.[17,18,22,23] Hexamethonium bromide, methylatropine bromide, and L-NAME were dissolved in saline and administered in volumes of 1 mL/kg body weight, while ACh chloride (dissolved in saline) was given in a volume of 100 µL. Each i.v. injection was followed by a 60-µL (catheter volume) flush of physiological saline to ensure complete delivery of the dosage. For in vitro experiments, Eug and ACh were prepared directly in the perfusion medium and sonicated just before use. The perfusion medium used was fresh modified Tyrode solution (pH = 7.4) containing (mM): NaCl 136, KCl 5, $MgCl_2$ 0.98, $CaCl_2$ 2, NaH_2PO_4 0.36, $NaHCO_3$ 11.9, and glucose 5.5.

Statistical Analysis

All results are expressed as means ± SEM. Maximal changes in MAP and HR (expressed as a percentage of baseline values) after each dose of the Eug were used to construct a dose-response curve. The IC_{50} value, defined as the Eug concentration (mM) required to produce half maximum reduction of the potassium perfusion effects on basal mesenteric flow, was used to evaluate vascular sensitivity to Eug, and was determined graphically in each individual experiment. The mean IC_{50} was calculated by averaging the IC_{50}s of each concentration-effect curve. Statistical significance ($P < 0.05$) of the results was assessed by means of paired and unpaired Student *t* tests, Mann-Whitney *U*-tests, and one-way (groups, doses, or time) or two-way (treatment × dose or treatment × time) analysis of variance (ANOVA), followed by Dunnett tests where appropriate.

RESULTS

In Vivo Experiments

Studies in Pentobarbital-Anesthetized Rats

In this series of experiments, baseline MAP and HR before injection of each dose of Eug did not vary in magnitude ($P > 0.05$). Therefore, mean values of baseline MAP and HR in this group of animals were 102 ± 3 mm Hg and 399 ± 7 beats/min, respectively (pooled data from 19 rats). These baseline values remained unchanged after i.v. administration of 0.3 mg/kg of Eug (data not shown). However, intravenous injections of Eug (1 to 10 mg/kg) induced immediate and dose-dependent decreases in MAP and HR ($P < 0.001$, Fig. 1). These effects became significant at the dose of 1 mg/kg ($P < 0.05$, Fig. 1), and were maximal within the first 20 to 30 seconds after Eug treatment. After all doses tested of Eug, pre-dose values of MAP were fully recovered within the first 1 minute following Eug treatment, but MAP remained significantly reduced 1, 3, and 5 minutes following administration of the highest dose (10 mg/kg) of Eug ($P < 0.05$, Fig. 2). After 1 or 3 mg/kg i.v. of Eug, pre-dose values of HR were fully recovered within the first 1 minute following Eug treatment, but HR remained significantly ($P < 0.05$) reduced at 3 (−12.55 ± 3.31%) and 5 (−14.3 ± 4.70%) minutes following administration of 5 mg/kg of Eug, and at 3 (−17.28 ± 5.64%), 5 (−21.26 ± 5.35%), and 10 (−21.54 ± 5.76%) minutes following administration of 10 mg/kg of Eug. Bilateral vagotomy did not affect baseline MAP (100 ± 6 vs. 102 ± 5 mm Hg in intact rats), but induced a significant ($P < 0.05$) increase in baseline HR (454 ± 16 vs. 395 ± 10 beats/min in intact rats). Bilateral vagotomy did not alter the Eug dose-hypotensive response curve ($P > 0.05$, Fig. 1) while it significantly reduced Eug-induced bradycardia ($P < 0.001$, Fig. 1).

The positive reference drug "ACh (5 mg/kg, i.v.)" also induced a significant decrease in MAP, the magnitude of which was maximal (−47.86 ± 1.68%) within the first 20 seconds after drug treatment ($P < 0.01$, Fig. 2), as was observed with Eug. However, unlike Eug, pre-injection values of MAP were fully recovered within the first 1 minute following ACh treatment (Fig. 2). A two-way analysis of variance revealed that time course of ACh-induced changes in MAP was significantly different from that of Eug (10 mg/kg) ($P < 0.05$, Fig. 2).

Studies in Conscious Rats

As in experiments with anesthetized rats, baseline MAP and HR before any treatment in conscious rats remained essentially invariant ($P > 0.05$). Mean values of MAP and HR in this group of animals were 116 ± 2 mm Hg and 375 ± 10 beats/min, respectively (pooled data from 27 rats). Only baseline MAP was significantly different from that measured in intact, pentobarbital-anesthetized rats ($P < 0.001$). In rats pretreated with vehicle, i.v. injections of Eug (1 to 10 mg/kg) induced immediate and dose-dependent decreases in MAP and HR ($P < 0.001$, Fig. 3). These decreases became also significant at the dose of 1 mg/kg ($P < 0.05$, Fig. 3) and were maximal within the first 20 to 30 seconds post-injection. Maximal percent decreases in MAP elicited by Eug in conscious rats did not differ from those measured in pentobarbital-anesthetized rats ($P > 0.05$). However, the bradycardia response to the highest dose of Eug was significantly ($P < 0.05$) greater than that recorded in anesthetized rats. After all doses tested of Eug, pre-dose values of MAP were fully recovered within the first 1 minute following Eug administration. However, a remaining significant ($P < 0.05$) bradycardia was observed at 1 (−7.42 ± 1.81%), 3 (−9.78 ± 3.26%), and 5 (−8.74 ± 2.33%) minutes following

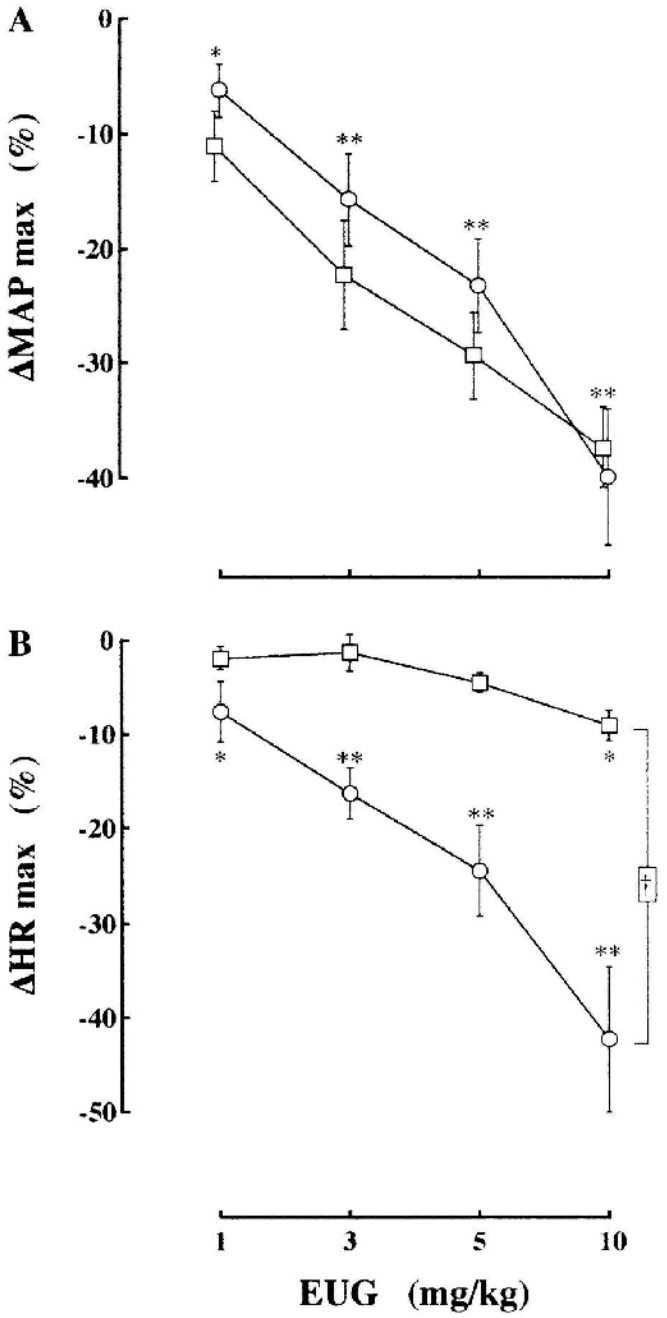

FIGURE 1. Maximal decreases in mean aortic pressure (ΔMAP max; A) and heart rate (ΔHR max; B) elicited by increasing bolus doses of intravenous eugenol (Eug; 1 to 10 mg/kg) in pentobarbital-anesthetized rats with (□) or without (○) bilateral vagotomy. Values are means of changes expressed as a percentage of baseline and vertical bars indicate SEM (6–7 rats per group). In both groups studied, maximal decreases in MAP and HR were significantly related to the dose of Eug ($P < 0.001$, one-way ANOVA). Bilateral vagotomy significantly reduced the Eug-induced bradycardia (†$P < 0.001$, two-way ANOVA) without affecting the hypotension. *$P < 0.05$, **$P < 0.01$ by Dunnett test.

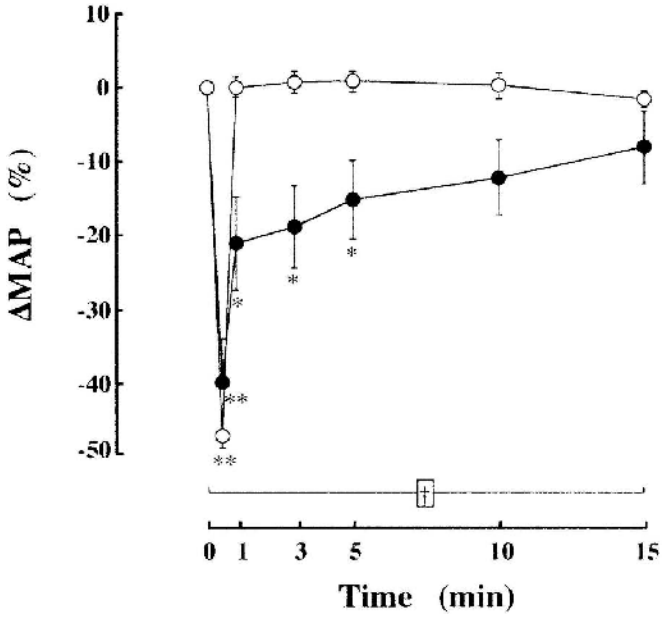

FIGURE 2. Time course of the changes in mean aortic pressure (ΔMAP) elicited by an intravenous injection of the positive reference drug acetylcholine (ACh; 5 μg/kg) (○) and eugenol (Eug; 10 mg/kg) (●) in pentobarbital-anesthetized rats. Values are means of changes expressed as a percentage of baseline. Vertical bars indicate SEM (6–7 rats per group). Time course of Eug-induced changes in MAP was significantly different from that obtained with ACh (†$P < 0.05$, two-way ANOVA). *$P < 0.05$, **$P < 0.01$ by Dunnett test.

administration of 5 mg/kg of Eug, and at 1 (−21. 06 ± 6.16%), 3 (−26.75 ± 5.63%), 5 (−23.33 ± 6.41%), and 10 (−27.92 ± 6.67%) minutes following administration of 10 mg/kg of Eug.

Pretreatment with hexamethonium (30 mg/kg, i.v.) induced significant ($P < 0.001$) decreases in baseline MAP (76 ± 4 vs. 115 ± 4 mm Hg) without affecting significantly the baseline HR (394 ± 15 vs. 393 ± 14 beats/min). Pretreatment with methylatropine (1 mg/kg, i.v.) did not alter baseline MAP (116 ± 5 vs. 114 ± 5 mm Hg), while it significantly increased (451 ± 17 vs. 379 ± 15 beats/min) baseline HR ($P < 0.05$). Pretreatment with either methylatropine or hexamethonium did not modify significantly the dose-dependent decreases in MAP elicited by Eug ($P > 0.05$, Fig. 3A); however, both pretreatments reduced significantly the magnitude of Eug-induced bradycardic effects ($P < 0.001$, Fig. 3B). Two-way ANOVA revealed that Eug-induced decreases in HR after methylatropine pretreatment were not statistically ($P > 0.05$) different from those obtained in animals pretreated with hexamethonium.

Pretreatment with L-NAME (20 mg/kg, i.v.) induced significant ($P < 0.01$) increases in baseline MAP (158 ± 5 vs. 121 ± 3 mm Hg) and decreases in baseline HR (244 ± 7 vs. 368 ± 5 beats/min). Nitric oxide synthase inhibition by L-NAME did not modify significantly the dose-dependent decreases in MAP and HR elicited by Eug ($P > 0.05$, Fig. 4).

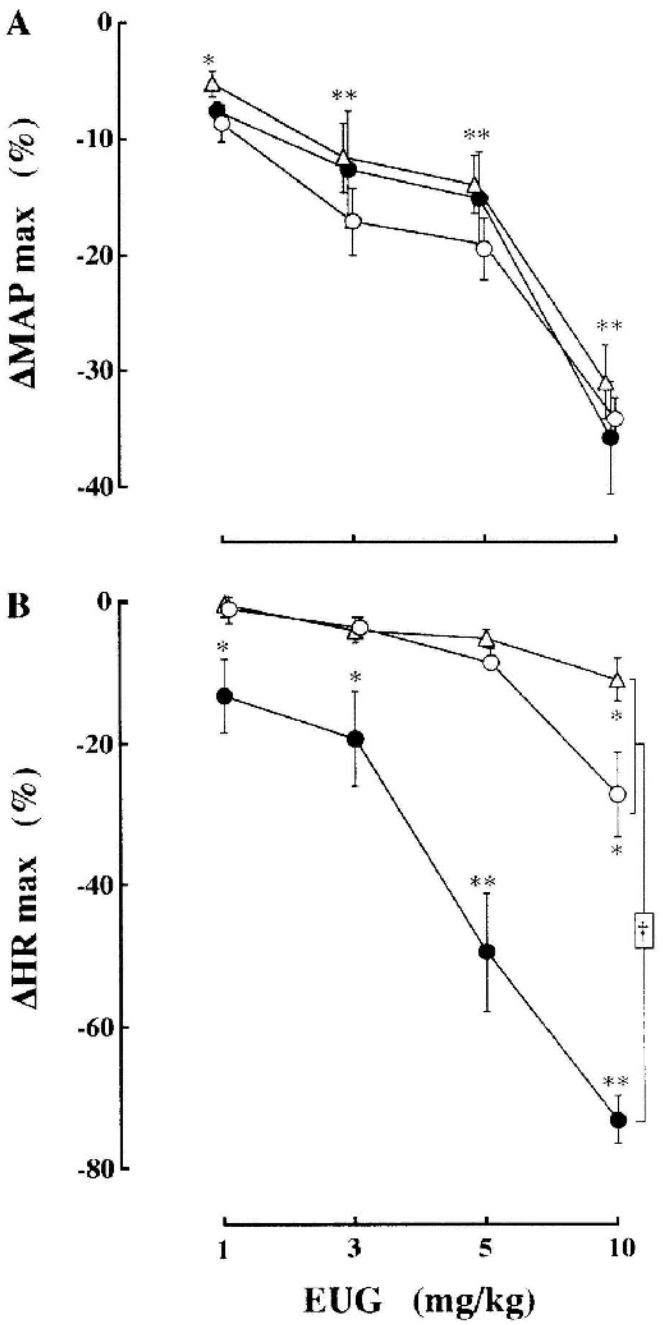

FIGURE 3. Maximal decreases in mean aortic pressure (ΔMAP max; A) and heart rate (ΔHR max; B) elicited by increasing bolus doses of intravenous (i.v.) eugenol (Eug; 1 to 10 mg/kg) in conscious, freely moving rats subjected to i.v. pretreatment with vehicle (1 mL/kg, ●), hexamethonium (30 mg/kg, ○), or methylatropine (1 mg/kg, △). Values are means of changes expressed as a percentage of baseline and vertical bars indicate SEM (6–7 rats per group). In all groups studied, maximal decreases in MAP and HR were significantly related to the dose of Eug ($P < 0.001$, one-way ANOVA). Pretreatment with either hexamethonium or methylatropine significantly reduced the Eug-induced bradycardia (†$P < 0.001$, two-way ANOVA) without affecting the hypotension. *$P < 0.05$, **$P < 0.01$ by Dunnett test.

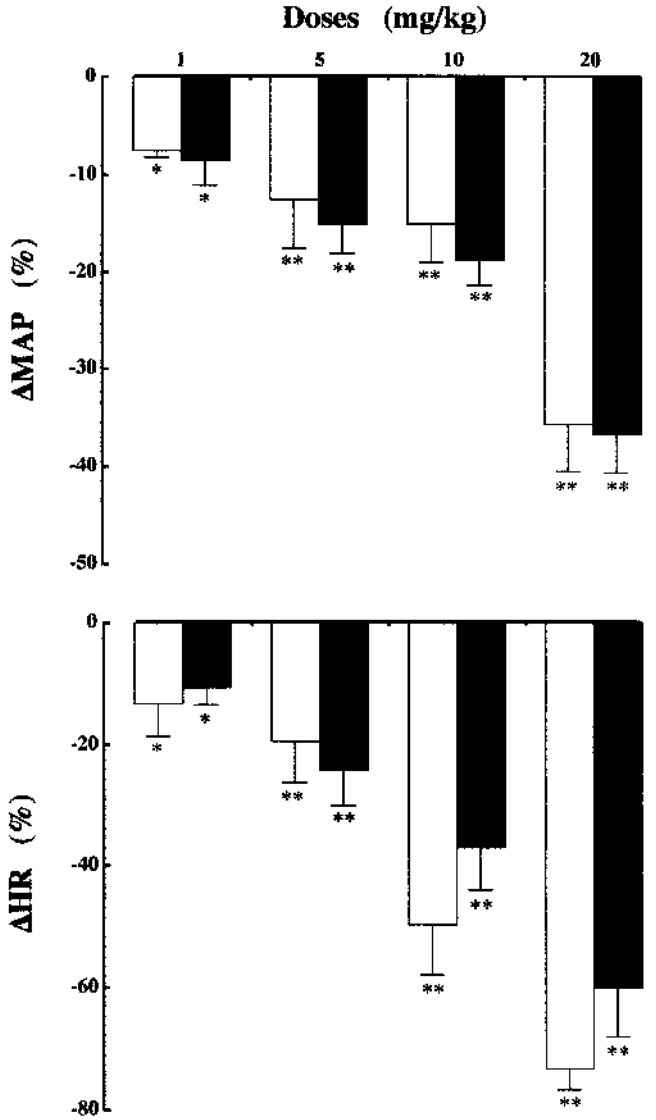

FIGURE 4. Maximal decreases in mean aortic pressure (ΔMAP max; A) and heart rate (ΔHR max; B) elicited by increasing bolus doses of intravenous (i.v.) eugenol (Eug; 1 to 10 mg/kg) in conscious, freely moving rats subjected to i.v. pretreatment with vehicle (1 mL/kg) (white columns) or L-NAME (20 mg/kg) (black columns). Values are means of changes expressed as a percentage of baseline and vertical bars indicate SEM (7 rats per group). In both groups studied, maximal decreases in MAP and HR were significantly related to the dose of Eug ($P < 0.001$, one-way ANOVA). Pretreatment with i.v. L-NAME affected neither the hypotension nor the bradycardia elicited by Eug. *$P < 0.05$, **$P < 0.01$ by Dunnett test.

In Vitro Experiments

Basal mesenteric flow (3.40 ± 0.19 mL/min) was reduced by perfusion of 60 mM potassium to a stable plateau of 1.78 ± 0.22 mL/min (Fig. 5). Bolus injections of Eug (0.1–2 mM) enhanced the mesenteric flow in a concentration-

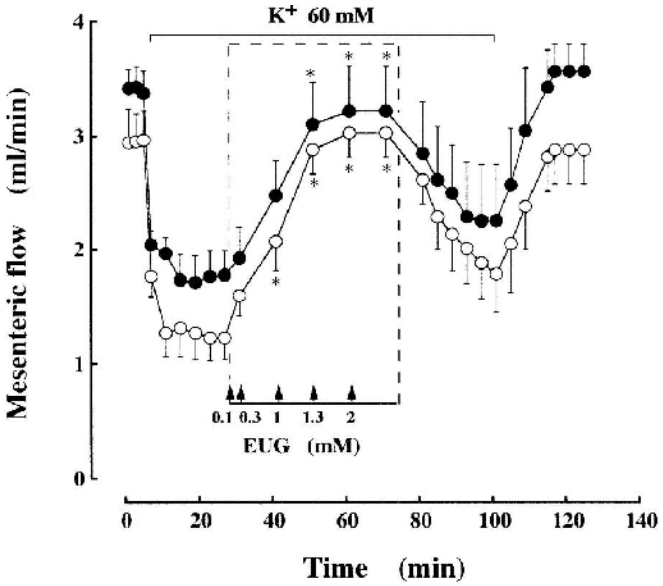

FIGURE 5. Vasodilator responses of increasing concentrations (0.01–2 mM) of eugenol (Eug) on potassium (60 mM)-precontracted rat mesenteric vascular beds in the absence (●) or in the presence (○) of atropine (1 μM). Vertical bars indicate SEM (6 rats per group). Eug elicited reversible and concentration-dependent vasodilator responses ($P < 0.001$, one-way ANOVA), which remained unaffected by atropine. *$P < 0.05$, by Dunnett test.

dependent and reversible manner ($P < 0.001$, Fig. 5); an effect indicative of vasodilation. Such an effect became significant at a concentration of 1 mM ($P < 0.05$, Fig. 5) and the IC_{50} values for the vasodilator effects of Eug were 0.31 ± 0.05 mM. In the presence of atropine, perfusion of potassium (60 mM) induced a stable reduction of mesenteric flow from 2.97 ± 0.25 to 1.24 ± 0.20 mL/min (Fig. 5). In these conditions, Eug also enhanced the mesenteric flow in a concentration-dependent and reversible manner ($P < 0.001$, Fig. 5); an effect that became significant at a concentration of 0.3 mM ($P < 0.05$, Fig. 5). However, the IC_{50} values for Eug-induced vasodilation in the presence of atropine (0.30 ± 0.04 mM) were similar ($P > 0.05$) to those obtained in preparations without atropine.

DISCUSSION

Baseline MAP and HR values of anesthetized or conscious freely moving, normotensive rats were of the same order of magnitude as those previously reported in the same preparation.[17,18,22,23] In both groups, i.v. treatment with Eug induces immediate and dose-dependent decreases in MAP and HR. This result is in accord with a previous report regarding the blood pressure effects of Eug in dogs[24] and rats.[25] Different kinds of anesthesia have been reported to alter cardiovascular responses to neurotransmitters, such as norepinephrine, in rats.[26] It is possible that norepinephrine may interfere with the cardiovascular responses to Eug. However, in the current study, magnitude of the depressor effect of i.v. Eug did not differ greatly between pentobarbital-anesthetized and conscious rats. This result suggests that the mechanism by which Eug decreases blood pressure is not altered by general anesthesia with sodium pentobarbital. It seems unlikely that Eug-induced hypotension and bradycardia could be related to a putative toxic effect of this compound. Such a conclusion is supported by the results of acute toxicity test showing that the Eug could be classified in the group of moderately toxic substance on the basis for classification of chemical substances. In fact, the LD_{50} value ranged from 1.9 to 3.0 g/kg body weight for rats.[27,28]

In the present study, an attempt was made to determine the role of the autonomic nervous system in Eug-induced cardiovascular effects in rats. Treatment of conscious rats with Eug was able to decrease MAP in rats even when the central sympathetic nerve drive, contributing to the maintenance of blood pressure, was eliminated by ganglionic blockade by hexamethonium. Under these experimental conditions, Eug-induced hypotension was of the same order of magnitude as that measured in vehicle-pretreated rats. This indicates that Eug hypotension is not dependent upon the presence of an operational central autonomic drive to the vascular system, because this effect occurs irrespective of whether vessels are constricted by the sympathetic neural drive. This conclusion is supported by the observation that although the basal level of sympathetic nervous system activity is lower in pentobarbital-anesthetized rats,[29] Eug-induced hypotension was of the same order of magnitude as that observed in conscious rats. These findings with i.v. hexamethonium are in line with those previously observed in normotensive rats treated with the essential oil of *O. gratissimum* (EOOG) (unpublished data), and suggest that the hypotensive response to Eug may be due to an active vascular relaxation rather than to a withdrawal of sympathetic tone. Such a hypothesis is corroborated by the present finding that Eug induces a concentration-dependent vasodilator effect on potassium (60 mM)-precontracted rat mesenteric vascular beds, as was recently reported.[8] It is unlikely that vascular muscarinic receptors are involved since neither i.v. pretreatment with methylatropine affected the Eug-induced hypotension nor pretreatment of endothelium-intact mesenteric bed preparations with atropine altered the vasodilator responses to Eug.

The effects of Eug on rat mesenteric vascular bed are not peculiar to that tissue alone since Eug has been reported to induce vasorelaxant effects on either rat[6] or rabbit[7] thoracic aorta preparations as well as to exert a concentration-dependent, reversible antispasmodic effect on rat ileum.[5] The antispasmodic activity of Eug would appear to support the popular therapeutic use of plants whose essential oil have a high Eug content, such as *O. gratissimum* and *C. zehntneri* var. *eugenoliferum* for the treatment of intestinal disorders.[30] The

present investigation did not attempt to assess the putative mechanisms underlying the Eug-induced relaxation in isolated mesenteric bed preparations. Previous in vitro studies using visceral[5] or vascular[6–8] smooth muscle have suggested that Eug act directly at the intracellular level to induce its pharmacological effects. For instance, the myorelaxant effect of Eug in rat isolated ileum was demonstrated to be independent upon an operational neural plexus activity since it was not blocked by the blocker of membrane sodium channels tetrodoxin or the neural ganglionic blocker hexamethonium, and occurred in the presence of 60 mM KCI[5], a situation in which action potentials are unlikely to occur due to inactivation of the fast sodium channel.[31] Furthermore, as was observed with the EOOG,[32] Eug showed no obvious selectivity between different contractile stimuli, such as high potassium (depolarizing stimulus) and the neurotransmitter ACh, since their responses were reversibly inhibited with similar IC_{50} values.[5] This may reflect an ability of Eug to depress ileal smooth muscle contraction at some stage distal to the receptor transduction process. Finally, it was reported that ACh-induced contraction in smooth muscle is mediated by a release of intracellular Ca^{2+} from the sarcoplasmic reticulum and by Ca^{2+} entry via voltage-dependent and -independent mechanisms.[33] Both the nifedipine-resistant component of ACh-induced contraction and the transient contraction of ACh in Ca^{2+}-free solution were shown to be significantly inhibited by Eug.[5] Such findings point to an important intracellular mechanism in the mediation of the antispasmodic effects of Eug although they did not exclude the possibility that Eug may also inhibit Ca^{2+} entry via voltage-dependent Ca^{2+} channels by direct or indirect action. In this respect, Eug was suggested to act, at least, as a Ca^{2+} channel antagonist either through voltage-dependent or through receptor-operated Ca^{2+} channels to induce its vasodilatory effects in the rat isolated aorta[6,7] but not in the small resistance vessels.[8] This is due probably to the fact that each vascular bed is characterized by specific function and structure of vascular cells.

It is well known that ACh causes generalized vasodilatation, which is an indirect effect mediated by released nitric oxide from vascular endothelial cells.[34] Given the fact that time to maximal hypotensive effect was similar for both Eug and ACh, it was postulated that hypotensive effect of Eug is mediated, at least in part, by an endothelial L-arginine/nitric oxide pathway. This hypothesis is not supported by 3 lines of evidence. First, time course of Eug-induced changes in MAP was significantly different from that obtained with ACh. In fact, only Eug-induced hypotension remained still significant during a period of 5 minutes post-injection. Second, i.v. pretreatment of conscious rats with the nitric oxide synthase inhibitor L-NAME was without significant effects on the Eug-induced hypotension. Finally, it was reported that pretreatment of endothelium-intact mesenteric bed preparations with L-NAME attenuated the vasodilator responses to ACh without affecting those elicited by Eug.[8]

Hypotensive effects of Eug are associated with a significant and dose-dependent bradycardia, which appears to be partly dependent upon the presence of an operational autonomic drive to the heart, as demonstrated by its attenuation in hexamethonium-pretreated rats. In rats subjected to either cervical bivagotomy or i.v. pretreatment with methylatropine, bradycardia elicited by Eug was significantly reduced, indicating that this effect is of vagal origin. Because attenuation of Eug-induced bradycardia by methylatropine was of the same order of magnitude as that induced by hexamethonium, it seems unlikely that sympathetic inhibition is also involved. Such findings with methylatropine and hexamethonium point to independent mechanisms for Eug-induced hypotension and bradycardia and preclude any possibility that Eug-induced hypotension may result from the concomitant bradycardia. In fact, if the hypotensive response to Eug resulted from the bradycardia, any change in HR would be expected to induce a quantitatively and qualitatively similar change in blood pressure.

The present study, using a combined in vivo and in vitro approach, shows that i.v. treatment of either anesthetized or conscious rats with Eug lowers blood pressure probably through an active vascular relaxation rather than withdrawal of sympathetic tone. This relaxation does not seem primarily mediated by an endothelial L-arginine/nitric oxide pathway. Such findings may suggest that Eug contributes to mediation of the hypotensive effects of essential oils of some aromatic plants popularly used for the treatment of hypertension. Further studies are presently underway in our laboratory to assess the cardiovascular effects of this compound in hypertensive rats.

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5.2 ANEXO 2

Cardiovascular effects of the essential oil of *Ocimum gratissimum* leaves in rats: role of the autonomic nervous system.

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CARDIOVASCULAR EFFECTS OF THE ESSENTIAL OIL OF *OCIMUM GRATISSIMUM* LEAVES IN RATS: ROLE OF THE AUTONOMIC NERVOUS SYSTEM

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SUMMARY

1. The cardiovascular effects of intravenous (i.v.) administration of the essential oil of *Ocimum gratissimum* (EOOG) were investigated in rats. In addition, the present study examined: (i) whether the autonomic nervous system is involved in the mediation of EOOG-induced changes in mean aortic pressure (MAP) and heart rate (HR); and (ii) whether these changes could be attributed, at least in part, to the actions of eugenol, the major constituent of EOOG.

2. In both pentobarbitone-anaesthetized and conscious rats, i.v. bolus injections of EOOG (1–20 mg/kg) elicited immediate and dose-dependent decreases in MAP and HR. These responses to EOOG were of the same order of magnitude irrespective of whether the animal was under general anaesthesia.

3. Pretreatment of anaesthetized rats with bilateral vagotomy did not significantly modify the EOOG-induced dose-dependent hypotension, whereas it significantly reduced the bradycardia at the highest dose used.

4. In conscious rats, i.v. injections of bolus doses (1–10 mg/kg) of eugenol also elicited immediate and dose-dependent decreases in MAP and HR. Intravenous pretreatment of conscious rats with either methylatropine (1 mg/kg) or hexamethonium (30 mg/kg) significantly reduced the EOOG-induced dose-dependent bradycardia without affecting the hypotension.

5. These data show, for the first time, that i.v. administration of EOOG to either anaesthetized or conscious rats induces an immediate and significant hypotension and bradycardia, which appear to be due, at least in part, to the actions of the major constituent of EOOG, eugenol. These cardiovascular effects appear to be mediated by different pathways because only EOOG-induced hypotension appears to be independent of the presence of an operational autonomic nervous system. This may suggest that the hypotensive activity of EOOG results from its vasodilatory effects directly upon vascular smooth muscle.

Key words: autonomic nervous system, blood pressure, essential oil, eugenol, heart rate, *Ocimum gratissimum* Labiatae, rat, vascular smooth muscle.

INTRODUCTION

Ocimum gratissimum L. (Labiatae) is an aromatic plant abundant in north-eastern Brazil, where it is commonly known as ‘alfavaca’. The plant is commonly used in cooking for flavouring. In folk medicine, infusions or decoctions of leaves from *O. gratissimum* are commonly used for the treatment of digestive problems and as a mouth antiseptic.[1] In West Africa, extracts of this plant are used as diaphoretics, stomachics, laxatives and also as a treatment for cough, fever and conjunctivitis.[2]

Leaves of *O. gratissimum* have an essential oil content of 0.2% of the plant dry weight, comprised principally of mono- and sesquiterpenes.[3] Previously, the essential oil of *O. gratissimum* (EOOG) and its main constituent, eugenol, were reported to have antibacterial activity.[4] Recently, EOOG and eugenol have been reported to be efficient in inhibiting the eclodibility of *Haemonchus contortus* eggs, suggesting a possible use in the treatment of gastrointestinal helmintosis of small ruminants.[3] Madeira *et al*. showed that EOOG exerts relaxant effects on intestinal smooth muscle, justifying the use of the plant in folk medicine for the treatment of gastrointestinal disorders.[5] Such a myorelaxant activity has also been demonstrated for eugenol in rat isolated ileum, suggesting that the myorelaxant activity of EOOG could be attributed, in part, to the actions of eugenol.[6] However, no reports published in the international literature have studied systematically the cardiovascular effects of EOOG in rats. Therefore, the aims of the present study were to assess the cardiovascular effects of intravenous (i.v.) EOOG in either anaesthetized or conscious rats and to determine whether eugenol could be the active principle mediating these cardiovascular effects. In addition, the role of the autonomic nervous system in mediating the EOOG-induced changes in mean aortic pressure (MAP) and heart rate (HR) was investigated.

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METHODS

Plant material

Aerial parts of *O. gratissimum* were collected between March and June 2002, at the Experimental Farm of the Federal University of Ceará, Fortaleza, Ceará, Brazil. The identification of the plants was confirmed by Dr FJ Abreu Matos (Laboratory of Natural Products, Federal University of Ceará). A voucher specimen (No. 23929) is deposited in the herbarium of Prisco Viana, Federal University of Ceará.

Extraction and chemical analysis

The EOOG was prepared from freshly chopped leaves by steam distillation and analysed chemically as described previously.[7] Briefly, analytical conditions were as follows: EOOG analysis was performed by gas chromatography and mass spectrometry (GC/MS; model 6971; Hewlett-Packard, USA). The column was a dimethylpolysiloxane DB-1 fused silica capillary column (20 m × 0.25 mm; 0.1 μm), the carrier gas was helium (1 mL/min), the injector temperature was 250°C, the detector temperature was 280°C and the column temperature was 50–180°C at 4°C/min then 180–250°C at 20°C/min. The mass spectra was an electronic impact at 70 eV. The composition of the EOOG and the retention indices of the various constituents are given in Table 1. These compounds were identified using a mass spectral library search and [^{13}C]-nuclear magnetic resonance spectroscopy.[8]

Solutions and drugs

Eugenol (Sigma Chemical, St Louis, MO, USA) and EOOG were dissolved in Tween (2%), brought to the chosen volume with sterile isotonic saline and sonicated just before use. Control preliminary experiments showed that this vehicle (100 μL) had no effects on either baseline MAP or HR over a period of 20 min. Sodium pentobarbitone (Sanofi, Libourne, France) and heparin (Laboratoires Léo SA, Montigny-le-Bretonneux, France) were used as commercially available injectable solutions. Methylatropine bromide, acetylcholine chloride and hexamethonium bromide were purchased from Sigma Chemical and dissolved in saline just before use. Both methylatropine bromide and hexamethonium bromide were administered in a volume of 1 mL/kg bodyweight, whereas acetylcholine chloride was given in a volume of 100 μL.

Catheterization procedure

Male Wistar rats (260–330 g) were kept under conditions of constant temperature (22 ± 2°C) with a standard light/dark cycle (12 h light/12 h dark) and free access to food and water. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html). Rats were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.) and catheters (PE-10 fused to PE-50) were implanted in the abdominal aorta (for the recording of arterial blood pressure) and in the inferior vena cava (for drug administration) through the left femoral artery and vein, respectively. These catheters, filled with heparin–saline solution (125 IU/mL), were exteriorized at the dorsal neck level. Postoperatively, rats were housed individually in plastic cages and allowed to recover for 48 h before any circulatory experiments.

Recording of MAP and HR

At the time of experiment, the arterial catheter was connected to a blood pressure transducer (Statham P23 ID; Gould Instruments, Oxnard, CA, USA) coupled to a polygraph recorder; HR was obtained from a cardiotachometer triggered by the pressure pulses. Both signals were recorded on a Gilson recorder (model 5/6H; Medical Electronics, Middletown, WI, USA). The MAP was calculated as diastolic + ((systolic – diastolic)/3).

Experimental protocol

Before each experiment, blood pressure and HR were allowed to stabilize and were recorded for a period of 10–15 min (depending on the duration of effects) after i.v. administration of EOOG or eugenol. When subsequent doses of EOOG or eugenol were administered, MAP and HR were first allowed to return to baseline levels, obtained prior to the first injection of the compound. Doses of agonists and antagonists were chosen according to the those recommended in the literature. Two series of experiments were performed as follows.

Series 1

This series of experiments was performed in anaesthetized rats to establish a dose–effect relationship. Rats were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.). Rectal temperature was kept close to 37°C by placing animals on a thermostatically controlled table. Each animal received a series of increasing bolus (100 μL) doses of EOOG (1, 5, 10 and 20 mg/kg) via the i.v. catheter and the time-course of changes in MAP and HR was recorded. These experiments were performed in both intact rats (n = 6) and in rats (n = 6) that had been subjected to a bilateral vagotomy performed at the cervical level 15 min earlier. In another group of rats (n = 6), the time-course of decreases in MAP elicited by i.v. injection of acetylcholine (5 μg/kg), used herein as a positive control,[9,10] was determined and compared with EOOG (20 mg/kg).

Series 2

This series of experiments was performed in conscious rats in order to establish a dose–effect relationship and to assess the role of the autonomic nervous system in the mediation of EOOG-induced cardiovascular changes. Therefore, the time-course of changes in MAP and HR elicited by i.v. injections of EOOG (1, 5, 10 and 20 mg/kg; n = 7) or eugenol (1, 5 and 10 mg/kg; n = 6) was determined in conscious rats. The time-course of changes in MAP and HR elicited by EOOG (1–20 mg/kg) was also determined in rats that had been pretreated 10 min earlier with one of the following: vehicle (1 mL/kg, i.v.; n = 7), hexamethonium (30 mg/kg, i.v.; n = 6) or methylatropine (1 mg/kg, i.v.; n = 7).[11] Each rat received increasing bolus doses as described above. It should be noted that the dose of hexamethonium chosen (30 mg/kg) was sufficient to achieve complete ganglionic blockade.[12]

Table 1 Chemical composition and retention indices of the constituents of the essential oil of *Ocimum gratissimum* (data bank of the Department of Physics and Chemistry of the Federal University of Ceará)

Compounds	Composition (% of total weight)	Retention indices
α-Pinene	0.95	936
β-Pinene	3.02	976
β-Myrcene	0.70	992
1,8-Cineole	32.70	1035
cis-Ocimene	6.20	1042
Linalool	0.50	1106
α-Terpineol	0.60	1201
Eugenol	43.70	1389
β-Elemene	0.50	1402
trans-Caryophyllene	4.10	1428
α-Humulene	0.50	1458
Germacrene-D	1.30	1485
β-Selinene	4.00	1491
α-Selinene	1.30	1489
Total identified	100	

Statistical analysis

All results are expressed as the mean±SEM. Maximal changes (expressed as a percentage of baseline values) in MAP and HR after each dose of EOOG or eugenol were used to construct a dose–response curve. The significance of results was assessed by means of unpaired or paired Student's *t*-tests, Mann–Whitney *U*-test and one- or two-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Studies in pentobarbitone-anaesthetized rats

In this series of experiments, baseline MAP and HR before injection of each dose of EOOG did not vary in magnitude ($P > 0.05$, one-way ANOVA). Therefore, mean values of baseline MAP and HR in this group of animals were 107 ± 2 mmHg and 395 ± 7 b.p.m., respectively (pooled data from 18 rats). Intravenous injections of EOOG (1–20 mg/kg) induced immediate and dose-dependent decreases in MAP and HR (Fig. 1; $P < 0.001$, one-way ANOVA). These effects became significant at a dose of 1 mg/kg EOOG (Fig. 1; $P < 0.05$, paired Student's *t*-test) and was maximal within the first 20–30 s after the administration of EOOG. After all doses had been tested, predose values of MAP were fully recovered within the first 1 min after the administration of EOOG, except that MAP remained significantly reduced 1 min after the administration of the highest dose of EOOG (20 mg/kg; Fig. 2; $P < 0.05$, paired Student's *t*-test). Predose values of HR were fully recovered within the first 1 min after the administration of 1 and 5 mg/kg, i.v., EOOG, whereas HR remained significantly reduced 1, 3 and 5 min after 10 mg/kg EOOG (-8.48 ± 3.03, -6.86 ± 2.53 and -10.52 ± 3.64%, respectivley; $P < 0.05$, paired Student's *t*-test) and 3, 5 and 10 min after 20 mg/kg EOOG (-5.32 ± 1.82, -6.22 ± 1.84 and -8.04 ± 2.02%, respectively; $P < 0.05$, paired Student's *t*-test).

Bilateral vagotomy did not affect baseline MAP (108 ± 6 *vs* 107 ± 5 mmHg in intact rats), but induced a significant increase in baseline HR (440 ± 11 *vs* 400 ± 7 b.p.m. in intact rats; $P < 0.05$, paired Student's *t*-test). Bilateral vagotomy did not affect the dose-hypotensive response curve to EOOG (Fig. 1a; $P > 0.05$, two-way ANOVA), but it did significantly reduce the EOOG-induced bradycardia at the highest dose used (Fig. 1b; $P < 0.05$, Mann–Whitney *U*-test).

The positive reference drug acetylcholine (5 μg/kg) also induced a significant decrease in MAP, the magnitude of which was maximal (-45.71 ± 1.38%) within the first 20 s after drug administration (Fig. 2; $P < 0.001$, paired Student's *t*-test). Pre-injection values of MAP were fully recovered within the first 1 min following the administration of acetylcholine, as was observed with EOOG. However, unlike EOOG, no residual decreases in MAP were observed 1 and 3 min after the administration of acetylcholine (Fig. 2). Two-way ANOVA revealed that the time-course of acetylcholine-induced changes in MAP was significantly different from that following 20 mg/kg EOOG ($P < 0.05$).

Studies in conscious rats

As in experiments with anaesthetized rats, baseline MAP and HR before the injection of each dose of EOOG in conscious, vehicle-pretreated rats remained essentially invariant ($P > 0.05$, one-way ANOVA). Mean values of MAP and HR in this group of animals before any treatment were 118 ± 2 mmHg and 375 ± 10 b.p.m., respectively (pooled data from 26 rats). Only baseline MAP was

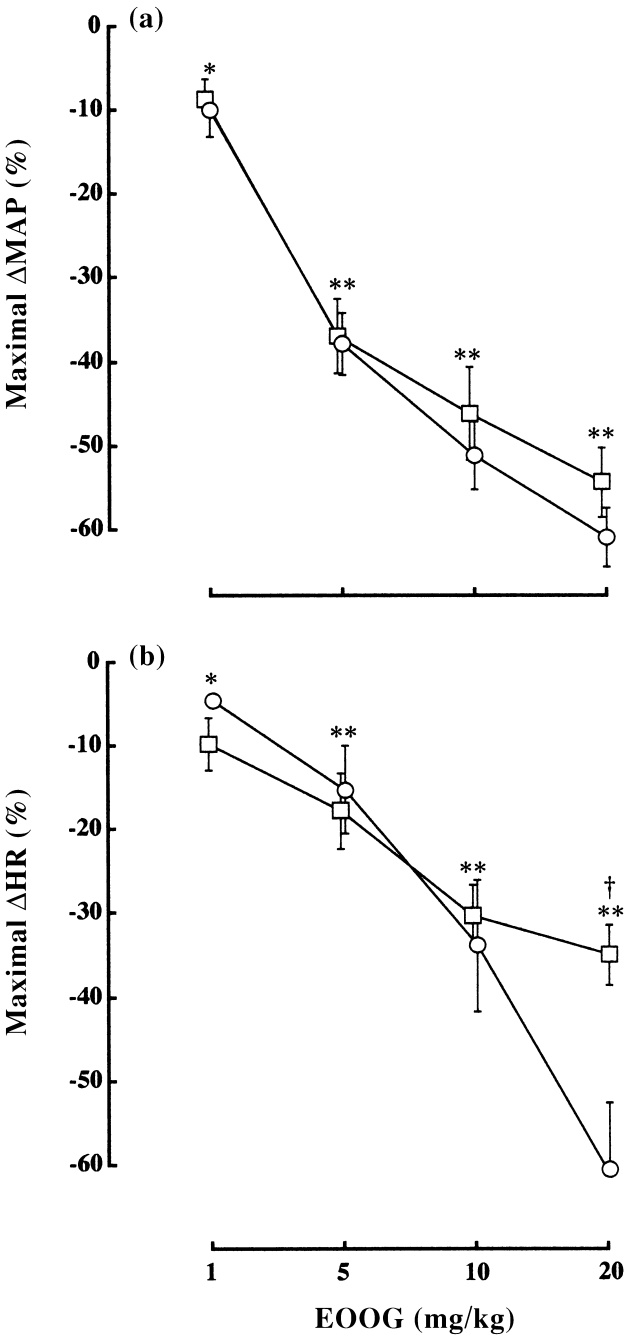

Fig. 1 Maximal decreases in (a) mean aortic pressure (MAP) and (b) heart rate (HR) elicited by increasing bolus doses (1–20 mg/kg, i.v.) of essential oil of *Ocimum gratissimum* (EOOG) in pentobarbitone-anaesthetized rats with (□) or without (○) bilateral vagotomy. Values are the mean±SEM of changes, expressed as a percentage of baseline ($n = 6$ rats per group). Baseline MAP and HR were 107 ± 5 mmHg and 390 ± 10 b.p.m. in intact rats, respectively, and 107 ± 6 mmHg and 440 ± 11 b.p.m. in bivagotomized rats, respectively. In both groups studied, maximal decreases in MAP and HR were significantly related to the dose of EOOG ($P < 0.001$, one-way ANOVA). Bilateral vagotomy had no significant effect on EOOG-induced dose-dependent hypotension, whereas bradycardia was reduced at the highest dose tested (10 mg/kg; $^{\dagger}P < 0.05$, Mann–Whitney *U*-test). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with pre-injection values (paired Student's *t*-test).

significantly different from that measured in intact, pentobarbitone-anaesthetized rats ($P < 0.01$, unpaired Student's *t*-test). Intravenous administration of EOOG (1–20 mg/kg) produced immediate and dose-dependent decreases in MAP and HR (Fig. 3; $P < 0.001$, one-way ANOVA), which became significant at a dose of 1 mg/kg EOOG (Fig. 3; $P < 0.05$, paired Student's *t*-test) and peaked during the first 20–30 s after administration. For all doses studied, predose values of MAP were fully recovered within the first 1 min following the administration of EOOG. However, HR remained significantly reduced 1, 3 and 5 min after 5 mg/kg EOOG (–11.70 ± 2.72, –10.90 ± 3.59 and –9.67 ± 3.80%, respectively; $P < 0.05$, paired Student's *t*-test), 1, 3 and 5 min after 10 mg/kg EOOG (–11.08 ± 3.38, –12.14 ± 3.23 and –12.75 ± 3.63%, respectively; $P < 0.05$, paired Student's *t*-test) and 1, 3, 5 and 10 min after 20 mg/kg EOOG (–9.28 ± 2.42, –10.52 ± 1.98, –17.22 ± 3.44 and –13.72 ± 1.62%, respectively; $P < 0.05$, paired Student's *t*-test). Maximal percentage decreases in MAP and HR elicited by EOOG in conscious rats did not differ significantly from those measured in pentobarbitone-anaesthetized rats ($P > 0.05$, two-way ANOVA).

Intravenous injections of eugenol (1–10 mg/kg) also induced immediate and dose-dependent decreases in MAP and HR (Fig. 4; $P < 0.001$, one-way ANOVA), the magnitude of which were maximal within the first 20–30 s after the administration of eugenol. These effects became significant at a dose of 1 mg/kg eugenol ($P < 0.05$, paired Student's *t*-test). For all doses studied, predose values of MAP were fully recovered within the first 1 min after the administration of eugenol, as was observed for EOOG. However, HR remained significantly reduced 1, 3 and 5 min after 5 mg/kg eugenol (–7.42 ± 1.81, –10.26 ± 2.97 and –9.58 ± 1.73%, respectively; $P < 0.05$, paired Student's *t*-test) and 1, 3, 5 and 10 min

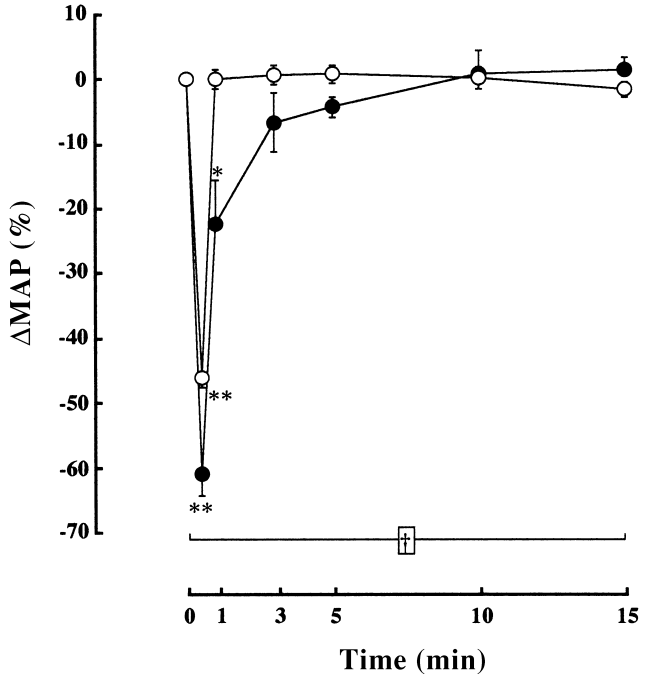

Fig. 2 Time-course of changes in mean aortic pressure (MAP) elicited by an intravenous injection of the positive reference drug acetylcholine (5 μg/kg; ○) and essential oil of *Ocimum gratissimum* (EOOG; 20 mg/kg; ●) in pentobarbitone-anaesthetized rats. Values are the mean±SEM of changes, expressed as a percentage of baseline (n = 6–7 rats per group). Baseline MAP in EOOG- and acetylcholine-treated rats was 107 ± 5 and 106 ± 2 mmHg, respectively. The time-course of EOOG-induced changes in MAP was significantly different from that obtained with acetylcholine ($^{\dagger}P < 0.05$, two-way ANOVA). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with pre-injection values (paired Student's *t*-test).

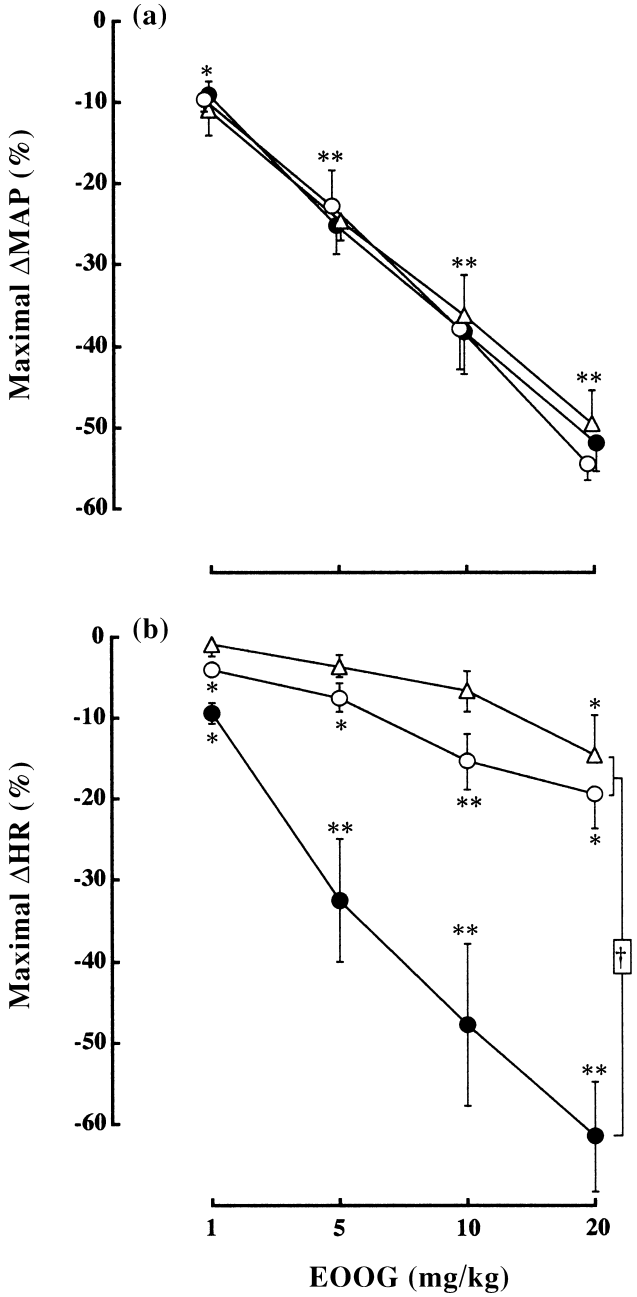

Fig. 3 Maximal decreases in (a) mean aortic pressure (MAP) and (b) heart rate (HR) elicited by increasing bolus doses (1–20 mg/kg, i.v.) of essential oil of *Ocimum gratissimum* (EOOG) in conscious rats subjected to i.v. pre-treatment with vehicle (Tween (2%) and isotonic saline; 1 mL/kg; ●), hexamethonium (30 mg/kg; ○) or methylatropine (1 mg/kg; △). Values are the mean±SEM of changes, expressed as a percentage of baseline (n = 6–7 rats per group). Baseline MAP and HR in vehicle-, hexamethonium- and methylatropine-pretreated rats were 118 ± 4 mmHg and 361 ± 20 b.p.m., 87 ± 5 mmHg and 366 ± 19 b.p.m. and 119 ± 4 mmHg and 464 ± 17 b.p.m., respectively. Pretreatment with i.v. hexamethonium or methylatropine significantly reduced the EOOG-induced bradycardia ($^{\dagger}P < 0.01$, two-way ANOVA) without affecting the dose-dependent hypotension. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with pre-injection values (paired Student's *t*-test).

after 10 mg/kg eugenol (–20.05 ± 5.14, –26.75 ± 5.36, –23.33 ± 6.41 and –32.62 ± 8.11%, respectively; $P < 0.05$, paired Student's *t*-test). Two-way ANOVA revealed that maximal percentage decreases in MAP elicited by eugenol were not significantly different from those evoked by EOOG (Fig. 4a). At the highest dose (10 mg/kg), the bradycardia elicited by eugenol was significantly greater than that evoked by the same dose of EOOG ($P < 0.05$, Mann–Whitney *U*-test; Fig. 4b).

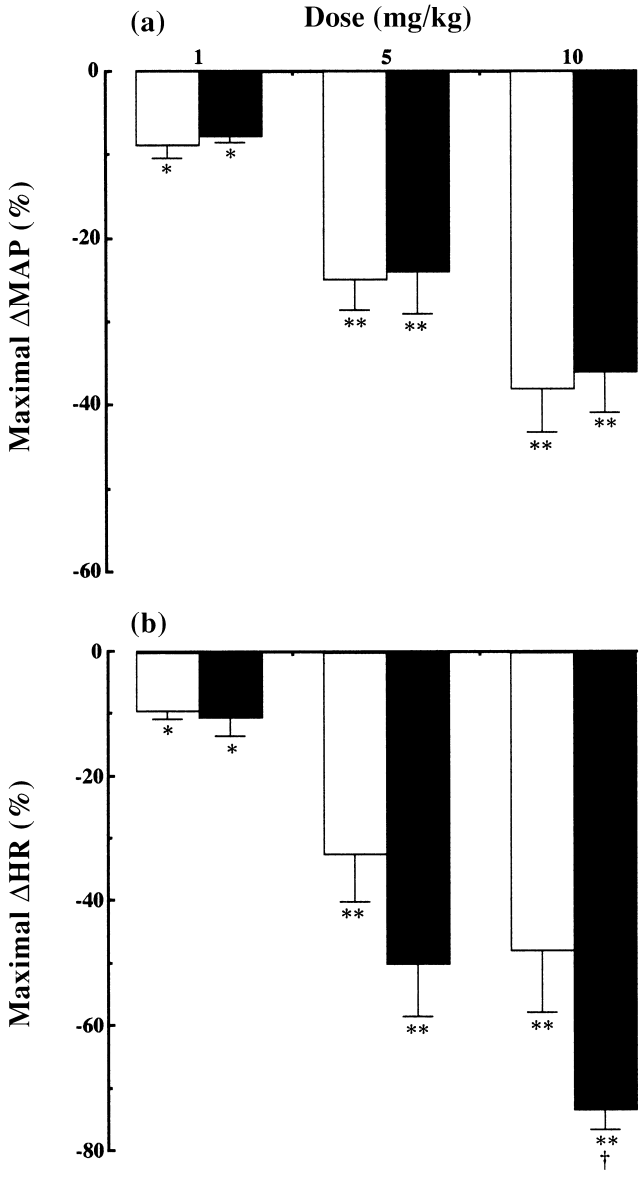

Fig. 4 Maximal decreases in (a) mean aortic pressure (MAP) and (b) heart rate (HR) elicited by increasing i.v. bolus doses (1–10 mg/kg) of essential oil of *Ocimum gratissimum* (EOOG; □) and its major constituent eugenol (■) in conscious rats subjected to i.v. pretreatment with vehicle (Tween (2%) and isotonic saline; 1 mL/kg). Values are the mean±SEM of changes, expressed as a percentage of baseline (n = 6–7 rats per group). Baseline MAP and HR in EOOG- and eugenol-treated rats were 118 ± 4 mmHg and 361 ±20 b.p.m. and 115 ±2 mmHg and 362 ±13 b.p.m., respectively. In both groups studied, maximal dose-dependent decreases in MAP and HR were of the same order of magnitude, except that the bradycardic response to eugenol at the highest dose (10 mg/kg) was significantly enhanced compared with EOOG ($^{\dagger}P < 0.05$, Mann–Whitney *U*-test). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with pre-injection values (paired Student's *t*-test).

Pretreatment with hexamethonium induced significant ($P < 0.001$, paired Student's *t*-test) decreases in baseline MAP (87 ± 5 *vs* 122 ± 6 mmHg) without any significant effect on baseline HR (366 ± 19 *vs* 400 ± 10 b.p.m.). However, pretreatment with methylatropine significantly ($P < 0.001$, paired Student's *t*-test) increased baseline HR (464 ± 17 *vs* 388 ± 15 b.p.m.) without affecting baseline MAP (119 ± 4 *vs* 115 ± 3 mmHg). Pretreatment with either methylatropine or hexamethonium did not significantly modify the dose-dependent decreases in MAP elicited by EOOG (Fig. 3a; $P > 0.05$, two-way ANOVA); however, both pretreatments significantly reduced the magnitude of maximal EOOG-induced bradycardic effects (Fig. 3b; $P < 0.01$, two-way ANOVA). Two-way ANOVA revealed that EOOG-induced decreases in HR after methylatropine pretreatment were not statistically different from those recorded in animals pretreated with hexamethonium. In a separate group of animals (n = 5), hypotensive responses to eugenol (1, 5 and 10 mg/kg, i.v.) also remained unaffected by i.v. hexamethonium (–9.25 ± 2.04, –19.00 ± 3.57 and –32.54 ± 2.20%, respectively, *vs* –7.80 ± 0.71, –24.08 ± 5.00 and –35.66 ± 4.88%, respectively, in vehicle-pretreated rats). However, as for EOOG, such pretreatment significantly ($P < 0.01$, two-way ANOVA) reduced the bradycardic responses to eugenol (–1.06 ± 2.21, –8.00 ± 1.27 and –27.10 ± 6.24%, respectively, *vs* –10.30 ± 3.10, –49.80 ± 8.40 and –73.15 ± 3.38%, respectively, in vehicle-pretreated rats). Baseline MAP and HR values in hexamethonium-pretreated rats just before eugenol injection were 76 ± 5 mmHg and 395 ± 20 b.p.m., respectively.

DISCUSSION

Baseline MAP and HR values of conscious or anaesthetized normotensive rats were of the same order of magnitude as those reported previously in the same preparation.[10] In both groups, i.v. administration with EOOG induced immediate and dose-dependent decreases in MAP and HR. To be the best of our knowledge, this is the first time that such cardiovascular effects of EOOG have been reported in rats. Different kinds of anaesthesia have been reported to alter cardiovascular responses to neurotransmitters, such as noradrenaline, in rats.[13] It is possible that noradrenaline may interfere with the cardiovascular responses to EOOG. However, in the present study, both the magnitude and time-course of the depressor effects of i.v. EOOG did not differ greatly between pentobarbitone-anaesthetized and conscious rats. This suggests that the mechanism by which EOOG decreases blood pressure is not altered by general anaesthesia with sodium pentobarbitone.

The cardiovascular effects of EOOG can be partly attributed to the actions of eugenol, the major constituent of EOOG. Two lines of evidence support such a conclusion. First, hypotensive and bradycardic responses to i.v. eugenol became significant at a dose of 1 mg/kg and peaked within the first 20–30 s after eugenol administration, as was observed for EOOG. Second, pretreatment with i.v. hexamethonium partially, but significantly, decreased the bradycardic effects of eugenol without affecting the hypotension, as was observed for EOOG. Such a partial contribution of eugenol to the antispasmodic[5] and anthelmintic[3] effects of EOOG has been proposed recently. Recent studies from our laboratory have shown that i.v. administration of 1,8-cineole, a terpenoid oxide present in

many plant essential oils that is the second major constituent (32.70%) of EOOG, also dose-dependently decreased MAP in both conscious and anaesthetized rats, with a time-course similar to that of EOOG.[10] Taken together, these data suggest that the cardiovascular effects of EOOG in rats can be attributed in part to the actions of eugenol and in part to the actions of 1,8-cineole, the two main constituents of EOOG. It seems unlikely that EOOG-induced cardiovascular changes could be related to a putative toxic effect of this essential oil. In fact, the oral acute toxicity LD_{50} values for eugeol[14] and 1,8-cineole,[15] the major constituents of EOOG, have been found to be greater than 2000 mg/kg.[14,15]

In the present study, an attempt was made to determine the role of the autonomic nervous system in EOOG-induced cardiovascular effects in rats. The administration of EOOG was able to decrease MAP in rats, even when the central sympathetic nerve drive, contributing to the maintenance of blood pressure, was eliminated by ganglionic blockade with hexamethonium. Under these experimental conditions, EOOG-induced hypotension was of the same order of magnitude as that measured in vehicle-pretreated rats. This indicates that EOOG hypotension is not dependent upon the presence of an operational central autonomic drive to the vascular system, because this effect occurs irrespective of whether vessels are constricted by the sympathetic neural drive. This conclusion is supported by the observation that although the basal level of sympathetic nervous system activity is lower in pentobarbitone-anaesthetized rats,[16] EOOG-induced hypotension was of the same order of magnitude as that observed in conscious rats. As was previously proposed for 1,8-cineole-induced hypotension,[10] these findings with i.v. hexamethonium suggest that the hypotensive response to EOOG may be due to an active vascular relaxation rather than to a withdrawal of sympathetic tone. Vascular muscarinic receptors that normally mediate hypotension are probably not involved, because pretreatment with methylatropine did not affect EOOG-induced hypotension.

Previous *in vitro* studies[5,6,10,17–20] have suggested that EOOG and its main constituents act directly on the smooth muscle rather than indirectly on neurotransmitter release to induce their pharmacological effects. For example, EOOG was able to completely reverse acetylcholine-induced contractions with an IC_{50} value different to that obtained in the presence of high potassium (60 mmol/L).[5] This may reflect the ability of EOOG to depress ileum smooth muscle contraction at some stage distal to the receptor transduction process. Furthermore, 1,8-cineole has been reported to induce concentration-dependent relaxation of potassium (60 mmol/L)-induced contractions in both rat isolated aorta[10] and guinea-pig isolated ileum.[17] Finally, eugenol has been reported to act directly at the intracellular level to induce its myorelaxant and antispasmodic effects[6] and its vasorelaxant effects on rat[18] and rabbit[19] thoracic aorta, as well as on rat mesenteric vascular bed.[20] Further studies are presently underway in our laboratory to assess the mechanism(s) underlying the vasorelaxant effects of EOOG.

Acetylcholine causes generalized vasodilatation, which is an indirect effect mediated by nitric oxide released from vascular endothelial cells.[21] In the present study, the time-course of EOOG-induced changes in MAP was significantly different from that obtained with acetylcholine. If fact, only EOOG-induced hypotension remained significant during a period of 1–3 min post-injection. However, in view of the observation that time to maximal hypotensive effect was similar for both EOOG and acetylcholine, it is possible that the hypotensive effect of EOOG is mediated, at least in part, by an endothelial L-arginine/nitric oxide pathway. Further studies using analogues of L-arginine (i.e. N^G-monomethyl-L-arginine or N^G-nitro-L-arginine methyl ester) that inhibit nitric oxide formation are presently underway in our laboratory to test the latter possibility.

The hypotensive effects of EOOG are associated with a significant and dose-dependent bradycardia, which appears to be partly dependent upon the presence of an operational autonomic drive to the heart, as demonstrated by its attenuation in hexamethonium-pretreated rats. In rats subjected to either cervical bivagotomy or i.v. pretreatment with methylatropine, the bradycardia elicited by EOOG (10 mg/kg) was reduced, indicating that this effect is of vagal origin. Such a vagal origin has been also attributed to the bradycardic responses to i.v. 1,8-cineole in normotensive rats.[10] Because attenuation of EOOG-induced bradycardia by methylatropine was of the same order of magnitude as that induced by hexamethonium, it seems unlikely that sympathetic inhibition is also involved. Such findings with methylatropine and hexamethonium point to independent mechanisms for EOOG-induced hypotension and bradycardia and preclude any possibility that EOOG-induced hypotension may result from the concomitant bradycardia. In fact, if the hypotensive response to EOOG resulted from the bradycardia, any change in HR would be expected to induce a quantitatively and qualitatively similar change in blood pressure.

In conclusion, this is the first physiological evidence that i.v. administration of EOOG to either conscious or pentobarbitone-anaesthetized rats results in hypotensive and bradycardic effects, which appear to be attributable, at least in part, to the actions of eugenol, the major constituent of EOOG. These cardiovascular responses occur through separate mechanisms because only EOOG-induced hypotension is independent of the presence of an operational central autonomic drive to the cardiovascular system. These findings may add a putative antihypertensive activity to the list of therapeutic uses for *O. gratissimum* in folk medicine and suggest that the hypotensive activity of EOOG may result from its direct vasodilatory effects on vascular smooth muscle. Further experiments using hypertensive rats are presently underway in our laboratory to test this hypothesis.

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