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UNIVERSIDADE ESTADUAL DE CAMPINAS

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"ALTERAÇÕES MORFOLÓGICAS E ATIVIDADES E EXPRESSÃO DE CITOCROMOS P450 NO FÍGADO DE RATOS TRATADOS COM L-NAME"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Christiane Ap. Badin Tarsitano

e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural na área de Histologia.

Orientador: **Prof. Dr. Stephen Hyslop**

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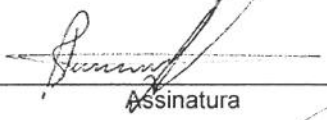

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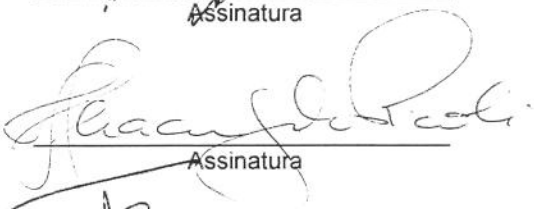

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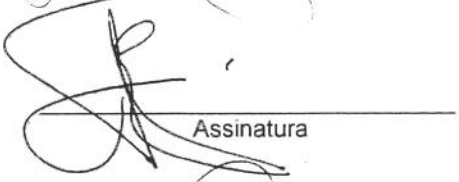

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Dedicatória

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Obrigado! Chris

CYP450 -	citocromo P450
CYP1A1/2 -	citocromo P450 subfamília 1A
CYP2B1/2 -	citocromo P450 subfamília 2B
CYP2C11 -	citocromo P450 subfamília 2C
CYP2E1 -	citocromo P450 subfamília 2E
ECA -	enzima conversora de angiotensina
EROD -	Etoxiresorufina – O - Deetilase
FAD -	flavina-adenina-dinucleotídeo
FMN –	flavina-mononucleotídeo
HE -	hematoxilina e eosina
β-NADPH -	nicotinamida adenina dinucleotídeo fosfato
eNOS –	óxido nítrico sintase endotelial (ou NOS III)
iNOS –	óxido nítrico sintase induzível (ou NOS II)
nNOS -	óxido nítrico sintase neuronal (ou NOS I)
L-NAME -	N^{ω}-nitro-L-arginina metil éster
NO -	óxido nítrico
NOS -	óxido nítrico sintase
O_2 -	oxigênio molecular
$O_2^{\cdot-}$	radical anion superóxido
P450 -	citocromo P450
PAGE -	eletroforese de gel de poliacrilamida
PAS -	reativo de ácido periódico de Schiff-PAS
PROD -	pentoxiresorufina – O - Deetilase
PSA -	persulfato de amônio
RE -	retículo endoplasmático
REL -	retículo endoplasmático liso
RER -	retículo endoplasmático rugoso
SDS -	dodecilsulfato de sódio
SRA -	sistema renina angiotensina

O óxido nítrico (NO), um co-produto do metabolismo do aminoácido L-arginina para L-citrulina pela NO sintase (NOS), exerce importantes funções no controle do tôno vascular, no processo inflamatório, e na modulação da expressão gênica e atividade enzimática de diversas enzimas, tais como citocromos P-450 e NOS. A inibição crônica da biossíntese de NO por N^{ω}-nitro-L-arginine metil éster (L-NAME) leva à hipertensão e produz alterações morfológicas que incluem hipertrofia e remodelamento vascular, efeitos estes que são revertidos por inibidores da enzima conversora de angiotensina I (ECA) e antagonistas dos receptores AT_1 de angiotensina II. O NO exerce papel fundamental na hemodinâmica e função celular do fígado, e pode regular a atividade dos citocromos P450 monooxigenases que são importantes na biotransformação de substâncias endógenas e exógenas. Neste trabalho, examinamos a influência do tratamento com L-NAME sobre a morfologia hepática, o conteúdo de glicogênio, colesterol e triglicérides, e a atividade e expressão das isoformas CYP1A1/2, CYP 2B1/2, CYP2C11 e CYP2E1 de citocromo P450. No tratamento agudo estudamos também a expressão de duas metaloproteinases de matriz (MMP-2 e MPP-9). Ratos Wistar machos foram tratados com L-NAME (20 mg/rato/dia, administrado na água de beber) por quarto e oito dias (tratamento agudo) e duas, quatro e oito semanas (tratamento crônico) e os fígados foram removidos para análise. A indução enzimática foi realizada tratando-se os ratos com fenobarbital (para induzir CYP2B1/2), β-naphthoflavone (para induzir CYP1A1/2) (80 mg/kg/dia, i.p., 4 d) ou pirazole (200 mg/kg/dia, i.p., 2 d) (para induzir CYP2E1). O tratamento com L-NAME elevou significativamente a pressão sangüínea e esta foi revertida pelo tratamento concomitante com enalapril (25 mg/kg/dia, p.o., inibidor da ECA) ou losartan (30 mg/kg/dia, p.o., antagonista dos receptores AT_1). L-NAME causou hipertrofia vascular em artérias hepáticas, com depósito de colágeno perivascular e fibrose intersticial. Houve também um aumento significativo no conteúdo de glicogênio hepático. Todas estas alterações foram completamente revertidas por enalapril ou losartan. O tratamento com L-NAME não alterou o conteúdo de colesterol e triglicérides hepáticos, e não afetou as atividades basais e induzidas

ou a expressão das isoformas de citocromo P450. O tratamento agudo com L-NAME aumentou a expressão de MMP-2 e MMP-9 na parede vascular e em células inflamatórias (leucócitos, macrófagos). Esses resultados mostram que o tratamento agudo e crônico com L-NAME produz alterações morfológicas na vasculatura hepática e aumenta o conteúdo hepático de glicogênio, sem porém alterar o metabolismo de lípides ou a atividade e expressão de citocromos P450. A ausência de um efeito sobre os citocromos P450 indica que estas enzimas, não são influenciadas por níveis baixos de NO. Já a capacidade do enalapril e losartan em proteger contra as alterações morfológicas e o aumento de glicogênio indica um papel importante para o sistema renina-angiotensina nestas respostas.

Nitric oxide (NO), a co-product of the metabolism of L-arginine to L-citrulline by NO synthases (NOS), has an important role in regulating vascular tone, inflammatory responses, and the gene expression and enzymatic activity of various proteins, including cytochrome P450 and NOS. The chronic inhibition of NOS by N^{ω}-nitro-L-arginine methyl ester (L-NAME) produces sustained arterial hypertension and morphological alterations that include arterial wall hypertrophy and vascular remodeling. These changes can be prevented by concomitant treatment with angiotensin-converting enzyme (ACE) inhibitors and angiotensin II AT_1 receptor antagonists. NO is an important modulator in the hepatic vasculature and of hepatic cell function, and may regulate the activity of cytochrome P450 monooxygenases involved in the biotransformation of endogenous and exogeous substances. In this work, we examined the influence of L-NAME on rat liver morphology, hepatic glycogen, cholesterol and triglyceride content, and the activities and expression of the cytochrome P450 isoforms CYP1A1/2, CYP2B1/2, CYP2C11 and CYP2E1. We also examined the expression of two matrix metalloproteinases (MMP-2 and MPP-9) after short-term treament with L-NAME. Male Wistar rats were treated with L-NAME (20 mg/rat/day, administered in the drinking water) for four and eight days (short-term treatment) and two, four and eight weeks (chronic treatment) and the livers were then removed for analysis. Enzymatic induction was produced by treating rats with phenobarbital (to induce CYP2B1/2), β-naphthoflavone (to induce CYP1A1/2) (80 mg/kg/day each, i.p., 4 d) or pyrazole (200 mg/kg/day, i.p., 2 d) (to induce CYP2E1). Treatment with L-NAME significantly elevated the blood pressure and this was reversed by concomitant treatment with enalapril (25 mg/kg/day, p.o., ACE inhibitor) or losartan (30 mg/kg/day, p.o., angiotensin II AT_1 receptor antagonist). L-NAME caused vascular hypertrophy in hepatic arteries, with perivascular and interstitial fibrosis involving collagen deposition. There was also a significant increase in the hepatic glycogen content. All of these changes were completely reversed by concomitant treatment with enalapril or losartan. L-NAME had no effect on the hepatic cholesterol and triglyceride content, nor did it affect the basal or drug-induced

activities and protein expression of the cytochrome P450 isoforms. Short-term treatment with L-NAME enhanced the expression of MMP-2 and MMP-9 in vessel walls and inflammatory cells (mast cells, macrophages). These results show that acute or chronic treatment with L-NAME produces morphological alterations in the hepatic vasculature and increases the hepatic glycogen content without affecting lipid metabolism or the activity or expression of cytochrome P450 isoforms. The lack of effect on cytochrome P450 activities and expression indicates that these enzymes are not significantly influenced by low levels of NO. In contrast, the ability of enalapril and losartan to prevent the morphological alterations and the increase in hepatic glycogen indicates an important role for the renin-antiogensin system in these responses.

1. INTRODUÇÃO

1.1. FÍGADO

1.1.1. Anatomia hepática

O fígado é a maior glândula do organismo humano e encontra-se na cavidade abdominal, abaixo do diafragma. Situado nos cruzamentos entre o trato digestivo e o resto do corpo, mantém a homeostase metabólica corporal, esse órgão é essencial para a vida, funcionando tanto como glândula exócrina quanto endócrina. Sua capacidade de regeneração é extraordinária, como é demonstrado após hepatectomia subtotal, no qual ocorre total regeneração do volume do fígado (JUNQUEIRA, 1999).

Anatomicamente, o fígado possui quatro lobos: o direito (o maior), o esquerdo, o quadrado, o caudado. O órgão é totalmente recoberto pelo peritônio e unindo os lobos esquerdo e direito, há o ligamento falciforme; e fazendo a junção entre o fígado e o músculo diafragma, há dois ligamentos: o triangular e o coronário. É irrigado principalmente pelas artérias hepáticas, e é drenado pela veia cava inferior e veia porta, sendo esta última a principal via de comunicação do fígado com o corpo. Abaixo do lobo direito situa-se a vesícula biliar, uma bolsa de 9 cm, aproximadamente, que tem a capacidade de coletar cerca de 50 ml de bile produzida pelo fígado (figura 1).

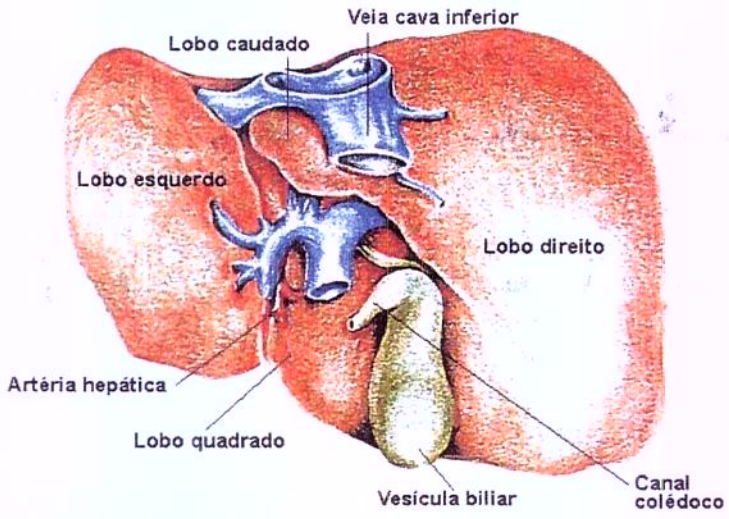


Figura 1: Lobos hepáticos, fígado posterior (site: http://wikipedia.org.com.br)

Os hepatócitos constituem a maioria das células parenquimatosas do fígado, que tem a função de secretar a bile, além de ser importante para o armazenamento de glicose sob a forma de glicogênio, sintetizado a partir dos carboidratos absorvidos no intestino delgado e transportados através da veia porta. Outras atividades dos hepatócitos incluem a síntese proteíca, acúmulo de metabólitos, e a remoção e degradação (metabolização) de substâncias endógenas e/ou tóxicas do organismo (ROBBINS, 2000).

1.1.2. Organização histológica do fígado

Os hepatócitos constituem 80% da população celular do fígado humano; as quais se agrupam em lâminas hepatocelulares, organizadas em fileiras formando placas que se anastomosam entre si formando unidades morfológicas chamadas lóbulos hepáticos. Nestes os hepatócitos se dispõem em placas orientadas radialmente, os lóbulos encostam uns aos outros em quase toda sua extensão. No entanto, em algumas regiões, os lóbulos ficam separados por tecido conjuntivo e vasos. Estas regiões recebem o nome de espaços-porta; este é composto por uma vênula e uma arteríola (ramos da veia porta e da artéria hepática, respectivamente), um ducto biliar, vasos linfáticos e nervos. Este conjunto é cercado por uma capa de tecido conjuntivo, contínua com a cápsula de Glisson.

O espaço-porta também pode receber o nome de tríade porta, pois, suas estruturas predominantes são a vênula, a arteríola e o ducto biliar. Da tríade, o sangue atravessa a placa limitante através de canais controlados por esfíncter. Esses canais descarregam o sangue numa rede de capilares chamada de sinusóides (ROBBINS, 2000) (figura 2).

Os sinusóides são capilares que ocupam o espaço entre as placas de hepatócitos. Suas paredes são revestidas de células endoteliais típicas e macrófagos que, no fígado, recebem o nome de células de Kupffer, estas têm função fagocitária. O estreito espaço que separa o sinusóide dos hepatócitos

recebe o nome de espaço de Disse o qual é composto por fibras reticulares. Um terceiro tipo de célula na parede do sinusóide é a chamada célula de Ito ou "células esteladas" (células perisinusoidais ou células ricas em vitamina A) Os capilares sinusóides desembocam em uma veia localizada no centro do lóbulo chamada veia centrolobular a qual é o ramo inicial da veia hepática (JUNQUEIRA, 1999).

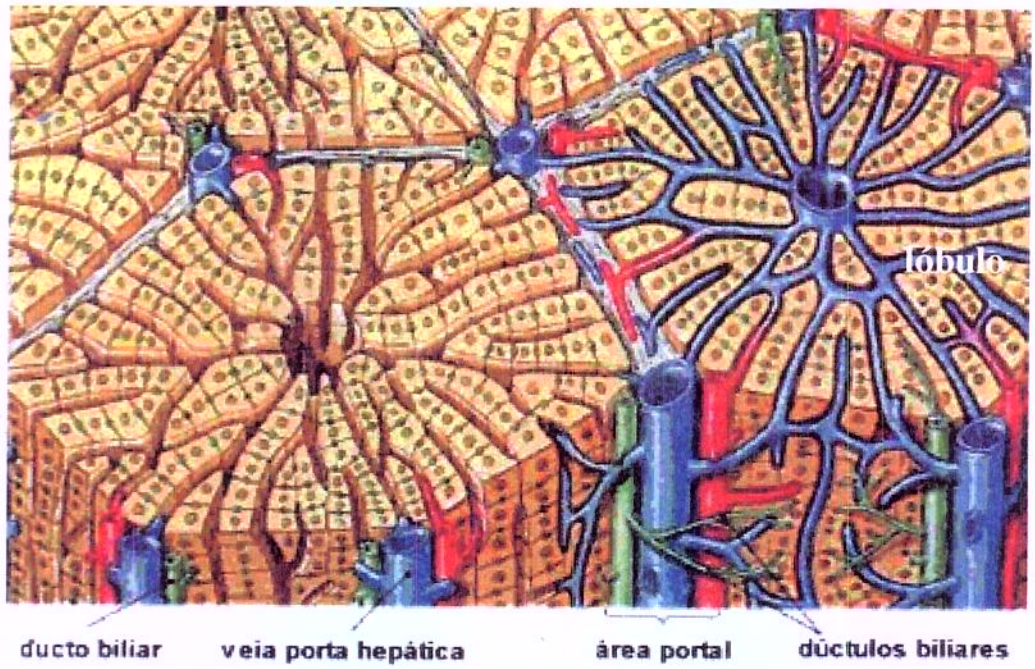
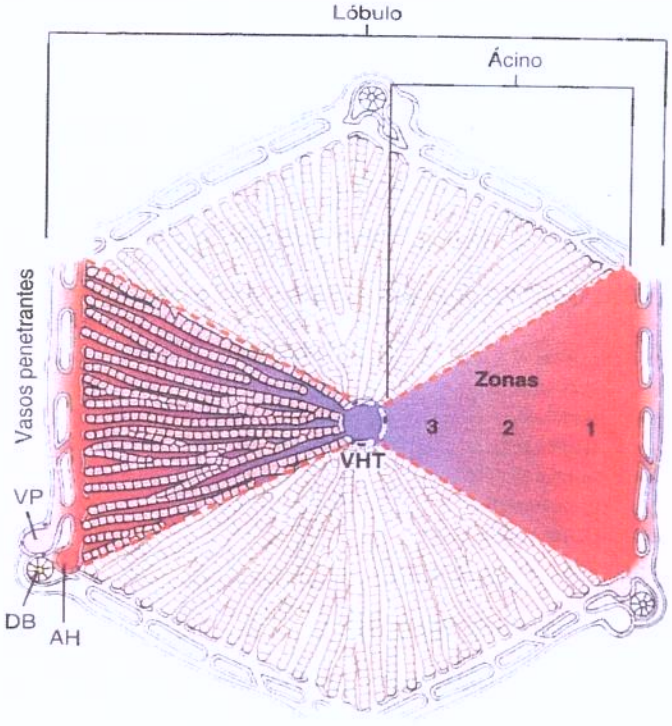


Figura 2: Esquema da organização hepática (retirado do site www.hepcentro.com.br).

O suprimento sangüíneo hepático ocorre através da artéria hepática (sangue arterial) e da veia porta (sangue venoso) (SHERLOCK, 1989, JUNQUEIRA, 1999), este percorre os sinusóides da periferia para o centro dos lóbulos, os hepatócitos estão sob um gradiente de composição sangüínea. Os mais periféricos recebem em primeiro lugar tanto nutrientes quanto oxigênio, com eventuais toxinas trazidas pela veia porta e artéria hepática. Isto explica as diferenças entre as células centrolobulares e as perilobulares.

O ácino é a unidade funcional deste órgão, sendo a massa de parênquima dependente do suprimento sangüíneo através da tríade portal. As células estão dispostas em zonas concêntricas que cercam os vasos aferentes terminais. Zona 1 (periportal) – mais próxima ao espaço porta, é a primeira a receber sangue com alto conteúdo de oxigênio, insulina e glucagon. Tem alta taxa metabólica e é a última a sofrer necrose e a primeira a mostrar sinais de regeneração. Zona 3 (centrilobular) – mais próxima às veias hepáticas terminais, recebe sangue por último. Aqui estão muitas das enzimas que participam de biotransformação (NADPH citocromo P450-redutase). Zona 2 (mediolobular) – recebe sangue com conteúdo intermediário de oxigênio (figura 3B) (ROBBINS, 2000). Então, resumindo o fígado pode ser estudado usando-se dois critérios: um o morfológico: o lóbulo, que apresenta um formato hexagonal, e o outro funcional: o ácino, região de intensa atividade metabólica, veja figura abaixo (3A esquema e 3B foto microscopia óptica).

A



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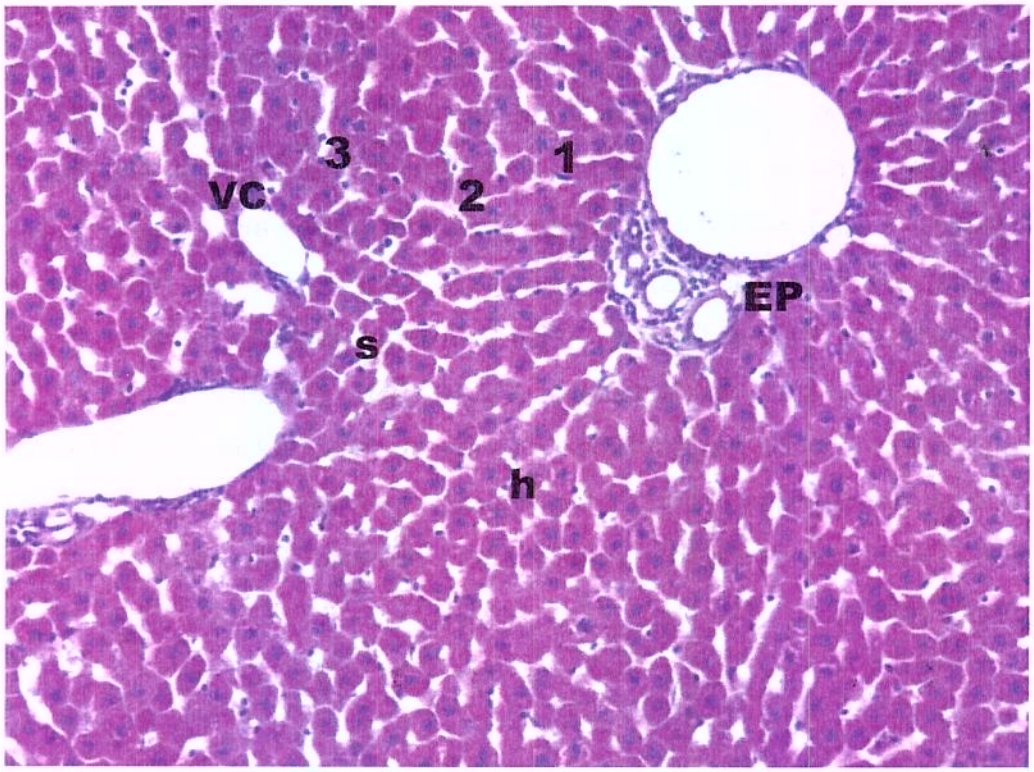

Figura 3: A) Esquema de lóbulo e ácino hepático (Robbins, 2000). **B)** Corte histológico de fígado de rato (coloração PAS), mostrando as delimitações das zonas acinares, o espaço porta e veia central, onde as letras e os números representam: VC – veia central, s – sinusóide, h – hepatócitos, EP – espaço porta, 1 - zona 1, 2 – zona 2 e 3 – zona 3) (aumento: 300 x, de Christiane A Badin-Tarsitano).

No citoplasma do hepatócito, a presença de grande quantidade de mitocôndrias e retículo enodoplasmático (RE). Seus núcleos são grandes, arredondados ou ovalados, apresentam um ou mais nucléolos e cromatina frouxa.

A organela mais evidente do hepatócito é o RE, tanto na sua forma rugosa (R) como na lisa (L) (figura 4). É nessa organela que ocorre a síntese das várias proteínas plasmásticas produzidas pelo fígado, entre as quais a albumina e o fibrinogênio do sangue. Outro componente importante da célula hepática é o glicogênio, que funciona como um depósito de carboidrato que a célula mobiliza quando ocorre uma queda na concentração de glicose no sangue (CARVALHO, 2005). Entretanto, é no REL, que contém uma variedade de enzimas relacionadas aos processos de oxidação, metilação e conjugação de substâncias, visando a inativação (detoxicação).

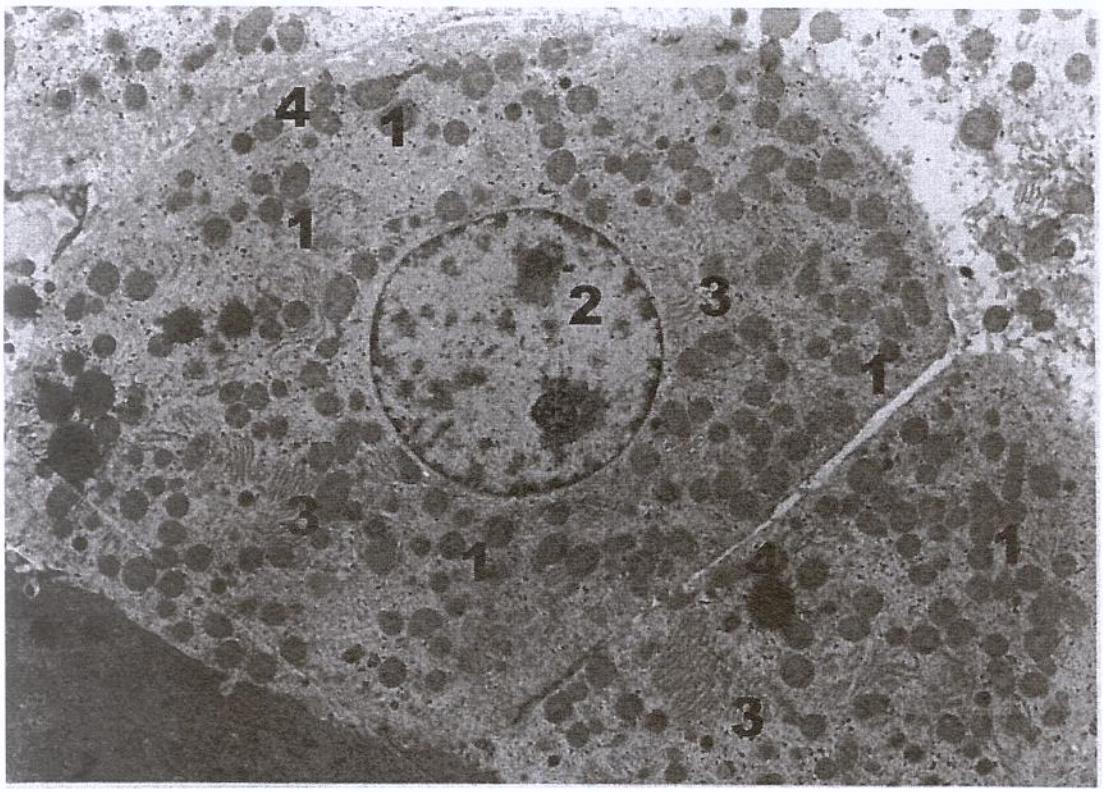

Figura 4: Foto de microscopia eletrônica de transmissão, onde os números representam: 1- mitocrôndrias, 2- núcleo, 3- RE e 4- membrana plasmática (aumento: 4646x, de Christiane A Badin-Tarsitano).

1.1.3. Papel fisiológico

As funções mais importantes do fígado estão relacionadas às suas características de glândula endócrina e exócrina. Sendo, a primeira favorecida pelo duplo suprimeto sanguíneo, que traz uma grande quantidade de substâncias endógenas e xenobióticas, dentre estas nutrientes e substâncias tóxicas (CARVALHO, 2005). Podemos resumir as principais funções do fígado em:

1. Síntese proteica: o hepatócito renova suas próprias proteínas e sintetiza várias outras para exportação como albumina, fibrinogênio, protrombina e lipoproteínas.
2. Secreção de bile: função exócrina. Os principais componentes da bile são a bilirrubina (resultante da digestão da hemoglobina pela célula de Kupffer) e os ácidos biliares (90% oriundos da circulação enterohepática e 10% produzidos pelo hepatócito).
3. Função metabólica: depósito de metabólitos como glicogênio, vitamina A, gorduras neutras.
4. Metabolismo: a glicose se origina por glicogenólise do glicogênio armazenado ou por gliconeogênese, a partir de lactato, piruvato, glicerol, entre outros.
5. Desintoxicação e Neutralização: muitas toxinas são neutralizadas pelos processos de oxidação, acetilação, metilação e conjugação. As enzimas que participam deste processo estão localizadas no retículo endoplasmático liso.

1.2. BIOTRANSFORMAÇÃO

A biotransformação é um processo que visa converter um substrato lipofílico em um composto mais polar e, conseqüentemente, ter seus metabólitos rapidamente excretados. As enzimas envolvidas no metabolismo de compostos tóxicos atuam sobre uma variedade de substratos e a sua atividade depende da semelhança entre os compostos endógenos e xenobióticos (TIMBRELL, 2000).

O fígado exerce um papel fundamental na biotransformação (ativação ou degradação) de substâncias endógenas (hormônios, esteroides, etc) e exógenas (drogas, substâncias tóxicas) no organismo. As enzimas envolvidas na biotransformação estão localizadas principalmente no REL e estão presentes em número menor nas mitocôndrias e em outras organelas (VROLIJK *et al.*, 1994; TIMBRELL, 2000; YASUI *et al.*, 2005).

A biotransformação inclui numerosos sistemas enzimáticos diferentes, os quais atuam em diversos tipos de substratos. Muitas dessas enzimas tem a função de converter estruturas tóxicas para menos tóxicas, e converter estruturas lipofílicas em estruturas hidrofílicas e, rapidamente excretadas. Os compostos passam por uma série de reações, com várias enzimas participando em etapas seqüenciais para ativação, desintoxicação e por último a excreção do xenobiótico. A biotransformação geralmente envolve duas fases (fase I e II).

As reações da fase I (reações de oxidação, hidrólise ou redução) da biotransformação ocorre com a alteração da estrutura da molécula original através da adição de grupos funcionais (-OH, COOH, etc), ou grupos funcionais já presentes na molécula original são expostos, que a tornam menos hidrofóbica (STEGEMAN e LECH, 1991). A inserção destes grupos leva à produção de metabólitos com alta solubilidade, podendo provocar não só a inativação do composto, mas também a ativação de compostos pro-carcinogênicos (STEGEMAN e LECH, 1991; GRAHAM e PETERSON, 1999). A ativação metabólica de pró-carcinógenos pode dar origem a metabólitos eletrofílicos que se ligam ao DNA, causando mutações que, uma vez "fixadas" pela célula podem iniciar o processo de carcinogênese (OMURA, 1999). Na fase II, a molécula já estruturalmente modificada é conjugada a compostos endógenos e polares, tornando-a mais hidrofílica, menos tóxica e facilmente excretável (VROLIJK *et al.*, 1994; TIMBRELL, 2000).

As reações da fase I da biotransformação são catalisadas por dois grupos de monooxigenases: as flavoproteínas monooxigenases e o sistema microssomal P450, sendo que este último é o principal e está localizado na membrana do

retículo endoplasmático liso das células. A complexidade desta organela está relacionada à sua superfície bidimensional que possui uma distribuição heterogênica de proteínas e lipídios, sendo a densidade de proteínas correspondente a 70% do peso total da membrana (HIRD *et al.*, 1964; DEVLIN, 1997).

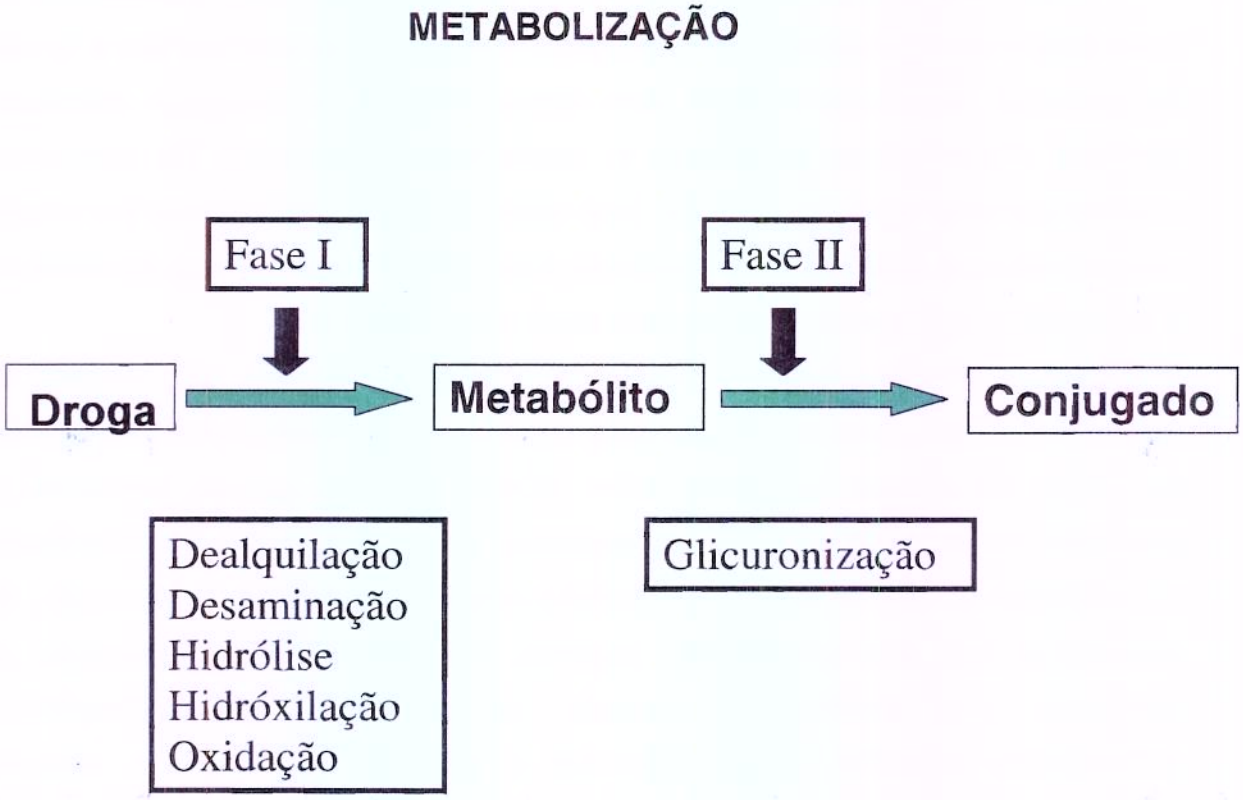

Figura 5: Resumo das fases I e II (metabolização)

1.3. CITOCROMOS P450 MONOOXIGENASES (CYP-450)

1.3.1. Nomenclatura e classificação de citocromos P450

O termo "citocromo P450" foi usado pela primeira vez em 1962 para identificar uma hemeproteína com características únicas, que recebe este nome por exibir um pico em 450 nm proeminentes em espectros ópticos numa posição peculiar (450 nm) quando reduzido por ditionito de sódio e ligado ao monóxido de carbono ("microssomal carbon monoxide-binding pigment") (OMURA e SATO, 1964a,b; BERNHARDT, 1996; OMURA, 1999); como resultado da formação Fe(II)-CO – surgindo desta constatação o nome P450 (OMURA e SATO, 1964a,b; MANSUY, 1998) (Figura 6).

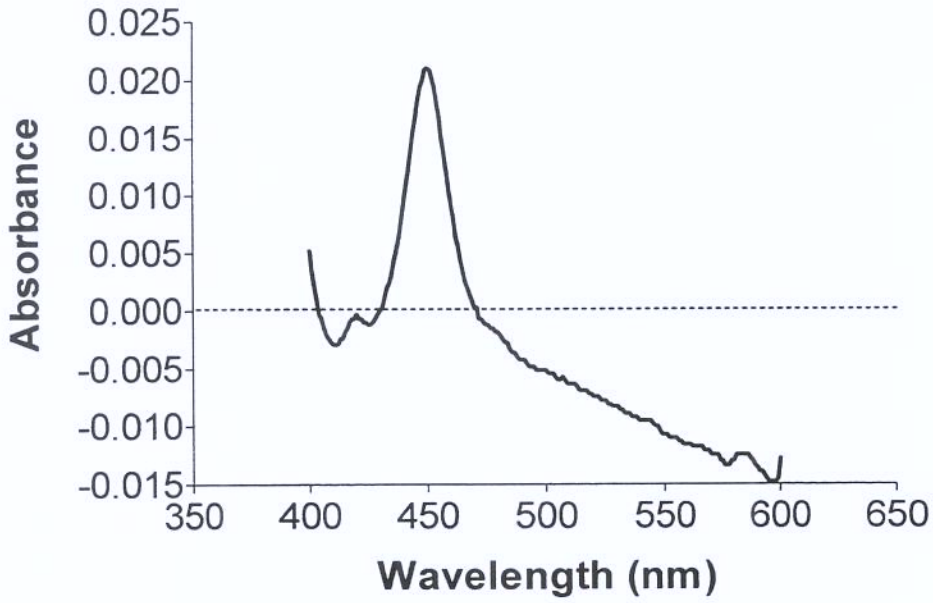

Figura 6: Espectro de absorbância do citocromo P450 ligado ao monóxido de carbono (de Christiane A Badin-Tarsitano).

Atualmente, os citocromos P450 (CYP-450) constituem uma superfamília contendo 99 famílias e mais de 150 isoformas (GUENGRICH, 1987; GONZALEZ, 1990; PORTER e COON, 1991; COON *et al*, 1992; DEGTYARENKO e ARCHAKOVA, 1993; NELSON *et al.,* 1993; BERNHARDT, 1996). Destas famílias, as principais envolvidas na biotransformação de drogas são os CYP1, CYP2 e CYP3, enquanto que as outras famílias têm papel importante na metabolização de compostos endógenos (figura 7 A e B).

A

Família de P450	Função
CYP1-CYP3	Metabolismo de esteróides e xenobióticos
CYP4	Metabolismo dos ácidos gordos, prostaglandinas e leucotrienos
CYP5	Síntese de tromboxanos
CYP7	Hidroxilação 7α do colesterol
CYP11	Clivagem da cadeia lateral do colesterol+hidroxilase 11β do colesterol
CYP17	Hidroxilação do carbono 17α de esteróides
CYP19	Aromatização de esteróides
CYP21	Hidroxilase do carbono 21 de esteróides
CYP24	Hidroxilação da vitamina D
CYP27	Hidroxilase do carbono 27 do colesterol

B

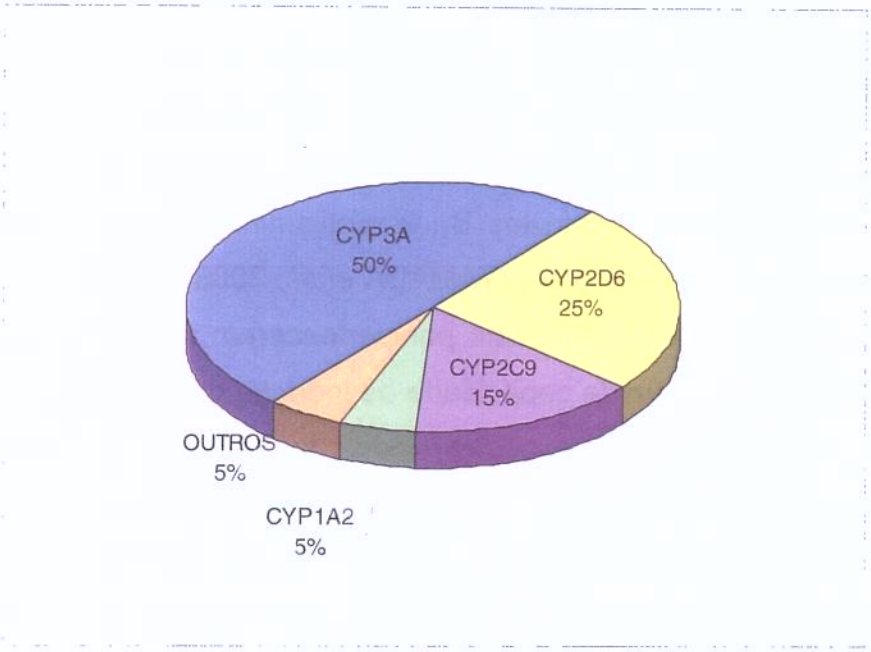

Figura 7: A) Principais famílias e funções do CYP450. B) Quantidades de CYP450 no fígado. (site: www.xenobióticop450.com)

Tanto em seres humanos como em animais de laboratório, a atividade e a expressão dos P450 podem ser influenciadas (geralmente aumentadas) por diversos fatores tais como infecções e processos inflamatórios (AITKEN *et al.*, 2006). Como conseqüência disso, pode ocorrer alteração na depuração (*clearance*) de fármacos que dependem do metabolismo hepático e da atividade dos CYP. Assim, a modulação da formação de metabólitos de substâncias endógenas fisiologicamente ativas poderia estar relacionada a mecanismos homeostáticos e fisiopatológicos envolvidos nestas condições de infecção e inflamação (MORGAN, 2000).

Em ratos, as isoformas mais estudadas são a CYP1A1/2, CYP2B1/2, CYP2E1 e CYP2C11. As CYP1A1 e CYP1A2 possuem uma alta similaridade em suas estruturas primárias, bem como em suas características físico-químicas (KAWAJIRI *et al*, 1996), tem um papel importante na ativação de xenobióticos a metabólitos tóxicos [carcinógenos químicos, como hidrocarbonetos aromáticos policíclicos (*e.g.*, benzo[*a*]pireno, encontrado na fumaça do cigarro e em alimentos queimados - literalmente carbonizados), aminas aromáticas e heterocíclicas, derivados do azobenzeno, aflatoxina B_1, 2-acetilaminofluoreno], incluindo vários medicamentos (*e.g.*, acetaminofen) (YAMADA *et al.*, 2006)

A subfamília 2B é reconhecida por pertencerem as isoenzimas induzidas pelo fenobarbital. A acentuada capacidade de indução da subfamília CYP2B, pelo fenobarbital, na maioria das espécies, induz também muitas outras enzimas (resposta pleiotrópica) (YAMADA *et al.*, 2006; NIMS e LUBET, 1996).

Na isoforma 2E1 (CYP2E1), várias substâncias de baixo peso molecular são substratos para reações de oxidação catalisadas por CYP2E1 e, muitos delas, como a N-nitrosodimetilamina (NDMA), são pró-carcinógenos potentes que requerem ativação metabólica. Outras substâncias capazes de induzir CYP2E1 são a isoniazida, a piridina, o etanol, a acetona, o éter dietílico, o *m*-xileno e o pirazol. CYP2E1 parece exercer também um papel importante na doença hepática induzida pelo álcool (NEAU *et al*,1990).

O citocromo P450 2C11 (CYP2C11) é uma isoforma constitutiva, e também com maior componente microssomal em fígado de rato, tem sido muito estudada em modelos de inflamação e infecção (MORGAN *et al*, 2002).

1.3.2. Estrutura e função de citocromos P450

O complexo enzimático P450 pode ser isolado em frações microssomais, vesículas de aproximadamente 100 nm obtidas através de uma seqüência de homogeinização e ultracentrifugações que levam à fragmentação do retículo

endoplasmático em vesículas conhecidas como microssomas que mantêm as mesmas características e composição do retículo endoplasmático na célula (DE PIERRE e ERNSTER, 1977; ALBERTS *et al.*, 1994). O sistema microssomal hepático é composto por três flavoproteínas (NADPH-citocromo P450 redutase, NADH-citocromo b5-redutase e N-oxidase) responsáveis pela transferência de elétrons, e duas hemeproteínas, o citocromo P450, uma monooxigenase, e o citocromo b5, que quando reduzido é capaz de doar seus elétrons para o oxi-P450 (DEVLIN, 1997) (Figura 8).

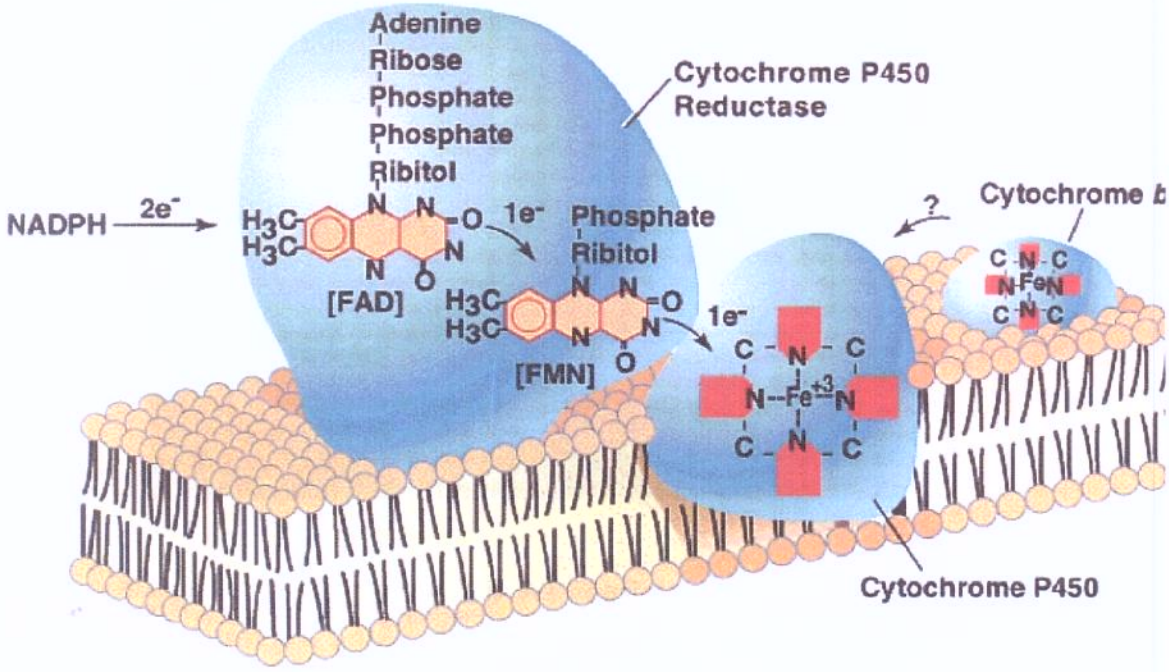

Figura 8: Componentes do sistema citocromo P450 de retículo endoplasmático. A NADPH-citocromo P450 redutase é ligada pela sua cauda hidrofóbica à membrana, enquanto o citocromo P450 é profundamente incrustado na membrana. Também é mostrado o citocromo b5, que pode participar em reações selecionadas mediadas pelo citocromo P450. (DEVLIN, 1997).

O grupamento heme do citocromo P450 é resultante da protoporfirina IX, o que contribui para as suas características espectrais únicas (MANSUY, 1998; OMURA, 1999). Tais características espectrais também são atribuídas à presença de cisteína, histidina e do ânion tiolato ligados ao ferro-heme (figura 9). O 5° ligante do ferro-heme é o responsável por gerar o espectro do P450 reduzido e complexado com monóxido de carbono e graças aos avanços das técinicas de cristalografia de proteínas e da biologia molecular hoje se sabe que este quinto ligante do ferro é o ânion tiolato de um resíduo de cisteína (OMURA, 1999).

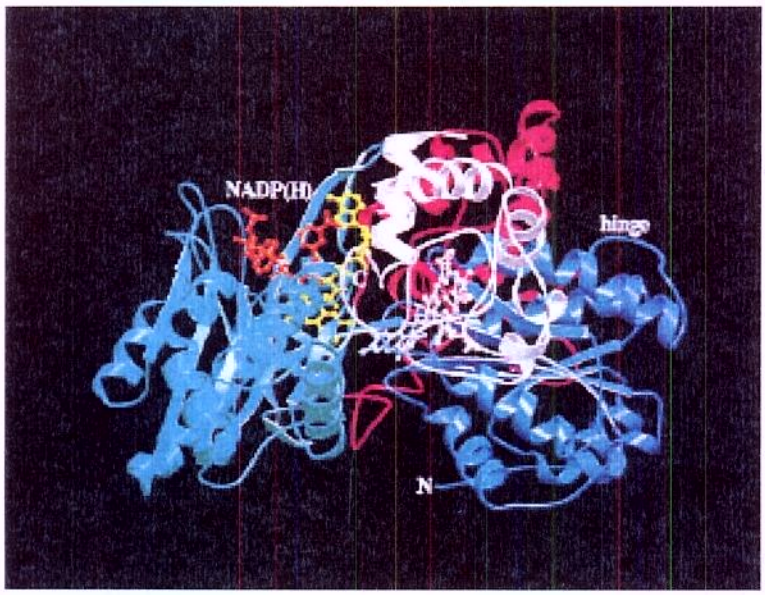

Figura 9: Estrutura do citocromo P450 (WANG *et al*, 1997)

1.3.3. Reações

Entre as reações catalisadas pelos citocromos P450 destaca-se a oxidação de moléculas lipofílicas sendo, também denominadas monooxigenases e oxidases de função mista. As monooxigenases podem catalisar um amplo espectro de reações tais como *N*-oxidação, sulfoxidação, epoxidação, *N*-, *S*-, e *O*-desalquilação, peroxidação, desaminação, dessulfuração e desalogenação. Além

de participarem da metabolização de xenobióticos, os citocromos P450 são essenciais para a homeostase sangüínea, biossíntese do colesterol e esteroidogênese (DEVLIN, 1997).

O CYP 450 é capaz de realizar uma série de reações de oxidação, como demonstrado na Figura 10. Isto é possível graças a duas propriedades desta heme-proteína: (1) habilidade do ferro em apresentar um grande número de estados de oxidação com diferentes reatividades, e (2) acessibilidade do seu grupamento ferro a um grande número de substratos (MANSUY, 1998).

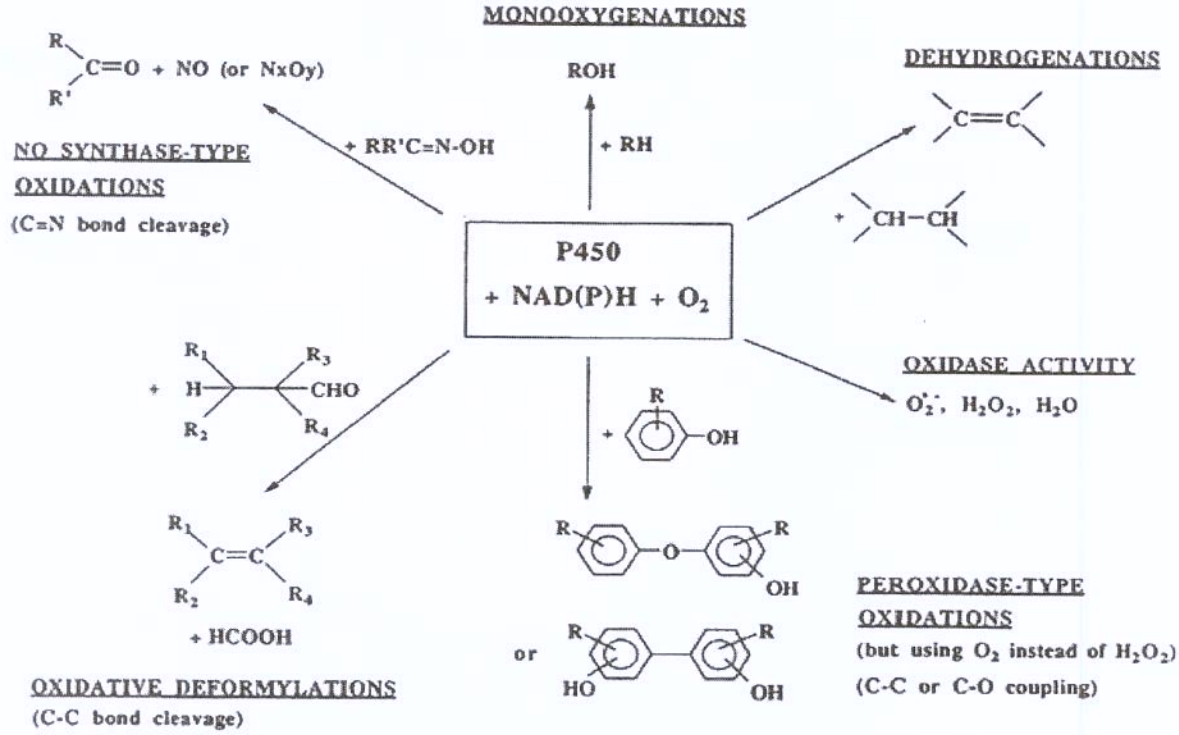

Figura 10: Esquema das reações catalisadas pelo citocromo P450 (MANSUY, 1998).

A reação básica catalisada pelo CYP 450 é a monooxigenção, que envolve a transferência dos seus átomos de oxigênio para o substrato (RH) e outro reduzido a água, sendo os elétrons para a redução fornecidos pelo NADPH (PARKISON, 2001). Estas transferências geram espécies reativas do oxigênio na forma de ânion superóxido (O_2^-) e peróxido de hidrogênio (H_2O_2) (Figura 11), que por sua vez podem causar danos ao organismo por serem precursores do radical hidroxil (COON *et al.*, 1992; OMURA, 1999).

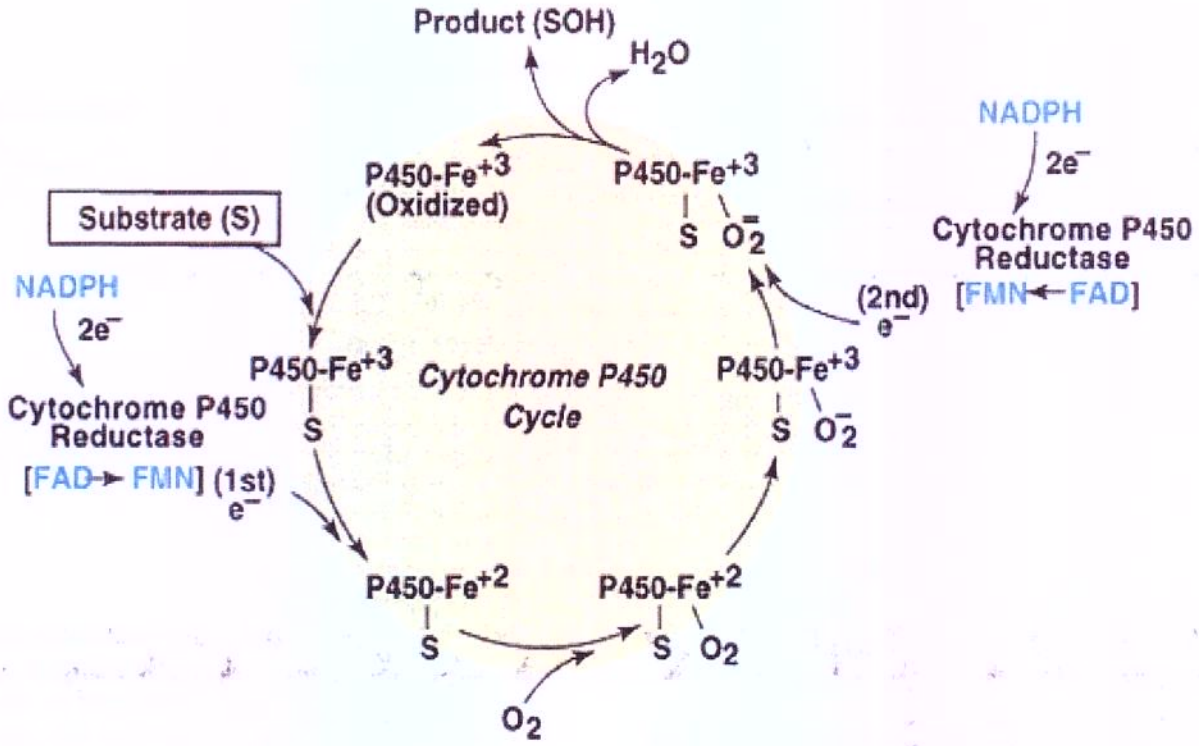

Figura 11: Ciclo catalítico do citocromo P450. (1) Ligação do substrato (RH) ao citocromo P450 (Fe^{3+}), (2) redução monoeletrônica do complexo enzima-substrato pela enzima NADPH citocromo P450 redutase (NCR), (3) ligação do oxigênio molecular (O_2) ao complexo enzima-substrato. Enquanto um átomo do oxigênio

molecular é introduzido no substrato, o outro é reduzido até água, gerando espécies reativas do oxigênio, tais como o ânion superóxido (O_2^-) e o peróxido de hidrogênio (H_2O_2), (4) transferência do segundo elétron para o complexo enzima-substrato através do citocromo b5, (5) protonação do complexo enzima-substrato com liberação de peróxido de hidrogênio, (6) clivagem da ligação O-O gerando um intermediário reativo, (7) inserção de um átomo de oxigênio no substrato (monooxigenação), (8) dissociação do produto e liberação do citocromo P450, e (9) reação de hidroxilação sem a necessidade de equivalentes redutores. (DEVLIN, 1997).

Estruturalmente, o CYP 450 redutase compartilha homologia com a região do C-terminal de NOS (BREDT *et al.*, 1991; LYONS *et al.*, 1992) e a co-distribuição destas duas enzimas tem sido relatada em alguns estudos histológicos (NORRIS *et al.*, 1994; YOUNG *et al.*, 1997). Há também relação estrutural entre as NOS e outras CYP-450 (MCMILLAN *et al.*, 1996).

1.4. ÓXIDO NÍTRICO (NO)

1.4.1 Aspectos gerais

O óxido nítrico (NO), é uma molécula gasosa, liberada como co-produto do metabolismo do aminoácido L-arginina para L-citrulina (figura 12) numa reação catalizada por enzimas denominadas óxido nítrico sintases (NOS). A biossíntese de NO já foi relatada em diversos tipos de células, tais como células endoteliais, miócitos, neurônios, macrófagos, monócitos, neutrófilos, plaquetas, e hepatócitos, entre outros (BARRETO *et al.*, 2005; MONCADA *et al.*, 2006, 1991; NATHAN 1992; FUKUTO e CHAUDHURI, 1995).

Pelo menos três tipos de NOS são reconhecidos: nNOS (neuronal, NOS I), eNOS (endotelial, NOS III) e iNOS (induzível, NOS II) (TAYLOR *et al.*, 1998). A nNOS e a eNOS são enzimas de expressão constitutiva, cuja atividade depende

do complexo Ca^{2+}/calmodulina, enquanto que a iNOS é uma isoforma induzível por citocinas e endotoxinas bacterianas e cuja atividade independe de Ca^{2+} (FUKUTO e CHAUDHURI, 1995). A reação envolvendo as NOS requer diversos cofatores, como NAPDH, FAD, FMN, tetrahidrobiopterina, calmodulina e heme. A nNOS e a eNOS liberam baixos níveis de NO por períodos curtos enquanto que a iNOS produz altos níveis de NO por períodos prolongados de tempo (MONCADA *et al.*, 1991).

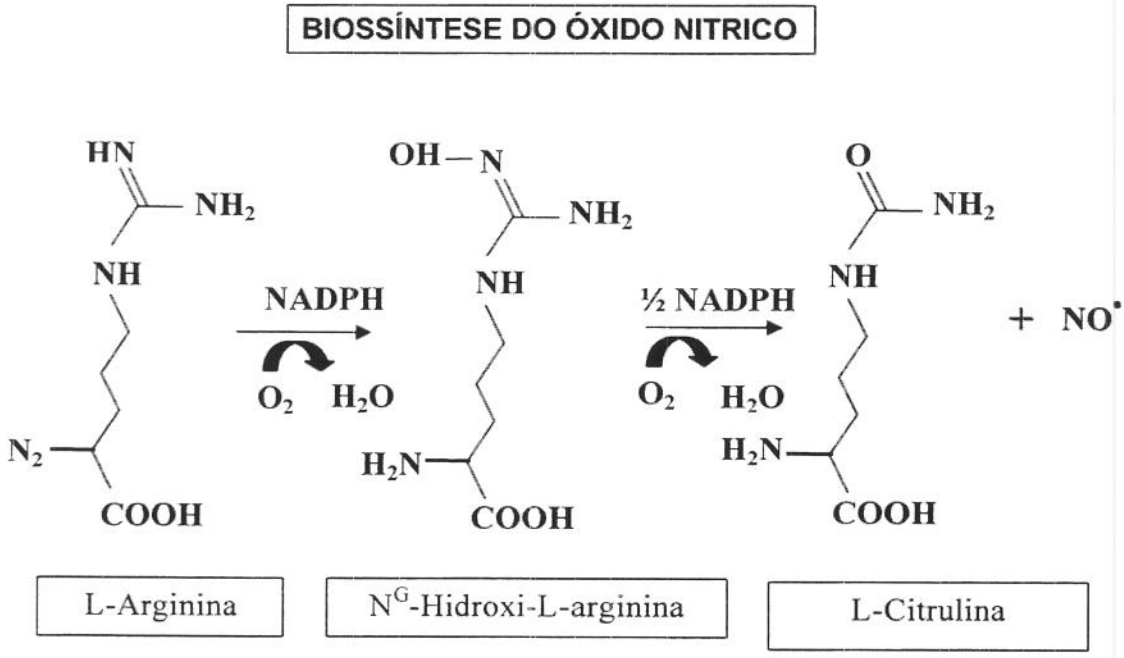

Figura 12: Esquema biossíntese óxido nítrico

As NOs possuem em cada lado da molécula duas porções funcionalmente complementares. Na porção carboxiterminal, existe um domínio redutase (recebe elétrons do NADPH) homólogo ao citocromo P450 e na porção aminoterminal, existe o domínio oxidase (que abstrai um elétron do substrato L-arginina), que

possui sítios de ligação para heme, para o co-fator tetradrobiopterina (BH_4) e para o substrato L-arginina (LUZ *et al.*, 2003).

O NO exerce amplo espectro de atividades biológicas *in vivo*, inclusive na apoptose, citotoxicidade (DRAPIER e HIBBS, 1988; BECKMAN e KOPPENOL, 1996; BRÜNE *et al.*, 1998a,b), inflamação, dor (CATTELL e JANSEN, 1994), neurotransmissão e no tôno vascular (FLEMING *et al.*, 1996). No sistema cardiovascular, o NO possui potente ação vasodilatadora e é responsável por pelo menos parte da resposta hipotensora à acetilcolina e peptídeos como a bradicinina. Muitos efeitos do NO são mediados principalmente pela guanosina-3',5'-monofosfato cíclico (cGMP), produzida após estimulação da enzima guanilil ciclase solúvel pelo NO (MONCADA *et al*, 2006,1991). Além disso, a interação de NO com intermediários reativos de oxigênio pode ter um papel importante na modulação de respostas vasculares (BECKMAN e KOPPENOL, 1996; WOLIN *et al.*, 1998).

1.4.2. Inibição da biossíntese de NO

Substâncias análogas à L-arginina, tais como a L-nitro-arginina (L-NA; ISHII *et al.*, 1990; MAGGI *et al.*, 1991), N^{ω}-amino-L-arginina (L-NAA), N^{ω}–nitro-L-arginina metil éster (L-NAME; MOORE *et al.*, 1990), N-iminoetil-L-ornitina (L-NIO), e N^{G}-monometil-L-arginina (L-NMMA; MARLETTA *et al.*, 1998) inibem a atividade de NOS, tanto *in vitro* como *in vivo*, embora existam diferenças na especificidade e potência (FUKUTO e CHAUDHURI, 1995). Os isômeros L são inibidores mais efetivos da NOS do que os isomêros D correspondentes (MONCADA *et al.*, 2006, 1997). Entre os inibidores disponíveis, o L-NMMA e o L-NAME são ativos por via oral. Outros compostos não derivados da L-arginina, tais como guanidinas, imidazóis, 7-nitroindazóis e azul de metileno (SOUTHAN e SZABÓ, 1996), também têm sido descritos como inibidores da NOS. Neste grupo, existem compostos "seletivos" para a NOS, como é o caso da aminoguanidina que inibe

preferencialmente a iNOS (CORBETT *et al.*, 1992) e o 7-nitroindazol que inibe preferencialmente a nNOS (MOORE *et al.*, 1993). Através do uso de inibidores de NOS, tem sido possível estudar o papel do NO endógeno em diversos eventos fisiopatológicos. Dentre os inibidores de NOS, destaca-se o L-NAME, um inibidor amplamente usado devido ao seu baixo custo comercial e por ter uma potente ação inibitória não-específica sobre as NOS.

1.4.3. Alterações morfológicas e sistema renina-angiotensina

A administração aguda de L-NAME em diversas espécies animais resulta em hipertensão arterial acompanhada por aumento da resistência vascular sistêmica, bradicardia e redução do débito cardíaco (GARDINER *et al.*, 1990; RICHARD *et al.*, 1991; KLABUNDE *et al.*, 1991; VAN GELDEREN *et al.*, 1993; BOWER e LAW, 1993; ZAPELLINI *et al.*, 1997), efeitos estes que podem ser revertidos pela administração de L-arginina (REES *et al.*, 1988). De forma semelhante, ratos tratados cronicamente com L-NAME (20 mg/kg/dia) durante até 8 semanas desenvolvem uma hipertensão prolongada (ARNAL *et al.*, 1992; BAYLIS *et al.*, 1992; RIBEIRO *et al.*, 1992). As alterações hemodinâmicas e morfológicas associadas a este modelo de hipertensão têm sido bastante estudadas, principalmente nos tecidos cardíaco (MORENO Jr. et al., 1995, 1997) e renal (para revisão, ver ZATZ e BAYLIS, 1998) e JOVER e MIMRAM (2001). Nestes dois órgãos, a inibição crônica afeta não somente as propriedades vasculares destes, mas também resulta em dano tecidual com necrose seguido de fibrose. Doses menores de L-NAME (7,5 mg/kg/dia) também são eficazes em inibir as NOS e causar hipertensão, porém sem produzir dano morfológico intenso (OLIVEIRA *et al.*, 2000).

Além de promover hipertensão, a inibição crônica da biossíntese de NOS causa lesão isquêmica glomerular, glomeruloesclerose, fibrose intersticial e lesões vasculares (HROPOT *et al.*, 2003; RIBEIRO *et al*, 1992; FUJIHARA *et al*, 1999;

ZATZ e BAYLIS, 1998). Estas lesões podem levar a um colapso glomerular. O aumento das áreas hialinizadas e a expansão intersticial podem ser acompanhados por uma infiltração de fibroblastos e deposição de colágeno. Em tecido cardiáco, o tratamento crônico com L-NAME resulta em isquemia miocárdica, talvez devido à vasoconstrição e fluxo coronário reduzido, o que leva ao infarto e subsequentemente necrose (MANDARIM-DE-LACERDA *et al.*, 1999, PESSANHA e MANDARIM-DE-LACERDA, 2000; HROPOT *et al.,* 1994; MORENO *et al.,* 1995, 1997; ver PECHÁNOVÁ e BERNÁTOVÁ, 1996, e SIMKO e SIMKO, 2000). Ocorre também um aumento de fibroblastos e de granulações com infiltrado de células inflamatórias (SAMPAIO *et al.,* 2002). O aumento na espessura da parede do vaso e a hipertrofia do ventrículo esquerdo são devidos principalmente à fibrose perivascular e intersticial e refletem o remodelamento do tecido (MANDARIM-DE-LACERDA *et al*, 2001; KRISTEK *et al.,* 1996; TAKEMOTO *et al.,* 1997; SIMKO e SIMKO, 2000; PEREIRA, 2003) que é acompanhado de angiogênese (OKRUHLICOVÁ *et al.,* 1999). Estudos mais recentes têm mostrado que alterações semelhantes a estas descritas em tecido cardíaco e renal também ocorre no cérebro (HSIEH *et al.,* 2004).

Embora o efeito do tratamento crônico com L-NAME tem sido estudado principalmente por períodos 2 a 8 semanas, alterações semelhantes na pressão arterial e na morfologia (isquemia, hipertrofia e aumentos de células inflamatórias) também têm sido observadas em tratamentos curtos de três dias à uma semana (KATAOKA *et al.,* 2004; NI *et al.,* 2001; IKEGAKI *et al.,* 2001). De fato, há relatos na literatura mostrando que tratamento com L-NAME durante até uma semana resulta em alterações morfológicas em alguns tecidos (KATAOKA *et al*, 2004; NI *et al*, 2001; IKEGAKI *et al*, 2001), e que também ocorrem mudanças na expressão de algumas proteínas, sendo assim, USUI *et al.* (1998) mostrou que a inibição da síntese de NO pelo L-NAME em ratos aumentou a expressão de RNAm para receptores do tipo I (AT_{1A}, AT_{1B}) de angiotensina II na glândula adrenal e, conseqüentemente, elevou o número de receptores expressos neste tecido, efeito

este observado na primeira semana de tratamento e não era resultado da hipertensão que acompanhava a inibição de NOS.

Vários trabalhos têm demonstrado o envolvimento do sistema renina angiotensina (SRA) na reposta pressora após o tratamento crônico com L-NAME. A administração crônica concomitante de um antagonista de receptores AT_1 (losartan) com L-NAME preveniu a hipertensão neste modelo (RIBEIRO *et al.*, 1992). Após este trabalho envolvendo a administração de antagonistas de receptores AT_1 ou inibidores da ECA (enzima conversora de angiotensina) (enalapril) confirmaram esses resultados (PEREIRA *et al.*, 2004; POLLOCK *et al.*, 1993; NAVARRO-CID *et al.*, 1996; TAKEMOTO *et al.*, 1997).

A inibição crônica de NO promove danos estruturais renais severos e progressivos em ratos Wistar, como isquemia glomerular, glomeruloesclerose e expansão intersticial (BAYLIS *et al.*, 1992; RIBEIRO *et al.*, 1992; ARCOS *et al.*, 2000; PEREIRA e MANDARIN-DE-LACERDA, 2001).

1.5. INTERAÇÃO NO - CYP-450

Diversos tipos de células no fígado produzem NO através dos três NOS, sendo que a eNOS e a iNOS são as mais importantes fontes deste mediador (IVANAGI, 2005; ALEXANDER, 1998; TAYLOR *et al.*, 1998; CLEMENS, 1999; MURIEL, 2000). No fígado normal, o NO exerce múltiplas funções, principalmente no controle do tôno vascular e na citoproteção. Por outro lado, o NO tem sido implicado em várias alterações celulares (inclusive citotoxicidade) associadas a diversas patologias, tais como hipertensão sistêmica portal, choque hemorrágico, isquemia e reperfusão, tumores hepáticos, cirrose e dano por toxinas (HARTLEB *et al.*, 1997; JONES E CZAJA, 1998; TAYLOR *et al.*, 1998; CLEMENS, 1999; LI e BILLIAR, 1999; WIEST e GROSZMANN, 1999; KAPLOWITZ, 2000).

O NO possui alta afinidade para grupos heme e é capaz de formar complexos com proteínas contendo heme, especialmente enzimas, tais como a

guanilil ciclase solúvel (IGNARRO, 1990), aconitase (STADLER *et al.*, 1991), citocromo *c* oxidase (CLEETER *et al.*, 1994), ciclooxigenase (KANNER *et al.*, 1992) e a própria nNOS (ABU-SOUD *et al.*, 1995). Em algumas enzimas, (guanilil ciclase e ciclooxigenase), a ligação de NO resulta em estimulação, porém, na maioria de enzimas ou proteínas contendo heme ou ferro (citocromos - inclusive os P450, hemoglobina, ferritina, oxigenases, algumas oxidases NADPH-dependentes, etc.), a ligação de NO resulta em uma diminuição de atividade (através da formação de hemo-proteínas nitrosiladas), e em alguns casos, de expressão também (HENRY *et al.*, 1991; KHATSENKO, 1998).

No que diz respeito aos citocromos P450, sabe-se desde a década de 60 que o NO interage com estas enzimas para produzir complexos ferrosos (MIYAKE *et al.*, 1968; EBEL *et al.*, 1975; O'KEEFE *et al.*, 1978). De fato, NO liberado de moléculas doadoras exógenas, tais como a NOC7, nitroprussiato de sódio, S-nitroso-N-acetilpenicilamina (SNAP) e outras, inibe citocromos P450 (DONATO *et al.*, 1997; GERGEL *et al.*, 1997; MINAMIYAMA *et al.*, 1997) e pode causar hepatotoxicidade (NIKNAHAD e O'BRIEN, 1996). Em hepatócitos isolados, a formação de NO endógeno é responsável pela perda rápida de atividade P450 destas células em cultura (LÓPEZ-GARCÍA, 1998). Alguns estudos indicam que os próprios CYP-450 também são capazes de liberar NO de moléculas exógenas (SERVENT *et al.*, 1989; ANDRONIK-LION *et al.*, 1992; SCHRÖDER, 1992; BOUCHER *et al.*, 1992; MINAMIYAMA *et al.*, 1999) e endógenas, embora a eficiência neste último caso seja menor do que com a própria NOS devido a algumas diferenças e restrições estruturais (KESERU *et al.*, 2000).

Atualmente, acredita-se que a ação inibitória do NO se dá 1) através da capacidade desta molécula de reduzir a forma férrica da enzima para a forma ferrosa, 2) por competição com os sítios de ligação de ligantes endógenos ou exógenos no ferro das enzimas e 3) por atuar como um "scavenger" de radicais livres (STADLER *et al.*, 1996; KHATSENKO, 1998). Neste último caso, a formação de peróxinitrito (resultado da interação de NO com ânion superóxido) poderia mediar o efeito inibitório (ROBERTS *et al.*, 1998; DAIBER *et al.*, 2000a,b). Apesar

da capacidade de NO em inibir os citocromos P450, a sensibilidade destas enzimas à esta inibição varia entre (KHATSENKO *et al.*, 1993; WINK *et al.*, 1993) e dentro de (STADLER *et al.*, 1994) subfamílias.

Em culturas de células, a inibição da atividade e expressão de citocromos P450 (CYP1A2, CYP1B1/2, CYP2C11 e CYP3A2) por citocinas tais como TNF-α, IL-1β e IFN-γ (STADLER *et al.*, 1994; OSAWA *et al.*, 1995; CARLSON e BILLINGS, 1996; DONATO *et al.*, 1997) e lipopolissacarídeo (LPS) de *Escherichia coli* (OSAWA *et al.*, 1995; TAKEMURA *et al.*, 1995) é mediada principalmente pela produção aumentada de NO, embora alguns estudos (RENTON, 2004; MONSHOUWER *et al.*, 1996; SEWER e MORGAN, 1997, 1998; SEWER *et al.*, 1998) não conseguiram demonstrar um papel importante para o NO na ação de IL-1α, IL-1β, TNF-α e LPS; o NO tem pouco envolvimento na ação inibitória da IL-6 (CARLSON e BILLINGS, 1996; SEWER e MORGAN, 1997). Este efeitos também foram observados em pacientes que tiveram rejeição de fígados transplantados (WESTERHOLT *et al.*, 2004). De modo geral, o efeito inibitório de NO sobre a atividade e expressão de citocromos P450 pode ser prevenido ou abolido por inibidores de NOS, tais como a L-NMMA (STADLER *et al.*, 1994; CARLSON e BILLINGS, 1996; DONATO *et al.*, 1997). As observações de que nem todas as isozimas de CYP-450 têm sua atividade restaurada após inibição da biossíntese de NO (STADLER *et al.*, 1994) e que, mesmo quando se consegue uma inibição total desta produção, a atividade das CYPs não é totalmente restaurada (DONATO *et al.*, 1997), indicam que outros mecanismos também podem modular a atividade e expressão dos CYP-450.

Entretanto, em algumas situações de inflamação, tais como aquela produzida por endotoxina (lipopolissacarídeo) da bactéria *Escherichia coli*, a inibição de CYP-450 observada é aparentemente independente da produção elevada de NO (SEWER e MORGAN, 1998; AITKEN *et al.*, 2005).

Os efeitos de NO sobre a atividade e expressão de citocromos P450 observados *in vitro* têm sido confirmados *in vivo*. Assim, a supressão da atividade e da expressão (KHATSENKO *et al.*, 1993; KIM *et al.*, 1995a,b; MÜLLER *et al.*,

1996; KHATSENKO e KIKKAWA, 1997; KHATSENKO *et al.*, 1997) dos CYP-450 após tratamento de ratos com citocinas ou LPS pode ser revertida tratando-se os animais com inibidores de NOS (KIM *et al.*, 1995a; MÜLLER *et al.*, 1996; KHATSENKO e KIKKAWA, 1997).

2. OBJETIVOS

Embora sabe-se que o NO exerce papel fundamental na hemodinâmica e função celular do fígado, a influência da inibição prolongada da formação de NO sobre a morfologia e alguns parâmetros bioquímicos (glicogênio e lípides) deste órgão e sobre a atividade e expressão dos citocromos P450 ainda não foi avaliada de forma sistemática. Assim, neste projeto, propomos:

1. Avaliar as alterações morfológicas e bioquímicas hepáticas decorrentes do tratamento agudo e crônico com L-NAME através de microscopia de luz e dosagens bioquímicas, respectivamente.

2. Investigar a influência da inibição da biossíntese de NO sobre a atividade e expressão de citocromos P450 hepáticas em ratos tratados aguda e cronicamente com L-NAME, usando as técnicas de ensaio enzimático e imunoblot. Para isso, foram estudadas uma isoforma com expressão constitutiva (CYP2C11) e três isoformas induzíveis, CYP1A1/2, CYP2B1/2 e CYP2E1, que podem ser induzidas por ß-naftoflavona (Stadler *et al.*, 1994), fenobarbital (Khatsenko *et al.*, 1993), e pirazol (Gergel *et al.*, 1997), respectivamente.

3. CAPÍTULOS

Artigo submetido

"HEPATIC MORPHOLOGICAL ALTERATIONS AND CYTOCHROME P450 ACTIVITIES IN RATS TREATED CHRONICALLY WITH N$^{\omega}$-NITRO-L-ARGININE METHYL ESTER (L-NAME)"

Cell & Tissue Research

HEPATIC MORPHOLOGICAL ALTERATIONS AND CYTOCHROME P450 ACTIVITIES IN RATS TREATED CHRONICALLY WITH N^{ω}-NITRO-L-ARGININE METHYL ESTER (L-NAME)

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Running title: Hepatic alterations in L-NAME-treated rats

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Abstract

The chronic treatment of rats with N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) biosynthesis, results in hypertension mediated partly by enhanced angiotensin I-converting enzyme (ACE) activity. In this study, we examined the influence of L-NAME on rat liver morphology, hepatic glycogen, cholesterol and triglyceride content, and activities of the cytochrome P450 isoforms CYP1A1/2, CYP2B1/2, CYP2C11 and CYP2E1. Male Wistar rats were treated with L-NAME (20 mg/rat/day, administered in the drinking water) for two, four and eight weeks and the livers then removed for analysis. Enzymatic induction was produced by treating rats with phenobarbital (to induce CYP2B1/2) or β-naphthoflavone (to induce CYP1A1/2) (80 mg/kg/day each, i.p., 4 d) or pyrazole (200 mg/kg/day, i.p., 2 d) (to induce CYP2E1). L-NAME significantly elevated the blood pressure and this was reversed by concomitant treatment with enalapril (25 mg/kg/day, p.o., ACE inhibitor) or losartan (30 mg/kg/day, p.o., angiotensin II AT_1 receptor antagonist). L-NAME caused vascular hypertrophy in hepatic arteries, with perivascular and interstitial fibrosis involving collagen deposition. There was also a significant increase in the hepatic glycogen content. All of these changes were completely reversed by concomitant treatment with enalapril or losartan. L-NAME had no effect on the hepatic cholesterol and triglyceride content, nor did it affect the basal or drug-induced activities and protein expression of the cytochrome P450 isoforms. These results show that the chronic inhibition of NO biosynthesis produced hepatic morphological alterations and changes in carbohydrate metabolism that were mediated by the renin-angiotensin system, without significantly affecting the basal or induced activities of cytochrome P450 isoforms.

Keywords: cytochrome P450, hypertension, liver, nitric oxide inhibition, vascular hypertrophy

Introduction

Nitric oxide (NO), a co-product of the metabolism of L-arginine to L-citrulline by NO synthases (NOS), plays a central role in cardiovascular regulation, including blood pressure, in vertebrates (Moncada et al. 1991). The chronic inhibition of NOS by L-arginine analogs such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) produces sustained arterial hypertension that is mediated to a large extent by the renin-angiotensin system (RAS) since treating rats with angiotensin-converting enzyme (ACE) inhibitors such as captopril and enalapril, or with angiotensin II AT_1 receptor antagonists such as losartan, restores blood pressure to near normal levels (Ribeiro et al. 1992; Zatz and Baylis 1998).

The chronic inhibition of NO biosynthesis also causes marked morphological alterations in various structures, particularly the vasculature. In cardiac tissue, there is ischemia, possibly caused by vasoconstriction and a consequent reduction in coronary flow, and this leads to infarction, necrosis and apoptosis (Moreno et al. 1996, 1997; Takemoto et al. 1997a,b; Pereira and Mandarim-de-Lacerda 1999; Pessanha and Mandarim-de-Lacerda 2000). There is also arterial wall and left ventricular hypertrophy (Takemoto et al. 1997a,b; Simko and Simko 2000; Pereira and Mandarim-de-Lacerda 2001; Pereira et al. 2003) and angiogenesis (Okruhlicová et al. 2000). In renal tissue, there is glomerular ischemia with glomerulosclerosis, interstitial fibrosis (involving infiltration by fibroblasts and deposition of collagen) and vascular lesions that may result in glomerular collapse (Zatz and Baylis 1998; Jover and Mimran 2001). The vascular changes seen in cardiac and renal vessels have also been described in cerebral vessels (Hsieh et al. 2004).

In addition to its role in regulating blood pressure and tissue remodeling, NO can modulate the expression of numerous genes (Pfeilschifter et al. 2001; Pilz and Casteel 2003). The chronic inhibition of NO formation affects the expression and activities of receptors (Katoh et al. 1998), intracellular signaling molecules such as ERK1/2 kinases (Martens et al. 2002), $p70^{S6K}$ kinase (Minamino et al. 2000) and

Rho kinase (Ikegaki et al. 2001; Kataoka et al. 2002), and various enzymes, including matrix metalloproteinases (Gonzalez et al. 2000), and angiotensin-converting enzyme (Takemoto et al. 1997a,b; Gonzalez et al. 2000).

In liver, NO is produced mainly via constitutive NOS (endothelial or eNOS) and inducible NOS (iNOS) in several cell types and, under normal conditions, is involved in the control of vascular tone and in cytoprotection (Alexander 1998; Muriel 2000). Alterations in the levels of NO production have been implicated in cellular alterations (including cytotoxicity) associated with pathologies such as systemic portal hypertension, hemorrhagic shock, ischemia and reperfusion injury, hepatic tumors, cirrhosis, and damage by toxins (Clemens 1999; Wiest and Groszmann 1999; Kaplowitz 2000).

Cytochromes P450 (CYP-450) comprise a superfamily of hemoproteins (more than 150 isoforms classified in 12 families) that serve as the terminal oxidases in the mixed function oxidase system that metabolizes various endogenous substrates such as steroids, as well as xenobiotics such as drugs, toxins, and carcinogens (Guengerich 1987; Bernhardt 1996). Cytochromes P450 are located in the smooth endoplasmic reticulum and show some structural homology with the C-terminal region of NOS. The expression and activity of these enzymes is regulated by a variety of factors such as cytokines, growth factors, NO and bacterial endotoxin (lipopolysaccharide, LPS), particularly in inflammation (Khatsenko, 1998; Morgan et al., 1998, 2002; Aitken et al., 2005). Increased NO production inhibits CYP-P450 activity *in vitro* (Stadler et al. 1994; Carlson and Billings, 1996; Donato et al. 1997), although some studies have suggested that NO-independent mechanisms may be involved (Monshouwer et al. 1996; Sewer and Morgan 1997). Experiments *in vivo* have indicated that the downregulation of CYP-450 expression and activity in response to interferon inducers (Hodgson and Renton 1995) and LPS (Sewer et al. 1998; Sewer and Morgan 1998; Li-Masters and Morgan 2002) is independent of enhanced NO production.

In contrast to these studies, little is known about the influence that a chronic reduction or absence of basal NO production has on the expression and activities

of these enzymes. In this study, we investigated the influence of a prolonged inhibition of basal NO biosynthesis on liver morphology, lipid and glycogen levels, and on the activities and expression of the cytochrome P450 isoforms CYP1A1/2, CYP2B1/2, CYP2C11 and CYP2E1 in rats treated with L-NAME for 2, 4 and 8 weeks.

Material and methods

Reagents

L-Arginine, L-citrulline, L-aminoguanidine, bovine serum albumin, calmodulin, dithiothreitol, D-glucose 6-phosphate dehydrogenase, D-glucose-6-phosphate disodium hydrate, ethoxyresorufin, flavine-adenine-dinucleotide (FAD), flavine-adenine-mononucleotide (FMN), β-nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), β-nicotinamide adenine dinucleotide phosphate (NADPH, reduced form), β-naphthoflavone, 4-nitrocatechol, *p*-nitrophenol, N^{ω}-nitro-L-arginine methyl ester (L-NAME), 7-pentoxyresorufin and pyrazole were from Sigma Chemical Co. (St. Louis, MO, USA). [U-^{14}C]Arginine, acrylamide, ammonium persulfate, Coomassie brilliante blue R250, donkey anti-mouse IgG-peroxidase conjugate, glycerol, Hybond-P PVDF membrane (0.45 μm), *N,N'*-methylene bis-acrylamide, sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethylenediamine (Temed), and Tris base were from Amersham Biosciences (Piscataway, NJ, USA or Amersham, Buckinghamshire, UK). Anti-mouse IgG monoclonal antibodies to CYP1A1/2, CYP2B1/2B2, CYP2C11 and CYP2E1 were from Oxford Biomedicals (Oxford, OH, USA). Tienilic acid was a generous gift from Dr. Patrick M. Dansete (Laboratoire de Chemie et de Biochime Pharmacologiques et Toxicologiques, Paris, France). Flat-bottomed 96-well plates were from Corning (Corning, MA, USA), chemiluminescence kits (SuperSignal, West Pico) were from Pierce Chemicals (Rockford, IL, USA) and photographic film was from Kodak (São José dos Campos, SP, Brazil). Sodium pentobarbital (Hypnol®) was obtained from Cristália Indústria Farmacêutica (Itapira, SP, Brazil). Formalin (10%) and solvents

for histology were from Synth (São Paulo, SP, Brazil). Paraffin (Histosec), hematoxylin, eosin, periodic acid-Schiff reagent and entellan were from Merck (Rio de Janeiro, RJ, Brazil). Kits for the quantification of HDL cholesterol and triglycerides were from Laborlab (São Paulo, SP, Brazil) and those for LDL cholesterol were from Wiener (Rosario, Argentina). Other reagents of analytical grade were obtained from Baker or Merck.

Animals

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

Treatment with L-NAME and blood pressure measurements

Rats were treated with L-NAME (20 mg/rat/day) given in their drinking water for 2, 4 and 8 weeks (Ribeiro et al. 1992). The amount of L-NAME ingested was calculated based on the water intake of the rats which was monitored daily and the dose adjusted accordingly. Control rats received tap water alone. Once a week, the rats were weighed and tail blood pressure was measured by a tail-cuff method (Kent Instruments).

In some experiments, rats treated with L-NAME also received losartan (30 mg/kg/day) or enalapril (25 mg/kg/day) to block angiotensin II AT_1 receptors and inhibit ACE activity, respectively. These inhibitors were given in the drinking water together with L-NAME.

Cytochrome P450 induction

To induce selected cytochrome P450s, control and L-NAME-treated rats received phenobarbital (to induce CYP2B1/2) or β-naphthoflavone (to induce CYP1A1/2) (80 mg/kg/day each, i.p., for 4 d prior to sacrificing) (Suzuki et al., 1992) or pyrazole (200 mg/kg/day, i.p., for 2 d prior to sacrificing) (to induce CYP2E1) (Clejan and Cederbaum 1992).

NOS activity

Liver NOS activity was measured as described by Ribeiro Jr. et al. (1999) using [^{3}H]L-arginine as substrate. Enzyme activity was expressed as pmol of [^{3}H]L-citrulline formed/mg of protein/min.

Histological analysis and vessel wall morphometry

At the end of each period of treatment, the rats were anesthetized with sodium pentobarbital (>50 mg/kg, i.p; Hypnol®) and perfused via the aorta with heparinized saline to remove blood from the liver. The liver was then excised and 5x10 mm fragments were fixed in 10% formalin for 24 h. Fragments of liver were subsequently dehydrated in an ethanol series, cleared in xylene, embedded in Histosec and sections 5 μm thick were cut on a Leica microtome and stained with hematoxylin-eosin (HE) or picrosirius red. The stained sections were examined by light microscopy using a Nikon Eclipse E800 microscope and images were captured and processed with Image Pro Plus software. Arterial wall thickness was expressed as the ratio of the vessel total cross-sectional area divided by the vessel lumen cross-sectional area (10 vessels/rat, 5 rats/group; total of 50 vessels/group) (Borzychowski et al. 2003). All measurements and analyses were done using the microscope and software described above.

Determination of hepatic lipid and glycogen levels

Pentobarbital-anesthetized rats were perfused with heparinized saline and the livers were collected and frozen in liquid nitrogen prior to storage at -80°C until used. Hepatic lipids were extracted by the method of Folch et al. (1957) and the concentrations of triglycerides, HDL-cholesterol and LDL-cholesterol were measured using commercial kits.

The hepatic glycogen content was determined according to Lo et al. (1970), with minor modifications. Liver samples were weighed and immersed for 30 min at 100°C in 1 ml of 30% KOH saturated with Na_2SO_4. Ethanol (95%) (1.5 ml) was added to precipitate the glycogen from the alkaline digest. The samples were then centrifuged and the supernatants were carefully aspirated. The pellets were resuspended in 1 ml of distilled water containing phenol (5%) and concentrated H_2SO_4, and the resulting absorbance was read in a Beckman spectrophotometer (Fullerton, CA, USA) at 490 nm. The results were calculated using a standard curve of glucose and were expressed in mg of glycogen/100 mg of wet weight of tissue.

Preparation of microsomes

Livers were collected from pentobarbital-anesthetized rats as described above. Liver samples were homogenized in 5-10 volumes of ice-cold 0.1 M potassium phosphate, pH 7.4, containing 1 mM EDTA and 0.15 M KCl, and then centrifuged (10,000 *g* for 20 min at 4°C) (Beckman JA 25.5 rotor). The resulting supernatant (S9) was subsequently centrifuged at 105,000 *g* (Beckman L5-75Ki rotor) for 60 min to obtain microsomes. The microsomal pellets were gently resuspended in phosphate buffer containing 20% glycerol and stored at -80°C (Haugen and Coon 1976; Khatsenko and Kikkawa 1997). The microsomal cytochrome P450 content was determined according to Omura and Sato (1964) using an absorption coefficient of 91 mM^{-1} cm^{-1} for P450. Spectral scans were obtained from 400 nm to 700 nm in a Beckman DU70 spectrophotometer.

Protein concentrations

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Enzymatic assays

The activities of CYP1A1/2 and CYP2B1/2 were determined using the substrates 7-ethoxyresorufin and 7-pentoxyresorufin (5 μM each), respectively, in a reaction mixture containing 0.1 M Tris-HCl, pH 8, and microsomal protein (50-200 μg). The reaction was started by adding NADPH (final concentration, 250 μM). The reaction rate was monitored by the increase in fluorescence using a SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA, USA), with excitation and emission at 530 nm and 580 nm, respectively (Burke and Mayer 1983; Burke et al. 1985). Activity was expressed in arbitrary fluorescence units/mg of protein/min.

The activity of CYP2C11 was measured by the 5-hydroxylation of tienilic acid (TA). Microsomes (0.2-1.5 mg of protein/ml) were suspended in 0.1 M phosphate buffer, pH 7.4, containing TA (0.1 mM). The reaction was started by adding an NADPH-generating system (10 mM glucose-6-phosphate, 1 mM NADP and 2 units of glucose-6-phosphate/ml) and the increase in absorbance at 390 nm was monitored for 30 min at 37°C (Neau et al. 1990). Activity was expressed as the increase in absorbance/mg of protein/min.

CYP2E1 activity was determined in a reaction mixture containing 0.2 M *p*-nitrophenol as substrate, microsomes (1.0-2.0 mg of protein/ml) and 500 μl of 0.1 mM phosphate buffer, pH 6.8. The reaction was initiated by adding NADPH (final concentration, 1 mM) at 37°C and was terminated after 30 min by adding 250 μl of 0.6 N perchloric acid followed by centrifugation (4000 rpm). NaOH (50 μl of a 1 M solution) was added to 500 μl of the resulting supernatant and the absorbance then read at 510 nm. The amount of 4-nitrocatechol released was determined from a

standard curve (Koop 1986) and the activity was expressed as nmol/mg of protein/min.

SDS-PAGE and immunoblotting

Aliquots (100 μg) of microsomes prepared as described above were electrophoresed (100 V constant) in 10% polyacrylamide gels in the presence of SDS (Laemmli 1970) and the proteins then electrotransferred (70 mA) to Hybond-P PVDF membranes (Towbin et al. 1979). The membranes were subsequently blocked by incubation with 5% milk for 2 h at room temperature, followed by three washes with buffer (0.01 M Tris-HCl, pH 6.8, 0.17 M NaCl, 0.02% Tween 20) prior to incubation with appropriate primary antibodies for each cytochrome P450 isoform and detection by chemioluminescence. The immunoreactive bands were subsequently scanned and analyzed by densitometry using Scion Image® software. The levels of cytochrome P450 were expressed as arbitrary densitometric units.

Statistical analysis

The results were expressed as the mean ± SD. Statistical comparisons were done using analysis of variance (ANOVA) followed by the Bonferroni test, with values of $P<0.05$ indicating significance.

Results

Arterial blood pressure measurements

Table 1 shows the body weights and arterial blood pressures of the various groups of rats studied. There were no significant differences among the body weights of the different groups. Similarly, there were no significant differences in the liver wet weight:body weight indicies (in %) (control rats: 3.9±0.1, 4.2±0.3 and 3.2±0.4 *versus* L-NAME-treated rats: 4.2±0.2, 4.2±0.2 and 4.3±0.6, for 2, 4 and 8 weeks, respectively; n=5-8 rats/group). Treatment with L-NAME significantly

elevated the blood pressure in the three intervals studied. This increase in blood pressure was attenuated by treatment with the ACE inhibitor enalapril or the AT_1 receptor antagonist losartan, neither of which alone affected blood pressure. None of the three enzymatic inducers (β-naphthoflavone, phenobarbital and pyrazole) had any significant effect on the basal or L-NAME-induced increase in blood pressure.

As confirmation of the efficacy of treatment with L-NAME, the activity of NOS in the liver of control and L-NAME-treated rats was measured. NOS activity was significantly inhibited (≥95%, $p<0.05$) in the latter animals compared to the controls (Controls: 3.50 ± 0.20, 2.92 ± 0.14 and 4.04 ± 0.48 *versus* L-NAME: 0.1 ± 0.06, 0.15 ± 0.05, and 0.1 ± 0.004 pmol/mg/min, for 2, 4 and 8 weeks, respectively; n=5 each).

Histological alterations and morphometric analysis

Figure 1 shows the histological appearance of liver parenchyma in control and L-NAME-treated rats. The hepatic acini of control rats showed a normal morphology in zones I (Fig. 1A), II (Fig. 1C) and III (Fig. 1E), with hepatocytes radially disposed in interconnected plates and liver sinusoids located among these plates. In contrast, the livers of L-NAME-treated rats showed a reduction in the sinusoidal capillary spaces and extensive "vacuolation" around the cell nucleus (Fig. 1B,D,F), particularly in zones I and II. The extensive hepatocyte perinuclear vacuolation, which probably resulted from the loss of accumulated glycogen during tissue processing (hepatic glycogen is elevated by treatment with L-NAME; see below), and the interstitial expansion, were generally prevented by concomitant treatment with enalapril or losartan (data not shown). In addition, non-polyhedric hepatocytes and random foci of cellular infiltrates without collagen deposition were also seen in various regions of the liver (Fig. 2).

Compared to control rats, treatment with L-NAME resulted in marked thickening of the muscular tunica of hepatic arterial vessels, with no significant

difference in the degree of hypertrophy among the intervals studied (Fig. 3). In addition, there was extensive perivascular fibrosis involving the deposition of collagen around portal vessels, as shown by staining with picrosirius red (Fig. 4). Both the vessel wall hypertrophy and the extracellular matrix deposition were completely prevented by concomitant treatment with enalapril or losartan (Figs. 3 and 4); neither of these drugs alone had any effect on vessel wall thickness (Fig. 3E) or collagen deposition (data not shown).

Hepatic lipid and glycogen content

The hepatic levels of HDL cholesterol (27.1±4.3, 28.0±12 and 38.8±11 mg/dl), LDL cholesterol (22.3±5.3, 19.7±5.7 and 25.0±12 g/l) and triglycerides (126±29, 154±20 and 152±13 mg/dl) in control rats after 2, 4 and 8 weeks, respectively, were not significantly altered by treatment with L-NAME (data not shown). In contrast, there was a significant increase in the hepatic glycogen content that was independent of the duration of treatment, and this increase was completely prevented by concomitant treatment with enalapril or losartan (Fig. 5).

Cytochrome P450 activities and expression

Figure 6 shows the activities of various CYP450 in control and L-NAME-treated rats. Treatment with L-NAME had no significant effect on the basal activities of the CYP isoforms, nor did it affect the induction of these activities in the various time intervals studied. This lack of effect was confirmed by western blotting, which showed that the expression of these enzymes was unaltered by L-NAME (Fig. 7).

Discussion

Our results show that the chronic treatment of rats with L-NAME caused hypertrophy of hepatic arterial vessel walls (tunica media), with perivascular fibrosis involving the deposition of collagen fibers. In addition, compactation of the sinusoidal lumen and interstitial expansion (increase in cellularity, mainly fibroblasts, and connective tissue in the portal space) were observed. The hypertrophy and fibrosis were similar to seen observed in other organs (brain, heart and kidney) in this model (Zatz and Baylis 1998; Jover and Mimran 2001; Hsieh et al. 2004).

The finding that the hepatic morphological alterations were attenuated by enalapril and losartan agrees with the enhanced activity of the renin-angiotensin system in this model and with the ability of ACE inhibitors and AT_1 receptor antagonists to protect against morphological damage in cardiac and renal tissue (Zatz and Baylis 1998; Pereira and Mandarim-de-Lacerda 2001; Sanada et al. 2001; Pereira et al. 2003). The vascular remodeling stimulated by angiotensin II occurs largely through the mitogenic activity of this peptide and is mediated by intracellular signaling molecules, including kinases such as ERK1/2 (Martens et al. 2002; Sanada et al. 2001, 2003), $p70^{S6K}$ (Minamino et al. 2000) and Rho kinase (Ikegaki et al. 2001; Kataoka et al. 2002). In agreement with these studies, Dupuis et al. (2004) have reported the enhanced expression of genes associated with the regulation of cellular proliferation, extracellular matrix remodeling, and NO/cGMP signaling in aortic tissue of rats treated with L-NAME for 15 or 30 days.

Treatment with L-NAME also significantly increased the hepatic glycogen content. This observation agrees with the findings that the inhibition of NOS by L-NAME in cultured hepatocytes increases the intracellular levels of glycogen (Donato et al. 2001), and that NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) inhibit the biosynthesis of glycogen (Sprangers et al. 1998) and may cause discreet inhibition of glycogenolysis stimulated by glucagon (Brass and Vetter 1993). In contrast, Hropot et al. (2003) observed that the treatment of rats with L-

NAME (25 mg/rat/day for six weeks) decreased the cardiac tissue content of glycogen, ATP and creatine phosphate, and Kitano et al. (2002) reported that NO had little effect on glycogen synthesis by hepatocytes. The inhibition of glycogen synthesis by NO involves a reduction in glycogen synthase activity (Sprangers et al. 1998) and in the expression (mRNA levels) of the glucose transporter GLUT-2 and of phosphoenolpyruvate carboxykinase (Casada et al. 1996). Inter-organ variation in the effect of NO on glycogen stores could be an important factor in determining the responses observed. In agreement with this idea, the effects of NO on glycogen metabolism in rat isolated liver (Borgs et al. 1996) and rat skeletal muscle (Young et al. 1997) may be stimulatory or inhibitory.

Concomitant treatment with enalapril or losartan prevented hepatocyte glycogen accumulation in L-NAME-treated rats, although neither of these inhibitors alone had any effect on the basal glycogen levels. This finding indicates that angiotensin II, the levels of which are elevated in this model (Zatz and Baylis 1998), modulates the accumulation of glycogen since the inhibition of ACE (leading to reduced angiotensin II formation) and the blockade of AT_1 receptors (preventing the binding of angiotensin II) protected against glycogen accumulation. The mechanism by which this occurs is unclear, particularly considering that angiotensin II generally stimulates glycogenolysis (Hothi et al. 1988; Machado et al. 1995). In cardiac tissue, ACE inhibition (Hropot et al. 1994) and AT_1 blockade (Hropot et al. 2003) protect against the decrease in glycogen, ATP and creatine phosphate seen following treatment with L-NAME.

Treatment with L-NAME had no significant effect on the hepatic cholesterol and triglyceride levels when compared to control rats. These findings agree with those of Khedara et al. (1996) who also reported no significant variations in the hepatic content of triglycerides, phospholipids and total cholesterol in rats fed with L-N^{ω}-nitroarginine (L-NNA) in their diet (0.2 g/kg for five weeks). However, these authors observed an increase in serum triglyceride levels and body fat in rats fed this L-NNA-enriched diet, with a reduction in the activity of hepatic carnitine palmitoyltransferase but no effect on glucose-6-phosphate dehydrogenase and

fatty acid synthase (Khedara et al. 1996, 1999). In contrast, Zheng et al. (2002) reported that parenterally fed rats treated with L-NAME for seven days had increased hepatic triglyceride and cholesterol levels. In rabbits fed with a semipurified, cholesterol-free, casein diet, NO donors such as sodium nitroprusside prevented an increase in LDL cholesterol but did not alter hepatic lipid levels after four weeks (Kurowska and Carroll 1998). In agreement with this, NO-donating morpholine derivatives can act as hypolipemic and antioxidant agents by inhibiting lipid peroxidation and reducing cholesterol (especially LDL) and triglyceride levels (Chrysselis et al. 2002). The mechanisms by which NO modulates lipid accumulation in hepatic tissue have not been fully elucidated but probably involve the stimulation of soluble guanylate cyclase with the formation of cGMP and subsequent activation of a cGMP-dependent protein kinase (PKG), and the mobilization of intracellular calcium (Garcia-Villafranca et al. 2003).

NO can modulate the activity of CYP-450 by forming complexes with the iron in the heme moiety of these enzymes (Ebel et al. 1975), and NO released from NO donors, such as NOC7, sodium nitroprusside and SNAP inhibits CYP450 (Donato et al. 1997; Gergel et al. 1997). In agreement with this finding, endogenous NO formation contributes to the rapid loss of CYP-450 activity in cultured hepatocytes (López-García 1998), and enhanced NO production accounts for the inhibition of CYP-450 expression and activity in response to cytokines (TNF-α, IL-1β and IFN-γ) (Stadler et al. 1994; Carlson and Billings 1996; Donato et al. 1997) and *Escherichia coli* lipopolysaccharide (LPS) (Takemura et al. 1995) *in vitro*. The inhibitory action of NO on CYP-450 seen *in vitro* (Stadler et al. 1994; Carlson and Billings 1996; Donato et al. 1997) and *in vivo* (Müller et al. 1996; Khatsenko and Kikkawa 1997) can be prevented or reversed by NOS inhibitors such as L-NAME and L-N^G-monomethyl-arginine (L-NMMA).

Whereas various studies have investigated the importance of NO formation on CYP-450 expression and activities *in vitro* (Stadler et al. 1994; Takemura et al. 1995; Carlson and Billings 1996; Donato et al. 1997) and *in vivo* (Sewer and Morgan 1998; Sewer et al. 1998) in response to stimuli such as cytokines and LPS,

few reports have examined the possible influence of a long-term reduction in NO availability on CYP450 expression and activity. As shown here, treatment with L-NAME for 2-8 weeks did not alter the basal or drug-induced expression and activities of the CYP450 isoforms in any of the intervals studied. These observations indicate that a prolonged reduction in NO availability does not adversely affect basal CYP450 activities or the ability of the liver to respond to enzyme inducers. These findings also agree with studies indicating that elevated rather than reduced levels of NO are important in regulating CYP450s *in vitro* and *in vivo*.

In conclusion, the chronic inhibition of NO biosynthesis in rats resulted in hepatic vascular alterations similar to those seen in cardiac, cerebral and renal tissues, and were mediated largely by angiotensin II. In addition, a prolonged reduction in NO formation enhanced hepatic glycogen levels but had no adverse effect on the basal and induced expression and activities of selected CYP450 isoforms.

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Figure legends

Figure 1. Histological alterations in hepatic tissue of L-NAME-treated rats. L-NAME (20 mg/rat/day, p.o.) was administered in the drinking water for two weeks. Control rats received water without inhibitor. The panels show the appearance of hepatic zones I (periportal zone), II (intermediary zone) and III (centrolobular zone) in control (**a,c,e**) and L-NAME-treated (**b,d,f**) rats. Note the loss of the hepatic acinar architecture and the polyhedral aspect of hepatocytes, mainly in zones I and II. Note also the marked perinuclear unstained areas or "vacuolation" (resulting from increased glycogen storage) in hepatocytes of zones I and II, the parenchymal compactness resulting from collapsed sinusoidal capillaries, and the poorly visible hepatic plates. Similar changes were seen in rats treated with L-NAME for four and eight weeks. In all cases, the L-NAME-induced alterations were reverted by concomitant treatment with enalapril (25 mg/kg/day, p.o.) or losartan (30 mg/kg/day, p.o.) (not shown). HE. Bar corresponds to 40 µm.

Figure 2. Focal cellular infiltration (arrow) in the portal space (zone I) after treatment with L-NAME for four weeks (HE). Staining with picrosirius showed that this infiltration was not accompanied by extracellular matrix (collagen fiber) deposition (not shown). Bar corresponds to 12.5 µm.

Figure 3. Micrograph of a portal space showing arterial vessel wall hypertrophy induced by L-NAME. Panels **a-d** show liver sections from control rats (**a**) and rats treated with L-NAME (LN, 20 mg/rat/day, p.o., four weeks) (**b**), LN + enalapril (Enal, 25 mg/kg/day, p.o.) (**c**) and LN + losartan (Los, 30 mg/kg/day, p.o.) (**d**). Note that treatment with enalapril and losartan reverted the vessel wall hypertrophy, which affected mainly the endothelium and muscle fibers (the tunica intima and tunica media, respectively). Similar changes were seen in rats treated with L-NAME for two and eight weeks. HE. Bar corresponds to 12.5 µm. Vessel wall

thickness was expressed as the ratio of the total vessel cross-sectional area divided by the vessel lumen cross-sectional area (**e**). The columns represent the mean ± SD of 50 vessels from five rats/group. *p<0.001 compared to the corresponding controls (ANOVA followed by the Bonferroni test).

Figure 4. Perivascular deposition of collagen in L-NAME-treated rats. Panels **a-d** show liver sections from control rats (**a**) and rats treated with L-NAME (LN, 20 mg/rat/day, p.o., four weeks) (**b**), LN + enalapril (25 mg/kg/day, p.o.) (**c**) and LN + losartan (30 mg/kg/day, p.o.) (**d**). Note the extensive collagen fiber deposition in LN-treated rats and that treatment with enalapril and losartan prevented this phenomenon. Similar changes were seen in rats treated with L-NAME for two and eight weeks. Picrosirius staining. Bar corresponds to 150 μm.

Figure 5. Glycogen accumulation in the liver of L-NAME-treated rats. Rats were treated with L-NAME, enalapril and losartan as described in Figure 3 and glycogen was quantified as described in Methods. The columns represent the mean ± SD of five rats/group. *p<0.001 compared to the corresponding controls (ANOVA followed by the Bonferroni test).

Figure 6. Cytochrome P450 activities in control and L-NAME-treated rats. The rats were treated with L-NAME (20 mg/kg/day, p.o.) as described in Methods. In some experiments, the rats were also treated concomitantly with phenobarbital (Pheno; 80 mg/kg/day each, i.p., 4 d; to induce CYP2B1/2, β-naphthoflavone (β-Naphtho; 80 mg/kg/day each, i.p., 4 d; to induce CYP1A1/2) or pyrazole (Pyraz; 200 mg/kg/day, i.p., 2 d) (to induce CYP2E1). Note that CYP2C11 activity (constitutive) is shown only for control and L-NAME-treated rats without inducers The columns represent the mean ± SD of six rats/group. *p<0.001 compared to the corresponding controls. AFU, arbitrary fluorescence units.

Figure 7. Cytochrome P450 expression in control and L-NAME-treated rats. The rats were treated with L-NAME (20 mg/kg/day, p.o.) and the livers then processed for western blotting as described in Methods. One hundred micrograms of protein were applied to each lane. The columns represent the mean ± SD of five rats/group. There was no significant differences in the expression levels of the various isoforms between the two groups in a given treatment interval.

Table 1: Body weights and arterial blood pressure measurements in the different groups of rats studied.

		Body weight (g)			Systolic blood pressure (mm Hg)		
Treatment	n	2	4	8	2	4	8
Control	8	233 ± 15	280 ± 20	302 ± 10	118 ± 9	120 ± 9	117 ± 7
L-NAME	8	245 ± 16	273 ± 33	305 ± 36	168 ± 10*	171 ± 13*	189 ± 10*
Losartan	5	224 ± 15	270 ± 29	339 ± 27	127 ± 5	117 ± 9	108 ± 8
L-NAME + Losartan	5	218 ± 10	272 ± 29	317 ± 16	143± 10*,#	142 ± 6*,#	129 ± 10
Enalapril	5	228 ± 16	260 ± 14	308 ± 3	125 ± 9	115 ± 5	105 ± 5
L-NAME+ Enalapril	5	232 ± 21	272 ± 22	303 ± 9	140 ± 4*,#	141 ± 6*,#	123 ± 7
Phenobarbital	6	218 ± 7	269 ± 15	369 ± 12	118 ± 8	119 ± 7	109 ± 9
L-NAME + Phenob	6	225 ± 11	250 ± 10	335 ± 12	166 ± 13*	172 ± 9*	183 ± 8*
Pyrazole	6	244 ± 6	276 ± 19	359 ± 17	116 ± 9	113 ± 8	122 ± 6
L-NAME +Pyrazole	6	220 ± 11	262 ± 22	345 ± 27	175 ± 6*	175 ± 7*	183 ± 9*
β-Naphthoflavone	6	244 ± 4	287 ± 11	351 ± 11	125 ± 9	124 ± 9	123 ± 6
L-NAME + β-Naphtho	6	247 ± 6	255 ± 8	350 ± 18	158 ± 18*	177 ± 12*	182 ± 11*

The values are the mean ± SD of the number of rats (n) indicated. *$p<0.05$ compared to the corresponding control group. #$p<0.05$ compared to L-NAME alone.

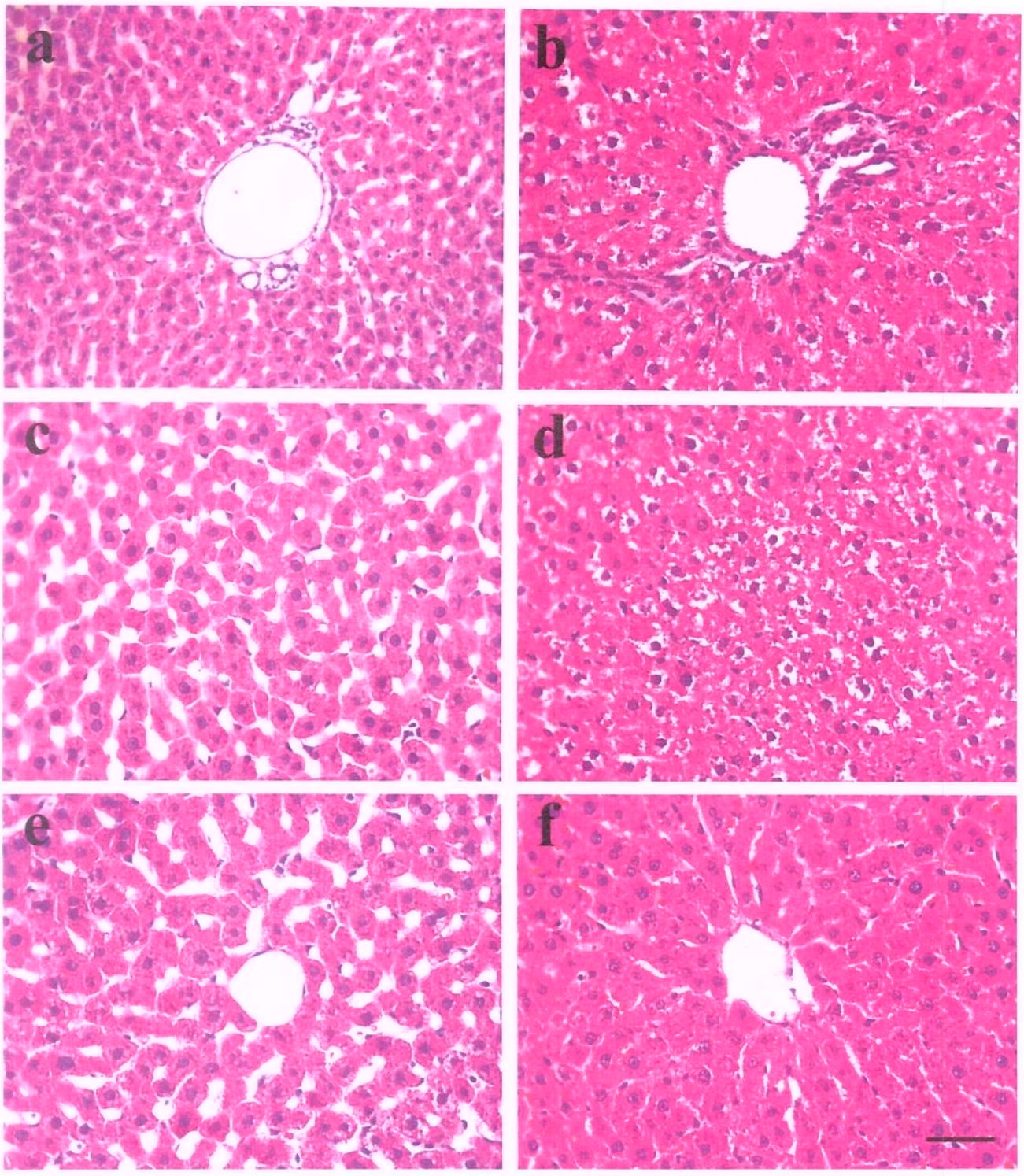


Figure 1

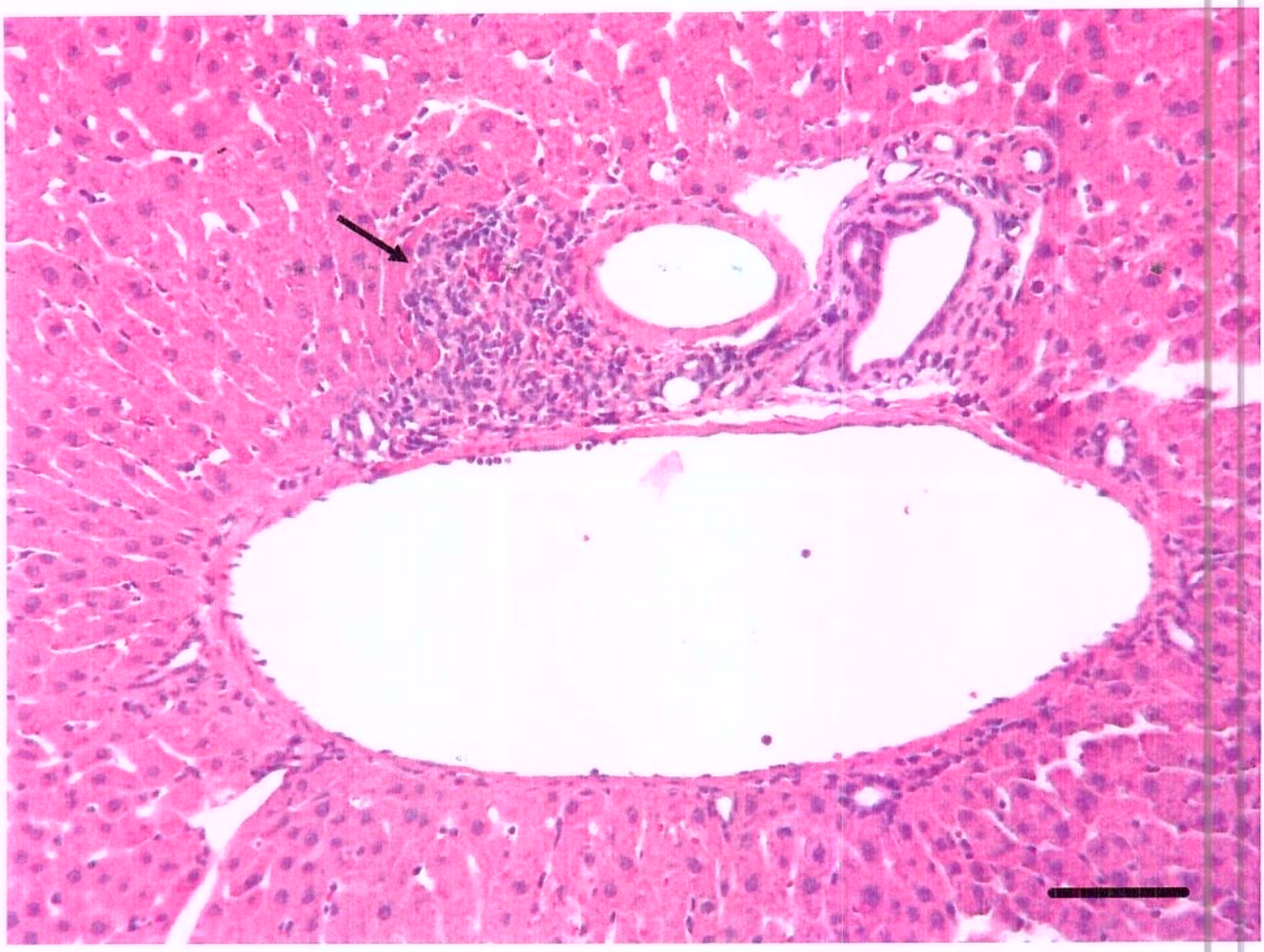
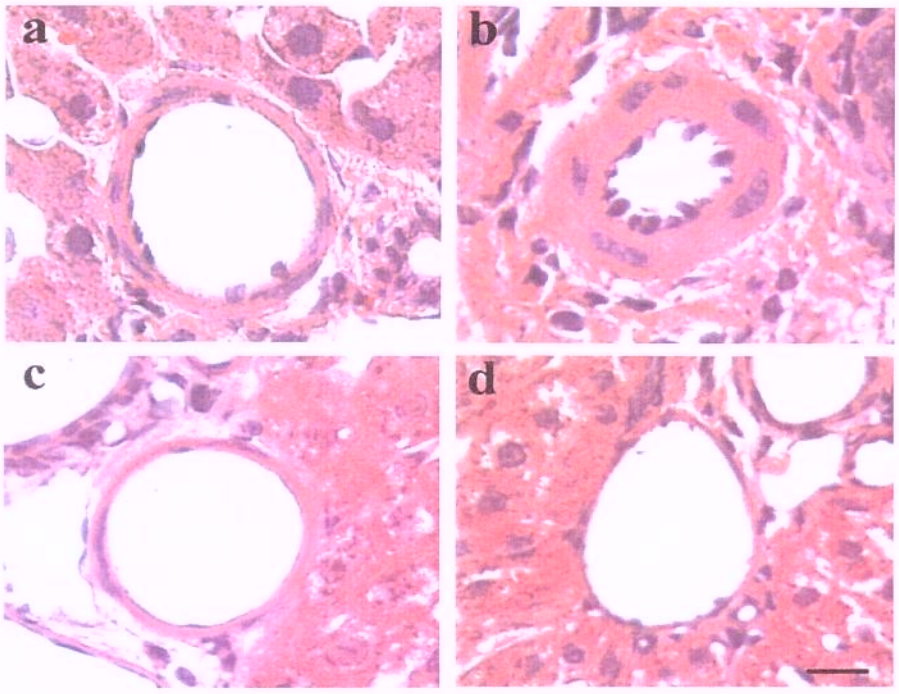


Figure 2


a
b
c
d

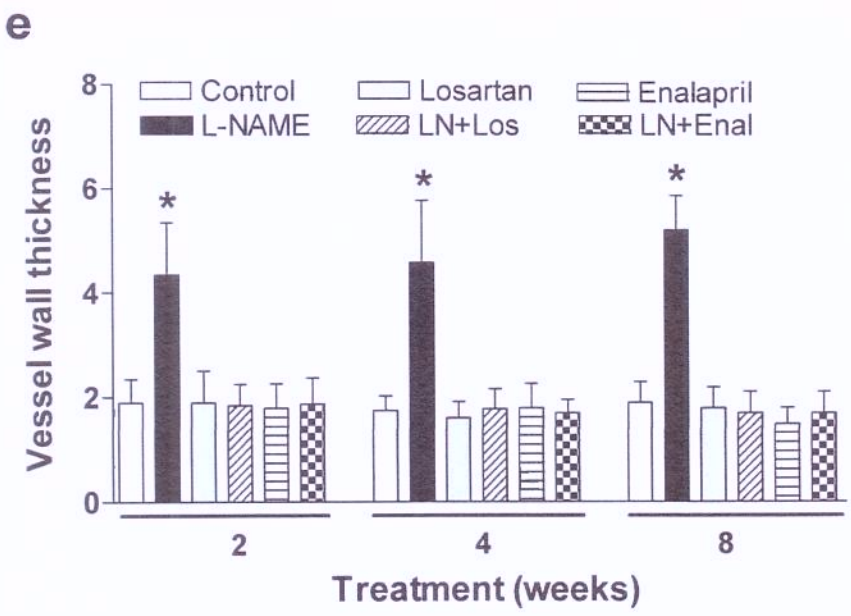

e
Control
L-NAME
Losartan
LN+Los
Enalapril
LN+Enal
Vessel wall thickness
0
2
4
6
8
*
*
*
2
4
8
Treatment (weeks)

Figure 3

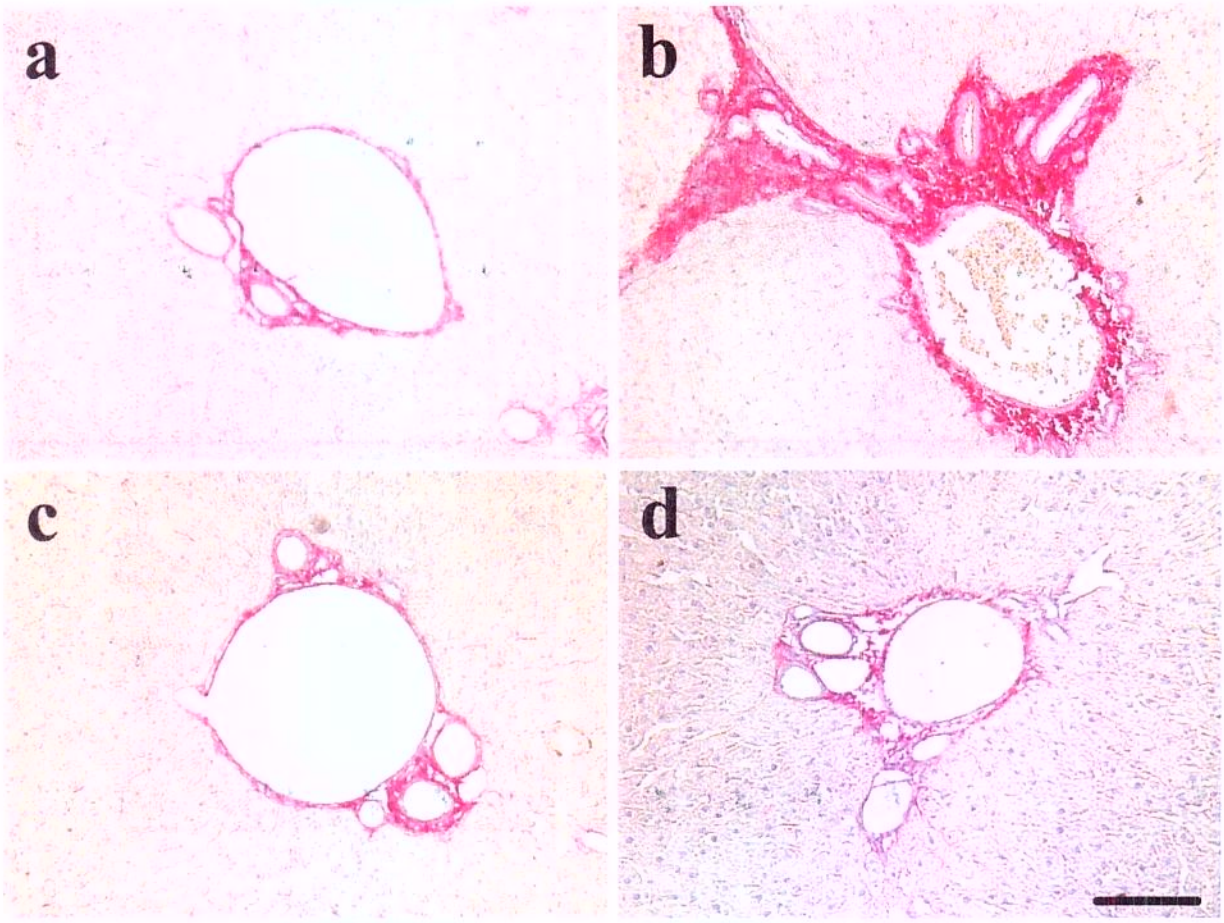


Figure 4

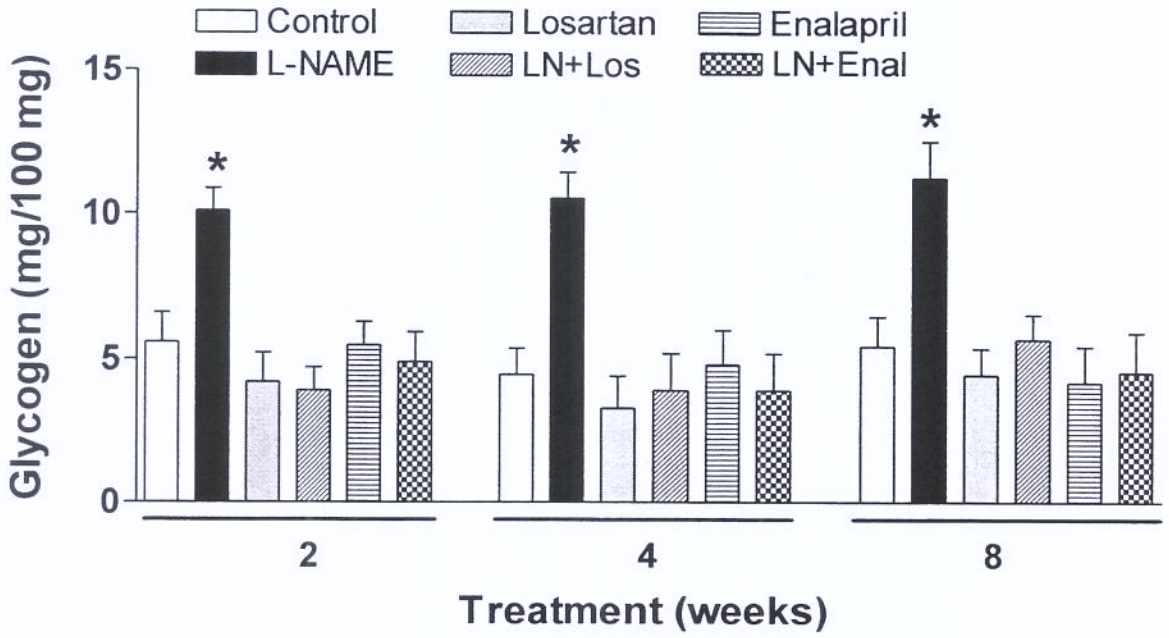

Control
L-NAME
Losartan
LN+Los
Enalapril
LN+Enal
Glycogen (mg/100 mg)
15
10
5
0
*
*
*
2
4
8
Treatment (weeks)

Figure 5

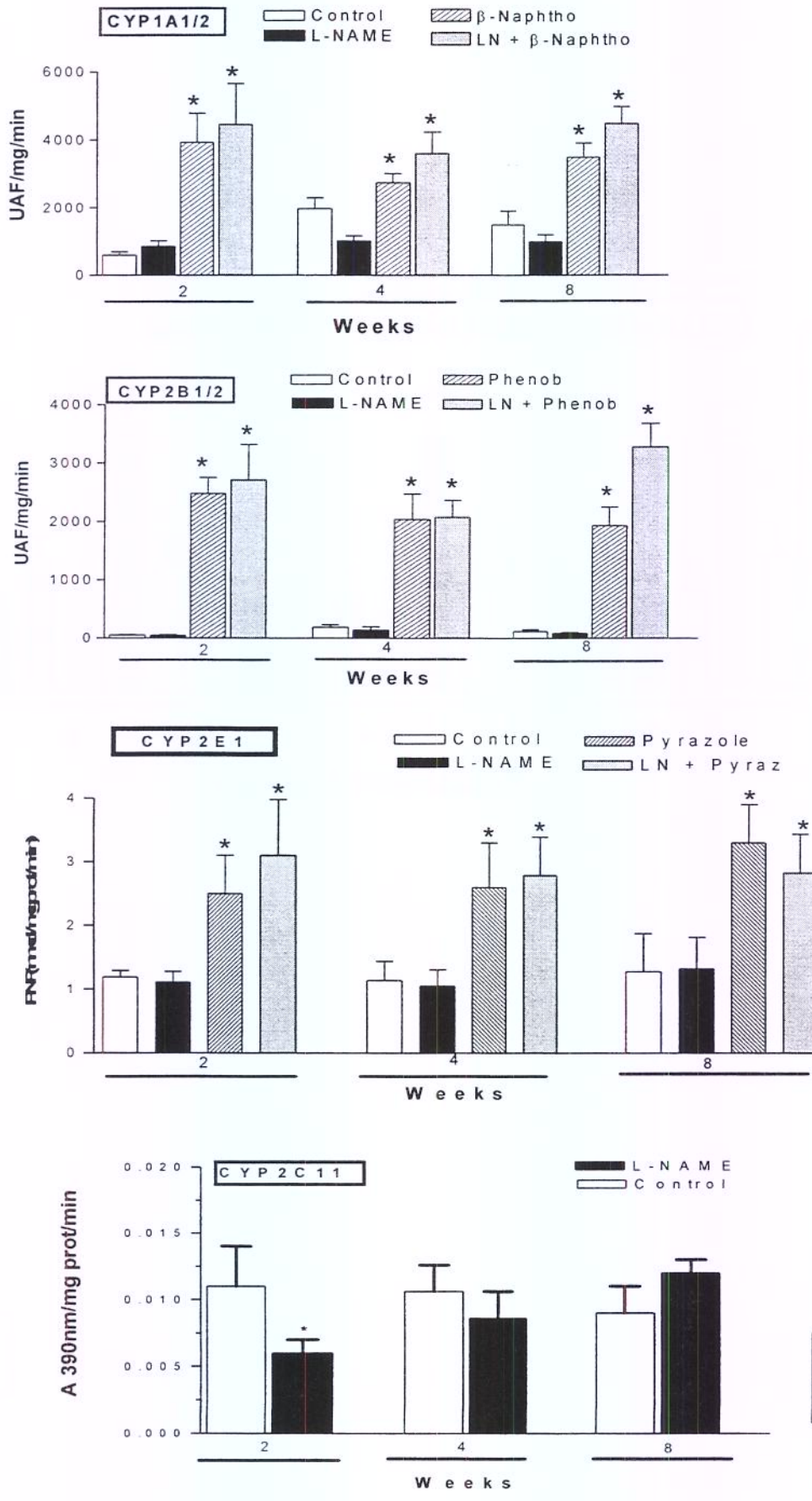

CYP1A1/2
Control
L-NAME
β-Naphtho
LN + β-Naphtho
UAF/mg/min
6000
4000
2000
0
2
4
8
Weeks
CYP2B1/2
Control
L-NAME
Phenob
LN + Phenob
UAF/mg/min
4000
3000
2000
1000
0
2
4
8
Weeks
CYP2E1
Control
L-NAME
Pyrazole
LN + Pyraz
4
3
2
1
0
2
4
8
Weeks
CYP2C11
L-NAME
Control
A 390nm/mg prot/min
0.020
0.015
0.010
0.005
0.000
2
4
8
Weeks

Figure 6

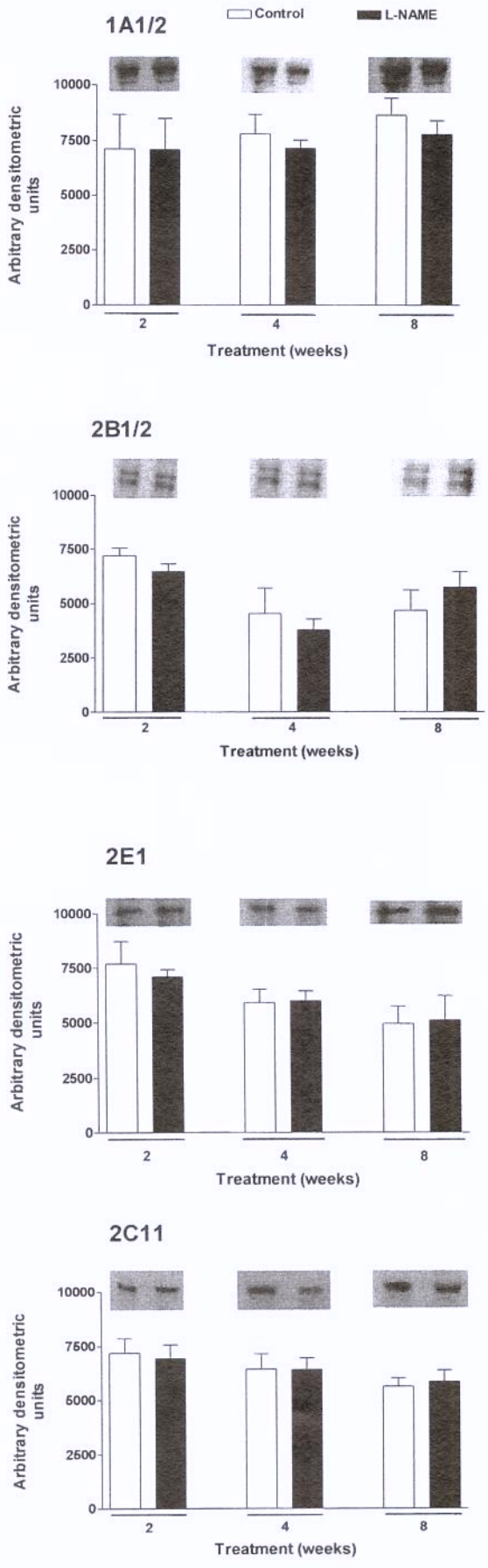

1A1/2
Control
L-NAME
Arbitrary densitometric units
10000
7500
5000
2500
0
2
4
8
Treatment (weeks)
2B1/2
Arbitrary densitometric units
10000
7500
5000
2500
0
2
4
8
Treatment (weeks)
2E1
Arbitrary densitometric units
10000
7500
5000
2500
0
2
4
8
Treatment (weeks)
2C11
Arbitrary densitometric units
10000
7500
5000
2500
0
2
4
8
Treatment (weeks)

Figure 7

Trabalho em redação

"SHORT-TERM TREATMENT WITH L-NAME AFFECTS VASCULAR MORPHOLOGY, GLYCOGEN CONTENT AND MATRIX METALLOPROTEINASE EXPRESSION IN RAT LIVER"

SHORT-TERM TREATMENT WITH L-NAME AFFECTS VASCULAR MORPHOLOGY, GLYCOGEN CONTENT AND MATRIX METALLOPROTEINASE EXPRESSION IN RAT LIVER

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Running title: Hepatic alterations in L-NAME-treated rats

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Abstract

The short-term inhibition (≤8 days) of nitric oxide (NO) biosynthesis by treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of NO synthase, produces hypertension and alters the expression of some proteins and receptors. In this study, we examined the effect of short-term treatment with L-NAME on rat liver morphology, glycogen and lipid content, matrix metalloproteinase (MMP-2 and MPP-9) expression, and cytochrome P450 (CYP1A1/2, CYP2B1/2, CYP2C11 and CYP2E1) activities and expression. Male Wistar rats received L-NAME (20 mg/rat/day, administered in the drinking water) for four and eight days after which the livers were removed and processed for analysis. Treatment with L-NAME significantly elevated the blood pressure and caused vascular hypertrophy in hepatic arteries, with perivascular and interstitial fibrosis involving collagen deposition. All of these changes were reversed by concomitant treatment with enalapril (25 mg/kg/day) or losartan (30 mg/kg/day). Treatment with L-NAME enhanced the expression of MMP-2 and MMP-9 in vessel walls, but did not affect the hepatic content of cholesterol or triglycerides, or the basal or drug (phenobarbital, β-naphthoflavone or pyrazole)-induced activities and protein expression of cytochrome P450 isoforms. These results show that the short-term inhibition of NO biosynthesis produces selective changes in hepatic morphology and some biochemical parameters. The ability of enalapril and losartan to attenuate these effects indicates involvement of the renin-angiotensin system.

Keywords: angiotensin-converting enzyme, chronic inhibition, cytochrome P450, hypertension, liver, nitric oxide, vascular hypertrophy

1. Introduction

Numerous studies have shown that the prolonged inhibition of nitric oxide (NO) biosynthesis leads to hypertension and a variety of morphological alterations in various structures, particularly the vasculature (Arnal *et al.,* 1992; Baylis *et al.,* 1992; Ribeiro *et al.,* 1992; see Zatz and Baylis, 1998, and Jover and Mimran, 2001). The cellular effects observed include infarction, necrosis and apoptosis (Moreno *et al.,* 1995, 1997; Takemoto *et al.,* 1997a,b; Pechánová and Bernátová, 1996; Pereira and Mandarim-de-Lacerda, 2001; Pessanha and Mandarim-de-Lacerda, 2000), as well as an increase in arterial wall thickness and left ventricular hypertrophy with tissue remodeling (Kristek *et al.,* 1996; Takemoto *et al.,* 1997a,b) and angiogenesis (Okruhlicová *et al.,* 2000). In renal tissue, there is glomerular ischemia with glomerulesclerosis, interstitial fibrosis (involving the infiltration by fibroblasts and deposition of collagen) and vascular lesions that may result in glomerular collapse (Baylis *et al.,* 1992; Ribeiro *et al.,* 1992; Fujihara *et al.,* 1994; Yamada *et al.,* 1996; Zatz and Baylis, 1998; Jover and Mimran, 2001; Hsieh *et al.,* 2004). Many of these alterations, particularly the arterial wall thickness and left ventricular hypertrophy, are mediated to a large extent by the renin-angiotensin system (RAS) since treating rats with angiotensin-converting enzyme (ACE) inhibitors such as captopril and enalapril, or with angiotensin II AT_1 receptor antagonists such as losartan restores blood pressure to near normal levels (Zatz and Baylis, 1998; Jover and Mimran, 2001).

Some studies have also examined the physiological effects of short-term NO inhibition. Usui *et al.* (1998) reported an increase in the mRNA levels and number of AT_{1A} and AT_{1B} receptors (but not of AT_2 receptors) for angiotensin II in adrenal gland after one week of treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME). Katoh *et al.* (1998) described a similar phenomenon for AT_1 and AT_2 receptors in cardiac tissue of rats after one week of treatment with L-NAME, with the changes disappearing after four weeks of treatment. In addition, treatment with L-NAME for up to one week results in augmented inflammatory responses, including enhanced monocyte adhesion to the endothelium, infiltration of monocytes, increased

expression of monocyte chemoattractant protein (MCP-1), P-selectin, intracellular adhesion molecule (ICAM)-1 and TGF-β_1, vascular endothelial growth factor (VEGF), and type I collagen in cardiac tissue and blood vessels (Tomita *et al.*, 1998a,b; Koyanagi *et al.*, 2000a,b; Zhao *et al.*, 2002; Kataoka *et al.*, 2004).

Since NO has an important role in modulating hepatic vascular tone and in cytoprotection (Alexander, 1998; Taylor *et al.*, 1998; Clemens, 1999; Muriel, 2000), and since NO can influence the expression of numerous genes (Pfeilschifter *et al.*, 2001; Piz and Casteel, 2003), enzyme activities (Takemoto *et al.*, 1997a,b; Khatsenko, 1998; Gonzalez *et al.*, 2000) and intracellular signaling molecules (Minamino *et al.*, 2000; Martens *et al.*, 2002; Ito *et al.*, 2004), we have investigated the effect of inhibiting NO biosynthesis for up to eight days on liver morphology, glycogen and lipid content, matrix metalloproteinase (MMP-2 and MMP-9) expression, and the activities and expression of selected cytochrome P450 isoforms (CYP1A1/2, CYP2B1/2, CYP2C11 and CYP2E1).

2. Material and methods

2.1. Reagents

L-arginine, L-citrulline, L-aminoguanidine, bovine serum albumin, calmodulin, dithiothreitol, D-glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate disodium hydrate, ethoxyresorufin, FAD (flavine-adenine-dinucleotide), FMN (flavine-adenine-mononucleotide), NADP (β-nicotinamide adenine dinucleotide phosphate, sodium salt), NADPH (β-nicotinamide adenine dinucleotide phosphate), β-naphthoflavone, 4-nitrocatechol, *p*-nitrophenol, N^{ω}-nitro-L-arginine methyl ester (L-NAME), 7-pentoxyresorufin and pyrazole were from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, ammonium persulfate, [U-^{14}C]arginine, Coomassie brilliant blue R250, donkey anti-mouse IgG-peroxidase conjugate, glycerol, Hybond-P PVDF membrane (0.45 μm), *N,N'*-methylene bis-acrylamide, sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethylenediamine (Temed) and Tris base were from Amersham Biosciences (Piscataway, NJ, USA). Anti-mouse IgG monoclonal antibodies to CYP1A1/2, CYP2B1/2, CYP2C11 and

CYP2E1 were from Oxford Biomedicals (Oxford, OH, USA). Tienilic acid was a generous gift from Dr. Patrick M. Dansete (Laboratoire de Chemie et de Biochime Pharmacologiques et Toxicologiques, Paris, França). Flat-bottomed 96-well plates were from Corning (Corning, MA, USA), chemiluminescence kits (SuperSignal, West Pico) were from Pierce Chemicals (Rockford, IL, USA) and photographic film was from Kodak (São José dos Campos, SP, Brazil). Sodium pentobarbital (Hypnol®) was obtained from Cristália Indústria Farmacêutica (Itapira, SP, Brazil). Formalin (10%) and solvents for histology were from Synth (São Paulo, SP, Brazil). Paraffin (Histosec), hematoxylin, eosin, periodic acid-Schiff reagent and entellan were from Merck (Rio de Janeiro, RJ, Brazil). Kits for the quantification of HDL cholesterol and triglycerides were from Laborlab (São Paulo, SP, Brazil) and those for LDL cholesterol were from Wiener (Rosario, Argentina). Other reagents of analytical grade were obtained from Baker or Merck.

2.2. *Animals*

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

2.3. *Treatment with L-NAME and blood pressure measurements*

Rats were treated with L-NAME (20 mg/rat/day) given in their drinking water (Ribeiro *et al.*, 1992) for four and eight days. The amount of L-NAME ingested was calculated based on the water intake of the rats which was monitored daily. Control rats received tap water alone. The rats were weighed and tail blood pressure was measured by a tail-cuff method (Kent Instruments).

In some experiments, rats treated with L-NAME also received losartan (30 $mg.kg^{-1}.day^{-1}$) or enalapril (25 $mg.kg^{-1}.day^{-1}$) to block angiotensin II AT_1 receptors

and inhibit ACE activity, respectively. These inhibitors were given in the drinking water together with L-NAME (Hropot *et al.*, 2003; Pereira *et al.*, 2003).

2.4. Cytochrome P450 induction

To induce selected cytochrome P450s, control and L-NAME-treated rats received phenobarbital (to induce CYP2B1/2), β-naphthoflavone (to induce CYP1A1/2) (80 mg/kg/day each, i.p., 4 d) (Suzuki *et al.*, 1992) or pyrazole (200 mg/kg/day, i.p., 2 d) (to induce CYP2E1) (Clejan and Cederbaum, 1992) prior to sacrificing.

2.5. NOS activity

Liver NOS activity was measured by the method of Rees *et al.* (1995), as described by Ribeiro Jr. *et al.* (1999) using [^{3}H]L-arginine as substrate. Enzyme activity was expressed as pmol of [^{3}H]L-citrulline formed/mg of protein/min.

2.6. Histological analysis

At the end of each period of treatment, the rats were anesthetized with sodium pentobarbital (>50 mg/kg, i.p; Hypnol®) and perfused via the aorta with heparinized saline to remove blood from the liver. The liver was then excised and fixed in 10% formalin for 24 h. Fragments of liver were subsequently embedded in paraffin and sections 5 μm thick were cut on a Leica microtome and stained with hematoxylin-eosin or picrosirius red. The stained sections were examined by light microscopy using a Nikon Eclipse E800 microscope and images were captured and processed with Image ProPlus software. Arterial wall thickness was expressed as the ratio of the vessel total cross-sectional area divided by the vessel lumen cross-sectional area (10 vessels/rat, 5 rats/group; total of 50 vessels/group) (Borzychowski *et al.*, 2003). All measurements and analyses were done using the microscope and software described above.

2.7. Determination of hepatic lipid and glycogen content

Pentobarbital-anesthetized rats were perfused with heparinized saline and the livers were collected and frozen in liquid nitrogen prior to storage at -80°C until used. Hepatic lipids were extracted by the method of Folch *et al.* (1957) and the concentrations of triglycerides, HDL-cholesterol and LDL-cholesterol were measured using commercial kits.

The hepatic glycogen content was determined according to Lo *et al.* (1970), with minor modifications. Liver samples were weighed and immersed for 30 min at 100°C in 1 ml of 30% KOH saturated with Na_2SO_4. Ethanol (95%) (1.5 ml) was added to precipitate the glycogen from the alkaline digest. The samples were then centrifuged and the supernatants were carefully aspirated. The pellets were resuspended in 1 ml of distilled water containing phenol (5%) and concentrated H_2SO_4, and the resulting absorbance was read in a Beckman spectrophotometer (Fullerton, CA, USA) at 490 nm. The results were calculated using a standard curve of glucose and were expressed in mg of glycogen/100 mg of wet weight of tissue.

2.8. Preparation of microsomes

Livers were collected from pentobarbital-anesthetized rats as described in section 2.7. Samples of liver tissue were homogenized in 5-10 volumes of ice-cold 0.1 M potassium phosphate, pH 7.4, containing 1 mM EDTA and 0.15 M KCl, and then centrifuged (10,000 *g* for 20 min at 4°C) (Beckman JA 25.5 rotor). The resulting supernatant (S9) was then centrifuged at 105,000 *g* (Beckman L5-75Ki rotor) for 60 min to obtain the microsomes. The microsomal pellets were gently resuspended in phosphate buffer containing 20% glycerol and stored at -80°C (Haugen and Coon, 1976; Khatsenko and Kikkawa, 1997). The microsomal cytochrome P450 content was determined according to Omura and Sato (1964a,b) using an absorption coefficient of 91 $mM^{-1}cm^{-1}$ for P450. Spectral scans were obtained from 400 nm to 700 nm in a Beckman DU70 spectrophotometer.

2.9. Protein concentrations

Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

2.10. Enzymatic assays

The activities of CYP1A1/2 and CYP2B1/2 were determined using the substrates 7-ethoxyresorufin and 7-pentoxyresorufin (5 μM each), respectively, in a reaction mixture containing 0.1 M Tris-HCl, pH 8, and microsomal protein (50-200 μg). The reaction was started by adding NADPH (final concentration, 250 μM). The reaction rate was monitored by the increase in fluorescence using a SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA, USA), with excitation and emission at 530 nm and 580 nm, respectively (Burke and Mayer, 1983; Burke *et al.*, 1985). Activity was expressed in arbitrary fluorescence units/mg of protein/min.

The activity of CYP2C11 was measured by the 5-hydroxylation of tienilic acid (TA). Microsomes (0.2-1.5 mg of protein/ml) were suspended in 0.1 M phosphate buffer, pH 7.4, containing TA (0.1 mM). The reaction was started by adding an NADPH-generating system (10 mM glucose-6-phosphate, 1 mM NADP and 2 units of glucose-6-phosphate/ml) and the increase in absorbance at 390 nm was monitored for 30 min at 37°C (Neau *et al.*, 1990). Activity was expressed as the increase in absorbance/mg of protein/min.

CYP2E1 activity was determined in a reaction mixture containing 0.2 M *p*-nitrophenol as substrate, microsomes (1.0-2.0 mg of protein/ml) and 500 μl of 0.1 mM phosphate buffer, pH 6.8. The reaction was initiated by adding NADPH (final concentration, 1 mM) at 37°C and was terminated after 30 min by adding 250 μl of 0.6 N perchloric acid followed by centrifugation (4000 rpm). NaOH (50 μl of a 1 M solution) was added to 500 μl of the resulting supernatant and the absorbance then read at 510 nm. The amount of 4-nitrocatechol released was determined from a

standard curve (Koop, 1986) and the activity was expressed as nmol/mg of protein/min.

2.11. SDS-PAGE and immunoblotting

Aliquots (100 μg) of microsomes prepared as described above were electrophoresed (100 V constant) in 10% polyacrylamide gels in the presence of SDS (Laemmli, 1970) and the proteins then electrotransferred (70 mA) to Hybond-P PVDF membranes (Towbin *et al.,* 1979). The membranes were subsequently blocked by incubation with 5% milk for 2 h at room temperature, followed by three washes with buffer (0.01 M Tris-HCl, pH 6.8, 0.17 M NaCl, 0.02% Tween 20) prior to incubation with appropriate primary antibodies for each cytochrome P450 isoform and detection by chemioluminescence. The immunoreactive bands were subsequently scanned and analyzed by densitometry using Scion Image® software. The levels of cytochrome P450 were expressed as arbitrary densitometric units.

2.12. Immunohistochemical detection of MMP-2 and MMP-9

Immunohistochemistry for the detection of MMP-2 and MMP-9 was done as described by Nikkari *et al.* (1996), using tissue sections prepared as described in section 2.6, with primary antibodies against rat MMP-2 (diluted 1:150) and MMP-9 (diluted 1:100) (NeoMarkers, LabVision, USA). A peroxidase-conjugated secondary antibody (diluted 1:500; Amersham) and (DAB) were used to detect the MMP antibodies. The labeled sections were viewed and documented as described in section 2.6.

2.13. Statistical analysis

The results were expressed as the mean ± SD. Statistical comparisons were done using analysis of variance (ANOVA) followed by the Bonferroni test, with values of $P<0.05$ indicating significance.

3. Results

3.1. Arterial blood pressure measurements

Table 1 shows the body weights and arterial blood pressures of the various groups of rats studied. There were no significant differences among the body weights of the different groups. Treatment with L-NAME significantly elevated the blood pressure in the two intervals studied. This increase in blood pressure was reversed by treatment with the ACE inhibitor enalapril or the AT_1 receptor antagonist losartan, neither of which alone affected blood pressure. None of the three enzymatic inducers (β-naphthoflavone, phenobarbital and pyrazole) had any effect on the basal or L-NAME-induced increase in blood pressure. Treatment with L-NAME significantly ($p<0.05$) inhibited the NOS activity of hepatic tissue by ~90% (data not shown).

3.2. Histological alterations and morphometrical analysis

Figure 1 shows the histological appearance of liver parenchyma in control and L-NAME-treated rats. The hepatic acini of control rats showed a normal morphology in zones I (Fig. 1A), II (Fig. 1B) and III (Fig. 1C), with hepatocytes radially disposed in interconnected plates and interspersed with hepatic sinusoids. The livers of L-NAME-treated rats showed a reduced sinusoidal capillary space and extensive "vacuolation" around the hepatocyte nucleus (Fig. 1D,E,F). The extensive hepatocyte perinuclear vacuolation and the interstitial expansion were generally prevented by concomitant treatment with enalapril or losartan (data not shown).

Compared to control rats, treatment with L-NAME resulted in marked hepatic arterial vessel wall hypertrophy, with no significant difference in the degree of hypertrophy among the intervals studied (Fig. 3). In addition, there was extensive perivascular fibrosis involving the deposition of collagen around portal vessels, as shown by staining with picrosirius red (Fig. 2). The vessel wall

hypertrophy and the extracellular matrix deposition were completely prevented by concomitant treatment with enalapril or losartan.

3.3. *Hepatic lipid and glycogen content*

The hepatic levels of HDL cholesterol (23.0±9 and 26.8±11 mg/dl), LDL cholesterol (20.5±4.3 and 22.0±8 g/l) and triglycerides (134±14 and 127±9 mg/dl) in control rats after 4 and 8 days, respectively, were not significantly altered by treatment with L-NAME (data not shown). However, the hepatic glycogen content increased significantly with L-NAME, and this increase was completely prevented by concomitant treatment with enalapril or losartan (Fig. 4).

3.4. *Cytochrome P450 activities and expression*

Treatment with L-NAME had no significant effect on the basal or induced CYP activities in the two time intervals studied (Fig. 5). In agreement with this, western blotting showed that L-NAME did not affect the expression of these enzymes (Fig. 6).

3.5. *Immunohistochemical detection of MMP-2 and MMP-9*

MMP-2 expression was detected in the cytoplasm of hepatocytes in control and L-NAME-treated rats, although there was no marked difference in the staining between the control group and those treated with L-NAME for 4 or 8 days. In contrast, whereas little MMP-2 was detected in normal vessel walls, the presence of this enzyme was increased in the walls of hypertrophic vessels in L-NAME-treated rats (Fig. 7A-F). There was little staining for MMP-9 in the liver of control and L-NAME-treated rats; in the treated groups, staining was associated mainly with specific cells (mast cells, macrophages) in the vessel walls and surrounding regions of fibrosis (Fig. 8A-F).

4. Discussion

Treatment with L-NAME for four or eight days increased the arterial blood pressure of rats, as also observed by others (Katoh *et al.,* 1998; Tomita *et al.,* 1998a,b; Usui *et al.,* 1998; Koyanagi *et al.,* 2000a,b; Zhao *et al.,* 2002; Kataoka *et al.,* 2004). This hypertension was attenuated by concomitant treatment with enalapril or losartan, indicating involvement of the RAS (Zatz and Baylis, 1998). The hypertension was accompanied by vascular changes that included vessel wall hypertrophy and interstitial fibrosis, which were most marked after eight days. Again, these changes were prevented by concomitant treatment with enalapril or losartan.

Matrix metalloproteinases (MMPs) are extensively involved in turnover of the extracellular matrix and in tissue remodeling (Lindsey, 2004), and the activity and expression of some MMPs in aortic tissue are altered during long-term inhibition of NO biosynthesis (Gonzalez *et al.,* 2000). In agreement with this, we observed enhanced immunodetection of MMP-2 and, to a lesser extent, MMP-9, in the hepatic arterial vessel walls of rats treated with L-NAME for four and eight days. Increased levels of these enzymes in vessel walls may be mediated by enhanced formation of reactive oxygen species (ROS) in the vessel walls of L-NAME-treated rats (Kataoka *et al.,* 2004) since ROS can stimulate the expression of MMPs (Siwik and Colucci, 2004). The increased expression of MMPs may contribute to vascular and perivascular remodeling (Lindsey, 2004) by modulating the levels of extracellular matrix components such as type I collagen, the expression of which is augmented in this experimental model (Koyonagi *et al.,* 2000a; Tomita *et al.,* 1998b) .

In contrast to the changes noted above, there were no alterations in the hepatic content of lipids or in the activities and expression of CYP450 isoforms. CYP450 are activated by increased levels of NO (Khatsenko and Kikkawa, 1997; Khatsenko, 1998), particularly during pathological conditions such as inflammation (Aitken *et al.,* 2005). Our results indicate that a reduction in NO formation does not adversely affect the activity or expression of these enzymes. The lack of effect on

CYP450 activity and expression contrasts with the enhanced expression of certain receptors (Katoh *et al.*, 1998; Usui *et al.*, 1998), and the increased expression and activity of signaling proteins and pathways in this model (Depuis *et al.*, 2004), including kinase signaling pathways (Ikegaki *et al.*, 2001; Kataoka *et al.*, 2002; Martens *et al.*, 2002; Minamino *et al.*, 2000) and inflammatory proteins and mediators (Tomita *et al.*, 1998a,b; Koyanagi *et al.*, 2000a,b; Zhao *et al.*, 2002; Kataoka *et al.*, 2004).

In conclusion, short-term inhibition of NO biosynthesis produces vascular changes and selectively affects different enzymes or pathways in rat liver. These findings agree with the ability of NO to differentially affect the gene expression and activity of various proteins and enzymes (Pfeilschifter *et al.*, 2001; Piz and Casteel, 2003), including during prolonged inhibition of NO biosynthesis (Depuis *et al.*, 2004).

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Figure legends

Figure 1. Histological alterations in hepatic tissue of L-NAME-treated rats. L-NAME (20 mg/rat/day, p.o.) was administered in the drinking water for four and eight days. Control rats received water without inhibitor. The panels show the appearance of hepatic zones I (periportal zone), II (intermediary zone) and III (centrolobular zone) in control (**A,B,C**) and L-NAME-treated (**D,E,F**) rats. Note the marked perinuclear unstained areas (resulting from increased glycogen storage) in hepatocytes of zones I, II and III, the parenchymal compactness caused by collapse of the sinusoidal capillaries, and the poorly visible liver plates. All of the L-NAME-induced alterations were reverted by concomitant treatment with enalapril (25 mg/kg/day, p.o.) or losartan (30 mg/kg/day, p.o.) (results not shown). HE. Bar corresponds to 40 µm.

Figure 2. Perivascular deposition of collagen in L-NAME-treated rats. Panels **A-C** show liver sections from control rats (**A**) and from rats treated with L-NAME (20 mg/rat/day, p.o.) for four (**B**) and eight (**C**) days. In all cases, the L-NAME-induced alterations were reverted by concomitant treatment with enalapril (25 mg/kg/day, p.o.) or losartan (30 mg/kg/day, p.o.) (results not shown). Picrosirius staining. Bar corresponds to 35 µm.

Figure 3. Arterial vessel wall hypertrophy induced by L-NAME. Panels **A** and **B** show liver sections from a control rat (**A**) and a rat treated with L-NAME (LN, 20 mg/rat/day, p.o.) for four days (**B**). Concomitant treatment with enalapril (Enal, 25 mg/kg/day, p.o.) or losartan (Los, 30 mg/kg/day, p.o.) prevented the vessel wall hypertrophy (not shown). Similar but more marked changes were seen in rats treated with L-NAME eight days. Panel (**C**) shows the vessel wall thickness expressed as a ratio of the total vessel cross-sectional area divided by the vessel lumen cross-sectional area. The columns represent the mean $\pm$ SD of 50 vessels

from five rats/group. *p<0.001 compared to the corresponding control (ANOVA followed by the Bonferroni test). HE. Bar corresponds to 50 μm.

Figure 4. Glycogen accumulation in the liver of L-NAME-treated rats. Rats were treated with L-NAME, enalapril and losartan as described in Figure 3 and glycogen was quantified as described in Methods. The columns represent the mean ± SD of five rats/group. *p<0.001 compared to the corresponding control (ANOVA followed by the Bonferroni test).

Figure 5. Cytochrome P450 activities in control and L-NAME-treated rats. The rats were treated with L-NAME (20 mg/rat/day, p.o.) for four or eight days. In some experiments, the rats were also treated concomitantly with phenobarbital (Pheno; 80 mg/kg/day each, i.p., 4 d; to induce CYP2B1/2), β-naphthoflavone (β-Naphtho; 80 mg/kg/day each, i.p., 4 d; to induce CYP1A1/2) or pyrazole (Pyraz; 200 mg/kg/day, i.p., 2 d; to induce CYP2E1). Note that CYP2C11 activities (constitutive) are shown only for control and L-NAME-treated rats without inducers The columns represent the mean ± SD of six rats/group. *p<0.001 compared to the corresponding control or L-NAME alone group. AFU, arbitrary fluorescence units.

Figure 6. Cytochrome P450 expression in control and L-NAME-treated rats. The rats were treated with L-NAME (20 mg/rat/day, p.o.) for four or eight days and the livers then processed for western blotting as described in Methods. One hundred micrograms of protein were applied to each lane. The columns represent the mean ± SD of five rats/group. There were no significant differences in the expression levels of the various isoforms between the control and treated groups. CYP450 expression in rats treated with inducers was not assessed.

Figure 7. Immunochemical detection of MMP-2 (**A-F**) in liver of control and L-NAME-treated (20 mg/rat/day, p.o., four or eight days) rats. The proteins were

detected (brown staining) as described in Methods. **A,B** – control, **C,D** – L-NAME (four days) and **E,F** – L-NAME (eight days).

Figure 8. Immunochemical detection of MMP-9 (**A-F**) in liver of control and L-NAME-treated (20 mg/rat/day, p.o., four or eight days) rats. The proteins were detected (brown staining) as described in Methods. **A,B** – control, **C,D** – L-NAME (four days) and **E,F** – L-NAME (eight days).

Table 1: Body weight and arterial blood pressure in the groups of rats studied.

Treatment	Body weight (g)		Systolic blood pressure (mm Hg)	
	4	8	4	8
Control	215 ± 5	240 ± 19	118± 9	120 ± 9
L-NAME	220 ± 6	230 ± 6	168 ± 10*	170 ± 10*
Losartan	212 ± 9	228 ± 12	127 ± 5	127 ± 5
L-NAME + Losartan	212 ± 8	222 ± 10	141 ± 8*,#	142 ± 6*,#
Enalapril	218 ± 7	233 ± 10	125 ± 10	125 ± 10
L-NAME + Enalapril	214 ± 5	238 ± 6	135 ± 4*,#	138 ± 3*,#
Phenobarbital	219 ± 5	235 ± 12	118 ± 8	119 ± 7
L-NAME + Phenobarbital	220 ± 6	235 ± 11	160 ± 8*	165 ± 9*
Pyrazole	216 ± 5	238 ± 7	116 ± 9	113 ± 8
L-NAME + Pyrazole	212 ± 4	245 ± 17	170 ± 6*	170 ± 8*
β-Naphthoflavone	215 ± 6	251 ± 11	125 ± 9	124 ± 9
L-NAME + β-Naphthoflavone	225 ± 8	250 ± 8	160 ± 8*	170 ± 9*

The values are the mean ± S.D. of five rats in each group. *,#$p<0.05$ compared to the control (*) and L-NAME-treated (#) rats.

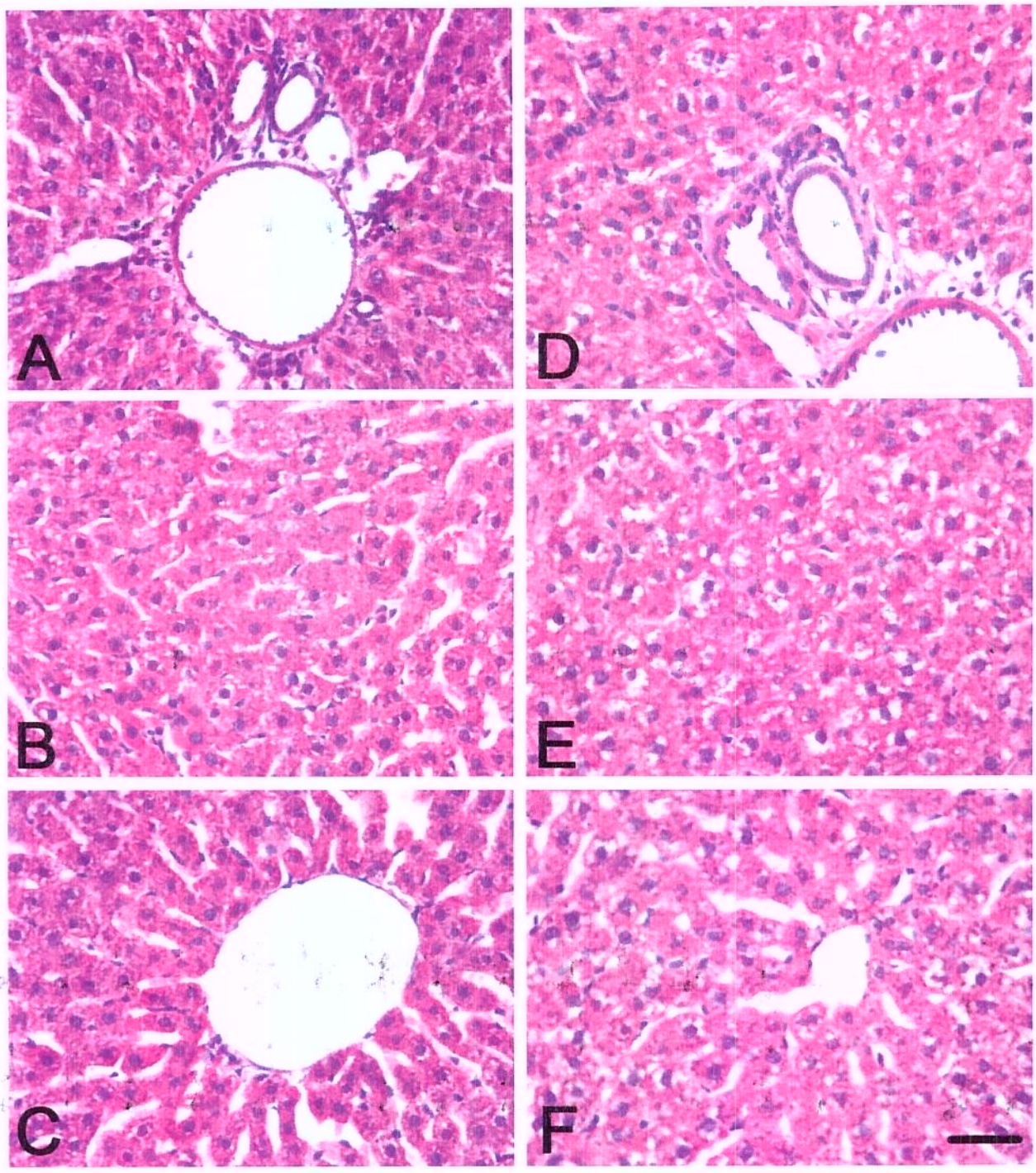


Figure 1

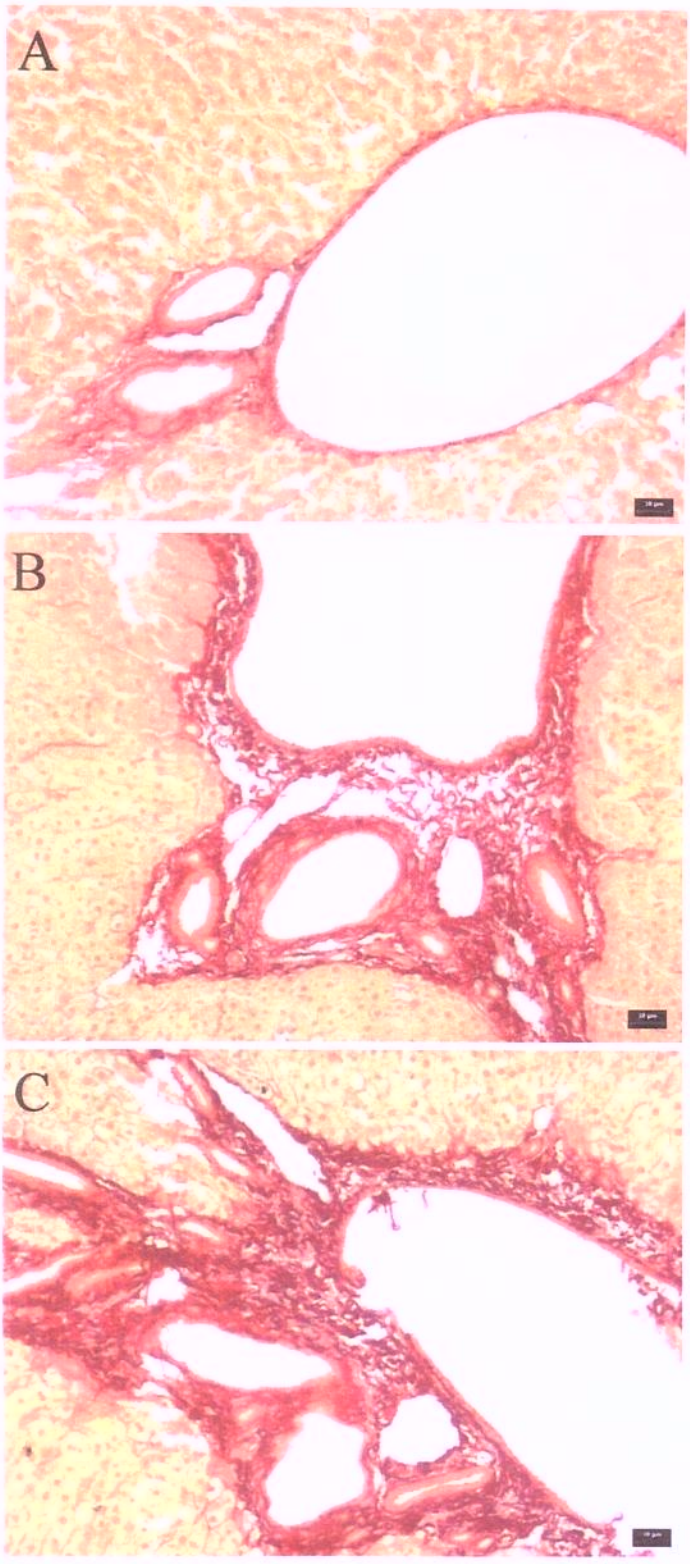
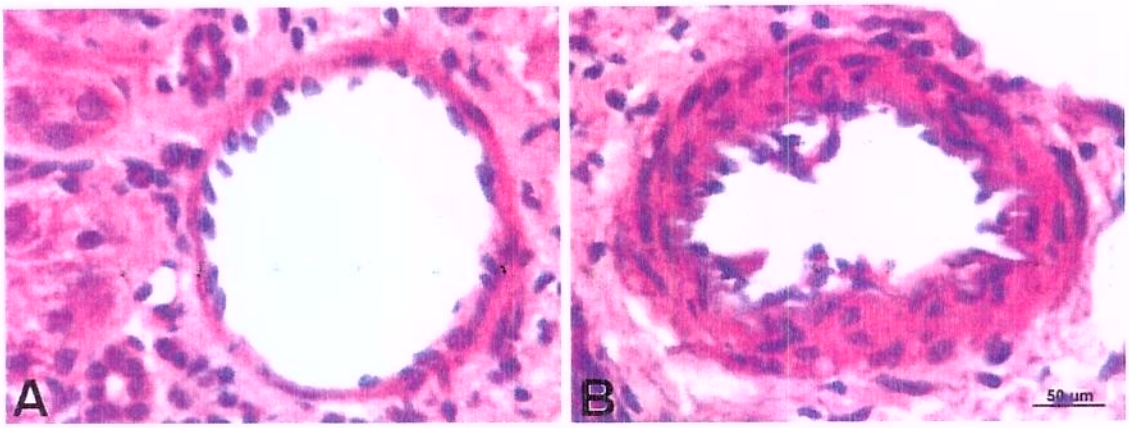


Figure 2


A
B
50 µm

C

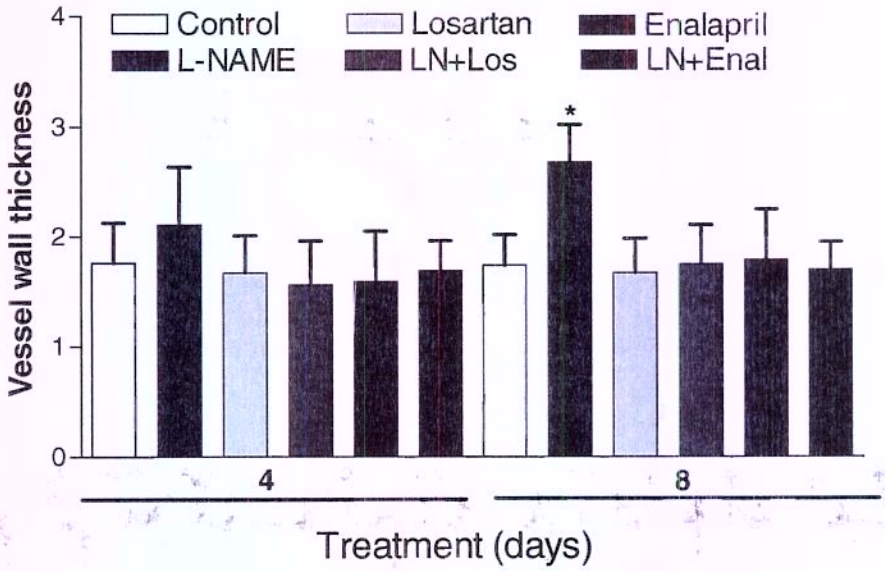

Control
L-NAME
Losartan
LN+Los
Enalapril
LN+Enal
Vessel wall thickness
0
1
2
3
4
*
4
8
Treatment (days)

Figure 3

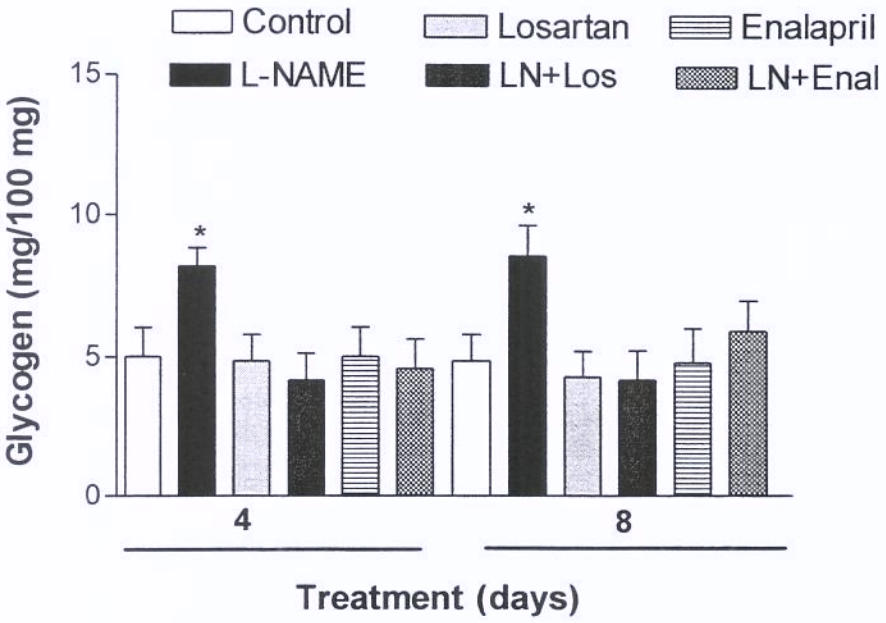

Control
Losartan
Enalapril
L-NAME
LN+Los
LN+Enal
Glycogen (mg/100 mg)
15
10
5
0
*
*
4
8
Treatment (days)

Figure 4

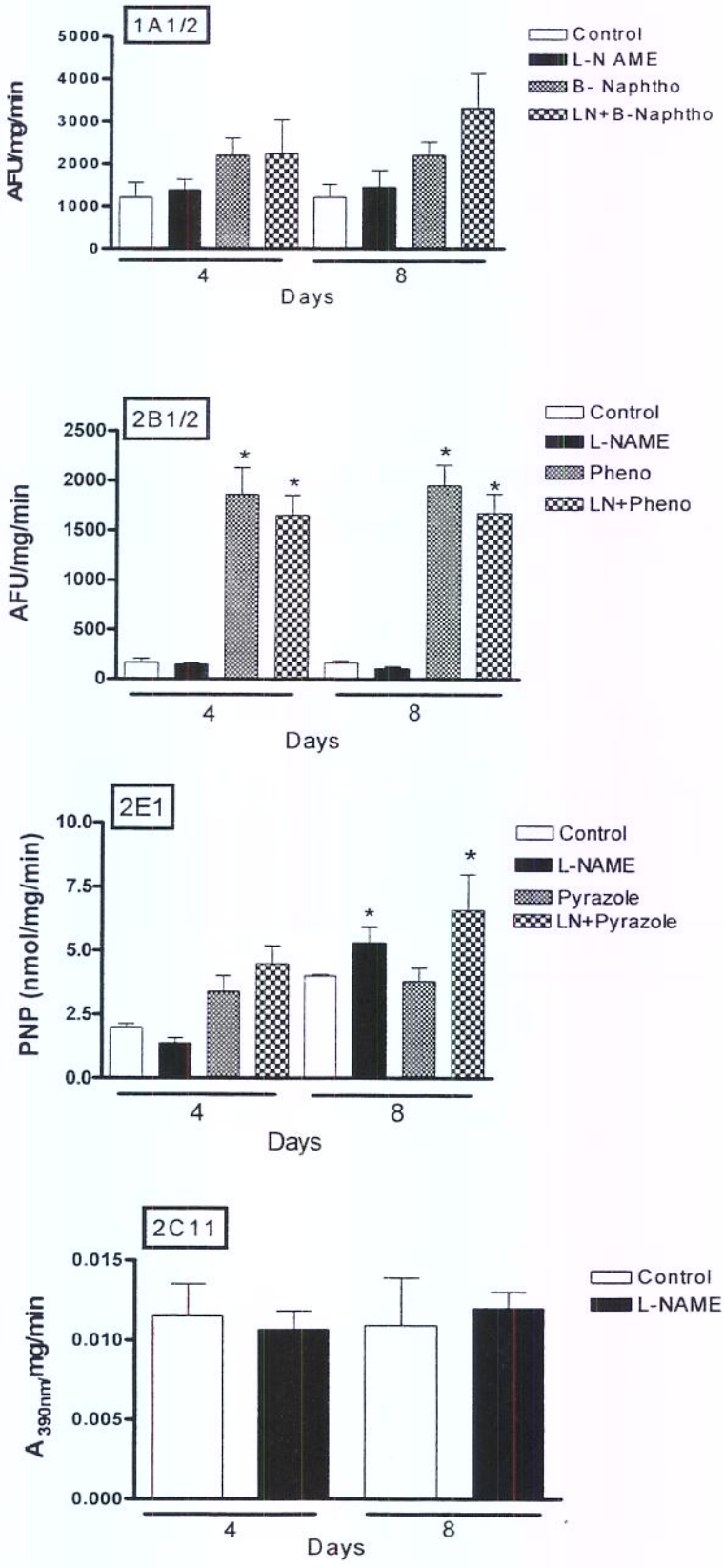

1A1/2
AFU/mg/min
5000
4000
3000
2000
1000
0
4
8
Days
Control
L-N AME
B- Naphtho
LN+B-Naphtho
2B1/2
AFU/mg/min
2500
2000
1500
1000
500
0
*
4
8
Days
Control
L-NAME
Pheno
LN+Pheno
2E1
PNP (nmol/mg/min)
10.0
7.5
5.0
2.5
0.0
*
4
8
Days
Control
L-NAME
Pyrazole
LN+Pyrazole
2C11
A 390nm/mg/min
0.015
0.010
0.005
0.000
4
8
Days
Control
L-NAME

Figure 5

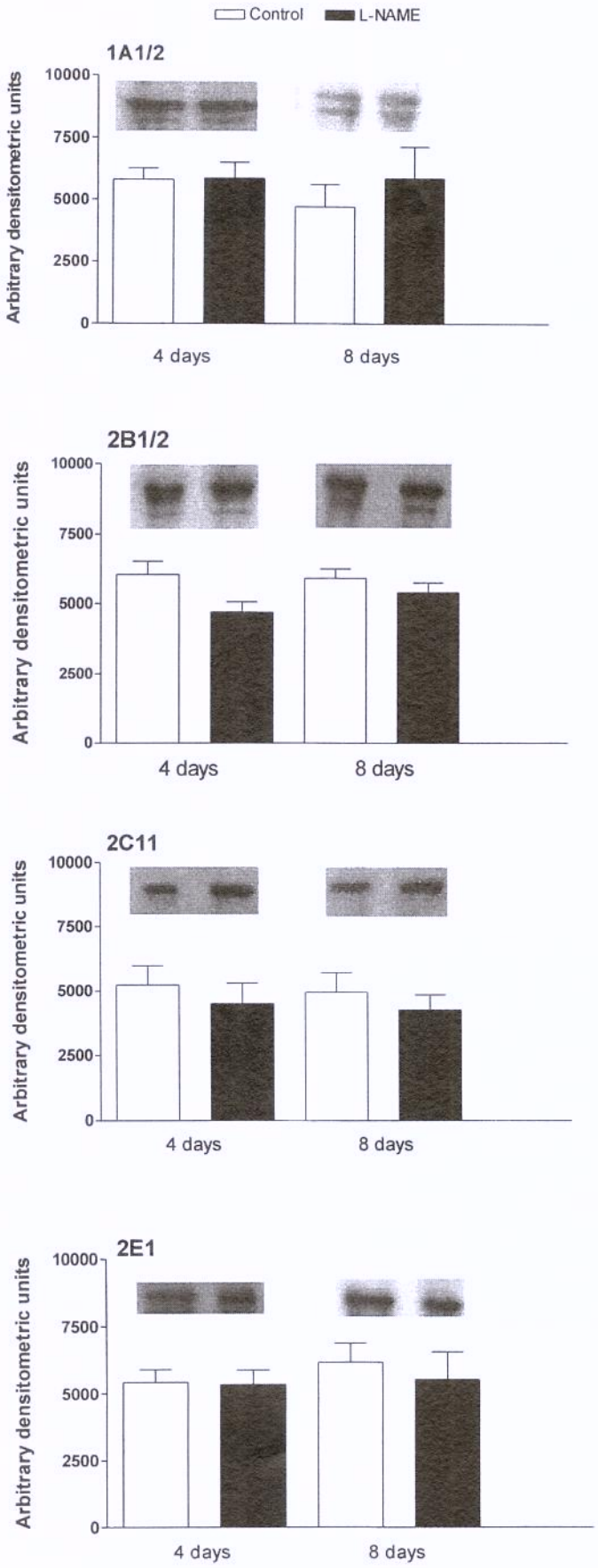

Control
L-NAME
1A1/2
2B1/2
2C11
2E1
Arbitrary densitometric units
10000
7500
5000
2500
0
4 days
8 days

Figure 6

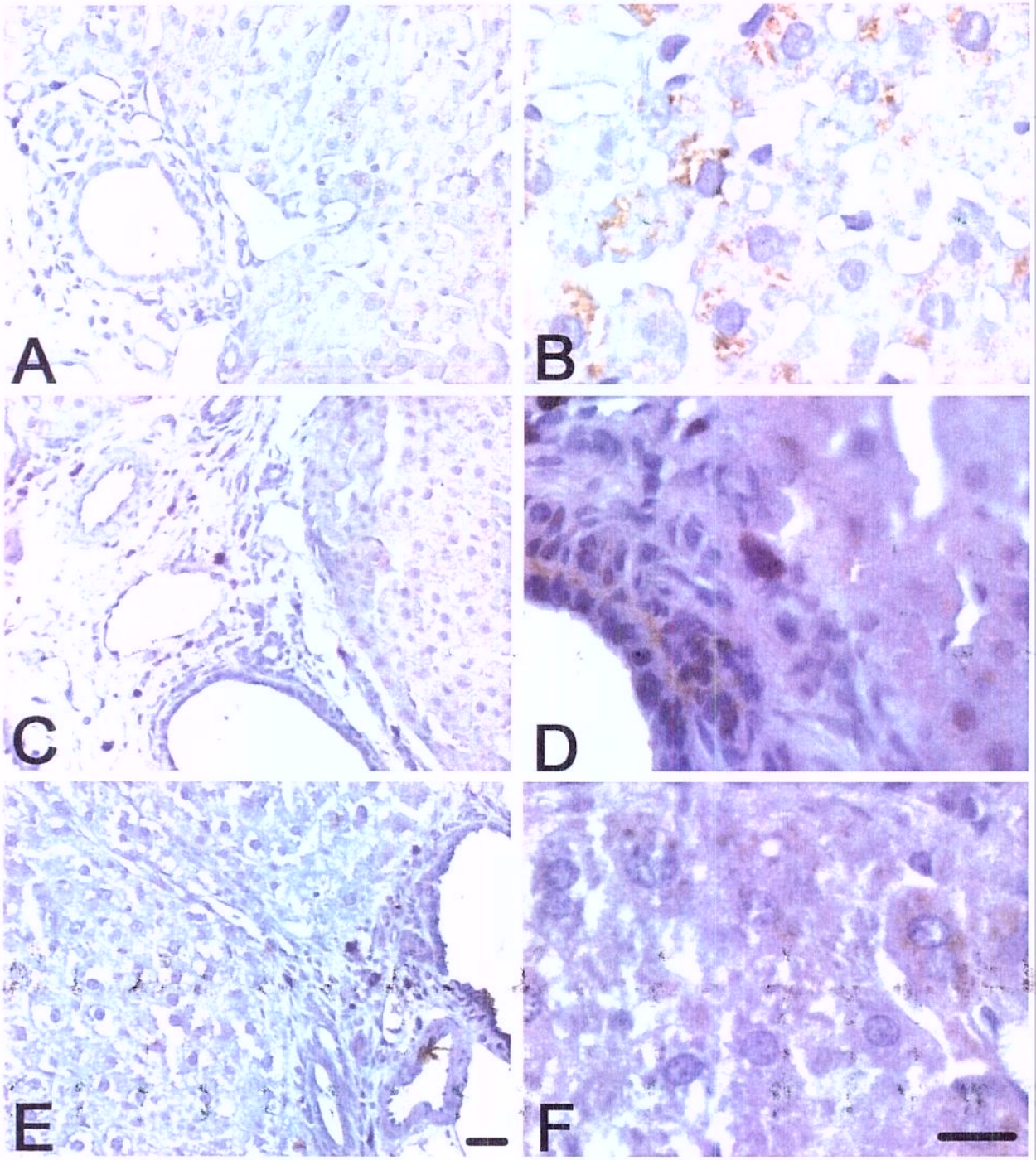


Figure 7

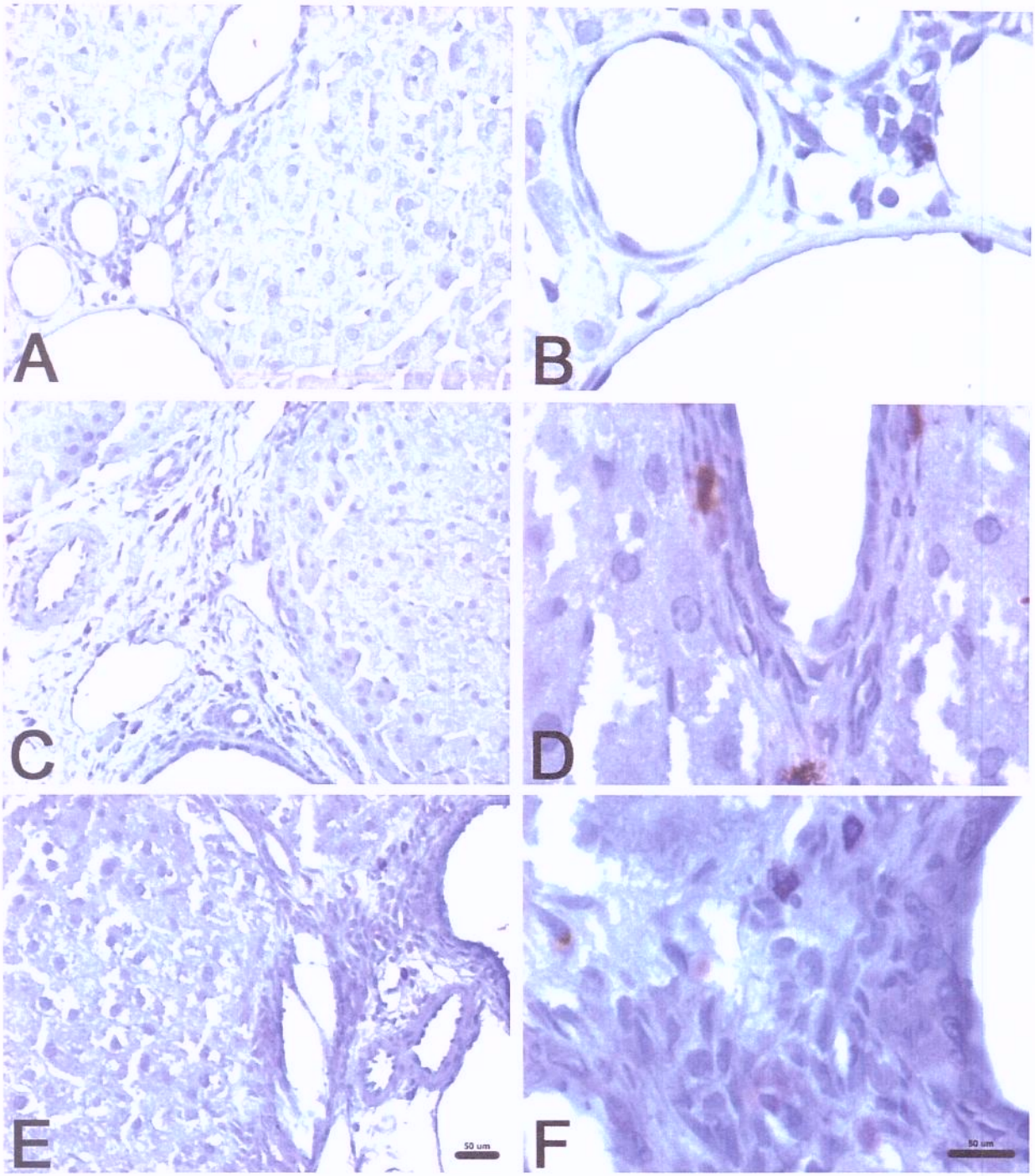


Figure 8

4. CONCLUSÕES

Os resultados deste trabalho mostram que:

1. O tratamento agudo ou crônico com L-NAME resulta em alterações morfológicas hepáticas que incluem hipertrofia da parede vascular, deposição de matriz extracelular (principalmente colágeno) e infiltração de celulas inflamatórias. Estas alterações são semelhantes às observadas em tecido cardíaco, renal e cerebral.

2. O tratamento agudo ou crônico com L-NAME leva a um acúmulo de glicogênio hepático, sem alterar significativamente o conteúdo de colesterol (HDL e LDL) e triglicérides.

3. As alterações morfológicas (hipertrofia vascular e deposição de matriz extracelular) e o aumento de glicogênio hepático são abolidos por tratamento concomitante com enalapril (inibidor da ECA) ou losartan (antagonista de receptores AT_1), indicando um papel importante do sistema RAS nestas respostas.

4. O tratamento com L-NAME (crônico ou agudo) não afeta as atividades (basal e induzida) e expressão das isoformas de citocromo P450, indicando que níveis reduzidos de NO por tempos variados não influenciam a atividade e expressão destas enzimas. Este achado contrasta com diversos estudos mostrando que um aumento na produção de NO leva a um aumento na atividade e expressão de citocromos P450.

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