Cytokine-mediated production of nitric oxide in isolated rat hepatocytes is dependent on cytochrome P-450III activity

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Abstract To investigate the role of the cytochrome P-450 system in NO synthesis, cytochrome P-450IIIA, IIE and IA activities were specifically inhibited by cimetidine (IIIA), clotrimazole (IIIA), benzoflavone (IA) and disulfiram (IIE) in a model of cultured rat hepatocytes. Cytokine-induced NO synthesis was significantly decreased in the presence of cimetidine and clotrimazole. Kinetic analysis revealed a non-competitive mode of inhibition (K_i = 21 mM, cimetidine; K_i = 13 μM, clotrimazole). Reverse transcriptase-PCR and immunoblot analysis revealed no significant change in steady state levels of iNOS mRNA and protein expression with P-450IIIA inhibition. Purified iNOS enzyme activity was not altered. These data suggest that cytokine-mediated hepatocyte synthesis of NO is dependent upon P-450IIIA activity, which functions in a post-translational capacity.

Key words: Nitric oxide; Cytochrome P-450; Cimetidine; Clotrimazole; Rat hepatocyte

1. Introduction

Nitric oxide (NO) is a short lived, readily diffusible free radical with a multitude of organ-specific functions [1]. The monoxygenase enzyme, NO synthase (NOS), catalyzes a five electron oxidation of L-arginine with oxygen and NADPH as co-substrates to ultimately yield L-citrulline and NO via the intermediate, *N*-hydroxy-L-arginine (NOHA) [2,3]. Recently, using a system of rat liver microsomes devoid of NOS activity, Boucher and colleagues have described an alternative mechanism whereby cytochrome P-450-dependent monooxygenases catalyze the oxidation of NOHA to NO and citrulline [4,5]. To further characterize this observation, we examined the effect of specific inhibition of cytochrome P-450IIIA, IIE or IA activities on NO synthesis in a model of rat hepatocytes in short term primary culture. Inhibition of CP-450IIIA activity by cimetidine or clotrimazole was associated with significantly decreased NO synthesis, while steady state levels of iNOS mRNA and protein expression did not change. In contrast, inhibition of P-450IIE or IA activity did not alter hepatocyte NO production. Addition of 7-ethoxyresorufin, a non-specific inhibitor of cytochrome P-450 activity, also decreased cytokine-mediated NO synthesis. Our results suggest that (i) hepatocyte synthesis of NO is dependent upon cytochrome P-450 IIIA activity, (ii) P-450IIIA activity functions in a post-translational capacity, and (iii) imidazole-derived inhibitors of P-450IIIA activity modulate NOS activity by a non-competitive mode of inhibition.

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2. Materials and methods

2.1. Rat hepatocyte isolation

Male Sprague–Dawley rats (200–300 g) were fed water and chow ad libitum. Hepatocyte isolation, as described by Schuetz and Guzelian, was performed using the technique of in situ collagenase perfusion followed by sequential Percoll gradient centrifugation [6]. Hepatocytes were resuspended in Williams' E media with 1 mM L-arginine, 1 μM insulin, 15 mM HEPES, pH 7.4, penicillin, streptomycin, and 10% heat-inactivated low endotoxin newborn bovine serum (NBS). Hepatocyte purity was assessed by leukocyte esterase staining, while viability was assessed by Trypan blue exclusion. Preparations were routinely >90% viable and >98% pure. The cell suspension was incubated in plastic wells for 30 min to remove residual Kupffer cells. Hepatocytes were then plated at a density of 5.0×10^5 cells/ml onto gelatin-coated wells. After 2 h, the media were changed to remove unattached cells. After 24 h of incubation at 37°C in 95% O_2/5% CO_2, cells were washed twice and fresh media applied for experimental use.

2.2. Preparation of microsomes from cultured rat hepatocytes

Samples of isolated rat hepatocytes were homogenized in 4 vols. of 250 mM sucrose, 0.1 M potassium phosphate, pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol. After centrifugation at 10,000 × *g* for 25 min, the supernatant was again centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet was homogenized in 0.1 M sodium pyrophosphate, pH 7.25, 23 μM butylated hydroxytoluene, 1 mM EDTA and again, centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting microsomal preparation was suspended in 0.1 M potassium phosphate, pH 7.4, glycerol (20%, v/v), 1 mM EDTA, 0.1 mM DTT. Microsomal preparations were kept at −70°C until ready for use [7].

2.3. Generation of NO in isolated rat hepatocytes

Isolated rat hepatocytes were stimulated to produce NO by incubation in Williams' E media with 10% heat inactivated NBS, and 1 mM L-arginine supplemented with 25 ng/ml IL-1, 500 U/ml TNF, 100 U/ml h IFN, and 12 μg/ml LPS (*E. coli* 0111:B4) [8]. In selected instances, the competitive substrate inhibitor of NO synthase, N^G-monomethyl-L-arginine (L-NMMA) was added to inhibit NO synthesis. Cimetidine (0–50 mM) or clotrimazole (0–50 μM) were added as specific inhibitors of CP-450IIIA; similarly, disulfiram (0–100 μM) or benzoflavone (0–100 μM) were added as specific inhibitors of CP-450 IIE and IA activities, respectively. After incubation for 18 h at 37°C in 95% O_2/5% CO_2, the supernatant was aspirated and the cells washed twice with DPBS. Following treatment with trypsin/EDTA, the hepatocytes were harvested for biochemical assays.

2.4. Measurement of NO

NO release from cells in culture was quantified by measurement of the NO metabolite, nitrite, using the technique of Snell and Snell [9]. Conditioned media was mixed with 1% sulfanilamide in 0.5N HCl (50%; v/v). After a five minute incubation at room temperature, an equal volume of 0.02% naphthylenediamine was added. Following incubation at room temperature for 10 minutes, absorbance at 570 nm was compared with that of an $NaNO_2$ standard.

2.5. Determination of monooxygenase activities

Cytochrome P-450 isoform-specific enzyme activities, ethoxyresorufin deethylase (IA), aniline hydroxylase (IIE) and erythromicin demethylase (IIIA), were assayed using previously published techniques [7,10,11]. Microsomes were diluted to 0.2 mg/ml in 0.1 M potassium phosphate, pH 7.4, and incubated at 37°C for 5 min in the presence of the appropriate substrate. The reaction was initiated by addition of

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1 mM NADPH and allowed to proceed at 37°C. Final substrate concentrations were 5 μM ethoxyresorufin, 1 mM erythromicin and 1 mM aniline. Turnover (pmol of product formed/mg/unit time) was determined from the initial part of the reaction where substrate oxidation was linear as a function of time.

2.6. Immunoblot analysis of inducible NOS

The hepatocytes were washed three times in phosphate buffered saline and incubated with boiling 2 × non-reducing electrophoresis sample buffer for 2 min. Separation was performed on a 12% SDS-PAGE, and then electrotransferred to a polyester-supported nitrocellulose membrane for 90 min at 150 mA. The membrane was blocked overnight at 4°C in TBS (10 mM Tris-HCl at pH 7.5, 150 mM NaCl) containing 3% BSA. Blocked membranes were incubated with immunoblotting iNOS Ab, washed three times in TBS/0.1% Tween, and incubated with biotinylated sheep anti-murine IgG for 1 h. After washing again three times, membranes were then incubated with strepavidin–horseradish peroxidase conjugate. Following washing, bound Abs were detected by the ECL detection system (Amersham, Arlington Heights, IL).

2.7. RNA extraction and reverse transcriptase-PCR

Total cellular RNA was extracted by a commercial modification of the phenol/chloroform/isoamyl alcohol extraction method (Promega RNAgents Total RNA Isolation System; Promega Corp., Madison, WI) [12]. Upon completion of RNA extraction, all samples were treated with 10 U/μl RNase-free DNase (Stratagene Cloning Systems, La Jolla, CA) for 10 min at 37°C, followed by inactivation at 100°C for 15 min. Cellular RNA (0.5 μg) was reverse transcribed by incubating samples for 45 min at 42° C in 10 μl reverse transcriptase buffer containing 5 mM $MgCl_2$, 10 mM Tris, 0.25 mM dNTP, 0.5 U/ml RNase inhibitor, 2.5 nM random hexamer, and 10 U/μl Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD). The reaction was terminated by heating to 95°C for 5 min. The mRNA-PCR method was performed (20–30 cycles) by using a Perkin-Elmer GeneAmp PCR System 9600 with 10 μl of the reverse transcription mix, 2.5 U/100 μl *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD), and 0.5 μM oligonucleotide primer pairs (Operon Technologies Inc., Alameda, CA). The sequence of primer pairs for human vascular smooth muscle cell iNOS have been described [13] and are: TAGAGGAACATCTGGCCAGG and TGGCCGACCTGATGTTGCCA. The sequence primer pairs for rat β-actin mRNA, used to determine constitutive expression, have also been described [14] and are: CATCGTGGGCCGCTCTAGGCAC and CCGGCCAGCCAAGTCCAGACGC. Amplification was initiated by 1 min of denaturation at 94°C for 1 cycle followed by 20–30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min. After the last cycle of amplification, the samples were incubated for 10 min at 72°C and then held at 4°C. Verification of the amplified PCR products was performed by automated DNA sequencing (Applied Biosystems, Foster City, CA) for both rat iNOS and rat β-actin mRNA.

The PCR products were visualized by UV illumination after electrophoresis through 1.0% agarose (UltraPure, Sigma Chemical Co.) at 120 V for 45 min and staining in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA, pH 8.2) containing 0.5 μg/ml ethidium bromide. DNA was visualized on a UV illuminator and gels were photographed with type 55 positive/negative film (Polaroid, Cambridge, MA). The negative films of the gel photographs were scanned with a computerized laser densitometer (Hoeffer Scientific Instruments, San Francisco,CA) and the area under the curve was normalized for β-actin mRNA content.

2.8. Purification of NOS

Hepatocyte homogenates were prepared by washing the cells twice in PBS (pH 7.4); the cells were scraped off the culture wells and resuspended in sonication buffer containing 20 mM Tris, pH 7.4, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, 1 μM leupeptin, 1 μM BH_4 and 0.2 mM phenylmethanesulfonyl fluoride. All manipulations were performed at 4°C. Cells were sonicated and centrifuged at 100,000 × *g* for 15 min at 4°C. The 100,000 × *g* supernatant was applied to a 2′,5′-ADP Sepharose column equilibrated with sonication buffer with 1 mM $MgCl_2$ and 100 mM NaCl. The column was washed with sonication buffer with 0.5 M NaCl, and NOS activity was eluted in sonication buffer supplemented with 5 mM NADPH and 10% glycerol (v/v). The eluate was concentrated (approximately 10-fold) by ultrafiltration through a cell with a 50 kDa cutoff membrane (Amicon). Enzyme, in the presence of 5 μM BH_4, was used immediately for determination of iNOS activity.

2.9. Determination of iNOS activity

NOS enzyme activity was determined by the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, as previously described [15]. The assay was performed in the presence and absence of cimetidine (50 mM) or clotrimazole (50 μM).

2.10. Determination of AST, LDH and protein

AST and LDH in the cell culture supernatant were measured by chromogenic assay. Total protein was determined by the method of Lowry [16]. All measured values were normalized to total cell protein to correct for variation in cell number from well-to-well. Total cell protein varied by less than 10% among the various treatment groups.

2.11. Statistical methods

Data were analyzed with the two-way Student's *t*-test or analysis of variance (ANOVA) with Newman-Keuls post-hoc comparison, as appropriate. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of P-450 isoform activity on cytokine-mediated hepatocyte synthesis of NO

Hepatocytes in primary culture were cytokine-stimulated to produce NO in the presence and absence of the P-450 isoform inhibitors: cimetidine (IIIA), clotrimazole (IIIA), benzoflavone (IA) and disulfiram (IIE). Unstimulated cells served as controls. In the setting of cytokine stimulation, NO production was found to increase by 2–3 fold in comparison to controls. Addition of cimetidine (0–50 mM) or clotrimazole (0–50 μM) did not alter steady state nitrite levels in control cells. In contrast, the presence of cimetidine or clotrimazole resulted in significantly decreased NO synthesis in stimulated cells (Fig. 1). For both agents, kinetic analysis was consistent with a non-competitive model of inhibition: K_i = 21 mM, $V_{max\,i}$ = 0.05 nm NO/mg protein/min for cimetidine and K_i = 13 μM, $V_{max\,i}$ = 0.08 nm NO/mg protein/min for clotrimazole. In the presence of 7-ethoxyresorufin (0–50 μM), a non-specific inhibitor of P-450 reductase, cytokine-induced NO synthesis by hepatocytes was found to decrease in a significant fashion, while release of NO in control

Table 1
Microsomal cytochrome P-450 isoform activity in cultured rat hepatocytes

	ED (IA)	AH (IIE)	ER (IIIA)
Control	3.2 ± 0.6	407 ± 35	89 ± 10
Stimulated	2.8 ± 1.2	375 ± 67	86 ± 13
Stimulated + L-NMMA	3.7 ± 1.0	395 ± 70	98 ± 16
Control + cimetidine (50 mM)	2.5 ± 0.8	389 ± 59	10 ± 8#
Stimulated + cimetidine (50 mM)	2.0 ± 0.7	350 ± 45	8 ± 5*
Control + disulfiram (100 μM)	2.3 ± 0.5	54 ± 32#	72 ± 25
Stimulated + disulfiram (100 μM)	2.6 ± 0.7	43 ± 27*	83 ± 17
Control + benzoflavone (100 μM)	ND	394 ± 37	87 ± 19
Stimulated + benzoflavone (100 μM)	ND	373 ± 46	91 ± 13

# $P < 0.01$ vs. Control; *$P < 0.01$ vs. Stimulated. Data presented as mean ± S.E.M. of three experiments performed in duplicate; ND, not detectable. P-450 isoform-specific activities of ethoxyresorufin deethylase (ED), aniline hydroxylase (AH) and erythromicin demethylase (ER) are expressed as pmol/mg/min. Cultured rat hepatocytes were incubated for 16 h at 37°C in the presence of IL-1, TNF, IFN and LPS to stimulate NO synthesis. L = NMMA (100 μM) was added as a specific substrate inhibitor of NO synthesis. Untreated hepatocytes served as controls.

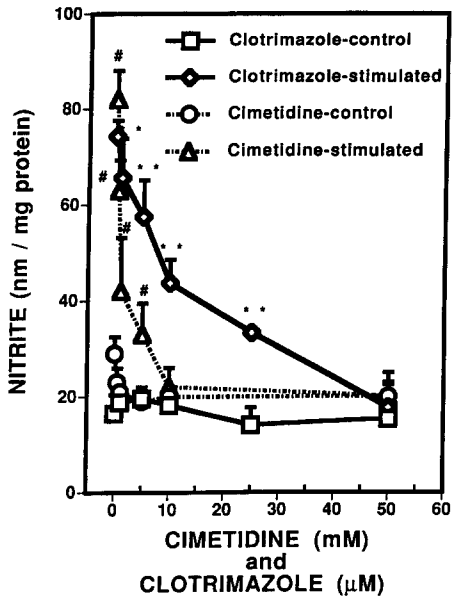

Fig. 1. Nitric oxide production in control and cytokine-stimulated hepatocytes in the presence of the cytochrome P-450 IIIA inhibitors, cimetidine (0–50 mM) and clotrimazole (0–50 μM). Data are presented as mean ± S.E.M. of four experiments. $^{**}P < 0.01$ clotrimazole-stimulated vs. clotrimazole control; $^{\#}P < 0.01$ cimetidine-stimulated vs. cimetidine control. ANOVA: $P < 0.001$ clotrimazole-stimulated; $P < 0.001$ cimetidine-stimulated.

cells was unaltered (Fig. 2). In the presence of the P-450IA inhibitor, benzoflavone, or the P-450IIE inhibitor, disulfiram, NO synthesis was not significantly altered in either control or cytokine-stimulated hepatocytes (Fig. 3). At these concentrations, the P-450 inhibitors were not intrinsically hepatotoxic, as demonstrated by AST and LDH measurements in the cell culture supernatant (data not shown). These data indicate that cytokine-mediated hepatocyte synthesis of NO is decreased in the setting of either generalized inhibition of P-450 reductase function or specific inhibition of P-450IIIA activity.

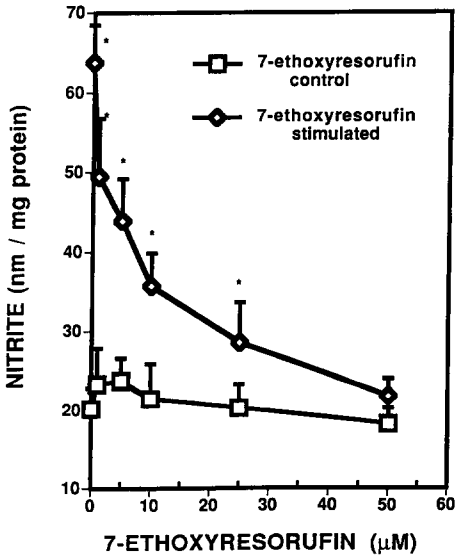

Fig. 2. Nitric oxide production in control and cytokine-stimulated hepatocytes in the presence of the non-specific cytochrome P-450 reductase inhibitor, 7-ethoxyresorufin (0–50 μM). Data are presented as mean ± S.E.M. of four experiments. $^{**}P < 0.01$ control vs. stimulated hepatocytes. ANOVA: $P < 0.001$ cytokine-stimulated cells.

3.2. Cytochrome P-450 activity and cytokine-mediated NO synthesis

P-450 isoform-specific enzyme activity was measured to confirm inhibition of P-450 activity (Table 1). Cytochrome P-450 IA, IIE and IIIA activities were not significantly different in control and cytokine stimulated hepatocytes. NO production in stimulated cells was associated with marginally decreased P-450 isoform activity; however, this did not reach statistical significance. Similarly, cytokine stimulation in the absence of NO synthesis, as represented by the 'Stimulated + L-NMMA' group, did not have significantly altered P-450 isoform activities. In control and stimulated hepatocytes, cimetidine (50 mM) was associated with a 10-fold decrease in P-450IIIA activity ($P < 0.01$), while IA and IIE activity was not altered. In a similar fashion, disulfiram (100 μM) treatment resulted in a 6–8 fold decrease in IIE activity without significant alteration in IA and IIIA activities in either control or stimulated hepatocytes. Finally, benzoflavone (100 μM) was associated with non-detectable P-450IA activity in both control and stimulated hepatocytes. These results suggest that specific inhibition of P-450IA, IIE and IIIA activities occurred with benzoflavone, disulfiram and cimetidine, respectively.

3.3. Effect of P-450IIIA inhibition on iNOS mRNA and protein expression

cDNA from isolated rat hepatocytes was amplified using a single set of primers specific for iNOS and β-actin. DNA sequence analysis of the amplified PCR products revealed complete homology to rat iNOS and β-actin. Amplification of cDNA isolated from hepatocytes incubated with cytokines exhibited an iNOS PCR product of the anticipated size (approx. 250 bp) after 15 cycles. Further amplification showed a linear

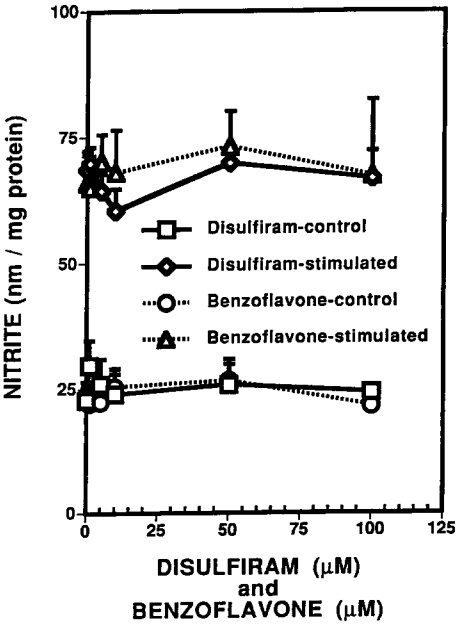

Fig. 3. Nitric oxide production in control and cytokine-stimulated hepatocytes in the presence of the cytochrome P-450 IA inhibitor, benzoflavone (0–100 μM) and the IIE inhibitor, disulfiram (0–100 μM). Data are presented as mean ± S.E.M. of four experiments.

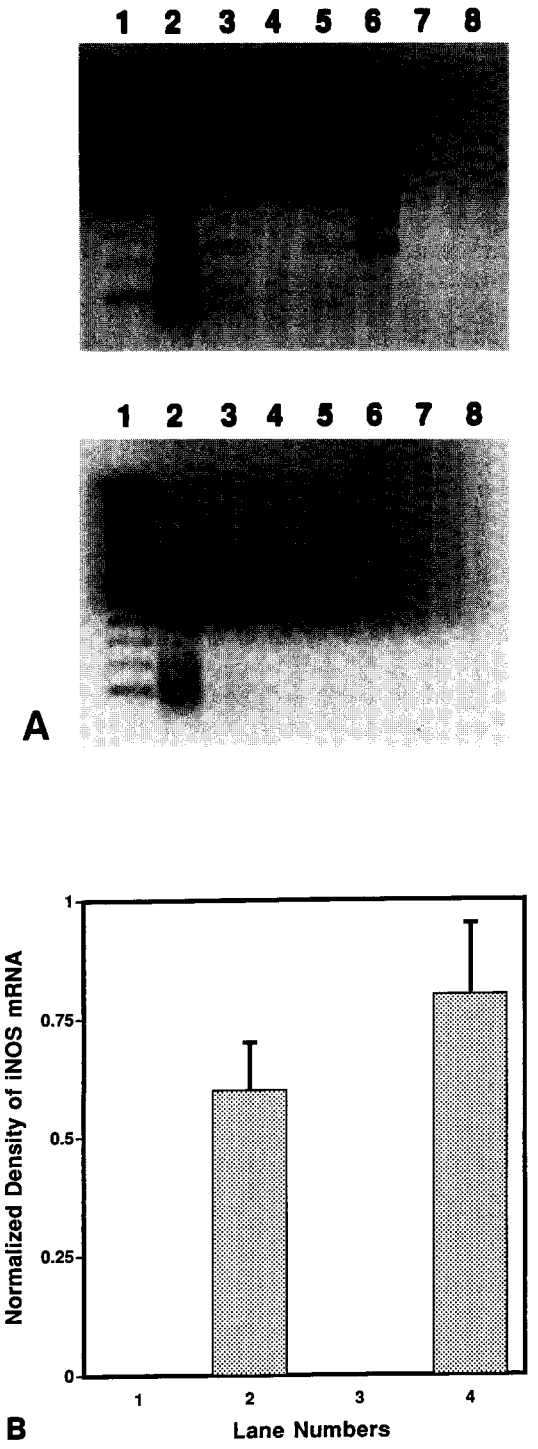

Fig. 4. Effect of cimetidine on steady state hepatocyte iNOS mRNA expression. (A) PCR analysis of inducible NOS (upper gel) and β-actin (lower gel) mRNA in isolated rat hepatocytes. cDNA prepared by reverse transcription of 500 ng RNA was amplified by PCR, and products electrophoresed on a 1% agarose gel and stained with ethidium bromide. Lane 1, DNA 100 bp molecular weight marker; lanes 2,4, control hepatocyte in the absence and presence of cimetidine (50 mM), respectively; lanes 3,5, cytokine-stimulated cells in the absence and presence of cimetidine (50 mM). In the iNOS gel only: lane 6, control PCR reaction (no DNAse); lane 7, control PCR reaction (no reverse transcriptase); lane 8, control PCR reaction (no template). Gels are representative of three seperate experiments. (B) Densitometric analysis of iNOS mRNA PCR products normalized to β-actin mRNA. Data are presented as mean ± S.E.M. of three seperate experiments.

increase in PCR product between 15 and 25 cycles without further increase in PCR product beyond 25 cycles of amplification. Therefore, 23 amplification cycles were used in subsequent studies; negative controls included specimens without reverse transcriptase, DNase or template.

RT-PCR of rat hepatocytes was performed in the presence and absence of 50 mM cimetidine (Fig. 4). Control hepatocytes did not exhibit iNOS mRNA expression (lane 2) and this finding was not altered by the presence of cimetidine (lane 4). iNOS mRNA was noted in cytokine-stimulated cells in both the absence and presence of the P-450IIIA inhibitor (lanes 3, 5). When iNOS mRNA expression was normalized to the β-actin mRNA standard, cytokine-stimulated hepatocytes were found to express statistically equivalent levels of iNOS mRNA. These results suggest that inhibition of P-450IIIA isoform activity does not alter steady state iNOS mRNA expression in this model of isolated rat hepatocytes in primary culture.

To determine the effect of P-450IIIA activity inhibition on iNOS protein levels, immunoblot analysis was performed (Fig. 5). No iNOS protein was detected in control hepatocytes in the presence or absence of 50 mM cimetidine (lanes 1, 3). In the setting of cytokine stimulation, immunoblot staining for hepatocyte iNOS was noted in the absence and presence of cimetidine (lanes 2, 4). The addition of the alternative P-450IIIA inhibitor, clotrimazole (50 μM), did not significantly alter iNOS protein content in stimulated cells (lane 5) and was not statistically different from that found in the presence of cimetidine. These results indicate that the decrease in cytokine-induced NO synthesis mediated by P-450IIIA inhibition is not the result of decreased iNOS protein mass.

3.4. Effect of P-450IIIA inhibition on iNOS enzyme activity

iNOS was isolated and partially purified from cytokine-stimulated hepatocytes (Table 2). In the presence of the P-450IIIA inhibitors, cimetidine (50 mM) or clotrimazole (50 μM), there was no detectable difference in activity in comparison to that in the absence of the P-450IIIA inhibitors. This suggests that the decreased NO production noted in the presence of cimetidine and clotrimazole is not the result of direct inhibition of iNOS enzyme activity.

4. Discussion

Nitric oxide is generated by the monooxygenase, nitric oxide synthase, which is homologous to cytochrome P-450 reductase [1]. Like P-450, NOS contains iron-protoporphyrin IX with cysteine as the axial ferric ion. In the presence of NADPH and oxygen, L-arginine is oxidized to L-citrulline and NO [2,3]. The exact molecular pathway is yet to be fully clarified, but

N-Hydroxy-L-arginine has been determined to be an intermediate in NO synthesis. Work by Boucher and colleagues has

Table 2
Effect of cimetidine and clotrimazole on 2′,5′-ADP Sepharose purified iNOS enzyme activity

	Specific activity (ng/mg/min)
iNOS	180
iNOS + cimetidine (50 mM)	167
iNOS + clotrimazole (50 mM)	199

ND, none detected. Activity was measured in duplicate by the citrulline assay. Data are representative of three preparations.

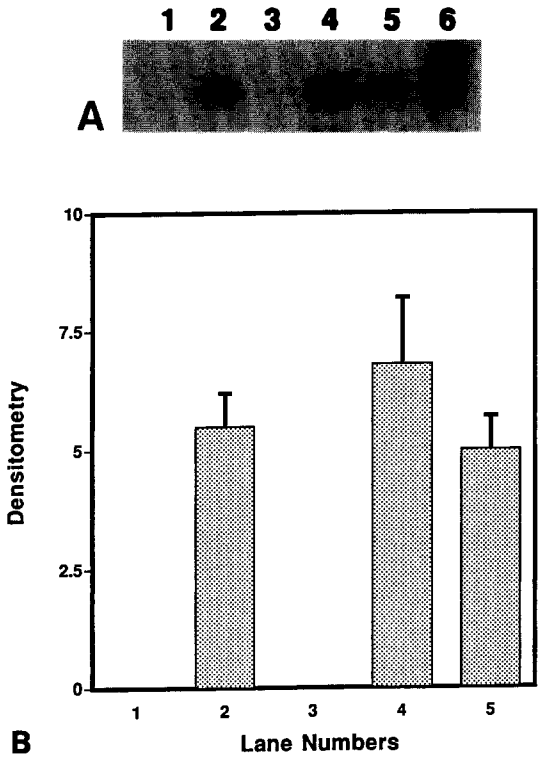

Fig. 5. Effect of cimetidine and clotrimazole on steady state hepatocyte iNOS protein levels. (A) Immunoblot of inducible iNOS protein in isolated rat hepatocytes. Protein extracts were electrophoresed on a 12% polyacrylamide gel, electrotransferred to a nitrocellulae membrane and incubated with a murine monoclonal antibody to iNOS. Visualization was performed with biotinylated sheep anti-murine IgG secondary antibody and strepavidin–horseradish peroxidase conjugate. Bound antibodies were detected by the ECL detection system (Amersham, Arlington Heights, IL). Lanes 1,3, control cells in the absence and presence of cimetidine (50 mM), respectively; lanes 2,4, cytokine-stimulated cells in the absence and presence of cimetidine (50 mM), respectively; lane 5, cytokine-stimulated cells in the presence of clotrimazole (50 μM); lane 6, positive control, murine macrophage iNOS. The gel is representative of three seperate experiments. (B) Densitometric analysis of iNOS immunoblots. Data are presented as mean ± S.E.M. of three seperate experiments.

demonstrated that cytochrome P-450s may be involved in the final oxidation of NOHA to produce NO [4,5]. Also, in a rat liver microsome system devoid of NOS activity, specific induction of P-450IIIA by dexamethasone is associated with significantly increased NOHA conversion to NO [4,17]. However, the applicability of this observation to the physiology of the intact hepatocyte is unknown.

In this study, specific inhibition of P-450IIIA activity by cimetidine and clotrimazole was associated with decreased cytokine-mediated NO synthesis in a system of rat hepatocytes in primary culture. In addition, these imidazole-derivatives were found to behave as non-competitive inhibitors of NO synthesis. In contrast, inhibitors of P-450IIE and IA activities did not alter NO production. This would suggest that cytokine-mediated hepatocyte NO synthesis is dependent upon P-450IIIA activity. However, because NOS and P-450s both contain heme components and cimetidine and clotrimazole are both imidazole-derivatives which act by coordinating with a heme moiety, the possibility exists that NO synthesis is decreased as the result of inhibition of NOS rather than P-450 [18]. 7-Ethoxyresorufin is a cytochrome P-450 substrate which acts as a non-specific inhibitor of P-450 function [19]. In the presence of 7-ethoxyresorufin, NO synthesis was noted to be significantly reduced. In addition, measurement of purified iNOS enzyme activity was not altered by incubation with cimetidine or clotrimazole. This serves as evidence that non-specific inhibition of cytochrome P-450 activity results in diminished NO production and that cimetidine and clotrimazole act to decrease hepatocyte NO synthesis by specific inactivation of P-450IIIA rather than NOS.

The effect of P-450IIIA inhibition on steady state iNOS mRNA and protein levels was characterized by RT-PCR and immunoblot analysis. Expression of iNOS mRNA and protein was not altered in the presence of these P-450 inhibitors. This would suggest that P-450IIIA activity plays a post-translational role in the synthesis of NO by hepatocyte iNOS.

The role of the cytochrome P-450 system in NO synthesis by the intact hepatocyte has not been previously addressed. In a model of rat hepatocytes in primary culture, specific inhibition of P-450IIIA but not IIE or IA activity was associated with significant diminution of cytokine-induced NO production. These observations extend Boucher's findings regarding the stimulatory effect of P-450IIIA induction on hepatocyte microsomal NO production [4,17]. The physiological significance of these observations in the whole organism remain unknown. However, given the emerging evidence concerning the protective effect of hepatocyte NO synthesis in oxidative injury, strategies to induce or augment cytochrome P-450IIIA activity would be of potential utility [20].

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