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Cytokine-mediated Down-regulation of CYP1A1 in Hepa1 Cells

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ABSTRACT. The activation of host defense mechanisms down-regulates microsomal cytochrome P450 in cell culture, humans, and animals. Investigation into various aspects of this effect using *in vivo* models has yet to define clearly the role that cytokines play in this phenomenon. The mechanism of down-regulation by immunostimulants, such as lipopolysaccharide (LPS), is explored with an *in vitro* model, utilizing a murine hepatoma (Hepa1) and a murine macrophage (IC-21) cell line. It is hypothesized that down-regulation of P450 activity by immunostimulants involves the activation of immune cells and the subsequent release of cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). The effects of immunostimulation on P450 activity are assessed by ethoxyresorufin *O*-dealkylase, an assay that measures CYP1A activity in Hepa1 cells. Initial studies demonstrated that LPS added directly to hepatoma cells had no effect on the levels of CYP1A1 activity. In contrast, a significant down-regulation in CYP1A1 activity occurred when hepatoma cells were incubated with monocyte conditioned medium obtained by incubating LPS with IC-21 cells. When pentoxifylline, a TNF-α synthesis inhibitor, was co-administered with LPS to macrophages, the down-regulation of CYP1A1 activity was prevented. The direct administration of murine recombinant TNF-α to hepatoma cells resulted in a down-regulation of CYP1A1 activity. These results implicated the release of TNF-α from macrophages as an important step in the down-regulation of CYP1A1 by LPS. BIOCHEM PHARMACOL **55**;11:1791–1796, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cytochrome P450; cytochrome P450 down-regulation; cytokines; lipopolysaccharide

Cytochrome P450 is a group of heme-containing proteins known for their ability to metabolize a wide variety of endogenous and exogenous compounds [1]. It is well known that the expression of these P450s can be influenced by many factors, which, in turn, can affect the rate of drug detoxification and/or activation by these enzymes [2]. The stimulation of the acute phase response by viral or bacterial infection is known to affect the levels of P450s [3–5].

Administration of LPS† to animals has been shown to down-regulate levels of P450 enzymes, with a resultant lowering of drug metabolism [6, 7]. In administering LPS to the whole animal, it is unknown as to whether LPS exerts its effect directly on hepatocytes or indirectly through the stimulation of immunocompetent cells. Evidence suggests that the effect of immune stimulation on P450 levels is via an indirect manner involving the activation of immunocompetent cells and the subsequent release of cytokines [8].

In vivo administration of immunostimulants, such as viruses, interferon inducers, and bacterial endotoxins, to animals results in the depression of P450-dependent drug metabolism [3–5]. In addition to these immunostimulants, the *in vivo* and *in vitro* administration of recombinant acute phase cytokines, such as IL-1, IL-6, and TNF-α, demonstrates a depression in P450-mediated drug metabolism [2, 8–15]. *In vitro* administration of these cytokines results in the selective suppression of some isoforms of P450 enzymes [2]. IL-1α was shown to have minimal effects on the levels of CYP1A1, 1A2, and 2B1/2 in cultured rat hepatocytes [15] and only minor effects on the levels of CYP1A1, 1A2, and 3A4 in cultured human hepatocytes [2]. In contrast, *in vitro* administration of IL-1β resulted in a depression of CYP1A1, 1A2, 2B1/2, 2C11, and 3A2 activity in cultured rat hepatocytes [9, 15] and demonstrated a depression in levels of CYP1A1, 1A2, 2C, 2E1, and 3A4 in cultured human hepatocytes [2, 13]. IL-6, when added to cultured human hepatocytes, has a more potent effect on levels of CYP3A4 than on CYP1A1 or 1A2 [2, 13], but in rat hepatocytes, some investigators reported a depression in levels of both CYP1A1 and 1A2, whereas others reported that IL-6 had no effect [9, 11, 14, 16]. TNF-α administration, as with IL-6, showed a somewhat selective type of behaviour with potent depression in levels of CYP1A1 and 1A2 activity and moderate effects on levels of 2B1/2, 2C, 2E1, 3A2, and 3A4 [2, 13, 15]. Given the complex interplay between cytokines and their multitude of effects on the isoforms of P450, it has been difficult to precisely determine the mechanism by which immunostimulants depress the levels

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† *Abbreviations:* DBA, dibenz[*a*,*h*]anthracene; EROD, ethoxyresorufin **O**-dealkylase; LPS, lipopolysaccharide; IL, interleukin; MCM, monocyte conditioned medium; PNTX, pentoxifylline; TNF-α, tumor necrosis factor-α; and UCM, unconditioned medium.

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of P450s. In this investigation, a murine hepatoma cell line (Hepa1) was used to investigate the mechanism by which LPS affects levels of induced CYP1A1 activity.

MATERIALS AND METHODS

Reagents

DBA, *Escherichia coli* LPS, pentoxifylline, ethoxyresorufin, and murine TNF-α (purity > 99%) were purchased from the Sigma. Anti-CYP1A1 antibody was purchased from Human Biologics Inc., and the cell culture reagents were obtained from Life Technologies.

Cell Culture and Treatment

A murine-derived hepatoma cell line (Hepa1 cells) was cultured as a monolayer of cells incubated at 37°, 5% CO_2 and 95% air. The Hepa1 cells were propagated in α-Minimum Essential Medium supplemented with 5% fetal bovine serum (FBS). Cells were grown to confluence in 75 cm^2 culture flasks (Falcon), detached, collected into 10 mL of medium for each flask, and then pooled. The pooled cells were plated in tissue culture dishes at a concentration of 0.5 mL of cells to 4.5 mL of medium yielding approximately 1×10^6 cells/plate. Cells were allowed to incubate for 24 hr prior to the addition of drugs or MCM.

IC-21 cells, a murine-derived macrophage cell line, were propagated in RPMI-1640 medium supplemented with 10% FBS and were incubated at 37° and 5% CO_2. In preparation for the experiments, flasks of cells, approximately 80% confluent, were collected in 10 mL of PBS (no calcium or magnesium) per flask and pooled. Each plate containing 4.5 mL of fresh medium was inoculated with 0.5 mL of cells and incubated for 48 hr. Following the incubation period, cells were either incubated with LPS (10 and 50 μg/mL final concentration of LPS in 50 μL of sterile water), or unstimulated (50 μL of sterile water) for 24 hr. The incubation was carried out in the presence of 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg/mL of amphotericin B. The medium obtained from the IC-21 cells incubated with LPS is defined as MCM and that obtained from the cells treated with water is defined as UCM.

Following the 24 hr incubation of IC-21 cells with LPS, the medium was removed and the cells were discarded. This medium, termed MCM, was added to Hepa1 in addition to DBA (50 nM), a CYP1A inducer, and a fresh dose of antibiotic. Hepa1 cells, which received UCM, were incubated with 50 nM of DBA, 0, 10, or 50 μg/mL of LPS, and antibiotic.

EROD Assay and Protein Determination

Following the final 24-hr incubation of Hepa1 cells, the medium was removed and replaced with fresh medium containing 0.60 μM of ethoxyresorufin. Cells were incubated for 30 min and then 2.5 mL of medium was removed for the determination of CYP1A1 activity as judged by the amount of resorufin formed in 30 min [17].

Following the removal of a sample for EROD analysis, the remaining medium was removed, and cells were washed with PBS and then scraped with a rubber policeman into 2 mL of PBS. Cells were lysed by 3 min of sonication followed by removal of 250 μL of the cell sonicate for protein determination using the method of Lowry *et al.* [18]. The CYP1A1 activity was determined for each plate and expressed as picomoles of resorufin formed per milligram of protein per 30-min incubation period. Prior to collection of cells, a trypan blue exclusion test was performed on one plate from each treatment to determine cell viability. In each case, the cell viability was 90% or greater.

Western Blots

Proteins were concentrated by pooling cell sonicates from four replicate plates of each treatment. The pooled cell sonicates were centrifuged at 105,000 *g* for 1 hr at 4° [19]. The proteins were separated by electrophoresis on a 7.5% stacking gel under nonreducing conditions [20]. Separated proteins were electrophoretically transferred to a nitrocellulose membrane using a semi-dry transfer process (Tyler Research). Bands were immunologically detected using a monoclonal antibody against murine CYP1A1 and visualized with an alkaline phosphatase conjugated secondary antibody using nitro blue tetrazolium as the substrate [21]. Band intensities were measured with a Macintosh OneScanner™ using the software Ofoto™ and Scananalysis™.

Statistical Analysis

All data are reported as means ± SEM. Comparisons between treatment groups were carried out using one-way ANOVA.

RESULTS

Induction of CYP1A1 Activity in Hepa1 Cells

Hepa1 cells constitutively express low levels of CYP1A1; however, the activity can be greatly enhanced by treatment with inducers such as the aromatic hydrocarbon DBA [22]. Exposure to 50 nM of DBA for 24 hr led to a 3.5-fold increase in CYP1A1 activity over cells that received vehicle alone (DMSO). In all subsequent experiments, CYP1A1 activity was induced in Hepa1 cells with DBA.

Effect of LPS Added Directly to Hepa1 Cells

LPS (5–100 μg/mL) had no effect on the levels of CYP1A1 when added to Hepa1 cells for a 24-hr period.

Effect of LPS-treated MCM on Hepa1 Cells

When LPS-stimulated MCM was added to Hepa1 cultures, the level of CYP1A1 activity was decreased significantly

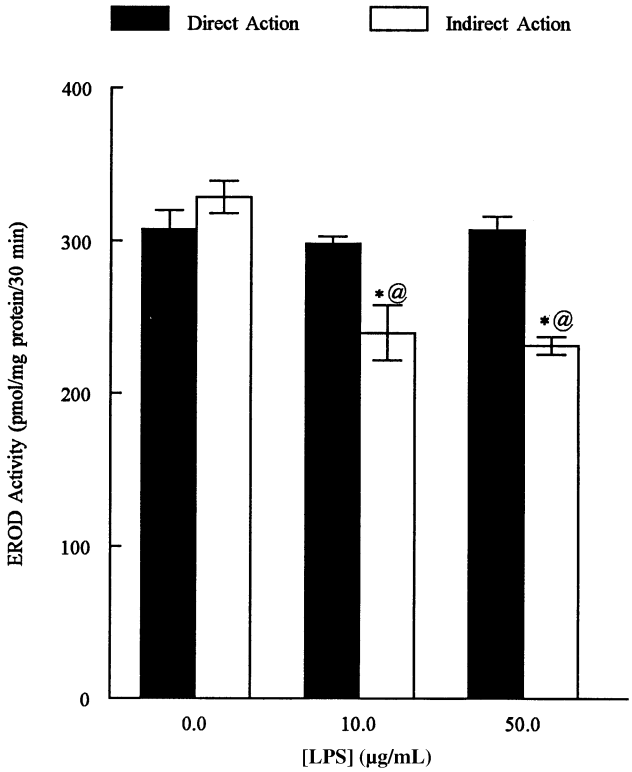

FIG. 1. Action of LPS on CYP1A1 activity in Hepa1 cells. The direct action of LPS on Hepa1 cells was examined by adding LPS to Hepa1 cells containing unconditioned medium. The indirect action of LPS on Hepa1 cells was determined by adding medium from macrophages (MCM) treated with 0, 10, or 50 μg/mL of LPS and incubating for 24 hr. Each value is the mean ± SEM for 4 individual cultures. *Significantly different from corresponding controls containing zero LPS, $P < 0.05$; and @significantly different from cells treated directly with LPS, $P < 0.05$.

(Fig. 1). A 27 and 30% decrease in CYP1A1 activity compared with control was observed for 10 and 50 μg/mL of LPS, respectively. In this same experiment, LPS at concentrations of 10 and 50 μg/mL was added directly to hepatoma cells and again found to have no significant effect on CYP1A1. CYP1A1 protein levels, determined by Western blot, mirrored the results obtained with the EROD assay, namely a down-regulation in protein levels in cells treated with conditioned medium, whereas levels of CYP1A1 protein remained unchanged when LPS was added directly (Fig. 2A).

Effect of PNTX on LPS-induced Down-regulation of CYP1A1

In Hepa1 cells treated with MCM alone, a 21 and 27% decrease in EROD activity was observed for 10 and 50 μg/mL of LPS. In comparison, MCM from macrophages incubated with LPS and PNTX, a TNF-α synthesis inhibitor, had no effect on EROD activity (Fig. 3). Levels of CYP1A1 protein, as determined by western blot, were down-regulated in cells treated with MCM alone, whereas PNTX prevented the down-regulation in CYP1A1 protein, a result that mirrored the EROD activity (Fig. 2B).

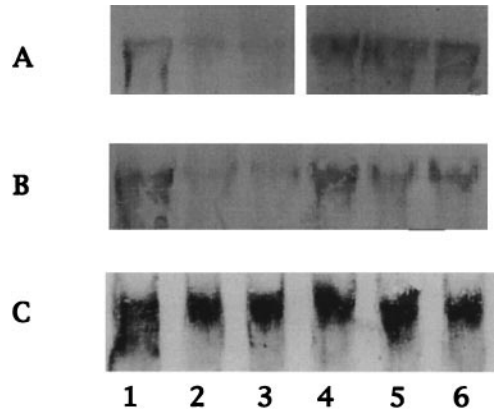

FIG. 2. (A) Representative Western blot for CYP1A1 protein levels in Hepa1 cells treated with LPS conditioned medium. Lanes 1–3: Hepa1 cells treated with MCM, produced using 0, 10, and 50 μg/mL of LPS, respectively. Lanes 4–6: Hepa1 cells treated directly with 0, 10, and 50 μg/mL of LPS. Each lane contained material pooled from the 4 individual incubations used in Fig. 1. In the case of MCM-treated cells, protein levels, as determined by densitometry, were 17 and 15% of control for 10 and 50 μg/mL of LPS, respectively. In the case of UCM, protein levels were 99 and 92% of control. (B) Representative Western blot of CYP1A1 protein levels in Hepa1 cells treated with LPS and PNTX. Lanes 1–3: protein levels in cells exposed to MCM, produced using 0, 10, and 50 μg/mL of LPS, respectively. Lanes 4–6: protein levels in cells exposed to MCM, produced using 0, 10, and 50 μg/mL of LPS, respectively, and PNTX. Each lane contained material pooled from the 4 individual incubations used in Fig. 3. In cells treated with MCM alone, protein levels, as determined by densitometry, were depressed to 43 and 29%, respectively, by 10 and 50 μg/mL of LPS. In cells exposed to PNTX, protein levels were depressed by LPS to 66 and 80%, respectively. (C) Representative Western blot in Hepa1 cells treated with TNF-α. Lanes 1–3: protein levels in cells treated directly with 0, 5, and 10 ng/mL of TNF-α. Lanes 4–6: protein levels in cells treated with medium, produced using 0, 5, and 10 ng/mL of TNF-α. Each lane contained material pooled from the 4 individual incubations used in Fig. 4. Following direct addition of TNF-α, protein levels, as determined by densitometry, were 104 and 105% of control for 5 and 10 ng/mL, respectively. Following the addition of TNF-α, conditioned medium protein levels were 105 and 86% of control for 5 and 10 ng/mL.

Ability of TNF-α to Decrease CYP1A1 Activity

When TNF-α was added directly to Hepa1 cells, the EROD activity was decreased by 29 and 44% for 5 and 10 ng/mL of TNF-α. Conditioned medium from TNF-α-stimulated macrophages also down-regulated CYP1A1 activity by 26 and 35% for 5 and 10 ng/mL of TNF-α, respectively (Fig. 4). In contrast to experiments with LPS, however, Western blot analysis indicated that TNF-α had no effect on the levels of CYP1A1 protein (Fig. 2C).

DISCUSSION

The addition of LPS to Hepa1 cells demonstrated that LPS had no direct effect on the induced activity of CYP1A1 in these cells. In experiments designed to produce a model of the *in vivo* situation, Hepa1 cells were exposed to medium

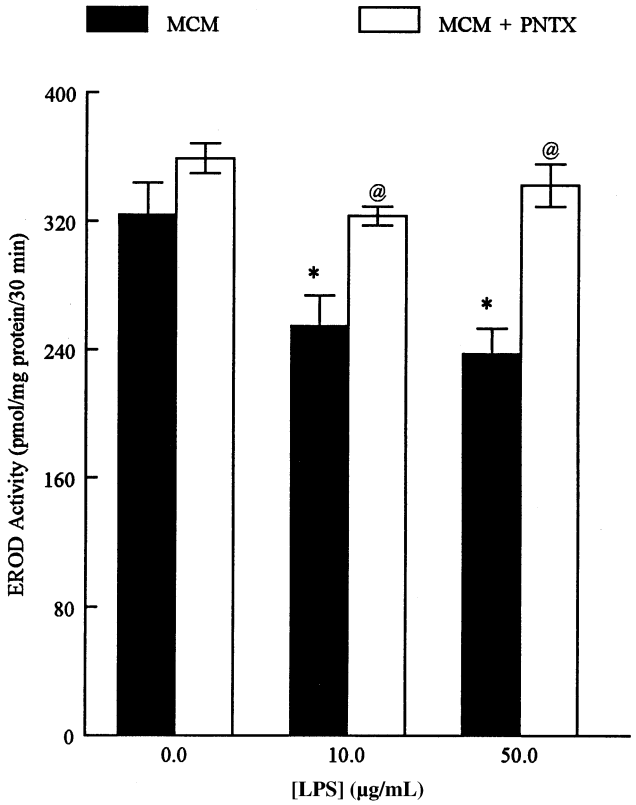

FIG. 3. Ability of PNTX to block LPS-mediated down-regulation of CYP1A1. Macrophages, plated and incubated for 48 hr, were stimulated with 0, 10, or 50 μg/mL of LPS or with 0, 10, or 50 μg/mL of LPS and PNTX added concurrently. These are designated as MCM and MCM + PNTX in the figure, respectively. These conditioned media were removed from macrophages and added to Hepa1 cells along with DBA and incubated for 24 hr. Each value is the mean ± SEM for 4 individual cultures. *Significantly different from corresponding controls containing zero LPS, $P < 0.05$; and @significantly different from corresponding cells treated with MCM alone, $P < 0.05$.

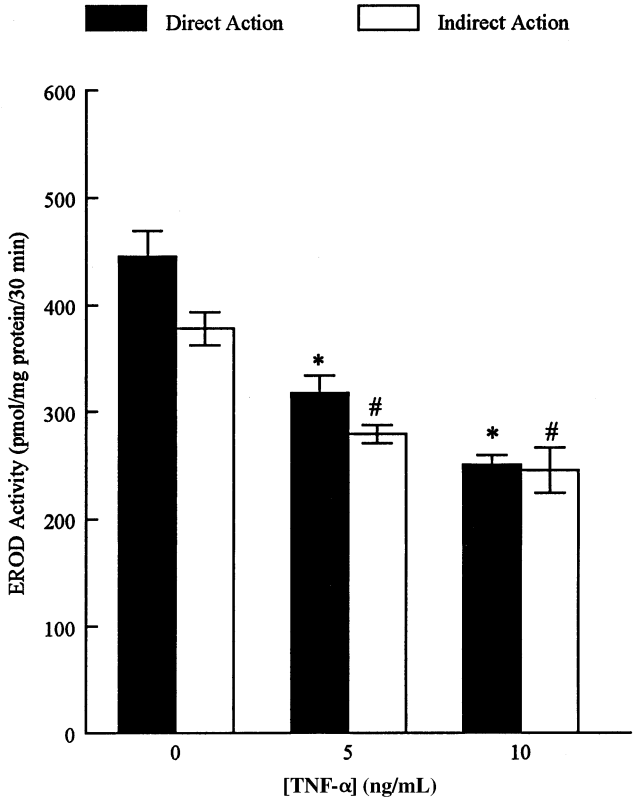

FIG. 4. Ability of TNF-α to down-regulate CYP1A1 activity. Macrophages were plated and incubated for 48 hr and then were stimulated with 0, 5, or 10 ng/mL of TNF-α or with vehicle alone (medium) for 24 hr. Hepa1 cells were plated and incubated for 24 hr and then were subjected to TNF-α conditioned medium with DBA (indirect action) or to unconditioned medium with TNF-α and DBA added concurrently (direct action). Following an additional 24-hr incubation period, the EROD activity levels for Hepa1 cells were determined. Each value is the mean ± SEM for 4 individual cultures. *Significantly different from corresponding controls containing normal medium, $P < 0.05$; and #significantly different from corresponding controls containing medium from macrophages treated with vehicle, $P < 0.05$.

obtained from LPS-stimulated macrophages. Macrophages are known to contain CD14 receptors and do respond to LPS challenge [23]. The addition of this MCM to hepatoma cells led to a down-regulation of CYP1A1, an effect that mirrors *in vivo* administration of LPS. Direct administration of LPS to Hepa1 cells did not lead to a decrease in CYP1A1 activity, an observation in agreement with previous reports [8, 24]. The loss of enzyme activity caused by treatment with MCM was accompanied by a concomitant decrease in CYP1A1 protein levels. We hypothesize that this effect resulted from cytokines released into the medium by LPS-stimulated macrophages.

Chensue *et al.* [25] mapped the *in vivo* release of cytokines following the administration of LPS to mice. They found that TNF-α was consistently the first to be released followed by IL-1β then IL-6 and IL-1α, an observation in agreement with the commonly held view that TNF-α and IL-1β are the early phase cytokines. It has been postulated that the early release of TNF-α is required to stimulate the release of IL-1 and IL-6; therefore, the blockade of TNF-α synthesis would be expected to attenuate the effect of LPS on CYP1A1 activity. Indeed, medium from macrophages treated concurrently with LPS and PNTX, a TNF-α synthesis inhibitor [26–28], attenuated the down-regulation of CYP1A1 activity and CYP1A1 protein by LPS. PNTX is known for its ability to inhibit the production of TNF-α [26–28], but there is some debate about the specificity of this drug. D'Hellencourt *et al.* [29] reported that 1.0 mM of PNTX could inhibit the production of IL-β and IL-10, in addition to TNF-α; at concentrations of 0.1 mM, only the production of TNF-α is inhibited. Thus, at the concentration used in the present experiments (0.6 mM), there would only be minimal effects on IL-β or IL-10, with a significant inhibition of TNF-α. This is also supported by the results indicating that TNF-α added directly to Hepa1 cells caused a significant down-regulation of CYP1A1. Although these experiments support the idea that TNF-α is an important mediator of this effect, they do not reveal whether this

action results from blocking the direct action of TNF-α on hepatocytes or preventing the release of other acute phase cytokines by TNF-α.

Interestingly, Western blot analysis demonstrated that PNTX was able to block the down-regulation of CYP1A1 activity and protein levels caused by LPS. On the other hand, the direct and indirect effects of TNF-α were to decrease CYP1A1 activity, but this was not accompanied by a loss in protein levels. Clearly, these results imply that the mode of regulation of CYP1A1 activity by LPS and TNF-α occurs through two separate mechanisms. From Western blots, it appears as though LPS mediates its effects through a pretranslational mechanism such as transcriptional inhibition, whereas TNF-α mediates its effects though a post-translational mechanism in which the enzyme protein is not active but is still recognized by the antibodies. The discrepancy between these results suggests that TNF-α is not the sole mediator of LPS-induced down-regulation of CYP1A1; rather, it must result from a combination of cytokines. Residual LPS would not be responsible for this effect, as LPS does not have a direct effect in hepatoma cells (Fig. 1). Both IL-1 and IL-6 are capable of transcriptional inhibition of CYP1A1 and 1A2 [2, 8, 9, 12–14], implicating these two cytokines as prime mediators in LPS-induced down-regulation of CYP1A1 activity and protein levels. The role of TNF-α in the down-regulation of CYP1A1 by LPS may be as a stimulus for the release of IL-1 and IL-6, in addition to some direct action on hepatoma cells.

It is well known that endotoxin and the resultant cytokines, TNF-α in particular, can induce the release of NO from macrophages and hepatocytes [30–33]. NO is capable of binding to the heme portion of P450 enzymes, blocking the binding of O_2 and thus inhibiting the activity of the enzyme. Wink *et al.* [34] reported that action of NO on CYP1A1 and 2B1 has both a reversible and irreversible phase. They postulated that NO binds to the heme portion of P450 blocking enzyme catalysis, an effect that can be overcome by increasing the concentration of O_2. Because this binding is reversible, it cannot account for the lasting suppression in activity seen following a 24-hr incubation. However, NO can react with oxygen to form reactive nitrogen oxides capable of oxidizing amino acids critical to the functioning of the enzyme with resultant irreversible loss of catalytic activity. If this alteration in the enzyme occurs with direct stimulation of hepatoma cells by TNF-α, the inhibition of enzymatic activity would account for the western blot results, as cytokine treatment inhibits activity of the enzyme and not its transcription. Experiments by Stadler *et al.* [35] found that the stimulation of hepatocytes with conditioned medium results in a functional inhibition of NO and, to a lesser degree, suppression of enzyme expression via transcriptional inhibition. Work by Khatsenko *et al.* [36] also confirms these findings, as they have demonstrated that addition of the NO synthase inhibitor L-nitroarginine methylester attenuates the loss of CYP1A1 activity by LPS but could not entirely block it, implying that down-regulation is not mediated by NO alone. Carlson and Billings [16] found that decreases in the constitutive isoforms of P450s mediated by IL-1β or TNF-α were associated with increased NO generation and could be blocked by inducible NO synthase inhibitors. In addition, they found that down-regulation by IL-6 was independent of NO generation and, thus, was acting via a different mechanism. These results, along with observations reported here, demonstrate that the effect of LPS on hepatocytes occurs through two separate mechanisms. The induction of NO release by cytokines such as TNF-α and IL-1β leads to an inhibition of CYP1A1 catalytic activity, whereas the primary action of IL-1α and IL-6 would be the transcriptional inhibition of CYP1A1, leading to a down-regulation in protein levels.

In summary, we have shown that LPS down-regulates CYP1A1 activity in an indirect manner. LPS stimulated macrophages to release cytokines, which down-regulate the activity of CYP1A1. It was also demonstrated that down-regulation occurs via two separate mechanisms that are cytokine dependent. TNF-α administration decreased CYP1A1 activity through a post-transcriptional mechanism, whereas the combination of cytokines released by LPS down-regulated CYP1A1 activity through a pretranslational type of mechanism.

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