Pentoxifylline Attenuates Bacterial Lipopolysaccharide-Induced Enhancement of Allyl Alcohol Hepatotoxicity

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Small amounts of exogenous lipopolysaccharide (LPS) (10 ng/kg–100 μg/kg) enhance the hepatotoxicity of allyl alcohol in male Sprague-Dawley rats. This augmentation of allyl alcohol hepatotoxicity appears to be linked to Kupffer cell function, but the mechanism of Kupffer cell involvement is unknown. Since Kupffer cells produce tumor necrosis factor-alpha (TNFα) upon exposure to LPS, and this cytokine has been implicated in liver injury from large doses of LPS, we tested the hypothesis that TNFα contributes to LPS enhancement of allyl alcohol hepatotoxicity. Rats were treated with LPS (10–100 μg/kg iv) 2 h before allyl alcohol (30 mg/kg ip). Co-treatment with LPS and allyl alcohol caused liver injury as assessed by an increase in activity of alanine aminotransferase in plasma. Treatment with LPS caused an increase in plasma TNFα concentration, which was prevented by administration of either pentoxifylline (PTX) (100 mg/kg iv) or anti-TNFα serum (1ml/rat iv) 1 h prior to LPS. Only PTX protected rats from LPS-induced enhancement of allyl alcohol hepatotoxicity; anti-TNFα serum had no effect. Exposure of cultured hepatocytes to LPS (1–10 μg/ml) or to TNFα (15–150 ng/ml) for 2 h did not increase the cytotoxicity of allyl alcohol (0.01–200 μM). These data suggest that neither LPS nor TNFα alone was sufficient to increase the sensitivity of isolated hepatocytes to allyl alcohol. Furthermore, hepatocytes isolated from rats treated 2 h earlier with LPS (i.e., hepatocytes which were exposed in vivo to TNFα and other inflammatory mediators) were no more sensitive to allyl alcohol-induced cytotoxicity than hepatocytes from naive rats. These data suggest that circulating TNFα is not involved in the mechanism by which LPS enhances hepatotoxicity of allyl alcohol and that the protective effect of PTX may be due to another of its biological effects.

Key Words: tumor necrosis factor-alpha (TNFα); liver damage; Kupffer cells; allyl alcohol.

Lipopolysaccharide (LPS, endotoxin), an important component of gram-negative bacterial cell membranes (Raetz et al., 1988, 1991; Rietschel et al., 1994), elicits strong host defenses in mammals. These defenses include a pronounced inflammatory response characterized by activation of inflammatory cells and release of soluble mediators (Watson et al., 1994). In most instances, this response is beneficial to the host; however, it can also be detrimental. LPS has been associated with conditions such as septic shock and multiple organ system failure (Siegel et al., 1993), and large doses of LPS cause tissue injury (Hewett et al., 1992; Hirata et al., 1980; Jaeschke et al., 1991; Shibayama, 1987; Wang et al., 1995). In addition, exposure to endogenous or exogenous LPS enhances the toxicity of several xenobiotics, including carbon tetrachloride (Chamulitrat, et al., 1994), alpha-naphthylisothiocyanate (ANIT) (Calcagni et al., 1992), halothane (Lind et al., 1984), allyl alcohol (Sneed et al., 1997), ethanol (Hansen et al., 1994) and galactosamine (Galanos et al., 1979). This augmentation of toxicity likely involves LPS-invoked inflammatory mediators. For example, ANIT-induced hepatotoxicity, a component of which involves increased exposure of the liver to endogenous LPS (Calcagni et al., 1992), is prevented by prior depletion of blood neutrophils (Dahm et al., 1991), suggesting that these inflammatory cells and their mediators are central to the toxic response.

We have recently demonstrated that the hepatotoxicity of allyl alcohol is enhanced by pretreatment with quite small doses of LPS and that this augmented response is prevented by inhibition of the function of another cellular mediator of inflammation, Kupffer cells (Sneed et al., 1997). These results indicate that properly functioning Kupffer cells are important in the mechanism of LPS-induced enhancement of allyl alcohol hepatotoxicity and evoke interest in whether inflammatory mediators released by these cells participate in augmenting toxicity.

Kupffer cells are the resident macrophages of the hepatic sinusoids (Bouwens and Wisse, 1992; Jones and Summerfield, 1988; Wisse et al., 1996) and have a major role in clearing the hepatic portal blood of intestinally derived LPS (Fox et al., 1989; Toth and Thomas, 1992). These macrophages respond to LPS with production of mediators such as cytokines (e.g., tumor necrosis factor-alpha [TNFα], interleukin-1, and interleukin-6), reactive oxygen species, and prostaglandins (Decker, 1990). Kupffer cells play a critical role in liver injury from
large doses of LPS as evidenced by the observation that inhibition of their function with gadolinium chloride (GdCl₃) affords protection (Brown et al., 1997; Iimuro et al., 1994; Pearson et al., 1997). Cytokines are also essential to LPS-induced responses. For example, inhibition of TNFα synthesis or activity attenuates LPS-mediated liver injury and lethality in baboons (Tracey et al., 1987), mice (Beutler et al., 1985) and rats (Hewett et al., 1993). These results indicate that TNFα is important in the pathogenesis of tissue injury from large doses of LPS and raise the possibility that TNFα may be a factor in the ability of LPS to enhance the hepatotoxicity of xenobiotics. The present study was undertaken to test the hypothesis that TNFα participates in the potentiation of allyl alcohol hepatotoxicity by LPS. Two approaches were taken to inhibit the effects of TNFα in animals treated with LPS and allyl alcohol: pentoxifylline (PTX) was given to inhibit synthesis of TNFα, and an antisemdirer directed against TNFα was administered to neutralize TNFα activity.

MATERIALS AND METHODS

Animals. Male, Sprague-Dawley rats (ICR; CD-CD-(SD)BR VAF/Plus; Charles River, Portage, MI) weighing 200–300 g were used in these studies. The animals were allowed food (Rodent Chow, Teklad, Madison, WI) and water ad libitum. They were maintained on a 12-h light and dark cycle under conditions of controlled temperature and humidity.

Isolation of hepatocytes. Hepatocytes were isolated by collagenase digestion (Klaunig et al., 1981; Seglen, 1973), placed in Williams’ medium E supplemented with 10% fetal calf serum and 0.1% gentamicin, and plated in 6-well primaria plates (Falcon Laboratories) at a density of 5 × 10⁵ cells per well. In some experiments, hepatocytes were obtained from the livers of rats treated 2 h earlier with LPS (4 mg/kg iv). For all experiments, viability of the isolated cells was ≥ 85% as measured by trypan blue exclusion.

The hepatocytes were allowed to stabilize in culture for 3 h, the medium was removed, and the cells were washed once with Williams’ medium E supplemented only with 0.1% gentamicin. A final volume of 2 ml per well of the latter medium was used in the remainder of the study. Alkyl alcohol was added to the hepatocyte cultures at the concentrations indicated in the figures and in the Results section. Hepatocyte injury was assessed 90 or 180 min after treatment with LPS (100 µg/kg iv). Two h after treatment with LPS or saline vehicle, animals were killed. The liver was removed and homogenized in a solution of 0.05 M HEPES (pH 8.4) and 0.33 mM dithiothreitol. The homogenate was centrifuged at 100,000 × g for 45 min. The supernatant fluid was collected, and activity of alcohol dehydrogenase (ADH) was measured spectrophotometrically (366 nm) by monitoring the reduction of nicotinamide adenine dinucleotide (NAD) using ethanol as a substrate (Krebs et al., 1969). Protein concentration in the supernatant fluid was determined using the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL), which uses the method of Smith and coworkers (1985).

Treatment of animals with anti-TNFα serum. Serum directed against TNFα (anti-TNFα serum) was produced in New Zealand White rabbits (Hewett et al., 1993). Rats were treated intravenously with anti-TNFα serum (1 ml diluted with 1 ml of saline) 1 h before treatment with LPS (10 µg/kg iv). This treatment protocol has been shown previously to prevent the LPS-induced increase in plasma TNFα activity (Hewett et al., 1993). Two h after administration of LPS, allyl alcohol (30 mg/kg) or sterile saline was injected intraperitoneally. Liver injury was assessed 6 h later.

Assessment of hepatotoxicity in vivo. Rats were anesthetized with sodium pentobarbital (50 mg/kg ip), and blood was collected from the abdominal aorta into syringes containing sodium citrate (final concentration, 0.38%). ALT activity was determined in plasma using Sigma Diagnostics Kit No. 59-UV.

Determination of activity of TNFα. Ninety min after administration of LPS or its saline vehicle, as described above for experiments with PTX and anti-TNFα serum, blood was collected from rats for determination of the activity of TNFα. Plasma was prepared, serially diluted, and incubated for 22 h in the presence of the TNFα-sensitive fibrosarcoma cell line, WEHI 164 clone 13 (Eskandari et al., 1990; Espevik and Nissen-Meyer, 1986). The extent of cell lysis was measured with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) using a Bio-Tek plate reader.

Statistical analysis. Data are expressed as means ± SEM. For all in vitro studies, N represents the number of repetitions, each performed on a different day with cells from different animals. For all in vivo studies, n represents the number of individual animals used, and experiments were repeated at least once. Homogeneous data were analyzed by one-way or two-way analysis of variance (ANOVA) for in vivo studies and by repeated measures ANOVA for in vitro studies. ALT content varies among isolations of hepatocytes, thus introducing variability among experimental days. The repeated measures ANOVA was used to remove the contribution of this variability from the statistical analysis to allow detection of treatment-related differences. Individual means for all data analyzed by ANOVA were compared using Tukey’s omega test. When variances were not homogeneous, data were analyzed using Kruskal-Wallis ANOVA on ranks, and Dunn’s test was used to assess significance. Data expressed as percentages were transformed by the arc sine square root method prior to analysis. The criterion for statistical significance was p ≤ 0.05.

RESULTS

Effect of In Vitro Exposure to LPS on Allyl Alcohol-Induced Cytotoxicity in Isolated Hepatocytes

To test whether LPS had direct effects on hepatocytes that contribute to enhancement of allyl alcohol toxicity, isolated hepatocytes were pretreated with LPS for 2 h, then exposed to...
allyl alcohol (Fig. 1). This experimental design was selected to mimic the dosing regimen for LPS and allyl alcohol that results in LPS enhancement of allyl alcohol toxicity \textit{in vivo} (Sneed et al., 1997). In hepatocytes not exposed to LPS, allyl alcohol caused a concentration-related increase in ALT release at 90 min; statistical differences were observed at concentrations of allyl alcohol $\geq 100 \mu$M. In hepatocytes treated with LPS, a significant rise in ALT release was also seen at concentrations of allyl alcohol $\geq 100 \mu$M, and there were no significant differences in ALT release among groups at any concentration of allyl alcohol. LPS was not cytotoxic in the absence of allyl alcohol (Fig. 1). Similar experiments were performed in which cytotoxicity was assessed at 180 min after addition of allyl alcohol. No further increase in toxicity was observed at this time, and no differences were observed among the LPS-treated groups (data not shown).

**Protection from LPS-Induced Enhancement of Allyl Alcohol Hepatotoxicity by PTX**

PTX decreases the synthesis of TNF$\alpha$ at the mRNA level (Dezube et al., 1993). Control animals treated with saline only, or with PTX and saline only, did not have detectable plasma activity of TNF$\alpha$ (Table 1). TNF$\alpha$ activity was increased significantly in the plasma of animals treated 90 min earlier with LPS. Pretreatment with PTX significantly reduced the circulating activity of TNF$\alpha$.

Plasma ALT activity was low in rats in the saline/saline or LPS/saline groups irrespective of PTX pretreatment (Fig. 2). In animals that received the vehicle for PTX and then were treated with saline and allyl alcohol, there was an increase in plasma ALT activity, but this increase was not statistically significant.
Animals treated with the vehicle for PTX and then cotreated with LPS and allyl alcohol had significantly elevated plasma ALT activity. Pretreatment with PTX attenuated the increase in plasma ALT activity in cotreated animals.

Allyl alcohol hepatotoxicity requires bioactivation by ADH to acrolein. Accordingly, we examined the effect of PTX on the activity of ADH in the livers of rats pretreated with LPS in order to determine if PTX afforded protection by inhibition of the bioactivation of allyl alcohol. As shown in Table 2, pretreatment with PTX did not affect the activity of ADH in rat liver.

Lack of Effect of Inactivation of TNFα on LPS-Induced Potentiation of Allyl Alcohol Hepatotoxicity

To verify that reduction of circulating TNFα protects rats from LPS-potentiated allyl alcohol hepatotoxicity, animals were treated with an anti-serum specific for TNFα prior to treatment with LPS. In preliminary studies, the efficacy of the anti-TNFα antibody was determined. A marked increase in plasma TNFα activity was observed in LPS-treated animals pretreated with vehicle (3.5 ± 1.4 ng/ml). The administration of 1 ml of anti-TNFα serum prevented the rise in plasma TNFα such that TNFα activity in all samples (n = 4) was below the limit of detection (which was approximately 2 pg/ml).

There was no significant elevation in plasma ALT activity in animals in the saline/saline, LPS/saline or saline/allyl alcohol groups, irrespective of pretreatment with control or anti-TNFα serum (Fig. 3). Plasma ALT activity was significantly elevated in animals cotreated with LPS and allyl alcohol compared to animals treated with LPS alone or allyl alcohol alone. There was no significant difference in ALT activity between co-treated animals pretreated with control serum and anti-TNFα serum.

Effects of in Vitro Exposure to TNFα on Allyl Alcohol-Induced Cytotoxicity in Isolated Hepatocytes

To test whether TNFα alone can enhance the hepatotoxicity of allyl alcohol in isolated hepatocytes, cells were pretreated with TNFα for 2 h before exposure to allyl alcohol. Two concentrations of TNFα were used: 15 ng/ml to replicate the TNFα activity found in peripheral plasma of rats treated with LPS (Table 1), and 150 ng/ml to estimate a greater TNFα activity potentially found in the liver sinusoids after treatment with LPS. As in experiments depicted in Figure 1, allyl alcohol caused a concentration-dependent increase in release of ALT (Fig. 4). Cytotoxicity of allyl alcohol was unaffected by pretreatment with TNFα.

**Allyl Alcohol-Induced Cytotoxicity in Isolated Hepatocytes from LPS Treated Rats**

TNFα reaches a maximal concentration in plasma 90-min after treatment with LPS in vivo. To examine whether in vivo exposure to TNFα increased sensitivity of hepatocytes to allyl alcohol, hepatocytes were isolated from rats treated with LPS 2 h earlier and were exposed to allyl alcohol as described in Figure 1. Allyl alcohol caused a concentration-dependent increase in ALT release in hepatocytes isolated from LPS-treated rats (Fig. 5). Maximal release of ALT was about 70% of total in cells from naïve rats or from LPS-treated rats. The concentration of allyl alcohol at which half maximal cytotoxicity was observed was about 33 μM in cells from naïve rats (similar to

![FIG. 3. Lack of protection from LPS enhancement of allyl alcohol hepatotoxicity by anti-TNFα serum. Animals were pretreated with control or anti-TNFα serum (1 ml iv) 1 h prior to treatment with LPS (10 μg/kg iv). Allyl alcohol (30 mg/kg ip) was given 2 h after LPS. Liver damage was measured 6 h after allyl alcohol treatment. This experiment was performed 3 times with comparable results. Data are combined results from all three days. Data are expressed as mean ± SEM; (a) indicates a significant difference from respective value in absence of LPS; (b) indicates a significant difference from respective values in the absence of allyl alcohol; n = 3–13.](image)
results presented in Fig. 1). In hepatocytes taken from LPS-treated rats, this value was about 64 µM.

DISCUSSION

We have reported previously that very small amounts (10 ng/kg–100 µg/kg) of LPS potentiate the hepatotoxicity of allyl alcohol (Sneed et al., 1997), and the studies presented here were performed to begin to explore the mechanism of potentiation. Hepatic injury resulting from exposure to relatively large doses of LPS is dependent upon several factors. These factors include, but are not limited to, the release of inflammatory mediators by activated macrophages and the influx of inflammatory cells into the liver. Blockade or inhibition of any one of these factors prevents the hepatic injury associated with large doses of LPS (Chang et al., 1993; Hewett et al., 1993; Imuro et al., 1994; Jaeschke et al., 1991; Sato et al., 1993; Tracey et al., 1987). Although LPS damages the liver through indirect means via inflammatory cells and soluble mediators, direct effects of LPS on hepatocytes have been reported. For example, LPS decreases bile formation (Uttiri et al., 1977) and increases fatty acid synthesis in the liver (Feingold et al., 1992). It is unlikely that the direct effects of LPS contribute to the enhancement of hepatotoxicity of allyl alcohol, because allyl alcohol-induced cytotoxicity in isolated hepatocytes was not altered by pretreatment of cells with LPS (Fig. 1). Thus, these results support the hypothesis that factors other than LPS alone are responsible for the enhancement of hepatotoxicity seen in vivo.

This hypothesis is consistent with results of studies in which inhibition of Kupffer cell function prevented enhancement of allyl alcohol hepatotoxicity by LPS (Sneed et al., 1997). One of the inflammatory mediators produced by LPS-activated Kupffer cells is the proinflammatory cytokine, TNFα, which plays a critical role in liver injury from large doses of LPS (Beutler et al., 1985; Hewett et al., 1993; Tracey et al., 1987). Accordingly, we examined the role of TNFα in LPS potentiation of allyl alcohol hepatotoxicity.

The methylxanthine, PTX, inhibits the synthesis of TNFα (Dezube et al., 1993; Doherty et al., 1991; Han et al., 1990; Noel et al., 1990; Semmler et al., 1993; Zabel et al., 1989, 1993), and results presented here (Table 1) confirm this. Administration of PTX prior to LPS treatment protected animals from the enhanced hepatotoxicity of allyl alcohol. These data suggested that TNFα might be involved in the mechanism by which LPS augments the hepatotoxicity of allyl alcohol. PTX, however, has multiple pharmacological effects; therefore, a
more specific approach, neutralization of TNFα with an anti-TNFα serum, was used to test further whether inhibition of TNFα afforded protection. The anti-TNFα serum did not diminish LPS enhancement of allyl alcohol hepatotoxicity despite complete neutralization of circulating TNFα activity. Protection by PTX and lack of protection by antisera-duced neutralization of TNFα have also been observed in a rat model of intestinal injury induced by nonsteroidal anti-inflammatory drugs (Reuter and Wallace, 1999) and in a model of bacteria-induced lung injury in rabbits (Miyazaki et al., 1999).

One explanation for the disparate results observed with PTX and anti-TNFα serum in these studies is that, since PTX inhibits synthesis of TNFα, it affords a more complete blockade of TNFα action in the liver, whereas TNFα is still produced by Kupffer cells after treatment with anti-TNFα serum and can act locally before neutralization by the anti-serum. Thus, autocrine or paracrine hepatic effects of TNFα may still occur. An alternative explanation is that TNFα is not involved in the mechanism by which LPS enhances the hepatotoxicity of allyl alcohol. This explanation is supported by results from two series of in vitro experiments presented here. In the first, exposure of isolated hepatocytes to TNFα did not alter the cytotoxic response to allyl alcohol (Fig. 4), indicating that direct effects of TNFα on hepatocytes are not sufficient to increase sensitivity to allyl alcohol. Others have also shown that TNFα alone is not cytotoxic to isolated hepatocytes, and that cell damage requires the addition of other cytokines or induction of oxidative stress in the cells (Adamson and Billings, 1992; Sieg and Billings, 1997). In the second series of experiments, hepatocytes isolated from rats treated 2 h earlier with LPS were used. Since TNFα activity in plasma reaches a peak 90 min after administration of LPS, these hepatocytes were exposed to TNFα in vivo. Despite this exposure to TNFα and other mediators evoked by treatment with LPS, allyl alcohol was neither more potent nor more toxic in these cells. Maximal cytotoxicity was observed at the same concentration of allyl alcohol (100 μM) in both cell populations, and the concentration of allyl alcohol required to achieve half-maximal cytotoxicity was greater, not less, in hepatocytes from LPS-treated rats compared to those from naïve rats. These results suggest that exposure in vivo to LPS-duced mediators for up to 2 h is not sufficient to increase sensitivity of hepatocytes toward allyl alcohol.

If TNFα is not involved in the mechanism by which LPS enhances allyl alcohol hepatotoxicity, then the protective effect produced by PTX is due to one or more of the other pharmacological properties of this drug. One possibility explored in this study was that PTX inhibited toxicity by decreasing activity of ADH and thereby decreasing the formation of the toxic metabolite of allyl alcohol. In fact, hepatic ADH activity was not different in vehicle- and PTX-treated rats (Table 2). Another possibility is that PTX decreased Kupffer cell function through its inhibition of phosphodiesterase, which increases intracellular levels of cyclic adenosine monophosphate (cAMP). Increases in cAMP have been associated with inhibition of macrophage function (Taffet et al., 1989), and decreased Kupffer cell function protects against hepatotoxicity from LPS plus allyl alcohol (Sneed et al., 1997). PTX also improves blood flow in tissues (Ward and Clissold, 1987). This effect of PTX has been demonstrated to be protective in one model of sepsis in which high mortality was associated with hemodynamic shock (Yang et al., 1999). In addition, PTX reduces the levels of toxic free radicals, attenuates the expression of inducible nitric oxide synthase (Wu et al., 1999) and decreases the respiratory burst of neutrophils (Kowalski et al., 1999). A combination of the above factors may be involved in the ability of PTX to protect animals from LPS-enhanced allyl alcohol hepatotoxicity.

In summary, inflammatory mediators may participate in the ability of LPS to enhance the hepatotoxicity of certain xenobiotics. In LPS-induced enhancement of allyl alcohol hepatotoxicity, however, circulating TNFα does not appear to play a major role. The observation that TNFα may not be involved in the mechanism by which LPS enhances the toxic response to allyl alcohol is interesting, because it suggests (1) that although hypotheses can be formulated based on what is known about organ injury from larger, toxic doses of LPS, the mechanisms may not be the same for smaller doses that augment the toxicity of other chemicals, and (2) that select, and not all, components of inflammation are critical to this enhanced response. The drug PTX protects animals from the LPS-mediated enhancement of allyl alcohol-induced liver injury and may do so by affecting the responses of Kupffer cells to the presence of LPS.

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REFERENCES


