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Estriol Enhances Lipopolysaccharide-Induced Increases in Nitric Oxide Production by Kupffer Cells via Mechanisms Dependent on Endotoxin

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Background: Estriol causes sensitization of Kupffer cells to lipopolysaccharide (LPS) via mechanisms dependent on gut-derived LPS. Accordingly, this study examines the effect of estriol treatment on nitric oxide (NO) production from Kupffer cells.

Methods: Rats were given estriol (20 mg/kg body weight) intraperitoneally, and Kupffer cells were isolated 24 hr later. Some rats were treated for 4 days with 150 mg/kg/day of polymyxin B and 450 mg/kg/day of neomycin to prevent growth of intestinal bacteria, the primary source of endotoxin in the gastrointestinal tract. After addition of LPS, NO production by Kupffer cell was detected using a fluorescence indicator, DAF-2.

Results: Twenty-four hours after estriol administration, LPS-induced NO production by Kupffer cells was enhanced as compared with control Kupffer cells. Sterilization of the gut with antibiotics blocked this enhancement.

Conclusions: Estriol treatment in vivo enhances LPS-induced NO production in Kupffer cells.

KUPFFER CELLS, THE resident macrophages in the liver, play a pivotal role in removal of gut-derived endotoxin (lipopolysaccharide [LPS]) from blood (Nolan, 1981). During this process, endotoxin activates Kupffer cells to release active substances such as eicosanoids, inflammatory cytokines, superoxide, and nitric oxide (NO) (Decker, 1990). These mediators have been implicated in chemical-induced hepatotoxicity (Laskin and Pendino, 1995) and are partially responsible for the pathophysiology of alcoholic liver disease (Adachi et al., 1995).

NO is a potent vasodilator synthesized from L-arginine by NO synthase, which, in the liver, exists in at least two functionally distinct forms (Hibbs et al., 1987; Stamler et al., 1992). One form, constitutively active in the vascular endothelium, regulates vasomotor tone and tissue perfusion (Moncada et al., 1988; Palmer et al., 1988). A genetically distinct form of NO synthase is induced in the vascular endothelium, macrophages, smooth muscle cells, and hepatocytes in response to inflammatory cytokines and endotoxin (Gaillard et al., 1991). We hypothesized that modulation of cytotoxic effect by NO could explain some of the liver changes induced by ethanol.

It is still unclear, however, why women develop alcoholic liver injury more rapidly and to a greater extent than men; hormonal effects are an obvious possibility. We showed that estriol increases portal endotoxin via mechanisms dependent on gut permeability (Enomoto et al., 1999), consistent with the hypothesis that higher plasma endotoxin levels lead to more extensive Kupffer cell activation in women than in men. Iimuro et al. (1997) reported that female rats develop hepatic injury more rapidly and with exposure to less ethanol than male rats. In the study, they showed that plasma endotoxin levels in female rats were significantly higher than in male rats. It is therefore possible that treatment with estriol potentiates LPS-induced NO production by Kupffer cells. The purpose of this study was to test this hypothesis.

MATERIALS AND METHODS

Animals and Treatments

Female Wistar rats that weighed between 200 and 250 g were used in this study. All animals were given humane care in compliance with institutional guidelines. Twelve rats were given estriol (20 mg/kg body weight intraperitoneally) 24 hr before experiments. They were divided into three groups, and one group was treated for 4 days with polymyxin B and neomycin (Sato et al., 1983) to prevent growth of intestinal bacteria, the primary source of endotoxin in the gastrointestinal tract, before estriol treatment. On the basis of the results of preliminary experiments, 150 mg/kg/day of polymyxin B and 450 mg/kg/day of neomycin were given orally. Under these conditions, gut sterilization was achieved (Adachi et al., 1995).

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Kupffer Cell Preparation, Culture, and Measurement of NO Production

Kupffer cells were isolated by collagenase digestion (Pertoft and Smedsrød, 1987) and cultured in RPMI 1640 (GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL of penicillin G and 100 μg/mL of streptomycin sulfate) at 37°C with 5% CO_2. Cells were incubated with fresh media containing LPS (10 μg/mL supplemented with 5% rat serum) for an additional 12 hr. Kupffer cells were loaded with DAF-2DA (20 μM) for 1 hr. The glass-bottom culture dish was mounted on an inverted fluorescence microscope (Diaphot, Nikon, Tokyo, Japan) equipped with xenon lamp, an excitation filter (470–490 nm), a diachronic mirror (505 nm), and an emission filter (515–550 nm). Optical signals were recorded with an ARGUS-50 imaging system (Hamamatsu Photonics, Hamamatsu, Japan) with a charge-coupled device camera (Kojima et al., 1998). Fluorescence intensity of DAF-2 was quantified every 1 min for 10 min in user-selected regions of uniform pixel value with a 256-level gray scale corresponding to a uniform site in cytoplasm. Background fluorescence and cellular autofluorescence were not detectable at the setting used for gain and sensitivity. Moreover, DAF-2 measurement was performed in the control and estriol groups under the same conditions.

Statistical Analysis

All results were expressed as mean ± standard error of the mean. Statistical differences between means were determined using ANOVA and Bonferroni's post hoc test. $P < 0.05$ was selected before the study to reflect significance.

RESULTS

Fluorescence images of Kupffer cells loaded with DAF-2 in the presence of LPS (10 μg/ml) are shown in Fig. 1. Time intervals between the images are 3 to 4 min. The fluorescence intensity of DAF-2-loaded Kupffer cells was elevated with time as a result of an increase in the triazole forms of DAF-2 converted from DAF-2 by reaction with NO. Accordingly, the increased intensity correlated with the amounts of NO production. NO production was much higher in Kupffer cells from estriol-treated rats (Fig. 1B) as compared with cells from control rats (Fig. 1A). These results indicated that estriol treatment enhances NO production by Kupffer cells. However, Nakatsubo et al. (1998) reported the effect of various concentrations of L-NAME (Nω-Nitro-L-arginine methyl ester), a known inhibitor of NOS, on DAF-2 fluorescence generation. L-NAME blocked the increase of fluorescence intensity in a dose-dependent manner. We measured fluorescence intensity after a 120-min incubation with 1 mM of L-NAME; L-NAME blocked the increase of fluorescence intensity (data not shown).

The time course of the fluorescence intensity data is quantitatively analyzed in Fig. 2. In the absence of LPS, NO production was minimal and not different between the groups studied. However, after Kupffer cells were exposed to 10 μg/mL of LPS, NO production from estriol-treated rats increased at a rate twice as high as that by control Kupffer cells. Treatment of rats with antibiotics abolished the increase in LPS-induced NO production observed 24 hr after estriol treatment.

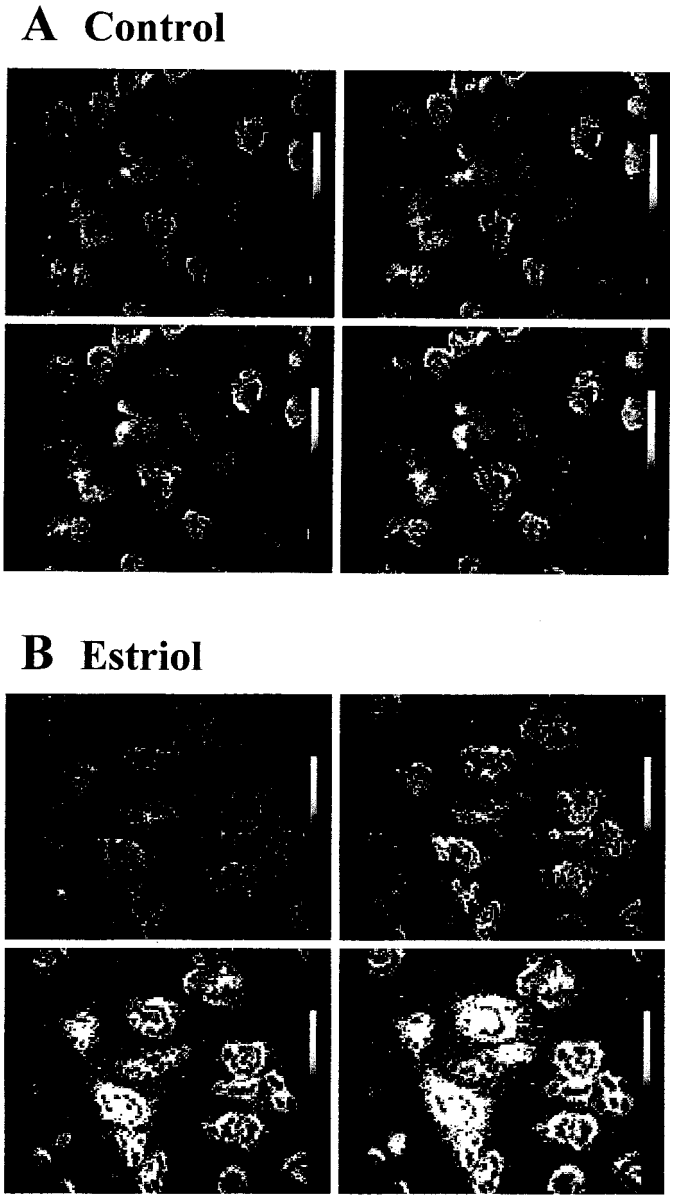

Fig. 1. Image analysis of LPS-induced NO production in isolated Kupffer cells from estriol-treated rats. Fluorescence images of the same group of activated rat Kupffer cells with DAF-2 in the presence of L-Arg (1 mM). (A) Control. (B) Estriol-treated rats. These images are shown in black and white and correspond to the fluorescence intensity data in Fig. 2. Time intervals between the images are 3 to 4 min. Images are serially shown from the top left to the bottom right. Representative data are shown from at least 20 experiments in each group.

DISCUSSION

Activation of Kupffer cells is a prominent event in the initiation of alcoholic liver disease (Martinez et al., 1992; Stahnke et al., 1991). Kupffer cells are activated by endotoxin leading to rapid increases in intracellular calcium (Enomoto et al., 1998) followed by release of inflammatory cytokines, superoxide, and NO. However, the effect of ethanol on NO production by Kupffer cells remains controversial (Enomoto et al., 2000). For example, Kimura et al. (1996) reported that long-term alcohol feeding suppresses LPS-induced NO production (Greenberg et al.,

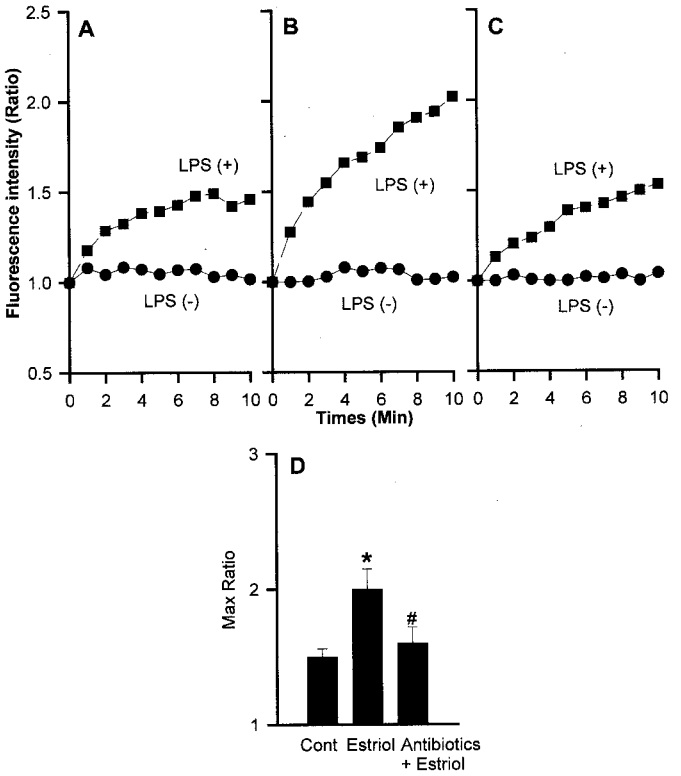

Fig. 2. Effect of estriol on LPS-induced NO production by isolated Kupffer cells. Some rats were treated with antibiotics before experiments as described in "Materials and Methods." Kupffer cells were isolated from rats that were treated with estriol for 24 hr and cultured on glass-bottom culture dish with RPMI 1640 + 10% fetal bovine serum for 24 hr (see "Materials and Methods"). NO production was measured with fluorescence indicator DAF-2. (A) Control. (B) Estriol-treated rats. (C) Estriol- and antibiotic-treated rats. Data are representative traces from experiments repeated four times. (D) Maximal ratio of fluorescence intensity. Data represent mean ± standard error of the mean of preparations from four rats. *$p <$ 0.05 versus control; #$p < 0.05$ versus estriol by ANOVA and Bonferroni's post hoc test.

1994; Spolarics et al., 1993). Moreover, Horie et al. (2000) reported that long-term ethanol feeding enhances NO production (Nanji et al., 1995; Wang et al., 1995).

In this study, LPS-induced NO production from Kupffer cells from estriol-treated rats was compared with that from control Kupffer cells. The LPS-stimulated NO production was enhanced in Kupffer cells isolated from rats that were treated with estriol for 24 hr (Fig. 1). This effect was completely blocked by treatment with antibiotics. Thus, it is concluded that increased NO production by estriol was caused by gut-derived endotoxin. These findings are in line with our previous study that showed that Kupffer cells from rats that were treated with estriol were sensitized to LPS via mechanisms dependent on gut-derived endotoxin (Enomoto et al., 1999). Moreover, our data provide further insight into the mechanism by which estriol causes aggravation of alcoholic liver disease in women.

Arguably, Drew and Chavis (2000) showed that estriol and progesterone treatment in vitro inhibited LPS-induced NO production from microglial cells . Although it has been reported that some macrophages contain estrogen receptors (Cutolo et al., 1996; Gulshan et al., 1990), they have not been identified on Kupffer cells. It is interesting that addition of estrogen in vitro failed to change LPS-induced NO and tumor necrosis factor-α (TNF-α) production from Kupffer cells, supporting the idea that the effect of estrogen in vivo is indirect (Ikejima et al., 1998). Moreover, it was reported that estriol in vivo indeed increased permeability of the isolated small intestine (Enomoto et al., 1999), leading to an increase in plasma endotoxin level, a phenomenon that was prevented by treatment with antibiotics. Antibiotics prevented LPS-induced TNF-α production. Collectively, it is concluded that Kupffer cells isolated from rats that were treated with estriol exhibited sensitization to LPS involving the mechanism of gut derived endotoxin.

Although the precise roles for NO in the pathogenesis of alcoholic liver injury are yet to be elucidated, it seems reasonable to postulate that NO, which is produced in a large amount by activated Kupffer cells, acts as hepatic toxicants synergistically with TNF-α. In summary, Kupffer cells from estriol-treated rats exhibited sensitization to LPS assessed by LPS-induced NO production.

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