Effect of a potent iNOS inhibitor (ONO-1714) on acetaminophen-induced hepatotoxicity in the rat

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Received 22 May 2003; accepted 23 September 2003

Abstract

Overproduction of nitric oxide (NO) in the liver has been implicated as an important event in endotoxin shock and in other models of hepatic inflammation and injury. The present study was undertaken to evaluate the effect of ONO-1714, a potent and specific inhibitor of inducible NO synthase (iNOS), on acetaminophen-induced hepatotoxicity in the rats. Oral administration of ONO-1714 dose-dependently inhibited NOx (NO\textsubscript{2} and NO\textsubscript{3}) accumulation in rat plasma after lipopolysaccharide (LPS) treatment. Intraperitoneal acetaminophen at 1 g/kg caused damage to the centrilobular regions of the liver and increase in serum alanine and aspartate transaminase (ALT and AST, respectively) levels accompanied by elevated plasma NOx levels after 24 h. Oral administration of ONO-1714 at 10 and 100 \textmu g/kg dose-dependently reduced the acetaminophen-induced hepatic tissue damage and the increases in serum ALT and AST levels. ONO-1714 also blocked the increase in plasma NOx concentrations. These findings demonstrate that oral ONO-1714, an iNOS inhibitor, protects against acetaminophen-evoked hepatic inflammation/injury, strongly suggesting that NO produced by iNOS plays a key role in the pathogenesis of this drug-induced hepatotoxicity.

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Keywords: Inducible nitric oxide synthase (iNOS); Acetaminophen; Hepatotoxicity

Introduction

Inflammatory stimuli, such as endotoxin and inflammatory cytokines, can induce a Ca\textsuperscript{2+}-independent NO synthase (NOS) in a variety of cells including macrophages and neutrophils (Radomski et al., 1990;
The expression of inducible NOS (iNOS) leads to overproduction of NO, resulting in damage to endothelial, neuronal, and epithelial cells (Tepperman et al., 1993; Minc-Golomb et al., 1994; Stoclet et al., 1998). In the liver, Kupffer cells and neutrophils are major sources of reactive nitrogen and oxygen species, and of proinflammatory cytokines, which promote oxidative stress (McCuskey et al., 1995; Wang et al., 1999; Remirez et al., 2002; Spitzer et al., 2002). Overproduction of NO in the liver plays an important role in endotoxin shock and in various models of hepatic inflammation and injury (Geller et al., 1994; Laskin et al., 1995; Pastor and Billiar, 1995; Aono et al., 1997; Rockey and Chung, 1997; Saetre et al., 1998). NO reacts with superoxide radicals, forming peroxynitrite, an even more potent oxidizing agent. Peroxynitrite causes protein nitration and tissue injury (Beckman et al., 1990; Radi et al., 1991a,b). Acetaminophen, a mild analgesic and antipyretic agent, could cause centrilobular hepatic necrosis at toxic doses. N-acetyl-p-benzoquinoneimine, a reactive metabolite of acetaminophen, binds to centrilobular hepatic proteins, leading to cellular damage (Bartolone et al., 1988; Roberts et al., 1991; Gibson et al., 1996). On the other hand, a large number of activated macrophages also accumulate at the injured regions of the liver and release high concentrations of NO formed by iNOS, as well as other inflammatory mediators including TNF-α, IL-1, and reactive oxygen intermediates, which would promote hepatotoxicity (Laskin and Pilaro, 1986; Laskin et al., 1986; Bourdi et al., 2002; Ishida et al., 2002). The possibility that NO is crucial for hepatotoxicity has been suggested by the fact that aminoguanidine, a relatively iNOS-preferential inhibitor, attenuated acetaminophen-induced hepatotoxicity (Gardner et al., 1998), although this iNOS inhibitor is less potent and poorly specific. ONO-1714, the structure of which is shown in Fig. 1, is one of the most potent iNOS inhibitors in vitro and in vivo, which is useful to study pathophysiological roles of iNOS and may be clinically available as a therapeutic agent (Naka et al., 2000). In this context, we examined the effect of ONO-1714 on acetaminophen-induced hepatotoxicity.

Materials and methods

Animals

Male SD rats (5–6 weeks old) were obtained from Charles River Japan, Inc. (Osaka, Japan) or Japan SLC Co. (Shizuoka, Japan). All animal experiments were conducted with approval from the
Kinki University School of Pharmaceutical Sciences’ Committee for the care and use of laboratory animals.

**Chemicals**

The structure of ONO-1714 (Ono Pharmaceutical Co. Ltd., Osaka, Japan) is shown in Fig. 1. Lipopolysaccharide (LPS) (B E. coli 026: B6 LPS) was obtained from Difco Lab. (Detroit, USA), and acetaminophen was from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

**Nitrate/Nitrite (NOx) accumulation induced by lipopolysaccharide (LPS)**

LPS (1.0 mg/kg) was administered i.v. to the rat. ONO-1714, dissolved in distilled water, at 10, 30, 100 and 300 μg/kg, was administered orally 3 h after LPS treatment. Blood was collected from the abdominal aorta under ether anesthesia 3 h and 6 h after LPS treatment. Plasma was obtained by centrifugation and plasma NOx concentrations were determined as described below. The effects of ONO-1714 are expressed as % inhibition of accumulation of NOx for 3–6 h after LPS challenge.

**Hepatotoxicity induced by acetaminophen**

Rats received i.p. administration of acetaminophen at 1 g/kg (Gardner et al., 1998), which was dissolved in a 20% Tween-80 saline solution. It has been shown that iNOS induction became remarkable 6 h after acetaminophen, and that pre-treatment with aminoguanidine, an iNOS-preferential inhibitor, significantly suppressed the hepatotoxicity (Gardner et al., 1998). Based on these findings, in the present study, ONO-1714 at 10 and 100 μg/kg or distilled water (vehicle) was administered orally 30 min before and 6 h after acetaminophen treatment. Blood was collected 24 h after acetaminophen treatment for determination of plasma NOx concentrations and serum levels of alanine and asparate transaminase (ALT and AST, respectively), and then the liver was excised for histological examination.

**Measurement of ALT and AST**

The levels of serum ALT and AST were measured using clinical kits for measurements of ALT and AST activities (Wako Pure Chemical Industries Ltd, Osaka, Japan) and an automatic analyser (model 7070, Hitachi Ltd, Tokyo, Japan).

**Measurement of NOx**

Nitrite and nitrate, oxidized forms of NO, were determined by the use of the nitrite/nitrate colorimetric assay kit (Cayman Chemical, Michigan, USA). Briefly, the nitrate in the sample was converted into nitrite by nitrate reductase, and the total nitrite levels were determined spectrophotometrically as the total nitrate/nitrite (NOx) concentration.
Histology

The liver was fixed in 10% neutral buffered formalin. From each sample, approximately 3 μm-thick paraffin sections of the internal and left lateral lobes were prepared according to standard manners and were stained with hematoxylin and eosin (HE). The HE-stained sections were evaluated histopathologically.

Statistics

Data were represented as means ± SEM. Statistical significance was evaluated by ANOVA followed by Dunnett’s’ multiple comparison test, and accepted when \( P < 0.05 \).

Results

Effect of ONO-1714 on nitrate/nitrite (NOx) accumulation induced by LPS

Oral administration of ONO-1714 at 10–300 μg/kg inhibited LPS-induced NOx accumulation in the plasma in a dose-dependent manner, and the ID\(_{50}\) value was 39 μg/kg. The maximal dose, 300 μg/kg, of ONO-1714 exhibited almost complete inhibition (Fig. 2).

Effect of ONO-1714 on hepatotoxicity and increased plasma NOx levels induced by acetaminophen

Serum ALT and AST levels were dramatically increased 24 h after i.p. acetaminophen at 1 g/kg. ONO-1714 at 10 and 100 μg/kg inhibited the acetaminophen-evoked increases in ALT and AST levels in a dose-dependent manner (Fig. 3). Plasma NOx concentrations also significantly increased 24 h after i.p. acetaminophen. ONO-1714 at 10 and 100 μg/kg inhibited the increase in NOx levels in a dose-dependent manner (Fig. 4). Thus, the increased plasma ALT and AST levels and the concomitant increase in NOx levels after acetaminophen treatment were blocked by ONO-1714 in the same dose range.

![Graph](image_url)

Fig. 2. Inhibitory by ONO-1714 of NOx accumulation in LPS-treated rats. ONO-1714 was administered orally 3 h after administration of LPS at 1.0 mg/kg. Accumulation of NOx in plasma for 3 hours was determined. Each column represents the mean with SEM from 7 animals.
Fig. 3. Effect of ONO-1714 on serum levels of ALT and AST in acetoaminophen-treated rats. ONO-1714 at 10 and 100 μg/kg was administered orally 30 min before and 6 h after acetaminophen treatment. Serum ALT and AST levels were determined 24 h after acetaminophen. Each column represents the mean with SEM from 6–10 animals. ##p < 0.01 versus the normal group; *p < 0.05 versus the control group.

Fig. 4. Effect of ONO-1714 on plasma NOx concentrations in acetoaminophen-treated rats. ONO-1714 at 10 and 100 μg/kg was administered orally 30 min before and 6 h after acetaminophen treatment. Plasma NOx concentrations were determined 24 h after acetaminophen. Each column represents the mean with SEM from 6–10 animals. ##p < 0.01 versus the normal group. **p < 0.01 versus the control group.
In the typical microphotograph of the liver in the rat treated with acetaminophen, extensive zonal necrosis of centrilobular hepatocytes was observed (Fig. 5A). In contrast, neither hepatic necrosis nor abnormality was observed in the rat pretreated with ONO-1714 (B).

Fig. 5. Typical light micrographs of liver sections stained with hematoxylin and eosin in the acetaminophen-treated rats with or without oral preadministration of ONO-1714 at 100 μg/kg. Extensive zonal necrosis of the centrilobular hepatocytes was observed in the rats treated with 1 g/kg acetaminophen (A). In contrast, neither hepatic necrosis nor abnormality was seen in the rat pretreated with ONO-1714 (B).

Discussion

The liver is a major target organ for toxicity of xenobiotics and drugs, because most orally ingested xenobiotics and drugs pass through the liver, and some chemicals are metabolized into toxic intermediates in the liver (Jaeschke et al., 2002). A variety of cells in the liver including hepatocytes
Kupffer cells (Mustafa et al., 1999) and endothelial cells (Laskin et al., 1994) produce NO that plays a dual role, being cytoprotective and cytotoxic (Li and Billiar, 1999; Laskin et al., 2001). The small amount of NO produced by endothelial NOS (eNOS) acts to maintain homeostasis and has a cytoprotective effect (Albrecht et al., 2003), whereas excessive NO formation by iNOS that is induced by cytokines (Nakayama et al., 1994) and LPS (Zhang et al., 2000) in hepatocytes or Kupffer cells, exerts cytotoxic actions via lipid peroxidation (Shibuki et al., 2000), activation of poly(ADP-ribose) polymerase (Khandoga et al., 2002), apoptosis (Lin et al., 1999; Zhuang and Simon, 2000) and nitration of tyrosine residues (Haddad et al., 1994; Demiryurek et al., 2000). The present study demonstrated that a toxic dose of acetaminophen produced accumulation of NOx in the plasma, in addition to increased ALT/AST levels and extensive liver necrosis. Moreover, we found that a potent iNOS inhibitor, ONO-1714, inhibited acetaminophen-induced hepatotoxicity and increases in plasma NO levels and serum ALT/AST levels, suggesting a critical role of iNOS in the development of this drug-induced hepatic damage.

ONO-1714 is 10-fold more selective inhibitor for iNOS (Ki value = 1.88 nmol/L) than eNOS (Ki value = 18.8 nmol/L), and the mode of inhibition by ONO-1714 of both iNOS and eNOS is competitive (Naka et al., 2000). It has been reported that ONO-1714 improves glomerulonephritis (Ogawa et al., 2002), intestinal ischemia-reperfusion (Wu et al., 2002), cardiac dysfunction (Funakoshi et al., 2002), septic shock (Beilman, 2001), pancreatitis (Mikawa et al., 2001) and colitis (Naito et al., 2001) in animal models. In the present study, oral ONO-1714 inhibited LPS-induced NOx accumulation, the ID_{50} value being 39 μg/kg. In our previous study, s.c. administration of ONO-1714 inhibited the NOx accumulation, the ID_{50} value being 10 μg/kg (Naka et al., 2000). Therefore, ONO-1714 is considered a potent iNOS inhibitor available orally as well as parenterally.

Acetaminophen, when used at high doses, could cause acute liver injury most probably via formation of N-acetyl-p-benzoquinoneimine, a toxic metabolite, by cytochrome P4502E1 (CYP2E1). N-acetyl-p-benzoquinoneimine is usually inactivated by hepatic glutathione, but it, when produced excessively, covalently binds to centriobular hepatic proteins, contributing to hepatic toxicity (Gardner et al., 1998; Gardner et al., 2002; Jaeschke et al., 2002). It has been confirmed in our preliminary experiments that ONO-1714 did not inhibit P4502E1 (data not shown). Therefore, our data from experiments using ONO-1714 strongly suggest the importance of NO formed by iNOS in the pathogenesis of acetaminophen-induced hepatotoxicity, in agreement with some other studies (Laskin et al., 1995; Gardner et al., 1998; Gardner et al., 2002). Subcutaneous administration of aminoguanidine, a relatively iNOS-preferential inhibitor, also reduces acetaminophen-induced hepatotoxicity, whereas this effect of aminoguanidine can be obtained at a very high dose, 100 mg/kg (Gardner et al., 1998), which is one thousand higher than the effective doses of oral ONO-1714 in the present study. Although the exact mechanisms underlying the hepatotoxicity induced by NO have yet to be elucidated, peroxynitrite, a reaction product of NO with superoxide, is now considered, at least in part, responsible for the acetaminophen-induced hepatotoxicity. Peroxynitrite is capable of producing nitrated tyrosine accompanied by tissue injury (Michael et al., 1999; Michael et al., 2001; Hinson et al., 2002; Knight et al., 2002). In the liver, nitrated tyrosine increases in the centrilobular cells after acetaminophen administration (Hinson et al., 1998).

In conclusion, our data demonstrate that oral administration of ONO-1714 has a beneficial action on the acetaminophen-evoked hepatic inflammation and injury, further suggesting that NO produced by iNOS plays a critical role in the development of this drug-induced hepatotoxicity.
References


Ishida, Y., Kondo, T., Ohshima, T., Fujiiwara, H., Iwakura, Y., Mukaida, N., 2002. A pivotal involvement of IFN-gamma in the

Toxicological Sciences 65, 166–176.


hepatic macrophages, endothelial cells following acute exposure of rats to endotoxin. Journal of Leukocyte Biology 56, 751–758.


