URINARY EXCRETION OF OXIDATIVE METABOLITES OF BILIRUBIN IN FENOFIBRATE-TREATED RATS

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ABSTRACT — Bilirubin oxidative metabolites (BOM) were shown to be excreted into the urine in rats in which exaggerated oxidative stress was induced. We measured bilirubin (BR) and biopyrrins in the urine of rats treated with fenofibrate, a peroxisome proliferator, which is known to cause oxidative stress. Male Crl:CD(SD)IGS rats aged 6 weeks were treated orally with fenofibrate at 10, 400 and 800 mg/kg for 2 weeks. Urinary excretion of BR and BOM, and the plasma BOM levels were determined after the first dose and after 1-week and 2-week treatment.

Urinary excretion of BOM was significantly and dose-dependently increased by fenofibrate treatment at 400 and 800 mg/kg. This became more prominent as the dosing period progressed and reached an 8-fold increase in the 400 mg/kg group and 11-fold increase in the 800 mg/kg group compared with the data before dosing on Day 14. Plasma BOM levels were increased 1.8-fold and 2.7-fold, respectively, at 400 and 800 mg/kg in fenofibrate-treated rats. At 800 mg/kg, there was also increased urinary excretion of BR (2-fold) on Day 14. These changes of BOM in the urine and plasma indicated that BR was oxidized by reactive oxygen species (ROSs), which were produced by treatment with fenofibrate. In conclusion, urinary excretion of BOM, which is a marker for oxidative stress, urinary excretion of BR and the plasma BOM levels were increased in rats treated with fenofibrate. Increased urinary excretions of BR and BOM, and increased plasma BOM levels are likely to be the consequence of physiological protection against the oxidative stress produced by fenofibrate. These findings suggest a possibility that analysis of BOM in the urine and plasma could be helpful in evaluating the degree of oxidative stress in vivo.

KEY WORDS: Bilirubin, Biopyrrins, Oxidative stress, Rats

INTRODUCTION

There is evidence indicating a close relationship between oxidative stress and the pathogenesis of various toxic changes (Winterbourn, 1995; Taniguchi et al., 1999). Many compounds, such as ciprofibrate, clofibrate and NiCl₂, have been shown to produce reactive oxygen species (ROS) in the body, which are considered to cause cellular and tissue damage (Dhaunisi et al., 1994; Elliott et al., 1986; Chan-Yu et al., 1998). Thus, the quantitative analysis of oxidative stress is highly important to characterize the toxicity of these compounds.

Recently, bilirubin oxidative metabolites (BOM) have been shown to be excreted into the urine in rats in which oxidative stress was exaggerated by lipopolysaccharide (LPS) administration (Yamaguchi et al., 1997). Urinary BOM were found to increase in rats treated with LPS and in patients who had undergone prolonged laparotomy (Yamaguchi et al., 1997, 1994). In addition, Yamaguchi et al. (1997) reported that LPS induced heme oxygenase-1 (HO-1), which is a rate-limiting enzyme of BR biosynthesis, in rats. These results suggest that bilirubin has a protective effect.
against oxidative stress caused by the reactive oxygen species induced by LPS. In addition, Dore et al. (1999) reported that BR is probably the most abundant endogenous antioxidant in mammalian tissues and in the circulation (Dore et al., 1999).

Lipid-lowering agents such as fenofibrate, clofibrate and ciprofibrate have been shown to increase the number of peroxisomes or peroxisomal enzymes, such as catalase, which can be used as marker enzymes for peroxisomes (Rao et al., 1997; Cattley et al., 1998; Gonzalez et al., 1998). These peroxisomal proliferators have been proposed to cause oxidative stress through overproduction of hydrogen peroxide and other reactive oxygen species due to an increase in the functions of cytochrome P450 or the β-oxidation system enzymes, particularly hydrogen peroxide (H₂O₂)-generating oxidase (Dhaunisi et al., 1994; Elliott et al., 1986; Gonzalez et al., 1998). In the present study, we measured urinary excretion of BR and its oxidative metabolite, BOM, as a stress marker in rats treated with fenofibrate, which was expected to increase ROS in vivo.

MATERIALS AND METHODS

Animal

Five-week-old male CD(SD)IGS rats were purchased from Charles River Japan Inc. (Kanagawa, Japan). The animals were housed individually in wire-mesh cages kept in an air-conditioned room with a 12-hr light-dark cycle at a temperature of 23 ± 1°C, a relative humidity of 55 ± 5% and a ventilation rate of about 15 times per hour. The rats were allowed free access to a commercial diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum.

Treatment

Fenofibrate purchased from Sigma Chemical Co. (St. Louis, MO, USA) was suspended in 0.5% sodium carboxymethyl cellulose (CMC; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) solution. Fenofibrate, at doses of 10, 400, 800 mg/kg/day, or vehicle was given orally to rats at a constant volume of 5 mL/kg body weight to (starting at 6 weeks of age) for 14 days.

Determination of BR and biopyrrins in the urine or plasma

Groups of 5 rats were housed individually in metabolic cages during the pretreatment period at days 1, 7, and 14 of the dosing period. The urine was collected over 24 hr. After measurement of the urine volume, the urine was assayed for determination of BR and biopyrrins. BR was measured by the diazo method and biopyrrins were measured by EIA using an antibody (24G7), highly specific for the BR dipyrrole epitope (Yamaguchi et al., 1996). Determination of the plasma biopyrrins level was done the same manner. The unit µmol is used for BR and biopyrrins in this report. The numerical expression, BOM, was calculated as follows; [BOM] = [biopyrrins] – [BR].

Isolation of peroxisome-enriched fraction

All animals were euthanized under ether anesthesia 24 hr after the last treatment and the livers were removed and weighed. Peroxisome was prepared by differential centrifugation of a 20% liver homogenate in 0.25 M sucrose solution; the homogenate was centrifuged at 1,100 g for 3 min. The supernatant was saved, while the sediment of the nuclear fraction was resuspended in 0.25 M sucrose solution and centrifuged at 1100 g for 3 min. All of the combined supernatant fractions were centrifuged at 9,000 g for 15 min. The resulting peroxisome-enriched pellets (containing peroxisomes, mitochondria and lysosomes) were then resuspended in 0.25 M sucrose solution and centrifuged again at 20,200 g for 15 min (Elliott et al., 1986; Esbenshade et al., 1990; Pedersen et al., 1978). The pellets obtained as the peroxisome-enriched fraction were suspended in 0.25 M sucrose solution and frozen at −80°C until assayed for catalase activity.

Enzyme assays

Catalase activity was measured by degradation of hydrogen peroxide at room temperature (Aebi, 1974).

Statistical analysis

The mean values and standard deviations in each group were calculated for the liver weights and biochemical parameters. Statistical analyses were conducted using Dunnnett’s multiple comparative test (Yoshimura, 1997) for the effects of fenofibrate.

RESULTS

Plasma BOM levels

Plasma BOM levels were significantly increased in the 400 and 800 mg/kg groups from Day 1 and throughout the dosing period (Fig. 1). However, the increase in plasma BOM level at 400 mg/kg on Day 14 was not statistically significant. The plasma BOM levels were increased dose-dependently, being increased by up to 2.0-, 2.6-, or 1.9-fold higher in the 800 mg/kg
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Group than those of the control group on Days 1, 7 and 14, respectively. This was not observed in the 10 mg/kg group at any sampling points in this study. In addition, no clear increase in plasma BR levels was observed at any dose levels throughout the dosing period (data not shown).

**Urinary excretion of BR**

Urinary BR excretion was significantly increased in rats treated with 800 mg/kg fenofibrate on Days 1 and 14 (Fig. 1). The increase in urinary excretion of BR at 800 mg/kg was only about 2-fold as compared with the control group on Day 14. In addition, no significant increase in urinary BR excretion was observed in the 10 and 400 mg/kg groups throughout the dosing period in this study.

**Urinary excretion of BOM**

A significant and dose-dependent increase in urinary excretion of BOM was observed after single administration in rats treated with fenofibrate at 400 and 800 mg/kg (Fig. 1). Urinary excretion of BOM at these dose levels became more prominent as the dosing period progressed and reached about 8-fold increase in the 400 mg/kg group and 11-fold increase in the 800 mg/kg group compared with the data before dosing. No significant increase in urinary excretion of BOM was observed in the 10 mg/kg group in which the animals did not show increased catalase activity in the peroxisome fraction in the liver (Table 1).

**Liver weight and catalase activity**

On Day 14, the liver weights were more than doubled in the groups treated with fenofibrate at 400 and 800 mg/kg when compared with the control values (Table 1). Catalase activity of the peroxisome fraction from fenofibrate-treated animals' liver was also increased at 400 mg/kg (Table 1). The activities were 2-fold (activity per g tissue) higher than the control. No significant increases in liver weights or catalase activity were observed at 10 mg/kg on Day 14.

**DISCUSSION**

The present study was designed to examine the changes in urinary BOM in rats treated with fenofibrate, which has been suggested to cause oxidative stress. Although recently there have been many papers published about oxidative stress in vivo, reports regarding the relation between BOM and chemical agents were few. In this study, BOM was calculated from BR and biopyrazines, which constitute a group of compounds which are identified by anti-bilirubin monoclonal anti-

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Liver Weight (g)</th>
<th>Catalase activity (x10^4U/g tissue)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>11.2 ± 1.3</td>
<td>18.0 ± 3.4</td>
</tr>
<tr>
<td>10</td>
<td>11.9 ± 3.2</td>
<td>22.4 ± 4.6</td>
</tr>
<tr>
<td>400</td>
<td>23.4 ± 2.7**</td>
<td>33.2 ± 3.5**</td>
</tr>
<tr>
<td>800</td>
<td>21.8 ± 3.5**</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Values are mean ± SD of five rats.

**Significantly different from control, p<0.01 (Dunnett's test). N.D.: Not determined.**

![Fig. 1](image-url) Changes of plasma BOM levels, urinary BR and BOM excretion after oral administration of fenofibrate in rats. Points indicate mean values and a vertical bar at each point indicates the SD. ○: control, ●: 10 mg/kg, ▲: 400 mg/kg, ■: 800 mg/kg. **Significantly different from control, p<0.01 (Dunnett's test).
body (24G7). Since 24G7 is a highly specific antibody for the BR dipyrrole epitope, 24G7 can detect both BR and BOM. In addition, Yamaguchi et al. (1997) reported that BOM, i.e., biopyrrins, were highly excreted into the urine in rats in which oxidative stress had been exaggerated by administration of LPS, and that it was reduced by feeding ascorbic acid, an antioxidant. In the present study, urinary excretion of BR and BOM, as well as plasma BOM levels were significantly increased in fenofibrate-treated rats. In addition, urinary excretion of BOM and plasma BOM were increased in both the 400 and 800 mg/kg groups, which showed increases in liver weight and catalase activity. Furthermore, although increased urinary excretion of BR was only 2-fold as compared with the control group on Day 14 at 800 mg/kg, urinary BR excretion on Day 14 was increased 8- and 11-fold at 400 and 800 mg/kg, respectively. These results suggest that administration of fenofibrate caused oxidative stress in the rats. These findings are consistent with other studies that demonstrated that peroxisome proliferators, such as fenofibrate, clofibrate and ciprofibrate, cause oxidative stress through overproduction of hydrogen peroxide and other reactive oxygen species (Dhaunisi et al., 1994; Elliott et al., 1986; Gonzalez et al., 1998). Peroxisome proliferation in the fenofibrate-treated rats was indicated by increased catalase activity and liver weights in this study. Plasma BOM levels were increased only about 2-fold as a maximum; however, there was a significant alteration in the 400 and 800 mg/kg groups. One reason for this small but significant change is probably that rats have a very low renal threshold for BOM, and as a result BOM in plasma is rapidly excreted in the urine. Yamaguchi et al. (1997) also reported that the concentration of BOM in urine increased at only 3 hr after administration of LPS. The results in the report support our hypothesis of the reason for the low plasma BOM levels.

An increase in urinary excretions of BR in fenofibrate-treated rats appears to be due to an increase in BR biosynthesis by HO-1, a rate-limiting enzyme of BR biosynthesis, because some experiments have demonstrated that HO-1 is induced by oxidative stress (Applegate et al., 1991; Otani et al., 2000; Yamaguchi et al., 1997). This thesis is also supported by the fact that BR has a protective effect against oxidative stress (Stoker et al., 1987). Furthermore, cholestasis in the liver has not been reported in rats treated with fenofibrate. However, there is no clear evidence regarding a relationship between an induction of HO-1 and treatment with fenofibrate at this time.

In conclusion, urinary excretion of BOM, which is a marker for oxidative stress, urinary excretion of BR and plasma BOM levels were increased in rats treated with fenofibrate, which causes oxidative stress. Increased urinary excretion of BR and BOM, and increased plasma BOM levels are likely to be the consequence of physiological protection against the oxidative stress produced by fenofibrate. These findings suggest a possibility that analysis of BOM in the urine and plasma could be helpful in evaluating the degree of oxidative stress in vivo.

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