Expressions of Protein Oxidation Markers, Dityrosine and Advanced Oxidation Protein Products in Acetaminophen-Induced Liver Injury in Rats

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(Received 23 February 2011/Accepted 15 April 2011/Published online in J-STAGE 28 April 2011)

Acetaminophen (N-acetyl-p-aminophenol; APAP) is a widely-used analgesic and antipyretic drug. Although safe at the therapeutic levels, an overdose of APAP causes liver injury in humans [21] and experimental animals [6, 28]. Despite substantial efforts in the past, the mechanism of APAP-liver injury has not been still fully understood [14].

The toxicity is brought about following the metabolic acti-
vation of APAP via cytochrome P450 system, which leads to the formation of an electrophilic reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI)[24]. Although NAPQI can be reactily metabolized by glutathione (GSH), when produced at an excessive amount, it may cause over-use or depletion of GSH storage in the liver leading to apoptosis or necrosis of hepatocytes [4, 26]. NAPQI also increases the formation of reactive oxygen species (ROS) including superoxide anions, hydroxyl radicals and hydrogen peroxide, which may result in reduction of antioxidant enzymes and enhancement of lipid peroxidation [12, 33]. Moreover, it is known that increased expression of oxidative stress markers, such as nitrotyrosine and 8-hydroxydeox-
yguanosine (8-OHdG), was associated with the severity of APAP-induced liver injury and the pathogenesis of this toxic effect [13, 15].

Recently, the protein oxidation markers, such as dity-
rosine and advanced oxidation protein products (AOPP), have been given more attention in monitoring the tissue damage by an overdose of xenobiotics. However, it is unclear if these biomarkers can be also useful for monitoring the development of APAP-induced liver injury.

Dityrosine is one of the cross-linkers of proteins [3] and produced by the reaction of 2 tyrosyl radicals following the oxidation of tyrosine [1, 3, 8, 17]. It has been identified as a universal biomarker of protein oxidation because it can be generated by various ROS, such as peroxynitrite, metal-catalyzed oxidates, and UV-irradiation [23].

AOPP which are also the cross-linking proteins containing dityrosine in their moiety, are formed during oxidative stress on plasma proteins by chlorinated oxidants [2, 7, 31]. As plasma AOPP concentration is closely correlated with oxidized proteins [2, 7, 31], they have been considered as markers of oxidant-mediated protein damage [2, 7, 31].

In the present study, we investigated the changes in the immunohistochemical expression of dityrosine in the liver and plasma AOPP concentration in rats during 24 hrs following a single injection of APAP.

All experiments were performed using male Wistar Imamichi rats, 7 week old, 179.12 ± 1.84 g (mean ± SE) in weight which were obtained from the Institute of Animal Reproduction (Ibaraki, Japan). The animals were reared in a room controlled temperature at 22 ± 2°C and lighting of 12:12-hr light/dark cycle (light cycle, 7:00–19:00) and given standard chow (CE-2; Nihon Clea, Tokyo, Japan). The present experiments were performed following the provisions approved by the Animal Research Committee at Tottori University.

A total of 30 animals were divided into two different groups as follows: (1) control rats (n=6), (2) APAP-treated rats (n=24). APAP (Sigma-Aldrich, Co., St. Louis, MO, U.S.A.) was dissolved in the vehicle, 40% polyethylene glycol 400 solution (Wako, Osaka, Japan). Animals were fasted overnight before the experiments with free access to water. The rats received intraperitoneal injection (i.p.) with APAP (1 g/kg body weight) or the vehicle (the control). Blood and liver samples were collected under pentobarbital anesthesia (100 mg/kg, i.p.) 3, 6, 12 and 24 hr after APAP
injection (each group; n=6). Plasma ALT, AST, urea nitrogen and creatinine levels were determined by a biochemical autoanalyzer (Dri-Chem 3000; Fuji Film Company, Tokyo, Japan). Plasma AOPP concentration was assayed by a colorimetric method with a commercial kit (Immundiagnostik AG, Bensheim, Germany).

The samples of the liver (right and left median lobes) and kidney were fixed in 10% buffered formalin, processed through a conventional histological method, and stained with hematoxylin and eosin (HE).

Apoptotic bodies in the liver were detected by terminal deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL), which was performed using an in situ apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD, U.S.A.). More than 1,500 hepatocytes were examined on at least 25 high-power fields chosen randomly and analyzed with a histometric analysis software (Olympus Corporation, Tokyo, Japan). The apoptotic index was calculated as the percentage of TUNEL-positive cells out of the total number of hepatocytes counted.

For immunohistochemistry, an anti-dityrosine mouse monoclonal antibody (Nikken Seil Co., Ltd., Shizuoka, Japan) was used as the primary antibody. All sections were dewaxed, rehydrated, rinsed with 0.05 M tris-buffered saline (TBS; pH 7.6), treated with 3% hydrogen peroxide, and then rinsed again with TBS. Slides were incubated with the primary antibody (1:100 dilution) at 4°C overnight, rinsed with TBS, and treated with Simple Stain MAX-PO (M) (Nichirei, Tokyo, Japan) for 30 min at room temperature. They were then rinsed with TBS before being treated with a 3,3'-diaminobenzidine solution containing 0.01% hydrogen peroxide to facilitate a peroxidase color reaction. After a further wash with TBS, the slides were counterstained with Mayer’s haematoxylin. The positive control for the detection of dityrosine was the liver sections from the rats receiving carbon tetrachloride (CCl4) treatment. These liver sections were also used as the negative control for the antibody reaction on addition to those from the normal rats. These sections were incubated with the antibody diluents (Dakocytomation, Tokyo, Japan) instead of anti-dityrosine antibody.

The extent of immunohistochemical staining of dityrosine in the tissue was measured with histometric analysis software (Olympus Corporation, Tokyo, Japan) and determined as a percentage of dityrosine-positive area in a total of 50 fields on the tissue, at × 40 magnification, which were randomly chosen from at least five independent sections.

All data were expressed as means ± SE in each group, compared by 1-way analysis of variance (ANOVA) and then analyzed by Tukey’s multiple comparison test with a statistical software (SSRI Co., Ltd., Tokyo, Japan). *P<0.05 was considered to be statistically significant.

Plasma ALT and AST increased significantly 12 and 24 hr after exposure to APAP, respectively (Fig. 1A and B). At 3 hr APAP post-treatment, vacuolated hepatocytes were observed in zone 3 (Fig. 2a, b), and, at 6 hrs, they became to increase in number and expand their distribution to zone 2 (Fig. 2c). Clumping of chromatin was occasionally observed in nuclei of centrilobular hepatocytes of this group. Then, at 12 hr, coagulative necrosis and single cell necrosis were observed in all zones (Fig. 2d). There were nuclear changes of clumping of chromatin, pyknosis and karyorrhexis in this group. Congestion in sinusoids was prominent, especially at the periphery of the necrotic areas. At 24 hr, coagulative necrosis became more prominent (Fig. 2e). The cytoplasmic membranes of affected hepatocytes were frequently obscure, and it was difficult to distinguish individual cell borders. Pyknosis of nuclear material and karyorrhexis were frequently observed in this group. There

![Fig. 1. Time course of hepatic function and apoptotic index in APAP-induced liver injury. (A) Plasma ALT. (B) Plasma AST. (C) Apoptotic index. Values are expressed means ± SE (n=6). *: P<0.05, compared with control group. †: P<0.05, compared with 3 hr group. ‡: P<0.05, compared with 6 hr group.](image-url)
Fig. 2. Time course of liver injury induced by APAP. a; control group, b; 3 hr group, c; 6 hr group, d; 12 hr group, e; 24 hr group. 1: HE stain, Bar=100 μm. 2: HE stain, Bar=50 μm. 3: TUNEL stain, Bar=50 μm. *: central vein.

Fig. 3. Changes of dityrosine immunoexpression in APAP-induced liver injury. a: control group, b: 3 hr group, c: 6 hr group, d: 12 hr group, e: 24 hr group. Bar=100 μm. *: central vein.
was a significant increase in the apoptotic index at 12 and 24 hr (Figs. 1c and 2).

Hepatocytes in the control were negative for dityrosine. At 3 hr, dityrosine was positive in the cytoplasm of damaged hepatocytes in the centrilobular region (Fig. 3). The extent of positive area of the immunostaining increased significantly at 6, 12 and 24 hr (Figs. 3 and 4A). At 3 hr, the plasma AOPP level remained as low as that of the control (Fig. 4B). Plasma AOPP increased after 6 hr, showing statistically significant differences in the level from the control at 12 and 24 hr (Fig. 4B).

Although there was slight inflammatory reaction during the observation period of 24 hr after APAP treatment, only a few neutrophils and plasma cells could be seen in the lesions at 12 and 24 hr but no inflammatory cells were at 3 and 6 hr.

Significant increases in plasma urea nitrogen and creatinine were observed in at 12 and 24 hr (Plasma urea nitrogen; control 12.2 ± 0.76 mg/dl, 3 hr 23.6 ± 3.77 mg/dl, 6 hr 33.3 ± 1.48 mg/dl, 12 hr 55.7 ± 7.04 mg/dl, 24 hr 100.0 ± 13.11 mg/dl. Plasma creatinine; control 0.1 ± 0.00 mg/dl, 3 hr 0.2 ± 0.03 mg/dl, 6 hr 0.2 ± 0.02 mg/dl, 12 hr 0.3 ± 0.04 mg/dl, 24 hr 1.2 ± 0.19 mg/dl). In the kidney, there were no histopathological changes at 3 hr. At 6 hr, a slight cytoplasmic vacuolization and tubular dilation were observed in the straight portion of the proximal tubule. These histopathological changes became severe at 12 hr. At 24 hr, desquamation, necrosis and single cell necrosis of the tubular epithelial cells at the corticomedullary area were noted, predominantly in the straight portion of the proximal tubule.

The mechanism of dityrosine formation begins with the generation of a tyrosyl radical, radical isomerization followed by diradical reaction, and finally enolization [9]. The evidence that dityrosine was found in the different types of protein after exposed to a variety of oxidants may support more availability of its use as a protein oxidation marker [10]. Dityrosine has been detected in diverse pathological situations in humans [16] and experimental animals [1, 8, 17, 19, 22, 32]. Dityrosine was detected immunohistochemically in lipofuscin of pyramidal neurons of aged human brains [16], atherosclerotic lesions of apolipoprotein E-deficient mice [17] and cholesterol-fed rabbits [8], and in renal tubular lesions in rat treated with cisplatin [19]. The dityrosine concentration significantly increased in the liver of rats chronically intoxicated with ethanol [1], cardiac and skeletal muscle proteins in aging mice [22] and cooking-oil fume-induced acute lung injury in rats [32]. To our knowledge, this is the first report about expression of dityrosine in APAP-induced liver injury. Immunohistochemically, dityrosine was expressed in degenerative hepatocytes 3 hr after APAP injection, earlier than elevation of plasma ALT and AST. These findings suggested that dityrosine immunoreaction may be useful as an early biomarker in acute liver injury induced by APAP.

Increase of plasma or serum AOPP concentration has been observed in various pathological situations related to oxidative stress in humans and experimental animals, peri-

toneal dialysis [20], obese children [29], chronic diabetic in rats [18], cisplatin-induced nephrotoxicity in rats [19] and protein malnutrition in mice [30]. In the present study, plasma AOPP levels were higher in rats examined in the later period of the study and were well comparable to the severity of hepatotoxicity, suggesting that plasma AOPP levels may be useful as an oxidative stress marker for monitoring the development of APAP-induced liver injury.

In the present study, the levels of plasma AOPP concentration were comparable to the extent of dityrosine immunostaining in the liver. However, the plasma AOPP level remained as low as that of the control 3 hr after APAP injection, while dityrosine was already evident in the liver. This finding suggested dityrosine produced in hepatocytes might not leak into blood significantly 3 hr after APAP injection.

In the present study, plasma urea nitrogen and creatinine levels were significantly 24 hr after APAP injection, suggesting the evidence of renal injury. Tubular cell death was observed in rats 24 hr after injection. These findings are consistent with the results of the previous study in which an overdose of APAP was administered to experimental animals [5, 11]. APAP-induced nephrotoxicity is due to NAPQI produced as a result of the metabolic conversion of
APAP by the microsomal P-450 enzyme system, similarly to liver injury [11, 25, 27]. Oxidative stress is reported to play a role in the pathogenesis of APAP-induced nephrotoxicity, as evidenced by an increase in the lipid peroxidation and depletion of intracellular glutathione, similarly to APAP-induced liver injury [5].

In the present study, dityrosine immunostaining in the liver was observed earlier than elevation of plasma ALT and AST. The extent of dityrosine immunostaining in the liver along with elevation of plasma AOPP concentration in rats was comparable to the severity of hepatotoxicity by APAP. These findings suggest that expression of dityrosine and AOPP associate closely with development of APAP-induced liver injury and that they might be useful biomarkers for early detection or estimating the degree of oxidant-mediated protein damage in APAP-induced liver injury.

REFERENCES


