Immunohistochemical Detection of Polyunsaturated Fatty Acid Oxidation Markers in Acetaminophen-Induced Liver Injury in Rats

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ABSTRACT. To clarify whether polyunsaturated fatty acid (PUFA) oxidation is involved in the mechanism of acetaminophen (APAP)-induced apoptotic cell death, the production and localization of PUFA oxidation markers N\(^\epsilon\)-propanoyl-modified lysine, N\(^\epsilon\)-hexanoyl-modified lysine, 4-hydroxyhexenal-modified histidine and crotonaldehyde-modified lysine were evaluated in the development of APAP-induced liver injury. The immunohistochemical expression of these markers in the liver was examined up to 24 hr post-APAP intraperitoneal injection in rats (1 g/kg body weight). The histopathological changes in the liver appeared 3 hr after APAP injection and became exacerbated with time. Proapoptotic protein Bax immunoreactivity was first detected in the degenerative hepatocytes 3 hr after the injection and areas positively immunostained for Bax reached a peak level at 6 hr, and then decreased at 12 and 24 hr. There was a significant increase in the TUNEL-positive rate at 12 and 24 hr. Immunohistochemical expression of all these oxidation markers was first detected in the degenerative hepatocytes 3 hr after the injection, and earlier than the occurrence of hepatocyte apoptosis. Immunohistochemical expression of these markers were observed in almost all degenerative hepatocytes 3–24 hr after APAP injection. Areas positively immunostained for these markers reached a peak level at 6 hr, and then decreased at 12 and 24 hr. The results thus suggest that the generation of PUFA oxidation markers may be the signature of early events preceding the induction of liver cell apoptosis and thus useful for early detection of oxidative stress-related liver cell injury.

KEY WORDS: acetaminophen, liver injury, oxidation, polyunsaturated fatty acid.


Acetaminophen (N-acetyl-p-aminophenol; APAP) is a safe and effective analgesic and antipyretic drug when used at therapeutic levels [36]. However, an overdose of APAP causes liver injury in humans [27] and experimental animals [5, 37]. The initial step in toxicity is the cytochrome P450 metabolism to N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive metabolite [16, 31, 32]. NAPQI depletes the intracellular storage of glutathione (GSH) in the liver [4, 16, 32]. After the cellular GSH content is exhausted, NAPQI covalently binds to cellular proteins including mitochondrial proteins [2, 16, 17, 37]. The covalent binding of NAPQI to cellular proteins is a critical initiating event, which requires amplification to cause cell death [2, 16, 18]. Since GSH is depleted by NAPQI in APAP-induced liver injury and GSH is the cofactor for GSH-peroxidase detoxification of peroxides, a main mechanism of peroxide detoxification is compromised in APAP-induced liver injury. Thus, GSH depletion may be expected to lead to increased intracellular peroxide and increased oxidative stress [14]. APAP may also cause hepatotoxicity by mechanisms leading to the formation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical, reactive nitrogen species such as nitric oxide and peroxynitrite, and peroxidation reaction products [4, 8, 17]. Previous studies have demonstrated that lipid peroxidation is involved in APAP-induced liver injury mechanism [42, 43]. However, in APAP-induced liver injury, the role of lipid peroxidation on mechanism of hepatocytes apoptotic cell death remains to be elucidated.

Recently, polyunsaturated fatty acid (PUFA) oxidation markers, such as N\(^\epsilon\)-propanoyl-modified lysine (PRL), N\(^\epsilon\)-hexanoyl-modified lysine (HEL) 4-hydroxyhexenal-modified histidine (4-HHE-histidine) and crotonaldehyde–modified lysine (CRA-lysine), have been given more attention as factors related to the processes of diverse pathological situations [9, 15, 19, 21, 23, 33, 38–40, 44]. PUFA is easily peroxidized by free radicals and enzymes, and peroxidation of PUFA results in compromised integrity of cellular membrane and leads to lipid hydroperoxide as a major reaction product, and lipid hydroperoxide is decomposed into aldehyde [20]. PRL and HEL can be classified as a group of alkylamide-type adducts [20]. PRL is formed by the reaction of lysine with lipid hydroperoxide derived from n-3 PUFA such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and α-linolenic acid [15]. HEL is formed by the reaction of lysine and lipid hydroperoxide derived

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from n-6 PUFA, such as linoleic acid, arachidonic acid and γ-linolenic acid [20]. 4-HHE and CRA are the α,β-unsaturated aldehydes that are end products of oxidation of n-3 fatty acids [6, 29]. These highly reactive aldehydes readily attack and modify the protein amino acid residues to protein-bound 4-HHE/CRA, such as 4-HHE-histidine adduct and CRA-lysine adduct [39, 41]. However, the production and localization of these PUFA oxidation markers are unclear in the development of APAP-induced liver injury.

In the present study, we investigated the immunohistochemical localization of PUFA oxidation markers PRL, HEL, 4-HHE-histidine and CRA-lysine in the liver of rats during 24 hr following a single injection of APAP. The objective of this investigation is to evaluate whether PUFA oxidation is involved in the mechanism of APAP-induced apoptosis.

MATERIALS AND METHODS

All experiments were performed using male Wistar Imamichi rats, 7 weeks 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, humidity of 50 ± 5%, ventilation of 11 times per hour 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)[3] 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 and AST levels were determined by a biochemical autoanalyzer (Dri-Chem 3000; Fuji Film Company, Tokyo, Japan). Samples of the liver (right and left median lobes) 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 [26] and analysed with a histometric analysis software (Olympus Corporation, Tokyo, Japan). TUNEL-positive rate was calculated as the percentage of TUNEL-positive cells out of the total number of hepatocytes counted.

For immunohistochemistry, mouse monoclonal antibodies for Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.), PRL (prepared as described previously [10]), HEL (Nikken Sei Co., Ltd., Shizuoka, Japan), 4-HHE-histidine (NOF Corporation, Tokyo, Japan) and CRA-lysine (NOF Corporation, Tokyo, Japan) were used as the primary antibodies. 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 at 4°C overnight (Bax, HEL, 4-HHE-histidine) or at room temperature for 30 min (PRL, CRA-lysine), 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 PRL, HEL, 4-HHE-histidine and CRA-lysine 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. The sections were then incubated with the antibody diluents (Dako cytomation, Tokyo, Japan) instead of these antibodies.

Areas positively immunostained for Bax, PRL, HEL, 4-HHE-histidine and CRA-lysine in the tissue were measured with histometric analysis software (Olympus Corporation, Tokyo, Japan) and determined as a percentage of positive area in a total of 50 fields on the tissue, at ×40 magnification, which were randomly chosen from at least five independent sections. Necrotic area was excluded from immunohistochemical evaluations of these PUFA oxidation markers.

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

RESULTS

Plasma ALT and AST tended to increase 6 hr and showed significant increase 12 and 24 hr after exposure to APAP, respectively (Table 1). At 3 hr APAP post-treatment, vacuolated hepatocytes were observed in zone 3 (Figs. 1b-1 and 2), and, at 6 hr, they increased in number and expanded their distribution to zone 2 (Figs. 1c-1 and 2). Morphological features of apoptosis such as clumping and margination of chromatin in the nuclei and cell shrinkage was occasionally observed in hepatocytes in zone 3 of this group. Then, at 12 hr, coagulative necrosis, single cell necrosis and apoptosis were observed in all zones (Figs. 1d-1 and 2). There were nuclear changes of clumping and margination of chromatin, pyknosis and karyorrhexis in this group. Congestion in
sinusoids was prominent, especially at the periphery of the necrotic areas. At 24 hr, necrotic and apoptotic changes became more prominent (Figs. 1e-1 and 2). The cytoplasmic membranes of affected hepatocytes were frequently obscure, and it was difficult to distinguish individual cell borders. Pyknosis of nuclear material, karyorrhexis and cell shrinkage were also frequently observed in this group. The TUNEL-positive rate tended to increase 6 hr and showed significant increase 12 and 24 hr (Figs. 1d-3, 1e-3 and Table 1).

Hepatocytes in the control were negative for Bax, PRL, HEL, 4-HHE-histidine and CRA-lysine (Figs. 2, 3 and 4). The immunohistochemical expression of Bax and all these PUFA oxidation markers was also first detected in the cytoplasm of degenerative hepatocytes in zone 3 at 3 hr after the injection and earlier than the occurrence of hepatocyte apoptosis (Figs. 2, 3 and 4). In 12 and 24 hr, degenerative hepatocytes and surrounding intact hepatocytes reacted specifically to Bax and these oxidation markers, while necrotic cells showed non-specific reactions (Figs. 2, 3 and 4). Areas positively immunostained for Bax and these oxidation markers reached a peak level at 6 hr, and then decreased at 12 and 24 hr (Tables 1 and 2). These time-course changes of areas positively immunostained for Bax and these oxidation markers are dependent on the development of necrosis in APAP-induced liver injury.

**DISCUSSION**

PRL, HEL, 4-HHE-modified protein and CRA-lysine has been immunohistochemically detected in diverse pathological situations in humans [19, 21, 23, 38, 39, 44] and experimental animals [9, 15, 33, 40]. PRL has been immunohistochemically detected in atherosclerotic lesions of humans [19], cholesterol-fed rabbits [9] and lipopolysaccharide-induced liver injury in D-galactosamine-sensitized mice [33] has already been reported. 4-HHE-modified protein immunoreactivity was localized in liver of patients with chronic hepatitis C [23], spinal cords from patients with amyotrophic lateral sclerosis [39], in human atherosclerotic aorta [34] and intense light-exposed rat retina [40]. CRA-lysine has been immunohistochemically detected in spinal cord of ALS patients [38] and Alzheimer’s disease brain [21]. To our knowledge, this is the first report about expression of PRL, HEL, 4-HHE-histidine and CRA-lysine in APAP-induced liver injury. Immunohistochemical expression of these PUFA oxidation markers was detected in degenerative hepatocytes 3 hr after APAP injection, earlier than the occurrence of hepatocyte apoptosis or necrosis and significant elevation of plasma ALT and AST. Immunohistochemical expression of these PUFA oxidation markers was observed in almost all degenerative hepatocytes 3–24 hr after APAP injection. These results suggest that PUFA oxidation may be involved in the pathogenesis of APAP-induced liver injury, and the production of these oxidation markers may be early events that precede hepatocyte apoptosis.

In the present study, TUNEL-positivity was observed in the necrotic foci 12 and 24 hr after APAP injection. Since TUNEL reportedly marks cells that have undergone not only apoptosis but also necrosis and autolysis, it may thus overestimate apoptosis [11]. However, in the present study, TUNEL-positivity 12 and 24 hr after injection may be almost specific to apoptosis, because almost all TUNEL-positive cells showed morphological features of apoptosis such as cell shrinkage and clumping and margination of chromatin.

Several previous studies demonstrated that apoptosis and necrosis coexist in APAP-induced liver injury [24, 35]. Ray et al. [35] found that, following a toxic dose of APAP to mice, 40% of the dead hepatocytes were apoptotic and 60% necrotic. However, the role of apoptosis in APAP-induced cell death is still controversial. Some reports demonstrate that APAP-induced cell death involves signaling mechanisms which are thought to be characteristic of apoptosis such as: mitochondrial translocation of Bax and Bid [7], mitochondrial release of cytochrome c [16], activation of JNK [14], DNA fragmentation as indicated by a DNA ladder [34], morphological chromatin fragmentation and margination in the nucleus [34], cells staining positive for TUNEL assay [24], and activation of apoptosis-related cysteine peptidases, caspase-3 [24]. Other reports have indicated that the percentage of apoptosis is low and that oncotic necrosis is the principal mechanism in APAP-induced cell death [12]. Kon et al. [24] demonstrated that APAP-induced mitochondrial permeability transition (MPT) leads to both ATP depeletion-dependent oncotic necrosis and caspase-dependent apoptosis via cytochrome c release in hepatocytes. Pathways leading to apoptosis and necrosis share the MPT. Such sharing of these pathways reflects and

**Table 1. Time course of liver function markers and apoptotic markers in APAP-induced liver injury**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3 hr</th>
<th>6 hr</th>
<th>12 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma AST (U/l)</td>
<td>107.0 ± 29.5</td>
<td>116.2 ± 18.9</td>
<td>361.2 ± 33.6</td>
<td>3991.0 ± 963.8*†</td>
<td>3983.2 ± 657.9*†</td>
</tr>
<tr>
<td>Plasma ALT (U/l)</td>
<td>26.8 ± 2.3</td>
<td>35.2 ± 4.3</td>
<td>86.0 ± 8.9</td>
<td>1276.8 ± 341.7*†</td>
<td>2223.5 ± 499.9*†</td>
</tr>
<tr>
<td>TUNEL-positive rate (%)</td>
<td>0.36 ± 0.23</td>
<td>1.12 ± 0.14</td>
<td>5.80 ± 1.43</td>
<td>24.53 ± 5.91*†</td>
<td>29.70 ± 4.50*†</td>
</tr>
<tr>
<td>Areas positively immunostained for Bax (%)</td>
<td>0.00 ± 0.00</td>
<td>8.21 ± 0.99*</td>
<td>30.38 ± 3.91*†</td>
<td>7.72 ± 0.93†</td>
<td>7.09 ± 0.87†</td>
</tr>
</tbody>
</table>

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.
explains the coexistence of apoptosis and necrosis in APAP-induced liver injury.

Bax is a pro-apoptotic protein allowing apoptosis to occur through the intrinsic, damage-induced pathway, and amplifying one occurring via the extrinsic, receptor-mediated pathway \[10\]. Bax protein is activated and translocates from the cytosol to the outer mitochondrial membrane, forming high conductance channels \[1, 10\]. This permits release of cytochrome c from the mitochondria into the cytosol \[1, 10\]. Cytosolic cytochrome c leads to caspase activation and subsequent cell death \[1, 10\]. APAP overdose reportedly induced increase of mitochondrial Bax pro-
Fig. 3. Changes of PRL and HEL immunoeexpression in APAP-induced liver injury. a: PRL, b: HEL. 1: control group, 2: 3 hr group, 3: 6 hr group, 4: 12 hr group, 5: 24 hr group. Bar=50 μm. *: central vein.

Fig. 4. Changes of 4-HHE-histidine and CRA-lysine immunoeexpression in APAP-induced liver injury. a: 4-HHE-histidine, b: CRA-lysine. 1: control group, 2: 3 hr group, 3: 6 hr group, 4: 12 hr group, 5: 24 hr group. Bar=50 μm. *: central vein.
tein levels in hepatocytes [2]. In the present study, immunohistochemical localization and timing of expression of PUFA oxidation markers were in accord with those of Bax protein. These findings suggest PUFA oxidation is associated with the mitochondrial pathway to apoptosis.

Previous studies postulated that an APAP metabolism triggering lipid peroxidation was responsible for liver injury [42, 43]. Lipid peroxidation is known to produce many reactive species, such as lipid hydroperoxides and α,β-unsaturated aldehydes that are biologically deleterious. Lipid hydroperoxides enhance ROS-mediated DNA fragmentation [13]. Lipid peroxidation-derived α,β-unsaturated aldehydes such as 4-HHE and CRA are known to be capable of inducing cellular stress-responsive processes such as cell signaling and apoptosis [28, 29]. In the intense light-exposed rat retina, protein modification by 4-HHE was an early event that preceded photoreceptor cell apoptosis [40]. Lee et al. [28] demonstrated that 4-HHE-induced endothelial apoptosis was mediated by the enhancement of apoptotic Bax and the suppression of anti-apoptotic Bcl-2 by peroxynitrite generation. Kristal et al. [25] demonstrated that 4-HHE-induced mitochondrial permeability transition (MPT), led to the breakdown of the mitochondrial membrane potential, the inability to synthesize ATP, and finally cell death [22, 25]. Long et al. [30] reported that 4-HHE also depletes neuronal glutathione (GSH) content and neuronal reactive oxygen species (ROS) in rat cerebral cortical neurons. CRA can penetrate through the cell membrane and bind to GSH without metabolic activation [29]. A reduced GSH level leads to imbalance of cellular redox and causes increases of ROS that could explain its apoptotic properties [29]. CRA-induced apoptosis was mediated via cytochrome c release and caspases cascade [29]. CRA causes both apoptosis and necrosis, and there is a transition from apoptosis to necrosis with increasing CRA concentration [29].

In conclusion, immunohistochemical expression of these PUFA oxidation markers, PRL, HEL, 4-HHE-histidine and CRA-lysine, was detected in degenerative hepatocytes before hepatocyte apoptosis. These PUFA oxidation markers were expressed immunohistochemically in almost all degenerative hepatocytes 3–24 hr after APAP injection. The results thus suggest that the generation of PUFA oxidation may be the signature of early events preceding the induction of liver cell apoptosis and thus useful for early detection of oxidative stress-related liver cell injury.

### REFERENCES


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### Table 2. Areas positively immunostained for PUFA oxidation markers in APAP-induced liver injury

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>3 hr</th>
<th>6 hr</th>
<th>12 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRL (%)</td>
<td>0.00±0.00</td>
<td>8.35±1.17*</td>
<td>39.94±3.73**</td>
<td>7.04±1.08†</td>
<td>5.45±1.19†</td>
</tr>
<tr>
<td>HEL (%)</td>
<td>0.00±0.00</td>
<td>14.56±3.64*</td>
<td>41.10±4.51**</td>
<td>6.68±1.20‡</td>
<td>3.79±0.53‡</td>
</tr>
<tr>
<td>4-HHE-histidine (%)</td>
<td>0.00±0.00</td>
<td>9.25±1.44*</td>
<td>36.21±4.05**</td>
<td>7.42±1.06′</td>
<td>5.59±0.75′</td>
</tr>
<tr>
<td>CRA-lysine (%)</td>
<td>0.00±0.00</td>
<td>11.39±1.88*</td>
<td>35.78±5.12**</td>
<td>8.49±0.87†</td>
<td>4.84±0.95†</td>
</tr>
</tbody>
</table>

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.


