Peroxynitrite-Induced Mitochondrial and Endonuclease-Mediated Nuclear DNA Damage in Acetaminophen Hepatotoxicity

Cathleen Cover, Abdellah Mansouri, Tamara R. Knight, Mary Lynn Bajt, John J. Lemasters, Dominique Pessayre, and Hartmut Jaeschke

Liver Research Institute, College of Medicine, University of Arizona, Tucson, Arizona (C.C., T.R.K., M.L.B., H.J.); Institut National de la Santé et de la Recherche Médicale Unite 481, Faculté de Médecine Xavier Bichat, Paris, France (A.M., D.P.); and Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, North Carolina (J.J.L.)

Accepted May 6, 2005; accepted August 1, 2005

ABSTRACT

Intracellular sources of peroxynitrite formation and potential targets for this powerful oxidant and nitrating agent have not been identified after acetaminophen (AAP) overdose. Therefore, we tested the hypothesis that peroxynitrite generated in mitochondria may be responsible for mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage. C3Heb/FeJ mice were treated with 300 mg/kg AAP and monitored for up to 12 h. Loss of mtDNA (assayed by slot blot hybridization) and substantial nDNA fragmentation (evaluated by anti-histone enzyme-linked immunosorbent assay, terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, and agarose gel electrophoresis) were observed as early as 3 h after AAP overdose. Analysis of nitrotyrosine protein adducts in subcellular fractions established that peroxynitrite was generated predominantly in mitochondria beginning at 1 h after AAP injection. Delayed treatment with a bolus dose of glutathione (GSH) accelerated the recovery of mitochondrial glutathione, which then effectively scavenged peroxynitrite. However, mtDNA loss was only partially prevented. Despite the absence of nitrotyrosine adducts in the nucleus after AAP overdose, nDNA damage was almost completely eliminated with GSH administration. A direct comparison of nDNA damage after AAP overdose with nDNA fragmentation during tumor necrosis factor receptor-mediated apoptosis showed similar DNA ladders on agarose gels but quantitatively different results in three other assays. We conclude that peroxynitrite may be partially responsible for mtDNA loss but is not directly involved in nDNA damage. In contrast, nDNA fragmentation after AAP overdose is not caused by caspase-activated DNase but most likely by other intracellular DNase(s), whose activation is dependent on the mitochondrial oxidant stress and peroxynitrite formation.

Acetaminophen (AAP) is a safe drug at therapeutic levels, but an overdose can cause serious liver injury in experimental animals and in humans (Lee, 2004). AAP overdose is the most frequent cause of acute drug-induced liver failure in the United States (Lee, 2004). Key features of the toxic mechanism include the formation of a reactive metabolite, presumably N-acetyl-p-benzoquinone imine (NAPQI), which depletes glutathione and then binds to cellular proteins (Nelson, 1990; Cohen and Khairallah, 1997). The alkylation of proteins, especially mitochondrial proteins (Tirmenstein and Nelson, 1989; Qiu et al., 2001), seems to be an initiating event for the formation of reactive oxygen species selectively in mitochondria (Jaeschke, 1990; Tirmenstein and Nelson, 1990; Knight et al., 2001, Jaeschke et al., 2003). The mitochondrial oxidant stress precedes cell death (Bajt et al., 2004). Although AAP overdose does not cause cell injury through excessive lipid peroxidation (Knight et al., 2003), the oxidant stress is a critical event in the opening of mitochondrial membrane permeability transition pores and the breakdown of the mitochondrial membrane potential (Kon et al., 2004a,b; Reid et al., 2005), which leads to ATP depletion and cell death by oncotic necrosis (Gujral et al., 2002). The importance of mitochondrial dysfunction in the pathophysiology of proteins, especially mitochondrial proteins (Tirmenstein and Nelson, 1989; Qiu et al., 2001), seems to be an initiating event for the formation of reactive oxygen species selectively in mitochondria (Jaeschke, 1990; Tirmenstein and Nelson, 1990; Knight et al., 2001, Jaeschke et al., 2003). The mitochondrial oxidant stress precedes cell death (Bajt et al., 2004). Although AAP overdose does not cause cell injury through excessive lipid peroxidation (Knight et al., 2003), the oxidant stress is a critical event in the opening of mitochondrial membrane permeability transition pores and the breakdown of the mitochondrial membrane potential (Kon et al., 2004a,b; Reid et al., 2005), which leads to ATP depletion and cell death by oncotic necrosis (Gujral et al., 2002). The importance of mitochondrial dysfunction in the pathophysiology
of AAP hepatotoxicity is supported by the protective effect of cyclosporine in vivo (Masubuchi et al., 2005).

In recent years, it was recognized that peroxynitrite is being generated in hepatocytes, which undergo oncotc necrosis (Hinson et al., 1998; Knight et al., 2001). A reduced injury in inducible nitric-oxide synthase gene knockout mice (Gardner et al., 2002) and after delayed treatment with GSH (Knight et al., 2002; Bajt et al., 2003) or N-acetylcysteine (James et al., 2003b; Bajt et al., 2004) suggests that peroxynitrite is a critical mediator in the injury mechanism. However, the intracellular sources of peroxynitrite formation and potential targets for this potent oxidant and nitrating agent have not been identified. It was shown that a mitochondrial oxidant stress can cause depletion of mitochondrial DNA (mtDNA) in several in vivo models, including alcohol binge drinking (Mansouri et al., 1999) and tacrine hepatotoxicity (Mansouri et al., 2003). Since the fate of the mtDNA during AAP hepatotoxicity is unknown, one of the objectives of this investigation was to evaluate the intracellular location of peroxynitrite formation and to test whether peroxynitrite could modify mtDNA in hepatocytes.

In contrast to mtDNA, fragmentation of nuclear DNA (nDNA) is a well described early phenomenon in the pathophysiology of AAP hepatotoxicity (Ray et al., 1990). Nuclear DNA damage can be seen as karyolysis by histology and DNA laddering (Ray et al., 1990), positive staining with the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Lawson et al., 1999; Gujral et al., 2002), and detection of DNA fragments in the cytosol (Lawson et al., 1999; Cover et al., 2005) and in plasma (Jahr et al., 2001). Although a general endonuclease inhibitor attenuated nDNA fragmentation and liver injury (Shen et al., 1992), which would implicate enzymatic degradation of nDNA, a potential role of peroxynitrite in the nDNA damage has not been investigated. Despite its reactivity, peroxynitrite can hit targets some distance away from its point of formation (Denicola and Radi, 2005). Therefore, the second major objective of this study was to test whether peroxynitrite can nitrate nuclear proteins and could be involved in nDNA fragmentation.

Materials and Methods

Animals. Male C3HeB/FeJ mice with an average weight of 18 to 20 g were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in an environmentally controlled room with 12-h light/dark cycle and allowed free access to food (certified rodent diet 8640; Harlan, Indianapolis, IN) and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee and followed the criteria of University of Arizona and the National Research Council for the care and use of laboratory animals in research. All animals were fasted overnight before the experiments. Animals received an intraperitoneal injection of 300 mg/kg AAP (Sigma-Aldrich, St. Louis, MO) between 8:00 and 9:00 AM. AAP was dissolved in warm saline (15 mg/ml). Some animals received a single intravenous bolus dose of glutathione (200 mg/kg GSH) dissolved in phosphate-buffered saline (PBS) at 1.5 h after AAP injection (Knight et al., 2002; Bajt et al., 2003).

Experimental Protocols. At selected times after AAP treatment, animals were killed by cervical dislocation. Blood was drawn from the vena cava into heparinized syringes and centrifuged. The plasma was used for determination of alanine aminotransferase (ALT) activities. Immediately after collecting the blood, the livers were excised and rinsed in saline. A small section from each liver was placed in 10% phosphate-buffered formalin to be used in immunohistochemical analysis. The remaining liver was either used to isolate subcellular fractions or frozen in liquid nitrogen and stored at −80°C. In a positive control experiment for nitrotyrosine adduct formation, livers were flushed free of blood with saline, and then 1 ml of 1 mM freshly diluted peroxynitrite solution in PBS (Cayman Chemical, Ann Arbor, MI) was injected into the portal vein. After 10 min, the liver was removed, and subcellular fractions were isolated.

Methods. Plasma ALT activities were determined with the kinetic test kit 68-B (Biotron Diagnostics, Inc., Herent, CA) and expressed as IU/liter. Protein concentrations were assayed using the bicinchoninic acid kit (Pierce Chemical, Rockford, IL). Total soluble GSH and glutathione disulfide (GSSG) were measured in the liver homogenate with a modified method of Tietze as described in detail previously (Jaeschke, 1990; Knight et al., 2002). Briefly, the frozen tissue was homogenized at 0°C in 3% sulfosalicylic acid containing 0.1 mM EDTA. After dilution with 0.01 N HCl, the sample was centrifuged, and the supernatant was diluted with 100 mM potassium phosphate buffer, pH 7.4. The samples were assayed using dithionitrobenzoic acid. All data are expressed in GSH-equivalents.

DNA fragmentation was evaluated using the cell death detection ELISA (anti-histone ELISA) (Roche Diagnostics, Indianapolis, IN) as described in detail previously (Bajt et al., 2002). In this assay, the kinetics (\(V_{\text{max}}\)) of product generation is a measure of DNA fragmentation. The \(V_{\text{max}}\) values obtained for untreated controls (100%) are compared with those in treated groups. The assay allows the specific quantitation of cytoplastmic histone-associated DNA fragments. For the TUNEL assay, formalin-fixed tissue samples were imbedded in paraffin and 5-μm sections were cut. Sections of liver were stained with the in situ cell death detection kit AP (Roche Diagnostics) as described in the manufacturer’s instructions.

Isolation of Subcellular Fractions. The detailed protocol has been described previously (Knight et al., 2001). Briefly, the liver was homogenized in ice-cold isolation buffer, pH 7.4, containing 220 mM mannitol, 70 mM sucrose, 2.5 mM HEPES, 10 mM EDTA, 1 mM EGTA, and 0.1% bovine serum albumin. Mitochondria were isolated by differential centrifugation (10,000g) and washed with 2 ml of isolation buffer. The supernatant was centrifuged at 100,000g. The pellet (microsomes) was resuspended and centrifuged again; the supernatant represented the cytosolic fraction. Nuclear extracts were obtained by homogenizing liver tissue (1:2 (g/v)) in ice-cold 10 mM HEPES-KOH, pH 7.6, 25 mM KCl, 1 mM EDTA, pH 8.0, 2 M sucrose, and 10% glycerol (v/v). Lysis was monitored by trypan blue, and samples were homogenized until greater than 90% of cells were broken. Homogenate was diluted to 17 ml with sucrose buffer and layered onto 3.15 ml of sucrose buffer in Beckman Ultraclear thin wall tubes (Beckman Coulter, Palo Alto, CA). Nuclei were collected as a pellet after centrifugation at 24,600 rpm in the Beckman SW41 Ti rotor for 41 min at 4°C. In previous experiments, we verified the purity of the subcellular fractions isolated by the described procedures using lactate dehydrogenase (cytosol), succinate dehydrogenase, and cytochrome c (mitochondria), histone (nuclei), and cytochrome 2E1 (microsomes).

Cytosol was diluted and pellets of mitochondria, microsomes, and nuclei were suspended in 300 μl of PBS, and nitrosylated proteins were measured with the nitrotyrosine assay kit (Kamiya Biomedical Company, Seattle, WA) according to the manufacturer’s directions. For determination of mitochondrial GSH and GSSG, the mitochondrial pellet was resuspended in 3% sulfosalicylic acid containing 0.1 mM EDTA, vigorously vortexed, and centrifuged to sediment the precipitated protein. A part of the supernatant was diluted to 100 mM potassium phosphate buffer, pH 6.5, for the determination of total glutathione (GSH + GSSG), and another part was added to 10 mM N-ethylmaleimide in potassium phosphate buffer for the determination of GSSG as described previously (Knight et al., 2001).

Analysis of nDNA and mtDNA Levels. Total hepatic DNA was isolated with Genomic-tip 100G columns (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s recommendations. DNA solutions were split into aliquots that were either stored at 4°C or...
frozen once at −80°C. DNA concentration was assessed from absorption at 260 nm. To quantify mtDNA and nDNA, slot blot hybridization was performed as described previously (Mansouri et al., 1999, 2003). Hepatic DNA purified from 5 mg of tissue was blotted onto a Hybond-N+ nylon membrane (GE Healthcare, Piscataway, NJ) and first hybridized with a 10.9-kilobase mtDNA probe (nucleotides 4964–15896) (Bibb et al., 1981) generated by long polymerase chain reaction and labeled by random priming (Multiprime DNA labeling system; GE Healthcare). Membranes were stripped and hybridized again with a mouse C.t-1 nDNA probe (Invitrogen, Cergy Pontoise, France). mtDNA and nDNA were assed by densitometric analysis of autoradiographs. To look for nDNA fragmentation, hepatic DNA (5 μg) was loaded on 2% agarose gels (1.4% NuSieve GTG agarose plus 0.6% Seakem GTG agarose; FMC Bioproducts, Rockland, ME) stained with ethidium bromide. Electrophoresis was performed for 5 h at 50 V, and gels were photographed under UV transillumination (Mansouri et al., 1999, 2003).

Statistics. All results were expressed as mean ± S.E. The Student’s t test was used for the comparison of two groups. Comparisons between multiple groups were performed with one-way analysis of variance followed by Bonferroni t test. If the data were not normally distributed, we used the Kruskal-Wallis test (nonparametric analysis of variance) followed by Dunn’s multiple comparisons test. P < 0.05 was considered significant.

Results

Acetaminophen-Induced mtDNA and nDNA Damage. Using the slot blot hybridization technique, we observed a progressive loss of hepatic mtDNA after treatment with a hepatotoxic dose of 300 mg/kg AAP (Fig. 1A). Densitometric analysis showed a significant reduction of intact mtDNA between 3 and 12 h after AAP administration (Fig. 1B). Since slot blot hybridization did not show a significant loss of nDNA (Fig. 1A), the sharp decline of the mtDNA-to-nDNA hybridization ratio at 3 h after AAP treatment and beyond reflects mainly the loss of intact mtDNA (Fig. 1C). On the
other hand, using an anti-histone ELISA to sensitively measure nDNA damage in the cytosol, we observed increased nDNA damage at 3 h after AAP with further progression up to 12 h (Fig. 2A). The nDNA damage correlated with the release of ALT into plasma as an indicator for necrotic cell death (Fig. 2B). Nuclear DNA damage (DNA strandbreaks) after AAP is also shown by the increased number of TUNEL-positive centrilobular hepatocytes (Fig. 3, A and B). In contrast to TNF-mediated apoptosis, where the TUNEL staining is concentrated in the apoptotic nuclei (Fig. 3, E and F), hepatocytes exposed to an overdose of AAP stained in the nucleus and in the cytosol (Fig. 3B).

**Mitochondrial Peroxynitrite Formation and mtDNA versus nDNA Damage.** It is well established that AAP overdose leads to peroxynitrite formation mainly in those hepatocytes that eventually die by onecotic necrosis (Hinson et al., 1998; Knight et al., 2001). However, the intracellular localization of peroxynitrite formation and protein nitration remained unclear. Therefore, we isolated different cell organelles at various times after AAP administration in vivo. Nitrotyrosine protein adducts could be observed in mitochondria as early as 1 h after AAP overdose (Fig. 4). Peak levels of protein nitration were seen at 2 h with no further significant change up to 6 h (Fig. 4). In contrast, there was no significant increase in nitrotyrosine adduct formation in the cytosol, the nucleus or in microsomes (Fig. 4). As a positive control experiment, we infused 1 ml of 1 mM peroxynitrite solution into the liver through the portal vein and isolated subcellular fractions after 10 min. Peroxynitrite caused a 3-fold increase of NT adducts in the cytosol and microsomes compared with untreated controls. However, there was no change of NT adducts over baseline in mitochondria and nuclei. These data indicate that nitration can occur in any subcellular compartment dependent on the route of peroxynitrite exposure. The increase in mitochondrial NT adduct levels after AAP overdose strongly suggests that mitochondria are the main location of peroxynitrite formation in hepatocytes. Based on these findings, we hypothesized that peroxynitrite might be responsible for the loss of mtDNA. To test this hypothesis, we treated animals with a bolus dose of GSH at 1.5 h after AAP as described previously (Knight et al., 2002; Bajt et al., 2003). GSH injected intravenously is rapidly hydrolyzed to its amino acids, which are taken up by hepatocytes and used to resynthesize GSH (Wendel and Jaeschke, 1982). GSH treatment, which accelerated the recovery of the depleted mitochondrial glutathione levels (Fig. 5B), eliminated nitrotyrosine staining in mitochondria (Fig. 5A) but caused a dramatic increase of the mitochondrial GSSG content compared with animals treated only with AAP (Fig. 5C). Despite the very effective scavenging of peroxynitrite by mitochondrial GSH, the loss of the mtDNA (Fig. 6A) and the reduction of the mtDNA-to-nDNA hybridization ratio (Fig. 6C) were only partially prevented. On the other hand, if GSH was injected immediately after AAP administration, the recovering GSH levels in the liver are used to scavenge NAPQI and therefore prevent any mitochondrial oxidant stress and peroxynitrite formation (Knight et al., 2002). Under these conditions, there was no loss of mtDNA or nDNA (Fig. 6, A–C). Interestingly, when DNA fragments were measured in the cytosol with the anti-histone ELISA, both GSH treatment regimens inhibited nDNA fragmentation (Fig. 7A). Thus, early treatment with GSH (GSH), which is used to scavenge NAPQI, prevents mitochondrial dysfunction and oxidant stress and eliminates nDNA fragmentation. However, delayed treatment with GSH (1.5 h after AAP) does not prevent mitochondrial dysfunction but scavenges mitochondrial peroxynitrite and effectively prevents nDNA fragmentation. In support of these findings, there were either no TUNEL-positive hepatocytes (GSH) (data not shown) or only few hepatocytes (GSH) that stained with the TUNEL assay (Fig. 3, C and D). Consequently, both GSH treatment regimens were highly effective in reducing ALT release as an indicator of onecotic necrosis at 6 h after AAP (Fig. 7B).

**TNF-Mediated Apoptosis and mtDNA versus nDNA Damage.** Since our data do not seem to support a role for peroxynitrite in nDNA damage, we compared the extent of nDNA fragmentation after AAP with a model of TNF-induced apoptosis. At 6 h after Gal/ET treatment, about 15 to 20% of hepatocytes are undergoing apoptosis with no interference of the later neutrophil-induced injury phase (Jaeschke et al., 1998). Consistent with these data, there was massive caspase-3 activation (data not shown) but only very minor ALT release at 6 h after Gal/ET administration (Table 1). However, DNA fragmentation was increased 16-fold.

![Figure 2](image-url)
above baseline (Table 1), which is about three times higher than values observed 6 h after AAP (Fig. 7A). In addition, the TUNEL staining was fundamentally different between these models. Gal/ET induced nuclear staining in many hepatocytes across the entire liver lobules (Fig. 3, E and F) compared with the nuclear and cytosolic staining in strictly centrilobular hepatocytes after AAP (Fig. 3, A and B). Using the slot blot hybridization technique, there was a 46% loss of nDNA after Gal/ET but no loss of mtDNA (Table 1). This is again fundamentally different to the results after AAP, where only loss of mtDNA was observed (Fig. 1). Interestingly, a direct comparison of DNA fragments on an agarose gel showed very similar DNA ladders in samples from AAP- and Gal/ET-treated animals (Fig. 8).

Fig. 3. Acetaminophen-induced DNA strandbreaks as assessed by the TUNEL assay. There are no TUNEL-positive cells in livers of untreated control animals (not shown). A and B, extensive TUNEL staining in predominantly centrilobular hepatocytes 6 h after treatment with 300 mg/kg acetaminophen. Cellular staining included the nucleus and the cytosol. C and D, dramatic reduction of the number of TUNEL-positive hepatocytes after AAP treatment in animals, which received a bolus dose of GSH 1.5 h after AAP. E and F, characteristic TUNEL staining of individual hepatocytes undergoing apoptosis 6 h after treatment with 700 mg/kg galactosamine/100 μg/kg endotoxin. Original magnification, 100× (A, C, and E) and 400× (B, D, and F).

Fig. 4. Time course of protein nitration in mitochondria, cytosol, microsomes, and nuclei of untreated controls (t = 0) and at different time points after administration of 300 mg/kg acetaminophen. Nitrotyrosine protein adducts were measured by ELISA and are expressed as nanomoles of adducts per milligram of protein. Data points represent means ± S.E. of n = 4 animals per time point. * P < 0.05 (compared with control; t = 0).

Discussion

The major objectives of this investigation were to 1) evaluate the intracellular sources of peroxynitrite formation and assess whether peroxynitrite could deplete mtDNA in hepatocytes and 2) test whether peroxynitrite can nitrite nuclear proteins and could be responsible for nDNA fragmentation.
Our data showed that protein nitration after AAP treatment occurred in mitochondria but was not observed in any other cellular compartment. Thus, within the detection limit of the anti-NT ELISA, mitochondria are the predominant source of peroxynitrite formation after AAP overdose. These data are consistent with previous observations of an oxidant stress in mitochondria as indicated by the increased levels of mitochondrial GSSG (Jaeschke, 1990; Knight et al., 2001, 2002; James et al., 2003a). The fact that no biliary efflux of GSSG was observed suggested that GSSG formation was restricted to mitochondria (Jaeschke, 1990). Since NO can diffuse across membranes, NO was either generated inside mitochondria or in the cytosol by inducible nitric-oxide synthase (Gardner et al., 2002). Independent of the source of NO, our data suggest that the rapid spontaneous reaction between superoxide and NO to form peroxynitrite must have taken place inside the mitochondria. Mitochondrial peroxynitrite
formation was observed as early as 1 h after AAP overdose. These findings are consistent with the hypothesis that the oxidant stress started at the time of maximal GSH depletion and protein binding of NAPQI (Bajt et al., 2004). Thus, the increased generation of superoxide favored peroxynitrite formation and the very low GSH levels in mitochondria at that time allowed nitration of proteins.

**Mitochondrial versus Nuclear DNA Damage.** Mitochondrial DNA and associated proteins represent a potential target for nitration and oxidative modifications. Indeed, we could demonstrate a substantial loss of mtDNA after the onset of protein nitration. Loss of mtDNA refers to the fact that mtDNA is no longer detectable with the slot blot hybridization assay presumably due to structural modifications such as nitration and alkylation or due to fragmentation. Delayed treatment with GSH, which does not affect the mitochondrial oxidant stress (Knight et al., 2002) or protein binding (Salminen et al., 1998; James et al., 2003b), eliminated protein nitration as assessed by immunohistochemistry in tissue sections (Knight et al., 2002) and by ELISA in isolated mitochondria (Fig. 4). However, the mtDNA loss was only prevented by about 50%. These data suggest that the potent oxidant and nitrating agent peroxynitrite was only partially responsible for the mtDNA loss. Other potential mechanisms of mtDNA modifications include oxidation reactions due to reactive oxygen species (Mansouri et al., 1999) or alkylation of DNA by NAPQI (Rogers et al., 1997). A more detailed analysis of the mtDNA is necessary to evaluate the structural changes and to provide conclusive proof that peroxynitrite and/or other agents are directly responsible for the mtDNA loss. The hypothesis that either alkylation or oxidation by reactive oxygen species may be the cause of some of the mtDNA modifications is supported by the fact that improved detoxification of NAPQI and prevention of the mitochondrial oxidant stress and peroxynitrite formation by early injection of GSH (GSH₀) eliminated any mtDNA loss. Interestingly, no nDNA damage was observed with the slot blot hybridization technique. However, the anti-histone ELISA, the TUNEL assay, and the DNA ladder on agarose gel indicated substantial nDNA fragmentation after AAP overdose. Indeed, AAP hepatotoxicity is associated with the generation of high-molecular-weight DNA fragments com-

**TABLE 1**

<table>
<thead>
<tr>
<th>Galactosamine/endotoxin-induced mitochondrial and nuclear DNA damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ALT activities were measured as indicator of liver injury and DNA fragmentation was assessed with an anti-histone ELISA at 6 h after treatment with 20 ml/kg saline (control) or 700 mg/kg galactosamine and 100 μg/kg endotoxin (Gal/ET). In addition, mtDNA and nDNA were evaluated with specific probes using the slot blot hybridization technique. Data are expressed as means ± S.E. of n = 5 animals per group.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Gal/ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/l)</td>
<td>21 ± 7</td>
<td>139 ± 22*</td>
</tr>
<tr>
<td>DNA fragmentation (% control)</td>
<td>100 ± 9</td>
<td>1624 ± 218*</td>
</tr>
<tr>
<td>mtDNA (O.D. units)</td>
<td>1150 ± 105</td>
<td>1304 ± 51</td>
</tr>
<tr>
<td>nDNA (O.D. units)</td>
<td>1801 ± 202</td>
<td>892 ± 62*</td>
</tr>
<tr>
<td>mtDNA-to-nDNA ratio</td>
<td>0.61 ± 0.02</td>
<td>1.47 ± 0.06*</td>
</tr>
</tbody>
</table>

O.D., optical density.  
* P < 0.05 (compared with control).
pared with Fas receptor-mediated apoptosis (Jahr et al., 2001). Nevertheless, the appearance of small DNA fragments in the cytosol and the nucleus (DNA ladder) supports the hypothesis that the nDNA damage was caused by DNases. This conclusion is further supported by the fact that no nitration of nuclear or cytosolic proteins was found. Thus, there is no evidence that peroxynitrite generated inside mitochondria was able to leave this cell organelle to cause DNA damage in the nucleus.

**Mechanisms of nDNA Fragmentation after AAP versus TNF-Mediated Apoptosis.** It is clearly established that nDNA fragmentation during apoptosis induced by TNF receptor family members is caused by the caspase-activated DNase (CAD). This enzyme is activated when the associated inhibitor is proteolytically cleaved by activated caspase-3 and dissociates from CAD, allowing the active enzyme to enter the nucleus (Nagata et al., 2003). The protective effect of a general endonuclease inhibitor against AAP-induced liver cell injury suggests that nDNA damage is an important step in the pathophysiology (Shen et al., 1992). However, the actual enzyme involved has not been identified. The direct comparison of nDNA damage after treatment with AAP or Gal/ET revealed some similarities but also fundamental differences. Both hepatotoxins induce internucleosomal DNA cleavage leading to the characteristic DNA ladder on an agarose gel. However, despite the higher number of injured hepatocytes after AAP treatment (50%) (Knight et al., 2002; Bajt et al., 2003) compared with Gal/ET (20%) (Jaeschke et al., 1998), the anti-histone ELISA indicated that substantially less low-molecular-weight DNA fragments entered the cytosol after AAP treatment. On the other hand, staining of the cytosol with the TUNEL assay would indicate the presence of large DNA fragments, which could be used as primer for the terminal deoxynucleotidyl-transferase in this assay. As discussed above, the slot blot hybridization technique indicated a loss of nDNA only after Gal/ET but not after AAP treatment. Again, this is consistent with the generation of predominantly larger DNA fragments during AAP-induced cell death. Thus, all three assays (anti-histone ELISA, TUNEL assay, and slot blot hybridization) point toward formation of large amounts of low-molecular-weight DNA fragments through CAD activation during TNF-mediated apoptosis. In contrast, AAP-induced oncocytic necrosis is associated with the dominant formation of high-molecular-weight fragments together with the appearance of some smaller fragments. This conclusion is directly supported by a study where molecular weights of DNA fragments in plasma were evaluated after AAP overdose and after treatment with the agonistic anti-Fas antibody Jo-2 (Jahr et al., 2001). Fas receptor-mediated apoptosis was associated with the release of low-molecular-weight fragments, and AAP-induced oncocytic necrosis correlated with the release of large size fragments (Jahr et al., 2001). Since only TNF and Fas receptor-induced apoptosis, but not AAP-mediated oncocytic necrosis, involves massive activation of caspases (Lawson et al., 1999; Gujral et al., 2002), nDNA damage after AAP overdose is unlikely to be mediated by CAD. AAP overdose triggers both lysosomal contents release (Kon et al., 2004a) and the release of proteins from the intermembrane space of mitochondria (Adams et al., 2001; Knight and Jaeschke, 2002; El-Hassan et al., 2003). Thus, other potential candidates could be lysosomal endonucleases and/or mitochondrial endonuclease G. Further studies using specific inhibitors or gene knockout mice are required to address these questions. However, the fact that scavenging mitochondrial peroxynitrite effectively prevented nDNA damage suggests that the activation of this DNase(s) is dependent on the mitochondrial oxidant stress and peroxynitrite formation.

In summary, our data showed nitration of mitochondrial proteins and depletion of mtDNA after AAP overdose in mice. Since scavenging of mitochondrial peroxynitrite only partially prevented the loss of mtDNA, our data suggest that peroxynitrite may be only one of several mediators responsible for mtDNA damage. In contrast, nDNA damage was not directly caused by peroxynitrite. Based on the lack of caspase activation and quantitatively different results in three of four assays evaluating DNA fragmentation, we conclude that nDNA damage after AAP overdose is likely caused by DNase(s) unrelated to the caspase-activated DNase, which is typically responsible for DNA fragmentation during apoptosis. Although the specific DNase(s) remain to be identified, our data suggest that the activation of these DNase(s) is dependent on the mitochondrial oxidant stress and peroxynitrite formation.

**References**


Knight TR, Farhood MW, Farhood A, and Jaeschke H (2003) Role of lipid peroxidation...


**Address correspondence to:** Dr. Hartmut Jaeschke, Liver Research Institute, University of Arizona, 1501 N. Campbell Ave., Room 6309, Tucson, AZ 85724. E-mail: jaeschke@email.arizona.edu