Calpain Inhibition but not Reticulum Endoplasmic Stress Preconditioning Protects Rat Kidneys from p-Aminophenol Toxicity

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p-Aminophenol (pAP, 225 mg/kg) administration to rats induced renal failure and has been associated with markers of endoplasmic reticulum (ER) stress, as well as calpain and caspase-12 activation in kidneys. To determine the importance of ER stress and calpain during pAP-induced nephrotoxicity, rats were pretreated with low, nontoxic, doses of ER stress inducers or with the selective calpain inhibitor PD150606 (3 mg/kg). Prior ER stress induced by tunicamycin and oxidized dithiothreitol did not result in protection against renal failure, but PD150606 administration was protective and decreased significantly the rise in creatinine and blood urea nitrogen observed after 24-h post-pAP administration. pAP-induced XBP1 upregulation was not modified by calpain inhibition, but a trend to lower GRP94 induction was determined, suggesting that pAP-induced ER stress was mostly calpain independent. In contrast, pAP-induced caspase-12 cleavage products were significantly decreased with PD150606 pretreatment, demonstrating that caspase-12 activation was calpain dependent. This study reveals the importance of calpain in pAP-induced renal failure. Further research with other nephrotoxicants needs to be performed to determine if calpain activation is a common feature of drug-induced renal failure.

Key Words: p-a-minophenol; XBP1; calpain; caspase-12; nephrotoxicity; endoplasmic reticulum stress.

p-Aminophenol (pAP) is a nephrotoxic metabolite of acetaminophen (APAP), inducing acute renal failure, elevated blood urea nitrogen (BUN) concentration, and proximal tubular damage within 24 h of administration to rats (Davis et al., 1983; Henry et al., 1990; Newton et al., 1982; Shao and Tarloff, 1996). Glutathione (GSH) depletion, mitochondrial dysfunction, and covalent binding of pAP to renal proteins have reported in the late 1970s following pAP exposure (Crowe et al., 1977, 1979). It was later proposed that pAP was bioactivated by oxidation and GSH conjugation leading to oxidative stress and covalent binding (Harmon et al., 2005; Klos et al., 1992). We recently observed that pAP administration to Sprague-Dawley rats was associated with induction of markers of endoplasmic reticulum (ER) stress, such as increased XBP1 (X-box binding protein 1) and GRP78 expression, and evidence of caspase-12 and calpain activation (Peyrou et al., 2007). The involvement of the ER in pAP toxicity was also supported in vitro by the protective effect of ER stress preconditioning against subsequent exposure of renal cells to pAP. However, this protective effect was observed in LLC-PK1 (porcine) and HEK293 (human) renal cell lines, but not in the rat NRK-52E cell line for which the toxicity was increased (Peyrou and Cribb, 2007). The role of calpain in drug-induced toxicity remains unclear, but it has been shown in vitro that calpain inhibition protected renal cells from the toxicity of several model toxicants and of the ER stress inducer tunicamycin (TUN), suggesting its involvement in drug-induced cell death (Muruganandan and Cribb, 2006; Obatomi et al., 2001; Schnellmann and Williams, 1998). Furthermore, calpain inhibition prevented the induction of GRP78, a marker of ER stress, after exposure to model toxicants, suggesting a link between calpain and ER stress (Muruganandan and Cribb, 2006).

To determine the involvement of the ER and the calpain system in pAP nephrotoxicity, we studied the effect of ER stress preconditioning and calpain inhibition on the nephrotoxicity of pAP in Sprague-Dawley rats. We hypothesized that ER stress preconditioning and calpain inhibition would both protect the rat kidneys from pAP toxicity and that calpain inhibition would decrease pAP-induced ER stress.

MATERIALS AND METHODS

Chemicals and materials. pAP, TUN, and oxidized dithiothreitol (DTTox) were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada). A sterile drug solution of pAP (100 mg/ml) was prepared in phosphate-buffered saline (PBS) with 10% dimethyl sulfoxide (DMSO) and pH was adjusted to 7.4. Calpain inhibitor PD150606 was purchased from VWR CANLAB (Mississauga, ON, Canada) and diluted (1 mg/ml) in PBS with 10% DMSO. TUN and DTTox were prepared in PBS with pH adjusted to 7.4. Sterile materials were purchased from VWR. Routine chemicals were obtained from Sigma or Fischer Scientific (Nepean, ON, Canada).

Animals, treatments and samples collection. The protocol of this study was approved by the Atlantic Veterinary College Animal Care Committee and followed the regulations of the Canadian Council on Animal Care. Male Sprague-Dawley rats of 175–250 g body weight were purchased from Charles
pAP administration with a single dose of 225 mg/kg body weight resulted in increased concentrations of BUN and creatinine in rat blood samples, indicating a drug-induced renal failure (Fig. 1). Renal tubular epithelial damage was confirmed by light microscopic examination: pAP exposure was associated with extensive coagulation necrosis of tubular epithelium, with many dilated tubules filled with proteinaceous content (Fig. 2B).

To determine adequate in vivo doses of TUN and DTTox that increase the expression of ER stress proteins in the kidneys without inducing systemic toxicity, a pilot study was performed (data not shown) and a dose of 25 μg/kg and 200 mg/kg for TUN and DTTox, respectively, was chosen for further assessment. Using these doses, TUN and DTTox did not result in any clinical signs, increased BUN or creatinine, or significant alterations of other biochemistry markers (alanine aminotransferase, γ-glutamyltranspeptidase, sorbitol-dehydrogenase, and alkaline phosphatases) (data not shown). There was no significant change of the hematology parameters (white blood cells and differential counts, red blood cells, hematocrit, platelets count) (data not shown). There was a trend to observe lower counts of granulocytes and dose-dependent increase in ALT activities after administration of higher doses of TUN (data not shown). DTTox proved to be nontoxic with all used doses and a dose of 200 mg/kg was selected for the sake of comparison with a previously published study (Asmellash et al., 2005). ER stress was assessed by the induction of GRP78 and GRP94 in microsomal fractions of kidney, liver, and brain at 24 and 72 h after administration of TUN or DTTox. GRP78 and GRP94 were strongly induced in kidney microsomal fraction (Fig. 3A) with both ER stress inducers, but no significant induction was observed in the liver tissue with the same doses (Fig. 3B). In the brain microsomal fractions, TUN resulted in 2.3- and 2.5-fold induction of GRP78 and GRP94, respectively, but DTTox failed to increase either protein expression (Fig. 3C). Twenty-four hours after TUN or DTTox administration, pAP was injected and kidney function was assessed 24 h later. TUN and DTTox pretreatment did not alter the rise of BUN and creatinine...
concentrations after pAP administration (Fig. 1). Histological damage was not modified by TUN pretreatment (Fig. 2D) or DTTox pretreatment (data not shown).

PD150606 had no significant effect on any of the biochemical and hematology parameters (data not shown). There was no effect of PD150606 pretreatment on BUN and creatinine concentrations 6-h post-pAP administration (Fig. 4A). However, PD150606 administration prevented further rise of BUN and creatinine concentrations at 24 h after pAP administration, so that BUN and creatinine values were significantly lower than after pAP administration without PD150606 pretreatment (Fig. 4B). Light microscopic examination revealed that PD150606 pretreatment before pAP administration was associated with milder damage to the tubular cells, with focal areas of coagulation necrosis and no dilated tubules (Fig. 2C) at 24 h.

To determine the effect of PD150606 administration on the induction of ER stress, we first examined the expression of XBP1, a transcription factor specifically upregulated upon ER stress. XBP1 expression was increased to the same extent after pAP administration with or without pretreatment with calpain inhibitor (Fig. 5A). GRP78 and GRP94 expression was increased after pAP administration alone, but induction was not significantly different from control with PD150606 pretreatment (Fig. 5B). Finally, we determine indirectly the activation of caspase-12 by Western blotting with appearance of cleavage products. There was both a significant decrease in the expression of cleavage products and in the upregulation of procaspase-12 in PD150606 pretreated animals compared to animals without pretreatment prior pAP administration (Fig. 6).

DISCUSSION

pAP is a metabolite of acetaminophen and produced acute tubular necrosis similar to that caused by acetaminophen in rats, suggesting that acetaminophen nephrotoxicity was due to pAP or one of its derivatives (Tarloff et al., 1989). Several authors have proposed that pAP nephrotoxicity is, at least in part, linked with the formation of toxic GSH conjugates leading to oxidative stress and covalent binding (Harmon et al., 2005; Klos et al., 1992). We recently observed that administration of pAP in rats resulted in induction of markers of ER stress, namely increased expression of XBP1 and GRP94 and evidence of caspase-12 activation, as suggested by appearance of caspase-12 cleavage products (Peyrou et al., 2007). Furthermore, we observed a decrease of the 80-kDa m-calpain band that is suggestive of the activation/consumption of m-calpain during pAP toxicity (unpublished observation). Additional evidence of involvement of the ER in pAP toxicity comes from in vitro experiments. Pretreatment of LLC-PK1 and HEK293 cells (porcine and human renal cell line, respectively) with TUN and oxidized DTTox to increase the
expression of GRP78 and GRP94 resulted in decreased toxicity of pAP (Peyrou and Cribb, 2007). However, this cytoprotective effect was not observed with the rat renal cell line NRK-52E (Peyrou and Cribb, 2007). Therefore, it appeared important to complete in vivo experiments in order to study the involvement of the ER and its relationship with calpain activation in the toxicity of pAP.

We chose to determine first if ER stress preconditioning could decrease the toxicity of pAP at a dose of 225 mg/kg ip, with a similar experimental design to that used in previous in vitro studies (Peyrou and Cribb, 2007). At this dose, pAP causes kidney damage without evidence of liver damage (Tarloff et al., 1989). ER stress preconditioning was achieved by pretreatment with an ER stress inducer, TUN, or DTTox, 24 h prior to pAP injection, at doses that increased expression of GRP78 and GRP94 in the kidney tissues without evidence of systemic toxicity (Fig. 3). Despite a significant induction of GRP94 and GR78 in rat kidney microsomal fractions, TUN and DTTox pretreatments did not protect against pAP-induced renal failure, as demonstrated by a similar increase in creatinine and BUN, as well as no histological changes with or without ER stress preconditioning (Figs. 1 and 2). These data demonstrated that increased GRP78 and GRP94 expression, with our rat experimental model, was not sufficient to afford protection to renal cells against pAP toxicity, in accordance with in vitro results obtained in NRK-52E cells. Interestingly, pretreatment of rats with DTTox at the same dose resulted in decreased toxicity of the nephrotoxicant S-(1,1,2,2-tetrafluoroethyl)-L-cysteine (Asmellash et al., 2005). This previous study suggests that the dose of DTTox employed is sufficient to induce a protective response under some circumstances. These differences are not dissimilar to what is observed in vitro: pretreatment of NRK cells resulted in protection against some toxicants and not others (Peyrou and Cribb, 2007). The failure

![Graph bars of density analysis data from Western blots of kidney (A) and liver (B) microsomal fractions of rats exposed to TUN (25 μg/kg) and oxidized DTTox (200 mg/kg), 24- and 72-h postadministration. Brain samples (C) have been collected 24-h post-TUN and DTTox administration. * **: p < 0.05 and p < 0.01, respectively.](image-url)
FIG. 4. BUN and creatinine (CREAT) concentrations in blood samples of rats injected with control vehicle (C), or pAP (225 mg/kg) with or without 30-min pretreatment by PD150606 (PD, 1 mg/kg) at 6 (panel A) and 24 h (panel B) post-pAP administration. *, **, ***: $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

FIG. 5. Markers of ER stress and ER-mediated cell death 24 h after pAP administration, with or without 30-min pretreatment by PD150606. XBP1 expression was induced similarly with or without pretreatment (A). Without pretreatment, pAP administration resulted in a significant increased expression of GRP94 (B). Prevention of GRP94 by PD150606 pretreatment failed to achieve statistical significance (B). *$p < 0.05$. 

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to observe protection of rat tubular cells in vitro and in vivo might be interpreted to suggest that damage to the ER is not critical in acute PAP nephrotoxicity in the rat. However, the effects of prior ER stress on ER function and the effects of damage to the ER are complex. It is not possible to rule out involvement of the ER based on a lack of protection by prior induction of ER stress. Moreover, the results obtained in the rat would not rule out the involvement of the ER or damage to the ER in other species and/or at later time points.

Previous experiments demonstrated evidence of calpain and caspase-12 activation after PAP administration (Peyrou et al., 2007) and calpain is known to cleave caspase-12 and promote caspase-12 activation during ER stress-induced apoptosis (Nakagawa and Yuan, 2000; Tan et al., 2006) and is reported to be involved in renal cell death (Muruganandan and Cribb, 2006; Tan et al., 2006). In LLC-PK1 renal cells, we have observed a clear link between calpain activation and cell death following exposure to cytotoxins targeting the ER (Muruganandan and Cribb, 2006; Obatomi et al., 2001; Schnellmann and Williams, 1998). There is evidence that calpain activation may also lead to increased ER stress (Muruganandan and Cribb, 2006). We therefore hypothesized that calpain inhibition would decrease PAP nephrotoxicity and would interfere with PAP-induced ER stress.

Calpain inhibition was achieved by ip administration of the selective nonpeptide cell permeable calpain inhibitor PD150606 (Wang et al., 1996). The dose and time of pretreatment was selected from the literature: when injected at 3 mg/kg 30 min prior ischemia-reperfusion, PD150606 could prevent calpain-mediated ischemia-reperfusion renal injury (Chatterjee et al., 2005). Kidney failure was not significantly different with or without PD150606 pretreatment at 6-h post-PAP administration (Fig. 4A), but was significantly improved at 24 h (Fig. 4B). Therefore, with our paradigm, calpain inhibition did not prevent the acute toxic effect of PAP, but appeared to significantly reduce medium term renal damage mechanisms. This observation is in favor of a role for calpain in injury progression, as previously observed in S-(1,2-dichlorovinyl)-L-cysteine renal toxicity or carbon tetrachloride-induced liver damage. Calpain leakage from necrotic cells was demonstrated and it was suggested that calpain may damage neighboring cells (Limaye et al., 2003; Reddy et al., 2006). The role of calpain in necrotic cell death has been reviewed elsewhere (Liu et al., 2004).

In order to assess the relationship of calpain activation with ER stress, we determine the effect of PD150606 pretreatment on various markers of ER stress and ER-mediated cell death. During ER stress, XBP1 unspliced mRNA is spliced and

**FIG. 6.** Typical blot (A) and densitometric analysis (B) of caspase-12 expression in kidney S9 fraction 24 h after PAP administration, with or without 30-min pretreatment by PD150606. PD150606 prevented procaspase-12 increased expression and appearance of cleavage products associated with PAP administration. *p < 0.05.
translated to a potent transcription factor which upregulates ER stress genes, including GRP78 and GRP94. We observed that XBP1 expression was not altered by administration of PD150606 (Fig. 5A). The increases in GRP94 and GRP78 protein expression following pAP treatment were not significant compared to controls when pAP exposed rats had been pretreated with PD150606 (Fig. 5B). However, there was not a significant difference between GRP94 and GRP78 expression in pAP alone and the pAP-PD150606 groups (significant difference between GRP94 and GRP78 expression in pretreated with PD150606 (Fig. 5B). However, there was not a significant compared to controls when pAP exposed rats had been administered by pAP or its immediate covalent binding/oxidative stress effects. While it was not directly assessed here, the knowledge that ER stress can lead to calpain activation and subsequent caspase-12 activation suggests that calpain activation is a downstream signaling event, possibly triggered by calcium release from the ER (Goll et al., 2003).

Evidence is accumulating that the ER is an important player during drug-induced renal failure (reviewed by Cribb et al., 2005). Cisplatin, gentamicin, and pAP are three clinically relevant nephrotoxic drugs that have been associated with markers of ER stress in vitro and/or in vivo (Mandic et al., 2003 and Peyrou et al., 2007), however, the in vivo significance of suggested ER stress-mediated proapoptotic mechanisms (caspase 12 and JNK activation, GADD153 upregulation) in cell death remains unclear (Szegedi et al., 2006). In this study, we have shown that calpain activity was important in medium-term renal damage following pAP exposure and was required for caspase-12 cleavage. These data also highlight a putative relationship between calpain activation and ER stress. It seems now important to study the role of calpain with other nephrotoxins, such as cisplatin or gentamicin, to determine if calpain activation is a common step of drug-induced renal failure.

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**REFERENCES**


