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Echinomycin Decreases Induction of Vascular Endothelial Growth Factor and Hepatocyte Regeneration in Acetaminophen Toxicity in Mice

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Abstract

Up-regulation of vascular endothelial growth factor (VEGF) is important to hepatocyte regeneration in the late stages of acetaminophen (APAP) toxicity in the mouse. The following study was conducted to examine the relationship of hypoxia inducible factor 1 α (HIF-1 α) to VEGF and hepatocyte regeneration in APAP toxicity by using an inhibitor of HIF-1 α DNA-binding activity, echinomycin (EC). B6C3F1 male mice were treated with APAP (200 mg/kg IP), followed by EC (0.15 mg IP) and killed at 4 hr. Serum ALT, necrosis, hepatic glutathione (GSH) and APAP protein adducts were comparable in the APAP/EC and the APAP/veh mice at 4 hr. Additional studies showed that high dose EC (0.3 mg) reduced hepatic VEGF but also lowered hepatic GSH. Subsequent studies were performed using the 0.15 mg dose of EC. Although EC 0.15 mg had no effect on hepatic VEGF levels at 8 hr, by 24 hr VEGF levels were decreased by 40%. Toxicity (ALT and histopathology) were comparable in the APAP and APAP/EC groups at 24 and 48 hr. Proliferating cell nuclear antigen expression was reduced by both western blot analysis and immunohistochemical staining in the APAP/EC mice at 48 hr. The data support the hypothesis that induction of HIF-1 α , its binding to DNA and subsequent expression of vascular endothelial growth factor are important factors in hepatocyte regeneration in APAP toxicity in the mouse.

Acetaminophen (paracetamol, N-acetyl-p-aminophenol, APAP) overdose is one of the most common causes of drug poisoning in the United States. APAP is also the leading drug-related cause of acute hepatic failure in adults (1). Recent reports indicate that APAP is responsible for approximately 56,000 emergency room visits, 26,000 hospitalizations and 500 deaths per year (2). In large doses, APAP produces a centrilobular hepatic necrosis in experimental animals. The mechanisms involved in the acute toxic response to APAP have been previously examined in the mouse, including the role of metabolism, glutathione depletion and covalent modification of proteins (3), inflammatory cytokines (4,5), reactive nitrogen species (6), mitochondrial dysfunction and permeability transition (7,8) and

oxidative stress (3,7). However, mechanisms that control the recovery of the liver and the initiation of hepatocyte regeneration in APAP toxicity are poorly understood.

Hypoxia-inducible factor (HIF) is a transcription factor important in activating over 100 genes involved in angiogenesis, glucose metabolism, inflammation, cell survival, and proliferation (9,10). The subunit HIF-1 α is constitutively expressed, but during normoxic conditions is hydroxylated and degraded via the proteasome. During hypoxia, these mechanisms are inhibited and the HIF-1 α subunit accumulates in the cell and binds to HIF-1 β (another constitutively expressed subunit which is also known as ARNT) (9,10). The two subunits dimerize, translocate to the nucleus and activate genes that are needed to maintain cellular homeostasis in the hypoxic microenvironment. The induction of HIF-1 α in APAP toxicity in mice has been reported to occur as early as 1 hr after APAP (11). While hypoxia is a well characterized mechanism for the induction of HIF-1 α , the normoxic induction of HIF-1 α has also been described (12). In addition to thrombin, inflammatory cytokines and lipopolysaccharide, oxidative stress has been implicated as a mechanism for HIF-1 α induction (13,14). Reactive oxygen species have been shown to inhibit HIF-1 α degradation, resulting in up-regulation of HIF-1 α even in normoxic conditions (15). We previously reported the induction of HIF-1 α in freshly isolated hepatocytes exposed to a toxic dose of APAP under an oxygen atmosphere (7,11).

Vascular endothelial growth factor (VEGF) is a well-known gene target of HIF induction (16). This endothelial cell-specific mitogen is up-regulated in various models of liver injury, such as ischaemia-reperfusion and partial hepatectomy (17–19). In a mouse model of APAP toxicity, hepatic levels of VEGF were significantly elevated in the late stages of toxicity (20). In addition, treatment of mice with a VEGF receptor inhibitor resulted in decreased expression of proliferating cell nuclear antigen (PCNA), a marker of hepatocyte regeneration (20). In subsequent studies, exogenous treatment with human recombinant VEGF enhanced PCNA expression and hepatocyte regeneration, resulting in reduced toxicity in mice treated with APAP (21). Manipulation of the VEGF axis may represent a future therapeutic target in the treatment of liver toxicity (22) and thus examining the regulation of VEGF in APAP toxicity in the mouse model has implications for the possible future development of new strategies for the treatment of liver injury.

To further dissect mechanisms important in the repair response in APAP toxicity, we hypothesized that pharmacological inhibition of HIF-1 α binding activity to DNA would reduce VEGF expression and subsequent hepatocyte regeneration in APAP toxicity in the mouse. To test the above hypothesis, we investigated the effect of echinomycin (EC), a small molecule inhibitor of HIF DNA binding activity (23), on the acute and later stages of APAP toxicity. EC has distinct inhibitory actions on HIF-1 α in other experimental models. For example, it has been used in cancer models to alter HIF-1 α -induced cellular migration (24,25). The studies described herein demonstrate that EC (0.15 mg) reduced VEGF expression and subsequent hepatocyte regeneration in APAP toxicity in the mouse without altering the metabolism of APAP.

Materials and Methods

Drugs and reagents

APAP was obtained from Sigma Chemical Co. (St. Louis, MO, USA). EC was obtained from Alexis Biochemicals (San Diego, CA, USA). Because of its insolubility in water, EC was initially dissolved in dimethyl sulfoxide (DMSO; Pharmco Products Inc., Brookfield, CT, USA) at a concentration of 50 mg/ml. This was subsequently diluted with a saline solution containing 10 mg/ml bovine serum albumin to a concentration of 0.01875 mg/ml; 0.2 ml was administered to a 25 gm mouse (0.15 mg/kg). Coomassie Plus Protein Assay

Reagents were purchased from Pierce Chemical Co. (Rockford, IL, USA). DTT (dithiothreitol; Cleland's reagent) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Gills Hematoxylin II and PermMount were acquired from Fisher Scientific, Inc. (Pittsburgh, PA, USA). Anti-HIF-1 α monoclonal antibody was purchased from Novus Biologicals (Littleton, CO, USA) and diluted 1:1000 immediately before use. Anti-proliferating cell nuclear antibody (PCNA) monoclonal antibody was obtained from DakoCytomation (Carpinteria, CA, USA) and diluted 1:500.

Experimental animals

Six-week-old male B6C3F1 mice (mean weight, 25.1 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). All animal experimentation was in accordance with the criteria of the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences. Protocols for animal experimentation were approved by University of Arkansas for Medical Sciences Animal Care and Use Committee. Mice were acclimatized one week prior to the planned experiments and fed *ad libitum*. Animals were housed 3 per cage and maintained on a 12-hr light : dark cycle. On the day prior to experiments, mice were fasted overnight and dosing studies began at 0800 the following morning. Food was given to the mice 4 hr after APAP. Mice were administered APAP (200 mg/kg IP), immediately followed by echinomycin (EC; 0.15 mg IP) and killed at 4 hr (n=5/group).

To examine the effect of the EC dose on the VEGF response, a dose response study was conducted in which mice were treated with APAP (200 mg/kg IP) followed by EC 0.08, 0.15 or 0.3 mg IP and killed at 8 hr (n=5/group). In the final experiment, mice were administered APAP (200 mg/kg IP), followed by EC (0.15 mg IP) and killed at 24 and 48 hr (n=5–6/group). A second dose of EC (0.15 mg IP) was administered at 24 hr to the mice that were killed at 48 hr. The treatment control group received APAP followed by a DMSO/BSA solution in saline (herein referred to as "vehicle" [veh]), and another control group received BSA in saline only. A preliminary experiment showed no difference in toxicity (ALT) between the saline and DMSO/BSA-treated mice. Animals were anaesthetized with CO₂ for blood sampling. Blood was removed from the retro-orbital plexus, allowed to coagulate at room temperature, centrifuged, and the serum was used for measurement of alanine aminotransferase (ALT). Mice were then euthanized under a CO₂ atmosphere followed by cervical dislocation and removal of the livers. The livers were weighed and a portion was preserved in formalin for histological sections. The remaining livers were snap frozen in liquid nitrogen and stored at –80°C for additional analyses.

Liver histology

Haematoxylin and eosin staining was performed for histological examination of the liver samples. To standardize the assessment of necrosis, the interactive spline measuring tool in the AxioVision 4.6.3 program (Carl Zeiss Inc., Germany) was used. Three photographic images of centrilobular areas were obtained of each liver section at 10X magnification. The spline measuring tool was used to outline the necrotic regions around the central veins of each field to obtain the total area of necrosis for that field. The central veins were measured in the same manner for control mice. The mean central vein area of the control mice was subtracted from the necrotic regions for each mouse. The necrotic areas for each section were expressed relative to the total area of a 10X field as a percent.

Metabolism and toxicity assays

Serum ALT levels were measured using a Cobas Mira (Roche Diagnostics, Indianapolis, IN, USA). APAP covalently bound to protein in the liver was measured by initial protease treatment of liver homogenates followed by high performance liquid chromatography-electrochemical analysis for APAP-cysteine as previously described (26).

Growth factor assays

Snap-frozen liver samples were thawed, weighed and homogenized in solutions containing 1 ml of protease inhibitor cocktail (Complete; Boehringer Mannheim, Indianapolis, IN, USA). The resulting supernatants were analysed in duplicate and standardized to the weight of the homogenized liver sample. Levels of murine VEGF were measured in the supernatants of liver homogenates using a highly specific ELISA kit, as per the manufacturer's instructions (R & D, Minneapolis, MN, USA).

HIF-1 α nuclear extraction and PCNA expression

HIF-1 α and PCNA expression were measured by western blot as previously described (11,20). Liver homogenates (30 μ g) were separated by SDS-PAGE and then transferred to nitrocellulose membranes by electro-blotting. Membranes were blocked overnight at 4°C in blocking buffer (5% nonfat dry milk) and then incubated for 2 hr at room temperature with a monoclonal anti-PCNA antibody (1:500). Next, a peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used at a 1:2000 dilution for 1 hr at room temperature. Band detection was performed using ECL Plus detection (Amersham, Piscataway, NJ, USA). Liver tissue for HIF-1 α was prepared using Active Motif's (Carlsbad, CA, USA) Nuclear Extract kit. 30 μ g of protein was separated by SDS-PAGE and blocked for 1 hr at room temperature and then incubated in the primary antibody (1:1000) overnight at 4°C.

Morphometric analysis

The number and intensity of PCNA-stained hepatocyte nuclei were analysed employing a semi-automated method that used Scion Image (a PC port on NIH image software), as previously described (20). Mean nuclear intensities and the percent of nuclei above a set threshold (165) were calculated using a minimum of 3 sections per animal at 20X magnification.

Statistical analysis

Results are expressed as means $\pm$ SE. A p value of 0.05 was considered significant for all analyses. Comparisons between multiple groups were performed by one-way analysis of variance followed by the Tukey HSD post-hoc test. For the morphometric analysis, the Holm-Sidak method was applied for pairwise multiple comparison procedures, with values of $p < 0.05$ considered significant. SPSS Version 10.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Effect of echinomycin on the early stages of acetaminophen toxicity

In initial experiments, mice were treated with APAP (200 mg/kg IP) followed by EC (0.15 mg IP) or APAP followed by vehicle and killed at 4 hr, a time after maximal covalent binding of APAP (27). Serum ALT levels were significantly increased in both APAP groups and no differences were detected between the APAP groups (fig. 1A). In addition, the extent of necrosis was comparable between the APAP/EC and APAP/veh mice (figs. 1B–1E). To examine the possible effects of EC on the metabolism of APAP, the supernatants of liver homogenates were analysed for APAP protein adducts (fig. 1F). APAP-protein adducts were increased in both APAP groups and no differences were detected between the APAP groups. Fig. 1G shows that HIF-1 α was significantly induced at 4 hr and EC did not alter the relative amount of induction. Thus, EC did not alter toxicity, metabolism or HIF-1 α induction in the early stages of APAP toxicity.

Effect of echinomycin dose on VEGF expression

In previous studies, we found that VEGF levels were initially increased at 8 hr at APAP toxicity in the mouse (20). Therefore, a dose response study of EC (0.08, 0.15 and 0.3 mg IP) was performed to test the effect of EC dose on hepatic VEGF expression. As demonstrated in fig. 2A, no differences in toxicity or APAP protein adducts were detected at 8 hr among the APAP/EC and APAP/veh mice (fig. 2C). However, hepatic GSH was lower in the APAP/EC 0.3 mg mice than the other APAP mice, implying that this dose of EC had some effect on the metabolism of APAP. Thus, in subsequent studies, the 0.15 mg dose of EC was used. In addition, hepatic VEGF levels were lower in the APAP/EC 0.3 mg IP mice compared to the other APAP groups ($p < 0.05$) (fig. 2B, 2D).

Effect of echinomycin on the late stages of acetaminophen toxicity

In the final study, the effect of EC 0.15 mg IP on VEGF expression and hepatocyte regeneration at 24 and 48 hr was examined. ALT levels were significantly increased ($p < 0.05$) in both APAP groups at 24 and 48 hr and no differences in ALT were detected between the APAP groups (fig. 3A). Review of the histology from the mice was consistent with the ALT data (fig. 3B-D). Hepatic VEGF levels were significantly increased at 24 and 48 hr in the APAP/EC and the APAP/veh mice (fig. 4), but VEGF expression was reduced by 40% in the APAP/EC mice compared to the APAP/veh mice at 24 hr ($p < 0.05$). Since EC alters DNA binding of HIF-1 α to the VEGF promoter, these data are consistent with a postulated role for HIF-1 α in the regulation of VEGF expression in APAP toxicity.

To examine the downstream effects of VEGF modulation by EC, PCNA expression was analysed by immunoblot (fig. 5) and through quantitative image analysis of immunohistochemistry of PCNA in liver sections. PCNA is an auxiliary protein of DNA polymerase delta and is widely used as a marker of cell cycle activation (20,21,28,29). Hepatic PCNA expression peaks at 48 hr in APAP toxicity in the mouse (unpublished data). PCNA induction was apparent at 24 and 48 hr in the APAP/veh and APAP/EC mice (fig. 5). While the expression was comparable in the two groups of APAP mice at 24 hr, by 48 hr, PCNA expression was substantially reduced in the APAP/EC mice compared to the APAP/veh mice. These data were confirmed by immunohistochemical analysis of PCNA in liver sections in which hepatocytes were selectively examined for PCNA expression using quantitative image analysis (fig. 6A-C). Increased expression of PCNA in hepatocytes was apparent in the APAP/vehicle-treated mice at 24 and 48 hr (fig. 6D). In comparison to the APAP/veh groups, PCNA expression was reduced in hepatocytes in the 48-hr APAP/EC group but not the 24-hr APAP/EC group (fig. 6D).

Discussion

In the present study, we examined the effect of echinomycin, a small molecule HIF-1 α inhibitor on APAP toxicity. We found that EC treatment of mice with APAP toxicity resulted in the reduced expression of hepatic levels of VEGF at 24 hr in APAP-treated mice. In addition, EC lowered PCNA expression, a marker of hepatocyte regeneration. Importantly, EC at a dose of 0.15 mg had no effect on the metabolism of APAP.

HIF is the primary mediator of the cellular response to hypoxia. This transcription factor activates the expression of over 100 genes involved in the cell's response to hypoxia, including pro-angiogenic genes such as VEGF (30). The induction of HIF results in binding of the active complex to the hypoxia-responsive element of the VEGF promoter (30,31). VEGF is best known for its trophic effects on endothelial cells, an effect that has been shown to be primarily mediated by VEGF receptor 2 (22,32). In addition, VEGF has been shown to have proliferative effects on other cell types. Activation of VEGF receptor 1

induces the expression of mitogens for hepatocytes (eg., interleukin 6 and hepatocyte growth factor) by endothelial cells, which in turn elicit proliferative effects on hepatocytes (22). These data demonstrate that VEGF, via activation of VEGF receptor 1, has cytoprotective effects beyond those initially described for endothelial cells and suggest that disruption of this growth factor may be adversely affects the recovery of the toxin-injured liver.

Manipulation of the HIF-1 α – VEGF axis has been examined in a number of studies employing animal models of disease including cancer, cardiac ischaemia, atherosclerosis and corneal disease (33–37). However, few studies have examined this relationship in animal models of liver injury and disease. Consistent with our previous data reporting temporal associations between HIF-1 α induction and VEGF expression in the mouse model of APAP toxicity (11,20), HIF-1 α expression was also found to precede VEGF expression in a rat model of partial hepatectomy (38). To further test these temporal associations, we hypothesized that modulation of HIF-1 α would alter VEGF expression and hepatocyte regeneration in APAP toxicity in the mouse. For this purpose, the effect of EC on the early and late stages of APAP toxicity in the mouse was examined. EC has been demonstrated to inhibit HIF-1 binding activity to the hypoxia – responsive element of the VEGF promoter (23). Initial reports from *in vitro* studies showed that EC induced a sequence-specific effect on HIF-1; EC did not affect the expression of other proteins, but specifically inhibited the induction of VEGF mRNA expression. In studies using cell lines, EC blocked morphological changes caused by the induction of HIF-1 in stem cells and decreased HIF-1 expression and the activation of HIF-1 response genes (e.g., EPO, *p21* and VEGF) (39). More recently, EC was used to test the effect of HIF on oxygen consumption within tumours in mouse models of cancer (40).

The metabolism of APAP is a critical component in the development of toxicity (41). Therefore, it was important to assess the effects of HIF-1 α inhibition on the early stages of APAP toxicity (fig. 1). HIF induction occurs early in APAP toxicity (1 hr) (11), before biochemical evidence of toxicity (e.g., increased ALT levels) is evident. In the current study, EC (0.15 mg IP) did not alter the toxicity or metabolism of APAP or the induction of HIF-1 α . Thus, ALT levels, liver histology, levels of APAP protein adducts in the liver and levels of HIF-1 α in hepatic nuclei were all comparable in the APAP/vehicle mice and APAP/EC mice at 4 hr (fig. 1). Mice treated with APAP/EC 0.3 mg had reduced levels of hepatic GSH compared to the APAP, the APAP/EC 0.15 mg and the APAP/EC 0.08 mg mice (fig. 2). Thus, due to the concern that higher doses of EC would alter the metabolism of APAP, the 0.15 mg dose of EC was used in subsequent studies.

Treatment of mice with EC reduced VEGF expression at 24 hr (fig. 4). We previously found that VEGF protein expression was initially apparent at 8 hr and maximally induced at 24 hr (20). A similar temporal profile for VEGF expression was reported in APAP toxicity in the rat (42). In addition to lowering VEGF protein expression at 24 hr, EC also caused reduced expression of PCNA at 48 hr as determined by immunoblots of whole liver homogenates and immunohistochemical assays for PCNA in which the hepatocytes were analysed for staining intensity (figs. 5–6). In unpublished data, we have found that PCNA expression, as determined by immunoblots, peaks at 48 hr in APAP toxicity.

In previous work, we postulated that oxidative stress was the mechanism for the induction of HIF-1 α in APAP toxicity (7,11). Pre-treatment of mice with N-acetylcysteine (NAC) lowered HIF-1 α expression in APAP toxicity in the mouse at 12 hr (11). NAC is well known for its anti-oxidant properties. Also, HIF-1 α induction was observed in freshly isolated hepatocytes exposed to APAP under an oxygen atmosphere and induction could be blocked by the addition of inhibitors of oxidative stress (11), supporting the postulation that HIF-1 α induction in APAP toxicity is secondary to oxidative stress. Peroxynitrite formation

in endothelial cells at early time points in APAP toxicity suggests that oxidative stress is occurring in vascular cells (43). Alternatively, hypoxia or other mechanisms such as inflammatory cytokines (13,14) may contribute to HIF-1 α induction in APAP toxicity *in vivo*. Alterations of sinusoidal blood flow have been reported to occur in APAP toxicity and may result in hypoxia (44,45). In addition, the increased expression of inflammatory cytokines has been demonstrated in APAP toxicity (46,47) and inflammatory cytokines may regulate HIF expression. Time course studies with the hypoxia marker pimonidazole showed that staining was present in the later time points of toxicity but not at 1- and 2-hr time points, suggesting a non-hypoxic mechanism for the induction of HIF-1 α in APAP toxicity in the early stages of toxicity (48). In addition, we have shown that HIF-1 α induction occurs as early as 1 hr in APAP toxicity (48).

In summary, the data from this study demonstrate that EC, a HIF inhibitor, had no effect on mechanisms important in the early stages of toxicity but reduced VEGF expression and subsequent PCNA expression in the later stages of APAP toxicity in the mouse. The data support the hypothesis that HIF-1 α induction and the subsequent expression of VEGF are important to the recovery of the liver in APAP toxicity (11,20,21,42). Understanding mechanisms that trigger the induction of HIF-1 α in APAP toxicity and modulate the expression of its downstream targets has application for the future development of new therapeutic strategies in the treatment of APAP toxicity in the clinical setting.

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Figure 1. Effect of echinomycin (EC) on the early stages of acetaminophen toxicity

Mice were treated with APAP (200 mg/kg IP) plus vehicle, APAP plus EC (0.15 mg IP) or saline and killed at 4 hr. **A.** Serum ALT Levels. ALT values were determined in as an indicator of hepatotoxicity (hepatocyte lysis). *Significantly different from saline mice (* $p < 0.05$). **B-D.** Representative liver sections stained with haematoxylin and eosin. **B.** Saline-treated mouse. **C.** APAP/EC mouse. **D.** APAP/vehicle mouse. **E.** Assessment of liver necrosis by quantitative image analysis. Necrotic areas are shown as a percentage of the entire histological field. *Significantly different from saline mice (* $p < 0.05$). **F.** APAP protein adducts obtained by HPLC-EC analysis of proteolytically digested liver homogenates were increased in the APAP and APAP/EC mice (* $p < 0.05$). **G.** HIF-1 α Induction. HIF-1 α levels were determined by western blot analysis and the density of the proteins determined. The bar graph represents the mean density of each group. *Significantly different from saline mice (* $p < 0.05$) for all comparisons.

Figure 2. Dose response of echinomycin (EC) on VEGF expression in APAP toxicity

Mice were treated with APAP (200 mg/kg IP) plus vehicle, or APAP plus EC (0.08, 0.15 or 0.3 mg IP), or saline and killed at 8 hr. **A.** Serum ALT levels, indicating no difference between the APAP groups compared to saline mice (* $p < 0.05$). **B.** Hepatic glutathione (GSH) levels were reduced in the APAP/EC 0.3 mg mice, compared to the other APAP and APAP/EC groups (* $p < 0.05$). **C.** APAP proteins adduct levels were comparable in all APAP and APAP/EC groups. **D.** Hepatic VEGF expression was increased in the APAP, APAP/EC 0.08 and APAP/EC 0.15 groups compared to the saline group (* $p < 0.05$). Hepatic VEGF expression in the APAP/EC 0.3 mg mice was comparable to that of saline mice.

Figure 3. Effect of echinomycin (EC) on the late stages of acetaminophen toxicity

Mice were treated with APAP (200 mg/kg IP) plus vehicle, APAP plus EC (0.15 mg IP) or saline and killed at 24 hr. Another group of mice received a 2nd dose of EC at 24 hr and was killed at 48 hr. **A.** Serum ALT values. Serum was analysed for ALT at 24 and 48 hr.

*Significantly different from saline mice. **B-D.** Representative H & E stained liver sections of mice killed at 24 hr. **B.** Saline-treated mouse. **C.** APAP/EC-treated mouse. **D.** APAP/vehicle-treated mouse. **E.** Assessment of liver necrosis by quantitative image analysis. Necrotic areas are shown as percentage of the entire histological field. *Significant difference from saline mice ($p < 0.05$).

Figure 4. Effect of echinomycin (EC) on hepatic VEGF expression in APAP-treated mice
Mice were treated with APAP (200 mg/kg IP), immediately followed by EC (0.15 mg IP) or saline and then killed at 24 hr. Another group of mice received a 2nd dose of EC at 24 hr and was killed at 48 hr. *Significantly increased (* $p < 0.05$) compared to the saline group. #Significantly reduced (# $p < 0.05$) compared to the APAP/vehicle group at 24 hr.

Figure 5. Effect of Echinomycin (EC) on PCNA expression at 24 and 48 hr

Immunoblot analysis for PCNA was performed on liver homogenates of the treated mice described in fig. 3. Each group consisted of 6 mice. Each lane represents the pooled liver homogenates from three mice.

Figure 6. Immunohistochemical Analysis of PCNA expression

Mice were treated with APAP (200 mg/kg IP), immediately followed by EC (0.15 mg IP) or saline and killed at 24 hr. Another group of mice received a 2nd dose of EC at 24 hr and was killed at 48 hr. **A-C.** Immunohistochemical staining for PCNA showing representative images at 48 hr. The nucleoli of regenerating hepatocytes are shown by white arrows. **A.** Saline-treated mouse. **B.** APAP/vehicle-treated mouse. **C.** APAP/EC mouse. **D.** Quantitative image analysis of PCNA immunostained slides showing colour intensity of hepatocyte nuclei. *Significantly different from saline-treated mice at 48 hr ($p < 0.05$); #significantly different from APAP/vehicle group at 48 hr.