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Yours sincerely,



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**Title: The role of prostaglandin E<sub>2</sub> in acute acetaminophen hepatotoxicity in mice**

**Original scientific paper**

**Running title: PGE<sub>2</sub> in Acute APAP Hepatotoxicity**

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## Summary

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which is synthesized by many cell types, has a cytoprotective effect in <sup>THE</sup> gastrointestinal tract and in several other tissues and cells. On the other hand, overdose or chronic use of a high dose of acetaminophen (Paracetamol, APAP) is a major cause of acute liver failure in the western world. These observations prompted us to investigate whether PGE<sub>2</sub> <sup>PLAYS</sup> ~~is playing~~ a role in host defence to toxic effect of APAP. (CBAT6T6xC57Bl/6)F<sub>1</sub> hybrid mice of both sexes were intoxicated with <sup>A</sup> single lethal or high sublethal dose of APAP, which was administered to animals by oral gavage. Stable analogue of PGE<sub>2</sub>, 16,16-dimethyl PGE<sub>2</sub> (dmPGE<sub>2</sub>), or inhibitor of its production, CAY10526, were given intraperitoneally (i.p.) 30 minutes before or 2 hours after APAP administration. The toxicity of APAP was determined by observing <sup>THE</sup> survival of mice during 48 hours, by measuring concentration of alanine-aminotransferase (ALT) in plasma 20-22 hours after APAP administration and by liver histology. The results have shown that PGE<sub>2</sub> exhibits a strong hepatoprotective effect when it <sup>IS</sup> ~~was~~ given to mice either before or after APAP, while CAY10526 demonstrated mainly <sup>THE</sup> opposite effect. Immunohistochemical or immunofluorescent examinations in the liver tissue generally support these findings, <sup>A</sup> suggesting that PGE<sub>2</sub> inhibited APAP-induced activation of nuclear factor kappa B (NF-κB). Similarly, PGE<sub>2</sub> down regulated the activity of inducible nitric oxide synthase (iNOS), which was up regulated by APAP. Thus, by these and perhaps by other mechanisms, PGE<sub>2</sub> contributes to <sup>THE</sup> defence of <sup>THE</sup> organism to noxious effects of xenobiotics on the liver.

**Key words:** prostaglandin E<sub>2</sub>, acetaminophen, liver injury, NF-κB, immunohistochemistry

## Introduction

Acetaminophen (Paracetamol, N-acetyl-p-aminophenol, APAP), the most ~~common~~<sup>COMMONLY</sup> used analgesic and antipyretic drug, is very safe at therapeutic doses. However, overdose or chronic use of a high dose of APAP has been shown as a major cause of acute liver failure in the western world (reviewed in Lee, 2003; Bernal 2003). After an overdose of APAP, the reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), is generated by hepatic cytochromes P450 (CYPs). Although, the precise biochemical mechanism of cell death is not fully understood it is generally recognized that NAPQI triggers hepatic toxicity via mitochondria injury, apoptosis and cell necrosis. These effects of NAPQI are mediated through its covalently binding with nucleophilic macromolecules such as DNA or proteins, glutathione (GSH) depletion, lipid peroxidation and generation of oxidative stress molecules (Jaeschke, 2000; James et al., 2003; Mazer and Perrone, 2008). It is believed that inflammatory mechanisms play a role in the later phases of liver APAP toxicity (O'Connor and Bennet, 2005). Thus, it was shown that nuclear factor kappa B (NF- $\kappa$ B) is also activated during APAP-induced hepatotoxicity (Bauer et al., 2000; Dambach et al., 2006). Oxidative stress has been shown to activate signal transduction pathways involving transcription factors such as NF- $\kappa$ B and activator protein-1 (AP-1) (Jaeschke, 2000; Janssen-Heininger et al., 2000). NF- $\kappa$ B is known to regulate the expression of genes controlling inflammatory mediators, including inducible nitric oxide synthase (iNOS) and consequent production of nitric oxide (NO), which is a highly reactive oxidant produced in the liver in response to different inflammatory stimuli and has been implicated in hepatotoxicity (Gardner et al., 2002; James et al., 2003; Kamanaka et al., 2003). Prostaglandins (PGs) are lipid-derived autacoids generated by sequential metabolism of arachidonic acid by the cyclooxygenase (COX) and prostaglandin synthase enzymes. PGs are ubiquitously produced and have been implicated in a broad array of diseases, including cancer, inflammation, cardiovascular disease and hypertension (reviewed in Hata and Breyer, 2004; reviewed in Matsuoka and Narumiya, 2007).

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is produced by many cells of the body and exerts its actions by binding to one (or a combination) of its four subtypes of receptor, EP1, EP2, EP3 and EP4 (Harris et al., 2002). PGE<sub>2</sub> is considered to be ~~as~~ very important for normal physiological functions, especially for the function of gastrointestinal tract (Dey et al., 2006) and kidney (Hao and Breyer, 2008). Thus, in the stomach of rat PGE<sub>2</sub> was essential for conferring protection against indomethacin or ethanol-induced injury (Araki et al., 2000; Suzuki et al., 2001). Protective effects of exogenous PGE<sub>2</sub> and prostacyclin (PGI<sub>2</sub>) has been demonstrated in various models of liver injury induced with lipopolysaccharide (LPS), D-galactosamine (DGalN), concanavalin A (Con A), carbon tetrachloride (CCl<sub>4</sub>), virus and ischemia-reperfusion (reviewed in Quiroga and Prieto, 1993; Yin et al., 2007). The protective effect of PGE<sub>2</sub> was also observed, but not directly proven, in APAP-induced liver injury in mice (Renić et al., 1995; Reilly et al., 2001). Various mechanisms for cytoprotective effects of PGE<sub>2</sub> are proposed (reviewed in Quiroga and Prieto, 1993). Data *in vitro* have revealed ~~X~~ plenty of cell signaling pathways by which this protection could be mediated. Most often investigated is the effect of PGE<sub>2</sub> on activation of NF-κB, by which it <sup>HAVE AN</sup> may influence on the synthesis of inflammatory hepatotoxic cytokines and generation of oxidative radicals (Tran-Thi et al., 1995; Laskin et al., 2001; Dambach et al., 2006; Ogawa et al., 2009), activation of iNOS (Laskin et al., 2001; James et al., 2003), stimulation of synthesis of inhibitory (suppressive) cytokine – IL-10 (Cheon et al., 2006), endogenous antioxidants (Enomoto et al., 2001) and cyclic adenosine monophosphate (cAMP), which has various immunosuppressive and anti-inflammatory effects (Bourne et al., 1974; Nakano et al., 1994). However, some of these mechanisms are far from being definitively proven, since their role in toxicity of APAP is not firmly established and <sup>THE</sup> obtained effects of PGE<sub>2</sub> were sometimes contradictory. This particularly relates to effects on activation of NF-κB and iNOS.

Based on these data, the present studies ~~were~~ aimed to investigate the role of exogenously applied PGE<sub>2</sub> and its derivatives on APAP-induced hepatotoxicity *in vivo*, as well as to examine their effects on expression of NF-κB and iNOS in liver tissue.

## Materials and Methods

### Animal model

Male or female (CBAT6T6xC57Bl/6)F<sub>1</sub> hybrid mice aged 12-16 weeks and weighing 20-30 g were used in all experiments. They were raised in an animal facility unit at the Department of Physiology, School of Medicine, University of Zagreb. <sup>THE</sup> Animal colony unit had regulated 12 h light/dark cycle and the temperature and relative humidity in the animal room were 22 ± 2 °C and 50 ± 5%, respectively. The cages were sanitized twice weekly and mice were allowed free access to tap water and standard mouse chow diet (No. 4RF21, Diet Standard, Milano, Italy). All animal protocols were approved by the Ethics Committee of the University of Zagreb, School of Medicine (Zagreb, Croatia).

### Chemicals and treatments of animals

Pure APAP substance was a kind gift from Belupo (Koprivnica, Croatia). Phenobarbitone-sodium was obtained from Kemika (Zagreb, Croatia). Since the PGE<sub>2</sub> is rapidly converted to an inactive metabolite, 13,14-dihydro-15-keto PGE<sub>2</sub>, its stable structural analog, 16,16-dimethyl PGE<sub>2</sub> (dmPGE<sub>2</sub>), was used for *in vivo* experiments. Stock solution of dmPGE<sub>2</sub> in methyl acetate (No. 14750, Cayman Chemical, Ann Arbor, MI, USA) was first evaporated under <sup>A</sup> gentle stream of nitrogen and <sup>THE</sup> remaining substance was dissolved (1.0 mg/mL) in phosphate buffered saline (PBS, pH=7.2). DmPGE<sub>2</sub> was administered to animals (0.2 mg/kg, i.p.) 30 min before or 2 h after APAP. CAY10526 (an inhibitor of PGE<sub>2</sub> production through the selective modulation of microsomal PGE synthase-1 expression, mPGES-1) was supplied as a crystalline solid (No. 10010088, Cayman Chemical). Since the CAY10526 is sparingly soluble in aqueous buffers, it was first dissolved in an organic solvent, dimethyl formamide (DMF, 25 mg/mL), diluted in PBS (pH=7.2) and finally injected <sup>INTO</sup> (2.0 mg/kg, i.p.) ~~to~~ animals 30 min before or 2 h after APAP. The doses of the drugs for application *in vivo* were chosen from scarce data in the literature or



according to the toxicity data in our preliminary experiments, in which the effects of the drugs on survival of mice and gross macroscopic changes of liver and other visceral organs were observed.

Animals in control groups received appropriate vehicle. Survival of mice was followed for 48 h after APAP administration, since almost all mice either died within this period or fully recovered thereafter. For immunohistochemical or immunofluorescent examinations, PGE<sub>2</sub> or vehicle was given to mice 30 min before, and CAY10526 was given 2 h after APAP administration.

### **Assessment and measurement of hepatotoxicity induced with APAP**

In order to induce hepatic cytochromes P450 (CYPs), mice were given phenobarbitone-sodium in drinking water for 7 days (0.3 g/L). Thereafter, mice were fasted overnight and APAP was given by oral gavage in a volume of 0.4 to 0.6 mL. APAP was dissolved under mild magnetic stirring in warm PBS, to which 1-2 drops of Tween 20 were added. Animals were allowed free access to food 4 h later (Guarner et al., 1988; Renić et al., 1995). <sup>TO OBSERVE THE</sup> ~~For~~ <sup>THE</sup> ~~observing~~ survival of mice, APAP was administered in a dose of 250-300 mg/kg, which in our previous experiments induced 43 to 72% mortality of untreated animals. <sup>TO DETERMINE</sup> ~~For determination~~ of plasma alanine aminotransferase (ALT) and NO concentration in plasma, as well as for histopathological, immunohistochemical or immunofluorescent evaluation of liver slices and measurement of PGE<sub>2</sub> production by liver fragments, mice were treated with high sublethal dose of APAP (150 mg/kg). Experimental and control groups of mice contained 12-13 animals (for observation of survival) or 6-10 animals (for all other measurements).

*Plasma ALT activity.* ALT levels were determined 20-22 h after APAP administration. Mice were given 250 U heparin i.p. 15 min before bleeding and blood was collected by puncture of the medial eye angle with heparinized glass capillary tubes. After centrifugation, separated plasma was stored at -80 °C for 24 h before ALT determination. ALT concentrations were measured by standard laboratory techniques (Renić et al., 1995).

*Liver histology.* Mice were sacrificed under light ether anesthesia by cervical dislocation 20-22 h after APAP administration. Liver lobes of each animal (9-10 animals per group) were fixed in 4% buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Thereafter, sections of tissue were cut at 5  $\mu$ m on a rotary microtome, mounted on clean glass slides and dried overnight at 37 °C. The sections were cleared, hydrated and stained with hematoxyllin and eosin. Microscopically, the liver damage was classified using arbitrary scale from 0 to 5 as follows: degree 0—there was no damage; degree 1—minimal lesions involving single to few necrotic cells; degree 2—mild lesions, 10-25% necrotic cells or mild diffuse degenerative changes; degree 3—moderate lesions, 25-40% necrotic or degenerative cells; degree 4—marked lesions, 40-50% necrotic or degenerative cells; degree 5—severe lesions, more than 50% necrotic or degenerative cells. Sections, with scores higher than 2, were considered to exhibit significant liver injury (Silva et al., 2001).

#### **Influence of CAY10526 on *ex vivo* production of PGE<sub>2</sub>**

Mice were sacrificed 6 h after APAP administration and samples of liver tissue, kept on ice, were minced in small fragments (1-2 mm<sup>3</sup>) in PBS. After sedimentation at unit gravity, they were washed 2 times more in fresh PBS, transferred into preweighed tubes and centrifuged at 500 g at +4 °C for 3 min. The sediment was quickly weighed, resuspended in Minimal Essential Medium (MEM, 5  $\mu$ l MEM/mg tissue) and incubated in a water bath at 37 °C for 1 h. The samples were then centrifuged as above and supernatants stored at -80 °C until analysis. Concentration of PGE<sub>2</sub> was determined using appropriate PGE<sub>2</sub> EIA Kit according to the manufacturer's instructions (No. 514010, Cayman Chemical) (Čulo et al., 1995).

#### **Immunohistochemistry**

Mice were sacrificed under light ether anesthesia by cervical dislocation 6 h after APAP administration. Immediately, liver lobes were taken from each animal (6 mice per group) and tissue samples were fixed

in 4% paraformaldehyde in phosphate buffer and dehydrated in 100% ethanol. They were embedded in paraffin wax, serially sectioned as 7  $\mu\text{m}$  thick sections and mounted on glass slides. After removing paraffin with xylene, the sections were rehydrated in ethanol and water. In order to quench endogenous peroxidase activity, sections were incubated for 10 min in 0.3%  $\text{H}_2\text{O}_2$ , washed in PBS and then cooked in sodium citrate buffer for 17 min at 95  $^\circ\text{C}$ . After being cooled to room temperature, sections were incubated with diluted (1:100) polyclonal rabbit anti-NF- $\kappa\text{B}$  (p65 subunit) or anti-iNOS antibodies (No. sc-109 and sc-651, respectively, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at room temperature in a humidified chamber. After being washed with PBS, sections were incubated with a biotinylated secondary antibody (rabbit UniTect ABC Kit, Oncogene, Boston, Mass., USA) for 30 min at room temperature. Afterwards, sections were washed in PBS and incubated with avidin biotinylated horseradish peroxidase complex (ABC) for 30 min, washed again with PBS and stained with diaminobenzidine (DAB). Finally, sections were rinsed in distilled water, counterstained with hematoxyllin and dehydrated in ethanol and xylol. Cells positive to NF- $\kappa\text{B}$  had brown-stained cytoplasm or nuclei (depending of NF- $\kappa\text{B}$  activation) and cells positive to iNOS had brown-stained cytoplasm. Positive internal controls for NF- $\kappa\text{B}$  and iNOS staining were smooth muscle cells in vascular walls. Negative controls were prepared by substituting Dako ChemMate antibody diluent for secondary antibody. Images were captured with digital camera (SPOT Insight, Diagnostic Instruments, USA) mounted on an Olympus BX51 microscope using the SPOT software.

### **Immunohistochemical quantification**

Liver tissue specimens of four experimental groups of mice were analyzed. The number of cells was evaluated quantitatively by two independent investigators and classified as negative (not stained) cells, nuclear positive for NF- $\kappa\text{B}$  counting and cytoplasm positive for iNOS counting. Counts were made along the length of the liver only for DAB staining sections. DP-SOFT version 3.1 software was used to divide each liver section <sup>INTO</sup> ~~in~~ squares of  $100\mu\text{m}\times 100\mu\text{m}$  at  $40\times$  magnifications. The cells <sup>BELOW</sup> ~~below~~ the left

and upper border of squares were not taken into account, but only those at the right and lower border. To avoid counting ~~of~~ the same cell twice, we used every other consecutive section. The examination was performed on an Olympus BX51 microscope equipped with a DP11 digital camera. Images were analyzed using DP-SOFT version 3.1 software.

### **Immunofluorescence**

After deparaffinization and rehydration, the sections were treated in a microwave oven at 95 °C for 17 minutes in sodium citrate buffer (pH 6.0) for antigen retrieval. After being cooled to room temperature, sections were washed in PBS. Sections were incubated with goat serum (Normal Goat Serum, X0907 DAKO, Glostrup, Denmark) for 1 h to block non-specific antibody binding. Sections were then incubated with primary antibodies for 1 h at room temperature. Polyclonal rabbit anti-NF- $\kappa$ B p65 antibody from Santa Cruz Biotechnology (No. sc-109), diluted 1:100, was used. After multiple washes in PBS, sections were incubated for 1 h with diluted (1:200) secondary antibody (Texas Red, No. sc-2780, Santa Cruz Biotechnology). Following secondary antibody incubation, the sections were washed in PBS and counterstained with diamidino-2-phenylindole (DAPI) to stain nuclei. After final rinsing in PBS, sections were mounted, air-dried and coverslipped (Immuno-Mount, Shandon, Pittsburgh, PA, USA). Control of specificity included omitting primary antibody from the staining procedure. Sections were examined by using a fluorescence microscope (Olympus BX61, Tokyo, Japan). Immunofluorescent images were obtained with <sup>A</sup>digital camera (DP71) mounted on an Olympus BX61 microscope using the <sub>^</sub>Olympus Cell software and Adobe Photoshop.

### **Measurement of NO in plasma**

Mice were sacrificed under light ether anesthesia and plasma samples were taken 6 h after treatment of mice with APAP. Thereafter, samples were stored at -80 °C until determination of NO synthesis. For quantification of total NO production, nitrate ( $\text{NO}_3^-$ ) was first reduced into nitrite ( $\text{NO}_2^-$ ) using copper-coated cadmium granules (NITRALYZER-II, Nitrate to Nitrite reduction kit, World Precision Instruments,

Sarasota, FL, USA). Concentration of nitrite was measured by Griess reaction according to the manufacturer's instructions. Finally, absorbance readings were performed with the use of an automated microplate reader (Dynatech MR 5000, Dynatech Technology, Inc., Horsham, PA, USA) at 540 nm.

## Statistical Analysis

Results are expressed as mean  $\pm$  SEM. Differences in survival between groups of mice were compared by chi-square test using Yate's correction when indicated. Statistical comparisons between two groups were made using a Student's t-test. Comparisons between multiple groups were carried out using one-way analysis of variance (ANOVA) with a *post hoc* test of significance between individual groups. Significance was accepted at  $p < 0.05$ .

## Results

### Effects of dmPGE<sub>2</sub> and CAY10526 on APAP-induced mortality and plasma ALT concentration in mice

~~For determining~~ <sup>TO DETERMINE</sup> the survival of animals, mice were treated with 300 mg/kg of APAP. DmPGE<sub>2</sub> (0.2 mg/kg, i.p.) and CAY10526 (2.0 mg/kg, i.p.) were given either 30 min before or 2 h after APAP administration. Administration of dmPGE<sub>2</sub> 30 min before APAP significantly improved the survival of animals (Fig. 1A,  $p < 0.05$ ). When given 2 h after APAP, dmPGE<sub>2</sub> increased the survival of mice, but without statistical significance (Fig. 1A,  $p > 0.05$ ). CAY10526 decreased the survival of animals when given either 30 min before or 2 h after APAP, ~~however~~ <sup>ALTHOUGH</sup> the differences did not reach statistical significance (Fig. 1B,  $p > 0.05$  for both comparisons). ~~For determination~~ <sup>TO DETERMINE</sup> the plasma ALT concentration, mice ~~have been~~ <sup>WERE</sup> treated as <sup>THE</sup> in previous experiment, except that mice received <sup>A</sup> lower dose of APAP (150 mg/kg). Fig. 2A and B show mean ALT levels ( $\pm$ SEM) obtained in 8-10 mice per group 20-22 h after APAP administration. Pretreatment or post treatment of mice with dmPGE<sub>2</sub> significantly reduced ALT

level (Fig. 2A,  $p < 0.05$  for both comparisons). Fig. 2B shows that CAY10526, if given 2 h after APAP, increased ALT concentration, but, due to high intra group variability, the difference was not significant ( $p > 0.05$ ).

### **Effect of CAY10526 on *ex vivo* production of PGE<sub>2</sub>**

PGE<sub>2</sub> production was determined in supernatants of incubated liver fragments taken from normal mice and mice treated with CAY10526 (2 mg/kg, i.p.) or vehicle 2 h after APAP administration. In comparison to normal (non-treated) mice, treatment with APAP alone (vehicle group) significantly increased production of PGE<sub>2</sub>, while treatment with CAY10526 reduced that increase in PGE<sub>2</sub> production (Fig. 3,  $p < 0.05$  for both comparisons).

### **Liver histology**

Macroscopically, the whole liver surface of some APAP treated animals had a mottled appearance; dark red hemorrhagic-necrotic spots were regularly scattered on the yellowish background. Microscopically, the liver damage was graduated using arbitrary scale from 0 to 5 as described in Materials and Methods (Fig. 4). The severity of necrosis was quite variable both between animals and also within different parts of the same liver. However, dmPGE<sub>2</sub> significantly decreased the number and size of necrotic foci in the liver, which could be easily seen by macroscopic observation and on histological analysis. Macroscopic and microscopic damages of the liver parenchyma appeared more pronounced in mice injected with CAY10526 (Table 1).

### **Immunohistochemistry to NF- $\kappa$ B p65**

In *normal (non-treated) mice*, NF- $\kappa$ B was expressed in liver cells around blood vessels in Kiernan spaces (afferent arterioles), where numerous small granules were diffusely scattered in the cytoplasm. NF- $\kappa$ B immunostaining was also expressed in individual cells in the lobules and around the central vein

(efferent arterioles) (Fig. 5A). Almost all NF- $\kappa$ B immunoreactivity was seen in cytoplasm and there were only 3.7% positive cells with nuclear positivity to NF- $\kappa$ B (Table 2). In <sup>WHICH</sup> mice received APAP alone (vehicle group), the whole liver tissue was infiltrated with reactive cells. Normal cells were rarely observed. Around blood vessels, there were layers of cells with numerous vacuoles in cytoplasm and nuclei (Fig. 5B). In the central part of lobules, perimembranous staining pattern and dark colored vacuoles in nuclei were observed. Smaller cells could represent Kupffer cells. Besides cytoplasmic positivity to NF- $\kappa$ B, immunoreactivity was also seen in nuclei (21.8% positive cells) of hepatocytes, indicating significantly higher activity of NF- $\kappa$ B in comparison to normal mice (Table 2,  $p < 0.001$ ). In mice treated with PGE<sub>2</sub>, the whole tissue was also infiltrated with reactive cells. However, a lot of cells were in the different mitotic periods, <sup>IMPLIED</sup> which ~~implying~~ liver regeneration. Around the blood vessels, there were reactive cells with small <sup>THE</sup> diffusely scattered granules giving to individual cells a strong positivity in cytoplasm in comparison to nuclei (Fig. 5C). Immunohistochemical localization of NF- $\kappa$ B, especially around the blood vessels, was mostly seen in <sup>THE</sup> cytoplasm, while its nuclear expression (8.0% cells with NF- $\kappa$ B positive nuclei) was significantly reduced in comparison to mice <sup>WHICH</sup> received APAP alone (Table 2,  $p < 0.001$ ). In mice treated with CAY10526, immunohistochemical expression of NF- $\kappa$ B in liver cells showed mostly perimembranous staining pattern. In the central part of lobules, NF- $\kappa$ B positivity was observed in <sup>THE</sup> cytoplasm and in nuclei with vacuoles in both cell compartments (Fig. 5D). <sup>COMPARED</sup> Comparing to <sup>WHICH</sup> mice received APAP alone, nuclear positivity to NF- $\kappa$ B in liver cells was higher (23.0% positive cells), but did not reach statistical significance (Table 2,  $p > 0.05$ ).

### Immunofluorescence to NF- $\kappa$ B p65

Immunofluorescent analysis of NF- $\kappa$ B expression in liver cells generally confirmed previous investigation done by immunohistochemistry. In group of *normal mice*, only a few cells showed nuclear positivity to NF- $\kappa$ B (Fig. 6A). Nuclear expression of NF- $\kappa$ B was significantly higher in <sup>WHICH</sup> mice received APAP alone in comparison to normal mice (Fig. 6B), whereas *treatment with PGE<sub>2</sub>* reduced that

expression (Fig. 6C). In mice treated with CAY10526, nuclear positivity to NF-κB revealed similar pattern as in <sup>THE</sup> <sup>WHICH</sup> group received APAP alone (Fig. 6D).

### Immunohistochemistry to iNOS

*Normal mice.* Granular accumulations of iNOS were seen in the cytoplasm of hepatocytes organized in small groups, while other hepatocytes were devoid of iNOS reactivity (Fig. 7A). Cells on the inner surface of sinusoid capillaries (endothelial or Kupffer cells) also displayed reactivity to iNOS in their cytoplasm. There were 3.9% positive cells with cytoplasmic positivity to iNOS (Table 2). *Mice received APAP alone.* iNOS was expressed in the cytoplasm of hepatocytes in the form of dense small granules or large single vacuoles. Some endothelial (or Kupffer) cells expressed iNOS in their cytoplasm as well (Fig. 7B). The number of hepatocytes with cytoplasmic immunoreactivity to iNOS (41.6% positive cells) was significantly higher in comparison to normal mice (Table 2,  $p < 0.001$ ). *PGE<sub>2</sub> treated mice.* Diffuse expression of iNOS was found in the cytoplasm of hepatocytes, particularly of those situated around the lumen of blood vessels such as central vein (Fig. 7C). The number of liver cells positive to iNOS (20.5% positive cells) was significantly lower in comparison with <sup>THE</sup> <sup>WHICH</sup> group received APAP alone (Table 2,  $p < 0.001$ ). *CAY10526 treated mice.* Fine granular accumulations of iNOS were seen in the cytoplasm of some hepatocytes. In other hepatocytes, iNOS was expressed in the form of large vacuoles in the cytoplasm (Fig. 7D). The number of hepatocytes, which showed positive immunoreactivity to iNOS (42.5% positive cells), was similar as in <sup>THE</sup> <sup>WHICH</sup> group received APAP alone (Table 2,  $p > 0.05$ ).

### Effects of PGE<sub>2</sub> and CAY10526 on the plasma concentration of nitrite/nitrate (Fig. 8)

NO synthesis by hepatocytes, measured as a concentration of nitrite/nitrate in mice plasma, was almost undetectable in normal animals. Treatment with APAP significantly elevated nitrite concentration, whereas pretreatment with PGE<sub>2</sub> reduced that level, but not significantly. In CAY10526 treated animals,



nitrite level was elevated in comparison to <sup>THE</sup> group <sup>WHICH</sup> received APAP alone, but ~~also~~ <sup>AGAIN</sup> the difference was not significant.

## Discussion

Drug toxicity is a severe complication to drug therapy and new drug development. Because ~~the~~ APAP is a major cause of acute liver failure in the western world (reviewed in Lee, 2003; Bernal, 2003), we used it as a model of drug-induced liver injury for examination the influence of PGE<sub>2</sub> and its derivatives on drug toxicity mechanisms.

Although ~~the~~ protective role of PGE<sub>2</sub> and its analogues is well proven in other models of liver injury, especially in those considered as immune system-mediated (reviewed in Quiroga and Prieto, 1993; Muntané et al., 2000; Yin et al., 2007), the protective effect of PGE<sub>2</sub> in a model of APAP-induced toxicity was shown only indirectly. It was reported that <sup>A</sup> lack of PGE<sub>2</sub> in COX-2 deficient mice (COX-2<sup>-/-</sup> and <sup>-/-</sup> mice) and inhibition of COX-2 by selective COX-2 inhibitory drug, celecoxib, increased <sup>THE</sup> hepatotoxic effect of APAP (Reilly et al., 2001). In our previous experiments, we ~~have~~ demonstrated <sup>THE</sup> that protective effect of interleukin 1 $\alpha$  (IL-1 $\alpha$ ) on APAP-induced toxicity in mice was abolished by administration of specific anti-PGE<sub>2</sub> antibodies (Renić et al., 1995). The presented results clearly demonstrate that dmPGE<sub>2</sub> improves the survival of mice, especially <sup>INTO</sup> when it was injected ~~to~~ mice before an APAP overdose. Furthermore, hepatic damage, as assessed by plasma ALT concentration and liver histology, was alleviated when animals received dmPGE<sub>2</sub> either before or after an APAP administration.

Our findings are in accordance with previous studies describing beneficial protective effects of PGE<sub>2</sub> against a variety of hepatotoxic agents other than APAP (ethanol, CCl<sub>4</sub>, aflatoxin, Con A, DGalN, LPS alone, or combined with DGalN) (reviewed in Quiroga and Prieto, 1993; Yin et al., 2007) or with our recently observed data obtained by protective effect of PGI<sub>2</sub> in the same model of hepatotoxicity (Ćavar

et al., 2009, unpublished data). CAY10526 is known as an inhibitor of PGE<sub>2</sub> production through the selective modulation of mPGES-1 expression. In ~~presented~~<sup>THE PRESENT</sup> experiments, treatment of mice with CAY10526 aggravated the liver damage as shown by ~~increase~~<sup>THE</sup> in mortality of animals, elevation of serum ALT level and histopathological changes in liver morphology. As far as we know, this is the first time that CAY10526 ~~was~~<sup>HAS BEEN</sup> used *in vivo* in a model of experimental liver damage induced by ~~noxious~~<sup>A</sup> agent. The mechanism of its hepatotoxic action is most probably due to the inhibition of PGE<sub>2</sub> synthesis, because it significantly inhibited *ex vivo* production of PGE<sub>2</sub> by liver homogenates and it displayed the strongest effect when given to mice 2 h after APAP. This indirectly points to PGE<sub>2</sub> as an endogenously produced hepatoprotective agent, which is supported by our preliminary observation that APAP alone increases synthesis of PGE<sub>2</sub> and PGI<sub>2</sub> in the liver (data not shown).

Our investigations demonstrated that administration of APAP resulted in a higher expression of NF-κB in liver cells from control animals. This is in agreement with previous studies, in which it was shown that APAP-induced liver damage was influenced by oxidative stress (Jaeschke, 2000; James et al., 2003) and that this is dependent, in part, on NF-κB (Bauer et al., 2000; Dambach et al., 2006).

Immunohistochemistry and immunofluorescence to NF-κB revealed that PGE<sub>2</sub> inhibited to greater part activation of NF-κB and its translocation from cytoplasm to the nucleus of hepatocytes, while CAY10526 had ~~the~~<sup>THE</sup> opposite effect. In ~~one~~<sup>ANOTHER</sup> ~~the~~<sup>THE</sup> animal model, it was shown that selective EP4 agonist (EP4Rag) suppressed production of proinflammatory cytokines, chemokines, adhesion molecules and NF-κB (Ogawa et al., 2009). In LPS-treated cultured rat liver macrophages (Kupffer cells), PGE<sub>2</sub> reduced TNF-α production due to down activation of NF-κB (Tran-Thi et al., 1995). NF-κB is known to up regulate expression of genes controlling inflammatory mediators including iNOS, TNFα, IL-1, IL-10 and COX-2, for each of has been implicated in hepatotoxicity (Dambach et al., 2006). However, it is not always clear whether NF-κB plays a positive or negative role in the pathogenesis of toxicity in our and above described models; our results just indicate that ~~protective~~<sup>A</sup> effect of PGE<sub>2</sub> could be expressed through inhibition of activation of NF-κB.

<sup>THE PRESENT</sup>

<sup>THE PERCENTAGE</sup>

~~Presented~~ results also showed that treatment with APAP high significantly increased ~~percent~~ of iNOS-positive cells and that PGE<sub>2</sub> significantly diminished expression of iNOS in hepatocytes and reduced NO production, which was reflected by lower (albeit statistically not significant) nitrite/nitrate concentration in plasma in comparison to animals receiving APAP alone. CAY10526 showed slightly inverse action on iNOS production. Our results are in agreement with findings provided by others, which reported ~~about~~ <sup>A</sup> significant increase of NO synthesis in APAP toxicity (Hinson et al., 1998; Jaeschke et al., 2003) indicating that increased synthesis of iNOS is a part of toxic mechanism imparted by APAP. However, data in the literature on role of iNOS in liver damage, i.e. whether it is a part of regenerative (protective) or toxic (pathogenic) mechanisms, are highly controversial. This was shown in various models of hepatic damage using different experimental designs, including toxic hepatitis induced by APAP (Bourdi et al., 2002; Gardner et al., 2002; Ito et al., 2003; Fiorucci et al., 2005), CCl<sub>4</sub> (Morio et al., 2001), immune-mediated hepatitis (Willuweit et al., 2001), etc. Explanation for these conflicting results might be in conception that iNOS is pathogenic and constitutive NOS (cNOS) protective (Fiorucci et al., 2005), or by observation that the action of NO and its derivatives depends on precise targets on which it is acting and the level of oxidative tissue injury done by superoxide (Laskin et al., 2001). It was shown in this model that NO scavenges superoxide anion to produce peroxynitrite, which then causes protein nitration and tissue injury (Jaeschke 2000; Jaeschke et al., 2003). Despite its controversial role in hepatotoxicity, ~~the~~ <sup>A</sup> NO is generally considered as proinflammatory mediator and our results, at least in part, support that opinion.

It has been shown that the major producers of endogenous PGs in the liver are Kupffer cells and extra hepatic inflammatory cells recruited to liver by chemoattractants (Decker, 1990). APAP-induced hepatotoxicity was paralleled by significant elevation in synthesis of PGI<sub>2</sub>, PGE<sub>2</sub> and TXA<sub>2</sub> from liver homogenates or fragments of treated animals (Guarner et al., 1988, our unpublished data). Possible cytoprotective mechanisms of PGE<sub>2</sub> could be, besides <sup>THE</sup> above discussed pathways, due to vasodilatation (by which it can reduce or reverse hepatic vascular congestion), increase in intracellular cAMP level and

stimulation of mitogenesis in hepatocytes (reviewed in Quiroga and Prieto, 1993). cAMP suppresses as well as PGE<sub>2</sub> the release of potentially hepatotoxic inflammatory cytokines: TNF- $\alpha$ , IL-1 and IFN- $\gamma$  (Oh-ishi et al., 1996; Schroer et al., 2002). Taken together with our previous investigations, these findings support the view that PGE<sub>2</sub> has a cytoprotective effect and is involved in defence of organism to noxious effects of xenobiotics on liver. According to our results, this protection is mediated, at least partially, through down activation of NF- $\kappa$ B and iNOS. Therefore, much remains to be done ~~for~~ <sup>to</sup> elucidate precise mechanisms underlying cytoprotective effects of PGs in acute liver injury.

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**Figure 1.** Influence of dmPGE<sub>2</sub> and CAY10526 on survival of mice with APAP-induced liver injury. APAP (300 mg/kg) was given by oral gavage and survival was recorded 48 h later. A. dmPGE<sub>2</sub> (0.2 mg/kg, i.p.) and B. CAY10526 (2.0 mg/kg, i.p.) were given either 30 min before or 2 h after APAP administration. Vehicle was given 30 min before APAP administration. Results represent mean ± SEM of 12-13 mice per group. \*p<0.05 in comparison to vehicle group.

**Figure 2.** Influence of dmPGE<sub>2</sub> and CAY10526 on plasma ALT concentrations in mice with APAP-induced liver injury. APAP (150 mg/kg) was given by oral gavage and plasma ALT levels were determined 20-22 h later. ALT concentration in normal mice was 28 ± 4 U/L (data not shown). A. dmPGE<sub>2</sub> (0.2 mg/kg, i.p.) was given either 30 min before or 2 h after, and vehicle 30 min before APAP administration. B. CAY10526 (2.0 mg/kg, i.p.) and vehicle were given 2 h after APAP administration. Results represent mean ± SEM of 8-10 mice per group. \*p<0.05 in comparison to vehicle group.

**Figure 3.** Effect of CAY10526 on PGE<sub>2</sub> production by the liver fragments. CAY10526 (2.0 mg/kg, i.p.) and vehicle were given 2 h after APAP administration (150 mg/kg). Liver samples were taken 6 h after APAP administration. PGE<sub>2</sub> concentration was determined in supernatants obtained after 1 h incubation of liver fragments. Results represent mean ± SEM of 6 mice per group. \*p<0.05 in comparison to normal or vehicle group.

**Figure 4.** Histopathological changes in livers from normal and APAP-intoxicated mice.

Livers were collected 20-22 h after APAP administration (150 mg/kg). Graduation of liver damage was determined according to the arbitrary scale as follows: degree 0 (A), degree 1 (B), degree 2 (C), degree 3 (D), degree 4 (E) and degree 5 (F). Descriptions of each degree are explained in Materials and Methods. Sections were stained with hematoxyllin and eosin (original magnification, 100x).

**Figure 5.** Immunohistochemical expression of NF- $\kappa$ B p65 in mice liver cells. A. *Normal*

*mice.* NF- $\kappa$ B positivity showed mostly diffuse staining pattern within cell cytoplasm (white

arrows), particularly in areas close to the central vein,  $\times 40$ , scale bar 25  $\mu$ m. B. *Mice received*

*APAP alone* (vehicle group). NF- $\kappa$ B staining of liver cells showed perimembranous

accumulations like vacuoles both in nuclei (white arrows) and cytoplasm (black arrows),

$\times 100$ , scale bar 10  $\mu$ m. C. *Mice treated with PGE<sub>2</sub>.* Expression pattern of NF- $\kappa$ B positive liver

cells (white arrows) was similar to that observed as in group of normal mice (see A),  $\times 40$ ,

scale bar 25  $\mu$ m. D. *Mice treated with CAY10526.* NF- $\kappa$ B positivity showed similar pattern of

staining as in <sup>THE WHICH</sup> group received APAP alone (see B). NF- $\kappa$ B immunostaining was observed both

in nuclei (white arrows) and cytoplasm (black arrows),  $\times 40$ , scale bar 25  $\mu$ m. vc- central vein.

Immunohistochemical staining to NF $\kappa$ B. N=6 animals per group.



**Figure 6.** Immunofluorescence to NF- $\kappa$ B p65 in mice liver cells. A. *Normal mice*. B. *Mice*  
<sup>which</sup> received APAP alone. C. *Mice treated with PGE<sub>2</sub>*. D. *Mice treated with CAY10526*. White  
<sup>^</sup> arrows show red colored nuclear or perinuclear positivity to NF- $\kappa$ B. Immunofluorescent  
staining to NF- $\kappa$ B, x100, scale bar 10  $\mu$ m. vc- central vein. N=6 animals per group.

**Figure 7.** Immunohistochemical expression of iNOS in mice liver cells. A. *Normal mice*.  
iNOS positive liver cells showed diffuse granular staining pattern in cytoplasm (black  
arrows). iNOS expression was also seen in the cytoplasm of endothelial cells (white arrows),  
x40. B. *Mice received APAP alone*. iNOS positivity showed diffuse granular (black arrows)  
and vacuolar (white arrows) staining pattern in the cytoplasm of hepatocytes, x40. C. *Mice*  
*treated with PGE<sub>2</sub>*. Diffuse staining pattern of iNOS was seen in the cytoplasm (black arrows)  
of hepatocytes close to the central vein, x40. D. *Mice treated with CAY10526*. iNOS positivity  
showed <sup>A</sup> similar pattern as in <sup>THE</sup> group of mice <sup>WHICH</sup> received APAP alone (see B), with diffuse  
granular (black arrows) and vacuolar (white arrows) staining patterns in the hepatocytes, x40.  
vc-central vein. Immunohistochemical staining to iNOS, scale bar 25  $\mu$ m. N=6 animals per  
group.

**Figure 8.** Effects of PGE<sub>2</sub> and CAY10526 on nitrite/nitrate concentration in mice plasma. APAP (150 mg/kg) was given by oral gavage and plasma nitrite/nitrate levels were determined 6 h later. Nitrite concentration in normal mice was 0.50 ± 0.11 U/L. PGE<sub>2</sub> (0.2 mg/kg, i.p.) and vehicle were given 30 min before, while CAY10526 (2.0 mg/kg, i.p.) was given 2 h after APAP administration. Results represent mean ± SEM of 6 mice per group. Nitrite concentrations in vehicle, PGE<sub>2</sub> and CAY10526 treated groups were significantly higher in comparison to normal mice (p<0.05 or better). There were no significant differences between other groups of mice (p>0.05).

**Table 1.** Effects of PGE<sub>2</sub> and CAY10526 on APAP-induced liver injury

Treatment <sup>a</sup>	Histopathological scores <sup>c</sup>						
	0	1	2	3	4	5	%>2 <sup>d</sup>
Vehicle + APAP	1	2	2	2	2	1	50
PGE <sub>2</sub> + APAP	6	4	0	0	0	0	0*
APAP + PGE <sub>2</sub>	6	3	1	0	0	0	0*

  

Treatment <sup>b</sup>	Histopathological scores <sup>c</sup>						
	0	1	2	3	4	5	%>2 <sup>d</sup>
APAP + vehicle	1	3	2	1	2	0	33
APAP + CAY10526	0	1	1	2	3	2	78

<sup>a</sup>PGE<sub>2</sub> (0.2 mg/kg, i.p.) was given either 30 min before or 2 h after, and vehicle 30 min before APAP administration. <sup>b</sup>CAY10526 (2.0 mg/kg, i.p.) and vehicle were given 2 h after APAP administration. Mice were sacrificed and livers were collected 20-22 h after mice received APAP (150 mg/kg). <sup>c</sup>Histopathological scores were determined and graded by intensity of hepatocellular necrosis from 0 to 5 as described in Materials and Methods. <sup>d</sup>Scores greater than 2 were considered as significant necrosis. N=9-10 mice per group. \*Statistically significant in comparison to vehicle group (p<0.05).

**Table 2.** Quantification of immunohistochemistry to NF- $\kappa$ B and iNOS in mice liver cells

Group	% of liver cells <sup>a</sup> positive to:	
	NF- $\kappa$ B	iNOS
Normal	3.7 $\pm$ 0.7	3.9 $\pm$ 0.5
Vehicle + APAP	21.8 $\pm$ 3.8 <sup>b</sup>	41.6 $\pm$ 4.4 <sup>b</sup>
PGE <sub>2</sub> + APAP	8.0 $\pm$ 1.4 <sup>b,c</sup>	20.5 $\pm$ 4.0 <sup>b,c</sup>
APAP + CAY10526	23.0 $\pm$ 4.0 <sup>b,d</sup>	42.5 $\pm$ 4.9 <sup>b,d</sup>

<sup>a</sup>In each area of the liver (100 $\mu$ m $\times$ 100 $\mu$ m), the percentage of positive cells to NF- $\kappa$ B or iNOS was calculated and expressed as mean  $\pm$  SEM. <sup>b</sup>Significantly different from normal mice.

<sup>c</sup>Significantly different from vehicle group. <sup>d</sup>Significantly different from PGE<sub>2</sub> treated group.

Differences were significant at p<0.001 level.

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