

The Role of Prostacyclin in Modifying Acute Hepatotoxicity of Acetaminophen in Mice

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ABSTRACT

Prostaglandins (PGs) are lipid compounds that mediate the variety of physiological and pathological functions in almost all body tissues and organs. Prostacyclin (prostaglandin I₂, PGI₂), which is synthesized by the vascular endothelium, is a potent vasodilator, inhibits the aggregation of platelets *in vitro* and has cytoprotective effect on gastrointestinal mucosa. The aim of this study was to determine whether PGI₂ is playing a role in host defense to toxic effect of acetaminophen (APAP). This was investigated in C57Black/6 mice which were intoxicated with single lethal or high sublethal dose of APAP. APAP was administered to mice by gastric lavage and PGI₂ agonists or antagonists were given intraperitoneally (*i.p.*) 30 minutes before or 2 hours after administration of APAP. The toxicity of APAP was determined by observing the survival of mice during 48 hours, by measuring the concentration of alanine-aminotransferase (ALT) in plasma 20–24 hours after APAP administration, and by liver histology. Mice were given either pure PGI₂ (PGI₂ sodium salt), its stable agonist (iloprost) or inhibitor of prostacyclin (IP)-receptor (CAY-10441). The results have shown that PGI₂ exhibits a strong hepatoprotective effect when it was given to mice either before or after APAP (both increase of survival of mice and decrease of plasma ALT levels were statistical significant). Iloprost has not shown a similar effect and CAY-10441 increased toxic effect of APAP if given 2 hours after its administration. Histopathological changes in liver generally support these findings. These investigations support the view that PGI₂ is involved in defense of organism to noxious effects of xenobiotics on liver.

Key words: prostacyclin, acetaminophen, liver, toxicity, mice

Introduction

Acetaminophen (Paracetamol, N-acetyl-p-aminophenol, APAP), the widely used antipyretic and analgesic drug, is very safe at therapeutic doses¹. However, overdose or chronic use of a high dose of APAP is major cause of acute liver failure (ALF) in the western world^{2,3}. Therefore, intoxication of laboratory animals with high dose of APAP has become most frequently used experimental model for the study of mechanisms and prevention of acute hepatotoxicity of xenobiotics. Acetaminophen is primarily metabolized in the liver by glucuronidation and sulphation; however, a small proportion undergoes cytochrome P450 (CYP450)-mediated bioactivation to reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which is rapidly quenched by glutathione (GSH)^{4,5}. After an overdose of APAP, elevated levels of the toxic NAPQI metabolite are generated, which can extensively deplete hepatocellular GSH, covalently bind to

cellular macromolecules with sequent modification of its function, and finally cause hepatocyte death. The precise biochemical mechanism of cell necrosis is not fully understood. However, it is generally recognized that there is simultaneous involvement of covalent binding, lipid peroxidation and oxidative stress, each contributing to hepatocellular damage^{6,7}.

Prostaglandins (PGs) are lipid-derived autacoids generated by sequential metabolism of arachidonic acid by the cyclooxygenase (COX) and prostaglandin synthase enzymes, which are responsible for the production of the five principal bioactive prostaglandins generated *in vivo*: PGE₂, PGF_{2α}, PGD₂, PGI₂ (prostacyclin), and TXA₂ (thromboxane)⁸. Prostaglandins are ubiquitously produced and act locally in an autocrine or juxtacrine manner to elicit a diverse set of pharmacological effects modulat-

ing many physiological systems. The disorders in prostaglandin synthesis or production have been implicated in a broad array of diseases including cancer, inflammation, cardiovascular disease, and hypertension⁹.

Prostacyclin (PGI₂) is the primary prostaglandin produced by endothelial cells and plays an important role in vascular homeostasis as a result of its potent vasodilatory and antithrombotic effects¹⁰. Thus, prostacyclin functionally opposes the effects of TXA₂ and has been shown to specifically inhibit platelet activation and TXA₂-induced vascular proliferation following vascular injury¹¹. The vasodilatory actions of prostacyclin have enabled its clinical use for reducing pulmonary vascular resistance in individuals suffering from primary pulmonary hypertension¹². There are also evidences that prostacyclin has beneficial effect on liver injury induced by various toxic agents and conditions, such are hypoxia in perfused liver *ex vivo*¹³ and hepatic injury *in vivo* due to galactosamine¹⁴ or carbon tetrachloride¹⁵. Moreover, studies on isolated rat hepatocytes¹⁶ or human leucocytes¹⁷ demonstrated a protection by prostacyclin against carbon tetrachloride induced necrosis. Prostacyclin, also, shows cytoprotective effect on gastrointestinal mucosa¹⁸. However, the effect of PGI₂ and its analogs or antagonists on APAP-induced hepatotoxicity has not been systematically studied.

These results prompted us to investigate the influence of administration of PGI₂, its stable analogue (iloprost), and inhibitor of IP-receptor (CAY-10441) on APAP-induced liver injury in mice.

Materials and Methods

Animals

C57Black/6 mice were raised in an animal colony unit at the Department of Physiology, School of Medicine, Zagreb. Mice of both sexes aged 12–16 weeks and weighing 20–25 g were used in all experiments. The cages were stored in rooms with a 12 h light period from 6 a.m. to 6 p.m., and the temperature and relative humidity in the animal room were 21±2 °C and 50±5%, respectively. The cages were sanitized twice weekly. All mice were given free access to tap water and standard mouse chow diet (Diete Standard, Milano, Italy).

Chemicals

Pure APAP substance was a kind gift from the Belupo pharmaceutical company (Koprivnica, Croatia). Phenobarbitone-sodium was obtained from Kemika (Zagreb, Croatia). Since the PGI₂ is rapidly bioconverted to PGF_{1α}¹⁹ ($t_{1/2}$ =2–3 min), in certain experiments we used besides the pure PGI₂ (sodium salt) also its stable structural analog-iloprost. PGI₂ sodium salt was dissolved (1 mg/mL) in Tris buffer (1M, pH=8.5) and after appropriate dilution in 5% bicarbonate administered at a dose of 10 µg/kg of b.w. (body weight), i.p., 30 min before or 2 h after APAP. Iloprost, was supplied as a solution in methyl acetate. In order to change the solvent, we evaporated

the methyl acetate under gentle stream of nitrogen and dissolved the remaining substance (1 mg/mL) in phosphate buffered saline (PBS, pH=7.2). Iloprost was administered to animals (0.1 or 0.5 mg/kg of b.w., i.p.) 30 min before APAP. Antagonist of IP-receptor (CAY-10441) was supplied as a crystalline solid. Since CAY-10441 is sparingly soluble in aqueous buffers, it was first dissolved in an organic solvent, dimethyl formamide (DMF; 25 mg/mL), then diluted in aqueous solution (PBS, pH=7.2), and finally injected to animals 30 min before or 2h after APAP (2.0 mg/kg of b.w., i.p.). All these compounds were purchased from Cayman Chemical, USA. The doses of drugs for application *in vivo* were chosen from scarce data in literature or according to 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.

Induction of hepatitis with APAP

The procedure of Guarner et al.²⁰ was followed with slight modifications²¹. To induce hepatic drug-metabolizing enzymes, mice were given phenobarbitone-sodium in drinking water during 7 days (0.3 g/L). Thereafter, mice were fasted overnight and APAP was given by a gastric lavage in a volume of 0.4 to 0.5 mL. Before application, APAP was dissolved in heated PBS to which 1–2 drops of Tween 20 were added. Animals were allowed for food 4 hours later. In all experiments, a dose of 200–300 mg/kg of APAP was administered, which in our previous experiments induced 63–70% mortality in control mice. Experimental and control groups of mice counterbalanced 12 animals (for determining survival of animals) or 6–10 animals (for determining ALT activity and liver histology). Control animals received appropriate vehicle.

Plasma ALT activity

Alanine aminotransferase (ALT) levels were measured 20–24 h after APAP administration. Plasma samples were obtained by a procedure in which haemolysis was undetectable. Mice were given 250 U heparin i.p. 15 min before bleeding. Blood was collected by puncture of the medial eye angle with heparinized glass capillary tubes. Plasma was stored at –70 °C for 24h before ALT determination. ALT concentrations were measured by standard laboratory techniques²¹.

Liver histology

Mice were scarified under light ether anesthesia by cervical dislocation 24 h after APAP administration. Liver lobes of each animal (6 to 8 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 mm on a rotary microtome, mounted on clean glass slides and dried overnight at 37 °C. The sections were cleared, hydrated, and stained with haematoxylin and eosin. Microscopically, the liver damage was classified as follow: degree 0. – there was no damage; degree 1. – occasional vacuolar and fat changes; degree 2. – occasional evidence

of hepatocytic necrosis with minimal inflammatory reaction; degree 3. – spotty necrosis of hepatocytes with inflammatory reaction widely distributed throughout the liver; and degree 4. – severe diffuse hepatocellular necrosis with panlobular acute inflammatory cell infiltration and complete lobular disarray.

Statistical analysis

Results are expressed as $\bar{X} \pm \text{SEM}$. Parametric methods were used, including students t-test. Differences in survival between groups of mice were compared by χ^2 -test, using Yates's correction of the test when indicated.

Results

The effects of PGI₂, CAY-10441 and iloprost on APAP-induced mortality

In three separate experiments, APAP was always given in dose of 300 mg/kg of b.w. PGI₂ (10 $\mu\text{g}/\text{kg}$ of b.w., i.p.) and CAY-10441 (2.0 mg/kg of b.w., i.p.) were given either 30 min before or 2h after APAP administration. Iloprost was given to mice only 30 min before APAP in two different doses: 0.1 mg/kg and 0.5 mg/kg of b.w., i.p. Control mice were given vehicle at the same time points. The survival of mice was followed for 48h, as we and others observed that almost all control mice either die within this period or fully recover thereafter. Administration of either PGI₂ 30 min before or 2h after APAP significantly

improved the survival of animals (5/12 and 6/12 *vs.* controls: 0/12, $p < 0.05$ for both comparisons, Figure 1a). CAY-10441 decreased the survival of animals when given either 30 min before or 2h after APAP (7/12 and 4/12 *vs.* 8/12); however, the differences did not reach statistical significance ($p > 0.05$ for both comparisons, Figure 1b). Iloprost shows slight hepatoprotection against APAP-induced hepatotoxicity when given before APAP. In doses of 0.1 mg/kg and 0.5 mg/kg it increased survival of mice in comparison to control mice (6/12 and 4/12 *vs.* 3/12), but the differences were not statistical significant ($p > 0.05$ for both comparisons, Figure 1c).

Mice had been treated as in previous experiment, except that mice were given lower dose of APAP (200 mg/kg of b.w.). Blood was collected 20–24 h after APAP administration. Figure 2 shows mean ALT levels ($\pm \text{SEM}$) obtained in 7 to 10 mice *per* group. As seen (Figure 2a), pretreatment of mice with PGI₂ significantly reduced ALT level (1624 \pm 528 U/L *vs.* 3791 \pm 825 U/L, $p < 0.05$). If given 2 hours after APAP, PGI₂ only slightly reduced ALT level (3605 \pm 504 U/L *vs.* 3791 \pm 825 U/L in control group, $p > 0.05$). Figure 2b shows that CAY-10441 increased ALT concentration either if given before or after APAP; however, the elevation of ALT level was significant only if it has been given to mice 2h after APAP (2006 \pm 476 U/L *vs.* 436 \pm 124 U/L, $p < 0.05$). PGI₂ stable analog, iloprost, decreased, but not significantly, plasma level of ALT if given in lower dose (0.1 mg/kg) before APAP (Figure 2c, 1494 \pm 477 U/L *vs.* 2447 \pm 1326 U/L, $p > 0.05$). However, when given in

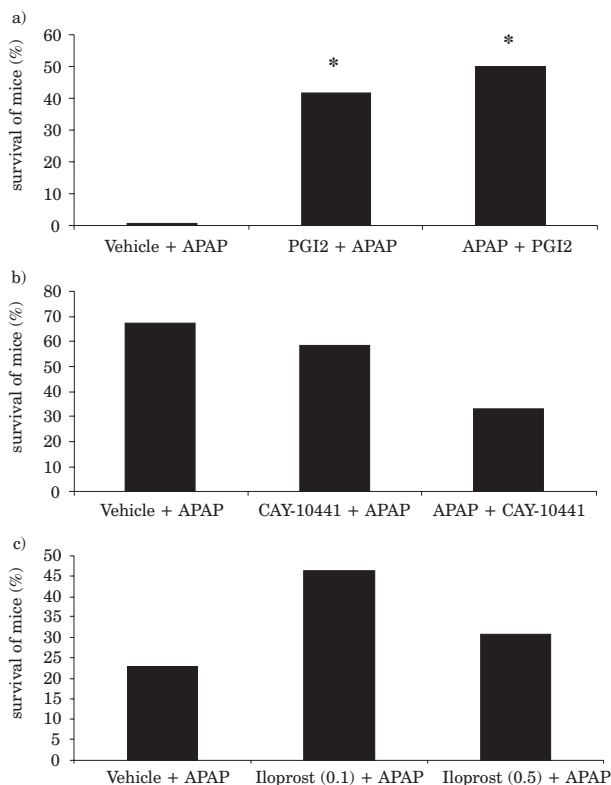


Fig. 1. Influence of PGI₂, CAY-10441 and iloprost on survival of mice with APAP induced hepatitis.

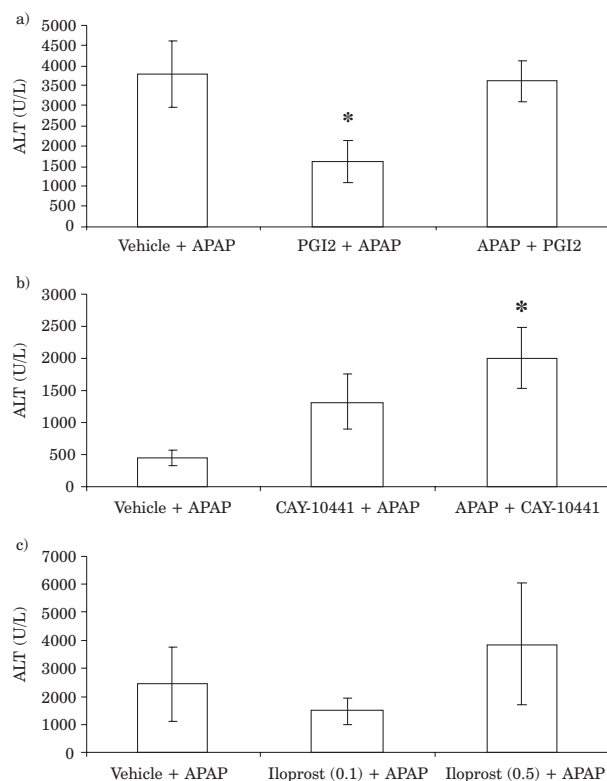


Fig. 2. Influence of PGI₂, CAY-10441 and iloprost on plasma ALT levels in mice with APAP induced hepatitis.

a higher dose (0.5 mg/kg), iloprost supremely raised the level of ALT.

All livers from the APAP-treated mice showed well described centrilobular necrosis, which in some cases was also accompanied by congestion of the sinusoids with blood. Macroscopically, the whole liver surface of APAP treated animals had a mottled appearance – dark red hemorrhagic-necrotic spots were regularly scattered on the yellowish background. Microscopically, severity of the necrosis was considerably variable, both between animals and also within different parts of the same liver. However, the necrosis appeared less marked in those mice which had been treated with PGI₂, and microscopic damages of the liver parenchyma were more pronounced in mice injected by CAY-10441 (Table 1).

TABLE 1
MEAN SCORE OF HISTOPATHOLOGICAL CHANGES IN LIVER

Treatment ^a	$\bar{X} \pm \text{SEM}^c$
Experiment 1 ^b	
Vehicle+APAP	2.87±0.40
PGI ₂ +APAP	2.50±0.23 ^d
Experiment 2 ^b	
Vehicle+APAP	3.25±0.31
APAP+CAY-10441	3.50±0.34 ^d

^a Dose of APAP was 200 mg/kg

^b N=6–8 animals per group, PGI₂ was given 30 min before and CAY-10441 2 h after APAP

^c Determined 20–24 h after APAP administration

^d p>0.05

Discussion

The presented results clearly show that PGI₂ improves the survival of mice before and after an APAP overdose. Furthermore, hepatic damage, as assessed by serum ALT concentration, was alleviated especially when PGI₂ was administered before APAP. Histopathological findings, although not statistically significant, also point at hepatoprotective effect of PGI₂. These data are in agreement with previous studies describing beneficial protective effects of PGI₂ and PGE₂ against a variety of hepatotoxins other than acetaminophen: ethanol, carbon tetrachloride¹⁵, aflatoxin²², concanavalin A²³, D-galactosamine¹⁴, LPS (endotoxin) alone, or associated with D-galactosamine²⁴. In the present study the results differ from data obtained by Guarner and colleagues²⁰, which obtained hepatoprotection only if PGI₂ was given after APAP. We gotened hepatoprotection with PGI₂, both when it was injected to mice before or after APAP. We have not rational explanation for this difference, since the dose of the drug and design of experiment was practically identical (besides mouse strains used, all other experimental conditions were practically identical in two experiments).

There are insufficient data about *in vivo* action of iloprost in a model of APAP-induced mortality in laboratory animals. Most data on protective effect of iloprost on action of chemical toxicants are obtained in a model of hepatocytes grown *in vitro*^{25–27}. According to these findings *in vitro* and data on hepatoprotective effect of PGI₂, we supposed that iloprost, a stabile analog of PGI₂, will show a strong hepatoprotective effect in our experiments. However, neither 0.1 mg/kg nor 0.5 mg/kg iloprost had any significant effect on the decrease of mortality and serum ALT concentrations in mice treated with APAP. The possible reason for that could be that iloprost was injected to mice in a single and, perhaps, too high dose. Bursch et colleagues have shown that iloprost protects cultured rat hepatocytes in very narrow range of doses (10⁻⁹–10⁻¹² M) and in whole animal experiments it was administered by continuous i.v. infusion (0.1 µg/kg/min)^{25,27}.

CAY-10441 is known as one of the most potent high-affinity ligands and functional antagonists for the human IP-receptor. In presented experiments it displayed hepatodamaging action, as shown by significant increase in mortality of animals, elevation in serum ALT level and changes in liver morphology. To our best knowledge, this is first time that CAY-10441 was used *in vivo* in a model of experimental liver damage induced by toxic agent. The mechanism of CAY-10441 hepatotoxic action is probably due to the blockage of IP receptor, because the major of its hepatotoxicity was expressed when it was given to mice 2h after APAP administration. This indirectly supports the role of PGI₂ as an endogenously produced hepatoprotective agent. This is supported by our recent preliminary observation that APAP alone increases synthesis of PGI₂ in the liver (Čavar et al., unpublished observations). Nevertheless, we could not exclude its interaction with hepatic drug metabolism.

The origin of the prostanoids in APAP-induced liver injury is not completely understood. APAP-induced hepatotoxicity was followed by significant elevation in prostanoid biosynthesis (PGI₂, PGE₂ and TXA₂) from liver homogenates or fragments of treated animals^{20,21}. Hepatocytes are PG-metabolizing rather than PG-synthesizing cells that produce low amounts of prostanoids, which probably act as autocrine modulators or participate in cell-to-cell communications between contiguous hepatocytes²⁸. Liver endothelial cells produce PGI₂ as the predominant metabolite, but also minor amounts of PGE₂ and TXA₂²⁹. The major producers of prostanoids are Kupffer cells and extrahepatic inflammatory cells recruited to liver by chemoattractants³⁰. Although the precise mechanisms underlying the cytoprotective effects of PGs in acute liver injury remain to be precisely defined, studies on isolated rat hepatocytes¹⁶ or human leucocytes¹⁷ demonstrated a direct cellular protection by PGI₂ against carbon tetrachloride induced necrosis, possibly by stabilization of membranes or inhibition of lysosomal enzyme release^{13,17,23}. As a result of its potent vasodilatory and antithrombotic effects (by inhibiting platelet aggregation)¹⁴, PGI₂ functionally opposes the ef-

fects of TXA₂ and thus may reduce or reverse the hepatic vascular congestion observed in APAP toxicity. Guarner et colleagues also have reported that thromboxane (TX) blockade by itself does not protect against hepatic necrosis induced by APAP, but the increased PGI₂ production after selective TX synthetase inhibition may play a role in preventing liver damage²⁰. However, our investigations imply that PGI₂ prevents hepatic damage also by other mechanism, since it showed hepatoprotective effect also when applied before APAP administration. We

are presently investigating the possible mechanism of protective effect of PGI₂ and its derivatives on sub-cellular and biochemical level.

Taken together with our previous investigations, these findings support the view that PGI₂ is, similarly to more extensively studied PGE₂, involved in defense of organism to noxious effects of xenobiotics on liver.

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ULOGA PROSTACIKLINA U MEHANIZMIMA AKUTNOG TOKSIČNOG OŠTEĆENJA JETRE ACETAMINOFENOM U MIŠEVA

SAŽETAK

Prostaglandini (PG) su spojevi koji nastaju razgradnjom lipida stanične membrane te posreduju u mnogim fiziološkim i patofiziološkim zbivanjima u gotovo svim organima i tkivima u organizmu. Prostacyclin (prostaglandin I₂, PGI₂), kojeg stvara endotel krvnih žila, je snažan vazodilatator i inhibitor agregacije trombocita *in vitro*. PGI₂ također štiti sluznicu probavnog sustava od toksičnog djelovanja različitih agenasa (citoprotektivni učinak). Cilj ovog istraživanja bio je ispitati ulogu PGI₂ u obrani organizma od toksičnog učinka acetaminofena (APAP). Pokusi su bili obavljani na visokosrodnim miševima soja C57Black/6 kojima je gastričnom sondom uštrcana letalna ili visoka subletalna doza APAP. Agonisti (čisti PGI₂-natrijeva sol ili stabilni analog PGI₂-iloprost) i antagonist (CAY-10441) PGI₂-receptora uštrcani su intraperitonealno (i.p.) 30 minuta prije ili 2 sata nakon primjene APAP. Toksičnost APAP određivala se na temelju 48-satnog praćenja preživljenja životinja, mjerenja koncentracije alanin aminotransferaze (ALT) u plazmi 20–24 sata nakon aplikacije APAP i određivanja histološkog stupnja oštećenja jetre. Rezultati su pokazali da PGI₂ ima snažan hepatoprotektivni učinak (statistički značajno povećanje preživljenja životinja i smanjenje razine ALT u plazmi u odnosu na kontrolne skupine životinja). Iloprost nije pokazao značajan učinak na toksičnost APAP, a CAY-10441 je povećao hepatotoksični učinak APAP kad je bio uštrcan 2 sata nakon njegove primjene. Patohistološke promjene u jetri općenito potvrđuju prethodne rezultate. Ovo istraživanje potkrjepljuje tezu da je PGI₂ jedan od endogenih posrednika u obrani organizma od štetnog djelovanja hepatotoksičnih agenasa.