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

Ivan Ćavar1, Tomislav Kelava1,2, Renata Heinzel3 and Filip Ćulo1,2

1 Department of Physiology, School of Medicine, University of Mostar, Mostar, Bosnia and Herzegovina
2 Department of Physiology, School of Medicine, University of Zagreb, Zagreb, Croatia
3 Department of Pathology, University Hospital Dubrava, Zagreb, Croatia

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 doses. However, overdose or chronic use of a high dose of APAP is major cause of acute liver failure (ALF) in the western world. 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). 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.

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, PGF2α, PGD2, PGI2 (prostacyclin), and TXA2 (thromboxane). 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 prosta-
glandin 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 pro-
duced by endothelial cells and plays an important role in
vascular homeostasis as a result of its potent vasodi-
latory and antithrombotic effects10. Thus, prostacyclin
functionally opposes the effects of TXA2. However, the effect of PGI2 and its analogs or antago-
nists on isolated rat hepatocytes16 or human leucocytes17
shows cytoprotective effect on gastrointestinal mucosa18.
Moreover, studies on isolated rat hepatocytes19 or human leucocytes20 demonstrated a protection by prostacyclin against
carbon tetrachloride induced necrosis. Prostacyclin, also,
shows cytoprotective effect on gastrointestinal mucosa18.
However, the effect of PG12 and its analogs or antago-
nists on APAP-induced hepatotoxicity has not been system-
atically studied.

These results prompted us to investigate the influence
of administration of PG12, its stable analogue (iloprost),
and inhibitor of IP-receptor (CAY-10441) on APAP-in-
duced liver injury in mice.

Materials and Methods

Animals

C57 Black/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 weigh-
ing 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). Pheno-
barbitone-sodium was obtained from Kemika (Zagreb,
Croatia). Since the PGI2 is rapidly bioconverted to
PGF1a19 (t1/2=2–3 min), in certain experiments we used
besides the pure PG12 (sodium salt) also its stable struc-
tural analog-iloprost. PG12 sodium salt was dissolved (1
mg/mL) in Tris buffer (1M, pH=8.5) and after appropri-
ate 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 phos-
phate buffered saline (PBS, pH=7.2). Iloprost was ad-
ministered 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 dissol-
ved 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 pre-
liminary 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-metaboliz-
ing 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 experi-
ments induced 65–70% mortality in control mice. Experi-
mental and control groups of mice countered 12 animals
(for determining survival of animals) or 6–10 animals
(for determining ALT activity and liver histology). Con-
rol animals received appropriate vehicle.

Plasma ALT activity

Alanine aminotransferase (ALT) levels were measu-
red 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 determi-
nation. 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. Li-
ver 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, hy-
drated, and stained with haematoxylin and eosin. Micro-
scopically, the liver damage was classified as follow: de-
gree 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 $X \pm$ SEM. Parametric methods were used, including students t-test. Differences in survival between groups of mice were compared by $\chi^2$-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 μg/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 (±SEM) obtained in 7 to 10 mice per group. As seen (Figure 2a), pre-treatment of mice with PGI2 significantly reduced ALT level (1624±528 U/L vs. 3791±825 U/L, p<0.05). If given 2 hours after APAP, PGI2 only slightly reduced ALT level (3605±504 U/L vs. 3791±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±476 U/L vs. 436±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±477 U/L vs. 2447±1326 U/L, p>0.05). However, when given in
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**

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>X̄±SEMc</th>
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</thead>
<tbody>
<tr>
<td>Experiment 1b</td>
<td></td>
</tr>
<tr>
<td>Vehicle+APAP</td>
<td>2.87±0.40</td>
</tr>
<tr>
<td>PGI2+APAP</td>
<td>2.50±0.23d</td>
</tr>
<tr>
<td>Experiment 2b</td>
<td></td>
</tr>
<tr>
<td>Vehicle+APAP</td>
<td>3.25±0.31</td>
</tr>
<tr>
<td>APAP+CAY-10441</td>
<td>3.50±0.34d</td>
</tr>
</tbody>
</table>

| 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 tetrachloride, aflatoxin, concanavalin A, D-galactosamine, LPS (endotoxin) alone, or associated with D-galactosamine. In the present study the results differ from data obtained by Guarner and colleagues, which obtained hepatoprotection only if PGI2 was given after APAP. We goten hepatoprotection with PGI2, both when it was injected to mice before or after APAP. We have no 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*. According to these findings *in vitro* and data on hepatoprotective effect of PGI2, we supposed that iloprost, a stable 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). 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 PG12 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. 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 hepatocytes. Liver endothelial cells produce PGI2 as the predominant metabolite, but also minor amounts of PGE2 and TXA2. The major producers of prostanoids are Kupffer cells and extrahepatic inflammatory cells recruited to liver by chemoattractants. Although the precise mechanisms underlying the cytoprotective effects of PGs in acute liver injury remain to be precisely defined, studies on isolated rat hepatocytes or human leukocytes demonstrated a direct cellular protection by PGI2 against carbon tetrachloride induced necrosis, possibly by stabilization of membranes or inhibition of lysosomal enzyme release. As a result of its potent vaso-dilatory and antithrombotic effects (by inhibiting platelet aggregation), PGI2 functionally opposes the ef-
flicts of TXA2 and thus may reduce or reverse the hepatic vascular congestion observed in APAP toxicity. Guarnier 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 damage. 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 subcellular 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.

REFERENCES


I. Ćavard

Department of Physiology, School of Medicine, University of Mostar, Bijeli brije bb, 88000 Mostar, Bosnia and Herzegovina

email: ivancavarsb@yahoo.com

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 zbijenjima u gotovo svim organima i tkivima u organizmu. Prostaciklin (prostaglandin I2, PGI2), kojeg stvara endotel krvnih čila, 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 obavljeni na visokosrodnim miševima soja C57Black/6 kojima je gastričnom sondom učtranje letalna ili visoka subletalna doza APAP. Agonisti (~isti PGI2-natrijeva sol ili stabilni analog PGI2-iloprost) i antagonist (CAY-10441) PGI2-receptora učtreni 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 te u obrani od 30 minuta nakon aplikacije APAP i određivanja histološkog stupnja oštećenja jetre. Rezultati su pokazali da PGI2 ima snažan hepatoprotaktivni 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 učtran 2 sata nakon njegove primjene. Patohistološke promjene u jetri pojavile su se srednje u crveno-žutim žljetovima, a toksičnost je na učtanima snažno smanjenja.