Anti-thromboxane B₂ antibodies protect against acetaminophen-induced liver injury in mice

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Abstract

Prostanoids are lipid compounds that mediate a variety of physiological and pathological functions in almost all body tissues and organs. Thromboxane (TX) A₂ is a powerful inducer of platelet aggregation and vasoconstriction and it has ulcerogenic activity in the gastrointestinal tract. Overdose or chronic use of a high dose of acetaminophen (N-acetyl-p-aminophenol, APAP) is a major cause of acute liver failure in the Western world. We investigated whether TXA₂ plays a role in host response to toxic effect of APAP. CBA/H Zg mice of both sexes were intoxicated with a single lethal or high sublethal dose of APAP, which was administered to animals by oral gavage. The toxicity of APAP was determined by observing the survival of mice during 48 h, by measuring concentration of alanine-aminotransferase (ALT) in plasma 20-22 h after APAP administration and by liver histology. The results have shown that anti-thromboxane (TX) B₂ antibodies (anti-TXB₂) and a selective inhibitor of thromboxane (TX) synthase, benzylimidazole (BZI), were significantly hepatoprotective, while a selective thromboxane receptor (TPR) antagonist, daltroban, was slightly protective in this model of acute liver injury. A stable metabolite of TXA₂, TXB₂, and a stable agonist of TPR, U-46619, had no influence on APAP-induced liver damage. Our findings suggest that TXA₂ has a pathogenic role in acute liver toxicity induced with APAP, which was highly abrogated by administration of anti-TXB₂. According to our results, this protection is mediated, at least in part, through decreased production of TXB₂ by liver fragments ex vivo.

Introduction

Overdose or chronic use of a high dose of acetaminophen (N-acetyl-p-aminophenol, APAP) represents the most prevalent cause of acute liver failure in the Western world today. APAP, a commonly used analgesic and antipyretic drug, is considered safe at therapeutic doses and in overdose the elevated levels of the toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), are generated by hepatic cytochromes P450 (CYPs). Several CYPs, including CYP1A2, CYP2A6, CYP2E1 and CYP3A, have been established as APAP-metabolizing enzymes. Among them, CYP2E1 was identified as the major isof orm responsible for the biotransformation of APAP to NAPQI. Although the pathophysiological events that occur in the early phase of APAP toxicity have been well established, the precise biochemical mechanisms leading to cell death are not fully understood. It is recognized that NAPQI covalently binds with nucleophilic macromolecules such as DNA or proteins with subsequent loss of their activity or function. It has been proposed that primary cellular targets of NAPQI toxicity are mitochondrial proteins as well as proteins involved in cellular ion control. NAPQI extensively reduces the level of hepatic cellular glutathione (GSH) and this event results in a subsequent generation of reactive oxygen or nitrogen species, which mediate toxicity by protein oxidation, enzyme inactivation and damage of cell membranes via lipid peroxidation. However, the toxic sources of APAP-induced liver injury are not limited to APAP itself because of various hepatic biotransformation routes of APAP. It has been shown that the oxidative stress caused by CYP2E1-mediated APAP metabolism might significantly contribute to APAP-induced hepatotoxicity. Necrosis is recognized as the mode of cell death—rather than apoptosis and hepatic necrosis mainly occurs in the centrilobular region, where CYP2E1 is highly expressed. Prostanoids, consisting of the prostaglandins (PGs) and thromboxanes (TXs), are lipid compounds produced by sequential metabolism of membrane phospholipids (arachidonic acid) by the cyclooxygenase (COX) and specific PG/TX synthase enzymes. Prostanoids mediate a variety of physiological and pathological functions in almost all body tissues and organs, i.e. they regulate kidney function, platelet aggregation and neurotransmitter release, modulate function of immune system and are implicated in a broad array of diseases including inflammation, hypertension, cardiovascular disease and cancer. Thromboxane (TX) A₂ is a potent mediator of platelet aggregation and stimulates the contractile activity of smooth muscle in blood vessels and trachea. Increased TXA₂ synthesis has been linked to cardiovascular diseases, such as angina and myocardial infarction, asthma and certain ulcerative disorders in the stomach.

Concerning the role of TXA₂ in liver diseases, there are some reports suggesting that TXA₂ is involved in hepatorenal syndrome and it could promote acute liver injury caused by xenobiotics. Thus, it was shown that the production of TXA₂ by liver homogenates ex vivo is significantly elevated in liver injury induced with carbon tetrachloride (CCl₄), lipopolysaccharide (LPS) or ethanol. Similarly, the overproduction of endogenous TXA₂ has been found to be involved in APAP-induced liver damage. Administration of OKY-046 or OKY-1581, a selective thrombox-
ane (TX) synthase inhibitors, and ONO-3708, a thromboxane receptor (TPR) antagonist, highly ameliorated liver injury in the above-mentioned models of toxic hepatitis.25-28 However, data from the study of Guarner et al. suggest that TXA₄ inhibition per se does not reduce hepatic necrosis induced by APAP.²⁵ Therefore, selective inhibition of the TX synthase may, besides decreasing synthesis of TXA₄, increase production of PGI₂ or PGE₂, the protective effects of which have been demonstrated in various models of liver injury.²⁹,³⁰ Based on these data, the present studies aimed to investigate the role of exogenously applied TX and its derivatives on APAP-induced hepatotoxicity in vivo.

Materials and Methods

Animals CBA/H Zg mice were raised in an animal colony unit at the Department of Physiology, School of Medicine, University of Zagreb. Mice of both sexes aged 12-16 weeks and weighing 20-25 g were used in all experiments. The animal colony unit had regulated temperature at 22±2°C and relative humidity in the animal room were 50±5%, respectively. The cages were sanitized twice weekly and mice were allowed free access to tap water and standard mouse chow diet (n. 4RF21, Diet Standard, Milan, Italy). All animal protocols were approved and conducted in accordance with the Ethics Committee of the University of Zagreb, Faculty of Medicine (Zagreb, Croatia).

Chemicals and treatments of animals

Pure APAP substance was a kind gift from the Belupo Pharmaceutical Company (Koprivnica, Croatia). Phenobarbitone-sodium was obtained from Kemika (Zagreb, Croatia). Since the TXA₄ has a half-life of about 30 s, in certain experiments we used its stable metabolite, TXB₂. TXB₂ (n. 19030, Cayman Chemical, Ann Arbor, MI, USA) was supplied as a crystalline solid, dissolved in PBS (100 µg/mL, pH=7.2) and thereafter injected into mice 30 min before APAP administration. Polyclonal anti-thromboxane (TX) B₂ antibodies (anti-TXB₂) were supplied as a lyophilized powder (n. P7291, Sigma-Aldrich, St. Louis, MO, USA), which was dissolved in 5 mL of PBS (pH=7.2) and finally injected (40 µg/kg, i.p.) into animals 3 h before APAP. Daltroban (n. D7441, Sigma-Aldrich), a selective TPR antagonist, was dissolved in Tris buffer (2.0 mg/mL, pH=7.4) and injected (5.0 mg/kg, i.p.) into mice 30 min before APAP. U-46619 (n. 16450, Cayman Chemical), a stable analog of the endoperoxide PGH₂ and a TPR agonist, was purchased as a solution in methyl acetate. Organic solvent-free aqueous solution of U-46619 was prepared by evaporating stock solution under a gentle stream of nitrogen and by dissolving the remaining substance directly in PBS (250 µg/mL, pH=7.2). Thereafter, U-46619 was administered to animals (0.2 and 0.8 mg/kg, i.v.) 30 min before APAP. Benzylimidazole (BZI), a selective inhibitor of TX synthase, was purchased from Sigma Aldrich (n. 116416) as a crystalline solid, dissolved in PBS (1 mg/mL, pH=7.2) and injected into animals (50 mg/kg, i.p.) 2 h after APAP administration. The doses of the drugs for application in vivo were chosen from the data available 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.

Assessment and measurement of hepatotoxicity induced with APAP

In order to induce hepatic CYPs, mice were given phenobarbitone-sodium in drinking water for seven 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, into which 1-2 drops of Tween 20 were added. Animals were allowed free access to food 4 h later. To observe the survival of mice, APAP was administered in a dose of 250-300 mg/kg. In order to determine the concentration of plasma alanine aminotransferase (ALT), as well as for histopathological evaluation of liver slices and measurement of 11-dehydro TXB₂ 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 the survival) or 6-10 animals (for all other measurements).

Plasma ALT activity

Plasma ALT levels were determined 20-22 h after APAP administration. Mice were given 250 U of 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. Samples of liver tissue were fixed in 4% buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Thereafter, sections of liver lobes of animals (8 animals per group) were fixed in 10% buffered formaldehyde solution under a gentle stream of oxygen before overnight at 37°C. Sections were cleared, hydrated and stained with hematoxylin and eosin. Microscopically, the liver damage was classified using an 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 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 be showing significant liver injury.

Production and measurement of TXB₂ concentration ex vivo

Mice were sacrificed 6 h after APAP administration. Blood was collected by puncture of the medial eye angle with heparinized glass capillary tubes. After centrifugation, separated plasma was stored at -80°C. Samples of liver tissue were given phenobarbitone-sodium in drinking water for seven 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, into which 1-2 drops of Tween 20 were added. Animals were allowed free access to food 4 h later. To observe the survival of mice, APAP was administered in a dose of 250-300 mg/kg. In order to determine the concentration of plasma alanine aminotransferase (ALT), as well as for histopathological evaluation of liver slices and measurement of 11-dehydro TXB₂ 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 the survival) or 6-10 animals (for all other measurements).

Liver histology

Mice were sacrificed under light ether anesthesia by cervical dislocation 20-22 h after APAP administration. Liver lobes of each animal (8 animals per group) were fixed in 4% buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Thereafter, sections of liver lobes 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 hematoxylin and eosin. Microscopically, the liver damage was classified using an 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 be showing significant liver injury.

Statistical analysis

Data were expressed as means±SEM. Differences in survival between the groups of mice were compared by χ² test using Yates’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. P<0.05 was considered statistically significant.
Results

Effects of TXB₂ and anti-TXB₂ on APAP-induced mortality and plasma ALT concentration in mice

To determine the survival of animals, mice were treated with 250 mg/kg of APAP TXB₂ (2.0 mg/kg, i.p.) and vehicle were given 30 min, and anti-TXB₂ (40 mg/kg, i.p.) 3 h before APAP. Administration of TXB₂ had no effect on the survival of mice (Figure 1A, P>0.05). Administration of anti-TXB₂ significantly improved the survival of animals (Figure 1A, P<0.05). To determine the plasma ALT concentration, mice were treated as in the previous experiment, except that mice received a lower dose of APAP (150 mg/kg). Figure 1B shows mean ALT levels (±SEM) obtained 20-22 h after APAP administration. Treatment of animals with TXB₂ slightly decreased ALT concentration in plasma (Figure 1B, P>0.05), while pretreatment of mice with anti-TXB₂ significantly reduced ALT level (Figure 1B, P<0.01).

Effect of daltroban on APAP-induced mortality and plasma ALT concentration in mice

To determine the effect of daltroban on APAP-induced hepatotoxicity, the survival of animals was estimated after mice were treated with 300 mg/kg of APAP. To measure the plasma ALT level, mice received 150 mg/kg of APAP. Daltroban (5.0 mg/kg, i.p.) and vehicle were given 30 min before APAP administration. Pretreatment of mice with daltroban increased the survival of mice and reduced ALT level, but the differences did not reach statistical significance (Figure 2A and B, P>0.05 for both comparisons).

Effect of U-46619 on survival of mice and plasma ALT concentration

To determine the survival of animals, mice received 250 mg/kg of APAP and for the measurement of plasma ALT level mice were given 150 mg/kg of APAP. U-46619 (0.2 and 0.8 mg/kg, i.v.) and vehicle were given 30 min before APAP administration. Pre-treatment of mice with U-46619 did not significantly change the survival and plasma ALT level (Figure 3A and B; P>0.05 for all comparisons).

Effect of BZI on survival of mice and plasma ALT concentration

In order to determine the survival of animals and plasma ALT level, mice were treated with 300 mg/kg or 150 mg/kg of APAP, respectively. BZI (50 mg/kg, i.p.) and vehicle were given 2 h after APAP administration. Administration of BZI significantly improved the survival of ani-
mals (Figure 4A; P<0.01) and decreased plasma ALT level (Figure 4B; P<0.001).

Liver histology
Macroscopically, the whole liver surface of some APAP-treated mice had a mottled appearance; dark red hemorrhagic-necrotic spots were regularly scattered on the yellowish background. Microscopically, the liver damage was graduated using an arbitrary scale from 0 to 5 as described in Materials and Methods (Figure 5). The severity of necrosis was quite variable both between animals and also within different parts of the same liver. However, administration of anti-TXB2 significantly decreased the number and size of necrotic foci in the liver in comparison to the vehicle group, which could be easily seen by macroscopic observation and by histological analysis (Table 1, P<0.05). In mice injected with BZI, macroscopic and microscopic damages of the liver parenchyma appeared less pronounced, although the differences did not reach statistical significance (Table 1, P>0.05).

Effect of anti-TXB2 on the production of TXB2 ex vivo
TXB2 production was determined in plasma and supernatants of incubated liver fragments taken from normal (non-treated) mice and mice treated with anti-TXB2 (40 μg/kg, i.p.) or vehicle 3 h before APAP administration. In comparison to normal mice, treatment with APAP alone (vehicle group) significantly increased production of TXB2, while treatment with anti-TXB2 reduced that increase in TXB2 production (Figure 6, P<0.05 for both comparisons).

Discussion
Intoxication with APAP is a major cause of acute liver failure in the Western world and therefore we used it as a model of drug-induced liver injury to examine the influence of anti-TXB2 and derivatives of TXA2 on drug toxicity mechanisms. The presented results clearly demonstrate that treatment of mice with anti-TXB2 significantly improved the survival of mice and alleviated hepatic damage, as assessed by plasma ALT concentration and liver histology. To our best knowledge, this is the first study in which anti-TXB2 has been used in an in vivo model of experimental liver damage induced by a noxious agent. However, the mechanism of its protective action is not known and our results indicate that it might somehow be related to the inhibition of production of TXB2 by liver homogenates taken from mice intoxicated with APAP. This points
to TXA₂ as an endogenously produced hepatotoxic agent. This is supported by our finding that administration of APAP alone increased synthesis of TXB₂ in the liver. In our previous research, it was shown that APAP also increased hepatic synthesis of PGE₂ and PGI₂, which have both been shown to have a significant hepatoprotective effect. The elevated levels of TXB₂ have been found in liver and systemic circulation after hepatic injury induced by different agents, such as endotoxemia, hepatic ischemia-reperfusion, hepatectomy, liver transplantation, hepatic cirrhosis, ethanol and CCl₄. Due to the unstable nature and very short half-life of TXA₂, its stabile metabolite, TXB₂, was used in our investigations. Although TXB₂ is considered to be a biologically inactive metabolite of TXA₂, it has been shown that TXB₂ has several biological activities such as increasing pulmonary arterial pressure, increasing arterial pressure in dogs, inducing or decreasing myocardial contractility on isolated rat heart and increasing the rate of apoptosis in cultured rat hepatocytes. Our experiments, treatment of mice with TXB₂, in various doses, did not cause elevation in platelet aggregation induced with ADP or arachidonic acid in mice plasma (data not shown). Hepatoprotective effects of anti-TXB₂ remain unclear; however, they might be exerted through its cross-reactivity with TXA₂ and TXB₂, and the consequent inactivation of these compounds. In the present experiments, treatment of mice with daltroban, a selective antagonist of TPR, alleviated the liver damage induced by APAP as shown by the decrease in mortality of animals and plasma ALT level. Numerous animal studies have shown that other specific TPR antagonists protected the liver from injury after toxic dose of ethanol and CCl₄, endotoxemia, warm ischemia, ischemia-reperfusion, etc. On the other hand, the administration of the well-known stabile analog of TXA₂, U-46619, to normal mice or animals which received APAP did not produce or aggravate liver injury. It was previously demonstrated that U-46619, injected i.v. into mice, caused clear elevation of serum ALT and AST levels and enhanced the histopathological score of liver necrosis. We are not able to offer an appropriate explanation for the biological inefficacy of U-46619, except that a different mice strain was used in our experiments. Our results clearly show that BZI, a selective inhibitor of TX synthase, when given to mice 2 h after APAP significantly improved the survival and decreased plasma ALT level of treated animals. In the study by Guarner et al., it has been demonstrated that BZI had a strong protective effect on APAP overdose-induced liver damage in mice, but only when it was given to animals 30 min before the lethal dose of APAP. Furthermore, the same authors showed that BZI interfered with hepatic drug metabolism as assessed by prolongation of the pentobarbitone sleeping time. Data in this study demonstrate that interference with hepatic drug metabolism is not the major mechanism of protective effect of BZI, since the drug was protective when administered to animals 2 h after APAP, i.e., at the time when its conversion to toxic metabolite is almost complete. Also, in our experiments the dose of BZI was much higher in comparison to that used in the previously described study. Therefore, the mechanism of its protective action is most probably due to the inhibition of TXA₂ synthesis. Supporting this assertion, several studies have shown that a decrease in TXA₂ synthesis by inhibition of TX-synthase or COX-2 has beneficial effect on liver injury induced by various toxic agents other than APAP, some of which are not dependent on hepatic drug metabolism.

The presented results indicate that TXA₂ plays an important role in the onset and development of APAP-induced liver injury in mice. Among the eicosanoids, TXA₂ promotes inflammatory processes in the liver, has vasoconstrictive effect in the portal venous system and induces leukocyte adhesion in the sinusoids, all of which lead to hepatic tissue injury. In endotoxemia (LPS)-induced liver damage in mice, enhanced release of TXA₂ from the liver is responsible for the high production of a representative proinflammatory cytokine, tumor necrosis factor alpha (TNF-α). In addition, TXA₂ is associated with the increased expression of intercellular adhesion molecule.
(ICAM) 1 in the hepatic microvasculature, which facilitates leukocyte adhesion and contributes to the hepatic inflammatory process. Several studies have shown that TXA₂ induces the release of endothelin, which contributes to the liver injury through its potent vasoconstrictive action. Furthermore, TXA₂ up-regulates other vasoconstrictors such as angiotensin II, down-regulates vasodilators such as PGI₂ and NO, and these actions further exacerbate the vasoconstrictive effect of TXA₂. In an animal model of ischemia-reperfusion liver injury, an increase in the level of lipid peroxide and a decrease in the concentration of antioxidant glutathione and alpha-tocopherol have been shown. In this regard, administration of different COX inhibitors, specific TX synthase inhibitors and specific TPR antagonists has been shown to abrogate these negative effects of TXA₂ and protect from severe hepatic injury elicited with various noxious agents. It seems that PGI₂/TXA₂ ratio is critical for the maintenance of hepatic microcirculation, since its decrease has been observed in several models of liver injury. PGI₂ antagonizes the effects of TXA₂ by inducing vasodilatation and by inhibiting platelet aggregation. Thus, exogenously applied PGI₂ or its stable agonists significantly increased PGI₂/TXA₂ ratio and improved the outcome of liver injury induced by APAP, as well as by other noxious agents, in vivo and in vitro. Data in the literature reveal that other types of eicosanoids, including leukotrienes (LTs) and F₂-isoprostanes (F₂-IsopPs), may mediate tissue damage in several models of liver injury. It has been shown that cysteinyl LTs (LTC₄, LTD₄ and LTE₄) contribute to the development of ischemia-reperfusion liver injury in rats by causing vasocstriction, tissue edema and cell toxicity and that LTD₄/LTβ receptor pathway mediates hepatic microcirculatory dysfunction in a murine model of endotoxemia. Accordingly, inhibition of the enzyme 5-lipoxygenase, which is responsible for the generation of LTs from arachidonic acid, promoted liver regeneration and decreased hepatic hyaluronate in LPS and CCl₄-treated laboratory animals. F₂-IsopPs, a PGE₂-like compound formed by free radical-mediated lipid peroxidation, have been shown to be potent renal vasoconstrictors via TX-like receptor, increase portal pressure in normal and cirrhotic rats, and mediate the liver damage in CCl₄-treated mice. Nevertheless, there are no data in the literature on the role of LTs and F₂-IsopPs in liver injury induced with APAP. This may merit investigation in further experiments.

Based on previous research, in which we have shown that PGE₂ and PGI₂ have a hepato-protective effect, our present findings support the hypothesis that TXA₂ plays a pivotal role in producing hepatic injury induced by APAP overdose, as well as in other forms of hepatic stress. However, much remains to be done to clarify the precise mechanisms underlying pathogenic/toxic effects of TXA₂ in acute liver injury.

Table 1. Effects of TXB₂, anti-TXB₂ and BZI on APAP-induced liver injury.

| Treatment* | Histopathological scores | %<sup>-2</sup> |
|------------|--------------------------|--------------|
| Vehicle + APAP | 0  1  2  3  4  5 | 63 |
| TXB₂ + APAP | 0  2  1  1  3  1 | 50 |
| Anti-TXB₂ + APAP | 2  3  2  0  1  0 | 13 |
| APAP + BZI | 1  3  1  1  1  3 | 37 |

Mice were sacrificed and livers were collected 20-22 h after mice received APAP (150 mg/kg). *Anti-TXB₂ (40 μg/kg, i.p.) was given 1 h, while TXB₂ (2.0 mg/kg, i.p.) and vehicle were given 30 min before APAP administration. BZI (50 mg/kg, i.p.) was given 2 h after APAP administration. Histopathological scores were determined and graded by intensity of hepatocellular necrosis from 0 to 5 as described in Materials and Methods. Scores greater than 2 were considered to be significant necrosis. N=8 mice per group. Statistical significance in comparison to vehicle group (P<0.05).

Figure 6. Effect of anti-TXB₂ on TXB₂ production by the plasma and liver fragments. Anti-TXB₂ (40 μg/kg, i.p.) and vehicle were given 3 h before APAP administration (150 mg/kg). Plasma and liver samples were taken 6 h after APAP administration. Concentration of TXB₂ was determined in plasma and supernatants obtained after 1 h incubation of liver fragments. Results represent means±SEM of 6 mice per group. *P<0.05 in comparison to normal mice. #P<0.05 in comparison to group which received anti-TXB₂.

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