Basic Studies

Beraprost sodium, a prostacyclin (PGI\textsubscript{2}) analogue, ameliorates concanavalin A-induced liver injury in mice

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Abstract: Background/Aims: Prostacyclin (PGI\textsubscript{2}) is a potent mediator in the inflammatory and coagulation processes. The aim of this study was to test whether beraprost sodium, a PGI\textsubscript{2} analogue, could prevent experimental hepatic injury induced by concanavalin A (Con A), which is a model of fulminant hepatic failure. Methods: Beraprost (100 \textmu g/kg) was administered intraperitoneally simultaneously with Con A (40 mg/kg) in C57B6J mice. Blood circulation in the liver was determined by laser-Doppler flowmetry. Plasma levels of alanine aminotransferase (ALT), tumor necrosis factor (TNF)-\textalpha, interferon (IFN)-\gamma, and interleukin (IL)-6 were determined. Levels of TNF-\textalpha and IFN-\gamma in culture supernatant of splenocytes were also determined. Results: Beraprost administration reduced the incidence of death following hepatic failure (76.5% vs. 29.4%, \textit{P}<0.05). Plasma levels of ALT were significantly lower in the beraprost-treated group than in the control group, and in the former, there was concomitant suppression of the histological features of injury. Beraprost significantly increased hepatic blood flow volume in Con A-treated mice. Plasma levels of TNF-\textalpha and IFN-\gamma were significantly reduced at 6 and 12 h after Con A injection, respectively, but the levels of IL-6 were increased at 6 h. In vitro, beraprost also suppressed Con A-induced TNF-\textalpha production in splenocytes, while it stimulated IFN-\gamma production. Conclusion: These findings imply that beraprost suppresses Con A-induced liver injury. These data also suggest that beraparost, which is clinically effective in treating pulmonary hypertension, may have therapeutic potential for preventing hepatic injury.

Prostacyclin (PGI\textsubscript{2}) is a modulator of several biological processes, such as antiplatelet aggregation (1), vasodilation (2), and cytoprotection (3). Beraprost sodium, a stable PGI\textsubscript{2} analogue (4), shows a similar pharmacological action to PGI\textsubscript{2} (5, 6), and has a long biological half-life and high oral bioavailability. Beraprost was shown to have beneficial effects on patients with peripheral arterial occlusive disease (7) and on patients with primary pulmonary hypertension (8). It was recently reported that beraprost attenuated glomerulonephritis (9, 10), and warm ischemia/reperfusion damage of liver grafts in rats (11). Although this evidence suggests the therapeutic potential of beraprost in several diseases, it remained unknown as to whether beraprost could ameliorate liver diseases such as fulminant hepatitis.

The lectin concanavalin A (Con A) is a T cell activator and induces T-cell-dependent liver injury in mice (12), which is relevant to the pathophysiology of human liver diseases such as fulminant hepatitis and autoimmune hepatitis (13, 14). Administration of Con A triggers rapid depletion of natural killer T cells in the liver, and leads to an influx of immune-activated cells, mainly CD4-positive T lymphocytes, accompanied by an increase in the production of several inflammatory cytokines.

Key words: Beraprost – Concanavalin A (Con A) – Hepatic blood flow – Inflammatory cytokines – Prostacyclin

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Received 6 August 2004, accepted 17 January 2005

Abbreviations: Con A, concanavalin A; ALT, alanine aminotransferase; TNF-\textalpha, tumor necrosis factor; IFN-\gamma, interferon; IL-6, interleukin.
cytokines, such as tumor necrosis factor (TNF-\(\alpha\)) and interferon (IFN-\(\gamma\)). In this model, TNF-\(\alpha\) and IFN-\(\gamma\) play central roles in the development of massive hepatocellular apoptosis and necrosis (15–25). In addition, TNF-\(\alpha\) and IFN-\(\gamma\) cause sinusoidal endothelial cell damage, leading to intrasinusoidal hemostasis which is another important mechanism of liver damage (26, 27). In contrast, interleukin (IL)-6 alleviates liver damage through decrease in production of TNF-\(\alpha\) and IFN-\(\gamma\) (15, 17).

It has been recently reported that treprostinil, another PGI2 analogue, inhibits the production of inflammatory cytokines (TNF-\(\alpha\), IL-6, and IL-1\(\beta\)) through the blockade of NF-\(\kappa\)B nuclear translocation in human alveolar macrophage (28), which is a key transcriptional factor for inflammatory cytokines. PGI2 and its analogs also increase the levels of CAMP via prostaglandin receptors, which then activates protein kinase A (PKA) signal transduction. The activation of PKA inhibits T cell proliferation and cytokine production (29) through inhibition of NF-\(\kappa\)B transcriptional activity (30). In this study, we evaluated the effects of beraprost on hepatic injury in the Con A model of fulminant hepatic failure, and found that beraprost alleviated Con A-induced liver injury. To investigate the hepatoprotective mechanisms, we also investigated the effects of beraprost on TNF-\(\alpha\) and IFN-\(\gamma\) production by splenic cells in vitro.

**Materials and Methods**

**Animal experiments**

Male C57B6J mice (8 weeks old) (Kyudo Co., Ltd., Saga, Japan) were used in this experiment. Protocols were carried out according to the Guidelines for Animal Experiments in Kyushu University and The Law and Notification of the Japanese Government. Liver injury was induced in mice by treatment with Con A using a modified version of a method reported previously (12). Con A [Type IV] (Sigma, St. Louis, MO) was dissolved in pyrogen-free saline and injected into mice via the jugular vein (i.v.) at a dose of 40 mg/kg in a volume of 0.2 ml. Beraprost (Toray, Kanagawa, Japan) was dissolved in saline and injected intraperitoneally (i.p.) at a dose of 100 \(\mu\)g/kg in a volume of 0.1 ml, immediately after injection of Con A. The dose of 100 \(\mu\)g/kg was selected according to previous reports in several rat models showing that this dose is non-toxic (11, 31). Preliminary experiments employing control rats confirmed that beraprost had no significant side effects, including liver damage, at this dose.

**Experiment 1**

To assess the effect of beraprost on the survival rate of Con A-treated mice, 34 mice were randomized into two experimental groups \((n = 17\ \text{per group})\), the Con A group (injection of Con A i.v. and 0.1 ml saline i.p. as vehicle) and the Beraprost group (injection of Con A i.v. and beraprost i.p.). Mice were observed at 6, 12 and 24 h after injection of Con A.

**Experiment 2**

To measure tissue blood flow in the liver, mice were assigned to three groups \((n = 10\ \text{per group})\): the Normal group (saline i.v., injection of saline i.p.), the Con A group, and the Beraprost group. Liver tissue blood flow was measured using a laser-Doppler flowmeter (ALF2100; Advance, Tokyo, Japan) as described previously (32). The laser probe (P7c; Monte System Co., Tokyo, Japan) was placed on the right lobe, and blood flow was measured three times for each mouse 24 h after Con A injection.

**Experiment 3**

To evaluate the severity of the liver injury and the levels of cytokine (TNF-, IFN-, and IL-6) in this model, 40 mice were assigned to two groups \((n = 20\ \text{per group})\), with or without beraprost administration (Beraprost group and Con A group, respectively). Blood was collected by retro-orbital puncture under anesthesia at 0, 2, 6, 12, and 24 h after Con A injection. Mice were sacrificed after the blood collection at 24 h, and liver specimens for light microscopic study were rapidly excised and fixed in 10% formalin. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for histological evaluation.

**Splenic cell culture experiments**

Spleens from male C57B6J mice were excised and ground in a 1 cm dish with 5 ml RPMI 1640 medium (Life Technologies, Inc., Rockville, MD). The spleens were ground using sterile glass slides to prepare splenic cell suspensions. Splenocytes were isolated using Lympholyte-M CL5030 (Cedarlane, Ont., Canada) according to the manufacturer’s instructions. The isolated splenocytes were plated into 96-well microtiter plates at 5 \(\times\) 10^5 cells per well. The splenocytes were stimulated with 10 \(\mu\)g/ml Con A either with or without 100 \(\mu\)M beraprost. Incubations were carried out in an incubator at 5% CO2 and 37 \(^\circ\)C. Twenty-four hours after addition of the stimulus,
cell culture supernatant was collected for the measurement of TNF-α and IFN-γ.

**Measurement of TNF-α, IFN-γ, and IL-6**

Plasma and cell culture supernatant concentrations of TNF-α, IFN-γ, and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) (BioSource International, Inc., Camarillo, CA).

**Aminotransferase assay**

L-Alanine aminotransferase (ALT) activity in the plasma was assayed by standard spectrophotometric methods using commercial test reagents (GPT-OA test; Wako, Tokyo, Japan).

**Statistical analysis**

All results are shown as the mean ± SEM. Comparisons were made using one-way analysis of variance (ANOVA) followed by Scheffe’s test or the Mann–Whitney test. Survival curves were calculated by the Kaplan-Meier method, and differences in survival were evaluated by log-rank test.

**Results**

**Effect of beraprost on lethality and liver injury induced by Con A**

The 6, 12 and 24 h survival rates were all significantly higher in the group treated with beraprost (Beraprost group) than in the control group (Con A group) [24 h survival, 76.5% (13/17) vs. 29.4% (5/17), \( P < 0.05 \) (Fig. 1)]. This result indicated that Con A-induced lethality was significantly reduced by beraprost administration. In addition, beraprost significantly suppressed the increase in plasma ALT levels at 12 and 24 h induced by Con A (24 h ALT levels in the Beraprost group were 6650 ± 320 U/l vs. 3520 ± 280 U/l in the Con A group, \( P < 0.01 \)) (Fig. 2). To eliminate the possibility that beraprost directly interfered with Con A, we injected beraprost 30 min before \((n = 5)\) or after \((n = 5)\) injecting Con A, and measured the levels of ALT at 12 h after Con A injection. We found that the pre- or posttreatment with beraprost significantly reduced ALT levels as compared with the Con A group, and the levels did not differ significantly as compared with the Beraprost group (data not shown).

Histological examination of specimens obtained at 24 h showed that mice in the Con A group had focal or massive necrosis and moderate-to-severe infiltration with mononuclear and polymorphonuclear cells in portal areas (Fig. 3A and C). In contrast, beraprost suppressed the necrosis and cell infiltration (Fig. 3B and D).

**Effect of beraprost on tissue blood flow in the liver**

Because PGI₂ is known to affect antiplatelet aggregation (1) and vasodilation (2), and because Con A induces sinusoidal endothelial cell damage and intrasinusoidal hemostasis in the liver (26, 27), we next evaluated the effect of beraprost on tissue blood flow in this model. Con A injection dramatically decreased blood flow to 20% of normal
flow (Con A group, 4.8 ± 1.0 ml/min/100 g vs. Normal group, 23.0 ± 1.2 ml/min/100 g, \( P < 0.01 \)). In contrast, blood flow in the beraprost-treated group was increased to twice that of the Con A group (Beraprost group, 10.1 ± 0.8 ml/min/100 g, \( P < 0.05 \)) (Fig. 4), although beraprost did not completely restore blood flow. These results indicate that beraprost partially attenuated ConA-induced blood flow insufficiency.

Effect of beraprost on serum levels of TNF-\( \alpha \), IFN-\( \gamma \), and IL-6

Proinflammatory cytokines, such as TNF-\( \alpha \), play an important role in the pathogenesis of liver injury, particularly in the Con A model (15, 16, 20, 21). We therefore evaluated the kinetics of TNF-\( \alpha \) levels during Con A-induced liver injury. In the Con A group, the serum level of TNF-\( \alpha \) reached its peak at 6 h after the Con A injection, and beraprost significantly inhibited the induction of TNF-\( \alpha \) at 6 h by Con A (Con A group, 10.0 ± 4.8 pg/ml vs. Beraprost group, 65.2 ± 23.8 pg/ml, \( P < 0.05 \)) (Fig. 5A). Because IFN-\( \gamma \) is also an important modulator of liver injury in this model (17–19), we evaluated serum levels of IFN-\( \gamma \). In contrast to the rapid induction of TNF-\( \alpha \), IFN-\( \gamma \) levels peaked at 24 h after Con A injection, and beraprost significantly decreased IFN-\( \gamma \) levels at 12 h (Con A group, 372 ± 228 pg/ml vs. Beraprost group, 128 ± 82 pg/ml, \( P < 0.05 \)) (Fig. 5B). IL-6, like TNF-\( \alpha \) and IFN-\( \gamma \), is an inflammatory cytokine and has been
Antia Laboratories Inc. reported to ameliorate hepatitis through mechanisms that include reduced production of TNF-α and IFN-γ (15, 17). In the Con A group, serum levels of IL-6 increased time-dependently, and reached maximum levels at 24 h. Interestingly, beraprost significantly enhanced IL-6 production at 6 h (Con A group, 2240 ± 50 pg/ml vs. Beraprost group, 4420 ± 580 pg/ml, P<0.05), and then the levels decreased time-dependently (Fig. 5C).

Fig. 5. Time course of plasma tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-6 levels in concanavalin A (Con A)-treated mice with or without beraprost. Beraprost significantly suppressed the Con A-induced increase in plasma TNF-α (A) and IFN-γ (B) levels at 6 and 12 h after Con A injection, respectively. In contrast, beraprost enhanced the levels of IL-6 (C) at 6 h. Closed squares and closed circles represent the Con A group (Con A alone) and Beraprost group (Con A plus beraprost), respectively. Data are expressed as mean ± SEM. *Indicates a statistically significant difference (P<0.05) as compared with mice not treated with beraprost.

Beraprost inhibits Con A liver injury

Fig. 6. Effects of beraprost on tumor necrosis factor (TNF)-α and interferon (IFN)-γ production in splenic cells. Beraprost suppressed TNF-α production but enhanced IFN-γ production in splenic cells. Data are expressed as mean ± SEM. **Indicates a statistically significant difference (P<0.01) as compared with splenic cells not treated with beraprost.

Effect of beraprost on TNF-α and IFN-γ production by Con A-stimulated splenic cells

Since our in vivo experiments showed that beraprost suppresses the induction of TNF-α and IFN-γ, we next evaluated the effect of beraprost on the levels of these cytokines in isolated splenic cells. Under basal conditions without Con A stimulation, splenic cells produced small amounts of TNF-α, and this effect was inhibited by beraprost. Con A-stimulated TNF-α production to four times that of the unstimulated condition, and beraprost significantly inhibited Con A-induced TNF-α production (Con A, 562 ± 83 pg/ml vs. Beraprost, 443 ± 61 pg/ml, P<0.05) (Fig. 6A). In contrast, beraprost stimulated IFN-γ production when splenic cells were stimulated by Con A in vitro (Con A, 245 ± 35 pg/ml vs. Beraprost, 472 ± 43 pg/ml, P<0.05) (Fig. 6B).
Discussion

In the present study, we demonstrated that beraprost attenuated Con A-induced liver injury and lowered the mortality rate, plasma ALT levels, and histological signs of liver damage (Figs 1–3). There are two possible mechanisms by which beraprost ameliorated liver damage; namely, maintenance of hepatic blood flow and suppression of cytokine production. In our model, Con A injection reduced hepatic blood circulation to about 20% of normal; the values in beraprost-treated mice were two times that of the Con A-treated mice (Fig. 4). Con A treatment led to prominent intrasinusoidal hemostasis, which consisted of erythrocyte agglutination, lymphocyte/neutrophil adhesion to endothelial cells, and platelet aggregation and degranulation, resulting in a marked decrease in intrahepatic blood flow (26). These agglutination activities of Con A were independent of T cell activation mediated by TNF-α/IFN-γ production, suggesting that intrasinusoidal hemostasis is important for the development of Con A-induced hepatic injury. The biological effects of PGI2 include antiplatelet aggregation (1), vasodilation (2), and anti-leukocyte adherence (33). It was reported that antithrombin III, an anticoagulant factor, prevented Con A-induced liver injury through production of PGI2 (34). These findings, together with those of the present study, indicate that beraprost ameliorates Con A-induced liver injury in part through a direct effect on hepatic sinusoidal circulation. Recently, beraprost was reported to increase the production of nitric oxide (NO) in endothelial cells by increasing the expression of endothelial nitric oxide synthase (eNOS), which is a main regulatory enzyme catalyzing the production of NO from arginine (35). Since NO is a major regulator of vascular tone, it is of interest to study the effects of beraprost on NO production and eNOS expression in hepatic sinusoidal endothelial cells and the accompanying changes in hepatic blood circulation.

In the Con A-induced model, TNF-α and IFN-γ play central roles in the development of massive hepatocellular apoptosis and necrosis (15–25). In contrast, IL-6 alleviates the hepatitis through mechanisms which include the reduced production of TNF-α and IFN-γ (15, 17). In our Con A model, maximal TNF-α and IFN-γ levels were observed at 6 and 24 h after injection, respectively, which is in agreement with previous reports (16, 18, 19). Beraprost significantly reduced both TNF-α and IFN-γ levels, suggesting that beraprost attenuated Con A-induced liver damage via inhibition of these key cytokines. In contrast, the beraprost-treated group showed transient but significant increases in IL-6 levels at 6 h after injection, which decreased thereafter, while in the control group IL-6 levels continually increased over time. Previous studies have shown that IL-6 is protective when administrated prior to Con A injection (15, 17, 36). However, Con A-induced liver damage itself triggers IL-6 expression, and there is a close correlation between the increase in transaminases and IL-6 levels (37) (Figs 2 and 5). Although we do not know the precise reasons why IL-6 increased significantly in the Beraprost group compared with that of control group 6 h after injection, there are two possible explanations. First, beraprost might directly stimulate IL-6 production in cells involved in Con A-induced liver damage, such as Kupffer cells (hepatic macrophages), as suggested by a report that PGI1 and its analogues (cilostam and cicaprost) increased IL-6 production in peritoneal macrophages in vitro (38). Second, the early increase in IL-6 levels at 6 h and decrease at 12 h in the Beraprost group might reflect the early regeneration of the liver, since IL-6 has been identified as an essential mediator of liver regeneration (39). It seems reasonable that liver regeneration began earlier in the Beraprost group than in the Con A group, because less liver damage was observed in the Beraprost group than in the Con A group. It is also noteworthy that the IL-6 levels at 24 h in the Con A group were much higher than those at 6 h in the Beraprost group. At present, the ultimate role of IL-6 in the Con A model is unclear, but the results of our study and those of previous studies suggest that IL-6 has cytoprotective effects prior to and/or in the early phase of Con A-induced liver damage.

In regard to a link between increased blood flow and reduced cytokine production by beraprost, TNF-α, which was reduced by beraprost, might play an important role since TNF-α upregulates cell surface adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), during Con A-induced liver damage (40). As the inflammatory response progresses, upregulation of ICAM-1 in hepatic sinusoidal endothelial cells contributes to the recruitment of leukocytes including monocytes and lymphocytes, and is also associated with impaired hepatic parenchymal microcirculation (41). In fact, an anti-ICAM monoclonal antibody was reported to inhibit Con A-induced hepatitis (40). Therefore, beraprost might increased hepatic blood flow via inhibition of TNF-α as well as by its direct vasodilatory action.

To investigate the effects of beraprost on cytokine production, we used cultured splenic
cells. As we expected, beraprost significantly inhibited TNF-α production in these cells (Fig. 6A). PGI₂ and its analogs increase cAMP levels via prostaglandin receptors, which then activates PKA signal transduction. The activation of PKA inhibits T cell proliferation and cytokine production (29) through inhibition of the transcriptional activity of NF-κB (30), which plays a key role as a transcriptional factor for inflammatory cytokines. However, beraprost increased Con A-induced IFN-γ production in splenic cells in vitro (Fig. 6B). As we described above, beraprost in vivo significantly suppressed Con A-induced IFN-γ production, and it is difficult to explain the discrepancy between the in vivo and in vitro results. In the Con A model, hepatocytes, CD4-positive T cells, liver natural killer T (NKT) cells, polymorphonuclear cells, and Kupffer cells are involved in liver cell damage (12, 42, 43). Recently, it has been shown that liver NKT cells are essential in the early phase of Con A-induced liver failure, since genetic elimination of liver NKT cells results in unresponsiveness to Con A injection (42, 44). In a later phase, after liver NKT cells are eliminated, there is an influx of immune-activated cells, such as CD4-positive T cells and Kupffer cells, which become activated and cause an increase in the levels of several cytokines, e.g. TNF-α, IFN-γ and IL-6 (12, 16, 17). Therefore, further in vivo and in vitro studies on different cell types in the liver, such as NKT and Kupffer cells, will be required to determine whether the effect of beraprost on cytokine production is therapeutically effective for the treatment of hepatic injury.

Beraprost has been already approved clinically for peripheral arterial occlusive disease (7) and for patients with primary pulmonary hypertension (8). Recently, beraprost has also been reported to attenuate glomerulonephritis (9, 10) and warm ischemia/reperfusion damage of liver grafts in rats (11). These studies, together with the results of our own investigations, suggest that beraprost may have therapeutic potential for various conditions, including liver injury. Further clinical studies will be required to determine whether beraprost is effective for the treatment of liver injury.

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