Effect of Prostaglandin E1 on the Expression of Monocyte Chemotactic Protein-1 in Rats Kupffer Cells of Hepatic Ischemia-Reperfusion Injury

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Abstract **Objective** To study the expression of monocyte chemotactic protein-1 (MCP-1) in rats kupffer cells (KCs) of hepatic ischemia-reperfusion injury (IRI) and the effect of prostaglandin E_1 (PGE_1) on it. **Methods** seventy-two SD rats were divided at random into three groups: the sham operation group (control group), the ischemia-reperfusion group (IRI group) and the PGE_1 treatment group (PGE_1 group). A model of partial warm ischemia/reperfusion injury in the rat liver was established. PGE_1 were given ten minutes before the operation. One hour, six hours, twelve hours and twenty-four hours after the reperfusion, Kupffer cells (KCs) were isolated and incubated. Tumor necrosis factor alpha (TNF-α)and interleukin-1β (IL-1β) in the supernatants were measured by enzyme linked immunosorbent assay (ELISA). The expression of MCP-1 in KCs was detected by immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR). **Results** The mRNA and protein expression of MCP-1 and the level of TNF-α and IL-1β in the IRI group significantly increased in the course of the reperfusion and slightly decreased at 24 h, but were still significantly higher than those in the control group ($P<0.05$). The expression of these factors were markedly decreased after PGE_1 treatment compared with the IRI group ($P<0.05$). **Conclusion** PGE_1 protect against ischemia/reperfusion injury of the rat liver partially by restraining the KCs activation, reducing the excess release of TNF-αand IL-1βfrom KCs and decreasing the high expression of MCP-1 protein and mRNA in KCs.

Key word Prostaglandin E_1; Liver; Ischemia-reperfusion; Kupffer cell; Monocyte chemotactic protein-1; Cytokine

Hepatic ischemia/reperfusion injury (IRI) is a common pathological process of traumatic surgical diseases in the liver, such as severe liver trauma, extensive hepatic lobus excision, liver transplantation, shock and infection [1]. It can cause a series of injuries on metabolism, structure, and function in hepatic tissues and cells, and even liver function failure. It is also one of the major factors influencing the prognosis, operative success and survival of patients. The mechanisms of liver injury caused by I/R resemble those observed in other organs that are transiently deprived of oxygen. These mechanisms involve a series of events that include Kupffer cells (KCs) activation, cytokine release, neutrophil activation, increased expression of adhesion molecules, sinusoidal endothelial cell death, and hepatocyte injury [2]. Among these, the activation and the excessive release of cytokine in KCs play a major role.

Prostaglandin E_1 (PGE_1) is a pulmonary and systemic vasodilator with anti-inflammatory properties [3]. Studies [4,5] show that PGE_1 have beneficial effects on liver injury after ischemia/reperfusion and can depress the level of hepatic transaminases in the blood serum of the rats during the liver transplantation. But the complex mechanisms are unclear until now. We postulate that PGE_1 might have beneficial effects on inflam-

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matory responses through restraining the KCs activation. In order to test this hypothesis, we establish a model of rat hepatic ischemia/reperfusion injury at the room temperature, isolate the KCs at different times, that is, 1h, 6h, 12h and 24h after the onset of reperfusion, to evaluate whether PGE_1 attenuates the release of some cytokines (TNF-α, IL-1β and MCP-1) from KCs, which is probably an important factor in the protection mechanism against ischemia/reperfusion injury.

MATERIALS AND METHODS

Materials

Prostaglandin E_1 was obtained from Harbin White Swan Pharmaceutical Co., Ltd (China). Type Ⅳ collagenase, percoll and MCP-1 polyclonal antibody were purchased from Sigma, Inc (America). Triozol was from Invitrogen, Inc (America). RNA PCR Kit and DNA Maker were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd (China). Lysozyme polyclonal antibody and SABC kit were produced by Wuhan Boshide Biological Engineering Co.,Ltd, (China). TNF-α and IL-1β ELISA kit was obtained from Shanghai Sengxiong Biotech Industry Co.,Ltd,(China).

Animals

Male Sprague Dawley rats weighing 250~300g were used in the study. They were obtained from the Experimental Animal Center of the Xi´an Jiaotong University. All animals were housed in a macroion cage in rooms maintained at 22~24℃ using a 12/12-h light/dark cycle. The animals were given a standard rat chow and fasted over night before the experiment with water allowed *ad libitum.* Care was provided in accordance with the procedure outlined in the "Guide for the care and use of Laboratory Animals" (NIH publication No.85-23, revised 1996). The study was approved by the subcommittee on research animal care at our institution.

Hepatic IRI Model in Rats

Seventy-two SD rats were divided at random into three groups: the sham operation group (control group), the ischemia/reperfusion injury group (IRI group) and the PGE_1 treatment group (PGE_1 group). The trial rats were starved for 12 h but were allowed water. The model of the partial warm hepatic ischemia/reperfusion was based on the Nauta et al method [6]. In the experiment, the rats were anesthetized with aether. For the control group and the IRI group,

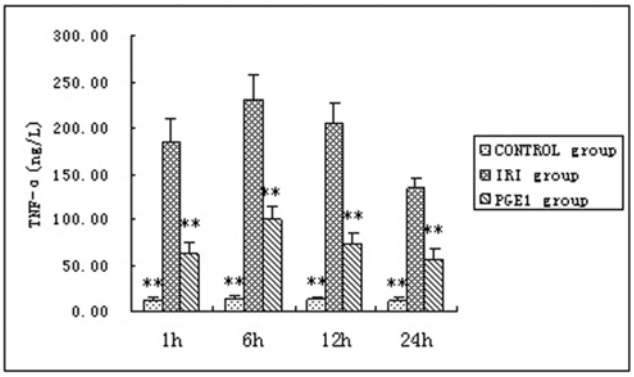

Figure 1 Comparison of TNF-α production in supernatant of KCs. (n=6, mean±S.E)

In the IRI group, the TNF-α level in the supernatant was increased with time, and reached the maximum (230.6±26.3 μg/L) at 6 h following reperfusion (P<0.01). But in the PGE_1 group, the production of TNF-α was obviously inhibited compared with that in the IRI group (P<0.01).

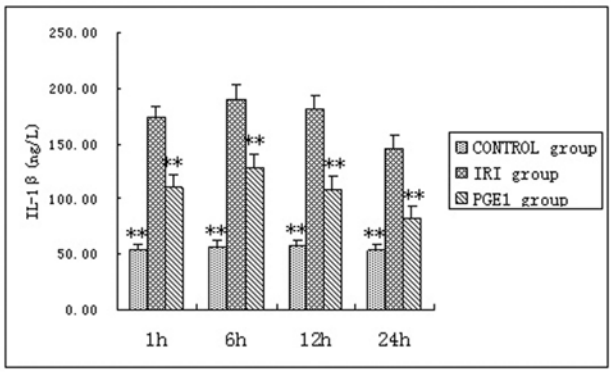

Figure 2 Comparison of IL-1β production in supernatant of KCs. (n=6, mean±S.E)

The concentrations of IL-1β were significantly increased with time, reaching its maximum (189.8±13.13μg/L) at 6h following reperfusion (P<0.01). Significant decreases were observed in the PGE_1 group when compared to the IRI group (P<0.01)

3mL saline containing heparinate (125U/mL) were injected through the penile vein. The rats in the PGE_1 group were injected with 3mL saline containing PGE_1 (50μg/kg) and heparinate (125U/mL). The liver was exposed by a midline incision. The left lateral and median hepatic lobes were occluded with a microvascular clamp for 45 min. After 45 min of ischemia, reperfusion was induced by removing the vascular clamp. The sham control rats underwent the same treatment, but without vascular occlusion.

KCs isolation and incubation

KCs were isolated 1h, 6h, 12h and 24h after the reperfusion by a collagenase perfusion technique described by Knittel et al [7]. Briefly speaking, the liver was perfused for 30 min with D-Hanks containing 0.01%EDTA and removed to smash by scissors. The resulted stuff was digested for 45 min in a solution of 0.5g/L type Ⅳ collagenase and was filtered through 200-mesh strainer. The filtrate was washed thoroughly and suspended in 3mL PBS, layered on 3mL 25% percoll solution and 3mL 50% percoll solution, centrifuged at 2500r/min for 25 min to pellet the cells. The freshly isolated cells were suspended in DMEM~ medium supplemented with 25mM HEPES, 10% fetal bovine serum, penicillin (100U/mL), streptomycin (100μg/mL), and L-glutamine (2mM). Cell suspensions (4mL, 8×10^6cells) were plated on 60-mm culture dishes and maintained in an incubator at 37℃ in a humidified atmosphere of 90% air-10% CO_2 for 1h. The supernatants were discarded and the adherent cells were incubated for 4h. The cellular components of adherent cells were examined by Lysozyme immunohistochemical staining to determine the percentage of KCs. The percentage of KCs in the adherent cells was over 90%. The cells were counted after the trypan blue exclusion. The viability of the cells was over 90% in the adherent cells. The KCs and supernatants in all the groups were collected and stored in the refrigeration at -70℃ for subsequent analysis.

Expression of TNF-α and IL-1β in the supernatants

TNF-α and IL-1β in the supernatants were measured by ELISA kits. The concentrations of TNF-α and IL-1β were estimated, based on the absorbance read by an ELISA reader (EL800) at 450 nm.

Immunohistochemical detection of MCP-1 protein in KCs

Immunohistochemical staining of KCs was performed with the strept- avidin-biotin-peroxidase complex (SABC) method. PBS takes the place of MCP-1 anti-body as the negative staining control. By using an image analysis system, the values of the mean optical density were measured under the same magnification (original magnification×400).

Determination of MCP-1 mRNA by semiquantitative RT-PCR

Total RNA was isolated from rat KCs by using Trizol reagent. One microgram of RNA was reverse-transcribed to complementary DNA by using RNA PCR Kit according to the manufacturer´s instructions. The

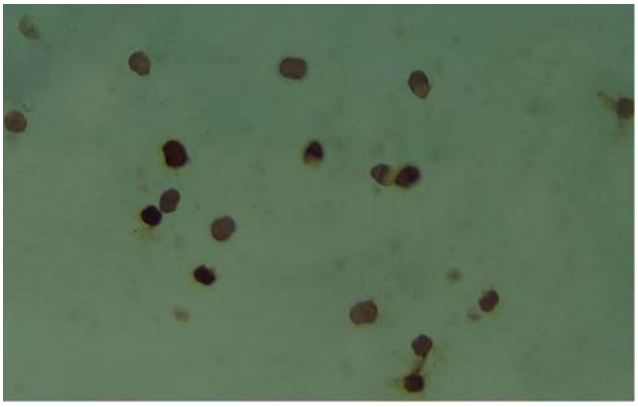

Figure 3-A Immunohistochemical staining of MCP-1 in KCs in IRI group at 12h interval (SP×400)

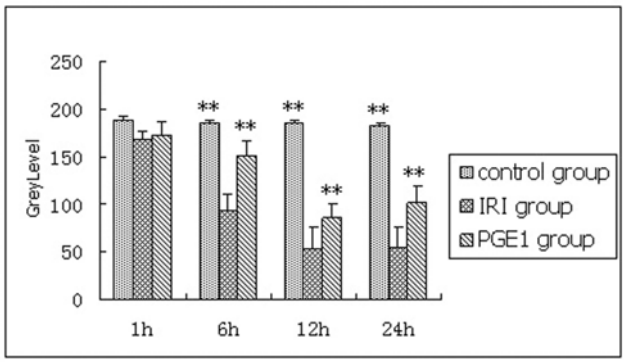

Figure 3-B The synthesis of MCP-1 protein in KCs tested by immunohistochemical(n=6, mean±S.E)
**P<0.01 as compared with IRI group

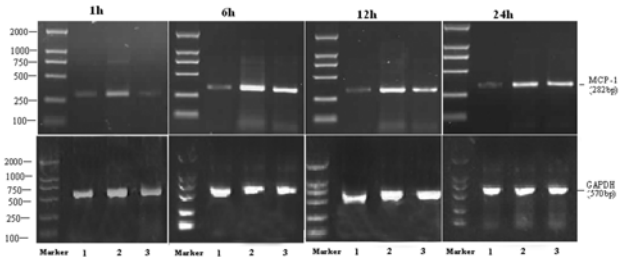

Figure 4-A Expression of MCP-1 mRNA in KCs tested by RT-PCR

PCR products were electrophoresed on agarosegels and photographed. The track numbered 1 is for the control group, numbered 2 for the IRI group and 3 for the PGE_1 group.

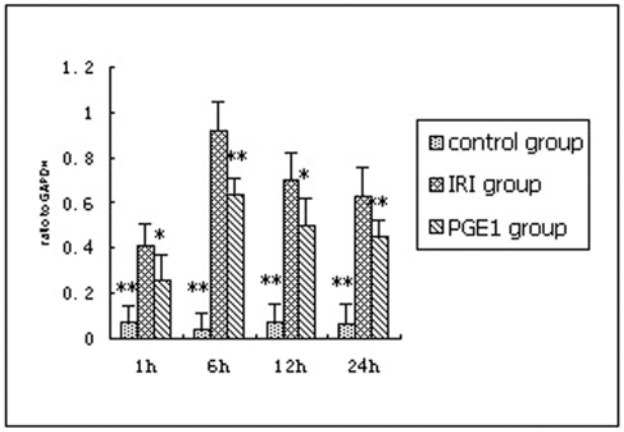

Figure 4-B Quantitative date of MCP-1mRNA levels (n=6, mean±S.E)

* P<0.05 as compared with IRI group, **P<0.01 as compared with IRI group.

Quantitative data of MCP-1mRNA levels were represented by the ratio of relative absorbance and expressed as mean±SD. The expression of MCP-1 mRNA significantly increased in the IRI group compared with that in the control group (P<0.01), and in the PGE_1 group it slightly declined compared with the IRI group (P<0.05), but was still significantly higher than that in the control group (P<0.01).

PCR primers were synthesized on the basis of GenBank data. The primers were chemically synthesized by using DNA synthesizer (Tiangen Biotech Co., Ltd, Beijing China). Their sequences were 5-CCAATGAGTCGGCTGGAGAA-3, 5-GCTTGAGGTGGTTGTGGAAA-3 for MCP-1, and 5-CATAGACAAGATGGTGAAGG-3 ,5-TCCACAGTCTTCTGAGTGGC-3for GAPDH. All the PCR reactions had an initial denaturation step at 94℃ for 3 min, and a final extension at 72℃ for 5 min by using PTC-100 (MJ Research, Inc. U.S.A.).The PCR amplification cycling conditions were 94℃ 30s, 55℃ 30s, 72℃ 30s, 40 cycles for MCP-1; 94℃ 30s, 53℃ 30s, 72℃ 30s, 35 cycles for GAPDH. Following RT-PCR, 5 μL samples of amplified products were resolved by electrophoresis in 1.5% agarose gel, and stained with ethidium bromide. The intensity of each PCR product was semi-quantitatively evaluated by using the labworks analysis software (UVP, Inc, Upland, CA, U.S.A.).

Statistical analysis

Results are expressed as mean±S.E. Statistical calculations were done by using the SPSS10.0 software package. One-factor analysis of variance was applied in order to establish whether the differences among the three groups were statistically significant. In all the cases, $P < 0.05$ were considered to be statistically significant.

RESULTS

Measurement of the levels of TNF-α, IL-1β in the supernatants

As shown in the Figure 1 and Figure 2, the concentrations of TNF-α, IL-1β were significantly increased with time, reaching its maximum at 6 h following reperfusion (P<0.01). Significant decreases were observed in the PGE_1 group when compared to the IRI group (P<0.01)

Measurement of the levels of MCP-1 protein in KCs

Immunohistochemical studies (Figure 3) showed MCP-1 did not or weakly expressed in the control group. Its expressions in the IRI group had a significant increase at 6h, 12h and 24h interval (P<0.01), which were contrary to the control group, but there were no difference at 1h interval (P>0.05). The levels of MCP-1 protein lessened in the PGE_1 group at 6h, 12h and 24h intervals when compared with the IRI group (P<0.01), but there was no difference at 1h interval (P>0.05).

Expression of MCP-1 mRNA in KCs

RT-PCR analysis (Figure 4) showed that KCs from the control group had low but detectable levels of MCP-1 mRNA. The mRNA level was significantly increased with time, reaching its maximum at 6 h following IR, and slightly declined 24 h after I/R, but was still significantly higher than that in the control group ($P<0.01$). However, in the PGE_1 group, these changes were significantly inhibited by the treatment of PGE_1 ($P<0.01$).

DISCUSSION

Kupffer cells, the most multitude resident macrophage in corpora, play a central role in mediating liver after ischemia/reperfusion. Being their topological localization within the liver sinusoids, they represent the first line of defense against viruses entering the liver through the portal circulation. The functions of Kupffer cells are activated by a variety of particles and substances, including viruses, bacterial, lipopolysaccharide (LPS), muramyl dipeptide, gamma interferon, and tumor necrosis factor alpha (TNF-α). The phagocytosis of parasites by Kupffer cells is accompanied by the release of proinflammatory cytokines that act primarily as a paracrine signal on neighboring hepatocytes and induce chemotaxis and aggregation of neutrophils. Furthermore, Kupffer cells express class II major histocompatibility complex molecules, as well as processing and presenting antigens. [8] Therefore, Kupffer cells are the main sources of cytokine and chemokine formation during the reperfusion period.

Chemokines are members of a large and expanding family of proteins that interfere with normal (leukocyte homing, hematopoiesis, angiogenesis) and pathological (inflammation, HIV-1 infection, atherosclerosis) processes [9~13]. Depending on the positioning of the cysteine residues, chemokines are classified as C, CC, CXC and CX3C chemokines. MCP-1 is from the CC (cysteinecysteine) chemokine subfamily. These soluble factors are involved in the regulation of cell-mediated immunity [14]. MCP-1 is produced by monocytes, macrophages, lymphocytes, and other cell types. Kupffer cells are both a target and a source of chemokines. Therefore, we examined the expression of MCP-1 in Kupffer cells following ischemia/reperfusion injury. The MCP-1 proteins were not detected by immunohistochemical staining in the cells of the control group and the cells at 1 h following reperfusion. Likewise, synthesizing of MCP-1 mRNA in the cells of the control group was not active. Following reperfusion injury, expression of MCP-1 protein and mRNA significantly increased. This may indicate that the genes MCP-1 are inactive in unactivated Kupffer cell. Some inflammatory factors stimulate MCP-1 gene in Kupffer cells during the reperfusion injury. This finding is in agreement with that of the research by Bukara M et al [15]. The roles of MCP-1 in the pathological process of hepatic ischemia/reperfusion injury are not well known. But previous study has proved MCP-1 can induce mononuclear macrophage to secrete TNF-α and IL-1β [16]. Therefore we suppose MCP-1 may play a negative role in the Kupffer cell activation following hepatic ischemia/reperfusion injury.

In the present study we find that hepatic ischemia/reperfusion injury is related with the increased toxic amounts of TNF-α and IL-1β released from activated Kupffer cells. Each of these inflammatory factors is involved in the pathogenesis of liver injury. TNF-α and IL-1β appear to be a key mediator of the inflammatory response in Kupffer cells, and it functions as a survival factor in hepatocytes under certain physiological conditions, for example, liver regeneration. Consequently, suppression of TNF-α and IL-1β activity can have beneficial or deleterious effects depending on the condition being studied. Our study has presented evidence that activated Kupffer cells produce toxic mediators during the process of hepatic ischemia/reperfusion injury. The mechanism of induction of TNF-α and IL-1β bioactivity after hepatic ischemia/reperfusion is complex. To make the most of its function, TNF-α needs the participation of other inflammatory cytokine including IL-1 and IL-12. Likewise, TNF-α can evoke the release of IL-1 from Kupffer cells and vice versa.[17] Studies show that LPS, IL-1β and TNF-α can induce the expression of MCP-1 [18,19]. On the

contrary, the high expression of MCP−1 activates mononuclear macrophage to release superoxide anion and lysosomal enzyme, and to produce TNF−α, IL−1β and other inflammatory factors [16]. Therefore, TNF−α、IL−1β、MCP−1 and other inflammatory factors compose a complicated cytokine network and the hepatic injury is aggravated through this infernal circle.

PGE_1 is a pulmonary and systemic vasodilator with anti−inflammatory properties.[3] Studies [20] showed it can ameliorate microcirculation, abate acid−intoxication, and degrade partial pressure of carbon dioxide in artery, but it will not cause hepatic portal vein vasorelaxation, or rather, it has no effect on the pressure of hepatic portal vein. Therefore, the blood supply of liver has no decrease and will not lead to the deficiency of nutritional ingredients. Recent researches [3−5, 21] have presented evidence showing PGE_1 has benefits to the liver injury of ischemia/reperfusion. Similarly, the remarkable protection against ischemia/reperfusion injury displayed by PGE_1 in our model conforms favorably to the previous putative treatments. Our study demonstrates that PGE_1 protects against the development of hepatitis by suppressing TNF−α and IL−1β produced by rat Kupffer cells. Further researches show that the gene of MCP−1 in Kupffer cells is also inhibited. Therefore, the liver damage caused by reperfusion would be effectively attenuated by PGE_1.

In conclusion, the present study shows that PGE_1 is an ideal drug to attenuate hepatic ischemia/reperfusion injury by inhibiting the releasing of TNF−α and IL−1β and the expression of MCP−1 in Kupffer cells. Further studies are necessary to determine its optimal dosage, administration time, and combination with other anti−inflammatory regimens to obtain the most effective protection from hepatic ischemia/reperfusion injury, especially in larger animals. These therapies may provide a better understanding of the protection function of PGE_1 in the fields of hepatic surgery, preservation, and rejection.

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