

RAPID COMMUNICATION

Type I inositol 1, 4, 5-triphosphate receptors increase in kidney of mice with fulminant hepatic failure

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Abstract

AIM: To delineate the mechanisms of renal vasoconstriction in hepatorenal syndrome (HRS), we investigated the expression of type I inositol 1, 4, 5-triphosphate receptors (IP₃R I) of kidney in mice with fulminant hepatic failure (FHF).

METHODS: FHF was induced by lipopolysaccharide (LPS) in D-galactosamine (GalN) sensitized BALB/c mice. There were 20 mice in normal saline (NS)-treated group, 20 mice in LPS-treated group, 20 mice in GalN-treated group, and 60 mice in GalN/LPS-treated group (FHF group). Liver and kidney tissues were obtained at 2, 6, and 9 h after administration. The liver and kidney specimens were stained with hematoxylin-eosin for studying morphological changes under light microscope. The expression of IP₃R I in kidney tissue was tested by immunohistochemistry, Western blot and reverse transcription (RT)-PCR.

RESULTS: Kidney tissues were morphologically normal at all time points in all groups. IP₃R I proteins were found localized in the plasma region of glomerular mesangial cells (GMC) and vascular smooth muscle cells (VSMC) in kidney by immunohistochemical staining. In kidney of mice with FHF at 6 h and 9 h IP₃R I staining was up-regulated. Results from Western blot demonstrated consistent and significant increment of IP₃R I expression in mice with FHF at 6 h and 9 h ($t = 3.16, P < 0.05$; $t = 5.43, P < 0.01$). Furthermore, we evaluated IP₃R I mRNA expression by RT-PCR and observed marked up-regulation of IP₃R I mRNA in FHF samples at 2 h, 6 h and 9 h compared to controls ($t = 2.97, P < 0.05$; $t = 4.42, P < 0.01$; $t = 3.81, P < 0.01$).

CONCLUSION: The expression of IP₃R I protein increased in GMC and renal VSMC of mice with FHF, possibly caused by up-regulation of IP₃R I mRNA.

INTRODUCTION

Patients with fulminant hepatic failure (FHF) are at high risk for the serious complication of hepatorenal syndrome (HRS), which is an important cause of death^[1]. HRS is a functional renal failure secondary to the liver failure itself^[2,3]. Renal failure will recover when there is recovery of liver function, and in the absence of a spontaneous hepatic recovery, liver transplantation will reverse the HRS. Until now, its precise pathogenesis is not clear. It is considered generally that the decrement of renal plasma flow (RPF) caused by renal vasoconstriction is a key factor^[4]. Multiple mechanisms are involved in renal vasoconstriction. Cell contraction is closely related to changes of intracellular calcium ([Ca²⁺]_i) signaling. The inositol 1, 4, 5-trisphosphate receptors (IP₃Rs) is the primary cytosolic target for the initiation of [Ca²⁺]_i. Many cell types, often nonexcitable, including glomerular mesangial cells (GMC) and renal vascular smooth muscle cells (VSMC), depend on this pathway to couple external signals to intracellular Ca²⁺ release^[5]. IP₃Rs not only triggers the release of calcium from intracellular stores but also opens plasma membrane calcium channels^[6,7]. This is thought to be a crucial step in allowing the cell to contract to some agonists. Many factors can affect the level of IP₃Rs expression^[8,9]. Is there a relationship between the changes in IP₃Rs expression level and HRS progression in FHF? In order to explore the potential mechanisms, we conducted a series of studies on IP₃R I expression in the kidney of mice with FHF.

MATERIALS AND METHODS

Materials

BALB/c mice were provided by Laboratory Animal Center in China Medical University. D-galactosamine

(GalN) and lipopolysaccharide (LPS) were obtained from Sigma. Polyclonal antibody of IP₃R I was purchased from US biological. BCIP/NBT liquid substrate system was purchased from Sigma. RT-PCR kit was purchased from Tskara.

Mouse models of FHF

One hundred and twenty six-week-old male BALB/c mice were divided into 4 groups: 20 mice in NS-treated group, 20 mice in LPS-treated group, 20 mice in GalN-treated group, and 60 mice in GalN/LPS-treated group (20 mice at 2, 6, 9 h, respectively). Mice with FHF were given an intraperitoneal injection of D-galactosamine (GalN, 800 mg/kg body weight), followed by lipopolysaccharide (LPS, 10 µg/kg body weight)^[10]. In control groups, mice were given an intraperitoneal injection of GalN (800 mg/kg body weight), LPS (10 µg/kg body weight), NS (similar volume to GalN and LPS), respectively. Liver and kidney tissues were fixed for histopathologic analysis and immunohistochemistry under light microscopy. Frozen specimens were stored for quantitative analysis of IP₃R I by Western blot and RT-PCR.

Immunohistochemistry for IP₃R I

Sections were indirectly immunolabeled with an ABC kit according to the manufacturer's instructions. Sections were deparaffined, blocked with normal goat serum for 30 min, and incubated with primary antibody (rabbit anti-mouse IP₃R I diluted in phosphate-buffered saline (PBS) 1:100) for 12 h in a humidified chamber at 4°C. These sections were then rinsed thrice for 10 min in PBS, and the secondary antibody (goat anti-rabbit IgG diluted 1:100 in PBS) was applied for 2 h at room temperature. Sections were rinsed in PBS and then in distilled water. Fresh peroxidase reaction mixture containing equal amounts of 0.02% hydrogen peroxide in H₂O and 0.1% diaminobenzidine in PBS were prepared. We chose four fields of vision at random, and analyzed the optical density using the "multi-system color/RGB monitor" computer image processing system.

Western blot for IP₃R I

Total protein of kidney tissues were extracted in lysis buffer (50 mmol/L Tris-HCl (pH 7.2), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L PMSF, and 5 mg/mL each of aprotinin and leupeptin). Protein concentrations were determined by BCA protein assay and equal amounts of protein were run on a 6% SDS-PAGE gel, and transferred to PVDF membrane. The PVDF membrane was then blocked with 5% nonfat dry milk for 2 h. After being blocked, PVDF membrane was immunoblotted with the primary antibody (IP₃R I antibody) for 2 h. The membrane was washed three times with TBS to remove unbound primary antibody. Then the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG for 2 h, washed three times with TTBS to remove unbound secondary antibody, and incubated with BCIP/NBT liquid substrate reagent for 5 min, the reaction was stopped by H₂O. β-actin was used for internal control, and 230KD bands of

IP₃R I and 45KD bands of β-actin were quantitated using a densitometer (model GS-700, Bio-Rad Laboratories). Band volumes were calculated with the ratio of IP₃R I to β-actin bands.

RT-PCR for IP₃R I mRNA

Total RNA was extracted using TRIzol following manufacturers' protocol. RNA concentrations were determined by UV analysis and diluted to 100 ng/µL with DEPC water. RNA was incubated at 30°C for 10 min followed by 42°C for 30 min and 99°C for 5 min for reverse transcription (RT). cDNA underwent 35 cycles of PCR (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min). The sense and antisense primers used were 5'-GGTTTCATCTGCAAGCTAATAAAA -3' and 5'-AATGCTTTTCATGGAATACTCGGTC -3', respectively. After PCR, 5 µL sample was run on the gel and a PCR amplified product of 525 bp was observed under an ultraviolet illuminator. GAPDH was used for internal control. The sense and antisense primers of GAPDH were 5'-GACAACCTTTGGCATCGTGGA-3' and 5'-ATGCAGGGATGATGTTCTGG-3'. A PCR product of 133 bp was observed, 525 bp bands of IP₃R I and 133 bp bands of GAPDH were quantitated using a densitometer. Band volumes were calculated with the ratio of IP₃R I to GAPDH bands.

Statistical analysis

Software SPSS 11.0 was used in statistical analysis. Each parameter was expressed as mean ± SE, and compared using Student's *t*-test. *P* < 0.05 was considered statistically significant.

RESULTS

GalN/LPS induced FHF models

Administration of LPS (10 µg/kg body weight) and GalN (800 mg/kg body weight) can induce FHF. Nine hours after GalN/LPS administration, the mortality of mice reached 60%. Liver tissues stained with hematoxylin-eosin presented severe hemorrhage and hepatic necrosis in 2, 6 and 9 h FHF groups. Kidney tissues were morphologically normal at all time points in all groups. Therefore, we concluded that the animal models of FHF were established successfully.

Expression of IP₃R I protein with immunohistochemistry

IP₃R I proteins were localized to plasma region of GMC and renal VSMC. IP₃R I protein expressed at low levels in 3 control groups. There was no difference between 2 h FHF group and 3 control groups. IP₃R I -staining was up-regulated in 6 h, 9 h FHF groups. The maximal effect was seen at 9 h (Figure 1).

Western blot for IP₃R I protein expression

In order to quantify the changes of IP₃R I protein, we carried out Western blot analysis for IP₃R I protein expression. An antibody raised to the C terminus of the mouse IP₃R I recognized a 230-kDa polypeptide. The expression of the IP₃R I protein was similar among 3

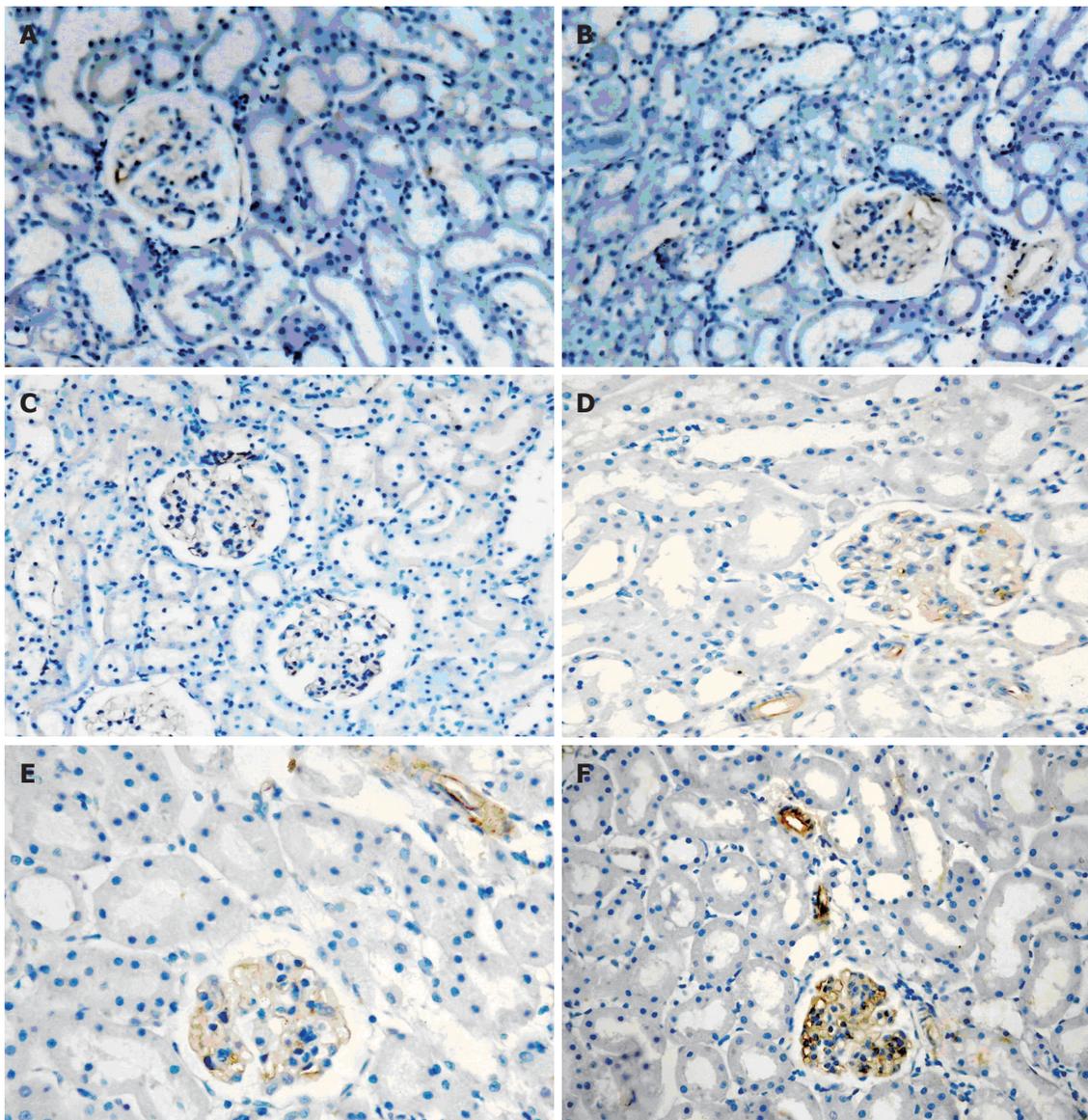


Figure 1 Kidney immunohisto-chemical staining for IP₃R I × 400. **A:** NS-treated group; **B:** LPS-treated group; **C:** GalN-treated group; **D:** 2hGalN/LPS-treated group; **E:** 6hGalN/LPS-treated group; **F:** 9hGalN/LPS-treated group.

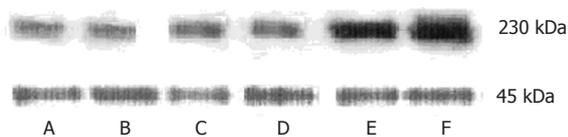


Figure 2 Western blot analysis for IP₃R I protein in kidney. **A:** NS-treated group; **B:** LPS-treated group; **C:** GalN-treated group; **D:** 2hGalN/LPS-treated group; **E:** 6hGalN/LPS-treated group; **F:** 9hGalN/LPS-treated group.

control groups. There was no difference between 2 h FHF group and 3 control groups. The expression of the IP₃R I protein increased to 167% ± 4% by 6 h, 196% ± 5% by 9 h with respect to control values ($t = 3.16, P < 0.05; t = 5.43, P < 0.01$). The maximal effect was seen at 9 h, which is identical to the result by immunohistochemistry (Figure 2).

RT-PCR for the expression of the IP₃R I mRNA

To determine if the effects of increment of the IP₃R I protein expression are caused by an increment of

new protein synthesis, we evaluated possible regulation at the mRNA level of IP₃R I. Quantitative analysis demonstrated an increase to 155% ± 12% of control IP₃R I mRNA expression as early as 2h FHF, and it remained increased at 9 h FHF (173% ± 10%) ($t = 2.97, P < 0.05; t = 3.81, P < 0.01$). The maximal effect was seen at 6 h, and IP₃R I mRNA expression was increased to 192% ± 4% of control values ($t = 4.42, P < 0.01$). Thus, the increment in mRNA for the IP₃R I preceded the increment in protein (Figure 3).

DISCUSSION

HRS is a common complication in patients with FHF and end stage cirrhosis. HRS develops in approximately 55% of all patients with FHF^[11]. There are only functional changes and relatively few histological changes in the kidneys. The pathogenesis of HRS involves in the development of hyperdynamic circulation, lowering of renal perfusion pressure, the activation of sympathetic

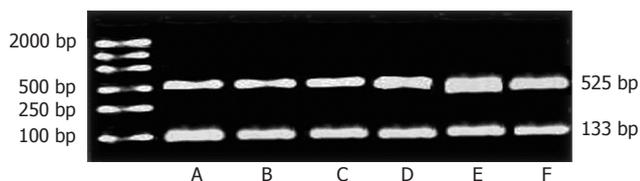


Figure 3 RT-PCR for the expression of IP₃R I mRNA. A: NS-treated group; B: LPS-treated group; C: GalN-treated group; D: 2hGalN/LPS-treated group; E: 6hGalN/LPS-treated group; F: 9hGalN/LPS-treated group.

nervous system, and increased synthesis of a variety of vasoactive mediators, which renders the kidneys more susceptible to decrease in perfusion pressure. Patients with HRS have an obvious reduction in renal blood flow and the glomerular filtration rate (GFR). The hallmark of HRS is renal vasoconstriction. Multiple mechanisms may be involved in renal vasoconstriction^[12]. The circulating concentrations of many vasoconstrictors including endothelin and angiotensin II were markedly high in HRS^[13,14]. These mediators can cause renal vasoconstriction, but more importantly they can also decrease the glomerular capillary ultrafiltration coefficient (Kf), thus causing a decline of GFR over and above that caused by renal vasoconstriction alone. As it is known, contraction of renal VSMC can result in a reduction in renal blood flow, while contraction of GMC can lower the filtration fraction and glomerular Kf^[15]. Both GMC and renal VSMC are sensitive to vasoconstrictor. As a result, GFR was decreased obviously in HRS. Both endothelin and angiotensin II were important renal vasoconstrictors that stimulate IP₃-mediated [Ca²⁺]_i mobilization via the IP₃Rs, followed by release of stored intracellular Ca²⁺ and Ca²⁺ entry through plasma membrane channels^[16]. IP₃Rs are the intracellular Ca²⁺ release channels that play a key role in Ca²⁺ signal in cells^[17]. Cell contraction is closely connected with changes in intracellular Ca²⁺ concentration. IP₃Rs is localized to endoplasmic reticulum. There is also evidence suggesting the presence of functional IP₃Rs on the plasma membrane^[18]. IP₃Rs contains four independent ligand binding sites that are cooperative with respect to calcium channel opening^[19]. In mammalian cells, there are at least three isoforms of the IP₃R derived from three distinct genes. IP₃R I is predominant in the GMC and renal VSMC, but absent in other renal cells^[20]. Therefore, our study focused on IP₃R I as a candidate protein that may facilitate intracellular Ca²⁺ release. The level of IP₃R I expression can be affected by many factors such as some cytokines. There might be a relationship between the changes in IP₃R I expression level and renal vasoconstriction in HRS. Theoretically, changes in IP₃R I expression level are linked to regulation of functional [Ca²⁺]_i stores and [Ca²⁺]_i concentration. Wang JY *et al.*^[21] had discovered the up-regulation of expression of IP₃R I on rat glomerular and afferent arterioles in a model of liver cirrhosis by immunohistochemical method^[21]. Does IP₃R I of kidney also increase in mice with FHF? In order to explore the possible pathogenesis of HRS, we examined the expression level of IP₃R I of kidney in mice with FHF by immunohistochemistry, Western blot and RT-PCR.

Our study shows that IP₃R I protein was localized to

the plasma region of GMC and renal VAMC, which is consistent with previous studies. IP₃R I expressed at low levels in control groups. There was no difference between NS-treated group, LPS-treated group and GalN-treated group. Its expression level was still low in 2 h FHF, while increased obviously in 6 h and 9 h FHF. This phenomenon was testified by Western blot quantitative analysis. The results from Western blot showed that the expression of the IP₃R I protein increased to 167% ± 4% by 6 h, and 196% ± 5% by 9 h with respect to control values. The maximal effect was seen at 9 h, and IP₃R I expression was increased to 196% ± 5% of control values. In order to elucidate the reason of increased protein expression of IP₃R I, we evaluated the expression level of IP₃R I mRNA of kidney in mice with FHF by RT-PCR. We concluded that increased protein expression of IP₃R I is caused by increased synthesis of the new IP₃R I protein as an increase in mRNA levels was found in mice with FHF at 2 h, 6 h and 9 h. Up-regulation of the IP₃R I mRNA was demonstrated as early as 2 h FHF. The maximal effect 192% ± 4% of control values was seen at 6 h. There was mild decrement at 9 h in contrast to the level at 6 h, but it was still higher than control values. Our result that the increment in mRNA for IP₃R I precedes the increment in protein is consistent with the time sequence of increased mRNA levels prior to occurrence of increased protein levels. Our finding demonstrates that up-regulation of IP₃R I mRNA levels can be explained by the increase in transcription of the IP₃R I gene. Our observation also indicates that factors that can not directly induce Ca²⁺ flux may also regulate [Ca²⁺]_i concentration by affecting IP₃R I expression. Increased IP₃R I protein expression provided more ligand binding sites for IP₃ that is more beneficial to [Ca²⁺]_i release. Consequently, kidney became more sensitive to agonists that stimulate IP₃-mediated [Ca²⁺]_i mobilization *via* the IP₃R I. Overexpression of IP₃R I may lead to modulatory influences on renal blood flow and GFR. Interaction between IP₃R I up-regulation and agonists may enhance the contracting ability of GMC and VSMC to vasoconstrictors. Overexpression of IP₃R I may be an important factor in contributing to HRS progression.

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