Original Article

PKCα signaling pathway involves in TNF-α-induced IP_3R1 expression in human mesangial cells

Yu-rong Wang, Huan Zhang, Hui Sun, Pei Liu

Department of Digestion, Jiujiang First People's Hospital, Jiujiang 332000, China

Corresponding Author: *Yu-rong Wang, Email: hldwyr2009@163.com*

BACKGROUND: This study aimed to explore the effects of TNF-α on the expression of IP_3R1 mRNA and protein in human mesangial cells (HMCs), and to elucidate the mechanism of TNF-α relating to IP_3R1 expression in the occurrence of hepatorenal syndrome (HRS).

METHODS: HMCs were stimulated by tumor (TNF-α) with 100 ng/mL for different hours (2, 4, 8, and 24 hours). The expression changes of IP_3R1 mRNA and protein were detected by quantitative real-time polymerase chain reaction and immunoblotting. Several inhibitors including D609, U73122, PP1, safingol, rottlerin and non-radioactive protein kinase C (PKC) were used to examine the mechanism of signal transduction of TNF-α-regulated IP_3R1 in HMCs.

RESULTS: The levels of IP_3R1 mRNA at 2 hours after TNF-α exposure were significantly enhanced and peaked at 8 hours in HMCs (P<0.01), then descended at 24 hours (P<0.01). The levels of IP_3R1 protein at 4 hours after TNF-α exposure were obviously increased and peaked at 24 hours after TNF-α exposure (P<0.01). Compared to the control group, safingol (PKCα inhibitor) and D609 (phosphatidylcholine-specific phospholipase C inhibitor) significantly blocked the TNF-α-induced expression of IP_3R1 mRNA (3.30±0.81 vs. 1.95±0.13, P<0.05; 2.10±0.49, P<0.01) and IP_3R1 protein (3.09±0.13 vs. 1.86+0.39, P<0.01; 1.98±0.02, P<0.01). TNF-α promoted PKCα activation with maximal PKCα phosphorylation that occurred 8 hours after stimulation measured by non-radioactive PKC assay, and the effect was markedly attenuated by pretreatment with D609 or safingol.

CONCLUSION: TNF-α increased the expression of IP_3R1 and this was mediated, at least in part, through the PC-PLC/PKCα signaling pathways in HMCs.

KEY WORDS: TNF-α; Hepatorenal syndrome; Human mesangial cells; Protein kinase C; Phosphatidylcholine-specific phospholipase C

World J Emerg Med 2012;3(4):282–286
DOI: 10.5847/ wjem.j.issn.1920-8642.2012.04.008

INTRODUCTION

Hepatorenal syndrome (HRS) is a severe complication in patients with fulminant hepatitis and liver failure, which is associated with the worst prognosis and has been considered as a terminal event of the disease. The hallmark of HRS is renal vasoconstriction.[1,2] Human glomerular mesangial cells (HMCs) are smooth muscle-like cells within the glomerulus, and play a key role in maintaining glomerular capillary integrity and regulating the glomerular filtration area.[3] Little is known about the mechanisms involving in the molecular signaling of decreased glomerular filtration rate (GFR).

The inositol 1,4,5-trisphosphate receptor (IP_3R) is an intracellular Ca^{2+} channel that releases Ca^{2+} from internal Ca^{2+} stores in response to IP_3. Cell contraction is closely connected with changes in intracellular Ca^{2+} concentration.[4] Researches found that increased IP_3R1 expression promotes Ca^{2+} release and cell contraction, which is associated with many diseases such as atrial fibrillation, Alzheimer's disease, Parkinson's disease and asthma.[5–7] A *in vitro* study proved that TNF-α could up-regulate IP_3R1 expression.[7] TNF-α is closely related to

severe hepatitis.[8,9]

Therefore, we hypothesize that the increased IP_3R1 expression induced by TNF-α may provide more ligand binding sites for IP_3 that is more beneficial to Ca^{2+} release, augments the contracting ability of HMC, and modifies the progression of HRS. In the present study, we determined our hypothesis whether TNF-α affected IP_3R1 expression in HMCs, and if so, what kind of mechanisms are involved in TNF-α-induced IP_3R1 expression. It is essential to understand this mechanism of the action of TNF-α in the exacerbation of renal ischemic injury in order to identify target molecules that could modulate the pathogenesis of HRS.

METHODS

Materials

Primary culturing HMCs and mesangial cell medium (MCM 4201) were from the Science Cell Research Laboratories (San Diego, CA). TNF-α were from R&D System (Minneapolis, MN, U.S.A.); pp1, rottlerin, U73122, D609 and safingol were from Calbiochen (Schwalbach, Germany); anti-IP_3R1 and anti-β-actin were from Chemicon International. The PepTag non-radioactive protein kinase C (PKC) assay system was from Promega (Madison, Wisconsin). The Trizol reagent was from Invitrogen. The primer synthesis, reverse transcription kit, ExScriptTM RT Reagent Kit was from Takala (Japanese).

Cell culture and chemical treatment of HMCs

HMCs were grown in MCM containing 2% fetal calf serum (FCS) until reaching 80%–90% confluency, followed by MCM without FCS for 24 hours before TNF-α stimulation for 2 to 24 hours. Then total RNA or protein extraction at various time points were harvested for quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis.

Further experiments were conducted to investigate the regulatory role of PLC, PKC, PP1 in TNF-α-stimulated IP_3R1 expression by incubating cells with selective protein kinase inhibitors. An inhibitor of the phosphatidylinositol-specific phospholipase (PI-PLC) U73122 (5 μmol), an inhibitor of the phosphatidylcholine-specific phospholipase (PC-PLC) D609 (50 μmol), a non-receptor tyrosine kinase inhibitor PP1 (10 μmol), a selective inhibitor of protein kinase C (PKCα), safingol (5 μmol) or a selective inhibitor of protein kinase C (PKCδ), rottlerin (5 μmol) were preincubated with cells for one hour. Then cells were stimulated with TNF-α 100 ng/mL for 8 hours or 24 hours before harvesting for RNA or protein extraction.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from HMCs by the Trizol RNA isolation system in accordance with the instructions of the manufacturer (Invitrogen). The concentration and purity of mRNA were determined by spectrophotometry at 260 and 280 nm. One mg of RNA was reversely transcribed as a template in qRT-PCR on an iCycler (BioRad, Munich, Germany). The PCR reaction mixture at a 25 μL volume contained 12.5 μL of 2×SYBR Green PCR Master Mix, 2 μL RT product, and 0.5 μL sense and anti-sense primer sets. After sequential incubations at 37 °C for 5 minutes and 85 °C for 10 minutes, the amplification protocol consisted of 45 cycles of denaturation at 95 °C for 15 seconds, annealment at 57 °C for 15 seconds, and extension at 72 °C for 20 seconds. Each sample was added in triplicate. The primer sequences were as follows: IP_3R1: 5-TGCCTCCACAATTCTACGACTGA (sense), 5-TCCCACAGTTGCCCACAAAG (anti-sense); β-actin: 5-AGGCATCCTCACCCTGAAGTA (sense), 5-CACACG-CAGCTCATTGTAGA (anti-sense).

A standard curve was constructed from serial dilutions of cDNA synthesized from a known quantity of total RNA for IP_3R1 or β-actin. IP_3R1 and β-actin values in unknown samples were quantified by the measurement of Ct and reading the corresponding value off the standard curve. IP_3R1 expression was then normalized to β-actin expression, and IP_3R1 expression level in medium treated control cells were considered to be "1". All experiments were conducted in triplicates.

Western blot analysis

To detect IP_3R1 protein, cells were treated and washed with ice-cold PBS. Afterwards, the cells were collected and lysed in RIPA buffer. The protein concentration was determined with a BCA protein assay reagent kit. Eighty micrograms were used for 7% (for IP_3R1) sodium dodecyl sulfate (SDS)-polyacryl-amide gels, electrophoresed, and then transferred onto a polyvinylidine difluorid (PVDF) membrane. After being blocked with Tris-buffer containing 5% skim milk and probed with polyclonal rabbit anti-human IP_3R1 antibody followed respectively by HRP-conjugated secondary antibody, they were incubated with an enhanced chemiluminescent substrate and exposed to X-OMAT film. The protein expression was quantified by densitometry.

PKC activity assay

The PKC activity assay was carried out in accordance with the instructions of the PepTag® Non-Radioactive Protein Kinase C Assay Kit. The cells were washed once with PBS and lysed in lysis buffer, which contained 20 mmol Tris-HCl, 0.5 mmol EGTA, 2 mmol EDTA, 2 mmol dithiothreitol, 1 mmol phenylmethanesulfonyl fluoride (PMSF), and 10 mg/mL leupeptin (pH 7.5). Assays were then performed at 30 °C in a total volume of 25 μL that included 5 μL PKC reaction 5×buffer, 5 μL PLSRTLSVAAK peptide, 5 μL PKC activator, and 1 μL peptide protection solution. Reactions were initiated by the addition of 9 μL of the sample and terminated after 30 minutes by incubating the reaction mixture at 95 °C for 10 minutes. After the addition of 1 μL of 80% glycerol, each sample was separated by 0.8% agarose gel electrophoresis at 100 V for 15 minutes.

Data analysis

The results were representative of at least three separate experiments. SPSS version 13.0 Software was used to perform the statistical analyses. All quantitative data were given as mean ± SEM and were analyzed using analysis of variance (ANOVA). *P* values<0.05 were considered statistically significant.

RESULTS

Effects of TNF-α time-course stimulation on IP_3R1 mRNA and protein expression

QRT-PCR revealed that the expression of IP_3R1 mRNA was up-regulated at 2 hours after 100 ng/mL TNF-α treatment, peaked at 8 hours, and then descended at 24 hours compared with the basal levels (P<0.01, Table 1). Consistent with the result of qRT-PCR, western blotting showed that the expression of IP_3R1 protein was up-regulated at 4 hours after TNF-α exposure and significantly enhanced at 24 hours compared with the basal levels (P<0.01, Figure 1 and Table 1).

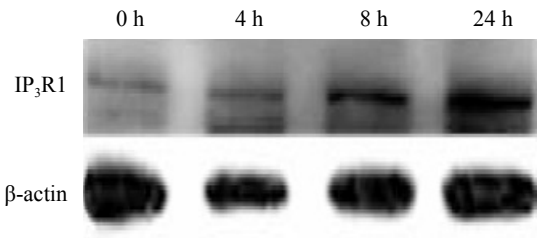

Figure 1. IP_3R1 protein expression in HMCs treated with TNF-α.

Table 1. Real-time PCR and Western blotting of IP_3R1 mRNA (n=3)

Groups	0-hour	4-hour	8-hour	24-hour
Real-time PCR	1.05±0.09*	2.27±0.01*	3.45±0.11*	1.76±0.26*
Western blotting	1.19±0.03	1.46±0.22	1.69±0.14	2.90±0.55*

Compared with control group (0-h group), *P<0.01.

Table 2. Effects of cell signaling inhibitors on TNF-α stimulated IP_3R1 mRNA expression

Groups	n	IP_3R1 mRNA
pp1	3	2.84±0.32
D609	3	1.32±0.27
Safingol	3	1.78±0.16
Rottlerin	3	2.84±0.84
u7	3	2.44±0.94
t0h	3	1.00±0.00
t8h	3	3.30±0.81Δ
PP1+TNF-α	3	2.60±0.77
D609+TNF-α	3	2.10±0.49*
Safingol+TNF-α	3	1.95±0.13#
Rottlerin+TNF-α	3	2.43±0.42
U73122+TNF-α	3	2.66±0.43

Compared to the 0-hour group, ΔP<0.01; compared to the 8-hour group, *P<0.05, #P<0.01.

Table 3 Effects of cell signaling inhibitors on TNF-α-induced IP_3R1 protein expression

Groups	n	IP_3R1 mRNA
T0h	3	1.54±0.10
T24h	3	3.09±0.13#
PP1+TNF-α	3	2.78±0.30
Rottlerin+TNF-α	3	2.70±0.30
U73122+TNF-α	3	2.80±0.13
D609+TNF-α	3	1.98±0.02*
Safingol+TNF-α	3	1.86±0.39*

Compared to the 0-hour group, #P<0.01; compared to the 24-hour group, *P<0.01.

Effects of cell signaling inhibitors on TNF-α stimulated IP_3R1 mRNA and protein expression

QRT-PCR showed that the level of IP_3R1 mRNA expression markedly increased at 8 hours after TNF-α treatment compared with the basal levels (P<0.01). Inhibitors alone had no significant effect on the basal expression of IP_3R1 (P>0.05, Figure 2). D609 (PC-PLC inhibitor) and safingol (PKCα inhibitor) suppressed TNF-α-induced expression of IP_3R1 mRNA levels compared to the control 8-hour group (3.30±0.81 vs. 1.95±0.13, P<0.05; 2.10±0.49, P<0.01, Table 2), and they also markedly blocked TNF-α-induced IP_3R1 protein levels compared to the control 24-hour group (3.09±0.13 vs. 1.86±0.39, P<0.01; 1.98±0.02 vs. 1.86±0.39, P<0.01, Figure 2 and Table 3). The effects of TNF-α were not affected by U73122, rottlerin or PP1. These results

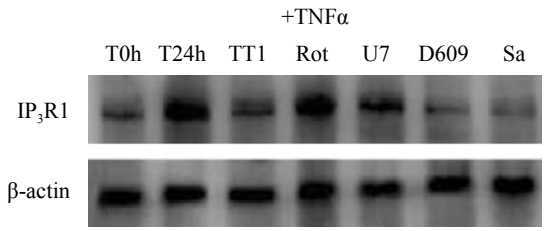

Figure 2. Effects of cell signaling inhibitors on TNF-α-induced IP_3R1 protein expression.

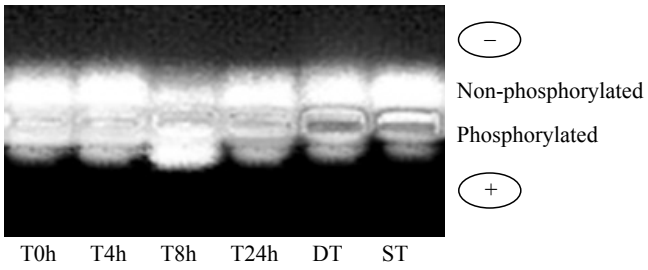

Figure 3. Effects of TNF-α and signaling inhibitors on intraocular PKCα activity.

showed a positive regulatory role of PC-PLC and PKCα signals in TNF-α-induced expression of IP_3R1 in HMCs.

PKCα activity detection

A weak specific phosphorylated stripe in cathode occurred at 0, 4, 8, and 24 hours after TNF-α treatment and the phosphorylated stripe was remarkably enhanced in density and width in the 8-hour group compared to the basal level (Figure 3), suggesting TNF-α promoted autophosphorylation. Hence, the activation of PKCα with maximal phosphorylation occurred at 8 hours after stimulation. While the phosphorylated stripe was significantly weakened in the group pretreated with D609 and safingol (Figure 3), suggesting D609 and Safingol could inhibit the effect of TNF-α on the PKCα activity.

DISCUSSION

Inositol 1,4,5-trisphosphate receptor (IP_3R) is an intracellular calciumion (Ca^{2+}) channel that triggers the release of Ca^{2+} from internal Ca^{2+} stores in response to inositol 1,4,5-trisphosphate (IP_3). IP_3R plays a crucial role in many biological activities, such as fertilization, cell contraction, proliferation, and neuronal signaling transmission. IP_3R1, a subtype of IP_3R, is predominant in the GMCs and renal VSMC.[10]

Overexpression IP_3R1 would enhance IP_3 binding activity, sensitivity, and Ca^{2+} releasing activity, which might be causally related to initiating or perpetuating diseases such as atrial fibrillation, Alzheimer's disease, Parkinson's disease and asthma.[5–7] The increased IP_3R1 expression in GMCs and afferent arteriole VSMC were causally related to low kidney perfusion in animal model of liver cirrhosis.[11,12] In contrast, reduction in IP_3R1 expression induced by transforming growth factor-β might lead to diminished vascular responsiveness of GMCs and smooth muscle cells, which related to low diabetic kidney perfusion and involved in the pathogenesis of diabetic nephropathy.[13]

It may be concluded that alteration in IP_3R number and construction would affect intracellular amplitude and frequency of Ca^{2+} signaling, resulting in cell contraction dysfunction. Therefore, we hypothesize that it is possible that increased IP_3R1 induced by TNF-α would lead to increased IP_3 sensitivity and Ca^{2+} releasing activity in response to a variety of vasoconstrictors when HRS occurred, promoting GMCs and renal VSMCs contraction, GFP reduction and HRS occurrence and development.

Accumulated evidence suggests that the serum level of TNF-α increases significantly in patients with severe liver injury.[8] Glomerular mesangial cells (GMCs) modify GFP via alternations in glomerular capillary filtration surface area owing to the ability of contract. In addition, MCs are influenced by autocrine and paracrine effects of a variety of active substances, which crucially contribute to most pathological processes of the renal glomerulus. Our results showed that TNF-α increased IP_3R1 mRNA and protein expression in HMCs, which was consistent with the finding that TNFa increased IP_3R1 expression in primary murine neurons.[7] In our study, that TNF-α increased IP_3R1 mRNA levels can be explained either by an effect on transcription of the IP_3R1 gene or by the reduced degradation rate of mRNA and needs further studies.

PTK, PLC and PKC were indispensable signaling factors and participate in many gene expressions induced by TNF-α in GMCs. It had been reported that TNF-α could activate PKCα, PKCε and PKCζ in GMCs, and PKCα and PKC β in vascular smooth muscle cells and endothelial cells.[14,15] PKC activity was closely related to PLC, which decomposes of cell membrane phospholipids and generates DAG to activated PKC.[16,17] Further experiments were conducted to determine the regulatory role of PLC, PKC, PTK in TNF-α-stimulated IP_3R1 expression. An selective inhibitor of the phosphatidylinositol-specific phospholipase (PI-PLC) U73122 (5 μmol), the phosphatidylcholine-specific phospholipase (PC-PLC) D609 (50 μmol), the non-receptor tyrosine kinase PP1 (10 μmol), the protein kinase C (PKCα) safingol (5

μmol) or the protein kinase C (PKCδ) rottlerin (5 μmol) was preincubated with cells for 1 hour. Then the cells were stimulated with TNF-α 100 ng/mL for 8 hours or 24 hours before harvesting for RNA or protein extraction. All the signaling inhibitors were in safe doses,[14–16] which have no influence on cellular activity and could effectively block corresponding signal protein activity. Our results showed that the expression of IP_3R1 mRNA and protein was markedly reduced when HMCs were pretreated with D609 and Safingol, wherears the effect was not affected by U73122, rottlerin or PP1. Obviously, D609 and safingol participated in the IP_3R1 expression induced by TNF-α. We also found that nearly 40% of the IP_3R1 protein expressions were blocked in the group pretreated with D609 and safingol, suggesting that another pathway may be involved except for the PC-PLC/PKCα signal pathway.

Non-radioactive assay proved that activated PKCα played a crucial role in TNF-α-up-regulated IP_3R1 expression. It showed that PKC was markedly activated at 8 hours after TNF-α treatment, while D609 inhibited TNF-α-stimulated activation of PKCα. These results were consistent with the findings that PC-PLC, which is a molecule that produces DAG and phosphorylcholine (Pchol) from PC hydrolysis, is involved in the TNF-α-activated PKCα signaling in various cells.[19]

In conclusion, TNF-α could increase the production of IP_3R1 by the control of the PC-PLC/PKCα signaling pathway in HMCs. Overexpression of IP_3R1 enhanced by TNF-α promotes HMCs contraction, GFP reduction, and HRS occurrence and development.

Funding: This work is supported by a grant from Health Bureau of Jiangxi Province.

Ethical approval: Not needed.

Conflicts of interest: There is no conflict of interest.

Contributors: Wang YR proposed the study and wrote the paper. All authors contributed to the design and interpretation of the study and to further drafts.

REFERENCES

1 Venkat D, Venkat KK. Hepatorenal syndrome. South Med J 2010; 103: 654–661.
2 Liu LQ, Ke X. Octreotide acetate treat liver cirrhosis with hepatorenal syndrome. World J Emerg Med 2005; 14: 956.
3 Yao J, Kitamura M, Oite T. Characteristics of glomerular mesangial cells. Nippon Jinzo Gakkai Shi 2008; 50: 554–560.
4 Taylor CW, da Fonseca PC, Morris EP. IP(3) receptors: the search for structure. Trends Biochem Sci 2004; 29: 210–219.
5 Brightling C, Xie H, Zhu PH. Targeting TNF-alpha: a novel therapeutic approach for asthma. J Allergy Clin Immunol 2008; 121: 5–10.
6 Liang X, Xie H, Zhu PH, Hu J, Zhao Q, Wang CS, et al. Enhanced activity of inositol-1,4,5-trisphosphate receptors in atrial myocytes of atrial fibrillation patients. Cardiology 2009; 114: 180–191.
7 Park KM, Yule DI, Bowers WJ. Impaired TNF-alpha control of IP_3R-mediated Ca^{2+} release in Alzheimer's disease mouse neurons. Cell Signal 2010; 22: 519–526.
8 Lu JW, Wang H, Yan LJ, Zhang C, Ning H, Li XY, et al. Differential effects of pyrrolidine dithiocarbamate on TNF-alpha-mediated liver injury in two different models of fulminant hepatitis. J Hepatol 2008; 48: 442–452.
9 Zhu C, Li Y, Li W, Wu Q, Gao R. Gene transfer of c-met confers protection against D-galactosamine/lipopolysaccharide-induced acute liver failure. Dig Dis Sci 2012; 57: 925–934. Epub 2012 Jan 24.
10 Monkawa T, Hayashi M, Miyawaki A, Sugiyama T, Yamamoto-Hino M, Hasegawa M, et al. Localization of inositol 1,4,5-trisphosphate receptors in the rat kidney. Kidney Int 1998; 53: 296–301.
11 Wen Y, Cui W, Liu P. Type I inositol 1, 4, 5-triphosphate receptors increase in kidney of mice with fulminant hepatic failure. World J Gastroenterol 2007; 13: 2344–2348.
12 Wang J, Sun J, Lu S, Liu P. Tumor necrosis factor-αenhances type I inositol 1,4,5--trisphosphate receptor expression in rat glomerular afferent arterioles smooth muscle cells. China J Intern Med 2002; 41: 86–89.
13 Sharma K, Wang L, Zhu Y, DeGuzman A, Cao GY, Lynn RB, et al. Renal type I inositol 1,4,5-trisphosphate receptor is reduced in streptozotocin-induced diabetic rats and mice. Am J Physiol Renal Physiol 1999; 276: F54–61.
14 Nee L, Tuite N, Ryan MP, McMorrow T. TNF-alpha and IL-1 beta-mediated regulation of MMP-9 and TIMP-1 in human glomerular mesangial cells. Nephron Exp Nephrol 2007; 107: e73–86.
15 Huang WC, Chen JJ, Inoue H, Chen CC. Tyrosine phosphorylation of I-kappa B kinase alpha/beta by protein kinase C-dependent c-Src activation is involved in TNF-alpha-induced cyclooxygenase-2 expression. J Immunol 2003; 170: 4767–4675.
16 Das Evcimen N, King GL. The role of protein kinase C activation and the vascular complications of diabetes. Pharmacol Res 2007; 55: 498–510.
17 Eun JC, Moore EE, Mauchley DC, Johnson CA, Meng X, Banerjee A, et al. The 5-lipoxygenase pathway is required for acute lung injury following hemorrhagic shock. Shock 2012; 37: 599–604.
18 Soh JW, Weinstein IB. Roles of specific isoforms of protein kinase c in the transcriptional control of cyclin d1 and related genes. J Biol Chem 2003; 278: 34709–34716.
19 Chen C, Chou C, Sun Y, Huang W. Tumor necrosis factor alpha-induced activation of downstream NF-kappaB site of the promoter mediates epithelial ICAM-1 expression and monocyte adhesion. Involvement of PKCalpha, tyrosine kinase, and IKK2, but not MAPKs, pathway. Cell Signal 2001; 13: 543–553.

Received May 11, 2012
Accepted after revision August 25, 2012