Involvement of decreased myo-inositol transport in lipopolysaccharide-induced depression of phosphoinositide hydrolysis in vascular smooth muscle

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Abstract The mechanism underlying lipopolysaccharide (LPS)-induced depression of phosphoinositide (PI) hydrolysis was investigated using rat aortas. In LPS-pretreated aortas, the 5-hydroxytryptamine-stimulated accumulation of inositol monophosphate and incorporation of exogenous myo-inositol into PIs were significantly less than those in control aortas. Both sodium-myo-inositol cotransporter (SMIT) and phosphatidylinositol transfer protein (PITP) genes were constituently expressed in rat aortas. The mRNA level of SMIT was remarkably lower in LPS-pretreated aortas, while that of PITP mRNA was not affected by LPS. These results suggest that LPS-induced depression of SMIT expression is involved in inhibition of agonist-stimulated PI hydrolysis by LPS. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Septic shock; Phosphatidylinositols; Inositol 1,4,5-trisphosphate; Vasoconstriction; Gene expression

1. Introduction

Circulatory shock is a serious complication of sepsis, and the biological action of endotoxin, i.e. lipopolysaccharide (LPS), is greatly involved in the pathophysiology of septic shock. Arteries dissected from animal models of endotoxemic shock show hypocontractility of vascular smooth muscle, and this vascular abnormality is a major mechanism of endotoxemic shock [1]. Similarly, incubation of isolated blood vessels with LPS in vitro induced hypocontractility of vascular smooth muscle [2]. Accumulation of cGMP due to a large amount of inducible nitric oxide (NO), which is produced through a pathway of LPS-stimulated inducible NO synthase, has been demonstrated to be involved in hypocontractility of blood vessels during endotoxemic shock [3].

In vascular smooth muscle, agonist-stimulated G-protein activation is coupled with phospholipase C (PLC) activation and is followed by hydrolysis of phosphatidylinositol 4,5-bisphosphate. Its two metabolites, inositol 1,4,5-trisphosphate and diacylglycerol, play critical roles in the signal transduction cascade of vascular smooth muscle contraction [4]. Decreased agonist-induced phosphoinositide (PI) hydrolysis was proposed to be involved in hypocontractility of vascular smooth muscle during endotoxemic shock [5,6], although the mechanism of the decreased PI hydrolysis has not been clarified. Since exogenous NO is known to inhibit PI hydrolysis [7], the decrease in PI hydrolysis of endotoxemic arteries could be considered to be due to inducible NO. However, our recent study has shown that inducible NO can only partially explain LPS-induced decrease in PI hydrolysis [8].

The purpose of the present study was to elucidate the NO-independent mechanism of depressed PI hydrolysis in blood vessels during endotoxemic shock. We focused on transport of myo-inositol, a substrate of phosphatidylinositol synthase that is the rate-limiting enzyme of PI synthesis [9], and investigated whether LPS affects utilization of myo-inositol, which is taken up from blood into cytoplasm via sodium-myo-inositol cotransporter (SMIT), converted into phosphatidylinositol in the endoplasmic reticulum, and then transported to the plasmalemma by phosphatidylinositol transfer protein (PITP) to synthesize phosphatidylinositol 4,5-bisphosphate [10,11].

2. Materials and methods

2.1. Animals and tissue preparation

The study protocols regarding treatment of animals were in accordance with the 'Guidelines for Experiments Using Laboratory Animals in Yamagata University School of Medicine'. Male Wistar rats aged 10–12 weeks were anesthetized with sodium pentobarbital (50 mg/kg) and killed by exsanguination. The thoracic aortas were removed and ring-shaped aortic strips (4 mm long) were prepared as described previously [8]. Each aortic strip was then put in a well of a 24-well plate containing 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum. The strips were incubated in a humidified atmosphere at 37°C under 5% CO_2–95% air and then used for the experiments.

2.2. Animal model of endotoxemic shock

Rats were anesthetized with ether. Peritonitis was produced by a modified method of cecal ligation and puncture (CLP) [12]. Through a 2 cm midline incision, cecum was exposed and ligated just distally to the ileocecal valve. Care was taken not to produce intestinal obstruction. Then, a 5 mm blade incision was made on the cecum, and the abdominal wound was closed in two layers. Sham-operated rats underwent the same surgical procedure as the above, except that the cecum was neither ligated nor punctured. Saline (4 ml per 100 g of body weight) was injected subcutaneously to each rat following CLP

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Abbreviations: PI, phosphoinositide; LPS, lipopolysaccharide; SMIT, sodium-myo-inositol cotransporter; PITP, phosphatidylinositol transfer protein; NO, nitric oxide; 5-HT, 5-hydroxytryptamine; IP, inositol monophosphate; PLC, phospholipase C; DMEM, Dulbecco's modified Eagle's medium; CLP, cecal ligation and puncture; cDNA, complementary DNA; PCR, polymerase chain reaction; TNFα, tumor necrosis factor-α

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or sham operation for fluid replacement. At 5–6 h after the operation, all the rats undergoing CLP appeared severely lethargic and showed piloelection, cyanosis and tachypnea which are typical symptoms of endotoxemic shock.

2.3. Measurement of inositol monophosphate (IP) accumulation and [^{3}H]IP generation

The strips were stabilized in DMEM, and then LPS (1 μg/ml) or a vehicle (distilled water) was added to the medium. Myo-[2-^{3}H]inositol (0.3 μM) was also added to the medium simultaneously with LPS or the vehicle. After 24 h of incubation with LPS or the vehicle, the strips were used for measurement of IP accumulation and PI generation. The strips from rats receiving CLP or sham operation were incubated with myo-[2-^{3}H]inositol (0.3 μM) for 3 h. IP accumulation was measured according to the method described previously [13]. Aortic strips were rinsed three times with fresh warm (37°C) DMEM and then transferred to individual glass tubes containing 0.4 ml DMEM. LiCl at a final concentration of 10 mM was added to each tube, and 5-hydroxytryptamine (5-HT, 100 μM) or the vehicle was added to the tube 10 min later and further incubated at 37°C for 60 min. The reaction was then terminated by addition of 0.9 ml of chloroform–methanol solution (1:2, v/v), followed by addition of 0.3 ml of chloroform and vortexing. Water (0.3 ml) was then added, followed by vigorous vortexing. The tubes were centrifuged at $1000\times g$ for 5 min, allowing the aqueous and chloroform phases to separate. An aliquot of 0.9 ml of the upper phase was then loaded onto AG 1×8 (formate form) resin packed in a disposable column. The columns were then sequentially washed with 9 ml of water, 9 ml of 60 mM sodium formate/5 mM sodium borate, and 9 ml of 1 M ammonium formate/0.1 M formic acid to selectively elute [^{3}H]IP. Aliquots (3 ml) of the eluant were mixed with scintillant and counted in a liquid scintillation spectrophotometer.

The total lipid layer remaining after removal of the aqueous phase was transferred to individual tubes. Then, 3 ml of $CHCl_3/CH_3OH$ (2:1, v/v) and 2 ml of CH_3OH/KCl (1 M)-myo-inositol (0.01 M) (1:1, v/v) were added to each tube, followed by vortexing. The aqueous and chloroform phases were separated by centrifugation. After the upper phase was removed and discarded, the residual lipid layer was washed twice with 3 ml of CH_3OH/KCl (1 M)–myo-inositol (0.01 M) (1:1, v/v). The lipid phase was then removed, placed in a scintillation vial, and left to evaporate overnight. Scintillant was added to each vial, and the radioactivity was counted in a liquid scintillation counter.

The aortic strips were blotted gently with a filter paper and weighed on a microbalance. [^{3}H]IP and [^{3}H]PI levels were corrected by mg wet tissue weight (dpm/mg).

2.4. Measurement of SMIT and PITP mRNA levels

The thoracic aorta was harvested to investigate the expression of SMIT and PITP-α mRNA. The aorta was cleaned of adherent connective tissues and intimal layer, and then cut into rings (5 mm long). The total RNA was prepared from each aortic strip using Isogen (Nippon Gene). The quality and yield of the RNA were assessed by the 260/280 nm optical density ratio.

The total RNA (0.5 μg) from each sample was reversely transcribed into complementary DNA (cDNA) using a commercial kit (River Tra Dash TM from Toyobo). The polymerase chain reaction (PCR) amplification for SMIT, PITP-α and β-actin was carried out in an automatic DNA thermal cycler (PCR Thermal Cycler PERSONAL TP 240, Takara, Shuzo) using Taq DNA polymerase and oligonucleotide primers. The sequences of the specific primers were 5′-ACACACAACCTTTCCAACAC (sense) and 5′-TCTGCTTCCACACACTTGC (antisense) for SMIT and 5′-GGATGAGATGAGACAAAAGGAC (sense) and 5′-TTGGAAATAAAGGGGGAGGG (antisense) for PITP-α. The sequences of the β-actin-specific primers were 5′-TTGTAACCAACTGGGACGATATGG (sense) and 5′-GATCTTGATCTTCATGGTGCTAGG (antisense). The PCR amplification of SMIT and PITP-α was carried out through 24 cycles for each at 95°C for 30 s, at 64°C for 30 s and at 70°C for 1 min for SMIT, and each at 95°C for 30 s, at 63°C for 30 s and at 70°C for 1 min for PITP-α. The PCR amplification of β-actin was performed through 18 cycles for each at 98°C for 10 s, at 63°C for 2 s and at 70°C for 20 s.

The amplified cDNAs were separated by agarose (2%) gel electrophoresis, stained with ethidium bromide, and visualized using an ultraviolet transilluminator (ATTO DENSITOGRAPH, AE-6900M, ATTO). The intensities of the SMIT- and PITP-α-specific bands were quantified by densitometry, and the mRNA levels of SMIT and PITP-α were corrected to arbitrary units using the level of β-actin as 100%. In the preliminary experiments, we confirmed a linear relationship between the total RNA levels of the tissue extracts and the density of the PCR products from SMIT, PITP-α and β-actin mRNA (data not shown).

2.5. Chemicals

The drugs used were LPS and 5-HT creatinine sulfate obtained from Sigma Chemical Co. LPS was dissolved in distilled water to make up a stock solution of 100 μg/ml and stored at −20°C. 5-HT was dissolved in distilled water to make up a stock solution of 10 mM, and this solution was stored at 4°C. The concentration of each drug was expressed as the final concentration in the wells and the tubes.

2.6. Statistical analysis

The data are shown as means ± S.E.M. Statistical analysis was done with Student's *t*-test, and *P* values less than 0.05 were regarded as significant.

3. Results

3.1. Changes in IP accumulation and PI generation in rat aortas after LPS exposure

LPS exposure for 24 h resulted in diminution of aortic PI generation compared with that in the control aortas (Fig. 1A). 5-HT-stimulated IP accumulation was strongly correlated with the level of aortic PI generation (correlation coefficient = 0.85, $P < 0.05$). LPS exposure for 24 h inhibited 5-HT-stimulated IP accumulation but did not affect the basal IP level (Fig. 1B).

3.2. Changes in IP accumulation and PI generation in aortas isolated from endotoxemic rats

In the aortas from endotoxemic rats, basal and 5-HT-induced IP accumulation as well as PI generation were significantly lower than those from the sham-operated rats (Fig. 2A,B). The levels of 5-HT-stimulated IP accumulation were significantly correlated with those of PI generation (correlation coefficient = 0.85, $P < 0.05$).

3.3. Effects of LPS in vitro and endotoxemia on genetic expression of SMIT and PITP-α in rat aortas

In rat aortas, SMIT mRNA was expressed constituently and significantly inhibited by LPS exposure in vitro for 24 h

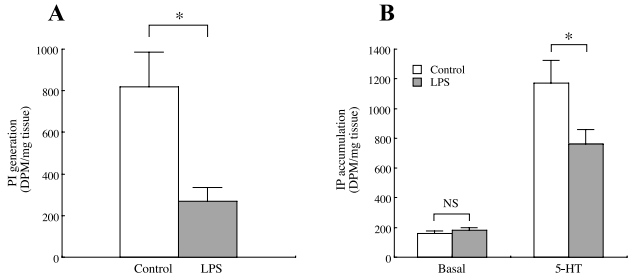

Fig. 1. A: Effects of incubation with LPS for 24 h on [^{3}H]PI generation in the aortic strips. The strips were incubated with [^{3}H]myo-inositol (0.3 μM) and LPS (1 μg/ml) or a vehicle for 24 h. An asterisk denotes significant difference within the indicated data set. $n = 7$. B: Effects of incubation with LPS for 24 h on basal and 5-HT-stimulated IP accumulation in aortic strips. After the aortic strips had been incubated with [^{3}H]myo-inositol (0.3 μM) and LPS (1 μg/ml) or a vehicle for 24 h, the strips were stimulated with 5-HT (100 μM) for 60 min. An asterisk denotes significant difference within the indicated data set. NS, not significant. $n = 7$.

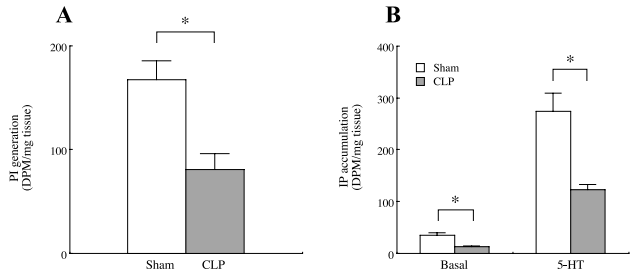

Fig. 2. A: [^{3}H]PI generation in the aortic strips. The aortic strips were dissected from the rats suffering from endotoxemic shock produced by CLP. The control aortic strips were dissected from sham-operated rats (Sham). An asterisk denotes significant difference within the indicated data set. $n=6$. B: Basal and 5-HT-stimulated IP accumulation in aortas from the rats suffering from endotoxemic shock produced by CLP. The control aortic strips were dissected from sham-operated rats (Sham). Some strips were stimulated with 5-HT (100 μM) for 60 min. Asterisks denote significant difference within the indicated data sets. $n=6$.

(Fig. 3A,B). Similarly, SMIT mRNA levels were markedly lower in aortic strips isolated from the rats suffering from endotoxemic shock (Fig. 3C,D) than in those isolated from control rats that underwent a sham operation. SMIT mRNA levels were increased by a high-osmotic stimulus with raffinose at 150 mM (data not shown).

On the other hand, LPS exposure in vitro (Fig. 4A,B) did not affect PITP-α mRNA levels of rat aortas. The PITP-α mRNA levels were not significantly different in aortas from the rats with endotoxemic shock and aortas from the control rats (Fig. 4C,D).

4. Discussion

Incubation of rat aortas with LPS in vitro for 24 h caused a decrease in myo-inositol incorporation into PIs as well as a decrease in 5-HT-induced IP accumulation that results from PLC-coupled PI hydrolysis. 5-HT-stimulated IP accumulation was strongly correlated with basal myo-inositol incorporation into PIs in the control and LPS-pretreated aortas. Thus, a decrease in myo-inositol uptake is closely related to LPS-induced depression of agonist-stimulated PI hydrolysis. Myo-inositol is utilized for the synthesis of PIs, which play integral roles in signal transduction pathways by virtue of release of secondary messengers upon activation of PI-specific PLC [9,14]. Moreover, myo-inositol functions as an osmolyte, serving to protect cells from hyperosmolyte stress. Myo-inositol uptake across the plasmalemma is mediated by SMIT, which has been reported to be constituently expressed in mammalian cells such as kidney cells, endothelial cells, neural cells and adipocytes, and its expression is regulated by extracellular osmolarity [15,16]. The present study is the first study to demonstrate genetic expression of SMIT in vascular tissues without the endothelium. In cultured endothelial cells and 3T3-L1 adipocytes, tumor necrosis factor-α (TNFα) has been reported to cause a decrease in SMIT mRNA [17,18]. The present study clearly demonstrated that SMIT mRNA levels were decreased in blood vessels after LPS stimulation.

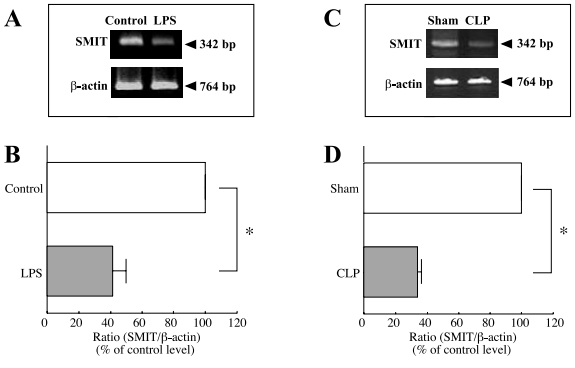

Fig. 3. A: Representative gel photograph of PCR-amplified cDNA derived from SMIT and β-actin mRNA in rat aortas. The aortic strips were incubated with LPS (1 μg/ml) or a vehicle for 24 h. B: Densitometric analysis of the above gel photograph. The mRNA levels of SMIT on the gel photograph were quantified by densitometry. The results are expressed as a percentage of the control without LPS stimulation for the ratio of SMIT to β-actin. An asterisk denotes significant difference within the indicated data set. $n=3$. C: Representative gel photograph of PCR-amplified cDNA derived from SMIT and β-actin mRNA in aortas. The aortic strips were dissected from rats with endotoxemic shock produced by CLP. The control aortic strips were dissected from sham-operated rats (Sham). D: Densitometric analysis of the above gel photograph. The mRNA levels of SMIT on the gel photograph were quantified by densitometry. The results are expressed as a percentage of the control without LPS stimulation for the ratio of SMIT to β-actin. An asterisk denotes significant difference within the indicated data set. $n=3$.

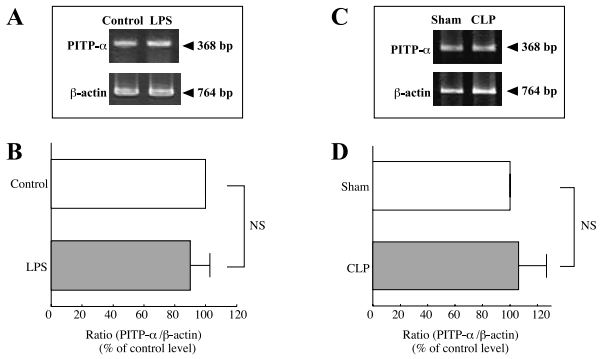

Fig. 4. A: Representative gel photograph of PCR-amplified cDNA derived from PITP-α and β-actin mRNA in rat aortic strips incubated for 24 h with a vehicle (Control) or LPS (LPS, 1 μg/ml). B: Densitometric analysis of the above gel photograph. The mRNA levels of PITP-α on the gel photograph were quantified by densitometry. The results are expressed as a percentage of the control without LPS stimulation for the ratio of PITP-α to β-actin. NS, not significant. $n=3$. C: Representative gel photograph of PCR-amplified cDNA derived from PITP-α and β-actin mRNA in the aortas dissected from rats with endotoxemic shock produced by CLP. The control aortic strips were dissected from sham-operated rats (Sham). D: Densitometric analysis of the above gel photograph. The mRNA levels of PITP-α on the gel photograph were quantified by densitometry. The results are expressed as a percentage of the control without LPS stimulation for the ratio of PITP-α to β-actin. NS, not significant. $n=3$.

PITPs are abundant cytosolic proteins of mammalian cells [11] and are known to act as specific carriers of single phospholipid molecules between membranes [10]. Recently, G-protein-stimulated PLC activity has been shown to be critically dependent on PITP activity [19]. The present study is also the first study to demonstrate that PITP mRNA is constituently expressed in blood vessels but is not affected by LPS stimulation. Moreover, we also confirmed that both SMIT and PITP mRNA was constituently expressed in A7r5 rat aortic smooth muscle cells (data not shown).

Using aortic strips isolated from rats of an animal model of endotoxemia, we examined the effects of endotoxemia in vivo

on the above PI pathway. Similar to the above-described effects of LPS in vitro, IP accumulation, myo-inositol incorporation into PIs, and genetic expression of SMIT were inhibited in the aortas from endotoxemic rats compared with those in the sham-operated rats, while genetic expression of PITP was not affected. Therefore, the direct inhibitory effect of LPS on myo-inositol transport may be involved in attenuation of PI hydrolysis in endotoxemic vessels in vivo.

While LPS inhibited agonist-induced IP accumulation, its basal level was not affected by LPS in vitro. Stimulation of G-protein with 5-HT strongly accelerated PI hydrolysis, which may require a greater supply of myo-inositol for PI generation. Based on this speculation, it is thought that the amount of myo-inositol required for basal PI hydrolysis is sufficient even in LPS-exposed aortas, while myo-inositol supply is insufficient when PI turnover is accelerated by agonists. On the other hand, the aortic strips isolated from endotoxemic rats showed a decrease in the levels of both basal and agonist-stimulated IP accumulation. This difference in the results of basal IP accumulation from in vitro and in vivo studies may be explained by the additional in vivo effects of endotoxemia, possibly due to endogenous mediators such as cytokines (e.g. TNFα).

Our recent study has shown that 5-HT-stimulated IP accumulation is attenuated in LPS-pretreated vessels, and this attenuation is mainly due to inducible NO at the early stage after LPS incubation (for 10 h), while inducible NO is only partially involved in the attenuation of IP accumulation at the later stage after LPS incubation (for 24 h) [8]. The present study has shown that the decrease in SMIT-mediated myo-inositol incorporation is also involved in attenuation of IP accumulation in aortas pretreated with LPS for 24 h.

In conclusion, we propose decrease in SMIT-mediated myo-inositol uptake into vascular smooth muscle as a mechanism of diminished PI hydrolysis in endotoxemic vessels, in addition to the inducible NO-related mechanism.

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