Modification of Hepatic Protein Kinase C with Phorbol Myristate Acetate and Staurosporine Alters Hemodynamics in the Perfused Rat Liver

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Activation of protein kinase C (PKC) has been implicated in the pathogenesis of endotoxicosis and severe sepsis. Since hepatic blood flow and metabolism have been known to be altered in endotoxicosis and sepsis, we studied the hemodynamic effect of PKC modulation with phorbol 12-myristate 13-acetate (PMA) and staurosporine (St) on the perfused rat liver. The liver was isolated from overnight-fasted male Sprague-Dawley rats and placed in a recirculating perfusion apparatus. The liver was perfused with Krebs-Ringer-bicarbonate solution at a constant pressure of 12 cmH_2O. Flow to the liver was continuously monitored with an electric magnetic flowmeter. PMA at an initial concentration of 2×10^{-8}M significantly decreased hepatic flow. Staurosporine (St), a potent PKC inhibitor at 4×10^{-7}M produced a small increase in hepatic flow. Pretreatment with St significantly attenuated the flow reduction by PMA. St significantly suppressed the flow reductions by 4×10^{-6}M of prostaglandin E_2 and D_2. These results suggest that the PKC inside the liver may play an important role in the regulation of hepatic blood flow during endotoxicosis and sepsis. (Key words: phorbol 12-myristate 13-acetate, staurosporine, prostaglandin E_2, prostasglandin D_2, protein kinases)

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Evidence has been accumulated that the activation of protein kinase C (PKC) is involved in the development of endotoxicosis and sepsis. Lipid A, a toxic principle of endotoxin (ETX) has been reported to activate PKC in macrophages[1]. Prostaglandin synthesis by macrophages[2] and tumor necrosis factor production by Kupffer cells[3] are mediated by a PKC-dependent pathway. PKC has been proposed as a common mediator of human endothelial cell activation by ETX, tumor necrosis factor and interleukin-1[4]. Inhibitors of PKC block the activation of B-lymphocytes by ETX[5]. PKC in human platelets has been reported to be modulated by endotoxic lipid A[6]. *In vivo* treatment of animals with phorbol 12-myristate 13-acetate (PMA), a specific activator of PKC has been shown to

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augment endotoxic lethality and associated glucose dyshomeostasis[7]. Furthermore endotoxic alterations of glucose metabolism are attenuated by treatment of animals with PKC inhibtors, H-7 and polymyxin B[8]. K252a, another inhibitor of PKC has been reported to improve endotoxic lethality and glucose dyshomeostatis[9].

PKC is distributed in a variety of organs, tissues and cells including parenchymal and non-parenchymal liver cells[10]. Endotoxicosis and sepsis are associated with alteration of hepatic blood flow. Maintenance of adequate blood flow to the liver is essential in the management of critically ill patients with sepsis. To elucidate the role of PKC in mediating the alteration of hepatic blood flow during sepsis, we studied the effect of PKC modulation on hepatic flow using isolated liver perfusion at a constant pressure. We employed phorbol 12-myristate 13-acetate (PMA) as a PKC activator[11] and staurosporine (St) as a PKC inhibor[12]. In addition, we tested whether St may antagonize the flow reductions by prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2), possible mediators of the effect of PMA[13–15].

Materials and Methods

Animals and care

Male Sprague-Dawley rats weighing 280–330g were obtained from Nihon SLC (Shizuoka, Japan). They were acclimated to our research facilities for at least 1 week at 24–26°C. A 12:12h light-dark cycle was maintained, and standard laboratory chow and water were provided ad libitum. Animals were fasted overnight before the experiment. The experiments were performed in adherence to the NIH guidelines for the use of experimental animals.

Chemicals

PMA and fraction V bovine serum albumin (BSA) were purchased from Sigma Chemial (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Wako Pure Chemical (Osaka, Japan). PMA was dissolved in DMSO and was stored as small aliquots at −25°C. St was a generous gift from Yuzuru Matsuda, Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., LTD (Tokyo, Japan). It was dissolved in DMSO and was stored in the dark at 4°C. PGE_2 and PGD_2 were obtained from Ono Pharmaceutical Co., LTD (Osaka, Japan). They were dissolved in phosphate buffered saline (PBS) and were stored as small aliquots in a freezer.

PMA and St dissolved in 50 μl of DMSO, and PGE_2 and PGD_2 in 100 μl of PBS were added to the perfusate reservoir to achieve initial concentrations desired.

Solutions

Krebs-Ringer-bicarbonate (KRB) solution was used as the perfusion medium. The solution contained 117 mM of NaCl, 4.7 mM of KCl, 2.46 mM of $CaCl_2$, 1.19 mM of KH_2PO_4, 1.44 mM of $MgSO_4$ and 24.8 mM of $NaHCO_3$. KRB solution was saturated with a 95% O_2/5% CO_2 gas mixture, and pH was adjusted to 7.35 ± 0.05 at 37°C using 1 N $NaHCO_3$ and NaOH.

Recirculating perfusion of the rat liver with a constant pressure

Rats were anesthetized with pentobarbital sodium at 30 $mg \cdot kg^{-1}$ i.v. The abdomen was opened through midline and midtransverse incisions. The inferior vena cava and portal vein were isolated. The animals were treated with 500 units of heparin sodium, and their abdominal inferior vena cava was ligated above the renal vein. A polyethylene catheter (PE-240) was inserted into the portal vein and secured in place with 2–0 silk suture. The non-recirculating perfusion of the liver with glucose-free

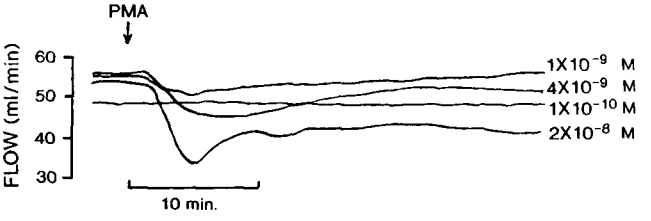

Fig. 1. Dose-dependent decrease in hepatic flow by phorbol 12-myristate 13-acetate (PMA).

PMA at initial concentrations of 1×10^{-9} to 2×10^{-8} was given at the time indicated by arrow.

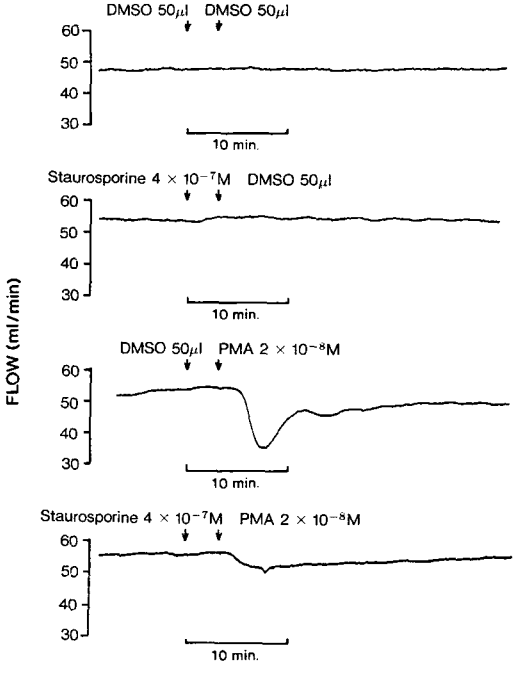

Fig. 2. Typical recordings in the PMA-staurosponine experiment.

KRB solution was immediately initiated, and was continued during the following surgical procedure. Thus, the anoxic time never exceeded 1 min. The thorax was opened by transverse and longitudinal cephalad incisions. A polyethylene catheter (PE-260) was inserted through the right atrium and secured in place in the thoracic inferior vena cava. The liver was gently excised and placed on a liver platform. Finally the liver on the platform was placed in a modified Miller-type recirculating perfusion-aeration chamber with temperature control system by which the temperature of perfusate was kept at 37°C. The liver was perfused at a constant pressure of 12 cmH_2O with KRB containing 0.5% albumin and 10 mM D(+)-glucose. The recirculating perfusate volume was 200 ml. The flow to the liver was continuously measured by an electromagnetic flow transducer (Nihon Koden, Tokyo, Japan).

Antagonism of PMA-induced decline of hepatic flow by St

A preliminary study disclosed that PMA at initial concentrations of 1×10^{-9} to 2×10^{-8}M decreased the hepatic flow in a dose-dependent fashion (fig. 1). We employed 2×10^{-8}M of PMA in the following experiments. After the flow became stable, we added either St at an initial concentration of 4×10^{-7}M or 50 μl of DMSO to the perfusate reservoir at Time = 0 min. PMA at an initial concentration of 2×10^{-8}M or 50 μl of DMSO was administered at Time = 3 min. The flow was recorded till Time = 30 min.

Antagonism of PGE_2- and PGD_2-induced flow reduction by St

Either St at an initial concentration of 4×10^{-7}M or 50 μl of DMSO was added to the reservoir at Time = 0 min. PGE_2 or PGD_2 at an initial concentration of 4×10^{-6}M was administered at Time = 3 min. Flow to the liver was recorded till Time = 30 min.

Statistical analysis

Comparisons among the treatment groups were made by one-way ANOVA followed by Duncan's multiple range

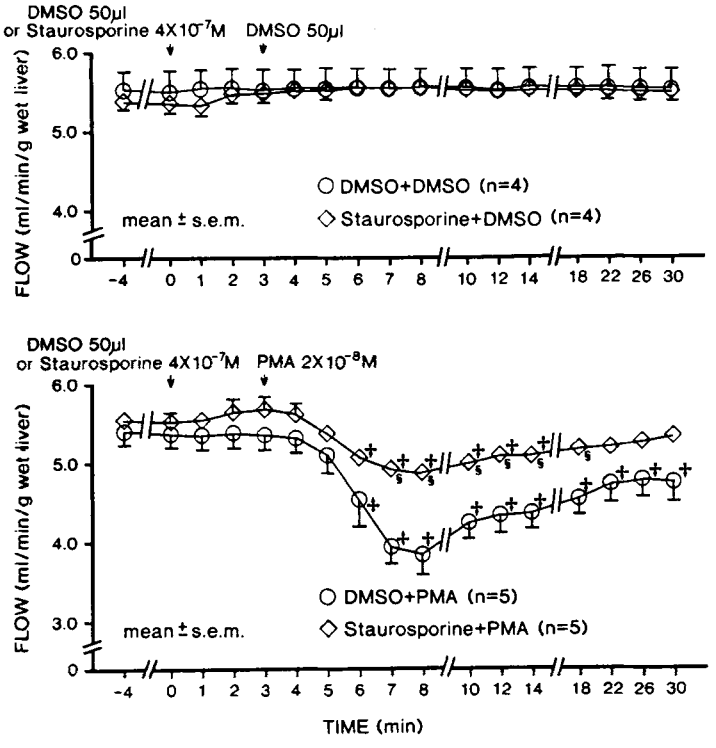

Fig. 3. Antagonism of PMA-induced flow reduction by staurosporine (St).

Fifty μl of DMSO or 4×10^{-7}M of St was administered at Time = 0 min. Fifty μl of DMSO or 2×10^{-8}M of PMA was added at Time = 3 min.

§ significantly different from DMSO-PMA-treated group ($P < 0.05$, one-way ANOVA followed by Duncan's multiple range test).

+ significantly different from the corresponding control group ($P < 0.05$, one-way ANOVA followed by Duncan's multiple range test).

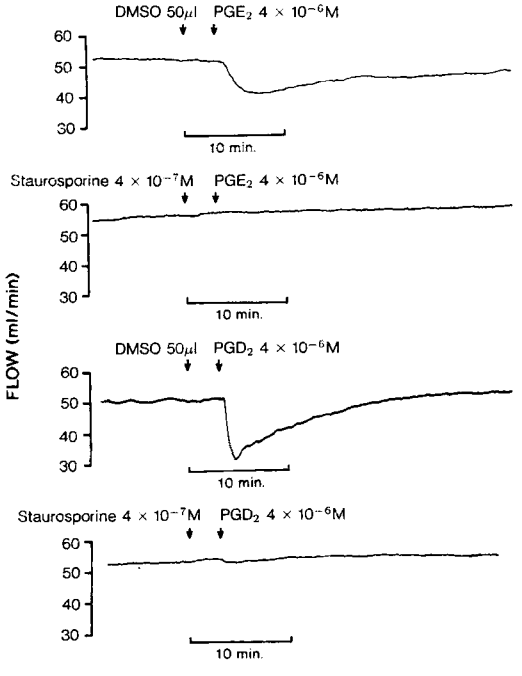

Fig. 4. Typical recordings in the staurosporine-prostaglandins experiment.

test. Differences were considered to be significant when probability (P) values were less than 0.05. All values in Figures are expressed as mean ± s.e.m.

Results

Antagonism of PMA-induced decline of hepatic flow by St

Typical recordings of flow in 4 treatment groups are shown in figure 2. Upper panel of figure 3 demonstrates the alterations of flow in the two control groups treated with DMSO at Time = 3 min. St produced a very small but sustained increase in hepatic flow. However, there was no significant difference in flow between St-treated and DMSO-treated groups. Thus, St did not have a major effect on basal flow. As shown in the lower panel of figure 3, 2×10^{-8}M of PMA significantly reduced hepatic flow. Maximal reduction of flow occurred 5 min after PMA administration or at Time = 8 min.

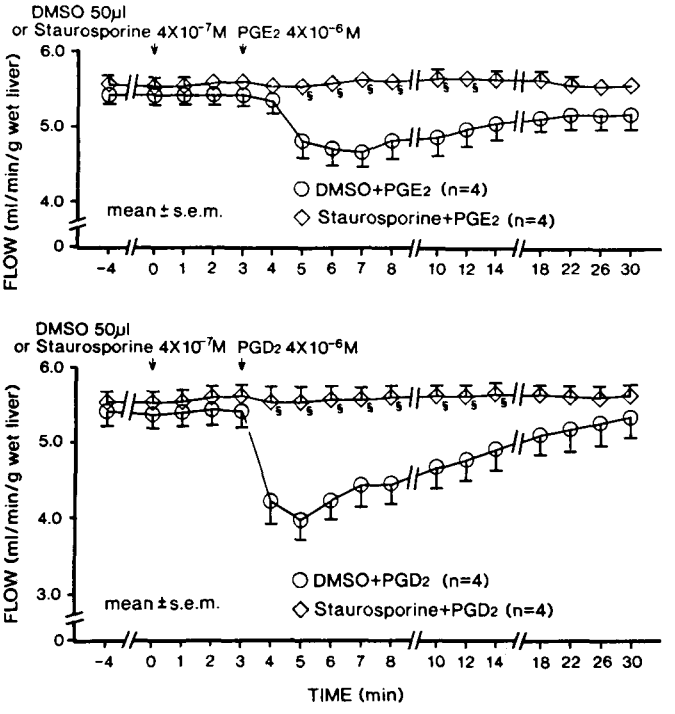

Fig. 5. Antagonism of PGE_2- and PGD_2-induced flow reductions by St.

Fifty μl of DMSO or 4×10^{-7}M of St was added at Time = 0 min. PGE_2 or PGD_2 at an initial concentration of 4×10^{-6}M was administered at Time = 3 min.

§ significantly different from PGE_2- or PGD_2-treated group ($P < 0.05$).

From Time = 7 to 18 min, there was a significant difference in flow between St-treated and DMSO-treated groups. Thus pretreatment with 4×10^{-7}M of St significantly attenuated the flow reduction by PMA.

Antagonism of PGE_2- and PGD_2-induced flow reduction by St

Typical recordings of flow in 4 treatment groups are demonstrated in figure 4. As shown in figure 5, both PGE_2 and PGD_2 at 4×10^{-6}M decreased the flow in the 2 groups pretreated with DMSO. Apparently PGD_2 produced a greater and sharper decline in hepatic flow than PGE_2. Pretreatment with St at 4×10^{-7}M significantly antagonized both PGE_2-induced (upper panel) and PGD_2-mediated (lower panel) reductions of hepatic flow.

Discussion

PKC was first discoverd as a proteolytically activated enzyme and has been characterized to be phospholipid- and Ca^{2+}-dependent[10,16]. When cells are stimulated by extracellular signals, inositol phospholipids in the cell membranes are hydrolyzed, producing two second messengers, diacylglycerol and inositol triphosphate. Diacylglycerol activates PKC by translocating the enzyme to the membrane and increasing the enzyme affinity to Ca^{2+}, while inositol triphosphate acts to mobilize intracellular calcium. PKC can be activated by tumor-promoting phorbol esters such as PMA, and is considered to be a major receptor for the phorbol esters. Thus, PMA has been widely applied to elucidate the wide variety of physiological roles of the enzyme. It has been clarified that PKC, distributed in various organs, tissues and cells, play an important role in the regulation of cellular responses to biologically active substances.

Recently the activation of PKC has been implicated in the development of endotoxin (ETX) shock and severe gram-negative sepsis. ETX-induced ac-

tivation of macrophages[1-3], endothelial cells[4], platelets[6] have been shown to involve the activation of PKC. Furthermore, *in vivo* modulation of PKC with PMA and inhibitors of PKC has been reported to influence the endotoxic lethality and/or associated disturbance of carbohydrate metabolism[7-9].

Since endotoxicosis and sepsis are associated with the alteration of hepatic blood flow, and since the blood flow to the liver can be regulated by constriction of the portal venules and by sinusoids[17,18], it is of interest to investigate whether the modulation of PKC in the liver may affect the intrahepatic flow regulation. In the present study, we showed that the modulation of PKC with PMA and St exerts a significant effect on hepatic flow in the perfused rat liver.

The effect of PMA on hemodynamics and carbohydrate metabolism has been studied in the rat liver which is isolated from fed rats and perfused with a constant flow rate[14,19]. PMA increased the perfusion pressure and stimulated glycogenolysis in these experiments. Since the effect of PMA was blocked by indomethacin, a cyclooxygenase inhibitor, and since PMA failed to stimulate glycogenolysis in isolated parenchymal liver cells or hepatocytes, it was suggested that prostaglandins produced by Kupffer and endothelial cells mediate the action of PMA by directly acting on the hepatocytes[14] or by inducing the flow redistribution and local hypoxia[15].

In the present study, PMA decreasd hepatic flow in a dose-dependent manner. The decline of flow by PMA was antagonized by St, a PKC inhibitor. Since prostaglandin synthesis by Kupffer cells can be mediated by a PKC-dependent pathway[1], PKC in Kupffer cells may regulate hepatic flow by altering prostaglandin synthesis. Interestingly St attenuated not only PMA-induced but also PGE_2- and PGD_2-induced declines of flow. Since prostaglandins can directly act on vasoactive cells, PKC in vasoactive smooth muscle or endothelial cells may also be involved in the regulation of hepatic flow. However, like other inhibitors of PKC, St inhibits not only PKC but also other protein kinases including myosinlight-chain kinase[12,20]. Thus, it is also likely that the antagonism of PGE_2- and PGE_2-induced contraction by St may be attributed to non-specific action of St on other protein kinases.

Since we employed KRB as the perfusion medium, the interaction of blood cells and liver cells was excluded in the present study. Infusion of PMA to rabbits has been reported to cause neutropenia, thrombocytepenia and severe respiratory distress[21]. PKC inhibitors have been shown to inhibit platelets aggregation induced by various stimuli[22], and to suppress lysosomal enzyme release from neutrophils[23]. Thus, *in vivo* activation of PKC may produce a more severe deterioration of microcirculation in the liver, which may precipitate hepatic dysfunction or failure.

In summary, we showed that the modulation of PKC with PMA and St has a significant influence on intrahepatic regulatory mechanisms of hepatic flow. Since the activation of PKC has been implicated in the pathogenesis of endotoxicosis and severe sepsis, the alterations of hepatic blood flow and metabolism during sepsis may be mediated by PKC-dependent pathway. PKC is a large family of related enzymes that may have different enzymological characteristics and distinct functions in the modulation of physiological and pathological responses. It is possible that global inhibition of PKC may have some deleterious effects on sepsis. Thus, the identification and modulation of specific subspecies of the enzyme in severe sepsis

may serve as a new therapeutic approach to hepatic dysfunction in septic patients. Among clinically available drugs, phospholipid-interacting agents such as polymyxin B[24], chlorpromazine, imipramine, phentolamine, verapamil, dibucaine and tetracaine[25] have been reported to be indirect inhibitors of PKC. It remains to be clarified whether these agents are effective for the preservation of hepatic blood flow and function in septic patients.

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