Calcium-Dependent Synthesis of Prostacyclin in ATP-Stimulated Venous Endothelial Cells

Jaehwa Choi, Leah W. Hammer, Robert L. Hester

Abstract—Prostacyclin is a powerful vasodilator that is released from vascular endothelial cells. Previous studies in our laboratory have indicated that arachidonic acid metabolites from venous endothelium play an important role in the dilation of adjacent arterioles during muscle stimulation. Furthermore, recent studies have suggested that ATP released from red blood cells during hypoxia promotes dilation of arterioles. We tested the hypothesis that an ATP-induced increase in intracellular Ca\(^{2+}\) in venous endothelium promotes prostacyclin synthesis. Small branches of femoral veins were isolated from male golden hamsters, placed in a 1 mL bath, and cannulated for perfusion with 3-(N-morpholino) propanesulfonic acid (MOPS)-buffered physiological salt solution at 37°C. Prostacyclin synthesis was determined by enzyme immunoassay of bath solution. Perfusion of veins with ATP increased prostacyclin synthesis from 50±5 to 627±46 pg/mL (n=49). ATP-induced prostacyclin synthesis was inhibited by removal of extracellular Ca\(^{2+}\), chelation of intracellular Ca\(^{2+}\) with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (10 μmol/L for 10 minutes), and preincubation with cytosolic phospholipase A\(_2\) (PLA\(_2\)) inhibitors, AACOCF\(_3\), and bromoenol lactone. Changes in intracellular Ca\(^{2+}\) in cultured human venous endothelial cells were assessed by fura-2 spectrofluorometry. ATP induced a transient Ca\(^{2+}\) peak within seconds, and the subsequent Ca\(^{2+}\) plateau was abolished by removal of extracellular Ca\(^{2+}\). An increase in prostacyclin synthesis was detected in these cells 2 minutes after application of ATP. These findings suggest that the ATP-induced increase in intracellular Ca\(^{2+}\) stimulates prostacyclin synthesis in venous endothelial cells. (Hypertension. 2002;39[part 2]:581-585.)

Key Words: microcirculation ■ prostacyclin ■ veins ■ endothelium

Local regulation of vascular tone through the production of vasoactive substances is dependent on endothelial function. In vivo studies of the regulation of the microcirculation in the hamster cremaster muscle have revealed an obligatory role of the venular endothelium and intact venular flow in the dilation of its paired arteriole in response to stimulation of the muscle.\(^1,2\) In these studies, disruption of the endothelium of venules paired with arterioles attenuated the arteriolar vasodilation that was observed in response to electrical stimulation of the muscle.

Vasodilators released from the endothelium include the arachidonic acid metabolite prostacyclin, nitric oxide, and endothelium-derived hyperpolarizing factor. However, the quantitative role of these vasodilators in different regions of the vasculature has not been characterized. Nitric oxide appears to have a minimal role in regulating arteriolar diameter during an increase in metabolic rate.\(^3,5\) Previous studies from our laboratory have shown that arachidonic acid metabolites make a major contribution to the regulation of arteriolar diameter during muscle stimulation.\(^6,7\)

Arachidonic acid is released from membrane phospholipids through the direct action of phospholipase A\(_2\) (PLA\(_2\)). Further metabolism of arachidonic acid generates three main groups of eicosanoids: prostaglandins and thromboxanes by cyclooxygenase; leukotrienes and lipoxins by lipooxygenase; and epoxides by cytochrome P-450 epoxygenase. Prostacyclin (prostaglandin I\(_2\)) is a very powerful vasodilator that is produced primarily in vascular endothelial cells.\(^4\) There are both secretory and intracellular forms of PLA\(_2\).\(^8\) The cytosolic form of PLA\(_2\) includes both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent isozymes. However, it is not yet certain which of these enzymes is responsible for the release of arachidonic acid for the regulation of arteriolar diameter during muscle stimulation.

Ellsworth et al have suggested a role for ATP released from red blood cells during hypoxia.\(^10,11\) Application of ATP at a physiological concentration to the lumen of arterioles and venules in vivo induces increases in blood flow and tissue PO\(_2\).\(^10\) A recent in vivo study from our laboratory has demonstrated that administration of ATP in venules will result in a vasodilation of the adjacent arteriole.\(^12\) The present study was designed to determine the cellular processes involved in prostacyclin release from venular endothelial cells in response to increases in ATP. We tested the hypothesis that an ATP-induced increase in intracellular Ca\(^{2+}\) in the venous endothelium was necessary to stimulate prostacyclin synthesis.

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From the Department of Physiology and Biophysics, University of Mississippi Medical Center (J.C., L.W.H., R.L.H.), Jackson, Miss.

Correspondence to Dr Robert L. Hester, Department of Physiology and Biophysics, University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216-4505. E-mail rhester@physiology.umsmed.edu

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Methods

Isolation of Veins for In Vitro Perfusion
The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and were carried out in accord with both the Guide for the Care and Use of Laboratory Animals from the National Institute of Health and the guidelines of the Animal Welfare Act. Male golden hamsters (140g to 170g, Charles River Laboratories, Wilmington, Mass) were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg). The left jugular vein was cannulated for continuous infusion of pentobarbital sodium in 0.9% saline solution (5 mg/mL at 0.01 mL/min). Deep esophageal temperature was maintained at 37°C to 38°C by convectional heating. The hindlimb vasculature of the hamsters was exposed by a skin incision and the tissue was kept moist by superfusion with physiological salt solution (PSS) of the following composition (in mmol/L): 132 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.5 MgCl₂, 1.2 NaH₂PO₄, 5 glucose, and 3 MOPS (pH 7.4 at 37°C). To ensure adequate dissolution of oxygen in the buffer and thus to avoid a possible effect of hypoxia on prostacyclin synthesis, MOPS-buffered PSS was bubbled with 10% O₂/5% CO₂ balance instead of 6% O₂ (normal O₂ level in venous blood). Our preliminary data show no significant difference in prostacyclin synthesis with 5% or 10% O₂. Blood vessels were perfused at a pressure of 25 mm Hg, which resulted in a flow rate of approximately 1 mL/min. Prostacyclin release into the bath solution was determined through measurement of the stable metabolite 6-keto-prostaglandin F₁α using an enzyme-immunoassay kit from Neogen Corp.

Protocols for In Vitro Perfusion Studies
Isolated veins were perfused with MOPS-PSS for a 50-minute equilibration period. The bath was then replaced with fresh MOPS-PSS every 10 minutes, with the entire 1 mL bath solution being collected for measurement of prostacyclin release. The protocols involved a 10-minute perfusion of the vessel with MOPS-PSS containing 100 μM/L ATP. The perfusate was changed to include either a Ca²⁺ free solution or a variety of inhibitors before a second 10-minute perfusion with MOPS-PSS containing 100 μM/L ATP. For the Ca²⁺ dependence studies, there was a 10-minute wash period between the two ATP perfusion periods. For the PLA₂ inhibitor studies, there was a 20-minute wash period between the two ATP perfusion periods. The concentration of 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA/AM) used to chelate intracellular calcium was chosen on the basis of previous studies and our Ca²⁺ studies in human umbilical vein endothelial cells (HUVEC). The concentrations of the phospholipase A₂ inhibitors, AACOCF₃, and bromoelcolactone (BEL), were chosen on the basis of previously published studies.

Culture of Human Umbilical Vein Endothelial Cells
Primary cultures of HUVEC were purchased from Clonetics (Walkersville, Md) and further propagated according to the manufacturer’s directions. The cells were examined for positive immunofluorescence staining of von Willebrand factor (vWF) and for uptake of acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Di-Ac-LDL, Molecular Probes), which are characteristic features of endothelial cells. As preliminary studies showed negative staining of the cells (<30%) after 4 to 5 passages in cell culture, cells after only 2 to 3 passages were used throughout these experiments.

Measurement of Intracellular Free Ca²⁺ Concentration
Changes in intracellular free Ca²⁺ concentration [Ca²⁺]ᵢ, in HUVEC were assessed using the fluorescent Ca²⁺ indicator fura2 (Molecular Probes). Cells were plated onto glass coverslips at a density of 5x10⁴ cells/cm² and incubated overnight. To load the Ca²⁺ indicator, the cells were washed with extracellular solution containing (in mmol/L): 125 NaCl, 6 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose. The cells were loaded for 30 minutes at 37°C with fura2-AM diluted to a final concentration of 2 μmol/L in the extracellular solution. The coverslip with fura2-loaded cells was washed with extracellular solution and positioned diagonally in a quartz cuvette maintained at 37°C. The fura2-loaded cells were excited alternately at 340 nm and 380 nm and the emitted fluorescence was measured at 510 nm using a Photon Technology International RatioMeter RC-D spectrofluorometer. The [Ca²⁺]ᵢ was estimated using the equation previously described.

Prostacyclin Release from HUVEC Cultures
Cultured HUVEC were plated on 24-well tissue culture plates at a density of 10⁶ cells/cm², incubated overnight in EGM BulletKit medium (Clonetics), and serum starved for 16 hours. The cells were washed twice with PSS before stimulation with the appropriate agent in 300 μL MOPS-PSS. The tissue culture supernatant (200 μL) was collected after stimulation to determine prostacyclin synthesis.

Data Analysis
For statistical analysis of data from the vessel perfusion studies, the prostacyclin response to the second application of ATP was calculated as a percentage of the release following the first application of ATP. The responses to the treatments were compared with the control responses using the Mann-Whitney rank-sum test to determine statistical significance.

Chemicals
Arachidonyl trifluoromethyl ketone (AACOCF₃), BEL, and BAPTA/AM, were purchased from Calbiochem, Cayman Chemical Company, and Molecular Probes, Inc, respectively. Concentrated (10⁴-fold) stock solutions were prepared in dimethyl sulfoxide (DMSO) and frozen at −80°C. Reagents for cell culture were purchased from Clonetics. All other reagents were obtained from Sigma.

Results

Ca²⁺-Dependent Synthesis of Prostacyclin in ATP-Perfused Veins
Perfusion of isolated veins with 100 μmol/L ATP produced an increase in prostacyclin synthesis from a basal level of 50±5 to 627±46 pg/mL (mean±SE, n=49). To determine whether the ATP-induced prostacyclin synthesis was mediated by an increase in [Ca²⁺]ᵢ, vessels were perfused with ATP before and after removal of extracellular Ca²⁺ or chelation of intracellular Ca²⁺ with BAPTA. As a time control, veins were stimulated repetitively with 100 μmol/L ATP under identical conditions, with a 10-minute intervening wash period; prostacyclin synthesis during the second stimulation averaged 46±13% (n=6) of that during the original stimulation (Figure 1A). Removal of extracellular Ca²⁺ from both the bath and the perfusion solution (†ATP) or the perfusion solution alone (‡ATP) significantly inhibited prostacyclin synthesis during the second ATP stimulus (Figure 1B, P<0.01), indicating that ATP-induced Ca²⁺ influx is essential for prostacyclin synthesis. Chelation of intracellular Ca²⁺, by perfusion of 10 μmol/L BAPTA/AM for 10 minutes, also significantly inhibited ATP-induced prostacyclin synthesis (Figure 1D, P<0.01), demonstrating the importance of intracellular Ca²⁺ in ATP-induced prostacyclin synthesis.
Role of Cytosolic PLA2 in ATP-Induced Prostacyclin Synthesis

We determined if the Ca\(^{2+}\)-dependent process in ATP-induced prostacyclin synthesis was mediated by cytosolic PLA2. To ensure that the veins had recovered more completely from the first ATP stimulation, the washing period was extended to 20 minutes (Figure 2A); under these conditions the second response averaged 87±13% (n=6) of the initial response. Treatment of the vessels with the Ca\(^{2+}\)-dependent cytosolic PLA2 inhibitor AACOCF3 (30 μmol/L) abolished ATP-induced prostacyclin synthesis (Figure 2B, *P* < 0.05). The Ca\(^{2+}\)-independent PLA2 inhibitor BEL (10 μmol/L) also significantly inhibited this response (Figure 2C, *P* < 0.05). These results suggest that both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent PLA2 contribute to ATP-induced prostacyclin synthesis in the venous endothelium.

ATP-Induced Changes in [Ca\(^{2+}\)]\(i\) and Prostacyclin Synthesis in HUVEC

The preceding observations suggest that an ATP-induced increase in [Ca\(^{2+}\)]\(i\) in venous endothelial cells was likely to be responsible for the stimulation of prostacyclin synthesis. To verify our hypothesis, we initially aimed to measure ATP-induced [Ca\(^{2+}\)]\(i\) changes in endothelial cells isolated from small veins from the hamster. Due to technical difficulties culturing these cells, changes in [Ca\(^{2+}\)]\(i\) were measured in HUVECs instead. As shown in Figure 3A, ATP induced a sustained increase in [Ca\(^{2+}\)]\(i\), in HUVECs. The transient Ca\(^{2+}\) peak arose within seconds of ATP stimulation. Removal of extracellular Ca\(^{2+}\) abolished the sustained increase in [Ca\(^{2+}\)]\(i\) induced by ATP (Figure 3C). Chelation of intracellular Ca\(^{2+}\) with 10 μmol/L BAPTA (10 minutes loading) abolished both the transient peak and the sustained increase in [Ca\(^{2+}\)]\(i\) (Figure 3D). Stimulation of HUVECs with ATP increased prostacyclin synthesis (Figure 4A). Furthermore, the ATP-induced prostacyclin synthesis reached a peak level 2 minutes after application of 10 μmol/L ATP (Figure 4B).

**Discussion**

ATP has been shown to stimulate prostacyclin release from endothelial cells. Recent evidence from our laboratory has shown, in vivo, that ATP infusion into venules results in a cyclooxygenase dependent vasodilation of the adjacent arteriole. The current study shows that exposure of venous endothelial cells to ATP results in a significant production of prostacyclin. Although several prior studies have also shown that ATP induces prostacyclin synthesis by the endothelium, those studies were performed on cultured cells or perfused arterial segments. Stimulation of cells with ATP induces an increase in [Ca\(^{2+}\)]\(i\), by both activation of Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores and Ca\(^{2+}\) influx through ion channels in the plasma membrane. Removal of extracellular Ca\(^{2+}\) pre-
vent the Ca\(^{2+}\) influx that is essential for maintaining a sustained increase in \([\text{Ca}^{2+}]\); such removal was found to abolish ATP-induced prostacyclin synthesis in these vessels. This finding is consistent with previous observations in cell culture systems.\(^{22-24}\) Treatment of the vessel with the Ca\(^{2+}\)-dependent cytosolic PLA\(_2\) inhibitor AACOCF\(_3\), also inhibited prostacyclin synthesis, implicating the cytosolic PLA\(_2\) as the \(\text{Ca}^{2+}\)-dependent process in ATP-induced prostacyclin synthesis. An increase in \([\text{Ca}^{2+}]\) is required for translocation of the \(\text{Ca}^{2+}\)-sensitive cytosolic PLA\(_2\) from the cytosol to the endoplasmic reticulum and the nuclear membrane. Furthermore, it has been suggested that a sustained elevation of intracellular \(\text{Ca}^{2+}\) and phosphorylation of PLA\(_2\) by mitogen-activated protein kinase are important for full activation of this PLA\(_2\).\(^{24,25}\) Several isotypes of PLA\(_2\) have been characterized and their role in generating arachidonic acid in specific cell types still needs further investigation. We also found that the \(\text{Ca}^{2+}\)-independent PLA\(_2\) inhibitor BEL substantially inhibited ATP-induced prostacyclin synthesis in the venous endothelium. Because our data show a similar pattern with the \(\text{Ca}^{2+}\)-dependent PLA\(_2\) inhibitor, we speculate that \(\text{Ca}^{2+}\)-dependent and \(\text{Ca}^{2+}\)-independent isozymes of PLA\(_2\) play a synergistic role in ATP-induced prostacyclin synthesis.

The in vivo studies that we have performed previously have examined the role of arachidonic acid metabolites released from \(\approx 100 \mu\text{m}\) venules in the dilation of arterioles during muscle stimulation.\(^{5,7,12}\) The ideal approach to investigating the contribution of ATP to functional hyperemia in hamster cremaster vessels would be to conduct studies on isolated venules from the hamster cremaster muscle. We thus conducted preliminary studies on such venules. Those studies revealed an increase in prostacyclin synthesis in response to ATP, as in our present study with the larger veins. However, the basal level of prostacyclin synthesis was below the detection limit of the assay, thus limiting our ability to detect changes in prostacyclin synthesis. Because such microvessels are very difficult to isolate and perfuse, the present study characterized ATP-induced prostacyclin synthesis in a relatively large vein. Although this may limit generalization from our data, these data provide direct evidence that venous endothelium can release the vasoactive substance prostacyclin. Moreover, higher concentrations of prostacyclin might be attained in the smaller venules owing to their greater surface area to volume ratio. Additionally, we have not been able to isolate endothelial cells from small veins or venules. Therefore we used HUVECs to examine the intracellular \(\text{Ca}^{2+}\) changes during ATP treatment. The measurements of prostacyclin synthesis in cultured HUVECs (Figure 4) corroborate the data from the isolated veins presented in Figures 1 and 2. The cytosolic concentration of ATP in erythrocytes is in the range 4 to 5 mmol/L. Previous studies have reported that released ATP can reach a concentration in the range 0.2 to 20 \(\mu\text{mol/L}\) under various experimental conditions.\(^{26}\) Higher concentrations may be attained locally. Moreover, previous studies from our laboratory have demonstrated that venular application of both 1 \(\mu\text{mol/L}\) and 100 \(\mu\text{mol/L}\) ATP induced arteriolar dilation.\(^{12}\) We used 100 \(\mu\text{mol/L}\) ATP in the present study, rather than 1 \(\mu\text{mol/L}\) ATP, to better characterize the effect of the inhibitors.
The experiments presented in this paper are a continuation of our studies determining the role of the release of arachidonic acid metabolites from venular endothelium in the control of arteriolar diameter. We have presented evidence that, in vivo, the venular endothelium does release products of the cyclooxygenase pathway, presumably prostacyclin. The stimuli for the release of prostacyclin are not known. On the arteriolar side of the circulation, several metabolic factors, including increased $\text{PCO}_2$, decreased $\text{PO}_2$, and acidosis, have been shown to stimulate prostanooid release.27–29 Recent studies have indicated that erythrocytes release ATP under conditions of hypoxia.10 Such release should occur predominantly in the venules where $\text{PO}_2$ levels decline as a result of muscle stimulation. Future studies will be needed to determine the overall quantitative importance of ATP release from erythrocytes in the stimulation of prostanooid release from the venular endothelium.

In conclusion, our study shows that ATP induces significant production of prostacyclin by the venous endothelium. A $\text{Ca}^{2+}$-dependent cytosolic PLA$_2$signaling pathway is essential to this process. These data provide more evidence that release of ATP from erythrocytes may be important in the regulation of arteriolar diameter in the microcirculation.

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