Effects of Bradykinin on Prostaglandin I₂ Synthesis in Human Vascular Endothelial Cells

Seiki Yamasaki, Shohei Sawada, Sumio Komatsu, Takeshi Kawahara, Yutaka Tsuda, Toshiyuki Sato, Akihisa Toratani, Yoshihito Kono, Tadashi Higaki, Hitoshi Imamura, Yusuke Tada, Naoaki Akamatsu, Toshiyuki Tamagaki, Hajime Tsuji, Masao Nakagawa

Abstract—The effects of bradykinin on the regulatory mechanisms of prostacyclin synthesis in endothelial cells were investigated in association with intracellular Ca²⁺ kinetics, cytosolic phospholipase A₂ (cPLA₂) activity, and mRNA expression of cPLA₂ and prostaglandin H synthase (PGHS) isoforms. Bradykinin enhanced prostacyclin release from endothelial cells time-dependently, but pretreatment with EGTA H-7 or HOE 140 inhibited bradykinin-induced prostacyclin release. Bradykinin increased both the influx of extracellular Ca²⁺ and Ca²⁺ release from the intracellular Ca²⁺ storage sites. These reactions occurred within 5 minutes after bradykinin stimulation. Within 15 minutes, bradykinin activated cPLA₂ to 1.3-fold the control level. The constitutive expressions of mRNA of cPLA₂, PGHS-1, and PGHS-2 was 87, 562, and 47 amol/µg RNA, respectively. With the stimulation of bradykinin, cPLA₂ mRNA increased to 746 amol/µg RNA in 15 minutes, PGHS-1 mRNA increased to 10 608 amol/µg RNA, and PGHS-2 mRNA increased to 22 400 amol/µg RNA in 180 minutes. Pretreatment with cycloheximide superinduced cPLA₂ and PGHS-2 mRNA expression but almost completely inhibited PGHS-1. Pretreatment with EGTA had effects similar to pretreatment with cycloheximide in the case of cPLA₂ and PGHS-1 but did not affect PGHS-2. These findings suggest that the elevation of cPLA₂ activity caused by the increase of intracellular Ca²⁺ concentration is important in the early phase of bradykinin-induced prostacyclin synthesis and that the mechanisms regulating cPLA₂ are different from those regulating PGHS isoforms in endothelial cells. (Hypertension. 2000;36:201-207.)

Key Words: bradykinin ■ prostacyclin ■ kinetics, calcium ■ phospholipases A ■ prostaglandin H synthase ■ polymerase chain reaction

B radykinin (BK) has blood pressure–lowering effects that can be induced by either the direct action of BK on target organs or the secretion of secondary agents. BK in plasma mainly acts on endothelial cells and stimulates them to release various agents associated with vascular tonus and blood coagulation, such as prostaglandins, endothelium-derived relaxation factor, catecholamines, and cytokines, resulting in the maintenance of circulation homeostasis. BK is resolved into inactive peptides by 2 kininases, carboxypeptidase N (kininase I) and dipeptide hydrase (kininase II). Kininase II is the same enzyme as angiotensin I–converting enzyme (ACE) and is secreted by endothelial cells. The clinical observation that ACE inhibitors lower blood pressure, even under low renin conditions, suggests a renin-angiotensin system–independent mechanism for evoking their actions. From reports that the blood pressure–lowering effect of ACE inhibitors correlates with the increase of intravascular concentration of kinin and prostacyclin (prostaglandin I₂, PGI₂), it appears that this mechanism is due to the local accumulation of BK and BK-induced vasodilators, especially PGI₂ rather than to the reduction of vasoconstrictor angiotensin II²; however, the effects of ACE inhibition on BK and prostaglandins are unclear. PGI₂ is synthesized in response to release of arachidonic acid (AA) from phospholipids followed by transformation of AA to PGH₂ by prostaglandin H synthase (PGHS=cyclooxygenase; COX) and subsequent transformation of PGH₂ by PGI₂ synthase. Recent studies have revealed that PLA₂ and PGHS have several isoforms and that many agents affect their expression at the level of genome transcription or protein translation. However, there have been few reports about the relation between BK-induced PGI₂ synthesis and signal transduction mechanisms in endothelial cells.

In the present study, we investigated the effects of BK on the regulatory mechanisms of PGI₂ generation in human umbilical vein endothelial cells (HUVEC) with reference to intracellular Ca²⁺ kinetics, cytosolic PLA₂ activity, and mRNA expression of cPLA₂ and PGHS isozymes.

Methods

Culture of HUVEC

Endothelial cells were obtained from human umbilical cord vein and cultured according to our previously described method.¹ We used primary cultured HUVEC that formed confluent monolayers in the...
following experiments. Cells were identified as vascular endothelial cells by electron microscopic findings of Weibel-Palade bodies in their cytoplasm and detection of ACE activity.

Assay of PGI₂ and Inositol 1,4,5-Trisphosphate Concentration

The concentration of PGI₂ and inositol 1,4,5-trisphosphate (IP₃) was measured with the use of previously described methods. Briefly, the amount of PGI₂ released from HUVEC was measured as the stable metabolite 6-keto prostaglandin F₁₃₀₆ with the use of a [³H] 6-keto prostaglandin F₁₃₀₆ radioimmunoassay kit (New England Nuclear), and the IP₃ in HUVEC was measured with the use of the D-myo-inositol 1,4,5-trisphosphate [²H] Biotrack radioimmunoassay system (Amersham Pharmacia Biotech).

Measurement of cPLA₂ Activity

After incubation in tissue culture medium (TCM) for 12 hours, the cells were scraped from the dish and collected into glass test tubes. The contents were centrifuged at 3000g for 5 minutes at 4°C. The cells were resuspended in TCM and centrifuged under the same conditions. The cells were then resuspended in TCM at a final concentration of 10⁶ cells/mL and sonicated at 4°C for 3×10 seconds. The sonicated cells were centrifuged at 3000g for 10 minutes to remove unlysed cells. The supernatant (soluble fraction) was transferred to another container and used as a source for the assay of cPLA₂. The activity of cPLA₂ was measured by the method of Kramer, with some modification. Each substrate was dried under nitrogen gas and suspended in distilled water by sonication at room temperature. The standard incubation system for the assay of cPLA₂ contained, in 0.25 mL, 50 mmol/L HEPES (pH 7.4), 0.25 mL of a [³H] 6-keto prostaglandin F₁₃₀₆ reagent, and 100 mg of silicic acid powder. After the tubes were shaken in a vortex mixer for 5 minutes and centrifuged at 3000g for 60 minutes, the supernatant and the IP₃ in HUVEC were measured with the use of the D-myo-inositol 1,4,5-trisphosphate [²H] Biotrack radioimmunoassay system (Amersham Pharmacia Biotech).

Measurement of Ca²⁺ Release From Ca²⁺ Storage Sites

First, the cells were scraped from the dish and permeabilized with saponin according to our previously described method. The saponized HUVEC were subsequently incubated for 15 minutes for the addition of Ca²⁺, phosphocreatine (PC), CPK, succinic acid disodium salt (Suc), and adenosine 5'-triphosphate magnesium salt (ATP). After the supernatant was removed by centrifugation, the cells were resuspended in 3×10⁻⁶ mol/L Ca²⁺-EGTA buffer solution containing PC, CPK, Suc, and ATP and then were aliquoted into several tubes (2×10⁶ cells/200 µL). After stimulation of the cells with the test agent for a certain period, 200 µL of cell suspension was filtered on a filter unit using a diaphragm vacuum pump and 1225 Sampling Manifold (Millipore Co) with 30 mL of a rinsing buffer solution. The radioactivity of HUVEC on the vacuum pump filter was measured as residual Ca²⁺ in the cells by means of liquid scintillation spectrometry. The Ca²⁺ release from Ca²⁺ storage sites induced by agents was calculated at 10 seconds and compared with the Ca²⁺ release induced by Ca²⁺ ionophore A23187.

Quantitative Measurement of mRNA

Synthesis of Primers for Genomic DNA Preparation, Reverse Transcription Reaction, and Competitive Polymerase Chain Reaction

Primers used for genomic DNA (gDNA) amplification, reverse transcription, and for competitive polymerase chain reaction (PCR) were synthesized on the Gene Assembler Plus (Pharmacia LKB Biochorm Ltd). The sequences of 5'- and 3'-flanking oligonucleotide primers used for PCR amplification of gDNA and mRNA were 5'-GGAAGAGACGTGGCCAGTGCCAGCTC3'-PGHS-1-Fw, 5'-TTGTCCTCAATAATGGGCCAGTCTACCC3'-PGHS-1-Rv, 5'-TTCTTGCTATTAGACCTGCTGCCC3' (PLA₂-Fw), 5'-TACAATTAGGCCAGACAAATGTTGAAGGGC3'-PLA₂-Rv, 5'-ATGTTCCACCGCACTACAG3'-PGHS-2-Fw, and 5'-GCCTCCAGTTATGGCAAT3'-PGHS-2-Rv.

RNA Isolation and Reverse Transcription

Total cellular RNA was isolated from HUVEC and was reverse transcribed to complementary DNA (cDNA) by Moloney murine leukemia virus reverse transcriptase according to our previously published method. The concentrations of total RNA were determined by spectrophotometer at a 260-nm wavelength.

Amplification of Genomic PGHS-1 DNA and Its Purification for Internal Standard of Competitive PCR

Genomic DNA samples were extracted from heparinized blood and amplified by PCR following the method of Saiki et al. in the presence of 25 pmol of each primer (PGHS-1-Fw and -Rv), 100 µmol of each dNTP, 20 µg genomic DNA, 1× buffer (5 mmol/L Tris-HCl [pH 8.3], 20 mmol/L MgCl₂, 50 mmol/L KCl, 0.01% gelatin), and 5 U of AmpliTaq (Takara Co), for a total volume of 200 µL. With the use of the Gene ATAQ controller (Pharmacia LKB Biochorm Ltd), 40 PCR cycles were performed as follows: denaturation at 94°C for 1 minute, annealing at 62°C for 2 minutes, and extension at 72°C for 3 minutes. Five microliters of the reaction mixture containing the PCR product was subjected to electrophoresis on 2% agarose gel for confirmation of the product size. The remaining 195 µL was loaded onto a 10% polyacrylamide gel (acylamide/bis-acrylamide: vol/vol, 29:1) and electrophoresed for 40 minutes. The segment of the gel containing the DNA band was cut out by visualizing the ethidium-bromide–stained gels in long-wavelength ultraviolet light. The gel slice then was transferred to a microtube containing elution buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mL EDTA, 0.2% SDS, 0.3 mmol/L NaCl). After the tube was incubated at 37°C for 12 hours, the supernatant was transferred to another microtube. Two volumes of 100% ethanol were added to the tube at 4°C, and the tube was stored at -20°C for 30 minutes. The DNA was recovered as a pellet by centrifugation at 12000 g for 10 minutes at 4°C in a microfuge. Then the pellet was rinsed twice with 70% ethanol and redissolved in 50 µL of dH₂O.
Effect of Inhibitors on Constitutive and Bradykinin-Induced PGI\textsubscript{2} Release From HUVEC

<table>
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<tr>
<th>Inhibitors</th>
<th>Control (15 min)</th>
<th>15 min</th>
<th>60 min</th>
<th>180 min</th>
<th>Bradykinin (10^{-1} mol/L)</th>
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<tr>
<td></td>
<td>6-keto PGF\textsubscript{1a} concentration (ng/2×10^5 cells)</td>
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<td>Buffer</td>
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<td>18.22±3.51</td>
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<td>63.37±3.42^*</td>
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<td>Mepacrine, 10^{-4} mol/L</td>
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<td>1.93±0.18^†</td>
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<td>Aspirin, 10^{-3} mol/L</td>
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<td>1.54±0.13^†</td>
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<td>EGTA, 10^{-3} mol/L</td>
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<td>16.65±0.77^†</td>
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<tr>
<td>H-7, 10^{-3} mol/L</td>
<td>16.18±0.57</td>
<td>16.63±1.03^†</td>
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<tr>
<td>U-73122, 10^{-5} mol/L</td>
<td>6.82±1.21^*</td>
<td>7.84±1.07^†</td>
<td>19.77±0.79^*</td>
<td>32.8±1.18^†</td>
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<td>RHC80267, 5×10^{-5} mol/L</td>
<td>6.20±1.75^*</td>
<td>5.75±0.7^†</td>
<td>9.93±0.5^†</td>
<td>7.56±0.52^†</td>
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<td>NS-398, 10^{-4} mol/L</td>
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<td>58.2±3.66^*</td>
<td>74.23±4.89^*</td>
<td>65.09±2.67^*</td>
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<td>des-Arg&lt;sup&gt;6&lt;/sup&gt;-bradykinin, 10^{-4} mol/L</td>
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<td>78.59±4.19^*</td>
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<td>HOE 140, 10^{-4} mol/L</td>
<td>18.83±1.25</td>
<td>21.16±0.60^†</td>
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Data are expressed as mean±SEM. ^*P<0.01 vs buffer (control, 15 minutes), †P<0.01 vs buffer (bradykinin, 15 minutes). n=6.

Synthesis of Mutant-cDNA of cPLA\textsubscript{2} and PGHS-2 by Site-Directed Mutagenesis With PCR Used for Internal Standard of Competitive PCR

Complementary DNA of a mutant-cDNA of cPLA\textsubscript{2} and PGHS-2 were prepared by PCR-based, site-directed mutagenesis following the method of Higuchi et al.\textsuperscript{8} The mutagenic primer of sequence 5'‘-GTTGTTATCATGCATGTATGCCACGAGGTTGTTCTC-3' (PLA\textsubscript{2}-mFw), 5'‘-ATGTTCCACCCGAGCTACA- GAAAG-TATCACA-3' (PGHS-2-mFw), which created a unique Hind\textsubscript{III} site. In this manner, cPLA\textsubscript{2} and PGHS-2 were divided into 2 fragments by Hind\textsubscript{III}– digested site. In this manner, competitive PCR products of native and mutant-cDNA of cPLA\textsubscript{2} and PGHS-2 did not have the Hind\textsubscript{III}–digested site. In this manner, competitive PCR products of native and mutant-cDNA of cPLA\textsubscript{2} and PGHS-2 were differentiated by digesting them with Hind\textsubscript{III} before electrophoresis. The samples then were loaded onto an 8% polyacrylamide gel (acrylamide/bis-acrylamide: vol/vol, 29:1) and analyzed by electrophoresis. The gel was stained with ethidium bromide (0.4 μg/ml) and photographed. The bands corresponding to the amplified products of the internal standard (gDNA or digested mutant cDNA) and mRNA were cut out, and their radioactivity was counted. The data were analyzed by plotting the logarithm of the gDNA cpm/cDNA cpm ratio as a function of the logarithm of the gRNA concentration.

Statistical Analysis

Data are expressed as mean±SEM. ANOVA was used to make comparisons. For F ratios significant at the level of ≤5%, Duncan’s multiple range test was applied to determine differences between any 2 groups. Differences of ≤5% (P<0.05) were considered statistically significant. Percent changes, for which a normal distribution cannot be assumed, were compared by means of the Kruskal-Wallis nonparametric method for ANOVA. Whenever the χ² test results were significant, the Mann-Whitney test was used to determine the significance of the differences between pairs of means.

Results

The addition of BK increased PGI\textsubscript{2} release dose-dependently at concentrations from 10^{-10} mol/L through 10^{-5} mol/L, and the release reached a plateau at the concentration of 10^{-5} mol/L, so we used this concentration (10^{-5} mol/L) of BK in the following experiments. The stimulation of BK remarkably increased PGI\textsubscript{2} generation within 15 minutes, and the effect was sustained through 180 minutes. This increase was not inhibited by pretreatment with the B\textsubscript{2} receptor antagonist des-Arg<sup>6</sup>-BK(10^{-4} mol/L) or the PGHS-2 inhibitor NS-398 (10^{-6} mol/L) but was inhibited by pretreatment with the B\textsubscript{2} receptor antagonist HOE 140 (10^{-4} mol/L) and was reduced to the constitutive level by pretreatment with the extracellular Ca\textsuperscript{2+} chelator EGTA (10^{-3} mol/L) or protein kinase C (PKC) inhibitor H-7 (10^{-3} mol/L). Pretreatment with a phospholipase C (PLC) inhibitor, U-73122 (10^{-3} mol/L), or a diacylglycerol (DAG) lipase inhibitor, RHC80267 (5×10^{-5} mol/L), reduced PGI\textsubscript{2} release to the level under the constitutive generation and pretreatment with a PLA\textsubscript{2} inhibitor, mepacrine (10^{-4} mol/L), or a PGHS inhibitor, aspirin (10^{-3} mol/L), reduced PGI\textsubscript{2} release to almost zero in control as well as in BK stimulation experiments (Table). The concentration of BK in incubation fluid measured by radioimmunoassay- dextran coated charcoal method at 15, 60, 180 minutes after BK addition was 1.12±0.06 (10^{-5} mol/L), 1.16±0.02 (10^{-5} mol/L), and 1.10±0.11 (10^{-5} mol/L), respectively. These data were not statistically different from each other.

Within 15 minutes of BK addition, the cytosolic PLA\textsubscript{2} activity was increased to 132±10.3% of control. A further
increase to 199.4 ± 6.5% of control was found at 60 minutes, and this high level was maintained for 180 minutes.

The \([\text{Ca}^{2+}]_{i}\) in HUVEC was 86.37 ± 8.34 nmol/L in the steady state. BK caused a transient increase in \([\text{Ca}^{2+}]_{i}\) with the peak at 30 seconds after addition and the return to basal level within 5 minutes (Figure 1A). BK also increased \(^{45}\text{Ca}\) uptake to 145 ± 5.3% that of control. Pretreatment with EGTA (10\(^{-3}\) mol/L) remarkably decreased the increase of both \([\text{Ca}^{2+}]_{i}\) and \(^{45}\text{Ca}\) uptake induced by BK (Figure 1B).

The generation of IP\(_{3}\) in HUVEC was increased by BK from 0.213 ± 0.038 (pmol/2 × 10\(^{5}\) cells) to 0.476 ± 0.052 (pmol/2 × 10\(^{5}\) cells). Pretreatment with EGTA (10\(^{-3}\) mol/L) inhibited the BK-induced IP\(_{3}\) generation (Figure 2A). The addition of IP\(_{3}\) (10\(^{-5}\) mol/L) increased the \(^{45}\text{Ca}\) release from the intracellular calcium storage sites in saponized HUVEC. The increase of \(^{45}\text{Ca}\) release induced by IP\(_{3}\) was 35.7% less than that caused by the calcium ionophore A23187 (10\(^{-3}\) mol/L) (Figure 2B).

The constitutive expression of cPLA\(_{2}\) mRNA was 86.8 ± 6.8 amol/μg RNA in HUVEC. BK increased it to 746.5 ± 43.3 amol/μg RNA at 15 minutes after stimulation, and the same level of mRNA expression was maintained for 180 minutes. The constitutive expression of PGHS-1 mRNA was 562.2 ± 21.8 amol/μg RNA in HUVEC. Whereas BK had increased the expression only to 773.2 ± 15.4 amol/μg RNA after 15 minutes of stimulation, it reached to 10 608 ± 467.4 amol/μg RNA in 180 minutes. The constitutive expression of PGHS-2 mRNA was only 46.8 ± 3.0 amol/μg RNA in HUVEC. BK did not affect its expression until 60 minutes after stimulation; however, the increase reached 22 400 ± 2650 amol/μg RNA at 180 minutes (Figure 3).

Pretreatment with cycloheximide (5 μg/mL) for 60 minutes superinduced the mRNA expression of cPLA\(_{2}\) and PGHS-2 to 2183 ± 193 and 6891 ± 547 amol/μg RNA, respectively, but it suppressed the mRNA expression of PGHS-1 to 16.6 ± 2.5 amol/μg RNA. Pretreatment with EGTA (10\(^{-3}\) mol/L) for 60 minutes superinduced the mRNA expression of cPLA\(_{2}\) to 2718 ± 309 amol/μg RNA, but it suppressed that of PGHS-1 to 1.1 ± 0.8. The mRNA expression of PGHS-2 was increased by EGTA pretreatment from 46.8 ± 3.0 amol/μg RNA to 746.5 ± 43.3 amol/μg RNA at 15 minutes after stimulation.
89.5±23.7 amol/μg RNA; however, the difference of PGHS-2 mRNA expression between control and EGTA pretreatment was not statistically significant (Figure 4).

**Discussion**

ACE inhibitors are the most widely used drugs in the treatment of hypertensive patients. Their pharmacological effects depend not only on the reduction of the vasoconstrictor angiotensin II but also the local accumulation of kinins and kinin-related PGI₁ release, and the importance of the latter mechanisms has become apparent recently. Several kinds of BK-receptor subtypes have been identified and characterized according to their pharmacological and biochemical properties. Above all, the B₂ receptor is widely distributed over human tissues, especially smooth muscle cells and neurons, and mediates most of the cellular actions of BK.9 In the present study, pretreatment with the B₂-receptor antagonist des-Arg⁹-bradykinin did not inhibit the increase of BK-induced PGI₁ generation in HUVEC, but pretreatment with B₁-receptor antagonist HOE 140 inhibited the BK-induced increase in HUVEC. The increased effect of BK on PGI₁ release was sustained for 3 hours after stimulation, but the increase rate of PGI₁ release declined after 15 minutes of BK stimulation. Because the concentration of BK in incubation fluid did not change from the beginning of incubation to 180 minutes after BK addition, this suggests that the cause of the rate of decline of PGI₁ release was not due to the reduction of BK concentration in incubation fluid but to other factors, for instance, downregulation of BK receptors induced by long-term exposure of BK. Pretreatment with the calcium chelator EGTA, PKC inhibitor H-7, PLC inhibitor U-73122, or DAG lipase inhibitor RHC80267 remarkably reduced BK-induced PGI₁ release, but the effects of the latter 2 inhibitors were more suppressive. These findings suggest that PLC and DAG affect BK-induced PGI₁ synthesis in HUVEC not only by PKC but also the PKC-independent pathway. From these results, we speculate that BK acts on HUVEC through B₂ receptors and that extracellular Ca²⁺ and protein phosphorylation related to PKC, PLC, and DAG play important roles in the process of BK-induced PGI₁ synthesis.

The release of AA from phospholipids in the cell membrane is the first step of PGI₁ generation, and PLA₂ acts mainly in this step. PLA₂ has several isoforms. The genomic structures of type I and II of secretory PLA₂ (sPLA₂) and cytosolic PLA₂ (cPLA₂) have been determined. The release of AA from phospholipids of cell membranes is chiefly dependent on cPLA₂ and partially on sPLA₂.10 In the present study, BK elevated cPLA₂ activity to 132% of control within 15 minutes after stimulation, and this level of activity was maintained for 180 minutes. It is well known that cPLA₂ activity is affected by intracellular Ca²⁺ kinetics. BK increased [Ca²⁺], transiently to ≈240% of control. Pretreatment with the extracellular Ca²⁺ chelator EGTA reduced BK-induced Ca²⁺ influx and [Ca²⁺] increase, but this BK-induced [Ca²⁺] increase was not completely inhibited, and we could observe a small amount of increase in [Ca²⁺], despite EGTA pretreatment. This increase in [Ca²⁺], was thought to be mobilized from Ca²⁺ storage sites in cytosol. The intracellular Ca²⁺ storage sites consist of mitochondrial and nonmitochondrial sites, and the latter, especially for IP₃-induced Ca²⁺ release, is a main source of [Ca²⁺]. In our present study, BK significantly increased the concentration of cytosolic IP₃ in HUVEC, and the addition of IP₃ (10⁻⁵ mol/L) promoted the ⁴⁰Ca release from the Ca²⁺ storage sites in saponinized HUVEC. These results suggest that the BK-induced [Ca²⁺], increase consists of not only Ca²⁺ influx but also IP₃-induced Ca²⁺ release from the [Ca²⁺], storage sites in HUVEC. This elevated [Ca²⁺], in turn activates cPLA₂. It was recently reported that cPLA₂ contains the CalB domain, a Ca²⁺-dependent translocation domain in its N-terminal.11 and that cPLA₂ translocates from cytoplasm to cell membrane in the gradient of [Ca²⁺], concentration.12 It was also reported that the phosphorylation by MAP kinase raised PLA₂ activity to severalfold that of control.13 These mechanisms raised cPLA₂ activity, thereby promoting the release of AA from membrane phospholipids and its subsequent conversion into PGI₁ through the PGHS pathway.

The synthesis of PGI₁ can also be controlled by altering the activity or expression of the enzyme. Because the increase effect of BK on PGI₁ generation is sustained for 3 hours after stimulation, we believe that not only the raise of enzymatic activity but also the quantitative increase of cPLA₂ or PGHS protein through mRNA transcription is involved in BK-induced PGI₁ synthesis in HUVEC. PGHS is another rate-limiting enzyme in the biosynthesis of prostanoids. Recently, 2 isoforms of PGHS, PGHS-1 (COX-1) and PGHS-2 (COX-2), were detected, and their gene structures and proteins were examined. PGHS-1 is expressed constitutively in most tissues, but PGHS-2 is highly and rapidly induced in response to cell activation.14 In our present study, pretreatment with the PGHS-2 inhibitor NS-398 did not affect BK-induced PGI₁ release until 3 hours after BK stimulation, and the mRNA of PGHS-1, quantified by competitive PCR, was more markedly expressed than that of cPLA₂ or PGHS-2. This finding indicates that PGI₁ generation of the constitutive phase and early phase of BK stimulation in HUVEC depends on PGHS-1, not PGHS-2. The importance of PGHS-1 in the constitutive generation of prostanoids has been reported in most tissues including endothelial cells,15 and our results support these findings.

The stimulation of BK provided interesting results. The mRNA expression of cPLA₂ increased to ≈8-fold that of control at 15 minutes after BK stimulation and maintained this level for 180 minutes. In contrast, that of PGHS-1 was unchanged until 60 minutes after BK stimulation but then had increased remarkably by 180 minutes. Although many investigators have evaluated the effects of substances on PGHS-1 mRNA expression, an increase of PGHS-1 mRNA was reported in a few studies, and their ratios of increase in expression of PGHS-1 mRNA were only ≈2-fold to 4-fold that of control level.14 It was, therefore, assumed that the role of this enzyme was to produce prostaglandins that regulate normal cellular processes. Contrary to these former reports, in our study the level of PGHS-1 mRNA expression increased to ≈18-fold that of control at 180 minutes. Thus, it is possible that PGHS-1 acts as not only a constitutive enzyme but also an inducible enzyme in the regulation of PGI₁ synthesis in BK-stimulated HUVEC. The expression of PGHS-2 mRNA
was only 44 amol/μg RNA in an unstimulated HUVEC but had increased to ∼500-fold of control level at 180 minutes. The changes in PGHS-2 mRNA induced by BK were typical of an inducible enzyme. From our present findings, we speculate that there are 3 steps in the process of BK-induced PGI₂ synthesis in HUVEC. The first step is an immediate activation of cPLA₂ mediated by increase of intracellular Ca²⁺ and protein phosphorylation of the already-existing enzyme. The second is a new synthesis of cPLA₂ protein by gene transcription. In these 2 steps, the main reaction is the supply of AA by cPLA₂ and the synthesis of PGI₂ by PGHS. Further examinations to determine the relation of an inducible enzyme. From our present findings, we speculate that there are 3 steps in the process of BK-induced PGI₂ synthesis in HUVEC. The first step is an immediate activation of cPLA₂ mediated by increase of intracellular Ca²⁺ and protein phosphorylation of the already-existing enzyme. The second is a new synthesis of cPLA₂ protein by gene transcription. In these 2 steps, the main reaction is the supply of AA by cPLA₂ and the synthesis of PGI₂ by PGHS. Further examinations to determine the relation of intracellular Ca²⁺-sensitive. The report that the Ca²⁺ ionophore A23187 induces PGHS-2 mRNA expression in IL-1β-stimulated chondrocytes shows the important role of intracellular Ca²⁺ in mRNA expression. The elucidation of these phenomena must await further investigations of the intracellular Ca²⁺-dependent signaling mechanisms involved with cPLA₂ and PGHS mRNA expression.

This is the first report to investigate the effects of BK on the regulatory mechanisms of PGI₂ generation by cPLA₂, activity and the mRNA expression of cPLA₂ and PGHS isoforms in human vascular endothelial cells. We determined that the elevation of cPLA₂ activity caused by the increase of intracellular Ca²⁺ concentration is an important event in the early phase of BK-induced PGI₂ synthesis, and we conclude that the mechanisms for the regulation of cPLA₂ and PGHS isoforms in endothelial cell are different. However, because we could not directly measure the protein and PGHS activity, further evaluations are needed to elucidate the regulatory mechanisms of BK-induced PGI₂ synthesis in HUVEC.

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