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

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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

Bradykinin (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 II; 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. 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 F₃α with the use of a [H] 6-keto prostaglandin F₃α radioimmunoassay kit (New England Nuclear), and the IP₃ in HUVEC was measured with the use of the D-myo-inositol 1,4,5-trisphosphate [H] 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 finally suspended 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 resulting lysate was then centrifuged at 10 000g for 60 minutes. 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), 150 mmol/L NaCl, 1 mmol/L CaCl₂, 2 mmol/L 2-mercaptoethanol, 2×10⁻⁶ mol/L 1-palmitoyl-2-[1-¹⁴C]arachidonyl-phosphatidylcholine and enzyme preparation. The incubation was for 15 to 180 minutes at 37°C in the presence of BK (final concentration 10⁻⁵ mol/L). The reaction was stopped by adding 1.25 mL of Dole’s reagent. After 0.5 mL of H₂O and 0.75 mL of n-heptane were added, the contents were spun in a vortex mixer for 5 minutes and centrifuged at 3000g for 5 minutes. An aliquot (0.8 mL) of the upper phase was pipetted into another tube containing 1 mL of heptane and 100 mg of silicic acid powder. After the tubes were shaken in a vortex mixer for 5 minutes and centrifuged as above, the heptane phase was transferred into scintillation vials. The radioactivity was measured by liquid scintillation spectrometry (TRI-CARB 460C Automatic Liquid Scintillation System, Packard Instrument Co Inc) with 10 mL of Atomlight (New England Nuclear) as a scintillator agent.

**Measurement of Intracellular Ca²⁺ Concentration**

Intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was measured by the method of Grynkiewicz et al., with some modification. The cells were scraped from the dish and collected in centrifugation tubes. After being suspended in 3 mL of buffer A containing 1.0% BSA a, the cells were incubated in the presence of 3.2×10⁻⁶ mol/L fura-2/AM at 37°C for 45 minutes. After resuspension in 1.0% BSA-containing solution (cell count 10⁶/mL), they were centrifuged at 250g for 10 minutes. The fluorescence intensity from the endothelial cell suspension was then recorded in UV-competitive cuvettes with a Ca²⁺-analyzer (CAF-100: Japan Spectroscopic Co, Ltd) at excitation wavelength of 340 nm and 380 nm and at an emission wavelength of 510 nm.

**Ca²⁺ Uptake**

After the cells were washed 3 times with buffer A, they were incubated with buffer A containing 1.85×10⁻⁸ Bq/mL of ⁴⁰CaCl₂ (7.148×10⁸ Bq/mg CaCl₂, New England Nuclear) for 10 minutes. After incubation, the cells were washed 3 times with buffer A, and 1.0 mL of 6% trichloroacetic acid then was added to the dish. The cells were then rubbed off the dish with a rubber spatula, and the radioactivity of intracellular ⁴⁰Ca was measured by means of liquid scintillation spectrometry.

**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 after the addition of ⁴⁰Ca²⁺, phosphocreatine (CP), 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 apportioned into several tubes (2×10⁴ 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 holder 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 ⁴⁰Ca 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 Biochrom Ltd). The sequences of 5′- and 3′-flanking oligonucleotide primers used for PCR amplification of gene and mRNA transcription were 5′- GGAAGAGCGATGTCAGACATCCAGCTC-3′ (PGHS-1-Fw), 5′-TTGTCCCTAATATGTCGGAGATC-3′ (PGHS-1-Rv), 5′-GTGTTCTATGAGATCCAGCTC-3′ (PLA₂-Fw), 5′-TAGAATGAGCCACAAATTTGGAAGGCGC-3′ (PLA₂-Rv), 5′-ATGTGCCGACGATCAGAGCT-3′ (PGHS-2-Fw), and 5′-GC-CCTTCAGGTATTGCAGAT-3′ (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 Bioc hem 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 2 h. 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, 1.0 mol/L EDTA, 0.2% SDS, 0.3 mol/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 12 000 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
Release From HUVEC

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Control</th>
<th>Bradykinin (10(^{-5}) mol/L)</th>
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<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Buffer</td>
<td>19.96±2.00</td>
<td>18.22±3.51</td>
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<tr>
<td>Mecaprine, 10(^{-4}) mol/L</td>
<td>1.29±0.08*</td>
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<td>Aspirin, 10(^{-3}) mol/L</td>
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<td>17.43±0.82</td>
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<td>16.18±0.57</td>
<td>16.63±1.03†</td>
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<td>6.82±1.21*</td>
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<td>RHC80267, 5×10(^{-5}) mol/L</td>
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<td>NS-398, 10(^{-4}) mol/L</td>
<td>20.16±0.57</td>
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<td>des-Arg(^6)-bradykinin, 10(^{-4}) mol/L</td>
<td>18.09±1.19</td>
<td>78.59±4.19*</td>
</tr>
<tr>
<td>HOE 140, 10(^{-4}) mol/L</td>
<td>18.83±1.25</td>
<td>21.16±0.60†</td>
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</table>

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\(_2\) and PGHS-2 by Site-Directed Mutagenesis With PCR Used for Internal Standard of Competitive PCR

Complementary DNA of a mutant-cDNA of cPLA\(_2\) and PGHS-2 were prepared by PCR-based, site-directed mutagenesis following the method of Hiraguchi et al. The mutagenic primer of sequence 5’-GTGTTCTATTGAGATTGCACCCAGAGAACACAGGTTGCTCTC-3’ (PLA\(_2\)-mFw), 5’-AGTGGCCACCCAGCTGACCAAAAGTATCACA-3’ (PGHS-2-mFw) was synthesized on the Gene Assembler Plus. Amplification of PLA\(_2\)– or PGHS-2–cDNA with each mutagenic primer and Rv-primer by PCR showed a single change, C to T (italicized in PLA2 -mFw) or G to A (italicized in PGHS-2-mFw), which created a unique Hind III site. The amplification and purification then were performed.

Competitive PCR for Quantification of PGHS-1 or -2 and cPLA\(_2\) mRNA With Amplified Genomic DNA or Mutant cDNA Fragments Used as Internal Standard

In a series of tubes, PCR amplification was performed on reaction mixtures (10 µL) containing 1 µL of cDNA of PGHS-1, PGHS-2, or cPLA\(_2\); and an increasing concentration of amplified genomic DNA of PGHS-1, mutant-cDNA of PGHS-2 (0.01, 0.1, 1.0, 10, 100, 1000 amol), or mutant-cDNA of cPLA\(_2\). Amplification of PLA\(_2\)- or PGHS-2-cDNA with each mutagenic primer and Rv-primer by PCR showed a single change, C to T (italicized in PLA\(_2\)-mFw) or G to A (italicized in PGHS-2-mFw), which created a unique Hind III site. The amplification and purification then were performed.

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 χ\(^2\) 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\(_2\) 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\(_2\) generation within 15 minutes, and the effect was sustained through 180 minutes. This increase was not inhibited by pretreatment with the B\(_1\) receptor antagonist des-Arg\(^6\)-BK (10\(^{-4}\) mol/L) or the PGHS-2 inhibitor NS-398 (10\(^{-6}\) mol/L) but was inhibited by pretreatment with the B\(_2\) receptor antagonist HOE 140 (10\(^{-4}\) mol/L) and was reduced to the constitutive level by pretreatment with the extracellular Ca\(^2+\) chelator EGTA (10\(^{-5}\) 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\(_2\) release to the level under the constitutive generation and pretreatment with a PLA\(_2\) inhibitor, mepacrine (10\(^{-4}\) mol/L), or a PGHS inhibitor, aspirin (10\(^{-3}\) mol/L), reduced PGI\(_2\) 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\(^{-3}\) mol/L), 1.16±0.02 (10\(^{-5}\) mol/L), and 1.10±0.11 (10\(^{-7}\) mol/L), respectively. These data were not statistically different from each other.

Within 15 minutes of BK addition, the cytosolic PLA\(_2\) 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 [Ca^{2+}]_{i} in HUVEC was 86.37±8.34 nmol/L in the steady state. BK caused a transitory increase in [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}}Ca uptake to 145±5.3% that of control. Pretreatment with EGTA (10^{-3} mol/L) remarkably decreased the increase of both [Ca^{2+}]_{i} and {^{45}}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}}Ca release from the intracellular calcium storage sites in saponized HUVEC. The increase of {^{45}}Ca release induced by IP_{3} was 35.7% less than that caused by the calcium ionophore A23187 (10^{-5} 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 mg/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^{-2} 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

![Figure 1. A, Practical records of the change in [Ca^{2+}]_{i} induced by BK (10^{-5} mol/L) with or without EGTA (10^{-3} mol/L) pretreatment in HUVEC. B, Effect of bradykinin (10^{-5} mol/L) with or without EGTA (10^{-3} mol/L) pretreatment on the change in {^{45}}Ca^{2+} uptake in HUVEC. Data are expressed as percent change (mean±SEM), with 100% uptake equal to uptake in unstimulated state in HUVEC. *P<0.01 vs control. n=6.](image1)

![Figure 2. A, Effect of BK (10^{-5} mol/L) with or without EGTA (10^{-3} mol/L) on change in cytosolic IP_{3} in HUVEC. Data are expressed as mean±SEM. *P<0.01 vs control. n=6. B, Effect of calcium ionophore A23187 (10^{-5} mol/L) or IP_{3} (10^{-6} mol/L) on {^{45}}Ca^{2+} release from calcium storage site in saponized HUVEC. Data are expressed as percent change (mean±SEM), with 100% change equal to A23187-induced {^{45}}Ca^{2+} release. *P<0.01 vs control. n=6.](image2)

![Figure 3. Effect of BK (10^{-5} mol/L) on time course of cPLA_{2} and PGHS isoforms mRNA expression in HUVEC. Data are expressed as mean±SEM. n=4. *P<0.01 vs 0 minutes (level of constitutive expression).](image3)

![Figure 4. Effect of inhibitors on constitutive and BK-induced mRNA expression of cPLA_{2} and PGHS isoforms in HUVEC. HUVEC were pretreated with cycloheximide (5 μg/mL) or EGTA (10^{-3} mol/L) for 10 minutes and then stimulated by bradykinin (10^{-5} mol/L) for 60 minutes. Data are expressed as mean±SEM. n=4. *P<0.01 vs control, respectively.](image4)
BK-induced PGI$_2$ release, but the effects of the latter mechanisms have become apparent recently. Several kinds of BK-receptor subtypes have been identified and characterized according to their pharmacological and biochemical properties. Above all, the B$_2$ receptor is widely distributed over human tissues, especially smooth muscle cells and neurons, and mediates most of the cellular actions of BK. In the present study, pretreatment with the B$_2$-receptor antagonist des-Arg$^9$-bradykinin did not inhibit the increase of BK-induced PGI$_2$ generation in HUVEC, but pretreatment with B$_1$-receptor antagonist HOE 140 inhibited the BK-induced increase in HUVEC. The increased effect of BK on PGI$_2$ release was sustained for 3 hours after stimulation, but the increase rate of PGI$_2$ 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 PGI$_2$ 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 PGI$_2$ release, but the effects of the latter 2 inhibitors were more suppressive. These findings suggest that PLC and DAG affect BK-induced PGI$_2$ synthesis in HUVEC not only by PKC but also the PKC-independent pathway. From these results, we speculate that BK acts on HUVEC through B$_2$ receptors and that extracellular Ca$^{2+}$ and protein phosphorylation related to PKC, PLC, and DAG play important roles in the process of BK-induced PGI$_2$ synthesis.

The release of AA from phospholipids in the cell membrane is the first step of PGI$_2$ generation, and PLA$_2$ acts mainly in this step. PLA$_2$ has several isoforms. The genomic structures of type I and II of secretory PLA$_2$ (sPLA$_2$) and cytosolic PLA$_2$ (cPLA$_2$) have been determined. The release of AA from phospholipids of cell membranes is chiefly dependent on cPLA$_2$ and partially on sPLA$_2$. In the present study, BK elevated cPLA$_2$ 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 cPLA$_2$ activity is affected by intracellular Ca$^{2+}$ kinetics. BK increased [Ca$^{2+}$]i, transitorily to $\approx 240\%$ of control. Pretreatment with the extracellular Ca$^{2+}$ chelator EGTA reduced BK-induced Ca$^{2+}$ influx and [Ca$^{2+}$]i, increase, but this BK-induced [Ca$^{2+}$]i increase was not completely inhibited, and we could observe a small amount of increase in [Ca$^{2+}$]i, despite EGTA pretreatment. This increase in [Ca$^{2+}$]i, was thought to be mobilized from Ca$^{2+}$ storage sites in cytosol. The intracellular Ca$^{2+}$ storage sites consist of mitochondrial and nonmitochondrial sites, and the latter, especially for IP$_3$-induced Ca$^{2+}$ release, is a main source of [Ca$^{2+}$]i. In our present study, BK significantly increased the concentration of cytosolic IP$_3$ in HUVEC, and the addition of IP$_3$ (10$^{-5}$ mol/L) promoted the $^{45}$Ca release from the Ca$^{2+}$ storage sites in saponized HUVEC. These results suggest that the BK-induced [Ca$^{2+}$]i increase consists of not only Ca$^{2+}$ influx but also IP$_3$-induced Ca$^{2+}$ release from the [Ca$^{2+}$]i storage sites in HUVEC. This elevated [Ca$^{2+}$]i, in turn activates cPLA$_2$. It was recently reported that cPLA$_2$ contains the CaLB domain, a Ca$^{2+}$-dependent translocation domain in its N-terminal, and that cPLA$_2$ translocates from cytoplasm to cell membrane in the gradient of [Ca$^{2+}$]i. This was also reported that the phosphorylation by MAP kinase raised PLA$_2$ activity to severalfold that of control. These mechanisms raised cPLA$_2$ activity, thereby promoting the release of AA from membrane phospholipids and its subsequent conversion into PGI$_2$ through the PGHS pathway.

The synthesis of PGI$_2$ can also be controlled by altering the activity or expression of the enzyme. Because the increase effect of BK on PGI$_2$ generation is sustained for 3 hours after stimulation, we believe that not only the raise of enzymatic activity but also the quantitative increase of cPLA$_2$ or PGHS protein through mRNA transcription is involved in BK-induced PGI$_2$ 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. In our present study, pretreatment with the PGHS-2 inhibitor NS-398 did not affect BK-induced PGI$_2$ release until 3 hours after BK stimulation, and the mRNA of PGHS-1, quantified by competitive PCR, was more markedly expressed than that of cPLA$_2$ or PGHS-2. This finding indicates that PGI$_2$ 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, and our results support these findings.

The stimulation of BK provided interesting results. The mRNA expression of cPLA$_2$ increased to $\approx 8$-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 $\approx 2$-fold to 4-fold that of control level. 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 $\approx 18$-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 PGI$_2$ 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 PGI₁ 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 cycloheximide and EGTA pretreatment remarkably increased the level of cPLA₂ and PGHS isoforms. We observed no increase in PGHS-1 and PGHS-2 mRNA until 180 minutes. This time lag of BK-induced mRNA expression of cPLA₂ and PGHS isoforms is notable. It seems likely that cPLA₂ protein is generated by an induction of cPLA₂ mRNA before PGHS and provides PGHS with AA and that PGHS, which is synthesized belatedly for cPLA₂, in turn synthesizes PGI₁ through the PGHS pathway. The newly synthesized cPLA₂ and PGHS proteins apparently act mainly in the later phase (after 180 minutes of BK stimulation) and maintain the sustained phase of PGI₁ generation. Their time course appears to be a reasonable reaction; however, we could not quantify the protein of cPLA₂ and PGHS. Further examinations to determine the relation of mRNA expression and protein synthesis are necessary.

To investigate the mechanism of intracellular signal transduction in BK-stimulated HUVEC, we evaluated the effects of cycloheximide and EGTA on mRNA expression. As speculated, BK stimulation under the condition of cycloheximide pretreatment remarkably increased the level of cPLA₂ and PGHS-2 mRNA expression in HUVEC. The cycloheximide-induced superinduction of cPLA₂ mRNA was previously reported in rat mesangial cells incubated with epidermal growth factor.16 Cytosolic phospholipase A₂ mRNA, similar to immediate early genes, contains an AU-rich sequence in its 3′-untranslated region, and cycloheximide superinduces immediate early genes by altering the stability of mRNA and inhibiting the synthesis of AU-binding factor, which increases the instability of mRNA. In the case of PGHS-2, cycloheximide also superinduces mRNA expression to about 90-fold that of the control level. Because PGHS-2 mRNA also contains an AU-rich sequence in its 3′-untranslated region,14 mechanisms similar to those of cPLA₂ are thought to be involved in its cycloheximide-induced superinduction. Moreover, Srivastava et al.17 detected a new protein induced by interleukin-1β in rat mesangial cells and reported that this protein stabilizes PGHS-2 mRNA by binding its AU-rich region. Interestingly, the finding was completely different for PGHS-1 mRNA expression. As its expression is almost completely inhibited by cycloheximide, we speculate that there exists an enhancer protein that is indispensable for the transcription of PGHS-1 mRNA. In our present study, EGTA pretreatment inhibited the Ca²⁺ influx by chelating extracellular Ca²⁺, resulting in a decrease in the BK-induced increase of [Ca²⁺]ᵢ by ~50%. Although the importance of intracellular Ca²⁺ as a second messenger in intracellular signal transduction has been recognized, a detailed account of the relation between the second messenger, including intracellular Ca²⁺, and mRNA has not been provided. EGTA pretreatment increased cPLA₂ mRNA expression to the same extent as pretreatment with cycloheximide but decreased PGHS-1 mRNA expression to almost zero. The finding that PGHS-2 mRNA expression was not affected by EGTA pretreatment is unexpected because protein phosphorylation by PKC is or protein tyrosine kinase activation is causally linked to expression of mRNAs and because some kinds of PKC isoforms are Ca²⁺-sensitive. The report that the Ca²⁺ ionophore A23187 induces PGHS-2 mRNA expression in IL-1β-stimulated chondrocytes20 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 PGI₁ 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 PGI₁ 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 PGI₁ synthesis in HUVEC.

References


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