Effects of Bradykinin on Prostaglandin I$_2$ 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 Ca$^{2+}$ kinetics, cytosolic phospholipase A$_2$ (cPLA$_2$) activity, and mRNA expression of cPLA$_2$ 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 Ca$^{2+}$ and Ca$^{2+}$ release from the intracellular Ca$^{2+}$ storage sites. These reactions occurred within 5 minutes after bradykinin stimulation. Within 15 minutes, bradykinin activated cPLA$_2$ to 1.3-fold the control level. The constitutive expressions of mRNA of cPLA$_2$, PGHS-1, and PGHS-2 was 87, 562, and 47 amol/μg RNA, respectively. With the stimulation of bradykinin, cPLA$_2$ 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 cPLA$_2$ 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 cPLA$_2$ and PGHS-1 but did not affect PGHS-2. These findings suggest that the elevation of cPLA$_2$ activity caused by the increase of intracellular Ca$^{2+}$ concentration is important in the early phase of bradykinin-induced prostacyclin synthesis and that the mechanisms regulating cPLA$_2$ 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 intravenous concentration of kinin and prostacyclin (prostaglandin I$_2$, PGI$_2$), it appears that this mechanism is due to the local accumulation of BK and BK-induced vasodilators, especially PGI$_2$, rather than to the reduction of vasoconstrictor angiotensin II$^{2}$ however, the effects of ACE inhibition on BK and prostaglandins are unclear. PGI$_2$ is synthesized in response to release of arachidonic acid (AA) from phospholipids followed by transformation of AA to PGH$_2$ by prostaglandin H synthase (PGHS=cyclooxygenase; COX) and subsequent transformation of PGH$_2$ by PGI$_2$ synthase. Recent studies have revealed that PLA$_2$ 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 PGI$_2$ synthesis and signal transduction mechanisms in endothelial cells. In the present study, we investigated the effects of BK on the regulatory mechanisms of PGI$_2$ generation in human umbilical vein endothelial cells (HUVEC) with reference to intracellular Ca$^{2+}$ kinetics, cytosolic PLA$_2$ activity, and mRNA expression of cPLA$_2$ 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) were 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 [¹³C] 6-keto prostaglandin F₃α radioimmunoassay kit (New England Nuclear), and the IP₃ in HUVEC was measured with the use of the D-myoinositol 1,4,5-trisphosphate [¹³C] 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 10⁵ 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 PCR amplification of gene and mRNA transcription were 5'-GGAGAAGACGGCTGAGGATGCAGTC-3' (PGHS-1-Fw), 5'-TTGTCTCCAAATAATTGCGGAGTCTAC-3' (PGHS-1-Rv), 5'-GTGGTCTATATTAGAATTTCTGAG-3' (PLA₂-Rv), 5'-TAGAAATGGACGACAAAAAGTGTGAAGGCTC-3' (PLA₂-Fw), 5'-ATGTTCACCCCGATAGCAG-3' (PGHS-2-Fw), and 5'-GCCCTCCAGTTATGGCAAT-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 wave length.

**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 Biocom 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, 1.0 mmol/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 000g 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.

**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 ⁴⁶Ca²⁺, phosphocreatine (PC), CPK, succinic acid dehydrogenase (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 (2×10⁶ 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 bed 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 ⁴⁶Ca 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.
Effect of Inhibitors on Constitutive and Bradykinin-Induced PGI₂ Release From HUVEC

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>6-keto PGF₁α concentration (ng/2×10⁵ cells)</th>
<th>Bradykinin (10⁻¹ mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Bradykinin (10⁻¹ mol/L)</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Buffer</td>
<td>19.96±2.00</td>
<td>20.32±2.15</td>
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<tr>
<td>Mepacrine, 10⁻⁴ mol/L</td>
<td>1.29±0.08*</td>
<td>16.65±0.77†</td>
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<td>Aspirin, 10⁻³ mol/L</td>
<td>0.64±0.13*</td>
<td>5.75±0.74†</td>
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<tr>
<td>EGTA, 10⁻³ mol/L</td>
<td>17.43±0.82</td>
<td>58.2±3.66*</td>
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<td>16.18±0.57</td>
<td>8.02±0.02</td>
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<td>U-73122, 10⁻⁵ mol/L</td>
<td>6.82±1.21†</td>
<td>7.84±1.07†</td>
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<tr>
<td>RHC80267, 5×10⁻⁶ mol/L</td>
<td>6.20±1.75*</td>
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<td>NS-398, 10⁻⁴ mol/L</td>
<td>16.05±0.57</td>
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<tr>
<td>des-Arg⁶-l-bradykinin, 10⁻⁴ mol/L</td>
<td>18.09±1.19</td>
<td>78.59±4.19*</td>
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<tr>
<td>HOE 140, 10⁻⁴ mol/L</td>
<td>18.83±1.25</td>
<td>21.16±0.60†</td>
</tr>
</tbody>
</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 cPLA₂ and PGHS-2 by Site-Directed Mutagenesis With PCR Used for Internal Standard of Competitive PCR

Complementary DNA of a mutant-cDNA of cPLA₂ and PGHS-2 were prepared by PCR-based, site-directed mutagenesis following the method of Higuchi et al. The mutagenic primer of sequence 5′-GGGATCCATATTGAGATCTGAGCTCCCTAGA-GAAAGTCTGCTGTT-3′ (PLA₂-mFw), 5′-GGGATCCACCCGCTGTAAGGAAATCTA-AGCTTTTGGTCTGTC-3′ (PLA₂-mRv) was synthesized on the Gene Assembler Plus. Amplification of PLA₂- 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 cPLA₂ 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 cPLA₂ 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 cPLA₂ (0.25, 1.0, 16, 256, 4096 amol), 250×10⁻¹⁶ mol/L of each dNTP plus 3.7×10⁻⁹ Bq of [α-³²P]dCTP (1.11×10⁸ Bq/mmol: New England Nuclear), 0.5×10⁻⁶ mol/L of each paired primer (Fw and Rv), 0.25 U of Taq DNA polymerase (AmpliTaq), in PCR buffer (100 mMol/L Tris-HCl [pH 8.3], 500 mMol/L KCl, 15 mMol/L MgCl₂, 0.01% [wt/vol] gelatin). The PCR cycle was repeated 40 times following the protocol described above. Before loading the PCR products on gels for electrophoresis, cPLA₂ or PGHS-2 PCR products were digested by 10 U of Hind III for 60 minutes at 37°C. The PCR products of mutant-cDNA of cPLA₂ and PGHS-2 were divided into 2 fragments by Hind III digestion, but the PCR products of cDNA of native cPLA₂ and PGHS-2 did not have the Hind III-digested site. In this manner, competitive PCR products of native and mutant-cDNA of cPLA₂ and PGHS-2 were differentiated by digesting them with Hind III before electrophoresis. The samples then were loaded onto 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 c-DNA) and mRNA were cut out, and their radioactivity was counted. The data were analyzed by plotting the logarithm of the gDNA cpm/cDNA cDNA 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 PGI₂ release dose-dependently at concentrations from 10⁻⁶ mol/L through 10⁻⁵ mol/L, and the release reached a plateau at the concentration of 10⁻⁴ mol/L, so we used this concentration (10⁻⁵ mol/L) of BK in the following experiments. The stimulation of BK remarkably increased PGI₂ generation within 15 minutes, and the effect was sustained through 180 minutes. This increase was not inhibited by pretreatment with the B₁ receptor antagonist des-Arg⁶-BK (10⁻⁴ mol/L) or the PGHS-2 inhibitor NS-398 (10⁻⁴ mol/L) but was inhibited by pretreatment with the B₂ receptor antagonist HOE 140 (10⁻⁴ mol/L) and was reduced to the constitutive level by pretreatment with the extracellular Ca²⁺ chelator EGTA (10⁻² mol/L) or protein kinase C (PKC) inhibitor H-7 (10⁻⁵ 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 PGI₂ release to the level under the constitutive generation and pretreatment with a PLA₂ inhibitor, mepacrine (10⁻⁴ mol/L), or a PGHS inhibitor, aspirin (10⁻³ mol/L), reduced PGI₂ 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 (10⁻⁴ mol/L), respectively. These data were not statistically different from each other.

Within 15 minutes of BK addition, the cytosolic PLA₂ 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 \(^{45}\text{Ca}\) uptake to 145±5.3% that of control. Pretreatment with EGTA \((10^{-5}\text{ mol/L})\) remarkably decreased the increase of both \([Ca^{2+}]_i\), 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 \((10^{-5}\text{ mol/L})\) inhibited the BK-induced IP\(_3\) generation (Figure 2A). The addition of IP\(_3\) \((10^{-5}\text{ mol/L})\) 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 \((10^{-5}\text{ mol/L})\) (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).

The mRNA expression of cPLA\(_2\) and PGHS isoforms in HUVEC. Data are expressed as mean±SEM. n=4. *P<0.01 vs 0 minutes (level of constitutive expression).

Figure 1. A, Practical records of the change in \([Ca^{2+}]_i\) induced by BK \((10^{-5}\text{ mol/L})\) with or without EGTA \((10^{-5}\text{ mol/L})\) pretreatment in HUVEC. B, Effect of bradykinin \((10^{-5}\text{ mol/L})\) with or without EGTA \((10^{-5}\text{ mol/L})\) pretreatment on the change in \(^{45}\text{Ca}\) 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.

Figure 2. A, Effect of BK \((10^{-5}\text{ mol/L})\) with or without EGTA \((10^{-5}\text{ mol/L})\) 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 \((10^{-5}\text{ mol/L})\) or IP\(_3\) \((10^{-6}\text{ mol/L})\) on \(^{45}\text{Ca}\) release from calcium storage site in saponized HUVEC. Data are expressed as percent change (mean±SEM), with 100% change equal to A23187-induced \(^{45}\text{Ca}\) release. *P<0.01 vs control. n=6.

Figure 3. Effect of BK \((10^{-5}\text{ mol/L})\) 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).

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 \((10^{-5}\text{ mol/L})\) for 10 minutes and then stimulated by bradykinin \((10^{-5}\text{ mol/L})\) for 60 minutes. Data are expressed as mean±SEM. n=4. *P<0.01 vs control, respectively.
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 on the local accumulation of kinins and kinin-related PGI₂ 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.⁸ In the present study, pretreatment with the B₂-receptor antagonist des-Arg¹⁰-bradykinin did not inhibit the increase of BK-induced PGI₂ generation in HUVEC, but pretreatment with B₂-receptor antagonist HOE 140 inhibited the BK-induced increase in HUVEC. The increased effect of BK on PGI₂ release was sustained for 3 hours after stimulation, but the increase rate of PGI₂ 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 PGI₂ 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 PGI₂ release, but the effects of the latter 2 inhibitors were more suppressive. These findings suggest that PLC and DAG affect BK-induced PGI₂ 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 PGI₂ synthesis.

The release of AA from phospholipids in the cell membrane is the first step of PGI₂ 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. 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 PGI₂ 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 PGI\textsubscript{2} synthesis in HUVEC. The first step is an immediate activation of cPLA\textsubscript{2} mediated by increase of intracellular Ca\textsuperscript{2+} and protein phosphorylation of the already-existing enzyme. The second is a new synthesis of cPLA\textsubscript{2} protein by gene transcription. In these 2 steps, the main reaction is the supply of AA by cPLA\textsubscript{2} and the synthesis of PGI\textsubscript{2} by constitutively existing PGHS-1. The third step is the gene transcription and synthesis of new proteins of the 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 cPLA\textsubscript{2} and PGHS isoforms is notable. It seems likely that cPLA\textsubscript{2} protein is generated by an induction of cPLA\textsubscript{2} mRNA before PGHS and provides PGHS with AA and that PGHS, which is synthesized belatedly for cPLA\textsubscript{2}, in turn synthesizes PGI\textsubscript{2} through the PGHS pathway. The newly synthesized cPLA\textsubscript{2} and PGHS proteins apparently act mainly in the later phase (after 180 minutes of BK stimulation) and maintain the sustained phase of PGI\textsubscript{2} generation. Their time course appears to be a reasonable reaction; however, we could not quantify the protein of cPLA\textsubscript{2} 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 cPLA\textsubscript{2} and PGHS-2 mRNA expression in HUVEC. The cycloheximide-induced superinduction of cPLA\textsubscript{2} mRNA was previously reported in rat mesangial cells incubated with epidermal growth factor.\textsuperscript{16} Cytosolic phospholipase A\textsubscript{2} 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,\textsuperscript{14} mechanisms similar to those of cPLA\textsubscript{2} are thought to be involved in its cycloheximide-induced superinduction. Moreover, Srivastava et al.\textsuperscript{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 Ca\textsuperscript{2+} influx by chelating extracellular Ca\textsuperscript{2+}, resulting in a decrease in the BK-induced increase of [Ca\textsuperscript{2+}]\textsubscript{i} by ~50%. Although the importance of intracellular Ca\textsuperscript{2+} as a second messenger in intracellular signal transduction has been recognized, a detailed account of the relation between the second messenger, including intracellular Ca\textsuperscript{2+}, and mRNA has not been provided. EGTA pretreatment increased cPLA\textsubscript{2} 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\textsuperscript{18} or protein tyrosine kinase activation is causally linked to expression of mRNAs\textsuperscript{19} and because some kinds of PKC isoforms are Ca\textsuperscript{2+}-sensitive. The report that the Ca\textsuperscript{2+} ionophore A23187 induces PGHS-2 mRNA expression in IL-1β-stimulated chondrocytes\textsuperscript{20} shows the important role of intracellular Ca\textsuperscript{2+} in mRNA expression. The elucidation of these phenomena must await further investigations of the intracellular Ca\textsuperscript{2+}-dependent signaling mechanisms involved with cPLA\textsubscript{2} and PGHS mRNA expression.

This is the first report to investigate the effects of BK on the regulatory mechanisms of PG\textsubscript{I\textsubscript{2}} generation by cPLA\textsubscript{2} activity and the mRNA expression of cPLA\textsubscript{2} and PGHS isoforms in human vascular endothelial cells. We determined that the elevation of cPLA\textsubscript{2} activity caused by the increase of intracellular Ca\textsuperscript{2+} concentration is an important event in the early phase of BK-induced PG\textsubscript{I\textsubscript{2}} synthesis, and we conclude that the mechanisms for the regulation of cPLA\textsubscript{2} 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 PG\textsubscript{I\textsubscript{2}} synthesis in HUVEC.

References


