A Novel Inhibitory Effect on Prostacyclin Synthesis of Coupling Factor 6 Extracted from the Heart of Spontaneously Hypertensive Rats*

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Tomohiro Osanai‡, Takaatsu Kamada, Naoto Fujiwara, Takeshi Katoh, Koki Takahashi, Masao Kimura, Kiyohiko Satoh, Koji Magota§, Shiho Kodama§, Takaharu Tanaka§, and Ken Okumura

From the Second Department of Internal Medicine, Hirosaki University School of Medicine, Hirosaki 036-8562 and §Pharmaceutical Research Laboratories, Suntory Institute for Biomedical Research, Osaka 618-8503, Japan

The possible presence of an unknown prostacyclin synthesis inhibitory substance has been reported in some strains of rats. We purified the inhibitory substance from the heart of spontaneously hypertensive rats by collecting active fractions after gel-filtration column chromatography and two steps of reverse-phase high performance liquid chromatography. The amino acid composition and automated gas-phase sequencing of the full-length substance and fragments cleaved by AspN indicated that the prostacyclin-inhibitory peptide was identical to coupling factor 6. Recombinant rat coupling factor 6, which was synthesized using a cleavable fusion protein strategy, attenuated base-line and bradykinin-induced [3H]AA release in human umbilical vein endothelial cells in a dose-dependent manner (10−8−10−7 M). Exogenous AA- and prostaglandin H2-induced prostacyclin synthesis were unchanged even after treatment with 10−7 M recombinant coupling factor 6. Base-line and bradykinin-induced [3H]AA release were suppressed by arachidonyltrifluoromethyl ketone, a relatively specific inhibitor of cytosolic phospholipase A2 at 40 μM, and simultaneous administration of coupling factor 6 showed no further effect. Neither oleoyloxyethyl phosphorylcholine at 1 μM nor bromoeno lactone at 1 μM affected AA release. Preincubation (1 min) with 10−7 M recombinant coupling factor 6 had no influence on adenosine diphosphate- and collagen-induced platelet aggregations. We conclude that coupling factor 6 possesses a novel function of prostacyclin synthesis inhibition in endothelial cells via suppression of Ca2+ dependent cytosolic phospholipase A2, although it is unclear whether coupling factor 6 functions in normal conditions or only in pathophysiological states.

Prostacyclin, a potent vasodilator and the most potent endogenous inhibitor of platelet aggregation known, is synthesized from arachidonic acid (AA)1 by various stimuli in many types of cells, including vascular endothelial cells and smooth muscle cells. Of a number of stimuli, bradykinin (1, 2) and arginine vasopressin (3), whose receptors are coupled to GTP-binding proteins, enhance AA release via Ca2+-dependent translocation of cytosolic phospholipase A2 (PLA2) (4) and the activation of PLA2 due to phosphorylation (5, 6). In contrast, growth factors (7, 8), whose receptors are coupled to tyrosine kinase, activate PLA2 by the additional mechanism of de novo protein synthesis. A novel substance, designated prostacyclin-stimulating factor (9), has recently been discovered to be a stimulus for AA release, and the physiological and pathophysiological roles of this new substance in health and disease are being investigated.

The in vivo function of endogenous prostacyclin was unknown until quite recently. Mice lacking the gene for encoding the prostacyclin receptor (10), which was cloned from a human lung library (11), demonstrated increased susceptibility to thrombosis, reduced pain perception, and decreased inflammatory response. These functions seem to be related to the amount of prostacyclin, which is regulated not only by stimuli that augment synthesis but also by the clearance from the circulating system and synthesis inhibitors. A prostaglandin (PG) transporter (12), which was isolated from the protein encoded by the rat matrin complementary DNA, mediates the vascular clearance of classical PGs on passage through the pulmonary circulation but does not mediate the clearance of prostacyclin. Thus, it could not contribute to the regulation of prostacyclin effects. On the other hand, lipomodulin or macrocortin (13–15), which is known to be an inhibitory protein of PLA2, inhibits the inflammatory effect of prostacyclin, but it is limited only when anti-inflammatory glucocorticoids were administered and only to the inside of the cell.

In recent years, exposure to a high salt diet has been reported to increase the plasma concentration of an unknown substance that inhibits AA release from the plasma membrane in Dahl salt-sensitive rats (16) and Wistar rats (17). In addition, urinary excretion of 2,3-dinor-6-keto-PGF1α, an indicator for endogenous prostacyclin levels, was demonstrated to be similar in both spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats, despite the enhanced activity of prostacyclin synthesis in SHR-derived aortic strips (18, 19). Thus, these lines of accumulating evidence strongly suggest that there may be undiscovered endogenous prostacyclin synthesis inhibitor(s). Based on this reasoning, we tried to isolate and identify the prostacyclin synthesis inhibitory substance(s) by using SHR, which appear to be a suitable source for isolated rats: OPC, oleoyloxyethyl phosphorylcholine; BEL, bromoeno lactone; DMEM, Dulbecco’s modified Eagle’s medium.

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‡ To whom correspondence should be addressed: Second Dept. of Internal Medicine, Hirosaki University School of Medicine, Zaifu-cho 5, Hirosaki 036-8562, Japan. Tel.: 81-172-39-5057; Fax: 81-172-35-9190.

§ The abbreviations used are: AA, arachidonic acid; PLA2, phospholipase A2; HUVEC, human umbilical vein endothelial cells; HPLC, high performance liquid chromatography; PGF, prostaglandin F; AACCQTF, arachidonyltrifluoromethyl ketone; SHR, spontaneously hypertensive rats; OPC, oleoyloxyethyl phosphorylcholine; BEL, bromoeno lactone; DMEM, Dulbecco’s modified Eagle’s medium.
Inhibition of Prostacyclin Synthesis

**EXPERIMENTAL PROCEDURES**

**Materials**—SHR (16–20 weeks old) were obtained from Charles River, Japan. [3H]-Keto-PGF1α and [3H]AA were purchased from NEN Life Science Products. Antibodies and standards for 6-keto-PGF1α were kindly provided by Ono Pharmaceutical Co., Ltd., Japan. Archichondroitin-2 trifluoromethyl ketone (AACOCF3), Ca2+-dependent cytosolic PLAA inhibitor was purchased from Biomol. PGH2, oleyloxyethyl phospholipid (OPC, Ca2+-dependent secretory PLA2 inhibitor), and bromoeno lactone (BEL, Ca2+-independent cytosolic PLA2 inhibitor) were purchased from Cayman Chemicals, Ann Arbor, MI. Ionomycin was from Calbiochem. Cell culture media were purchased from Life Technologies, Inc., except that HuMedia was from Kurnobu, Osaka, Japan. EcoRI, SalI, and T4 DNA ligase were purchased from Takara Shuzo, Kyoto, Japan. All other reagents were of the finest grade available from Sigma.

**Isolation and Determination of Prostacyclin Synthesis Inhibitory Substance**—Brain, heart, lung, kidney, liver, and intestine obtained from 10 SHR were heat-treated in boiling water for 10 min and extracted with 1 M acetic acid (AcOH). After desalting through a Sep-Pak C18 column, fractionated by acetone precipitation at a concentration of 66%, the resulting supernatant was then evaporated to dryness. This inhibitory activity of each crude extract was determined by measuring the amount of 6-keto-PGF1α released from vascular smooth muscle cells exposed to the extract, by using the following method. Vascular smooth muscle cells were isolated from the mesenteric arteries of SHR and were grown to subconfluence on 24-well plates in 10% fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM). The cells were washed twice with serum-free DMEM and incubated in 250 µl of serum-free DMEM in the presence or absence of crude extract (10 µg protein/ml) at 37 °C. After 30 min, the medium was collected and the concentration of 6-keto-PGF1α was measured by direct radioimmunoassay in duplicate, as reported previously (20).

Next, the dry material of 187 SHR hearts, extracted by the same procedure and dissolved in 1 M AcOH, subjected to a column of Sephadex G-25 (1.5 × 30 cm) at 3 ml/h flow rate, and collected into 0.5-ml/tube fractions. The active fraction (number 19–21) from the gel-filtration column chromatography was applied on reverse-phase high performance liquid chromatography (HPLC) using an Inertsil ODS-2 C18 column (4.6 × 250 mm, GL Science Inc.) and an acetonitrile/0.05% trifluoroacetic acid elution at 10% acetonitrile over the first 10 min and followed by linear gradient of acetonitrile ranging from 50% over 40 min at 1 ml/min flow rate. The active fraction shown with a black bar was further subjected to an ODS-80A C18 column (4.6 × 250 mm, GL Science Inc.) and an acetonitrile, 0.05% trifluoroacetic acid elution at 20% acetonitrile over the first 5 min followed by linear gradient of acetonitrile ranging from 20% to 60% over 30 min with a flow rate of 1 ml/min, and eventually purified as a single peptide peak. The amino acid composition of the purified peptide was determined by amino acid analyzer (JLC-300, Japan) after hydrolysis of the peptide in 6 N HCl containing 0.005% phenol at 110 °C for 20 h. The amino acid sequence of the purified full-length peptide was first analyzed by automated gas-phase peptide sequencing. Next, after 1.6 µg of the peptide was digested with 36 ng of AspN (endopeptidase) in 89 µl of 1 M urea (pH 8.0) containing 200 µM tetrahydrobiopterin at 37 °C for 6 h, the peptide fragments were separated by reverse-phase HPLC using YMC A-302 (4.6 × 150 mm). Elution was carried out with a linear gradient of 0–80% acetonitrile in a 0.1% trifluoroacetic acid solution at a flow rate of 1 ml/min for 80 min. The amino acid sequence of the peptide fragments was then determined by the same procedure.

**Synthesis of Recombinant Coupling Factor 6**—A recombinant rat coupling factor 6, to which the prostacyclin synthesis inhibitory peptide was found to be identical, was synthesized and provided by Suntory Institute for Biomedical Research, Japan. Briefly, recombinant coupling factor 6 was obtained from Escherichia coli using a cleavable fusion protein strategy (21). Chemically synthesized rat coupling factor 6 gene, which possesses DNA fragment encoding the factor Xa recognition peptide sequence (aprotinin recognition peptide sequence in EPGLUGR), was ligated to the EcoRI- and SalI-digested pG9754DhCTG (plasmid) (22) with T4 DNA ligase. E. coli JM109 cells harboring the constructed plasmid were grown at 37 °C for 16 h in 100 ml of LB medium composed of 0.27% yeast, 0.12% (NH4)2SO4, 0.02% NH4Cl, 2 mg/ml l-methionine, 0.2% MgSO4·7H2O, and the mineral solution reported previously (23). E. coli cells that had been cultured as just described were collected and mechanically disrupted with the Manton-Gaulin homogenizer (model 15MSTBA) at a pressure of 500 kg/cm2. The inclusion bodies were collected by centrifugation and washed twice with deionized water. The washed inclusion bodies were then suspended in 2 ml urea solution (50 mM Tris-HCl at pH 8.0, 100 mM NaCl, and 2 mM CaCl2). After precipitation at 30 °C for 10 min, factor Xa was added to the solution at an enzyme/substrate ratio of 1:1000 (w/w), which was then incubated for 120 min. Cleaved rat coupling factor 6 was purified by HPLC using YMC A-302 (4.6 × 150 mm). Elution was carried out with a linear gradient of 25.5–47.2% acetonitrile in a 0.1% trifluoroacetic acid solution at flow rate of 1 ml/min for 54 min. Finally, the amino acid sequence and molecular mass of this recombinant peptide were checked by mass spectrometry and sequence analysis.

**Effects of Recombinant Coupling Factor 6 on Prostacyclin Synthesis**—Dose-dependent effects of recombinant coupling factor 6 (6 × 10–7 M) on base line and bradykinin (10–6 M)-induced prostacyclin production were measured by assessing the amount of 6-keto-PGF1α released from confluent human umbilical vein endothelial cells (HUVEC) on 24-well plates for 30 min. Briefly, HUVEC were grown to subconfluence on 24-well plates in complete medium (HuMedia) supplemented with 2% fetal bovine serum, 10 ng/ml recombinant epidermal growth factor, 1 µg/ml hydrocortisone, 5 ng/ml recombinant basic fibroblast growth factor, and 10 µg/ml heparin. The cells were washed twice with 700 µl of serum-free DMEM and incubated with various concentrations of coupling factor 6 in the absence or presence of 10–6 M bradykinin in 250 µl of serum-free DMEM for 30 min at 37 °C. To determine the site of action of coupling factor 6, the effects of this recombinant peptide at 10–7 M on conversion of both exogenous AA (10 ng/ml) to prostacyclin and exogenous PGH2 (10 ng/ml) to prostacyclin were assessed by the same method. In addition, prostacyclin synthesis due to calcium-dependent PLA2 activation was measured after the addition of ionomycin (2 µg/ml) in both the absence and presence of coupling factor 6.

**Effects of Recombinant Coupling Factor 6 on AA Release—HUVEC** were grown to subconfluence on 24-well plates in the complete medium. The cells were then labeled for 24 h with [3H]AA (0.25 µCi in 250 µl of complete medium/well at 37 °C), washed twice with 700 µl of serum-free DMEM, and preincubated for 10 min in the same medium. This medium was removed, and 250 µl of serum-free DMEM was added for 120 min. Cleaved rat coupling factor 6 was purified by HPLC using a gradient of 25.5–47.2% acetonitrile in a 0.1% trifluoroacetic acid solution. The active fraction (number 19–21) from the gel-filtration column chromatography in both the absence and presence of 10–6 M bradykinin in 250 µl of serum-free DMEM at 37 °C. In some experiments, the cells were incubated with AACOCF3 at 40 µM, OPC at 1 µM, or BEL at 1 µM in both the absence and presence of 10–6 M bradykinin. After 30 min, the medium was collected and the [3H] level was counted with a liquid scintillation counter. In addition, the effect of co-administration of coupling factor 6 at 10–7 M and AACOCF3 was also assessed by the same method.

**Effects of Recombinant Coupling Factor 6 on Platelet Function**—Platelets were harvested as described previously (24). Briefly, platelet-rich plasma was prepared from SHR by centrifugation of blood samples containing 10% sodium citrate at 190 × g for 7 min and platelet-poor plasma by centrifugation of platelet-rich plasma at 900 × g for another 10-min period at room temperature. Platelet aggregation in platelet-rich plasma was examined with an aggregometer (NKK hematocrit, Japan) in siliconized cuvettes with continuous stirring at 37 °C. Platelet aggregation was induced with 2.5 or 5 µM adenosine diphosphate (ADP) and 0.5 µg/ml collagen after pretreatment with 10–6 M recombinant coupling factor 6 for 1 min, and the maximum aggregability was observed at 37 °C.

**Statistics**—All data are shown as mean ± S.E. An unpaired t test for comparison of two variables and analysis of variance for repeated measures for multiple comparison were used for statistical analysis. The level of significance was <0.05.

**RESULTS**

**Purification and Determination of Prostacyclin Synthesis Inhibitory Peptide**—As shown in Fig. 1A, 6-keto-PGF1α production was most strongly suppressed by the heart extract, and its suppression was abolished by pretreatment with trypsin and chymotrypsin (data not shown). Therefore, the prostacyclin-bradykinin inhibitor was purified from 187 hearts (152 g) of SHR by collecting active fractions after gel-filtration column chromatography (Fig. 1B). The fraction numbers 19–21 that are approximately equivalent to relative molecular mass 9000 Da showed the highest inhibitory activity. Thus, after being evaporated in vacuo to dryness, the active fraction was applied to two steps of reverse-phase HPLC (Fig. 1, c and d) and eventu
ally purified as a single peak. Purity of the single peak (9.5 µg) eluted from the final HPLC (Fig. 1d) was verified by reverse-phase HPLC with different kinds of elution and by gel electrophoresis. The amino acid composition, determined by acid hydrolysis, was (mol/mol of peptide) as follows: Asx, 9.4; Ser, 2.2; Glx, 13.3; Val, 4.3; Met, 2.3; Ile, 1.0; Leu, 6.6; Tyr, 2.8; Phe, 6.2; Lys, 10.8; Thr, 1.9; Arg, 3.3; Gly, 5.1; Pro, 6.3; Ala, 2.2. His, Cys and Trp were undetected. Automated gas-phase peptide sequencing of the purified full-length peptide (Fig. 1e) was interpretable up to the 40th amino acid from the N terminus and was completely identical to coupling factor 6.

Four of the 8 fragment peptides cleaved by AspN were found to have amino acid sequences compatible with the amino acid sequence up to the 39th described above. The sequences of the other four fragments were DRELFLKQMYGKGM, DKFPTFNFE, DPKFKEVL, and DKFQS, which were completely identical to the 40–55th, the 56–64th, the 65–71st, and the 72–76th amino acid sequences of rat coupling factor 6, respectively. These results indicated that the prostacyclin-inhibitory peptide was of relative molecular mass 9000 Da, comprised of 76 amino acid residues, and identical to coupling factor 6.

**Effects of Recombinant Coupling Factor 6 on Prostacyclin Synthesis**—Prostacyclin synthesis at base line was 234 ± 36 pg/well/30 min in HUVEC. As shown in Fig. 2, recombinant coupling factor 6 suppressed it in a dose-dependent manner (10⁻⁹–10⁻⁷ M). Treatment with bradykinin at 10⁻⁶ M elicited an increase in prostacyclin synthesis from 272 ± 45 to 469 ± 24 pg/well/30 min (Fig. 3). Simultaneous administration of coupling factor 6 suppressed bradykinin-induced prostacyclin production in a dose-dependent manner. Ionomycin-induced increase in prostacyclin production above the base line was suppressed from 857 ± 9 to 83 ± 23 pg/well/30 min (n = 4, p < 0.05) by recombinant coupling factor 6 at 10⁻⁷ M. In contrast, exogenous AA-induced increase in prostacyclin synthesis above
the base line was unchanged even after treatment with 10^{-7} M recombinant coupling factor 6 (764 ± 25 before and 758 ± 35 pg/well/30 min after coupling factor 6, n = 4, p = not significant). An increase in prostacyclin production induced by exogenous PGH2 also was not affected by coupling factor 6 at 10^{-7} M (504 ± 64 before and 432 ± 18 pg/well/30 min after PGH2, n = 4, p = not significant).

**Effects of Recombinant Coupling Factor 6 on AA Release—** Base-line [3H]AA release count was 678 ± 53 cpm/well/30 min in HUVEC. As shown in Fig. 4, recombinant coupling factor 6 suppressed base-line [3H]AA release in a dose-dependent manner. Base-line [3H]AA release was suppressed from 610 ± 39 to 453 ± 75 cpm/well/30 min (p < 0.05) by 40 μM AACOCF3 but not by 1 μM OPC and 1 μM BEL (Fig. 5). The addition of 10^{-7} M coupling factor 6 to 40 μM AACOCF3 showed no further suppressant effect on [3H]AA release induced by bradykinin (555 ± 26 cpm/well/30 min).

**Effects of Recombinant Coupling Factor 6 on Platelet Function—** ADP at 2.5 and 5 μM and collagen at 0.5 μg/ml all induced significant platelet aggregation. The maximum aggregation was 30 ± 1% with 2.5 μM ADP, 39 ± 1% with 5 μM ADP, and 63 ± 8% with collagen. Preincubation (1 min) with 10^{-7} M of recombinant coupling factor 6 had no influence on ADP (2.5 and 5 μM)- and collagen-induced maximum platelet aggregation (2.5 μM of ADP, 30 ± 1% (n = 3); 5 μM of ADP, 40 ± 3% (n = 3); collagen, 63 ± 5% (n = 3)).

**Discussion**

In the present study, a prostacyclin synthesis inhibitory substance was purified from the heart of SHR, and its structure was determined. It was a 76-amino acid peptide, and the sequence from the N terminus to the 40th amino acid was completely identical to the known peptide designated coupling factor 6. Further analysis of fragment peptides cleaved by AspN revealed that the amino acid sequences of the initial four fragments were identical to the 1–39th sequences and the other four to the 40–55th, the 56–64th, the 65–71st, and the 72–76th...
sequences of rat coupling factor 6. Thus, the purified peptide was completely identical to rat coupling factor 6. Then, we reconstructed rat coupling factor 6 using a cleavable fusion protein strategy and confirmed the effect of this recombinant peptide on prostacyclin synthesis. Recombinant coupling factor 6 manifested the same effect as the purified substance, and prostacyclin synthesis inhibitory substance was verified as identical to coupling factor 6.

Coupling factor 6 (25) is known to act inside the cell as an energy transducer in mitochondrial adenosine triphosphate synthase that consists of three domains, namely the extrinsic and intrinsic membrane domains, Fₐ and F₀, respectively, joined by a stalk. Fₒ is the proton channel of the complex that spans the inner mitochondrial membrane, and the protons are conducted from Fₒ through the stalk to the catalytic sites in Fₐ composed of five kinds of polypeptides (26, 27). Four subunits of the stalk have been identified and designated as follows: the oligomycin sensitivity conferral protein, coupling factor 6, and subunit b and d (28–30), of which coupling factor 6 is reported to be essentially required for energy transduction (25). Coupling factor 6 is first synthesized as an immature form in the rough endoplasmic reticulum and matures by proteolytic processing. The mature form is composed of 243 amino acids, and the intramembrane domains, F₁ and F₀, are joined by a stalk. F₀ is the proton channel of the complex that exhibits 500-fold greater potency to cPLA₂ than to sPLA₂ (33, 34) but also inhibits macrophage iPLA₂ with IC₅₀ value of 15 μM (35). BEL (iPLA₂ inhibitor) manifests greater than 1000-fold selectivity for iPLA₂ than for sPLA₂ and cPLA₂ (36, 37). However, BEL has been found to block M₉P₂⁻-dependent phosphatidate phosphohydrolase, a key enzyme in cellular phospholipid metabolism (38). Base-line and bradykinin-induced AA releases were both suppressed by AACOCF₃ but not by OPC (sPLA₂ inhibitor) and BEL, suggesting that cPLA₂ contributes to AA release under these conditions. Coupling factor 6 suppressed base-line and bradykinin-induced AA releases, and the simultaneous administration of AACOCF₃ and coupling factor 6 had no additive effect to that by AACOCF₃, suggesting that coupling factor 6 inhibits AA release from the plasma membrane via suppression of cPLA₂ activity.

A distinct difference in the responsiveness to coupling factor 6 was clearly observed between endothelial cells and platelets. We demonstrated that the acting site of coupling factor 6 was AA release from the plasma membrane and not prostacyclin synthase. Therefore, it is reasonable to presume that this peptide may inhibit thromboxane A₂ synthesis in platelets and suppress aggregation of platelets. However, pretreatment of platelets with coupling factor 6 had no influence on the maximal value of platelet aggregation. This lack of effect suggests that coupling factor 6 may exert an AA release-inhibitory effect on platelet aggregation.

Because prostacyclin receptor is expressed in many tissues such as aorta, lung, atrium, ventricle, and kidney (20), and because PG transporter (12) does not mediate the vascular clearance of prostacyclin, the endogenous prostacyclin synthe-
sis inhibitory peptide may have inhibitory effects against widespread biological actions of prostacyclin. This peptide also may counteract a biological action of AA such as inhibition of voltage-gated Ca\(^{2+}\) current, because a major acting site of coupling factor 6 is the inhibition of AA release from the plasma membrane. Further investigations, especially on the in vivo effects of exogenous coupling factor 6, will be required to establish the effect and role of this peptide.

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REFERENCES
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