Molecular Cloning of Human Prostacyclin Receptor cDNA and Its Gene Expression in the Cardiovascular System

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Background Prostacyclin elicits a potent vasodilation and inhibition of platelet aggregation through binding to its membrane receptor. The impairment of prostacyclin receptor activity is implicated in various human cardiovascular diseases. In the present study, we succeeded in the isolation and characterization of human prostacyclin receptor cDNA and elucidated its gene expression in human tissues.

Methods and Results We isolated a cDNA clone encoding the human prostacyclin receptor from a human lung cDNA library. The isolated cDNA clone encodes a 386-amino acid protein with seven putative transmembrane domains, which belongs to the G protein-coupled receptor superfamily. [3H]Iloprost, a prostacyclin receptor agonist, specifically bound to the receptor transiently expressed in COS-7 cells. The binding was inhibited in the rank order of iloprost = cicaprost, another prostacyclin receptor agonist, > prostaglandin E1, (PGE2) > PGE2 (PGE2), PGD2, STA2. In addition, iloprost dose-dependently stimulated cAMP generation in these COS-7 cells. These results are consistent with the characteristics of the human prostacyclin receptor. Northern blotting analysis on human tissues revealed that prostacyclin receptor mRNA is abundantly expressed in the aorta, lung, atrium, ventricle, and kidney.

Conclusions We cloned human prostacyclin receptor cDNA and elucidated its abundant gene expression in the human cardiovascular system. The present study will lead to better understanding of the significance of prostacyclin in humans and further facilitate the clinical application of prostacyclin. (Circulation. 1994;90:1643-1647.)

Key Words • prostaglandins • cDNA • cloning • mRNA

The prostanooids are oxygenated derivatives of fatty acids, principally arachidonic acid. They show various biological activities and work mainly as local regulators in many organs.1 Prostacyclin (prostaglandin I2, PGI2), predominantly produced by vascular endothelial cells, is a potent vasodilator and inhibitor of platelet aggregation. The actions of prostacyclin generally counteract those of thromboxane A2 (TXA2) and are implicated in the control of platelet/endothelium/vascular smooth muscle interaction.1 The impairment of prostacyclin receptor activity is regarded as one of the important pathogenic and deteriorating factors in various human diseases including atherosclerosis and ischemic heart disease.2,3 In addition, the therapeutic usefulness of prostacyclin and its stable analogues has been widely accepted.4,5 It is of great significance, therefore, to elucidate the molecular mechanism of prostacyclin actions in humans.

We and others have determined the structures of TXA2 receptors and PGE2 receptors.6-11 These receptors share the structural features and constitute a novel family in the G protein-coupled receptor superfamily. It is known that prostacyclin expresses biological actions through binding to its membrane-associated receptor and subsequent increase in intracellular cAMP concentration.1,12 Recently, we cloned mouse prostacyclin receptor cDNA using polymerase chain reaction and screening of a cDNA library.13 In the present study, we have succeeded in the isolation and characterization of human prostacyclin receptor cDNA and elucidated the gene expression of the prostacyclin receptor in various human tissues.

Methods

Isolation and Sequencing of Human Prostacyclin Receptor cDNA

Approximately 1x10^6 plaques of a human lung cDNA library constructed in bacteriophage Agt 10 (Clontech Laboratories Inc) were screened using a 32P-labeled EcoRV fragment of mouse prostacyclin receptor cDNA, which covers its entire coding region.13 Plaque hybridization was carried out at 37°C in a solution containing 25% formamide, 5xSSC, 0.1% SDS, 1x Denhardt’s solution,14 and 200 μg/mL heat-denatured salmon sperm DNA. The membranes were washed twice at room temperature in 2xSSC, 0.1% SDS. The hybridization-positive clone (AhIPR1) was purified, and a restriction fragment of the cDNA insert was subcloned into Bluescript SKII (+) vector (Stratagene) (pHPr1). DNA sequencing was performed by the dideoxy-mediated chain termination method15 and was confirmed by reading both DNA strands.

Expression of Human Prostacyclin Receptor in COS-7 Cells

A HindIII-PstI fragment of pHIPR1 was subcloned into the expression vector pRC/RSV (Invitrogen) (pRC/RSV-hIPR1).
COS-7 cells were transfected with pRC/RSV-hIPR1 (10 μg of DNA per 100-mm dish) using a lipopolamine derivative, Transfectam (Sepracor Inc).10

Crude Membrane Preparation and Binding Assay
Sixty hours after transfection, the COS-7 cells were harvested and crude membranes were prepared as described.17 In brief, the cells were homogenized in a solution containing 20 mmol/L Tris/HC1 (pH 7.5), 10 mmol/L MgCl2, 1 mmol/L EDTA, 250 mmol/L sucrose, 200 μmol/L phenylmethylsulfonyl fluoride, and 20 μmol/L indomethacin. The homogenate was centrifuged at 800g for 10 minutes and the supernatant was centrifuged at 35000 g for 30 minutes. The pelleted crude membrane was suspended in the binding assay buffer composed of 20 mmol/L MES/NaOH (pH 6.0), 10 mmol/L MgCl2, and 1 mmol/L EDTA.

For saturation binding assay, 40 μg of crude membrane was incubated with various concentrations (1 to 100 nmol/L) of [3H]iloprost (Amersham International) in 100 μL of the binding assay buffer at 30°C for 1 hour. For displacement binding assay, 40 μg of crude membrane was incubated with 20 nmol/L of [3H]iloprost with or without unlabeled iloprost (Amersham), cicaprost (Schering AG), PGE2, PGE1, PGF2α, (Nacalai Tesque), PGD2, (Sigma Chemical), and STA2 (a gift from Ono Pharmaceutical). The incubation mixture was filtered through a Whatman GF/C filter, and the radioactivity associated with the filter was measured using a liquid scintillation counter.17

cAMP Generation Assay
Thirty-six hours after transfection, the COS-7 cells were seeded at a density of 1.2×106 cells per well in 24 well plates. Thirty hours later, the cells were washed with phosphate-buffered saline and were preincubated for 10 minutes in Dulbecco’s modified Eagle’s medium containing 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma) and 0.1% (wt/vol) bovine serum albumin (Sigma) at 37°C. Various concentrations (10 pmol/L to 100 nmol/L) of iloprost was then added and incubated for 30 minutes at 37°C. The reaction was terminated by the addition of trichloroacetic acid in a final concentration of 6%, and the cAMP content was determined by radioimmunoassay, as previously reported.18 Statistical analysis was performed using the Student’s t test.

Northern Blotting Analyses
Total RNA was extracted from human tissues obtained at autopsy or operation using the guanidinium thiocyanate–cesium chloride method, and poly(A)+ RNA was purified using an oligo(dT) latex (oligoTEX dT30 Super, Daichi Chemical).14 Informed consent was obtained from each patient or family, and the study was approved by the ethical committee on human research of Kyoto University (No. 61-98).

Three-microgram aliquots of poly(A)+ RNA were size-fractionated by electrophoresis on a 1.0% agarose-formaldehyde gel and transferred to Biodyne A nylon membranes (Pall). The membranes were prehydrized in a solution containing 4×SSC, 10×Denhardt’s solution, 0.5% SDS, and 250 μg/mL of salmon sperm DNA at 65°C for 12 hours. The hybridization was performed at 42°C for 24 hours in a solution composed of 50% formamide, 4×SSC, 5× Denhardt’s solution, 0.5% SDS, 10% dextran sulfate, and 250 μg/mL salmon sperm DNA, using a 32P-labeled, 1.9-kb EcoRI fragment of pIPR1 as a probe. An internal control, the membranes were rehybridized with a 32P-labeled human β-actin genomic probe (Wako Pure Chemical).19

Results
Isolation and Sequence Analysis of Human Prostacyclin Receptor cDNA
We screened overall 1×106 recombinants from a human lung cDNA library using mouse prostacyclin receptor cDNA as a probe, and isolated one positive clone. Fig 1A shows the 1862-nucleotide sequence of the cloned cDNA containing a 1158-nucleotide open reading frame encoding a 386-amino acid protein with a relative molecular mass of 40 956. The nucleotide sequence surrounding the putative initiation codon agrees well with the consensus sequence,20 and the putative initiation site is preceded by an in-frame termination codon. Hydrophathy analysis of the deduced amino acid sequence revealed that it contains seven hydrophobic clusters of 21 to 27 residues, each separated by stretches of hydrophilic residues, indicating that the cDNA encodes a protein with seven membrane-spanning domains, which is consistent with the structure of the G protein–coupled receptor superfamily,21,22 as shown in Fig 1A, the deduced amino acid sequence of the isolated cDNA clone shares several features of G protein–coupled receptors22,22: consensus sites for N-glycosylation, cysteine residues in the N-terminus of the cytoplasmic C tail, one of which may be palmitoylated as in the β, adrenergic receptor. There are two cysteine residues in the first and second extracellular loops. The disulfide linking between them is considered important in the formation of the tertiary structure of the receptors. In addition, a basic residue (Arg 279) exists in the seventh transmembrane region, which is possibly assigned for the binding of an acidic ligand, as is the case with retinal attachment of rhodopsin.24 There are several serine and threonine residues in the cytoplasmic regions, which can be phosphorylated by protein kinase C. In contrast, no potential consensus sites for the phosphorylation by cAMP-dependent protein kinase exist in this receptor.25

The nucleotide sequence of the open reading frame is 80% identical to that of mouse prostacyclin receptor cDNA, and the deduced amino acid sequence is 79% identical to that of the mouse prostacyclin receptor.11 The sequence homologies of the isolated cDNA clone to the human and mouse TXA2 receptors and PGE2 receptors are less than 50% in the amino acid level.6–11

Ligand Selectivity of Human Prostacyclin Receptor Transiently Expressed in COS-7 Cells
To demonstrate that the isolated cDNA clone encodes a receptor specific for prostacyclin, we performed saturation and displacement binding assays of the transiently expressed receptors in COS-7 cells using pRC/RSV-hIPR1. [3H]iloprost, a prostacyclin receptor agonist,26 specifically bound to the crude membrane prepared from transfected cells, and Scatchard analysis yielded a dissociation constant of 24 nmol/L and a maximal binding of 5.7 pmol/mg protein. Fig 1B represents the result of the displacement binding assay. The specific [3H]iloprost binding was inhibited by the unlabeled PGs and related compounds in the relative potency rank order of iloprost>cicaprost (another prostacyclin receptor agonist1) > PGE2, > PGE1, > PGE2, PGD2, STA2 (a TXA2 receptor agonist).4 The specific [3H]iloprost binding was not observed in the crude membrane of untransfected COS-7 cells. We also performed an intact cell binding assay using COS-7 cells that were transfected with pRC/RSV-hIPR1 and obtained essentially the same binding profiles (data not shown). These results are consistent with the pharma-
cAMP Generation in COS-7 Cells Transiently Expressing Human Prostacyclin Receptor

We further examined the effect of iloprost treatment on cAMP formation in COS-7 cells transiently transfected with pRC/RSV-hiPR1. The treatment with iloprost induced cAMP generation in a dose-dependent fashion, as seen in the Table. The increase was detectable at 10^{-11} mol/L and reached a maximal level at 10^{-8} mol/L, with an EC_{50} value of 2\times10^{-10} mol/L. No response was detected in untransfected COS-7 cells. These observations were compatible with the previous studies concerning the intracellular signaling of prostacyclin actions.\(^{1,12}\)

Distribution of Prostacyclin Receptor mRNA in Human Tissues

To elucidate the gene expression of the human prostacyclin receptor, we performed Northern blotting analysis on various human tissues (Fig 2). A major mRNA band with a size of 2.5 kb was detected. We detected prostacyclin receptor mRNA most abundantly in the aorta. We also detected high mRNA expression in the lung, atrium and ventricle of the heart, and kidney. The modest expression of prostacyclin receptor mRNA was observed in the liver, intestinal tract, spleen, adrenal gland, and placenta. We observed no detectable band in the cerebral cortex, cerebellum, or thymus.

Discussion

In the present study, we succeeded in the isolation and characterization of human prostacyclin receptor cDNA from a human lung cDNA library. The cDNA clone isolated in the present study encodes a protein with seven hydrophobic regions, which is consistent with receptors of the G protein–coupled superfamily.\(^{21-25}\) The binding studies revealed that the cDNA clone encodes a membrane-associated receptor showing li-
gand selectivity that is compatible with that of the human prostacyclin receptor.26-29 The iloprost treatment of COS-7 cells transiently expressing the receptor dose-dependently stimulated the formation of cAMP, which has been known as an intracellular second messenger of prostacyclin actions.1,13 In addition, the nucleotide and deduced amino acid sequences of the cDNA clone are highly homologous with those of the mouse prostacyclin receptor.13 Taken together, we concluded that the cDNA clone isolated in the present study encodes the human prostacyclin receptor.

The human and mouse prostacyclin receptors show higher sequence homology in the putative transmembrane domains (89%) compared with the extracellular and intracellular regions (72%).13 These transmembrane domains exhibit considerable homologies to those of other prostanoïd receptors, whereas the overall homologies are low between them.6-11 Of notable interest, the human prostacyclin receptor possesses no potential consensus sites for the phosphorylation by cAMP-dependent protein kinase,22 which have been reported to exist in the mouse prostacyclin receptor and other prostanoïd receptors cloned so far.6-11,13 There might possibly be some differences in the regulation of signal transduction, including the desensitization mechanism.

We further investigated the gene expression of the prostacyclin receptor on various human tissues. We detected prostacyclin receptor mRNA most abundantly in the aorta. The expression of prostacyclin receptor mRNA also was observed in the atrium and ventricle. The major biological role of prostacyclin in the cardiovascular system is considered to be the regulation of platelet and vascular homeostasis, which is impaired in various disorders such as atherosclerosis and ischemic heart disease.1 The abundant expression of prostacyclin receptor mRNA in the aorta is quite consistent with the concept. It is also proposed that prostacyclin possesses direct actions for cardiomyocytes, such as a cytoprotective effect in experimental and clinical acute myocardial infarction.4,5 The demonstration of prostacyclin receptor mRNA expression in the human heart will give a clue in investigating the molecular mechanism of such novel biological roles of prostacyclin. High-level gene expression of the prostacyclin receptor also was observed in the lung and kidney. In these organs, prostacyclin may elicit some organ-specific actions in addition to the modulation of vascular homeostasis. Further studies should clarify the detailed distribution of prostacyclin receptor mRNA in these organs and its alteration in disease states. In the present study, no mRNA expression was detected in the thymus of a 1-year-old girl with endocardial cushion defect. This result is in contrast to extremely high gene expression of the prostacyclin receptor in mouse thymic medulla.13 It remains open to question whether the difference is species dependent or not.

cDNA cloning of the human prostacyclin receptor and the elucidation of its gene expression in human tissues will lead to better understanding of the clinical implication of prostacyclin and will give helpful information for the therapeutic application of related agents.

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