Impaired Receptor Binding and Activation Associated with a Human Prostacyclin Receptor Polymorphism*

Jeremiah Stitham, Aleksandar Stojanovic, and John Hwa‡
From the Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755

The human prostacyclin receptor (hIP) is a seven transmembrane-spanning G-protein-coupled receptor that plays an important role in vascular homeostasis. Recent genetic analyses (SNP database, NCBI) have revealed the first two polymorphisms within the coding sequence, V25M and R212H. Here we present structure-function characterization of these polymorphisms at physiological pH (7.4) and at an acidic pH (6.8) that would be encountered during stress such as renal, respiratory, or heart failure. Through a series of competition binding and G-protein activation assays (measured by cAMP production), we determined that the V25M polymorphism exhibited agonist binding and G-protein activation similar to wild-type receptor at normal pH (7.4). However, the R212H variant demonstrated a significant decrease in binding affinity at lower pH (R212H at pH 7.4, Kᵢ = 2.2 ± 1.2 nM; pH 6.8, Kᵢ = 45.6 ± 12.0 nM). The R212H polymorph also exhibited abnormal activation at both pH 7.4 and pH 6.8 (pH 7.4, R212H EC₅₀ = 2.8 ± 0.5 nM versus wild-type hIP EC₅₀ = 0.5 ± 0.1 nM; pH 6.8, R212H EC₅₀ = 3.2 ± 1.6 nM versus wild-type hIP EC₅₀ = 0.5 ± 0.2 nM). Polymorphisms of the human prostacyclin receptor potentially may be important predictors of disease progress during biological stressors such as acidosis in which urgent correction of bodily pH may be required to restore normal hemostasis and vasodilation. This study provides the mechanistic basis for further research into genetic risk factors and pharmacogenetics of cardiovascular disease associated with hIP.

Similar to other prostanoids, prostacyclin is a derivative of the C-20 unsaturated fatty acid arachidonic acid (5,8,11,14-eicosatetraenoic acid), and its cellular action is conveyed through cell surface G-protein-coupled receptors that predominantly couple to the heterotrimeric G-protein Gₛ stimulating the production of cAMP (1). The human prostacyclin receptor (hIP)¹ is expressed on platelets, where it mediates inhibition of platelet aggregation and on vascular smooth muscle cells, where it mediates vascular smooth muscle relaxation. Dysfunctional prostacyclin activity has been implicated in the development of a number of cardiovascular diseases including thrombosis, myocardial infarction, stroke, myocardial ischemia, atherosclerosis, and systemic and pulmonary hypertension (2). Accordingly, IP receptor knock-out mice exhibit increased thrombosis and reduced inflammatory and pain responses (3).

Limited studies have begun to identify generalized regions within the IP and other prostanoloid receptors that appear crucial for ligand-binding specificity and affinity. Studies using chimeric combinations of mouse prostaglandin D (mDP) and prostaglandin I (mIP) receptors have shown that protein segments within transmembrane domains VI and VII (TMVI and TMVII) are involved in distinct binding interactions with prostacyclin side chains. In addition, TMII (along with a portion of the first extracellular loop) confers broader binding functions, incorporating recognition and interaction with the cyclopentane ring of prostacyclin (1, 4). Glycosylation at Asn-17 and Asn-78 in the extracellular domain (see Fig. 1), has also been demonstrated to be essential for proper binding and G-protein activation (5).

As observed with other G-protein-coupled receptors, genetic variants of the hIP receptor may act as predisposing and/or modifying factors for disease states or therapeutic response. In this investigation, we have undertaken a functional analysis of the first polymorphisms identified in the coding region of the hIP receptor, recently identified in the SNP database (6). The goal of this study is to determine the effects of these polymorphisms on agonist binding and G-protein activation at physiologic and pathological pH levels. Our results indicate that the V25M polymorph had no significant effects on agonist binding or Gₛ activation, functioning in a manner consistent with the wild-type hIP. In contrast, the R212H polymorph showed a significant decrease in signal transduction activation, requiring a 6-fold increase of agonist to elicit a wild-type-like response at both pH 7.4 and 6.8. Furthermore, under acidic conditions (pH 6.8), a defect in binding was also observed for R212H.

EXPERIMENTAL PROCEDURES

Materials—Illoprost ligands, radiolabeled [³H]iloprost (17.0 Ci/mmol), and non-radiolabeled iloprost as well as the cAMP radiomunnoassay system were purchased from Amersham Biosciences. Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). The hIP cDNA was a generous gift from Dr. Mark Abramovitz (Merck Frost, Quebec, Canada).

Construction of Mutant Receptors and Expression in COS-1 Cells—Human IP cDNA was cloned along with a C-terminal 1D4 epitope tag (native nine C-terminal amino acids from rhodopsin) into the pMT4 expression vector. Point mutations were generated using conventional methods of PCR mutagenesis as previously described (7). Complementary oligonucleotide primers were designed extending 10–12 nucleotides 3’ and 5’ from the desired mutation sites (V25 or R212). All mutant constructs were confirmed via PCR DNA dideoxynucleotide chain termination sequencing (Dartmouth Medical School Molecular Biology Core Facility). Transient transfections of COS-1 cells were performed initially at a DNA concentration of 2.0 µg/ml followed by decreasing concentrations of 1.0, 0.5, 0.25, 0.05, and 0.025 µg/ml using diethylaminoethyl-dextran (DEAE-Dextran; Sigma) as previously described (7).

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‡To whom correspondence should be addressed: Dept. of Pharmacology and Toxicology, Dartmouth Medical School, 7650 Remsen, Hanover, NH 03755; Tel.: 603-650-1813; Fax: 603-650-1129; E-mail: John.Hwa@Dartmouth.edu.

¹The abbreviations used are: hIP, human prostacyclin receptor; TM, transmembrane domain; SNP, single nucleotide polymorphism.
Membrane Preparations—Preparations of COS-1 cell membranes were carried out as follows. Cells were washed in phosphate-buffered saline and harvested by scraping. Subsequent washes in 0.25 % sucrose solution were followed by vigorous vortexing (providing shear forces) for 3 min. A low speed spin (1,200 × g) was performed for 5 min, and the supernatant was collected. After a high speed centrifugation (~30,000 × g for 15 min) the pellet was washed twice in 1× HEM (20 mM Hepes pH 7.4, 1.5 mM EGTA, and 12.5 mM MgCl2) followed by resuspension in 1× HEM containing 10% glycerol and was stored at −70 °C. A Bradford protein assay was performed to quantitate membrane proteins.

Ligand Binding—Ligand-binding characteristics for the expressed receptors were determined through a series of competition binding assays using radiolabeled [3H]iloprost (fixed concentration), an IP receptor-specific agonist, versus non-radiolabeled iloprost (varied concentrations). Mock transfected COS-1 cell membranes revealed no specific binding to iloprost. Reaction mixtures (performed in duplicate) contained 50 μg of protein, 1× HEM buffer (pH 7.4, 6.8, and 5.9), 15 nm [3H]iloprost, and one of 12 different concentrations (10 μM to 1 nM) of cold (non-radiolabeled) iloprost. After a 1.5-h incubation at 4 °C, reactions were stopped by the addition of ice-cold 10 mM Tris/HCl buffer (pH 7.4), and the reaction mixture was filtered onto Whatman® GF/C glass fiber filters using a Brandel® cell harvester. The filters were washed five times with ice-cold Tris/HCl buffer, and radioactivity remaining on the filter paper (trapped membranes) was measured in the presence of 5 ml of Liquisint™ scintillation fluid (National Diagnostics, Atlanta, GA). Non-specific binding was determined by the addition of a 500-fold excess of non-radiolabeled iloprost, whereas the concentration of [3H] iloprost was varied from 1 to 100 nM for saturation binding studies. Data were analyzed using GraphPad Prism® software. IC50 values were converted to Kd using the Cheng-Prusoff equation, and Kd values were expressed as means ± S.E. An analysis of variance (post-test Newman-Keuls) and Student’s t tests were used to determine statistically significant differences (p < 0.05).

Determination of cAMP Levels—The wild-type hIP with the epitope tag hIP1D4 and mutant constructs were analyzed for signal transduction capabilities. COS-1 cells were transiently transfected with 2.0 μg/ml receptor DNA in 25-cm plates as described above. After 72 h, cells were washed twice with phosphate-buffered saline plus 4 mM EDTA and 2 mM IBMX (Sigma) (pH 7.4, 6.8, or 5.9) and incubated at 20 °C for 10 min. This was followed by addition of defined concentrations of iloprost to selected plates. Dose-response curves were determined by the addition of six different concentrations (1 μM to 10 μM) in duplicate. After 20 min, the cells were harvested and boiled for 3 min, followed by high speed (10,000 rpm) centrifugation. Fifty microliters of the resultant supernatant (a total of 300 μl) was used to determine cAMP production in the competition assay. cAMP levels were measured using the radio-receptor competition assay (Amersham Biosciences). In brief, [3H]cAMP was used in competition for a cAMP-binding protein against known concentrations of non-radiolabeled cAMP, followed by determination of the unknowns. The reaction was allowed to proceed for 2 h at 4 °C. Charcoal was used to remove excess unbound cAMP. Samples were counted in 5 ml of Liquisint™ (National Diagnostics).

Results were analyzed with GraphPad Prism® software. Mean ± S.E. was calculated for basal and maximal cAMP production. For the dose response, a non-linear, curve-fitting program (GraphPad Prism®) was used, and the EC50 was determined for wild-type hIP1D4 and mutant constructs. An analysis of variance (post test Newman-Keuls) and Student’s t tests were used to determine statistically significant differences (p < 0.05).

RESULTS

Two polymorphisms in the coding region of the hIP were recently identified and appeared on the SNP database (6). Using PCR mutagenesis we have reproduced these polymorphisms, V25M and R212H (Fig. 1). The overall goal of our study was to determine whether these naturally occurring mutations would modify hIP receptor function. In particular, we analyzed binding of the high-affinity agonist iloprost (a stable derivative of the native hIP ligand prostacyclin), activation of the native Gs pathway, and cell surface expression of the receptor.

Normal Iloprost Binding in the V25M and R212H Polymorphism Mutants at pH 7.4—Receptor binding was initially evaluated at physiological pH 7.4 with iloprost, a stable high-affinity analogue of prostacyclin. No significant difference was detected in the Kd values for wild-type hIP1D4, V25M, or R212H (Table I). All binding curves were best fit by a one-site model. Thus, iloprost binding for both polymorphism mutants remained unaffected as compared with the wild-type hIP1D4 receptor. Saturation binding performed on the three constructs showed expression levels of 1.8 ± 0.3 pmol/mg membrane protein for the hIP1D4 (n = 3) and 1.5 ± 0.4 pmol/mg membrane protein for the V25M (n = 3). However, the R212H expressed significantly (p < 0.05) lower (0.8 ± 0.2 pmol/mg membrane protein; n = 3) than the hIP1D4.

Defective Receptor Activation for the R212H Polymorphism Mutant at pH 7.4—Receptor activation, as measured by increases in the production of cAMP, revealed a significant defect associated with the R212H polymorph, which exhibited an EC50 (2.8 ± 0.5 nM; p < 0.01) 6-fold greater than that of the wild-type hIP1D4 receptor (EC50 = 0.5 ± 0.1 nM) (Table II). Conversely, the V25M mutant did not show any significant difference from the wild-type hIP1D4 in regards to cAMP generation (Table II). Thus, with respect to both ligand binding and activation the V25M variant exhibited wild-type-like characteristics. In contrast, at pH 7.4 the R212H mutant had adverse effects upon receptor activation exclusively, with no significant effect on agonist binding.

Functional Defects Detected under Acidotic Conditions—During conditions of stress such as those observed with renal, cardiac, or respiratory failure, severe acidosis (both metabolic and respiratory) can ensue, lowering in vivo pH levels nearly
concentrations per assay). Unpaired Student’s t significant decrease in EC50 for both wild-type hIP1D4 (EC50 constructs (Table II, Fig. 3). The R212H still differed from significant change in activation from pH 7.4 to 6.8 for all three pH on receptor activation was then determined. Constructs in binding from the change in pH (Fig. 2, Table I). The effect of hIP1D4 nor the V25M variant showed any detrimental effects persisted at a lower pH of 5.9. However, neither the wild-type R212H, we did not observe a further reduction of the already abnormal EC50.

Comparison of Receptor Expression with cAMP Levels and EC50—Our cAMP activity assays showed equivalent maximal levels for all three constructs using 2.0 μg/ml hIP1D4 DNA for transfection (Fig. 3). The R212H still differed from wild-type hIP1D4 by 6-fold. At pH 5.9, however, there was a significant decrease in EC50 for both wild-type hIP1D4 (EC50 = 2.1 ± 0.7 nM, p < 0.05) and V25M (EC50 = 7.5 ± 2.6 nM, p < 0.05) (Table II, Fig. 3). The R212H still remained abnormal at 7.0 ± 1.6 nM. For both the wild-type hIP1D4 and the two variants, activation was impaired at lower pH. However, only at pH 5.9 was a defect observed with wild-type hIP1D4 and V25M. (Table II). Despite the change in affinity at lower pH for R212H, we did not observe a further reduction of the already abnormal EC50.

Comparison of Receptor Expression with cAMP Levels and EC50—Our cAMP activity assays showed equivalent maximal levels for all three constructs using 2.0 μg/ml hIP1D4 DNA for transfection (Fig. 3). However, using the same concentration of DNA our saturation binding indicated that R212H expressed at half the levels of V25M and hIP1D4. We hypothesized that this apparent difference arose from our overexpression system. We thus sequentially titrated the plasmid DNA used for our transfection (0.025–2.0 μg of DNA/ml), using equal concentrations for hIP1D4 wild-type and R212H (Fig. 4). DNA concentrations of 2.0, 1.0, and 0.5 μg of DNA/ml yielded no significant differences in maximal cAMP produced. However, lower DNA concentrations (0.25, 0.05, and 0.025 μg/ml) showed a significant difference that correlated with receptor expression. At 0.05 μg of DNA/ml, the Bmax was 0.3 pmol/mg membrane protein for hIP1D4 and 0.2 pmol/mg membrane protein for R212H. At 0.025 μg of DNA/ml expression was 0.2 pmol/mg for hIP1D4 and 0.1 pmol/mg for R212H. Further experiments were performed to assess whether EC50 was affected by the reduction in expression (Fig. 5). At 0.6 pmol/mg membrane protein the maximal cAMP values were the same for both hIP1D4 and R212H. The EC50 for wild-type hIP1D4 was 0.6 nM in comparison to 2.5 nM for R212H. At lower expression levels for the R212H (0.2 pmol/mg membrane protein) the EC50 was 3.1 nM, and at 0.1 pmol/mg membrane protein the EC50 was 7.1 nM (Fig. 5). Although a change was noted in maximal cAMP produced, there were no significant differences in EC50 for both hIP1D4 and R212H at lower cell surface expression.

Discussion

Receptor polymorphisms are emerging as important contributors to the understanding of both disease pathophysiology and therapeutics (8–10). Numerous naturally occurring variants have been found in virtually all domains of G-protein-coupled receptors, altering ligand binding and coupling to G-protein (11). Transmembrane domain variants in rhodopsin (7), the dopamine D4 receptor (12), and the vasopressin V2 receptor (13) show marked impairment on ligand (or chromophore) binding. Similarly, variants detected in the intracellular loops in the dopamine D2 receptor (14), the endothelin ETB receptor (15), and the vasopressin V2 receptor (16) exhibit impairment in G-protein coupling. Such studies have uncovered many new functionally important residues (11). There are, however, many polymorphisms that may be silent under normal physiological

Table I

|        | pH 7.4 | pH 6.8 | pH 5.9 |
|--------|--------|--------|--------|
| K<sub>n</sub> (nM) |        |        |        |
| hIP1D4 | 5.9 ± 1.9 | 7.1 ± 3.2 | 7.3 ± 3.0 |
| V25M  | 6.1 ± 2.3 | 6.8 ± 3.6 | 10.3 ± 3.1 |
| R212H | 2.2 ± 1.2 | 45.6 ± 12.0 | 33.7 ± 7.7 |

<sup>a</sup> Number in parentheses is the number of experiments (n).
<sup>b</sup> p < 0.05.

Table II

|        | pH 7.4 | pH 6.8 | pH 5.9 |
|--------|--------|--------|--------|
| EC<sub>50</sub> (nM) |        |        |        |
| hIP1D4 | 0.5 ± 0.1 | 0.5 ± 0.2 | 2.1 ± 0.7 |
| V25M  | 0.9 ± 0.4 | 0.4 ± 0.2 | 7.5 ± 2.6 |
| R212H | 2.8 ± 0.5 | 3.2 ± 1.6 | 7.0 ± 1.6 |

<sup>a</sup> Number in parentheses is the number of experiments (n).
<sup>b</sup> p < 0.05.
<sup>c</sup> p < 0.01.
DNA were used at different concentrations (0.025–15442 nM). C50 were determined from the best-fit curve with non-linear regression (GraphPad Prism®). A, production of cAMP performed at pH 7.4. B, production of cAMP performed at pH 6.8. C, production of cAMP at pH 5.9.

FIG. 3. cAMP activation at pH 7.4, 6.8, and 5.9. Mean ± S.E. of cAMP production (pmol/10^6 cells) from at least three identical experiments in which each construct was performed in parallel with wild-type hIP1D4 is shown. The stimulation by iloprost ranged from 1 μM to 0.01 nm. EC50 were determined from the best-fit curve with non-linear regression (GraphPad Prism®). A, production of cAMP performed at pH 7.4. B, production of cAMP performed at pH 6.8. C, production of cAMP at pH 5.9.

FIG. 4. Titration of transfected DNA and corresponding cAMP response to 1 μM iloprost. Wild-type hIP1D4 (gray bars) and R212H (black bars) DNA were used at different concentrations (0.025–20 μg/ml) to transfet COS-1 cells as described under “Experimental Procedures.” Basal levels in the absence of iloprost were subtracted from cAMP levels (pmol/10^6 cells) upon stimulation with 1 μM iloprost. Shown are means ± S.E. from three experiments. Significant differences (*, p < 0.05, **, p < 0.01) between hIP1D4 and R212H were determined from unpaired Student’s t tests.

conditions with the underlying functional abnormalities becoming apparent only in the diseased state (17, 18). Defects in hIP receptor structure and function caused by such naturally occurring mutations may ultimately lead to explanations concerning the intrinsic differences observed in the pathophysiology of cardiovascular disease and responses to therapy (e.g., variable responses to iloprost in the treatment of pulmonary hypertension) (19, 20). In this study, we characterize the effects of the V25M and R212H polymorphisms on hIP function.

Detection of hIP Polymorphisms—Two hIP polymorphisms in the coding region of the hIP, were recently identified and appeared on the SNP database (6). The V25M variation was found to originate from a single guanine-to-adenine mutation at codon position 1 (corresponding to the amino acid position number 25), and multiple PCR reactions from a sample size of 62 chromosomes (i.e., 31 chromosomal pairs) were used to confirm this change. Homozygotes were detected, implying an ample rate of mutant incidence; however, actual prevalence and population frequency requires further determination. This polymorphism is located in transmembrane helix I in the region of the putative agonist binding pocket (Fig. 1). The R212H mutation arose from a single guanine-to-adenine change at codon position 2 (corresponding to the amino acid position 212) with a sample size of 40 chromosomes (i.e., 20 chromosomal pairs). Homozygous samples were also detected for this variant, which is located in the important third intracellular loop (Fig. 1). The goal of our study was to determine the effects of these naturally occurring mutations on hIP receptor function. In particular, we analyzed agonist binding, activation of the native Gs pathway, and cell surface expression of the receptor.

Defective Receptor Activation Detected in the R212H Polymorphism—Receptor binding was initially evaluated at physiological pH 7.4 with iloprost as described under “Experimental Procedures.” No significant difference was observed in agonist binding (Kd) between wild-type hIP1D4 and the V25M or R212H polymorphisms. Receptor activation, as measured by increases in the production of cAMP, revealed that there was a significant defect associated with the R212H mutation. Conversely, the V25M mutant did not show any significant difference from the wild-type hIP1D4 in regards to cAMP generation. Thus, with respect to both ligand binding and activation, the V25M variant exhibited wild-type-like characteristics, indicating that the valine-to-methionine mutation was well tolerated despite a significant change in amino acid size. In contrast, at pH 7.4 the R212H mutant (located in the important third intracellular loop) had adverse effects upon receptor activation exclusively, with no significant effects on agonist binding.

Acidosis Induces Further Functional Defects—A variety of pathophysiological conditions (e.g., cardiac failure) can result in severe acidosis, drastically reducing in vivo pH levels. The hIP receptor located on the plasma membrane is thus vulnerable to such pH changes. Positive charges in the third intracellular loop play an important role in receptor activation, the V25M variant exhibited wild-type-like characteristics, indicating that the valine-to-methionine mutation was well tolerated despite a significant change in amino acid size. In contrast, at pH 7.4 the R212H mutant (located in the important third intracellular loop) had adverse effects upon receptor activation exclusively, with no significant effects on agonist binding.

The R212H mutation arose from a single guanine-to-adenine change at codon position 2 (corresponding to the amino acid position 212) with a sample size of 40 chromosomes (i.e., 20 chromosomal pairs). Homozygous samples were also detected for this variant, which is located in the important third intracellular loop (Fig. 1). The goal of our study was to determine the effects of these naturally occurring mutations on hIP receptor function. In particular, we analyzed agonist binding, activation of the native Gs pathway, and cell surface expression of the receptor.

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mental effects in binding caused by the change in pH (Fig. 2, Table I).

Despite the binding defect in R212H there was no significant change in activation from pH 7.4 to 6.8 for any of the three constructs (Table II, Fig. 3), and the R212H polymorphism still differed from wild-type hIP1D4 by 6-fold. Activation was impaired at lower pH levels for wild type as well as both variants, but only at pH 5.9 was a defect observed for hIP1D4 and V25M (Table II). Despite the change in affinity at lower pH for R212H, we did not observe a significant lowering of the already abnormal EC50. This may be related to the sensitivity of our assay system in detecting small but significant changes in EC50. However, mutations have been found in the prostacyclin receptor that significantly decrease agonist binding affinity without an equivalent effect on activation (21). We believe that this stems from amino acid-ligand interactions (receptor binding pocket) that contribute to affinity but do not contribute to receptor conformational changes required for Gi activation. Reduced pH (6.8) may alter such critical residues (protonation) mimicking such mutations. The defect in receptor activation for R212H remains markedly abnormal at acidic pH. 

Receptor Expression Affects cAMP Levels but Not EC50—Our cAMP activity assays revealed equivalent maximal activation for all three constructs using 2.0 μg/ml DNA for transfection. However, using the same concentration of DNA saturation binding indicated that R212H expresses at half the levels of V25M and hIP1D4. This apparent difference may be attributable to our overexpression system in which the stoichiometry of components in G-protein-coupled receptor signaling plays an important role (22). It has been shown that adenylyl cyclase is able to our overexpression system in which the stoichiometry of components in G-protein-coupled receptor signaling plays an important role (22).

In conclusion, this study highlights the resultant structural and functional defects associated with the first known naturally occurring human prostacyclin receptor polymorphisms V25M and R212H in conjunction with a common in vivo pathophysiological stressor, acidosis. Important clinical corollaries may arise during episodes of acute severe acidosis in patients possessing hIP polymorphisms such as R212H. In these situations an urgent correction of bodily pH may be required to restore normal hemostasis and vasodilation, as well as to improve therapeutic responses. This study provides the mechanistic basis for further research into genetic risk factors and pharmacogenetics of human prostacyclin receptor-associated diseases.

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