Clinical Research

Acceleration of Cardiovascular Disease by a Dysfunctional Prostacyclin Receptor Mutation
Potential Implications for Cyclooxygenase-2 Inhibition


Abstract—Recent increased adverse cardiovascular events observed with selective cyclooxygenase-2 inhibition led to the withdrawal of rofecoxib (Vioxx) and valdecoxib (Bextra), but the mechanisms underlying these atherothrombotic events remain unclear. Prostacyclin is the major end product of cyclooxygenase-2 in vascular endothelium. Using a naturally occurring mutation in the prostacyclin receptor, we report for the first time that a deficiency in prostacyclin signaling through its G protein–coupled receptor contributes to atherothrombosis in human patients. We report that a prostacyclin receptor variant (R212C) is defective in adenylyl cyclase activation in both patient blood and in an in vitro COS-1 overexpression system. This promotes increased platelet aggregation, a hallmark of atherothrombosis. Our analysis of patients in 3 separate white cohorts reveals that this dysfunctional receptor is not likely an initiating factor in cardiovascular disease but that it accelerates the course of disease in those patients with the greatest risk factors. R212C was associated with cardiovascular disease only in the high cardiovascular risk cohort (n=980), with no association in the low-risk cohort (n=2293). In those at highest cardiovascular risk, both disease severity and adverse cardiovascular events were significantly increased with R212C when compared with age- and risk factor–matched normal allele patients. We conclude that for haploinsufficient mutants, such as the R212C, the enhanced atherothrombotic phenotype is likely dependent on the presence of existing atherosclerosis or injury (high risk factors), analogous to what has been observed in the cyclooxygenase-2 inhibition studies or prostacyclin receptor knockout mice studies. Combining both biochemical and clinical approaches, we conclude that diminished prostacyclin receptor signaling may contribute, in part, to the underlying adverse cardiovascular outcomes observed with cyclooxygenase-2 inhibition. (Circ Res. 2008;102:986-993.)

Key Words: prostacyclin ■ eicosanoid ■ cyclooxygenase-2 ■ G protein coupled receptor ■ mutation

The direct cost of treating cardiovascular disease is estimated at $431.8 billion annually for the 79,400,000 individuals who have cardiovascular disease (2007 NHLBI Morbidity and Mortality Chart Book). The recent withdrawal of rofecoxib (Vioxx), following the results of the VIGOR, APPROVe, and other cyclooxygenase (COX)-2 inhibitor clinical outcomes trials,1–4 and the development of cardiovascular disease in predisposed prostacyclin receptor knockout mice,5,6 underscores the necessity to better understand the effects of COX-2–derived metabolites on cardiovascular health. Endothelial prostacyclin synthesis requires the COX-2 enzyme7 and may serve a role in protection from atherothrombosis.8,9 This cardioprotective role has been supported by recent prostacyclin receptor knockout mice studies showing that the absence of the prostacyclin receptor (IP) (International Union of Pharmacology Receptor classification) leads to intimal hyperplasia, atherosclerosis, and hypercoagulability,5,6 as well as reperfusion injury,10 and premenopausal atherogenesis.11 Despite such accumulating information, controversy remains as to whether prostacyclin deficiency is the etiology of the cardiovascular events observed with COX-2 inhibition,12 particularly as no human studies have directly implicated defective prostacyclin signaling in the development of cardiovascular disease.

The human prostacyclin receptor (hIP) gene (PTGIR) spans approximately 7000 bases along chromosome 19 (locus...
19q13.3) and is comprised of 3 exons separated by 2 introns, 1 intron lying upstream from the ATG start codon and the other at the end of the sixth transmembrane (TM) helix. It encodes a G protein–coupled receptor (GPCR) composed of 386 amino acids and has a molecular mass ranging from 37 to 41 kDa, depending on different states of glycosylation. The hIP is most commonly associated with coupling to the \( \alpha \) subunit of the heterotrimeric G protein, which on receptor activation stimulates membrane-bound adenylyl cyclase to catalyze the formation of the second messenger, cAMP. Like other prostanoid receptors, the hIP has been categorized (based on sequence homology, ligand structure, and overall receptor functionality) as a class A rhodopsin-like GPCR, and shares many structural commonalities with rhodopsin, the class A representative and “prototypical” GPCR. These common traits can be divided into 3 major receptor domains: (1) the extracellular domain, consisting of a short amino N-terminal tail and 3 extracellular loops (exoloops); (2) a TM domain, comprised of 7-TM–spanning \( \alpha \)-helices, whose upper third \( \alpha \)-helices contain the putative binding pocket; and (3) the cytoplasmic or intracellular domain that is made up of 3 helix-joining intracellular loops (cytoloops), a fourth loop produced by lipid anchoring (palmitoylation) of intracellular cysteines, and a fairly lengthy carboxyl terminus (Figure 1). Genetic variants may, therefore, have differential effects on binding, expression, and activation, dependent on localization within the protein structure, analogous to the retinitis pigmentosa rhodopsin mutations.

We now report that defective prostacyclin signaling appears to accelerate atherothrombosis in human subjects leading to increased cardiovascular disease and events, analogous to COX-2 inhibition. Importantly, we conclude that the association of dysfunctional prostacyclin signaling with cardiovascular disease is cardiovascular risk factor–dependent, potentially explaining the variability in association with cardiovascular disease observed with COX-2 inhibition tri-
The mechanism arises from defective activation, inducing relative states of increased thrombosis, a hallmark of cardiovascular events.

**Patients and Methods**

**Whole Blood Assessment**

Ten milliliters of whole blood (preserved in EDTA) was collected from patients. Ligand-binding characteristics for the platelet-expressed hIP and human thromboxane receptor (hTP) were determined through saturation-binding assays, using 30 μL of human blood (~1×10^8 platelets) and 1 of 6 different concentrations (1 to 200 nmol/L) of [H]-iloprost (hIP agonist, specific activity 16 Ci/mmol, Amershams) and [H]-SQ 29548, hTP antagonist, specific activity 48 Ci/mmol, PerkinElmer) to detect the hIP and hTP, respectively. Human IP receptor activation was determined through the [H]-cAMP radio-receptor competition assay (Amersham), using 100 μL of human blood (~3.3×10^8 platelets) at maximal doses of iloprost stimulation (10 mmol/L to 1 μmol/L). Data were analyzed using GraphPad Prism software (GraphPad Software Inc, San Diego, Calif). We believe that whole blood analysis is more physiological and directly pertinent to the patients. Patient variability from the use of medications and other concomitant diseases are important considerations, and thus the addition of COS-1 cell experiments (expressing the wild type or mutant receptors) were used to further support or refute our observations.

Functional assays of platelet aggregation were performed using a Chronolog aggregometer to comparing platelet-rich plasma with platelet-poor plasma (control) generated by differential centrifugation. Dose-response of prostacyclin agonists (10 μmol/L to 1 mmol/L) was determined in parallel with standard controls (eg, ADP). cAMP production was determined for each response (percentage inhibition) and correlated to the expected response for the reduced cAMP observed with R212C. Direct R212C patient blood analysis could not be performed as all patients were on multiple antiplatelet therapy including aspirin (and clopidogrel). Our aggregation analysis was, therefore, indirect, based on the observation that reduced cAMP production leads to decreased inhibition of aggregation (a relative state of hyperaggregation).

**In Vitro Assessment in COS-1 Cell System**

Details of the mutagenesis procedure including random mutagenesis at the 212 position can be found in the expanded Materials and Methods in the online data supplement. Paradoxically, hIP receptor activation, and confocal microscopy can be found in our previous publication.

**Cohorts**

Details about each of the Dartmouth-Hitchcock Medical Center (DHMC), Nurses Health Study (NHS), and Health Professional Follow-Up Study (HPFS) cohorts studied and genomic analysis are found in the expanded Materials and Methods in the online data supplement.

**Statistical Analysis**

Baseline characteristics were compared among groups using Student’s t tests and χ² tests as appropriate. The association of the R212C with coronary heart disease (CHD) was analyzed using the Mantel–Haenszel test and an estimate of the odds ratio, which aggregated the odds ratio from each of the 3 study cohorts. The homogeneity of the odds ratio across the three studies was tested using the Q statistic. To adjust for risk factors present in the R212C group a control group was randomly selected (n=20) from the DHMC cohort. The percentage of adverse events occurring in patients found to be positive for the R212C mutation compared with risk-matched control patients was analyzed using a Wilcoxon rank-sum test for 2 independent samples. The analysis was limited to 3 years, analogous to the time periods observed in the COX-2 inhibition studies. Analysis of variance (ANOVA with post test Newman–Keuls) was used for multiple group comparisons and unpaired Student’s t tests were used to directly compare 2 sets of samples.

Analysis of the DHMC cohort was stopped at 1036 (980 used in the study) after clear statistical significance was reached. The HPFS cohort was powered greater (n=2293, double that of the DHMC cohort) to establish whether there was truly a lack of association in a low cardiovascular risk factor group.

**Results**

We recently screened a multiracial population to detect novel hIP genetic variants. An R212C was found in low frequency in both white (1/125 samples, 0.8%) and Asian (1/127 samples, 0.8%) cohorts. The R212C (rs4987262) mutation (Figure 1A and 1B), located in the critical third intracellular loop, represented a significant change in both size and charge. On analysis of our 3D homology model of the hIP (based on the 2.8-Å crystal structure of rhodopsin and site-directed mutagenesis data), the R212C appears to be located at the end of an α-helical conformation within the critical third intracellular loop, which is known in other GPCRs to interact with G protein (Figure 1C).

**R212C Exhibits Defective Function in Patients Blood (Ex Vivo)**

We have also detected this R212C variant in a human cardiology patient population. We were fortunate in obtaining a limited number of whole blood samples from heterozygote R212C patients (n=4, CGC/TGC) and from a single homozygote patient (n=1, TGC/TGC) (described in detail in expanded Results section in the online data supplement). No such mutant hIP structure/function analysis has ever been performed on human tissue. Our studies revealed significant defects in both agonist binding (Kᵢ) (Figure 2A and the Table) and receptor activation (cAMP production) (Figure 2B), with all mutant patient samples exhibiting clear defects. Figure 2A shows saturation binding from individual patients, and the Table summarizes the results. The hTP, also found in abundance in human blood was used as an internal control for both the assessment of binding affinities and hIP receptor expression. Binding affinities (Kᵢ) were 28.7±5.2 and 196.5±23.4 nmol/L for the wild-type hIP and R212C, respectively (P=0.0004, Student’s t test) (Table). After further detailed studies, it appears that this binding defect is, at least in part, related to age and disease severity (expanded Results section in the online data supplement). Paradoxically, hIP receptor numbers were increased in relation to hTPs (also found on platelets) in the R212C heterozygous group (P=0.007, Student’s t test) (Table). This was reflected in the reversed hIP/hTP receptor ratio (wild type=0.44±0.10; R212C=1.36±0.15; P=0.003, Student’s t test). Despite the increased receptor expression, receptor activation (cAMP production) remained severely impaired for the R212C variants (100 nmol/L iloprost wild type=1.53±0.63 pmol versus R212C=0.07±0.12 pmol cAMP: P=0.03, Student’s t test) (Figure 2B). Both heterozygote and homozygote patient blood samples exhibited defective function and increased receptor expression compared with age- and cardiovascular risk factor–matched control patients.

**R212C Exhibits Defective Function in COS-1 Cells (In Vitro)**

Because samples from patients were limited, we sought to biochemically validate the effects of the R212C mutation in a...
COS-1 cell overexpression system. As with patient samples, functional assessment of the R212C variant in COS-1 revealed a consistent reduction in cAMP production compared with wild-type receptor when expressed at equivalent levels (1.0 pmol/mg membrane protein). A significant defect was observed for agonist potency (wild-type EC₅₀ = 0.8 ± 0.1 nmol/L, n = 6; R212C = 2.6 ± 0.7 nmol/L, n = 7; P = 0.035, Student’s t test) (Figure 2C). The reason for this could be further explored with a combination of saturation binding (Bₘₐₓ). Western blot analysis, and confocal microscopy. R212C showed a significant reduction in cell surface expression using saturation binding (wild-type = 1.05 ± 0.06 pmol/mg membrane protein versus R212C = 0.52 ± 0.03 pmol/mg membrane protein; P = 0.01, Student’s t test). Western blot analysis of cell surface membrane preparations also showed a marked reduction in protein levels for R212C (Figure 3A). Confocal analysis staining for receptor (red, anti-1D4 antibody) endoplasmic reticulum (green, anti-calnexin antibody) and nucleus (blue, 4’,6-diamidino-2-phenylindole [DAPI]) showed that there was a marked reduction in cell surface expression for R212C in comparison with wild type, as seen from the reduced red surface staining (white arrows) on the R212C versus the wild type (Figure 3B). There was significant endoplasmic reticulum retention for R212C (Figure 3B, red surrounding nucleus). Because, in overexpression systems, it is usually the endogenous signal transduction effectors that are limiting and not receptor numbers, our results suggest that the R212C that is expressed at the surface is greatly impaired given 3.25-fold reduction in potency. Thus, the in vitro results confirm the ex vivo studies showing that the R212C is functionally defective. The acute expression of R212C results in endoplasmic reticulum retention and

### Table. Summary of Saturation-Binding Results for Age- and Risk Factor–Matched Wild-Type Patients, Diseased Heterozygote Patients, and Our Single Homozygote Patient

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>R212C</th>
<th>R212C</th>
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<tbody>
<tr>
<td></td>
<td>CGC/GGC</td>
<td>TGC/GGC</td>
<td>TGC/TGC</td>
</tr>
<tr>
<td>KD hIP (nmol/L)</td>
<td>28.7 ± 5.2</td>
<td>196.5 ± 23.4*</td>
<td>213.0</td>
</tr>
<tr>
<td>KD hTP (nmol/L)</td>
<td>119.0 ± 34.1</td>
<td>67.8 ± 10.8</td>
<td>155.3</td>
</tr>
<tr>
<td>Bₘₐₓ hIP (fmol/10⁷ plts)</td>
<td>63.0 ± 9.7</td>
<td>142.3 ± 17.5†</td>
<td>140</td>
</tr>
<tr>
<td>Bₘₐₓ hTP (fmol/10⁷ plts)</td>
<td>156.3 ± 24.0</td>
<td>107.3 ± 17.5†</td>
<td>65</td>
</tr>
<tr>
<td>Ratio (hIP/hTP)</td>
<td>0.44 ± 0.10</td>
<td>1.36 ± 0.15‡</td>
<td>2.15</td>
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For wild-type patients, CGC/GGC (n = 5); diseased heterozygote patients, TGC/GGC (n = 4); and our single homozygote patient, TGC/TGC. Shown are the mean ± SE dissociation constant (Kᵤ) (nmol/L), the Bₘₐₓ for each receptor and the ratio of prostacyclin to thromboxane ratio (Ratio). Unpaired Student’s t tests were used to directly compare the wild-type vs heterozygote group. *P = 0.0004, †P = 0.007, ‡P = 0.003; §P = 0.007; n = 4; DHMC 918 (wild-type), DHMC 826 (heterozygote R212C), and DHMC 726 (homozygote R212C) to demonstrate changes in binding and expression. [³H]-Iloprost was used for detection of the hIP and [³H]-SQ 29548 for the hTP. Arrows indicate relative changes in receptor numbers comparing hIP to hTP. B, cAMP determination from the patient samples. Picomoles of cAMP were corrected for changes in receptor numbers. C, cAMP determination from COS-1 cell overexpression experiments for wild-type and R212C constructs. A dose-response for iloprost was established (1 μmol/L, 0.1 μmol/L, and 0.01 μmol/L).

Figure 2. Patient blood analysis. A, Representative saturation binding curves on patient bloods for 3 individual patients DHMC 918 (wild-type [WT]), DHMC 826 (heterozygote R212C), and DHMC 726 (homozygote R212C) to demonstrate changes in binding and expression. B, Corresponding confocal microscopy overlay images (x63 resolution) showing predominant membrane trafficking only for wild-type protein. The hIP receptors both wild-type and mutants are labeled red (1D4 monoclonal antibody), and the overlay picture additionally has blue nuclear staining (DAPI) and a phase-contrast microscopic image of the cell to localize the cells perimeter. White arrows are used to localize areas of cell surface membrane.

Figure 3. R212C expression in a COS-1 system. A, Results of saturation binding and Western blot analysis performed on COS-1 membrane preparations containing R212C and wild-type constructs. B, Corresponding confocal microscopy overlay images (x63 resolution) showing predominant membrane trafficking only for wild-type protein. The hIP receptors both wild-type and mutants are labeled red (1D4 monoclonal antibody). The endoplasmic reticulum is labeled green (anti-calnexin antibody), and the overlay picture additionally has blue nuclear staining (DAPI) and a phase-contrast microscopic image of the cell to localize the cells perimeter. White arrows are used to localize areas of cell surface membrane.
reduced cell surface expression (reduced $B_{\max}$); however, with patient blood samples, there is an interesting paradoxical increase in receptor expression (increased $B_{\max}$), most probably secondary to a compensatory response. Although both in vitro and ex vivo analyses showed reduced function, these studies highlight the importance of using human patient tissue with the mutation of interest to observe pathophysiologically relevant results.

**R212 Stabilizes the Critical Third Intracellular Loop**

We then performed random mutagenesis (expanded Materials and Methods section in the online data supplement) at the R212 position to determine the structural role played by the native Arg at this position. In addition to the 3 naturally occurring mutations at the R212 position (R212C, TGC, R212H, CAC, and R212R CGT), which we had previously identified, random mutagenesis led to the production of an additional 5 mutations (R212R, CGA, R212L, CTC, R212S, AGC, R212P, CCC, and R212T ACT) (Table I and Figure I in the online data supplement). All synonymous mutations (no change in amino acids) showed no difference in binding and activation in comparison with wild-type protein. In contrast, all nonsynonymous mutations exhibited an activation deficiency with evidence of normal binding. Interestingly, R212L had a significant proportion of binding-deficient receptor. These data, when combined with molecular modeling, suggested that the 212 position is critical, by virtue of both its size and charge, in stabilizing the third intracellular loop through interaction with S205 (Figure 1C). Additional mutation of S205 to alanine displayed defective activation with an EC$_{50}$ of 19.1 ± 8.1 nmol/L (wild type=0.8±0.1 nmol/L; supplemental Table I), further supporting an R212-S205 hydrogen-bonding stabilizing interaction.

**R212C Defective Signaling Leads to an Increased Thrombotic State**

We proceeded to address the question as to whether the defective signaling could affect platelet function, leading to a state of increased thrombosis. Because the R212C patients from whom blood samples were obtained were all receiving aspirin therapy, it was necessary to conduct these experiments on wild-type platelets from human volunteers. Dose–response experiments (agonist-induced inhibition of thrombosis) were performed on human platelet–rich plasma. A sigmoidal dose–response was achieved with the addition of increasing concentrations of iloprost (EC$_{50}$=7.1 ± 1.4 nmol/L, n = 4). Corresponding cAMP production was determined and plotted against the percentage inhibition of aggregation (Figure 4). We superimposed the range of cAMP production previously determined in multiple samples from R212C and wild-type patients as bars along the x axis of this graph. These experiments indicate that the very low levels of hIP-stimulated cAMP generated by a defective receptor correspond to a pronounced decrease in inhibition of aggregation, which would result in a relative hyperthrombotic state (Figure 4), a critical component in the development of atherothrombosis.

**R212C Is Associated With Coronary Heart Disease in a Risk Factor–Dependent Manner**

We demonstrated that the R212C leads to defective hIP signaling, promoting key components in the development of atherothrombosis. To determine whether these defects are associated with clinical disease, we examined 3 separate white cohorts (total n=3970) (the R212C was found initially in only whites and Asians). The prevalence of the R212C genotype (heterozygote or homozygote) was comparable among the 3 cohorts (2.14% in DHMC, n=980; 1.72% in NHS, n=697; and 1.92% in HPFS, n=2293). Overall, the R212C was significantly associated with an increased risk for CHD (OR=1.68 [1.03 to 2.76], $P=0.04$) (supplemental Table II). Interestingly, analogous to the COX-2 inhibition studies, there was considerable variability in the odds ratio for individual cohorts, with only the DHMC cohort being significantly associated with CHD (OR=4.71 [1.09 to 20.36], $P=0.022$). An initially surprising observation was the lack of association with the HPFS, which had no association with CHD (OR, 1.15 [0.61 to 2.17], $P=0.66$). Of importance, however, we noted that the prevalence of cardiovascular risk factors was much greater in the DHMC group in comparison with the HPFS group (Figure 5 and supplemental Table III). Furthermore, a group with intermediate risk (NHS) exhibited a trend toward CHD (OR, 2.02 [0.65 to 6.28], $P=0.21$). The 3 cohorts followed the same rank order: DHMC>NHS>HPFS for both R212C associations with CHD and risk factors (Figure 5 and supplemental Table III). In the high risk DHMC cohort, there are significantly more R212C in the CHD cases (19/660=2.9%) compared with the controls (2/320=0.6%). Thus, the association of R212C with coronary artery disease appears to be risk factor–dependent, suggesting that development of atherothrombosis with R212C was dependent on existing underlying disease (promoted by the large number of risk factors). This observation is strongly supported by the prostacyclin knockout mouse studies, which also demonstrated that injury or increased risk factors (concurrent LDL receptor knockout, balloon injury to artery, or precipitation of
thrombosis) was required to observe a cardiovascular phenotype.5,6 Additionally, with COX-2 inhibition (leading to reduced levels of prostacyclin), cardiovascular events appeared to be most evident in those patients most predisposed to cardiovascular disease (highest cardiovascular risk factors).9 This led to our hypothesis that reduced prostacyclin signaling was accelerating existing disease, rather than causing cardiovascular disease de novo.

**R212C Increases CHD Severity, Disease Burden, and Cardiovascular Events**

To address the hypothesis (existing underlying disease was accelerated by R212C), it was mandatory to compare age- and risk factor–matched controls with and without R212C in the DHMC cardiology population for which we had coronary angiographic results. We assessed both disease severity (number of major vessels score of 1 to 3 with significant obstructions) and clinical cardiovascular events (myocardial infarction, percutaneous coronary angioplasty, stroke, documented peripheral vascular disease, unstable angina, and cardiac bypass surgery). For the DHMC cohort, there was a mean±SE of 1.5±0.04 vessels occluded per patient, in comparison with 2.1±0.2 vessels occluded per patient in the R212C cases (P=0.019, Student’s t test) (Figure 6A). The R212C cohort had a lower incidence of patients with no disease and a higher incidence of triple-vessel disease (Figure 6B). The number of adverse events was significantly higher for the R212C group (4.6±0.7 events) versus control (2.7±0.4 events) (P=0.026, Student’s t test; Figure 6C), despite the use of aspirin in all patients except 1 (allergic to aspirin, on clopidogrel). To determine whether the R212C variant predisposed patients to a higher frequency of adverse events, we used a Wilcoxon rank-sum test, in which we limited the assessment of events to the number of follow-up years available for all patients (first 3 years from the initial event, 60 patient years in total for both groups). We found a significant difference between the R212C and risk factor–matched control cohort with regard to adverse event frequency (P=0.016, Wilcoxon rank-sum test) (Figure 6D). Again, these results are supported by the knockout mice studies in which the exaggerated atherothrombotic response in these knockout mice studies required vascular injury initiation (eg, concurrent LDL receptor knockout11 or balloon injury5). Similarly, the hazards associated with COX-2 inhibition are most apparent in patients with the greatest cardiovascular risk factors (ie, predisposition to atherothrombosis).9 The most dramatic example of coronary artery disease acceleration could be observed in our single homozygote patient (expanded Results and Figure II in the online data supplement).

**Discussion**

Only recently have IP polymorphisms been reported and characterized.20,23,27 All characterizations, however, have been in in vitro COS-1 overexpression systems. Our present studies in human patient tissues highlight the importance of such analysis, because we are able to detect functional

![Figure 5](image.png)

**Figure 5.** Clinical analysis of 3 white cohorts. A graph representing the R212C frequencies in the 3 white cohorts, the DHMC, NHS, and HPFS. The white bars represent no CHD, and the shaded bars the CHD groups. The ORs are described and confidence limit observed in supplemental Table II.

![Figure 6](image.png)

**Figure 6.** R212C association with accelerated disease. A, Comparison of disease severity (vessels with significant obstruction, left anterior descending, left circumflex, and right coronary artery) in the cardiology cohort vs the R212C (both heterozygote and homozygote) patients. B, An analysis of disease severity in the cardiology control cohort vs the R212C cohort focusing on percentage of cohort with the number of vessels obstructed. C, Total cardiovascular adverse events recorded for risk-matched cardiology control patients (n=20) and the R212C patients (n=20). D, Number of cardiovascular events recorded for patients from either the risk-matched control patients or the R212C patients. Events were recorded for 3 years from the initial hospital visit/admission for a cardiovascular event.
effects, in addition to potential compensatory mechanisms for the defects (increase in hIP receptors on platelets). Furthermore, the mechanism that leads to clinical association can be deciphered, and the reason for lack of association in other populations may be determined. There has already been some evidence in the literature from COX-2 inhibition trials that the reduction in prostacyclin may be associated with systemic hypertension in human subjects; however, no study has directly linked such prostacyclin signaling deficiencies with human cardiovascular disease. Consistent with these early observations, 80% of the R212C patients in the DHMC study were hypertensive in comparison with the DHMC cardiology population overall, which had a 67% incidence of hypertension. Our goals herein were to assess whether defective prostacyclin signaling is associated with cardiovascular disease in human subjects, as was suggested from both the selective COX-2 inhibitor trials and genetic knockout mice.

**Underlying Disease or Injury Is Required for the R212C Phenotype**

We are rapidly becoming aware that genetic variants may remain silent under normal physiological conditions, or throughout childhood and early adulthood, with the underlying functional abnormalities becoming apparent only in the diseased state or under times of pathophysiological insult. For the prostacyclin signaling pathway, this was demonstrated most dramatically with the IP knockout mice, in which underlying stress or injury was required to reveal that the lack of this receptor promotes increased thrombosis or intimal hyperplasia. We now demonstrate in patients that cardiovascular risk factors likely provide the analogous underlying injury. In this context, the R212C accelerates cardiovascular disease. In the absence of significant risk factors, there appears to be no significant R212C phenotype. This is in parallel to COX-2 inhibition, where reduction in prostacyclin production appears to increase cardiovascular events in high cardiovascular risk patients but shows a lack of association in other study populations. This important principle may explain why many high-profile articles reporting polymorphism–disease associations are not uniformly confirmed by parallel studies. For such association studies, separate populations with differential risk factor profiles may now be necessary.

**Mechanism for R212C Modulation of CHD**

The mechanism for the accelerated atherothrombosis appears to be, at least in part, attributable to a combination of reduced ability to inhibit thrombosis from reduced cAMP signaling in platelets and an inability to reduce human coronary vascular smooth muscle cell (VSMC) proliferation and dedifferentiation, also from reduced cAMP production in VSMCs. For the R212C variant, this appears to arise from the disruption of a critical interaction between R212 and S205, required for stabilizing conformation in the critical G protein–interacting third intracellular loop. A decrease in the effective dose of prostacyclin, attributable either to receptor signaling defects or to inhibition of prostacyclin production (COX-2 inhibition), would also promote thrombosis and promote dedifferentiation and proliferation in human coronary VSMCs. These critical components of atherothrombosis were most clearly defective in our homozygote patient, who had significant disease acceleration at the age of 70 years, on a background of only statin controlled hyperlipidemia. Aspirin (predominant COX-1 inhibitor) itself appears insufficient to oppose the atherothrombotic state in our CHD R212C patients, because all the CHD R212C patients (except 1) were receiving low-dose aspirin therapy.

**Conclusion**

Our combination of in vitro tissue culture analysis, patient blood analysis (defective R212C activation), and the case–control study of R212C patients (showing increased disease and cardiovascular events), together with the published observations from COX-2 inhibition trials (increased cardiovascular events) and IP knockout mice studies (increased cardiovascular disease), all support the conclusion that defective prostacyclin signaling promotes cardiovascular disease in human subjects. Analogous to the knockout mice, we have now shown for the first time that prostacyclin signaling appears to contribute to cardiovascular phenotype in humans in a risk factor–dependent manner. Although the R212C hIP polymorphism is observed at low frequency (2% in our 3 cohorts), with more than 60 million cardiovascular patients in the United States, 2% represents more than 1 million patients. In addition to mandatory control of risk factors, the therapy of choice for R212C may ultimately be potent, stable prostacyclin analogs.

**Acknowledgments**

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**Disclosures**

None.

**References**


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ONLINE SUPPLEMENTARY METHODS

Dartmouth-Hitchcock Medical Center (DHMC), Nurses Health Study (NHS) and Health Professional Follow-Up Study (HPFS) Cohorts.

1036 consecutive, consenting Caucasian participants were recruited from the Dartmouth-Hitchcock Medical Center or Dartmouth Medical School, after informed consent (CPHS approval #14888). All cardiology patients undergoing a coronary angiogram were included. DHMC angiographic/PTCA data was scored with regard to number of diseased vessels (≥50% stenosis of left anterior descending, left circumflex and/or right coronary artery). Patients in shock or unable to consent were excluded. All cardiology subjects underwent a complete medical history and physical examination. 660 DHMC patients had confirmed coronary artery disease (≥50% occlusion, refer to Online Table 3 for risk factors) and 195 had no significant disease based upon coronary angiography (serves as an important internal control group for which we have angiographic results; hypertension 60%, hyperlipidemia 55%, smoking 44%, family history 42%, diabetes 19%). With consecutive Cardiology patients undergoing angiography only approximately 20% have normal angiograms and thus we had to supplement the remaining control cohort (125 for a total of 320) with healthy normal volunteers (no history of cardiovascular events or symptoms). R212C frequency in the 195 patients with normal angiograms (1/195, 0.5%) was equivalent to that of the 125 normal healthy volunteers (1/125, 0.8%). 56 patients (from 1036) were excluded due to indeterminate or incomplete coronary studies or indeterminant genomic analysis, giving a final DHMC study cohort of 980 (660 cases, 320 controls).

For the DHMC cohort, ten milliliters of whole blood (EDTA-preserved) was obtained for genomic DNA extraction (white blood cells). Genomic DNA was extracted from tissue samples (cheek brushing or EDTA-anticoagulated blood) using a commercially-available Puregene® system (Gentra Systems, Inc.). Blood samples were processed by Sorenson Genomics, Utah. The hIP coding gene segments were amplified from genomic DNA (Sorenson Genomics) and sequenced using exon II and exon III primers (PTGIR, GenBank accession number NM000960). All sequences were verified in both the sense and anti-
sense directions, with alternative DNA polymerases (e.g. Taq and Pfu). For the NHS and HPFS cohort, The R212C (rs4987262) polymorphism in exon II of the PTGIR gene on chromosome 19 was genotyped using Taqman™ single-nucleotide polymorphism allelic discrimination by means of an ABI 7900HT Sequence Detection System (Applied Biosystems).

We additionally conducted a prospective nested case-control study within the Nurses Health Study (NHS) and Health Professionals Follow-Up Study (HPFS). Among participants free of cardiovascular disease at baseline, 249 women (NHS) and 266 men (HPFS) developed nonfatal myocardial infarction or fatal CHD during 8 and 6 years of follow-up. As a secondary end point, we additionally identified 564 men (HPFS) who had coronary artery bypass graft surgery (CABG) or percutaneous transluminal coronary angioplasty (PTCA) during follow-up. For the NHS study, complete genotype data could be determined on 233 cases (16 indeterminant) and for the HPFS 761 cases (69 indeterminant). Online Table 3 describes the cardiovascular risk factors for both groups. Controls were selected 2:1 and matched on age, smoking, and month of blood draw (464 for NHS and 1532 for HPFS). The study protocol was approved by the institutional review board of the Brigham and Women’s Hospital and the Harvard School of Public Health Human Subjects Committee review board; all participants provided informed consent.

**PCR-based random mutagenesis at R212 in third cytoplasmic loop of hIP**

Random mutagenesis was performed using a conventional method of PCR mutagenesis, as previously described. For the current studies, complementary oligonucleotide primers were designed with equal amounts of A, T, G, and C at each of the three nucleotide positions at codon 212 (NNN). Thus, the oligonucleotide sequence (in the sense direction) reads 5’ C AGC CTC TGC NNN ATG TAC CGC C 3’ and 5’ G GCG GTA CAT NNN GCA GAG GCT G 3’ (in anti-sense direction). Since wild-type hIP cDNA template was used, the procedure cannot be deemed completely random. Nevertheless, assessments regarding nucleotides that are most compatible (or well-tolerated) within the wild-type Arg codon CGC can be inferred. The PCR reaction mixture contained 1x Pfu reaction buffer, 200ng of wild-
type DNA construct, 150ng of each of the two primers (sense and antisense), 10mM dNTPs, and 2.5 units of Pfu DNA polymerase (Stratagene). The cycling protocol involved heating and cooling at 95°C for 30 seconds, 45°C for 1 minute and 68°C for 10 minutes for 16 cycles. DNA was extracted from all clones found on 20 plates (166 colonies in total) and sequenced (Dartmouth Medical School Molecular Biology Core Facility). All mutant constructs were transfected into COS-1 cells for structure/function characterization.

Modeling of the third intracellular loop on the human prostacyclin receptor

We have recently described the development of an hIP three-dimensional homology model using the internet-based protein-modeling server, SWISS-MODEL (GlaxoSmithKline --- Geneva, Switzerland) and the 2.8Å-resolution x-ray crystallographic structure of bovine rhodopsin as the template (1HZX). The transmembrane domains were energy-minimized, utilizing the Gromos96 force field to improve the stereochemistry of the model and remove unfavorable clashes (SWISS-MODEL) and the loop peptides energy-minimized using the NAMD molecular dynamics simulator.
ONLINE SUPPLEMENTARY RESULTS

Prostacyclin receptor binding defect detected in patients with severe multi-vessel coronary artery disease.

It was intriguing that the $K_D$ values from our patient samples (both R212C and control patients) showed a defect in binding (Table 1) in addition to marked reductions in activation (Figure 2B) compared to the COS-1 cell expressed system (Figure 2C), which exhibited an activation defect with no binding abnormalities (Supplementary Table 1). This strongly suggested that the observed binding defect may be independent of the mutation. To address this we performed further analysis on the bloods of patients without R212C who had severe multivessel disease and compared the results to age matched patients without disease in addition to a younger healthy cohort. The $K_D$ values of randomly selected younger patients (mean age 39.8 ± 4.2 years) was 8.7 ± 2.1 nM (n=6), consistent with COS-1 cell results (5.5 ± 0.7 nM) and published reports (~10nM). Further analysis of older patients with no disease showed a $K_D$ value of 20.8 ± 4.3 nM (n=11, age 66.8 ± 6.8 years) and those age matched with significant multivessel disease 126.3 ± 59.7 nM (n=8, age 66.3 ± 2.3 years). Despite the binding defect there was no significant effect observed on cAMP production, with 1µM iloprost leading to 1.92 ± 0.33 pmol cAMP (age-matched with no disease) compared to 2.30 ± 0.38 pmol cAMP (age-matched with multivessel disease) (p=0.46, Student t test). This defect in binding with no significant effect on cAMP production most probably reflects the abundance (redundancy, spare receptors) of hIP receptors on platelets. These results suggest that both age and disease may affect hIP binding and warrants further investigations into the etiology. A significant defect in prostacyclin binding associated with age and disease explains at least in part, the unexpected binding defect associated with R212C.

The homozygote R212C patient shows CHD disease acceleration

Although we only found a single homozygote R212C, the patient history supported our hypothesis that R212C accelerates underlying disease. The single R212C homozygous patient presented at the age of seventy, with an acute coronary syndrome involving the left anterior descending (LAD, 50% occlusion)
and right coronary arteries (RCA, 80% occlusion) (Supplementary Figure 2A). Although this presentation is not unusual for this age group, it was notable that his only risk factor was mild statin-controlled hyperlipidemia. Similarly and importantly, the age of presentation of the heterozygote R212C patients was lower but not significantly different from the high risk DHMC cardiology patients (DHMC patients mean ± SEM, 64 ± 0.4 yrs versus R212C patients 61 ± 2.7 yrs). After failed procedures and double coronary bypass operation (LIMA and saphenous RCA), the homozygote patient re-presented 5 years later with total (100%) occlusions in two vessels and significant obstruction in his third vessel. Additionally, he had completely occluded one of his bypass grafts (Supplementary Figure 2B). As previously demonstrated, his platelets exhibited severe prostacyclin receptor functional defects (Figure 2A, 2B & Table 1, TGC/TGC), with decreased binding affinity (increased $K_D$), defective receptor activation (decreased cAMP), and increased hIP expression (reversed hIP/hTP ratio). Although somewhat anecdotal, this homozygous R212C individual illustrates an unusually accelerated course in the presence of only hyperlipidemia.
ONLINE SUPPLEMENTARY FIGURES

Supplementary Figure 1: In our theoretical energy minimized model, Arg212 forms an electrostatic interaction with Ser205, stabilizing the helical region formed by the proximal portion of the third cytoplasmic loop connecting transmembrane helices five and six. The random mutations generated (indicated by the one letter amino acid code) at this site all lack this stabilizing interaction and demonstrate impaired signaling with an increased EC$_{50}$ (shown below the structures). Although His212 carries a protonatable nitrogen, the side chain is not long enough to form a contact with Ser205. Leu212 has the additional property of adversely affecting agonist binding, likely due to hydrophobic interference between native interactions formed between the third and second intracellular loops.
Supplementary Figure 2: Angiograms from the R212C homozygote patient

PANEL A: Angiographic views of the left coronary system in our single homozygote patient. The 70 years old view shows only partial obstruction of the mid left anterior descending (LAD, green arrow), on a background of a partially occluded right coronary artery (RCA, not shown). Panel B: The 75 years old view is a rotated view to better visualize the left circumflex artery (LCx), which has now several sites of significant occlusion (white arrows). The mid LAD (green arrow) is 100% occluded. The yellow arrows show formation of collaterals to the occluded (100%) right coronary artery and occluded (100%) right saphenous vein graft. The gray arrows show the staples for the left internal mammary graft to the LAD which was the only vessel with no significant obstruction.
ONLINE SUPPLEMENTARY TABLES

Online Table 1: Analysis of binding (Ki nM) and activation (EC\textsubscript{50} nM) of mutations at the R212 position through random PCR mutagenesis. Degenerate oligonucleotides were used with equal GACT at each of the three nucleotide positions at position 212, giving a theoretical possibility of all amino acids. 166 colonies were picked from 20 plates and sequenced to determine what mutants were present. The three naturally occurring mutations at the R212 position are highlighted (#). The nucleotide changes are bolded and underlined. All results were obtained from at least three independent experiments. (*p<0.05, **p<0.01, ***p<0.001). @ The R212L was best fit to a 2 site binding model. Wild type binding constituted 36% while the second component constituted 64%. The binding for the second site was 134.2 ± 26.7 nM (p<0.001).

<table>
<thead>
<tr>
<th></th>
<th>Ki (nM)</th>
<th>EC\textsubscript{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R212R (CGC) WT</td>
<td>5.5 ± 0.7</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>R212R (CGT)#</td>
<td>8.0 ± 1.5</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>R212R (CGA)</td>
<td>6.8 ± 0.9</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>R212L (CTC)</td>
<td>10.2 ± 4.2@</td>
<td>5.7 ± 1.1**</td>
</tr>
<tr>
<td>R212H (CAC)#</td>
<td>6.1 ± 0.8</td>
<td>3.1 ± 1.0*</td>
</tr>
<tr>
<td>R212C (TGC)#</td>
<td>2.0 ± 0.6</td>
<td>2.6 ± 0.7*</td>
</tr>
<tr>
<td>R212S (AGC)</td>
<td>6.4 ± 0.9</td>
<td>3.9 ± 0.9**</td>
</tr>
<tr>
<td>R212P (CCC)</td>
<td>6.7 ± 3.5</td>
<td>3.2 ± 0.8**</td>
</tr>
<tr>
<td>R212T (ACT)</td>
<td>10.2 ± 3.4</td>
<td>2.1 ± 0.5**</td>
</tr>
</tbody>
</table>
Online Table 2: Analysis of the three cohorts. Weighted average of odds ratio (OR) estimates for coronary heart disease from the DHMC, HPFS and NHS studies (Review Manager (RevMan) [Computer program]. Version 4.2 for Windows. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2003). The overall effect from 3970 patients showed $Z = 2.07$ ($P = 0.04$)

<table>
<thead>
<tr>
<th>Study</th>
<th>Variant n/N</th>
<th>Wildtype n/N</th>
<th>OR (fixed) 95% CI</th>
<th>Weight %</th>
<th>OR (fixed) 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHMC</td>
<td>19/21</td>
<td>641/959</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHS</td>
<td>6/12</td>
<td>227/685</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HPFS</td>
<td>16/44</td>
<td>745/2249</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>77/3893</td>
<td></td>
<td></td>
<td>100.00</td>
<td>1.68 [1.03, 2.76]</td>
</tr>
</tbody>
</table>

Total events: 41 (Variant), 1613 (Wildtype)
Test for heterogeneity: $\chi^2 = 3.37$, df = 2 ($P = 0.07$), $I^2 = 40.8\%$
Test for overall effect: $Z = 2.07$ ($P = 0.04$)
Online Table 3: Comparison of risk factor profiles in Cases. Shown are the numbers of patients with the reported risk factor followed by the percentage of total cases. Statistical analysis was used to determine whether there were significant differences between the three groups to justify the high, medium and low risk categorization. ANOVA was used to compare the age differences between the 3 cohorts and chi-square analysis to compare the proportions between the three cohorts. Shown in the right columns are the respective p values.

<table>
<thead>
<tr>
<th></th>
<th>DHMC (high)</th>
<th>NHS (medium)</th>
<th>HPFS (low)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (cases)</td>
<td>980 (660)</td>
<td>697 (233)</td>
<td>2293 (761)</td>
<td></td>
</tr>
<tr>
<td>Age (yr ± sd)</td>
<td>64 ± 11.9</td>
<td>60 ± 6.5</td>
<td>65 ± 7.9</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>449 (68 %)</td>
<td>133 (57 %)</td>
<td>335 (44 %)</td>
<td>p&lt;0.00001</td>
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<tr>
<td>Hyperlipidemia</td>
<td>475 (72 %)</td>
<td>124 (53 %)</td>
<td>403 (53 %)</td>
<td>p&lt;0.00001</td>
</tr>
<tr>
<td>Smoking</td>
<td>337 (51 %)</td>
<td>75 (32 %)</td>
<td>61 (8 %)</td>
<td>p&lt;0.00001</td>
</tr>
<tr>
<td>Family History</td>
<td>304 (46 %)</td>
<td>51 (22 %)</td>
<td>114 (15 %)</td>
<td>p&lt;0.00001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>198 (30 %)</td>
<td>47 (20 %)</td>
<td>76 (10 %)</td>
<td>p&lt;0.00001</td>
</tr>
</tbody>
</table>
SUPPLEMENTARY REFERENCES


