A Loss-of-Function Polymorphism in the Human P2X4 Receptor Is Associated With Increased Pulse Pressure

Leanne Stokes, Katrina Scurrah, Justine A. Ellis, Brett A. Cromer, Kristen K. Skarratt, Ben J. Gu, Stephen B. Harrap, James S. Wiley

Abstract—The P2X4 receptor is involved in endothelium-dependent changes in large arterial tone in response to shear stress and is, therefore, potentially relevant to arterial compliance and pulse pressure. Four identified nonsynonymous polymorphisms in P2RX4 were reproduced in recombinantly expressed human P2X4. Electrophysiological studies showed that one of these, the Tyr315>Cys mutation (rs28360472), significantly reduced the peak amplitude of the ATP-induced inward current to 10.9% of wild-type P2X4 receptors in transfected HEK-293 cells (10 μmol/L of ATP; n=4–8 cells; P<0.001). Concentration-response curves for ATP and the partial agonist BzATP demonstrate that the 315Cys-P2X4 mutant had an increased EC50 value for both ligands. Mutation of Tyr315>Cys likely disrupts the agonist binding site of P2X4 receptors, a finding supported by molecular modeling based on the zebrafish P2X4 receptor crystal structure. We tested inheritance of rs28360472 encoding the Tyr315>Cys mutation in P2RX4 against pulse pressure in 2874 subjects from the Victorian Family Heart Study. The minor allele frequency was 0.014 (1.4%). In a variance components analysis we found significant association with pulse pressure (P=0.023 for total association) where 1 minor allele increased pulse pressure by 2.84 mm Hg (95% CI: 0.41–5.27). This increase in pulse pressure associated with inheritance of 315Cys-P2X4 receptors might reflect reduced large arterial compliance as a result of impaired endothelium-dependent vasodilation in large arteries. (Hypertension. 2011;58:1086-1092.) ○ Online Data Supplement

Key Words: P2X4 receptor ▪ polymorphism ▪ SNP ▪ P2RX4 ▪ pulse pressure ▪ ATP

Pulse pressure is an independent risk factor for cardiovascular disease1 and is determined by large artery compliance, cardiac stroke volume, and reflected pressure waves. Endothelial cell responses to blood flow have an important role in the control of vascular tone because they release ATP in response to flow-related shear stress.2 ATP is the physiological ligand for the purinergic receptor family composed of 7 ligand-gated ion channels (P2X1 to P2X7) and 7 metabotropic P2Y receptors.3 P2X4 receptors are expressed in vascular endothelial cells where this receptor functions in shear stress-induced Ca2+ signaling and the generation of NO.4–6 P2X4 receptors show fast activation kinetics with high permeability to Ca2+ ions.7 Antisense knockdown of P2X4 receptors abolishes shear stress-induced Ca2+ signals in endothelial cells,8 and the P2X4 receptor knockout mouse exhibits reduced shear stress-mediated Ca2+ influx into cultured endothelial cells and reduced production of NO.9 Endothelial responses to shear stress regulate the compliance of large arteries, with consequences for pulse pressure. Functional variations in the human P2X4 receptor may influence such shear stress-related parameters within the vasculature.

The human P2X4 receptor gene (P2RX4) is located on chromosome 12q24.32.10 We determined functional effects of 4 nonsynonymous coding-region single nucleotide polymorphisms (SNPs) in P2RX4, focusing our investigation on 1 SNP (rs28360472) that leads to loss of P2X4 receptor function through disruption of the agonist binding site and shows association with increased pulse pressure.

Methods

Detailed methods are available in the online Data Supplement (please see http://hyper.ahajournals.org).

For the Sydney cohort, genomic DNA was prepared from peripheral venous blood as described previously,11 and 4 nonsynonymous SNPs in P2RX4 were genotyped; rs1044249, rs28360470, rs25644, and rs28360472. Details of the recruitment of participants for the Victorian Family Heart Study have been published previously.12,13 For the Victorian Family Heart Study cohort, statistical association between genotype and pulse pressure was assessed using variance components modeling and the quantitative trait linkage disequilibrium approach.14
HEK-293 cells were transfected with wild-type or mutated enhanced green fluorescent protein–tagged human P2X4 receptor and functional responses measured by whole cell patch clamping. Surface staining was performed on transfected HEK-293 cells using a rabbit antihuman P2X4 antibody.

Peripheral venous blood was taken from healthy volunteers carrying no mutations in P2RX4 (wild-type) or carrying one allele of rs28360472. CD14+ monocytes were isolated and differentiated to macrophages. For functional experiments, data are presented as mean ± SEM. Statistical analysis was performed using t-tests or ANOVA where appropriate with Instat version 3 (GraphPad Software Inc, La Jolla, CA). A molecular model of the human P2X4 receptor was built by homology to the zebra-fish P2X4 receptor structure (pdb code 3I5D) using SwissModel to explore the putative ATP-binding pocket in wild-type P2X4 and 315Cys-P2X4 receptors.

**Results**

**Polymorphic Variants of the P2X4 Receptor**

Four nonsynonymous coding SNPs in the P2RX4 gene were identified in the National Center for Biotechnology Information Single Nucleotide Polymorphism database (build 126). The mutations were introduced into a human P2X4 receptor plasmid and expressed in HEK-293 cells to ascertain whether these mutations altered functional responses. We measured ATP-induced inward currents by whole cell patch clamp (Figure 1A) and found only the Tyr315>Cys mutation (rs28360472) to have a significant effect, reducing the amplitude of the P2X4 response to 10.9% of wild-type P2X4 receptors (n=4–8 cells; P<0.001). The mutations altering Ala6>Ser (rs1044249), Ile119>Val (rs28360470), and Ser242>Gly (rs256444) had no major effect (n=4–8 cells; Figure 1). As expected, all of the P2X4 receptor constructs displayed potentiation by the allosteric modulator ivermectin (data not shown). Cell surface expression of 315Cys-P2X4 receptors was reduced compared with wild-type P2X4 receptors, where mean fluorescent intensity was 62.7% of wild-type P2X4 receptors (please see Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org).

**Table 1. Characterisation of 4 Nonsynonymous SNPs in the Human P2X4 Receptor**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>10 μMol/L of ATP Peak Amplitude, pA±SEM</th>
<th>% Desensitization to 10 μMol/L of ATP*</th>
<th>ATP EC50†</th>
<th>BzATP EC50‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>111.1±11.1</td>
<td>30.3</td>
<td>7.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Ala6&gt;Ser</td>
<td>159.3±30.3</td>
<td>29.2</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>Ile119&gt;Val</td>
<td>101.3±8.4</td>
<td>30.2</td>
<td>6.8</td>
<td>ND</td>
</tr>
<tr>
<td>Ser242&gt;Gly</td>
<td>135.1±16.1</td>
<td>31.2</td>
<td>4.0</td>
<td>ND</td>
</tr>
<tr>
<td>Tyr315&gt;Cys</td>
<td>12.2±2.3§</td>
<td>20.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SNP indicates single nucleotide polymorphism; ND, not determined.

*Desensitization was measured as percentage decline over 4 s from the peak of the inward current as described.†EC50 value for ATP was determined from concentration-response curves generated from application of a single concentration of agonist per cell to eliminate any effect of desensitization.

‡EC50 value for BzATP was determined from concentration-response curves generated from application of a single concentration of agonist per cell to eliminate any effect of desensitization.

§P<0.01.

||P<0.05.
to an agonist binding domain in the P2X4 receptor; therefore, we used BzATP, a known partial agonist at P2X4 receptors, which also showed a right-shifted concentration-response curve and an increased EC$_{50}$ value in the 315Cys-P2X4 receptor compared with the wild-type P2X4 receptor (Figure 1C).

**Tyr315>Cys Polymorphism Affects P2X4 Receptor Ectodomain Structure**

P2X receptors have 10 conserved cysteine residues in the ectodomain, thought to stabilize the structure through 5 disulphide bonds. The Tyr315>Cys mutation in the P2X4 receptor introduces an additional cysteine residue into the ectodomain, which may destabilize the structure of the receptor and permit aberrant disulphide bonding contributing to the reduced maximal response. We mutated the Tyr315 residue to serine, which cannot form disulphide bonds, and this 315Ser-P2X4 mutant showed a similar right-shifted ATP concentration-response curve and higher EC$_{50}$ value (EC$_{50}$ ATP: 243 µmol/L) compared with the wild-type P2X4 receptor but did not show a reduced maximal response to ATP (Figure 2A and 2B). Cell surface expression of 315Ser-P2X4 receptors was similar to 315Cys-P2X4 receptors (please see Figure S2). Therefore, we suggest that there are 2 distinct effects with a mutation of Tyr315 to cysteine; mutation away from tyrosine affects agonist potency, and the introduction of an extra cysteine residue reduces the maximum response. We investigated this further by using a short treatment of the reducing agent dithiothreitol (DTT) to disrupt disulphide bonds and found that this rescued the maximum response in 315Cys-P2X4 receptors to a level similar to that of wild-type P2X4 and 315Ser-P2X4 receptors (Figure 2C and 2D). Similar DTT treatment did not affect peak current response to ATP for wild-type P2X4 receptors (data not shown), and DTT had no effect on P2X4 receptor surface expression (please see Figure S3).

**Inheritance of 1 Copy of rs28360472 Impairs P2X4 Receptor Responses**

We genotyped 200 to 430 normal white subjects from the greater Sydney area (Sydney cohort) at each of the 4 identified polymorphic positions. We found no subjects carrying the variant allele at rs1044249 or rs28360470, although these have been reported by others; rs25644 was found to have an allele frequency of 0.116 (n=268 subjects), and the loss-of-function SNP rs28360472 was found to have a frequency of 0.011 (n=430 subjects; Table 2). rs28360472 was found only in heterozygous dosage, and, therefore, to determine whether inheritance of 1 allele encoding Tyr315>Cys would have a significant functional effect, we performed cotransfection experiments in HEK-293 cells to mimic a heterozygous state. Coexpression reduced the ATP-induced inward current to 32% of wild-type P2X4 receptors alone (50.3±10.3 pA/pF for wild-type/P2X4 receptors alone; n=4–8 cells; P<0.01; Figure 3A and 3B). To determine whether this reduction in P2X4 response was also seen in native cells, we recorded ATP-induced inward cur-
rents from monocyte-derived macrophages isolated from wild-type P2X4 and 315Cys-heterozygous subjects. The mean current density for the P2X4 response in 315Cys heterozygote macrophages was 40% of the P2X4 response in wild-type macrophages (0.53±0.19 pA/pF; n=13 cells; 315Cys heterozygotes) compared with 1.32±0.13 pA/pF (n=15 cells, wild-type subjects; P<0.01; Figure 3C and 3D).

**Inheritance of rs28360472 (Tyr315>Cys) Is Associated With Increased Pulse Pressure**

A second cohort of 2874 subjects from the Victorian Family Heart Study13 was genotyped for rs28360472 to determine whether inheritance of this SNP was associated with pulse pressure. The variant allele was found to have an allele frequency of 0.014 in this cohort (Table 2) and was present in 89 heterozygotes and 1 homozygote. The variant allele was in Hardy-Weinberg equilibrium (P=0.42), and there was no evidence of population stratification (P=0.73). Using variance components models and the total association test, we found that rs28360472 was associated with a slightly higher systolic and slightly lower diastolic blood pressure and was associated with a greater pulse pressure (P=0.023). We estimated that 1 copy of the variant allele increased pulse pressure by 2.84 mm Hg (95% CI: 0.41–5.27; Table 3). Almost identical results were obtained for the Quantitative Trait Linkage Disequilibrium Test14 (P=0.025).

**Discussion**

In this study we present evidence that an uncommon genetic variant, rs28360472, encoding amino acid change Tyr315>Cys in the human P2X4 receptor gene reduces the receptor functional response and shows an association with increased pulse pressure in a large white cohort. These observations are consistent with the hypothesis that the loss-of-function variant depresses the normal endothelial vasodilatory mechanisms involving the P2X4 receptor and

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**Table 2. Frequencies of 4 Nonsynonymous SNPs in P2RX4**

<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Amino Acid Change</th>
<th>Minor Allele Frequency</th>
<th>No. of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1044249</td>
<td>Ala6&gt;Ser</td>
<td>0</td>
<td>200*</td>
</tr>
<tr>
<td>rs28360470</td>
<td>Ile119&gt;Val</td>
<td>0</td>
<td>200*</td>
</tr>
<tr>
<td>rs25644</td>
<td>Ser242&gt;Gly</td>
<td>0.116</td>
<td>268*</td>
</tr>
<tr>
<td>rs28360472</td>
<td>Tyr315&gt;Cys</td>
<td>0.011</td>
<td>430*</td>
</tr>
<tr>
<td>rs28360470</td>
<td>Tyr315&gt;Cys</td>
<td>0.014</td>
<td>2874†</td>
</tr>
</tbody>
</table>

dbSNP indicates Single Nucleotide Polymorphism database.

*Subjects were part of the Victorian Family Heart Study.

†Subjects were recruited from the greater Sydney area for genotyping.

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**Table 3. Blood Pressure Measurements Correlated With P2RX4 Genotype at rs28360472 for 2874 Subjects From The Victorian Family Heart Study**

<table>
<thead>
<tr>
<th>Variable</th>
<th>rs28360472</th>
<th>rs28360472 A/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>39.8±15.8</td>
<td>36.6±15.69</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>71.7±14.0</td>
<td>72.57±13.02</td>
</tr>
<tr>
<td>Height, mm</td>
<td>1690±93</td>
<td>1706.3±89.0</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>123.3±15.4</td>
<td>124.8±15.1</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>72.6±11.6</td>
<td>71.3±11.6</td>
</tr>
<tr>
<td>Pulse Pressure</td>
<td>50.7±12.0</td>
<td>53.5±12.5*</td>
</tr>
</tbody>
</table>

BP indicates blood pressure.

*Represents P<0.05.
The Tyr315>Cys mutation has 2 distinct effects on the ATP-induced human P2X4 receptor response. The first is a reduction in agonist potency, as demonstrated by an increase in EC50 values for the primary agonist ATP and the partial agonist BzATP. This confirms that Tyr315 contributes to the agonist binding site on the P2X4 receptor, as shown by several mutagenesis studies on rat P2X receptors19,24,25 and also by molecular modeling (Figure 4) based on the recently published crystal structure of the zebrafish P2X4 receptor.16

Our molecular modeling analysis of the human P2X4 receptor structure shows that Tyr315 contributes to a putative ligand-binding pocket. The molecular model of human P2X4 receptors was built by homology to zebra-fish P2X4 receptor (pdb code 3I5D16) with ATP computationally docked at the putative ligand-binding pocket. A. Surface rendering of the overall structure of the trimeric receptor model colored by subunit, oriented with the membrane-inserted domain at the bottom of the picture with the membrane normal to the plane of the page. ATP can be seen docked at the putative binding site at the interface between the green and the blue subunits. B. Enlargement of the ATP-binding pocket for the wild-type receptor with key residues labeled and colored by atom type. Tyr315 is shown as spheres with C atoms colored magenta. Docked ATP is shown as bonds, colored by atoms, with C atoms white. C. The same view as for B for the 315Cys-P2X4 mutant receptor with the 315Cys atom visible as a yellow surface patch. Docked ATP (with C atoms yellow) showed a different preferred docking pose relative to the wild-type P2X4 receptor.

The second effect of the Tyr315>Cys mutation is a reduction in the P2X4 receptor maximum response. This presence of an extra cysteine residue in the large ectodomain in addition to the conserved 10 cysteines important for P2X receptor structure may permit aberrant disulphide bond formation leading to a disruption in ion channel structure. This hypothesis is supported by experiments using the Tyr315>Ser P2X4 receptor mutant, which cannot form disulphide bonds and does not show a reduced maximum response, only a reduced agonist potency (Figure 2). In addition, experiments with the reducing agent DTT could rescue the reduction in 315Cys-P2X4 maximum response apparently by rescuing nonfunctional receptors at the cell surface rather than by increasing cell surface expression. The simplest explanation of these data would be that aberrant disulphides involving 315Cys render some P2X4 receptors nonfunctional, but this is reversible with reduction by DTT.

Genotyping of 2 groups of white subjects revealed that rs28360472 (Tyr315>Cys) in P2RX4 was found in only 9 of 430 subjects in the Sydney cohort (minor allele frequency: 0.011; Table 2) and in 90 of 2874 subjects in the Victorian cohort (minor allele frequency: 0.014, Table 2). The loss-of-function polymorphism was predominantly found in heterozygous dosage; therefore, to mimic heterozygous inheritance of the Tyr315>Cys mutation, we cotransfected HEK cells with a mixture of wild-type P2X4 and 315Cys-P2X4 receptor plasmids. This reduced the P2X4 response to 40% of wild-type, suggesting that this functional defect would not be fully rescued by the presence of a wild-type P2X4 receptor allele. This reduction was also replicated in monocyte-derived macrophages from 315Cys-P2X4 heterozygote subjects.

Our previous studies have identified a modest but significant genetic contribution to pulse pressure in the Victorian Family Heart Study.26 Recent genetic analyses of population blood pressures have revealed the existence of common (population frequency: >10%) alleles of small effect (<1 mm Hg)27 and rare alleles (population frequencies: <0.1%) of modest effect (±6 mm Hg).28 The former are typically in noncoding regions of DNA, whereas the latter are generally in amino acid coding sequences. Interest-
ingly, rs28360472, encoding the Tyr315>Cys amino acid mutation, falls somewhere between the common and rare alleles in terms of expected frequencies and estimated effect sizes.

Pulse pressure represents the amplitude of the arterial pressure waveform and is recognized as a risk factor for cardiovascular disease, independent of systolic and diastolic blood pressures, per se.\textsuperscript{20} Pulse pressure is determined by the compliance of large arteries, by the cardiac stroke volume, and by reflected pressure waves. The P2X4 receptor has an important role in determining large vessel tone through endothelial-dependent mechanisms, such that P2X4 receptor activation by ATP leads to endothelial cell NO release and arterial smooth muscle relaxation. Such reduced large arterial tone would increase vascular compliance, and, in the context of a dysfunctional 315Cys-P2X4 receptor, reduced endothelial NO release would favor decreased large arterial compliance and account for the higher pulse pressure seen in carriers of rs28360472.

\textbf{Perspectives}

Many of the genetic correlates of blood pressure have focused on renal mechanisms that control fluid and electrolyte balance. This study instead turns attention to vascular hemodynamic control mechanisms, as defined by the actions of ATP that are mediated by the P2X4 receptor. The frequency of rs28360472 and the estimated magnitude of the inherited functional effect distinguish themselves from the often-reported common variants of small effect and the more rare mutations of moderate effect. These observations will stimulate further investigations into the roles of ATP and P2X4 receptors in blood pressure and vascular homeostasis and a more detailed physiological study into the mechanisms by which pulse pressure and its underlying phenotypes might be altered by the dysfunctional 315Cys-P2X4 receptor.

\textbf{Acknowledgments}

We thank Angela Lamantia for her technical assistance with DNA extraction and genotyping. We acknowledge the assistance of the Murdoch Children’s Research Institute Sequenom Platform Facility within which the Victorian Family Heart Study genotyping was carried out.

\textbf{Sources of Funding}

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\textbf{Disclosures}

None.

\textbf{References}


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A loss-of-function polymorphism in the human P2X4 receptor is associated with increased pulse pressure

Leanne Stokes¹, Katrina Scurrah², Justine A. Ellis¹,⁶, Brett A. Cromer⁴,⁵, Kristen K. Skarratt¹, Ben J. Gu¹,⁴, Stephen B. Harrap⁶, James S. Wiley¹,⁴.
Supplemental Methods

Subjects

In the Sydney cohort, 430 adult Caucasian subjects were genotyped to determine the allele frequency at rs28360472. These subjects included healthy volunteers and patients from hospital clinics not related to cardiovascular diseases. The study was approved by Sydney West Area Health Service HREC Nepean with informed written consent obtained from all participants. The mean age of this cohort was 56.9 ± 19.4 years, 58.4% were female and 41.4% were male subjects. To determine allele frequencies at rs1044249 (Ala6>Ser), rs28360470 (Ile119>Val), and rs25644 (Ser242>Gly) a subset of this cohort was used for genotyping (n=200-268 subjects).

Subjects from the Victorian Family Heart Study (VFHS) consisted of 2911 healthy adult volunteers recruited from the general population and comprised 767 families with 2 parents (40-70 years old) and at least one natural offspring (18-30 years old). Recruitment was limited to Caucasian families. The VFHS study was approved by the Ethics Review Committee of the Alfred Hospital, Melbourne with informed consent obtained from all participants.

Genotyping

In the Sydney cohort, rs1044249 and rs28360470 were genotyped using pre-designed Taqman assays (Applied Biosystems, Carlsbad, CA, USA) with Taqman Universal PCR Mastermix No AmpErase UNG (Applied Biosystems, Foster City, CA, USA) on a Rotogene 3000 (Corbett Research, Qiagen, Germany). Cycling parameters were 95 ºC for 10 minutes followed by 45 cycles of 92 ºC for 15 seconds and 60 ºC for 60 seconds. Internal controls included no DNA and positive control plasmid DNA for each of the mutations. rs25644 and rs28360472 were genotyped by homogeneous mass-extension or iPLEX® GOLD chemistry using Sequenom MassArray on an Autoflex Spectrometer at the Australian Genome Research Facility (St. Lucia, Queensland, Australia).

In the Victorian cohort, genotyping of rs28360472 was performed using iPLEX® Gold single base primer extension of multiplex PCR products using matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF) chemistry on an in-house Sequenom MassARRAY® platform. Primers used were as follows:
Forward: 5’-ACG TTG GAT GGT ACT ACA GAG ACC TGG CT-3’;
Reverse: 5’-ACG TTG GAT GAC ACA ATG ATG TCG AAG CGG-3’;
Extension: 5’-TGT CGA AGC GGA TGC CA -3’.
Raw data was analyzed using the Typer 4.0 Analyser Software (Sequenom, San Diego, California).

Blood pressure measurements

Blood pressure measurements on participants of the VFHS were taken carefully using a
calibrated mercury sphygmomanometer by trained observers. After 10 minutes of supine rest, three measurements of systolic blood pressure and diastolic blood pressure were taken in the supine position, the last two of which were recorded. Pulse pressure was calculated as the difference between systolic and diastolic pressure. For participants receiving antihypertensive treatments the recorded pressures were adjusted by previously validated methods\textsuperscript{1}.

**Cell culture and molecular biology**

HEK-293 cells were maintained in DMEM:F12 medium containing 10% foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO\textsubscript{2} in a humidified incubator. Plasmid DNA encoding an EGFP-tagged human P2X4 receptor was mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and constructs were verified by sequencing (SUPAMAC, University of Sydney, Australia).

Transfections were performed using 0.1µg plasmid DNA and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Transiently transfected cells were plated onto 13 mm glass coverslips 24 hours before patch clamp recordings were performed.

Peripheral venous blood was taken from healthy volunteers carrying no mutations in \textit{P2RX4} (wild-type) or carrying rs28360472 in heterozygous dosage. Mononuclear cells were isolated using Ficoll-Paque gradient centrifugation and monocytes were magnetically separated using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany)\textsuperscript{2}. Monocytes were cultured for 6 days in RPMI 1640 media containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 25ng/ml M-CSF (Peprotech Inc., Rocky Hill, NJ, USA) to induce differentiation to macrophages.

**Electrophysiology**

Whole-cell patch-clamp recordings were performed at room temperature using an EPC10 amplifier and Patchmaster software (HEKA, Lambrecht, Germany). Agonists were delivered using an RSC-160 fast-flow (Bio-Logic Science Instruments, France). Membrane potential was clamped at –60 mV in all experiments\textsuperscript{2}. External solution was 145 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl\textsubscript{2}, 1 mmol/L MgCl\textsubscript{2}, 13 mmol/L D-glucose, 10 mmol/L HEPES, and internal solution was 145 mmol/L NaCl, 10 mmol/L HEPES, 10 mmol/L EGTA. Both were pH 7.3 and 300-310 mosm/L. For DTT experiments, 1 mmol/L DTT was added to the bath solution for 15 minutes prior to recording ATP-induced inward currents.

P2X4 receptors undergo substantial desensitisation and rundown to repeated application of agonists therefore concentration-response curves were generated from normalised responses from individual cells exposed to a single concentration of ATP or BzATP. Concentration-response curves were generated using Kaleidagraph software (Synergy software, Redaing, PA, USA) and EC\textsubscript{50} values determined by the least-squares curve fitting to the Hill equation: \(I/I_{\text{max}} = 1/[1+ EC_{50}/[A]^nH]\) where \(I\) is the current as a fraction
of the maximum current (Imax), [A] is the agonist concentration and $n_H$ is the Hill coefficient.

**Immunostaining and flow cytometry**

Surface staining was performed on transfected HEK-293 cells using a rabbit anti-human P2X4 antibody (a gift from Dr F. Koch-Nolte, Germany) at 1:100 dilution in PBS containing 0.5% BSA. Cells were incubated on ice for 1 hour with primary antibody, washed and incubated with goat anti-rabbit IgG-Alexa 647 (Invitrogen, Carlsbad, CA, USA) for 30 minutes on ice. Cells were acquired using a BD FACSCalibur flow cytometer using two channels, excitation 485nm and emission 510nm for EGFP fluorescence and excitation 633nm and emission 668nm for Alexa 647 fluorescence.

**Modeling of ATP binding pocket in human P2X4 receptors**

A molecular model of the human P2X4 receptor was built by homology to the zebra-fish P2X4 receptor structure (pdb code 3I5D) using SwissModel and quality checked using Verify3D. AutoDock4 software was used to explore the feasible interactions of ATP with the model within a 20Å cube surrounding the putative ATP-binding pocket. The Lamarckian genetic algorithm was used to produce 100 docked conformations of ATP for the wild-type P2X4, 315Cys-P2X4, 315Ser-P2X4, and 315Ala-P2X4 receptor models. Docks were grouped in clusters within a root-mean-squared deviation (rmsd) of 2.0 Å. In each case the largest low energy cluster (ranging 12-20/100) was selected as the best docked pose.

**Statistical testing**

For functional experiments, data are presented as mean ± S.E.M. Statistical analysis was performed using t-tests or ANOVA where appropriate with Instat version 3 (GraphPad Software Inc., La Jolla, CA, USA).

For the VFHS cohort statistical association between genotype and pulse pressure was assessed using variance components modelling and the quantitative trait linkage disequilibrium (QTLD) approach. Six models were fitted and four pairwise comparisons were performed to test total association (measured genotype analysis), quantitative trait transmission disequilibrium, population stratification and quantitative trait linkage disequilibrium. All models accounted for within-family relatedness and were fitted using the software SOLAR. The models fitted in this study were simple correlation models which allowed a single variance component $\sigma^2$, and separate correlations for each relative type present in the data; $\rho_{SP}$, $\rho_{PO}$, $\rho_{SIB}$, $\rho_{DZ}$ and $\rho_{MZ}$, depending on whether the relative pair was a spouse, parent-offspring, sibling, dizygotic or monozygotic twin pair, as described previously.
Supplemental References

2. Stokes L, Fuller SJ, Sluyter R, Skarratt KK, Gu BJ, Wiley JS. Two haplotypes of the P2X7 receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1β secretion. *FASEB J*. 2010;24:2916-2927
**Figure S1: Reduced cell surface staining for 315Cys P2X4 receptors.**

(A) Cell surface expression of all human P2X4 constructs in HEK-293 cells was determined using a rabbit anti-P2X4 antibody followed by a secondary anti-rabbit IgG Alexa647 antibody. Fluorescence was measured by flow cytometry and histograms shown are representative of three separate transfection experiments. (B) Overlay shows reduced mean fluorescence intensity for 315Cys-P2X4 compared to wild-type P2X4 receptors. (47.9 ± 5.5 vs 76.4 ± 6.8 arbitrary fluorescence units for 315Cys-P2X4 and wild-type P2X4 receptors respectively, \( P = 0.03 \)).
Figure S2: Surface expression of 315Ser-P2X4 receptors.
Cell surface expression of 315Ser-P2X4 receptors (yellow) was compared to wild-type P2X4 receptors (black). Cells were stained with a rabbit anti-P2X4 antibody followed by a secondary anti-rabbit IgG Alexa647 antibody. Negative control was rabbit IgG and is shown grey shaded. Fluorescence was measured by flow cytometry using a BD FacsCalibur. Histograms shown are representative of three separate transfection experiments. Mean fluorescence intensity was calculated using CellQuest software.
**Figure S3: DTT does not rescue surface expression of 315Cys-P2X4 receptors.** Cell surface expression of wildtype P2X4 (black), 315Cys-P2X4 (purple) and 315Ser-P2X4 receptors (yellow) was determined following treatment with DTT (1mM for 15 minutes). Negative control was rabbit IgG and is shown as grey shaded histogram. Staining was performed using a rabbit anti-P2X4 antibody followed by a secondary anti-rabbit IgG Alexa647 antibody. Fluorescence was measured by flow cytometry using a BD FacsCalibur and histograms shown are representative of three separate transfection experiments.