C2238 Atrial Natriuretic Peptide Molecular Variant Is Associated With Endothelial Damage and Dysfunction Through Natriuretic Peptide Receptor C Signaling

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Rationale: **C2238 atrial natriuretic peptide (ANP) minor allele (substitution of thymidine with cytosine in position 2238) associates with increased risk of cardiovascular events.**

Objective: **We investigated the mechanisms underlying the vascular effects of C2238-αANP.**

Methods and Results: **In vitro, human umbilical vein endothelial cell were exposed to either wild-type (T2238)- or mutant (C2238)-αANP. Cell survival and apoptosis were tested by Trypan blue, annexin V, and cleaved caspase-3 assays. C2238-αANP significantly reduced human umbilical vein endothelial cell survival and increased apoptosis. In addition, C2238-αANP reduced endothelial tube formation, as assessed by matrigel. C2238-αANP did not differentially modulate natriuretic peptide receptor (NPR)-A/B activity with respect to T2238-αANP, as evaluated by intracellular cGMP levels. In contrast, C2238-αANP, but not T2238-αANP, markedly reduced intracellular cAMP levels in an NPR-C–dependent manner. Accordingly, C2238-αANP showed higher affinity binding to NPR-C, than T2238-αANP. Either *NPR-C* inhibition by antisense oligonucleotide or NPR-C gene silencing by small interfering RNA rescued survival and tube formation of human umbilical vein endothelial cell exposed to C2238-αANP. Similar data were obtained in human aortic endothelial cell with NPR-C knockdown. NPR-C activation by C2238-αANP inhibited the protein kinase A/Akt1 pathway and increased reactive oxygen species. Adenovirus-mediated Akt1 reactivation rescued the detrimental effects of C2238-αANP. Overall, these data indicate that C2238-αANP affects endothelial cell integrity through NPR-C–dependent inhibition of the cAMP/protein kinase A/Akt1 pathway and increased reactive oxygen species production. Accordingly, C2238-αANP caused impairment of acetylcholine-dependent vasorelaxation ex vivo, which was rescued by NPR-C pharmacological inhibition. Finally, subjects carrying C2238 minor allele showed early endothelial dysfunction, which highlights the clinical relevance of our results.**

Conclusions: **C2238-αANP reduces endothelial cell survival and impairs endothelial function through NPR-C signaling. NPR-C targeting represents a potential strategy to reduce cardiovascular risk in C2238 minor-allele carriers. (*Circ Res*. 2013;112:1355-1364.)**

Key Words: Akt ■ atrial natriuretic peptide ■ endothelial dysfunction ■ natriuretic peptide receptor type C ■ T2238C gene variant

Atrial natriuretic peptide (ANP), a cardiovascular hormone with natriuretic, diuretic, and vasodilator activity,[1] contributes to the risk of cardiovascular events depending on either abnormal circulating concentrations or peptide structural alterations.[2] In the latter regard, the molecular variant of the *prepro-ANP* gene characterized by the substitution of thymidine with cytosine in position 2238 affects the incidence of cardiovascular events in different human populations.[2–6] We recently reported a significant

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Nonstandard Abbreviations and Acronyms

ANP	atrial natriuretic peptide
cANF(4–23)	des[Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}]ANP$^{4-23}$-NH$_2$ (deleted derivative of ANP)
CNP	C-type natriuretic peptide
HAEC	human aortic endothelial cell
HUVEC	human umbilical vein endothelial cell
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
NPR	natriuretic peptide receptor
PKA	protein kinase A
PTX	pertussis toxin
ROS	reactive oxygen species

increase in the incidence of acute coronary syndromes and cardiovascular mortality in subjects carrying the C2238 minor allele (hereafter C2238-MA) from 2 European cohorts of stable angina patients.[6] Furthermore, in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) population, despite high genetic heterogeneity, an association of C2238-MA with the incidence of stroke was observed in a univariate analysis.[7] Diuretic treatment led to optimal blood pressure control and a significant reduction of cardiovascular risk in hypertensive patients carrying C2238-MA, thus suggesting an impaired diuretic effect of this ANP variant.[7] We have demonstrated previously that C2238-αANP inhibits endothelial cell proliferation and tube formation through increased reactive oxygen species (ROS) production in vitro.[8] Taken together, these findings strongly indicate that the C2238-MA carrier status represents a novel genetic cardiovascular risk factor. Of note, C2238-MA frequency ranges from 13% to 23% in the general population, which indicates the large impact that this variant may have. However, the molecular mechanisms underlying the vascular effects of C2238-αANP are currently unknown.

ANP binds preferentially to natriuretic peptide receptor (NPR)-A and NPR-C, whereas NPR-B is mainly bound by C-type natriuretic peptide (CNP), which can also bind NPR-C. NPR-A mediates the ANP-dependent natriuretic, vasorelaxant, and antihypertrophic effects,[1] whereas NPR-B modulates cardiomyocyte growth.[1] Although NPR-C is believed to be mainly a clearance receptor, increasing evidence indicates that NPR-C signaling plays an active role in the regulation of cellular processes during both physiological and pathological conditions.[9] To date, it is not known which receptor mediates the detrimental effects of C2238-αANP.

The aim of the present study was to dissect the molecular mechanisms underlying the vascular effects exerted by C2238-αANP. Here, we provide novel evidence that NPR-C mediates the detrimental actions induced by C2238-αANP in endothelial cells. We consider our results to be clinically relevant because they suggest a novel therapeutic strategy to reduce cardiovascular risk in C2238-MA carriers.

Methods

In Vitro Studies

Commercially available human umbilical vein endothelial cell (HUVEC; Cambrex) and human aortic endothelial cell (HAEC; Lonza) were used within 4 passages for in vitro experiments, and they were cultured in endothelial growth medium-2. Synthetic wild-type (T2238)- and mutant (C2238)-αANP were purchased from Primm. Cell viability and apoptosis were assessed by Trypan blue, annexin V staining (Roche), and immunoblot of cleaved caspase-3. cGMP and cAMP measurements were performed with specific enzyme immunoassay kits (Biotrak System, Amersham), as per the manufacturer's instructions. C2238- and T2238-αANP binding affinity at NPR-C was assessed through the evaluation of their ability to displace biotinylated cANF$^{4-23}$ from the NPR-C by fluorescence-activated cell sorter analysis. cANF$^{4-23}$ is a specific ligand for NPR-C. In addition, potential interaction energies of the binding of T2238-αANP, C2238-αANP, and CNP with NPR-C were computed. Phospholipase C activity was evaluated by phosphoinositide hydrolysis assay. HUVEC tube formation was studied with matrigel (BD Biosciences), as previously reported.[8] NPR-C was inhibited in vitro by antisense oligonucleotide, purchased from Life Technologies and used as per the conditions described by Palaparti et al.[10] Alternatively, *NPR-C* gene silencing with small interfering RNAs (Sigma) was performed. Pertussis toxin (PTX) was used to inhibit Giα protein, whereas forskolin was used to activate adenylate cyclase. Protein kinase A (PKA) was inhibited by H89, whereas nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase was inhibited by apocynin; all reagents were purchased from Sigma Aldrich. Adenovirus expressing constitutively active Akt1 was used to activate Akt1. Adenovirus expressing β-galactosidase was used as control. Recombinant adenovirus vectors were constructed, propagated, and titered as previously reported.[11]

Ex Vivo and In Vivo Studies

Rat isolated mesenteric resistance arteries were used to test the effect of C2238-αANP on endothelium-dependent and -independent vascular responses in the presence and absence of selective pharmacological NPR-C blockade by M372049, as previously described.[9] Flow-mediated dilation was measured, according to recent guidelines,[12] in 3 groups of young healthy subjects, matched for age and sex and free of cardiovascular risk factors: subjects homozygous for the T2238 wild-type allele (controls, n=10), subjects homozygous for the C2238-MA (n=10), and heterozygous TC2238 subjects (n=15). Each subject gave a written informed consent to participate to the study.

Statistical Analysis

Continuous variables are expressed as mean±SEM. Comparisons between 2 groups were performed using Student *t* test. When the analysis was adjusted for the multiplicity of compared groups, 1-way ANOVA followed by Bonferroni post hoc test was performed. Normality of variable distribution was tested by Kolmogorov-Smirnov test. GraphPad Software (San Diego, CA, version 5.0) and SPSS statistical software (SPSS Inc, Chicago, IL, version 12.0) were used for statistical analysis. A $P<0.05$ was considered significant. An expanded version of the Methods section is available in the online Data Supplement file.

Results

C2238-αANP Is Associated With Increased Endothelial Cell Death and Apoptosis

We hypothesized that C2238-αANP reduces HUVEC survival. After 48 hours, both 10^{-11} mol/L and 10^{-9} mol/L C2238-αANP concentrations were associated with a significant reduction of HUVEC number (Figure 1A; Online Figure IA) as compared with both control and T2238-αANP, thus indicating that C2238-αANP may either affect proliferation or survival of HUVEC in vitro. C2238-αANP was also associated with a significant increase in cell death (Figure 1B; Online Figure IB and IC). In contrast, T2238-αANP did not affect HUVEC survival (Figure 1A and 1B). Apoptosis was also increased in HUVEC exposed to C2238-αANP (Figure 1C and 1D). Therefore, C2238-αANP directly reduces HUVEC survival and increases apoptosis in vitro.

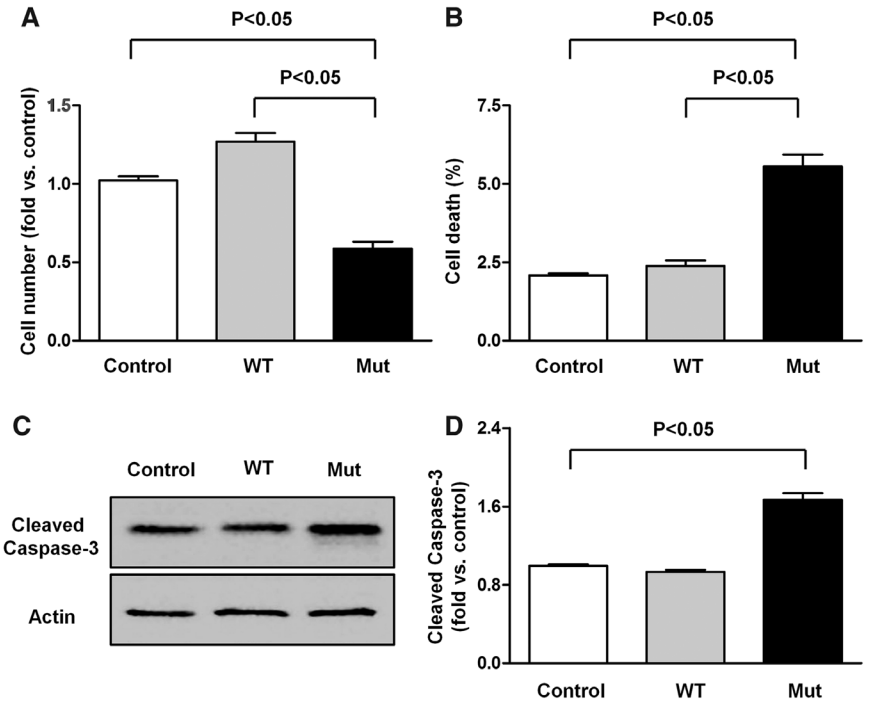

Figure 1. C2238-α atrial natriuretic peptide (ANP) affects endothelial cell survival in vitro. A and **B**, Human umbilical vein endothelial cell number (**A**) and cell death, as evaluated by Trypan blue assay (**B**), were assessed 48 hours after incubation with normal medium, with T2238-αANP, or with C2238-αANP. n=5. **C** and **D**, Cleaved caspase-3 accumulation was evaluated after exposure to C2238-αANP or T2238-αANP. Representative blot (**C**) and densitometric analysis (**D**) are presented. n=4 to 7. Control, cells cultured with normal medium; wild type (WT), T2238-αANP; and Mut, C2238-αANP.

C2238-αANP Is Associated With Endothelial Cell Impairment Through Stimulation of NPR-C and Inhibition of the Adenylate Cyclase/cAMP System

We investigated which NPR mediates the detrimental effects of C2238-αANP. NPR-A and NPR-B are coupled to the guanylate cyclase/cGMP system, thereby increasing cGMP intracellular levels on activation.[1] Increasing concentrations of T2238-αANP and C2238-αANP similarly increased intracellular levels of cGMP (Figure 2A). This result excludes that C2238-αANP differentially modulates NPR-A and -B, further confirming our previous observations.[8] In contrast, we found a significant progressive reduction of intracellular cAMP levels on short (30 minutes) stimulation with increasing concentrations of C2238-αANP, as compared with both control and T2238-αANP. The latter stimulated cAMP levels (Figure 2B), consistently with previous evidence.[13] This observation indicates an activation of NPR-C by C2238-αANP, because active NPR-C leads to reduction of cAMP intracellular levels through activation of Giα and inhibition of adenylate cyclase.[14] Remarkably, inhibition of NPR-C (Online Figure II), as well as inhibition of Giα protein by PTX, significantly restored cAMP intracellular concentration in HUVEC exposed to C2238-αANP (Figure 2C). Finally, our binding assays revealed that C2238-αANP displaced cANF,[4–23] which selectively binds NPR-C, more strongly than T2238-αANP (Figure 2D–2F), as indicated by lower dissociation constant (0.36±0.12 nmol/L for C2238-αANP versus 0.96±0.23 nmol/L for T2238-αANP, $P<0.01$) and higher B max (8605±387 sites per cell/nmol/L for C2238-αANP versus 7825±660 sites per cell/nmol/L for T2238-αANP, $P<0.01$). No cANF$^{4–23}$ displacement from NPR-C was observed with insulin coincubation, which was used as negative control. Consistent results were obtained through in silico computational analysis comparing the potential interaction energies of the binding between T2238-αANP or C2238-αANP or CNP and NPR-C (Online Table I), which showed that C2238-αANP has the highest affinity for the receptor. Overall, these data indicate that C2238-αANP significantly activates NPR-C, and it binds NPR-C with a higher affinity than T2238-αANP. Of note, no significant changes in the activity of phospholipase C were observed on HUVEC exposure to either C2238-αANP or T2238-αANP (T2238-αANP, 1.8±0.8; C2238-αANP, 1.9±0.7 fold versus baseline; P=NS), thus suggesting no changes in Gβγ subunit activity.

We hypothesized that C2238-αANP exerts its detrimental vascular effects through NPR-C signaling. Consistently, NPR-C inhibition abolished the effects of C2238-αANP on HUVEC number (Figure 3A) and death (Figure 3B) without affecting survival at baseline (data not shown). NPR-C knockdown by NPR-C–targeted small interfering RNA also restored viability and reduced cell death in HUVEC exposed to C2238-αANP (Online Figure III). Similar results were observed after PTX treatment (Online Figure IVA). Reactivation of adenylate cyclase abolished the detrimental effects of C2238-αANP on HUVEC survival (Figure 3A and 3B). The reduction of vasculogenic properties of HUVEC by C2238-αANP was rescued by NPR-C inhibition and by restoration of cAMP levels (Figure 3C and 3D), which did not show any effect on tube formation at baseline (data not shown). Similar effects were observed after PTX treatment (Online Figure IVB). We also validated our results in another cellular model. We found that C2238-αANP reduced HAEC number, increased HAEC death, and impaired HAEC vascular tube formation. All these detrimental effects induced by C2238-αANP were prevented by NPR-C knockdown (Online Figure V).

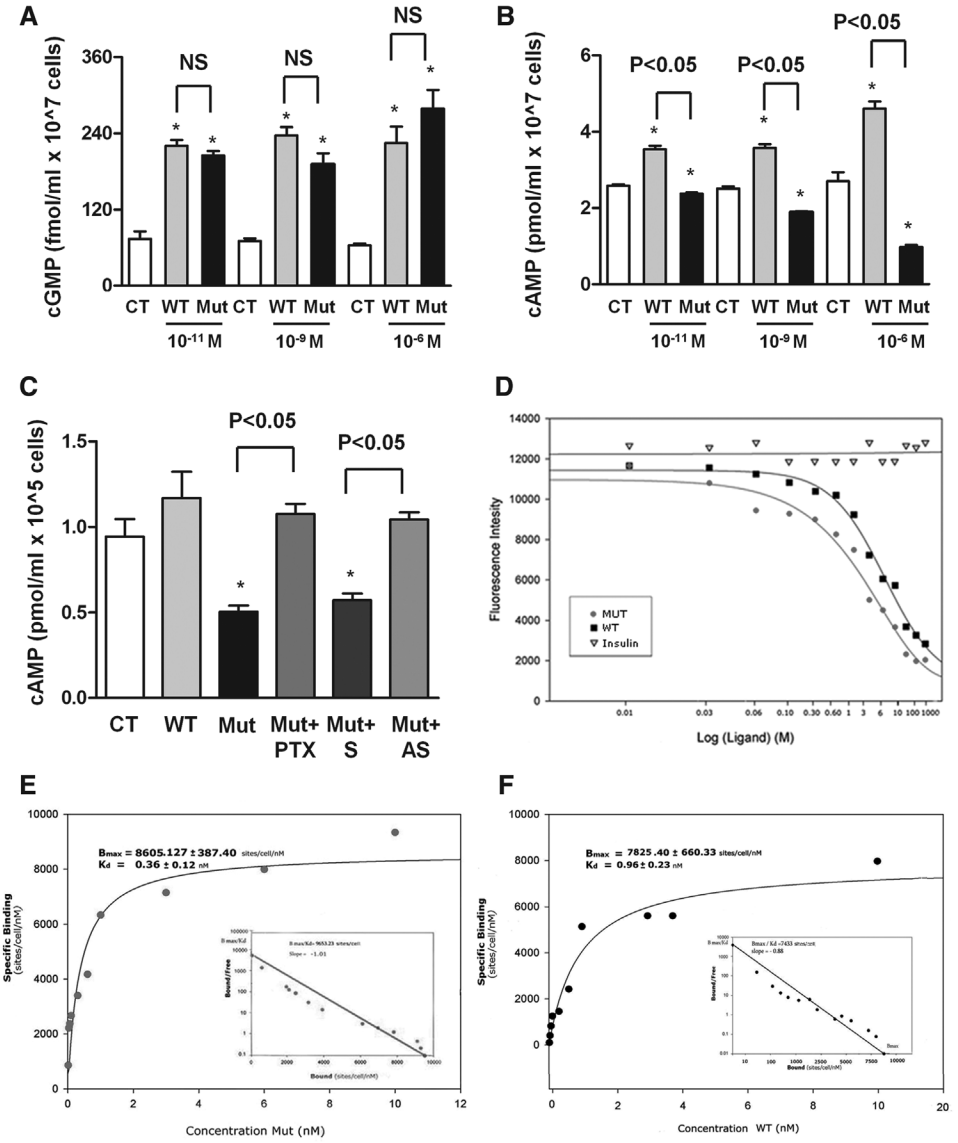

Figure 2. C2238-α atrial natriuretic peptide (ANP) differentially modulates natriuretic peptide receptor (NPR)-C. A and **B**, After 30 minutes. of incubation with ANP at 10^{-11}, 10^{-9}, 10^{-6} mol/L, intracellular concentrations of cGMP (**A**) and cAMP (**B**) were assessed. **P*<0.05 vs regular medium (CT). n=6. **C**, Human umbilical vein endothelial cell (HUVEC) were incubated with normal medium, T2238-αANP (wild type [WT]), or C2238-αANP (Mut), not pretreated or pretreated with NPR-C sense (S), antisense (AS) oligonucleotides or pertussis toxin (PTX; 50 ng/mL). Intracellular cAMP concentration was calculated after 30 minutes of exposure to WT and Mut. **P*<0.05 vs CT. n=6. **D**, HUVEC were incubated with 0.01 nmol/L of biotinylated ANF^{4-23} together with varying concentrations of nonbiotinylated C2238-αANP (closed red circles), T2238-αANP (closed black square) and insulin (closed white triangles). Mean fluorescence intensity at indicated concentrations of nonbiotinylated ligands was analyzed by fluorescence-activated cell sorter. Fluorescence intensity is represented against logarithmic concentration of biotinylated ligand ANF^{4-23}. Saturation curve and Scatchard analysis of the C2238-αANP (**E**) and T2238-αANP (**F**) binding to the NPR-C receptor. Saturation curve was obtained at varying concentrations of nonbiotinylated C2238-αANP and T2238-αANP. Kd value (0.36±0.12 nmol/L for C2238-αANP vs 0.96±0.23 nmol/L for T2238-αANP, *P*<0.01) and B max value (8605±387 sites per cell/nmol/L for C2238-αANP vs 7825±660 sites per cell/nmol/L for T2238-αANP, *P*<0.01) are presented as mean±SD. n=4 to 8.

Activation of the NPR-C/Giα/cAMP System by C2238-αANP Affects Endothelial Cell Survival and Angiogenetic Properties Through Inhibition of Akt Signaling and an Increase in Oxidative Stress

We hypothesized that NPR-C activation by C2238-αANP may result in inhibition of Akt1 through inhibition of the cAMP/PKA axis, which positively regulates Akt1. We found that C2238-αANP significantly reduced Akt phosphorylation, a recognized marker of Akt activity (Figure 4A). Interestingly, Akt phosphorylation was significantly restored by both NPR-C inhibition and adenylate cyclase activation (Figure 4A and 4B, Online FigureVI). Remarkably, adenylate cyclase activation failed to activate Akt in the presence of PKA inhibition (Figure 4B, Online Figure VI). Accordingly, levels of Akt-dependent phosphorylation of endothelial nitric oxide synthase and Bad (a Bcl-2–related family member) were decreased in HUVEC exposed to C2238-αANP, further indicating inhibition of Akt signaling and suggesting the involvement of Akt inhibition in

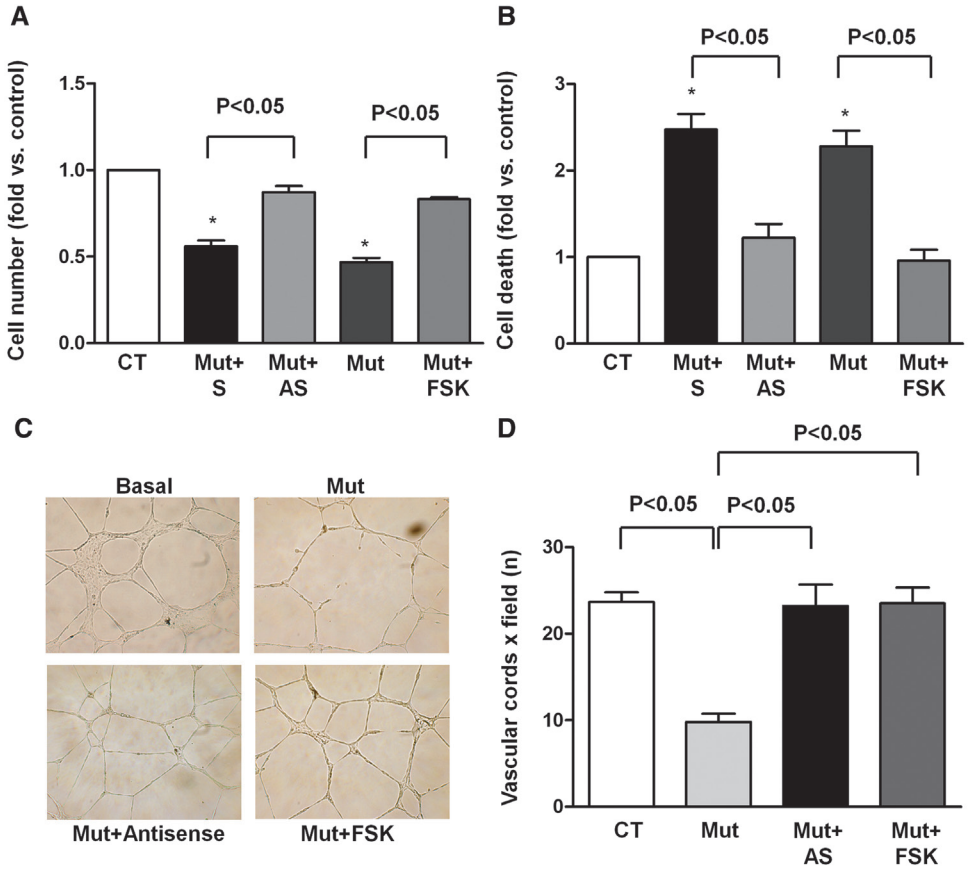

Figure 3. C2238-α atrial natriuretic peptide (ANP) affects endothelial cell survival and vascular cord formation through natriuretic peptide receptor (NPR)-C activation and reduction of intracellular cAMP levels. A and **B**, Human umbilical vein endothelial cell (HUVEC) were incubated with or without NPR-C sense (S) or antisense (AS) oligonucleotides for 48 hours. HUVEC were also treated with C2238-αANP (Mut) or control (normal medium, CT) during the final 12 hours of NPR-C inhibition, with or without forskolin (10 μmol/L), an activator of adenylate cyclase. HUVEC number and death were assessed 48 hours after the end of incubation. Results are shown as fold vs relevant control. n=5. In the same experimental conditions, vascular cord formation was evaluated at the end of peptide exposure. Representative images at magnification ×10 (**C**) and vascular cord number × field quantification (**D**) are presented. *$P<0.05$ vs relevant control. n=4.

the reduced survival and function and increased apoptosis of HUVEC exposed to C2238-αANP (Figure 4C).

To test the hypothesis that inhibition of Akt1, a master regulator of cell survival, by C2238-αANP is involved in the detrimental effects exerted by C2238-αANP, we reactivated Akt1 in HUVEC in the presence of C2238-αANP (Online Figure VII), as described in the Methods section. C2238-αANP increased cell death and inhibited vascular cord formation in control HUVEC, but failed to exert its detrimental effects in HUVEC expressing a constitutively active Akt1 (Figure 4D–4F).

We have previously shown that C2238-αANP promotes NADPH-dependent ROS production in HUVEC.[8] We evaluated whether NPR-C is involved in these effects. NADPH oxidase-dependent increase in HUVEC oxidative stress was blunted by NPR-C inhibition (Figure 5A). Basal expression level of gp91 phox subunit of NADPH was also reduced by NPR-C inhibition (Figure 5B). Moreover, inhibition of NADPH oxidase partially inhibited cell death induced by C2238-αANP (Figure 5C). However, apocynin failed to significantly rescue Akt inhibition induced by C2238-αANP (Figure 5D). These results suggest that NPR-C–dependent ROS production also contributes to the detrimental vascular effects exerted by C2238-αANP independently of Akt1 inhibition.

C2238-αANP Causes Endothelial Dysfunction In Vivo Through NPR-C–Dependent Mechanisms

We hypothesized that C2238-αANP affects endothelial function through NPR-C ex vivo. C2238-αANP induced blunted vasodilation of rat mesenteric vascular rings as compared with T2238-αANP (Figure 6A). C2238-αANP also impaired vasorelaxant responses to CNP, which is known to induce vasodilation through NPR-C activation[15] (Figure 6B). Furthermore, C2238-αANP impaired vascular responses to acetylcholine (Figure 6C). Selective pharmacological inhibition of NPR-C prevented the impairment of acetylcholine vascular responses in mesenteric arteries incubated with C2238-αANP (Figure 6D). In contrast, vascular responses to spermine–nitric oxide complex hydrate were preserved in the presence of C2238-αANP (Online Figure VIII). These data indicate that C2238-αANP induces endothelial dysfunction ex vivo through NPR-C–dependent mechanisms.

Importantly, we found that C2238-αANP induces endothelial dysfunction in humans. In fact, in a group of

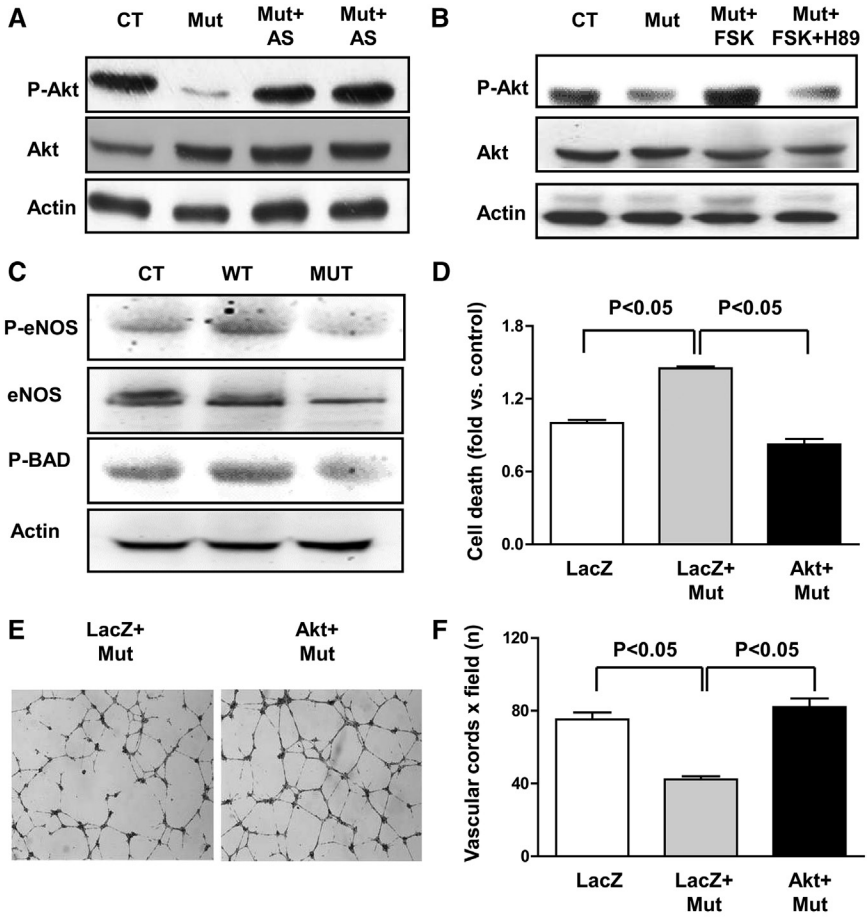

Figure 4. C2238-α atrial natriuretic peptide (ANP) affects endothelial cell survival and vascular cord formation through natriuretic peptide receptor (NPR)-C/cAMP/protein kinase A (PKA)–dependent inhibition of Akt signaling. **A** and **B**, Phosphorylation status of Akt1 (serine 473) was evaluated in human umbilical vein endothelial cell (HUVEC) pretreated or not with NPR-C antisense (AS) oligonucleotide and then incubated with regular medium, or C2238-αANP (Mut) with or without foskolin (FSK) or FSK plus H89, a protein kinase A inhibitor. Densitometric analyses are shown in Online Figure IV. n=5. **C**, HUVEC were exposed to regular medium, T2238-αANP (wild type [WT]) or C2238-αANP (Mut), and phosphrorylation statuses of endothelial nitric oxide synthase (eNOS) and Bad were evaluated. Representative immunoblots are shown. **D**–**F**, HUVEC were transduced with an adenovirus (10 mois) overexpressing constitutively active Akt1 (Akt) or β-galactosidase (LacZ) for 48 hours. HUVEC were then treated with Mut or control (normal medium, CT) for 12 hours. Cell death (**D**) and vascular cord formation were evaluated (**E** and **F**). Representative images and relative vascular cord quantification are presented at magnification ×2.5. n=6.

young healthy subjects free of cardiovascular risk factors (Online Table II), C2238-MA carriers (both heterozygous and homozygous) presented a significant reduction in endothelium-dependent flow-mediated dilation as compared with controls (Figure 7A). No differences were observed in the endothelium-independent vasodilation (Figure 7B).

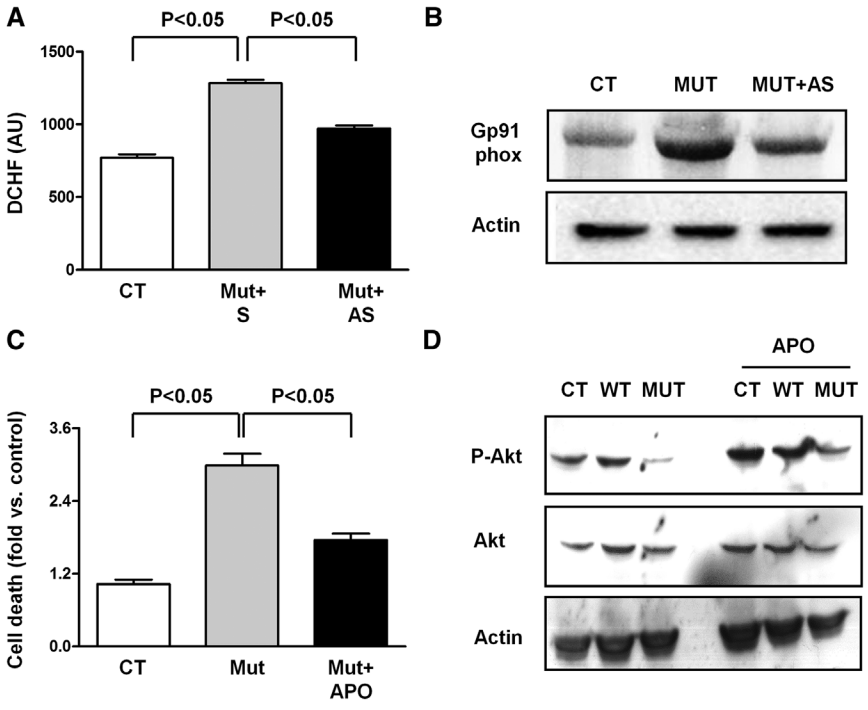

Figure 5. C2238-α atrial natriuretic peptide (ANP) induces natriuretic peptide receptor (NPR)-C–dependent oxidative stress. **A**, Reactive oxygen species production in human umbilical vein endothelial cell (HUVEC) exposed to regular medium (CT) or to C2238-αANP (Mut), pretreated with NPR-C sense (S) or antisense (AS) oligonucleotides. n=4. **B**, Expression levels of Gp91phox have been evaluated in HUVEC exposed to regular medium or C2238-αANP (Mut), with or without NPR-C AS. Representative immunoblot is presented. **C**, Cell death was assessed in HUVEC exposed to regular medium, Mut or Mut plus apocynin (APO) and expressed as fold vs CT. n=4. **D**, Phosphorylation status of Akt1 (serine 473) was evaluated in HUVEC exposed to regular medium, T2238-αANP (wild type [WT]) or C2238-αANP (Mut), with or without APO. Representative immunoblot is presented. DCHF indicates 5-(and-6)-Carboxy-2',7'-dichlorofluorescein ester.

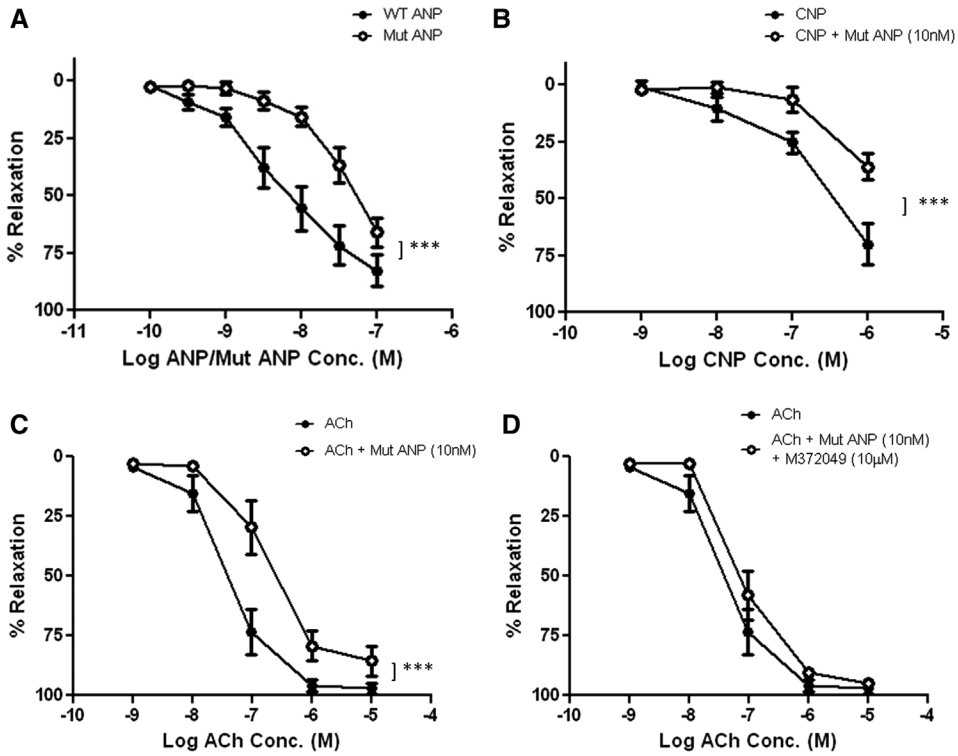

Figure 6. C2238-α atrial natriuretic peptide (ANP) induces endothelial dysfunction in vivo through natriuretic peptide receptor (NPR)-C–dependent mechanisms. A and **B**, Vasorelaxant responses of rat mesenteric arteries to T2238-αANP, C2238-αANP, C-type natriuretic peptide (CNP), or CNP plus C2238-αANP were evaluated. n=5. **C** and **D**, Vascular responses to acetylcholine were evaluated in mesenteric arteries exposed to C2238-αANP with or without M372049 (10 nmol/L). n=5. ****P*<0.05. M372049: NPR-C antagonist. WT indicates wild type.

Discussion

We demonstrate that a molecular variant of human ANP, C2238-αANP, reduces endothelial cell survival, increases apoptosis, and impairs endothelial cell growth in vitro. In addition, C2238-αANP inhibits endothelium-dependent vasorelaxation ex vivo and induces endothelial dysfunction in humans in vivo. This spectrum of detrimental cardiovascular actions is underpinned by inappropriate NPR-C/Giα signaling.

The frequency of the C2238-MA ranges from 13% to 23% in the general population. It does not affect circulating NT-proANP/ANP levels,[2,6] but rather it results in a structural alteration of the mature peptide by the addition of 2 arginines at the carboxy-terminal end of its primary structure. This ANP variant affects the incidence of cardiovascular events.[2–7] Notably, C2238-MA does not exert any effect on the development and progression of atherosclerosis,[6] whereas it increases the risk of plaque rupture, cerebrovascular events, acute coronary syndromes, and mortality.[4–7] In our recent study, C2238-MA was significantly associated with the risk of acute coronary syndrome and it significantly predicted the occurrence of major adverse cardiovascular events and mortality in patients with stable angina from 2 large European populations.[6]

Here, we demonstrate for the first time that C2238-αANP directly affects HUVEC survival and angiogenetic properties in vitro, and causes endothelial dysfunction in vivo through NPR-C–dependent mechanisms. It is known that ANP binds to all 3 types of NPRs with a significantly higher (>3 orders of magnitude) affinity for NPR-A than NPR-B.[1] These NPRs are coupled to the guanylate cyclase/cGMP system. In contrast, NPR-C, a clearance receptor of all natriuretic peptides, contains a 37-aa cytoplasmic domain with a Gα inhibitory-protein–activating sequence that negatively regulates the adenylate cyclase/cAMP system.[14] We found that NPR-A and NPR-B were not differentially modulated by C2238-αANP, thus corroborating our previous evidence.[8] T2238-αANP increased intracellular cAMP, consistent with recent evidence indicating that protein kinase G inhibits phosphodiesterase 3–mediated cAMP proteolysis.[13] In contrast, intracellular concentrations of cAMP were significantly reduced by C2238-αANP at all tested concentrations, consistent with NPR-C activation. Accordingly, reduction of cAMP levels on exposure to C2238-αANP was abrogated by inhibition of NPR-C, as well as by the Giα inhibitor PTX. Our competitive binding experiments also revealed an affinity binding of C2238-αANP to NPR-C significantly higher than that of T2238-αANP. The in silico binding studies also supported these observations. More importantly, either inhibition of NPR-C by AS oligonucleotide or NPR-C knockdown by RNA silencing rescued C2238-αANP–induced HUVEC death. Remarkably, we observed that C2238-αANP reduced survival and affected vasculogenic properties of HAEC. NPR-C knockdown was able to reverse the deleterious effects exerted by C2238-αANP also in these cells, thus extending our observations to a more clinically relevant endothelial cell type.

These findings provide striking evidence of the involvement of inappropriate activation of NPR-C in mediating the detrimental vascular effects of C2238-αANP, and indirectly support our previous work demonstrating that a preserved and physiological activity of NPR-C is required for normal vascular homeostasis.[9,15] Physiological activation of NPR-C by the endogenous ligand CNP is crucial for maintaining endothelial integrity and promoting cell proliferation.[9,15] In contrast, supraphysiological activation of NPR-C by cANF[4–23] has been shown to exert antigrowth properties in vascular smooth

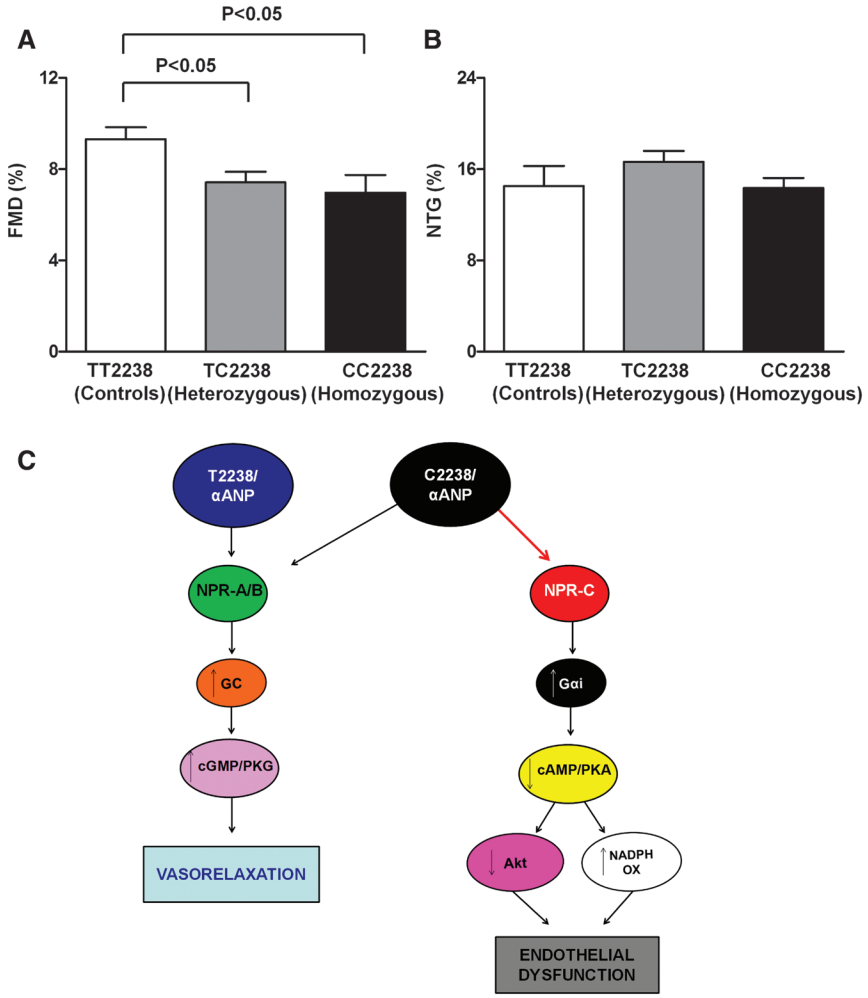

Figure 7. Subjects carrying C2238-MA display reduction of endothelial function. A and **B**, Flow-mediated dilation (FMD) and nitroglycerine (NTG)-induced arterial dilation were evaluated in heterozygous (TC2238) and homozygous (CC2238) for the C2238-α atrial natriuretic peptide (ANP) variant, as compared with control subjects not carrying the variant (TT2238). n=10 to 15. **C**, Schematic representation of mechanisms underlying the detrimental vascular effects of C2238-αANP. C2238-αANP activates natriuretic peptide receptor (NPR)-A and -B, similar to T2238-αANP, but it also exerts a deregulated agonist effect on NPR-C. NPR-C–deregulated activation inhibits the cAMP/Akt/protein kinase A (PKA) pathway and promotes nicotinamide adenine dinucleotide phosphate reduced form (NADPH)–dependent reactive oxygen species production, which are detrimental. However, T2238-αANP does not seem to significantly stimulate NPR-C in human umbilical vein endothelial cell. GC indicates guanylate cyclase; and PKG, protein kinase G.

muscle cells through inhibition of cAMP and Akt.[16,17] The remarkable difference in intracellular cAMP levels achieved after exposure of HUVEC to C2238-αANP versus wild-type T2238-αANP suggests that mutant ANP activates NPR-C in a unique, dysregulated manner that is detrimental. We speculate that the detrimental effects of NPR-C activation by C2238-αANP may be explained either by an excessive activation of the receptor or by an imbalance between NPR-C activation and activation of other NPRs. In addition, we propose that a deregulated activation of NPR-C by C2238-αANP may be explained either by its increased affinity for the receptor, or by unknown intrinsic properties of the peptide, which alter NPR-C conformation.

In fact, we demonstrate that C2238-αANP shows an increased affinity for NPR-C. In addition, our in silico calculations, showing higher potential interaction energy of the binding between C2238-αANP and NPR-C, with respect to the NPR-C binding with T2238-αANP or CNP, support the hypothesis that C2238-αANP activates NPR-C in a unique manner.

Our data suggest that deregulated activation of NPR-C by C2238-αANP is detrimental through inhibition of Akt1. cAMP positively regulates PKA, which in turn directly activates Akt signaling in endothelial cells.[18,19] Akt inhibition in endothelial cells is associated with endothelial damage and dysfunction.[20,21] We found that activation of NPR-C inhibited Akt signaling through inhibition of the cAMP/PKA axis. In addition, Akt reactivation was able to counteract the detrimental effects exerted on cell survival by C2238-αANP and rescue the angiogenetic properties of HUVEC.

We also observed that C2238-αANP exerts detrimental effects through NPR-C–dependent activation of NADPH oxidase and ROS production, independently of Akt1 inhibition. We speculate that the NPR-C–dependent activation of NADPH oxidase could be the consequence of lower cAMP levels, which have been previously shown to activate NADPH oxidase.[22] Of note, previous evidence indicated that NPR-C activation by cANF$^{4-23}$ reduces ammonium chloride–induced ROS production in astrocytes, thus suggesting that the effect of NPR-C activation on ROS accumulation is dependent on the level of receptor activation, on cell type, and on different cellular-stress conditions.[23]

C2238-αANP impaired acetylcholine-induced vascular responses in rat isolated resistance arteries, which was rescued by NPR-C pharmacological blockade. We have shown previously that CNP represents an endothelium-derived hyperpolarizing factor, and that many of its vasoprotective actions are mediated via activation of NPR-C.[9,24] Furthermore, NPR-C activation has been shown to reduce ROS production induced by angiotensin II.[25] Herein, we show that C2238-αANP

reduces vasorelaxant activity of both CNP and endothelium-derived hyperpolarizing factor. It is, therefore, likely that the deleterious effects induced by C2238-αANP are dependent, in these circumstances, on the antagonism exerted by C2238-αANP on the physiological activation of NPR-C by CNP.

We extended the in vitro functional observations to isolated vessels in human subjects. Flow-mediated dilation applied in the forearm, and used as an index of endothelial function, leads to a well-characterized and well-studied reactive hyperemia from the vascular endothelium, which seems to be significantly regulated by endothelium-derived hyperpolarizing factor, because subjects with a deficiency of tetrahydrobiopterin (an essential cofactor for synthesis of nitric oxide) have preserved flow-mediated dilation, which is relatively insensitive to nitric oxide synthase inhibitors.[26] In the present study, individuals carrying even 1 variant allele have a significantly reduced reactive hyperaemia (ie, endothelial function), as compared with subjects carrying the wild-type alleles. These data provide proof-of-concept evidence in the human vasculature that impairment of endothelium-derived hyperpolarizing factor bioactivity via NPR-C modulation partially underlies the endothelial dysfunction and associated increased risk of acute coronary events and stroke apparent in subjects carrying C2238-MA.[6] Of note, endothelial dysfunction is a known predictor of cardiovascular diseases, and its restoration is associated with a significant reduction of cardiovascular events.[27]

Conclusions

Our study provides the first clear-cut demonstration that C2238-αANP variant reduces endothelial cell survival and impairs endothelial function through binding to NPR-C and interference in the NPR-C/cAMP/Akt signaling pathway (Figure 7C).

Our in vitro and ex vivo results strongly suggest that pharmacological targeting of NPR-C represents a potential therapeutic strategy to reduce cardiovascular risk in C2238-MA carriers. Future clinical trials investigating this issue and translating our current results to human subjects are warranted.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Atrial natriuretic peptide (ANP) is a cardiovascular hormone that controls cardiorenal homeostasis mainly through the natriuretic peptide receptor (NPR) type A.
- NPR type C is a natriuretic peptide clearance receptor.
- In humans, a substitution of thymidine with cytosine in position 2238 of the ANP gene (with a frequency ranging from 13% to 23% in the general population), which alters ANP structure, is significantly associated with increased risk of cardiovascular events.

What New Information Does This Article Contribute?

- In comparison with wild-type ANP (T2238-αANP), ANP variant resulting from T2238C substitution (C2238-αANP) does not differently activate NPR type A, but binds to NPR type C with higher affinity and higher degree of activation.
- The C2238-αANP molecular variant reduces endothelial cell survival and impairs endothelial function through inappropriate activation of the NPR type C/Giα/cAMP signaling pathway.
- Healthy, young human subjects with low cardiovascular disease risk carrying the C2238-αANP variant display a significant reduction of endothelial-dependent vasorelaxation.

The common ANP molecular variant (C2238-αANP) is associated with higher rate of cardiovascular events, such as stroke and myocardial infarction, and it negatively affects the cardiovascular outcome in patients with coronary artery disease. The molecular mechanisms underlying the deleterious vascular impact of C2238-αANP are unknown. We studied the receptor and the signaling mechanisms through which C2238-αANP exerts its detrimental vascular effects. We found that C2238-αANP does not differently activate natriuretic peptide type A and B receptors, with respect to wild-type ANP, but binds to NPR type C with higher affinity and degree of activation. Importantly, we discovered that C2238-αANP reduces endothelial cell survival and vasculogenic properties in vitro, and it impairs vascular function ex vivo, through inappropriate activation of the NPR type C/Giα/cAMP pathway. NPR type C inhibition abrogates C2238-αANP–dependent detrimental vascular effects. Finally, we report that subjects carrying C2238-αANP, with low cardiovascular disease risk, show reduced endothelial function at an early age. These findings suggest that C2238-αANP behaves as a cardiovascular disease risk factor through NPR type C signaling. Therefore, selective pharmacological targeting of NPR type C may be a strategy to reduce cardiovascular risk in patients carrying C2238-αANP.

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C2238 Atrial Natriuretic Peptide Molecular Variant Is Associated With Endothelial Damage and Dysfunction Through Natriuretic Peptide Receptor C Signaling

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SUPPLEMENTAL MATERIAL

Materials and Methods

***In vitro* studies**

1. Effects of C2238-αANP on endothelial cell viability

a. Cell count by Trypan blue

Synthetic wild type (T2238) and mutant (C2238) αANP (Primm, Boston, MA, USA) were used as a stimulus for endothelial cells. Commercially available human umbilical vein endothelial cells (HUVEC; Cambrex, Ververs, Belgium) were used within four passages. The cells were seeded in 60-mm well plates (2 x 10^5 cells/well), cultured in endothelial growth medium-2 (EGM-2) for 24 hrs, and subsequently stimulated with either T2238 or C2238 αANP for 12 hrs in the presence of 10% fetal calf serum (FCS). ANP was tested at the concentrations of 10^{-11} M, defined as physiological, and 10^{-9} M, defined as therapeutical. However, since the endothelium actively produces ANP itself (1), the therapeutic concentration of ANP more likely mimics physiological condition. Control plates received αANP-free medium. Cell viability was assessed at 48 hrs after completion of either T2238 or C2238 αANP treatment. Viable and dead cells were counted using the Trypan blue exclusion method under an optical microscope.

b. Assessment of apoptosis induced by C2238- αANP by cleaved caspase-3 and by annexin staining.

Western blot for cleaved caspase-3. Protein cellular extracts were obtained at the end of peptide stimulation with C2238-αANP 10^{-9}M as previously reported (2). Western blotting was performed as previously described (2). The monoclonal anticleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA) was used at the concentration of 1 µg/ml in the presence of 5% BSA (bovine serum albumin, Sigma Aldrich, Milan, Italy). Following incubation with an antirabbit secondary antibody, signals were revealed with the enhanced chemiluminescence detection system (Ge Healthcare Life Science Europe, Milan, Italy) and visualized by autoradiography. β-actin was used as the housekeeping gene. Protein bands were finally scanned and quantified densitometrically.

Annexin-V staining. At the end of the cells exposure to 10^{-9} M C2238-αANP, HUVEC were washed twice with cold phosphate-buffered saline and then incubated with Annexin-V Biotin labeling solution (Roche, Milan, Italy) for 15 min. at room temperature. Subsequently, cells were incubated with streptavidin-conjugate staining solution (Alexa 488, Life Technologies, Monza, Italy) and DAPI (Prolong Gold, Life Technologies) for nuclear counterstaining at 4 °C, and then observed under a fluorescence microscope (Axiophot2, Carl Zeiss, Milan, Italy).

2. Identification of the receptor that mediates the effects of C2238-αANP

a. Measurement of cGMP and cAMP in cells exposed to wild-type or mutant ANP

HUVEC were seeded in six-well plates (2 x 10^5 cells/well) and cultured in EGM-2 for 24 hrs. At this time they were first starved for 2 hrs and then pre-treated for 30 min. with 0.1 mmol/L isobutylmethylxanthine (IBMX, Sigma Aldrich) before stimulation with the conditioned medium containing IBMX 0.1 mM and the specific αANP form at 10^{-11} M, 10^{-9} M, 10^{-6} M concentrations for 30 min. cGMP and cAMP measurements were performed with the specific enzymeimmunoassay Biotrak (EIA) System (Amersham), following the manufacturer's instructions.

b. Effects of C2238-αANP on endothelial cells under blockade with PTX

In an attempt to test the hypothesis that NPR-C could mediate the effects of C2238-αANP, PTX was used as a selective inhibitor of the Giα protein which is involved in NPR-C stimulation (3).

Cells were cultured as previously described. PTX (Sigma Aldrich) was used at a concentration of 50 ng/ml for 30 min. before overnight exposure to 10^{-9} M C2238-αANP. At the end of treatment, the following parameters were evaluated: cAMP measurement (as assessed above); cell viability (as assessed above); endothelial cell tube formation assay. For the latter purpose, 60 mm well plates (2x 10^5 cells/well) were

coated with 1 ml of Matrigel (BD Biosciences Europe, Buccinasco, Milan, Italy) and allowed to harden at 37°C for at least 30 min. At the end of treatment with PTX, HUVEC were removed by trypsin-EDTA and resuspended in growth media containing C2238-αANP. Cells treated only with C2238-αANP were used as control. Then, 2 ml of each solution was gently added to the corresponding plate and kept for 12 hrs. At the end of exposure, plates were photographed with a microscope fitted with a digital camera.

c. Effects of C2238-αANP on endothelial cells under NPRC inhibition with antisense oligonucleotide

Antisense, sense and missense oligonucleotides were purchased from Life Technologies. They were used following the conditions previously described by Palaparti et al. (4). First, to assess their efficacy, the protein level expression of NPR-C in the total protein extracts of HUVEC exposed to the three types of oligonucleotides was determined by western blotting with the use of a specific antibody (rabbit polyclonal, Abcam, Cambridge, UK). Based on the demonstration of a significant inhibition of NPR-C by this approach, the subsequent experiments were carried out by combining inhibition of NPR-C for 48 hrs with antisense oligonucleotide and treatment with 10^{-9} M C2238-αANP during the final 12 hrs of NPR-C inhibition. Missense and sense oligonucleotides were used as controls.
The following phenotypes were determined at the end of combined treatment with NPR-C/AS oligonucleotide and C2238-αANP: cAMP measurement (as assessed above); cell viability at 48 hrs (as assessed above); endothelial cell tube formation assay (as described above); oxidative stress production; gp91 phox subunit of NADPH protein expression [determined as previously reported (2)].
Oxidative stress was evaluated by applying the DCHF procedure, as previously described (2). Briefly, at the end of exposure of HUVEC to both AS oligonucleotide and C2238-αANP, cells were loaded with the permeable agent 5-(6)-carboxy-2'-7'-DCHF ester (Sigma Aldrich) for 30 min. Cells treated only with C2238-αANP were compared. In the presence of intracellular esterases, permeable DCHF ester is converted into its impermeable counterpart. The latter is reduced to the fluorescent dichlorofluorescein (DCF) by oxidants such as hydroxyl radicals. Therefore, oxidative stress can be quantified by monitoring intracellular DCF content using a fluorometer with excitation at 495 nm and emission at 525 nm. Fluorescence intensity was expressed as arbitrary units.

d. Modulation by forskolin of the effects of C2238-αANP in endothelial cells
Forskolin (Sigma Aldrich) was added at concentration of 10 μM when HUVEC were exposed to C2238-αANP peptide. Cell vitality and endothelial cell tube formation were determined, as reported above, at 12 hrs following peptide stimulation.

e. Evaluation of PLC activity under C2238-αANP exposure

Following exposure of HUVEC to either T2238-αANP or C2238-αANP, as reported above, PLC activity was measured by phosphoinositide hydrolysis assay as reported (5).

For this purpose, HUVEC were first incubated for 24 hrs with [*myo*-^{3}H]inositol (2 μCi/dish), followed by 10 min. incubation with 20 mM lithium to inhibit the conversion of inositol monophosphate (InsP) into free inositol. Then, either T2238 or C2238-αANP at a concentration of 10^{-9}M was added to the medium for 30 min. Cells were finally lysed, and cellular extracts were centrifuged at 10,000*g* for 20 min. The [^{3}H]InsP present in the supernatant was separated by anion exchange chromatography in 10-ml columns containing 1.5 ml of Dowex 1-X-8 resin (formate form, 100-200 mesh; Bio-Rad, Milan, Italy). Columns were washed twice with water, once with a solution of 5 mM sodium tetraborate and 40 mM sodium formate to elute cyclic InsP and glycerophosphoinositols, and then with 6.5 ml of 0.2 M ammonium formate and 0.1 M formic acid for the elution of InsP (5). Total radioactivity in the eluted cellular extracts was determined by counting through a beta counter, as reported (5).

f. *Evaluation of the effects of C2238-αANP in endothelial cells of arterial origin*

For this purpose, human aortic endothelial cells (HAEC, obtained from Lonza, Basel, Switzerland) were cultured in endothelial growth medium-2 (EGM-2) at 37°C in 95% O_2-5% CO_2. Cells were plated in 60 mm diameter dishes ($1.5x\ 10^{5}$), passaged upon reaching confluence with 2 ml of trypsin and used at the 5th passage for NPR-C gene silencing. In particular, once cells had reached 70% confluence, they were washed with phosphate-buffered-saline (PBS), and then OPTI-MEM-reduced serum medium (Invitrogen) was added

to the cells. Two NPR-C specific small interfering RNAs (Mission siRNA, Sigma Aldrich) and a nucleic acid transferring agent (RNA i MAX lipofectamine, Invitrogen) were incubated in OPTI-MEM reduced serum medium for 20 min. at room temperature to form a siRNA-lipofectamine complex. The siRNA-lipofectamine complex-containing medium was added to the cells to a final siRNA concentration of 50 nM. Five hours later, the complex-containing medium was replaced with EGM-2 supplemented with 10% FBS. Cells transfected with lipofectamine and nonsense siRNA (Sigma Aldrich) were used as controls. Twenty-four hours later, both silenced and not silenced cells were exposed overnight to *C2238-αANP* and subsequently analyzed for vitality by Trypan blue and for endothelial cell tube formation assay on Matrigel as described above.
HUVEC were used in parallel and compared to the HAEC under the same experimental protocol.

The efficiency of NPR-C gene silencing was verified by western blot, as reported above.

3. Characterization of the intracellular signaling pathway stimulated by C2238-αANP in endothelial cells

a. Evaluation of the role of Akt

In order to identify the main pathways involved in mediating the C2238-αANP effects in HUVEC, we characterized protein level expression of unphosphorylated and phosphorylated Akt after exposure to either T2238-αANP or C2238-αANP at a concentration of 10^{-9} M. Western blots were performed in total proteins of HUVEC using specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

An additional set of experiments was carried out to evaluate:

1) Akt phosphorylation during C2238-αANP exposure following treatment with forskolin at 10 μM concentration, as previously reported;

2) Akt phosphorylation following NPR-C inhibition with antisense oligonucleotide;

3) Akt phosphorylation during C2238-αANP exposure following treatment with apocynin at 200 μM concentration, as previously reported (2).

b. Effects of C2238-αANP in endothelial cells transfected with Akt

To investigate whether Akt inhibition is indeed responsible for the detrimental effects induced by C2238-αANP, we transduced HUVEC with an adenovirus overexpressing constitutively active Akt1 (ad-Akt) or beta-galactosidase (ad-LacZ) as control. Recombinant adenovirus vectors were constructed, propagated, and titered as previously described (6). Briefly, pBHGloxΔE1,3Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC316 shuttle vector containing the gene of interest into H293K cells using Lipofectamine 2000 (Life Technologies). Through homologous recombination, the test genes were integrated into the E1-deleted adenoviral genome. The viruses were then propagated in H293K cells. We made replication-defective human adenovirus type 5 (devoid of E1) harboring constitutively active Akt1 (Ad-Akt). Adenovirus harboring β-galactosidase (Ad-LacZ) was used as control. Then, HUVEC were transduced with 10 mois of Ad-LacZ or Ad-Akt for 48 hours, and they were stimulated with C2238-αANP for 12 hrs. At the end of stimulation, the following phenotypes were determined: cell viability; endothelial cell tube formation assay (as previously described).

c. Assessment of the role of PKA

Involvement of PKA was suggested by the modulation of forskolin on Akt phosphorylation under exposure to C2238-αANP. To test its involvement more deeply, PKA inhibitor (Dihydrochloride hydrate, H89, Sigma Aldrich) was used at the concentration of 15 μM in the presence of C2238-αANP and forskolin. Akt phosphorylation was determined at the end of stimulation by the above described procedure.

d. Binding assays

Experiments were performed according to previously published material (7). HUVEC were seeded in six-well plates (2 x 10^5 cells/well). For competition experiments cells were incubated at 4° C for 30 min. with

binding buffer (0.1% BSA/HANKS' balanced salt solution, Sigma) containing 0.1 pM biotinylated cANF(4-23) (Bachem) with varying concentrations (0.01 nM, 0.03 nM, 0.06 nM 0.10 nM, 0.30 nM, 0.60 nM, 1.0 nM, 3.0 nM, 6.0 nM, 10 nM) of either unlabeled T2238-αANP or unlabeled C2238-αANP. Insulin (Sigma Aldrich) was used as control. Cells were then washed twice with binding buffer, and incubated at 4° C for 30 min. with binding buffer containing streptavidin-fluorescein conjugated (Invitrogen). Cells were washed twice, and the fluorescence intensity of the samples was measured by a FACS C6 instrument (Accuri, Life Technologies). Binding affinity was calculated by using the GraphPad Prism software, which allowed estimation of Bmax and Kd values for each peptide.

e. In silico binding studies

Three complexes were prepared in silico, starting from the following crystallographic structures deposited in RCSB (http://www.rcsb.org): 1YK0 (NPR-C complexed with αANP), and 1JDP (NPR-C complexed with CNP22). Three different complexes were considered for comparative interaction energy calculations. A first complex between NPR-C and ANP was generated by completing the structure solved and stored in 1YK0. The unsolved ANP residues were added through the MOE (Molecular Operating Environment) builder: SLRRS amino acids were added at the ANP N-terminus and RY at the C-terminus (wild type T2238-αANP). A second complex of NPR-C with ANP-RR was generated by completing the 1YK0 structure: SLRRSRR residues were added instead of SLRRS at the C-terminus of ANP (to reproduce C2238-αANP). A third complex between NPR-C and CNP was available in 1JDP file. The unsolved amino acids of CNP were added through the MOE builder: GLSK residues were added at the N-terminus of CNP. All three complexes were then examined, prepared and completed through the MOE protein structure preparation for further computational analysis. The three systems were energy minimized until the root mean square (RMS) gradient fell below 10^{-5} kcal/mol/$Å^2$, in order to remove the steric clashes introduced during the completion of the ANP/CNP structures and to optimize the interactions in the complexes, removing bad geometries. The Amber12EH all-atom forcefield with the Generalized Born implicit solvation model were used both during energy minimization and single point potential energy calculations.

***In vivo* studies**

1. Effect of C2238-αANP on rat mesenteric artery endothelial function

Rat isolated mesenteric resistance arteries were used to test the effect of C2238-αANP on endothelium-dependent and independent vascular responses in the presence and absence of a selective pharmacological blockade of NPR-C. Previous studies have shown that NPR-C signaling represents a major component of the endothelium-dependent hyperpolarising factor (EDHF) pathway in these vessels (9); hence, they are a good functional model to investigate the role of NPR-C in mediating the effects of C2238-αANP.

Third order mesenteric arteries (200-250μm diameter) were collected from male Sprague-Dawley rats (~250g) culled by cervical dislocation. Vessels were dissected from the surrounding fat and mounted in an automated tension myograph (Danish Myotechnology, Denmark) containing Krebs solution (Composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.5 mM Glucose) and gassed with 95% O_2/5% CO_2. Vessels were allowed to equilibrate for 45 min prior to normalization using standard procedures. Subsequently, the arteries were contracted repeatedly with the thromboxane-A_2 mimetic U46619 (1 μM; Enzo Life Sciences, UK) until responses were reproducible. Following this, the vessels were washed with Krebs solution to restore basal tone before pre-contracting to approximately 50% of the maximum U46619 response. Once a stable contraction to U46619 was achieved, cumulative concentration-response curves to ANP (0.1 nM – 100 nM), C2238-αANP (0.1 nM – 100 nM), acetylcholine (ACh; 0.001 μM – 10 μM), spermine NONOate (SNO; 0.001 μM – 10 μM) and C-type natriuretic peptide (CNP; 0.001 μM – 1 μM; Genscript, UK) were constructed.

Vessels exposed to the endothelium-dependent vasodilator, ACh, were pre-treated with the nitric oxide synthase inhibitor, $_L$-NAME (300 μM), and the cyclooxygenase inhibitor, indomethacin (5 μM) for 30 min. prior to construction of the concentration-response curve. These inhibitors were used to abolish responses to nitric oxide and prostacyclin in order to investigate EDHF/NPRC-dependent relaxation in isolation. In certain experiments, tissues were treated with 10 nM C2238-αANP, alone, or in combination with the NPR-

C antagonist M372049 (10 μM; Astra Zeneca, UK) (8). Vessels were incubated with the mutant peptide for 30 min prior to commencing ACh, SNO and CNP vasodilator curves.

All chemicals for the myography experiments were purchased from Sigma Aldrich, UK, unless stated otherwise.

2. Analysis of endothelial function by FMD in young subjects without cardiovascular disease carrying C2238-MA

Ten young subjects free from cardiovascular diseases homozygous for the C2238-MA (ANP variant) were compared to ten control individuals homozygous for the T2238 wild type allele (controls) matched for age and sex. A group of fifteen age and sex-matched TC2238 heterozygous subjects were also included. Each subject gave an informed written consent to partecipate to the FMD study.

Genotype assessment for the ANP variant was performed as previously described (9). FMD was measured in each patient according to recent guidelines (10). Subjects were asked to fast overnight and to refrain from exercise, alcohol and caffeine intake for at least 12 hours. Measurements were performed by the same trained operator between 8:30 and 9:30 a.m. in a quiet, darkened and temperature-controlled room. After BP and HR measurement, subjects were required to lay supine and relax for 20 minutes before starting the evaluation. Right brachial artery was scanned by means of the Acuson Sequoia® ultrasound machine equipped with a 7.5-MHz linear-array transducer. A stereotactic probe-holding device was used to maintain arterial scan throughout the study. A computerized data acquisition system (FMD Studio®) was connected to the ultrasound machine to calculate final FMD values. To create a flow stimulus, a standard blood pressure cuff was placed below the antecubital fossa. The brachial artery was scanned longitudinally 5 to 10 cm above the antecubital fossa until the clearest visualization of anterior and posterior intimal interfaces between the lumen and the vessel wall. Continuous wall tracking was generated by automated edge-detection software allowing continuous diameter recording. Pulsed Doppler signal obtained from a mid-artery sample volume allowed us to record blood flow velocity at baseline and immediately after cuff deflation. The baseline artery diameter was acquired for 60 seconds. Thereafter, arterial occlusion was induced by cuff inflation to a pressure of at least 50 mmHg above SBP for 5 minutes. Subsequently, the cuff was rapidly deflated leading to a brief high-flow state (reactive hyperemia). Measurement of postdeflation greatest diameter was performed over a 4-minute period of time. FMD was expressed as the percentage change in peak vessel diameter from the baseline value before cuff inflation. After a 15-minute rest allowing reestablishment of baseline conditions, the subjects underwent endothelium-independent vasodilation with low-dose sublingual nitroglycerin (NTG). The arterial segment selected for this procedure was the same used for ischemia-induced FMD. After 60 seconds of baseline arterial diameter acquisition, low-dose NTG (0.4 mg) was administered sublingually and vessel diameter was continuously recorded. Peak vasodilation occurred 3 to 4 minutes after drug administration reflecting vascular smooth muscle function. Similarly, endothelium-independent vasodilation was expressed as the percentage change in peak vessel diameter from the baseline value. NTG was not administered in the presence of hypotension or bradycardia.

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Table I. *In silico* studies of the interaction energies between CNP or T2238-αANP or C2238-αANP and NPR-C.

	NPR-C::T2238-αANP	**NPR-C::C2238-αANP**	**NPR-C::CNP**
Complex	-33,498 kcal/mol	-33,880 kcal/mol	-34,817 kcal/mol
Peptide	-1,354 kcal/mol	-1,724 kcal/mol	-524 kcal/mol
interaction energy	**-290 kcal/mol**	**-305 kcal/mol**	**-235 kcal/mol**

Table II. Demographic and clinical characteristics of CC2238/ANP and TT2238/ANP healthy human carriers included in the FMD studies.

	CC2238 Homozygous (N=10)	**TC2238 Heterozygous (N=15)**	**TT2238 Controls (N=10)**
Age	34±7	32±4	35±7
Gender (M/F)	3/7	5/10	3/7
Body mass index (Kg/m^2)	24±1	25±2	25±1
Systolic blood pressure (mmHg)	107±3	111±3	112±2
Diastolic blood pressure (mmHg)	77±2	74±4	73±1
Heart rate (bpm)	63±5	68±3	68±4
Familial history	0	0	0
Pharmacological treatment (%)	0	0	0

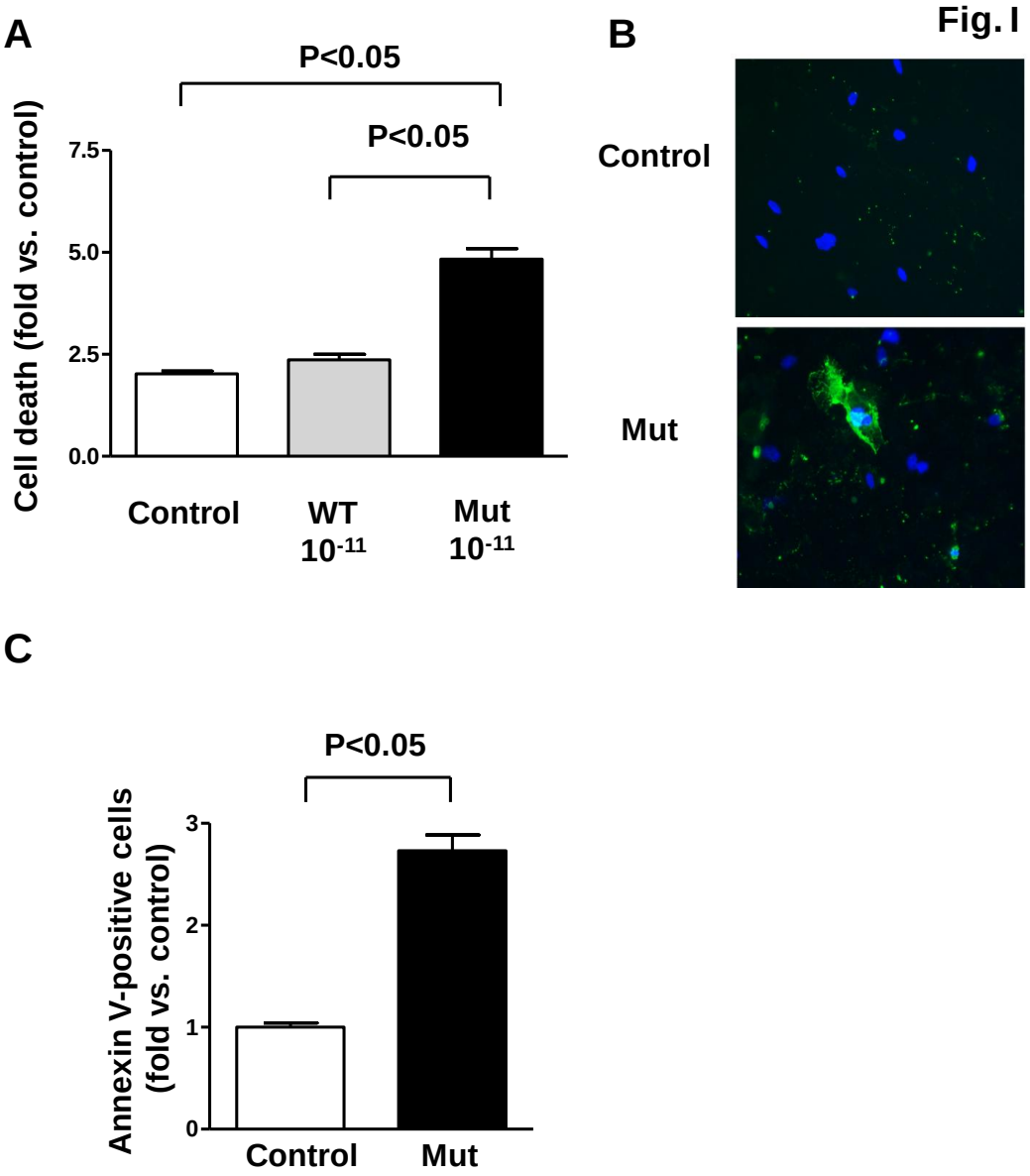

Figure I. A. HUVEC were incubated with normal medium, with T2238-αANP or with C2238-αANP for 12 hours at a concentration of 10^{-11}M. After 48 hours, C2238-αANP was found to be associated with a significant increase in cell death, as evaluated by Trypan blue assay. N=5. **B-C.** Percentage of annexin V-positive cells was calculated in HUVEC exposed to C2238-αANP (10^{-9} M) with respect to control. N=5.

Fig. II

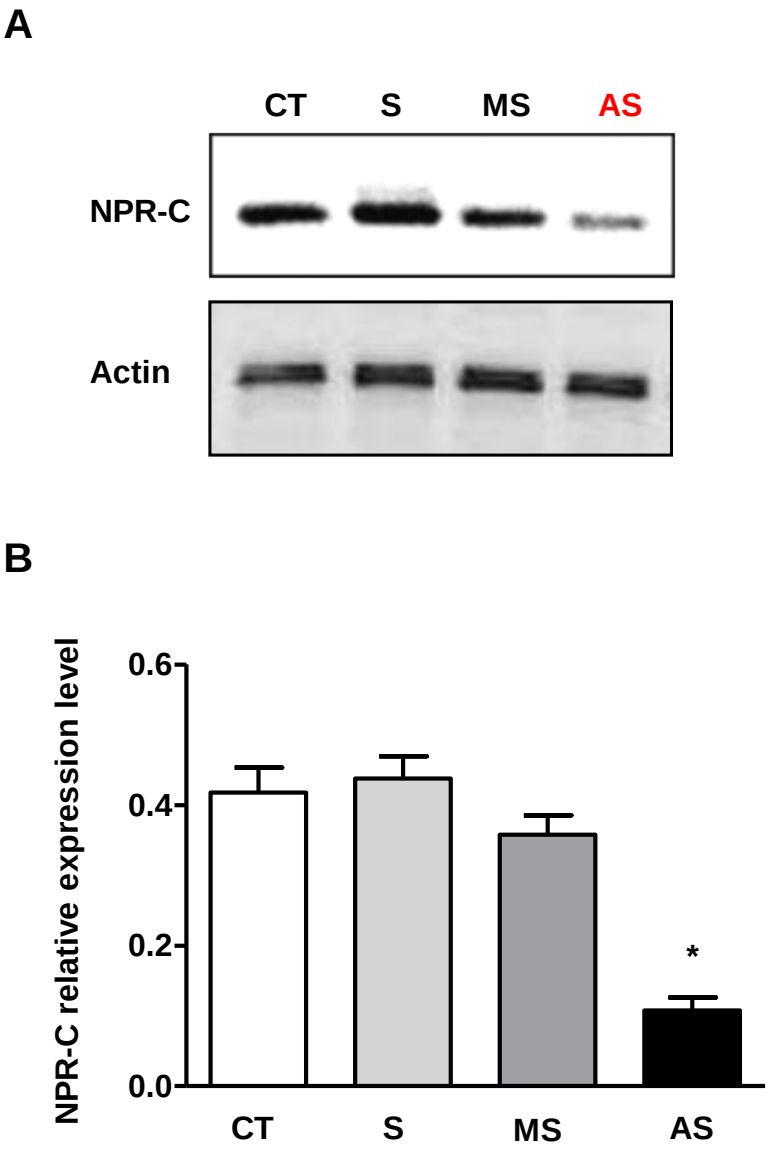

Figure II. A-B. HUVEC were incubated with CT or with medium plus antisense (AS), sense (S) or missense (MS) NPR-C oligonucleotides. Representative blot of NPR-C (B) and densitometric analysis (C) are presented. $P<0.05$ versus each of the other groups. N=4.

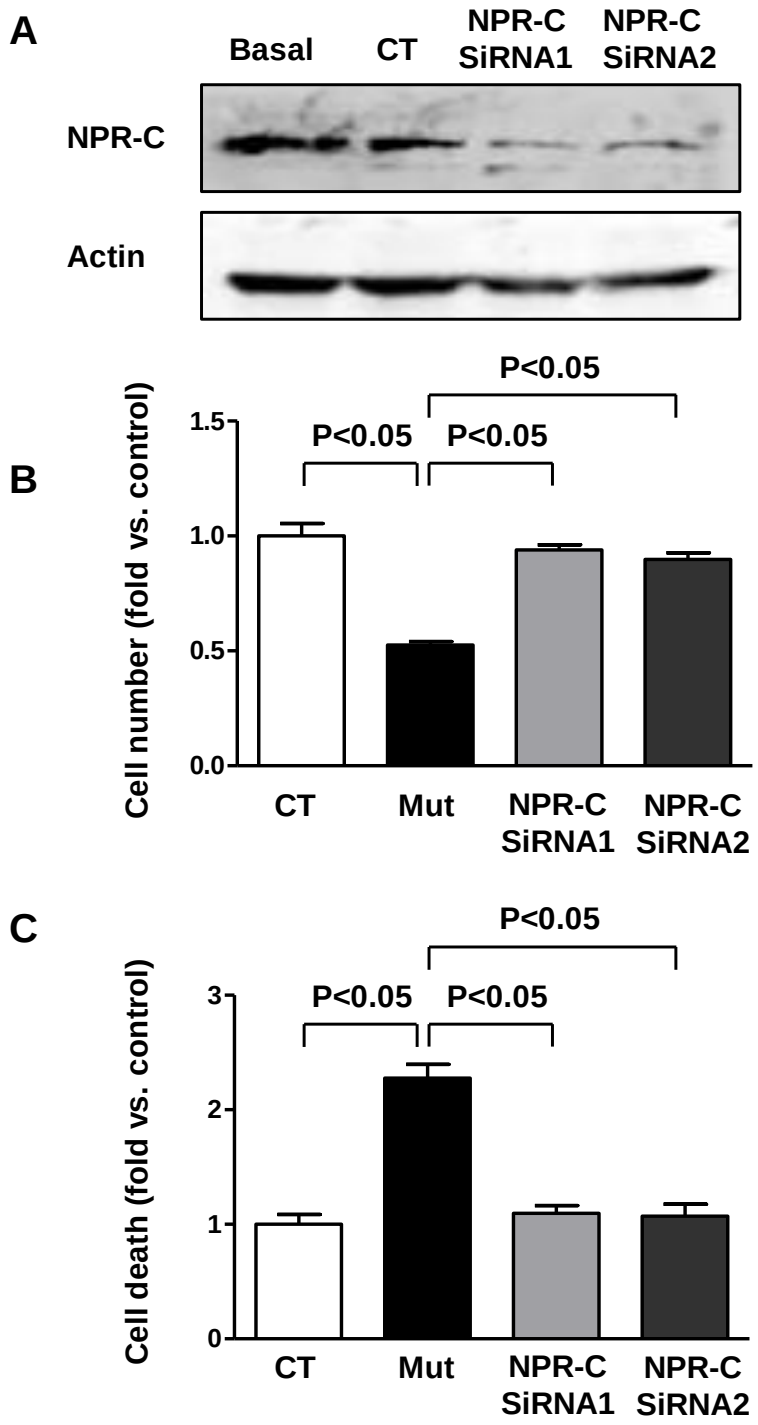

Fig. III

Figure III. A-C. HUVEC were transfected with lipofectamine and nonsense siRNA (CT), or with 2 NPR-C small interfering RNAs (Sigma Aldrich). NPR-C protein levels were evaluated (A). NPR-C-silenced and CT cells were exposed to C2238-αANP (Mut) for 12 hours. Number of viable cells (B) and rate of cell death (C) were evaluated. Data are expressed as fold vs.CT. N=4.

Fig. IV

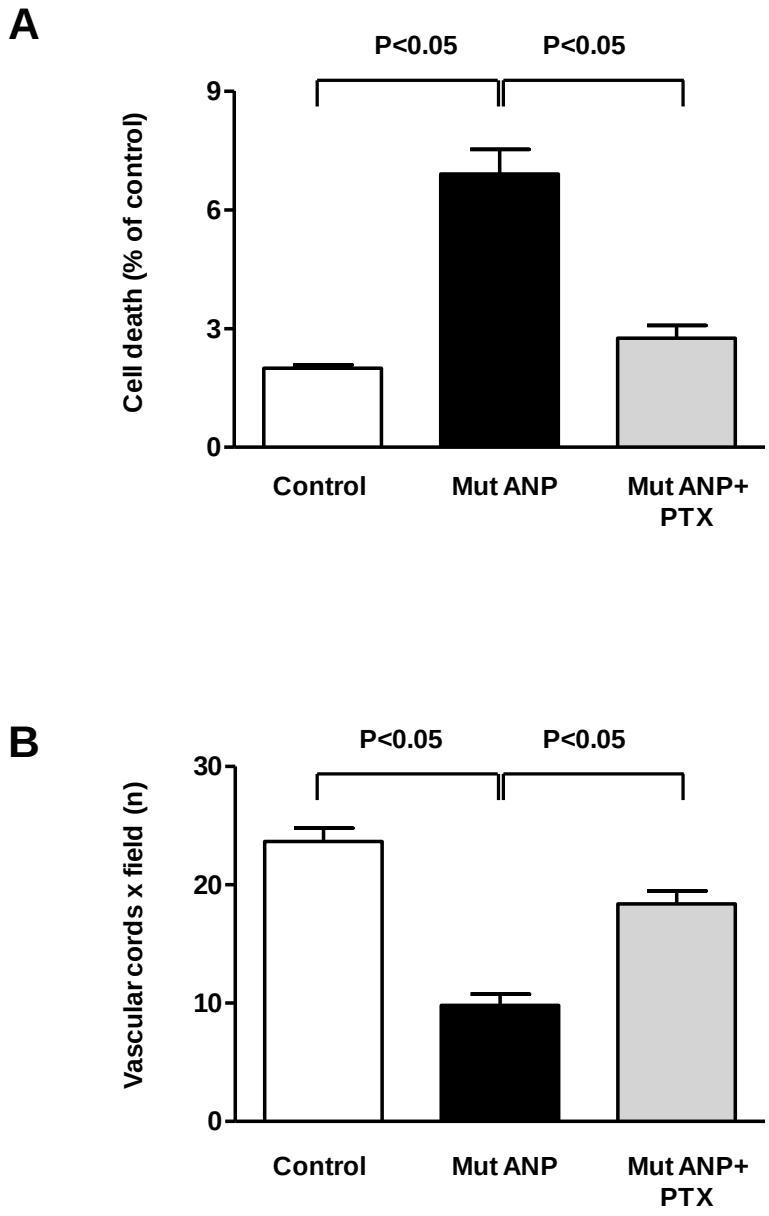

Figure IV. A-B. Pertussis toxin (PTX, 50 mg/ml) significantly reduced cell death induced by C2238-αANP (A), and increased vascular cord formation which was impaired by C2238-αANP (B). N=5.

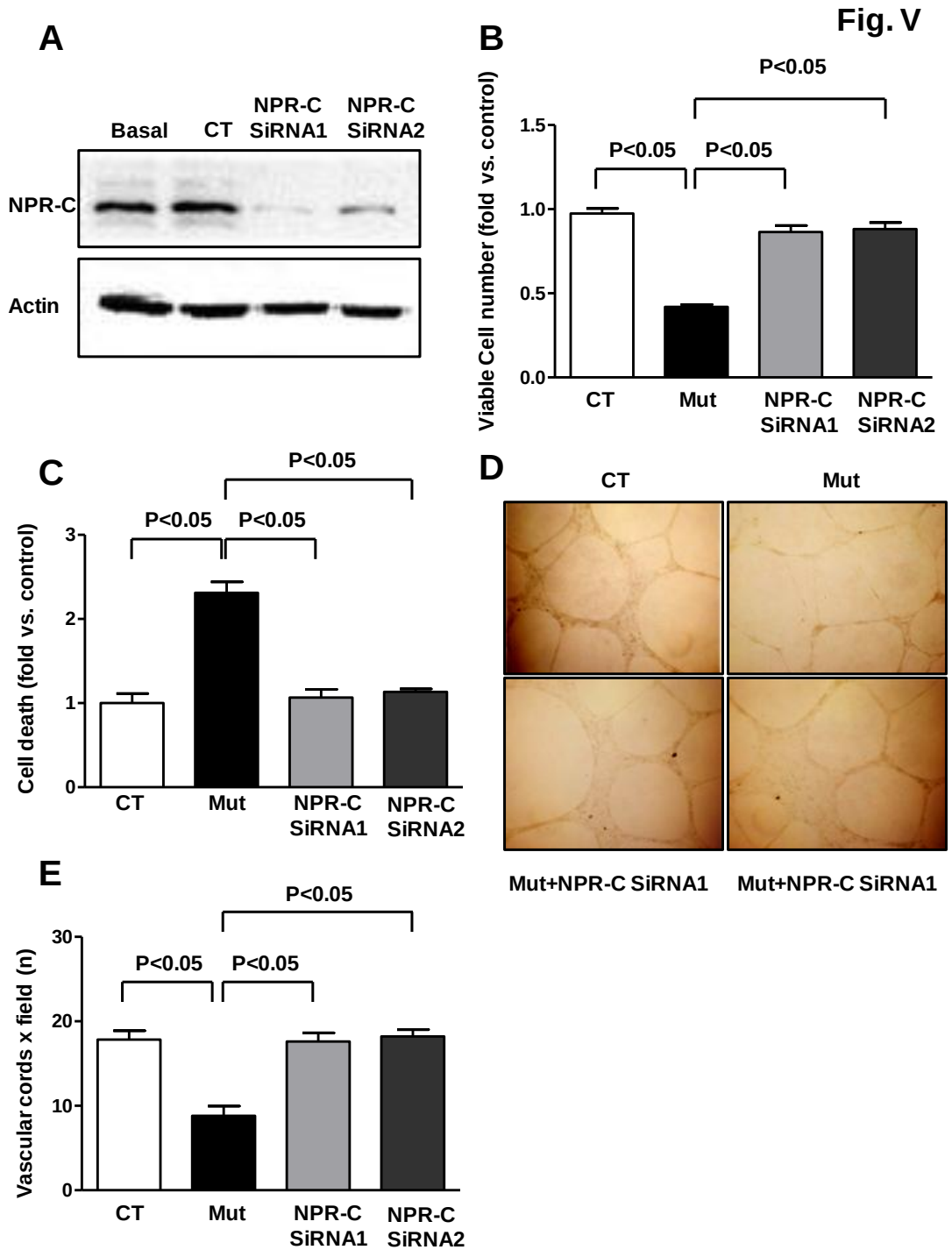

Figure V. A-E. HAEC were transfected with lipofectamine and nonsense siRNA (CT), or with 2 NPR-C small interfering RNAs (Sigma Aldrich). NPR-C protein levels were evaluated (A). NPR-C-silenced and CT cells were exposed to C2238-αANP (Mut) for 12 hours. Number of viable cells (B) and rate of cell death (C) were evaluated. In the same experimental conditions, vascular tube formation was evaluated (D-E). Data are expressed as fold vs.CT. N=4.

Fig. VI

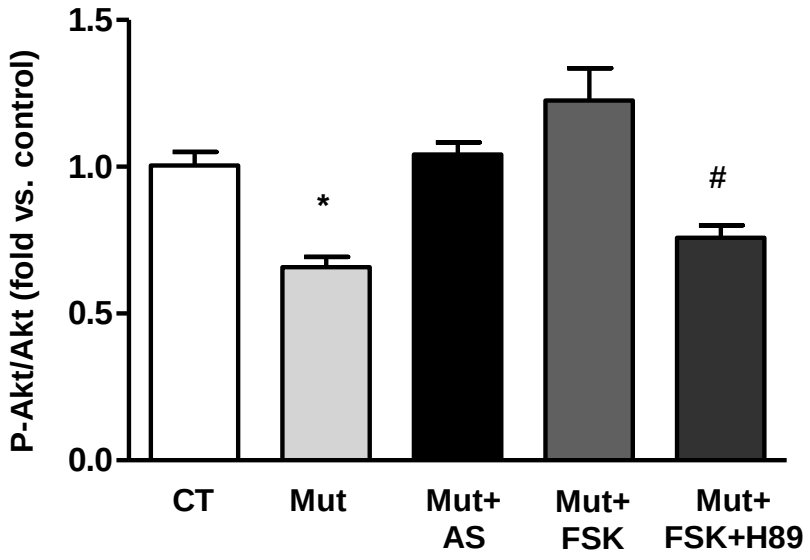

Figure VI. Densitometric analysis of phosphorylation levels (serine 473) of Akt1 in HUVEC exposed to regular medium, C2238-αANP (Mut), Mut+NPR-C antisense (AS) oligonucleotides, Mut+forskolin (FSK) and Mut+FSK+PKA inhibitor (H89). Representative immunoblots are shown in Fig. 4A-B. *$p<0.05$ vs. CT, Mut+AS and Mut+FSK. #$p<0.05$ vs. Mut+FSK+H89.

Fig. VII

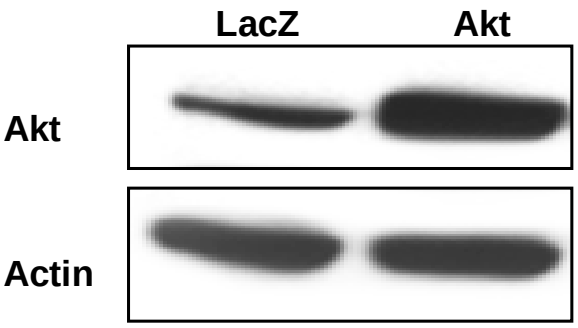

Figure VII. Akt protein expression was increased in HUVEC transduced with an adenovirus expressing constitutively active Akt with respect to HUVEC transduced with an adenovirus expressing β-galactosidase.

Fig. VIII

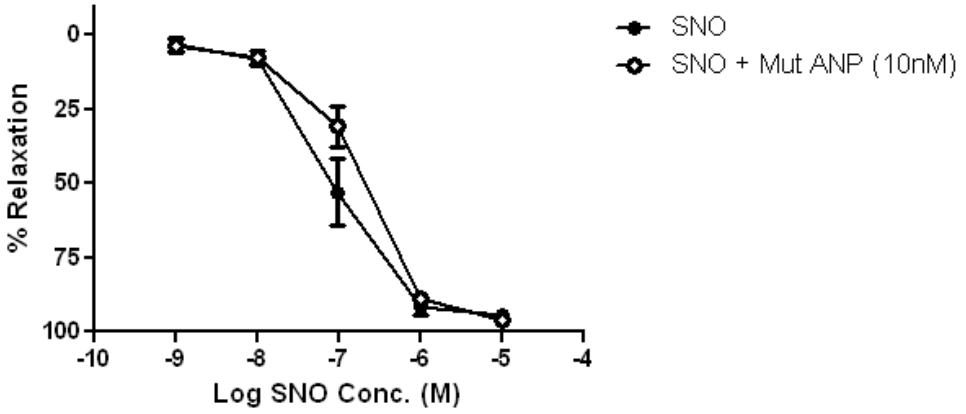

Figure VIII. A. SNO-dependent relaxation of rat mesenteric vascular rings was preserved either in the presence or in the absence of C2238-αANP. N=5.