Original Article

Association of Phagocytic NADPH Oxidase Activity With Hypertensive Heart Disease
A Role for Cardiotrophin-1?

María U. Moreno, Gorka San José, Álvaro Pejenaute, Manuel F. Landecho, Javier Díez, Óscar Beloqui, Ana Fortuño, Guillermo Zalba

Abstract—Left ventricular hypertrophy (LVH) is an independent marker of mortality in hypertension. Although the mechanisms contributing to LVH are complex, inflammation and oxidative stress may favor its development. We analyzed the association of the phagocytic NADPH oxidase–mediated superoxide anion release and LVH in patients with essential hypertension and the role of cardiotrophin-1 (CT-1) and interleukin-6 (IL-6), cytokines implicated in cardiac growth. Blood pressure, echocardiography data, and serum CT-1 and IL-6 levels were obtained in 140 subjects: 18 normotensives without LVH, 42 hypertensives without LVH, and 80 hypertensives with LVH. The NADPH oxidase–dependent superoxide production was assessed by chemiluminescence in peripheral blood mononuclear cells. Peripheral blood mononuclear cells were stimulated with CT-1 in vitro. Superoxide anion production by peripheral blood mononuclear cells associated with LVH and correlated with the left ventricular mass index. Serum CT-1 and IL-6 levels, which associated with the left ventricular mass index, correlated with superoxide production. Serum CT-1 and IL-6 levels were correlated. CT-1 stimulated NADPH oxidase superoxide production in peripheral blood mononuclear cells, which resulted in an increased release of IL-6. Our results show that superoxide anion production by the phagocytic NADPH oxidase associates with hypertensive heart disease, being significantly enhanced in hypertensive patients with LVH. This may be attributable to the activation of the NADPH oxidase by CT-1 and the subsequent release of IL-6. The phagocytic NADPH oxidase may be a therapeutic target in hypertensive heart disease. (Hypertension. 2014;63:00-00.) ● Online Data Supplement

Key Words: cardiotrophin 1 ■ hypertension ■ hypertrophy, left ventricular ■ NADPH oxidase ■ superoxides

Left ventricular hypertrophy (LVH), the major manifestation of hypertensive heart disease (HHD), is a strong, independent risk factor for cardiovascular morbidity and mortality.1 Although LVH has been attributed to many causes, such as hemodynamic and humoral factors, inflammation may exert a detrimental effect on myocardial structure favoring the development of LVH.2 However, the underlying mechanisms are not well understood. Such inflammatory processes include elevated levels of cytokines (such as interleukin-6 [IL-6])3 and increased reactive oxygen species generation by circulating cells.4

Oxidative stress associates with arterial hypertension and constitutes a potential mechanism that promotes LVH.5,6 A major source of superoxide anion in the cardiovascular system is the NADPH oxidase family, present in endothelial cells, smooth muscle cells, fibroblasts, cardiomyocytes, and phagocytes, including monocytes/macrophages.7 The phagocytic NADPH oxidase consists of 2 membrane-bound subunits (Nox2 and p22phox) and cytosolic subunits (p47phox, p67phox, p40phox, and rac2), which on stimulation are phosphorylated and translocate to membrane to form the catalytically active oxidase.8 Numerous evidences associate the activation of the NADPH oxidases with the pathophysiology of cardiovascular diseases, including human hypertension.5,7,8,9 Humoral factors such as angiotensin II, endo- therin-1,9 and insulin11 may activate the phagocytic NADPH oxidase. Experimental studies support the implication of circulating monocytes and lymphocytes in angiotensin II–induced hypertension, in part attributable to an NADPH oxidase–dependent mechanism.12 Among other effects, the NADPH oxidase overactivation results in increased IL-6 secretion in human monocytes.13 The NADPH oxidase also responds to cytokine stimulation.14

Cardiotrophin-1 (CT-1) is a member of the IL-6 superfamily of cytokines that signals via LIF (leukemia inhibitory factor) receptor-gp130–dependent pathways.15 Although originally characterized as a survival factor,16 chronically elevated CT-1 may contribute to left ventricular (LV) growth and dysfunction in hypertension. Plasma levels of CT-1 are elevated in hypertensive patients, namely in those with LVH.17 Regression of LVH and reduction of plasma levels of CT-1 associate in treated hypertensive patients.17 Finally, CT-1 is increased in the myocardium and plasma of hypertensive patients with heart failure.18

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To analyze whether the systemic superoxide generation participates in LVH in hypertensive patients and that CT-1 may be relevantly involved, we analyzed the relationship of the NADPH oxidase–dependent superoxide production by peripheral blood mononuclear cells (PBMCs) with the LVH in a cross-sectional study. Besides, we explored the role of CT-1 as an inducer of the NADPH oxidase in PBMCs and the subsequent proinflammatory phenotype.

Methods

Additional Methods are available in the online-only Data Supplement.

Subjects

According to institutional guidelines, subjects were aware of the research nature of the study and agreed to participate. The study was performed in accordance with the Declaration of Helsinki, and the Ethical Committee of the University Clinic of Navarra approved the protocol.

The study was performed in 140 unrelated white subjects who voluntarily came to our institution for a routine medical work-up after a 12-hour overnight fast. Blood pressure was measured on 3 occasions using a mercury sphygmomanometer, and the mean was recorded. Subjects were considered hypertensives (n=122) if they presented systolic blood pressure (SBP) and diastolic blood pressure of more than 139 and 89 mm Hg, respectively, or were under antihypertensive treatment. Patients had appropriate clinical, laboratory, and radiological evaluations to exclude secondary hypertension and chronic kidney disease. Normotensive subjects (n=18) presented repeated measurements of SBP and diastolic blood pressure below 140 and 90 mm Hg, respectively. The presence of LVH as determined by echocardiography, was established when LV mass index (LVMI) was >111 g/m² for men and >106 g/m² for women. Of the 122 hypertensives, 80 presented LVH. None of the hypertensive patients presented echocardiographic evidence of aortic stenosis or hypertrophic cardiomyopathy or clinical manifestations of heart failure. Thirty healthy volunteers were also recruited, and a sample of venous blood was obtained for in vitro studies.

Statistical Analysis

Quantitative variables are expressed as means±SEM or interquartile range with 95% confidence interval and categorical variables as numbers and percentages. To compare categorical variables, the χ² test was used. To compare numeric variables between patient groups, a 1-way ANOVA followed by a Student-Newman-Keuls test was performed with SPSS 15.0. To compare numeric variables between patient groups, a 1-way ANOVA followed by a Student-Newman-Keuls test was performed in accordance with the Declaration of Helsinki, and the Ethical Committee of the University Clinic of Navarra approved the protocol. The analyses were performed in 140 unrelated white subjects who voluntarily came to our institution for a routine medical work-up after a 12-hour overnight fast. Blood pressure was measured on 3 occasions using a mercury sphygmomanometer, and the mean was recorded. Subjects were considered hypertensives (n=122) if they presented systolic blood pressure (SBP) and diastolic blood pressure of more than 139 and 89 mm Hg, respectively, or were under antihypertensive treatment. Patients had appropriate clinical, laboratory, and radiological evaluations to exclude secondary hypertension and chronic kidney disease. Normotensive subjects (n=18) presented repeated measurements of SBP and diastolic blood pressure below 140 and 90 mm Hg, respectively. The presence of LVH as determined by echocardiography, was established when LV mass index (LVMI) was >111 g/m² for men and >106 g/m² for women. Of the 122 hypertensives, 80 presented LVH. None of the hypertensive patients presented echocardiographic evidence of aortic stenosis or hypertrophic cardiomyopathy or clinical manifestations of heart failure. Thirty healthy volunteers were also recruited, and a sample of venous blood was obtained for in vitro studies.

Results

Clinical and Echocardiographic Characteristics

The Table shows the clinical and echocardiographic characteristics of the subjects. Age, body mass index (BMI), and blood pressure values were significantly higher in the 2 groups of hypertensive patients as compared with normotensive subjects, whereas no differences existed for these parameters between hypertensives with LVH and hypertensives without LVH. No differences were found between hypertensives with LVH and hypertensives without LVH in the antihypertensive medication or in statin treatment. Among echocardiographic parameters, LV end-diastolic diameter, LVMI, left atrial anteroposterior diameter, and relative wall thickness were significantly higher in hypertensives with LVH as compared with normotensives, whereas the ratio of the early and late maximum transmitral velocity in diastole (VE/VA) was significantly lower in hypertensives with LVH as compared with normotensives. LV end-diastolic diameter, LVMI, left atrial anteroposterior diameter, and relative wall thickness were significantly higher in hypertensives with LVH as compared with hypertensives without LVH. No other differences among the 3 groups were observed in the remaining parameters.

NADPH Oxidase–Dependent Superoxide Anion Production in PBMCs

The superoxide anion production by PBMCs in response to phorbol myristate acetate (PMA) was increased in hypertensives with LVH compared with hypertensives without LVH and normotensive individuals (Figure 1). It was also higher in hypertensives without LVH than in normotensives. The chemiluminescence results were corroborated by measuring superoxide with the superoxide dismutase–inhibitable ferricytochrome c reduction (online-only Data Supplement and Figure S1 in the online-only Data Supplement). The chemiluminescent signal was inhibited by superoxide dismutase, thus indicating that lucigenin detected superoxide (Figure S2). The PMA-stimulated superoxide anion production was inhibited by diphenylene iodonium, gp91ds-tat, and apocynin (Figure S2). Oxyypurinol, rotenone, and L-NAME (L-N²-nitro-arginine methyl ester) did not inhibit superoxide production. Moreover, bisindolyl maleimide and wortmannin blunted PMA-induced superoxide production (Figure S2). Finally, PMA promoted the translocation of p47phox subunit from the cytosol to membranes, a direct index of NADPH oxidase activation in PBMCs (Figure S2). Collectively, these results substantiate the notion that NADPH oxidase is the major source of superoxide in PBMCs in response to PMA.11

Association of the NADPH Oxidase–Dependent Superoxide Anion Generation With Echocardiographic Parameters

The superoxide anion production by PBMCs correlated directly with the LVMI after adjusting for age, sex, BMI, and SBP (Figure 2A). A multivariate analysis adjusting for age, sex, BMI, and SBP indicates that an increase in 1 U (relative light units/s) of superoxide production resulted in an increase in 0.458 U of LVMI (P<0.033). The superoxide production correlated inversely with the deceleration time (Figure 2B) and directly with the left atrial anteroposterior diameter (Figure 2C). The correlations with the deceleration time (r=−0.323; P=0.005) and the left atrial anteroposterior diameter (r=0.259; P=0.023) were maintained in the hypertensives with LVH (HT+LVH) group.

Serum Concentration of Cytokines

CT-1 levels were increased in hypertensive patients with LVH compared with normotensive subjects and hypertensive patients without LVH (Figure 3A). Serum CT-1 levels were also higher in hypertensives without LVH than in normotensives, although the difference did not reach statistical significance. Moreover, serum CT-1 levels correlated directly with the superoxide production by PBMCs after adjusting for age, sex, SBP, and BMI (Figure 3B). This correlation was also evident when only the HT+LVH group was analyzed (r=0.478; P<0.001).
Taking into account that activated monocytes release IL-6, we investigated circulating IL-6 levels in our population. They were increased in hypertensives with LVH compared with normotensives and hypertensives without LVH and also higher in hypertensives without LVH than in normotensives (Figure S3). Serum IL-6 levels correlated with the superoxide production after adjusting for age, sex, SBP, BMI, superoxide production, and IL-6 levels (r=0.286; P<0.001).

In Vitro Studies on PBMCs
To evaluate the cause–effect relationship behind the correlation of circulating CT-1 levels and NADPH oxidase–dependent superoxide generation, we incubated human PBMCs isolated from 30 healthy individuals with recombinant human CT-1 for 10 minutes, detecting an increased superoxide production in a dose-dependent manner (Figure S4). This superoxide production was inhibited by diphenylene iodonium, gp91ds-tat, and apocynin (Figure 4A). Oxypurinol, rotenone, and L-NAME did not inhibit the superoxide generation.
Moreover, CT-1 promoted the translocation of p47phox subunit from the cytosol to membranes in PBMCs (Figure 4B). Collectively, these results support that CT-1 activates the phagocytic NADPH oxidase. CT-1–induced superoxide generation was blunted when PKC (protein kinase C) or PI3K (phosphatidylinositide 3-kinase) were inhibited or when gp130/LIF receptor was blocked (Figure 4C). Inhibition of MEK1/2 (MAP kinase kinase 1/2) did not affect the CT-1–induced superoxide generation (Figure 4C).

Stimulation of PBMCs with CT-1 significantly increased IL-6 secretion, which was inhibited in the presence of apocynin (Figure S5), suggesting that the CT-1–dependent release of IL-6 is mediated by the NADPH oxidase. Interestingly, 24-hour incubation with CT-1 upregulated Nox2 (Figure S6). CT-1 also increased superoxide production in human umbilical vein endothelial cells (Figure S7). Interestingly, IL-6 induced superoxide production in PBMCs, an effect that was inhibited by gp91ds-tat (Figure S8).

**Discussion**

The main finding of this study is that the NADPH oxidase–dependent superoxide anion production is enhanced in PBMCs from hypertensive patients with LVH as compared with cells from both hypertensives without LVH and normotensives. The NADPH oxidase–dependent superoxide anion production correlates with LV mass and function, as well as with left atrial morphology. Moreover, our results show that CT-1 can contribute to systemic oxidative stress by activating the NADPH oxidase in PBMCs and that, as a consequence, a further proinflammatory and prohypertrophic profile (IL-6 secretion) is induced. Taken together, our results support the notion that the PBMC NADPH oxidase overactivity may play a major role in LVH in hypertensive patients (Figure S9).

White cell numbers are increased in patients with hypertension and may be a marker of events. White cells present a primed phenotype: they are more prone to release reactive...
oxygen species, their gene expression is altered, and display a proadhesive phenotype. Target organs such as the heart display higher levels of proinflammatory molecules, which promote white cell infiltration. Such activated, infiltrated white cells turn into local sources of oxidative stress, proinflammatory cytokines, and matrix metalloproteinases, thus contributing to myocardial remodeling. Monocytes and lymphocytes may play a relevant role in the genesis of angiotensin II–induced hypertension, in part attributable to NADPH oxidase–dependent mechanisms, therefore supporting the role of inflammation in the basis of hypertension.

The NADPH oxidase system is a multimeric enzyme that was first described in phagocytes as a source of superoxide anion with bactericidal properties; thereafter, diverse variants of the enzyme have been described throughout the organism, with varied and relevant pathophysiological roles. Local NADPH oxidases play a role in the development and outcome of cardiovascular diseases. Likewise, the NADPH oxidase of circulating cells may be influenced by the systemic proinflammatory state and contribute to oxidative stress. In this regard, our results show that, paired to the development of LVH, there is an increase in circulating mononuclear cell NADPH oxidase activity. In fact, there is a direct correlation between the NADPH oxidase–dependent superoxide production by PBMCs and LVMI. Interestingly, in a study in hypertensives with LVH, the reduction in LVMI by treatment with antihypertensive drugs correlated with a reduction in reactive oxygen species produced by monocytes. Therefore, it may be suggested that humoral factors may have an effect on the NADPH oxidase of circulating white cells in patients with HHD.

In this regard, we have identified serum CT-1 as a potential candidate. CT-1 is a prohypertrophic cytokine that is increased in normotensives (NT, n=18; 133±13 fmol/mL [95% confidence interval (CI), 106 to 161 fmol/mL]), hypertensives without left ventricular hypertrophy (HT–LVH, n=42; 154±9 fmol/mL [95% CI, 135 to 173 fmol/mL]), and hypertensives with LVH (HT+LVH, n=80; 184±5 fmol/mL [95% CI, 174 to 195 fmol/mL]). *P=0.001 vs NT (CI, −75.90 to −25.93); †P=0.007 vs HT–LVH (CI, −49.92 to −11.10).

![Figure 3. A, Serum cardiotrophin-1 levels in normotensives (NT, n=18; 133±13 fmol/mL [95% confidence interval (CI), 106 to 161 fmol/mL]), hypertensives without left ventricular hypertrophy (HT–LVH, n=42; 154±9 fmol/mL [95% CI, 135 to 173 fmol/mL]), and hypertensives with LVH (HT+LVH, n=80; 184±5 fmol/mL [95% CI, 174 to 195 fmol/mL]). *P=0.001 vs NT (CI, −75.90 to −25.93); †P=0.007 vs HT–LVH (CI, −49.92 to −11.10). B, Correlation of serum cardiotrophin-1 levels and superoxide production by peripheral blood mononuclear cells after adjusting for age, sex, body mass index, and systolic blood pressure. Open circles indicate NT; closed circles, HT–LVH; and open squares, HL+LVH.](image)

![Figure 4. A, Superoxide production by peripheral blood mononuclear cells on 10-minute stimulation with human cardiotrophin-1 (hCT-1), in the presence or absence of Cu,Zn-SOD (enzymatic scavenger of superoxide), diphenylene iodonium (DPI, flavoprotein inhibitor), apocynin (inhibitor of phagocytic NADPH oxidase assembly), gp91ds-tat (inhibitor of phagocytic NADPH oxidase assembly), rotenone (inhibitor of mitochondrial chain), oxypurinol (inhibitor of xanthine oxidase), and L-NAME (L-N^G-nitro-arginine methyl ester; inhibitor of endothelial nitric oxide synthase; n=6). *P<0.01 vs control. †P<0.01 vs hCT-1. B, Representative image of p47phox translocation in the absence (baseline) and presence of hCT-1 (5 ng/mL). C, Evaluation of the blockade of the CT-1 receptor (anti-LIFR [leukemia inhibitory factor] and anti-gp130 antibodies) and signaling pathways (bisindolyl maleimide [BIS] -PKC [protein kinase C] inhibitor, -wortmannin -PI3K [phosphatidylinositol 3-kinase]/Akt inhibitor, and PD98059 -MEK1/2 [MAP kinase kinase 1/2] inhibitor) in peripheral blood mononuclear cells stimulated with hCT-1 (n=6). *P<0.01 vs control. †P<0.01 vs hCT-1.](image)
in HHD and may be a marker of LVH.\textsuperscript{27} We have now observed that serum CT-1 levels correlate with the NADPH oxidase–dependent superoxide production. Our in vitro studies show that CT-1 can activate the NADPH oxidase of PBMCs and increase Nox2 expression. We propose that in addition to its local, prohypertrophic effects, CT-1 may contribute to systemic oxidative stress. We cannot discard the effect on PBMC NADPH oxidase–dependent superoxide production of other humoral factors which may be relevant in hypertension. In fact, angiotensin II,\textsuperscript{9} endothelin-1,\textsuperscript{9} and insulin\textsuperscript{11} activate the phagocytic NADPH oxidase. In addition, our in vitro results show the release of IL-6 by PBMCs stimulated with CT-1, which is in agreement with Fritzenwanger et al\textsuperscript{28} who showed in monocytes from healthy volunteers that CT-1 induced IL-6 in a time- and concentration-dependent manner. However, IL-6 stimulation of PBMCs did not result in the release of CT-1. Finally, in our study, both CT-1 and IL-6 circulating levels correlate with the NADPH oxidase–dependent superoxide production and LVMI. Taken together, our results support the idea that the systemic proinflammatory status may have an impact on the LV in hypertension.

We see that as a consequence of the activation of the NADPH oxidase caused by CT-1, PBMCs release IL-6. IL-6 is a pleiotropic cytokine\textsuperscript{29} expressed in a multitude of cells, especially in monocytes and macrophages,\textsuperscript{30} that takes part in numerous processes, including differentiation of white cells, migration and proliferation of smooth muscle cells, insulin sensitivity in hepatocytes, phagocyte recruitment by endothelium, and secretion of metalloproteinases by fibroblasts.\textsuperscript{30} Interestingly, a clinical study showed a correlation between higher serum IL-6 levels and poorer cardiac function in subjects free of cardiovascular disease.\textsuperscript{30} In agreement with this, circulating IL-6 levels correlate with the LVMI in asymptomatic hypertensive patients.\textsuperscript{31} Moreover, a recent study in mice shows that IL-6 infusion induces cardiac hypertrophy and fibrosis, yielding a myocardial phenotype that resembles HHD.\textsuperscript{32}

Our finding showing that CT-1–induced IL-6 secretion can be blocked by apocynin suggests that therapies that directly or indirectly inhibit the NADPH oxidase may be beneficial in HHD. Interestingly, the treatment of patients with HHD with valsartan could both reduce monocyte–derived reactive oxygen species and LVH.\textsuperscript{27} We have recently showed that the EXP3179 metabolite of losartan diminished the NADPH oxidase activity of circulating white cells,\textsuperscript{33} which may be one of the factors that explain the beneficial effects of losartan, including LVH regression.\textsuperscript{34} It would be interesting to evaluate whether the effect of current medication for HHD is mediated by reduced systemic superoxide production by the NADPH oxidase.

One of the limitations of our study is the relatively small number of patients included in it. Further studies with larger samples are necessary to corroborate the association of the NADPH oxidase activity of circulating cells, cytokines, and cardiac structure and function, as well as to determine whether the phagocytic NADPH oxidase activity may be a marker of HHD. We are aware that other cells present in the cardiovascular system express the NADPH oxidase system as well as other pro-oxidant systems (such as the mitochondria or the uncoupled nitric oxide synthase), and therefore the PBMC NADPH oxidase may be one of many determinants of oxidative stress involved in LVH. Finally, we realize that pharmacological treatment may be a confounding factor in our study because antihypertensive drugs against the renin–angiotensin system as well as statins may inhibit the NADPH oxidases.\textsuperscript{35} Nevertheless, there were no significant differences in these treatments between the hypertensives without LVH (HT−LVH) and the HT+LVH groups.

**Perspectives**

Our findings suggest that, in addition to the important role of infiltrated white cells in cardiovascular pathophysiology, circulating phagocytes may contribute to the development of HHD via an increased superoxide generation by the NADPH oxidase and the subsequent release of IL-6 (Figure S9). In addition to its local, prohypertrophic effect on the heart, CT-1 may be one of the factors contributing to a systemic proinflammatory and pro-oxidant profile in patients with HHD, via an enhanced NADPH oxidase activity in PBMCs. Systemic oxidative stress and inflammation may be relevant mechanisms and therapeutic targets in HHD, thus drawing attention to the necessity of clinical studies to assess its state in the different stages of this cardiac disease.

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**Disclosures**

None.

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**Novelty and Significance**

**What Is New?**

- The systemic overactivity of the pro-oxidant phagocytic NADPH oxidase associates with cardiac structural and functional alterations in hypertensive patients.

- Cardiotrophin-1, a key prohypertrophic cytokine, promotes NADPH oxidase activation in circulating phagocytes.

**What Is Relevant?**

- The phagocytic NADPH oxidase may be a relevant novel therapeutic target in hypertensive heart disease.

**Summary**

In addition to the important role of infiltrated white cells in cardiovascular pathophysiology, circulating phagocytes may contribute to the progression of hypertensive heart disease via an increased superoxide generation by the NADPH oxidase. Cardiotrophin-1 may play a key role in the activation of the NADPH oxidase in circulating phagocytes, promoting their subsequent production of interleukin-6.
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ASSOCIATION OF PHAGOCYTIC NADPH OXIDASE ACTIVITY WITH HYPERTENSIVE HEART DISEASE: A ROLE FOR CARDIOTROPHIN-1?

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NADPH oxidase & hypertensive heart disease

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SUPPLEMENTARY METHODS

Echocardiographic studies
Two-dimensional echocardiographic imaging, targeted M-mode recordings, and Doppler ultrasound measurements were obtained in each patient as previously described. LV mass index (LVMI) and LV end-diastolic diameter (LVEDD) were calculated as previously described. The presence of LVH was established when LVMI was higher than 111 g/m² for men and higher than 106 g/m² for women. Of the 122 hypertensives, 80 presented LVH. The following pulsed Doppler measurements were obtained: maximum early transmitral velocity in diastole, maximum late transmitral velocity in diastole and the deceleration time of the early mitral filling wave. LV ejection fraction was calculated according to Quinones et al.

Cell isolation and culture
In all 140 subjects, PBMCs were isolated from venous blood samples with Lymphoprep with a high purity (99% were lymphocytes and monocytes) and immediately used for enzymatic and molecular measurements.

For in vitro studies, PBMCs from 30 healthy individuals were isolated similarly and used for enzymatic and molecular measurements in response to human recombinant CT-1 (Biotecnol). Besides, PBMCs were cultured in RPMI media (supplemented with 10 % fetal bovine serum, 1 % antibiotic/antimycotic, 2 mM glutamine, 5 mM sodium piruvate and 0.05 mM β-mercaptoethanol), in the presence or absence of recombinant human CT-1 or IL-6. The culture media was collected after 24 h and stored at -80°C for biochemical determinations.

Human umbilical vein endothelial cells (HUVECs) cultures were also used.

Determination of NADPH oxidase-dependent superoxide anion production
The superoxide anion production was measured in intact, freshly isolated PBMCs (4x10⁵ cells) in response to stimulation with phospholipid myristate acetate (PMA) (3.2 μmol/L) (Sigma) by chemiluminescence with 5 μmol/L lucigenin (Sigma), as previously published. Briefly, luminescence measurements (1 s) were recorded every 15-30 s along an interval of 1 hour in a plate reader luminometer (Luminoskan Ascent, Labsystem). The value of the area under the curve was used to quantify chemiluminescence. A buffer blank was subtracted from each reading. Measurements were expressed as relative light units (RLU)/s. In a small subgroup of the studied population the chemiluminescence measurements were validated against an independent measurement of superoxide production using superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction, which had previously
been shown to correlate well with the lucigenin superoxide determination.\textsuperscript{5} Briefly, PMBCs (4x10\textsuperscript{5}) were stimulated with 3.2 µmol/L PMA, in the presence of 50 µmol/L ferricytochrome c with or without 500 U/mL SOD, and incubated for 60 min at 37°C. After 1 hour, reduction of cytochrome c was determined at 550 nm in a spectrometer. The reduction of cytochrome c that was inhibitable with SOD reflected actual superoxide production.

The effect of Cu,Zn-SOD (an enzymatic scavenger of superoxide, 200 U/mL), diphenylene iodonium (DPI, a flavoprotein inhibitor, 10 µmol/L), apocynin (an inhibitor of phagocytic NADPH oxidase assembly, 100-500 µmol/L), gp91ds-tat (an inhibitor of NADPH oxidase assembly, 10\textsuperscript{-4} mol/L) rotenone (an inhibitor of the mitochondrial chain, 1 µmol/L), oxypurinol (an inhibitor of the xanthine oxidase, 1 µmol/L) and L-NAME (an inhibitor of endothelial nitric oxide synthase, 100 µmol/L) were examined. We also tested the effect of bisindolyl maleimide (BIS, a PKC inhibitor, 20 µmol/L), PD98059 (an inhibitor of MEK1/2, 10 µmol/L) and wortmannin (a PI3K/Akt inhibitor; 1 µmol/L).

In vitro studies were performed to analyze the effect of CT-1 on superoxide anion production. PBMCs (2x10\textsuperscript{5} cells) from healthy individuals were stimulated with recombinant human CT-1 (5, 10 and 50 ng/mL) for 10 minutes and superoxide production was determined as above. We also tested the effect on CT-1-induced superoxide generation of blocking the heterodimer receptor of CT-1, with the leukemia inhibitory factor receptor (LIFR, 1 ng/µL) or gp130 (1 ng/µL) antibodies (Santa Cruz Biotechnology). Finally, we evaluated CT-1-induced superoxide anion production in HUVECs.

We also analyse the effect of IL-6 on superoxide production. PBMCs (2x10\textsuperscript{5} cells) from healthy subjects were stimulated with recombinant human IL-6 (5 ng/mL) for 10 minutes and superoxide production was determined as above. The effect of gp91ds-tat (10\textsuperscript{-4} mol/L) was examined.

**p47phox translocation experiments**

p47phox translocation from cytosol to membranes, a direct index of NADPH oxidase activation, was measured in PBMCs in response to stimulation with PMA and human recombinant CT-1 (hCT-1), as previously published.\textsuperscript{4} Briefly, PBMCs (5x10\textsuperscript{6} cells) were stimulated with 3.2 µmol/L PMA or hCT-1 5 ng/mL for 15 minutes. Then, cells were centrifuged at 200g for 5 minutes and resuspended in 1 mL of Tris buffer saline (Tris 10 mmol/L pH 8, NaCl 150 mmol/L) containing a protease inhibitor cocktail Complete (Roche), incubated 10 minutes on ice, and centrifuged at 2000g for 10 minutes. The supernatant was collected and
centrifuged at 100000g for 60 minutes at 4°C. The supernatant, which corresponded to the cytoplasmatic fraction, was mixed with loading buffer (Tris 125 mmol/L, SDS 4%, glycerol 20%, β-Mercaptoethanol 10% and Bromophenol Blue 0.0125%). The pellet, which corresponded to the membranes, was resuspended with loading buffer in the same volume as cytoplasmatic fraction.

Protein levels of the p47phox subunit were determined in cytosolic and membrane fractions by Western blot with an antibody (sc-17845; 1:500) from Santa Cruz Biotechnology.

**Determination of Nox2 expression**

Nox2 expression was determined in PBMCs from healthy individuals as before. Briefly, PBMCs from (2x10^6 cells) from healthy individuals were stimulated with recombinant human CT-1 (5 ng/mL), in the presence or absence of gp91ds-tat (10^-4 mol/L) for 24 hours. Cells were homogenized in lysis buffer. Protein quantity was assessed by Lowry. Nox2 levels were assessed by western blot with a monoclonal anti-human Nox2 antibody (sc-130543, SantaCruz). Beta-actin (A5441, Sigma) were assessed as loading controls.

**Determination of cytokine concentration**

CT-1 concentration was measured in serum samples or in media from IL-6-stimulated PBMCs by ELISA according to the manufacturer's instructions (Antigenix America), as previously published.

IL-6 concentration was measured in serum samples or in media from CT-1-stimulated PBMCs by ELISA according to the manufacturer's instructions (R&D Systems).

**Superoxide anion determination in human umbilical vein endothelial cells (HUVECs)**

CT-1-induced superoxide anion was determined in human umbilical vein endothelial cells (HUVECs) to verify if the NADPH activation would be restricted to white cells or if other NADPH oxidase-expressing cell types could also be activated. In addition, being in constant contact with circulating humoral factors, activated endothelial cells may well contribute to a pro-inflammatory and pro-oxidative state in hypertensive heart disease.

HUVECs were isolated as previously described, and grown in MEM 199 (Invitrogen) supplemented with 10% fetal calf serum and 5% human serum (both PAA Laboratories). At passage 2-3 they were seeded in 6-well plates at 5x10^5 cells/well. They were kept for 12 hours in serum-free media and then hCT-1
ng/mL was added and incubated for 4 hours. After that time the cells were scraped and homogenated. The superoxide anion determination was performed with 1 μg of protein homogenate, lucigenin 5 µmol/L and NADPH 100 µmol/L, and the signal was recorded for 5 minutes.

**Statistical analysis**

Quantitative variables are expressed as mean±SEM and categorical variables as numbers and percentages. To compare categorical variables the chi-square test was used. To compare numerical variables between the normotensive group and the two groups of hypertensive patients, a 1-way ANOVA followed by a Student-Newman-Keuls test was performed once normality was checked (Shapiro–Wilks test); otherwise, the non-parametric Kruskal–Wallis test followed by a Mann–Whitney U test (adjusting the α-level by Bonferroni inequality) was used. The association between variables was tested calculating Pearson´s correlation coefficient and, when applicable, Spearman´s correlation coefficient. Adjustments for relevant confounding factors were performed in linear regression tests. Statistical significance was defined as two-sided P<0.05. The analyses were performed using the program SPSS 15.0.

**REFERENCES**


Figure S1. (A) The measurement of superoxide production using SOD-inhibitable ferricytochrome c reduction closely correlated the lucigenin measurements. (B) Available data of SOD-inhibitable ferricytochrome c superoxide generation in our population showed that peripheral blood mononuclear cells from hypertensive with left ventricular hypertrophy (HT+LVH, n=14) generated more superoxide than those of from hypertensive without left ventricular hypertrophy (HT-LVH, n=12) and normotensives (NT, n=6) (*P=0.042 vs NT; †P=0.009 vs NT; #P=0.044 vs HT-LVH).
Figure S2. (A) Determination by chemiluminescence with 5 µmol/L lucigenin of the NADPH oxidase activity in peripheral blood mononuclear cells by PMA in the absence or presence of Cu,Zn-SOD (an enzymatic scavenger of superoxide), diphenylene iodonium (DPI, a flavoprotein inhibitor), apocynin (an inhibitor of phagocytic NADPH oxidase assembly), gp91ds-tat (a specific inhibitor of NADPH oxidase assembly), rotenone (an inhibitor of the mitochondrial chain), oxypurinol (an inhibitor of the xanthine oxidase), L-NAME (an inhibitor of endothelial nitric oxide synthase), bisindolyl maleimide (BIS, a PKC inhibitor) and wortmannin (a PI3K/Akt inhibitor) (n=6). *P<0.001 vs control. †P<0.01 vs PMA. (B) Representative image and quantification of p47phox translocation in the absence (baseline) and presence of PMA 3.2 µmol/L (n=4). *P=0.01 vs baseline.
**Figure S3.** (A) Serum interleukin-6 concentration in normotensives (NT, n=18), hypertensives without left ventricular hypertrophy (HT-LVH, n=42) and hypertensives with left ventricular hypertrophy (HT+LVH, n=80). (*P=0.006 vs NT; †P<0.001 vs NT; #P=0.003 vs HT-LVH). (B) Correlation of serum interleukin-6 levels and superoxide anion production by peripheral blood mononuclear cells, after adjusting for age, sex, body mass index and systolic blood pressure.
Figure S4. (A) Determination by chemiluminescence with 5 µmol/L lucigenin of the NADPH oxidase activation in peripheral blood mononuclear cells by human recombinant CT-1 (hCT-1, n=6). *P<0.01 vs control. (B) Superoxide determination by the cytochrome c determination corroborated an increased superoxide generation upon stimulation with human recombinant CT-1 (hCT-1, 5 ng/mL, n=6). *P<0.05 vs baseline.
Figure S5. Interleukin-6 secretion by peripheral blood mononuclear cells upon 24 h stimulation with human recombinant CT-1 (hCT-1), in the presence or absence of apocynin (n= 6). *P<0.001 vs Basal and Apocynin.
Figure S6. Representative image of Nox2 expression at baseline, and after 24 h stimulation with human recombinant CT-1 (hCT-1, 5 ng/mL), in the presence or absence of gp91ds-tat ($10^{-4}$ mol/L).
Figure S7. Determination by chemiluminescence with 5 µmol/L lucigenin of the NADPH oxidase activity in human umbilical vein endothelial cells (HUVECs) after 4 hours of stimulation with human recombinant CT-1 (hCT-1, 5 ng/mL, n=4). *P<0.01 vs baseline.
Figure S8. Determination by chemiluminescence with 5 µmol/L lucigenin of the NADPH oxidase activation in peripheral blood mononuclear cells after 10 minutes of stimulation with human recombinant interleukin-6 (IL-6, 5 ng/µL, n=3). *P<0.05 vs basal condition.
Figure S9. Proposed contribution of cardiotrophin-1 (CT-1) to left ventricular hypertrophy (LVH) by its action on peripheral blood mononuclear cells (PBMC). In the present study (solid arrows) we propose that CT-1 activates, via its gp130/LIF receptor and the pathway PKC/PI3K, the PBMC NADPH oxidase, which results in the release of interleukin-6 (IL-6), all of which is associated with LVH. Other studies indicate that the phagocytic NADPH oxidase can be activated by other relevant cardiovascular agonists, or by other cytokines (IL-6 could exhibit an autocrine-paracrine effect on PBMCs). CT-1 exhibits also pro-hypertrophic actions through its direct effects on cardiac cells.


Between the original online posting on December 9, 2013, and the final issue posting, corrections were made to the Sources of Funding.

The Sources of Funding previously read as follows:

...“the Spanish Ministry of Science and Innovation (SAF2010-20367; RECAVA RD06/0014/0008); and the FP7-MEDIA project (75111002).”

This has been changed as follows:

...“the Spanish Ministry of Economy and Competitiveness (SAF-2010-20367; RECAVA RD06/0014/0008; RIC RD12/0042/0009); and the FP7-MEDIA project (HEALTH-2010-261409).”