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.\(^1\) LV mass index (LVMI) and LV end-diastolic diameter (LVEDD) were calculated as previously described.\(^1\) The presence of LVH was established when LVMI was higher than 111 g/m\(^2\) for men and higher than 106 g/m\(^2\) for women.\(^2\) 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.\(^3\)

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 (4x10\(^5\) cells) in response to stimulation with phorbol myristate acetate (PMA) (3.2 μmol/L) (Sigma) by chemiluminescence with 5 μmol/L lucigenin (Sigma), as previously published.\(^4\) 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.\(^5\) Briefly, PMBCs (4x10\(^5\)) were stimulated with 3.2 \(\mu\)mol/L PMA, in the presence of 50 \(\mu\)mol/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 \(\mu\)mol/L), apocynin (an inhibitor of phagocytic NADPH oxidase assembly, 100-500 \(\mu\)mol/L), gp91ds-tat (an inhibitor of NADPH oxidase assembly, 10\(^{-4}\) mol/L) rotenone (an inhibitor of the mitochondrial chain, 1 \(\mu\)mol/L), oxypurinol (an inhibitor of the xanthine oxidase, 1 \(\mu\)mol/L) and L-NAME (an inhibitor of endothelial nitric oxide synthase, 100 \(\mu\)mol/L) were examined. We also tested the effect of bisindolyl maleimide (BIS, a PKC inhibitor, 20 \(\mu\)mol/L), PD98059 (an inhibitor of MEK1/2, 10 \(\mu\)mol/L) and wortmannin (a PI3K/Akt inhibitor; 1 \(\mu\)mol/L).

In vitro studies were performed to analyze the effect of CT-1 on superoxide anion production. PBMCs (2x10\(^6\) 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/\(\mu\)L) or gp130 (1 ng/\(\mu\)L) 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\(^6\) 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\(^{-4}\) 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.\(^4\) Briefly, PBMCs (5x10\(^6\) cells) were stimulated with 3.2 \(\mu\)mol/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 cystosolic 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 5
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,\(^1,2\) 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.\(^3\)

