Hemin decreases cardiac oxidative stress and fibrosis in a rat model of systemic hypertension via PI3K/Akt signalling

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Received 3 October 2010; revised 1 March 2011; accepted 11 March 2011; online publish-ahead-of-print 15 March 2011

Time for primary review: 30 days

Aims Angiotensin II induces cardiac myocyte apoptosis and hypertrophy, which contribute to heart failure, possibly through enhanced oxidative stress. The aim of this work was to assess the impact of hemin (heme oxygenase-1 inducer) on NADPH oxidase activation, cardiac oxidative stress, and development of fibrosis in a rat model of renovascular hypertensive cardiomyopathy in comparison to an anti-hypertensive reference treatment with losartan.

Methods and results A 3 week hemin treatment was tested in an angiotensin II-dependent hypertensive rat model and a cellular model of neonatal rat cardiomyocytes stimulated by angiotensin II. Our findings demonstrate that hemin prevented development of intercellular fibrosis, expression of collagen I, and disorganization of intracellular fibres. Oxidative stress and apoptosis evaluated in hypertensive myocardial tissue were decreased by hemin. The reference treatment with the angiotensin II receptor (AT1) antagonist (losartan) was less effective than hemin in prevention of fibrosis and oxidative stress, although it was more effective in reducing hypertension. Rac-1 activation and, subsequently, NADPH oxidase activity were further decreased with hemin than with losartan. Hemin enhanced the expression of phosphoinositide 3-kinase (PI3K) p85 regulatory subunit, in contrast to losartan. The PI3K/Akt signalling pathway activation by hemin was related to heme oxygenase-1 activation and an increase in biliverdin reductase, and its inhibition by LY294002 reversed the effects of hemin on collagen I and caspase-3 expression. Finally, hemin increased Akt activation, and concomitantly decreased RhoA and p38 mitogen-activated protein kinase activation.

Conclusion We confirmed a positive effect of hemin on oxidative cardiac damage, apoptosis, and fibrosis induced by hypertension by modulating the NADPH oxidase activation through enhanced expression of the PI3K p85 regulatory subunit.

Keywords Cardiomyopathy • Hemin • Fibrosis • NADPH oxidase • Hypertension

1. Introduction

Hypertensive cardiomyopathies represent one of the more frequent aetiologies of cardiac insufficiency. Myocardial fibrosis, which is characterized by excessive deposition of extracellular matrix protein constituents, is one of the causes of heart failure. Myocardial fibrosis is induced by pressure overload that activates hormonal factor secretion (i.e. renin, aldosterone, angiotensin II and brain natriuretic peptide), stimulates the sympathetic system and adrenergic receptors, down-regulates calcium storage proteins, activates the fetal program and leads to structural modifications of contractile proteins. Ventricle remodelling by cardiac fibrosis is associated with a poor prognosis of cardiomyopathy leading to cardiac heart failure. It is known that alteration of the creatine phosphate/ATP ratio observed in patients with hypertensive cardiomyopathy is correlated with the presence of fibrotic areas in the myocardium of the left ventricle (LV).1

The role of angiotensin II in cardiac and smooth muscle contraction and salt retention is well known. In addition, it was recently shown to play a major part in the cardiac interstitial inflammatory response and in fibrosis accompanying cardiac failure.2,3 Numerous studies show that oxidative stress resulting from production of reactive oxygen...
species (ROS) plays an important role in cardiac remodelling and heart failure. ROS are derived from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, NO synthase (NOS), mitochondrial cytochromes. However, NADPH oxidase is the main source of ROS production in the heart and is important in signal transduction-dependent ROS. Several studies have shown that NADPH oxidase is important in many diseases related to cardiac remodelling. It is well established that oxidative stress could be a pro-fibrotic factor and that ROS derived from NADPH oxidase are involved in interstitial and perivascular fibrosis.

Following its induction by hemin, heme oxygenase 1 (HO-1) converts haem to free iron and carbon monoxide and also releases the antioxidant biliverdin. Biliverdin may be converted to bilirubin via biliverdin reductase. Heme oxygenase participates in the homeostatic control of cardiovascular functions, including the regulation of blood pressure and the prevention of cardiac fibrosis. Over-expression of HO-1 prevents extracellular matrix fibrosis, oxidative stress and finally heart failure in a transgenic rat model. It has been reported that the Rho/Rho kinase pathway is involved in different cardiovascular diseases, including hypertension. Rho GTPase controls several cellular functions, such as NADPH oxidase enzyme complex formation, which is responsible for production of oxygen free radicals during environmental stress. Stimulation of NADPH oxidase by pressure overload has been correlated with development of cardiac fibrosis. Previous studies have shown the protective effect of hemin on oxidative damage and fibrosis induction, but have not focused on its role in cardiac NADPH oxidase activation. The regulatory effect of hemin on NADPH oxidase activity could be mediated by bilirubin. We have previously shown that hemin acts on RhoA activation in aorta.

This study has been undertaken in order to evaluate the role of hemin treatment on cardiac oxidative stress and fibrosis in a two-kidney, one-clip hypertensive rat model and in a cellular model of cardiomyocytes isolated from newly born rats stimulated with angiotensin II. 

### 2. Methods

#### 2.1 Animals

All study protocols were approved by the Comité Régionale d’Ethique pour l’Experimenteration Animale (CREEA no. CL2007–013), Région Centre-Limousin, and carried out in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication no. 85–23, revised 1996), and European Directives (86/609/CEE). Forty-two adult male Wistar rats (10 weeks old, weighing 355 to 400 g, Laboratoire Janvier, Saint Berthevin, France) were randomized into a hypertensive group (n = 30) and a normotensive group (sham group, n = 12). The hypertensive group was divided into the following three experimental subgroups: (i) a hypertensive control (HTA group, n = 12); (ii) a hemin-treated group (hemin group, n = 12); and (iii) a losartan-treated group (losartan group, n = 6). Hemin (Sigma-Aldrich, Saint-Quentin, France) was injected at a dose of 50 mg/kg body weight every other day by intraperitoneal injection for 3 weeks (50 mg/mL dissolved in phosphate-buffered saline, pH 7.4). The rats received 20 mg/kg body weight/day of losartan (intraperitoneal). A two-kidney, one-clip unilateral renovascular hypertension model was developed by constricting the left renal artery. Animals were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7.5 mg/kg). Following laparotomy, the left renal artery was isolated and was partly constricted using a 3-mm-long, 1-mm-wide U-shaped silver clip with an internal gap of 0.25 mm. Doppler velocity was recorded at the end of the procedure in order to assess an increase of renal flow velocity; the flow velocity was about 6 m/s. In rats assigned to the sham group, surgical intervention was performed, but the left renal artery was not clipped. Experiments started following a 3 week surgical recovery period. The degree of hypertension was determined by invasive measurements of blood pressure (BP) at the time of killing, and the results were verified by cardiac ventricle weighing.

#### 2.2 In vitro model

Primary cultures of neonatal rat cardiac myocytes were prepared from 2- to 4-day-old Wistar rat hearts, as previously described. Cardiomyocytes were obtained from ventricular tissue by 10 dissociating steps of 8 min each at 37°C in 0.1% trypsin (Invitrogen, Cergy Pontoise Cedex, France). The dissociated cells were collected by centrifugation and suspended in a standard culture medium composed of Ham’s F-10 medium (Invitrogen) supplemented with 20% fetal bovin serum (Sigma), 20% human serum (Lonza, Levallois-Perret Cedex/Paris, France), streptomycin (150 UI/mL; Promocell, Sickingenstr. 63/65 69126 Heidelberg, Germany), and penicillin (200 UI/mL; Promocell). To increase the cardiac myocyte density selectively, a differential attachment technique was used, and cells expressed up to 99% troponin T in flow cytometry. Finally, cells were seeded in 150 cm² flasks (Corning Inc., Corning Incorporated, NY 14831, USA) at a final density of 7 × 10⁶ cells per flask and cultured at 37°C in a humidified atmosphere containing 5% CO2, 1% O2, and 76% N2. Cardiomyocytes (at passage 3–8) were grown to confluence and starved for 24 h before stimulation. In some experiments, cells were pre-treated for 2 h with 10⁻⁵ mol/L hemin, 10⁻⁵ mol/L losartan (angiotensin II type 1 receptor antagonist), 3 × 10⁻⁵ mol/L SnPP (Sn(IV) protoporphyrin IX dichloride Sn479–9; Frontier Scientific, P.O. Box 31, Logan, Utah 84323-0031, USA; an HO-1 inhibitor), 3 × 10⁻⁵ mol/L apocynin (Calbiochem, Merck Chemicals Ltd. Boulevard Industrial Park, Padgene Road, Beeston, Nottingham, NG9 2JR, UK; an NADPH oxidase inhibitor), or 5 × 10⁻⁵ mol/L of the PI3K inhibitor LY294002 (Cell Signalling Technology, Ozyme, Saint Quentin Yvelines Cedex, France), and then stimulated with 10⁻⁷ mol/L angiotensin II for 3 h.

#### 2.3 Transthoracic echocardiographic measurements

Left cardiac morphology and function were evaluated using a non-invasive transthoracic echocardiography method. Transthoracic echocardiographic measurement was performed using a commercially available echocardiograph (Sequoia 512 with 15 MHz transducer; Acuson, New York, USA). Echocardiography was performed in animals anaesthetized with ketamine (100 mg/kg intraperitoneal) and xylazine (7.5 mg/kg intraperitoneal) and consisted of two-dimensional mode, time–motion (TM) mode, and blood flow measurements. From TM mode measurements, LV hypertrophy index was calculated as [(diastolic septal wall thickness + diastolic posterior wall thickness)/2]/(end-diastolic diameter/2), and LV shortening fraction was calculated as [(end-diastolic diameter – end-systolic diameter)/end-diastolic diameter × 100]. The TEI index, an index of combined systolic and diastolic myocardial performance, was determined using Doppler tissue imaging. Recordings were performed by a physician blinded to the different groups.

#### 2.4 Haemodynamic measurements

Systolic blood pressure (SBP) was measured in conscious rats in the tail artery during the 3 week surgical recovery period. On the day of killing, BP was evaluated using arterial catheterization. Rats were anaesthetized with ketamine (100 mg/kg intraperitoneal) and xylazine (7.5 mg/kg intraperitoneal). The right carotid artery was cannulated with a polyethylene...
catheter filled with heparinized saline solution (0.9%) and connected to a Baxter uniflow gauge pressure transducer. Haemodynamic signals were collected on a strip-chart recorder (Hewlett Packard 78342A, Palo Alto, CA, USA). Mean arterial BP (MAP) was calculated as (SBP + DBP × 2)/3, where DBP is diastolic blood pressure, and pulse pressure (PP) as SBP minus DBP.

2.5 In vitro papillary experiments

The papillary muscles of the left ventricle were rapidly excised and placed in a chamber filled with saline solution (37°C, pH 7.4), attached to an isometric transducer and stimulated by an electric pulse of 15 V, 15 ms duration at a frequency of 200/min. Muscle contraction induced by electric stimulation was recorded via appropriate software (Labview 8, National Instruments Corporation, 11500 N Mopac Expwy, 78759-3504, USA). A piece of myocardium of roughly 5 mm length and width was dissected from the medial part of the left ventricle. The piece of myocardium was anchored with a hook at its two extremities, first at the bottom of a chamber filled with physiological saline solution (37°C, pH 7.4) and also to a rigid wire connected both to an isometric transducer (tension recording) and an isotonic transducer (length recording). The piece of myocardium was stretched at 3 g (first stretch), and the isotonic transducers were adjusted to zero. An increase of stretch up to 3 g was realized five times by recording the length variations. The maximal rate of tension development (contractility; (+d²/dt²)it in g/mm²/s) was measured from isometric contraction.

2.6 Histological analysis (fibrosis)

Following haemodynamic measurement, the animal’s thorax was opened, and the heart was rapidly excised and placed in a cold (4°C) physiological saline solution. The heart was dried out with a thin paper towel and weighed (Sartorius BP 160 P, Göttingen, Germany). Then, the atria, large vessels, and surrounding epicardial fat were carefully removed. Paraffin-embedded ventricles were cut into thin slices (4 μm) and observed in optical microscopy after haematoxylin, eosin, and safran staining. Masson staining allowed evaluation of interstitial fibrosis infiltration. Transmission electron microscopy was performed on fixed and dehydrated tissue cut into ultrathin sections and contrasted with uranyl acetate and lead citrate. Sections were observed at 80 kV on a JEOL 1010 transmission electron microscope.

2.7 Immunohistochemistry

LV myocardial samples were embedded in paraffin, and then sectioned at three levels from base to apex. The paraffin sections, 4 μm thick, were deparaffinized, and rehydrated with xylene and a graded alcohol series. Sections were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-HO-1 (1:1000 dilution; Santa Cruz Biotechnology, Inc.), rabbit anti-Pi3K p85 (1:1000 dilution; Cell Signaling Technology), rabbit anti-Akt (1:1000 dilution; Cell Signaling Technology), rabbit anti-phospho-Akt (Ser473; 1:5000 dilution; Cell Signaling Technology), rabbit anti-β-actin monoclonal antibody (1:500 dilution; Sigma-Aldrich) detection on autoradiographic film. Mouse anti-α-actin monoclonal antibody (1:500 dilution; Sigma-Aldrich) was used as a loading control.

2.8 Myocardial damage by oxidative stress and evaluation of apoptosis

Samples were incubated for 30–40 min in the dark at 37°C in the presence of 10 μM dihydroethidium (Invitrogen, USA). To estimate ROS, we measured changes in ethidium fluorescence, which is directly proportional to the oxidation of dihydroethidium by ROS. The sections of LV myocardial tissue were examined under fluorescence microscopy (Leica HC).

To determine the levels of fibrosis, a tissue apoptosis detection kit (ApopTag Plus Fluorescein; Chemicon International Inc., 28820 Single Oak Dr., Temecula, CA 92590, USA, Canada) was applied onto the LV myocardial tissue cryosections.

2.9 Measurement of NADPH oxidase activity

To evaluate the oxidant stress, we measured the activity of NADPH oxidase, the major source of ROS production. Following extraction of total NADPH/NADPH from to 2 × 106 cells, the NADPH oxidase activity was measured by using an NADPH/NADPH assay protocol (NADPH/ NADPH Assay Kit; Abcam, Cambridge, UK) according to the manufacturer’s instructions. The NADPH oxidase activity was expressed as the NADPH/NADPH ratio measured by reading absorbance at 450 nm on a microplate reader.

2.10 Western blotting

Samples were homogenized in a lysis buffer containing 50 mM Tris–HCl (pH 7.2), 1% Triton X-100, 1 mM EDTA, 0.5% deoxycholic acid, 500 mM NaCl, 0.1% sodium dodecyl sulphate, 10 mM MgCl2, 2 mM Na2VO4, and protease inhibitors. The homogenate was centrifuged at 9167 g for 5 min at 4°C and the supernatant collected as protein extracts. Protein concentration was determined by Bradford dye reagent (Bio-Rad, Marnes-la-Coquette, France) using bovine serum albumin as standard. Proteins (10–50 μg) were separated by 10–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked for 1 h at room temperature in 5% dry milk in Tris-buffered saline–Tween 20, and incubated for 1 h at room temperature under agitation or overnight at 4°C with one of the following primary antibodies: rabbit anti-HO-1 (1:1000 dilution; Santa Cruz Biotechnology, Inc.), rabbit anti-Pi3K p85 (1:1000 dilution; Cell Signaling Technology), rabbit anti-Akt (1:1000 dilution; Cell Signaling Technology), rabbit anti-phospho-Akt (Ser473; 1:500 dilution; Cell Signaling Technology), rabbit anti-β-actin monoclonal antibody (1:500 dilution; Sigma-Aldrich) detection on autoradiographic film. Mouse anti-α-actin monoclonal antibody (1:500 dilution; Sigma-Aldrich) was used as a loading control.

2.11 Rac-1 activation assay

An equal amount of proteins (500 μg) was used to assess the GTP-bound Rac-1 levels using p21-activated protein kinase p21 binding domain immobilized onto glutathione–agarose beads (Rac1/Cdc42 Activation Assay Kit; Upstate Biotechnology, Molsheim, Millipore, France) following the manufacturer’s instructions. The extent of Rac-1 activation (GTP–Rac-1) was expressed as the ratio of the density of the GTP–Rac-1 band to that of the total Rac-1 in each sample.

2.12 RhoA activation assay

An equal amount of proteins (500 μg) was used to assess the GTP-bound RhoA levels using Rhotekin rho binding domain immobilized onto glutathione–agarose beads (Rho Activation Assay Kit; Upstate Biotechnology, Millipore, France) following the manufacturer’s instructions. The extent of RhoA activation (GTP–RhoA) was expressed as the ratio of the density of the GTP–RhoA band to that of the total RhoA in each sample.
2.13 Quantitative RT-PCR

Total RNA from LV myocardial tissue was extracted using TRizol reagents (Invitrogen) according to the manufacturer’s instructions and quantified spectrophotometrically by its absorbance at 260 nm. One microgram of total RNA was treated with DNaseI (Invitrogen) and transcribed into complementary DNA using reverse transcriptase (Invitrogen) and random primers according to the supplier’s instructions. For PCR, 5 μL (20% of reverse transcription) of each cDNA preparation was subsequently used with SYBRGreen PCR Master Mix (Applied Biosystems, Courtaboeuf, France), and samples were run on a LightCycler480 (Roche, Meylan, France). The relative mRNA was normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Primer sequences were as follows: GAPDH forward, CTGCACCACA CACTGCTTAG, and reverse, GTCTTCTGGTGCGACTGAT; Rac-1 forward, GGACACGATTGAGAAGCTGA, and reverse, CAGCAGGC ATTTTCTCTTCC; and p38 MAPK forward, CCATCATTCACGC CAAAAAGG, and reverse, TCACATTCTGGCTTCTGATG.

2.14 Statistical analysis

Data were expressed as means ± SEM. Each variable was compared between the different groups using a two-way ANOVA. When an overall difference was found, a Mann-Whitney U-test was performed. A P-value of <0.05 was set as the criterion for significance in all comparisons.

3. Results

3.1 Impact of hemin on haemodynamic parameters and myocardial function

To demonstrate the effectiveness of hemin treatment, we evaluated HO-1 levels by western blot. As expected, hemin increased HO-1 levels (Figure 1A). In the HTA group, the systolic BP was significantly increased compared with the sham group (169 ± 6.4 mmHg, n = 12 vs. 125 ± 6.4 mmHg, n = 12, respectively; P < 0.05; Figure 1B). Hemin significantly decreased SBP (146 ± 5.7 mmHg in the hemin group, n = 12; P < 0.05; Figure 1B). Losartan decreased SBP more significantly than hemin (97 ± 3.7 mmHg in the losartan group, n = 6; P < 0.05; Figure 1B). Echocardiography showed an increase of LV hypertrophy index with HTA treatment (0.6 ± 0.02 in HTA group vs. 0.33 ± 0.02 in sham group; P < 0.05; Figure 1C). Left ventricular shortening fraction was not impaired in the HTA group, although TEI index was increased, indicating a diastolic dysfunction (TEI index = 0.37 ± 0.01 in HTA group vs. 0.30 ± 0.01 in sham group; P < 0.05; Table 1). Hemin decreased HTA-induced LV remodelling (LV hypertrophic index = 0.42 ± 0.04 in the hemin group).

Figure 1 Heme oxygenase 1 (HO-1) expression, haemodynamic parameters and myocardial function. (A) Western blot showed HO-1 overexpression in hemin-treated rats compared with the sham and losartan groups. (B) Systolic pressures measured in conscious rats in the tail (sham group n = 12, HTA group n = 12, and losartan group n = 6) during the 3 week surgical recovery period (right), and on the day of killing, and BP measured using arterial catheterization (left). (C) Left panel shows contractions with electrical external stimulation, illustrating a decrease of papillary contraction in the HTA group (b) that was reversed by hemin (a); right panel shows papillary active tension (normalized to initial value; T/T0) was measured after each elongation (normalized to initial value; L/L0). The length relation in the HTA group showed a progressive increase of tension, whereas an inverse relation was obtained with hemin, confirming a positive impact on myocardial relaxation. These results are supported by measurement of the contractility (dT/dt). *P < 0.05 vs. sham group; #P < 0.05 vs. HTA group; and †P < 0.05 vs. HTA + hemin group.
Table 1 Haemodynamic and echographic parameters

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<th>Sham</th>
<th>HTA</th>
<th>HTA + hemin</th>
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<tr>
<td>SBP (mmHg)</td>
<td>125 ± 6.45</td>
<td>169 ± 6.42*</td>
<td>146 ± 5.70†</td>
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<tr>
<td>DBP (mmHg)</td>
<td>93 ± 6.25</td>
<td>121 ± 5.31*</td>
<td>99.2 ± 2.88†</td>
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<td>PP (mmHg)</td>
<td>31.25 ± 1.25</td>
<td>47.72 ± 2.92</td>
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<td>Transthoracic echocardiographic measurements</td>
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<tr>
<td>Left ventricular hypertrophy index</td>
<td>0.33 ± 0.02</td>
<td>0.60 ± 0.02*</td>
<td>0.42 ± 0.04†</td>
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<tr>
<td>TEI index</td>
<td>0.30 ± 0.01</td>
<td>0.37 ± 0.01*</td>
<td>0.30 ± 0.03†</td>
</tr>
<tr>
<td>Left ventricular shortening fraction (%)</td>
<td>43.56 ± 2.12</td>
<td>36.72 ± 2.57</td>
<td>39.58 ± 3.50</td>
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Echographic measurements confirmed the effect of hemin on left ventricular hypertrophy and myocardial performance as shown by the decrease of left ventricular hypertrophy index and TEI index. Shortening fraction was not significantly different between the three groups of rats. HTA, hypertensive group; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; TEI, index, an index of combined systolic and diastolic myocardial performance.

*P < 0.05 vs. sham group; and †P < 0.05 vs. HTA group.

Figure 2 Evaluation of myocardial fibrosis. (A) Histological observations after Masson trichrome staining showed induction of fibrosis in the HTA group, while this fibrosis disappeared in the hemin group. (B) Immunohistochemistry showed that fibrosis deposits corresponded essentially to collagen I (brown staining). Hemin significantly decreased over-expression of collagen I compared with the HTA group, more than that observed with losartan. (C) Electronic microscopy showed intracellular remodelling with fibre disorganization into the cardiomyocytes in the HTA group, leading to an increase in distance between the contractile apparatus and mitochondria (red arrows). Hemin reduced both perivascular fibrosis and intracellular contractile fibre disorganization more than losartan. *P < 0.05 vs. sham group; †P < 0.05 vs. HTA group; and †P < 0.05 vs. HTA + hemin group.

3.2 Impact of hemin on infiltration of myocardial fibrosis

Histological observations after Masson trichrome staining confirmed induction of fibrosis in the HTA group corresponding to an extra-cellular fibrosis, while this fibrosis disappeared in hypertensive rats treated with hemin (Figure 2A). Fibrosis deposits corresponded essentially to collagen I, as shown by immunohistochemistry (Figure 2B). As shown in Figure 2B, hemin significantly decreased over-expression of collagen I compared with the HTA group, more than that observed with losartan. *P < 0.05 vs. sham group; and †P < 0.05 vs. HTA group.
with losartan. Likewise, electron microscopy showed that intracellular fibres were disorganized in the HTA group, leading to an increase in the distance between the contractile apparatus and mitochondria (Figure 2C). Hemin reduced both perivascular and extracellular fibrosis and intracellular fibre disorganization and had a more pronounced effect than losartan (fibrosis lesions (in arbitrary units) 3.27 in HTA group vs. 1.50 in hemin group, $P < 0.05$; and vs. 2.07 in losartan group, $P < 0.05$; Figure 2A).

### 3.3 Impact of hemin on oxidative stress and apoptosis

To evaluate ROS levels in myocardial tissue sections, we measured changes in ethidium fluorescence, which is directly proportional to the oxidation of dihydroethidium by ROS (Figure 3A). There was an increase in ethidium fluorescence in HTA rat sections compared with the sham group. Furthermore, ethidium fluorescence was significantly diminished in the hemin but not in the losartan group. (B) Analysis of myocardial apoptosis detection (apoptotic nuclei labelled with green fluorescence) showed a significant increase of the fluorescence in the HTA compared with the sham group. Fluorescence was also significantly attenuated in the hemin but not in the losartan group. (C) Hemin significantly decreased over-expression of caspase-3 compared with the HTA group, more than that observed with losartan. $\#P < 0.05$ vs. HTA group.

Fluorescence was significantly attenuated in hypertensive rats treated with hemin. To further investigate the implication of hemin in apoptosis, expression of caspase-3 was measured. Hemin significantly decreased over-expression of caspase-3 compared with the HTA group, more than that observed with losartan (Figure 3C), further corroborating the immunofluorescence results.

### 3.4 Impact of hemin on NADPH oxidase activity

To explain the effect of hemin treatment on oxidant stress, NADPH oxidase activity was evaluated in vitro after stimulating neonatal cardiomyocytes with angiotensin II for 3 h. Hemin significantly decreased NADPH oxidase activity compared with the HTA group, more than that observed with losartan. The modulation of NADPH oxidase activation by hemin was reversed by PI3K inhibition. As the enzymatic reactions allowed a relative NADP/NADPH ratio evaluation, we used apocynin, an NADPH oxidase inhibitor, as a control for total inhibition (Figure 4A).

Rac-1 is a subunit of the NADPH oxidase complex that allows cytoplasmic components to bind the membrane cytochrome b-245.
and to form the active form of NADPH oxidase. To examine NADPH activation, we evaluated Rac-1 gene expression and Rac-1 activity (Figure 4B and C). Rac-1 gene expression was significantly increased ($P < 0.05$) in the HTA group and decreased ($P < 0.05$) with losartan, although it was not modified by hemin (Figure 4B). Rac-1 activity, evaluated as the GTP–Rac-1/Rac-1 protein expression ratio, was increased in HTA rats and, interestingly, the treatment with hemin decreased GTP–Rac-1/Rac-1 protein expression more than treatment with losartan ($P < 0.05$ vs. HTA + hemin group).

### 3.5 Effect of hemin on signalling pathways implicated in hypertensive cardiomyopathies

The p38 MAPK, Rho/Rho kinase signalling pathways have been studied in relation to apoptosis and fibrosis induced by oxidative stress in HTA rats. Quantitative RT-PCR and western blot of p38 MAPK was significantly increased ($P < 0.05$) in the HTA group, and decreased ($P < 0.05$) by hemin treatment. In contrast, losartan did not significantly decrease p38 MAPK expression (Figure 5A).

RhoA activation was assessed as the ratio of the activated form of RhoA (GTP–RhoA) to total RhoA. Active RhoA was significantly increased ($P < 0.05$) in the HTA group but decreased in the hypertensive groups treated with hemin or losartan (Figure 5B).

Finally, the regulatory subunit p85 PI3K was increased in rats treated with hemin, and this was accompanied by PI3K/Akt activation (phospho-Akt protein expression; Figures 5C and 6A).

### 3.6 Biliverdin reductase activation and PI3K regulation

We found that treatment with hemin was associated with over-expression of biliverdin reductase, as shown in Figure 6A. This over-expression of biliverdin reductase in the presence of hemin was significantly reduced by an inhibitor of PI3K activity (LY294002) and by an inhibitor of HO-1 activity (SnPP). LY294002 reduces PI3K activity induced by hemin without modifying the expression of the protein. LY294002 abolished the effect of hemin on collagen I, caspase-3 and p38 MAPK expression (Figure 6B).

### 4. Discussion

The present study shows that hemin can limit oxidative damage due to activation of NADPH oxidase within the myocardium in a rat model of renovascular hypertensive cardiomyopathy through a particular effect on PI3K regulation due to induction of HO-1 and biliverdin reductase.

With the two-kidney, one-clip model, we observed the development of hypertensive cardiomyopathy, with an alteration in compliance that was reduced with hemin despite the mild effect on the level of hypertension. Histological examination confirmed the positive impact of hemin on collagen I deposits. Previous studies demonstrated the impact of hemin on pro-inflammatory/oxidative mediators, such as activating protein (AP-1, AP-2), nuclear factor-κB and c-Jun NH$_2$-terminal kinase (JNK). In this study, we focused on NADPH oxidase activation. Hemin prevented oxidative stress and apoptosis and decreased NADPH oxidase activation more than losartan. NADPH oxidase...
activation is involved in oxidative damage and cardiac failure. Previous studies demonstrated that hypertension induces Rac-1 activation. Rac-1 is a subunit of NADPH oxidase complex that allows cytoplasmic components to bind the membrane cytochrome b-245, and to form the active form of NADPH oxidase. Several reports have shown that this subunit is involved in oxidative stress in vascular pathology. We confirmed that oxidative damage and apoptosis were present in hypertensive myocardium but not in rats treated with hemin. We attributed this antioxidant effect of hemin to a decrease in Rac-1 activity, and NADPH oxidase activation in hypertensive rats despite a smaller anti-hypertensive effect compared to losartan, a reference treatment for prevention of hypertensive cardiomyopathy.

In a previous study, we showed that hemin decreased RhoA activation in smooth muscle cells and reversed hypertensive vascular reactivity alterations. RhoA, which activates ROCK protein, has already been implicated in the regulation of vascular tone, proliferation, inflammation, and oxidative stress. ROCK1-deficient mice displayed a reduction of perivascular fibrosis induced by pressure overload. We found the same effect of hemin on RhoA activation in the myocardium; however, this effect was similar to that obtained with losartan.

As Rac-1 activity can be blocked by PI3K/Akt inhibition, we evaluated the p85 subunit of PI3K. Hemin increased the PI3K p85 subunit, which regulates PI3K 110α catalytic subunit. Previous studies have shown that PI3K 110α subunit, contrary to the PI3K p110γ subunit, has a favourable effect on cardiac function and fibrosis. PI3Kγ is a key factor in tumour necrosis factor-mediated events, because tumour necrosis factor-induced superoxide production, matrix metalloproteinase expression and activity were significantly suppressed in cardiomyocytes and cardiofibroblasts deficient in PI3Kγ. Recent studies on mice deficient in cardiac PI3K (p110α) activity show a critical protective role of this pathway in cardiac disease. Mice deficient in PI3K (p110γ) have a more accelerated onset of heart failure in response to hypertrophic or dilated cardiomyopathy. An increase in PI3K (p110γ) activity had a favourable effect on cardiac function and fibrosis in the pressure-overload model, and attenuated pathological growth. We showed that modulation of NADPH oxidase activation by hemin was reversed by PI3K inhibition, and that this inhibition reduces PI3K activity without diminishing the over-expression of p38 MAPK.
of the protein in the presence of hemin. We propose that PI3K could exert its anti-apoptotic and anti-proliferative roles via a decrease of caspase-3 and p38 MAPK. We also found that hemin decreases p38 MAPK and phospho-Akt expression.

We demonstrated that hemin caused over-expression of biliverdin reductase. Co-activation of biliverdin reductase with PI3K p85 subunit has previously been shown in a model of hypoxic–reoxygenated cardiomyocytes. Inhibition of HO-1 and PI3K modified biliverdin reductase expression, indicating a feedback loop between HO-1, biliverdin reductase, and PI3K p85. The modification of the activity of NADPH oxidase by hemin was reversed by PI3K inhibitor, indicating that the particular regulation of PI3K p85 subunit by hemin could explain its supplemental antioxidative effects comparatively to losartan. Indeed, PI3K inhibition reversed the effect of hemin on collagen I, caspase-3, and p38 MAPK expression, which are implicated respectively in fibrosis, apoptosis, and proliferation.

In conclusion, hemin had antioxidant, anti-apoptotic, and anti-fibrotic effects, despite a moderate impact on the level of hypertension, involving PI3K/Akt pathway signalling regulation via HO-1/biliverdin reductase over-expression modifying NADPH oxidase activation.

Conflict of interest: none declared.

Funding
This study was supported by ‘Fondation de l’Avenir’ foundation for medical research (Paris, France).

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