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RESEARCH PAPER

Control of left ventricular mass by moxonidine involves reduced DNA synthesis and enhanced DNA fragmentation

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Background and purpose: Left ventricular hypertrophy (LVH) is a maladaptive process associated with increased cardiovascular risk. Regression of LVH is associated with reduced complications of hypertension. Moxonidine is an antihypertensive imidazoline compound that reduces blood pressure primarily by central inhibition of sympathetic outflow and by direct actions on the heart to release atrial natriuretic peptide, a vasodilator and an antihypertrophic cardiac hormone. This study investigated the effect of moxonidine on LVH and the mechanisms involved in this effect.
Experimental approach: Spontaneously hypertensive rats were treated with several doses of moxonidine (s.c.) over 4 weeks. Blood pressure and heart rate were continuously monitored by telemetry. Body weight and water and food intake were measured weekly. Measurements also included left ventricular mass, DNA content, synthesis, fragmentation, and apoptotic/anti-apoptotic pathway proteins.
Key results: The decrease in mean arterial pressure stabilized at ~ -10 mm Hg after 1 week of treatment and thereafter. Compared to vehicle-treated rats (100%), left ventricular mass was dose- and time-dependently reduced by treatment. This reduction remained significantly lower after normalizing to body weight. Moxonidine reduced left ventricular DNA content and inhibited DNA synthesis. DNA fragmentation transiently, but significantly increased at 1 week of moxonidine treatment and was paralleled by elevated active caspase-3 protein. The highest dose significantly decreased the apoptotic protein Bax and all doses stimulated anti-apoptotic Bcl-2 after 4 weeks of treatment.
Conclusions and implications: These studies implicate the modulation of cardiac DNA dynamics in the control of left ventricular mass by moxonidine in a rat model of hypertension.
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Keywords: left ventricular hypertrophy; hypertension; moxonidine; apoptosis; DNA fragmentation; DNA synthesis

Abbreviations: ABTS, 2,2′-azino-di-(3- ethylbenzthiazoline sulphonate; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; AT1, angiotensin receptor type 1; AT2, angiotensin receptor type 2; HPS, heamatoxylin, phloxine, saffron; LV, left ventricle; LVH, left ventricular hypertrophy; LVM, left ventricular mass; OD, optical density; RAAS, renin–angiotensin–aldosterone system; SHR, spontaneously hypertensive rat; SNS, sympathetic nervous system; TDT, deoxynucleotidyl transferase

Introduction

Left ventricular hypertrophy (LVH) occurs in response to altered mechanical load, humoral factors or tissue injury. LVH is characterized by a structural rearrangement of components of the normal chamber wall and involves cardiomyocyte hypertrophy, cardiac fibroblast proliferation and progression of interstitial and perivascular fibrosis, leading to ventricular stiffening, which impedes both cardiac contraction and relaxation. For several years, LVH has been considered as an adaptive process to preserve contractile function when cardiac workload is chronically increased, such as in hypertension, where a positive correlation between LVH and blood pressure is well established. However, recent studies have shown that LVH is a maladaptive process associated with increased cardiovascular risk. LVH represents an independent risk factor for cardiovascular morbidity and mortality in hypertensive subjects, and

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regression of cardiovascular hypertrophy is associated with reduced complications of hypertension (Okin *et al*., 2004; Ishimitsu *et al*., 2005; Peng *et al*., 2005; Yasunari *et al*., 2005).

The mechanisms implicated in the development of LVH in hypertension include stimulation of the renin–angiotensin–aldosterone (RAAS) and sympathetic nervous systems (SNS). The implication of activated SNS and noradrenaline release is based on several findings, such as a high sympathetic drive to the heart in hypertensive patients (Schlaich *et al*., 2003). Also, for similar blood pressure values, hypertensive patients with LVH display cardiac noradrenaline values and sympathetic nerve firing rates significantly greater than those found in individuals with uncomplicated hypertension (Grassi, 2004). Left ventricular mass is increased in normotensive obese subjects and correlates with heart sympathetic activity (Amador *et al*., 2004), and direct exposure to noradrenaline increases protein synthesis in cultured adult and neonatal cardiac myocytes (Calderone *et al*., 1998; Singal *et al*., 2004).

Antihypertensive compounds that interfere with the actions of the RAAS and the SNS have conferred beneficial effects in addition to the lowering of blood pressure. The benefits of angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) on regression of LVH have been well established (Ishimitsu *et al*., 2005; Peng *et al*., 2005; Yasunari *et al*., 2005). In the LIFE study, patients on losartan displayed significantly less hypertrophy and were less likely to suffer a major cardiovascular event. In contrast, persistence of cardiac hypertrophy predicted an adverse outcome. Beneficial effects provided by ARBs are related to blockade of the angiotensin AT1 receptor, in addition to co-stimulation of the angiotensin AT2 receptor and subsequent increase in nitric oxide and bradykinin-like effects. Mechanisms of LVH regression by ACE inhibitors and ARBs in spontaneously hypertensive rats (SHR) include the reduction of cardiomyocyte mass, fibroblast hyperplasia and collagen content (Tea *et al*., 1999; Varo *et al*., 1999; Der Sarkissian *et al*., 2003). Similarly, drugs that interfere with the release or action of noradrenaline have beneficial effects (Liao *et al*., 2004). Chronic treatment with moxonidine, a sympatholytic antihypertensive compound that binds preferentially to imidazoline receptors (Haxhiu *et al*., 1994), results in blood pressure reduction and regression of LVH in hypertensive patients and laboratory animals (Mall *et al*., 1991; Greenwood *et al*., 2000; Messerli, 2000; Menaouar *et al*., 2002) and prevents MI-induced left ventricle (LV) hypertrophy in rats (Van Kerckhoven *et al*., 2004). However, the mechanisms implicated in LVH regression by moxonidine have not been investigated. Because cardiac hypertrophy is the net product of cell growth and apoptosis, a gene-regulated process of physiological cell self-destruction, these studies were performed to investigate in SHR the temporal and dose-dependent effect of chronic *in vivo* moxonidine treatment on left ventricular DNA synthesis and fragmentation as well as on several regulatory proteins in the apoptotic pathway.

Methods

All animal procedures and experiments were performed following the approval of the Bioethics Committee of CHUM, according to the Canadian Guidelines. SHR (12–13 weeks old) with established hypertension were purchased from Charles River (St Constant, QC, Canada). The animals were housed in temperature- and light-controlled room with food and water *ad libitum* and maintained for at least 3 days before experimentation.

Alzet osmotic minipumps (2ML1, 2ML2, 2ML4; Alzet Corporation) were implanted subcutaneously in SHR, under isoflurane anaesthesia, as we have previously described (Menaouar *et al*., 2002). These minipumps allowed continuous delivery of moxonidine (kindly provided by Solvay Pharmaceuticals, Hannover, Germany) at doses of 0, 50, 100, 200 and 400 $\mu g\,kg^{-1}\,h^{-1}$ for different time points during 4 weeks. The solution of moxonidine was prepared by dissolving the drug in isotonic saline, pH < 6.5, then pH adjusted to 7.0–7.4 by NaOH.

Rats were euthanized after 1, 2 and 4 weeks of moxonidine or saline-vehicle treatment. Hearts were quickly isolated, LV were separated and weighed, then snap-frozen in liquid nitrogen and stored at −80° C.

In another group, blood pressure (systolic, diastolic and mean arterial pressures), heart rate and animal activity were continuously measured by telemetry (Data Sciences International, St Paul, MN, USA) as we have previously described (Menaouar *et al*., 2002) for 2 days before and 1 month after treatment. The rats were allowed to recover from the surgery for telemetry, for at least 10 days before implantation of the osmotic minipumps.

DNA extraction and content

Left ventricles of each group were homogenized in liquid nitrogen using a mortar and pestle, and aliquots of 50 mg were weighed and used for quantification of content, fragmentation and synthesis of DNA assays. The remaining cardiac tissues were immediately frozen in liquid nitrogen and stored at −80° C until processing.

The aliquot (50 mg) of the pulverized LV was digested in 300 µl lysis buffer (EDTA 20 $mmol\,l^{-1}$, Tris-HCl 50 $mmol\,l^{-1}$ and SDS 0.5% w/v) containing Proteinase K (20 $mg\,ml^{-1}$, GIBCO, Burlington, ON, Canada) for 3 h at 50° C. The aliquot was then treated with RNase (10 $mg\,ml^{-1}$; GIBCO) for 1 h at 37° C. Total tissue DNA was extracted by the phenol and chloroform procedure. DNA concentration was determined by UV spectrophotometry (260 nm) and DNA content per milligram of the tissue was calculated.

DNA fragmentation

DNA fragmentation was performed as previously described (Tea *et al*. 1999). One microgram of extracted DNA was labelled by enzymatic assay on 3′OH ends using **t**erminal **d**eoxynucleotidyl **t**ransferase (tdt; GIBCO) and [^{32}P]-dCTP (3000 $Ci\,mmol^{-1}$, Amersham, Baie, d'urfe, QC, Canada) for 1 h, at 37° C. Increasing quantities of radiolabelled DNA (1, 2, 4, 8 µl) were loaded on 1.5% agarose gel in the presence of 1 µg of standard DNA molecular weight (λ DNA/*Hind*III fragments; GIBCO) to control the variability of the procedure and to increase reproducibility. All samples were then electrophoresed at 100 V for 3.5 h. After electrophoresis,

DNA was transferred onto hybond N+ nylon membranes (Amersham) and exposed to a phosphor-sensitive cassette for 48 h, then scanned, visualized and quantified by PhosphorImager (ImageQuant, Molecular Dynamics, Sunnyvale, CA, USA). Radioactivity associated with 150–1500 bp DNA fragments quantified by PhosphorImager was divided by the value obtained by the standard DNA molecular weight of the same bp length. For each sample, the four values were plotted against the quantity of DNA loaded on the gel and the slope of the linear regression was defined as the DNA fragmentation index.

Another method was also used for quantification of DNA fragmentation. Quantification of cytosolic histone-associated DNA fragmentation was performed by the cell death detection ELISAplus kit (Roche Diagnostics, Laval, QC, Canada) following the manufacturer's instructions. Briefly, LV were homogenized in lysis buffer supplied with the kit followed by centrifugation at 200*g* for 10 min. A sample (20 μl) of the extracted cytoplasmic fraction was incubated with 80 μl of anti-histone-biotin/anti-DNA-POD reagent in a streptavidin-coated microplate for 2 h at room temperature under gentle shaking. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2′-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) as a substrate for 10–15 min at 20° C and then the reaction was stopped. The change in colour was measured at a wavelength of 405 nm against a blank reference wavelength of 490 nm by using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). All measurements were performed in duplicate and samples analysed on the same microtitre plate in the same setting with control DNA–histone complex sample as a positive control. The OD_{405} reading was then normalized to the total amount of protein in each sample. Data are reported as an apoptotic index (OD_{405} per mg protein) normalized to corresponding vehicle-treated rats, considered as 100%.

DNA synthesis

Other groups of rats were used to evaluate *in vivo* DNA synthesis. Rats were injected intraperitoneally with (methyl-^{3}H)-thymidine (0.5 mCi in 2 ml saline; MP Biomedicals Inc., Irvine, CA, USA) 18 h before euthanizing. DNA extraction was performed as described above. Radioactivity in 100 μg of DNA was counted by liquid scintillation to determine the extent of (methyl-^{3}H)-thymidine incorporation into DNA, as an estimate of DNA synthesis.

Apoptosis regulatory proteins

The protein levels of active caspase-3 fragments (17–20 kDa) as well as Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) in the remaining powder of LV were examined by immunoblot analysis.

Membranes of left ventricular tissue were prepared in radioimmunoprecipitation assay buffer as previously described (El-Ayoubi *et al*., 2003). Protein content was measured spectrophotometrically, using BSA as standard, according to the method of Bradford. Equal amounts of proteins (30 μg) separated on 12.5% SDS-polyacrylamide gel were transferred to Hybond-C extra membrane (Amersham). Membranes were blocked in 5% nonfat milk and incubated with anti-caspase-3 (1:1000; BD Transduction Laboratories, Mississauga, ON, Canada), anti-Bax (1:1000; Cell Signalling, Pickering, ON, Canada) or anti-Bcl-2 (1:1000; BD Transduction Laboratories). Bound antibodies were detected by a peroxidase-conjugated anti-rabbit IgG antiserum (1:5000, for caspase-3 and Bax) or by goat anti-mouse (1:5000, Bcl-2) and visualized by ECL-Plus chemiluminescence detection (Amersham ECL Hyperfilm).

Histological analysis

Cross-sections of heart ventricles were stored in neutral buffered formalin for 5 days. After ethanol dehydration and embedding in wax, 5 μm slices were obtained using a microtome. Sections were stained with heamatoxylin, phloxine, saffron (HPS). Microscopic visualization and photographs were obtained and cell surface measurements were performed by an investigator unaware of the treatments, using computer software (Micro Dimension Version 1.01, 1993). Cell surface was measured in 10 different areas of the sections. Results were obtained from two sections per rat from two rats; with each treatment selected at random from the various treatment groups.

Statistical analysis

Pressure data were collected every hour over 3 days before (baseline) and 28 days after treatment. Results of each 24 h were combined and presented as difference from baseline. All data obtained from moxonidine-treated rats were compared to corresponding saline-treated controls. In some, results were compared to pretreatment values. All data are reported as mean ± s.e.m. Comparisons were performed by ANOVA followed by Neuman–Keuls multiple comparison test or Dunnett's multiple comparison test, where applicable, using the computer program PRISM. Statistical significance was taken as $P<0.05$.

Results

Telemetric measurement of blood pressure showed that pressure did not vary significantly in SHR receiving saline (vehicle) over 4 weeks (MAP, 131.2 ± 1.0 vs 136.7 ± 0.6 mm Hg). However, moxonidine at 200 and 400 μg kg^{-1} h^{-1} resulted in immediate mild but significant reduction in MAP, reaching lowest levels after 3–4 days of treatment, then stabilizing at 1 week (Table 1). Table 1 also shows that at 1 week, diastolic pressure was significantly reduced ($P<0.01$) by both moxonidine doses, whereas only the 400 μg dose reduced ($P<0.01$) systolic pressure as compared to saline-vehicle-treated rats. Corresponding heart rates were reduced at 1 week by 200 and 400 μg moxonidine, significantly ($P<0.01$) different from vehicle-treated rats. Pressures and heart rates were maintained at these levels throughout the 4 weeks of treatment. These concentrations of moxonidine had no significant effect on animal activity during the study period.

Table 1 Telemetric measurement of blood pressures and heart rate (HR) after 1 week of treatment

	Diastolic (mm Hg)	*Systolic (mm Hg)*	*MAP (mm Hg)*	*HR (beats min^{-1})*
Vehicle	−0.8 ± 0.5	−1.1 ± 0.2	−4.0 ± 0.1	−8 ± 4
Moxonidine (200 μg kg^{-1} h^{-1})	−6.7 ± 0.5*	−9.6 ± 1.1	−9.0 ± 0.2*	−40 ± 12*
Moxonidine (400 μg kg^{-1} h^{-1})	−8.7 ± 0.4*	−11.1 ± 2.1*	−10.8 ± 0.2*	−48 ± 10*

Data obtained on days 6 and 7 of treatment are averaged and presented as difference from basal.
*$P<0.01$ vs corresponding vehicle-treated rats; MAP, mean arterial pressure.

Body weight increased in SHR during 4-week treatment with saline vehicle (Figure 1). The body weight gain over 4 weeks was not altered by 50 and 100 μg but was lowered by the higher doses of moxonidine (200 and 400 μg; Figure 1). The lower gain in body weight (<6%) may be secondary to improved metabolic profile (Velliquette and Ernsberger, 2003) as it was not influenced by food intake that remained unchanged during that period.

Left ventricular mass (LVM) increased in vehicle-treated SHR during the 4 weeks of study from 602 ± 9 to 670 ± 8 mg ($P<0.001$). The 50 μg dose had no effect, whereas moxonidine treatment with 100, 200 and 400 μg resulted in less increase in LVM over time, reaching 93 ± 2%, 88 ± 1%, and 86 ± 3% of corresponding vehicle-treated control ($P<0.001$) after 4 weeks, respectively (Figure 2a). The decrease in LVM persisted after normalization to corresponding body weight (3.33 ± 0.03 to 3.09 ± 0.03, 3.03 ± 0.03 and 3.06 ± 0.20 mg per g body weight ($P<0.02$), or 94 ± 1% ($P<0.01$), 92 ± 2% ($P<0.01$) and 94 ± 2% ($P<0.04$) of corresponding vehicle-treated rats, respectively). Left ventricular DNA content did not significantly increase with time in vehicle-treated rats 838.7 ± 58.4 vs 940.9 ± 27.5 μg per ventricle; representing 11 ± 3% increase. However, moxonidine resulted in progressive reduction in DNA content in SHR left ventricles that reached with 4-week 400 μg about 70% ($P<0.01$) of corresponding vehicle treatment (Figure 2b). The changes in DNA content positively correlated with changes in LVM at all doses and time points ($r^2=0.35$, $P<0.001$, not shown), with 4-week treatment showing the highest influence ($r^2=0.67$, $P<0.0001$) (Figure 2c).

DNA content may be influenced by synthesis and/or fragmentation. *In vivo* ^{3}H-thymidine incorporation in vehicle-treated SHR increased in a time-dependent manner from 1073 ± 58 to 1378 ± 12 c.p.m. 100 μg^{-1} DNA ($P<0.03$) by 4 weeks. Moxonidine decreased ^{3}H-thymidine incorporation in a dose-dependent manner, 66 ± 3% and 57 ± 2% of corresponding vehicle-treated (100%) at 4 weeks of 200 and 400 mg moxonidine ($P<0.01$) reaching 66 ± 3% and 57 ± 2% of corresponding vehicle-treated (100%) at 4 weeks of 200 and 400 mg moxonidine ($P<0.01$) (Figure 3). On the other hand, DNA fragmentation only increased with 50 μg moxonidine at 1 week, as compared to corresponding vehicle-treated ($P<0.01$) and 100 μg moxonidine-treated SHR ($P<0.05$), and then returned to basal values after 4 weeks of treatment (Figure 4a). In confirmation, a similar time- and dose-dependent pattern of DNA fragmentation was obtained by determining cytoplasmic histone-associated mono- and oligonucleosomes by ELISA (Figure 4b).

Western blot analysis of proteins involved in the apoptotic pathway revealed a transient increase in the expression of the apoptotic protein Bax, measured in moxonidine- vs vehicle-treated rats at 1 week (100 μg, 112 ± 5% of corresponding vehicle-treated rats, $P<0.05$); this progressively decreased to reach 82 ± 6% of corresponding saline-treated rats $P<0.02$) at 4-week treatment with 400 μg. The anti-apoptotic Bcl-2, which was not different from vehicle-treated control (100%) at 1 week, increased after 2 weeks of treatment with 200 μg moxonidine, and then further increased at 4 weeks with all moxonidine doses ($P<0.03$). Figure 5 shows that the ratio of Bax to Bcl-2 declined at 2 weeks by the 200 μg dose and at 4 weeks with all treatment doses ($P<0.01$).

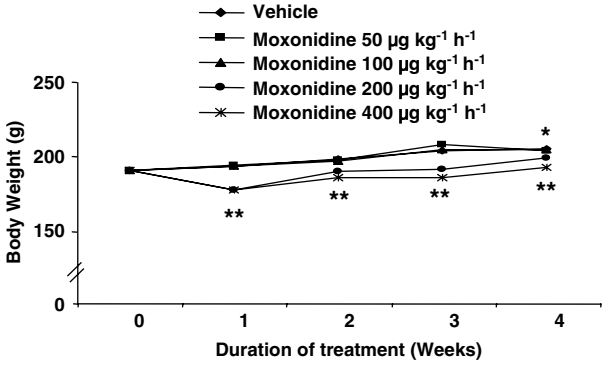

Figure 1 The effect of 4 weeks treatment with various concentrations of moxonidine on body weight. *$P<0.001$ vs basal; **$P<0.01$ vs corresponding vehicle-treated groups; $n=8$–23 rats per group.

Active caspase-3 protein increased after 1 week of treatment with moxonidine 50 and 100 μg ($P<0.05$) and at 2 weeks with 100 and 200 μg ($P<0.01$), and then decreased thereafter to levels not different from corresponding vehicle-treated SHR. Pro-caspase-3 proteins were not altered by treatment (Figure 6).

Histological analysis revealed that cardiomyocyte cell surface area increased from 465 ± 1 to 569 ± 3 μm^2 in SHR treated with vehicle for 1 and 4 weeks, respectively. Four-week treatment with moxonidine (50, 100, 200 and 400 μg) resulted in dose-dependent reduction in cardiomyocyte size to 565 ± 1, 527 ± 1, 531 ± 7 and 488 ± 7 μm^2, respectively, indicating prevention of cardiomyocyte hypertrophy.

Discussion

The findings of the present study demonstrate that in SHR, chronic antihypertensive treatment with moxonidine is associated with cardiac remodelling, as evidenced by a

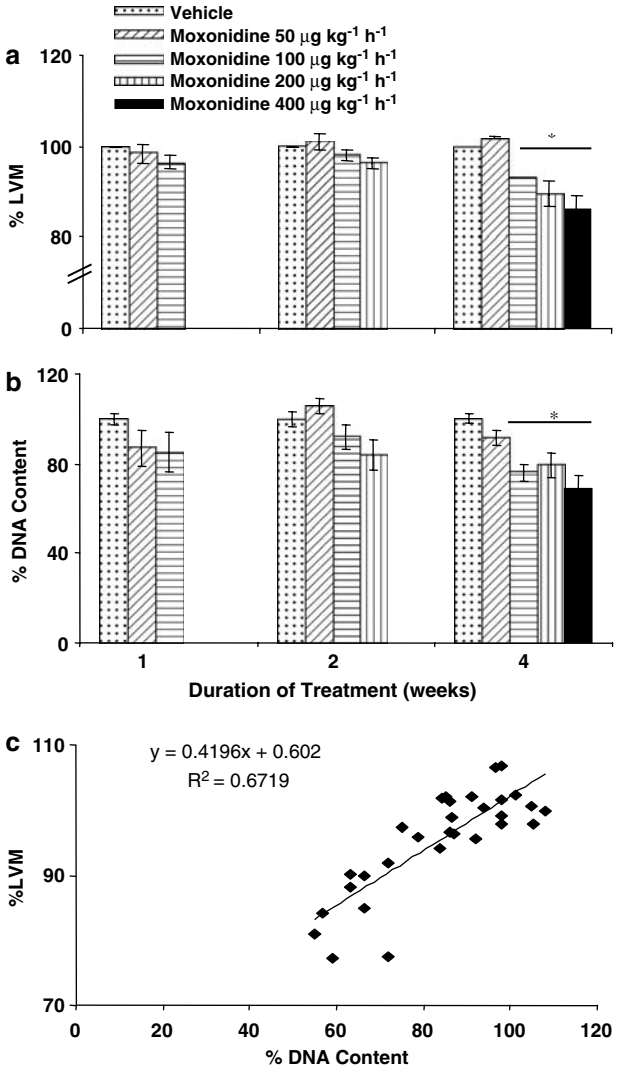

Figure 2 The effect of 1-, 2- and 4-week treatment with various concentrations of moxonidine on (**a**) left ventricular mass and (**b**) left ventricular DNA content, presented as percentage of corresponding vehicle-treated rats (100%). (**c**) Correlation between percentage decrease in left ventricular mass vs percentage decrease in DNA content in 4-week moxonidine-treated spontaneously hypertensive rat (SHR). *$P<0.01$ vs corresponding vehicle-treated groups. $n=6$–8 rats per group.

slower rate of left ventricular mass increase and a reduction in left ventricular DNA content over time. Changes in cardiac DNA content resulted from a sustained reduction in DNA synthesis and a transient increase in internucleosomal DNA fragmentation, a biological hallmark of apoptosis. The decrease in DNA content appeared during the fourth week of treatment. The mechanisms involved in the transient apoptosis induction included mild Bax and caspase-3 activation, opposed by later activation of Bcl-2. These studies show that moxonidine mainly inhibits DNA synthesis as part of the mechanisms involved in the control of cardiac mass.

Left ventricular hypertrophy is an independent risk factor for myocardial ischaemia, cardiac arrhythmia, congestive heart failure and sudden death. The prognostic significance

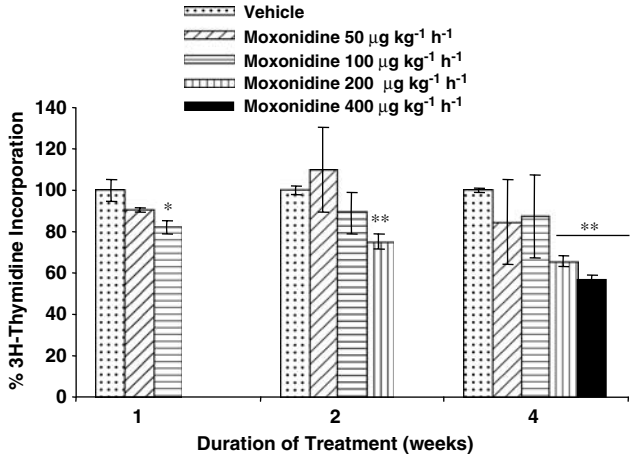

Figure 3 The effect of 1-, 2- and 4-week treatment with various concentrations of moxonidine on left ventricular ^{3}H-thymidine incorporation, presented as percentage of corresponding vehicle-treated rats (100%). *$P<0.04$; **$P<0.01$ vs corresponding vehicle-treated groups; $n=3$–5 rats per group.

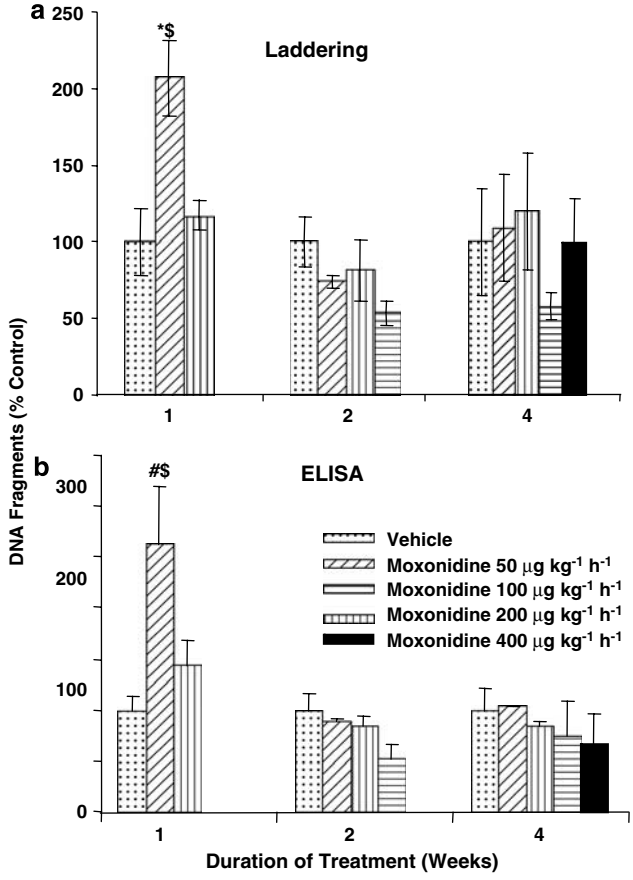

Figure 4 The effect of 1-, 2- and 4-week treatment with various concentrations of moxonidine on left ventricular DNA fragmentation. (**a**) DNA laddering, $n=3$–7 rats per group. (**b**) Cell death detection by ELISA, $n=3$–6 rats per group. *$P<0.01$; $^{\#}P<0.05$ vs corresponding vehicle-treated groups. $^{\$}P<0.05$ vs corresponding 100 μg kg^{-1} h^{-1} moxonidine-treated group. Data are presented as percentage of vehicle-treated control (100%).

is considerable, with an increased annual risk of cardiac death of up to 3% (Vakili *et al*., 2001). Regression of left ventricular mass with antihypertensive therapy is associated with improved diastolic function and overall reduction in cardiovascular events, independent of treatment modality and of decreases in blood pressure. These results support LVH regression as a therapeutic target in hypertension (Frey *et al*., 2004; Okin *et al*., 2004; Braz Nogueira, 2005).

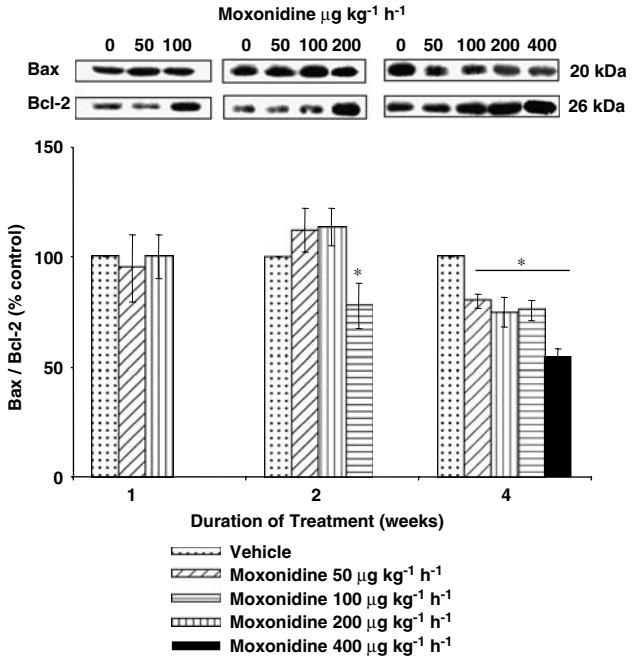

Figure 5 Representative western blot analysis of the effect of 1-, 2- and 4-week treatment with moxonidine on left ventricular Bax and Bcl-2 proteins. Column graph represents Bax/Bcl-2 ratio. *$P<0.01$ vs corresponding vehicle-treated spontaneously hypertensive rat (SHR); $n=5$–7 rats per group.

Left ventricular hypertrophy in hypertension involves both cellular hypertrophy (mainly in myocytes) and hyperplasia (mainly in fibroblasts), induced by angiotensin II, aldosterone, noradrenaline and endothelin. Cardiomyocyte hypertrophy is mediated by agonist stimulation of respective $G_{q/11}$-coupled receptors, which in turn activate phospholipase C (Singal *et al*., 2004), promoting cell growth. Schlaich *et al*. (2003) reported that in human hypertension, total systemic and cardiac noradrenaline spillover and LV mass index are increased and that LV mass index correlated with cardiac noradrenaline spillover, suggesting a growth-promoting effect of increased cardiac sympathetic tone on cardiomyocytes in hypertensive patients (Schlaich *et al*., 2003). Intravenous infusion of noradrenaline in normotensive rats resulted in increased ventricular myocyte TGF-β isoforms, which correlated with increased mRNA expression of collagens and matrix metalloproteinases. These effects were reduced by treatment with an α-adrenoceptor blocker, prazosin (Briest *et al*., 2004). *In vitro*, noradrenaline mimics many features of myocardial remodelling, including hypertrophy of individual myocytes and reinduction of fetal genes. Overexpression of β_1-adrenoceptors in the hearts of transgenic mice results in cardiomyocyte hypertrophy, fibrosis and progressive deterioration of cardiac performance (Engelhardt *et al*., 1999; Bisognano *et al*., 2000). Mice that lack dopamine β-hydroxylase, the essential enzyme for the synthesis of noradrenaline, exhibit a blunted hypertrophic

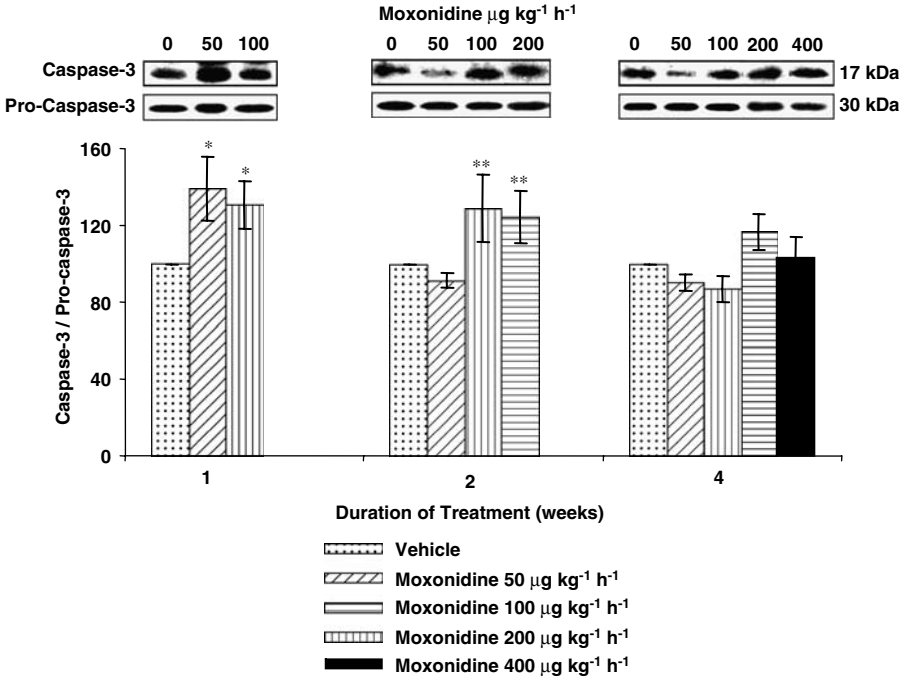

Figure 6 Western blot analysis of the effect of 1-, 2- and 4-week treatment with moxonidine on left ventricular pro- and cleaved caspase-3 protein expression. Column graph represents cleaved to pro-caspase-3 ratio. *$P<0.05$; **$P<0.01$ vs vehicle-treated spontaneously hypertensive rat (SHR). $n=6$–9 rats per group.

response (Esposito *et al*., 2002). On the other hand, fibroblast proliferation, also induced by active neurohormones in the heart, leads to fibrosis, which involves a disproportionate accumulation of the extracellular matrix proteins, collagen and fibronectin. The resulting increase in stiffness causes ventricular dysfunction and eventually heart failure. *In vivo* blockade of angiotensin II and catecholamines systems suppresses cardiac fibrosis (Masson *et al*., 2001; Peng *et al*., 2005).

Chronic treatment of hypertensive patients with the sympatholytic compounds, moxonidine and rilmenidine, results in blood pressure reduction and regression of LVH (Prichard, 1994; Bobik *et al*., 1998). Moxonidine also prevents development of LVH induced by myocardial infarction (Van Kerckhoven *et al*., 2004). In addition, moxonidine treatment of 6-month-old stroke- prone SHR (SHR-SP) reduced blood pressure and the degree of hypertrophy and myocardial fibrosis, capillarization and regressive changes of myocytes. Microarteriopathy and activation of nonvascular interstitial cells, which denote the first step in the development of interstitial myocardial fibrosis, were significantly suppressed by therapy (Amann *et al*., 1992).

Hamet *et al*. (1995) have shown that cardiac hypertrophy in SHR might be due, in part, to an imbalance between cell growth and apoptosis, favouring DNA accumulation. Consistent with this, the present studies show that moxonidine treatment was associated with inhibition of DNA synthesis as evidenced by lower ^{3}H-thymidine incorporation. Preliminary histological analysis revealed that cardiomyocyte size was dose-dependently reduced by treatment. These results indicate that moxonidine treatment hampers the growth signals involved in fibroblast proliferation and myocyte hypertrophy. On the other hand, moxonidine treatment is associated with transiently stimulated cardiac cell apoptosis, occurring early (within 1 week) after treatment and subsiding by 4 weeks. Both mechanisms lead to lower DNA accumulation and hence regression/prevention of LVH.

Apoptosis is a genetically regulated cell death involved in the deletion of cells in normal as well as malignant tissues. Apoptosis requires tightly regulated death pathways, including activation of cysteine proteases of the caspase family. The downstream apoptotic caspase-3 is in an inactive state ($\sim$30 kDa), and when an apoptotic stimulus occurs, it is cleaved into a biologically active peptide of $\sim$20 kDa and an inactive 12 kDa fragment. In the present study, moxonidine activated caspase-3 as early as 1 week of treatment, indicating stimulated apoptosis only early after treatment. Although moxonidine treatment was continued for 4 weeks, no further apoptosis occurred. The mechanisms underlying caspase activation are not entirely clear, but may include activation of cytochrome *c* released by mitochondria (James, 1998). Mitochondrial membrane is stabilized or destabilized by pro- and anti-apoptotic genes Bax and Bcl-2. Thus, higher Bax and lower Bcl-2 would stimulate the release of cytochrome *c,* which in turn, activates caspase-9 and subsequently, caspase-3 (Xu *et al*., 2005). The relative proportion of Bax to Bcl-2 after 1 and 2 weeks of treatment was not altered by the lower doses of moxonidine, but was reduced by 4 weeks, favouring anti-apoptosis. This response was contrasted by moxonidine-induced caspase-3 and Bax activation after 1 week of treatment, indicating a transient early apoptotic effect. These actions of moxonidine are similar, albeit of a lower magnitude, to those achieved by ACE inhibition in SHR, where enalapril-induced regression of cardiac hypertrophy was associated with increased apoptotic activity (Tea *et al*., 1999).

The increase in DNA fragmentation appeared larger with the 50 μg dose of the drug, a finding confirmed by two different methods. We postulate that a faster rate for transient apoptosis was induced in response to the higher dose (100 μg) that would be already decreased by 1 week. Further experiments measuring DNA fragmentation at lower doses and at time points earlier than 1 week may confirm this postulate. The time- and dose-dependent dynamic effect on DNA fragmentation was not reflected on DNA content, which was progressively reduced by all doses mainly after 4 weeks. This may be explained by the slightly larger inhibition of DNA synthesis with the higher doses of moxonidine, since DNA content is the result of a balance between DNA synthesis and degradation.

Prevention of LVH in these animals may be secondary to, albeit modest, reduction of blood pressure by moxonidine. However, these studies cannot rule out, at least in part, a pressure-independent effect. We have previously shown a pressure-independent control of left ventricular mass by moxonidine in comparison to hydralazine, a direct vasodilator. Despite equal blood pressure reduction, chronic treatment with hydralazine does not result in LVH regression (Menaouar *et al*., 2002). In addition, our studies have shown that in contrast to enalapril and losartan, reduction in cardiac workload by hydralazine was not associated with changes in cardiac mass, thymidine incorporation *in vivo*, cardiac DNA content or apoptosis within 4 weeks, despite the potent antihypertensive effect of hydralazine (Tea *et al*., 1999). These studies with hydralazine clearly demonstrate that cardiac growth and apoptosis can be dissociated from blood pressure regulation.

On the other hand, the sympatholytic effect of moxonidine was more evident on heart rate, causing long-lasting bradycardia. The bradycardia has been shown to result from moxonidine primarily acting centrally on imidazoline I_1-receptors and α_2-adrenoceptors in brainstem rostral ventrolateral medulla as well as on hindbrain sites such as the nucleus of the solitary tract, nucleus ambiguus and the dorsal motor nucleus of the vagus to facilitate vagal response (Haxhiu *et al*., 1994; Moreira *et al*. 2007). In contrast to its vasodilatory and hypotensive effects, centrally mediated bradycardia by moxonidine does not involve nitric oxide (Moreira *et al*., 2004). The reduction in sympathetic activity in the heart may have contributed to moxonidine-reduced LVH. Consistent with this, β-adrenoceptor blockade in SHR reduces left ventricular DNA via apoptosis during regression of cardiac hypertrophy (Tea *et al*., 1999). It is intriguing to speculate that the effect on cardiac apoptosis may also be locally mediated and specific to moxonidine activation of imidazoline receptors that we have identified in the heart (El-Ayoubi *et al*., 2002). However, the contribution of cardiac imidazoline receptors remains to be proven.

The cell type targeted by moxonidine treatment and resulting in apoptosis has not been determined in the

present studies, but it is unlikely that moxonidine resulted in loss of myocytes. The myocytes account for 70% of the normal cardiac mass; therefore, if the more than 30% reduction in DNA content, measured in the present study, occurred mainly in myocytes, the heart weight would be drastically reduced and the heart function would deteriorate, and then fail; these were not observed in moxonidine-treated rats. The moxonidine-treated rats showed activated Bcl-2 and reduced Bax, hence a significant reduction in Bax to Bcl-2 ratio after 4 weeks of treatment, which may protect cardiac myocytes from apoptosis and was associated with back to normal concentrations of atrial natriuretic peptide (ANP) mRNA and brain natriuretic peptide (BNP) mRNA in the LV (El-Ayoubi *et al*., 2003), markers of regression of LVH and treatment efficacy. Also, previous studies have shown that moxonidine has an antifibrotic effect (Ziegler *et al*., 1996), thus implicating fibroblasts in the action. In agreement, treatment of SHR with inhibitors of the renin-angiotensin system is associated with regression of cardiac hypertrophy by apoptotic mechanisms that occur in fibroblasts (Der Sarkissian *et al*., 2003).

In conclusion, these studies reveal a cardioprotective effect of moxonidine on the hypertensive rat heart. This effect is expressed as a diminished increase of left ventricular mass and a reduced DNA content, resulting from a sustained reduction of DNA synthesis and transient stimulation of DNA fragmentation that occur early after treatment. Future experiments should investigate the cardiac cell type targeted by moxonidine.

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Conflict of interest

The authors state no conflict of interest.

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