

**IMIDAZOLINE RECEPTORS BUT NOT ALPHA2 ADRENOCEPTORS  
ARE REGULATED IN SHR HEART BY CHRONIC MOXONIDINE  
TREATMENT**

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Running title: Cardiac Imidazoline I<sub>1</sub> receptors in SHR

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## Abstract

We have recently identified imidazoline I<sub>1</sub>-receptors in the heart. In the present study, we tested regulation of cardiac I<sub>1</sub>-receptors vs. α<sub>2</sub> adrenoceptors in response to hypertension and to chronic exposure to agonist. Spontaneously hypertensive rats (SHR, 12-14 weeks old) received moxonidine (10, 60 and 120 μg/kg/h, s.c.) for 1 and 4 weeks. Autoradiographic binding of <sup>125</sup>I-paraiodoclonidine (<sup>125</sup>I-PIC, 0.5 nM, 1h, 22°C) and inhibition of binding with epinephrine (10<sup>-10</sup> to 10<sup>-5</sup> M) demonstrated the presence of α<sub>2</sub>-adrenoceptors in heart atria and ventricles. Immunoblotting and RT-PCR identified α<sub>2A</sub>- α<sub>2B</sub>- and α<sub>2C</sub>-adrenoceptor proteins and mRNA, respectively. However, compared to normotensive controls, cardiac α<sub>2</sub>-adrenoceptor kinetic parameters, receptor proteins, and mRNAs were not altered in SHR with or without moxonidine treatment. In contrast, autoradiography showed that up-regulated atrial I<sub>1</sub>-receptors in SHR are dose-dependently normalized by 1 week, with no additional effect after 4 weeks of treatment. Moxonidine (120 μg/kg/h) decreased B<sub>max</sub> in right (40.0±2.9 to 7.0±0.6 fmol/unit area, p<0.01) and left (27.7±2.8 to 7.1±0.4 fmol/unit area, p<0.01) atria, and decreased the 85 and 29 kDa imidazoline receptor protein bands, in right atria, to 51.8±3.0% (p<0.01) and 82.7±5.2% (p<0.03) of vehicle-treated SHR, respectively. Moxonidine-associated percent decrease in B<sub>max</sub> only correlated with the 85 kDa protein (R<sup>2</sup> = 0.57; p<0.006), suggesting that this protein may represent I<sub>1</sub>-receptors. The weak but significant correlation between the two imidazoline receptor proteins (R<sup>2</sup> = 0.28; p<0.03), implies that they arise from the same gene. In conclusion, the heart possesses I<sub>1</sub>-receptors and α<sub>2</sub> adrenoceptors, but only I<sub>1</sub>-receptors are responsive to hypertension and to chronic *in vivo* treatment with a selective I<sub>1</sub>-receptor agonist.

## Introduction

Most of the centrally acting antihypertensive drugs, such as clonidine and related imidazoline derivatives mediate sympathoinhibition, not only via activation of central nervous  $\alpha_2$ -adrenoceptors, but also via imidazoline  $I_1$ -receptors (Bousquet, 1997; Bricca et al, 1989). Imidazoline  $I_1$ -receptors are non-adrenergic and non-cholinergic neurotransmitter receptors that possess low affinity for norepinephrine and other catecholamines.  $I_1$ -receptors are mainly found in the brainstem, adrenal chromaffin cells and kidneys. In addition, we have recently identified  $I_1$ -receptors in heart atria and ventricles, and shown that atrial  $I_1$ -receptors are up-regulated in rat hypertension and ventricular  $I_1$ -receptors are up-regulated in human and hamster heart failure (El-Ayoubi et al., 2002a). In other studies we demonstrated that acute injections of moxonidine, an imidazoline compound that shows 40 times higher affinity to  $I_1$ -receptor vs.  $\alpha_2$ -adrenoceptors, are associated with enhanced release of atrial natriuretic peptide (ANP) (Mukaddam-Daher and Gutkowska, 2000), a cardiac hormone involved in pressure and volume homeostasis. Taken together, these studies led us to suggest that heart  $I_1$ -receptors are functional and may be involved in cardiovascular regulation.

Previous binding studies reported  $^3\text{H}$ -idazoxan binding sites ( $I_2$ -receptors) but not  $I_1$ -receptors in human atrial appendage, but functionally, these receptors were different from presynaptic imidazoline receptors implicated in inhibition of noradrenaline release. Accordingly, atrial presynaptic imidazoline receptors were considered non- $I_1$  non- $I_2$  receptors, and the effects of moxonidine to inhibit noradrenaline release in atrial appendages were attributed to presynaptic  $\alpha_2$ -adrenoceptors (Molderings et al, 1999). In contrast, consistent with the presence of  $I_1$ -receptors in the heart, Schäfer et al (2003) have recently shown in isolated perfused rats hearts that moxonidine is able to decrease noradrenaline release independently of  $\alpha_2$ -adrenoceptors.

In fact, functional separation between imidazoline I<sub>1</sub>-receptors and  $\alpha_2$ -adrenoceptors is rather difficult, because these receptors are often co-localized and ligands with affinity to imidazoline I<sub>1</sub>-receptors also bind to  $\alpha_2$ -adrenoceptors (Bousquet, 1997). However, previous studies indicate that imidazoline receptors and  $\alpha_2$  adrenoceptors are subject to pathophysiological and pharmacological regulation (Yakubu et al., 1990; Ivanov et al., 1998; Zhu et al., 1997; Ernsberger et al., 1991). Therefore, the aim of the present studies was to test regulation of cardiac I<sub>1</sub>-receptors vs.  $\alpha_2$  adrenoceptors, by showing that I<sub>1</sub>-receptors, but not  $\alpha_2$  adrenoceptors are regulated in hypertension and in response to exposure to agonist. Accordingly, studies were performed to: 1) demonstrate the presence of  $\alpha_2$  adrenoceptors in the heart and their possible regulation in hypertension, and 2) to investigate the effect of chronic *in vivo* exposure to moxonidine on I<sub>1</sub>-receptors and  $\alpha_2$  adrenoceptors in hearts of normotensive rats and spontaneously hypertensive rats (SHR) with established hypertension.

## Methods

Female Spontaneously Hypertensive Rats (SHR, 12–14 weeks old) with established hypertension and age-matched normotensive Wistar-Kyoto (WKY) and Sprague Dawley (SD) rats were purchased from Charles River (St. Constant, QC). Animals were housed in temperature and light controlled room with food and water *ad libitum*, and maintained for at least 3 days before experimentation. Experiments were performed following the approval of the Bioethics Committee of CHUM, according to the Canadian Guidelines.

Alzet osmotic minipumps (2ML1 & 2ML4, Alzet Corp.) were implanted subcutaneously in SHR, under isoflurane anesthesia, as we have previously described (Menaouar et al., 2002). These mini-pumps allowed continuous delivery of moxonidine (Generous gift from Solvay Pharmaceuticals, Germany) or saline vehicle at the rate of 10  $\mu\text{L/h}$  (2ML1), for one week, and 2.5  $\mu\text{L/h}$  (2ML4) for 4 weeks. The concentrations of moxonidine were adjusted to allow delivery of 10, 60 & 120  $\mu\text{g/kg/h}$ . The solution of moxonidine was prepared by dissolving the drug in isotonic saline,  $\text{pH} < 6.5$ , then  $\text{pH}$  adjusted to 7.0–7.4 by NaOH. Rats were sacrificed after 1 and 4 weeks of vehicle and moxonidine treatment, and heart atria and ventricles were separated, snap-frozen in pre-chilled isopentane, then stored at  $-80^{\circ}\text{C}$ , for receptor analysis by autoradiographic binding, immunoblotting, and RT-PCR.

To rule out the influence of blood pressure on receptor regulation, another group of SHR was treated with hydralazine, given at 30  $\text{mg/kg/day}$ , in drinking water, for 1 week. The effectiveness of hydralazine was verified by tail cuff measurement of systolic blood pressure before and after 1-week treatment. Then, rats were sacrificed and tissues collected as described above.

### ***Autoradiography***

Autoradiography of heart I<sub>1</sub>-receptors and α<sub>2</sub>-adrenoceptors was performed on frozen heart sections from WKY and SD rats, and from saline- and moxonidine-treated SHR, using radiolabelled paraiodoclonidine (<sup>125</sup>I-PIC; 2200 Ci/mmol; New England Nuclear, Boston, MA) as we have previously described (El-Ayoubi et al., 2002a). Because <sup>125</sup>I-PIC binds to both receptor types, autoradiography was performed, separately, in conditions that favor α<sub>2</sub>- adrenoceptor binding and in conditions that favor I<sub>1</sub>-receptor binding. For α<sub>2</sub>-adrenoceptors, the slides were incubated for 1h at 22<sup>0</sup>C with 0.5 nM <sup>125</sup>I-PIC in incubation buffer: in mM 50 Tris-HC (pH 7.7), 5 EDTA, 5 EGTA, 10 MgCl<sub>2</sub>, and 50 μM phenylmethylsulfonyl fluoride (PMSF). Binding was inhibited by increasing concentrations of epinephrine (10<sup>-10</sup> to 10<sup>-5</sup> M). Binding in the presence of 10<sup>-4</sup> M piperoxan was considered non-specific. After several washes, the slides were dried, exposed in phosphor-sensitive cassette for 48h, then scanned, visualized, and quantified by PhosphorImager (ImageQuant, Molecular Dynamics, Sunnyvale, CA).

Autoradiography for I<sub>1</sub>-receptors was performed under identical incubation conditions, except for prior incubation of slides with 1 mM phenoxybenzamine and 0.5 mM ethylmaleimide for 35 min at room temperature, to irreversibly inhibit adrenoceptor binding; and by decreasing the concentration of MgCl<sub>2</sub> in the incubation buffer to 0.5 mM, conditions that favor binding to I<sub>1</sub>-receptors (Ernsberger et al., 1995)]. Binding of <sup>125</sup>I-PIC was competitively inhibited by increasing concentrations (10<sup>-12</sup> to 10<sup>-5</sup> M) of moxonidine

### ***Membrane preparation and Immunoblotting***

Membranes of ventricular and atrial tissues were prepared in sucrose buffer as previously described (El-Ayoubi et al, 2002a). Protein content was measured spectrophotometrically, using BSA as standard.

Immunoblotting was performed (El-Ayoubi et al., 2002a) using 30  $\mu\text{g}$  denatured protein samples from cardiac tissues and incubation of blots with rat  $\alpha_{2A}$ -,  $\alpha_{2B}$ -,  $\alpha_{2C}$ -adrenoceptor antiserum (1:500, Santa-Cruz Biotech), or anti-imidazoline receptor antiserum and non-immune antiserum (Generous gift from S. Regunathan, Jackson, MS) diluted 1:1000, or with anti- $\beta$ -actin (1:500). The blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antiserum (1:5000). Immunoreactive bands were visualized by Amersham's enhanced chemiluminescence (ECL) detection system (Amersham ECL hyperfilm), according to the manufacturer's instructions.

#### ***Total RNA Extraction and RT-PCR.***

Total RNA was extracted from the rat heart tissues using Trizol reagent (Life Technologies, Inc.,) according to the protocol described by the manufacturer. PCR reactions were performed (Zou and Cowley, 2000) using specific primer pairs for rat  $\alpha_{2A}$ -,  $\alpha_{2B}$ -,  $\alpha_{2C}$ - receptors, or  $\beta$ -actin (Operon Technologies, Alameda, CA, USA). After electrophoresis on agarose gel in the presence of ethidium bromide, fluorescent PCR products were scanned, counted and analyzed with the ImageQuant software. These data were normalized to the corresponding values of  $\beta$ -actin PCR product in the same samples.

#### ***Data Analysis***

The equilibrium dissociation constant (Kd) and maximum binding capacity (Bmax) for the ligands used in autoradiography were calculated by the non-linear method using the Ligand computer program (Elsevier-Biosoft, Cambridge, UK). Densitometric measurements of immunoblots were performed using Scion computer program (NIH, Bethesda, MD). Correlation coefficients were calculated from linear regression (GraphPad Prism; GraphPad Software, Inc.,



San Diego CA). Differences in data obtained from vehicle- or moxonidine-treated rats were compared by non-paired Student's t-test.  $P < 0.05$  was considered significant. All data are expressed as mean  $\pm$  SEM.

## Results

### Cardiac $\alpha_2$ -adrenoceptors

Autoradiographic binding of  $^{125}\text{I}$ -PIC to heart atrial and ventricular sections was inhibited by increasing concentrations of epinephrine. Kinetic parameters obtained from competitive inhibition curves (Table I) revealed that  $\alpha_2$ -adrenoceptor affinity ( $K_d \cong 2.5$  nM) and Bmax in right atria ( $12.8 \pm 0.7$  vs.  $13.4 \pm 0.9$  fmol/unit area), left atria ( $12.8 \pm 0.4$  vs.  $11.7 \pm 0.7$  fmol/unit area) and left ventricles ( $11.7 \pm 1.1$  vs.  $12.2 \pm 0.5$  fmol/unit area) were not altered in SHR as compared to WKY rats. Binding to cardiac  $\alpha_2$ -adrenoceptors in SHR was also not altered by chronic *in vivo* moxonidine treatment. Bmax remained in 120  $\mu\text{g}/\text{kg}/\text{h}$  moxonidine-treated SHR right atria at  $11.9 \pm 0.9$  fmol/unit area and  $K_d$  at  $2.3 \pm 0.3$  nM. Similarly, kinetic parameters obtained in left atria and left ventricles were not altered in vehicle- or moxonidine-treated SHR (Table I).

Three  $\alpha_2$ -adrenoceptor subtypes were identified in cardiac tissues of SD and SHR, by immunoblotting. Densitometric measurements of the bands corresponding to  $\alpha_{2A}$ - (Fig. 1),  $\alpha_{2B}$ - and  $\alpha_{2C}$ -adrenoceptors (not shown) were not significantly different in right atria and left ventricles of SHR vs. SD, nor in vehicle- and moxonidine-treated SHR, where variation did not exceed 10%. Furthermore, levels of three subtypes of  $\alpha_2$ -adrenoceptor mRNA detected in right and left atria and left ventricles of SHR were also not significantly different among vehicle- or moxonidine-treated groups, where variation did not exceed 10% (Fig. 2).

### Cardiac Imidazoline Receptors

Autoradiography showed that total specific binding of  $^{125}\text{I}$ -PIC to  $I_1$ -receptors was higher in SHR atria (162%) as compared to normotensive WKY rats, considered as 100%. Also, total specific

binding in atria decreased after treatment with moxonidine at 10, 60 and 120  $\mu\text{g}/\text{kg}/\text{h}$  for 1 week (Fig. 3).

Competitive inhibition curves were plotted from values obtained from normotensive and hypertensive vehicle- and moxonidine-treated rats and presented as percent  $B/B_0$ , where B and  $B_0$  represent respectively, binding with and without moxonidine (Fig. 3). Kinetic parameters calculated from these curves using the Ligand computer program, revealed that 1-week treatment dose-dependently decreased  $B_{\text{max}}$  in SHR right and left atria. At the lowest dose of 10  $\mu\text{g}$  moxonidine,  $B_{\text{max}}$  decreased from  $40.0 \pm 2.9$  to  $18.2 \pm 0.4$  fmol/unit area ( $p < 0.01$ ) in right atria, and from  $27.7 \pm 2.8$  to  $12.3 \pm 0.6$  fmol/unit area ( $p < 0.04$ ) in left atria. The doses of 60 and 120  $\mu\text{g}$  moxonidine decreased  $B_{\text{max}}$  in rat right and left atria to values not significantly different from 2 normotensive controls (Table II). Four-week treatment did not have additional effects, so that at 120  $\mu\text{g}$  moxonidine,  $B_{\text{max}}$  in right atria represented  $9.0 \pm 0.3$  fmol/unit area. Moxonidine treatment did not affect  $B_{\text{max}}$  and  $K_d$  of  $I_1$ -receptors in right and left ventricles of moxonidine- and vehicle-treated SHR (Table II).

The presence of three immunoreactive imidazoline receptor protein bands was shown in cardiac tissues by immunoblotting. The apparent molecular masses of these proteins were around 160, 85, and 29/30 kDa. Densitometric measurements of bands corresponding to the 160 kDa band was only slightly increased in atria of SHR, and almost not detected in normotensive SD rats and in moxonidine-treated SHR for 1 or 4 weeks. On the other hand, the density of bands corresponding to 85 kDa protein increased significantly ( $p < 0.05$ ) in right atria of SHR to  $134.6 \pm 3.3\%$  compared to normotensive control (considered as 100%).

Fig. 4 shows that, compared to vehicle-treated SHR (considered as 100%), chronic moxonidine treatment resulted in a significant ( $p < 0.01$ ) decrease in the intensity of the bands

corresponding to 85 kDa proteins to represent  $83.4 \pm 1.9\%$ ,  $59.9 \pm 2.7\%$ , and  $51.8 \pm 3.0\%$  in 10, 60 and 120  $\mu\text{g}$  moxonidine-treated SHR for 1 week, respectively. Treatment with moxonidine at 60 and 120  $\mu\text{g}/\text{kg}/\text{h}$  for 4 weeks, resulted in a mild additional decrease in the intensity of the 85 kDa bands to  $51.1 \pm 2.2\%$  and  $46.8 \pm 3.3\%$ , respectively (Fig. 4). A modest increase in the intensity of  $\sim 29$  kDa band (not shown) was observed in right atria of SHR ( $107 \pm 2\%$ ) that decreased to  $94 \pm 1\%$ ,  $87 \pm 3\%$ , and  $83 \pm 5\%$  after one week of moxonidine at 10, 60 and 120  $\mu\text{g}$ , respectively. In left ventricles, chronic treatment of SHR with moxonidine (120  $\mu\text{g}$ ) for 1 and 4 weeks did not alter the intensity of the bands corresponding to 85 kDa, but slightly decreased the 29 kDa band to  $89 \pm 3\%$ , and after 4 weeks to  $89 \pm 1\%$ .

Compared to corresponding WKY (considered as 100%), the percent increase in SHR right atrial Bmax correlated with the percent increase in the density of the 85 kDa band ( $R^2 = 0.7744$ ;  $p < 0.03$ ), but not with the 29 kDa protein band. Moxonidine treatment resulted in a dose-dependent decrease in Bmax and in the 85 kDa band as compared to corresponding saline-vehicle treated SHR (considered as 100%). Fig. 5 shows that the percent decrease in Bmax correlated with the percent decrease in the 85kDa band ( $R^2 = 0.5700$ ;  $p < 0.006$ ), but not with the 29 kDa band ( $R^2 = 0.1754$ ; N.S.), suggesting that the 85 kDa protein may represent imidazoline I<sub>1</sub>-receptors in the heart. A weak, but significant correlation was found between the 85 kDa and the 29 kDa protein band ( $R^2 = 0.2717$ ;  $p < 0.03$ ), in moxonidine-treated SHR, implying that the two receptor proteins arise from the same gene.

Treatment of SHR with hydralazine for 1 week, resulted in blood pressure reduction from  $193 \pm 8$  to  $135 \pm 5$  mmHg,  $p < 0.02$ , whereas blood pressure remained in control rats at  $186 \pm 11$  mmHg. However, hydralazine treatment did not alter imidazoline receptor proteins measured by immunoblotting.

## Discussion

The major findings of this study are: 1) First time localization of  $\alpha_2$ -adrenoceptors in heart atria and ventricles; and 2) demonstration that heart imidazoline I<sub>1</sub>-receptors but not  $\alpha_2$ -adrenoceptors are regulated in SHR, and in response to chronic *in vivo* exposure to a selective imidazoline receptor agonist, suggesting that heart I<sub>1</sub>-receptors are subject to regulation. In addition, 3) the parallel change in receptor B<sub>max</sub> and the 85 kDa imidazoline receptor protein, suggest that this protein may represent cardiac I<sub>1</sub>-receptors.

Pharmacologic and molecular cloning studies have revealed three  $\alpha_2$ -adrenoceptor subtypes:  $\alpha_{2A}$  ( $\alpha_{2D}$  in rats),  $\alpha_{2B}$ , and  $\alpha_{2C}$  (Link et al., 1996; Altman et al., 1999). In the human heart, mRNA for all 3  $\alpha_2$ -adrenoceptors subtypes have been detected by PCR (Brodde and Michel, 1999). However, probably due to very low expression relative to  $\alpha_1$ - and  $\beta_1$ -adrenoceptors, previous studies have not been successful in demonstrating  $\alpha_2$ -adrenoceptors in the heart at the protein level through radioligand binding studies (Brodde and Michel, 1999). In the present study, demonstration of  $\alpha_2$ -adrenoceptors in the heart was achieved by multiple approaches. Quantitative receptor autoradiography was used in conditions where binding of <sup>125</sup>I-PIC to adrenoceptors vs. I<sub>1</sub>-receptors was optimized by using high MgCl<sub>2</sub> (10 mM) concentration in the incubation buffer (Ernsberger et al., 1995). Furthermore, because radioligands cannot fully discriminate between  $\alpha_2$ -adrenoceptor subtypes, further identification was obtained by immunoblots and RT-PCR, using specific rabbit polyclonal antibodies and primers for each subtype (Zou and Cowley, 2000). However, cellular localization of these receptors needs further experiments, since receptor subtypes were detected in whole cardiac tissue which involves several cell types, including fibroblasts and myocytes, myocardial blood vessels, nerve terminals and intracardiac neurons (Armour, 1999).

The  $\alpha_{2A}$ , abundant in the CNS, mainly in brain stem, is directly involved in regulating sympathetic outflow, appears to be the major presynaptic autoinhibitory receptor subtype (Altman et al, 1999). The  $\alpha_{2B}$  is more abundant in arterial vascular smooth muscle cells and mostly responsible for vasoconstriction, and is responsive to altered salt handling. The function of  $\alpha_{2C}$  is not yet clear, but it may be the presynaptic autoreceptor in human atria (Rump et al., 1995; Hein, 2001).

The physiological significance of  $\alpha_2$ -adrenoceptors in various heart chambers is beyond the scope of the present study. This study, however, provides strong evidence that  $\alpha_2$ -adrenoceptors are present, albeit at low levels, in the rat heart atria and ventricles, at the levels of synthesis, protein expression and binding activity, but these receptors appear not to be regulated by moxonidine, a selective agonist of imidazoline I<sub>1</sub>-receptors. Since brain  $\alpha_2$ -adrenoceptors have been shown to be selectively down-regulated in response to  $\alpha_2$ -adrenoceptor agonists (Yakubu et al., 1990), and kidney imidazoline receptors to be down-regulated in response to imidazoline receptor agonists (Hamilton et al., 1993), the present findings imply that  $\alpha_2$ -adrenoceptors in the heart interact weakly with moxonidine.

Most importantly, the present study confirms our previous finding that I<sub>1</sub>-receptors are present in the heart, and extend to demonstrate that up-regulated atrial I<sub>1</sub>-receptors in SHR (El-Ayoubi et al., 2002a) are normalized by chronic *in vivo* exposure to I<sub>1</sub>-receptor agonist. These receptors appear to be unrelated to imidazoline I<sub>2</sub>-receptors, previously identified in the heart (Molderings and Gothert, 1999), because, by definition, the ligands used in the present study (<sup>125</sup>I-PIC and moxonidine) show very low affinity to I<sub>2</sub>-receptors (Bousquet, 1997).

Immunoblotting of heart membranes showed multiple molecular mass imidazoline receptor proteins similar to those so far described in brain and heart (El-Ayoubi et al., 2002a).

Levels of 85 kDa proteins were increased in atria of untreated SHR as compared to normotensive rats. Chronic moxonidine treatment, for short and long duration, was associated with decreased density of the 85 kDa bands in SHR atria. It is interesting to note that atrial 85 kDa but not the 29 kDa imidazoline receptor proteins vary in parallel to values of  $B_{max}$  for  $I_1$ -sites determined by  $^{125}I$ -PIC binding. This correlation leads us to propose that the 85 kDa protein may represent  $I_1$ -receptors in the heart, as has been suggested by Ivanov et al. (1998). Furthermore, the positive correlation between changes in the two receptor proteins, implies that they may arise from the same gene.

Regulation of imidazoline receptors has been previously reported in other tissues and under different physiological and pharmacological manipulations, usually in a manner distinct from  $\alpha_2$ -adrenoceptors. Chronic treatment with the prototypic antidepressant imipramine, down-regulates  $I_1$ -receptors in rat brainstem, without affecting  $\alpha_2$ -adrenoceptors (Zhu et al, 1997). Also, renal  $I_1$ -receptors are up-regulated by subpressor doses of angiotensin II infusion (Ernsberger et al., 1991), and in kidneys of SHR (El-Ayoubi et al., 2002b), while  $\alpha_2$ -adrenoceptors are either unchanged or decreased in 1K1C rat kidneys (Li et al., 1994).

In the present study, imidazoline receptors were not different in hearts of 2 normotensive strains, WKY and SD, but up-regulated in SHR hearts, then normalized and down-regulated after rat treatment with moxonidine, for short or long duration. The mechanisms involved in receptor up-regulation may include sympathetic over-activity, elevated blood pressure, increased cardiac mass and activated intracardiac neurohormones, such as angiotensin II and norepinephrine. Treatment with moxonidine inhibits or counteracts these effects, and eventually may indirectly lead to down-regulation of its receptor. We have previously shown that moxonidine dose-dependently reduced blood pressure in SHR (Menaouar et al., 2002). However, the dose of 10  $\mu$ g

moxonidine, which had no effect on blood pressure in those rats (Menaouar et al., 2002), significantly reduced I<sub>1</sub>-receptor protein and Bmax. Furthermore, treatment of SHR with hydralazine, a vasodilator antihypertensive compound that reduced blood pressure to a similar magnitude achieved by 120 µg moxonidine, had no effect on imidazoline receptor proteins. Further studies are needed to clarify the mechanisms involved, but the present results argue against receptor down-regulation occurring in response to reduction in blood pressure per se, and in favor of a direct effect of the ligand on the receptor. Other investigators demonstrated that stimulation of I<sub>1</sub>-receptor with moxonidine leads to activation of PC-PLC and generation of DAG, which activates several isoforms of protein kinase C (PKC) (Ernsberger, 1999). PKC results in functional desensitization of the I<sub>1</sub>-receptor through phosphorylation of serine and threonine residues in the receptor intracellular loop (Eason and Ligett, 1996)].

In conclusion, this study demonstrates that heart I<sub>1</sub>-receptors but not α<sub>2</sub>-adrenoceptors are up-regulated in SHR and normalized by chronic antihypertensive treatment with moxonidine. Cardiac I<sub>1</sub>-receptor normalization occurred after 1-week of treatment, the time point when moxonidine resulted in reversal of left ventricular hypertrophy in these rats (Menaouar et al., 2002). Also, the presence of I<sub>1</sub>-receptors in atria, tissues known to secrete or respond to natriuretic peptides, ANP and BNP, suggest a functional relationship between the two systems. Therefore, heart I<sub>1</sub>-receptors are subject to regulation by the cardiovascular environment. Future antihypertensive treatment with imidazoline drugs should consider the heart as a major target organ.



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## Figure Legends

**Fig. 1:** Representative immunoblot of  $\alpha_2$ -adrenoceptor subtypes ( $\alpha_{2A}$ ,  $\alpha_{2B}$ ,  $\alpha_{2C}$ ) and  $\beta$ -actin, and densitometric data of  $\alpha_{2A}$  measured in right atria and left ventricles of SD and SHR after treatment with moxonidine (0, 60 & 120  $\mu\text{g}/\text{kg}/\text{h}$ ) for 1 and 4 weeks. Data normalized to corresponding  $\beta$ -actin are presented as percent change from SD and vehicle-treated SHR.

**Fig. 2:** RT-PCR mRNA products of  $\alpha_2$ -adrenoceptors and  $\beta$ -actin in right and left atria and left ventricles of SHR treated with moxonidine (0, 60 & 120  $\mu\text{g}/\text{kg}/\text{h}$ ) for 4 weeks.

**Fig. 3:** Top: Representative autoradiography of total  $^{125}\text{I}$ -PIC binding to right atrial tissue sections (after irreversible inhibition of  $\alpha$ -adrenoceptor binding) in WKY and SHR after 1-week treatment with moxonidine (0, 10, 60, 120  $\mu\text{g}/\text{kg}/\text{h}$ ).

Bottom: Specific binding of  $^{125}\text{I}$ -PIC to right atrial sections in WKY and SHR after 1-week treatment with moxonidine (0, 10, 60, 120  $\mu\text{g}/\text{kg}/\text{h}$ ). Data are presented as  $\%B/B_0$ , where B and  $B_0$  represent binding in the absence and presence of increasing concentrations ( $10^{-12}$  to  $10^{-5}$  M) of inhibiting ligand.

**Fig. 4:** Representative immunoblot and densitometric measurement of the 85 kDa imidazoline receptor protein in right atria of SHR after treatment with moxonidine (0, 10, 60 & 120  $\mu\text{g}/\text{kg}/\text{h}$ ) for 1 week and 60 & 120  $\mu\text{g}/\text{kg}/\text{h}$  for 4 weeks. Data normalized to corresponding  $\beta$ -actin are presented as percent change from vehicle-treated SHR (considered as 100%). \* $p < 0.01$  vs. vehicle-treated SHR.

**Fig. 5:** Top: Correlation between percent decrease in moxonidine-treated SHR (vehicle-treated SHR considered as 100%) right atrial Bmax obtained from competitive binding assays vs. percent decrease in the density of the 29 kDa (dotted line) and 85 kDa (solid line) bands obtained by immunoblotting.

Bottom: Correlation between percent change in the density of right atrial 85 kDa vs. 29 kDa bands obtained by immunoblotting.

**Table 1. Kinetic Parameters of Cardiac  $\alpha_2$ -adrenoceptors.**

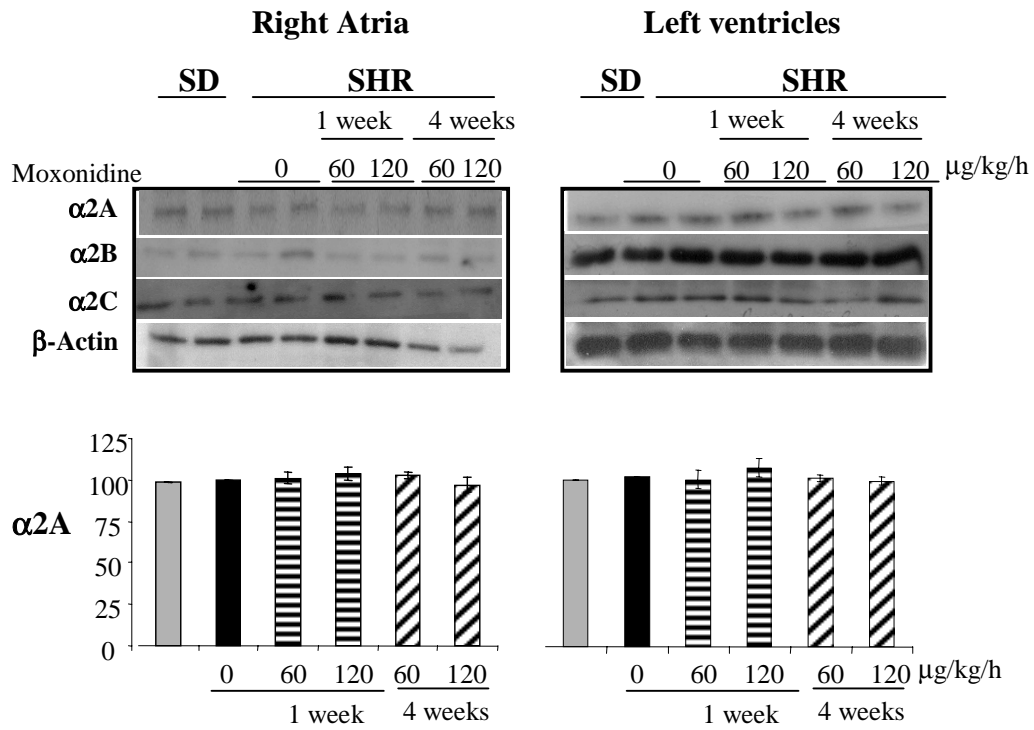
Moxonidine ( $\mu\text{g/kg/h}$ )	WKY	SHR		
	0	0	60	120
<b>Right Atria</b>				
Bmax (fmol/unit area)	12.8 $\pm$ 0.7	13.4 $\pm$ 0.9	11.5 $\pm$ 0.7	11.9 $\pm$ 0.9
Kd (nM)	2.3 $\pm$ 0.1	2.4 $\pm$ 0.3	2.2 $\pm$ 0.3	2.3 $\pm$ 0.3
<b>Left Atria</b>				
Bmax (fmol/unit area)	12.8 $\pm$ 0.4	11.7 $\pm$ 0.7	11.9 $\pm$ 0.7	12.5 $\pm$ 0.3
Kd (nM)	2.5 $\pm$ 0.3	2.1 $\pm$ 0.1	3.3 $\pm$ 0.5	2.2 $\pm$ 0.2
<b>Left Ventricles</b>				
Bmax (fmol/unit area)	11.7 $\pm$ 1.1	12.2 $\pm$ 0.5	11.6 $\pm$ 0.6	12.0 $\pm$ 0.4
Kd (nM)	2.5 $\pm$ 0.5	2.3 $\pm$ 0.3	2.9 $\pm$ 0.2	2.3 $\pm$ 0.4

**Table II. Kinetic Parameters of Cardiac I<sub>1</sub>-receptors.**

Moxonidine (µg/kg/h)	SD	WKY	SHR			
	0	0	0	10	60	120
<b>Right Atria</b>						
Bmax (fmol/unit area)	18.3±1.4	21.8±1.4	40.0±2.9*	18.2±0.4**	10.2±1.7**	7.0±0.6**
Kd (nM)	3.9±0.7	4.8±0.4	5.8±0.9	3.7±1.2	5.1±0.6	4.8±0.5
<b>Left Atria</b>						
Bmax (fmol/unit area)	16.0±2.6	12.2±1.1	27.7±2.8*	12.3±0.6**	7.9±1.1**	7.1±0.4**
Kd (nM)	4.3±0.8	4.5±0.5	5.8±0.9	2.1±0.8	3.8±0.4	4.8±0.1
<b>Right Ventricles</b>						
Bmax (fmol/unit area)	–	5.1±0.5	6.4±0.5	4.1±0.4	7.3±0.4	8.1±1.2
Kd (nM)	–	1.3±0.1	0.8±0.3	0.4±0.1	1.0±0.1	0.9±0.2
<b>Left Ventricles</b>						
Bmax (fmol/unit area)	–	13.1±2.5	10.5±0.8	–	11.7±0.6	10.3±0.7
Kd (nM)	–	3.7±0.6	4.1±0.7	–	3.9±0.4	3.6±0.3

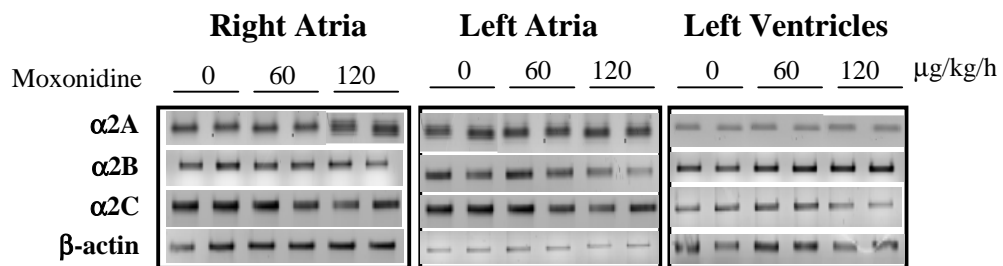
\*P < 0.01 vs. WKY

\*\*P < 0.01 vs. vehicle-treated SHR

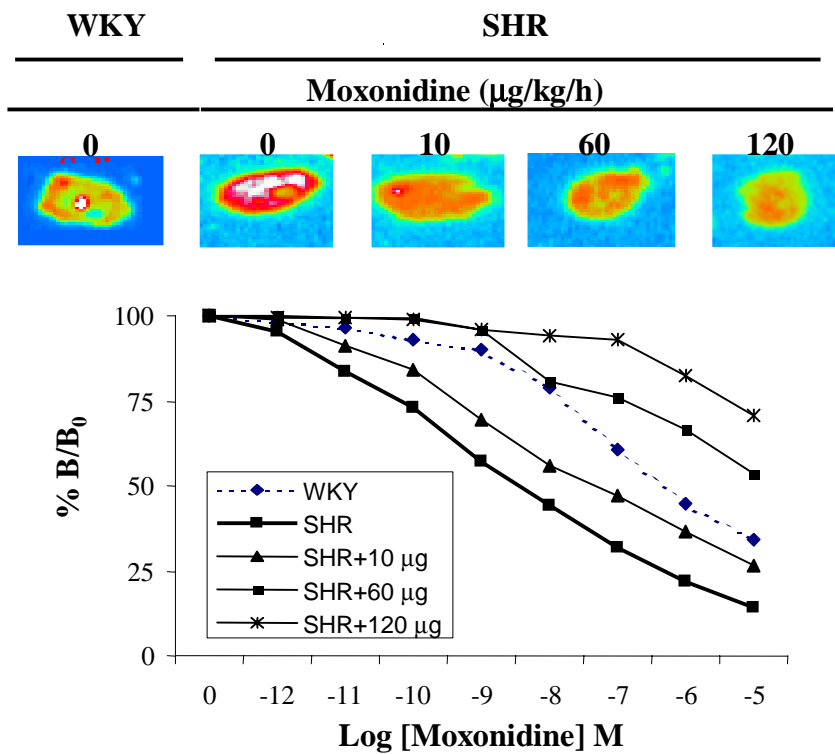


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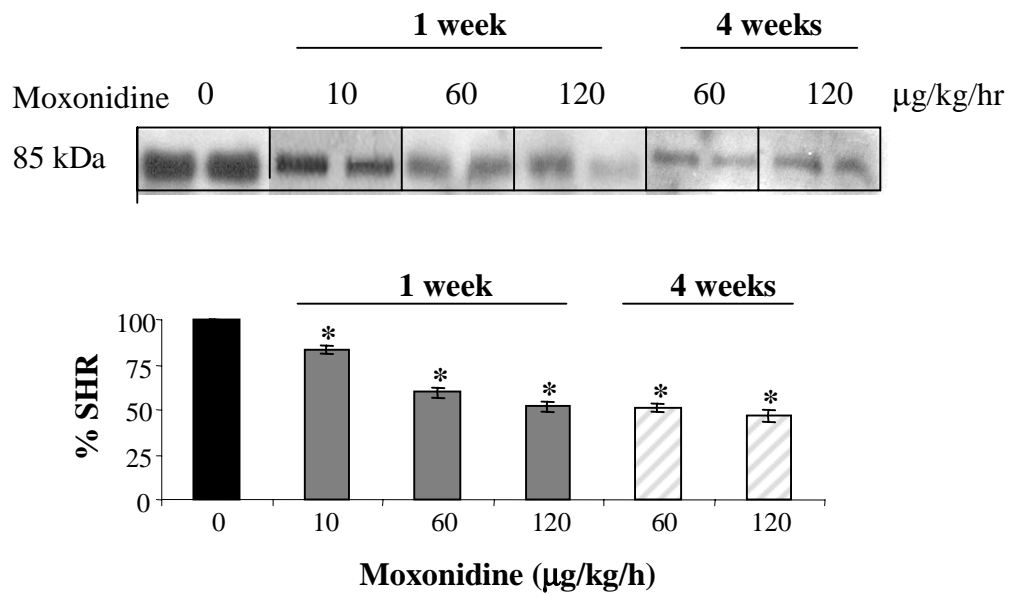




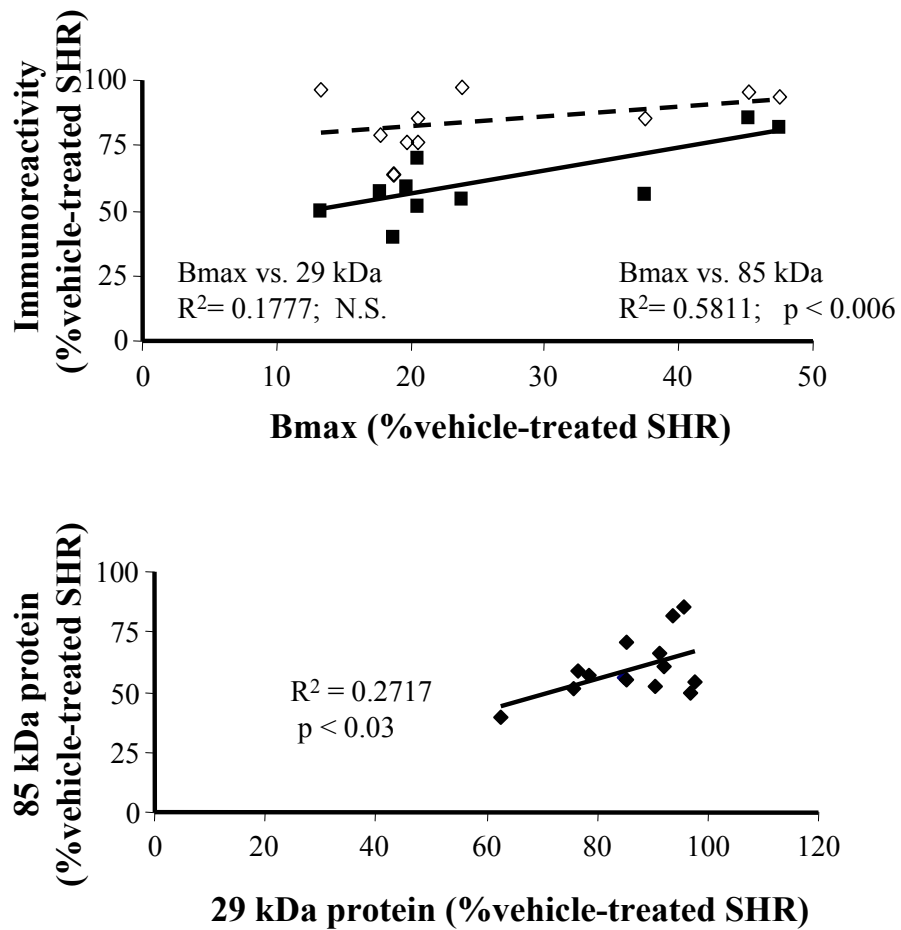
**Figure 2**  
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**Figure 3**  
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**Figure 4**  
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**Figure 5**  
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