JPET Fast Forward. Published on April 9, 2004 as DOI: 10.1124/jpet.104.067595

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

Rouwayda El-Ayoubi, Ahmed Menaouar, Jolanta Gutkowska,

and Suhayla Mukaddam-Daher

Laboratory of Cardiovascular Biochemistry, Centre Hospitalier de L'Université de Montréal Research Center, Campus Hotel-Dieu, Montréal (Quebec) Canada

This work was supported by grants from the Canadian Institutes for Health Research (MOP-57714) and the Heart and Stroke Foundation of Canada (to SMD and JG).

Running title: Cardiac Imidazoline I_1 receptors in SHR

Corresponding author: Suhayla Mukaddam-Daher, Ph.D., Laboratory of Cardiovascular Biochemistry, CHUM Research Center, 3840 St-Urbain Street (6-816), Montreal (Quebec), Canada, H2W 1T8. Tel: (514) 890-8000, ext: 12757; Fax: (514) 412-7199
E-mail: suhayla.mukaddam-daher@umontreal.ca

Text pages 18; 2 tables; 5 figures; 25 references;
Abstract 258 words
Introduction 415 words
Discussion 1113 words

Abstract

We have recently identified imidazoline I_1-receptors in the heart. In the present study, we tested regulation of cardiac I_1-receptors vs. α_2 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 ^{125}I-paraiodoclonidine (^{125}I-PIC, 0.5 nM, 1h, 22°C) and inhibition of binding with epinephrine (10^{-10} to 10^{-5} M) demonstrated the presence of α_2-adrenoceptors in heart atria and ventricles. Immunoblotting and RT-PCR identified α_{2A}- α_{2B}- and α_{2C}-adrenoceptor proteins and mRNA, respectively. However, compared to normotensive controls, cardiac α_2-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_1-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 Bmax 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 Bmax only correlated with the 85 kDa protein ($R^2 = 0.57$; $p<0.006$), suggesting that this protein may represent I_1-receptors. The weak but significant correlation between the two imidazoline receptor proteins ($R^2 = 0.28$; $p<0.03$), implies that they arise from the same gene. In conclusion, the heart possesses I_1-receptors and α_2 adrenoceptors, but only I_1-receptors are responsive to hypertension and to chronic *in vivo* treatment with a selective I_1-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 α_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. α_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}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 α_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 α_2-adrenoceptors.

In fact, functional separation between imidazoline I_1-receptors and α_2-adrenoceptors is rather difficult, because these receptors are often co-localized and ligands with affinity to imidazoline I_1-receptors also bind to α_2-adrenoceptors (Bousquet, 1997). However, previous studies indicate that imidazoline receptors and α_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_1-receptors vs. α_2 adrenoceptors, by showing that I_1-receptors, but not α_2 adrenoceptors are regulated in hypertension and in response to exposure to agonist. Accordingly, studies were performed to: 1) demonstrate the presence of α_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_1-receptors and α_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 μL/h (2ML1), for one week, and 2.5 μL/h (2ML4) for 4 weeks. The concentrations of moxonidine were adjusted to allow delivery of 10, 60 & 120 μg/kg/h. 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 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°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 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_1-receptors and α_2-adrenoceptors was performed on frozen heart sections from WKY and SD rats, and from saline- and moxonidine-treated SHR, using radiolabelled paraiodoclonidine (^{125}I-PIC; 2200 Ci/mmol; New England Nuclear, Boston, MA) as we have previously described (El-Ayoubi et al., 2002a). Because ^{125}I-PIC binds to both receptor types, autoradiography was performed, separately, in conditions that favor α_2- adrenoceptor binding and in conditions that favor I_1-receptor binding. For α_2-adrenoceptors, the slides were incubated for 1h at 22^0C with 0.5 nM ^{125}I-PIC in incubation buffer: in mM 50 Tris-HC (pH 7.7), 5 EDTA, 5 EGTA, 10 $MgCl_2$, and 50 μM phenylmethylsulfonyl fluoride (PMSF). Binding was inhibited by increasing concentrations of epinephrine (10^{-10} to 10^{-5} M). Binding in the presence of 10^{-4} 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_1-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 $MgCl_2$ in the incubation buffer to 0.5 mM, conditions that favor binding to I_1-receptors (Ernsberger et al., 1995)]. Binding of ^{125}I-PIC was competitively inhibited by increasing concentrations (10^{-12} to 10^{-5} 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 μg denatured protein samples from cardiac tissues and incubation of blots with rat α_{2A}-, α_{2B}-, α_{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-β-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 α_{2A}-, α_{2B}-, α_{2C}- receptors, or β-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 β-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 ± SEM.

Results

Cardiac α_2-adrenoceptors

Autoradiographic binding of ^{125}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 α_2-adrenoceptor affinity (Kd ≅ 2.5 nM) and Bmax in right atria (12.8±0.7 vs. 13.4±0.9 fmol/unit area), left atria (12.8±0.4 vs. 11.7±0.7 fmol/unit area) and left ventricles (11.7±1.1 vs. 12.2±0.5 fmol/unit area) were not altered in SHR as compared to WKY rats. Binding to cardiac α_2-adrenoceptors in SHR was also not altered by chronic *in vivo* moxonidine treatment. Bmax remained in 120 μg/kg/h moxonidine-treated SHR right atria at 11.9±0.9 fmol/unit area and Kd at 2.3±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 α_2-adrenoceptor subtypes were identified in cardiac tissues of SD and SHR, by immunoblotting. Densitometric measurements of the bands corresponding to α_{2A}- (Fig. 1), α_{2B}- and α_{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 α_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}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 μg/kg/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 Bmax in SHR right and left atria. At the lowest dose of 10 μg moxonidine, Bmax decreased from 40.0±2.9 to 18.2±0.4 fmol/unit area ($p<0.01$) in right atria, and from 27.7±2.8 to 12.3±0.6 fmol/unit area ($p<0.04$) in left atria. The doses of 60 and 120 μg moxonidine decreased Bmax 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 μg moxonidine, Bmax in right atria represented 9.0±0.3 fmol/unit area. Moxonidine treatment did not affect Bmax and Kd 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±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±1.9%, 59.9±2.7%, and 51.8±3.0% in 10, 60 and 120 μg moxonidine-treated SHR for 1 week, respectively. Treatment with moxonidine at 60 and 120 μg/kg/h for 4 weeks, resulted in a mild additional decrease in the intensity of the 85 kDa bands to 51.1±2.2% and 46.8±3.3%, respectively (Fig. 4). A modest increase in the intensity of ~29 kDa band (not shown) was observed in right atria of SHR (107±2%) that decreased to 94±1%, 87±3%, and 83±5% after one week of moxonidine at 10, 60 and 120 μg, respectively. In left ventricles, chronic treatment of SHR with moxonidine (120 μg) 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±3%, and after 4 weeks to 89±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_1-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±8 to 135±5 mmHg, $p<0.02$, whereas blood pressure remained in control rats at 186±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 α_2-adrenoceptors in heart atria and ventricles; and 2) demonstration that heart imidazoline I_1-receptors but not α_2-adrenoceptors are regulated in SHR, and in response to chronic *in vivo* exposure to a selective imidazoline receptor agonist, suggesting that heart I_1-receptors are subject to regulation. In addition, 3) the parallel change in receptor Bmax and the 85 kDa imidazoline receptor protein, suggest that this protein may represent cardiac I_1-receptors.

Pharmacologic and molecular cloning studies have revealed three α_2-adrenoceptor subtypes: α_{2A} (α_{2D} in rats), α_{2B}, and α_{2C} (Link et al., 1996; Altman et al., 1999). In the human heart, mRNA for all 3 α_2-adrenoceptors subtypes have been detected by PCR (Brodde and Michel, 1999). However, probably due to very low expression relative to α_1- and β_1-adrenoceptors, previous studies have not been successful in demonstrating α_2-adrenoceptors in the heart at the protein level through radioligand binding studies (Brodde and Michel, 1999). In the present study, demonstration of α_2-adrenoceptors in the heart was achieved by multiple approaches. Quantitative receptor autoradiography was used in conditions where binding of ^{125}I-PIC to adrenoceptors vs. I_1-receptors was optimized by using high $MgCl_2$ (10 mM) concentration in the incubation buffer (Ernsberger et al., 1995). Furthermore, because radioligands cannot fully discriminate between α_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 α_{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 α_{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 α_{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 α_2-adrenoceptors in various heart chambers is beyond the scope of the present study. This study, however, provides strong evidence that α_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_1-receptors. Since brain α_2-adrenoceptors have been shown to be selectively down-regulated in response to α_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 α_2-adrenoceptors in the heart interact weakly with moxonidine.

Most importantly, the present study confirms our previous finding that I_1-receptors are present in the heart, and extend to demonstrate that up-regulated atrial I_1-receptors in SHR (El-Ayoubi et al., 2002a) are normalized by chronic *in vivo* exposure to I_1-receptor agonist. These receptors appear to be unrelated to imidazoline I_2-receptors, previously identified in the heart (Molderings and Gothert, 1999), because, by definition, the ligands used in the present study (^{125I}-PIC and moxonidine) show very low affinity to I_2-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 Bmax 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 α_2-adrenoceptors. Chronic treatment with the prototypic antidepressant imipramine, down-regulates I_1-receptors in rat brainstem, without affecting α_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 α_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 μg

moxonidine, which had no effect on blood pressure in those rats (Menaouar et al., 2002), significantly reduced I_1-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_1-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_1-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_1-receptors but not α_2-adrenoceptors are up-regulated in SHR and normalized by chronic antihypertensive treatment with moxonidine. Cardiac I_1-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_1-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_1-receptors are subject to regulation by the cardiovascular environment. Future antihypertensive treatment with imidazoline drugs should consider the heart as a major target organ.

References

Altman JD, Trendelenburg AU, MacMillan L, Bernstein D, Limbird L, Starke K, Kobilka BK, and Hein L (1999) Abnormal regulation of the sympathetic nervous system in alpha2A-adrenergic receptor knockout mice. *Mol Pharmacol* **56**:154-161.

Armour JA (1999) Myocardial ischaemia and the cardiac nervous system. *Cardiovasc Res* **41**: 41-45.

Bousquet P (1997) Imidazoline receptors. *Neurochem Int* **30**:3-7.

Bricca G, Dontenwill M, Molines A, Feldman J, Belcourt A, and Bousquet P (1989) The imidazoline preferring receptor: binding studies in bovine, rat and human brainstem. *Eur J Pharmacol* **162**:1-9.

Brodde OE and Michel MC (1999) Adrenergic and muscarinic receptors in the human heart. *Pharmacol. Rev* **51**:651-689.

Eason MG and Liggett SB (1996) Chimeric mutagenesis of putative G-protein coupling domains of the alpha2A-adrenergic receptor. Localization of two redundant and fully competent Gi coupling domains. *J Biol Chem* **271**:12826-12832.

El-Ayoubi R, Gutkowska J, Regunathan S, and Mukaddam-Daher S (2002a) Imidazoline receptors in the heart: characterization, distribution, and regulation. *J Cardiovas Pharmacol* **39**: 875-883.

El-Ayoubi R, Menaouar A, Gutkowska J, and Mukaddam-Daher S (2002b) Effect of chronic moxonidine treatment on SHR renal imidazoline receptors. *Canadian J Cardiol* **18**:6. (Abstract)

Ernsberger P, Piletz JE, Graff LM, and Graves ME (1995) Optimization of radioligand binding assays for I_1-imidazoline sites. *Ann N Y Acad. Sci* **763**:163-168.

Ernsberger P, Sims C, and Douglas JG (1991) I_1-imidazoline sites in renal medulla: binding properties and up-regulation by subpressor angiotensin infusion. *Hypertension* **18**:380. (Abstract)

Ernsberger P (1999) The I_1-imidazoline receptor and its cellular signaling pathways. *Ann N Y Acad Sci* **881**:35-53.

Hamilton CA, Jardine E, and Reid JL (1993) Down-regulation of imidazoline sites in rabbit kidney. *Eur J Pharmacol* **243**: 95-97.

Hein L. (2001) The alpha2-adrenergic receptors: molecular structure and in vivo function. *Z Kardiol* **90**: 607-612.

Ivanov TR, Feng Y, Wang H, Regunathan S, Reis DJ, Chikkala DN, Gupta P, Jones JC, and Piletz JE (1998) Imidazoline receptor proteins are regulated in platelet-precursor MEG-01 cells by agonists and antagonists. *J Psychiatr Res* **32**:65-79.

Li P, Penner SB, and Symth DD (1994) Attenuated renal response to moxonidine and rilmenidine in one kidney-one clip hypertensive rats. *Br J Pharmacol* **112**:200-206.

Link RE, Desai K, Hein L, Stevens ME, Chruscinski A, Bernstein D, Barsh GS and Kobilka BK (1996) Cardiovascular regulation in mice lacking alpha2-adrenergic receptor subtypes b and c. *Science* **273**:803-805.

Menaour A, El-Ayoubi R, Jankowski M, Gutkowska J, and Mukaddam-Daher S (2002) Chronic imidazoline receptor activation in spontaneously hypertensive rats. *Am J Hypertens* **15**:803-808.

Molderings GJ and Gothert M (1999) Imidazoline binding sites and receptors in cardiovascular tissue. *Gen Pharmacol* **32**:17-22.

Mukaddam-Daher S and Gutkowska J (2000) Atrial natriuretic peptide is involved in renal actions of moxonidine. *Hypertension* **35**:1215-20.

Rump LC, Bohmann C, Schaible U, Schöllhorn J, and Limberger N (1995) Alpha2C-adrenoceptor-modulated release of noradrenaline in human right atrium. *Br J Pharmacol* **116**:2617-2624.

Schäfer U, Burgdorf C, Engelhardt A, Kurz T, and Richardt G (2002) Presynaptic effects of moxonidine in isolated buffer perfused rat hearts: role of imidazoline-1 receptors and α2-adrenoceptors. *J Pharmacol Exp Ther* **303**:1163-1170.

Tolentino-Silva FP, Haxhiu MA, Waldbaum S, Dreshaj IA, and Ernsberger P (2000) Alpha2-adrenergic receptors are not required for central antihypertensive action of moxonidine in mice. *Brain Res* **862**:26-35.

Yakubu MA, Deighton NM, Hamilton CA, and Reid JL (1990) Differences in the regulation of [^{3}H]idazoxan and [3H]yohimbine binding sites in the rabbit. *Eur J Pharmacol* **176**:305-311.

Zhu H, Halaris A, and Piltez JE (1997) Chronic imipramine treatment dowregulates IR_1-imidazoline receptors in rat brainstem. *Life Sci*. **61**:1973-1983.

Zou AP and Cowley AW, Jr. (2000) Alpha2-adrenergic receptor mediated increase in NO production buffers renal medullary vasoconstriction. *Am J Physiol Regul Integr Com. Physiol* **279**:R769-R777.

Figure Legends

Fig. 1: Representative immunoblot of α_2-adrenoceptor subtypes (α_{2A}, α_{2B}, α_{2C}) and β-actin, and densitometric data of α_{2A} measured in right atria and left ventricles of SD and SHR after treatment with moxonidine (0, 60 & 120 μg/kg/h) for 1 and 4 weeks. Data normalized to corresponding β-actin are presented as percent change from SD and vehicle-treated SHR.

Fig. 2: RT-PCR mRNA products of α_2-adrenoeceptors and β-actin in right and left atria and left ventricles of SHR treated with moxonidine (0, 60 & 120 μg/kg/h) for 4 weeks.

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

Bottom: Specific binding of ^{125}I-PIC to right atrial sections in WKY and SHR after 1-week treatment with moxonidine (0, 10, 60, 120 μg/kg/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 μg/kg/h) for 1 week and 60 & 120 μg/kg/h for 4 weeks. Data normalized to corresponding β-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.

JPET Fast Forward. Published on April 9, 2004 as DOI: 10.1124/jpet.104.067595
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Kinetic Parameters of Cardiac α_2-adrenoceptors.

	WKY	SHR		
Moxonidine (μg/kg/h)	**0**	**0**	**60**	**120**
Right Atria				
Bmax (fmol/unit area)	12.8±0.7	13.4±0.9	11.5±0.7	11.9±0.9
Kd (nM)	2.3±0.1	2.4±0.3	2.2±0.3	2.3±0.3
Left Atria				
Bmax (fmol/unit area)	12.8±0.4	11.7±0.7	11.9±0.7	12.5±0.3
Kd (nM)	2.5±0.3	2.1±0.1	3.3±0.5	2.2±0.2
Left Ventricles				
Bmax (fmol/unit area)	11.7±1.1	12.2±0.5	11.6±0.6	12.0±0.4
Kd (nM)	2.5±0.5	2.3±0.3	2.9±0.2	2.3±0.4

JPET Fast Forward. Published on April 9, 2004 as DOI: 10.1124/jpet.104.067595
This article has not been copyedited and formatted. The final version may differ from this version.

Table II. Kinetic Parameters of Cardiac I_1-receptors.

	SD	WKY	SHR			
Moxonidine (μg/kg/h)	**0**	**0**	**0**	**10**	**60**	**120**
Right Atria						
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
Left Atria						
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
Right Ventricles						
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
Left Ventricles						
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

JPET Fast Forward. Published on April 9, 2004 as DOI: 10.1124/jpet.104.067595
This article has not been copyedited and formatted. The final version may differ from this version.

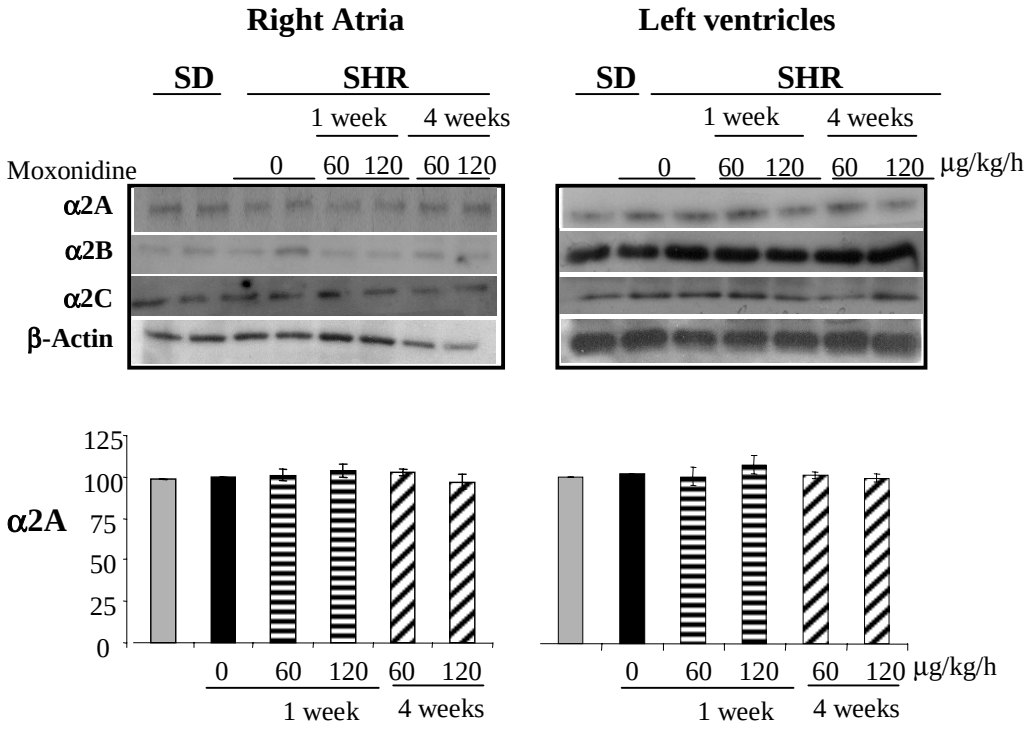

Figure 1
El-Ayoubi et al
JPET #67595

JPET Fast Forward. Published on April 9, 2004 as DOI: 10.1124/jpet.104.067595

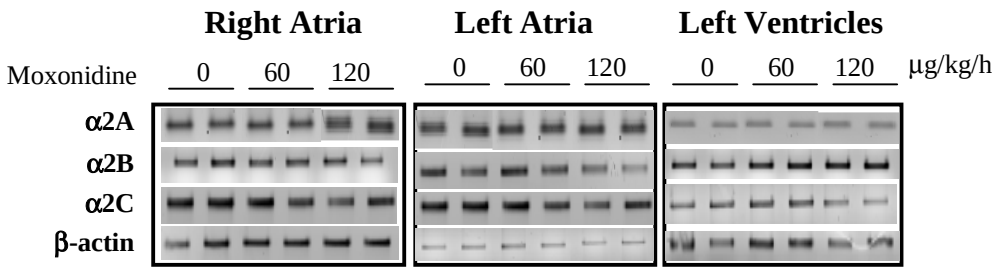

Figure 2
El-Ayoubi et al
JPET #67595

JPET Fast Forward. Published on April 9, 2004 as DOI: 10.1124/jpet.104.067595
This article has not been copyedited and formatted. The final version may differ from this version.

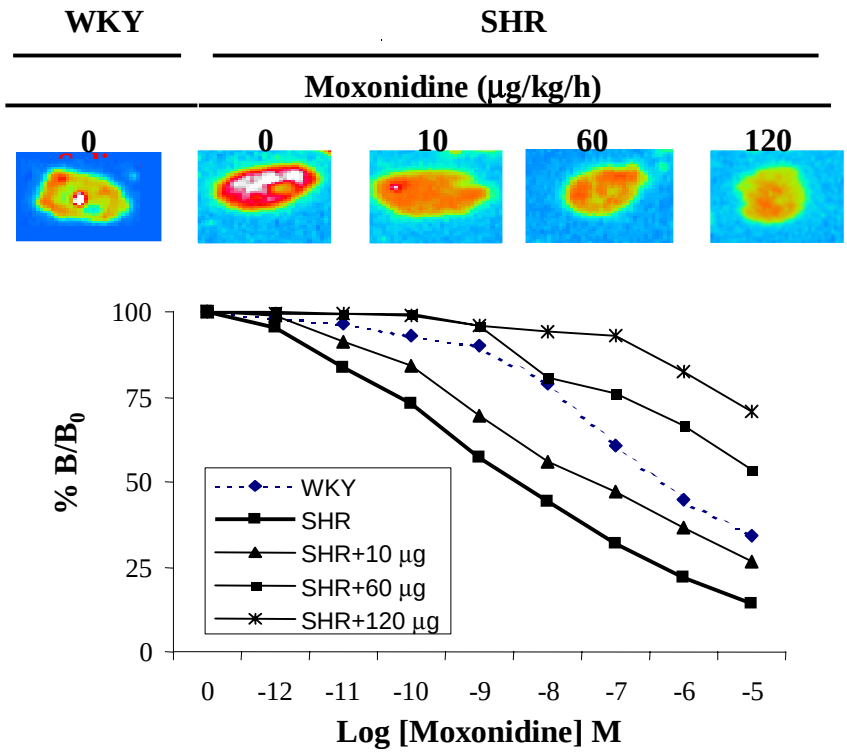

Figure 3
El-Ayoubi et al
JPET #67595

JPET Fast Forward. Published on April 9, 2004 as DOI: 10.1124/jpet.104.067595
This article has not been copyedited and formatted. The final version may differ from this version.

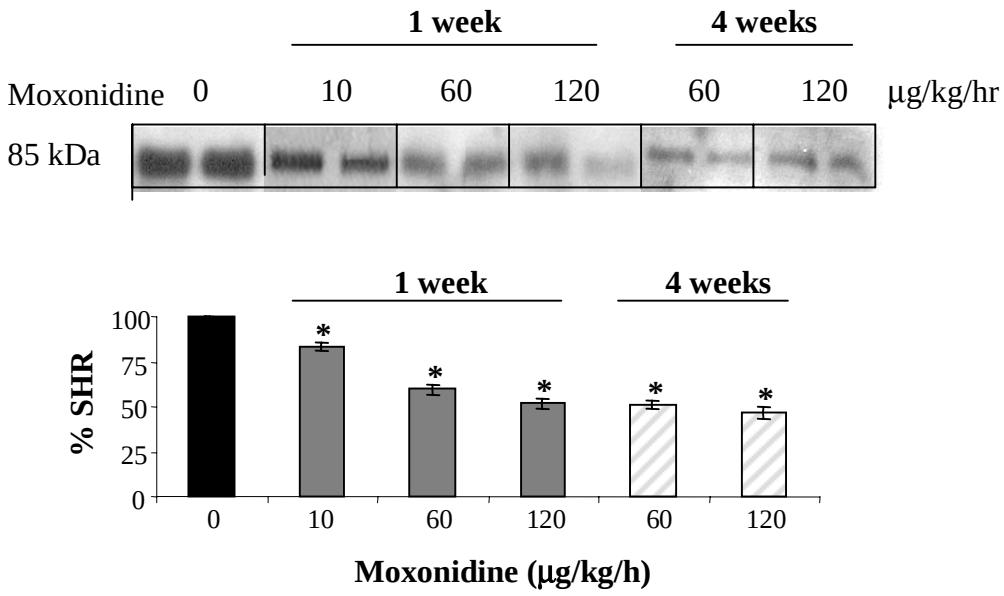

Figure 4
El-Ayoubi et al
JPET #67595

JPET Fast Forward. Published on April 9, 2004 as DOI: 10.1124/jpet.104.067595
This article has not been copyedited and formatted. The final version may differ from this version.

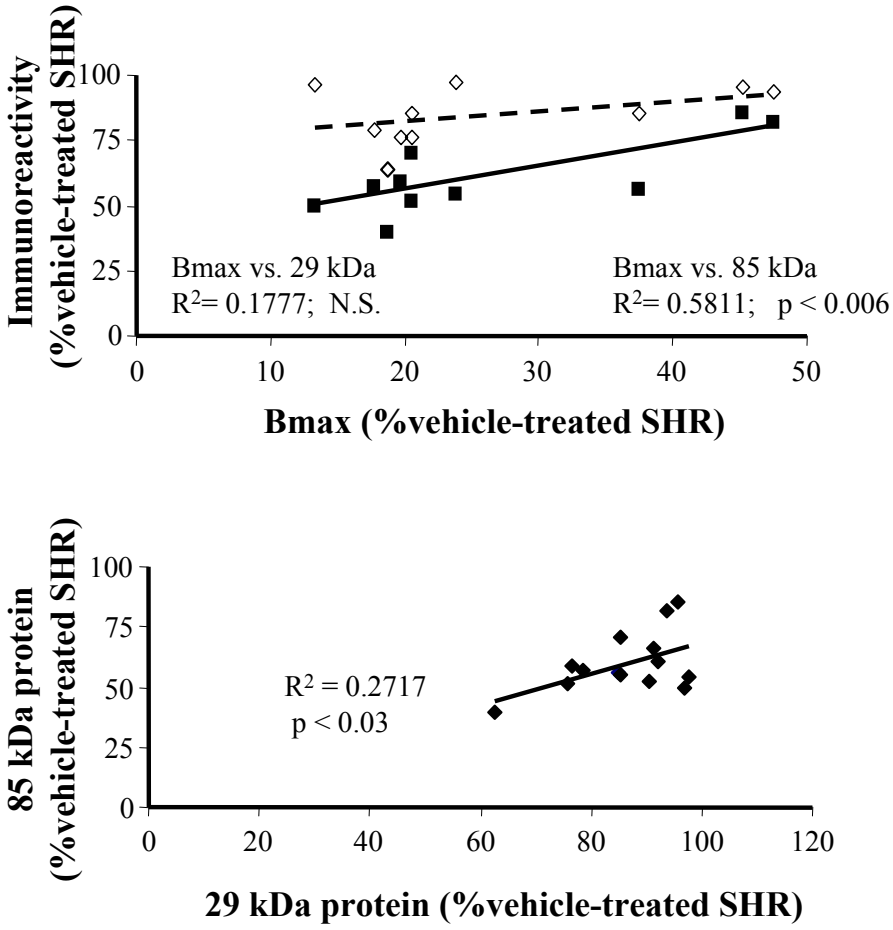

Figure 5
El-Ayoubi et al
JPET #67595