Imidazoline Receptors but Not α₂-Adrenoceptors Are Regulated in Spontaneously Hypertensive Rat 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

Received February 26, 2004; accepted April 8, 2004

ABSTRACT

We have recently identified imidazoline I₁-receptors in the heart. In the present study, we tested regulation of cardiac I₁-receptors versus α₂-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 s.c.) for 1 and 4 weeks. Autoradiographic binding of 125I-paraiodoclonidine (0.5 nM, 1 h, 22°C) and inhibition of binding with epinephrine (10⁻¹⁰–10⁻⁵ M) demonstrated the presence of α₂-adrenoceptors in heart atria and ventricles. Immunoblotting and reverse transcription-polymerase chain reaction identified I₁-receptor proteins and mRNA, respectively. However, compared with normotensive controls, cardiac α₂-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 I₁-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 ± 2.9–7.0 ± 0.6 fmol/unit area; p < 0.01) and left (27.7 ± 2.8–7.1 ± 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 percentage of decrease in Bₘax only correlated with the 85-kDa protein (R² = 0.57; p < 0.006), suggesting that this protein may represent I₁-receptors. The weak but significant correlation between the two imidazoline receptor proteins (R² = 0.28; p < 0.03) implies that they arise from the same gene. In conclusion, the heart possesses I₁-receptors and α₂-adrenoceptors, but only I₁-receptors are responsive to hypertension and to chronic in vivo treatment with a selective I₁-receptor agonist.

Most of the centrally acting antihypertensive drugs, such as clonidine and related imidazoline derivatives, mediate sympathoinhibition, not only via activation of central nervous α₂-adrenoceptors but also via imidazoline I₁-receptors (Bricca et al., 1989; Bousquet, 1997). Imidazoline I₁-receptors are nonadrenergic and noncholinergic neurotransmitter receptors that possess low affinity for norepinephrine and other catecholamines. I₁-receptors are mainly found in the brainstem, adrenal chromaffin cells, and kidneys. In addition, we have recently identified I₁-receptors in heart atria and ventricles and shown that atrial I₁-receptors are up-regulated in rat hypertension and ventricular I₁-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 I₁-receptor versus α₂-adrenoceptors, are associated with enhanced release of atrial natriuretic peptide (Mukaddam-Daher and Gutkowska, 2000), a cardiac hormone involved in pressure and volume homeostasis. Together, these studies led us to suggest that heart I₁-receptors are functional and may be involved in cardiovascular regulation.

Previous binding studies reported [³H]idazoxan binding sites (I₁-receptors) but not I₁-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-I₁ non-I₂ receptors, and the effects of moxonidine to inhibit noradrenaline...
release in atrial appendages were attributed to presynaptic \( \alpha_2 \)-adrenoceptors (Molderings and Gohert, 1999). In contrast, consistent with the presence of \( \alpha_1 \)-receptors in the heart, Schäfer et al. (2002) 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 \( \text{I}_1 \)-receptors and \( \alpha_2 \)-adrenoceptors is rather difficult, because these receptors are often colocalized and ligands with affinity to imidazoline \( \text{I}_1 \)-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; Ernsberger et al., 1991; Zhu et al., 1997; Ivanov et al., 1998). Therefore, the aim of the present studies was to test regulation of cardiac \( \text{I}_1 \)-receptors versus \( \alpha_2 \)-adrenoceptors, by showing that \( \text{I}_1 \)-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) investigate the effect of chronic in vivo exposure to moxonidine on \( \text{I}_1 \)-receptors and \( \alpha_2 \)-adrenoceptors in hearts of normotensive rats and spontaneously hypertensive rats (SHR) with established hypertension.

Materials and Methods

Female 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, Canada). Animals were housed in a 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 Centre Hospitalier de L’Université de Montréal, according to the Canadian Guidelines.

Alzet osmotic mini-pumps (2ML1 and 2ML4; Alzet, Cupertino, CA) were implanted subcutaneously in SHR, under isoflurane anesthesia, and analgesia. Rats were implanted subcutaneously in SHR, under isoflurane anesthesia, and analgesia.

Female 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, Canada). Animals were housed in a 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 Centre Hospitalier de L’Université de Montréal, according to the Canadian Guidelines.

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

Autoregulatory binding was performed 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 \( \text{I}_1 \)-receptors and \( \alpha_2 \)-adrenoceptors was performed on frozen heart sections from WKY and SD rats and from saline- and moxonidine-treated SHR, using radiolabeled paraiodoclonidine \( ^{125}\text{I}-\text{PIC} \; 2200 \text{Ci/mmol}; \text{PerkinElmer Life and Analytical Sciences, Boston, MA} \) as we have described previously (El-Ayoubi et al., 2002a). Because \( ^{125}\text{I}-\text{PIC} \) binds to both receptor types, autoradiography was performed, separately, in conditions that favor \( \alpha_2 \)-adrenoceptor binding and in conditions that favor \( \text{I}_1 \)-receptor binding.

For \( \alpha_2 \)-adrenoceptors, the slides were incubated for 1 h at 22°C with 0.5 nM \( ^{125}\text{I}-\text{PIC} \) in incubation buffer: 50 mM Tris-\( \text{HCl} \) (\( \text{pH} 7.7 \)), 5 mM EDTA, 5 mM EGTA, 10 mM MgCl\(_2\), and 50 \( \mu \)M phenylmethylsulfonyl fluoride. Binding was inhibited by increasing concentrations of epinephrine \( (10^{-10} \text{ to } 10^{-6} \text{ M}) \). Binding in the presence of 10 \( \mu \)M piperoxan was considered nonspecific. After several washes, the slides were dried, exposed in phosphor-sensitive cassette for 48 h, and then scanned, visualized, and quantified by PhosphorImager (ImageQuant; Amer sham Biosciences Inc., Piscataway, NJ).

Autoradiography for \( \text{I}_1 \)-receptors was performed under identical incubation conditions, except for prior incubation of slides with 1 mM phenoxybenzamine and 0.5 mM ethylmaelineide 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 \( \text{I}_1 \)-receptors (Ernsberger et al., 1995). Binding of \( ^{125}\text{I}-\text{PIC} \) was competitively inhibited by increasing concentrations \( (10^{-12} \text{ to } 10^{-8} \text{ M}) \) of moxonidine.

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

Immunoblotting was performed (El-Ayoubi et al., 2002a) using 30 \( \mu \)g of denatured protein samples from cardiac tissues and incubation of blots with rat \( \alpha_2\text{A}, \alpha_2\text{B}, \) and \( \alpha_2\text{C}\)-adrenoceptor antiserum (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-imidazoline receptor antiserum and nonimmune antiserum (generous gift from S. Regunathan, Department of Psychiatry, University of Mississippi Medical Center, 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 enhanced chemiluminescence detection system (ECL hyperfilm; Amersham Biosciences Inc.), according to the manufacturer’s instructions.

Total RNA Extraction and RT-PCR. Total RNA was extracted from the rat heart tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the protocol described by the manufacturer. PCR reactions were performed (Zou and Cowley, 2000) using specific primer pairs for rat \( \alpha_2\text{A}, \alpha_2\text{B}, \) and \( \alpha_2\text{C} \)-receptors, or \( \beta \)-actin (QIAGEN Operon, Alameda, CA). 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 sample.

Data Analysis. The equilibrium dissociation constant \( (K_d) \) and maximum binding capacity \( (B_{max}) \) for the ligands used in autoradiography were calculated by the nonlinear method using the Ligand computer program (Elsevier-Biosoft, Cambridge, UK). Densitometric measurements of immunoblots were performed using Scion computer program (National Institutes of Health, 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 nonpaired Student’s \( t \) test. \( P < 0.05 \) was considered significant. All data are expressed as mean \( \pm \) S.E.M.

Results

Cardiac \( \alpha_2 \)-Adrenoceptors. Autoradiographic binding of \( ^{125}\text{I}-\text{PIC} \) to heart atrial and ventricular sections was inhibited by increasing concentrations of epinephrine. Kinetic parameters obtained from competitive inhibition curves (Table 1) revealed that \( \alpha_2 \)-adrenoceptor affinity \( (K_d \approx 25 \text{ nM}) \) and \( B_{max} \) in right atria (12.8 \( \pm \) 0.7 versus 13.4 \( \pm \) 0.9 fmoI/unit area), left atria (12.8 \( \pm \) 0.4 versus 11.7 \( \pm \) 0.7 fmoI/unit area) and left ventricles (11.7 \( \pm \) 1.1 versus 12.2 \( \pm \) 0.5 fmoI/unit area) were not altered in SHR compared with WKY rats.
Binding to cardiac α2-adrenoceptors in SHR was also not altered by chronic in vivo moxonidine treatment. \( B_{\text{max}} \) remained in 120 \( \mu \)g/kg/h moxonidine-treated SHR right atria at 11.9 ± 0.9 fmol/unit area and \( K_d \) 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 1).

Three α2-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 (data not shown) were not significantly different in right atria and left ventricles of SHR versus 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 \( ^{125}\text{I}-\text{PIC} \) to \( \text{I}_1 \)-receptors was higher in SHR atria (162%) compared with normotensive WKY rats, considered as 100%. Also, total specific binding in atria decreased after treatment with moxonidine at 10, 60, and 120 \( \mu \)g/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 percentage of \( B/B_0 \), 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 \( \mu \)g of moxonidine, \( B_{\text{max}} \) 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 \( \mu \)g moxonidine decreased \( B_{\text{max}} \) in rat right and left atria to values not significantly different from two normotensive controls (Table 2). Four-week treatment did not have additional effects, so that at 120 \( \mu \)g of moxonidine, \( B_{\text{max}} \) in right atria represented 9.0 ± 0.3 fmol/unit area. Moxonidine treatment did not affect \( B_{\text{max}} \) and \( K_d \) of \( \text{I}_1 \)-receptors in right and left ventricles of moxonidine- and vehicle-treated SHR (Table 2).

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 (\( p < 0.05 \))

<table>
<thead>
<tr>
<th>Right Atria</th>
<th>Left ventricles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WKY</strong></td>
<td><strong>SHR</strong></td>
</tr>
<tr>
<td><strong>Moxonidine (( \mu )g/kg/h)</strong></td>
<td><strong>Moxonidine (( \mu )g/kg/h)</strong></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>
Moxonidine treatment resulted in a dose-dependent decrease in the 85-kDa band (considered as 100%). Figure 5 shows that the percentage of decrease in \( B_{\text{max}} \) correlated with the percentage of decrease in the 85-kDa band \( (R^2 = 0.5700; p < 0.006) \), but not with the 29-kDa band \( (R^2 = 0.1754; \text{N.S.}) \), suggesting that the 85-kDa protein may represent imidazole I₁-receptors in the heart. A weak, but significant correlation was found between the 85- 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 mm Hg \( (p < 0.02) \), whereas blood pressure remained in control rats at 186 ± 11 mm Hg. However, hydralazine treatment did not alter imidazoline receptor proteins measured by immunoblotting.

**Discussion**

The major findings of this study are as follows: 1) First time localization of \( \alpha_2 \)-adrenoceptors in heart atria and ventricles; and 2) demonstration that heart imidazoline I₁-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 I₁-receptors are subject to regulation. In addition, 3) the parallel change in receptor \( B_{\text{max}} \) and the 85-kDa imidazoline receptor protein, suggest that this protein may represent cardiac I₁-receptors. Pharmacological and molecular cloning studies have revealed three \( \alpha_2 \)-adrenoceptor subtypes: \( \alpha_{2A} \) (in rats), \( \alpha_{2B} \) and \( \alpha_{2C} \) (Link et al., 1996; Altman et al., 1999). In the human heart, mRNA for all three \( \alpha_2 \)-adrenoceptor 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 \(^{125}\text{I-PIC} \) to adrenoceptors versus I₁-receptors was optimized by using high \( \text{MgCl}_2 \) (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, because 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).
been shown to be selectively down-regulated in response to treatment with moxonidine (0, 10, 60, and 120 μg/kg/h) for 1 week and 60 and 120 μg/kg/h for 4 weeks. Data normalized to corresponding β-actin are presented as percentage of change from vehicle-treated SHR (considered as 100%). ∗, p < 0.01 versus vehicle-treated SHR.

The α2A, abundant in the central nervous system, mainly in brain stem, is directly involved in regulating sympathetic outflow and seems to be the major presynaptic autoinhibitory receptor subtype (Altman et al., 1999). The α2B is more abundant in arterial vascular smooth muscle cells and mostly responsible for vasoconstriction, and it is responsive to altered salt handling. The function of α2C is not yet clear, but it may be the presynaptic autoreceptor in human atria (Rump et al., 1995; Hein, 2001).

The physiological significance of α2-adrenoceptors in various heart chambers is beyond the scope of the present study. This study, however, provides strong evidence that α2-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 seem not to be regulated by moxonidine, a selective agonist of imidazoline I1-receptors. Because brain α2-adrenoceptors have been shown to be selectively down-regulated in response to α2-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 α2-adrenoceptors in the heart interact weakly with moxonidine.

Most importantly, the present study confirms our previous finding that I1-receptors are present in the heart and extend to demonstrate that up-regulated atrial I1-receptors in SHR (El-Ayoubi et al., 2002a) are normalized by chronic in vivo exposure to I1-receptor agonist. These receptors seem to be unrelated to imidazoline I2-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 I2-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 compared with 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
Cardiac Imidazoline I1 Receptors in SHR

At cardiovascular tissue, tissues known to secrete or respond to natriuretic peptides, atrial natriuretic peptide and brain natriuretic peptide, suggest a functional relationship between the two systems. Therefore, heart I1-receptors are subject to regulation by the cardiovascular environment. Future antihypertensive treatment with imidazoline drugs should consider the heart as a major target organ.

Reference


Address correspondence to: Dr. Suhayla Mukaddam-Daher, Laboratory of Cardiovascular Biochemistry, CHUM Research Center, 3840 St-Urbain St., Montreal, QC, Canada, H2W 1T8. E-mail: suhayla.mukaddam-daher@umontreal.ca