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Yohimbine-induced alterations in α_2-adrenoceptors in kidney regions of the spontaneously hypertensive rats: an autoradiographic analysis

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Abstract:
We have tested the hypothesis that a pharmacologically determined alteration in renal α_2-adrenoceptor (α_2-AR) density might be a pathophysiologically important factor of genetic hypertension in the spontaneously hypertensive rats (SHRs). First, we compared the regional distribution and biochemical parameters of α_2-ARs in SHRs and Wistar-Kyoto (WKY) rats, using the full agonist [^{3}H]UK 14304. Secondly, we evaluated the effect of selective blockade and stimulation of α_2-ARs on the development of hypertension and on renal α_2-AR density and regional distribution in SHRs. [^{3}H]UK 14304 binding was distributed predominantly over the outer medulla, less abundantly over the inner medulla and was almost absent from the renal cortex. Renal α_2-ARs were found to be increased in SHRs at the ages tested compared with their respective controls and the increase was completely localized to the outer medulla. In these rats, blood pressures immediately before sacrifice were significantly higher in the hypertensive group compared with normotensive controls. The daily administration of SK&F 86466 or clonidine significantly decreased the blood pressure but the autoradiographic studies showed that the prolonged administration of yohimbine to rats for two weeks resulted in a large increase in the density of α_2-ARs in some areas of the rat kidney but not in others. Taken together these data do not support the hypothesis that alteration in renal α_2-ARs (as measured by autoradiography) is crucial for the maintenance of hypertension in the SHR model.

Key words:
[^{3}H]brimonidine ([^{3}H]UK 14304), yohimbine, SK&F 86466, clonidine, autoradiography, renal α_2-adrenoceptors, SHR

Introduction

The sympathetic nervous system regulates cardiovascular physiology primarily by the release of catecholamines and activation of adrenoceptor (AR). Multiple subtypes of α_1-, α_2-, and β-AR regulate cardiovascular cell function and contribute to the pathophysiology of cardiovascular diseases [17]. For example, heritable differences in α_2-AR subtypes have been implicated in human [24] and rodent [9] hypertension. The α_2-AR family comprises three subtypes, namely α_{2A}, α_{2B} and α_{2C}, which mediate their effects through activation of Gi/Go proteins [6]. Although all receptors display similar affinity for their natural ligands, adrenaline and noradrenaline, they exhibit marked differences in pharmacological and biochemical properties.

Thus, unlike the other two subtypes, the α_{2B}-AR is not glycosilated and it exhibits a 10-fold higher affinity for prazosin than for oxymetazoline.

The three α_2-AR subtypes have been cloned in recent years. However, pharmacologic experiments and radioligand binding studies lack the selectivity to differentiate these subtypes and define their functional characteristics [2].

In the rat, the α_{2B}-AR is encoded by the ADRA 2B genes and is primarily expressed in the kidney of adult animals [23, 31, 39]. In agreement with this pattern of RNA distribution, radioligand binding experiments showed that α_{2B}-AR is the major subtype expressed in the rat kidney [37].

Until recently, the precise functions of the α_{2B}-AR remained rather elusive. Immunohistological studies on the rat kidney demonstrated that the α_{2B}-AR is predominantly located in the basolateral membrane of the proximal tubule [19] where it accelerates sodium reabsorption through the stimulation of Na^+/H^+ exchanger. On the other hand, the α_{2B}-AR subtype mediates the α_2-AR agonist-induced increase in blood pressure [22], increases the renal free water clearance [21], and is overexpressed in the kidney of spontaneously hypertensive rats (SHRs) [14, 15]. Because the physiologic activity of α_2-ARs depends at least in part on their density [10], alteration in renal α_2-ARs density could account for disruption in blood pressure control.

With the renewed interest in α_2-AR subtypes and to obtain a better understanding of the receptors' role in genetic hypertension in rats, the aim of the present study was to test regulation of α_2-AR in hypertension and in response to exposure to agonist and antagonist drugs. Accordingly, studies were performed to 1) demonstrate the presence and localization of α_2-AR in the kidney and their possible regulation in hypertension, and 2) investigate the effect of chronic *in vivo* exposure to clonidine, yohimbine and SK&F 86466 on the density and localization of α_2-AR in kidneys of normotensive and SHRs with established hypertension.

Materials and Methods

Animals and treatment

Male, 20-week-old SHRs and WKY rats (Iffa/Credo, L'Arbestre, France) weighing 300–350 g at the start of chronic drug administration were used. Animals were housed four per cage with free access to water and standard laboratory rat chow (Purina). The colony room had a temperature of 24 ± 1°C, humidity of 45–55% and light/dark cycle (lights on 07:00–19:00). Experiments were performed following the approval of Bioethics Committee of the University of Córdoba.

Four days after arrival, SHRs and WKY rats were randomly assigned to different treatments for 2 weeks (n = 6–8 per group). The rats were injected intraperitoneally with yohimbine (2 mg/kg/day), the α_2-receptor antagonist SK&F 86466 (10 mg/kg/day) or clonidine (0.5 mg/kg/day) in a final volume of 0.1 ml, twice daily (9 a.m. and 7 p.m.). Control rats received the same number of vehicle injections (saline) at the same time as the experimental animals were given drugs.

Clonidine is an agonist at α_2-ARs that also display high affinity for novel imidazoline receptor binding sites (IR sites) [11, 36]. Yohimbine is a potent selective α_2-AR antagonist with weaker α_1-antagonist activity [13]. Yohimbine has been predominantly used as a pharmacological tool to study the involvement of α_2-ARs in the regulation of autonomic function. The compound SK&F 86466, a selective α_2-AR antagonist, has been found to discriminate between pre- and postsynaptic α_2-ARs [18]. It has a low affinity for the prejunctional site (K_b = 17 nM) and a moderately high affinity for the postjunctional site (K_b = 42 nM).

Systolic blood pressure (SBP) was measured before drug administration, and at weekly intervals in warmed unanesthetized animals by the indirect tail-cuff method [5] using a programmed electrosphygmomanometer. Before the measurements, the rats were kept at 38°C for 10–15 min to make the pulsations of the tail artery detectable. Arterial blood pressure measurements were performed at the same time of day (between 7 a.m. and 9 a.m.) in order to avoid the influence of the circadian cycle, and the values of SBP were obtained by estimating the average reading of three measurements. Moreover, the person who measured the arterial blood pressure in the animals from the different groups did not know the drugs that had been administered to each of these groups.

A SBP of 150 mmHg or higher was the criterion for hypertension. Heart rate and body weight in the unanesthetized state were determined at the same time as the SBP.

Two weeks following the initiation of the treatments, groups of rats (n = 6–8) were killed by decapi-

tation without anesthesia. The kidneys were removed and were quickly frozen with dry ice-methanol and stored at –80°C for receptor analysis by autoradiographic binding.

Autoradiography of α_2-adrenoceptors

For autoradiographic studies, 10 μm-thick sagital sections from frozen kidneys from both WKY and SHRs were cut in a microtome cryostat and mounted onto gelatin-coated microscopic slides. Labeling of α_2-ARs was carried out with the full agonist [^{3}H]UK 14304 ([^{3}H]brimonidine), in order to selectively recognize the high affinity state of the α_2-AR [12], following the procedure previously described [27, 28]. Briefly, sections were preincubated at room temperature for 15 min in Tris-HCl buffer (50 mM, pH 7.7) containing 0.1 mM $MnCl_2$. This was followed by an incubation for 90 min in the same buffer containing 5 nM [^{3}H]UK 14304, also at room temperature. Finally, sections were washed for 5 min in ice-cold buffer and then dried under a cold air stream. Nonspecific binding was defined as that remaining in the presence of 10 μM phentolamine. Tissue sections from the different experimental groups were incubated together in these experiments. In selected cases, consecutive sections to those incubated for receptor labeling were used for routine histological examination.

In order to generate autoradiograms, radiolabeled sections were exposed at 4°C for 10 weeks by opposition against a tritium-sensitive film (^{3}H-Hyperfilm, Amersham Int., UK), together with the appropriate standards ([^{3}H]Microscales, Amersham Int., UK). Films were developed with Kodak HC.110 and fixed with AGFA G350. After the scanning of the films, the autoradiograms were analyzed as described by Unnerstall et al. [38], using a computerized image analysis system (NIH-IMAGE program, Bethesda, MA, USA). Autoradiographic values were finally expressed in fmol/mg of tissue equivalent (fmol/mg t.e.), as the mean ± SEM.

Chemicals

The drugs used and their sources of supply were as follows: [^{3}H]UK 14304 (62.7 Ci/mmol) was obtained from New England Nuclear (Dreieich, Germany). Yohimbine was obtained from Sigma Chemical Company (St. Louis, MO, USA). The following compounds were kindly donated by the indicated sources: phentolamine (Ciba-Geigy, Barcelona, Spain); clonidine (Boehringer-Ingelheim, Barcelona, Spain) and SK&F 86466 (6-chloro-3-methyl-2; 3,4,5-tetrahydro--1H-3-benzazepine) (Smith Kline & French Laboratories, Philadelphia, PA, USA). All other reagents were of analytical grade.

Data analysis

Results are expressed as the means ± SD. For the statistical evaluations, one-way analysis of variance followed by a Newman-Keuls multiple comparison test was used to isolate differences within and between groups. The level of significance was chosen as $p < 0.05$.

Results

Blood pressure

Mean SBPs for the 6–8 animal groups are presented in Table 1. At the time of sacrifice each of the experimental hypertensive groups had significantly higher pressures than its corresponding normotensive group, except for the clonidine group. The administration of SK&F 86466 and clonidine to SHRs decreased significantly their SBP, which declined to normotensive levels in the clonidine-treated group. There were no significant differences in SBPs among the four normotensive groups. Yohimbine, SK&F 86466 and clonidine reduced significantly the heart rate in SHRs. This effect was also observed in WKY rats treated with clonidine.

Tab. 1. Mean systolic blood pressure (SBP) and heart rate (HR) after different treatments in SHRs and WKY rats

Group	WKY		SHR	
	SBP	HR	SBP*	HR
Control	137 ± 5	359 ± 7	241 ± 3	428 ± 10
Yohimbine	136 ± 5	339 ± 12	224 ± 4	374 ± 6[a]
SK&F 86466	148 ± 5	357 ± 8	191 ± 3[a]	398 ± 18[c]
Clonidine	137 ± 2	230 ± 12[d]	140 ± 6[b]	250 ± 2[b]

Values represent the mean ± SE (n = 6–8), * SHR > WKY ($p < 0.01$) except clonidine group. [a] $p < 0.01$ compared with respective SHR control group; [b] $p < 0.01$ compared with the rest of SHR groups; [c] $p < 0.05$ compared with respective SHR control group; [d] $p < 0.01$ compared with respective rest of WKY groups

Autoradiographic studies

Autoradiographic localization of α_2-ARs with [^{3}H]UK 14304 showed a heterogeneous distribution of these receptors throughout the kidney in both normotensive and SHRs. While relatively low amounts of autoradiographic grains were found over the renal cortex and the papilla, high densities of [^{3}H]UK 14304 binding were present at the different levels of the renal medulla (Fig. 1A). In this region, while the inner strip of outer medulla and the inner medulla contained relevant autoradiographic densities, the concentration of total [^{3}H]UK 14304 binding over the outer strip of outer medulla was very high. However, the level of non-specific binding in this strip was also higher (30–40%) in comparison to the remaining areas of the kidney (15–25%). Thus, the real concentration of specific [^{3}H]UK 14304 binding (α_2-ARs) was fairly similar at the different medullary levels (Fig. 1B, Tab. 2, 3).

SHRs presented the same pattern of anatomical localization, but the densities of α_2-AR were higher in all regions analyzed, especially in the outer strip of the outer medulla and the inner medulla, where these differences reached statistical significance (Fig. 2A, D, Tab. 3). In the WKY rats, pretreatment with SK&F 86466 and clonidine did not produce modifications in the density of α_2-ARs in any of the regions studied. In contrast, in the animals pretreated with yohimbine a general response of increase in the number of receptors was observed, mainly over the outer strip of the outer medulla (+ 79%, $p < 0.05$) and the inner medulla (+ 41%) (Fig. 2A–C, Tab. 2).

In SHRs, only slight non-significant changes were observed in α_2-ARs in the animals pretreated with SK&F 86466. As found in WKY rats, pretreatment with yohimbine elicited a marked, significant increase in receptor density over the outer strip of the outer medulla (+ 74%, $p < 0.01$); however, in the remaining regions of the kidney, a tendency to a decrease was observed without statistical significance.

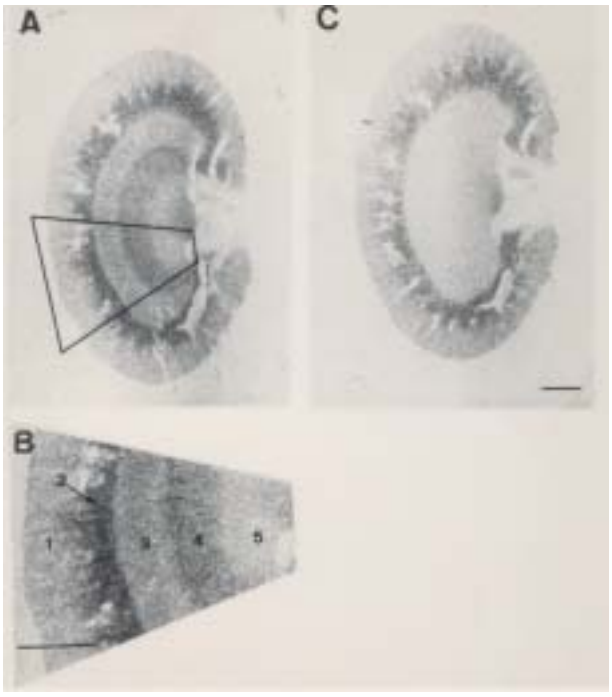

Fig. 1. Autoradiographic illustration of [^{3}H]UK 14304 binding in the WKY rat kidney. Dark areas are those enriched in binding sites. **A** and **B** – total binding (**B** corresponds to the same tissue, at a higher magnification). **1** – cortex, **2** – outer strip of outer medulla, **3** – inner strip of outer medulla, **4** – inner medulla, **5** – papilla. **C** – non-specific binding defined by co-incubation with 10 μM phentolamine. Bar – 0.2 cm

Tab. 2. Effect of yohimbine, SK&F 86466, and clonidine on [^{3}H]UK 14304 specific binding in the selected kidney regions of WKY rats studied by quantitative autoradiography

Regions	Control n = 12	Yohimbine n = 8	SK&F 86466 n = 6	Clonidine n = 8
Cortex	6.8 ± 0.6	8.1 ± 1.2	5.7 ± 1.2	6.0 ± 1.0
Outer medulla				
Outer strip	18.3 ± 1.0	32.8 ± 3.0[a]	19.2 ± 6.2	23.3 ± 3.8
Inner strip	20.6 ± 1.8	23.1 ± 1.2	18.4 ± 2.4	22.0 ± 4.2
Inner medulla	20.7 ± 2.5	29.2 ± 4.2	19.3 ± 4.9	22.5 ± 4.5

Values represent the mean ± SE and are expressed in fmol/mg tissue. [a] Significantly different from control, $p < 0.05$

Tab. 3. Effect of yohimbine, SK&F 86466, and clonidine on [^{3}H]UK 14304 specific binding in the selected kidney regions of SHRs studied by quantitative autoradiography

Regions	Control n = 8	Yohimbine n = 4	SK&F 86466 n = 7	Clonidine n = 7
Cortex	8.5 ± 0.7	5.2 ± 1.9	8.4 ± 1.7	3.6 ± 1.1
Outer medulla				
Outer strip	27.3 ± 0.8[b]	47.4 ± 11.2[a]	20.3 ± 2.7	27.7 ± 3.2
Inner strip	26.1 ± 1.9	18.5 ± 2.0	22.4 ± 2.5	18.3 ± 2.1
Inner medulla	29.1 ± 2.6[b]	18.9 ± 2.2	31.3 ± 4.3	20.0 ± 2.9

Values represent the mean ± SE and are expressed in fmol/mg tissue. [a] Significantly different from control, $p < 0.05$; [b] significantly different from WKY rats, $p < 0.01$

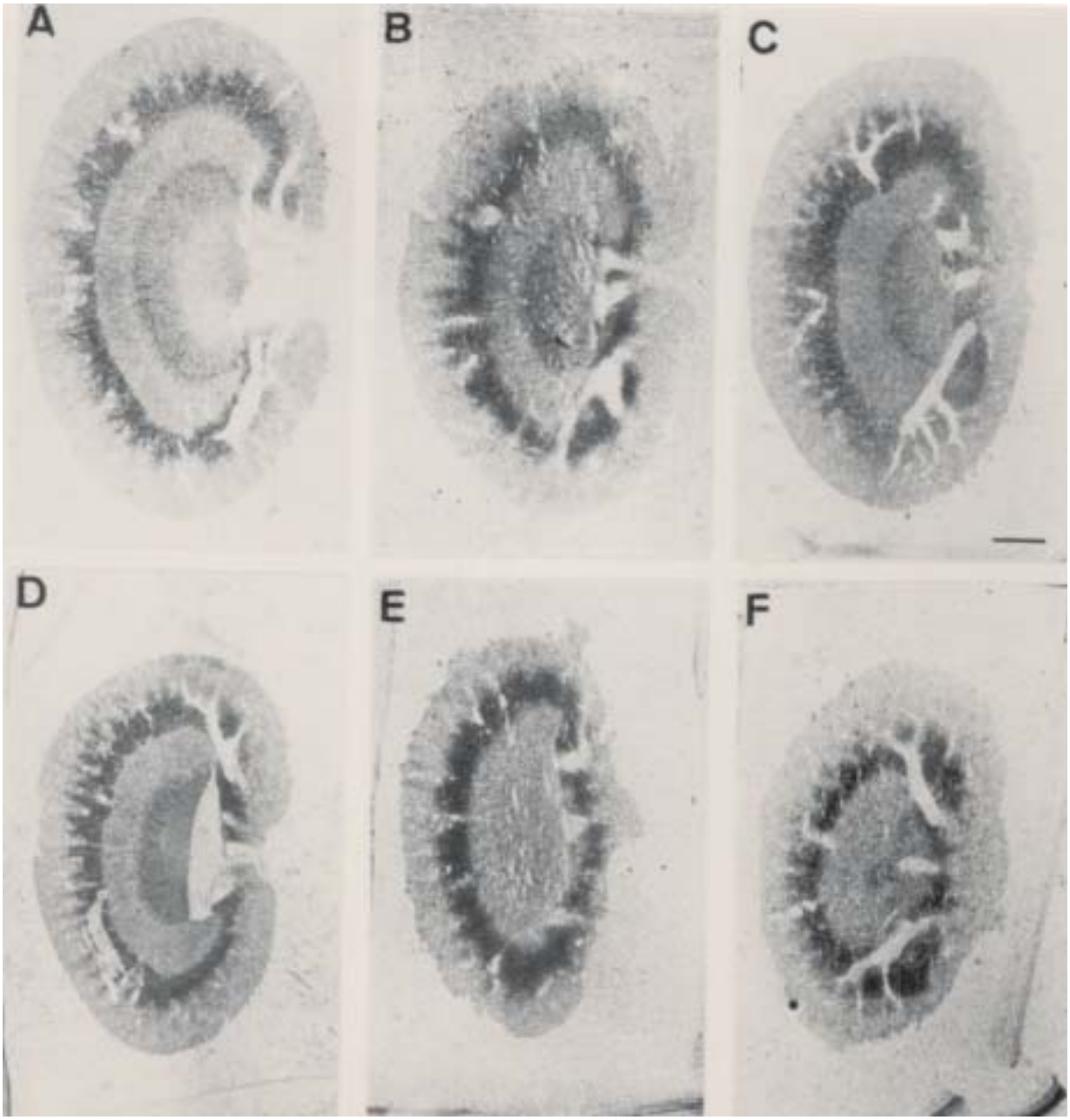

Fig. 2. Autoradiographic illustration of the modifications of α_2-ARs ([^{3}H]UK 14304 binding) induced by pharmacological pretreatment in WKY **(A–C)** and SHRs **(D–F)**. **A** and **D** – control animals, **B** and **E** – yohimbine-pretreated animals, **C** and **F** – clonidine-pretreated animals. Bar – 0.2 cm. Sections cut from different groups were processed in parallel

Finally, in SHRs pretreated with clonidine, the density of α_2-ARs over the cortex was reduced, although these modifications did not reach statistical significance; in the inner aspects of the medulla, a slight tendency to the decrease in the number of [^{3}H]UK 14304 binding sites was observed in this group (Fig. 2D–F, Tab. 3).

Discussion

Since previous studies with radioligands in rat kidney have demonstrated α-ARs in crude homogenates [30] but gave no indication where these are localized, this study demonstrates the feasibility of using [^{3}H]UK

14304 by autoradiographic procedures. Receptor autoradiography has two substantial advantages over biochemical studies, in providing specific anatomical resolution combined with high sensitivity.

Comparing the regions in the cortex of untreated WKY rats with SHRs, the autoradiography indicates that the density is very similar (6.8 *vs.* 8.5). Similarly although the outer strip of the medulla shows an increased binding with yohimbine, the other three regions in the kidney do not in the autoradiographic experiments.

Regarding the anatomical localization of α_2-ARs labeled with [^{3}H]UK 14304 in the kidney, our results show the predominance of these receptors at the medullary level, with clearly lower densities in the renal cortex. These results are in contrast with those previously published by Stephenson and Summers [35] and Calianos and Muntz [8] who found the cortex to contain the highest densities of α_2-ARs in the kidney using [^{3}H]rauwolscine as a ligand. However, they are closely similar to those reported by Sripairojthikoon and Wyss [34] with [^{3}H]para-aminoclonidine. This discrepancy between the histological localization of α_2-ARs depending on the radioligand used has also been found by others authors in the rat kidney [34] as well as in the brain [4]. This could be due to measuring total receptor number in one case (antagonists) and only high affinity receptors in the others (agonist). It could also result either from the labeling of different subtypes [7] or from the existence of an imidazoline binding component [25]. In this regard, preliminary data obtained in our laboratory appear to indicate that most of specific [^{3}H]UK 14304 binding in the rat brain and kidney does correspond to α_2-ARs. It is also worth mentioning that Uhlén and Wikberg [37] have reported that α_{2B}-ARs predominate in the rat kidney.

Although the renal cortex receives a denser sympathetic innervation when compared to the medulla [1], our results show that, in addition to proximal tubules and glomeruli, α_2-ARs are present in other areas, probably associated with collecting tubules, loops of Henle, and distal tubules. A certain localization of these receptors on blood vessels cannot be fully excluded in view of their innervations [16]. Although the exact relationship between this pattern of localization and the functional role of renal α_2-ARs is hard to establish, they appear to play a role in the regulation of both sodium reabsorption mechanisms and intrarenal blood flow [29–34].

Our autoradiographic data provide anatomical support to the increase in the density of renal α_2-ARs in SHRs found in binding studies [29, present data]. Therefore, receptors located over the outer strip of the outer medulla and over the inner medulla are significantly increased in SHRs. Moreover, α_2-ARs in these regions are those most clearly affected by the different pharmacological treatments in both SHRs and WKY rats. The low levels of [^{3}H]UK 14304 binding over the renal cortex preclude accurate analysis of the possible effects of pharmacological treatment of α_2-ARs in this area.

Chronic yohimbine treatment does increase α_2-AR density in the kidney, mainly in the medulla. We also examined the effect of chronic SK&F 86466 but found no effect on either α_2-AR density or localization. A possible explanation for these results is the presence of multiple α_2-AR subtypes modulating neurotransmitter release in the rat kidney, with dissimilar affinities for both yohimbine and SK&F 86466. Alternatively, it is possible that yohimbine and SK&F 86466 are antagonists at both receptors, pre- and postsynaptic subtypes and that, for unknown reasons, one of them is more resistant to the up-regulation phenomenon. The daily administration of SK&F 86466 and clonidine decreased SBP in SHR. This significant decrease in blood pressure was not accompanied by any modification in the number of α_2-AR sites. Clonidine in addition to producing a decrease in blood pressure elicited a marked decrease in heart rate due to its central action.

Since various imidazolines irrespective of their selectivity for the α-AR subtypes, have been shown to induce hypotensive effects when injected into the nucleus reticularis lateralis of the medulla oblongata [3] which suggested the existence of imidazoline binding sites which could be involved in the regulation of blood pressure, the decrease in blood pressure by clonidine without any alteration in the renal α_2-AR number suggests that clonidine can also act as an agonist at imidazoline receptors which is related to the control of arterial blood pressure. On the other hand, the blood pressure in SHRs was not altered by chronic yohimbine treatment. However, after a prolonged blockade in these rats treated with yohimbine for 2 weeks, there were approximately 2-fold increases in the number of [^{3}H]UK 14304 binding sites in kidney preparations. The molecular mechanism underlying the observed up-regulation of renal α_2-AR has yet to be elucidated, but could involve a direct action of yohimbine as well as effects of altered intrasynaptic norepinephrine levels. Also, the up-regulation of these receptors may be related to pathophysiological

processes which occur in disease states such as hypertension.

Although our data fail to detect a significant correlation between α_2-AR density and SBP in animals from the same colony, these data do not allow for conclusions regarding the possible relevance of alterations in α-AR signaling in hypertension. In contrast with the study of Michel et al. [26], it should be mentioned that the fact that they were not able to find a correlation between α_2-ARs and hypertension in SHRs from different breeding colonies does not exclude the possibility that it exists.

In conclusion, the role of the renal α-ARs remains controversial. Physiological studies indicate that renal α_2-AR stimulation causes sodium reabsorption at the proximal tubule [20]. In this study, we demonstrate that a large number of renal α_2-ARs reside in the medulla where they probably are associated with collecting tubules and the autoradiographic study revealed that the total density of α_2-ARs in the renal medulla, but not in the other parts of the kidney, was increased in SHRs. Thus, since the report by Smyth et al. [32] suggests that the extrajunctional α_2-ARs on the collecting ducts mediate sodium and water excretion by antagonizing the antidiuretic effect of vasopressin, the increased renal α_2-ARs may play both antinatriuretic and diuretic roles. Furthermore, the yohimbine-induced up-regulation was localized in the renal medulla and this alteration was not related to any modification in SBP. By contrast, clonidine-induced SBP decrease occured without any alteration in renal α_2-ARs. Thus, these data do not support the hypothesis that alteration in renal α_{2B}-ARs (as measured by autoradiography) is directly responsible of the elevated blood pressure in these animals. Further studies should be directed to the role of renal α_{2A}-ARs in SHRs.

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