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Angiotensin II AT2 Receptors Contribute to Regulate the Sympathoadrenal and Hormonal Reaction to Stress Stimuli

J. M. Saavedra¹I. Armando²

¹Department of Pharmacology and Physiology, Georgetown University Medical Center, 3900 Reservoir Road, Bldg. D, Room 287, Washington, DC 20007, USA

²The George Washington University School of Medicine and Health Sciences, Ross Hall Suite 738 2300 Eye Street, Washington, DC, USA

Abstract

Angiotensin II, through AT1 receptor stimulation, mediates multiple cardiovascular, metabolic, and behavioral functions including the response to stressors. Conversely, the function of Angiotensin II AT2 receptors has not been totally clarified. In adult rodents, AT2 receptor distribution is very limited but it is particularly high in the adrenal medulla. Recent results strongly indicate that AT2 receptors contribute to the regulation of the response to stress stimuli. This occurs in association with AT1 receptors, both receptor types reciprocally influencing their expression and therefore their function. AT2 receptors appear to influence the response to many types of stressors and in all components of the hypothalamic–pituitary–adrenal axis. The molecular mechanisms involved in AT2 receptor activation, the complex interactions with AT1 receptors, and additional factors participating in the control of AT2 receptor regulation and activity in response to stressors are only partially understood. Further research is necessary to close this knowledge gap and to clarify whether AT2 receptor activation may carry the potential of a major translational advance.

Keywords

Angiotensin II; Stress; AT2 receptors; Adrenal medulla; Catecholamines; Sympathoadrenal response

Introduction

Angiotensin II and Angiotensin II Receptors

Angiotensin II Contributes to the Control of Multiple Body Functions—The decapeptide Angiotensin II was discovered almost 70 years ago, and it was initially identified as the only effective principle of the Renin–Angiotensin System, a circulating factor enhancing vasoconstriction and a key participant in the mechanisms involved in

J. M. Saavedra, jms522@georgetown.edu.

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pathological blood pressure increase (Skrbic and Igic 2009). Subsequent studies clarified that Angiotensin II contributed to regulate additional multiple peripheral and brain functions, including the response to stress stimuli (Saavedra 1992, 2012; Bali and Jaggi 2013).

The Search for Angiotensin II Receptor Blockers—For a long time, research was oriented to develop compounds reducing excessive Angiotensin II activity, with the goal of controlling hypertension. Assuming the existence of a single Angiotensin II receptor, peptidic Angiotensin II receptor inhibitors were developed but their oral administration was not effective. Clinical success was reached in the 70s with the production of non-peptidic Angiotensin-Converting Enzyme (ACE) inhibitors, and compounds could be orally administered, reducing hypertension by decreasing Angiotensin II production (Moser 1986).

Discovery of Non-peptidic, Orally Active Receptor Blockers and Demonstration of Angiotensin II Receptor Heterogeneity—Further research demonstrated that Angiotensin II could selectively bind to two different sites, one sensitive or another insensitive to dithiothreitol (Chiu et al. 1990–1991; Wong et al. 1990). Additional non-peptidic ligands were developed and reported to selectively bind to one and not the other site. Angiotensin II binding sensitive to dithiothreitol was displaced by DuP753 (later called losartan). These sites were named AT1 receptors (Chiu et al. 1990). Angiotensin II binding resistant to dithiothreitol was not displaceable by losartan and instead selectively displaced by the non-peptidic ligand PD123177 (Chiu et al. 1990–1991; Wong et al. 1990). These sites were named AT2 receptors.

Limitations of the Present Review—While multiple factors regulate the response to stress stimuli (Carrasco and Van de Kar 2003), the present review is limited to address those factors that have been proposed to influence the expression and activity of AT2 receptors in response to stressors.

For this reason, we will not include an in-depth reexamination of the role of AT1 receptors and their blockade in the response to stress stimuli throughout the organism, and our description here has been limited to essential information to better assess their relationship to AT2 receptor function following exposure to stressors. To examine the response of AT1 receptors to stressors in further detail, readers may wish to consult recently published reviews (Armando et al. 2003a, b; Hunyady and Catt 2006; Saavedra (2005); Saavedra et al. 2011; Villapol and Saavedra 2015; Karnik et al. 2015).

A large body of research is progressively revealing a major complexity of the Angiotensin II system beyond the canonical RAS. Alternative pathways of Angiotensin II production and proposals of physiological effects of additional peptide fragments and receptors have been advanced. The increasing and hotly discussed complexity of the Angiotensin II system will not be addressed in the present review, and the readers are directed to authoritative recent publications on the most important novel and exciting advances (Batenburg and Danser 2012; Ferrario et al. 2014; Horiuchi et al. 2012; Kumar et al. 2012; Xu et al. 2011).

The Role of Angiotensin II AT₂ Receptors in the Response to Stress Stimuli

AT₂ Receptor Cloning, Molecular Structure, and Ligand Selectivity—The discovery of major therapeutic advantages of AT₁ receptor blockade raised questions on the role of the alternative AT₂ receptors and the consequences of their possible over-stimulation by Angiotensin II when AT₁ receptors were blocked (Porrello et al. 2009; De Gasparo and Siragy 1999; De Gasparo et al. 2000; Karnik et al. 2015; Guthrie 1995).

Cloning of AT₂ receptors revealed that they belong to the seven-transmembrane, G-protein-coupled receptor family, located on chromosome X (Inagami et al. 1994). AT₂ receptors are structurally and functionally different from the AT₁ receptor, with only a 32% homology between receptor types (Kambayashi et al. 1993, 1996; Inagami et al. 1994, 1995). This explained the initial findings of Angiotensin II binding resistant to dithiothreitol (Chiu et al. 1990–1991; Tsutsumi and Saavedra 1991a; Tsutsumi et al. 1992) and the selectivity of these binding sites for the non-peptidic ligand PD123319, related compounds such as PD123177 (Chiu et al. 1990–1991; Wong et al. 1990; Blankley et al. 1991; VanAtten et al. 1993) and the peptide derivative CGP42112A (Whitebread et al. 1991).

AT₂ Receptor Expression in Structures Significantly Involved in the Reaction to Stressors—Quantitative autoradiography with displacement of radio-labeled Angiotensin II or I¹²⁵ Sar¹-Angiotensin II with either PD123319 or CGP42112A, or direct binding with I¹²⁵ CGP42112A, qPCR, and in situ hybridization were extensively utilized to localize and quantify AT₂ receptor binding and gene expression, respectively (Kambayashi et al. 1993; Tsuzuki et al. 1994; Jöhren et al. 1995a, b, 1996; Tsutsumi and Saavedra 1991a, b; Israel et al. 1985a, b; Himeno et al. 1988; Heemskerk et al. 1993; Pucell et al. 1991).

It was early recognized that the AT₂ receptor expression is species dependent and that this dependency extends to the adrenal gland, the brain, and many other organs (Chang and Lotti 1991). For example, the expression of brain AT₂ receptors is extremely variable among species, higher in mice when compared to rats, lower in the rabbit, and very low in the human brain (Aldred et al. 1993; Allen et al. 1999; Häuser et al. 1998; Jöhren and Saavedra 1996; Jöhren et al. 1997; MacGregor et al. 1995; Saylor et al. 1992).

Most of the in vivo studies, however, have been performed in rat or mouse brain and pituitary and adrenal glands, and to what degree they extend to other species, particularly in humans, remains to be determined.

The hypothesis of a major role for AT₂ in the reaction to stressors was originated and is sustained by the discovery of the very significant expression of Angiotensin II receptors in the rat adrenal gland (Israel et al. 1984; 1985a), followed by the finding of a remarkable number of AT₂ receptors in the adrenal medulla, the tissue with the highest AT₂ receptor concentration in adult rodents (Kambayashi et al. 1993; Tsuzuki et al. 1994; Jöhren et al. 1995a, b; Tsutsumi and Saavedra 1991a, b; Israel et al. 1985b, 1995; Himeno et al. 1988; Heemskerk and Saavedra 1995; Heemskerk et al. 1993) (Fig. 1).

While it is possible that AT₂ receptors are localized both at their cellular membranes and the chromaffin granules, in adrenomedullary chromaffin cells, most of the studies have been

conducted in PC12 or pheochromocytoma cells and there are no localization studies on primary adrenomedullary chromaffin cells. For this reason, the precise cellular localization of adrenomedullary AT2 receptors has not been determined.

In the adrenal medulla, AT2 receptor expression markedly predominates over the limited AT1A receptor number (Macova et al. 2008). In the adrenal zona glomerulosa, AT2 receptors are expressed in approximately equal numbers with AT1B receptors (Macova et al. 2008). Whether the AT2, AT1A, and AT1B receptors are co-expressed in the same cells, either in the adrenal zona glomerulosa or adrenal medulla, has not been clarified.

Initial findings on the AT2 receptor distribution in the brain did not directly support the hypothesis of their participation in the response to stressors. While there is a widespread distribution of AT2 receptors in early development, in the adult rat initial studies indicated that their expression is restricted to a few brain nuclei such as the inferior olive and are detectable in the brain areas directly related to the response to stressors (Tsutsumi and Saavedra 1991b; Allen et al. 1999).

However, in the mouse there are AT2 receptors in amygdaloid nuclei and, in small numbers, in the paraventricular nucleus (PVN) and the locus coeruleus. In these areas, AT2 receptors may contribute to regulate peripheral sympathetic activity and behavioral responses to stress stimuli (Häuser et al. 1998; Jöhren et al. 1997; Saavedra et al. 2004). While there are no AT2 receptors in the rat pituitary gland (Tsutsumi and Saavedra 1991e), their presence in the mouse PVN suggests the possibility of an indirect effect.

Influence of Stress Stimuli in Adrenal AT2 Receptor Expression—Although initial studies reported that all effects of Angiotensin II in the adrenal gland were the result of AT1 receptor stimulation and that AT2 receptors played no role (Giacchetti et al. 1996), it was later demonstrated that changes in AT2 receptor number, AT2 receptor blockade, or deletion of the AT2 receptor gene influence the sympathetic, adrenomedullary, hormonal, and behavioral response to stressors.

The influence of stress in AT2 receptor expression has been for the most part studied in the rodent adrenal gland. All types of stressors studied affect AT2 gene expression, except for water deprivation and in spontaneously hypertensive rat (SHR) not submitted to stressors that are hypersensitive to stress stimuli, when compared to normotensive controls (Chatelain et al. 2003; Armando et al. 2001, 2003a, b; Leong et al. 2002; Nostramo et al. 2012, 2015; Sanchez-Lemus et al. 2008; Bucher et al. 2001; Lunardelli et al. 2015; Kitamura et al. 1998; Qiu et al. 1999; Bibeau et al. 2010; Jöhren et al. 2003) (Table 1).

In most cases of restraint, immobilization, and inflammatory stressors, whether inflammation is produced in adult or neonatal rats, and after ACTH administration, when determined, AT2 receptor gene expression was found to be reduced both in the zona glomerulosa and adrenal medulla or in the whole adrenal gland (Sanchez-Lemus et al. 2008; Lunardelli et al. 2015; Leong et al. 2002; Nostramo et al. 2015; Bucher et al. 2001; Sanchez-Lemus et al. 2008; Bregonzio et al. 2003; Kitamura et al. 1998) (Table 1; Fig. 2).

Exceptions were AT2 gene expression in the adrenal zona glomerulosa after repeated restraint (Leong et al. 2002), adrenomedullary AT2 gene expression after birth in rats submitted to growth restriction with a low-sodium diet during prenatal life (Bibeau et al. 2010), in the stressor-sensitive rat strain with low angiotensinogen not submitted to stressors (Müller et al. 2010), and following sympathectomy (Qiu et al. 1999). In these models, AT2 gene expression was increased (Table 1).

In the rat adrenal medulla, there were increases in AT2 receptor binding following isolation, a less intense stress stimulus prolonged for 24 h (Armando et al. 2001), after acute restraint in mice (Armando et al. 2003a, b), and after sympathectomy (Qiu et al. 1999) (Table 1).

It appears that alterations in adrenal AT2 receptor gene expression are dependent on whether the stressor is acute, like in single restraint, or repeated daily for several days. In the rat zona glomerulosa, repeated restraint reverses the initial decrease which occurs during acute restraint, and in the adrenal medulla, where the acute decrease in gene expression is normalized after repeated immobilization, indicating that adaptation mechanisms may be at play (Leong et al. 2002) (Table 1).

Alterations in adrenal AT2 receptor binding and gene expression are species dependent and are influenced by factors with additional strong influence in the response to stressors. There were no changes after maternal dietary restriction in lambs (Zhang et al. 2013), and while wild-type mice respond to acute restraint with decreased AT2 receptor binding in the adrenal medulla, the absence of the serotonin transporter (Sert^{-/-} mice) reverses this effect, and the adrenomedullary AT2 binding is enhanced (Armando et al. 2003a, b).

It is of note that, in rats, alterations in AT2 receptor gene or binding expression after acute and repeated restraint and after isolation were not complete and were not parallel with the respective AT2 gene expression or binding that were not modified (Leong et al. 2002; Armando et al. 2001) (Table 1). These findings may be interpreted as differential timing for AT2 receptor gene expression and binding, or by the influence of different stress stimuli in AT2 receptor recycling and/or translation, and are in need of clarification.

We can conclude that stress stimuli strongly influence AT2 receptor gene expression and binding both in the zona glomerulosa and adrenal medulla. These changes are dependent on the type of stressor, its intensity, and adaptation mechanisms, and are species dependent (Leong et al. 2002; Armando et al. 2001, 2003a, b; Bibeau et al. 2010; Zhang et al. 2013) (Table 1).

These apparent discrepancies noted may be the consequence of incomplete studies. In most cases, AT2 receptor gene expression and binding have not been determined simultaneously (Table 1), mechanisms of adaptation over time have not been considered, and simultaneous comparison or combination of different stressors has not been performed. For these reasons, it is difficult to determine how alterations in AT2 receptor transcription and production influence each other, and their role on the distinct phases of the response to stress stimuli.

Nevertheless, the significant although sometime opposite alterations in AT2 receptor gene expression and binding reported above correlate with the well-characterized increases in

adrenal catecholamine synthesis in both species, a hallmark of the response to stress stimuli (Armando et al. 2001, 2003a, b) (see below).

Influence of Stress Stimuli in Brain AT2 Receptor Expression—There are very few studies on the influence of stressors on brain AT2 receptors. In the stressor-sensitive strain, the SHR, cold-restraint decreased AT2 receptor binding in the ventrolateral nucleus of the thalamus. Conversely, AT2 receptor binding was enhanced in the inferior olivary complex (Bregonzio et al. 2008). The significance of these alterations has not been clarified.

Influence of Stressors in AT2 Receptor Expression in Other Organs—There are indications that stress stimuli may influence AT2 receptor expression throughout the organism, since cold-restraint decreases AT2 receptor binding in the stomach, and this is associated with massive ulcerations in the stomach mucosa (Bregonzio et al. 2003).

Relationship Between AT1 and AT2 Receptors—Changes in the expression or blockade of either AT1 or AT2 receptors reciprocally regulate the expression, and therefore the function, of the alternative receptor type. This has been reported in selective brain nuclei (Armando et al. 2002a, b; Bregonzio et al. 2008; Seltzer et al. 2004), the kidney (Saavedra et al. 2001a, b), the stomach (Bregonzio et al. 2003), and particularly in the adrenal gland (Saavedra et al. 2001a, b; Seltzer et al. 2004).

However, while increases in AT1 receptor expression in the adrenal gland are common to all types of stress stimuli studied, these changes occur whether the AT2 receptor expression is decreased, increased, or not changed. Examples include acute restraint (Leong et al. 2002), repeated restraint (Leong et al. 2002; Nostramo et al. 2015), neonatal inflammation (Lunardelli et al. 2015), acute inflammation in adult rats (Sanchez-Lemus et al. 2008), isolation (Armando et al. 2001), and water deprivation (Chatelain et al. 2003). The conclusion is that AT1 receptor expression, which is the rule in response to stressors, does not necessarily relate to AT2 expression. This makes the relationship between AT1 and AT2 receptors in response to stress stimuli not clear, the mechanisms involved not determined, and the results very difficult to interpret.

In the brain, AT1/AT2 receptor interactions may not be direct. In the adult rodent brain, AT2 receptor expression is extremely low, and for the most part is revealed in the brain areas devoid of AT1 receptor expression (Tsutsumi and Saavedra 1991a, b, c, d). There is no convincing demonstration of same-cell localization of AT1 and AT2 receptors not only in the brain but also, as stated earlier, in the adrenal gland, or in any other organ studied.

Notwithstanding the very limited AT2 receptor expression in the adult rodent brain and the lack of a demonstration of AT1/AT2 receptor co-expression in a single cell, numerous in vitro and in vivo studies have been performed to clarify AT2 receptor function, mechanisms of action, and the possible correlation with AT1 receptor activity in models not submitted to stressors (Danser et al. 2015). Based on these studies, it was proposed that while AT1 receptor stimulation carried most of the effects of Angiotensin II, AT2 receptor activation was protective, a counterbalance of excessive AT1 receptor function (Danser et al. 2015).

Effect of AT1 Receptor Blockade on Gene Expression and Binding of AT2

Receptors—The relationship between AT1 and AT2 receptors may be better understood addressing the effect of AT1 receptor blockade on the expression of AT2 receptor under stress stimuli, in rodent strains with increased susceptibility to stressors, and in non-stimulated control rats (Table 2).

AT1 receptor blockade, in vivo, generally modifies AT2 receptor gene expression and binding following the response to stressors. When determined, AT2 gene expression is enhanced by AT1 receptor blockade. This was reported to occur after isolation in both the adrenal zona glomerulosa and the adrenal medulla (Armando et al. 2001, 2003a, b; Jezova et al. 2003) and in the zona glomerulosa of salt-restricted rats (Gigante et al. 1997) (Table 2). On the other hand, AT2 receptor gene expression was not changed after acute inflammation in rats (Sanchez-Lemus et al. 2008) (Table 2).

The influence of in vivo AT1 receptor blockade was more frequently reported on AT2 receptor binding (Table 2). In most but not all cases, AT2 receptor binding is decreased by AT1 receptor blockade (Armando et al. 2001, 2003a, b; Jezova et al. 2003; Sanchez-Lemus et al. 2008; Zhuo et al. 1994) (Table 2).

In the adrenal medulla, there was a reduction of AT2 binding after AT1 receptor blockade during isolation (Armando et al. 2001, 2003a, b; Jezova et al. 2003), acute inflammation (Sanchez-Lemus et al. 2008), in SHR (Seltzer et al. 2004), and in control rats not submitted to stressors (Zhuo et al. 1994) (Table 2).

In the adrenal zona glomerulosa, AT1 receptor blockade reduced AT2 binding in control rats (Zhuo et al. 1994) and after acute inflammation (Sanchez-Lemus et al. 2008) (Table 2).

Exceptions are the SHR, where the AT2 receptor binding is increased without exposure to stressors in the locus coeruleus, inferior olive, and the adrenal zona glomerulosa (Seltzer et al. 2004), and in the adrenal zona glomerulosa of rats submitted to isolation, where both AT2 receptor gene expression and binding are increased (Armando et al. 2001). In the locus coeruleus and inferior olive, AT1 receptor blockade prevents the stressor-induced reduction in AT2 receptor binding (Saavedra et al. 2006) (Table 2).

The influence of brain AT1 receptors on AT2 receptor activity has been determined by systemic administration of AT1 receptor blockers, compounds passing the blood–brain barrier and reaching the brain parenchyma (Saavedra 2012, 2016).

In vivo AT1 receptor blockade enhanced AT2 receptor binding in the locus coeruleus and inferior olive (Seltzer et al. 2004) and prevented the decrease in AT2 receptor binding provoked by isolation (Saavedra et al. 2006) (Table 2).

Isolation, revealing the well-characterized enhanced pituitary–adrenal response, simultaneously increased AT1 receptor binding in the paraventricular nucleus (PVN) and enhanced adrenomedullary AT2 receptor binding (Armando et al. 2001). In turn, AT1 brain and peripheral AT1 receptor blockade normalized deisolation-induced changes in ACTH and AVP in the pituitary gland, the reduction in adrenal corticosterone, aldosterone, and

catecholamines, and decreased urinary vasopressin, corticosterone aldosterone, and catecholamines (Armando et al. 2001). Concomitantly, AT1 receptor blockade reversed the isolation-induced AT2 receptor binding in the adrenal medulla (Armando et al. 2001). While these results may be considered evidence of AT2 receptor participation in the stressor-induced pituitary–adrenal response in association with AT1 receptor activity, there is no definite proof of a causal relationship.

Unfortunately, excepting a few studies on isolation (Armando et al. 2001, 2003a, b; Jezova et al. 2003) and acute inflammation (Sanchez-Lemus et al. 2008), all other reports did not compare simultaneously AT2 receptor gene expression and binding (Table 2) during AT1 receptor blockade. Furthermore, the alterations in AT2 receptor gene expression and binding are to some extent depending on the type of stress stimuli administered and the organ studied.

The influence of AT1 receptor blockade on AT2 receptor activity is not restricted to the adrenal gland and the brain. In the thoracic aorta of SHR not submitted to stressors, AT1 receptor blockade unmasks vasodilator effects of AT2 receptors (Cosentino et al. 2005). This is evidence that the close interaction between AT1 and AT2 receptors extends to the peripheral vasculature. Regretfully, the role of AT2 receptors in the peripheral arteries or other peripheral organs has not been adequately addressed during exposure to stressors.

Cold-restraint significantly reduces AT2 receptor binding in the stomach mucosa, in association with the production of stomach ulcers (Bregonzio et al. 2003). The influence of AT1 receptor blockade on AT2 receptor expression in the vasculature and the stomach indicates that blocking AT1 receptors influences AT2 receptor expression and activity throughout the organism.

The overall conclusion is that in vivo AT1 receptor blockade significantly influences AT2 receptor gene expression and binding, not only during diverse types of stress stimuli and in a stressor-prone rat strain, but also under non-stimulated conditions in control rats. These results support the hypothesis that AT1 receptor activity not only influences the response of AT2 receptors to homeostatic challenges but also regulates their physiological activity.

Effects of AT2 Receptor Deletion—Studies on the influence of AT1 receptor blockade on AT2 receptor expression are complemented by research on the effects of AT2 receptors on AT1 receptor expression and function. Several studies analyzed either the effect of pharmacological blockade of AT2 receptors or that of deletion of the AT2 receptor gene.

The abolition of AT2 receptor activity results in whole-body permanent impairment of AT2 receptor expression (AT2 receptor knockout model, AT2 KO) (Hein et al. 1995; Ichiki et al. 1995; Watanabe et al. 1999; Okuyama et al. 1999) (Fig. 3) and enhances the response to hyperthermic, inflammatory, cage-switch and isolation (Saavedra et al. 2001a, b; Armando et al. 2002a, b; Watanabe et al. 1999). When compared to littermate controls, AT2 receptor-deficient mice exposed to stress stimuli further increase stressor-induced blood pressure, Angiotensin II-induced vasoconstriction, and adrenomedullary catecholamine synthesis and release (Hein et al. 1995; Ichiki et al. 1995) and exhibit anxiety behavior (Ichiki et al. 1995).

All these effects are associated with excessive AT1 receptor stimulation, since AT1 receptor overactivity participates in many responses to stress stimuli, and AT1 receptor blockade reduces inflammation and hyperthermia (Watanabe et al. 1999).

Increased anxiety in AT2 knockout mice may partially be the consequence of corticotropin-releasing factor (CRH) and central sympathetic system alterations following enhanced AT1 receptor activation (Macova et al. 2009) and involving $\alpha 1$ adrenergic receptors in the amygdala (Okuyama et al. 1999). This is of interest because, unlike the rat, mice express significant numbers of AT2 receptors in amygdaloid nuclei, an area playing a key role in the regulation of emotional behavior (Häuser et al. 1998).

These findings strongly suggest that the impairment of AT2 receptor expression enhances the effects of AT1 receptors. In non-stressed AT2 knockout mice, as it is the case in normal rats submitted to stressors, there is an enhanced AT1 receptor expression in the PVN, the median eminence (Armando et al. 2002a, b), and the adrenal gland (Saavedra et al. 2001a, b). For this reason, it is not surprising that the absence of AT2 receptor activity enhances the response of the entire hypothalamic–pituitary–adrenal axis, including increases in ACTH and glucocorticoid secretion and reduced adrenal aldosterone content (Armando et al. 2002a, b) (Fig. 4).

Moreover, upregulation of AT1 receptors in AT2 KO mice is a general phenomenon, since it has also been described in peripheral organs such as the kidney (Stegbauer et al. 2005; Saavedra et al. 2001a, b). This effect is also noted in the spleen and lung, organs devoid of AT2 receptors (Pavel et al. 2009). These results indicate that AT2 receptors regulate AT1 receptor expression indirectly, and probably throughout the organism. The mechanisms of such indirect interaction have not been addressed.

Effects of AT2 Receptor Blockade—The observations in AT2 knockout mice correlate well with the findings observed after peripheral administration of an AT2 receptor blocker in adult control rats (Macova et al. 2009). In this model, AT2 receptor blockade is universal, since it has been observed in the locus coeruleus, an area involved in the control of central sympathetic activity, and in the inferior olivary complex (Macova et al. 2009), and indicates that systemically administered AT2 receptor blockers reach the brain parenchyma in amounts sufficient to block parenchymal AT2 receptors.

In the locus coeruleus, reduction of AT2 binding correlates with decreased gene expression of TH, suggesting that AT2 receptor activity may contribute to regulate catecholamine synthesis in the brain, in balance with AT1 receptor activity (Macova et al. 2009). Pharmacologically induced AT2 receptor blockade enhances AT1 receptor binding and AT1 gene expression in the subfornical organ and the paraventricular nucleus, areas regulating central sympathetic activity (Macova et al. 2009).

In addition, as it is the case in AT2 KO mice, AT2 receptor blockade enhances AT1 receptor binding in the median eminence, an area intimately associated with the regulation of the pituitary gland, and therefore the hypothalamic–pituitary axis (Macova et al. 2009). The vasopressin and ACTH content in the pituitary gland are significantly decreased after AT2

receptor blockade, a finding that may indicate increased hormone release (Macova et al. 2009) since it correlates with the enhanced ACTH secretion to the circulation observed in AT2 receptor knockout mice (Armando et al. 2002a, b). Since there are no receptors in the pituitary gland, these alterations are indirect and may be the consequence of the enhanced AT1 receptor activity in the PVN and median eminence (Macova et al. 2009).

In a model of combined noise and footshock stressors applied to SHR, AT2 receptor blockade increases locus coeruleus stimulation by noradrenaline released in the hippocampus, and AT2 receptor stimulation inhibits noradrenaline release, reducing heart rate, indicating that AT2 receptors antagonize AT1 receptor-related effects (Gong et al. 2015).

The frequent reciprocal alterations in AT1 and AT2 receptor expression and activity support the hypothesis of AT2 receptor cross-talk with AT1 receptors under physiological conditions and in response to stressors, and not only in the adrenal gland but also in the brain and other peripheral organs (Leong et al. 2002; Bregonzio et al. 2008; Saavedra et al. 2001a, b; Armando et al. 2002a, b; Macova et al. 2009).

It may be concluded that the absence of AT2 receptors, and their universal blockade by systemic administration of AT2 receptor blockers, results in a stressed state, not essentially different than that induced in normal rodents by all stressors studied. This may be the most conclusive evidence of a role for AT2 receptors in response to stressors, their activation acting as a break for excessive AT1 receptor stimulation.

AT1/AT2 Interaction in Other Organs—Interactions between AT1 and AT2 receptors have been extensively explored in the vasculature, and it was established that vascular contractility to Angiotensin II is AT1 receptor dependent (Chung and Unger 1999). There are indications that AT2 receptor activity counteracts that of AT1 receptors in the vasculature. In the aorta of rats submitted to repeated exposure to stressors, AT2 blockade decreases the maximum contractile response to Angiotensin II (Baptista et al. 2014). In the human heart, AT2 receptors facilitate norepinephrine release by Angiotensin II (El Muayed et al. 2004). AT2 gene expression in the aorta of SHR is increased, and AT2 mediates vasodilatation after AT1 blockade (Cosentino et al. 2005).

However, the complexity of the effects of AT2 receptor activation is revealed by a report that the use of an AT2 receptor agonist may result not only in vasodilatation but also in vasoconstriction, depending on the conditions of the experiment, and by mechanisms unrelated to AT2 receptor activity (Verdonk et al. 2012).

Unfortunately, there is no definite conclusion on the characteristics of the AT1/AT2 interaction, and in conclusion the nature and the extent of the roles of AT2 receptors and the significance of their proposed regulation of AT1 receptor responses throughout the organism in health and disease have always been, and still are, a matter of controversy (Porrello et al. 2009; Miura et al. 2013; Akazawa et al. 2013; Clauser et al. 1996).

Regulation of Basal and Stressor-Induced Catecholamine Production and Release

Role of the Adrenal Gland—As a component of the hypothalamic–pituitary–adrenal system, the adrenal gland plays a fundamental role in the hormonal and sympathetic regulation of multiple organs, the maintenance of homeostasis, and the response to stressors. Enhanced catecholamine synthesis in the adrenal medulla and a remarkable increase in adrenaline release to the general circulation are hallmarks of the stress reaction. Such responses occur in all types of stressors studied, including inflammatory, restraint, isolation, maternal separation, and intrauterine growth restriction (Sanchez-Lemus et al. 2008; Leong et al. 2002; Armando et al. 2001; Bobrovskaya et al. 2013; Bibeau et al. 2010).

Catecholamine production and release from the adrenal medulla is tightly regulated under normal conditions. It is through the regulation of adrenal TH transcription and activity that AT2 and AT1 receptors participate in adrenaline production and release, under basal conditions and after submission to stressors and through complex synergistic regulations with common as well as distinct aspects.

It has been demonstrated that under basal conditions TH gene transcription is maintained and enhanced in response to stressors, by AT1 and AT2 receptor activation. Conversely, both AT1 and AT2 receptor blockade decreases, under basal conditions, TH gene expression and norepinephrine concentration in the adrenal medulla (Armando et al. 2004).

In vivo experiments in dogs revealed that while AT2 receptor blockade did not affect basal catecholamine release, it ameliorated Angiotensin II-induced catecholamine release (Martineau et al. 1999). In addition, after intrauterine growth restriction there was a correlation between enhanced AT2 receptor gene expression, increased TH mRNA, and lower catecholamine content, implying increased secretion (Bibeau et al. 2010). AT1 receptor blockade in rats exposed to stress not only decreases catecholamine production in the adrenal gland, but also enhances brain AT2 receptor expression, a possible compensatory mechanism (Bregonzio et al. 2008).

In vitro studies in PC12 cells, however, revealed direct but contradictory effects of AT1 and AT2 receptors; AT2 receptor activation decreases TH and dopamine- β -hydroxylase mRNA expression, while blockade of AT1 receptors abolishes Angiotensin II-induced increase in TH mRNA expression (Nostramo et al. 2012). Again, there appears to be a discordance between observations from in vivo and in vitro studies.

Regulatory Role of AT2 Receptors in Aldosterone Secretion

Increased activation of the adrenal zona glomerulosa during exposure to stressors increases the production and release of aldosterone, a sodium-retentive and proinflammatory hormone, in part through Angiotensin II and ACTH stimulation (Bollag 2014).

Initially, aldosterone production and release was reported to be the consequence of AT1 receptor stimulation (Belloni et al. 1998; Volpe et al. 1997; Hano et al. 1994; Wong et al. 1990; Balla et al. 1991; Tanabe et al. 1998a, b).

Subsequent experiments revealed a role of AT2 receptors in aldosterone production and release. While in dispersed zona glomerulosa cells Angiotensin II-stimulated aldosterone production was only blocked by AT1 receptor antagonists, both AT1 and AT2 blockers reduced Angiotensin II effects in adrenal slices containing chromaffin cells (Mazzocchi et al. 1998). This was interpreted as a paracrine effect of AT2 receptor activation, enhancing adrenaline production in chromaffin cells and leading to β -adrenoceptor-induced aldosterone production in the zona glomerulosa (Mazzocchi et al. 1998).

Moreover, in human adrenal glands, AT2 receptor stimulation increases aldosterone secretion independently of AT1 receptors (Tanabe et al. 1998a, b).

The results on the regulation of AT2 gene expression by aldosterone are also contradictory. While aldosterone was reported to decrease AT2 gene expression in the adrenal medulla (Wang et al. 1998) and these receptors are downregulated in the adrenal gland during DOCA-salt hypertension (Elijovich et al. 1997), another study failed to find aldosterone effects on AT2 receptor gene expression (Kitamura et al. 1998).

Molecular Mechanisms of AT2 Signaling

Regulation of AT2 Receptor Transcription and Translation—Little is known on factors regulating AT2 receptor transcription and translation. There are regulatory elements in the 5' flanking region containing cis-regulatory elements, such as AP-1, AP-2, C/ERB, NF-1, NF-IL6, NF-kappa B, and glucocorticoid and cAMP response elements (CRE). cAMP, through protein kinase A, downregulates AT2 mRNA by reducing mRNA destabilization, indicating the possible downregulation of AT2 translation by humoral factors transducing cAMP (Murasawa et al. 1996) and activating the protein kinase C–calcium pathway (Kijima et al. 1996).

Signal Transduction Mechanisms of AT2 Receptors—AT2 receptors were initially associated with G-protein-in-dependent mechanisms (Bottari et al. 1991) and stimulation of protein tyrosine phosphatase activity, in turn inhibiting particulate guanylate cyclase activity and rapidly dephosphorylating tyrosine residues of specific proteins associated with modulation of T-type Ca^{++} channels and regulation of cell proliferation and differentiation (Bottari et al. 1991, 1992) (Fig. 5). AT2-induced reduction of intracellular cyclic guanosine monophosphate (cGMP) (Israel et al. 1995) in turn decreased ERKs, JNK, and p38 MAPK via Ca^{2+} -dependent protein kinase C (PKC) as well as STATs 1, 3, and 5 (Ishii et al. 2001) (Fig. 5). These results explain how AT2 stimulation decreases atrial natriuretic peptide-stimulated cGMP concentration (Brechler et al. 1994a, b). Similar mechanisms may play a role in the increase in cortisol production, as revealed in bovine fasciculate cells, where AT2 receptor stimulation potentiated K^{+} -induced cortisol production in a manner independent of Gi (Defaye et al. 1995).

Conversely, other studies reported that AT2 receptor activation inhibits protein tyrosine phosphatase through a G-protein-coupled mechanism (Takahasi et al. 1994) and that signaling via a Gi protein was coupled to the activation of PP2A, PLA2, and K^{+} channels (Gelband et al. 1998).

Interaction with AT1 Receptor Signaling in Adrenomedullary Catecholamine

Production—AT1 and AT2 receptor stimulation activates different signal transduction mechanisms, sometimes resulting in similar and sometimes opposite effects. The complexity of these interactions is context dependent and may be best explained when analyzing their effects of adrenomedullary catecholamine production.

Although the activation of both AT1 and AT2 receptors increases catecholamine production, the molecular mechanisms involved in the regulation of TH mRNA expression are different for AT1 and AT2 receptors. AT1-related TH activation is associated with phosphorylation of the cAMP response element binding protein (pCREB) and ERK2, while AT2 receptor activation involves increased Fos-related antigen Fra-2 (Jezova et al. 2003; Armando et al. 2004; Peng et al. 2002) (Fig. 5). AT1 receptor blockade decreased both the pCREB/ERK2 and the Fra-2 mechanisms, resulting in reduced TH gene expression and adrenomedullary catecholamine production (Jezova et al. 2003). AT2 receptor blockade decreased TH gene expression, catecholamine production, and Fra-2 but does not affect the pCREB/ERK2 signaling (Jezova et al. 2003).

Reproductive hormones actively regulate the effects of AT1 and AT2 receptors on adrenomedullary catecholamine synthesis. Ovariectomy increased AT1 receptor expression while decreasing adrenomedullary catecholamine content and Fra-2 expression. Conversely, estrogen replacement normalizes AT1 receptor, TH, and Fra-2 gene expression and catecholamine content and increased AT2 receptor gene expression (Macova et al. 2008).

Opposite Effects of AT1 and AT2 Receptor Activation are Organ and Context

Dependent—In the adrenal zona glomerulosa, AT2 receptor stimulation prevented the AT1 receptor-mediated DNA synthesis increase (Mazzocchi et al. 1997), inhibits proliferation, and induce differentiation, effects mediated through hypoxia-inducible factor (HIF) and peroxisome proliferator-activated receptor gamma (PPAR γ) activation (Wolf et al. 2004; Zhao et al. 2005).

Conversely, in adrenal cortical cells, AT1 receptor stimulation downregulates AT2 receptor expression by decreasing mRNA stability through protein kinase C activation (Ouali et al. 1997).

However, it was also reported that the antimitogenic effect of AT2 receptor stimulation is additive to that of AT1 receptor stimulation (Liakos et al. 1997). In this case, different pathways are involved, AT1 receptor-dependent inhibition of bFGF-induced proliferation by prostaglandin synthesis stimulation and AT2 receptor-dependent protein tyrosine phosphatase activation (Liakos et al. 1997).

Different effects of AT2 receptor stimulation have been reported in the heart. Natriuretic peptide receptor/guanylyl cyclase-A (GCA) signaling inhibits AT2-mediated pro-hypertrophic signaling in the heart, protecting this organ from hypertrophy (Li et al. 2009).

Opposite effects of AT1 and AT2 receptor activation appear to depend on Angiotensin II concentrations. At low concentrations, Angiotensin II dilates adrenal cortical arteries through endothelial AT2 receptor activation and reduced NO production, while higher

Angiotensin II concentrations induce vasoconstriction through enhanced NO release and AT1 stimulation (Gauthier et al. 2005).

The cellular interactions between AT2 and AT1 receptor activities have been further analyzed in PC12 cells transfected with AT1 receptors. There was a report (AbdAlla et al. 2001) of direct binding of AT2 receptors to AT1 receptors under these conditions. These results are subject to criticism, because transfection of the AT1 receptor produces abnormally large receptor quantities in cells not normally expressing the receptor, and because of the use of non-characterized antibodies (see below). The results are further in doubt because a previous report of heterodimerization of AT1 with bradykinin B2 receptor (AbdAlla et al. 2000) has been found not to be correct (Hansen et al. 2009). Similar objections may be raised on the report that the hAT1-R/G(q)/extracellular signal-regulated kinase 1/2 pathway is involved in the downregulation of AT2-R using PC12 cells transfected with AT1-R (Saito et al. 2008).

The results outlined above demonstrate that the molecular mechanisms of AT2 signaling are incompletely understood and results are often contradictory. The studies reported isolated signaling aspects and many investigations were focused on PC12 cells, expressing AT2 receptors but not representing primary neurons or chromaffin cells. Furthermore, the participation of specific signal transduction mechanisms in the response to stressors has not been addressed.

Additional Factors Influencing the Response of AT2 Receptors to Stress

The physiological function of AT2 receptors and their reaction to stressors are regulated by multiple mechanisms in addition to AT1 receptors. Some of these factors are addressed below (Fig. 6).

Role of the Additional Components of the Renin–Angiotensin System—There is one study on a hypothetical role of Angiotensinogen on adrenal AT2 receptors, reporting that rats low in Angiotensinogen exhibit increased adrenal AT2 mRNA levels, in association with increased ACTH and corticosterone responses to the forced swim model (Müller et al. 2010). In this transgenic model, unlikely to represent physiological or pathological conditions, the results appear to be counterintuitive. Decreased levels of the Angiotensin II precursor should be reflected in decreased Angiotensin II production leading to reduced AT1 receptor stimulation and a diminished response to stressors. This interpretation is opposite to the body of literature demonstrating that increased susceptibility and vulnerability to stressors are associated with enhanced brain and peripheral AT1 receptor stimulation (Saavedra and Benicky, 2007).

Many RAAS components were identified both in the adrenal zona glomerulosa and in the adrenal medulla (Wang et al. 2002; Nostramo et al. 2015; Gupta et al. 1995; Horiba et al. 1990; Okamura et al. 1984; Peters 2012), suggesting that local adrenal production of Angiotensin II may be involved in the AT2 receptor regulation. However, there are no studies on the participation of these components, except AT1 receptors, in the regulation of AT2 receptor function after submission to stress stimuli.

In addition, AT₂ receptors may be directly stimulated by circulating Angiotensin II, and its production is increased after exposure to stressors (Saavedra and Benicky 2007). The relative role of the circulating and adrenal Angiotensin II in stimulating AT₂ receptors when exposed to stressors has not been determined (Fig. 7).

Central and Peripheral Sympathetic System—Activation of the central and peripheral sympathetic system after exposure to stress stimuli is well known. Injection of Angiotensin II in the brain of SHR stimulated sympathoadrenal catecholamine production and release (Seltzer et al. 2004). Brain and peripheral AT₁ receptor blockade prevents the enhanced sympathetic stimulation, increasing AT₂ receptor binding in the adrenal cortex and decreasing it in the adrenal medulla (Seltzer et al. 2004). The alterations in adrenal AT₂ binding resulting from injection of Angiotensin II in the brain are more likely the result of both AT₁ receptor and sympathetic stimulation, acting simultaneously or in sequence (Fig. 6).

In support of the close association between sympathetic activity and AT₁/AT₂ function, it was reported that sympathectomy increases AT₁ and AT₂ receptor expression in the adrenal gland (Qiu et al. 1999).

Conversely, AT₂ receptor activity may influence the organization of the sympathetic response. Stimulation of both AT₁ and AT₂ receptors increases insulin-induced adrenaline release from the adrenal medulla by modulating the sympathoadrenal response at several levels (Worck et al. 1998). In addition, AT₂ blockade increases baroreceptor-mediated bradycardia during insulin-induced hypoglycemia (Worck et al. 2001).

The precise mechanisms controlling the mutual influence between sympathetic activity and AT₂ receptor function have not been clarified.

ACTH and Glucocorticoids—AT₂ receptor expression and ACTH production, release, and effects on the adrenal zona glomerulosa regulate and balance each other under resting conditions and after exposure to stressors (Fig. 6).

Angiotensin II increased the expression of ACTH receptor mRNA and receptor number, increasing ACTH activity in the adrenal zona glomerulosa, effects initially attributed exclusively to AT₁ receptor activation (Lebrethon et al. 1994). However, under resting conditions, in vivo ACTH administration reduces binding and gene expression of AT₂ receptors in the adrenal zona glomerulosa (Kitamura et al. 1998). Conversely, simultaneous central and peripheral AT₂ receptor blockade decreases pituitary ACTH content, probably the consequence of increased hormone release and the indirect result of increased AT₁ receptor activity (Macova et al. 2009).

It appears that glucocorticoid receptor activity is important to maintain AT₁ and AT₂ receptor expression, as well as TH gene expression and catecholamine content in the adrenal medulla (Fig. 6). Transgenic mice with partial glucocorticoid receptor reduction show not only a decrease in adrenomedullary AT₂ receptor expression and TH mRNA and catecholamine content, but also, in the brain, decreased AT₁ and AT₂ receptor expression,

hypothalamic Corticosterone-Releasing Hormone (CRH) and vasopressin content, and reduced sympathetic tone (Jain et al. 2004).

Reproductive Hormones—The constitutive adrenal expression of AT1 and AT2 receptors, catecholamine synthesis, and aldosterone production are partially under the control of reproductive hormones (Macova et al. 2008) (Fig. 6).

In rats, ovariectomy increased AT1B receptor mRNA and AT1 receptor binding in the zona glomerulosa; estrogen replacement reversed these changes, increased the expression of AT2 mRNA and AT2 binding, and decreased aldosterone content (Macova et al. 2008). These results indicate that estrogen treatment decreases the production of aldosterone, a fluid-retentive, proinflammatory hormone (Bollag 2014), through AT1 receptor downregulation and AT2 receptor upregulation, partially explaining the protective effects of estrogen therapy (Macova et al. 2008).

In the adrenal medulla, ovariectomy increases AT1 receptor expression and decreases catecholamine content and Fra-2 expression; estrogen replacement normalized AT1 receptor expression, increased AT2 receptor expression and mRNA, enhanced TH mRNA expression, and normalized catecholamine content (Macova et al. 2008).

However, we cannot conclude that the effect of estrogens on AT2 receptor regulation in the adrenal gland is of physiological significance, because rat adrenal AT2 receptor expression was not modified during pregnancy (Forcier et al. 1995).

The effect of estrogens on AT2 receptor mRNA expression was not limited to the adrenal gland; it has also been demonstrated in the kidneys of ovariectomized rats and mice (Armando et al. 2002a, b; Baiardi et al. 2005), in mice deficient in apolipoprotein E (Brosnihan et al. 2008), and in human myometrium (Mancina et al. 1996). It has also been reported that in mouse smooth muscle cells, AT2 receptor-induced vascular relaxation requires both estrogen and the XX chromosome sex complement (Danser et al. 2015). However, these interacting factors have not been studied in the adrenal gland and in the context of stress.

Other reproductive hormones may participate in the regulation of AT2 receptor function, as revealed by the recent report that neonatal inflammation decreases the number of AT2 receptors only in male adrenal glands (Lunardelli et al. 2015).

Thyroid Hormones—Thyroid hormones influence the entire RAS system, including AT1 and AT2 receptor expression, demonstrated by the report that hyperthyroidism increases AT2 receptor and decreases AT1 receptor expression in the heart (Marchant et al. 1993) (Fig. 6). As it is the case with many other regulatory factors, the influence of thyroid hormones in the AT2 receptor response to stressors has not been studied.

Serotonin—Serotonin is a key factor in the adrenal AT2 and catecholamine response to stressors (Lefebvre et al. 1998). In wild-type mice, acute restraint increases not only noradrenaline and adrenaline levels and TH and AT2 mRNA expression, but also serotonin content (Armando et al. 2003a, b) (Fig. 6). In the absence of the serotonin transporter (SERT

–/– mice), there is increased adrenomedullary response to immobilization (Tjurmina et al. 2002) a decrease in serotonin content in mice not exposed to stressors, and the serotonin content does not increase after exposure to stressors. In addition, the stressor-induced increase in TH gene expression does not occur and catecholamine content decreases, both under resting conditions and after restraint, and there is a profound decrease in AT2 receptor expression (Armando et al. 2003a, b). These results demonstrate that SERT is necessary for the AT2 receptor expression and for the stressor-induced catecholamine production and release (Armando et al. 2003a, b; Tjurmina et al. 2004).

Inflammatory Cytokines and Nitric Oxide—There is a reciprocal interaction between severe inflammation and AT2 receptor activation, inflammation reducing AT2 receptor function, while AT2 activation enhances inflammatory stimuli (Fig. 6).

Exposure to sepsis downregulated adrenal AT2 receptor expression in vivo, an effect correlated with enhanced adrenal inflammatory cytokines and with nitric oxide synthase induction (Bucher et al. 2001). In PC12 cells, proinflammatory cytokines or nitric oxide donors decreased AT2 receptors, and this was reversed by blocking cytokine-induced nitric oxide synthesis. AT2 downregulation may reduce adrenal catecholamine secretion, a finding observed during severe sepsis (Bucher et al. 2001).

In rats submitted to systemic inflammation by endotoxin (lipopolysaccharide, LPS), AT2 receptor expression was reduced in the adrenal zona glomerulosa and medulla. This effect was associated with enhanced aldosterone, ACTH, and corticosterone synthesis and release (Sanchez-Lemus et al. 2008).

AT2 activation induces the chemokine RANTES and activates NF- κ B (Wolf et al. 2002), and in human umbilical vein endothelial cells AT2 stimulation has been proposed to be proinflammatory, activating reactive oxygen species (Castiñeiras-Landeira et al. 2016).

Conversely, AT2 receptor activation was reported to induce the activation of PPAR γ , a powerful anti-inflammatory factor (Zhao et al. 2005).

These contradictory findings indicate apparently the opposite effects of AT2 receptor function on different inflammatory models. While an interaction between inflammatory conditions and adrenal AT2 receptor function has been demonstrated, there is no clear interpretation of the findings.

Sodium Levels—AT2 receptor activity is sensitive to intracellular sodium levels (Fig. 6). Sodium restriction increased AT2 receptor binding in the adrenal medulla (Lehoux et al. 1997), and the intracellular sodium concentration modulates AT2 expression in PC12 cells (Tamura et al. 1999). In bovine adrenocortical cells, AT2 receptor activation increased ouabain synthesis with minimal cross-talk with AT1 receptors. Furthermore, ouabain increases AT2 receptor expression, and this is reduced by decreasing intracellular Na⁺ concentration (Laredo et al. 1997).

Acetylcholine—Acetylcholine regulates AT2 receptor expression, with nicotinic and muscarinic receptors exerting opposite effects (Fig. 6). Nicotine receptors are prominent in

adrenal chromaffin cells and contribute to regulate catecholamine release (Sala et al. 2008). AT2 receptor blockade potentiated nicotine-evoked dopamine release in hypothalamic and striatal brain slices (Narayanaswami et al. 2013), while in rats exposed to nicotine AT2 receptor expression is decreased (Tao et al. 2013). Conversely, in PC12 cells there is a muscarinic-mediated increase in AT2 dependent of protein kinase C and NO–cGMP activation (Shibata et al. 1998). Despite this information, the role of acetyl-choline and its nicotinic and muscarinic receptors in the regulation of AT2 receptors has not been adequately addressed.

A Critical Look at AT2 Receptor Research

Methodological Limitations—As mentioned above, while there is general agreement on the participation of AT2 receptors in response to stressors, most if not all studies include controversial findings, and the interpretation of the literature remains problematic.

One of the principal reasons is the limited scope of many research models. Results obtained in cultured cells may not have preclinical or clinical correlates, and more so when results obtained by studying pathological cell models, such as PC12 cells, are considered as representative of in vivo conditions. Additionally, studies based on receptor transfection in cells not expressing the native receptor do not represent natural conditions and must be considered with caution. Furthermore, many research groups, including ours, have studied the consequences of isolated AT1A or AT2 receptor deletion. Again, the results obtained with these models, while of great interest, may be confirmed with additional models, since deletion of one receptor is not limited to the receptor in question, but may have unclarified additional effects throughout the genome.

An additional methodological limitation is the uncritical interpretation of AT2 receptor blockade and activation. Initial studies demonstrated that the non-peptidic compound PD123177, an antagonist (Chiu et al. 1990–1991; Wong et al. 1990; Blankley et al. 1991; VanAtten et al. 1993), and the peptide derivative CGP42112A, an agonist (Whitebread et al. 1991), bound only to AT2 receptors and not the AT1 receptor type.

However, the consideration of PD123177 and CGP42112A as selective AT2 receptor ligands may be erroneous. For example, CGP42112A, an AT2 receptor agonist, also binds to a novel, non-AT2 binding site localized in macrophages and microglia and involved in the regulation of responses to inflammation (Saavedra and Pavel 2006; Jöhren et al. 1995b; Egidy et al. 1997; de Oliveira et al. 1994; Viswanathan et al. 1994a, b; Ciuffo and Saavedra 1995) (Table 3).

Moreover, AT2 receptor effects have been proposed, in multiple studies, on the basis of the administration or addition of the AT2 antagonist PD123177. Since a complete investigation of the selectivity of these compounds has never been performed, it is premature to conclude that all effects of PD123177 or CGP42112A are exclusively the result of influences on AT2 receptor activity (Table 3).

Compound 21 is proposed as a selective AT2 receptor agonist, and in vivo administration of this compound has been reported as beneficial in animal models of ischemia (Fouda et al.

2017; Alhusban et al. 2015; Joseph et al. 2014), diabetes (Chow et al. 2016), and cognitive decline (Iwanami et al. 2014, 2015). However, it has been demonstrated that Compound 21 is not a selective AT₂ receptor agonist (Verdonk et al. 2012) and the reported effects may be at least partially the result of mechanisms unrelated to AT₂ receptors (Table 3). The effects of Compound 21 have not been reported in stress models.

Determination of the localization and regulation of AT₂ receptor expression has been addressed with several methods. Quantification of receptor transcription has been analyzed using qPCR, and cellular and tissue receptor gene expression by radioactive or non-radioactive in situ hybridization (Jöhren et al. 1996; Obermüller et al. 1998). Quantification of the number of receptor sites has been achieved with the use of membrane binding of radiolabeled ligands and tissue localization with quantitative receptor autoradiography (Tsutsumi and Saavedra 1991a, b, c, d). While each technique has its applications, degree of complexity, and limitations, radiolabeled membrane binding, autoradiography, qPCR, and in situ hybridization are validated techniques.

Problems remain with commercially available antibodies used in immunocytochemistry and Western blotting to establish tissue localization, cellular expression, and quantity of AT₂ receptor protein (Hafko et al. 2013). The extreme variability, lack of selectivity, and false-positive results obtained with the use of these antibodies have been extensively documented (Hafko et al. 2013). The use of antibodies against the AT₂ receptor has already generated many discrepancies with the results obtained with the use of validated techniques such as quantitative autoradiography and in situ hybridization. For example, AT₂ immunoreactivity has been described in the anterior pituitary (Premier et al. 2013), reported negative in the adrenal gland (Reagan et al. 1996), and has been described even in the pyramidal tract but not in the brain areas known to express AT₂ receptors by autoradiography or in situ hybridization (Yiu et al. 1997).

For this reason, the literature based on the use of receptor antibodies must be revisited, and observations obtained with the use of AT₂ receptor antibodies, when these antibodies have not been appropriately characterized, may not be considered necessarily correct.

These limitations are not restricted to AT₂ receptor antibodies, but extend to AT₁ receptor antibodies (Benicky et al. 2012; Herrera et al. 2013) and to many, if not all GPCR antibodies (Saper 2009; Saper and Sawchenko 2003). Even with a well-characterized monoclonal antibody recognizing the AT₁ receptor, not a single band was detected in Western blots (Frei et al. 2001). Determination of positive immunofluorescence in cells transfected with the AT₂ receptor coupled with adrenomedullary positive control (Ozono et al. 1997) or the commonly used pre-absorption using the target peptide only shows that the antibody can recognize the AT₂ receptor, but says nothing regarding specificity.

Following the clear indications from the literature, in the United States, the National Institutes of Health has recently adopted a policy requiring to properly validate the use of antibodies in grant proposals (<https://nexus.od.nih.gov/all/2016/01/29/authentication-of-key-biological-andor-chemical-resources-in-nih-grant-applications/>). It is hoped that such policy will help advance the research using commercially available antibodies in the right direction.

A detailed procedure to identify incorrect or non-selective antibodies and the necessary remedies to avoid false results has been published earlier (Saper and Sawchenko 2003; Saper 2009; Bordeaux et al. 2010).

The questionable use of receptor antibodies leads to a question that at present is without a satisfactory answer. While there is sufficient evidence demonstrating interactions between AT1 and AT2 receptor expression and activity, there is no convincing evidence of same-cell co-localization of these receptor types. It may be assumed, therefore, that AT1–AT2 cross-talk is for the most part indirect. Unfortunately, the mechanisms of such indirect influence have not been clarified.

Although the number of AT2 binding sites can be determined using quantitative film autoradiography and in situ hybridization, localizing and quantitating receptor protein is of interest, and it is reasonable to continue the search for validated AT2 receptor antibodies.

Alternatively, localization and quantification of AT2 receptor protein may be achieved by photoaffinity labeling of the receptor (Servant et al. 1993; Bossé et al. 1993). However, this technique has not been widely used at present.

The Canonical Consideration of Angiotensin II or its Derivatives as Sole Endogenous Ligands for AT2 Receptors

Almost all the literature considers Angiotensin II as the only endogenous AT2 receptor ligand and the physiological and pathological determinant of AT2 receptor activity. Consequently, results obtained after exposure to Angiotensin II have been associated with physiological or pathological consequences of AT2 (and AT1) receptor activation. However, most experiments have been carried out using Angiotensin II concentrations manyfold higher than those measured in plasma or tissues. For this reason, these interesting experiments are unlikely to represent in vivo physiological or pathological events.

The canonical consideration of Angiotensin II as the sole endogenous AT2 receptor agonist has been recently challenged. As detailed above, AT2 receptors are expressed in very large numbers in adrenomedullary chromaffin cells, participating in catecholamine production and release during physiological conditions and in stress. Chromaffin granules present in adrenomedullary chromaffin cells co-store and co-release substantial amounts of chromogranin A, a marker of sympathoadrenal activity in addition to catecholamines (Mazza et al. 2010; Tota et al. 2014).

Chromogranin A is a precursor of several well-known cardioactive peptide derivatives such as vasostatin 1, catestatin, and serpinin (Mazza et al. 2010; Tota et al. 2014). Surprisingly, although chromogranin A and its peptide derivatives participate, in association with catecholamines, in complex interactions to maintain homeostasis during exposure to stressors and in cardiovascular disease, their association and possible interactions with adrenomedullary AT2 receptors have not been explored until recently.

A novel chromogranin A fragment, the Vasoconstriction-Inhibiting Factor (VIF), has recently been isolated from the adrenal glands. VIF is produced and released from the adrenal glands in substantial amounts, exerts major vasodilating effects, inhibiting

Angiotensin II-induced vasoconstriction, and effectively stimulates AT₂ receptors (Salem et al. 2015; Jugdutt 2015; Carney 2015) (Fig. 7). Because of the specific binding of VIF to AT₂ receptors, and its inhibition of AT₁ receptor vasoconstriction, VIF has become a recent trendy topic in cardiovascular research, and the hypothesis has been advanced of VIF as a natural regulator of blood pressure with possible significant protective cardiovascular effects.

Additional peptides, such as LKP, a tripeptide fragment of novokinin and AT₂ receptor agonist (B dzy ska et al. 2014), may play a role in AT₂ receptor activity. Specificity of LKP for AT₂ receptors, however, is not absolute, because the peptide may also bind to AT₁ receptors if AT₂ receptors are decreased (B dzy ska et al. 2014).

The role of VIF and other peptides in the response of AT₂ receptors during stress has not been explored, and it is likely that future analysis of AT₂ receptor regulation by VIF and perhaps other chromogranin A-related peptides may help clarify the role of AT₂ receptors when submitted to stressors and may reveal unexpected and interesting aspects of AT₂ function in health and disease.

Participation of AT₂ Receptors in Stressor-Related Disorders

It has long been established that the loss of homeostasis in response to major acute or chronic stressors is a major factor in the development of multiple illnesses across the medical spectrum.

This includes cardiovascular disorders, in particular events such as myocardial infarction, stroke, venous thromboembolism, psychiatric disorders including bipolar disorder and unipolar depression, diabetes and metabolic syndrome, and other disorders where the dysregulation of reproductive, growth, and immunity axes plays a significant role (Chrousos and Gold 1992; Ising and Holsboer 2006; Gradus et al. 2015).

Since the function of AT₂ receptors plays a role in the response to stressors, it is reasonable to speculate that AT₂ receptor activity may be involved in the initiation and development of many medical conditions when stressors play significant roles.

Based on preclinical studies in animal models and the effects of Compound 21, recent publications have proposed such a role (Alhusban et al. 2015; Sampson et al. 2016; Joseph et al. 2014; Gao et al. 2014; Castoldi et al. 2014; McCarthy et al. 2014; Chow et al. 2016; Iwanami et al. 2014; 2015).

Although Compound 21 has been shown to be neuro-protective in these studies, it does not selectively stimulate AT₂ receptors (Verdonk et al. 2012). Moreover, the influence of stress on the development of these conditions has not been determined. For this reason, it is not known whether Compound 21 effects are directly neuroprotective, effective because it reduces the associated stress, or both.

To date, there are no clinical studies in support of a role of AT₂ receptors in stressor-related medical conditions through modulation of the stressor response.

Conclusions

The collected evidence presented here conclusively demonstrates that AT₂ receptors participate in the reaction to stressors in close connection with AT₁ receptor activity.

However, the canonical interpretation of Angiotensin II as the sole endogenous AT₂ receptor agonist, the entire concept of the classical RAS, and the validity of the numerous published results obtained with not validated techniques must be revisited.

For these reasons, no definite conclusions may be drawn about the physiological importance of AT₂ receptor activity, their precise role in the response to stressors, and the proposed translational value of their modulation.

The field of AT₂ receptor role in response to stressors is in its infancy. It is hoped that in the future more complete studies using validated techniques will shed light on this subject of potential clinical relevance.

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Fig. 1.

Angiotensin II AT₂ receptor mRNA and AT₂ receptor binding in rat tissues. **a** AT₂ gene expression was determined in the adrenal gland, spleen, kidney cortex, liver, cerebellum, pituitary, and inferior olive of adult male Sprague Dawley rats. Data represent the mean $\pm$ SEM of three individual determinations. AT₂ mRNA is only expressed in the adrenal glands, inferior olive, and cerebellum, and it is not detectable in the spleen, kidney, liver, and pituitary. **b** AT₂-receptor expression was studied by quantitative autoradiography in sections from the adrenal gland and spleen, incubated in the presence [¹²⁵I] Sar¹-Ile⁸-Angiotensin II. *Figures* represent a typical result repeated in three different rats. Note abundant receptor binding in the adrenal medulla and spleen and lower expression in the adrenal zona glomerulosa and kidney cortex, localized to the glomeruli. Binding was partially displaced in the adrenal cortex and completely displaced in the spleen and kidney cortex, by incubation with the AT₁ receptor-specific antagonist losartan indicating the presence of AT₁ receptors. Most of the receptor binding in the adrenal medulla and part of the binding in the adrenal cortex are insensitive to losartan expression, revealing the expression of AT₂ receptors. **b** Non-specific binding was the result of incubation in the presence of non-radiolabeled Angiotensin II From Hakfo et al. 2013

Fig. 2.

Effect of peripheral inflammation on gene expression and binding of adrenal gland AT₂ receptors. Rats treated with vehicle (*open bars*) or the AT₁ receptor blocker candesartan for 3 days received an ip injection of sterile saline or bacterial endotoxin (lipopolysaccharide, LPS) on day 3. The animals were killed 3 h later. *Graphs on the left* represent the quantification of *in situ* hybridization. Values are mean $\pm$ SEM for groups of seven to nine animals measured individually. *Pictures on the right* show representative sections of hybridization on the adrenal zona glomerulosa and medulla. *** P < 0.001 compared with saline-treated groups; # P < 0.05 compared with vehicle–saline-treated group; one-way ANOVA and post hoc Tukey's test. *Scale bar* 1 mm From Sanchez-Lemus et al. (2008)

Fig. 3.

Angiotensin II AT₂ receptor mRNA and AT₂ receptor binding in wild-type and AT₂ knockout mice. **a** AT₂ receptor gene expression was determined in the adrenal gland, kidney cortex, spleen, and brainstem of wild-type and AT₂ knockout mice, and data represent the mean $\pm$ SEM of three individual determinations. Note that AT₂ mRNA is only expressed in the adrenal glands of wild-type mice and not in the kidney cortex, spleen, and brainstem of wild-type or AT₂ receptor knockout mice. **b** AT₂ receptor binding was studied in sections from the adrenal gland and spleen, incubated in the presence of [¹²⁵I] Sar¹-Ile⁸-Angiotensin II. Figures represent a typical result repeated in three different wild-type mice. Note abundant receptor binding in the adrenal medulla and spleen and lower expression in the adrenal cortex. Binding was partially displaced in the adrenal cortex and completely displaced in the spleen, by incubation with the AT₁ receptor-specific antagonist losartan, indicating AT₁ receptor expression. The results indicate that most of the receptor binding in the adrenal medulla and part of the binding in the adrenal cortex correspond to AT₂ receptors. Non-specific binding was the result of incubation in the presence of non-radiolabeled Angiotensin II From Hafko et al. (2013)

Fig. 4. Autoradiography of Angiotensin II AT1 receptor binding in the hypothalamic paraventricular nucleus and the median eminence of AT2 gene-deficient mice. **a, c** Sections from wild-type mice. **b, d** Sections from AT2 gene-deficient mice. Binding was performed by incubation in the presence of [125 I]Sar¹-Ang II and the AT2 receptor blocker PD-123319, to reveal binding to AT1 receptors. Note the increased binding in the parvocellular part of the paraventricular nucleus (*arrow* in **b**) and the median eminence (*arrowhead*) of AT2 gene-deficient mice when compared to wild-type controls. *Arrow* in **d** points to the third ventricle. *Scale bar* 1 mm From Armando et al. (2002a, b)

Fig. 5.

Signal transduction mechanisms of AT₂ receptors in adreno-medullary cells. AT₂ receptor stimulation enhances TH gene expression and catecholamine synthesis through increased Fra-2 phosphorylation. Additionally, AT₂ receptors activate protein tyrosine phosphatase (PTP) leading to decreased cGMP and the Erk/Junk/p38/MAPK and STATs pathways. In turn, PTP activation activates Ca⁺⁺ channels, regulating Ca⁺⁺ entry into the cell (Color figure line)

Fig. 6. Identified factors regulating AT2 receptor expression and activity. Stress influences AT2 receptor expression and activation, and AT2 receptor activity reciprocally modulates the response to stressors (*double arrow*). A number of factors activating or inhibiting AT2 receptor expression and/or activity have been identified. Inflammation, nicotinic receptors and sodium levels negatively influence AT2 receptors. ACTH, glucocorticoids, estrogens, the XX chromosome sex complement, thyroid hormones, and serotonin and muscarinic receptors enhance AT2 receptor expression and/or activity. The interaction between AT2 receptors and ACTH is reciprocal (*double arrow*) (Color figure online)

Fig. 7.

Principal AT2 receptor agonist factors. Circulating and intracellular Angiotensin II (*red arrow*) is the canonical endogenous AT2 receptor agonist, but may not be the only one. A novel agonist, the Vasoconstriction-Inhibiting Factor (VIF), derived from chromogranin A located in the chromatin granules of most of the adrenomedullary cells has been established as an additional AT2 receptor agonist. The AT2 receptors, the AT1 receptors, and the sympathetic system reciprocally influence each other. However, the precise mechanisms of these interactions are not sufficiently clarified and are in need of further exploration (Color figure online)

Table 1

Effects of stress on AT2 gene expression and receptor binding in the brain, adrenal zona glomerulosa, and adrenal medulla

Type of stress	Brain		Adrenal zona glomerulosa		Adrenal medulla		References
	mRNA	Binding	mRNA	Binding	mRNA	Binding	
Cold-restraint, ventrolateral nucleus of the thalamus (stress-sensitive strain: spontaneously hypertensive rats)		↓					Bregonzio et al. (2008)
		↑					Bregonzio et al. (2008)
			↓	=	↓	=	Leong et al. (2002)
Repeated restraint (rats)			↑	=	=	=	Leong et al. (2002)
Acute immobilization (rats)					↓		Nostramo et al. (2012, 2015)
Repeated immobilization (rats)					↓		Nostramo et al. (2012, 2015)
Acute restraint (wild-type mice)						↑	Armando et al. (2003a, b)
Acute restraint (Sert-/- mice)						↓	Armando et al. (2003a, b)
Acute inflammation (LPS parenteral injection) (rats)			↓		↓		Sanchez-Lemus et al. (2008)
Neonatal inflammation (parenteral LPS) (rats)			↓ whole adrenal (males)		↓ whole adrenal (males)		Lunardelli et al. (2015)
Isolation stress (rats)			=	=	=	↑	Armando et al. (2001)
Intrauterine growth restriction (low sodium diet) (rats)					↑		Bibeau et al. (2010)
Water deprivation (rats)				=		=	Chatelain et al. (2003)
Stress-sensitive strain: spontaneously hypertensive rat			=		=		Johren et al. (2003)
Stress-sensitive strain: low brainAngiotensinogen (rats)					↑		Muller et al. (2010)
In vivo ACTH administration (rats)					↓	↓	Kitamura et al. (1998)
Sympathectomy (rats)					↑	↑	Qiu et al. (1999)

↑ increased, ↓ decreased, = no statistically significant change empty squares: measurements not performed

Table 2

Effect of AT1 receptor blockade on AT2 receptor expression

Stress	Tissue	AT2 mRNA	AT2 binding	References
Isolation stress (rats)	Brain (locus coeruleus, inferior olive)		= prevents stress-induced ↓	Saavedra et al. (2006)
Stress-sensitive rat strain (SHR)	Brain (locus coeruleus, inferior olive)		↑	Seltzer et al. (2004)
Control rats	Brain (inferior olive)		=	Zhuo et al. (1994)
Isolation stress (rats)	Adrenal gland (zona glomerulosa)	↑	↑	Armando et al. (2001)
Isolation stress (rats)	Adrenal gland (adrenal medulla)	↑	↓	Armando et al. (2001, 2004), Jezova et al. (2003)
Acute inflammation (LPS parenteral injection) (rats)	Adrenal gland (zona glomerulosa)	=	↓	Sanchez-Lemus et al. (2008)
Acute inflammation (LPS parenteral injection) (rats)	Adrenal gland (adrenal medulla)	=	↓	Sanchez-Lemus et al. (2008)
Stress-sensitive rat strain (SHR)	Adrenal gland (zona glomerulosa)		↑	Seltzer et al. (2004)
Stress-sensitive rat strain (SHR)	Adrenal gland (adrenal medulla)		↓	Seltzer et al. (2004)
Salt-restricted, nephrectomized (rats)	Adrenal gland (zona glomerulosa)	↑		Gigante et al. (1997)
Control rats	Adrenal gland (zona glomerulosa)		↓	Zhuo et al. (1994)
Control rats	Adrenal gland (adrenal medulla)		↓	Zhuo et al. (1994)
Stress-sensitive rat strain (SHR)	Thoracic Aorta	↑		Cosentino et al. (2005)

↑ increased, ↓ decreased, = no statistically significant change, empty squares: measurements not performed

Table 3Questionable selectivity of AT₂ receptor ligands

Compound	Proposed effect on AT ₂ receptors	Selectivity	References
PD 123177	Antagonist	Blocks AT ₂ receptors; selectivity for additional receptors or additional activity not studied	Chiu et al. (1990–1991), Wong et al. (1990)
CGP 42112A	Agonist	Not selective for AT ₂ receptors; binds to uncharacterized receptors in macrophages/microglia	Saavedra and Pavel (2006), Roulston et al. (2004, 2005), Ciuffo and Saavedra (1995), de Oliveira et al. (1994), Viswanathan et al. (1994a, b)
Compound C21	Agonist	Not selective for AT ₂ receptors; blocks calcium transport into the cell, stimulates AT ₁ receptors, and reduces affinity of thromboxane A ₂ and α -adrenoceptors	Verdonk et al. (2012)

Compounds PD 123177, CGP 42112A, and C21 have been proposed as selective ligands for AT₂ receptors. Their selectivity for AT₂ receptors is questionable