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Angiotensin AT_1 – α_{2C}-adrenoceptor interaction disturbs α_{2A}-auto-inhibition of catecholamine release in hypertensive rats

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α_2-Adrenoceptors lower central sympathetic output and peripheral catecholamine release, and thus may prevent sympathetic hyperactivity and hypertension. α_2AR also influence vascular tension. These α_2AR are malfunctioning in spontaneously hypertensive rats (SHR). Here I tested if an interaction between α_2AR subtypes and the angiotensin AT1 receptor (AT_1R) precipitated these disorders. Blood pressure was monitored through a femoral artery catheter and cardiac output by ascending aorta flow in anesthetized rats. Catecholamine concentrations were determined in plasma collected at the end of a 15-min tyramine-infusion. Tyramine stimulates norepinephrine release through the re-uptake transporter, thus preventing re-uptake. Presynaptic control of vesicular release is therefore reflected as differences in overflow to plasma. Previous experiments showed surgical stress to activate some secretion of epinephrine, also subjected to α_2AR-auto-inhibition. Normotensive rats (WKY) and SHR were pre-treated with (1) vehicle or α_2AR-antagonist (L-659,066), followed by fadolmidine ($\alpha_{2C>B>A} + \alpha_1$AR-agonist), ST-91 ($\alpha_{2non\text{-}A}$-selective agonist), or *m*-nitrobiphenyline (α_{2C}AR-agonist + α_{2A+B}-antagonist), or (2) AT1R-antagonist losartan, losartan + L-659,066, or losartan + clonidine. In WKY, L-659,066 alone, L-659,066 + agonist or losartan + L-659,066 increased catecholamine overflow to plasma after tyramine and eliminated the norepinephrine-induced rise in total peripheral vascular resistance (TPR). In SHR, L-659,066 + fadolmidine/ST-91/*m*-nitrobiphenyline and losartan + L-659,066 greatly increased, and losartan + clonidine reduced, catecholamine concentrations, and L-659,066 + ST-91, losartan + L-659,066 and losartan + clonidine eliminated the tyramine-induced rise in TPR. Separately, these drugs had no effect in SHR. In conclusion, peripheral α_{2C}AR-stimulation or AT_1R-inhibition restored failing α_{2A}AR-mediated auto-inhibition of norepinephrine and epinephrine release and control of TPR in SHR.

Keywords: α_2-adrenoceptors, angiotensin AT1 receptor, sympathetic nervous system, norepinephrine, epinephrine, release-control, spontaneously hypertensive rats, total peripheral vascular resistance

INTRODUCTION

Sympathetic hyperactivity is a major force in initiating and sustaining spontaneous hypertension (Guyenet, 2006; Esler, 2011). α_2-adrenoceptors (AR) lower sympathetic output from the central nervous system (CNS), and inhibit release of norepinephrine from peripheral sympathetic nerves and catecholamines from the adrenal medulla (Starke, 2001). Their activation is tonic, and they hamper release even in the anesthetized rat without stimulation of norepinephrine release (Berg et al., 2012). They therefore represent the last line of defense against sympathetic hyperactivity, and, if not functioning, plasma norepinephrine levels and blood pressure (BP) will increase, as demonstrated in genetically modified mice (Makaritsis et al., 1999). In the spontaneously hypertensive rat (SHR), deficiencies have been detected in both central and peripheral α_2AR-mediated inhibition of release (Remie et al., 1992; Zugck et al., 2003). We have recently demonstrated that during tyramine-stimulated norepinephrine release, α_2AR failed to lower norepinephrine and epinephrine release in SHR, and also failed to control vascular tension (Berg and Jensen, 2013). These malfunctions were not detected without activation of norepinephrine release (Berg et al., 2012), indicating that they resulted from the released catecholamine itself, or another agent released by, or co-released with norepinephrine or epinephrine. Surprisingly, these peripheral disorders were repaired by the non-selective agonist clonidine, which reduced catecholamine release, and also, through a central action, normalized the high resting BP, heart rate (HR), and total peripheral vascular resistance (TPR) in SHR (Berg et al., 2012).

The restoring effect of clonidine may result from its central action or from an interaction between presynaptic receptors. α_2AR are divided into three subtypes, i.e., α_{2A}, α_{2B}, and α_{2C}. The α_{2A}- and α_{2C}-subtypes mediated the inhibition of central sympathetic output, whereas all three subtypes may reduce norepinephrine release from peripheral sympathetic nerves (Hein et al., 1999; Trendelenburg et al., 2003b) and the adrenal medulla (Brede et al., 2003; Moura et al., 2006). Inhibition of adrenal epinephrine

release involved the α_{2C}-subtype in the mouse (Brede et al., 2003; Moura et al., 2006), but the α_{2A}-subtype in rat and man (Lymperopoulos et al., 2007; Berg et al., 2012). It has been shown that on-going α_2AR-signaling markedly enhanced the stimulating effect of the angiotensin AT1 receptor (AT_1R) – phospholipase C – protein kinase C (PKC) pathway on norepinephrine release in the rat vas deferens (Talaia et al., 2006). Similarly, studies on tissues from genetically modified mice (Trendelenburg et al., 2003a) demonstrated that the enhancing effect of release-stimulating receptors, including the AT_1R, depended on active α_2AR-signaling. However, the interaction involved the α_{2C}AR-subtype only (**Figure 1**). Since the renin angiotensin system plays a significant role in hypertension pathology in SHR, I hypothesized that the clonidine-dependent restoration of α_2AR inhibition of release in SHR involved stimulation of the α_{2C}AR, thus counter-acting an excessive AT_1R-signaling.

The angiotensin II responsible for a possible AT_1R interference in SHR is not likely to origin from the sympathetic nerves themselves. Therefore, to have all components present, a role of the AT_1R in the α_2AR malfunction in SHR should be tested *in vivo*, which represents an experimental challenge. Due to synaptic uptake of norepinephrine through the norepinephrine re-uptake transporter (NET), presynaptic modulation of release is not reflected as differences in overflow and the plasma norepinephrine concentration (Berg et al., 2012). However, when NET-mediated re-uptake was blocked by desipramine, α_2AR-antagonists greatly increased the plasma concentration of norepinephrine in the resting, anesthetized rat, in which norepinephrine release was not stimulated. Overflow to plasma under resting conditions is low, and inhibition of release by α_2AR-agonist had no or little effect on the plasma norepinephrine concentration (Berg et al., 2012). In addition, the α_2AR malfunction in SHR was not observed unless norepinephrine release was activated. Peripheral norepinephrine release can be stimulated by tyramine, which activates reverse transport through NET. Most likely by engaging NET in release, thus preventing re-uptake, presynaptic α_2AR modulation altered tyramine-induced norepinephrine overflow to plasma, similar to that after desipramine in not-stimulated rats (Berg and Jensen, 2013). Restored α_2AR control of release after α_{2C}AR-stimulation or AT_1R-antagonist could therefore be tested by the ability of the non-selective α_2AR-antagonist L-659,066 to increase tyramine-induced norepinephrine overflow to plasma.

Epinephrine released in the adrenals is not subjected to re-uptake, and is not stimulated by tyramine. However, the stress induced by the surgical procedure activated some secretion of epinephrine, which was also subjected to α_2AR-mediated release-control (Berg et al., 2012; Berg and Jensen, 2013).

Due to the activation of norepinephrine release, tyramine in addition induced a sympathetic cardiovascular response. This response was not influenced by baroreceptor activation, demonstrated by that baroreceptor control of HR was abolished by the pentobarbital-anesthesia (Berg et al., 2012). Moreover, epinephrine secretion is not regulated by the baroreceptor reflex. Thus, by recording BP and cardiac output (CO), the implications of altered catecholamine release and a possible postsynaptic α_2AR/AT_1R interaction in the control of TPR could be evaluated.

The results will show that the failing α_2AR control of norepinephrine and epinephrine release and modulation of the norepinephrine-induced rise in TPR in SHR was restored by stimulation of peripheral α_{2C}AR or inhibition of the AT_1R.

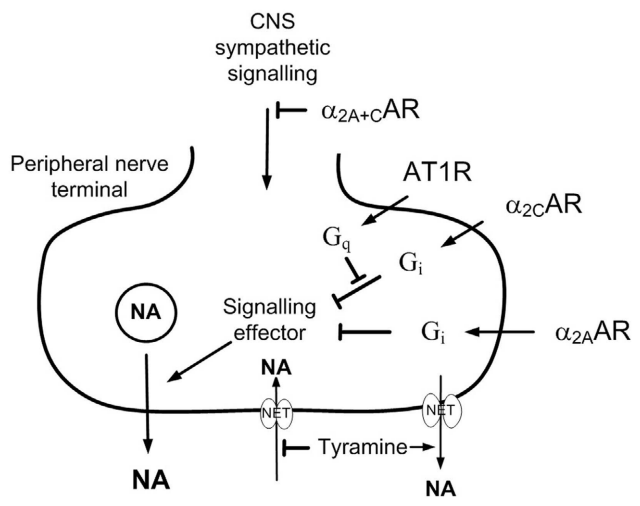

FIGURE 1 | The effect of presynaptic α_{2C}AR and AT_1R on norepinephrine release. AT_1R-G_q-signaling stimulates norepinephrine release by interfering with the down-stream signaling of G_i (Cox et al., 2000). The AT_1R/α_2AR interaction involved only the α_{2C}- and not the α_{2A}-subtype (Trendelenburg et al., 2003a). The present results show that α_{2C}AR-stimulation or AT_1R-inhibition was required for α_{2A}AR to effectively moderate peripheral norepinephrine release in SHR during tyramine-stimulated norepinephrine release. This malfunction may be due to excessive AT_1R-G_q-signaling in this strain, and α_{2A}-signaling was evidently not permitted as long as AT_1R-G_q-signaling interfered with the function of the α_{2C}AR. Tyramine stimulates reverse transport of norepinephrine through NET, and therefore also prevents synaptic NET re-uptake, allowing presynaptic control of release to be reflected as differences in overflow and the plasma norepinephrine concentration.

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURE

All experiments were approved by the institutional review committee, and conducted in accordance with the Directive 2010/63/EU of the European Parliament. About 12–14 weeks old, male normotensive rats (Wistar Kyoto, WKY, $n = 99$, 284 ± 3 g b.w.) and SHR (Okamoto, SHR/NHsd strain, $n = 107$, 288 ± 2 g b.w.) on 12/12 h light/dark cycles were allowed conventional rat chow diet (0.7% NaCl) and water *ad lib* until the time of the experiment. The rats were anesthetized with pentobarbital (70–75 mg/kg, i.p.). As previously described (Berg et al., 2010; Berg and Jensen, 2013), mean arterial BP [MBP = (systolic BP − diastolic BP)/3 + diastolic BP] was monitored through a catheter in the femoral artery, flushed with 0.15 ml PBS (0.01 M Na-phosphate, 0.14 M NaCl, pH 7.4) containing 500 IU heparin/ml. CO and HR were recorded by a flow probe on the ascending aorta. TPR (MBP/CO) was calculated. The rats were on a positive-pressure ventilator throughout the experiment, ventilated with air. Previous measurements of blood gas parameters demonstrated adequate ventilation in both strains (Berg, 2002, 2003).

Positive-pressure ventilation reduces right atrium ejection, and consequently lowered CO and MBP. This reduction was significant in SHR, but did not appear to influence the stimulated adrenergic responses, as previously discussed (Berg and Jensen, 2013). Body temperature was maintained at 37–38 °C by external heating, guided by a thermo sensor, inserted inguinally into the abdominal cavity.

EXPERIMENTAL DESIGN

Control rats were pre-treated with PBS and infused for 15 min with tyramine to induce NET-mediated norepinephrine release. Since subtype-selective α_2AR-agonists, which do not cross the blood-brain barrier, are not available, I used α_2AR-agonists with different subtype profiles and different ability to cross the blood-brain barrier. Rats were therefore pre-treated with PBS or the α_2AR-antagonist L-659,066, followed 10 min later by α_2AR-agonist, i.e., fadolmidine, ST-91, or (R)-(+)-*m*-nitrobiphenyline oxalate. Rats were also pre-treated with the AT_1R-antagonist losartan, alone or followed by L-659,066, clonidine, or ST-91. Drug specificity and dose are given in **Table 1**. Blood for the measurement of catecholamines was collected from the arterial catheter after the 15-min tyramine-observation period, but without discontinuing the infusion.

MEASUREMENT OF PLASMA CATECHOLAMINES

About 1.5 ml blood was collected into tubes containing 40 µl 0.2 M glutathione and 0.2 M EGTA (4 °C). Plasma was stored at −80 °C until the norepinephrine and epinephrine concentrations were determined, using 400 µl plasma and the 5000 Reagent kit for HPLC analysis of Catecholamines in plasma from Chromsystems GmbH, Munich, Germany, as described by the manufacturer.

DRUGS

Pentobarbital was from the Norwegian National Hospital, Oslo, Norway. L-659,066 was a kind gift from Merck, Sharp, and Dohme Labs, Rahway, NJ, USA, and fadolmidine HCl from Orion Corporation, Espoo, Finland. ST-91 was from TOCRIS bioscience, Bristol, UK; and (R)-(+)-*m*-nitrobiphenyline oxalate from Santa Cruz Biotechnology, Heidelberg, Germany. The remaining drugs were from Sigma Chemical Co., St. Louis, MO, USA.

STATISTICAL ANALYSES

Results are presented as mean values ± SEM. Changes in the cardiovascular parameters were expressed in % of baseline. Data were averaged every min in all experiments. For the narrow peak-pressor response to ST-91 and *m*-nitrobiphenyline, data were averaged every 5 s. The cardiovascular response-curves to agonists and tyramine were analyzed using Repeated Measures Analyses of Variance and Covariance, first as over-all tests within each strain, and subsequently for each group separately or between groups. Significant responses and groups differences were subsequently located using one- and two-sample Student's *t*-tests, respectively, at specific times. The plasma catecholamine concentrations, the cardiovascular baselines, and the effect of pre-treatment were first analyzed using one-way ANOVA, and group differences were subsequently located by two-sample Student's *t*-tests or, in the presence of out-liers, non-parametric Kruskal–Wallis tests. For all analyses, testing proceeded only when significant responses, differences and/or interactions were indicated. The *P*-value was for all tests and each step adjusted according to Bonferroni, except for the catecholamine data, where $P \leq 0.05$ was considered significant.

RESULTS

α_2AR- AND AT_1R-INFLUENCE ON THE PLASMA CATECHOLAMINE CONCENTRATIONS

Norepinephrine

Similar to that previously described (Berg and Jensen, 2013), the non-selective α_2AR-antagonist L-659,066 increased the tyramine-induced norepinephrine overflow to plasma in WKY ($P = 0.015$) (**Table 2**). A similar increase was not seen in SHR, where the plasma norepinephrine concentration was already elevated ($P < 0.001$, WKY compared to SHR controls). Pre-treatment

Table 1 | Mode of action and dose of the pharmacological agents used.

Drug	Mode of action	Crosses blood-brain barrier	Dose per kg
Tyramine	Norepinephrine efflux through NET	No	1.26 µmol/min (Berg et al., 2010)
Clonidine	α_2AR-agonist (non-selective)	Yes	151 nmol (Berg et al., 2012; Berg and Jensen, 2013)
Fadolmidine (Lehtimaki et al., 2008)	$\alpha_{2C>B>A}$AR-agonist (+α_1AR-agonist activity)	No	2 nmol[a]
ST-91 (Takano et al., 1992)	α_2AR-agonist (non-α_{2A})	No	24 nmol[a]
m-nitrobiphenyline (Crassous et al., 2007)	α_2AR-agonist (α_{2C}-selective) (+α_{2A+B}AR-antagonist activity)	Not known	12.4 nmol[a]
L-659,066 (Clineschmidt et al., 1988)	α_2AR-antagonist (non-selective)	No	4.4 µmol (Berg et al., 2012; Berg and Jensen, 2013)
Losartan	AT_1R-antagonist	Yes (Li et al., 1993)	79 µmol (Berg, 2002)

[a] *Concentration established in preliminary tests to give a substantial (50–100%) but sub-maximal increase in MBP. Tyramine was administered as a 15-min infusion, whereas the other drugs were administered as bolus injections (0.6–1.0 ml/kg) 10 min before tyramine, except clonidine, which was injected 15 min before. All drugs were dissolved in PBS, and administered through a catheter in the femoral vein. When pre-treatment consisted of two drugs, these were given 10 min apart.*

Table 2 | The plasma concentration of norepinephrine and epinephrine at the end of the tyramine-infusion period.

	WKY			SHR		
	N	**Norepinephrine (nM)**	**Epinephrine (nM)**	***N***	**Norepinephrine (nM)**	**Epinephrine (nM)**
PBS + tyramine	17	20.6 ± 0.7	2.0 ± 0.9	16	27.4 ± 1.8*	5.0 ± 0.6*
L-659,066$_{(non\text{-}selective)}$ + PBS + tyramine	6	26.3 ± 2.0†	7.0 ± 1.7†	7	30.3 ± 3.4	10.6 ± 2.7
PBS + fadolmidine ($\alpha_{2C>B>A}$) + tyramine	6	18.1 ± 1.3	7.4 ± 1.3†	6	23.9 ± 2.2	13.0 ± 2.2†
L-659,066 + fadolmidine + tyramine	5	26.6 ± 0.4†‡	12.8 ± 1.1†‡	6	70.1 ± 16.9†‡§	74.8 ± 20.7†‡§
PBS + ST-91($\alpha_{2non\text{-}A}$) + tyramine	6	26.5 ± 2.9†	5.5 ± 1.8†	8	24.0 ± 1.8	11.0 ± 4.1
L-659,066 + ST-91 + tyramine	6	25.4 ± 2.1†	12.7 ± 4.2†	7	58.3 ± 5.2†‡§	49.3 ± 8.0†‡§
PBS + *m*-nitrobiphenyline (α_{2C}) + tyramine	5	24.1 ± 1.7	4.6 ± 1.5	7	27.9 ± 2.2	8.5 ± 1.6
L-659,066 + *m*-nitrobiphenyline + tyramine	5	24.3 ± 2.0	15.8 ± 4.2†	7	50.1 ± 6.0†‡§	45.5 ± 15.0†‡§
Losartan + tyramine	9	18.4 ± 0.7	4.2 ± 1.5	6	28.4 ± 3.4	11.8 ± 4.1
Losartan + L-659,066 + tyramine	7	26.3 ± 1.9†‖	25.9 ± 10.4†‖	7	71.3 ± 10.1†‖§	41.2 ± 9.3†‖§
Losartan + clonidine + tyramine	7	17.7 ± 1.1†	1.1 ± 0.4†	6	19.7 ± 1.1†‖	1.6 ± 0.8†‖
Losartan + ST-91 + tyramine		Not done		7	27.4 ± 1.5	15.2 ± 4.4

*Differences were detected as indicated between corresponding SHR and WKY control groups (*), between the PBS + tyramine controls and corresponding experimental groups (†), between groups pre-treated with agonist alone (fadolmidine, ST-91, or m-nitrobiphenyline) and L-659,066 + the same agonist (‡), between groups pre-treated with losartan alone and losartan + L-659,066/clonidine/ST-91 (‖), and between groups pre-treated with L-659,066 alone and L-659,066 combined with agonist or losartan (§). N, number of rats per group. *, †, ‡, ‖, § - P ≤ 0.05.*

with α_2AR-agonist alone, i.e., fadolmidine ($\alpha_{2C>B>A}$), ST-91 ($\alpha_{2(non\text{-}A)}$), or *m*-nitrobiphenyline (α_{2C}) had no effect on overflow in either strain, except for an increase after ST-91 in WKY. After L-659,066 + agonist + tyramine, norepinephrine overflow was not different from that after L-659,066 + tyramine in WKY (P = NS), but was much higher in SHR ($P \leq 0.025$–0.004), also when compared to the SHR PBS + tyramine or corresponding PBS + agonist + tyramine groups ($P \leq 0.004$).

Losartan alone had no effect on the tyramine-induced norepinephrine overflow in either strain (P = NS compared to the controls). Losartan also did not influence the augmenting effect of L-659,066 in WKY (P = NS compared to the L-659,066 + tyramine group, and $P = 0.001$ compared to the WKY PBS + tyramine and losartan + tyramine groups). However, in SHR, losartan allowed L-659,066 to greatly increase norepinephrine overflow ($P \leq 0.005$ compared to PBS/L-659,066/losartan + tyramine groups). Pre-treatment with losartan + clonidine reduced the tyramine-induced norepinephrine overflow in SHR ($P \leq 0.048$ compared to the PBS/losartan + tyramine groups), and was lower than that in the controls, although not different from that in the losartan + tyramine group, in WKY. Norepinephrine overflow after pre-treatment with losartan + ST-91 was not different from that in the PBS + tyramine or losartan + tyramine groups (tested in SHR only).

Epinephrine

The effect of α_2AR-agonists and antagonist on the surgery-activated epinephrine secretion mostly paralleled their effect on the tyramine-induced norepinephrine overflow in both strains. However, pre-treatment with fadolmidine in both strains, and L-659,066 + *m*-nitrobiphenyline in WKY, increased circulating epinephrine without altering the concentration of norepinephrine.

THE CARDIOVASCULAR RESPONSES

The α_2AR- and AT$_1$R-influence on the cardiovascular baselines

L-659,066 reduced baseline MBP and TPR in both strains (**Table 3**). All α_2AR-agonists induced a transient rise in MBP and TPR (**Figure 2**, the response to clonidine was similar to that previously published, Berg et al., 2012). Pre-treatment with L-659,066 reduced these TPR-responses, except that of fadolmidine in SHR (**Figure 2A**), although the MBP-responses were not necessarily reduced. Only fadolmidine subsequently induced an L-659,066-sensitive reduction in MBP and TPR to below baseline in both strains, and also HR in SHR. The agonists had otherwise little effect on baseline HR. Losartan reduced baseline MBP in both strains, HR in WKY, and TPR in SHR (**Table 3**). Losartan + L-659,066 induced a significant reduction in both HR and TPR in both strains. Losartan increased the MBP-response to ST-91 (**Figure 2B**) and also the transient rise in CO and MBP in response to clonidine in SHR but had no effect on the HR- or TPR-response to clonidine in either strain (not shown).

The α_2AR- and AT$_1$R-influence on the cardiovascular response to tyramine

As previously documented (Berg et al., 2010; Berg and Jensen, 2013), tyramine induced an immediate, but transient rise in TPR (**Figure 3**) and a sustained increase in MBP, HR, and CO. The present results focused on the effect of pre-treatment on the TPR-response to tyramine, and the concomitant changes in MBP, HR, and CO (all expressed in % of baselines) are therefore shortly described but not shown.

Pre-treatment with α_2AR-agonist alone (**Figures 3A–C**), i.e., fadolmidine, ST-91, or *m*-nitrobiphenyline, had no effect on the TPR-response to tyramine in WKY (P = NS). In SHR, the TPR-response to tyramine was increased after fadolmidine ($P = 0.023$ at 15 min), not influenced by ST-91, and decreased

Table 3 | Cardiovascular baselines prior to tyramine and, in parenthesis, the response to pre-treatment.

Pre-treatment	WKY					SHR				
	N	MBP (mm Hg)	HR (beats/min)	CO (ml/min)	TPR (mm Hg/ml/min)	*N*	MBP (mm Hg)	HR (beats/min)	CO (ml/min)	TPR (mm Hg/ml/min)
PBS (pooled data)	27	69 ± 3	340 ± 5	32 ± 1	2.2 ± 0.1	26	94 ± 4*	381 ± 6*	19 ± 1*	5.2 ± 0.2*
		(−1 ± 2)	(−5 ± 3)	(2 ± 0)	(−0.3 ± 0.1)		(−3 ± 5)	(−17 ± 4)	(1 ± 1)	(−0.4 ± 0.2)
L-659,066 + PBS	6	62 ± 9†	338 ± 13	33 ± 2	1.8 ± 0.2	7	68 ± 6†	408 ± 8	18 ± 2	3.9 ± 0.4†
		(−16 ± 2)†	(−17 ± 9)	(1 ± 1)	(−0.6 ± 0.1)†		(−21 ± 4)	(−8 ± 9)	(−1 ± 1)	(−1.1 ± 0.2)
PBS + fadolmidine	6	70 ± 3	346 ± 7	35 ± 3	2.1 ± 0.2	7	73 ± 7†	352 ± 10†	18 ± 1	4.1 ± 0.2†
		(−13 ± 5)	(−12 ± 6)	(4 ± 0)	(−0.8 ± 0.2)		(−27 ± 5)†	(−60 ± 10)†	(1 ± 1)	(−1.8 ± 0.2)†
L-659,066 + fadolmidine	5	50 ± 2†	333 ± 11	33 ± 2	1.5 ± 0.1†	6	65 ± 7	401 ± 10	18 ± 1	3.7 ± 0.4
		(−23 ± 3)†	(−18 ± 9)	(3 ± 1)	(−0.9 ± 0.2)†		(−36 ± 10)†	(−17 ± 13)	(0 ± 1)	(−1.9 ± 0.4)†
PBS + ST-91	6	82 ± 5	349 ± 4	33 ± 5	2.8 ± 0.5	8	82 ± 4	378 ± 13	14 ± 1	6.1 ± 0.6
		(−6 ± 2)	(−30 ± 6)	(5 ± 1)	(−0.7 ± 0.0)†		(−14 ± 6)	(−35 ± 9)	(0 ± 1)	(−1.2 ± 0.4)
L-659,066 + ST-91	6	67 ± 6	345 ± 7	33 ± 2	2.0 ± 0.1	7	79 ± 10	412 ± 14	15 ± 2	6.4 ± 1.
		(−4 ± 5)	(−10 ± 9)	(8 ± 2)	(−0.9 ± 0.2)†		(−26 ± 7)	(−22 ± 8)	(0 ± 1)	(−1. ± 0.4)
PBS + *m*-nitrobiphenyline	5	85 ± 1	390 ± 25	31 ± 3	2. ± 0.2	7	137 ± 8	386 ± 6	18 ± 1	7.5 ± 0.5†
		(−9 ± 5)	(−4 ± 11)	(2 ± 1)	(−0.5 ± 0.2)		(39 ± 8)	(−10 ± 6)	(1 ± 2)	(1.8 ± 0.8)
L-659,066 + *m*-nitrobiphenyline	7	59 ± 2	349 ± 10	30 ± 4	1.9 ± 0.2	7	115 ± 8	420 ± 8	20 ± 1	5.8 ± 0.5
		(−11 ± 3)	(−12 ± 6)	(1 ± 2)	(−0.5 ± 0.1)		(19 ± 7)	(8 ± 7)	(1 ± 1)	(0.8 ± 0.4)
Losartan	9	53 ± 3	341 ± 12	31 ± 2	1.8 ± 0.1	6	72 ± 5†	376 ± 7	13 ± 1†	5.5 ± 0.3
		(−22 ± 4)†	(−25 ± 5)†	(0 ± 2)	(−0.7 ± 0.2)		(−24 ± 8)†	(−19 ± 6)	(−3 ± 1)	(−1.0 ± 1.0)†
Losartan + L-659,066	9	38 ± 3†‡	310 ± 7†	22 ± 2†‡	1.8 ± 0.1	7	41 ± 3†‡	348 ± 17†	10 ± 2†	5.2 ± 1.0
		(−23 ± 2)†	(−44 ± 8)†	(−2 ± 1)	(−0.9 ± 0.2)†		(−48 ± 8)†	(−73 ± 9)†‡	(−6 ± 2)†	(−0.6 ± 0.8)
Clonidine	7	65 ± 3	314 ± 7	40 ± 3	1.6 ± 0.1†	6	60 ± 4†	320 ± 11†	18 ± 1	3.4 ± 0.2†
		(−4 ± 6)	(−33 ± 8)	(11 ± 1)†	(−0.8 ± 0.2)†		(−35 ± 10)†	(−121 ± 19)†	(2 ± 1)	(−2.5 ± 0.6)†
Losartan + clonidine	6	54 ± 3	346 ± 7	35 ± 3	1.5 ± 0.1†	6	44 ± 3†‡	314 ± 8†‡	13 ± 3	4.2 ± 1.0
		(−22 ± 7)	(−34 ± 13)	(10 ± 2)†‡	(−1.4 ± 0.2)†‡		(−65 ± 9)†‡	(−134 ± 12)†‡	(−2 ± 2)	(−2.9 ± 1.3)†
Losartan + ST-91			Not done			7	54 ± 4†‡	358 ± 11	13 ± 1†	4.7 ± 0.6
							(−50 ± 7)†	(−69 ± 8)†‡	(−5 ± 1)†	(−1.5 ± 0.3)

*Cardiovascular baselines in the PBS-control groups are shown as pooled data from experiments run at different times. However, statistical evaluation of the effect of pre-treatment was done using control rats from the same set of experiments. Comparisons were made between the WKY and SHR controls (*), between the PBS-controls and the experimental groups (†), between the groups pre-treated with PBS + agonist (fadolmidine, ST-91, or nitrobiphenyline) and corresponding groups given L-659,066 + the same antagonist (significant differences not detected), and between the groups pre-treated with losartan alone and losartan + L-659,066/clonidine/ST-91 (‡). *$P \leq 0.0125$, †, ‡ $P \leq$ Bonferroni adjusted P-value for each set of experiment.*

after *m*-nitrobiphenyline ($P = 0.003$ at 3 min). L-659,066 alone (**Figure 3A**) virtually eliminated the TPR-response in WKY ($P \leq 0.008$), with no additional effect when combined with agonist (**Figures 3A–C**). In SHR, L-659,066 alone did not change the tyramine-induced rise in TPR, but abolished the response when combined with ST-91 (**Figures 3A,B**). The response to tyramine in L-659,066 + fadolmidine-pre-treated SHR was less than that after fadolmidine alone, although not different from that in the controls (**Figure 3A**). Moreover, ΔTPR was not further reduced after L-659,066 + *m*-nitrobiphenyline compared to that after *m*-nitrobiphenyline alone in SHR (**Figure 3C**).

A reduced MBP-response to tyramine after L-659,066, alone or combined with agonist (fadolmidine, ST-91, or *m*-nitrobiphenyline), was observed in WKY, but only after L-659,066 + agonist in SHR. *m*-Nitrobiphenyline alone reduced ΔMBP in both strains. The agonists had little effect on the tyramine-induced tachycardia, except fadolmidine which increased ΔHR in SHR. A lower tyramine-induced rise in CO was observed after fadolmidine and ST-91 in WKY, after fadolmidine in SHR, and in all groups given L-659,066 as part of the pre-treatment.

Losartan alone had no effect on the TPR-peak response to tyramine in either strain, but induced a vasodilatory TPR-response at the end of the tyramine-infusion in WKY (**Figure 4**). Like L-659,066 alone (**Figure 3A**), losartan + L-659,066 eliminated the TPR-peak response to tyramine in WKY (**Figure 4**), and in addition caused a fall in TPR to below baseline. Losartan + clonidine, like clonidine alone, had no effect on the TPR-response to tyramine in WKY (**Figure 4**). In SHR, losartan + L-659,066 and losartan + clonidine, unlike losartan, L-659,066 or clonidine alone, eliminated the TPR-response to tyramine. The TPR-peak response was reduced also after pre-treatment with losartan + ST-91 (tested in SHR only, **Figure 4**). Losartan did not alter the MBP-response to tyramine, but increased the CO-response in both strains. This increase was eliminated when losartan was combined with L-659,066, and in WKY also with clonidine. The tyramine-induced

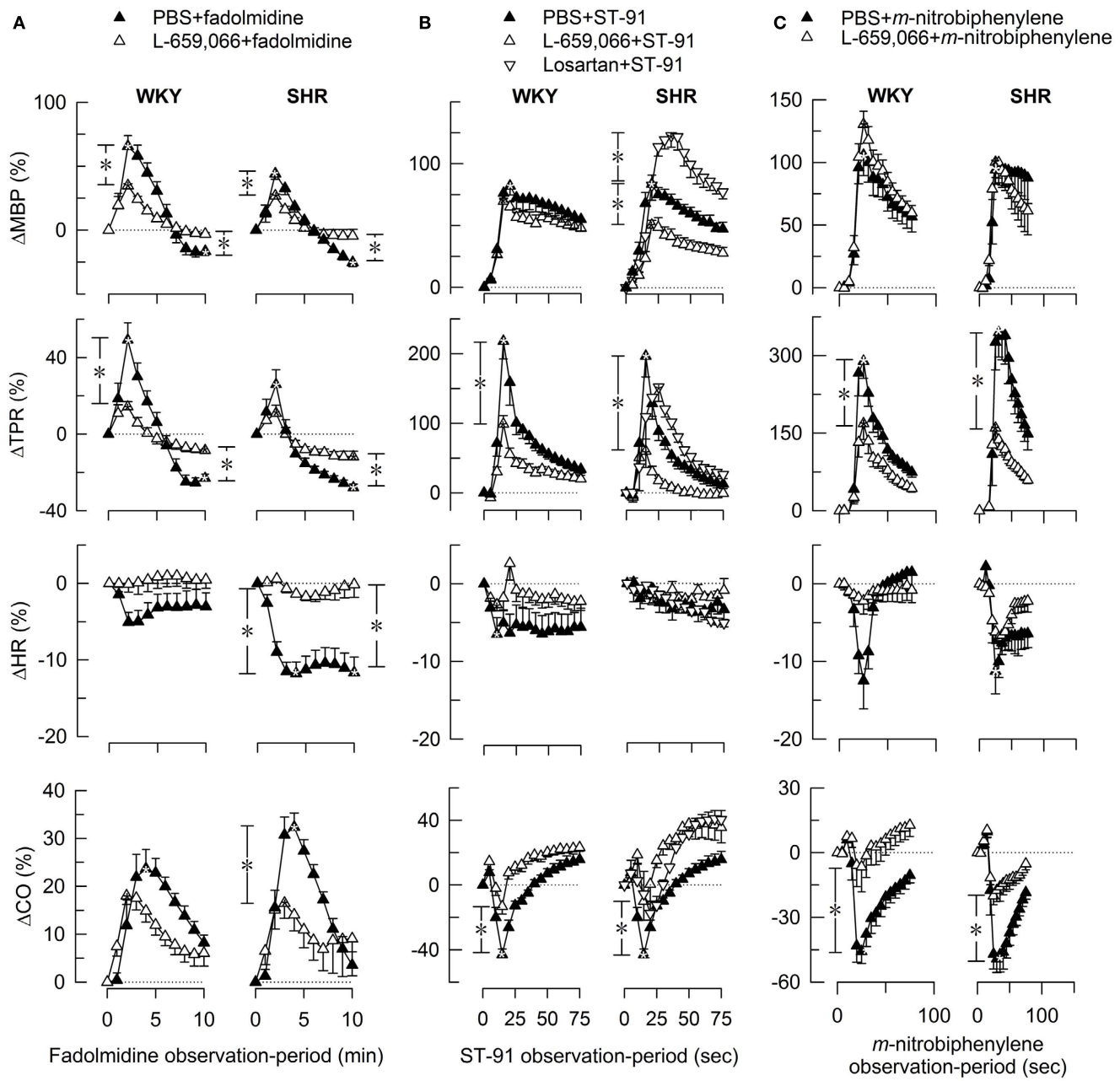

FIGURE 2 | The MBP-, TPR-, HR-, and CO-response to α2AR-agonists. Fadolmidine ($\alpha_{2C>B>A}$AR) **(A)**, ST-91 [$\alpha_{2(non\text{-}A)}$AR] **(B)**, and *m*-nitrobiphenyline (α_{2C}AR, with additional α_{2A+B}AR-antagonistic activity) **(C)** were injected alone or after pre-treatment with the non-selective α_2AR-antagonist L-659,066. The response-curves were analyzed using Repeated Measures Analyses of Variance and Covariance (please see Materials and Methods for details). Significant responses (*within symbols) and group differences (*in brackets) were located as indicated at peak response (all agonists) (brackets left of curves) and after 15 min (fadolmidine only) (brackets right of curves). *, $^{*}P \leq 0.025$ for **(A)**, and ≤ 0.05 for **(B,C)** after curve evaluations.

tachycardia was increased in SHR after losartan + clonidine, similar to that seen after clonidine alone.

DISCUSSION

The main finding in the present study was that the failing α_{2A}AR inhibition of peripheral norepinephrine and epinephrine release in SHR during tyramine-stimulated norepinephrine release was restored by stimulation of the α_{2C}AR or inhibition of the AT_1R. α_{2C}AR-stimulation and AT_1R-inhibition also restored the failing postsynaptic α_2AR control of vascular tension in SHR.

As previously described (Berg and Jensen, 2013), α_2AR-mediated auto-inhibition of peripheral catecholamine release was demonstrated in tyramine-stimulated WKY by an increased norepinephrine overflow to plasma after pre-treatment with

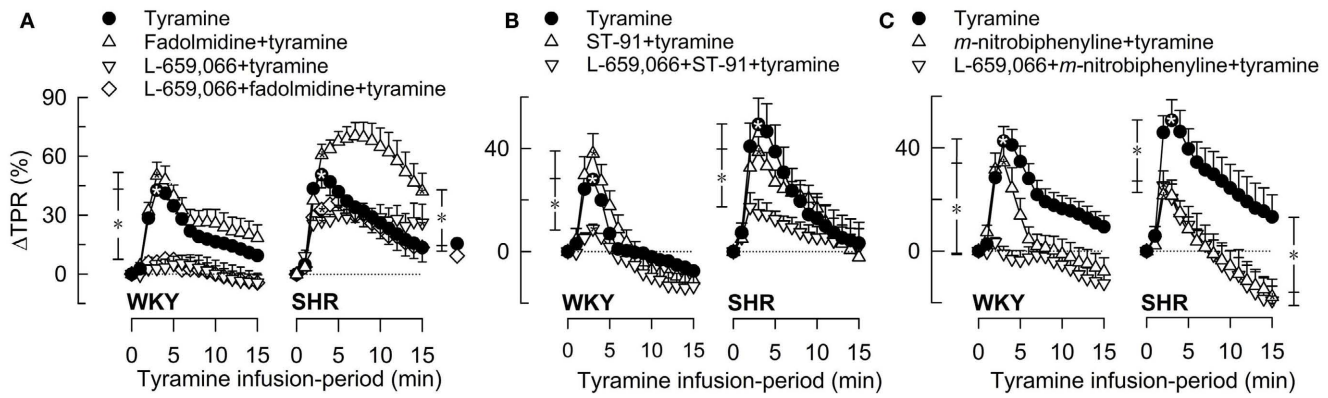

FIGURE 3 | The TPR-response to tyramine-induced norepinephrine release after pre-treatment with $\alpha_{2(non\text{-}A)}$AR-selective agonist, alone or combined with L-659,066. The peripherally restricted $\alpha_{2C>B>A}$AR-agonist fadolmidine **(A)**, the peripherally restricted $\alpha_{2(non\text{-}A)}$AR-selective agonist ST-91 **(B)**, and the α_{2C}-selective agonist *m*-nitrobiphenyline with additional α_{2A+B}AR-antagonistic activity **(C)** were injected alone or after pre-treatment with the peripherally restricted α_2AR-antagonist L-659,066. Baselines prior to tyramine are shown in **Table 3**. Significant responses (*within symbol) and differences between the control and experimental groups were located at peak response (*brackets left of curves) and at 15 min (*brackets right of curves). *, *$P \leq 0.025$ after curve evaluations.

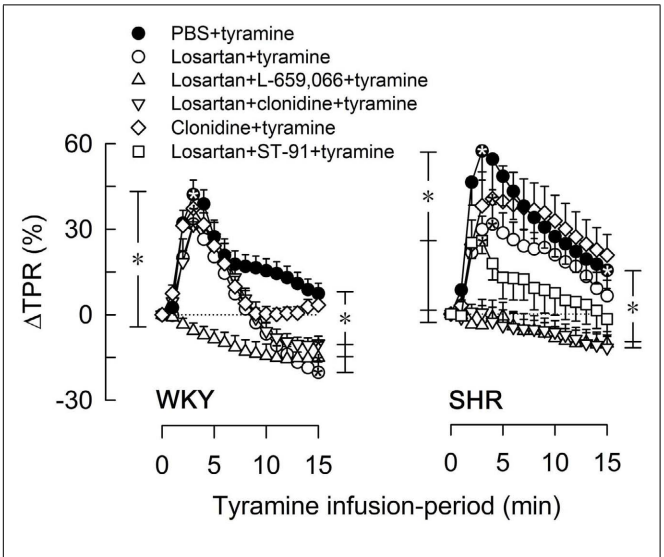

FIGURE 4 | The TPR-response to tyramine after pre-treatment with the AT_1R-antagonist losartan, alone or combined with L-659,066, clonidine, or ST-91. The effect of losartan + ST-91 was tested in SHR only. Baselines prior to tyramine are shown in **Table 3**. Significant responses (*within symbol) and group differences were detected at peak response (*brackets left of curves) and at 15 min (*brackets right of curves) as indicated. *, *$P \leq 0.025$ after curve evaluations.

the non-selective α_2AR-antagonist L-659,066. This increase was eliminated after addition of the non-selective α_2AR-agonist clonidine (Berg and Jensen, 2013), but not, as demonstrated by the present experiment, by agonists with less or no α_{2A}AR reactivity, such as fadolmidine, ST-91, or *m*-nitrobiphenyline. Clonidine reduced the tyramine-induced norepinephrine overflow in SHR, and this reduction was fully reversed by L-659,066 (Berg and Jensen, 2013), and, again, a similar decrease was not seen after fadolmidine, ST-91, or *m*-nitrobiphenyline. Both tyramine and L-659,066 are peripherally restricted, i.e., do not pass the blood-brain barrier (Oldendorf, 1971; Clineschmidt et al., 1988). Inhibition of tyramine-stimulated norepinephrine overflow therefore involved in both strains peripherally located α_2AR, predominantly of the α_{2A}-subtype, in agreement with that previously observed by others (Starke, 2001; Brede et al., 2004).

Epinephrine is secreted directly into blood and not subjected to local re-uptake, and release is therefore not stimulated by tyramine (Berg and Jensen, 2013). However, the stress induced by the surgical procedure activated some secretion of epinephrine from the adrenals (Berg et al., 2012). Clonidine precipitated an L-659,066-sensitive reduction in this secretion in both strains (Berg et al., 2012; Berg and Jensen, 2013), whereas fadolmidine, ST-91, or *m*-nitrobiphenyline did not. It therefore appeared that the α_{2A}AR inhibited also the secretion of epinephrine, in agreement with previous studies on the rat adrenal gland (Lymperopoulos et al., 2007). This differed from that in the mouse, where the α_{2C}-subtype inhibited epinephrine secretion (Brede et al., 2003; Moura et al., 2006).

Although clonidine reduced tyramine-induced norepinephrine overflow to plasma in SHR, the antagonist L-659,066 failed to increase overflow in this strain (Berg and Jensen, 2013). This malfunction depended on the tyramine-stimulated release of norepinephrine, since L-659,066, and also the α_2AR-antagonist yohimbine, clearly increased norepinephrine overflow in SHR not stimulated with tyramine but where NET-re-up-take was blocked by desipramine (Berg et al., 2012). However, norepinephrine overflow was greatly increased in tyramine-stimulated SHR when L-659,066 was combined with the α_{2C}AR-reactive agonist fadolmidine, which has a 35 and 10 times higher affinity for the α_{2C}- and α_{2B}AR than the rat α_{2A}-subtype, respectively (Lehtimaki et al., 2008). Overflow was also greatly increased when L-659,066 was combined with the non-A-selective ST-91 (Takano et al., 1992), or the α_{2C}AR-selective *m*-nitrobiphenyline, which in addition has an α_{2A+B}AR-antagonistic effect (Crassous et al., 2007).

Since fadolmidine and ST-91 do not cross the blood-brain barrier (Clineschmidt et al., 1988; Lehtimaki et al., 2008), stimulation of peripheral α_{2C}AR appeared to re-establish α_{2A}-auto-inhibition in SHR (**Figure 1**).

Augmented tyramine-induced norepinephrine overflow was also observed in SHR but not in WKY after pre-treatment with losartan + L-659,066, whereas losartan alone had no effect. G-protein G_q-signaling agents, including angiotensin II through the AT_1R, have been shown in isolated mouse atria to stimulate norepinephrine release by interfering with down-stream signaling of the inhibitory α_2AR-G_i pathway (**Figure 1**) (Cox et al., 2000; Trendelenburg et al., 2003a). The AT_1R interaction involved only the α_{2C}- and not the α_{2A}-subtype (Cox et al., 2000; Trendelenburg et al., 2003a). α_{2C}AR-agonist may therefore restore α_{2A}-auto-inhibition by counter-acting the AT_1R-G_q-interference, and losartan by eliminating the AT_1R-interference. Thus, as could be expected, ST-91 did not alter the tyramine-induced norepinephrine overflow after losartan in SHR. The present results were therefore compatible with studies showing that the reduced afferent renal nerve signaling observed in response to efferent renal sympathetic nerve activation was increased in SHR by the α_2AR-antagonist rauwolscine, and further potentiated when rauwolscine was combined with losartan, whereas losartan alone had no effect (Kopp et al., 2011).

However, the experimental approach is indirect and performed in the whole animal, and other explanations should therefore also be considered. For instance, α_{2C}AR-stimulation will hamper renal renin release (Michel and Rump, 1996), and, through that, may lower AT_1R-activation and stimulation of release. However, if this was the mechanism responsible, one might have expected losartan alone to lower the release of norepinephrine, which it did not. Unlike vesicular release, NET-mediated release has been considered not to be regulated by presynaptic receptors (Starke, 2001). However, recent studies show that NET may indeed be influenced by presynaptic control, as demonstrated by the hampering effect of muscarinic receptor activation on the NET transport rate (Parker et al., 2010), a response which in other cells is mediated through a PKC-dependent pathway (Apparsundaram et al., 1998). However, PKC did not seem to influence tyramine-induced transport through NET, since preliminary studies showed that the PKC-inhibitor staurosporine, like losartan alone, did alter norepinephrine overflow (plasma norepinephrine concentration = 19.8 ± 2.3 and 27.1 ± 2.3 nM in WKY and SHR, respectively, five rats/group, P = NS compared to the controls, Berg, unpublished observations). α_2AR-agonists have also been shown to bind to NET and to competitively inhibit re-uptake of a norepinephrine analog (Park et al., 2013). This response was not prevented by α_2AR-antagonist, and was therefore likely to result from their structural similarity to norepinephrine and not from α_2AR-signaling. Agonist inhibition of NET did not seem to alter the tyramine-induced reversed transport of norepinephrine through NET, since none of the present agonists lowered tyramine-induced overflow, and the reduction observed in SHR after clonidine was abolished by L-659,066 (Berg and Jensen, 2013).

The secretion of epinephrine mostly followed the same pattern as that of norepinephrine overflow, indicating that α_{2A}AR failed to inhibit also epinephrine secretion in SHR, and that this malfunction could be restored by α_{2C}AR-stimulation or AT_1R-inhibition.

The tyramine-stimulated norepinephrine overflow after L-659,066 + agonist and losartan + L-659,066 was about two times greater, and that of epinephrine 10 times greater, than that in the control or L-659,066-only groups in SHR, but not higher than that after pre-treatment with L-659,066 alone in WKY, i.e., 28% higher than in the controls. L-659,066 and yohimbine greatly increased the plasma concentration of norepinephrine and epinephrine also in desipramine-treated, non-stimulated SHR (Berg et al., 2012). These observations suggested an up-regulation of peripheral, presynaptic α_{2A}AR in SHR, in order to down-regulate the elevated sympathetic tone and/or to compensate for the failing α_2AR-auto-inhibition in this strain.

L-659,066 reduced baseline MBP and TPR in both strains, but abolished the tyramine-induced rise in TPR in WKY only. Also the G_i-inhibitor pertussis toxin eliminated the TPR-response to tyramine in this strain alone (Berg et al., 2009). The abolished TPR-response was most likely due to that L-659,066 inhibited postsynaptic, VSMC α_2AR-G_i-signaling, thereby allowing VSMC βAR-adenylyl cyclase-mediated dilatation to oppose the norepinephrine-induced, α_1AR-mediated vasoconstriction. Also this α_2AR-function failed in SHR. The malfunction appeared to be precipitated by the stimulated release of norepinephrine, since a strain-related difference was not seen in the moderating effect of L-659,066 on the TPR-response to exogenous α_1AR-agonist (Berg et al., 2012). Like the failing control of catecholamine release, also this disorder was repaired by AT_1R-inhibition or α_{2C}AR-stimulation, since losartan + L-659,066 and L-659,066 + ST-91 eliminated the TPR-response to tyramine. This may be due to the high norepinephrine and/or epinephrine release in these SHR groups, which, in the presence of the α_2AR-antagonist inhibiting VSMC α_2AR, may be sufficient to re-establish a βAR-mediated counter-action of the norepinephrine-induced α_1AR-mediated vasoconstriction. This conclusion is in agreement with our previous study showing that neuronally activated, β_1AR-mediated vasodilatation counter-acted the TPR-response to tyramine in WKY only, whereas β_{2+3}AR activated by epinephrine from the adrenals opposed the late half of the TPR-response in SHR (Berg et al., 2010). The TPR-response to tyramine in SHR was also eliminated after losartan + clonidine and reduced after losartan + ST-91, in spite of a normal plasma norepinephrine concentration. It is therefore possible that also the failing β_1AR contribution to TPR-control in SHR resulted from VSMC AT_1R-activation.

In agreement with studies on genetically modified mice, where the initial clonidine-induced vasoconstriction was due to activation of VSMC α_{2B}AR (Link et al., 1996), the present agonists, and as previously described also clonidine (Berg et al., 2012), induced a transient rise in TPR, which was reduced or eliminated by L-659,066, except that of fadolmidine in SHR. The L-659,066-sensitive fraction of this vasoconstriction may be mediated through the α_{2B}AR on VSMC, although the present experiments could not exclude a role of the α_{2A}AR. However, the L-659,066-sensitive fraction of the response to *m*-nitrobiphenyline was likely to be mediated through VSMC α_{2C}AR, since this α_{2C}-selective agonist also acted as an α_{2A+B}AR-antagonist (Crassous et al., 2007). Although VSMC α_{2C}AR did not contribute to BP control

in genetically modified mice (MacDonald et al., 1997), stimulated α_{2C}AR-mediated vasoconstriction has been demonstrated in veins and arterioles (Chotani et al., 2004; Görnemann et al., 2007). The L-659,066-insensitive part of the agonist-induced vasoconstriction was likely to be mediated through α_1AR, since at least fadolmidine contained α_1AR-agonistic activity (Lehtimaki et al., 2008). The latter component may also explain why fadolmidine increased the TPR-response to tyramine in SHR. This increase was absent after additional pre-treatment with L-659,066, possibly due to that L-659,066, by inhibiting the VSMC α_2AR-G_i pathway, allowed norepinephrine-stimulated, βAR-mediated vasodilatation, in that manner opposing the tyramine-induced, α_1AR-mediated vasoconstriction. Fadolmidine was the only agonist which induced a late L-659,066-sensitive fall in MBP, TPR, and HR in SHR, possibly due to its α_{2A}AR-component, which may lower catecholamine release prior to tyramine-stimulation and/or stimulate endothelial, vasodilatory α_{2A}AR (Shafaroudi et al., 2005). The TPR-response to tyramine was reduced by *m*-nitrobiphenyline. This reduction was not further influenced by additional pre-treatment with L-659,066, and was therefore likely to result from the α_{2A+B}AR-antagonistic effect of this agonist. The TPR-response was therefore more sensitive to the promiscuity of the α_2AR-agonists than the α_2AR-mediated control of catecholamine release.

CONCLUSION

Peripheral α_2AR represent the last line of defense against adrenergic hyperactivity. The α_{2A}-subtype played a dominating role in limiting peripheral catecholamine release in WKY, but failed to do so in SHR. This malfunction was restored after α_{2C}AR-stimulation or AT_1R-inhibition, suggesting that an AT_1R-G_q/α_{2C}AR-G_i-interaction disturbed normal α_{2A}AR-mediated control of catecholamine release in SHR. This α_{2C}AR-AT_1R-interaction may be responsible for the elevated plasma norepinephrine concentrations observed in SHR, and contribute to the sympathetic hyperactivity and hypertension in this strain. A loss-of-function α_{2C}AR deletion polymorphism has been shown to be more frequent in African–Americans and connected to a greater HR- and BP-response in the cold-pressor-test (Kurnik et al., 2008). An augmented sympathetic response to this stress-test is linked to increased cardiovascular morbidity (Matthews et al., 2004), and heart failure patients with the same α_{2C}AR polymorphism had a worsened prognosis and increased risk of heart failure (Small et al., 2002, 2003). Estrogen stimulated the expression of α_{2C}AR in human dermal arteriole VSMC (Eid et al., 2007), and may from the present results provide a mechanism whereby estrogen protects against hypertension. A failing α_{2A}AR auto-inhibition of catecholamine release due to an AT_1R-α_{2C}AR interaction may therefore be highly relevant for development of hypertension, the major risk factor for cardiovascular events.

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