British Journal of Pharmacology (2008) 155, 210–216

0007–1188/08 $32.00
www.brjpharmacol.org

RESEARCH PAPER

Mechanisms of augmented vasoconstriction induced by 5-hydroxytryptamine in aortic rings from spontaneously hypertensive rats

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Background and purpose: To test whether development of enhanced vasoconstriction to 5-hydroxytryptamine (5-HT; serotonin) in SHR was temporally related to hypertension, elevated vascular superoxide (O_2^-) levels, decreased NO bioavailability, or increased contractile effects of cyclooxygenase or rho-kinase and/or PKC.
Experimental approach: We examined systolic blood pressure (SBP), vascular O_2^-, and 5-HT-induced contractile responses of aortic segments from 4- and 8-week-old WKY and SHR.
Key results: SBP was 35% higher in SHR than WKY at 4 weeks and 60% higher at 8 weeks. Contractile responses to 5-HT were similar in WKY and SHR at 4 weeks, but were markedly augmented in SHR at 8 weeks. The NO synthase inhibitor, L-NAME, enhanced contractile responses to 5-HT markedly in both strains at 4 weeks and in WKY at 8 weeks, but only very modestly in SHR at 8 weeks. These functional differences were associated with higher O_2^- levels in SHR versus WKY at 8 weeks, but not at 4 weeks. The rho-kinase inhibitor, Y-27632, and the PKC inhibitor, Ro 31-8220, each only modestly attenuated contractions in WKY and SHR in each age group, and their effects in each strain were more pronounced at 8 weeks. The cyclooxygenase inhibitor, indomethacin, had no effect on contractile responses.
Conclusions and implications: Development of augmented vascular contractile responses to 5-HT in SHR is preceded by hypertension. It is associated with increased vascular O_2^- levels and reduced modulatory effects of NO, and is unlikely to be due to enhanced activity of rho-kinase, PKC or cyclooxygenase.
British Journal of Pharmacology (2008) **155,** 210–216; doi:10.1038/bjp.2008.247; published online 16 June 2008

Keywords: hypertension; nitric oxide; PKC; reactive oxygen species; rho-kinase

Abbreviations: eNOS, endothelial nitric oxide synthase; NO, nitric oxide; O_2^-, superoxide; PKC, protein kinase C; Ro 31-8220, (3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide); SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto; Y-27632, (R-(+)-trans-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide)

Introduction

Endothelial dysfunction is a common characteristic of advanced cardiovascular diseases associated with reduced bioavailability of the endogenous vasodilator, nitric oxide (NO), such as chronic hypertension. Endothelial NO is a critical modulator of vascular contractility, and its absence—for example, due to physical removal of the endothelium or endothelial NO synthase (eNOS) gene deletion—leads to enhanced vascular contractile responses, particularly to G protein-coupled receptor agonists such as 5-HT (Lamping *et al.*, 1985; Lamping and Faraci, 2003; Budzyn *et al.*, 2004). Increased levels of reactive oxygen species such as superoxide (O_2^-), which occurs in the vasculature of chronically hypertensive animals (Zalba *et al.*, 2000; Landmesser *et al.*, 2003; Matsuno *et al.*, 2005), can also contribute to compromised NO bioavailability and consequent vascular dysfunctions (Cai and Harrison, 2000).

It is also recognised that 'Ca^{2+} sensitisation'—the mechanism of vascular contraction that occurs independently of increasing intracellular Ca^{2+} levels is another major mechanism that regulates vascular contractility, particularly during hypertension (Soloviev and Bershtein, 1992; Shaw *et al.*, 1997). In particular, the role of rho-kinase—the predominant mediator of Ca^{2+} sensitisation—in

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Received 22 April 2008; revised 12 May 2008; accepted 15 May 2008; published online 16 June 2008

the regulation of vascular tone during hypertension has been studied extensively (Uehata *et al*., 1997; Chrissobolis and Sobey, 2001; Wehrwein *et al*., 2004; Jin *et al*., 2006). Furthermore, although early studies suggested that PKC might also contribute to increased vascular contractility during hypertension (Bruschi *et al*., 1988; Shibata *et al*., 1990; Secrest *et al*., 1991; Soloviev and Bershtein, 1992), very few studies have sought to clarify the relative roles of each kinase in hypertension.

Although many studies have described abnormalities in vascular function in established chronic hypertension (Shaw *et al*., 1997; Endemann *et al*., 2002; Jarajapu and Knot, 2005; Northcott *et al*., 2005), very little is known about the timing of the occurrence of certain abnormalities during its development. Hence, the main aims of this study were to test whether the development of enhanced vasoconstriction to 5-HT, early in genetic hypertension, is temporally related to increased blood pressure (BP), elevated vascular O_2^- levels, decreased NO bioavailability, or increased contractile effects of rho-kinase and/or PKC.

Methods

All experimental procedures were approved by the Monash University and the University of Melbourne Animal Experimentation Ethics Committees and complied with National Health and Medical Research Council of Australia guidelines. Male Wistar–Kyoto (WKY) and spontaneously hypertensive rats (SHR) were studied at 4 weeks ($n=47$) or 8 weeks ($n=48$) of age. Rats were housed under a 12-h light/dark cycle with access to food and water *ad libitum*. Systolic BP (SBP) was measured by tail-cuff plethysmography as described previously (Okuniewski *et al*., 1998).

In vitro *measurement of arterial* O_2^-

Rats were killed by inhalation of 80% CO_2:20% O_2 and decapitation. Segments of thoracic aorta were excised and cut into segments of equal length (~5 mm). O_2^- levels in the presence of NADPH (100 μM) were measured by 5 μM lucigenin-enhanced chemiluminescence as described previously (Paravicini *et al*., 2004; Miller *et al*., 2005). Vessels from age-matched WKY and SHR were studied in parallel on the same day. The 4- and 8-week-old rats were studied on separate days. Aortic O_2^- levels were normalised for dry tissue weight. Some additional experiments were performed using 100 μM L012-enhanced chemiluminescence (Wassmann *et al*., 2004) to compare basal O_2^- levels in aortae from 4-week-old WKY and SHR.

In vitro *measurement of contractile responses*

The thoracic aorta was isolated, cleaned and cut into 3–4 mm wide rings. Aortic segments from 4-week-old rats were mounted at 5 mN passive tension in 5 mL chambers of a small vessel myograph (Model 610 M, Multi Myograph, Denmark) containing Krebs-bicarbonate solution of the following composition (mmol L^{-1}): NaCl 118, KCl 4.5, $MgSO_4$ 0.45, KH_2PO_4 1.03, $NaHCO_3$ 25, glucose 11.1 and $CaCl_2$ 2.5, bubbled with 5% CO_2 in O_2 at 37 °C. Tension was continuously recorded on a chart recorder (Model 3721, Yokogawa, Japan). Aortic segments from 8 week-old rats were mounted at 5 mN passive tension in 10 mL organ chambers containing Krebs-bicarbonate solution bubbled with 5% CO_2 in O_2 at 37 °C. Tension was continuously recorded using a Grass FT03 force transducer and Powerlab Chart computer software (Version 5.2.2).

Following 45 min equilibration, arterial segments were exposed to an isotonic high K^+-containing physiological saline solution (KPSS; $[K^+]_{KPSS}=124$ mM). KPSS-induced contraction reached a stable level after 10–20 min. Following washout and return to a stable baseline, segments were precontracted to ~50% of their KPSS response with phenylephrine (1–3 μM). Relaxation in response to acetylcholine (10 μM) confirmed the presence of functional endothelium. Following washout and return to stable baseline, cumulative concentration–response curves were established to 5-HT (10 nM–0.3 mM).

Effects of NOS, cyclooxygenase, rho-kinase and PKC inhibition on contractile responses

We assessed the effect of 30 min pretreatment with the NO synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) on contractile responses to 5-HT. Similarly, the effects of the cyclooxygenase inhibitor, indomethacin (10 μM), or the rho-kinase inhibitor, Y-27632 (1 μM) and/or the PKC inhibitor, Ro 31-8220 (5 μM), on responses to 5-HT were also assessed after 30 min treatment in separate tissues.

Data analysis and statistical procedures

Contractile responses to 5-HT were normalised by expressing them as a percentage of the KPSS response of each arterial segment. Each *n* represents the number of animals used. All data were normally distributed, and expressed as mean ± s.e.mean. Single comparisons were made using Student's paired or unpaired *t*-test, as appropriate. Multiple comparisons were made using one-way ANOVA, followed by Tukey's *post hoc* test. GraphPad Prism (version 4) was used to perform all statistical analyses and $P<0.05$ was considered significant.

Drugs

Y-27632 (R-(+)-trans-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide) was obtained from Welfide Corporation (Osaka, Japan). Ro 31-8220 (3-[1-[3-(amidinothio) propyl-1*H*-indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide) was obtained from Calbiochem (La Jolla, CA, USA). All other drugs were obtained from Sigma Chemical Co. (St Louis, MO, USA). Ro 31-8220 was dissolved as a stock solution of 1 mM in 100% dimethyl sulphoxide and diluted in de-ionised water. Indomethacin was dissolved in 0.1 M Na_2CO_3 and diluted in de-ionised water. All other drugs were dissolved and diluted in de-ionised water or saline.

Results

Systolic blood pressure and aortic O_2^- levels

Systolic blood pressure was already higher in SHR than WKY at 4 weeks ($P<0.05$, unpaired *t*-test; Figure 1a), and this difference became even greater at 8 weeks ($P<0.05$, unpaired *t*-test; Figure 1a). Basal aortic O_2^- levels did not differ between WKY and SHR at 4 weeks using chemiluminescence assays based on either L-012 (WKY: 1318 ± 196 counts per mg versus SHR: 1094 ± 131 counts per mg; $P>0.05$, unpaired *t*-test) or lucigenin (Figure 1b) as a lumiphore. However, aortic O_2^- levels were significantly augmented at 8 weeks in SHR versus WKY ($P<0.05$, unpaired *t*-test; Figure 1b).

Contractile responses to 5-HT

Contractile responses of aortic segments to 5-HT were similar in WKY and SHR at 4 weeks (Figure 2a). However, at 8 weeks, maximum responses to 5-HT were augmented by ~50% in SHR relative to WKY ($P<0.05$, unpaired *t*-test; Figure 2b). There was also increased sensitivity to 5-HT-induced contractile responses in SHR versus WKY at 8 weeks ($P<0.05$, unpaired *t*-test; Figure 2b; Table 1). Moreover, absolute contractile responses to KPSS in aorta from 8 week-old rats was virtually identical in WKY (17.5 ± 0.06 mN; $n=24$) and SHR (16.8 ± 0.07 mN; $n=24$) ($P>0.05$, unpaired *t*-test), confirming that the responses to 5-HT were somewhat selectively augmented at 8 weeks.

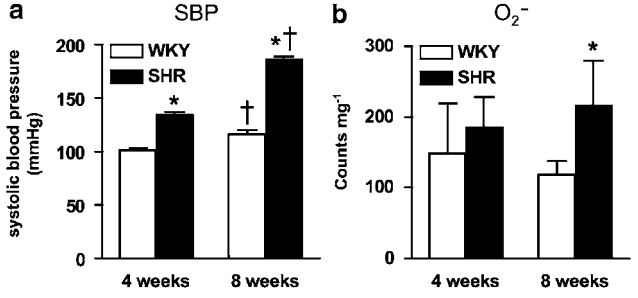

Figure 1 Systolic blood pressure and aortic O_2^- levels in WKY and SHR. (**a**) Measurement of systolic blood pressure in WKY and SHR at 4 and 8 weeks. (**b**) O_2^- levels in aortic segments from WKY and SHR at 4 and 8 weeks. All values are mean ± s.e.mean. $n=8$ of each strain per group. *$P<0.05$, unpaired *t*-test versus WKY, $^{\dagger}P<0.05$, unpaired *t*-test versus 4 weeks. SHR, spontaneously hypertensive rats; WKY, wistar–Kyoto.

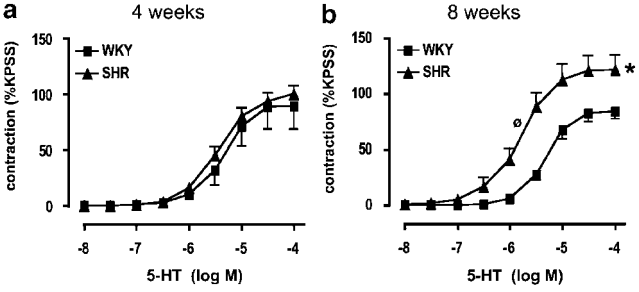

Figure 2 Contractile responses to 5-HT in WKY and SHR. Concentration–response curves to 5-HT in aortic segments from (**a**) 4-week- and (**b**) 8-week-old WKY and SHR. All values are mean ± s.e.mean. $n=15$–16 of each strain per group. *$P<0.05$ versus WKY maximum, $^{\oslash}P<0.05$ (unpaired *t*-test) versus WKY log EC_{50}. SHR, spontaneously hypertensive rats; WKY, wistar–Kyoto.

Effect of NOS and cyclooxygenase inhibition on contractile responses to 5-HT

L-NAME markedly increased contractile responses to 5-HT in WKY at both 4 and 8 weeks of age ($P<0.05$, paired *t*-test; Figures 3a and c). Furthermore, although L-NAME also augmented the sensitivity to 5-HT-induced contractions in SHR, at both 4 and 8 weeks, this effect was pronounced (~300-fold) at 4 weeks but minimal (~2-fold) at 8 weeks ($P<0.05$, paired *t*-test; Figures 3b and d; Table 2). In contrast, L-NAME had no overall effect on maximum contractile responses in SHR at either age (Figures 3b and d). Indomethacin had no effect on 5-HT-induced contractile responses in either WKY or SHR at 8 weeks (Figures 4a and b).

Effect of rho-kinase and PKC inhibition on contractile responses to 5-HT

Maximum contractile responses to 5-HT were only modestly reduced by either the rho-kinase inhibitor, Y-27632, or the PKC inhibitor, Ro 31-8220, in both WKY and SHR at 4 weeks of age ($P<0.05$, one-way ANOVA followed by Tukey's test; Figures 5a and b). Similarly, cotreatment of aortic segments from 4-week-old rats with Y-27632 and Ro 31-8220 also caused only a minimal inhibition of responses (Figures 5a and b). Y-27632 or Ro 31-8220 alone each caused a more pronounced attenuation of contractile responses to 5-HT at 8 weeks of age in both WKY and SHR (Figures 5c and d). Cotreatment with Y-27632 and Ro 31-8220 caused further inhibition of contractile responses than treatment with either inhibitor alone (Figures 5c and d) in WKY (Figure 5c; one-way ANOVA followed by Tukey's test).

Discussion and conclusions

Although many studies have described altered mechanisms of vascular function in SHR once hypertension is established, very few have examined temporal changes in such mechanisms during the developing phases of hypertension. We have investigated the degree to which NO, cyclooxygenase, rho-kinase and PKC influence vascular contractile responses to 5-HT during the developing phase of hypertension in SHR, as compared with normotensive WKY. Although SBP is elevated in SHR at 4 weeks, vascular O_2^- levels and

Table 1 Log EC_{50} values from concentration–response curves to 5-HT in arterial segments from WKY and SHR

Age	*WKY*	*SHR*
4 weeks	−5.32 ± 0.07 (15)	−5.32 ± 0.16 (16)
8 weeks	−5.26 ± 0.08 (16)	−5.72 ± 0.10* (16)

Abbreviations: SHR, spontaneously hypertensive rats; WKY, wistar–Kyoto. *$P<0.05$, unpaired *t*-test versus WKY.

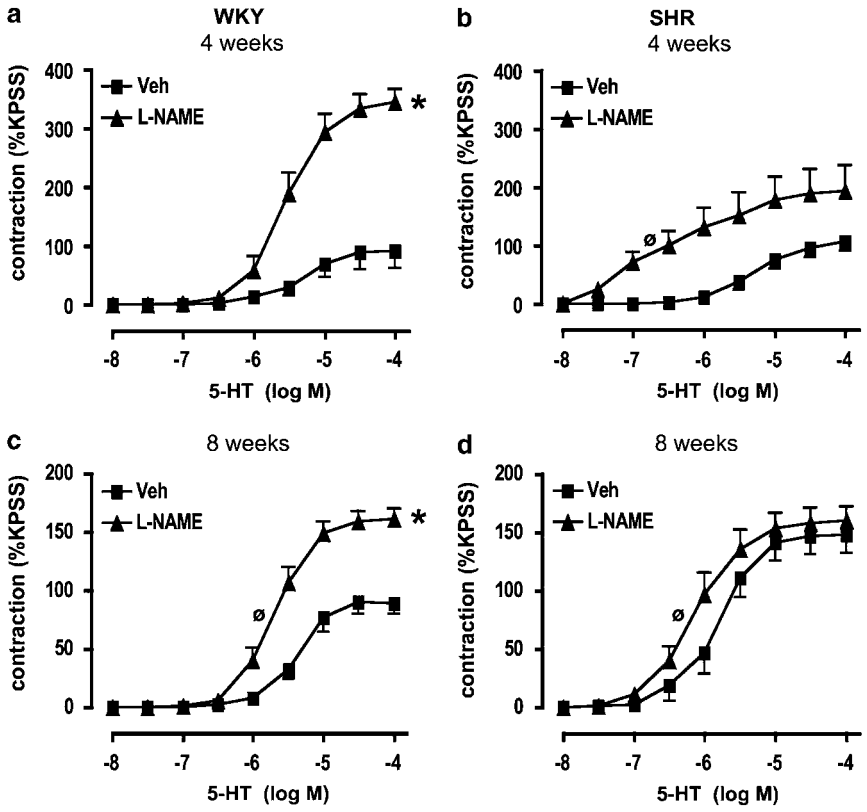

Figure 3 Effect of NOS inhibition on contractile responses to 5-HT. Concentration–response curves to 5-HT in aortic segments from (**a** and **c**) WKY and (**b** and **d**) SHR at 4 and 8 weeks of age, in the presence of vehicle (Veh; saline) or L-NAME (100 μM). All values are mean ± s.e.mean. $n=8$ per group. *$P<0.05$, unpaired *t*-test versus Veh maximum, $^{\varnothing}P<0.05$, unpaired *t*-test versus Veh log EC_{50}. NOS, nitric oxide synthase; SHR, spontaneously hypertensive rats; WKY, wistar–Kyoto.

Table 2 Log EC_{50} values from concentration–response curves to 5-HT in arterial segments from WKY and SHR

Treatment	*4 weeks*		*8 weeks*	
	WKY	*SHR*	*WKY*	*SHR*
Vehicle	−5.36 ± 0.12 (8)	−5.02 ± 0.27 (8)	−5.34 ± 0.07 (8)	−5.79 ± 0.12 (8)
L-NAME	−5.52 ± 0.12 (8)	−7.48 ± 0.81* (8)	−5.69 ± 0.08* (8)	−6.12 ± 0.12* (8)

Abbreviations: SHR, spontaneously hypertensive rats; WKY, wistar–Kyoto.*$P<0.05$, paired *t*-test versus vehicle.

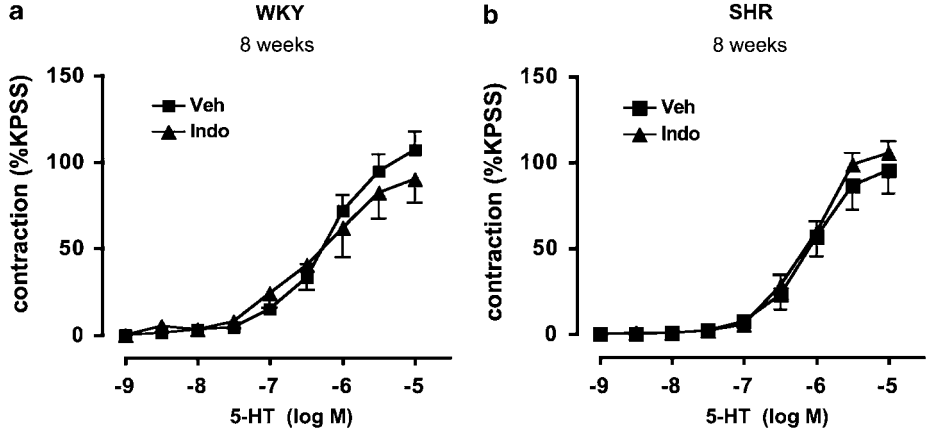

Figure 4 Effect of cyclooxygenase inhibition on contractile responses to 5-HT. Concentration–response curves to 5-HT in aortic segments from (**a**) WKY and (**b**) SHR at 8 weeks of age, in the presence of vehicle (Veh; 1 mM Na_2CO_3) or indomethacin (10 μM). All values are mean ± s.e.mean. $n=7$–8 per group.

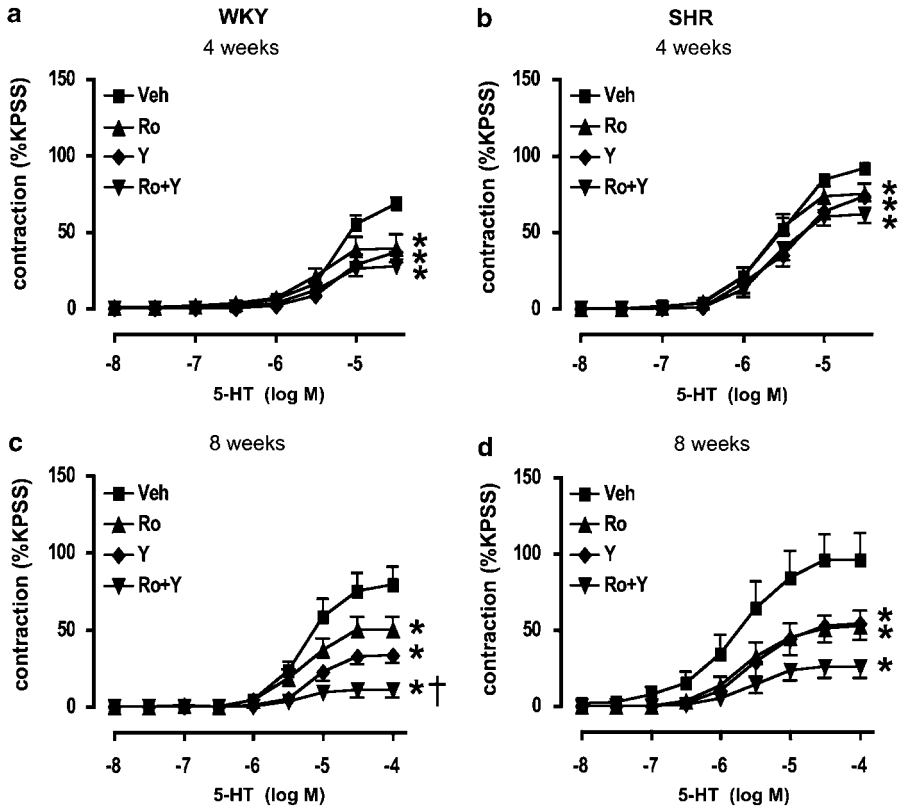

Figure 5 Effects of rho-kinase and PKC inhibition on contractile responses to 5-HT. Concentration–response curves to 5-HT in aortic segments from (**a** and **c**) WKY and (**b** and **d**) SHR at 4 and 8 weeks of age, in the presence of vehicle (Veh; 0.5% dimethyl sulphoxide), Ro 31-8220 (Ro, 5 μM) or Y-27632 (Y, 1 μM) alone, or in combination. All values are mean ± s.e.mean. $n=7$–8 per group. *$P<0.05$ versus Veh maximum, †$P<0.05$ versus Y-27632 maximum; one-way ANOVA followed by Tukey's test. SHR, spontaneously hypertensive rats; WKY, wistar–Kyoto.

contractility to 5-HT remain unchanged, relative to age-matched WKY. However, both become markedly enhanced in SHR by 8 weeks of age, in association with further elevation of SBP. In addition, endothelial NO contributes significantly to modulating responses to 5-HT in WKY at both 4 and 8 weeks of age, but only in SHR at 4 weeks, consistent with the possibility that NO is inactivated by O_2^- by 8 weeks in SHR. Furthermore, rho-kinase and PKC each contribute to vascular contraction in both WKY and SHR, albeit to similar degrees between each strain. Cyclooxygenase activity appears to play little or no role in contractile responses of either WKY or SHR to 5-HT in the first 8 weeks of life.

Blood pressure development in WKY and SHR

The present results indicate that SBP of SHR was already higher than in WKY at 4 weeks of age, consistent with other studies that have also used the tail cuff method of BP measurement (for example, Dickhout and Lee, 1998). This non-invasive method of measuring BP is reported to yield reproducible results similar to those obtained using intra-arterial methods in conscious rats (Widdop and Li, 1997; Ibrahim *et al*., 2006), and thus is thought to be valid. There is disagreement in the literature as to whether BP of SHR is indeed elevated at 4 weeks of age, or even earlier (Bruno *et al*., 1979; Cheng *et al*., 2004; Cruzado *et al*., 2005). However, several studies have reported that BP of WKY and SHR diverge from between 2 and 6 weeks of age (Morton *et al*., 1990; Dickhout and Lee, 1998; Christiansen *et al*., 2002).

Roles of cyclooxygenase or endothelial NO in contractile responses to 5-HT in WKY and SHR

Contractile responses to 5-HT were virtually identical in aortae from 4-week-old WKY and SHR, but became markedly augmented in SHR relative to WKY at 8 weeks. This difference at 8 weeks was apparently not related to augmented cyclooxygenase activity, because indomethacin had no effect on contractions to 5-HT in either WKY or SHR. Furthermore, although the inhibition of NOS by L-NAME caused a significant increase in contractions to 5-HT in all WKY and in 4-week-old SHR (increasing potency by ~300-fold and approximately doubling the maximum), it increased potency by a modest twofold and clearly had no effect on the magnitude of these responses in 8 week-old SHR. Previous studies have demonstrated that vascular sensitivity to 5-HT is augmented in the absence of endothelial NO, either as a consequence of eNOS gene deletion, physical removal of the endothelium or pharmacological inhibition of eNOS (Lamping *et al*., 1985; Lamping and Faraci, 2003; Budzyn *et al*., 2004). The present results are thus consistent with the notion that NO plays a substantial role in modulating contractions of aorta to 5-HT in the normotensive WKY, but not in the genetically hypertensive adult SHR.

The lack of modulation of contractile responses by NO is therefore likely to account, at least in part, for the increased reactivity to 5-HT in SHR seen at 8 weeks of age. Furthermore, it is conceivable that this relative lack of functional endothelial NO can be accounted for by increased generation of vascular O_2^-, and subsequent inactivation of eNOS/NO.

Role of vascular O_2^-

Increased O_2^- levels have been detected in blood vessels of hypertensive animals (Laursen *et al*., 1997; Schnackenberg *et al*., 1998; Kerr *et al*., 1999; Landmesser *et al*., 2003), and the generation of O_2^- is thought to play a major role in the development of hypertension. Interestingly, although SBP was higher in SHR than in WKY at both 4 and 8 weeks of age, higher aortic O_2^- levels were only detected in SHR at 8 weeks of age. Our data, therefore, suggest that an elevated vascular O_2^- level (and indeed vascular hypercontractility to 5-HT) does not necessarily precede the development of hypertension in SHR as previously suggested by others (Jameson *et al*., 1993; Cosentino *et al*., 1998; Nabha *et al*., 2005), but may be an effect rather than the cause of elevated BP.

Given that the aorta functions predominantly as a conductance vessel and whose contractility is also known to be greatly influenced by endothelial NO (Lamping *et al*., 1985; Lamping and Faraci, 2003; Budzyn *et al*., 2004), a possible extension to the present study could include investigating how contractility and vascular O_2^- production compare in resistance vessels, where the relative importance of endothelial NO in modulating contractility is diminished (Vanhoutte, 2004). We and others have previously demonstrated that the contractile properties of small resistance arteries such as the mesenteric artery, do indeed differ to those of larger conductance vessels such as the aorta (Asano and Nomura, 2003; Budzyn *et al*., 2006). Given that such vessels contribute to total peripheral resistance, and therefore BP regulation, it is possible that even more stark functional and biochemical differences could be observed in this vascular region.

Role of rho-kinase and PKC in contractile responses to 5-HT

The contribution of rho-kinase in the regulation of vascular tone is known to become enhanced in several animal models of established chronic hypertension (Uehata *et al*., 1997; Chrissobolis and Sobey, 2001; Wehrwein *et al*., 2004; Jin *et al*., 2006), however, this is the first study to investigate the functional contribution of rho-kinase to contractile responses at such an early stage of its development. The rho-kinase inhibitor, Y-27632, modestly reduced contractile responses to 5-HT in aorta from 4-week-old WKY and SHR, and importantly this occurred to a similar degree in each strain. Similarly modest effects were observed using the PKC inhibitor, Ro 31-8220, in 4-week-old rats. In contrast, at 8 weeks, Y-27632 and Ro 31-8220 each more effectively reduced responses to 5-HT in WKY and SHR, with the effects of each inhibitor being essentially equivalent in each strain. It should be noted that Ro 31-8220 may not be strictly selective for PKC and also may only inhibit certain isoforms of PKC (Davies *et al*., 2000), and so the data should be interpreted with care. Thus, although the contribution of both rho-kinase and PKC to 5-HT-induced vasoconstriction appears to increase with age, selectively augmented activity of either enzyme is unlikely to account for the selectively enhanced contractility to 5-HT in SHR at 8 weeks, even if this will eventually develop as chronic hypertension progresses (Soloviev and Bershtein, 1992; Satoh *et al*., 1994).

The possibility that the altered contractility observed in SHR at 8 weeks is also in part due to structural changes within the aorta at this stage of development cannot be ruled out. For instance, it is well documented that contractile properties of the aorta can become altered in parallel with vascular hypertrophy that occurs during the development of hypertension (Sudhir and Angus, 1990; van Gorp *et al*., 2000). Thus, it would be of interest to investigate whether any such structural changes have occurred in the aorta at the time points studied, and if so, whether these are temporally correlated with the onset of changes in the contractile properties of these vessels.

In summary, the present results suggest that the development of augmented contractile responses to 5-HT in SHR is preceded by hypertension and associated with increased vascular O_2^- levels, in parallel with reduced modulatory effects of NO, but is probably not due to excessive activity of cyclooxygenase, rho-kinase or PKC—at least during the earlier stages of hypertension.

Acknowledgements

This study was supported by funds from Project grants from the National Health and Medical Research Council of Australia (NHMRC ID 208969, 350477). KB was supported by a Melbourne University Research Scholarship and is currently supported by a CJ Martin Overseas Training Fellowship from the NHMRC. AAM is a Postdoctoral Fellow of the Foundation for High Blood Pressure Research, Australia. CGS is a Senior Research Fellow of the NHMRC.

Conflict of interest

The authors state no conflict of interest.

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