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RESEARCH PAPER

Mitochondrial monoamine oxidase-A-mediated hydrogen peroxide generation enhances 5-hydroxytryptamine-induced contraction of rat basilar artery

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monoamine oxidases; 5-hydroxytryptamine; mitochondrial reactive oxygen species; basilar artery; spontaneously hypertensive rats

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BACKGROUND AND PURPOSE
We evaluated the role(s) of monoamine oxidase (MAO)-mediated H_2O_2 generation on 5-hydroxytryptamine (5-HT)-induced tension development of isolated basilar artery of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats.

EXPERIMENTAL APPROACH
Basilar artery (endothelium-denuded) was isolated for tension measurement and Western blots. Enzymically dissociated single myocytes from basilar arteries were used for patch-clamp electrophysiological and confocal microscopic studies.

KEY RESULTS
Under resting tension, 5-HT elicited a concentration-dependent tension development with a greater sensitivity (with unchanged maximum tension development) in SHR compared with WKY (EC_{50}: 28.4 $\pm$ 4.1 nM vs. 98.2 $\pm$ 9.4 nM). The exaggerated component of 5-HT-induced tension development in SHR was eradicated by polyethylene glycol-catalase, clorgyline and citalopram whereas exogenously applied H_2O_2 enhanced the 5-HT-elicited tension development in WKY. A greater protein expression of MAO-A was detected in basilar arteries from SHR than in those from WKY. In single myocytes and the entire basilar artery, 5-HT generated (clorgyline-sensitive) a greater amount of H_2O_2 in SHR compared with WKY.

Whole-cell iberiotoxin-sensitive Ca^{2+}-activated K^+ (BK_{Ca}) amplitude measured in myocytes of SHR was approximately threefold greater than that in WKY (at +60 mV: 7.61 ± 0.89 pA·pF^{-1} vs. 2.61 ± 0.66 pA·pF^{-1}). In SHR myocytes, 5-HT caused a greater inhibition (clorgyline-, polyethylene glycol-catalase- and reduced glutathione-sensitive) of BK_{Ca} amplitude than in those from WKY.

CONCLUSIONS AND IMPLICATIONS

5-HT caused an increased generation of mitochondrial H_2O_2 via MAO-A-mediated 5-HT metabolism, which caused a greater inhibition of BK_{Ca} gating in basilar artery myocytes, leading to exaggerated basilar artery tension development in SHR.

Abbreviations

5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HTOL, 5-hydroxytryptophol; 5-HTT, 5-hydroxytryptamine transporter; BK_{Ca} channels, native iberiotoxin-sensitive large-conductance Ca^{2+}-activated K^+ channels; COX, cyclooxygenase; DMSO, dimethyl sulphoxide; GSH, reduced glutathione; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MAO-A, monoamine oxidase-A; MAO-B, monoamine oxidase-B; NADPH oxidases, reduced nicotinamide-adenine dinucleotide phosphate oxidases; PEG-catalase, polyethylene glycol-catalase; PEG-SOD, polyethylene glycol-superoxide dismutase; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats

Introduction

5-Hydroxytryptamine (5-HT), a potent vasoactive amine, involved in the regulation of cerebral circulation, and it is implicated in the aetiology of cerebral disorders such as migraine, vasospasm following acute subarachnoid haemorrhage and ischaemic brain diseases (Bonvento *et al.*, 1991). One of the endogenous sources of 5-HT is the circulating platelet and during aggregation, a large amount of 5-HT is released from the aggregated platelets into the plasma and causes aberrant vascular responses (Voldby *et al.*, 1982; Vanhoutte, 1983). 5-HT causes vasorelaxation (mainly via endothelial cells) and vasoconstriction (via vascular smooth muscle cells). At sites of atherosclerosis where endothelial damage has occurred, 5-HT released from aggregated platelets has direct contractile effects on the blood vessels and consequently decreases blood flow with serious outcomes.

The magnitude of the contractile response of the basilar artery in spontaneously hypertensive rats (SHR) a commonly used animal model for human essential hypertension, and stroke-prone SHR (SHRSP) in response to 5-HT is greater (but not to other vasoactive agents – U46619, endothelin-1, neuropeptide Y and angiotensin II) compared with normotensive Wistar-Kyoto (WKY) rats (Nishimura, 1996). It is mainly related to an attenuated NO-mediated relaxation (basal release of NO and/or 5-HT-induced NO release from endothelium) in SHR (Schoeffter and Hoyer, 1990; Schmuck *et al.*, 1996; Ullmer *et al.*, 1996). However, there was no differential contractile responses to 5-HT (topical application via the cranial window) of WKY and SHRSP (Paterno *et al.*, 1997).

In vascular smooth muscle cells, vascular tone is coupled to membrane potential (Trapani *et al.*, 1981; Haeusler, 1983) that is determined by potassium (K^+) conductance (Nelson *et al.*, 1990; Nelson and Quayle, 1995). Increased Ca^{2+}-activated K^+ channel (K_{Ca}) current amplitude was observed in arterial smooth muscle from rats with genetic, renal and salt-induced hypertension (Rusch *et al.*, 1992; England *et al.*, 1993; Liu *et al.*, 1994; 1995; Rusch and Runnells, 1994; Martens and Gelband, 1996). The arterioles of SHR constricted twofold to fourfold more intensely in response to iberiotoxin, a highly selective blocker of the large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}; ion channel nomenclature follows Alexander *et al.*, 2009) (Liu *et al.*, 1998). In cerebrovascular smooth muscle cells from SHR, there is a higher density (4.7-fold) of iberiotoxin-sensitive BK_{Ca} channels at physiological membrane potentials [plus a fourfold increase in BK_{Ca} channel α-subunit (a pore-forming subunit)], than in these cells from WKY. Iberiotoxin has a greater inhibitory effect on BK_{Ca} channel amplitude of the cerebral arteriole smooth muscle cells of SHR compared with WKY rats. Enhanced BK_{Ca} current amplitude plus an increased α-subunit of BK_{Ca} channels were observed in the aorta of SHR compared with WKY rats (Liu *et al.*, 1997; 1998). It has been suggested that the K_{Ca} current amplitude is positively correlated to the blood pressure level of animals, and the enhanced K_{Ca} channels act as a physiological brake to limit blood pressure elevation (Rusch and Runnells, 1994; Paterno *et al.*, 1997). Thus, agents that suppress the opening of vascular K_{Ca} channels of hypertensive animals abolish this beneficial compensatory mechanism and a greater increase in vascular tone is anticipated.

Monoamine oxidase (MAO)-containing nerve fibres is present in the major cerebral arteries including the basilar artery of rats (Shigematsu *et al.*, 1989) and humans (Kalaria and Harik, 1987). The MAOs, flavin-containing enzymes catalysing the oxidative deamination of endogenous monoamines (5-HT, dopamine, adrenaline and noradrenaline), are located in the outer mitochondrial membrane and exhibited in virtually all tissues of mammals. In humans, there are two types of MAO: MAO-A and MAO-B (based on genetic criteria, substrate specificity and inhibition by various synthetic compounds) (Youdim and Finberg, 1991). The physiological significance of MAO in the regulation of cardiovascular activities, reported so far, were mostly derived from the effects of MAO inhibitors used with administered catecholamines in different organs. However, less attention has been paid to the products of MAO activities, such as H_2O_2, one of the endogenous reactive oxygen species (ROS). MAO-dependent increases in ROS production appeared to be relevant in 5-HT-induced myocardial injury caused by post-ischaemic reperfusion (Bianchi *et al.*, 2005a) and myocyte hypertrophy *in vitro* (Bianchi *et al.*, 2005b). However, the role(s) of MAO in mediating the exaggerated, 5-HT-elicited, contractile responses of basilar artery of hypertensive animals is unknown.

Therefore, in this study, we tested the hypothesis that 5-HT caused a greater tension development of isolated basilar artery through the mediation of mitochondrial ROS, such as H_2O_2, generated via MAO-A, in SHR, than in normotensive WKY rats. To eliminate the possible contribution of NO to our results, we used arteries denuded of endothelium after isolation.

Methods

Animals

All animal care and experimental procedures conformed to guidelines for the use of laboratory animals and were approved by the Animal Research Ethics Committee of CUHK (Ref. #: 03/001/ERG). Every effort was made to limit animal suffering and the number of animals used in these experiments. SHR and normotensive WKY rats (22–26 weeks old, male) were used in this study.

Isometric tension development

Isolated basilar artery rings (length: 1 mm, endothelium denuded) were mounted under the optimum tension of 3 ± 0.3 mN in a 5 mL small vessel wire myograph containing physiological salt solution with the composition (mM) of NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 1.8, gassed with 16% O_2/6% CO_2 balanced with N_2, pO_2 ~100 mmHg (the physiological pO_2 level), in order to minimize the generation of ROS under the non-physiological conditions with the more commonly used gas mixture (carbogen): 95% O_2/5% CO_2 (pO_2 > 600 mmHg) (Farrow *et al.*, 2008). The endothelium was carefully removed by rubbing the intima of the artery with a human hair for ~5 min, and endothelium removal was confirmed by the failure of acetylcholine (10 μM)-induced relaxation as reported (Seto *et al.*, 2006). Four basilar artery rings were isolated from individual artery and experiments were performed on the same day. One concentration–response curve of 5-HT (with and without a particular blocker/inhibitor) was constructed in each arterial preparation of individual rats. In order to reduce the number of animals used, controls (inhibitor-free) and drug (blocker/inhibitor)-treated experiments were randomly conducted according to the incomplete block design/protocols (Hinkelmann and Kempthorne, 2005). In this regard, only one 'control curve' (i.e. 5-HT-induced contraction without the presence of inhibitor/blocker) of each strain was employed in this study for comparison and data analysis.

Isolation of rat cerebral vascular smooth muscle cells

Single basilar artery smooth muscle cells were enzymatically dissociated as reported (Wu *et al.*, 2005). Cells isolated were used within 8 h after isolation.

Patch-clamp electrophysiology

Conventional whole-cell native iberiotoxin-sensitive BK_{Ca} channel gating before, during and after 5-HT (and other drugs) challenge were recorded at room temperature (~22°C). Whole-cell, membrane-rupture recording of the macroscopic iberiotoxin-sensitive BK_{Ca} channels gating of single artery myocytes were recorded, as described by our group previously (Seto *et al.*, 2007). External physiological solutions for recording the BK_{Ca} channel amplitude contained (in mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 10 and HEPES 10 (pH 7.4 with NaOH). Internal pipette solution containing ~100 nM free [Ca^{2+}] (estimated using the computer programme: Maxchelator, Stanford University, Stanford, CA, USA) had the following composition (in mM): NaCl 10, KCl 110, $MgCl_2$ 5, $CaCl_2$ 2, EGTA 10, K_2ATP 5 and HEPES 10 (pH 7.2 with KOH). To measure the rate of onset of block and recovery from the block in response to drug challenge, the BK_{Ca} current was elicited with a test potential to +60 mV (500 ms duration) from a holding potential of –60 mV and stimulated at 0.0333 Hz.

Confocal microscopy

To estimate ROS levels, myocytes were incubated with mitochondrial H_2O_2-sensitive fluorescent probe: Reduced MitoTracker Red (Philipp *et al.*, 2006). Myocytes were incubated (37°C) with Reduced MitoTracker Red [5 μM in 0.05% dimethyl sulphoxide (DMSO)] for 1 h. After washing, the myocyte was imaged at 15 s intervals under a confocal microscope with a 60× objective (numerical aperture 1.45) (Eclipse CL Plus, Nikon, Japan) and the fluorescence emission (579–599 nm) from MitoTracker Red was acquired. Images were analysed using EZ-C1 3.5 programme (Nikon, Japan).

Chemiluminescence measurement of H_2O_2

No H_2O_2 was detected (with or without 5-HT) using one basilar artery when determined by chemiluminescence (Beckman LS-6000, Brea, CA, USA) (Gao *et al*., 2009). Twenty basilar arteries (cut longitudinally and endothelium denuded) of SHR and WKY were pooled together. Scintillation counting was performed 4–5 times after adding the basilar arteries (20 rats each group) to obtain a stable reading (baseline) before adding 5-HT (1 μM). Data were expressed as counts per minute (cpm) of 20 isolated basilar arteries (length: 8 mm each)·h^{-1}.

Western blots analysis

Basilar arteries were homogenized in the presence of protease inhibitors to obtain extracts of proteins. Different selective antibodies: anti-MAO-A (1:1000), anti-MAO-B (1:1000), anti-5-HT transporter (5-HTT; 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse HRP-conjugated IgG, 1:1000 and anti-rabbit HRP-conjugated IgG, 1:1000 (Bio-Rad Laboratories, Hercules, CA, USA) were used to detect the presence of protein of interest. The protein expression of MAO-A (61 kDa), MAO-B (60 kDa) and 5-HTT (70 kDa) was detected by Western blot analysis to generate chemiluminescent signals in the presence of the ImmueStar Reagent (Bio-Rad Laboratories). Intensity of individual protein (MAO-A, MAO-B and 5-HTT) bands was measured and quantified (at the corresponding molecular weight of each protein) using the Scion Image analysis programme (Scion Image Ltd., Frederick, MD, USA).

Statistical analysis

Data are expressed as mean ± SEM; *n* refers to number of basilar arterial ring preparations used in each experiment. Concentration of 5-HT causing 50% of the maximal contraction response (EC_{50}) observed was estimated using Prism (GraphPad Software, USA). Statistical comparisons were performed using one-way and two-way analysis of variance (ANOVA) or Student's *t*-test, where appropriate.

Materials

All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Citalopram hydrobromide and tomoxetine hydrochloride were obtained from Tocris Biosciences (Bristol, UK). All drugs were dissolved at the highest concentrations in either Nano-pure water [5-HT, 5-hydroxytryptophol (5-HTOL), 5-hydroxyindole-3-acetic acid (5-HIAA), clorgyline, pargyline, citalopram, tomoxetine, polyethylene glycol (PEG)-catalase, polyethylene glycol-superoxide dismutase (PEG-SOD), iberiotoxin, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and reduced glutathione (GSH)] or DMSO (indomethacin, apocynin and allopurinol), and diluted directly in external/internal recording solutions (in electrophysiological studies) and physiological salt solution (tension change measurements). Reduced MitoTracker Red was purchased from Invitrogen (Carlsbad, CA, USA), and iberiotoxin was obtained from Alomone Laboratories. (Jerusalem, Israel).

Results

Isometric tension development

5-HT elicited a concentration-dependent tension development of basilar arteries of normotensive WKY and SHR with similar maximum tension (~10 μM) (Figure 1A), with a significant leftward shift of the concentration–response curve for 5-HT (EC_{50}: WKY, 98.2 ± 9.4 nM; SHR, 28.4 ± 4.1 nM) ($P < 0.01$) in SHR when compared with that of WKY (Figure 1A). 5-HIAA and 5-HTOL (<30 μM) did not alter the tension of arterial rings from either strain of rat (Figure 1A).

Inhibition of MAO, 5-HTT and catecholamine uptake

Clorgyline (1 μM, a MAO-A inhibitor) did not alter the concentration–response curve of 5-HT [EC_{50}: 104.8 ± 6.7 nM (with clorgyline) vs. 98.2 ± 9.4 nM (control) ($P > 0.05$)] of WKY rats (Figure 1B). Interestingly, clorgyline caused a significant rightward shift (with no change in maximum contraction) of the concentration–response curve for 5-HT of basilar arterial rings from SHR (EC_{50}: 92.3 ± 5.5 nM (with clorgyline) vs. 28.4 ± 4.1 nM (control) ($P < 0.01$)] (Figure 1C), and the curve (with clorgyline) overlapped with that observed in WKY rats (control) (Figure 1C). Pargyline (10 μM, a MAO-B inhibitor) did not modify the 5-HT-induced tension development in WKY rats [EC_{50}: 96.1 ± 7.0 nM (with pargyline) vs. 98.2 ± 9.4 nM (control) ($P > 0.05$)] and SHR [EC_{50}: 33.5 ± 5.3 nM (with pargyline) vs. 28.4 ± 4.1 nM (control) ($P > 0.05$)]. Citalopram (0.1 μM, a potent 5-HTT inhibitor) attenuated 5-HT-induced tension development (a rightward shift of the curve with no change in maximum tension) of SHR [EC_{50}: 93.7 ± 10.3 nM (with citalopram) vs. 28.4 ± 4.1 nM

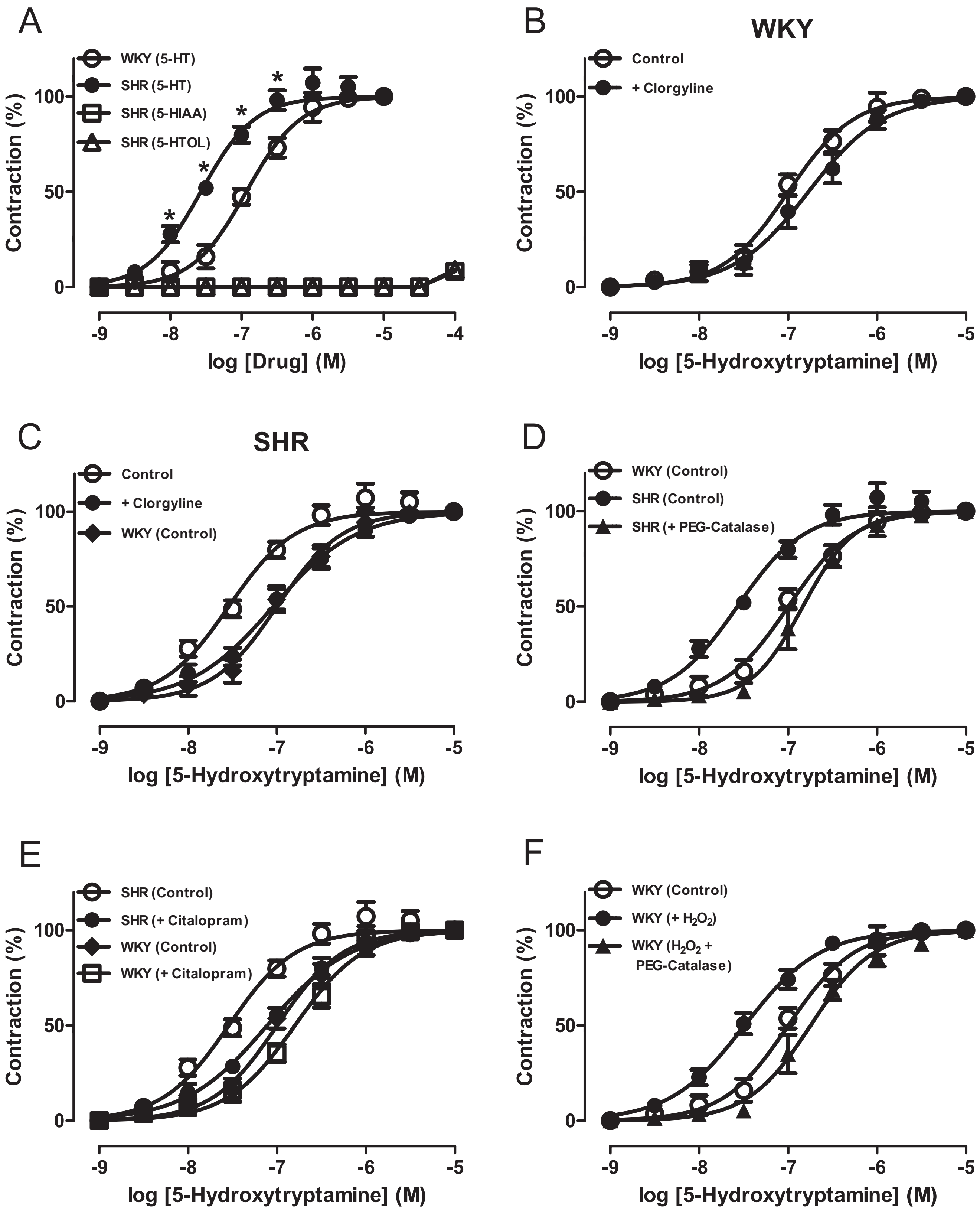

Figure 1

Concentration–response curves for the *in vitro* effects of 5-hydroxytryptamine (5-HT)-induced tension development of isolated basilar artery (endothelium-denuded) of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats in the absence or the presence of different agents/treatments. Results are expressed as mean ± SEM (n = 6–8). 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HTOL, 5-hydroxytryptophol; PEG-catalase, polyethylene glycol-catalase.

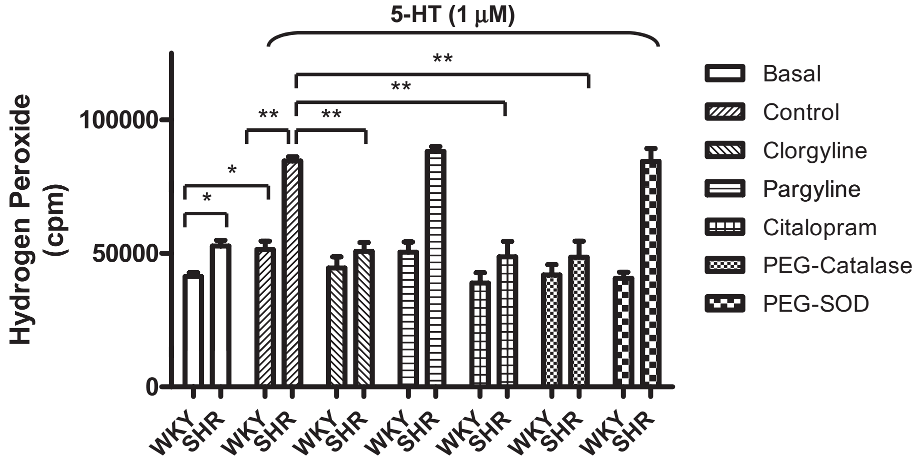

Figure 2

Assay, by chemiluminescence, of H_2O_2 generation (shown as cpm) in isolated basilar arteries (endothelium denuded) in response to 5-HT (1 μM) in the absence or presence of different agents (clorgyline, pargyline, citalopram, PEG-catalase and PEG-SOD). Results are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$. 5-HT, 5-hydroxytryptamine; cpm, counts per minute; PEG-catalase, polyethylene glycol-catalase; PEG-SOD, polyethylene glycol-superoxide dismutase; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

(control) ($P < 0.01$)] whereas a trend of rightward shift in WKY rats was observed [EC_{50}: 110.5 ± 8.8 nM (with citalopram) vs. 98.2 ± 9.4 nM (control) ($P > 0.05$)] (Figure 1E). Tomoxetine (10 nM, a potent, selective noradrenaline re-uptake inhibitor) did not modify 5-HT-induced tension development of WKY rats [EC_{50}: 103.7 ± 5.9 nM (with tomoxetine) vs. 98.2 ± 9.4 nM (control) ($P > 0.05$)] and SHR [EC_{50}: 33.8 ± 9.2 nM (with tomoxetine) vs. 28.4 ± 4.1 nM (control) ($P > 0.05$)].

Effects of PEG-catalase, H_2O_2 and PEG-superoxide dismutase

In WKY, PEG-catalase (100 U mL^{-1}, a cell-permeable enzyme that catalyses conversion of H_2O_2 to H_2O and O_2) did not modify 5-HT-induced tension development [EC_{50}: 103.4 ± 6.2 nM (with PEG-catalase) vs. 98.2 ± 9.4 nM (control) ($P > 0.05$)]. In SHR, the enhanced 5-HT-induced tension development was normalized by PEG-catalase (100 U·mL^{-1}) [EC_{50}: 101.9 ± 9.0 nM (with PEG-catalase) vs. 28.4 ± 4.1 nM (control) ($P < 0.01$)] (Figure 1F). In WKY, H_2O_2 (100 μM, 30 min) enhanced (PEG-catalase-sensitive) the 5-HT-induced tension development [EC_{50}: 25.7 ± 10.0 nM (with H_2O_2); 92.3 ± 7.7 nM (H_2O_2 plus PEG-catalase); 98.2 ± 9.4 nM (control)] which was similar to that observed in SHR. PEG-SOD (a cell-permeable enzyme that catalyses the dismutation of superoxide into O_2 and H_2O_2) (30 U·mL^{-1}) did not modify 5-HT-elicited tension development of both strains of rat [EC_{50}: WKY, 102.4 ± 7.3 nM (with PEG-SOD) vs. 98.2 ± 9.4 nM (control) ($P > 0.05$); SHR, 32.6 ± 5.6 nM (with PEG-SOD) vs. 28.4 ± 4.1 nM (control) ($P > 0.05$) Figure 1D].

Estimation of H_2O_2 generation

A higher basal level of H_2O_2 was detected in basilar arteries (a pool of 20 arteries) of SHR as compared with that of WKY rats. 5-HT (1 μM) elicited a stronger increase in H_2O_2 in SHR compared with WKY rats. Clorgyline (1 μM), PEG-catalase (100 U·mL^{-1}) and citalopram (0.1 μM), but not pargyline (10 μM) and PEG-SOD (30 U·mL^{-1}), abolished the 5-HT-induced H_2O_2 generation (Figure 2).

Protein expression of MAO-A, MAO-B and 5-HTT

The protein level of MAO-A in SHR was ~50% higher than that of WKY rats ($P < 0.01$) (Figure 3). However, the level of MAO-B and 5-HTT in WKY and SHR were not different (data not shown).

5-HT on BK_{Ca} channel gating

In single myocytes isolated from basilar arteries, the iberiotoxin-sensitive BK_{Ca} current amplitude (measured at +60 mV) in SHR was greater (approximately threefold) than that of WKY (Figure 4) [cell capacitance: 15.2 ± 1.1 pF (SHR) vs. 16.0 ± 0.9 pF (WKY)] ($P > 0.05$). 5-HT (1 μM) markedly suppressed the BK_{Ca} amplitude in SHR (54 ± 6% inhibition) whereas only a relatively small inhibition (12 ± 2%) was observed in WKY (Figure 4). The inhibitory effects of 5-HT on BK_{Ca} amplitude could not be reversed after washing (Figure 4). PEG-catalase (100 U·mL^{-1}), clorgyline (1 μM), citalopram (0.1 μM) and GSH (a physiological reductant) (5 mM, included in pipette solution) prevented the inhibitory effects of 5-HT on BK_{Ca} channels (Figure 4). Exogenous H_2O_2 (100 μM) (concentration

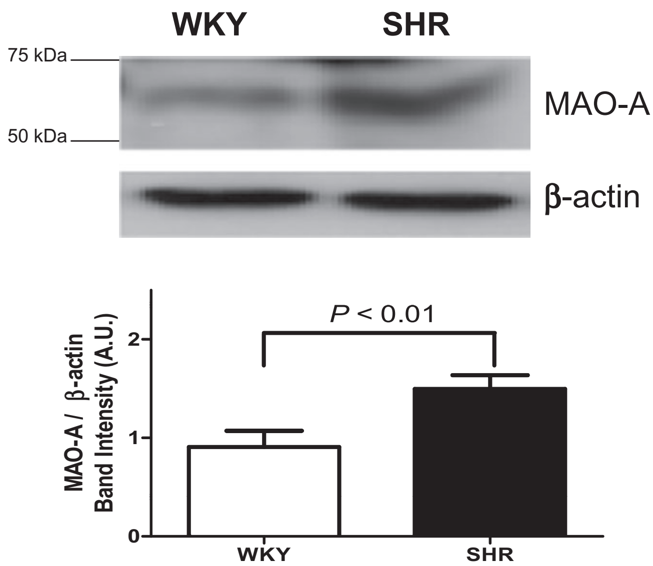

Figure 3
Western immunoblots analysis revealed monoamine oxidase-A (MAO-A, 61 kDa) expression of basilar artery (endothelium-denuded) of spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats. β-Actin was measured as a loading control. Results are normalized to β-actin expression and are expressed as mean (arbitrary units, AU) ± SEM of four independent experiments ($P < 0.01$, SHR vs. WKY).

at which it modified tension development of basilar artery and/or BK_{Ca} channels gatings) (Yang *et al.*, 1999; Dong *et al.*, 2008) inhibited BK_{Ca} amplitude of SHR (62 ± 8% inhibition; $n = 7$) and WKY (15 ± 6% inhibition; $n = 6$) with no recovery after washout (Figure 4). The rate of onset of the steady-state inhibition of BK_{Ca} amplitude by exogenous H_2O_2 (~8 min) was faster than that observed with 5-HT (~15 min) (Figure 4). 5-HIAA and 5-HTOL did not have significant effects on BK_{Ca} amplitude (data not shown).

Effects of 5-HT on mitochondrial ROS generation

5-HT (1 μM) caused an increase in mitochondrial ROS generation at ~5 min after 5-HT administration, and the peak amplitude of signal occurred at ~15 min. A relatively greater magnitude was detected in SHR when compared with that of WKY (Figure 5). ROS generation was inhibited by clorgyline (1 μM) and citalopram (0.1 μM) but not by pargyline (10 μM) (Figure 5). Indomethacin [10 μM, a cyclooxygenase (COX) inhibitor], L-NAME (100 μM, a nitric oxide synthase (NOS) inhibitor), allopurinol (10 μM, a xanthine oxidase inhibitor) and apocynin [100 μM, an inhibitor of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases] did not alter the 5-HT-elicited ROS generation (data not shown).

Discussion

Consistent with previous studies (Yokota *et al.*, 1994; Nishimura and Suzuki, 1995; Salomone *et al.*, 1997; Budzyn *et al.*, 2008), 5-HT-induced contraction of isolated basilar artery (endothelium-denuded) was greater in SHR than that in normotensive WKY rats. In isolated aorta (Budzyn *et al.*, 2008), the enhanced 5-HT-elicited contraction in SHR was due to an increase in vascular superoxide (O_2^-) and the destruction of NO. In our study, the exaggerated contraction observed in SHR could not be related to endothelium/NO as the endothelium was mechanically removed.

Exogenous H_2O_2 enhanced 5-HT-induced contractions (PEG-catalase-sensitive/PEG-SOD-insensitive) in basilar arterial rings from WKY rats, which overlapped with those observed in SHR. In SHR, PEG-catalase reversed the exaggerated 5-HT-induced contraction. Our results therefore suggested that the exaggerated contraction in SHR was mediated by H_2O_2 (and probably not O_2^-).

Arterial smooth muscle cells can take up 5-HT via the 5-HT transporter (5-HTT), and MAO is able to metabolize intracellular 5-HT (Small *et al.*, 1977; Brust *et al.*, 2000). In DOCA-salt and LNNA-induced hypertensive rats (Ni *et al.*, 2006), and in rats with pulmonary hypertension (Eddahibi *et al.*, 2001a,b), up-regulation of 5-HTT expression/function was observed. Similar to findings in the aorta of SHR and WKY rats (Ni *et al.*, 2006), our results failed to reveal a difference in the 5-HTT expression in basilar arteries between SHR and WKY rats. Citalopram (a potent 5-HTT blocker), but not tomoxetine (a potent noradrenaline uptake inhibitor), abolished the 'enhanced component' of 5-HT-elicited contraction in SHR suggesting that the uptake of 5-HT via 5-HTT plays an essential role in mediating the exaggerated contraction.

Both isoforms of MAO, MAO-A and MAO-B, have been demonstrated in cerebral microvessels in humans (Kalaria and Harik, 1987; Youdim and Finberg, 1991). As reported earlier, an augmented expression of MAO-A, but not of MAO-B, was detected in cerebral arteries of SHR (Lai and Spector, 1978; Guffroy and Strolin Benedetti, 1984). Given the fact that 5-HT is metabolized by MAOs, and H_2O_2 is generated (Sagin *et al.*, 2004), it is tempting to suggest that the enhanced MAO-A expression increased 5-HT metabolism and a higher H_2O_2 level was generated in SHR. Clorgyline (a MAO-A inhibitor) (Ulus *et al.*, 2000) [but not pargyline (a MAO-B

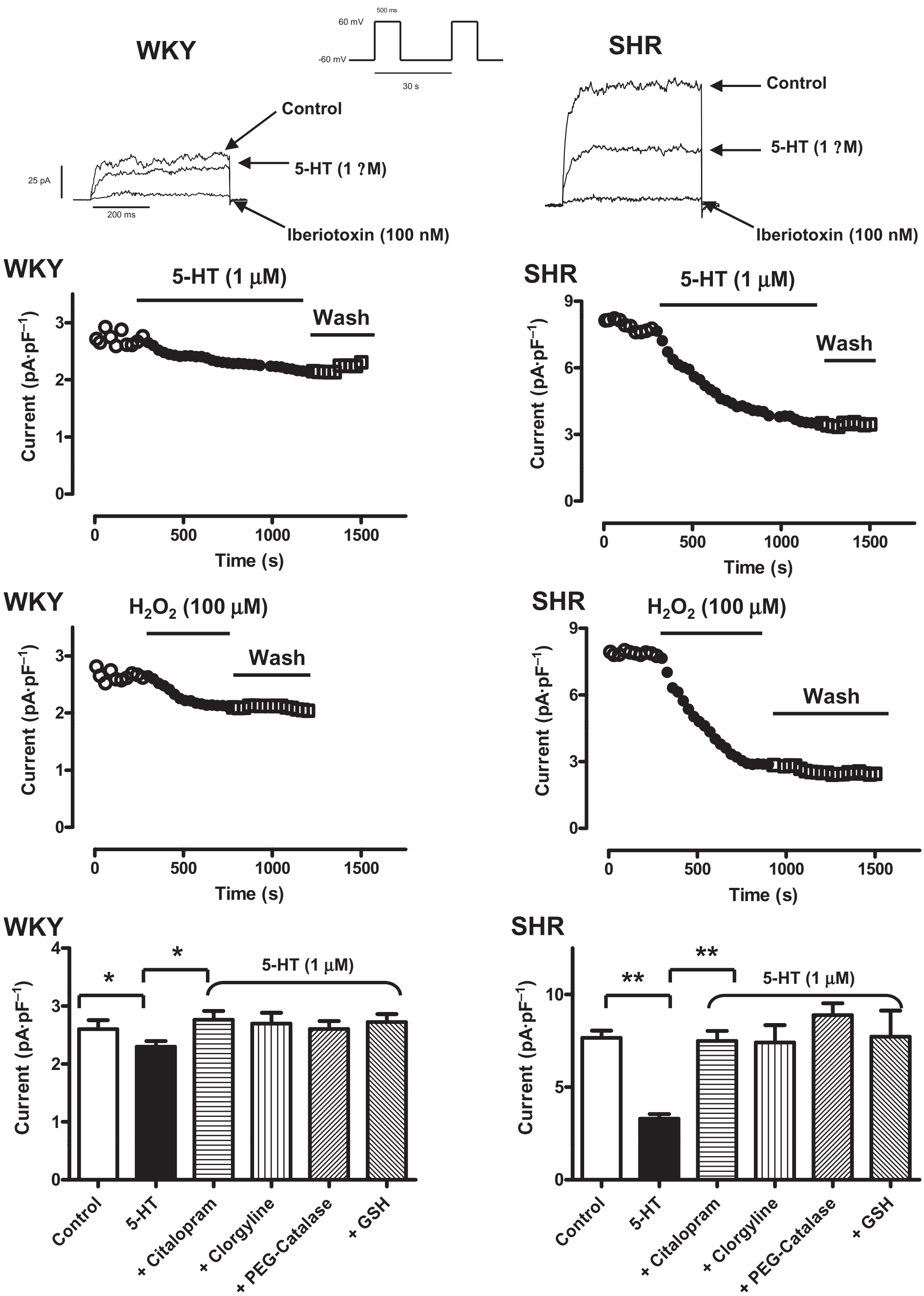

Figure 4

Time course of the inhibitory effects of 5-HT (1 μM, top panel) and H_2O_2 (100 μM, middle panel) on iberiotoxin-sensitive, large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) amplitude of single basilar artery myocytes of SHR and WKY rats. The BK_{Ca} current was elicited using a train-pulse protocol with a test potential of +60 mV (500 ms pulse duration) from a holding potential of −60 mV at 0.03 Hz. (Bottom panel): summary of the macroscopic BK_{Ca} current amplitude recorded [peak BK_{Ca} current ($pA{\cdot}pF^{-1}$) recorded at +60 mV from a holding potential of −60 mV for 500 ms duration at 0.0333 Hz] in response to 5-HT (1 μM) challenge in the absence or presence of different agents [citalopram, clorgyline, PEG-catalase and reduced glutathione (GSH)]. Mean ± SEM are indicated by columns and vertical bars respectively (*P < 0.05 and **P < 0.01). 5-HT, 5-hydroxytryptamine; PEG-catalase, polyethylene glycol-catalase; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

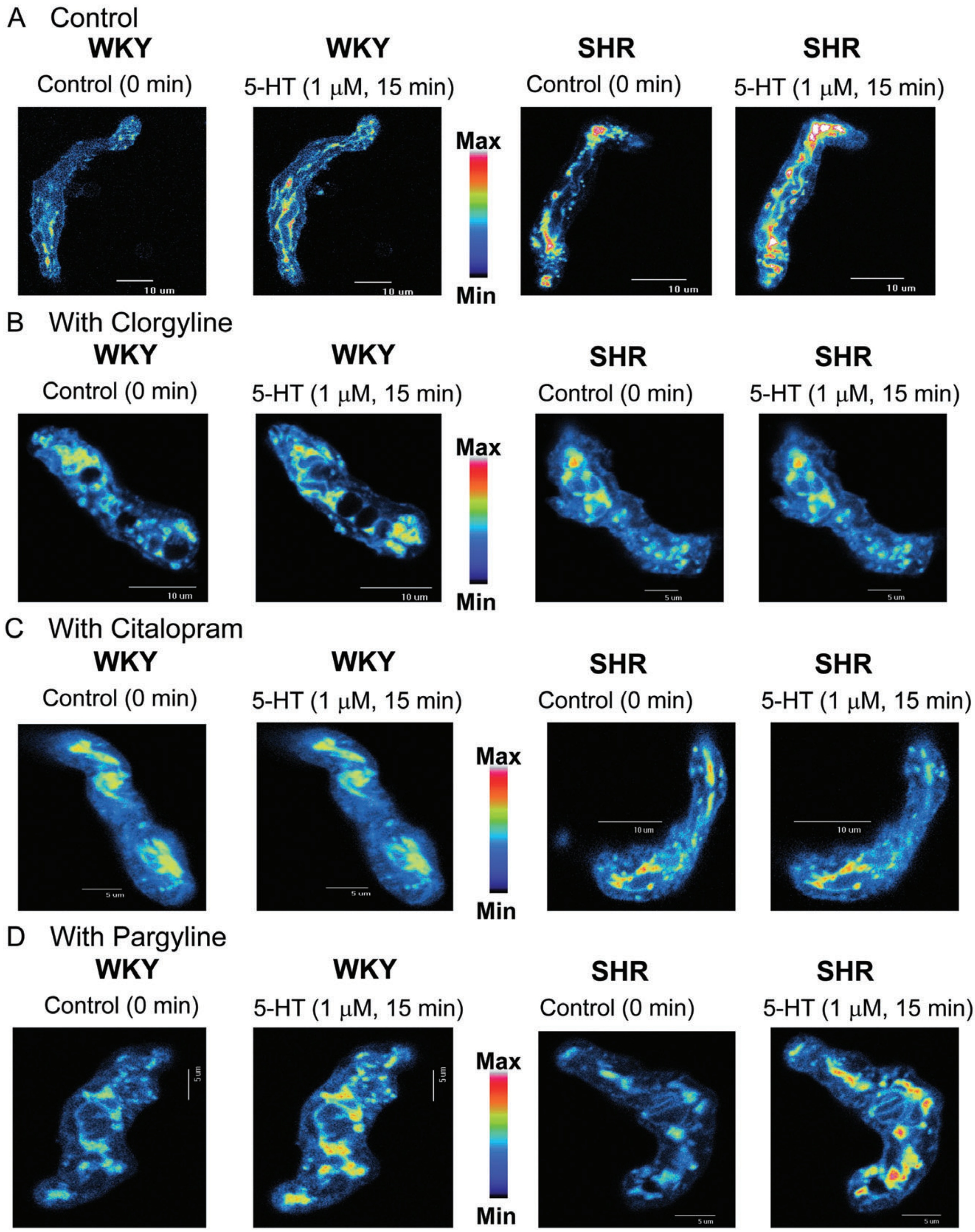

Figure 5

Effects of 5-HT (1 μM) on mitochondrial H_2O_2 generation estimated using reduced MitoTracker Red. Insets are representative images of single basilar artery myocytes of SHR and WKY rats (at least 20 cells in each condition) in response to 5-HT in the absence (control) (A) or the presence of clorgyline (1 μM) (B), citalopram (0.1 μM) (C) and pargyline (10 μM) (D). 5-HT, 5-hydroxytryptamine; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

inhibitor) (Nishimura, 1996), indomethacin, L-NAME, allopurinol and apocynin] reversed the enhanced contraction in SHR. These results therefore confirm the obligatory role of MAO-A in the enhanced contraction mediated by 5-HT in SHR.

Inside the cell, 5-HT is metabolized by MAO and, in addition to H_2O_2, 5-HIAA and 5-HTOL are formed (Sagin *et al.*, 2004). Consistent with a previous study performed on isolated mesenteric artery of DOCA-induced hypertensive rats (Thompson and Webb, 1987), neither 5-HIAA nor 5-HTOL ($\leq$30 μM) altered the basal tension development of the basilar artery of both strains of rat, arguing against the participation of 5-HT metabolites (i.e. 5-HIAA and 5-HTOL) in mediating the vascular effects of 5-HT observed.

In the cerebral circulation and other vascular beds, H_2O_2 is generally believed to be a vasodilator (Faraci, 2006; Modrick *et al.*, 2009). However, in canine basilar artery smooth muscle cells (Yang *et al.*, 1999), H_2O_2 caused an increase in $[Ca^{2+}]_i$ plus contraction of basilar artery myocytes whereas relaxation of pig coronary artery smooth muscle cells was observed (Hayabuchi *et al.*, 1998). Mitochondrial-derived H_2O_2 inhibits relaxation of bovine coronary arterial smooth muscle in response to hypoxia (Gao *et al.*, 2009). Using a specific fluorescent dye (reduced MitoTracker Red that is oxidized to a fluorescent form after exposure to ROS) (Oldenburg *et al.*, 2003) for monitoring mitochondrial H_2O_2 generation, we demonstrated, for the first time, that H_2O_2 was indeed generated (in a PEG-catalase-/citalopram-sensitive and PEG-superoxide dismutase-insensitive fashion) upon the addition of 5-HT, with a relatively greater magnitude of H_2O_2 generation detected in SHR. Generation of H_2O_2 in isolated basilar artery of both strains of rat was confirmed using chemiluminescence methods that further strengthens our conclusions on the participation of mitochondrial H_2O_2 in mediating the exaggerated vascular contractile effects of 5-HT observed in SHR. In addition, the generation of H_2O_2 by 5-HT was abolished by clorgyline strongly supporting an obligatory role of MAO-A in mitochondrial H_2O_2 generation after 5-HT challenge.

In K_{Ca} channels, a large number of cysteine residues are located within an extensive, presumably cytoplasmic, domain unique to these channels making them likely to be affected by redox modulation (Zhang *et al.*, 2006). In addition, several channel properties are changed when K_{Ca} channels are moved from the relatively reduced cellular environment to the more oxidized environments (e.g. ROS/oxidative stress). So far, our results demonstrate the generation of H_2O_2 during 5-HT metabolism via mitochondrial MAO-A. We therefore decided to assess the modulatory role(s) of H_2O_2 thus generated on BK_{Ca} channel gating. As previously reported (Liu *et al.*, 1998), the basal BK_{Ca} amplitude recorded in myocytes from SHR basilar arteries, was greater (approximately threefold) compared with that in myocytes from WKY. 5-HT and H_2O_2 elicited a greater magnitude of inhibition of BK_{Ca} amplitude in basilar artery myocytes of SHR than that observed in WKY. In addition, the rate of onset of steady-state maximum inhibition of BK_{Ca} channel caused by exogenous H_2O_2 (added into the external recording solution) was faster (~8 min) than that observed with 5-HT (~15 min). It is probably related to the time required for the generation of H_2O_2 via MAO-A (upon 5-HT addition) whereas exogenous applied H_2O_2 (it is freely diffusible) has 'direct/immediate effects' on BK_{Ca} channel gatings. A greater amplitude of basal BK_{Ca} currents in hypertensive states is thought to serve as a physiological brake in controlling blood pressure from rising too high (Liu *et al.*, 1998; Kamouchi *et al.*, 2002). Inhibition of the 'enhanced' BK_{Ca} channel gating, as by TEA and iberiotoxin, thus resulted in a greater magnitude of vascular contraction. Indeed, our results consistently demonstrate that 5-HT (1 μM) caused a greater degree of inhibition of BK_{Ca} amplitude of single basilar artery myocytes of SHR compared with those from WKY rats. It is probably correlated with a higher sensitivity (i.e. a lower EC_{50} value) of isolated basilar artery of SHR compared with WKY rats in response to 5-HT. In addition, modulation of 5-HT-induced inhibition of BK_{Ca} channels by GSH (a physiological reductant, delivered into the cytosol via the patch pipette) clearly illustrated that oxidant-mediated changes are responsible for 5-HT-elicited inhibition of BK_{Ca} amplitude. A persistent inhibition of BK_{Ca} channel gating by 5-HT was observed after extensive washout and may suggest that there was a long-lasting/permanent change of amino acids residues (e.g. methionine, tryptophan and cysteine) (Soto *et al.*, 2002) of BK_{Ca} channels by the H_2O_2 generated. Hence, our results clearly illustrated that there is a greater amount of mitochondrial H_2O_2 release upon 5-HT challenge in basilar artery myocytes of SHR, which is associated with a greater protein expression of MAO-A (and thus more 5-HT is metabolized). Interestingly, many MAO inhibitors have anti-hypertensive properties (MacCauley, 1980) that may be related to the inhibition of ROS formation as ROS generated inhibits vascular K^+ channels (as demonstrated in our study). It is important to point out that O_2^- stimulated K_{Ca} channels gating of rat basilar artery, leading to relaxation of the artery (Conde *et al.*, 1999) whereas in our study, inhibition of BK_{Ca} channels by 5-HT/H_2O_2 was consistently observed. In porcine renal artery myocytes (Brakemeier *et al.*, 2003), exogenous H_2O_2

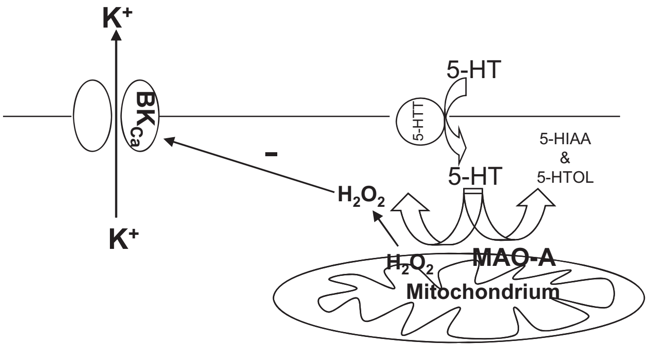

Figure 6

Proposed mechanisms underlying the exaggerated 5-HT-induced basilar artery contraction of spontaneously hypertensive rats (SHR). –, inhibition; 5-HT, 5-hydroxytryptamine; 5-HTT, 5-hydroxytryptamine transporter; 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HTOL, 5-hydroxytryptophol; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channels; H_2O_2, hydrogen peroxide; K^+, potassium ions; MAO-A, monoamine oxidase-A.

inhibited BK_{Ca} channels whereas in porcine coronary artery myocytes (Thengchaisri and Kuo, 2003) H_2O_2 activated BK_{Ca} channels. In our study, effects of 5-HT were not modified by PEG-SOD, and exogenously added H_2O_2 enhanced 5-HT-evoked contraction of basilar artery from WKY rats. Collectively, our results strongly suggest that H_2O_2 (but not O_2^-) is probably the ROS generated (via MAO-A) upon the addition of 5-HT to basilar artery myocytes.

In conclusion, we have provided convincing evidence that an enhanced MAO-A protein expression in the cerebral arteries is closely associated with/responsible for the exaggerated *in vitro* tension development, induced by 5-HT, of the isolated basilar artery of SHR. More importantly, in isolated cerebral artery myocytes from SHR and WKY rats, we have demonstrated, for the first time, an association between the generation of mitochondrial H_2O_2 (via metabolism of 5-HT by mitochondrial MAO-A) and the inhibition of BK_{Ca} channel gating caused by 5-HT and the H_2O_2 released (Figure 6).

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Conflicts of interest

None.

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