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# Altered Endothelin Receptor Expression and Affinity in Spontaneously Hypertensive Rat Cerebral and Coronary Arteries

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#### Abstract

**Background:** Hypertension is associated with arterial hyperreactivity, and endothelin (ET) receptors are involved in vascular pathogenesis. The present study was performed to examine the hypothesis that ET receptors were altered in cerebral and coronary arteries of spontaneously hypertensive rats (SHR).

**Methodology/Principal Findings:** Cerebral and coronary arteries were removed from SHR. Vascular contraction was recorded using a sensitive myograph system. Real-time PCR and Western blotting were used to quantify mRNA and protein expression of receptors and essential MAPK pathway molecules. The results demonstrated that both  $ET_A$  and  $ET_B$  receptor-mediated contractile responses in SHR cerebral arteries were shifted to the left in a nonparallel manner with increased maximum contraction compared with Wistar-Kyoto (WKY) rats. In SHR coronary arteries, the  $ET_A$  receptor-mediated contraction curve was shifted to the left in parallel with an increased pEC<sub>50</sub> compared with the arteries in WKY rats. There was no significant increase in  $ET_B$  receptor-mediated contraction in SHR coronary arteries.  $ET_A$  receptor mRNA and protein expression was increased in SHR cerebral arteries compared with the arteries in WKY rats. However,  $ET_A$  receptor mRNA and protein levels in coronary arteries and  $ET_B$  receptor protein levels in cerebral and coronary arteries remained unchanged in SHR compared with WKY rats. Meanwhile, phosphorylated ERK1/2 protein was significantly increased in SHR brain and heart vessels.

**Conclusions/Significance:** In SHR cerebral arteries,  $ET_A$  receptor expression was upregulated.  $ET_A$  receptor affinity was increased in coronary arteries, and  $ET_B$  receptor affinity was increased in cerebral arteries. The ERK1/2 activation may be involved in the receptor alterations.

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#### Introduction

Vascular smooth muscle cell receptors mediate vasoconstriction and vasodilatation, which are of key importance in vascular resistance and blood pressure (BP) regulation. Receptor metabolism updates constantly and is kept in a dynamic balance. Receptor regulation is an important factor for maintaining organismal homeostasis by changing receptor density and/or affinity and target cell sensitivity [1]. Numerous studies have demonstrated that receptor regulation plays a prominent role among adaptive changes in the cardiovascular system under pathological conditions [2]. Therefore, altered receptor expression is important for

regulating physiological function, which may contribute to diverse pathological processes and could be involved in vascular pathologies such as hypertension.

Hypertension is a major risk factor for many cardiovascular events [3]. There is a clear correlation between increased BP and cardiovascular risk because increased BP increases the risk of stroke, myocardial infarct and heart failure [4]. Pathological arteriole processes are the most significant features of hypertension. The endothelin (ET) system consisting of ligands and their receptors is an important modulator of vascular tone and blood pressure, which play an essential role in cardiovascular pathogenesis [5]. Endothelin-1 (ET-1) is a potent vasoconstrictor that is formed in endothelial cells and participates in vascular regulation such as controlling basal arterial tone, altering vessel diameter and modifying blood flow [6]. ET-1 induces its vasoactive response through two distinct transmembrane receptors: endothelin type A  $(ET_A)$ and endothelin type B ( $ET_B$ ) [7,8]. The  $ET_A$  receptor mediates vasoconstriction and vascular smooth muscle cell proliferation, whereas the  $ET_{\scriptscriptstyle B}$  receptor is involved in endothelial cell survival, ET reuptake, clearance, nitric oxide, and prostacyclin release. The ET<sub>A</sub> receptor is expressed in the vascular smooth muscle of most blood vessels [9-11]. While the ET<sub>B</sub> receptor predominates in endothelial cells, it is also present in the vascular smooth muscle of some vascular beds [12,13]. The prominent and long-acting vasoconstrictor effects of ET-1 may play a role in blood pressure regulation and hypertension pathophysiology [14]. Upregulation of the ET system is more commonly observed in severe hypertension and is associated with coronary artery disease, heart failure, and atherosclerosis. ET-1 plasma levels increase in some hypertensive patients. ET-1 mRNA expression is increased in the endothelium of subcutaneous resistance arteries from patients with moderate to severe hypertension [13].

Variability in the ET system in cardiovascular pathogenesis may not only involve ET-1 levels, but could also involve the amount of vascular ET receptors. Increased ET<sub>4</sub>/ET<sub>B</sub> receptor expression has been reported in blood vessels of cardiovascular disease patients [15-17]. Vascular ET receptor numbers could vary for different forms of hypertension and in various tissues isolated from subjects with the same form of hypertension. In a previous study, we reported that ET<sub>B</sub> and ET<sub>4</sub> receptor-mediated contractions were increased in the mesenteric artery of spontaneously hypertensive rats (SHR) and the colon marginal artery of hypertension patients [8]. Studies have demonstrated that ET-1-induced contraction is increased in rat heart coronaries during ischemia/reperfusion [18]. ET<sub>B</sub> receptors are upregulated in pulmonary hypertension [19]. ET receptors could also be downregulated when large amounts of ET are produced in the vasculature [9]. In addition, risk factors for vascular diseases, such as low-density lipoprotein and smoking, induce receptor upregulation in different vessels [20,21]. ET receptor antagonists reduce BP to a variable extent in hypertension [22], supporting the notion that the ET system is upregulated in hypertension. Therefore, we hypothesized that arterial ET receptor alterations may occur in hypertension.

In the present study, we focused on two key regions, the cerebral and coronary arteries. In SHR, we examined whether basal ET receptor levels were altered in cerebral and coronary arteries. Receptor-mediated vascular contractile responses together with receptor mRNA and protein expression levels were studied. We also addressed the possible mechanism behind the receptor modification by assessing the phosphorylation of the three main mitogen-activated protein kinase (MAPK) signaling pathways: extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK) and p38.

### **Materials and Methods**

#### Animals

In total, 20 male SHR and 24 Wistar-Kyoto (WKY) rats weighing 250-300 g (20 weeks) were obtained from Slac Laboratory Animal Co., Ltd. (Shanghai, China). The rats were acclimated for one week under standardized temperature (21-22°C) and humidity (50-60%) with free access to food and water before the experiment. All experimental protocols were approved by the Xi'an Jiaotong University Animal Ethics Committee.

#### **BP Measurement**

Arterial pressure was measured in SHR and WKY *via* a noninvasive tail-cuff plethysmography method [23] and monitored with the CODA 6 Non-Invasive Blood Pressure System (Kent Scientific, Torrington, CT, USA) before euthanasia. Systolic and diastolic tail arterial BP were 218  $\pm$  15/173 $\pm$ 9 mm Hg in SHR and 110  $\pm$  5/70 $\pm$ 4 mm Hg in the WKY rats. The recorded BP was an average of three readings.

#### **Artery Collection**

Rats were anesthetized with  $CO_2$  and decapitated. The brains and hearts were immediately removed and immersed in an ice-cold bicarbonate buffer solution [24]. The basilar artery, middle cerebral artery and circle of Willis artery were carefully removed from the brain. The left anterior descending (LAD) coronary artery, left circumflex artery and right coronary artery were isolated from the heart. Some basilar arteries and LAD coronary arteries were cut into cylindrical segments (1-2 mm in length) for *in vitro* pharmacology studies. Several of the remaining basilar arteries and LAD coronary arteries were frozen with other isolated arteries at -80°C for real-time PCR and Western blotting.

#### **Vascular Ring Myograph Studies**

Sensitive myographs (Danish Myo Technology A/S, Aarhus, Denmark) were used for recording isometric tension of isolated arteries [25]. The vessels were cut into cylindrical segments, threaded onto two 40 µm diameter stainless steel wires and mounted in the myograph chamber. One wire was connected to a force displacement transducer that was attached to a digital converter unit. Another wire was connected to a micrometer screw, which allowed for fine vascular tone adjustments by varying the distance between the wires. Measurements were recorded in a computer using a PowerLab Unit (ADInstruments, Oxford, UK). The segments were immersed in a temperature-controlled buffer solution (37°C), which was continuously equilibrated with a 5% CO<sub>2</sub> in O<sub>2</sub> gas mixture resulting in a stable pH of 7.4. The vessels were given an initial tension of 1-1.2 mN and were adjusted to this level of tension for at least 1 h. Potassium-rich (60 mM) buffer solution was used to determine segment contractile function as a contractile capacity reference.

The concentration–response curves of vascular segment were obtained by cumulative  $ET_B$  receptor agonist sarafotoxin 6c (S6c, 10<sup>-11</sup>-10<sup>-7</sup> M, Merck, Darmstadt, Germany)

administration and both  $\text{ET}_{A}$  and  $\text{ET}_{B}$  receptor agonist ET-1 (10<sup>-11</sup>-10<sup>-7</sup> M, NeoMPS, Strasbourg, France) administration. To evaluate  $\text{ET}_{A}$  receptor-mediated contraction, a concentration-contractile response to S6c (10<sup>-11</sup>-10<sup>-7</sup> M) was performed in advance to achieve  $\text{ET}_{B}$  receptor desensitization [26]. When a maximal contraction ( $\text{E}_{max}$ ) induced by S6c was reached, the segments remained in contact with the highest S6c concentration for 30 additional min until the contractile curves faded to baseline level, which was considered to be total desensitization. Thus, ET-1 (10<sup>-11</sup>-10<sup>-7</sup> M) induced a concentration-effect curve, which was only mediated by ET<sub>A</sub> receptors [27].

#### **Real-time PCR**

Cerebral and coronary arteries were collected as described above. Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) following the supplier's instructions. RNA purity was assessed with an Eppendorf Biophotometer (Hamburg, Germany), and the wavelength/ absorption ratio (260/280 nm) ranged from 1.7 to 2.0 for all of the preparations. Reverse transcription of total RNA to cDNA were performed in 40 µl reaction volume using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) in a PerkinElmer 2400 PCR machine (PerkinElmer, Waltham, MA, USA). Real-time PCR was performed using the GeneAmp SYBR® Green kit (Applied Biosystems) in a GeneAmp 7300 sequence Detection System (Applied Biosystems). Details were previously described [28]. Specific primers for the receptors were designed as follows: ET<sub>A</sub> receptor forward: 5'-GTC GAG AGG TGG CAA AGA CC-3', reverse: 5'-ACA GGG CGA AGA TGA CAA CC-3' and ET<sub>B</sub> receptor forward: 5'-GAT ACG ACA ACT TCC GCT CCA-3', reverse: 5'-GTC CAC GAT GAG GAC AAT GAG-3'. Elongation factor-1 (EF-1) was used as an endogenous reference standard. EF-1 primers were forward: 5'-GCA AGC CCA TGT GTG TTG AA-3' and reverse: 5'-TGA TGA CAC CCA CAG CAA CTG -3'. The data were analyzed using the comparative cycle threshold (C<sub>T</sub>) method [29].

#### Western Blotting

Cerebral and coronary arteries were harvested as mentioned above. Vessel proteins were extracted as described previously [30]. Equal amounts of protein (40 µg) were loaded onto a 4-15% Ready Gel Precast Gel (Bio-Rad Laboratories, Hercules, CA, USA) for electrophoresis. A molecular weight marker was loaded for protein band identification. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked in 5% non-fat milk and incubated with primary antibodies at 4°C overnight. Primary antibodies were as follows: 1:200 rabbit anti-ET receptor (sc-33535, Santa Cruz Biotechnology, CA, USA), 1:500 rabbit anti-ET<sub>B</sub> receptor (ab65972, Abcam, Cambridge, UK), 1:2000 rabbit anti-phospho-ERK1/2 (#4370, Cell Signaling Technology, Beverly, MA), 1:1000 rabbit anti-phospho-SAPK/JNK (#4668, Cell Signaling Technology), 1:1000 rabbit anti-phospho-p38 (#4631, Cell Signaling Technology), 1:1000 mouse anti-β-actin (#4970, Cell Signaling Technology) or 1:2000 mouse anti-ERK1/2 (#4696, Cell Signaling Technology). Next, membranes were incubated with 1:2000 HRP-conjugated anti-rabbit (#7074, Cell Signaling Technology) or anti-mouse (#7076, Cell Signaling Technology) secondary antibodies for 1 h at room temperature. Finally, membranes were developed and visualized using a Fujifilm LAS-1000 Luminescent Image Analyzer (Fujifilm, Stamford, CT, USA). Band intensity was quantified using ImageJ software (<u>http://rsb.info.nih.gov/ij/</u>).

#### **Statistical Analysis**

All of the data are expressed as the mean  $\pm$  SEM, and *n* refers to the number of rats. Contractile responses to receptor agonists in each segment were expressed as a percentage of the contraction that was induced by 60 mM K<sup>+</sup>-rich buffer solution. Target gene mRNA levels were expressed in relation to EF-1 levels. Target protein expression was determined relative to  $\beta$ -actin levels or total ERK1/2 protein levels. Unpaired Student's *t*-test was used to compare two data sets. 2-way ANOVAs were used to compare two corresponding data points at each concentration on the two curves. The data and statistical analysis was calculated using Graph-Pad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). *P* < 0.05 was considered to be statistically significant.

#### Results

# ET Receptor-Mediated Contractile Responses in Cerebral and Coronary Arteries

Cerebral basilar arteries and LAD coronary arteries were examined separately. K<sup>+</sup>-induced basilar artery or coronary artery contractile responses in SHR did not differ significantly from the responses in WKY (Table 1). However, K<sup>+</sup>-induced contraction on basilar arteries was stronger than in coronary arteries from SHR. Because KCI is a receptor-independent agonist, receptor agonist-mediated contractions relative to KCIinduced contractions (as a percentage of the KCI-mediated contraction) provided an indication of agonist sensitivity. Therefore, the K<sup>+</sup>-elicited contraction was used as a contractile capacity reference in each specific vessel.

The selective  $ET_B$  receptor agonist S6c induced weak contractile responses in WKY basilar (Figure 1A) and coronary arteries (Figure 1C).  $E_{max}$  values were approximately 5% of K<sup>+</sup>-induced contractions. Figure 1 demonstrates that S6c induced a significantly stronger contractile response in SHR ( $E_{max}$  17.10 ± 5.10%) compared with WKY ( $E_{max}$  of 5.41±2.16%, *P*<0.05) in cerebral arteries. However, S6c-induced maximal contraction was not significantly increased in SHR coronary arteries compared with WKY (11.80 ±3.70% vs. 4.85±3.18%, *P*>0.05). Additionally, there was no statistical difference of pEC<sub>50</sub> values in the cerebral or coronary artery between WKY and SHR (Table 1).

ET-1, which activates both  $ET_B$  and  $ET_A$  receptors, was applied to activate only  $ET_A$  receptors following a total  $ET_B$  receptor desensitization. Cumulative ET-1 administration induced potent contractions in basilar (Figure 1B) and coronary (Figure 1D) arteries. Basilar artery contractile responses were stronger in SHR than in WKY (Figure 1B) with a significantly elevated  $E_{max}$  (150 ± 7.85% *vs.* 112 ± 3.67%, *P* < 0.01). In

**Table 1.**  $E_{max}$  and pEC<sub>50</sub> values of the basilar and coronary arterial segment concentration-contractile curves isolated from endothelin receptor agonist-induced spontaneously hypertensive rats.

Group (n)		S6c		ET-1	
		WKY (14)	SHR (10)	WKY (14)	SHR (10)
Basilar	K <sup>+</sup> (mN)	2.53±0.27	3.27±0.33	2.55±0.26	3.33±0.31
	E <sub>max</sub> (% of K <sup>+</sup> )	5.41±2.16	17.1±5.10 <sup>*</sup>	112±3.67	150±7.85 <sup>**</sup>
	pEC <sub>50</sub>	8.18±0.58	8.31±0.63	8.96±0.06	8.92±0.10
Coronary	K <sup>+</sup> (mN)	1.84±0.22	1.61±0.28	1.98±0.18	1.72±0.24
	$E_{max}$ (% of K <sup>+</sup> )	4.85±3.18	11.8±3.70	156±5.78	152±5.00
	pEC <sub>50</sub>	7.41±0.46	8.37±0.50	8.31±0.05	8.75±0.05**

Dates are expressed as the means $\pm$ SEM, and *n* refers to the rat number. S6c: Sarafotoxin 6c; ET-1: endothelin-1; E<sub>max</sub>: maximal contraction; pEC<sub>50</sub>: negative logarithm of the agonist concentration that produces 50% of E<sub>max</sub>. E<sub>max</sub> are expressed as a percentage of 60 mM K<sup>+</sup>-induced contraction. \**P*<0.05, \*\**P*<0.01 *vs*. WKY group.

contrast, the  $\text{ET}_{A}$  receptor-mediated coronary artery contractile response curve in SHR was shifted toward the left in parallel with an increased pEC<sub>50</sub> and an unchanged  $\text{E}_{max}$  (Figure 1D, Table 1).

# ET Receptor mRNA and Protein Expression in Cerebral and Coronary Arteries

Relative  $\text{ET}_{\text{B}}$  and  $\text{ET}_{\text{A}}$  receptor mRNA levels were quantified by real-time PCR.  $\text{ET}_{\text{A}}$  receptor mRNA levels were significantly increased (Figure 2A) in SHR basilar segments. It appeared that  $\text{ET}_{\text{B}}$  receptor mRNA levels tended to increase, but this was not statistically significant (Figure 2A, P > 0.05). Figure 2B demonstrates that in SHR coronary arteries,  $\text{ET}_{\text{A}}$  receptor mRNA was not altered compared with WKY. Although there was a trend toward increased  $\text{ET}_{\text{B}}$  receptor mRNA expression, there were no statistically significant differences between SHR and WKY (*P*<0.05, Figure 2B).

 $\mathsf{ET}_{\mathsf{B}}$  and  $\mathsf{ET}_{\mathsf{A}}$  receptor protein expression was examined by Western blotting.

The results from cerebral artery segments demonstrated that  $ET_A$  receptor expression was increased while  $ET_B$  receptor expression was unaltered in SHR compared with WKY (Figure 3A). There was no significant change in  $ET_B$  or  $ET_A$  receptor protein expression in SHR coronary arteries (Figure 3B).

#### MAPK Activities in Cerebral and Coronary Arteries

To investigate the underlying intracellular signal transduction mechanisms involved in the vascular receptor alteration that occurred in SHR, MAPK activities were examined by Western blotting. We analyzed total and phosphorylated (p) ERK1/2, JNK and p38 MAPK protein expression to determine which pathways were activated during hypertension. The results demonstrated that p-ERK1/2 protein content was 1.8-fold higher in cerebral arteries and 2.1-fold higher in coronary arteries in SHR compared with vessels from WKY (Figure 4). However, neither p-JNK nor p-p38 proteins were altered in SHR and WKY vascular segments.

#### Discussion

The main finding of the present study is that there is significantly increased cerebral  $ET_A$  receptor expression in SHR compared with WKY.  $ET_A$  receptor mRNA and protein expression was increased in cerebral vessels, which was reflected by enhanced ET-1-induced smooth muscle contraction. However,  $ET_B$  receptor expression was unaltered in cerebral arteries, and  $ET_B$  and  $ET_A$  receptor expression was unchanged in coronary arteries. Coronary artery  $ET_A$  receptor and cerebral artery  $ET_B$  receptor affinities were increased. ERK1/2 phosphorylation was also increased in both cerebral and coronary arteries.

Resistance in arteries and arterioles profoundly influences BP. Relatively small changes in blood vessel diameter may dramatically alter vascular resistance. Blood vessel radius is maintained by a delicate balance of vasoconstrictive and vasodilatory inputs. As a major contributor to hypertension development, the vascular smooth muscle contractile state regulates the vessel radius thus modulating peripheral resistance [31].  $ET_B$  and  $ET_A$  receptor agonists can induce vasoconstriction or vasodilatation by binding to their respective receptors [9].

Lariviere et al. [32] studied ET-1 gene expression in blood vessels of adult SHR. Their results indicated reduced or normal vascular ET-1 content in the SHR aorta and mesenteric arteries. They concluded that ET-1 did not play an important role in the pathogenesis of elevated BP in the SHR model of genetic hypertension. Their results suggest that ET receptor density and/or activity might be upregulated because of reduced vascular ET-1 content, which is consistent with our present results.

Receptor agonist-induced concentration-response curves can be determined by two important parameters, E<sub>max</sub> and EC<sub>50</sub>. E<sub>max</sub> refers to receptor-mediated maximal response, which reflects receptor efficacy. Based on the receptor occupation theory, the receptor effect is proportional to receptor number. Thus, the  $E_{max}$  reflects receptor number.  $EC_{50}$ is the concentration of agonist that can produce a response equal to 50% of  $E_{max}$ , which is used to determine receptor affinity or potency. The present study revealed that ET<sub>A</sub> receptor-mediated contractile responses were enhanced in SHR cerebral arteries with an increased E<sub>max</sub> and an unchanged pEC<sub>50</sub> compared with WKY. These data suggest that the receptor number may be increased. Further results demonstrated that ET<sub>A</sub> receptor mRNA and protein expression in the cerebral artery was elevated compared with WKY. The results are in concert with contractile function studies, indicating that the cerebral artery ET<sub>4</sub> receptor is upregulated in SHR. Similar results have been documented in cerebral arteries of subarachnoid hemorrhage [33], demonstrating that the increased ET-1 sensitivity is caused by increasing smooth muscle receptor expression. In support of these data, ETA receptor density is increased in cerebral arterioles of the stroke-prone SHR [34]. The enhanced vasoconstriction that was observed may be attributed to transcription and de novo contractile receptor translation [35].



Figure 1. Concentration–response curves for cumulative sarafotoxin 6c (S6c, A and C) and endothelin-1 (ET-1, B and D) application in SHR and WKY cerebral (A, B) and coronary (C, D) arteries. Contractile responses were demonstrated as the percentage of K<sup>+</sup>-induced contraction. Each data point is derived from 10–14 animals and is expressed as the mean ± SEM. Statistical analysis was performed using a two-way ANOVA. \**P*<0.05, \*\**P*<0.01 *vs*. WKY group. doi: 10.1371/journal.pone.0073761.g001

Increased ET receptor expression has been found in coronary arteries of ischemic heart disease patients [17] and in cerebral artery of ischemic/hemorrhagic stroke patients [15,16], suggesting that ET receptor regulation is a novel and possibly important feature in many cardiovascular diseases [2]. Therefore, we speculated that ET receptor expression might be altered in SHR arteries. The present study focused on the cerebral and coronary arteries, which are in two of the most

important organs. Vascular ET receptor number may vary in different organs [36]. The present study demonstrated that  $ET_A$  receptor-mediated coronary artery contractile response curves in SHR were shifted toward the left in parallel with an increased pEC<sub>50</sub> and an unchanged  $E_{max}$ , suggesting that  $ET_A$  receptor affinity increases, whereas the receptor number may not be increased. The myograph results were consistent with mRNA and protein levels. There were no significant differences in ET<sub>A</sub>





**Figure 2.** ET<sub>B</sub> and ET<sub>A</sub> receptor mRNA expression levels in SHR and WKY cerebral (A) and coronary (B) arteries. ET<sub>B</sub> and ET<sub>A</sub> receptor mRNA levels were expressed relative to elongation factor-1 (EF-1) levels. Each data point is derived from 7–8 animals, and the data are expressed as the mean  $\pm$  SEM. Statistical analysis was performed using an unpaired Student's *t*-test. *P*<0.05 *vs*. WKY group.

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Figure 3. ET<sub>B</sub> and ET<sub>A</sub> receptor protein expression levels in SHR and WKY rat cerebral (A) and coronary (B) arteries.  $ET_B$  and  $ET_A$  receptor protein expression levels were expressed relative to  $\beta$ -actin levels. Each data point is derived from 7–8 animals and is expressed as the mean ± SEM. Statistical analysis was performed using an unpaired Student's *t*-test. '*P*<0.05 *vs*. WKY group.

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receptor mRNA and protein levels in coronary arteries of SHR compared with WKY. These data suggest that  $ET_A$  receptor number does not increase in SHR coronary arteries. Similar results appear in rat heart coronary arteries during ischemia/ reperfusion [18]. The augmented vascular reactivity to ET-1 in experimental hypertension may be related to increased intracellular free Ca<sup>2+</sup> concentration in vascular smooth muscles [37,38].

The ET<sub>B</sub> receptor agonist S6c-induced cerebral and coronary artery contractions were weak, and the mRNA and protein expression levels were low in WKY, which can be ignored. There was no direct functional response to the selective S6cmediated ET<sub>B</sub> stimulation. Beg et al. described a dimerization mechanism between the two receptors to form an ET<sub>A</sub>-ET<sub>B</sub> receptor heterodimer in ET-1 recognition that represents a functional ET<sub>B</sub> receptor [39]. ET<sub>B</sub> receptor-mediated cerebral artery contraction curves were obviously enhanced in SHR compared with WKY. However, cerebral arterial ET<sub>B</sub> receptor mRNA and protein expression in SHR were not markedly increased. These results suggest that the amount of ET<sub>B</sub> receptor may not change, whereas receptor susceptibility increases. Contrarily, in SHR coronary arteries, ET<sub>B</sub> receptormediated contraction, mRNA and protein expression levels were not significantly changed, suggesting that ET<sub>B</sub> receptor amounts and activity in coronary arteries were unaltered.

However, controversy exists about the ET-induced vascular response in hypertension, depending on the animal hypertension model, hypertension duration, the experimental conditions used, and the blood vessels studied [40]. Tschudi and Lüscher reported that SHR coronary artery contractile responses to ET-1 were decreased compared with WKY [40]. They determined that ET-1-induced tension was approximately 0.4 and 1.4 mN/mm in SHR and WKY, respectively. However, KCl-induced contraction also decreased. KCl-induced wall tension (0.28 ± 0.03 mN/mm) in SHR was approximately onethird of that in WKY (0.87 ± 0.05 mN/mm). ET-1 is a receptoroperated agonist, and KCI is a receptor-independent agonist. The relative contractions (as a percentage of the KCI-mediated contraction) indicated the agonist or receptor sensitivity. ET-1induced contractions relative to KCI were 143% and 161% in WKY and SHR, respectively. In our study, ET-1-induced contractions relative to KCI in the coronary artery were 156±5.8% and 152±5.0% in WKY and SHR, respectively. The relative contractions in the two experiments were similar. However, there was an important difference in ET-1-induced receptor-mediated contraction in the two experiments. Because ET<sub>B</sub> receptor desensitization was performed, only ET<sub>A</sub> receptormediated coronary contraction occurred in our experiment. However, in their experiment, coronary contraction was mediated by both ET<sub>A</sub> and ET<sub>B</sub> receptors. In their experiment,



**Figure 4. ERK1/2 protein phosphorylation in rat cerebral and coronary arteries.** Phosphorylated (p)-ERK1/2 levels in SHR group relative to the WKY group. Each data point is derived from 7–8 animals, and the data are expressed as the mean ± SEM. Statistical analysis was performed using an unpaired Student's *t*-test. \**P*<0.05 *vs*. WKY group. doi: 10.1371/journal.pone.0073761.g004

the contraction reflected the ET-1 function, whereas in our experiment, the contraction reflected the function of  $\mathsf{ET}_{\mathsf{A}}$ 

receptors. Thus, the two studies reflected two different ET family profiles.

MAPKs represent a serine/threonine protein kinase family that mediates fundamental biological processes and cellular responses to external stress signals. Many pathophysiological processes activate the MAPK pathway [41,42]. Previous studies demonstrated MEK/ERK1/2 activation in subarachnoid hemorrhages and following cigarette smoke exposure [28,43]. Molecular studies have demonstrated that the MEK/ERK1/2 pathway is responsible for upregulation of some G-protein coupled receptors such as ET receptors [44,45]. Therefore, the intracellular signal transduction pathway may be involved in hypertension-mediated receptor expression alteration. We determined that ERK1/2 phosphorylation was elevated in SHR cerebral and coronary arteries compared with WKY. Thus, basal ERK1/2 pathway activity is higher during hypertension, which might contribute to not only enhanced ET receptor expression but also to elevated ET receptor affinity in cerebral and coronary arteries in SHR. However, in SHR aortas, p38 MAPK activation plays an important role in ET-1-induced vasoconstriction maintenance [46]. These data suggest that the mechanism may be organ-specific.

In conclusion, the present study demonstrated that cerebral arterial  $ET_A$  receptor was upregulated in SHR.  $ET_A$  receptor affinity was increased, whereas receptor number was not

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increased in SHR coronary arteries. In addition,  $ET_B$  receptor susceptibility was increased, but the receptor amount may be not changed in cerebral arteries. In contrast,  $ET_B$  receptor expression levels and activity in coronary arteries were not altered. ERK1/2 pathway activation may play a role in hypertension-associated receptor alterations. Understanding receptor alterations may advance our knowledge of hypertension-associated cardiovascular disease, provide new insight for future analysis of the mechanisms underlying hypertension, and suggest possibilities for novel treatments.

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### **Author Contributions**

Conceived and designed the experiments: LC YXC CBX LE. Performed the experiments: LC. Analyzed the data: LC. Contributed reagents/materials/analysis tools: YXC LE. Wrote the manuscript: LC YXC LE.

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