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Benzo[*a*]pyrene alters vascular function in rat aortas *ex vivo* and *in vivo*

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ABSTRACT

Benzo[*a*]pyrene (BaP) is a polycyclic aromatic hydrocarbon found in tobacco smoke and air pollution products. BaP exposure has been recently suggested to be a risk factor for hypertension in coke oven workers. The mechanisms of BaP on vascular smooth muscle function remain unclear. Here, we examined the influence and possible mechanism of BaP on vasoconstriction in rat thoracic aortas *ex vivo* and *in vivo*. *In vivo* exposure of rats to BaP (20 mg/kg) for 8 weeks caused a significant enhancement in the systolic blood pressure and enhanced aortic hyperreactivity to α1-adrenoceptor selective agonist phenylephrine in aortas. BaP (1 and 10 μM) treatment for 18 h induced an enhancement of phenylephrine-induced vasoconstriction in the organ cultures of aortas. Aryl hydrocarbon receptor antagonist α-naphthoflavone, protein kinase C (PKC) inhibitor chelerythrine, mitogen-activated protein kinases (MAPK) inhibitor PD98059, myosin light chain kinase (MLCK) inhibitor ML-9, and Rho-kinase inhibitor Y-27632 significantly suppressed BaP-enhanced vasoconstriction. BaP time-dependently triggered reactive oxygen species (ROS) production in primary vascular smooth muscle cells. Both antioxidant *N*-acetylcysteine and NAD(P)H oxidase inhibitor diphenyleneiodonium significantly inhibited BaP-triggered ROS production and vasoconstriction. These results suggest that BaP enhances aortic vasoconstriction *via* the activation of ROS and muscular signaling molecules PKC, MAPK, MLCK, and Rho-kinase.

1. Introduction

Benzo[*a*]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) found in tobacco smoke and air pollution products, and is also a ubiquitous environmental pollutant and carcinogen. Environmental chemical carcinogens have been identified to be as a risk factor for atherosclerosis [1]. Both cigarette smoking and BaP have been shown to initiate and/or accelerate atherosclerosis [2,3], but related mechanism is not fully understood until now. Smoking is an independent risk factor of cardiovascular disease [2], and has also been shown to increase the cardiovascular risk resulting from the increased systolic blood pressure [4]. A cross-sectional study has found that PAHs have a positive association with hypertension among individuals of Mexican origin [5]. A recent study has reported that BaP and coke oven emissions exposures are associated with hypertension and abnormal electrocardiogram in coke oven workers [6]. Xu et al. showed that cigarette smoke extracts was capable of enhancing the cell proliferation of human vascular smooth muscle cells [7]. It has been shown that BaP alters smooth muscle cell phenotype from quiescent and differentiated to proliferated and synthetic state and by which leads to atherosclerosis [8,9]. Gan et al. (2012) have found that blood pressure is significantly increased in BaP-treated rats [10]. Recently, benzo[*a*]pyrene has been found to protect rat vascular smooth muscle cells (VSMCs) from nitric oxide-induced apoptosis [11]. However, the mechanisms underlying the effects of BaP in vascular smooth muscle function remain to be clarified.

Chronic hypertension has been observed to increase mortality in patients from coronary artery disease, stroke, cardiovascular disease, and renal disease [12,13]. Hypertension is also known as an

Abbreviations: BaP, benzo[*a*]pyrene; MAPK, mitogen-activated protein kinases; MLCK, myosin light chain kinase; PAH, polycyclic aromatic hydrocarbon; PKC, protein kinase C; ROS, reactive oxygen species; VSMCs, vascular smooth muscle cells

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independent risk factor of cardiovascular diseases [14]. Sustained increases in vascular tone of resistance arteries and arterioles contribute to the development of hypertension [15,16]. The role of VSMCs is to maintain normal vascular tone and regulate the size of the blood vessel lumen [17,18]. A balance between vasoconstriction and vasodilation is necessary for the maintenance of normal vascular structure and function. Once this balance is disrupted, it leads to vascular remodeling and injury, which may aggravate existing hypertension and accelerate the progression of atherosclerosis and other vascular diseases [19]. Moreover, when the balance of redox state in blood vessels tends to oxidation (oxidative stress), it may cause the dysregulation/dysfunction of signals in vascular smooth muscle cells and endothelial cells that may contribute to vascular pathologies, such as hypertension and atherosclerosis [20–23]. The increase of NAD(P)H oxidase-driven reactive oxygen species (ROS) generation has been found in VSMCs from spontaneously hypertensive rats during hypertension development [20]. In the present study, we investigated the effects of BaP on the contractile responses in rat aortas. We explored whether BaP has the capacity of influencing the function of vascular smooth muscle and further developing hypertension in rats. To determine the involved vasoconstriction-related signaling molecules and the role of ROS in the BaP-induced responses, the selective pharmacological inhibitors were used.

2. Materials and methods

2.1. Animals and blood pressure measurement

Male Wistar rats (200–250 g) were obtained from BioLASCO (Taipei, Taiwan). The animal study was approved by the institutional animal care and use committee of College of Medicine, National Taiwan University. The regulations of Taiwan and NIH guidelines on the care and welfare of laboratory animals were adopted in this animal study. Experimental animals were treated humanely to prevent or alleviate pain. Rats were allowed 1 week acclimation period at animal quarters under controlled environmental conditions (22 ± 2 °C, 12-h light/dark cycles) with standard chow diet and water *ad libitum*. Rats were given BaP (20 mg/kg) weekly by i.p. injection for 8 weeks. BaP was dissolved in corn oil. Systolic blood pressure was measured in each rat by use of the tail-cuff technique (Visitech Systems, Apex, NC, USA). Tests were performed on conscious rats restrained in a box with the cuff and the pulse-wave transducer set around the tail. The dosage for BaP was selected according to the previous study [10] and our preliminary experiments.

2.2. Aorta isolation and culture

The isolation and culture of aortas were performed as previously described [24]. The thoracic aorta was isolated from rats under anesthesia. The aortas were cut into ring segments of 4–5 mm length and cultured in an organ culture petri dish with sterile DMEM containing 10% fetal bovine serum at 37 °C. The aortic rings were used for organ bath study 18 h after culture. In some experiments, the endothelium-denuded rat aortic rings were prepared by removing endothelium using a cotton-tipped applicator to gently rub the luminal surface.

2.3. Organ bath study

The aorta rings were applied in organ bath to measure the vasoconstriction as previously described by Tzeng et al. [24]. For isometric constriction measurement, the aorta rings were suspended between two hooks connected to a transducer (Mantracourt Electronics Limited, Devon, Great Britain) and a high resolution laboratory data recorder (e-corder) (eDAQ Pty Ltd., Denistone East, NSW, Australia) in 10-ml organ baths containing Krebs solution (NaCl 118.3 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25.0 mM, and glucose 11.1 mM, pH 7.2–7.4) with 95% O_2 + 5% CO_2 at 37 °C. The basal tension of aortic ring was set at 1.0 g. Equilibration for 1 h before the dose-dependent responses to phenylephrine (0.0003–10 μM) were carried out. An isometric transducer (Grass FT.03) on a Biopac's MP 100 data acquisition system (Biopac Systems, Goleta, CA, USA) was used to record the tension. In some experiments, the pharmacological inhibitors for signaling molecules were administered simultaneously with the BaP treatment. The deficiency of endothelium was confirmed by lacking responses to acetylcholine. The force generation of aorta changes was normalized by the length of each ring. The vasoconstrictor responses to phenylephrine were expressed as g/mm.

2.4. Immunoblotting

Proteins (50 μg) were subjected for electrophoresis, and then proteins were transferred on to PVDF membranes blocked with 5% nonfat powdered milk. It was incubated overnight at 4 °C with anti-phosphorylated myosin light chain kinase (MLCK) (Abcam, Cambridge, MA, USA), anti-Rho-activated serine/threonine kinase (Rock)-1, and anti-α-tubulin (Santa Cruz Biotechnology, Dallas, TX, USA). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used to detect the signals that revealed with an enhanced chemiluminescence reagent (BioRad Laboratories, Redmond, WA, USA). The quantification was determined by densitometric analysis using the image J 1.48 software (National Institutes of Health, Bethesda, MD, USA).

2.5. Isolation of vascular smooth muscle cells (VSMCs)

VSMCs were prepared from isolated thoracic aortas of Wistar rats as previously described by [11,25]. In brief, the isolated thoracic aortas were cleaned of fat and adventitia and cut into small strips, and then incubated in 1 mg/ml collagenase and 0.125 mg/ml elastase at 37 °C for 60 min. The isolated cells exhibited characteristics of VSMCs and cultured in 10 ml of DMEM containing 10% fetal bovine serum at 37 °C. The third to sixth passages of VSMCs were used.

2.6. Detection of intracellular ROS

The detection of intracellular ROS in VSMCs was performed by flow cytometry using a peroxide-sensitive fluorescent probe (2′,7′-dichlorofluorescin diacetate, DCF-DA; Molecular Probes, Eugene, OR, USA) as previously described by [24]. In brief, subconfluent and serum-deprived VSMCs were loaded with 20 μM of DCFH-DA for 30 min and then chilled on ice and washed with cold PBS 3 times. The washed cells were detached from the culture plates by trypsin-EDTA digestion. The fluorescent intensity was analyzed by a flow cytometer (Becton-Dickinson, San Jose, CA, USA).

2.7. Statistical analysis

All data are shown as mean ± S.E.M of at least three independent experiments. Concentration-contractile response curves were fitted by nonlinear regression. The one way analysis of variance (ANOVA) followed by Dunnett's test was performed to assess the statistical significance. For blood pressure analysis, the statistical significance was determined by Mann-Whitney Rank Sum Test.

3. Results

3.1. The effect of BaP on developing hypertension

Hypertension is a clinical syndrome characterized by increased vascular tone. Gan et al. [10] have reported that BaP significantly elevates blood pressure in rats. We observed whether *in vivo* giving BaP to rats would cause hypertension. The rats were treated with 20 mg/kg

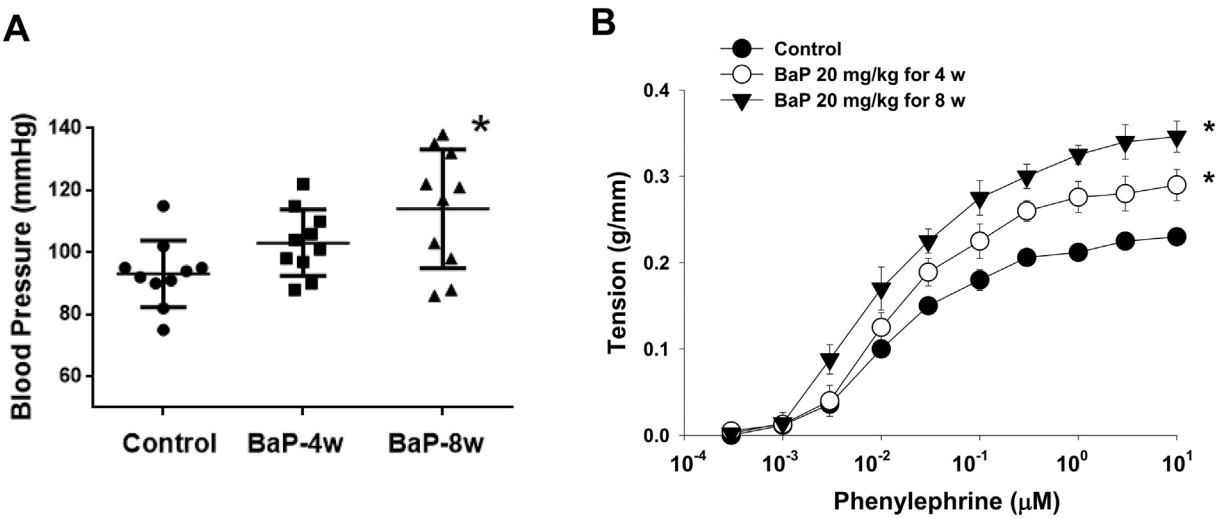

Fig. 1. Effects of BaP on systolic blood pressure and aortic vasoconstriction in rats. Rats were exposed to BaP (20 mg/kg) for 4 and 8 weeks. (A) The systolic blood pressure in rats was measured by tail-cuff method ($n = 10$ for each group). (B) Rats after exposure to BaP for 4 or 8 weeks were sacrificed and aortas were isolated to measurement of vasoconstriction. Phenylephrine (PE) was then added cumulatively to induce the contractile response. The force generation of aorta changes was normalized by the length of each ring. Each point represents mean ± SEM from six separate experiments. *$P < 0.05$ as compared to control.

BaP for 4 or 8 weeks, and then the systolic blood pressure was measured. The systolic blood pressure between vehicle control group (94.1 ± 3.6 mmHg, $n = 10$) and BaP group for 4 weeks (102.5 ± 3.4 mmHg, n = 10) had no significant difference ($P = 0.121$); but there was significant difference between vehicle control group (94.1 ± 3.6 mmHg, n = 10) and BaP group for 8 weeks (114.5 ± 6.0 mmHg, n = 10) ($P = 0.038$) (Fig. 1A). We next isolated the aortas from these rats to measure the contraction responses to phenylephrine, a α1-adrenoceptor selective agonist. A time-dependently enhancing vasoconstriction was observed in BaP-treated group compared to vehicle control group (Fig. 1B).

3.2. BaP enhances phenylephrine-elicited vasoconstriction in isolated aortic rings

We next investigated the effects of BaP on the contractile responses in isolated rat aortas. BaP (1 and 10 μM) challenge for 18 h enhanced the maximal contractile response elicited by phenylephrine in a concentration-dependent manner (Fig. 2A). In endothelium-denuded rat aortic rings, phenylephrine increased contractile responses compared to endothelium-intake aortic rings (Fig. 2B). Treatment with BaP (10 μM) for 18 h could also enhance the phenylephrine-induced contractile response in endothelium-denuded aortas (Fig. 2B). These results indicate that the stimulatory effect of BaP on aortic contractile response may be endothelium-independent. BaP by itself did not affect the basal tension of the aortas in these experimental conditions (data not shown).

3.3. Roles of AhR, PKC and ERK in BaP-enhanced vascular responses

We next tried to investigate the possible mechanisms of BaP on vascular responses in isolated aortic rings using pharmacological inhibitors. BaP is known as a ligand of aryl hydrocarbon receptor (AhR). Its stimulatory effects on aortic rings may be mediated by AhR signaling. As shown in Fig. 3A, treatment with AhR antagonist α-naphthoflavone (ANF; 2 μM) significantly inhibited BaP-enhanced contractile response.

The signals of Protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) are known to be involved in the regulation of vascular contraction [26]. PKC has been shown to be an upstream activator of MAPk in smooth muscle and non-muscle cells [26–28]. We next investigated whether PKC and MAPK are involved in the BaP-enhanced vascular responses. As shown in Fig. 3B, treatment with chelerythrine (1 μM), a selective PKC inhibitor, reversed the BaP-induced vascular changes in response to phenylephrine. Moreover, treatment with PD98059 (40 μM), a MAP kinase kinase (MEK) inhibitor, significantly abolished the stimulatory effect of BaP in the endothelium-denude aortas (Fig. 3C). These results indicate that PKC and MAPK participate in the BaP-enhanced vasoconstriction elicited by phenylephrine.

3.4. Roles of myosin light chain kinase (MLCK) and Rho-kinase in BaP-enhanced vascular responses

Smooth muscle contraction is mainly activated by phosphorylation of the 20-kDa myosin light chain (MLC_{20}) by Ca^{2+}/calmodulin–dependent MLCK [29,30]. Moreover, the Ca^{2+}-sensitizing Rho/Rho-kinase pathway also regulates smooth muscle contraction [30]. As shown in Fig. 4, treatment with BaP (1 and 10 μM) for 18 h significantly increased the MLCK phosphorylation and Rho-kinase ROCK-1 protein expression in isolated aortic rings. We further investigated the role of MLCK and Rho-kinase in BaP-triggered response. In the presence of ML-9 (5 μM), a selective MLCK inhibitor, BaP failed to enhance the phenylephrine-elicited vasoconstriction in organ cultures of rat aortas (Fig. 5A). Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride] is a specific inhibitor of the Rho-kinase [31]. Treatment with Y-27632 (1 μM) significantly inhibited BaP-enhanced vasoconstriction (Fig. 5B). These results indicate that MLCK and Rho-kinase participate in the BaP-enhanced vasoconstriction elicited by phenylephrine.

3.5. Role of ROS in BaP-enhanced vascular responses

It has been reported that BaP was capable of inducing oxidative stress in VSMCs [32]. We next investigated whether ROS is participated in BaP-enhanced vasoconstriction. We confirmed that ROS could be induced by BaP in VSMCs detected by a fluorescent dye DCFH-DA (Fig. 6A). BaP (10 μM) time-dependently stimulated DCF-sensitive ROS production peaking at 2 h compared to control (Fig. 5A). Stimulated DCF-sensitive ROS production by BaP could be inhibited in the presence of antioxidant *N*-acetylcysteine (NAC, 2 mM) and also attenuated by the pretreatment with diphenyleneiodonium (DPI, 1 μM), an inhibitor of NAD(P)H oxidase (Fig. 6B). Treatment with antioxidant *N*-acetylcysteine (NAC, 2 mM) significantly inhibited BaP-enhanced vasoconstriction in organ culture of rat aortas (Fig. 7A). Moreover, DPI (1 μM) also significantly suppressed the stimulatory effect of BaP on

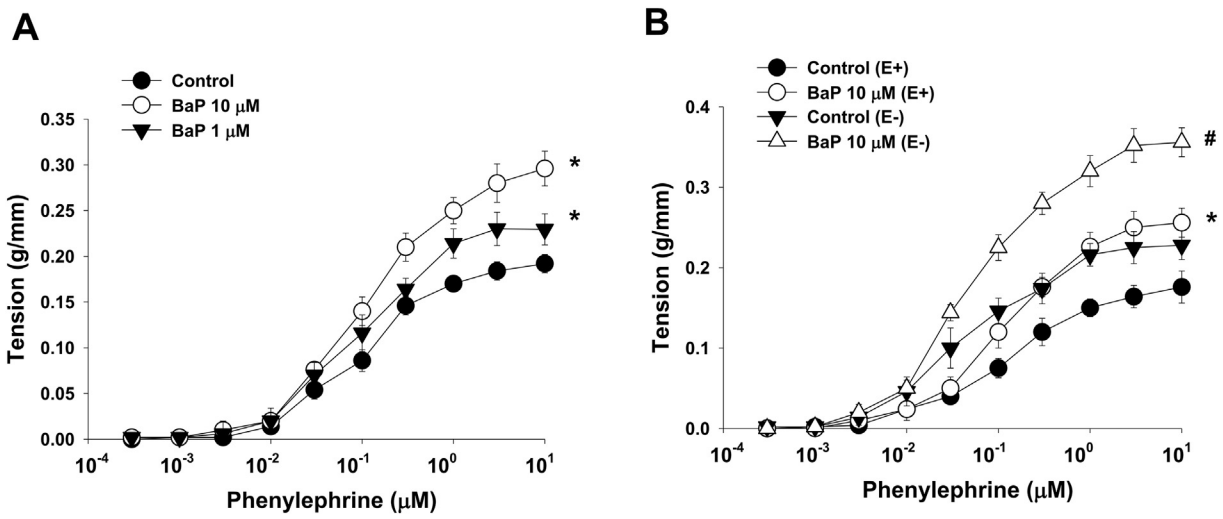

Fig. 2. Effects of BaP on the phenylephrine-elicited contraction in organ cultures of intact and endothelium-denuded rat aortas. (A) Each intact aortic ring was challenged with either vehicle (closed circle) or BaP (1 μM, open circle; 10 μM, closed triangle) for 18 h in organ culture condition, and then the contraction was measured in the presence of the indicated concentrations of phenylephrine (PE). (B) Both Endothelium-intact and -denuded aortic rings were challenged with vehicle (endothelium-intact (E+), closed circle; endothelium-denuded (*E*-), closed triangle) or BaP (10 μM; endothelium-intact (E+), open circle; endothelium-denuded (E-), open triangle) for 18 h, and then PE was added cumulatively. The force generation of aorta changes was normalized by the length of each ring. Each point represents mean ± SEM from six to ten separate experiments. *$P < 0.05$ as compared to control (E+). #$P < 0.05$ as compared to control (E-).

vasoconstriction triggered by phenylephrine (Fig. 7B). No significant difference in the contractile pattern was found between the control group and the NAC alone or DPI alone groups (data not shown). These results indicate that ROS is involved in the BaP-enhanced vasoconstriction elicited by phenylephrine.

4. Discussion

A recent cross-sectional study of chimney sweeps has shown that PAH exposure increases the levels of markers for cardiovascular disease [33]. They found that PAH metabolites 2-hydroxyphenanthrene, 3-hydroxybenzo[*a*]pyrene, and 3-hydroxybenzo[*a*]anthracene have a significant positive association with diastolic blood pressure in chimney sweeps. A recent systematic review has mentioned that PAH exposure is associated with increased risk of cardiovascular disease in which the major risk factors include elevated blood pressure and obesity [34]. Yang et al. [6] have recently shown that BaP is one of risk factors for hypertension in coke oven workers [6]. However, the mechanisms underlying the effects of BaP in vascular smooth muscle function and hypertension remain to be clarified. The present study provides evidence that organ culture of aortas to BaP and chronic BaP administration to aorta *in vivo* are linked to an enhancement in the vascular reactivity of the aorta to constrictor agent phenylephrine and further developing hypertension in rats. The involved signaling molecules in the BaP-enhanced contractile responses in aortas were further examined using selective pharmacological inhibitors.

Evidence has been presented that localized the P450 activity to the smooth muscle, as well as the endothelial layers of the aortic walls. One of the atherogenic activity of BaP is mediated by oxidative metabolism of the parent compound to BaP-7,8-diol-9,10-epoxide (BPDE), which covalently binds to cellular macromolecules, leading to formation of mutagenic DNA adducts [35]. BaP is also oxidized to 3-hydroxy- and 6-hydroxy-BaP, which further oxidized to form BaP quinines that can undergo redox cycling and generate ROS [36], which plays a pivotal role in atherosclerosis [17]. Moorthy et al. have also reported that CYP1B1 is induced in mouse VSMCs and catalyzed BaP metabolism to 3-hydroxy-BaP and 3,6-quinone-BaP, which are proximate genotoxic metabolites, and play an important role in BP genotoxicity and atherogenesis [37]. Recently, the PAH metabolites have been found to be positively associated with blood pressure among chimney sweeps [33]. In the present study, we found that the *in vivo* exposure of BaP (20 mg/kg) increased systolic blood pressure and *ex vivo* exposure of BaP enhanced aortic vasoconstriction. However, the role of BaP metabolites in hypertension and aortic vasoconstriction needs further investigation in the future.

Ginsberg and Atherholt [38] have shown that serum BaP levels fell steadily from 1980 nM at 1 h to 350 nM by 24 h in mice treated with BaP 200 mg/kg (i.p. injection); they also found that serum BPDE levels reached a plateau within 2.5 h and remained constant thereafter (10 to 11 nM) [38]. Gan et al. [10] have found that BaP injection for 4 weeks (10 mg/kg, weekly, i.p.) significantly increases blood pressure in rats, and decreases, but not statistically significant, the maximum contractile responses to phenylephrine in endothelium-intact aortic rings isolated from BaP-treated rats [10]. They also found that BaP at a concentration of 100 μM for 6 h did not decrease the phenylephrine-induced contractile response in isolated aorta rings under endothelium-intact and -denuded conditions. However, the mechanisms underlying the effects of BaP on vascular smooth muscle function still remain unclear. In the present study, results showed that BaP injection for 8 weeks (20 mg/kg, weekly, i.p.) could also increase blood pressure in rats. However, we found that BaP significantly enhanced the phenylephrine-induced contractile responses in aorta rings isolated from BaP-treated rats and in organ culture of aorta rings treated with BaP (1 and 10 μM) for 18 h. The dosage and duration of BaP exposure may cause these different results. We further demonstrated that BaP enhanced aortic vasoconstriction *via* the activation of ROS and muscular signaling molecules PKC, MAPK, MLCK, and Rho-kinase.

Various studies have shown the significant correlation between tail-cuff method and intraarterial blood pressure in conscious rats [39,40]. There were many studies using tail-cuff method to measure blood pressure in conscious animas such as Widdop and Li [41], Fritz and Rinaldi [42], Gan et al. [10], Choi et al. [43], and Jin et al. [44]. Gan et al. [10] have shown that benzo[*a*]pyrene exposure significantly increases both systolic and diastolic blood pressure in rats using the tail-cuff method [10]. Using the indirect method to monitoring blood pressure (tail-cuff method), Kurtz et al. [45] recommended it for measuring systolic blood pressure, but not recommended for measuring diastolic blood pressure [45]. In the present study, we repeated and verified that benzo[*a*]pyrene exposure also possessed the effects of increasing systolic blood pressure *in vivo* and affecting vasoconstriction *ex*

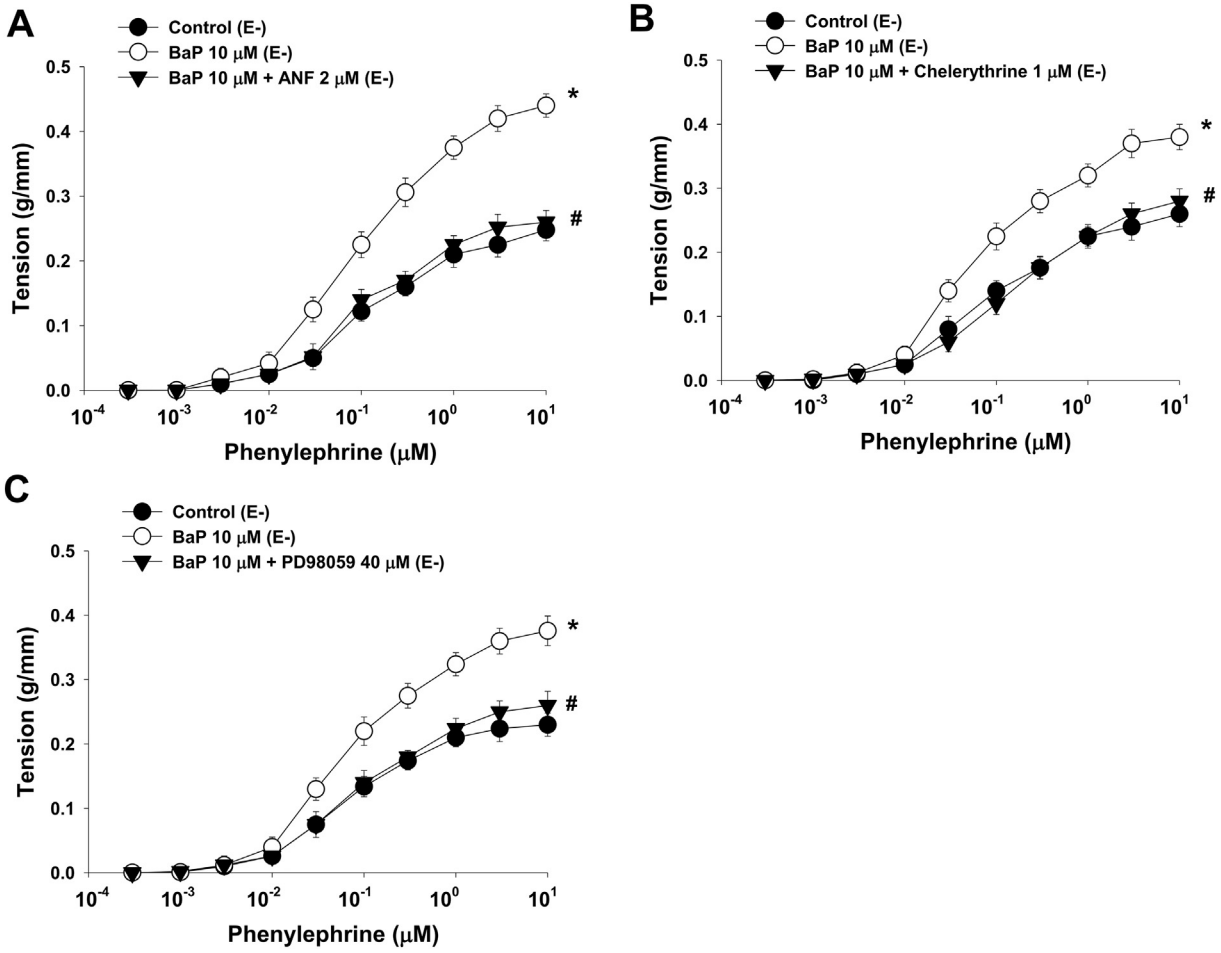

Fig. 3. Effects of AhR receptor antagonist α-naphthoflavone, PKC inhibitor chelerythrine, and MAPK inhibitor PD98059 on BaP-enhanced vasoconstriction in organ cultures of endothelium-denuded aortic rings. (A) Aortic rings were incubated with either vehicle control (closed circle), BaP (10 μM, open circle), or α-naphthoflavone (ANF, 5 μM; open triangle) plus BaP for 18 h. (B) Aortic rings were incubated with either vehicle control (closed circle), BaP (10 μM, open circle), or chelerythrine (5 μM, closed triangle) plus BaP for 18 h. (C) Aortic rings were incubated with either vehicle control (closed circle), BaP (10 μM, open circle) or PD98059 (40 μM, open triangle) plus BaP (10 μM) for 18 h. PE was added cumulatively to induce the contractile response. The force generation of aorta changes was normalized by the length of each ring. Each point represents mean ± SEM from four to six separate experiments. *$P < 0.05$ as compared to control. #$P < 0.05$ as compared to BaP alone.

vivo in our experimental condition.

In this study, we tried to explore the mechanisms underlying the effects of BaP on vascular contractile responses. We found that chelerythrine, a selective PKC inhibitor, was able to suppress the BaP-enhanced aortic vasoconstriction in organ culture condition, suggesting the involvement of PKC signaling in BaP-induced response. PKC is

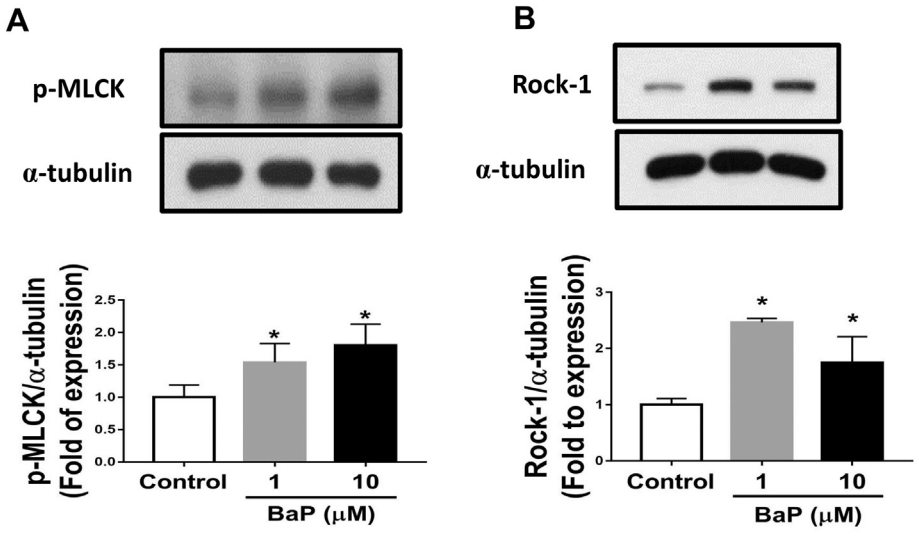

Fig. 4. Effects of BaP on the MLCK phosphorylation and Rho kinase protein expression in organ cultures of endothelium-denuded aortic rings. The aortic rings were incubated with vehicle or BaP (1 and 10 μM) for 18 h. The protein expressions were determined by Western blotting and quantified using densitometric analysis. The α-tubulin was regarded as a loading control. Results are expressed as mean ± SEM for at least three independent experiments. *$P < 0.05$ as compared to control.

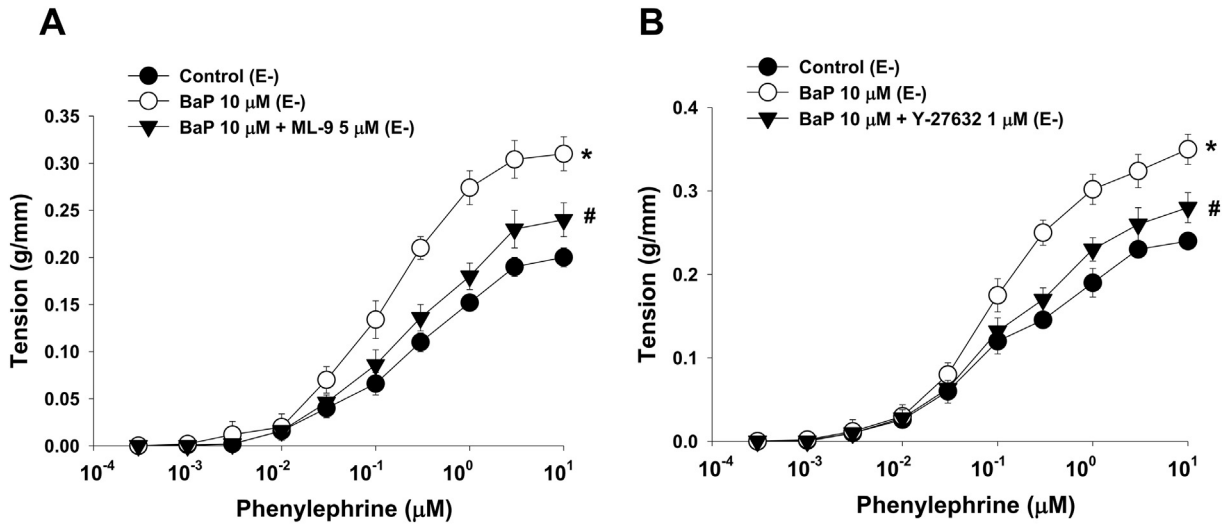

Fig. 5. Effects of MLCK inhibitor ML-9 and Rho kinase inhibitor Y-27632 on BaP-enhanced vasoconstriction in organ cultures of endothelium-denuded aortic rings. (A) The aortic rings were incubated with vehicle (closed circle), BaP (10 μM, open circle), or BaP plus ML-9 (5 μM, closed triangle) for 18 h. (B) Aortic rings were incubated with vehicle (closed circle), BaP (10 μM, open circle) or BaP plus Y-27632 (1 μM, open triangle) for 18 h. PE was then added cumulatively to induce the contractile response. The force generation of aorta changes was normalized by the length of each ring. Each point represents mean ± SEM from four to six separate experiments. *$P < 0.05$ as compared to control. #$P < .05$ as compared to BaP alone.

known as a major functional regulator of vascular smooth muscle [46]. The enhancement of prostaglandin-F_2-induced coronary smooth muscle contraction by endothelin-1 has been found to involve a selectively activation and translocation of PKCα [47]. This supports our results that BaP enhanced vasoconstriction triggered by phenylephrine was also through PKC activation. PKC is known to phosphorylate CPI-17 (C-kinase-activated protein phosphatase-1 inhibitor, 17 kDa), causing to suppression of MLC phosphatase, trigger the phosphorylation of MLC, and increase vascular smooth muscle contraction [46]. CPI-17 could be phosphorylated by PKCα and δ isoforms in histamine-induced vasoconstriction in porcine aortic smooth muscle [48]. The involvement of PKC isoforms in BaP-enhanced vasoconstriction remains to be further investigated. Moreover, the activation of MAPK also plays a role in vascular function and it can be through PKC-dependent and -independent pathway in vascular smooth muscle [26,46]. Khalil and Morgan reported that MAPK plays a role in the signal transduction cascade linking PKC activation to contraction of ferret aorta smooth muscle cells triggered by phenylephrine, indicating that MAPK activation is PKC-dependent [49]. Additionally, Birukov *et al* found that intraluminal pressure-induced ERK/MAPK activation is PKC-independent, but Src-family tyrosine kinase-dependent in organ culture of rabbit aortas [50]. In the present study, we also showed that PD98059, a MEK inhibitor, effectively inhibited the stimulatory effect of BaP on phenylephrine-induced vasoconstriction, suggesting that MAPK signaling is also involved in the BaP-enhanced vasoconstriction.

MLC_{20} phosphorylation is an important mechanism in regulation contractile activity of smooth muscle. The level of MLC_{20} phosphorylation depends on the balance of kinase and phosphatase activities, which could reflect either increased activity of the MLCK that phosphorylate myosin or inhibition of myosin light chain phosphatase

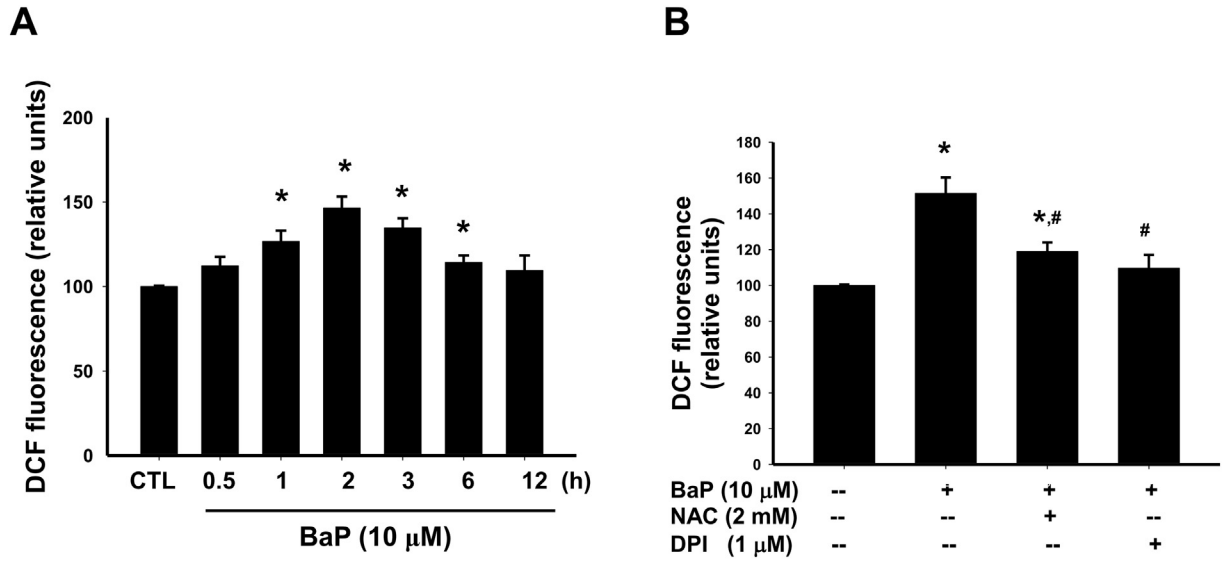

Fig. 6. BaP induced the 2′,7′-dichlorofluorescein (DCF)-sensitive ROS generation in VSMCs. Intracellular ROS generation was determined by fluorescence of DCFH-DA as described in "Materials and Methods." (A) The VSMCs were challenged with BaP (10 μM) for various time intervals. (B) Cells were pretreated with antioxidants NAC (2 mM) or DPI (1 mM) for 30 min followed by challenge of BaP (10 μM) for 2 h. The relative fluorescence intensity in VSMCs was quantitated by flow cytometry. Data are presented as mean ± SEM from three to five experiments performed in duplicate. *$P < 0.05$ as compared to control. #$P < 0.05$ as compared to BaP alone.

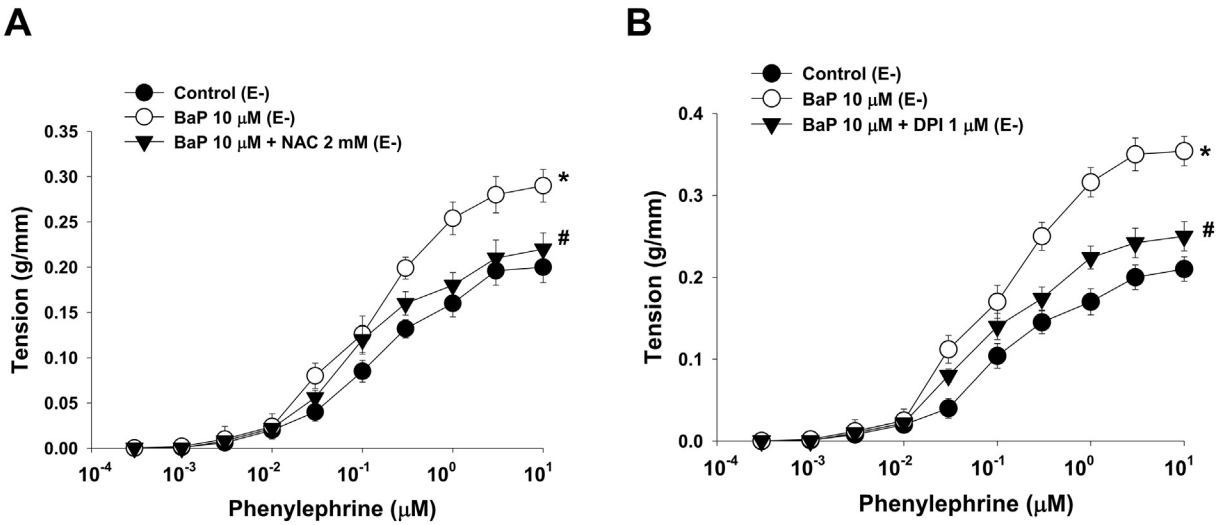

Fig. 7. Effects of antioxidants on BaP-enhanced vasoconstriction in organ cultures of endothelium-denuded aortic rings. (A) Aortic rings were incubated with vehicle (closed circle), BaP (10 μM, open circle), or BaP plus antioxidant NAC (2 mM, closed triangle). (B) Aortic rings were incubated with vehicle (closed circle), BaP (10 μM, open circle), or BaP plus Flavin-containing enzyme inhibitor DPI (1 μM, open triangle) for 18 h. PE was then added cumulatively to induce the contractile response. The force generation of aorta changes was normalized by the length of each ring. Each point represents mean ± SEM from four to six separate experiments. *$P < 0.05$ as compared to control. #$P < 0.05$ as compared to BaP alone.

(MLCP) [30]. ML-9 is a selective MLCK inhibitor with inhibitory constant values (Ki) of 3.8 μM (Ki values are 32 and 54 μM for protein kinase A (PKA) and PKC, respectively) [51]. In the present study, our results showed the inhibitory effect of ML-9 on BaP-enhanced vasoconstriction elicited by phenylephrine, suggesting that BaP-enhanced vasoconstriction was through MLCK signaling. On the other hand, the Rho/Rho-kinase pathway also plays an important physiological role in vascular smooth muscle [30]. During contractile stimulation, Rho kinase is activated by the small GTPase RhoA, which subsequently induces MLCP inhibition and trigger vascular contraction. Pathological activity of Rho-kinase in smooth muscle has been implicated in hypertension [52,53]. The inhibition of Rho-kinase by specific inhibitors inhibits neointimal formation in balloon-injured arteries [54]. Besides, both PKC and Rho kinase are capable of phosphorylating CPI-17 to inhibit MLCP activity, which maintain the vascular contractile state [30]. Y-27632 is specific to inhibit Rho-kinase activity with Ki of 0.14–0.22 μM, which are > 100-fold lower compared with PKA, PKC, and MLCK [31,55]. The present work found the inhibitory effect of Y-27632 on BaP-enhanced vasoconstriction elicited by phenylephrine, suggesting that Rho-kinase may also be involved in enhancing aortic vasoconstriction by BaP.

Redox regulation of vasoconstriction has been mentioned for various factors, including angiotensin II, oxidized-LDL, and mechanical stretch [56–58]. Moreover, ROS also possesses direct vasocontractile effects on blood vessels [59,60]. Production of superoxide radical in the vessel wall has been shown to inactivate nitrite oxide, leading to the production of peroxynitrite and impaired endothelium-dependent vasodilation and thus resultant vasoconstriction [61]. ROS is known as a vascular function modulator. Several protein kinase signaling pathways including receptor and non-receptor tyrosine kinases, PKC, MAPK, and Rho-kinase can be modified by ROS [21]. NAD(P)H oxidase-activated ROS production induced by angiotensin II has been found to be enhanced in VSMCs from spontaneously hypertensive rats during hypertension development [20]. H_2O_2-activated PKC activation possesses ability to stimulate arterial vascular smooth muscle L-type Ca^{2+} channels; hypoxia and angiotensin II can also induce PKC-mediated NADPH oxidase-activated mitochondrial ROS production [46]. In the present study, we showed that BaP-enhanced vasoconstriction of aortas in organ culture condition was also through ROS-dependent pathway. The antioxidant NAC was capable of attenuating the stimulatory effect of BaP on vasoconstriction evoked by phenylephrine. Besides, the ROS generation in VSMCs by BaP was also confirmed by flow cytometry using the fluorescent dye DCFH-DA. Moreover, NADPH oxidase inhibitor DPI could also abolish the stimulated ROS generation and vasoconstriction by BaP, suggesting that NAD(P)H oxidase may be one of the sources of ROS production under BaP stimulation by which BaP enhanced vasoconstriction. We also found that BaP enhanced vasoconstriction in endothelium-denuded aortas, suggesting that the stimulated ROS, other than interaction with nitric oxide from endothelium, may act as a secondary messenger to mediate vasoconstriction.

Small resistance arteries play an important role in the regulation of vascular tone and blood pressure [62]. However, the aortic tissue isolated from animals is a common pharmacological model to perform functional contractile response *ex vivo*. There were many studies using this aortic tissue model to examine vascular function such as Birukov et al. (1997; rabbit) [39], De Moudt et al. (2017; mouse) [63], Franchi-Micheli et al. (2000; rat) [64], Gan et al. (2012; rat) [10], and Rapacon-Baker et al. (2001; rat) [65]. In the present study, we verified that benzo[*a*]pyrene exposure possessed the effect of affecting aortic vasoconstriction *ex vivo* in our experimental condition. The major aim of this study is to examine the influence and possible mechanism of benzo[*a*]pyrene on vasoconstriction in rat thoracic aorta organ culture. We further demonstrated the possible molecular mechanism by which benzo[*a*]pyrene affected aortic vasoconstriction. Moreover, Rho kinase inhibitor has been found to prevent vascular contractions in the rat aorta [66]. We also found that Rho-kinase was involved in enhancing aortic vasoconstriction by benzo[*a*]pyrene. Nevertheless, the effects of BaP on vasoconstriction of small resistance arteries can be further investigated in the future.

5. Conclusion

In summary, *in vivo* exposure of rats to BaP enhanced the aortic contractility to phenylephrine and hypertension development. The alteration of vascular smooth muscle function in response to BaP exposure may participate in BaP-induced hypertension. Multiple signaling pathways might be triggered in response to BaP-enhanced vasoconstriction in aortas, including the production of ROS and the activation of PKC, MAPK, MLCK, and Rho-kinase. Based on the findings of previous studies, ROS may regulate the signals of PKC, MAPK, and Rho-kinase; PKC may also activate the ROS generation. These signals further

regulate the activity of MLCK and vascular smooth muscle contraction.

Declaration of Competing Interest

All authors have no conflict of interest.

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