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Oxygen Radicals and Substance P in Perinatal Hypoxia-Exaggerated, Monocrotaline-Induced Pulmonary Hypertension

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Abstract

Perinatal hypoxia has been observed to cause more aggressive pulmonary hypertension in human. Several mediators such as reactive oxygen species (ROS) and substance P are believed to be crucial in the mechanism of inducing pulmonary hypertension. This study was designed to test whether substance P and ROS play a role in perinatal hypoxia-exaggerated, monocrotaline (MCT)-induced pulmonary hypertension. Normoxic Wistar rats (weighing 258 ± 9 g, n = 31) were divided into two groups: control (n = 16) and MCT (n = 15). Perinatal hypoxia Wistar rats (weighing 260 ± 19 g, n = 49) were divided into six groups: Hypoxia (n = 8), Hypoxia+MCT (n = 8), Hypoxia+capsaicin (CP)+MCT (n = 7), Hypoxia+MCT+1,3-dimethyl-2-thiourea $(DMTU)_E$ (n = 10), Hypoxia+MCT+$DMTU_L$ (n = 9), and Hypoxia+MCT+ hexa(sulfobutyl) fullerenes (HSF) (n = 7). Rats in the control group received saline injections. MCT (60 mg/kg, s.c.) was given three weeks prior to the functional examination. Chronic capsaicin pretreatment was performed to deplete substance P. Hydroxyl radical scavenger DMTU (500 mg/kg) was intraperitoneally (i.p.) injected early ($DMTU_E$) or late ($DMTU_L$) after MCT. Antioxidant HSF (10 mg/kg, i.p.) was given once daily for three weeks following MCT. MCT treatment caused significant increases in pulmonary arterial pressure (Ppa) and substance P level in lung tissue in normoxic rats. The MCT-induced increase in pulmonary arterial blood pressure was exaggerated by perinatal hypoxia, but this exaggeration was attenuated by either capsaicin pretreatment or antioxidant administrations. These results suggest that both ROS and substance P are involved in perinatal hypoxia-augmented, MCT-induced pulmonary hypertension.

Key Words: substance P, reactive oxygen species, perinatal hypoxia, pulmonary hypertension, antioxidants

Introduction

Exposure to adverse conditions *in utero* can hamper the development of the fetus. It was hypothesized by Barker (2) that diseases of adults are of fetal and infant origins. It is proposed that alterations to the fetal environment can potentially interfere with fetal development. This, in turn, irreversibly impairs physiological functions and increases susceptibility to disease later in life (2, 3). Many clinical observations support this hypothesis. Smoking during pregnancy is probably the most documented example, and it is being associated with retardation of fetal growth (26, 31, 37, 43, 51). Despite the substances con-

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tained in a cigarette, another important consequence of smoking is perinatal hypoxia (9). Perinatal hypoxia is frequently seen in conditions of preeclampsia and living in high altitude. It is proved that during preeclampsia, harmful factors released from maternal circulation predispose to a pathological response in offspring's cardiovascular system in later life (23, 54). When living in high altitude, greater increase in pulmonary arterial pressure (Ppa) is found in the participants with perinatal hypoxemia than in the controls of matched for age and gender (42). Similar results are also obtained in an animal study (6).

Monocrotaline (MCT) is now widely used in animal studies to induce pulmonary hypertension (7, 47). It is a pyrrolizidine alkaloid, and can induce endothelial inflammation in pulmonary vessels hours after treatment (7, 20, 21, 53); muscularization of arterioles is then detectable within a week post injection (7, 34), finally ending up with pulmonary hypertension with right ventricular hypertrophy by 21 days (7, 34). MCT increases Ppa through the mechanism of pulmonary arterial tree remodeling. Caslin *et al*. (6) demonstrated that rats exposed to perinatal hypoxia exhibited an exaggerated response to MCT in the pulmonary arterial tree (6). However, the underlying mechanisms for the perinatal hypoxia exaggeration of MCT-induced pulmonary hypertension are still unclear. This study is designed to investigate the underlying mechanisms.

Reactive oxygen species (ROS) are presumed to be involved in the cellular mechanism that underlies the pneumotoxicity caused by MCT (1, 7). Animal studies have demonstrated that MCT-induced pulmonary hypertension can be prevented or attenuated by antioxidants (7, 8, 13). Hexa(sulfobutyl)fullerenes (HSF) are water-soluble polyhydroxylated antioxidants (55) that scavenge superoxide (55) and hydroxyl radicals (7, 8, 13). Additionally, 1,3-dimethyl-2-thiourea (DMTU) is a potent diffusing antioxidant with a long half-life that scavenges hydrogen peroxide and hydroxyl radicals (10). In our previous study, administration of HSF and DMTU protects the rats from MCT injury (8). Thus, both antioxidants were used in this study to evaluate the effects on perinatal hypoxia-exaggerated MCT-induced pulmonary hypertension.

Tachykinins, including neurokinin A and substance P (SP), are a family of peptides that share a common C-terminal sequence. SP induces proliferation of vascular smooth muscle cells (38). *In vitro* studies have demonstrated that SP elevates Ppa in a dose-dependent manner (48). SP is a vasoactive substance characterized by angiogenesis and is present in the afferent C-fibers arising from the mammalian lungs (4, 33). SP-ergic neurotransmission plays a role in the development of MCT-induced pulmonary hypertension. An increase in SP levels in lung tissues is observed in the MCT-induced pulmonary hypertension (56). In addition, depletion of tachykinins from afferent C-fibers by capsaicin prevents the development of MCT-induced pulmonary hypertension (8). These findings indicate a close relationship between the SP level and the induction of MCT-induced pulmonary hypertension. Accordingly, we explored whether SP also played a role in MCT-induced pulmonary hypertension in perinatal hypoxia rats.

The aims of the present study were to test the following two possibilities: 1. Perinatal hypoxia-exaggerated, MCT-induced pulmonary hypertension may be closely related to the SP levels of the lung tissue. 2. The exaggeration of MCT-induced pulmonary hypertension may be attenuated by antioxidants, namely DMTU and HSF, by lowering the lung tissue SP level.

Materials and Methods

Animal Preparation

All animal experiments and care were performed according to the principles and guidelines of the Canadian Council on Animal Care. The study was approved by the "Laboratory Animal Care Committee" of the College of Medicine National Taiwan University. Thirty-one normoxic male Wistar rats weighing 258 ± 9 g were divided into two groups: control (n = 16) and MCT (n = 15). Rats in the control group received saline injections. Three weeks before the functional study, when the rats were seven weeks old, MCT (60 mg/kg) was given subcutaneously. To breed perinatal hypoxia rats, pregnant Wistar rats were exposed to intermittent hypoxia by placing them in a decompression chamber with a barometric pressure of 380 Torr for 17 days, from the 5th day to the 21st day of pregnancy. They were exposed to hypoxia from 5 PM to 8 AM each day (intermittent exposure) and to the air the rest of the time. Forty-nine infants were born from these pregnant rats and grew up in normoxic conditions. These perinatal hypoxia rats (weighing 260 ± 19 g) were divided into six groups: Hypoxia (n = 8), Hypoxia+MCT (n = 8), Hypoxia+capsaicin (CP)+MCT (n = 7), Hypoxia+MCT+$DMTU_E$ (n = 10), Hypoxia+MCT+$DMTU_L$ (n = 9), and Hypoxia+MCT+HSF (n = 7). All perinatal hypoxia rats, except those in the Hypoxia+CP+MCT group, were treated starting from the age of 7 weeks. Perinatal hypoxia rats in the Hypoxia and the Hypoxia+MCT groups were treated in the same manner as the normoxic rats. For the Hypoxia+CP+MCT group, capsaicin pretreatment was performed according to the method of Zhou and Lai (56). Briefly, 6-week-old animals continuously received three daily subcutaneous (s.c.) injections of capsaicin; the consecutive daily doses of capsaicin were 50, 100 and 150 mg/kg. These doses were the same in terms of the total dosage as

those employed by Jancso *et al*. (22) to deplete tachykinins in adult rats. During the first two days, the capsaicin injections were administered in small doses as many as six to eight times per day, with at least a one-hour interval between any two injections. Four days after capsaicin pretreatment, the rats were injected with MCT (60 mg/kg, s.c.). DMTU (500 mg/kg, i.p.) treatments were carried out on the 3rd, 5th and 7th days (Hypoxia+MCT+$DMTU_E$), or on the 15th, 17th and 19th days (Hypoxia+MCT+$DMTU_L$) following MCT. Considering the toxicity and short half-life of HSF (50), rats in the Hypoxia+MCT+HSF group received daily injections of HSF (10 mg/kg, i.p.) for three weeks after MCT treatment.

Functional Study

Change in Ppa was used as the index of pulmonary hypertension. Functional measurement of Ppa and the sampling of tissues for measurement of SP were carried out three weeks after the MCT treatment. Rats in both the control and Hypoxia groups were kept for the same length of time as the MCT-treated animals. On the day of the functional tests, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After surgical insertion of a tracheal cannula and a right carotid arterial catheter, artificial ventilation with a respirator at a rate of 60 breaths/min and a tidal volume of 6-8 ml/kg was applied. The chest of the anesthetized-ventilated rat was opened *via* a midline incision. A 22-G needle filled with heparinized saline was inserted through the wall of the right ventricle and advanced into the pulmonary artery (7, 27). Ppa was measured with a Statham pressure transducer with its diaphragm located at the heart level and recorded on a MacLab/200 System. Mean Ppa was calculated according to the formula: diastolic pressure + 1/3 pulse pressure. The systemic mean blood pressure and heart rate were obtained through the right carotid arterial catheter connected with a Statham pressure transducer kept at the heart level and also recorded on the MacLab/200 System. After functional determination, the right ventricle (RV) was separated from the left ventricle and septum (LV+S); both portions were weighed, and the weight ratio, RV/(LV+S), was calculated as the index of right ventricular hypertrophy. To determine the SP level, right lung tissues were obtained immediately after Ppa measurement and stored at -70°C.

Determination of SP by Enzyme Immunoassay (EIA)

SP was first extracted from the thawed frozen lung tissues using the method of Saria *et al*. (41). We subsequently used a commercial EIA kit from Cayman Chemicals (Ann Arbor, MI, USA) to quantify the SP level. For the assay, a 96-well plate was pre-coated with mouse monoclonal antibody. Fifty microliters of SP standard or unknown sample, in duplicate, was added to each well. In addition, 50 µl of SP tracer (SP linked to an acetylcholinesterase molecule) and 50 µl of rabbit antiserum were added to each well. The plate was incubated at 4°C for 18 h. After washing, 200 µl of Ellman's reagent, consisting of acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid), was added to each well. Optimum development was obtained by using an orbital shaker equipped with a large, flat cover to allow the plate to develop in the dark for 90 min. The plate was then read in a microwell reader at 412 nm to detect the amount of 5-thio-2-nitrobenzoic acid. SP concentrations in the samples were determined from the simultaneously obtained SP concentration standard curve.

Morphometric Examinations

For morphometric evaluations, lung samples were taken from each group. The left lung was excised after the functional study and inflated with 4% formaldehyde to maintain 25 cm H_2O pressure for 30 min. Then, the trachea was tied and the lung lobe was immersed in formaldehyde. Tissue blocks were taken from the rostral, middle and caudal portions of the formaldehyde-immersed lung. These blocks were washed, fixed and vacuum embedded in paraffin. From each block, at least three nonconsecutive 2-µm sections were cut, stained with hematoxylin and eosin, and examined by light microscope for the thickness of pulmonary arteries (< 200 µm in diameter) according to the method of Zhou and Lai (56). The external diameter was measured as the distance between the outer edges of the bisected external elastic lamina, and the average external diameter was obtained from two perpendicular external diameter measurements. Thickness of the smooth muscle layer was determined as the distance from the luminal side of the internal elastic lamina to the outside of the external lamina by calculating the average of four measurements made at equidistant points around the artery. Arterial medial thickness was expressed as a ratio of thickness to external diameter (%). The total magnification of the light microscope was ×400. In each rat, at least 10 arteries were measured, and the resulting measurements were averaged.

Statistical Analysis

Data are presented as means ± SEM. Evaluations of differences in Ppa, weight ratio, body weight and hemodynamic parameters between normoxia and perinatal hypoxia rats were carried out by two-way analysis of variance with Scheffe's method for

Table 1. Body weight (BW), systemic hemodynamic parameters and weight ratio of the right ventricle to the left ventricle plus septum [RV/(LV+S)] in rats

	BW (g)	MBP (mmHg)	HR (beats/min)	RV/(LV+S) ratio
Normoxic groups				
C (n = 16)	253 ± 23	123 ± 4	403 ± 15	0.27 ± 0.01
MCT (n = 15)	272 ± 12	123 ± 7	355 ± 15	0.51 ± 0.05*
Perinatal hypoxia groups				
Hypoxia (n = 8)	243 ± 20	124 ± 12	384 ± 26	0.34 ± 0.04
Hypoxia+MCT (n = 8)	255 ± 24	133 ± 10	425 ± 21	0.35 ± 0.02
Hypoxia+CP+MCT (n = 7)	294 ± 19	110 ± 10	373 ± 15	0.51 ± 0.05*
Hypoxia+MCT+$DMTU_E$ (n = 10)	241 ± 20	130 ± 9	406 ± 10	0.40 ± 0.04
Hypoxia+MCT+$DMTU_L$ (n = 9)	251 ± 23	113 ± 7	388 ± 20	0.32 ± 0.02
Hypoxia+MCT+HSF (n =7)	242 ± 21	118 ± 9	404 ± 16	0.39 ± 0.04

Values are means ± SE. n, number of rats; MBP, mean blood pressure; HR, heart rate; C, control; MCT, monocrotaline; Hypoxia, perinatal hypoxic exposure; CP, capsaicin pretreatment; $DMTU_E$, 1,3-dimethyl-2-thiourea (DMTU) injected on the 3rd, 5th, and 7th days after MCT; $DMTU_L$, DMTU injected on the 15th, 17th and 19th days after MCT. HSF, hexa(sulfobutyl)fullerenes. *Significant difference ($P < 0.05$) compared to the respective control group.

post-hoc testing and estimating differences between groups. The differences in Ppa, weight ratio, pulmonary arterial medial thickness, and SP level among groups of MCT-treated perinatal hypoxia rats were established by one-way analysis of variance. Subsequently, significant differences between any two groups were established using Newman-Keuls multiple group comparisons. Differences were regarded as significant if $P < 0.05$.

Results

No significant difference in body weight (BW) or in hemodynamic parameters, including mean blood pressure (MBP) and heart rate (HR), between the experimental groups was found. The ventricular mass, the RV/(LV+S) ratio, showed no statistical difference between the control and Hypoxia groups. MCT administration elevated the right ventricular mass ratio significantly in normoxia but not in perinatal hypoxia rats when compared with the respective control groups. For perinatal hypoxia rats, MCT-induced right ventricular hypertrophy appeared only in rats receiving capsaicin pretreatment (Table 1).

The effects of MCT on Ppa in normoxia and perinatal hypoxia rats are summarized in Fig. 1. No significant difference in Ppa was observed between the control and Hypoxia groups. MCT administration significantly increased Ppa in both normoxia and hypoxia rats compared with their respective control groups. In addition, the increase in Ppa was larger in perinatal hypoxia rats than in normoxia rats indicating that the MCT-induced pulmonary hypertension was exaggerated by perinatal hypoxia exposure (Fig. 1). The Ppa values measured in all groups of perinatal hypoxia rats are summarized in Fig. 2. Compared with the Hypoxia group, MCT induced a significant increase in Ppa that was significantly attenuated by capsaicin, HSF and DMTU (both $DMTU_E$ and $DMTU_L$).

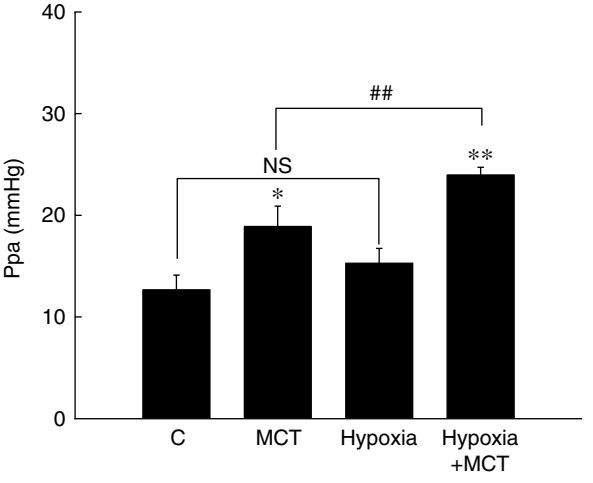

Fig. 1. Pulmonary arterial pressure (Ppa) in normoxia and perinatal hypoxia (Hypoxia) rats. C, control; MCT, monocrotaline; Bars indicate 1 SE. MCT administration caused a significant increase in Ppa in normoxia rats (*$P < 0.05$) and in perinatal hypoxia rats (**$P < 0.01$). A significant difference existed ($^{\#\#}P < 0.01$) between perinatal hypoxia and normoxia rats treated with MCT.

The levels of SP within lung tissues in all rats were investigated (Fig. 3). Control and Hypoxia groups had similar tissue SP levels. MCT induced a significant increase in the tissue SP level in the control rats, but

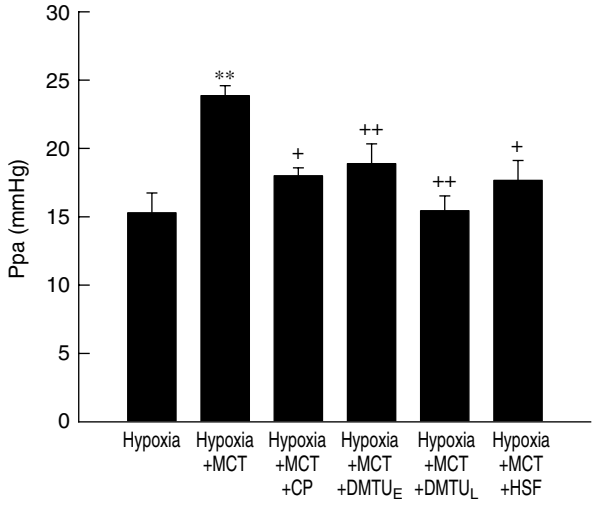

Fig. 2. Pulmonary arterial pressure (Ppa) in six groups of perinatal hypoxia (Hypoxia) rats. MCT, monocrotaline; CP, capsaicin pretreatment; DMTU$_E$, 1,3-dimethyl-2-thiourea (DMTU) was injected on the 3rd, 5th and 7th days after MCT; DMTU$_L$, DMTU was injected on the 15th, 17th and 19th days after MCT; HSF, hexa (sulfobutyl)fullerenes. Bars indicate 1 SE. **Significant difference ($P < 0.01$) compared with the Hypoxia group. Significant difference compared with the Hypoxia+MCT group: $^{+}P < 0.05$ and $^{++}P < 0.01$.

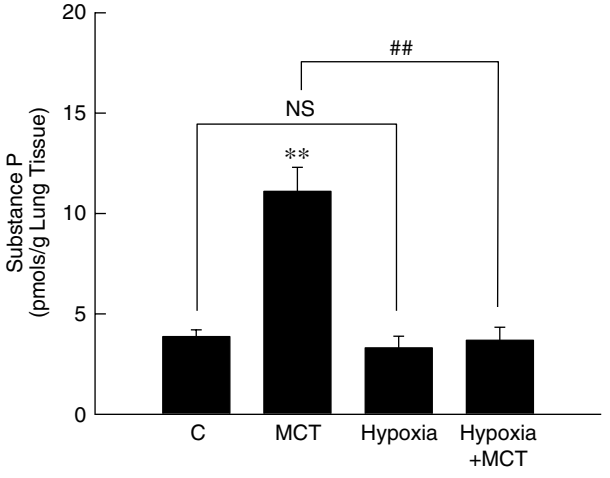

Fig. 3. Lung substance P levels in normoxic and perinatal hypoxia (Hypoxia) rats. C, control; MCT, monocrotaline. Bars indicate 1 SE. MCT administration caused a significant increase in substance P level in normoxia rats ($^{**}P < 0.01$). A significant difference ($^{\#\#}P < 0.01$) existed between perinatal hypoxia and normoxia rats treated with MCT.

not in the perinatal hypoxia rats. For the perinatal hypoxia rats, although the MCT-induced increase in the tissue SP levels were blunted, capsaicin pretreatment (Hypoxia+CP+MCT) and the antioxidants DMTU and HSF significantly decreased the SP levels when

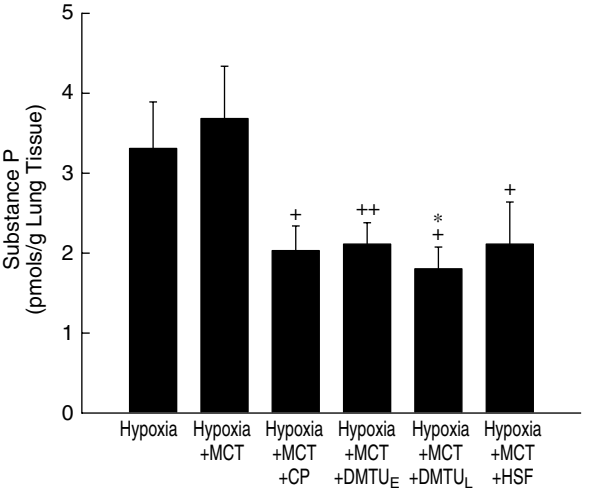

Fig. 4. Lung substance P levels in six groups of perinatal hypoxia (Hypoxia) rats. MCT, monocrotaline; CP, capsaicin pretreatment; DMTU$_E$, 1,3-dimethyl-2-thiourea (DMTU) was injected on the 3rd, 5th and 7th days after MCT; DMTU$_L$, DMTU was injected on the 15th, 17th and 19th days after MCT; HSF, hexa(sulfobutyl) fullerenes. Bars indicate 1 SE. *Significant difference ($P < 0.05$) compared with the Hypoxia group. Significant difference compared with the Hypoxia+MCT group: $^{+}P < 0.05$ and $^{++}P < 0.01$.

compared with the MCT-treated group (Fig. 4).

The magnitude of pulmonary arterial remodeling was evaluated by the arterial medial thickness (Figs. 5 and 6). As shown in Fig. 5, morphometry images revealed more severe proliferation in the smooth muscle layer of the pulmonary artery in the Hypoxia+MCT group (B) than in the Hypoxia group (A). Furthermore, capsaicin pretreatment and antioxidant administration (DMTU$_E$, DMTU$_L$ and HSF) prevented the progression of this proliferation. Mean vessel wall thicknesses from the groups of perinatal hypoxia rats are summarized in Fig. 6. Compared to the Hypoxia group, MCT injection significantly increased the arterial medial thickness, but this effect was significantly attenuated by capsaicin pretreatment and antioxidant administration (DMTU$_E$, DMTU$_L$ and HSF) (Fig. 6).

Discussion

This study confirmed that MCT administration in normoxia rats causes increases in Ppa and lung tissue SP levels (Figs. 1 and 3). Perinatal hypoxia exaggerated the MCT-induced increase in Ppa but did not affect the lung tissue SP levels (Figs. 1 and 3). Our study also yielded several notable findings. The perinatal hypoxia-exaggerated, MCT-induced pulmonary hypertension was attenuated significantly by capsaicin pretreatment and antioxidants (Fig. 2). Furthermore, in comparison with the Hypoxia+MCT

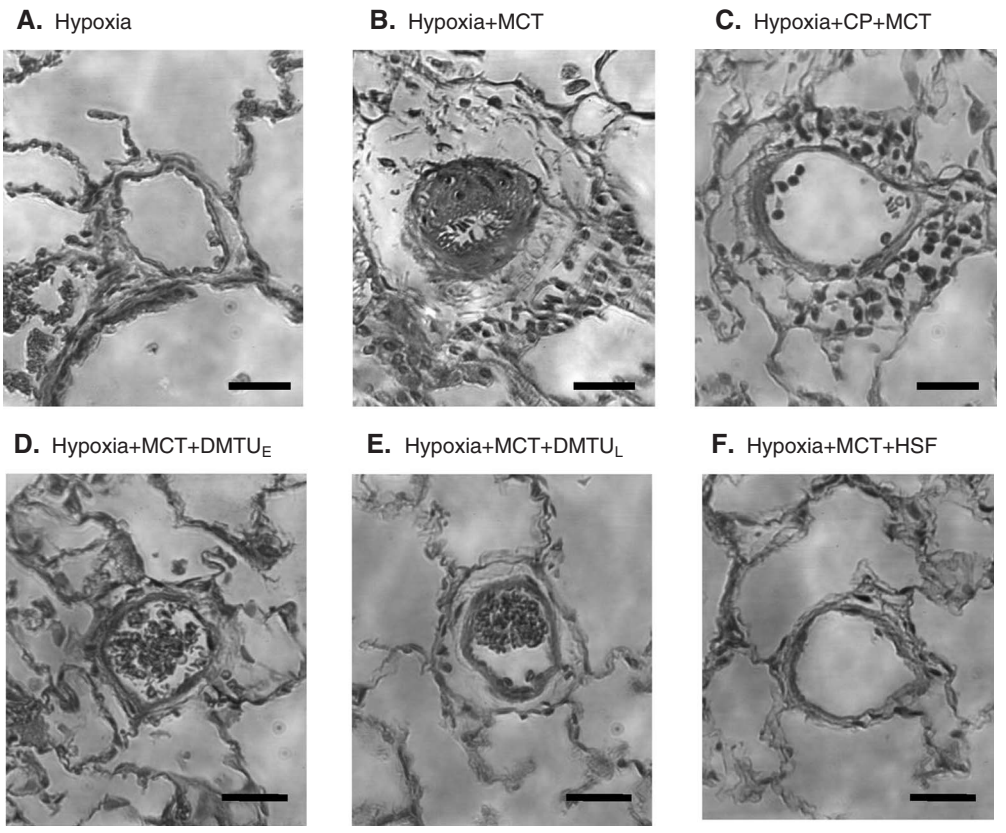

Fig. 5. Lung histology of perinatal hypoxia (Hypoxia) rats in six groups. MCT, monocrotaline; CP, capsaicin pretreatment; DMTU$_E$, 1,3-dimethyl-2-thiourea (DMTU) was injected on the 3rd, 5th and 7th days after MCT; DMTU$_L$, DMTU was injected on the 15th, 17th and 19th days after MCT. HSF, hexa(sulfobutyl)fullerenes (HSF). Bar indicates 50 μm, ×400.

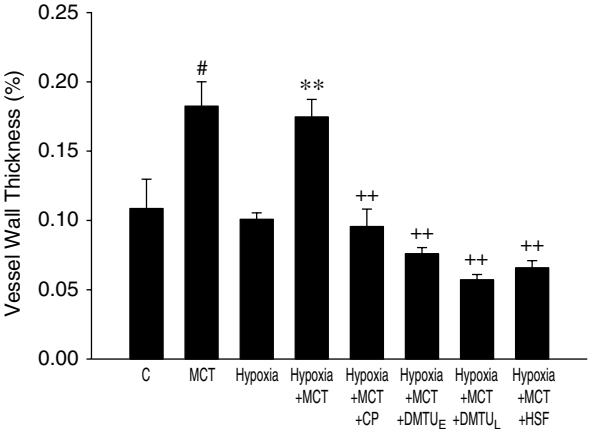

Fig. 6. Pulmonary artery (< 200 μm in diameter) medial thickness (%) in all groups of rats. C, control; MCT, monocrotaline; Hypoxia, perinatal hypoxia; CP, capsaicin pretreatment; DMTU$_E$, 1,3-dimethyl-2-thiourea (DMTU) was injected on the 3rd, 5th and 7th days after MCT; DMTU$_L$, DMTU was injected on the 15th, 17th and 19th days after MCT. HSF, hexa(sulfobutyl)fullerenes. Bars indicate 1 SE. $^{\#}$Significant difference ($P < 0.05$) compared with the Control group. **Significant difference ($P < 0.01$) compared with the Hypoxia group. $^{++}$Significant difference ($P < 0.01$) compared with the Hypoxia+MCT group.

group, both capsaicin pretreatment and antioxidants significantly decreased lung SP levels in MCT-treated perinatal hypoxia rats (Fig. 4).

Our study agrees with previous investigations that more severe pulmonary vascular disorders appear in tested animals that have ever been exposed to hypoxia perinatally, even though they are similar in resting conditions (6, 39, 42, 49). The increase in Ppa, measured by echocardiography at high altitude challenge, was greater in participants who had had perinatal hypoxemia than in the controls of matched age and gender (42, 54). These clinical results suggested that transient perinatal hypoxia, an insult to the pulmonary circulation, left a persistent imprint, which, when activated in adult life, predisposed to a greater pathological response than those without similar history (42). Similar results were obtained in different systems (39) and species (6, 49, 54). MCT-induced remodeling of pulmonary vessels was exaggerated in perinatal hypoxia rats compared to normoxia rats. However, the morphometric observations were similar in the two control groups prior to MCT (6, 7). Similar augmentation of Ppa in perinatal hypoxia rats was also performed in a hypoxia-induced pulmonary hypertension model (31, 49). In addition, the stress-

induced increases in the systemic blood pressure and heart rate were significantly enhanced in the perinatal hypoxic rats when compared with the normoxia rats, though those parameters did not alter under resting conditions (39). In this study, compared to normoxia rats, the MCT-induced increase in Ppa in perinatal hypoxia rats was statistically exaggerated (Fig. 1). Previous studies have demonstrated that rats exposed to hypoxia perinatally had elevated pulmonary vascular reactivity to hypoxia (14, 15). The elevated ROS as well as the decrease level of antioxidants were demonstrated recently in neonates from mother with preeclampsia (19). Since a decrease in O_2 tension in MCT-treated rats has been reported (17, 28), we propose that the exaggeration of the MCT-induced increase in Ppa is attributed to an augmentation of vascular reactivity to hypoxia in MCT-treated, perinatally hypoxia-exposed rats.

We suggest that the perinatal hypoxia-exaggerated, MCT-induced pulmonary hypertension is related to an enhanced effect of SP in the pulmonary arteries (8), which in turn activates the rennin-angiotensin system (12). The increased sensitivity to SP of pulmonary arterial tree in the MCT-rats was demonstrated in our previous study (8). SP is able to produce a receptor-mediated proliferative effect on connective tissues (35) and smooth muscle cells (38). The production of SP *via* leukotrienes is also related to ROS (40) subsequently resulting in peripheral vascular remodeling. Besides, it is able to induce the production of angiotensin II by a mixed population of isolated cardiac inflammatory cells, including mast cells, lymphocytes and macrophages (29), which are also identified in the MCT-induced pulmonary hypertension (35). Another piece of evidence is that the antenatal hypoxia results in enhancing the sensitivity of the rennin-angiotensin system in pulmonary circulation (12). In response to antenatal hypoxia for 48 h, the obvious increases in the mRNA and protein levels of angiotensin-converting enzyme, angiotensin II receptors, and rennin were identified in FVB/NJ mice (12). Our suggestion is supported by the fact presented in this study that decrease in SP by capsaicin or antioxidants prevented the development of perinatal hypoxia-exaggerated, MCT-induced pulmonary hypertension (Figs. 2 and 4). Incidentally, a lower level of nitric oxide synthase was detected in adult rats that experienced hypoxia in infancy (46), indicating impairment in the vasodilatation. Consistent with the above papers and our findings, the reduced SP level below the normal range prevents development of pulmonary hypertension.

We also demonstrated the involvement of ROS in perinatal hypoxia-exaggerated, MCT-induced pulmonary hypertension. ROS has been demonstrated to play a role in the development of MCT-induced pulmonary hypertension (7, 8). A rapid inflammatory response with notable increases in monocytes in the adventitia of pulmonary arterioles was found within 8-16 h after MCT injection (53). The proliferative muscularization of arterioles was then detectable as early as 3 and 7 days post injection (31, 34) and reached significance by 10 and 14 days. By compensatory reflex, the RV hypertrophy was apparent by 21 days (31, 34). Therefore, the increased amounts of ROS promote the development of pulmonary hypertension through the effects of inflammation (18, 44) in the early stage, and proliferation (16, 31) in the later stage. Another clue was performed in lung tissues which had had experienced oil smoking (30). The NK 1 receptor antagonist, L733060, prevented the increase in the amount of ROS in plasma (30). In addition, perinatal hypoxia decreased the expression and activity of superoxide dismutase (11), but increased the preproendothelin-1 mRNA in the lung (46). It is interesting that the production and function of endothelin-1, an important role in the development of MCT-induced pulmonary hypertension (17), are mediated by ROS (24). Accordingly, poor tolerance to ROS in perinatal hypoxia rats is reasonable. Hence, we used antioxidants to attenuate the syndrome of pulmonary hypertension. The water-soluble HSF shows not only a potent free radical scavenger (55) but also expresses antiproliferative effects through inhibiting protein tyrosine kinase (32). In the present study, treatment with HSF significantly attenuated the perinatal hypoxia-exaggerated, MCT-induced pulmonary hypertension (Table 1, Figs. 2 and 4). The rapid inflammatory response was found very soon after MCT injection (53), another antioxidant, DMTU, was designed to administer in the early phase. We suggest that the property of ROS scavenger should contribute to the attenuating effect on the MCT-induced pulmonary hypertension in the Hypoxia+MCT+$DMTU_E$ group. In addition to oxygen scavenging activities, DMTU inhibits cell mitogenesis by altering membrane integrity (45). Moreover, DMTU induces several effects, including reduction of cell growth rate (36), suppression of lung collagen synthesis (52) and promotion of cellular differentiation (36). These anti-proliferative (not antioxidant) and differentiation-promoting effects of DMTU are related to the characteristics occurring in the late phase of the development of MCT-induced pulmonary hypertension. Therefore, we propose that the attenuating effect on pulmonary hypertension in the Hypoxia+MCT+$DMTU_L$ group is contributed by the anti-proliferative and differentiation-promoting effects of DMTU. In contrast to our results, Brunner and colleagues investigated the effects of antioxidants catalase and dimethyl sulfoxide (DMSO) on MCT-induced pneumotoxicity and reported that DMSO

failed to prevent MCT-induced pneumotoxicity (5). Warren *et al*. (52) demonstrated that DMTU has a very long half-life in rats (> 24 hours) compared to the short half-life of DMSO and other scavengers. In addition, Bruner *et al*. (5) evaluated changes in the lung weight ratio one week after MCT pyrrole injection in normoxia rats, whereas we measured the change in Ppa three weeks after MCT injection in perinatal hypoxia rats. Therefore, we attribute the difference between our findings and those of Bruner *et al*. (5) to different experimental conditions and indicators.

The impairment of compensatory response in the perinatal hypoxia rats caused the disappearance of MCT-induced RV hypertrophy in this study. The perinatal hypoxia exposure caused a slight but persistent impairment of the development of the heart as well as the compensatory response to the increase in the Ppa (39). Comparing to the normoxic rats, the utilization rates of noradrenaline were significantly reduced in the heart, lungs, superior cervical ganglion, stellate ganglion and celiac ganglion in the perinatal hypoxia rats (39). Those ganglions were related to the development in cardiac structures and the adrenals (39). Unfortunately, the lowered metabolic activity persisted till adulthood (12-week-old) in the stellate ganglion and heart (39). Under stress challenges, the exaggerated variability of blood pressure is considered to be the main consequence of any intervention interrupting the baroreflex loop (39). Therefore, there was no difference in the weight ratio of RV/(LV+S) between the Hypoxia and Hypoxia+MCT groups (Table 1). On the other hand, capsaicin pretreatment depletes not only SP but also the calcitonin gene-related peptide. Complete depletion of calcitonin gene-related peptide from cardiac sensory nerves after systemic capsaicin treatment leads to deterioration of adriamycin-induced heart failure (25). The absence of calcitonin gene-related peptide in rats of the Hypoxia+CP+MCT group induced the RV hypertrophy (Table 1).

In summary, we have found that perinatal hypoxia can exaggerate MCT-induced pulmonary hypertension. Reducing the SP level in lung tissues and administering antioxidants are useful ways to attenuate the augmented MCT-induced pulmonary hypertension in perinatal hypoxia rats.

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References

1. Aziz, S.M., Toborek, M., Hennig, B., Mattson, M.P., Guo, H. and Lipke, D.W. Oxidative stress mediates monocrotaline-induced alterations in tenascin expression in pulmonary artery endothelial cells. *Int. J. Biochem. Cell Biol*. 29: 775-787, 1997.
2. Barker, D.J. The fetal and infant origins of adult disease. *Brit. Med. J*. 301: 1111, 1990.
3. Barker, D.J. Fetal origins of coronary heart disease. *Brit. Med. J*. 311: 171-174, 1995.
4. Barnes, P.J. and Liu, S.F. Regulation of pulmonary vascular tone. *Pharmacol. Rev*. 47: 87-131, 1995.
5. Bruner, L.H., Johnson, K., Carpenter, L.J. and Roth, R.A. Lack of effect of deferoxamine, dimethyl sulfoxide, and catalase on monocrotaline pyrrole pulmonary injury. *J. Toxicol. Environ. Health* 21: 205-217, 1987.
6. Caslin, A., Heath, D. and Smith, P. Influence of hypobaric hypoxia in infancy on the subsequent development of vasoconstrictive pulmonary vascular disease in the Wistar albino rat. *J. Pathol*. 163: 133-141, 1991.
7. Chen, K.H., Chen, Y.J., Yang, C.H., Liu, K.W., Chang, J.L., Pan, S.F., Lin, T.B. and Chen, M.J. Attenuation of the extract from *Moringa oleifera* on monocrotaline-induced pulmonary hypertension in rats. *Chinese J. Physiol*. 55: 22-30, 2012.
8. Chen, M.-J., Chiang, L.Y. and Lai, Y.-L. Reactive oxygen species and substance P in monocrotaline-induced pulmonary hypertension. *Toxicol. Appl. Pharmacol*. 171: 165-173, 2001.
9. Cnattingius, S. and Nordstrom, M.L. Maternal smoking and feto-infant mortality: biological pathways and public health significance. *Acta Paediatr*. 85: 1400-1402, 1996.
10. Fox, R.B. Prevention of granulocyte-mediated oxidant lung injury in rats by a hydroxyl radical scavenger, dimethylthiourea. *J. Clin. Invest*. 74: 1456-1464, 1984.
11. Giles, B.L., Suliman, H., Mamo, L.B., Piantadosi, C.A., Oury, T.D. and Nozik-Grayck, E. Prenatal hypoxia decreases lung extracellular superoxide dismutase expression and activity. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 283: L549-L554, 2002.
12. Goyal, R., Leitzke, A., Goyal, D., Gheorghe, C.P. and Longo, L.D. Antenatal maternal hypoxic stress: adaptations in fetal lung renin-angiotensin system. *Reprod. Sci*. 18: 180-189, 2011.
13. Guo, H., Hu, L.M., Wang, S.X., Wang, Y.L., Shi, F., Li, H., Liu, Y., Kang, L.Y. and Gao, X.M. Neuroprotective effects of scutellarin against hypoxic-ischemic-induced cerebral injury *via* augmentation of antioxidant defense capacity. *Chinese J. Physiol*. 54: 399-405, 2011.
14. Hakim, T.S. and Mortola, J.P. Pulmonary vascular resistance in adult rats exposed to hypoxia in the neonatal period. *Can. J. Physiol. Pharmacol*. 68: 419-424, 1990.
15. Hampl, V. and Herget, J. Perinatal hypoxia increases hypoxic pulmonary vasoconstriction in adult rats recovering from chronic exposure to hypoxia. *Am. J. Respir. Crit. Care Med*. 142: 619-624, 1990.
16. Herbert, J.M., Bono, F. and Savi, P. The mitogenic effect of H_2O_2 for vascular smooth muscle cells is mediated by an increase of the affinity of basic fibroblast growth factor for its receptor. *FEBS Lett*. 395: 43-47, 1996.
17. Hill, N.S., Warburton, R.R., Pietras, L. and Klinger, J.R. Nonspecific endothelin-receptor antagonist blunts monocrotaline-induced pulmonary hypertension in rats. *J. Appl. Physiol*. 83: 1209-1215, 1997.
18. Hotter, G., Closa, D., Prats, N., Pi, F., Gelpi, E. and Rosello-Catafau, J. Free radical enhancement promotes leucocyte recruitment through a PAF and LTB_4 dependent mechanism. *Free Radic. Biol. Med.* 22: 947-954, 1997.
19. Howlader, M.Z., Parveen, S., Tamanna, S., Khan, T.A. and Begum, F. Oxidative stress and antioxidant status in neonates born to pre-eclamptic mother. *J. Trop. Pediatr*. 55: 363-367, 2009.

20. Hurley, J.V. and Jago, M.V. Pulmonary oedema in rats given dehydromonocrotaline: a topographic and electron-microscope study. *J. Pathol*. 117: 23-32, 1975.
21. Ito, K.M., Sato, M., Ushijima, K., Nakai, M. and Ito, K. Alterations of endothelium and smooth muscle function in monocrotaline-induced pulmonary hypertensive arteries. *Am. J. Physiol. Heart Circ. Physiol*. 279: H1786-H1795, 2000.
22. Jancso, G., Kiraly, E., Joo, F., Such, G. and Nagy, A. Selective degeneration by capsaicin of a subpopulation of primary sensory neurons in the adult rat. *Neurosci. Lett*. 59: 209-214, 1985.
23. Jayet, P.Y., Rimoldi, S.F., Stuber, T., Salmòn, C.S., Hutter, D., Rexhaj, E., Thalmann, S., Schwab, M., Turini, P., Sartori-Cucchia, C., Nicod, P., Villena, M., Allemann, Y., Scherrer, U. and Sartori, C. Pulmonary and systemic vascular dysfunction in young offspring of mothers with preeclampsia. *Circulation* 122: 488-494, 2000.
24. Kahler, J., Mendel, S., Weckmuller, J., Orzechowski, H.D., Mittmann, C., Koster, R., Paul, M., Meinertz, T. and Munzel, T. Oxidative stress increases synthesis of big endothelin-1 by activation of the endothelin-1 promoter. *J. Mol. Cell. Cardiol.* 32: 1429-1437, 2000.
25. Katona, M., Boros, K., Sántha, P., Ferdinandy, P., Dux, M. and Jancsó, G. Selective sensory denervation by capsaicin aggravates adriamycin-induced cardiomyopathy in rats. *Naunyn-Schmiedebergs Arch. Pharmacol*. 370: 436-443, 2004.
26. Koren, G. Fetal toxicology of environmental tobacco smoke. *Curr. Opin. Pediatr*. 7: 128-131, 1995.
27. Lai, Y.-L., Chen, C.-F., Chien, C.-T., Shiao, H.-L., Thacker, A.A. and Zhang, H.Q. Capsaicin pretreatment attenuates chronic hypoxic pulmonary hypertension. *Respir. Physiol*. 99: 283-289, 1995.
28. Lai, Y.-L., Thacker, A.A. and Diana, J.N. Hypoxemia and elevated tachykinins in rat monocrotaline pneumotoxicity. *Lung* 174: 195-203, 1996.
29. Levick, S.P., Murray, D.B., Janicki, J.S. and Brower, G.L. Sympathetic nervous system modulation of inflammation and remodeling in the hypertensive heart. *Hypertension* 55: 270-276, 2010.
30. Li, P.-C., Lai, I.-J., Lin, Y.-C., Chang, L.-C. and Chen, W.-C. Substance P scavenger enhances antioxidant defenses and prevents prothrombotic effects on the rat lung after acute exposure to oil smoke. *J. Biomed. Sci*. 16: 58-68, 2009.
31. Liu, Y., Zhang, B., Dong, M.Q., Niu, W., Luo, Y., Gao, Y.Q. and Li, Z.C. Attenuation of pulmonary arterial smooth muscle cell proliferation following hypoxic pulmonary hypertensia by the Na^+/H^+ exchange inhibitor amiloride. *Chinese J. Physiol.* 53: 36-44, 2010.
32. Lo, Y.K. and Huang, H.T. A novel procedure for mediastinal vagotomy inhibits neurogenic inflammation in rat bronchial tree. *J. Auton. Nerv. Syst*. 67: 79-88, 1997.
33. Maggi, C.A., Giachetti, A., Dey, R.D. and Said, S.I. Neuropeptides as regulators of airway function: vasoactive intestinal peptide and the tachykinins. *Physiol. Rev*. 75: 277-322, 1995.
34. Meyrick, B. and Reid, L. Development of pulmonary arterial changes in rats fed *Crotalaria spectabilis*. *Am. J. Pathol*. 94: 37-50, 1979.
35. Nilsson, J., von Euler, A.M. and Dalsgaard, C.J. Stimulation of connective tissue cell growth by substance P and substance K. *Nature* 315: 61-63, 1985.
36. Nordenberg, J., Aloni, D., Wasserman, L., Beery, E., Stenzel, K.H. and Novogrodsky, A. Dimethylthiourea inhibition of melanoma cell growth *in vitro* and *in vivo*. *J. Natl. Cancer Inst*. 75: 891-895, 1985.
37. Olsen, J. Cigarette smoking in pregnancy and fetal growth. Does the type of tobacco play a role? *Int. J. Epidemiol*. 21: 279-284, 1992.
38. Payan, D.G. Receptor-mediated mitogenic effects of substance P on cultured smooth muscle cells. *Biochem. Biophys. Res. Commun*. 130: 104-109, 1985.
39. Peyronnet, J., Dalmaz, Y., Ehrström, M., Mamet, J., Roux, J.C., Pequignot, J.M., Thorén, H.P. and Lagercrantz, H. Long-lasting adverse effects of prenatal hypoxia on developing autonomic nervous system and cardiovascular parameters in rats. *Pflugers Arch.* 443: 858-865, 2002.
40. Sanderud, J., Kumlin, M., Granstrom, E. and Saugstad, O.D. Effects of oxygen radicals on cysteinyl leukotriene metabolism and pulmonary circulation in young pigs. *Eur. Surg. Res*. 27: 117-126, 1995.
41. Saria, A., Martling, C.R., Yan, Z., Theodorsson-Norheim, E., Gamse, R. and Lundberg, J.M. Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylphenyl piperazinium, and vagal nerve stimulation. *Am. J. Respir. Crit. Care Med.* 137: 1330-1335, 1988.
42. Sartori, C., Allemann, Y., Trube, L., Delabays, A., Nicod, P. and Scherrer, U. Augmented vasoreactivity in adult life associated with perinatal vascular insult. *Lancet* 26: 2205-2207, 1999.
43. Secker-Walker, R.H., Solomon, L.J., Flynn, B.S., Skelly, J.M., Lepage, S.S., Goodwin, G.D. and Mead, P.B. Smoking relapse prevention counseling during prenatal and early postnatal care. *Am. J. Prev. Med*. 11: 86-93, 1995.
44. Sen, C.K. and Packer, L. Antioxidant and redox regulation of gene transcription. *FASEB J*. 10: 709-720, 1996.
45. Shoker, A.S., Murabit, M.A., Georges, F.F., Qualtiere, L.F., Deneer, H.G. and Prasad, K. Inhibition of human lymphocyte function by organic solvents. *Mol. Cell. Biochem*. 171: 49-58, 1997.
46. Smith, A.P.L., Emery, C.J. and Higenbottam, T.W. Perinatal chronic hypoxia decreases endothelial nitric oxide synthase (NOS III) and increase preproendothelin-1 (ppET-1) mRNA levels in the rat (Abstract). *Eur. Respir. J*. 10: 433s, 1997.
47. Stenmark, K.R., Morganroth, M.L., Remigio, L.K., Voelkel, N.F., Murphy, R.C., Henson, P.M., Mathias, M.M. and Reeves, J.T. Alveolar inflammation and arachidonate metabolism in monocrotaline-induced pulmonary hypertension. *Am. J. Physiol.* 248: H859-H866, 1985.
48. Tanaka, D.T. and Grunstein, M.M. Vasoactive effects of substance P on isolated rabbit pulmonary artery. *J. Appl. Physiol*. 58: 1291-1297, 1985.
49. Tang, J.R., Le Cras, T.D., Morris, K.G., Jr. and Abman, S.H. Brief perinatal hypoxia increases severity of pulmonary hypertension after reexposure to hypoxia in infant rats. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 278: L356-L364, 2000.
50. Ueng, T.-H., Kang, J.-J., Wang, H.-W., Cheng, Y.-W. and Chiang, L.Y. Suppression of microsomal cytochrome P450-dependent monooxygenases and mitochondrial oxidative phosphorylation by fullerenol, a polyhydroxylated fullerene C60. *Toxicol. Lett*. 93: 29-37, 1997.
51. Wald, N.J. and Hackshaw, A.K. Cigarette smoking: an epidemiological overview. *Brit. Med. Bull*. 52: 3-11, 1996.
52. Warren, D.L., Hyde, D.M. and Last, J.A. Synergistic interaction of ozone and respirable aerosols on rat lungs. IV. Protection by quenchers of reactive oxygen species. *Toxicology* 53: 113-133, 1988.
53. Wilson, D.W., Segall, H.J., Pan, L.C. and Dunston, S.K. Progressive inflammatory and structural changes in the pulmonary vasculature of monocrotaline-treated rats. *Microvasc. Res*. 38: 57-80, 1989.
54. Wu, W.Z., Guo, X.M., Xie, M.L., Xiao, Z.F., Yang, Y. and Xiao, S.Z. Research on first heart sound and second heart sound amplitude variability and reversal phenomenon-a new finding in athletic heart study. *J. Med. Biol. Eng*. 29: 202-205, 2009.
55. Yu, C., Bhonsle, J.B., Canteenwala, T., Huang, J.-P., Shiea, J., Chen, B.J. and Chiang, L.Y. Novel water-soluble HSF as potent free radical scavengers. *Chem. Lett.* 455-465, 1998.
56. Zhou, K.R. and Lai, Y.-L. Capsaicin pretreatment attenuates monocrotaline-induced ventilatory dysfunction and pulmonary hypertension. *J. Appl. Physiol*. 75: 2781-2788, 1993.