Research Article

Electrical field stimulation-induced excitatory responses of pulmonary artery rings from monocrotaline-induced pulmonary hypertensive rats: influence of the endothelium

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ABSTRACT

Background: Nitric oxide-mediated endothelium-dependent relaxation is attenuated in pulmonary artery segments from monocrotaline (MCT)-induced pulmonary hypertensive rats. However, the influence of the endothelium on adrenergic neurotransmission in the rat pulmonary artery has not been investigated. The aim of the present study was to investigate the effect of the endothelium on electrical field stimulation (EFS)-induced excitatory responses of pulmonary artery segments from pulmonary hypertensive rats. Methods: Pulmonary hypertension was induced in rats with a single dose of monocrotaline (60 mg/kg) and 21 days later, arterial rings were set up for isometric tension recording. EFS-induced contractions were recorded in the presence or absence of drugs. Results: Electrical field stimulation (EFS) induced frequency-dependent contractions in artery segments from control rats and these contractions were not affected by removing the endothelium. L-NAME (10^{-4} M), a non-selective NO synthase inhibitor, but not 7-NI, a selective neuronal NO synthase inhibitor, potentiated EFS-induced contractions. In addition, L-NAME had no effect on EFS-induced contractions in artery segments without the endothelium indicating a role for endothelium-derived NO in modulating adrenergic neurotransmission in the pulmonary artery. EFS also induced frequency-dependent contractions of artery segments from pulmonary hypertensive rats. These contractions, expressed relative to KCl-induced contractions, were greater in artery segments from pulmonary hypertensive rats. L-NAME (10^{-4} M) potentiated EFS-induced contractions of artery segments from MCT-treated rats and did not discriminate between artery segments from control and MCT-treated rats. L-NAME potentiated noradrenaline-induced contractions in artery segments from both groups indicating that the effect of L-NAME was mediated post-junctionally. Conclusion: Monocrotaline-induced pulmonary hypertension is associated with enhanced contractile response to EFS. In addition, the modulatory effect of endothelial nitric oxide is unaltered in artery segments from pulmonary hypertensive rats.

INTRODUCTION

Pulmonary hypertension (PH) is associated with decreased vascular compliance, elevated pulmonary artery pressure, right heart failure and eventually, death. It is characterized by vasoconstriction of the pulmonary arteries, vascular remodeling and structural changes resulting from smooth muscle proliferation and migration (Salvi, 1999). Endothelial dysfunction is a prominent feature of pulmonary hypertension and several studies have documented decreased endothelium-dependent nitric oxide (NO)-mediated vascular smooth muscle relaxation in various models of pulmonary hypertension (Dinh-Xuan et al., 1990; Adnot et al., 1991; Nakazawa et al., 1999; Ito et al., 2000).

The pulmonary circulation is richly innervated by sympathetic nerves (Boe and Simonsson, 1980; Colucci et al., 1981; Salvi, 1999). It has been reported that α₁-
adrenoceptor antagonists reduce pulmonary vascular resistance (Colucci et al. 1981) which would explain why α1-adrenergic antagonists have been used successful in the treatment of pulmonary hypertension (Kanemoto et al., 1984; Lewczuk et al., 1992; Alpert et al., 1994; Inoue et al., 1994; Salvi, 1999; Ishikawa et al., 2009). The fact that chemical sympathectomy reduced right ventricular hypertrophy induced by monocrotaline in rats (Tucker et al., 1983) would support a role for enhanced sympathetic nerve activity in the pathobiology of pulmonary hypertension. Later studies have shown that noradrenaline in the pathobiology of pulmonary hypertension. Despite all these observations, the possible role of the sympathetic nervous system in the pathobiology of pulmonary hypertension has not received much consideration. This is probably because of the variable results obtained with respect to the circulating levels of noradrenaline (Haneda et al., 1983; Nagaya et al., 2000; Nootens et al., 1995; Richards et al., 1990; Velez-Roa et al., 2004). However, using microneurographic techniques, Velez-Roa et al. (2004) have reported elevated sympathetic nerve activity in patients with advanced pulmonary hypertension. Mak et al. (2011) have made similar observations using radiotracer techniques to measure cardiac and total body sympathetic activity in patients with cardiac failure. To date, most of the studies on vascular reactivity in pulmonary hypertension have focused mainly on agonist-induced (contraction/relaxation) responses and not much has been done on the effect of pulmonary hypertension on electrical field stimulation-induced contractions of the pulmonary artery. The main objective of the present study was therefore to investigate the effect of monocrotaline-induced pulmonary hypertension in rats on the excitatory response of the pulmonary artery to electrical field stimulation. Included in this study was the possible role of the endothelium in modulating these responses.

METHODS

Animals:
Adult male rats (200-300 g) were used in this investigation. These rats were bred and maintained under internationally accepted conditions in the animal resource center of the Faculty of Medicine, Kuwait University. This study was approved by the institution's research ethics committee. The rats had free access to food and water.

Induction of pulmonary hypertension:
Pulmonary hypertension was induced by treating the rats with a single intraperitoneal injection of monocrotaline (60 mg/kg) and used 3 wks later. Age-matched rats, not treated with monocrotaline, were used as controls.

Isolation and preparation of arterial ring segment:
On the day of the experiments, each rat was anaesthetized with sodium pentobarbitone (35 mg/kg i.v.). The thoracic cavity was opened up and the lungs together with the heart were removed en-bloc and placed in a Petri dish containing cold Krebs' solution. After carefully isolating the extralobar pulmonary arteries, the heart was weighed and the ratio of heart weight to body weight was used as an index of cardiac hypertrophy. The pulmonary artery segments were then carefully cleared of any adhering connective tissues without destroying the endothelium. However, in some experiments, the endothelium was removed by gently rubbing the artery lumen with fine wire to examine the role of the endothelium on vascular reactivity. In these instances, endothelial denudation was confirmed by the inability of acetylcholine (10⁻⁶ M) to relax artery segments contracted with noradrenaline (10⁻⁷ M). Artery ring segments (3-4 mm in length) were set up in 25.0 ml tissue baths containing Krebs’ solution (NaCl, 119; KCl, 4.7; NaHCO₃, 25; KH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 2.5 and glucose, 11 mM) at 37°C. The solution was continuously gassed with a 5% CO₂/95% O₂ mixture and the pH was approximately 7.4. Desipramine (10⁻⁷ M) and propranolol (10⁻⁶ M) were included in the Krebs' solution to block neuronal uptake and β-adrenoceptors respectively. The preparations were allowed to equilibrate under a resting tension of 1.0 g for up to 60 min during which the bath fluid was changed at least once. Isometric contractions were recorded through dynamometer UF1 transducers on a Lectromed 4-channel polygraph (MultiTrace 4P). After the period of equilibration, KCl (80 mM) was added to the bath to test for tissue viability. This concentration of KCl was repeated after 30 min. The tissues were then washed repeatedly over the next 30 min period.

Electrical field stimulation.
The tissues were set up between two platinum electrodes for electrical stimulation. They were stimulated using square wave pulses (pulse duration 0.5 ms, supramaximal voltage of 70 V for 20 s). The preparations were allowed to equilibrate under a resting tension of 1.0 g for up to 60 min during which the bath fluid was changed at least once. Isometric contractions were recorded through dynamometer UF1 transducers on a Lectromed 4-channel polygraph (MultiTrace 4P). After the period of equilibration, KCl (80 mM) was added to the bath and the contraction was recorded. At the peak of the contractions, the tissues were washed and allowed to return to the baseline. KCl was repeated after 30
Two consecutive frequency-response curves were separated by at least 60 min. EFS-induced (1-20Hz for 15sec) contractions were expressed relative to KCl-induced contractions.

**Effect of phentolamine on the contractile responses to EFS**

In order to determine the adrenergic nature of the response to electric field stimulation (EFS), the contractile responses to EFS were obtained in the absence and presence of phentolamine, a non-selective α-adrenoceptor blocker. After obtaining control frequency-response curve, phentolamine (10^-6 M) was added to the bath and allowed to equilibrate with the tissues for 30 minutes before the frequency-response curve was re-established.

**Effect of endothelial and neuronal NO on the contractile responses to EFS**

In order to study the influence of neuronal NO on the contractile responses to EFS, frequency-response curves for EFS were obtained in the absence and presence of L-nitroarginine methyl ester (L-NAME), a non-selective nitric oxide synthase (NOS) inhibitor, from endothelium-denuded ring segments of the pulmonary artery of control rats. After obtaining the control frequency-response curve for EFS, L-NAME (10^-4 M) was added to the bath and allowed to equilibrate with the tissues for 20 minutes before the EFS-response curve was re-established. In another experiment, the endothelium of the ring segments of the PA of control rats was kept intact and the frequency-response curves for EFS were obtained in the absence and presence of 7-nitroindazole (7-NI), a neuronal NOS inhibitor, and L-NAME. After obtaining the control frequency-response curve for EFS, 7-NI (10^-5 M) was added to the bath and allowed to equilibrate for 20 min before EFS-response curve was re-established. After obtaining EFS response curves in the presence of 7-NI, L-NAME (10^-4 M) was added to the bath and allowed to equilibrate for 20 min before EFS-response curve was re-established. EFS-induced contractile responses in the presence of L-NAME, 7-NI or both were expressed relative to the maximum response in the absence of these agents. In order to determine the effect of PH on the NO-mediated changes in the contractile responses to EFS, frequency-response curves for EFS were obtained in the absence and presence of 7-NI and L-NAME in both control and monocrotaline-treated rats.

**Effect of L-NAME on the contractile response to noradrenaline**

The effect of L-NAME on noradrenaline-induced contraction of the pulmonary artery segments was also investigated as follows: after establishing control concentration-response curve to noradrenaline, L-NAME (10^-4 M) was added to the bath and allowed to equilibrate with the tissue for 30 min. This was followed by adding increasing concentrations of noradrenaline to the bath to establish another concentration-response curve. In this series, the response to noradrenaline in the presence of L-NAME was expressed as a percentage of the maximum response to noradrenaline obtained in the absence of L-NAME.

**Drug solutions**

The following compounds were used in this study: monocrotaline (monocrotaline), phentolamine mesylate, carbamoylcholine chloride (carbachol), desipramine hydrochloride, (-)-noradrenaline (arterenol) bitartrate, (±)-propranolol hydrochloride, Nw-nitro-L-arginine methyl ester hydrochloride (L-NAME) and 7-nitroindazole (7-NI). All the compounds with the exception of 7-NI (Research Biochemicals Inc, MA) were obtained from Sigma chemicals (St. Louis, MO). Stock solution of monocrotaline was prepared as follows: 150 mg of monocrotaline was dissolved in 1.0 ml of 1 N HCl and 2.0 ml of distilled water was added and the pH was adjusted to 7.0 with NaOH. Thereafter, the volume was made up to 5.0 ml with distilled water to give a concentration of 30 mg/ml. Stock solutions of all other compounds were made in distilled water.

**Statistical analysis**

All graphs were analyzed using GraphPad Prism software. Data are presented as mean± S.E.M of ‘n’ observations where ‘n’ represents the number of rats. Where necessary, differences between mean values were tested for significance using student’s t test. Differences were assumed to be significant when P < 0.05.

**RESULTS**

Heart weight to body weight ratio increased from 3.19±0.08 (n = 10) in control rats to 3.99±0.17 (n = 14) 3 weeks after treatment with monocrotaline. These values were significantly (P<0.05) different from each other and indicate that cardiac hypertrophy was induced.

**Contractile responses**

Electrical stimulation of the rat pulmonary artery produced frequency-dependent contractions. The contractions started during the period of stimulation and in most cases (especially at the higher frequencies) continued for a short time post-stimulation. The contractions were reproducible as consecutive frequency-response curves could be established and tachyphylaxis was not observed in any of the
preparations. The electrically-induced contractions were almost abolished (not shown) by phentolamine (10^{-6} M) indicating that they were predominantly adrenergic in nature. EFS-induced contraction was greater in pulmonary artery segments from MCT-treated rats. This was characterized by a leftward shift of the frequency-response curve. However, there was no significant (p>0.05) difference in the maximum response between control and MCT-treated rats (Figure 1).

![Figure 1](image1.png)

**Fig. 1**
EFS-induced contractions of pulmonary artery ring segments isolated from control (■) and monocrotaline-treated pulmonary hypertensive rats (□). Each point on the graph is the mean ± S.E.M of 5 experiments. * - significantly different p < 0.05

**Modulation of EFS-induced contraction by nitric oxide (NO)**
L-NAME (10^{-4} M) had no effect on the resting tone of pulmonary artery segments from control rats. It however significantly enhanced EFS-induced contractions at all frequencies studied (Figure 2). Removal of the endothelium had no effect on the basal tone of the preparation or as shown in Figure 3, on the response of the artery segments to EFS. Under this condition, L-NAME (10^{-4} M) had no effect on EFS-induced contraction of the artery segments. The neuronal nitric oxide inhibitor, 7-NI had no effect on the basal tone of the preparation and as shown in Figure 4, also did not potentiate response of the artery segments to EFS.

![Figure 2](image2.png)

**Fig. 2**
Effect of L-NAME (10^{-4}M), a non-selective inhibitor of nitric oxide synthase, on EFS-induced contractions of pulmonary artery ring segments isolated from control (A) and monocrotaline-treated pulmonary hypertensive rats (B). ■ and □ represent EFS-induced contractions in the absence and in the presence of L-NAME, respectively. Each point on the graph is the mean ± S.E.M of 4 (monocrotaline-treated rats) and 10 (control rats) experiments. * - significantly different p < 0.05

**Effect of monocrotaline-induced pulmonary hypertension on the modulatory effect of NO**
L-NAME (10^{-4} M) produced a slight increase in basal tone of pulmonary artery segments from monocrotaline-treated rats. This contraction amounted to approximately 5% of the response to KCl. L-NAME (10^{-4} M) potentiated EFS-induced contractions of pulmonary artery segments from MCT-treated rats.

As shown in Figure 2, L-NAME (10^{-4} M) did not differentiate between artery segments from control and MCT-treated rats. The neuronal nitric oxide inhibitor, 7-NI had no effect on the basal tone of the artery segments from MCT-treated rats or on the response of the artery segment to EFS (Figure 4).

**Effect of L-NAME on noradrenaline-induced contractions of artery segments from control and MCT-treated rats.**
Noradrenaline (10^{-9} to 10^{-6} M) evoked concentration-dependent contractions of the pulmonary artery segments (Figure 5) with pD2 values of 6.70±0.11
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Fig. 4
Effect of 7NI, (10^{-4}M), a selective inhibitor of inducible nitric oxide synthase, on EFS-induced contractions of pulmonary artery ring segments isolated from control (A) and monocrotaline-treated pulmonary hypertensive rats (B). ■ and □ represent EFS-induced contractions in the absence and in the presence of 7NI, respectively. Each point on the graph is the mean± S.E.M of 4 (monocrotaline-treated rats) and 6 (control rats) experiments.

Fig. 5
Noradrenaline-induced contractions of pulmonary artery ring segments from control (A) and monocrotaline-treated pulmonary hypertensive (B) rats. ■ and □ represent noradrenaline-induced contractions before and in the presence of -NAME (10^{-4}M), respectively. Each point on the graph is the mean± S.E.M of 5 (control rats) and 7 (monocrotaline-treated rats) experiments. * - significantly different (p < 0.05)

(n=5), and 7.00±0.04 (n=10) in control and monocrotaline treated rats respectively. L-NAME (10^{-4} M) enhanced noradrenaline-induced contractions in artery segments from control and MCT-treated rats. The enhancement was greater in artery segments from MCT-treated rats (p<0.05) in the maximum contractile response to EFS of PA segments with intact endothelium from control and MCT-treated rats.

DISCUSSION

Even though the pulmonary artery is innervated by sympathetic nerves, which when stimulated produces an excitatory response, the effect of pulmonary hypertension on these responses has not been investigated. The main objective of the present study was therefore to investigate the effect of monocrotaline-induced pulmonary hypertension in rats on the excitatory response of the pulmonary artery to electrical field stimulation. In the present study, increased heart/body weight ratio (an index of cardiac hypertrophy) in rats treated with monocrotaline (compared with control rats) was taken as an indication of the development of pulmonary hypertension in these rats. Our results showed that electrical field stimulation of pulmonary artery segments from control rats produced reproducible and frequency-dependent contractions confirming previous reports in the literature (Cederqvist et al., 1991; Liu et al., 1991; Martinez et al., 1995; Segarra et al., 1999; Maclean and Morecroft, 2001). These contractions were almost abolished by phentolamine suggesting that they were predominantly adrenergic in nature. Similar reproducible and frequency-dependent contractions were also observed in artery segments from pulmonary hypertensive rats. These electrically induced contractions were significantly greater in artery segments from pulmonary hypertensive rats compared with similar preparations from control rats.

Previous studies have shown that electrically-induced contractions of pulmonary artery segments are modulated by nitric oxide (Cederqvist et al., 1991; Liu et al., 1991; Martinez et al., 1995; Segarra et al., 1999; Maclean and Morecroft, 2001). This was based on the ability of NO synthase inhibitors to potentiate electrically-induced contractions in these preparations (Cederqvist et al., 1991; Liu et al., 1991; Martinez et al., 1995; Segarra et al., 1999; Maclean and Morecroft, 2001) coupled with the fact that the potentiation was reversed by treatment with arginine. Endothelial dysfunction, characterized by impaired endothelium-dependent relaxation of the pulmonary artery to acetylcholine, has been reported by several authors (Altiere et al., 1985; Mathew et al., 1995; Nakazawa et al., 1999; Ito et al., 2000; Mam et al., 2010). We therefore tested the possibility that the enhanced electrically-induced contraction in artery segments from monocrotaline-treated rats could have been due to impaired release of NO by the vascular endothelium. The results from preliminary experiments showed that EFS-induced contractions in control rats were potentiated by L-NAME confirming modulation of EFS-induced contractions by NO in agreement with previous reports in the literature (Cederqvist et al., 1991; Liu et al., 1991; Martinez et al., 1995; Segarra et al., 1999; Maclean and Morecroft, 2001). The fact that the potentiating effect of L-NAME on EFS-induced contractions was lost in endothelium-denuded preparations would suggest a role for eNOS in this potentiation. Since L-NAME is a non-selective NOS inhibitor, we examined the effect of 7-NI, a selective inhibitor of neuronal NOS (nNOS) on EFS-induced contractions of the artery segments. It was observed that 7-NI had no effect on EFS-induced contractions confirming that the potentiating effect of L-NAME on
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EFS-induced contractions was mediated through inhibition of eNOS. A role for inducible NOS (iNOS) can also be excluded since aminoguanidine, an inhibitor of iNOS did not cause vasoconstriction of the perfused pulmonary hypertensive rat lung preparation (Tyler et al., 1999). Therefore L-NAME was used to investigate the effect of pulmonary hypertension on EFS-induced contractions of pulmonary artery segments. The results showed that L-NAME potentiated EFS-induced contractions of artery segments isolated from MCT-treated rats and that L-NAME did not differentiate between artery segments from control and monocrotaline-treated rats suggesting that modulation of EFS-induced contractions of the pulmonary artery was maintained in artery segments from monocrotaline-treated rats. In addition, L-NAME also potentiated NA-induced contractions of artery segments from control and MCT-treated rats. This is in contrast to failure by Mam et al. (2010) to demonstrate a potentiating effect of L-NAME on phenylephrine (PE)-induced contractions in MCT-treated rats even though it did potentiate PE-induced contractions in artery segments from chronically hypoxic rats, another experimental model of pulmonary hypertension. The role of NOS in the pathobiology of pulmonary hypertension is controversial. Many studies have reported impaired endothelium-dependent carbachol-induced relaxation of pulmonary artery segments and this has been interpreted as signifying impaired release of NO from the vascular endothelium (Nakazawa et al., 1999; Ito et al., 2000). However, it has also been shown that relaxation of hypertensive main pulmonary artery segments induced by sodium nitroprusside, an endothelium-independent relaxant was also reduced (Wanstall et al., 1992, 1993; Mam et al., 2010) suggesting that the decreased relaxation may not necessarily be due to impaired NO release from the vascular endothelium. Studies on the effect of pulmonary hypertension on the expression of NOS have also yielded conflicting results. Thus while Sahara et al. (2012) and Cheng et al. (2005) reported decreased expression of NOS in pulmonary hypertensive rats, Kim et al. (2012) have reported an up-regulation of NOS expression following induction of pulmonary hypertension in rats. This has been confirmed by other workers (Tyler et al., 1999; Cho et al., 2009). The reason for the conflicting reports is not known. However, using quantitative immunocytochemistry techniques, it has been convincingly shown that the intensity of eNOS staining was greater in pulmonary arteries from pulmonary hypertensive rats compared with controls (Resta et al., 1997; Tyler et al., 1999). Resta et al. (1999) suggested that the reduced protein expression in the lungs, even when the expression was enhanced in the arteries, could be due to dilution of NOS by other components of the whole lung. The observation that L-NAME produced a greater increase in pulmonary vascular resistance in monocrotaline-treated rats (Tyler et al., 1999; Leung et al., 2003) would support enhanced NOS expression in the pulmonary hypertensive rats. Our results indicating unaltered modulation of electrically-induced contractions of pulmonary artery segments isolated from monocrotaline-treated rats is consistent with these reports.

CONCLUSION

We conclude that pulmonary artery contractions to electrical field stimulation were enhanced in artery segments from pulmonary hypertensive rats and that the modulatory role of the vascular endothelium via release of nitric oxide was unaltered in these vessels.

REFERENCES


Tucker A, Bryant SE, Frost HH, Migally N (1983). Chemical sympathectomy and serotonin inhibition reduce monocrotaline-induced right ventricular...
