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Effect of pentoxifylline on diaphragmatic contractility in septic rats.

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Abstract

We investigated the effects of pentoxifylline (PTX) on endotoxin-induced diaphragmatic dysfunction in vitro. Seventy-two rats were divided into 3 groups: a group in which endotoxin (20 mg/kg) was injected intraperitoneally (endotoxin-group), a group in which PTX (100 mg/kg) was injected intraperitoneally 30 min before injection of endotoxin (endotoxin-PTX group), and a group in which only saline was given (sham group). Left hemidiaphragms were removed 4 h after injection of endotoxin. We evaluated the diaphragmatic contractility by twitch characteristics and force-frequency curves in vitro. We measured serum TNF-alpha concentrations, diaphragm malondialdehyde (MDA) levels (an index of oxygen-derived free radical-mediated lipid peroxidation), and diaphragm cAMP concentrations. Diaphragmatic force generation capacity was signifi cantly reduced after injection of endotoxin. Serum TNF-alpha concentrations and diaphragmatic MDA levels were significantly elevated after injection of endotoxin. PTX administration significantly improved diaphragmatic contractility and prevented the elevation in TNF-alpha concentrations and MDA levels after injection of endotoxin. There were no significant changes in the diaphragm cAMP concentrations among the 3 groups. These results demonstrated that PTX administration prevented endotoxin-induced diaphragmatic dysfunction without changing diaphragm muscle cAMP concentrations. The protective effects of PTX against endotoxininduced diaphragmatic contractile deterioration might be caused by attenuating TNF-alpha-mediated oxygen-derived free radical production.

KEYWORDS: endotoxin, diaphragm, pentoxifylline, TNF-alpha, MDA, cAMP

Original Article

Effect of Pentoxifylline on Diaphragmatic Contractility in Septic Rats

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We investigated the effects of pentoxifylline (PTX) on endotoxin-induced diaphragmatic dysfunction *in vitro*. Seventy-two rats were divided into 3 groups: a group in which endotoxin (20 mg/kg) was injected intraperitoneally (endotoxin-group), a group in which PTX (100 mg/kg) was injected intraperitoneally 30 min before injection of endotoxin (endotoxin-PTX group), and a group in which only saline was given (sham group). Left hemidiaphragms were removed 4 h after injection of endotoxin. We evaluated the diaphragmatic contractility by twitch characteristics and force-frequency curves *in vitro*. We measured serum TNF-α concentrations, diaphragm malondialdehyde (MDA) levels (an index of oxygen-derived free radical-mediated lipid peroxidation), and diaphragm cAMP concentrations. Diaphragmatic force generation capacity was significantly reduced after injection of endotoxin. Serum TNF-α concentrations and diaphragmatic MDA levels were significantly elevated after injection of endotoxin. PTX administration significantly improved diaphragmatic contractility and prevented the elevation in TNF-α concentrations and MDA levels after injection of endotoxin. There were no significant changes in the diaphragm cAMP concentrations among the 3 groups. These results demonstrated that PTX administration prevented endotoxin-induced diaphragmatic dysfunction without changing diaphragm muscle cAMP concentrations. The protective effects of PTX against endotoxin-induced diaphragmatic contractile deterioration might be caused by attenuating TNF-α-mediated oxygen-derived free radical production.

Key words: endotoxin, diaphragm, pentoxifylline, TNF-α, MDA, cAMP

Diaphragmatic dysfunction may contribute to the development of respiratory failure during sepsis [1-3]. Diaphragm muscle impairment and/or diaphragm muscle fatigue are thought to be the cause of diaphragmatic dysfunction occurring during sepsis [1].

Recent evidence suggests that tumor necrosis factor-alpha (TNF-α) plays an important role in the development of diaphragmatic dysfunction during sepsis. Intravenous administration of TNF-α resulted in impairment of diaphragmatic contractility in dogs [4]. Expression of TNF-α mRNA was observed in diaphragm muscle cells after injection of endotoxin. Preinjection of TNF-α antibody prevented the deterioration of diaphragmatic contractility after injection of endotoxin [5]. TNF-α is known as a potent activator of polymorphonuclear leukocytes (PMN), which trigger the production of oxygen-derived free radicals

[6]. Oxygen-derived free radicals are thought to be responsible for the development of diaphragmatic dysfunction elicited by TNF-α [3, 7].

Pentoxifylline (PTX) is a methylxanthine derivative and nonspecific phosphodiesterase inhibitor that has been used in the treatment of peripheral arterial disorders [8]. There are several lines of evidence that PTX has beneficial effects against sepsis [9–13]. PTX improves survival in animal models of endotoxic shock [14, 15]. It has been suggested that the beneficial effects of PTX against sepsis are due to inhibition of synthesis and bioactivity of TNF-α [14–17]. However, the effect of PTX against diaphragmatic contractility during sepsis remains unknown. Therefore, we hypothesized that PTX has a preventive effect on diaphragmatic dysfunction after injection of endotoxin by inhibiting the synthesis and bioactivity of TNF-α and that this effect is mediated by the inhibitory effects of oxygen-derived free radical production.

Thus, the present study was designed to evaluate the effects of PTX on diaphragmatic contractility after injection of endotoxin. We also measured the TNF-α concentration in serum and the diaphragm malondialdehyde (MDA) level, as an index of oxygen-derived free radical-mediated lipid peroxidation, after injection of endotoxin. Since PTX has multiple beneficial effects on the inflammatory cascade by increasing intracellular cyclic adenosine monophosphate (cAMP), we also measured diaphragm muscle cAMP concentrations.

Materials and Methods

This study was approved by the Committee on Animal Research of our institution. The care and handling of animals were in accord with National Institutes of Health guidelines.

Animals and protocol for induction of sepsis. Experiments were conducted on seventy-two 8- to 10-week-old male Wistar rats (weighing 250–300 g). Rats were randomly divided into 3 groups: a) sham group (n = 24), in which a sterile saline (2 ml) was administered intraperitoneally twice at 30 min intervals; b) endotoxin group (n = 24), in which a sterile saline (2 ml) was administered intraperitoneally 30 min before the intraperitoneal injection of endotoxin (lipopolysaccharides from Escherichia

coli; 055: B5 Sigma Chemical Co., St. Louis, MO, USA; 20 mg/kg) suspended in 2 ml saline; and c) endotoxin-PTX group (n = 24), in which PTX (Sigma Chemical Co.; 100 mg/kg) suspended in 2 ml saline was administered intraperitoneally 30 min before the intraperitoneal injection of endotoxin.

The left hemidiaphragm was removed 4 h after the injection of endotoxin or saline. Blood samples were taken 90 min after the injection of endotoxin or saline.

In vitro diaphragm muscle strip. The left hemidiaphragms were rapidly removed from the rats (n = 18) killed with deep O_2-isoflurane anesthesia and bleeding. Strips approximately 10 mm in width, not containing the phrenic nerves, were dissected from the medial aspect of the left hemidiaphragm of each rat. All strips were removed together with the associated ribs and central tendon. The isolated strips were placed in an organ bath containing oxygenated Krebs solution (27 °C). The strips were mounted vertically in a tissue chamber, with the central tendon superiorly positioned and attached to a Grass FT-10 force transducer (Grass Instruments, Quincy, MA, USA), which was connected to a micropositioner, and positioned between 2 platinum plates. The strips were stimulated with supramaximal currents (1.2 to 1.3 times the current required to elicit maximal tension) delivered via platinum field electrodes. The current was supplied by an amplifier driven by a Grass S48 stimulator. In these experiments, d-tubocurarine (Sigma Chemical Co.) at a concentration of 15 mM was added to the Krebs solution to eliminate the activation of intramuscular nerve branches.

Stimulation paradigm. The strips were allowed to equilibrate in the organ bath for 20 min. The optimal force-length (L0) relationship was then determined by adjusting the micropositioner between intermittent stimulations of the muscle strips. All stimulations during the study were performed at L0. Muscle contractile characteristics were then assessed according to twitch characteristics and the force-frequency relationship.

Twitch characteristics. Peak twitch tension, contraction time (time to peak tension), and half-relaxation time (time required for tension to decay from maximum to half-maximum) were calculated from single twitches (0.1 Hz stimulation) that were recorded at a high speed (100 mm/s).

Force-frequency relationship. Force-

frequency relationships were determined by stimulating the diaphragm strips tetanically at frequencies from 10 to 100 Hz over 10-Hz increments. An interval of 10 s was used between stimuli, and the pulses were 0.2 ms in duration with a train duration of 400 ms. Force-frequency relationships were determined 20 min after determination of L0.

Measurement of TNF-α concentration. Blood samples ($n = 18$) were centrifuged, and the plasma was frozen in liquid nitrogen. Plasma TNF-α concentrations were assayed with an enzyme-linked immunoabsorbent assay (Biosource, Camarillo CA, USA). According to the manufacturer, the sensitivity is 2.3 pg/ml.

Measurement of malondialdehyde levels. The MDA level was determined from the reaction of N-methyl-2-phenylindole with MDA using Bioxytech LPO-586 (Oxis International, Foster City, OR, USA). Briefly, diaphragm muscle samples ($n = 18$) were homogenized with 20 mM Tris buffer, pH 7.4, containing 5 mM butylated hydroxytoluene, to make a 20% homogenate. The homogenate was centrifuged at $3,000 \times g$ and for 10 min at 4 °C. Then, 650 μL of the R1 reagent, N-methyl-2-phenylindole in 25% methanol/75% acetonitrile, and 150 μL concentrated 12 N HCl were added to 200 μL of the supernatant and incubated at 45 °C for 60 min. Turbid samples were centrifuged at $15,000 \times g$ for 10 min. Measurement of 586 nm absorbance was performed. Final MDA levels were expressed as nmol MDA per mg protein. Protein concentrations were measured by the Lowry method [18, 19].

Measurement of cAMP concentrations. The diaphragmatic strips ($n = 18$) were homogenized in 10 volumes of Hank's balanced salt solution (without calcium and magnesium) containing 5 mM EDTA with a homogenizer. The homogenates were centrifuged at $1,000 \times g$ for 10 min at 4 °C. The extraction of cAMP from the supernatant was carried out by the solid-phase extraction method using Amprep SAX columns (Amersham, Buckinghamshire, UK). The concentrations of cAMP were measured by the Biotrak cAMP enzyme immunoassay system (Amersham). The resulting pellets were solubilized and used to measure protein concentrations by the Lowry method [18, 20].

Study design.

Experiment 1. Effect of pentoxifylline on diaphragmatic contractility.

Eighteen rats were used to measure diaphragmatic contractility after the injection of endotoxin.

Experiment 2. Effect of pentoxifylline on plasma TNF-α concentrations.

Eighteen rats were used to measure plasma TNF-α concentrations after the injection of endotoxin.

Experiment 3. Effect of pentoxifylline on diaphragmatic MDA levels.

Eighteen rats were used to measure diaphragmatic MDA levels after the injection of endotoxin.

Experiment 4. Effect of pentoxifylline on diaphragmatic cAMP concentrations.

Eighteen rats were used to measure diaphragmatic cAMP concentrations after the injection of endotoxin.

In these experiments, 6 animals each from the sham group, the endotoxin group and the endotoxin-PTX group were tested.

Data analysis. The cross-sectional area was calculated by dividing the muscle mass by the length in cm, and the density of muscle was assumed to be 1.056 g/cm^3. Tension was calculated as force per unit of cross-sectional area (kg/cm^2). Statistics were calculated using a software program (Macintosh StatView J 4.02; Abacus Concepts, Berkeley, CA, USA). Comparisons of force-frequency curves among the groups were made by repeated-measures analysis of variance (ANOVA). Comparisons of the diaphragm muscle twitch characteristics, peak tetanic tension, TNF-α concentration, MDA levels and cAMP concentration among the groups were made with one-way analysis of variance (ANOVA) combined with Scheffe's procedure used for post hoc comparison of data sets. Differences with a p value less than 0.05 were considered significant. Data are presented as means ± SEMs.

Results

Twitch characteristics. The twitch characteristics of the sham group, endotoxin group and endotoxin-PTX group are shown in Table 1. Peak twitch tension in the endotoxin group was significantly lower than that in the sham group ($p < 0.01$). Peak twitch tension in the endotoxin-PTX group was significantly higher than that in the endotoxin group ($p < 0.01$).

There were no significant differences in half RT and CT among the 3 groups.

Diaphragm force-frequency relationship. The force-frequency curves of the sham group, endotoxin group and endotoxin-PTX group are shown in Fig. 1. The force-frequency curve of the endotoxin group was significantly lower than that of the sham group ($p < 0.01$), and the force-frequency curve of the endotoxin-PTX group was significantly higher than that of the endotoxin group ($p < 0.01$).

Peak tetanic tensions were 2.23 ± 0.07 kg/cm^2 in the sham group, 1.53 ± 0.08 kg/cm^2 in the endotoxin group, and 2.18 ± 0.11 kg/cm^2 in the endotoxin-PTX group. Peak tetanic tension in the endotoxin group was significantly lower than that in the sham group ($p < 0.01$), and peak tetanic tension in the endotoxin-PTX group was significantly higher than that in the endotoxin group ($p < 0.01$).

TNF-α concentrations. Blood TNF-α concentrations are shown in Table 2. Blood TNF-α concentrations increased after endotoxin administration. Blood TNF-α concentration in the endotoxin-PTX groups was significantly lower than that in the endotoxin group ($p < 0.01$).

Table 1   Effect of pentoxifylline on diaphragmatic contractility

Group	TT (kg/cm^2)	CT (ms)	1/2RT (ms)
sham	0.45 ± 0.02	44.4 ± 0.85	30.3 ± 0.89
endotoxin	$0.33 \pm 0.02^{**}$	46.5 ± 0.76	35.0 ± 1.29
endotoxin-PTX	$0.41 \pm 0.02^{\#\#}$	46.9 ± 0.60	32.9 ± 1.97

CT, contraction time; TT, twitch tension; 1/2RT, half relaxation time. Values are means $\pm$ SEM. $^{**}p < 0.01$ vs sham group. $^{\#\#}p < 0.01$ vs endotoxin group.

Table 2   Effect of pentoxifylline on plasma TNF-α concentrations

Group	TNF-α (ng/ml)
Sham	NOT DETECTABLE
Endotoxin	23.1 ± 5.12
endotoxin-PTX	$0.24 \pm 0.03^{**}$

Values are means $\pm$ SEM. $^{**}p < 0.01$ vs endotoxin group.
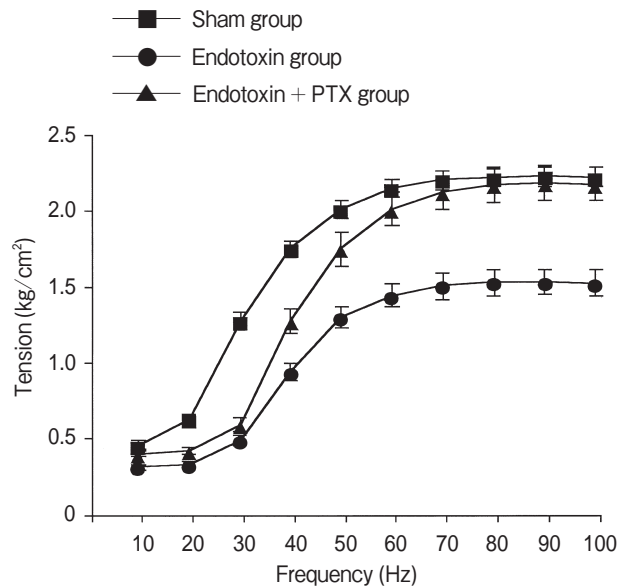

Fig. 1   Diaphragmatic force-frequency curves in 3 experimental groups.
Values are means $\pm$ SEM. The force-frequency curve of the endotoxin group was significantly lower than that of the sham group ($p < 0.01$). The force-frequency curve of the endotoxin-PTX group was significantly higher than that of the endotoxin group ($p < 0.01$).

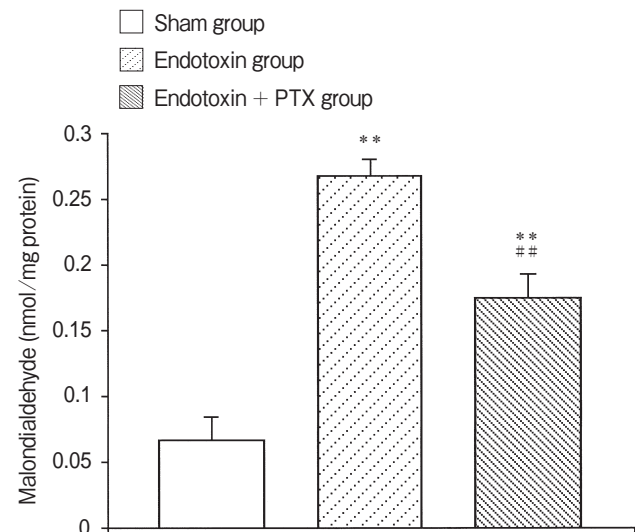**Fig. 2**   Diaphragmatic malondialdehyde levels in three experimental groups.
Values are means $\pm$ SEM. $^{**}p < 0.01$ vs sham group. $^{\#\#}p < 0.01$ vs endotoxin group. The malondialdehyde level of the endotoxin group was significantly higher than that of the sham group ($p < 0.01$). The malondialdehyde level of the endotoxin-PTX group was significantly lower than that of endotoxin group ($p < 0.01$).

MDA levels.   Diaphragmatic MDA levels are shown in Fig. 2. The diaphragmatic MDA level in the endotoxin group was significantly higher than that in the sham group ($p < 0.01$). The diaphragmatic MDA level in the endotoxin-PTX group was significantly lower than that in the endotoxin group ($p < 0.01$).

cAMP levels.   The cAMP concentrations are shown in Fig. 3. There were no significant differences in cAMP concentrations among the 3 groups.

Discussion

The present study demonstrated that diaphragmatic contractility was impaired after endotoxin administration, as manifested by reductions in twitch tension and a downward shift of force-frequency curves. Serum TNF-α and diaphragmatic MDA levels were elevated after endotoxin administration. PTX administration improved endotoxin-induced impairment of diaphragmatic contractility. PTX administration prevented the elevation in serum TNF-α and diaphragmatic MDA levels. These results indicated that PTX administration improved endotoxin-induced diaphragmatic dysfunction by attenuating TNF-α-mediated oxygen-derived free radical production.

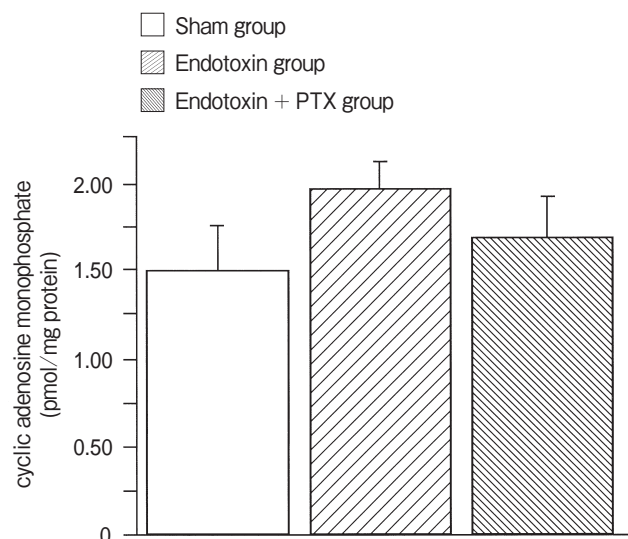***Endotoxin, TNF-α and free radical produc-***

Fig. 3   Diaphragmatic cyclic adenosine monophosphate concentrations in three experimental groups.
Values are means $\pm$ SEM. There were no significant differences in cAMP concentrations among the 3 groups.

tion.   Endotoxin activates macrophages, which produce cytokines such as interleukin-1 (IL-1) and TNF-α [21]. TNF-α is thought to be a central mediator of inflammatory response during endotoxemia and has been proposed to mediate sepsis-associated respiratory muscle dysfunction. TNF-α infusion in dogs caused deterioration of diaphragmatic contractility, similar to the effect of endotoxin injection [4]. TNF-α gene expression and production occurred in diaphragm muscle cells after injection of endotoxin, and pretreatment with TNF-α antibody prevented deterioration of diaphragmatic contractility [5]. However, TNF-α itself did not affect *in vitro* isolated diaphragm muscle contractility [22]. TNF-α is a potent primer of the oxidative activity of PMN [6]. TNF-α is thought to be responsible for the diaphragm muscle dysfunction by inducing oxygen radicals, such as superoxide, hydrogen peroxide and hydroxyl radicals [3, 5, 7, 19, 23, 24]. Several studies have demonstrated that free radical scavengers prevent the development of respiratory muscle dysfunction [19, 24–27]. Therefore, the oxygen-derived free radicals generated in this manner are thought to play an important role in endotoxin-induced diaphragm muscle dysfunction.

In this study, endotoxin administration caused reductions in diaphragmatic force generation at all frequencies. Diaphragmatic force generation was reduced without any changes in contraction time and half relaxation time. This pattern of dysfunction is thought to be consistent with either dysfunction of contractile proteins or altered sarcolemmal function [28, 29]. The elevation in MDA levels indicates that oxygen-derived free radicals promote the peroxidation of diaphragm lipids [6, 7]. These results suggested that oxygen-derived free radicals directly damage diaphragm muscle cells, resulting in a reduction in diaphragmatic contractility after endotoxin administration [3, 7].

Effects of PTX on TNF-α levels and free radical production.   In animal models of sepsis, the administration of PTX reduces serum TNF-α levels and the lethality of endotoxemia in mice [30]. PTX has multiple beneficial effects on the inflammatory cascade by modulating the inflammatory response [16]. PTX downregulates the production of various pro- and anti-inflammatory cytokines, especially by increasing intracellular cAMP and decreasing TNF-α

synthesis [16]. TNF-α is a potent primer of the oxidative activity of PMN [6]. PTX has been shown to inhibit the stimulated oxidative activity of PMN. PTX prevented endotoxin-induced degranulation of PMN [17]. In our study, it was suggested that PTX administration prevented endotoxin-induced oxygen-derived free radical production by reducing TNF-α production.

Effects of PTX on diaphragmatic dysfunction. This study demonstrated that PTX administration improved endotoxin-induced diaphragmatic dysfunction by attenuating oxygen-derived free radical production.

PTX improved muscle force generation mainly at high frequencies of stimulation. PTX did not improve muscle-force generation at low frequencies. PTX reduced diaphragmatic MDA levels after endotoxin administration, but the levels were still higher than the control levels. These results suggested that endotoxin-induced diaphragmatic contractile dysfunction remained after PTX administration.

There are several sources of oxygen-derived free radicals that might affect diaphragmatic contractility during sepsis [3, 7, 19, 24, 26]. Free radical formation during contraction is markedly enhanced in endotoxin-induced septic animals [3, 7]. Free radicals generated in this manner might account for the increased MDA levels after PTX administration.

PTX is known to inhibit phosphodiesterase, thereby increasing cAMP levels. Elevated intracellular levels of cAMP are observed following incubation of human cells, including PMNs [31], monocytes [31] and endothelial cells [32] with PTX. In this study, there were no significant changes in diaphragm muscle intracellular cAMP levels.

It is well known that β_2-adrenoceptor agonists improve septic diaphragmatic contractility *in vitro* by increasing diaphragm muscle intracellular cAMP levels [19, 33, 34]. Our study demonstrated that PTX improves endotoxin-induced diaphragmatic dysfunction by attenuating TNF-α-mediated oxygen-derived free radical production rather than by increasing diaphragm muscle intracellular cAMP levels.

It has been demonstrated that the elimination half-lives of PTX after intraperitoneal administration in rats and mice are almost within 15 min [35, 36]. Therefore PTX could not increase diaphragm muscle cAMP levels 4 h after endotoxin administration.

In conclusion, this study demonstrated that PTX administration prevented endotoxin-induced diaphragmatic dysfunction. PTX attenuated diaphragmatic dysfunction without changing diaphragm muscle cAMP concentrations. The protective effects of PTX against endotoxin-induced diaphragmatic contractile deterioration might be due to attenuation of TNF-α-mediated oxygen-derived free radical production.

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