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Protective Effects of Interleukin-10 on Diaphragm Muscle in a Septic Animal Model

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Taneda, A., Shindoh, C., Ohuchi, Y. and Shirato, K. *Protective Effects of Interleukin-10 on Diaphragm Muscle in a Septic Animal Model.* Tohoku J. Exp. Med., 1998, **185** (1), 45-54 —— The effects of Interleukin (IL)-10 intravenous injection after endotoxin administration on diaphragm muscle were studied using Wistar rats. The animals were divided into two treatment groups: A saline+endotoxin group as control and an IL-10+endotoxin group. *E. coli* endotoxin (10 mg/kg) was injected intraperitoneally 5 minutes after saline or IL-10 (1250 U, 0.25 mg) injection. The force-frequency curves, twitch kinetics and fatigability were measured at 0 and 4 hours after endotoxin injection. In the saline+endotoxin group, the force-frequency curves and half relaxation time were significantly decreased at 4 hours ($p<0.001$ and $p<0.05$, respectively) compared to those at 0 hour. In the IL-10+endotoxin group, the decrement in the force-frequency curves by endotoxin was prevented at 4 hours compared to that at 0 hour. Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry showed positive staining in the saline+endotoxin at 4 hours, but there was no significant staining at 0 or 4 hours in the IL-10+endotoxin group. These data suggest that IL-10 prevents the deterioration of contraction induced by endotoxin by inhibiting nitric oxide production in the diaphragm muscle. ———— endotoxin; nitric oxide; NADPH diaphorase

Interleukin (IL)-10, originally termed cytokine synthesis inhibitory factor (CSIF), is a 35-kDa protein produced as a result of immune activation by subpopulations of helper T cells (Th2), which express IL-4, IL-5 and IL-6. IL-10 profoundly suppresses the production of IL-2, IL-12 and IFN-γ by the other subpopulation of helper T cells (Th1) (Fiorentino et al. 1989). The encoding of murine IL-10 (mIL-10) cDNA has been reported (Moore et al. 1990), and human IL-10 (hIL-10) is known to have extensive homology to mouse and rat IL-10 (Vieira et al. 1991). In addition, IL-10 has recently become known for its beneficial effects on bacterial sepsis in human (Marchant et al. 1994) and mouse (Bogdan et al. 1991; Howard et al. 1993), presumably because of its ability to

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suppress tumor necrosis factor α (TNF-α), H_2O_2 and NO_2^- (a reflection of NO production) release from macrophages.

Nitric oxide (NO) is also known as a regulator molecule for leukocytes, endothelial cells of blood vessels, and neurons in the brain and peripheral nervous system (Furchgott and Zawadzki 1980; Dinerman et al. 1993), and NO synthase (NOS) was detected inside of diaphragm muscle fibers (Kobzik et al. 1994). Previous studies have shown that injection of endotoxin induces a decrement in diaphragm muscle contractility (Boczkowski et al. 1988; Shindoh et al. 1992), and that this deterioration may be caused by a network of TNF-α, NO and oxygen derived intermediates (free radicals) including superoxide and hydroxyl radical. Thus, it is suggested that this network plays an important role in the endotoxin induced deterioration of diaphragm muscle. Because we paid an attention to the suppressive effects of IL-10 described above, we hypothesized that IL-10 could block the deterioration of diaphragm muscle induced by endotoxin.

Therefore, we examined here whether IL-10 is able to reduce the contractile dysfunction induced by endotoxin in diaphragm muscle, and whether this is related to the suppression of NO production, using the histochemistry staining of NADPH diaphorase.

Methods and Materials

Animal preparation

Using Wistar rats weighing 250-320 g (Charles River Japan, Yokohama), experiments were performed on 28 animals divided into 2 groups. The saline+endotoxin group was given 0.5 ml of saline intravenously via the tail vein, followed 5 minutes later by an intraperitoneal injection of *E. coli* endotoxin (10 mg/kg) (055 : B5, Sigma Chemical Co., St. Louis, MO, USA) in 0.5 ml of saline ($n=14$). The IL-10+endotoxin group was given human IL-10 (1250 U: i.e., 0.25 mg, B50047, Genzyme Co., Cambridge, MA, USA) suspended with 0.5 ml of saline intravenously via the tail vein, followed 5 minutes later by an intraperitoneal injection of *E. coli* endotoxin (10 mg/kg) in 0.5 ml of saline ($n=14$). For the analysis of diaphragm muscle contractile properties, force-frequency curves, twitch kinetics and fatigability were measured immediately after (0 hour) and at 4 hours after injection of endotoxin in each experimental group. For the analysis of NO production, NADPH diaphorase histochemistry was performed using 0 hour and 4 hours diaphragm muscle samples from each group. Because the previous paper (Shindoh et al. 1995) showed that the force-frequency curves decreased maximally around 4 hours and then recovered at 6 hours after endotoxin (20 mg/kg) injection, we measured and analyzed these effects at 4 hours after endotoxin administration. Written approval was obtained from The Tohoku University Animal Facility.

Diaphragm muscle contractile measurements

Two muscle strips (3–4 mm wide) were dissected from the right and left hemidiaphragm under diethyl ether anesthesia and mounted in separate organ baths containing Krebs-Henseleit solution oxygenated with a 95% O_2- 5% CO_2 gas mixture (23.5±0.5°C, pH 7.40±0.05). The composition of the aerated Krebs-Henseleit solution in mEq/L was as follows: Na^+, 153.8; K^+, 5.0; Ca^{2+}, 5.0; Mg^{2+}, 2.0; Cl^-, 145.0; HCO_3^-, 15.0; HPO_4^{2-}, 1.9; SO_4^{2-}, 2.0; and glucose, 110 mg%; d-tubocurarine, 10 μM; regular crystalline zinc insulin, 50 U/L. Both muscle strips were simultaneously stimulated with supramaximal currents (i.e., 1.2 to 1.5 times the current required to elicit maximal twitch tension, 200–250 mA, 0.2 mseconds duration in pulses) by a constant current stimulus isolation unit (SS-302J, Nihon Kohden, Tokyo) driven by a stimulator (SEN-3201, Nihon Kohden). The elicited tensions were measured by a force transducer (UL-100GR, Minebea Co., Fujisawa). Each muscle strip length was changed by moving the position of the force transducer with a micrometer-controlled rack and pinion gear (accuracy of displacement, 0.05 mm, Mitutoyo Co., Kawasaki), and measured with a micrometer in close proximity to the muscle. The optimal length of the muscle (Lo) was defined as the muscle length at which twitch tension development was maximal, and this Lo was maintained in the following measurements.

The diaphragm force-frequency relationship was assessed by sequentially stimulating muscles at 1, 10, 20, 30, 50, 70 and 100 Hz. Each stimulus train was applied for approximately 1 second, and adjacent trains were applied at approximately 10 second intervals. The tensions of both muscle strips were recorded by a hot-pen recorder (RECTI-HORIZ-8K, Sanei, Tokyo). The force-frequency curves obtained from the studied groups were displayed as elicited tensions (kg/cm^2) in the Y-axis and stimulating frequencies in the X-axis.

Twitch contraction was elicited by single pulse stimulation (200 μseconds), and the trace of the twitch contraction was recorded at high speed (10 cm/sec). The twitch kinetics were assessed by (1) twitch tension (peak tension of twitch contraction, kg/cm^2), (2) contraction time (the time required to develop peak tension, mseconds) and (3) half relaxation time (the time required for peak tension to fall by 50%, mseconds) during a single muscle contraction.

Muscle fatigability was then assessed by examining the rate of fall of tension over 5 minutes of rhythmic contraction. Rhythmic contraction was induced by applying trains of 20 Hz stimuli (train duration, 0.22 sec; rest duration, 0.44 sec; train : rest ratios, 1 : 2) at a 60 train/min rate. Muscle fatigability was expressed as a percentage of the final remaining tension (%) from the initial tension. After completion of this protocol, the muscle strip was removed from the bath and weighed.

NADPH diaphorase histochemistry

NADPH diaphorase histochemistry was performed using the diaphragm muscles of 0 hour and 4 hours in the saline+endotoxin and IL-10+endotoxin groups. The diaphragm samples were excised quickly from the remaining part of the costal diaphragm muscle (after dissecting the muscle strips used for measuring muscle contractile properties), immersed in isopentane (Wako Pure Chemical Industries Ltd., Osaka) that had been cooled in liquid nitrogen, and embedded in mounting medium (OCT compound, Miles Inc., Elkhart, IN, USA). Diaphragm sample tissues were sectioned at 10 μm with a cryostat (BRIGHT Instrument, Huntingdon, UK) kept at -20°C, mounted on chrom-alum gelatin-coated glass slides, and immersed in 0.3% Triton X-100 containing phosphate buffer. Histochemical reaction for NADPH diaphorase consisted of dipping the sections in freshly prepared 1.0 mM β-NADPH (Oriental Yeast Co., Ltd., Tokyo) and 0.2 mM nitroblue tetrazolium (Wako Pharmaceutical Co., Osaka) in 100 mM Tris-HCl buffer pH 8.0, containing 0.2% Triton X-100 for 60 minutes at 37°C. The reaction was stopped by rinsing the sections in phosphate buffer saline (PBS). The sections were covered with a mixture of glycerol and PBS (2 : 1), and photographed with a Nikon microscope using black and white film (Neopan SS, FUJIFILM, Tokyo).

Data analysis

The strip cross-sectional area was calculated by dividing the muscle mass by the product of the strip muscle length and muscle density (1.06 g/cm^3) (Close 1972), and tension was calculated as force per unit area (kg/cm^2). Data obtained from both halves of the diaphragm in one animal were averaged, therefore, the sample number used was $n=7$ (animals) per treatment/time point for force-frequency curves, twitch kinetics and fatigability. The mean values for each frequency in force-frequency curves, twitch kinetics and fatigability were compared by Student t-test, and the entire force frequency curves among groups were compared by analysis of variance (ANOVA) with Fisher's PLSD post hoc test. All data are presented as means$\pm$S.D. Comparisons with a p value of less than 0.05 were considered statistically significant.

Results

Changes of force-frequency curves

Fig. 1 shows the force-frequency curves of the saline+endotoxin (A) and IL-10+endotoxin (B) groups. In the saline+endotoxin group, the force-frequency curves at 4 hours (1.39 ± 0.18 kg/cm^2 as a peak) were significantly decreased from those of 0 hour (2.02 ± 0.14 kg/cm^2 as a peak) by ANOVA ($p<0.001$). The symbol indicated significant difference at each frequencies compared to 0 hour ($p<0.001$). On the other hand, in the IL-10+endotoxin group, there

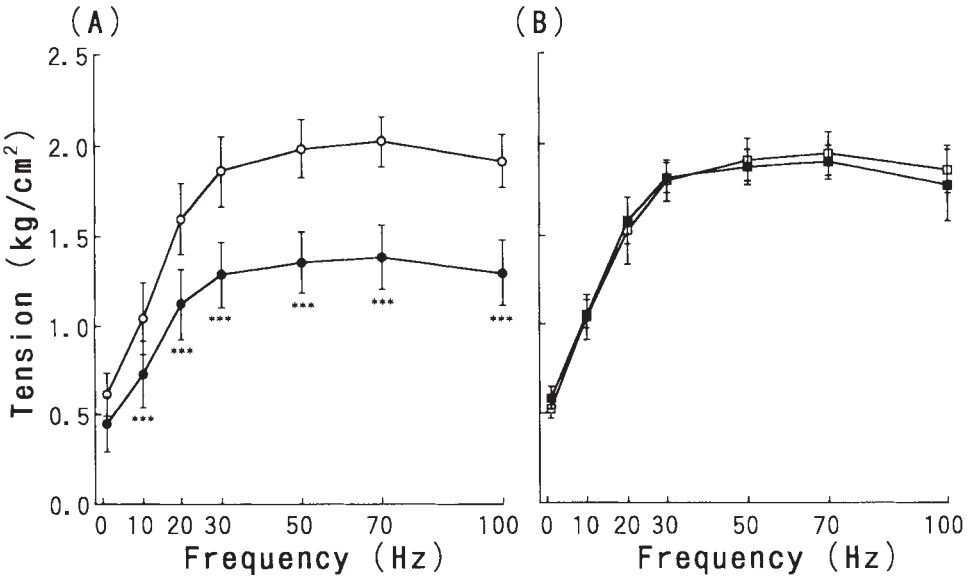

Fig. 1. Force-frequency curves of 0 hour (○) and 4 hours (●) in the saline+endotoxin group (A), and those of 0 hour (□) and 4 hours (■) in the IL-10+endotoxin group (B). Symbols indicate significant differences at given frequencies compared to 0 hour (***$p<0.001$).

were no significant changes at each frequency or in the whole force-frequency curves between 0 hour (1.95 ± 0.12 kg/cm^2 as a peak) and 4 hours (1.90 ± 0.09 kg/cm^2 as a peak). Comparing both groups at 4 hours, the entire force-frequency curves of the IL-10+endotoxin group increased significantly from those of the saline+endotoxin group by ANOVA ($p<0.001$).

Changes of twitch kinetics and fatigability

Fig. 2 shows the changes of twitch kinetics and fatigability in both groups. In the saline+endotoxin group (A), twitch tension, contraction time and half relaxation time at 4 hours showed a tendency of decrement from 0 hour, but this was significant only in the half relaxation time ($p<0.05$). These changes indicate that the contractile characteristics at 4 hours after endotoxin administration became faster than those at 0 hour. On the other hand, in the IL-10+endotoxin group (B), twitch tension, contraction time and half relaxation time at 4 hours showed no significant changes compared to those at 0 hour. The fatigability in both groups did not show significant changes between 0 hour and 4 hours.

NADPH diaphorase histochemistry

Fig. 3 shows photographs taken from 0 hour (A) and 4 hours (B) in the saline+endotoxin group, and 0 hour (C) and 4 hours (D) in the IL-10+endotoxin group. The saline+endotoxin group at 0 hour showed very slight staining, however, at 4 hour it showed positive staining (darkly staining) for NADPH diaphorase histochemistry in the diaphragm muscle fibers having a relatively smaller cross sectional area (dark arrows) compared to large ones (white arrows).

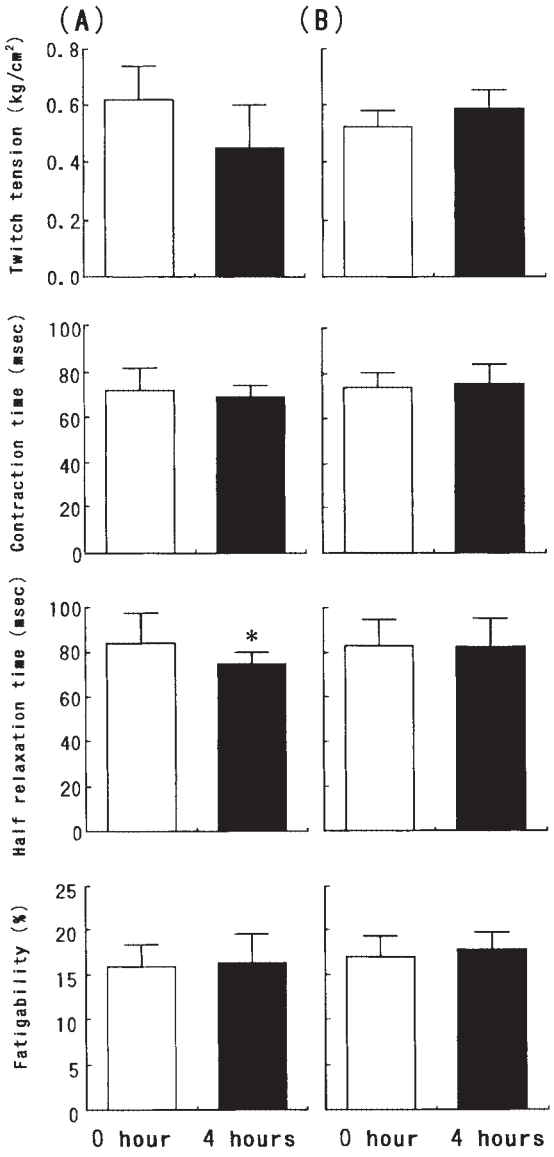

Fig. 2. Changes of twitch kinetics and fatigability of saline+endotoxin (A) and IL-10+endotoxin (B) groups. The symbol indicates a significant difference compared to 0 hour ($^*p<0.05$).

On the other hand, the IL-10+endotoxin group at neither 0 hour nor 4 hours showed significant positive staining. From these findings, it is likely to say that endotoxin induces NO production at 4 hours in the relatively smaller muscle fibers, but IL-10 inhibits this production.

Discussion

The present study showed that IL-10 prevented the decrement of force-frequency curves of diaphragm muscles and maintained the force-frequency curves at the same level as those of control, and that IL-10 also prevented NO production in diaphragm muscle fibers at 4 hours after endotoxin administration. These results suggest that this blocking of NO production is an important mechanism of IL-10 that reduces contractile deterioration in diaphragm muscle

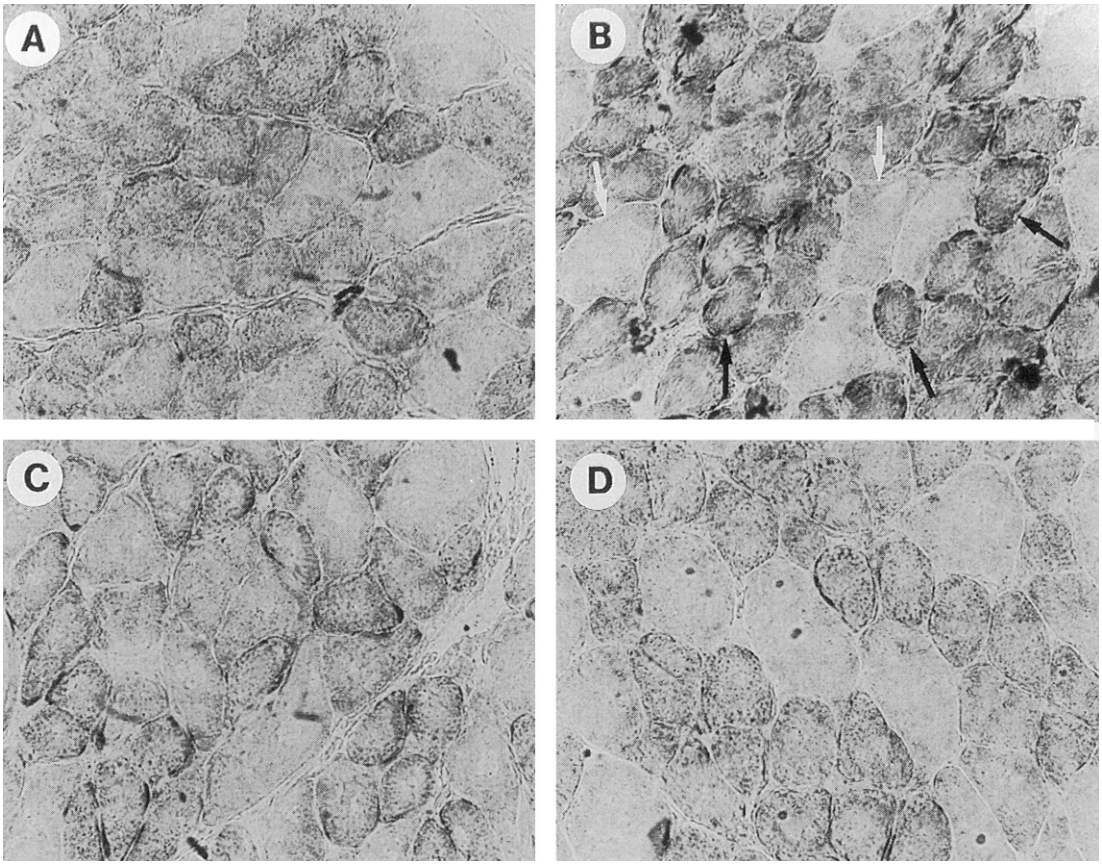

Fig. 3. NADPH diaphorase histochemistry of diaphragm muscles at 0 hour (A) and 4 hours (B) in the saline+endotoxin group, and 0 hour (C) and 4 hours (D) in the IL-10+endotoxin group. Dark arrows indicate positive staining in the smaller muscle fibers, and white arrows indicate negative staining in the larger muscle fibers. Magnification is×200.

induced by endotoxin.

In general, NO is suggested to be an important substance for vascular relaxation: NO blocks mitochondrial respiration by the inhibition of complex I (NADH: ubiquinone oxidoreductase) and complex II (succinate: ubiquinone oxidoreductase) activities of the mitochondrial respiratory chain. This NO-dependent inhibition might switch to anaerobic glycosis and reduce the energy production of the smooth muscle cells (Geng et al. 1992). In addition, a recent study showed that muscle-derived NO may play a role in modulating skeletal muscle carbohydrate metabolism, thereby contributing to the changes in contractile properties (Balon and Nadler 1994). Therefore, the impairment of muscle contraction occurs inside of the diaphragm muscle, which is supported by the evidence of NADPH diaphorase positive activity in muscle cell fibers, and it is also likely that the NO-dependent inhibition of mitochondrial respiration in the diaphragm muscle cell is responsible for the decrement of force-frequency curves after endotoxin administration.

The acute phase of sepsis is characterized by an excess of pro-inflammatory cytokines (IL-6, IL-8, TNF-α), while anti-inflammatory cytokines (IL-10, soluble

TNF receptor type I or II [TNFsrI, TNFsrII]) are predominantly present during the second phase (Groeneveld et al. 1997). IL-10 decreases the production of IL-1, IL-6, and TNF-α in mice (Howard et al. 1993), and IL-10 was exceedingly potent at suppressing the ability of mouse peritoneal macrophages to release TNF-α (Bogdan et al. 1991). In addition, high plasma levels of NO_2^-/NO_3^- (the stable end products of NO) were observed in septic patients (Ochoa et al. 1991). Since the present authors reported that TNF-α mRNA is expressed in diaphragm muscle cells after endotoxin administration (Shindoh et al. 1995), we also speculated that TNF-α and NO may contribute to the endotoxin induced diaphragm muscle contractile deterioration. It is possible that endotoxin and inflammatory cytokines can act as signals or key substances for increasing NO production.

Furthermore, the reaction of NO and superoxide could be of particular importance in the present endotoxin administration model because they form peroxynitrite anion ($ONOO^-$), which is a potent hydroxyl radical ($\cdot$ OH) like radical that causes cellular damage by increasing lipid peroxidation and inhibiting mitochondrial respiration (Pryor and Squadrito 1995). The line of evidence may suggest that TNF-α contributes to the production of not only superoxide but also NO, which then forms peroxynitrite anion to cause contractile deterioration in the diaphragm muscle cells after endotoxin administration.

NADPH diaphorase histochemistry showed positive staining in the smaller cross sectional area (CSA) muscle fibers of the saline+endotoxin group, as shown in Fig. 3. On the basis of the biochemical and physiological characteristics of skeletal muscle, muscle fibers are classified as either type I or type II by ATPase staining. Type I (slow-twitch, oxidative; SO) has a smaller CSA, and type II (fast-twitch) subtypes IIa (fast-twitch, oxidative, glycolytic; FOG), IIb (fast-twitch, glycolytic; FG) and IIc muscle fiber has a larger CSA, especially type IIb has the largest CSA (Dubowitz and Brooke 1973). If the smaller type I muscle fibers produce NO by endotoxin, the type I muscle fibers may be more easily damaged by NO and oxygen derived free radicals than type II muscle fibers. Therefore, it is speculated that the overall muscle contractile profiles will change to faster contraction and more fatigue by endotoxin. However, we observed positive changes in only two parameters of the force-frequency curves and the half relaxation time, and there were not significant changes in twitch tension, contraction time and fatigability at 4 hours in the saline+endotoxin group. Thus, there may be a limitation in assessing the changes of diaphragm muscle in terms of the contractile properties, however, it seems important for IL-10 that all contractile parameters were maintained at 4 hours as same as at 0 hour in the IL-10+endotoxin group.

Of particular interest was the observation that the NADPH diaphorase activity was inhibited by IL-10. It is known that the biosynthesis of NO from L-arginine by constitutive NO synthase (cNOS) occurs in endothelial cells (Zembowicz et al. 1991). Moreover, Kobzik et al. (1995) recently reported that

NOS occurs in the fatigued diaphragm muscle, and established that skeletal muscle cells can express two constitutive isoforms, neuronal-NOS and endothelial-NOS. Therefore, by the fact that the NO production was reduced by IL-10, it is suggested that IL-10 inhibited NOS in the diaphragm muscles to prevent the endotoxin induced deterioration of the force-frequency curves.

In summary, the present study is the first report showing that IL-10 is a protective cytokine for diaphragm muscles in a septic animal model. It is noted that IL-10 is classified as an anti-inflammatory cytokine in contrast to IL-6, IL-8 and TNF-α, which have deteriorative effects on diaphragm muscle. Recently, a report showed that IL-10 may have potential as a treatment for sepsis (Marchant et al. 1994). Although the protective effect of IL-10 on diaphragm muscle after endotoxin has been observed only in an animal model, we suggest that IL-10 could be useful in the treatment of patients with respiratory muscle failure caused by sepsis.

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