<Original Papers>Effects of Interleukin-8 on Diaphragm Muscle Contraction in Rats

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Effects of Interleukin-8 on Diaphragm Muscle Contraction in Rats

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横隔膜筋収縮に対するIL-8の影響

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Since interleukin-8 (IL-8) has been described as a neutrophil chemotactic factor produced by endotoxin-stimulated human blood mononuclear leukocytes, we examined whether IL-8 affects the diaphragm contractile properties in rats. For the *in vivo* study, we injected IL-8 (3 μg/kg) via the tail vein and measured force-frequency curves, twitch kinetics and fatigability at 2 and 4 hours after injection ($n=6$ each), using dissected diaphragm muscle strips. The force-frequency curves decreased to 1.63 ± 0.05 kg/cm^2 at 2 hours ($p<0.001$), and to 1.50 ± 0.04 kg/cm^2 at 4 hours ($p<0.001$), significant decreases from the level at 0 hours (1.98 ± 0.05 kg/cm^2). Twitch tension decreased from 0.59 ± 0.05 kg/cm^2 at 0 hours to 0.48 ± 0.02 kg/cm^2 at 2 hours ($p<0.05$), and to 0.44 ± 0.02 kg/cm^2 at 4 hours ($p<0.01$). However, contraction times, half relaxation times and fatigabilities at both 2 and 4 hours did not show significant differences. In NADPH diaphorase staining, diaphragm muscle fibers showed positive staining at 4 hours. For the *in vitro* study, we incubated muscle strips with IL-8 (0.01 μg/ml) in an organ bath for 4 hours. The force-frequency curves did not show significant changes. From these results, we suggest that IL-8 has no direct effect on diaphragm muscles *in vitro*, but has a significant effect via the vascular route *in vivo*. We suggest that IL-8 seems to play a significant role in endotoxin-induced diaphragm muscle deterioration.

Introduction

Monocyte-derived neutrophil chemotactic factor (MDNCF)[1], which is produced by human blood monocytes stimulated with lipopolysaccharide (LPS, i.e., endotoxin), has been described as interleukin-8 (IL-8)[2] and is known as a neutrophil chemotactic factor. IL-8 is characterized as a 10 kD molecular weight peptide and is a well-known member of a new class of

cytokines that are widely studied because of their ability to attract and activate leukocytes, and their probable role as mediators of inflammation. All of these cytokines have four conserved cysteins, and two subfamilies can be distinguished according to the position of the first two cysteins (C), which either are separated by one amino acid (X) (CXC proteins) or are adjacent (CC proteins)[3)4)].

In addition to its chemotactic action, IL-8 is also known as a mediator of the leukocyte-specific inflammatory response, since it is released as the result of inflammatory stimulation and has the selective capacity to attract neutrophils but not monocytes[1)]. It is also known to stimulate of IL-8 mRNA expression by IL-1, which augments the proliferation of lymphocytes in the thymus (T cells) and the production of tumor necrosis factor (TNF). These sequences suggest that the local pro-inflammatory action of TNF may be mediated by the induction of IL-8 secretion[5)6)]. Furthermore, IL-8 is suggested to cause inflammation with resultant tissue injury induced by the production of nitric oxide (NO) and/or oxygen-derived free radicals including superoxide ($O_2^{\cdot-}$) and hydroxyl radicals (• OH)[7)]. These processes are hypothesized to occur in the diaphragm muscle and to be related to the reduction of muscle contractile properties by administration of IL-8. Therefore, we examined whether IL-8 affects the diaphragm contractile properties in *in vivo* and *in vitro*, and performed NADPH diaphorase histochemistry to detect NO production in the diaphragm muscle *in vivo*.

Methods

Animal preparation

Experiments were performed using Wistar rats weighing 250-320 g (Charles River Japan, Kanagawa, Japan) after receiving written approval from the Tohoku University Animal Facility. We tested the effects of IL-8 on diaphragm muscle both *in vivo* and *in vitro*. For the *in vivo* study, we injected recombinant human IL-8 (3 μg/kg, Genzyme # 1588-00) via the tail vein, and measured force-frequency curves, twitch kinetics and fatigability at three points of time, namely, 0 hours (immediately after injection of IL-8), 2 and 4 hours after IL-8 injection ($n=6$, each), using dissected diaphragm muscle strips. For the *in vitro* study, we incubated muscle strips in a buffer with and without recombinant human IL-8 (0.01 μg/ml) in an organ bath for 4 hours. We measured force-frequency curves and twitch kinetics at 0 hours (immediately after adding IL-8 in organ buffer), 2 hours and 4 hours ($n=4$, each) after IL-8 administration.

Diaphragm muscle contractile measurements

For the *in vivo* study, the measurements of diaphragm muscle contractility were performed as previously reported[8)]. Briefly, 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. The organ baths were heated (37±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; glucose, 110 mg%; 10 μM d-tubocurarine; 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 ms duration in pulses) by a constant current stimulus isolation unit (SS-302J, Nihon Kohden) driven by a stimulator (SEN-3201, Nihon Koh-

den). The elicited tensions were measured by a force transducer (UL-100GR, Minebea Co.). The length of each muscle strip was changed by moving the position of the force transducer with a micrometer-controlled rack and pinion gear (accuracy of displacement, 0.05 mm), and measurement was carried out 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 frequencies of 1, 10, 20, 30, 50, 70 and 100 Hz. Each stimulus train was applied for approximately 1 s, and adjacent trains were applied at approximately 10-s intervals. The tensions of both muscle strips were recorded by a hot-pen recorder (RECTI-HORIZ-8K, San-ei).

Twitch contraction was elicited by a single pulse stimulation (0.2 milliseconds duration of pulses), and the trace of the twitch contraction was recorded at high speed (10 cm/seconds). The twitch kinetics were assessed based on (I) twitch tension (TT : peak tension of twitch contraction, kg/cm^2), (II) contraction time (CT : the time required to develop peak tension, milliseconds) and (III) half-relaxation time (HRT : the time required for peak tension to fall by 50%, milliseconds) during a single muscle contraction. For analysis of the contractile velocity of twitch contractions, TT/CT (slope during contraction time) and (1/2 TT)/HRT (slope during half-relaxation time) were calculated from the curve of the twitch contraction trace.

Muscle fatigability was then assessed by examining the rate of the fall of tension over a 5-minute period of rhythmic contraction. Such contraction was induced by applying trains of 20-Hz stimuli (train duration, 0.3 seconds ; rest duration, 0.7 seconds) at a rate of 60 trains/min. Muscle fatigability was expressed as a percentage of the final remaining tension (%) from the initial tension. After completion of this protocol, muscle strips were removed from the bath and weighed.

For the *in vitro* study, diaphragm muscle strips were prepared in a manner similar to that used in the *in vivo* study. We incubated muscle strips without or with IL-8 (0.01 mg/ml) in an organ bath for 4 hours. We measured force-frequency curves and twitch kinetics immediately after the addition of IL-8 to the organ buffer (i.e., 0 hours), 2 hours and 4 hours after such addition. These measurements were performed as completely as the same maneuvers in the *in vivo* studies.

NADPH diaphorase histochemistry

NADPH diaphorase histochemistry was performed at 0, 2 and 4 hours only in the *in vivo* groups ($n=4$, each). After resection of the muscle strips mentioned above, additional muscle tissue near the excision was quickly excised, and pieces of this tissue were immersed in 2% paraformaldehyde in PB at 4°C for 12 h and then stored in 10% sucrose containing PB for cryoprotection at 4°C for at least 24 h. Cryosections (10 μm in thickness) were cut from the diaphragm, mounted on chrome-alum gelatin-coated glass slides, and immersed in 0.3% Triton X-100 containing phosphate buffer for histochemistry. Histochemical reaction for NADPH diaphorase consisted of dipping the sections in freshly prepared Tris-HCl buffer (pH 8.0 ; 100 mM) containing 1.0 mM β-NADPH (Oriental Yeast Co., LTD., Tokyo, Japan), 0.2 mM nitroblue tetrazolium (Wako Pharmaceutical Co., Osaka, Japan) and 0.2% Triton X-100 for 60 min at 37°C[9]. The reac-

tion was stopped by rinsing the sections in 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, Fuji).

Data Analysis

The strip cross-sectional area was calculated by dividing muscle mass by the product of strip muscle length and muscle density (1.06 g/cm^3)[10], 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, and therefore, the sample number was $n=6$ (animals) per treatment/time point for force-frequency curves, twitch kinetics and fatigability. The mean values of tensions for each frequency in force-frequency curves, twitch kinetics and fatigability were compared by Student's t-test. To compare the entire configuration of each force-frequency curve at 0, 2 and 4 hours of the *in vivo* and *in vitro* groups, analysis of variance (ANOVA) with Fisher's PLSD (Protected Least Significant Difference) *post hoc* test was performed. Data are presented as means±SD (standard deviation). Comparisons with a p-value of less than 0.05 were considered to be statistically significant.

Results

Effects of IL-8 in vivo

The force-frequency curves of the *in vivo* group were obtained at 0, 2 and 4 hours after intravenous injections of IL-8 followed by intraperitoneal injection of endotoxin (20 mg/kg) (Fig. 1). The force-frequency curves at 2 hours were significantly decreased at 1 and 10 Hz (both $p<0.05$), at 20 Hz ($p<0.01$), and at 30, 50, 70 and 100 Hz ($p<0.001$, respectively) from those at 0 hours, and those at 4 hours were further decreased at 1 and 10 Hz (both $p<0.01$),

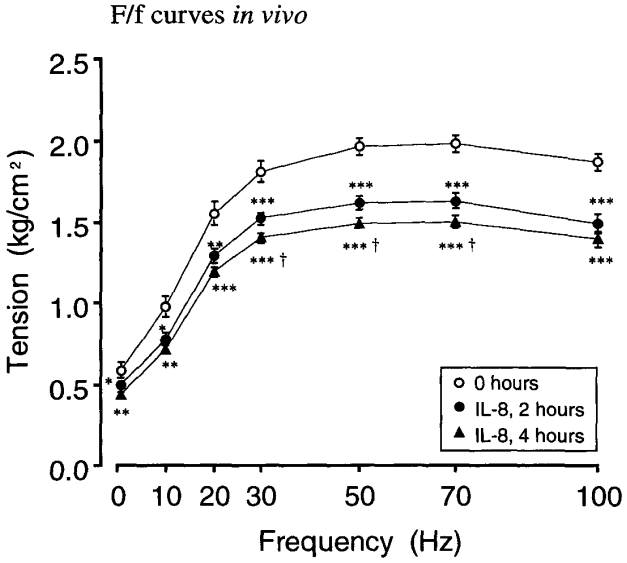

Fig. 1. Force-frequency curves *in vivo* immediately (0 hours), 2 hours, and 4 hours after IL-8 (20 mg/kg) injection. Symbols indicate significant differences at given frequencies compared with 0 hours (*$p<0.05$, **$p<0.01$, ***$p<0.001$), and compared with 2 hours ($^\dagger p<0.05$).

and at 20, 30, 50, 70 and 100 Hz ($p<0.001$, respectively) from those at 0 hours. The comparisons of whole force-frequency curves in each group were subjected to ANOVA and Fisher's *post hoc* test. The force-frequency curves were significantly decreased from those at 0 hours (1.98±0.05 kg/cm^2 as a peak) and those at 2 hours (1.63±0.05 kg/cm^2 as a peak, $p<0.001$), and were further decreased at 4 hours (1.50±0.04 kg/cm^2 as a peak, $p<0.001$). There were significant decreases of tension at 30, 50 and 70 Hz at 4 hours ($p<0.05$ each) from those at 2 hours; however, the force-frequency curves between 2 and 4 hours did not differ significantly by ANOVA.

Effects of IL-8 in vitro

In the *in vitro* study, we incubated muscle strips without (control group) and with IL-8 (0.01 μg/ml) in an organ bath for 4 hours (Fig. 2). The force-frequency curves decreased to

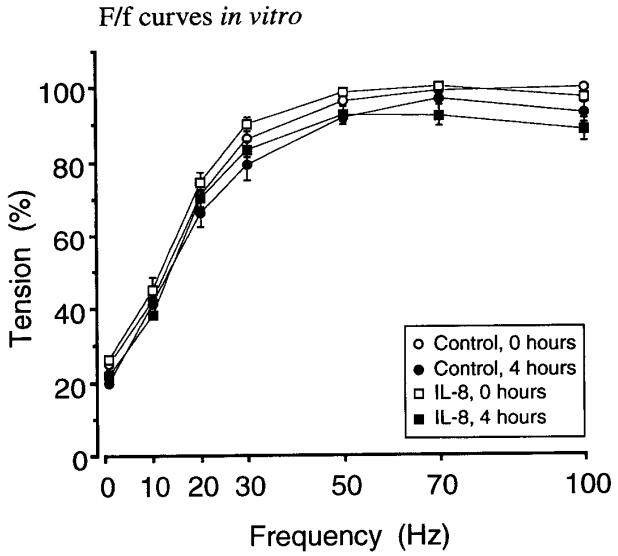

Fig. 2. Force-frequency curves *in vitro* immediately (0 hours) and 4 hours after IL-8 injection in organ bath. There were no significant changes in control and IL-8 groups between 0 and 4 hours.

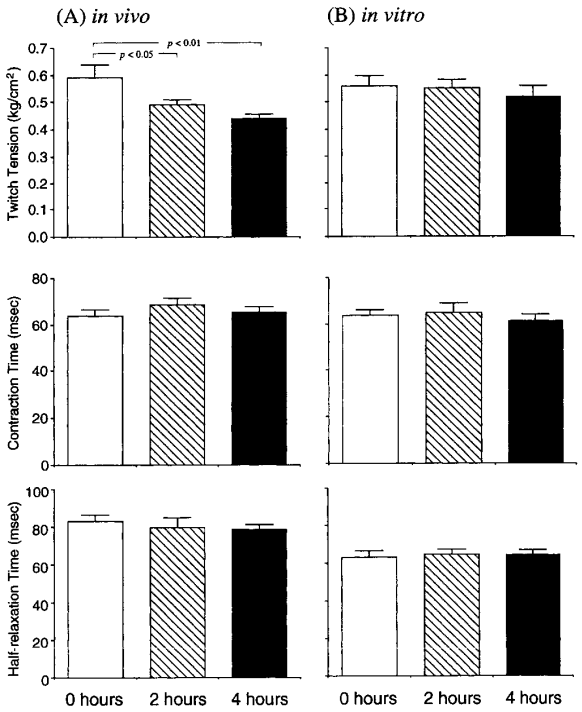

Fig. 3. Twitch kinetics of *in vivo* (A) and *in vitro* (B) groups. There were significant differences in twitch tension *in vivo*.

in the control group at 4 hours (95.8±0.95% as a peak) from the level at 0 hours, the peak tension of which was defined as 100%, but the changes were not significant. The force-frequency curves in the IL-8 group decreased at 4 hours (91.8±2.86% as a peak) from the level at 0 hours, but the change was not significant either. It may concluded that the diffusion from organ buffers of IL-8 did not have a significant effect on the diaphragm muscle.

Changes of twitch kinetics and fatigability

In the *in vivo* group, twitch tension decreased from 0.59±0.05 kg/cm² at 0 hours to 0.48±0.02 kg/cm² at 2 hours ($p<0.05$) and to 0.44±0.02 kg/cm² at 4 hours ($p<0.01$). Both contraction times and half relaxation times at each point of time did not show significant differences (Fig. 3A). In the *in vitro* groups, there were no significant changes in twitch tension, contraction time and half relaxation time (Fig. 3B). The fatigabilities at 0, 2, and 4 hours did not significantly change in either the *in vivo* group or the *in vitro* group (data not shown).

Changes of slopes of twitch contractions

In the *in vivo* group, the TT/CT of twitch contractions decreased from 0 hours (0.0093± 0.0007 kg/cm²/msec) to 2 hours (0.0072±0.0002 kg/cm²/msec, $p<0.01$) and to 4 hours (0.0068± 0.0004 kg/cm²/msec, $p<0.01$). Also, (1/2 TT)/HRT decreased from 0 hours (0.0036±0.0003 kg/cm²/msec) to 4 hours (0.0029±0.0002 kg/cm²/msec, $p<0.05$) significantly (Fig. 4A). These changes mean that IL-8 caused slowing of both contraction and relaxation at 4 hours.

In the *in vitro* group, both TT/CT and (1/2 TT)/HRT did not show significant changes. Hence, these findings mean that diffusion of IL-8 did not affect either twitch contraction or relaxation (Fig. 4B).

NADPH diaphorase histochemistry

NADPH diaphorase histochemistry was performed on diaphragm tissue taken at 0 hours

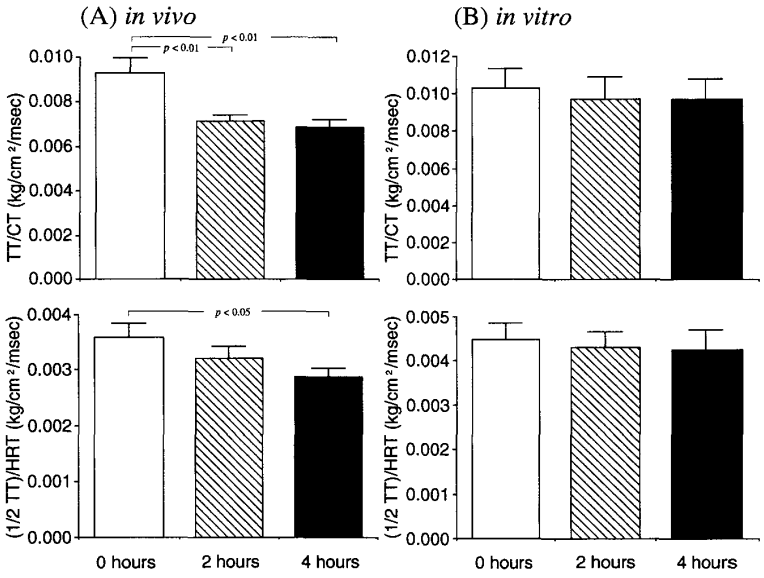

Fig. 4. Slopes of twitch contraction *in vivo* (A) and *in vitro* (B) groups. There were significant changes in TT/CT and (1/2TT)/HRT of *in vivo* group.

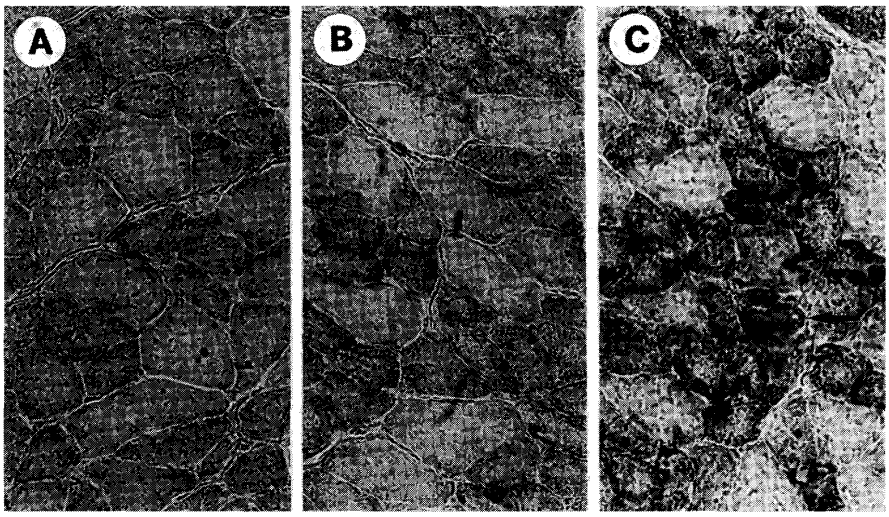

Fig. 5. NADPH diaphorase histochemistry at 0 hours (A), 2 hours (B) and 4 hours (C) after IL-8 injection in the *in vivo* group. Darker staining (black arrow) in the photo (C) indicates that NO was produced in the muscle fibers.

(A), 2 hours (B) and 4 hours (C) after IL-8 injection in the *in vivo* group only (Fig. 5). The 0-hour muscle tissue showed very little reaction along the muscle fibers, but some small muscle fibers at 4 hours showed positive staining (darker staining; black arrow) and increased in a time-dependent manner. This indicates that IL-8 causes NO production in the diaphragm muscle fibers at around 4 hours after injection.

Discussion

The *in vivo* study showed that IL-8 caused significant decreases in force-frequency curves, twitch tension and TT/CT time-dependently when it was given intravenously. Furthermore, NADPH diaphorase staining showed that

the induction of NO production occurred in the *in vivo* diaphragm muscle fibers at 4 hours after intravenous injection of IL-8. However, the *in vitro* study showed that IL-8 did not cause significant effects on force-frequency curves and twitch kinetics when absorbed from the surrounding organ buffer. From these results, it is suggested that IL-8 is a cytokine which causes deterioration of force-frequency curves and twitch contractions accompanying with NO production time-dependently.

It is noteworthy that the different administration routes of IL-8 in the *in vivo* and *in vitro* groups resulted in different responses of diaphragm muscle fibers. It is suggested that the effect of IL-8 on diaphragm muscle tissue is forthcoming mainly via the vascular routes not by diffusion from an organ bath. Middleton et al. showed that the prototype chemokine IL-8 is internalized by venular endothelial cells (ECs) and transcytosed to the luminal surface, as shown by electron microscopy, and that the intact C terminus of IL-8, the molecules' immobilization domain, is required for the EC binding and transcytosis[11]. The *in vivo* proemigratory activity of IL-8 indicates that the described subcellular interactions of IL-8 with the ECs are functionally relevant. Similarly, in the present study, the transcytosis of IL-8 is suggested to be an important mechanism for penetration of the vascular endothelial cells and immigration to subendothelial space and the diaphragm muscle tissue, and this is a reason that IL-8 requires intravenous injection rather than diffusion from an organ buffer. IL-8 transferred via endothelial cells probably directly results in the induction of NO productions in muscle tissues.

NADPH diaphorase staining showed that the production of NO increased at 4 hours due to IL-8 administration in the *in vivo* group. Since the density of NAFPH staining of the diaphragm muscle fibers was observed to be strong at 4 hours rather than at 2 hours, it seems that there was a slight delay in NO production compared with the changes of force-frequency curves and twitch kinetics at 2 and 4 hours. Biosynthesis of NO from L-arginine by constitutive NO synthase (cNOS) is known to occur in endothelial cells[12], NO synthase activity has been observed in the fatigued diaphragm muscle[13], and it has been established that skeletal muscle cells can express two constitutive isoforms of neuronal-NOS and endothelial-NOS[14]. Therefore, it is speculated that the delay of NO production was caused by the time necessary for the expressions of NO synthases in the diaphragm muscle fibers after IL-8 triggering. The NO production in the diaphragm muscle fibers is closely related to 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[15]. It is therefore likely that NO-dependent inhibition of mitochondrial respiration in the diaphragm muscle cells is also responsible for the decrement of force-frequency curves after IL-8 administration.

There are two types of IL-8 receptors, one called IL-8-RA (type A IL-8 receptor) or IL-8R1 (CXCR1), and the other called IL-8-RB (type B IL-8 receptor) or IL-8R2 (CXCR2). The priming effect of IL-8 on the neutrophil respiratory burst is predominantly mediated via IL-8-RA (type A IL-8 receptor), which is a high affinity receptor, whereas priming by melanoma growth-stimulatory activity (MGSA) and neutrophil-activating peptide

(NAP-2) is mediated by IL-8-RB (type B IL-8 receptor), which is a low affinity receptor[16]. It has also been reported that IL-8 (CC chemokine) binds with high affinity (Kd values between 0.5 to 3 nM) to both IL-8R1 (CXCR1, i.e. type A IL-8 receptor) and IL-8R2 (CXCR2, i.e. type B IL-8 receptor), whereas all the other CXC chemokines have high affinity for only IL-8R2 (CXCR2)[17]. The two receptors are quite similar in amino acid sequence identity within the seven transmembrane domains and connecting loops, but differ almost totally in their NH_2^- and $COOH^-$ terminal regions. In the neutrophils, responses followed by cytosolic-free Ca^{2+} changes and the release of granule enzymes are mediated through both receptors, whereas the respiratory burst and the activation of phospholipase D depend exclusively on stimulation via IL-8R1 (CXCR1). Therefore, because induction of NO production (i.e., respiratory burst) was observed in the diaphragm muscle tissue, it is speculated that IL-8 might trigger high affinity IL-8R1 (CXCR1, i.e. type A IL-8 receptor) in the diaphragm muscle tissue.

Clinical studies have shown that there is a significant relationship between IL-8 and variously caused tissue injury as follows. The serum level of IL-8 is increased in most patients with sepsis and correlates with some inflammatory parameters, suggesting a role of IL-8 in the pathophysiology of sepsis[18]. Circulating concentrations of cytokines measured in 44 patients with typhoid fever in the acute phase were observed to have increments of TNF-α (to 130 ± 50 pg/ml), IL-6 (to 96 ± 131 pg/ml) and IL-8 (to 278 ± 293 pg/ml)[19]. In the plasma from 33 patients at risk of multiple organ failure (MOF) after major trauma, the presence of platelet-activating factor (PAF) activity and IL-8 in the circulation was observed, suggesting that IL-8 may also be an early biochemical marker predicting the onset of MOF[20]. Concerning of the lungs, reperfusion of ischaemic lung causes neutrophil infiltration and destruction of pulmonary structure, as well as local production of IL-8. However, the administration of a neutralizing monoclonal antibody against IL-8 was found to prevent neutrophil infiltration and tissue injury, proving a causal role of locally produced IL-8 in this model[21]. The production of IL-8 mRNA occurs in most patients during CPB (cardiopulmonary bypass) in both the myocardium and skeletal muscle, and the high local IL-8 concentration augments neutrophil recruitment and contributes to subsequent tissue injury after CBP[22]. In addition, reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) can serve as intracellular second messengers to induce IL-8 gene expression in cultured fibroblasts stimulated by endotoxin[23]. As in this model, oxygenic substances such as NO and/or superoxide cause tissue injury in endotoxin-induced diaphragmatic tissue injury, and furthermore, the relationship of IL-8 and tissue injury of MOF and CBP seems to be an important mechanism in tissue injury coexisting with inflammation. Combining these results and the results of the present study, it is indicated that IL-8 is involved in diaphragm muscle contractile deterioration and NO production after endotoxin administration.

In conclusion, because IL-8 induces NO and NO contributes to the deterioration of diaphragm muscle contractile properties, IL-8 may play an important role in endotoxin-induced diaphragm muscle deterioration in a septic animal model. For clinical applications of our results, it is postulated that blocking the production of IL-8 or its circulation in the tissues would prevent diaphragm muscle deterioration in septic shock.

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