Down-regulation of Insulin-like Growth Factor I (IGF-I) in the Mouse Diaphragm during Sepsis

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Background: Diaphragm muscle impairment is an important cause of respiratory failure during sepsis. Insulin-like growth factor I (IGF-I) is an anabolic growth factor which prevents muscle degradation and wasting during sepsis, but its role in the diaphragmatic muscle during sepsis is unknown. The aim of this study was to investigate the expression of IGF-I in the diaphragmatic muscle in a murine model of sepsis induced by lipopolysaccharide (LPS).

Methods: Male B67 mice were peritoneally injected with LPS, and were killed and studied at different time-points, 24 h, 48 h, 72 h, and 96 h after injection. Diaphragm sarcolemmal damage was visualized by orange tracer dye infusion, and the expression of IGF-I, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in diaphragm tissue extracts were measured using ELISA.

Results: LPS induced sarcolemmal damage in diaphragm myofibers from 24 h to 96 h, which was accompanied by a significant increase in IL-1β expression in the tissues while IGF-I levels were down-regulated. No change in TNF-α was observed. Body weights of animals were also reduced, especially at 96 h.

Conclusions: The expression of IGF-I in diaphragm tissues was down-regulated during sepsis-induced diaphragm myofiber damage, suggesting that IGF-I may be an important factor in the regulation of diaphragm myofiber repair. Further studies are needed to examine the mechanisms involved.

(Chang Gung Med J 2010;33:501-8)

Key words: diaphragm myofiber, insulin-like growth factor-I, interleukin-1β, lipopolysaccharide, sarcolemmal damage, sepsis

References:

1. Respiratory failure, is frequently observed in patients with severe sepsis syndrome and is a major factor contributing to the high mortality rate. (1)

2. The diaphragm is a major primary muscle for respiration; diaphragm dysfunction characterized by a decrease in both diaphragmatic force production and endurance has been previously demonstrated in animal models of sepsis. (2,6) An inability of the diaphragm to ventilate the lungs, rather than pulmonary involvement per se, was seen in animals dying of respiratory failure in a septic shock model. (3)

Therefore, respiratory muscle dysfunction plays an
important role in the pathogenesis of respiratory insufficiency in sepsis syndrome.

The mechanism of respiratory failure in septic syndrome is not clear yet. Observations in studies of sepsis in animal models have proposed different mechanisms of pathogenesis such as an imbalance in the supply and utilization of energy by diaphragmatic muscle cells, and the effects of inflammatory mediators released in the inflammatory response in sepsis. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were reported to induce catabolic effects on skeletal muscle cells and may also be up-regulated in sepsis. Continued muscle protein breakdown could therefore result in muscle wasting and fatigue. Severe protein breakdown in respiratory muscle such as the diaphragm in sepsis can therefore lead to an insufficiency in respiratory ventilation and respiratory failure.

Studies have demonstrated that insulin-like growth factor I (IGF-I) could slow the catabolic response in skeletal muscle by inhibiting a ubiquitin-dependent pathway. IGF-I is a peptide hormone secreted from many different cells and the liver is the main source of synthesis with stimulation by pituitary growth hormone (GH). It is an anabolic effector hormone of GH. IGF-I was also shown to increase protein synthesis in the skeletal muscle of septic rats. Another source of extra-hepatic expression is myoblasts from skeletal muscles. There is also evidence showing that IL-1β is an endogenous mediator of the sepsis-induced changes in IGF-I, suggesting that the accompanying changes in muscle protein synthesis are partially mediated via changes in IGF-I.

Therefore, this study investigated the expression of IGF-I in the diaphragmatic muscle in a murine model of sepsis induced by lipopolysaccharide (LPS). We also examined the relationship between the expression of IGF-I and the amount of myofiber injury and the level of IL-1β.

METHODS

Animals

Pathogen-free male B57 eight week-old mice weighing 20-25 g were killed on the designated days by dislocation of the cervical vertebrae. The studies were performed within Taiwan Institutional Review Board (IRB) guidelines and were approved by the animal experiments committee for animal welfare at Chang Gung Memorial Hospital.

Study design

Mice were injected with LPS 20 mg/kg peritoneally and were randomly assigned to different time points, 24 h, 48 h, 72 h, and 96 h, after injection of a single dose of LPS. They were compared to control animals (n = 4 at each time point and controls). Diaphragms were excised for examination on the day of study. Parameters for the study included the expression of IGF-I, TNF-α and IL-1β in diaphragm tissues. Diaphragm damage was examined by fluorescent tracer dye under a microscope.

Tissue preparation

Frozen diaphragmatic samples of about 0.2 g of tissue were homogenized in 4 separate 500 µL volumes of buffer solution (0.9% NaCl, 10 mM Na₂HPO₄, 1 mmol PMSF, 1 mg/l each of pepstatin, aprotinin, and leupeptin, 0.5% Triton X-100, and 0.05% sodium azide, pH 7.2). Samples were sonicated briefly. The crude homogenates were centrifuged at 4°C for 20 min at 14,000 rpm and the supernatants were collected.

Morphologic assessment of diaphragmatic sarcolemmal injury

The level of sarcolemmal injury was studied, as previously described. Briefly, diaphragms were excised and perfused with a low-molecular weight (FW = 631) fluorescent tracer dye, Procion Orange (Chemicals, Poole, U.K.). Tissues were then immediately submerged in 1% oxygenated Procion dye/Ringer’s solution for an additional 90 min at room temperature. Diaphragm tissues were then snap-frozen. Cryostat sections of 10 µm thickness were embedded in mounting medium and were viewed under a fluorescence microscope (magnification 100X). Damaged myofibers were identified by the penetration of dye into the cytoplasm. Images were then counted by using the program ImageJ from the National Institutes of Health, Bethesda, MD, U.S.A.. Myofiber damage was measured by the percentage of cells that penetrated with dye out of the total number of cells. An average was calculated from measurement of a minimum of 300 myofibers from 3-5 randomly-chosen fields for each section.
IGF-I level determination by ELISA

The expression of IGF-I in diaphragm extracts was measured by a double sandwich ELISA using monospecific, polyclonal mouse antibodies to the respective recombinant cytokines. Briefly, Immuno-Maxisorp plates (Nunc™, Thermo Fisher Scientific, Rochester, NY, U.S.A.) were coated with protein-A affinity-purified anti-IGF-I mouse IgG before adding 50 µl of the samples. Non-specific IgG binding was inhibited by adding 50 µl PBS supplemented with 10% normal mouse serum (Dako, Glostrup, Denmark), EDTA 10 mM, aprotinin 2000 KIE/ml, and DL-dithiothreitol 10 mM. Biotinylated mouse antibody (Sigma-Aldrich, St. Louis, MO, U.S.A.) was used as a detecting antibody along with streptavidin–peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.). Assays were developed with substrate solution 1,2-phenylenediamine dihydrochloride and measured at 492 nm. The minimum detectable dose of the mouse IGF-I was 3.5 pg/mL.

TNF-α and IL-1β detection by ELISA

Analysis of TNF-α and IL-1β was carried out on the extracts of diaphragm tissues using a commercially available ELISA kit (Biosource International, Camarillo, CA, U.S.A.) specific for mice. Sensitivities of the kit for TNF-α and IL-1β were 4 and 1 pg/ml, respectively. Absorbance of the wells was read at 450 nm with a MR600 microplate reader (Thermomax-Molecular Devices; Fisher Scientific Instrument, Nepean, ON, Canada). Background absorbency of the blank wells was subtracted from the standards and unknowns prior to the determination of sample concentrations.

Statistical analysis

Data are presented as the mean ± SEM. For comparisons of different groups, the Kruskal-Wallis test for analysis of variance was used first. If this test was significant, a post-hoc Dunn’s test was applied for comparison between two individual groups. p values < 0.05 were considered significant.

RESULTS

Body weight change

The weights of the mice were reduced from 24 h to 96 h after injection of LPS compared to controls. Body weights were significantly reduced especially at 96 h (–6.1 ± 0.69 g) compared with the controls (0.0 ± 0.84 g, p < 0.05, Fig. 1).

Diaphragmatic sarcolemmal injury

Normally, undamaged myofiber sarcolemma are not permeable to the fluorescent tracer dye Procion Orange (Fig. 2A). After myofibers were damaged, the cytoplasm of the cells was penetrated and enhanced with dye, indicating the cell membrane integrity had been interrupted (Fig. 2B).

IL-β & TNF-α concentration

In our model of septic response induced by LPS injection, we measured IL-1β and TNF-α expression in diaphragm extracts. There were no changes in TNF-α expression in diaphragmatic myofibers at any time point (24 h 39.6 ± 0.88 ng/mg, 48 h 40.4 ± 1.54 ng/mg, 72 h 40.9 ± 2.03 ng/mg and 96 h 41.8 ± 2.39 ng/mg respectively, p > 0.05) compared to the controls (38.1 ± 1.03 ng/mg). The IL-1β concentration increased from 24 h (17.0 ± 1.90 ng/mg, p < 0.05) to 96 h (55.0 ± 18.00 ng/mg, p < 0.01) after injection of LPS compared with the controls (8.5 ± 0.45 ng/mg, Fig. 3).

IGF-I concentration

Our results demonstrated that after LPS injection, IGF-I expression in diaphragm tissues was increasingly suppressed from 48 h (4.5 ± 0.20 ng/mg, p < 0.01) to 96 h (2.6 ± 0.30 ng/mg, p <
This study also determined whether LPS induces diaphragm muscle cell damage. Our results demonstrated that after LPS injection, the percentage of injured myofiber cells increased from 24 h, 48 h, and 72 h (10.0 ± 1.00%, p < 0.05; 17.1 ± 1.10 ng/mg, p < 0.001; 44.2.0 ± 2.0%, p < 0.001 representatively) to 96 hrs (49.2 ± 3.50%, p < 0.001) compared with the controls (0.9 ± 0.20%, Fig. 5).

**Fig. 2** Morphologic assessment of diaphragmatic myofiber cell injury 24 h after LPS administration. (A) Only a small amount of orange tracer dye is found inside the myofiber cells in the control group. (B) Penetration of dye into myofiber cells indicates that cell membranes have been damaged by endotoxins.

**Fig. 3** Concentration of IL-1β in diaphragm tissues measured by ELISA. LPS injection has decreased the IL-1β level in the diaphragms after 24 h, 48 h, 72 h, and 96 h. Error bars represent the SEM. *: p < 0.05 and **: p < 0.01 compared to the controls. N = 4 for experimental animals at each time point and controls.

**Fig. 4** Concentration of IGF-I in the diaphragms. Following injection of LPS, the concentration of IGF-I was measured by ELISA at 24 h, 48 h, 72 h and 96 h. Error bars represent the SEM. **: p < 0.01 and ***: p < 0.001 compared to the controls. N = 4 for experimental animals at each time point and controls.
Correlations

After LPS injection, there was an upregulation of IL-1β expression in the diaphragms, indicating an increase in the inflammatory response. This coincided with the increased percentage of myofiber damage induced by LPS and IL-1β. IGF-I has anabolic effects on muscle. The IGF-I levels were down-regulated with time and demonstrated a negative correlation with the amount of myofiber injury (Spearman r: \(-0.91; p < 0.001\), Fig. 6A). The expression of IGF-I was inversely correlated with that of IL-1β (Spearman r: \(-0.57; p < 0.01\), Fig. 6B) and could actually be suppressed by the increasing expression of IL-1β in sepsis.

DISCUSSION

In the present study, we demonstrated that IGF-I expression in diaphragmatic myocytes was down-regulated in sepsis and this was inversely correlated with the sarcolemmal damage in the diaphragmatic myofibers, suggesting IGF-I may exhibit a protective effect on myofibers during sepsis. We also observed

![Graph showing correlations](image)

**Fig. 6** Correlation of IGF-I, myofiber cell injury and IL-1β. Figures are the correlation of IGF-I (ng/mg) with (A) myofiber cell injury (%), and (B) IL-1β concentration (ng/mg). \(r\) is Spearman’s correlation coefficient and \(p\) is the significance value. The dotted lines represent the upper and lower 95% confidence intervals. \(N = 4\) for experimental animals at each time point and controls.
IL-1β expression in the diaphragm was up-regulated following LPS injection, while TNF-α was not affected. The upregulation of IL-1β expression, the suppression of IGF-I and the increased myofiber damage also coincided with the body weight loss in this sepsis model.

IGF-I is an anabolic growth factor which is responsible for normal growth and development. Most secreted IGF-I is bound to its serum-binding protein with rather constant plasma levels while less than 10% of circulating IGF-I is in free form. In the bound form, IGF does not cross-react with the insulin receptor and does not exert an insulin-like effect. IGF-I can act either as a hormone or as a local factor. Autocrine IGF-I production has been demonstrated to play a crucial role in muscle growth. It also exerts anti-apoptotic effects in muscle, favoring the survival of differentiated cells. Our results showed that a decreased expression of IGF-I in diaphragmatic myofibers coincided with an increased level of myofiber damage in the diaphragm, which is supported by another study which observed that sepsis decreased IGF-I levels in the blood, liver, and gastrocnemius muscle in rodents. To our knowledge, this is the first study to demonstrate that IGF-I may play an important role in regulating the function of diaphragmatic muscle myofibers.

IL-1β is an endogenous mediator in sepsis which attenuates both basal and growth hormone-stimulated IGF-I synthesis and secretion in rat hepatocytes. One study showed that central administration of IL-1β produced changes in the IGF system which mimic that induced by endotoxin. Blockade of IL-1β may prevent a decrease in the skeletal muscle protein synthesis induced by the septic state partly mediated via the IGF-1 axis. Our results demonstrated that LPS administration induced an increase in endogenous IL-1β expression in diaphragmatic myofibers with a concomitant decrease in IGF-I expression. This suggests that IGF-I expression is suppressed by an increased IL-1β level.

TNF-α is an inflammatory cytokine which is reported to be upregulated by LPS in cultured rat myocytes and may play an important role in LPS-induced myocyte apoptosis. We therefore examined TNF-α expression in myofibers following injection of LPS in rats. We observed that LPS administration upregulated the expression of IL-1β but not TNF-α. This indicated that LPS-induced myofiber damage was different from that of myocytes and was not a TNF-α dependent pathway. The actual pathway is not known and we did not examine the mechanism in this study. Perhaps, IL-1β and TNF-α upregulation occurs by two independent pathways. This was supported by a study that used murine peritoneal macrophages pretreated in vitro for 4 hr with LPS or PMA or LPS plus protein kinase C inhibitor (PKCi) or 8-bromo-cAMP. In this study, IL-1β and TNF-α were released by independent signal transduction pathways. In another study by Fernández-Celemin and co-workers, IL-1β failed to decrease the IGF-I mRNA level significantly in C2C12 myotubes, but TNF-α strongly inhibited IGF-I mRNA and protein; this may further support this independent release theory. However, their results were different from what we observed in our study. This may be explained by differences in animal models, muscle measurement or study design. This could be seen in studies measuring muscle weight changes in sepsis, in which the weight of the gastrocnemius was significantly reduced by sepsis while the weight of the soleus was unaffected.

In conclusion, the expression of IGF-I in diaphragm tissues was down-regulated in sepsis together with increased diaphragmatic sarcolemmal damage. Although the mechanism is not yet clear, this suggests that the inhibition of IGF-I plays an important role in the regulation of diaphragm tissue damage in sepsis, and may perhaps contribute to the loss of myofibers in sepsis. Also, endogenous IL-1β but not TNF-α induced by LPS may act as a mediator that regulates murine diaphragm metabolism concomitantly with the suppression of IGF-I. The mechanisms for these muscle fiber changes and the role of IGF-I in muscle atrophy during sepsis are not entirely clear. There was a limitation in this study in that we did not further measure changes in serum cytokines and the timing of expression of transcriptional factors. A deeper understanding of the loss of muscle fibers and related changes may be important for further insight into the mechanisms underlying diaphragmatic atrophy in sepsis.

Acknowledgements
This study was supported by a grant from the National Science Council of Taiwan NSC 91-2314-B-182A-088.
REFERENCES


敗血症將導致老鼠橫膈肌肌肉中第一型類胰島素生長因子 (IGF-I) 的降低

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背景: 橫膈膜肌肉的損傷是敗血症中導致呼吸衰竭的重要因素之一。第一型類胰島素生長因子 (Insulin-like growth factor-I, IGF-I) 是一種促進蛋白合成的生長因子，可以防止肌肉的破壞與耗損。然而我們對於敗血症發生時，第一型類胰島素生長因子在橫膈肌所扮演的組織仍不是很了解。本研究主要在探討當老鼠發生敗血症反應時，第一型類胰島素生長因子在橫膈肌的表現，並且更進一步觀察其與橫膈膜傷纖維損傷程度以及間白素-1β (Interleukin-1β) 濃度之間的關係。

方法: 實驗模組選用 B57 的公老鼠，並以腹腔注射的方式給予脂多醣 (Lipopolysaccharide, LPS) 來使其產生敗血症的反應。然後依照不同的時點 (4, 48, 72 與 96 小時)，採取老鼠橫膈的樣本進行測試。對於橫膈肌纖維使用染的狀況，將以硫色逐及染料注入 (orange tracer dye infusion) 的方式來觀測。至於在橫膈組織上，第一型類胰島素生長因子、間白素-1β及腫癈壞死因子α (tumor necrosis factor-α, TNF-α) 等之濃度表現則採用酶免疫分析法 (enzyme-linked immunosorbent assay, ELISA) 來測量。

結果: 老鼠經由脂多醣刺激後的 24 到 96 小時內，即可發現橫膈肌纖維有損傷的現象產生，而第一型類胰島素生長因子在橫膈組織上之濃度則是呈現降低的趨勢，並且隨間白素-1β的濃度有明顯上升的表現。然而腫癈壞死因子α的濃度則無明顯變化。特別是在 96 小時的時候，發現老鼠的體重有明顯的下降現象。

結論: 當敗血症發生時，第一型類胰島素生長因子在橫膈組織上有降低的現象。而這可能是構成敗血症發生時。肌纖維損傷以及呼吸力衰竭的原因。

(長庚醫誌 2010;33:301-8)

關鍵詞: 橫隔膜肌纖維，第一型類胰島素生長因子，間白素-1β、脂多醣，肌纖維損傷，敗血症

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