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Protective Effects of Gabexate Mesilate (FOY) on Diaphragm Muscle after Endotoxin Administration

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エンドトキシン投与後におけるメシル酸ガベキセート (FOY) の横隔膜筋への防御効果

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Since it is known that endotoxins induce disseminated intravascular coagulation (DIC), we examined whether gabexate mesilate (FOY), a synthetic serine protease inhibitor, prevents diaphragm muscle contractile deterioration after endotoxin administration. We divided a total of 24 Wistar rats into a saline group as control, an endotoxin group (20 mg/kg, iv), an endotoxin+FOY group (endotoxin 20 mg/kg, iv, then FOY 20 mg/kg/hour), and a thrombin group (25 U/kg/hour) (n=6, respectively). In the endotoxin group, the force-frequency curves were significantly decreased at 4 hr (1.31±0.09 kg/cm^2 as a peak, $p<0.001$) compared with those of the control group (2.15±0.12 kg/cm^2). However, in the endotoxin+FOY group, the force-frequency curves were not decreased (1.93± 0.09 kg/cm^2, NS) from the control, and those of the thrombin group (1.66±0.11 kg/cm^2, $p<0.01$) showed a decrease intermediate between those of the control and endotoxin group. In the endotoxin+FOY groups, both contraction and half relaxation times became longer and more fatigue resistant than those of the control group, however, FOY improved contractile velocity. These results indicate that endotoxins may cause an acceleration of coagulation which partially contributes to diaphragm muscle contractile deterioration, and that such deterioration can be prevented by FOY. Therefore, we speculate that FOY may possibly prevent respiratory muscle failure accompanying septicemia.

Introduction

It is well known that endotoxin induces disseminated intravascular coagulation (DIC) in both experimental animals[1] and septicemic patients[2]. In experimental animals[1], it has

been reported that the administration of bacterial endotoxin activates intravascular coagulation and induces fibrin deposition in the glomerulus leading to renal cortical necrosis, recently termed multiple organ failure[3].

Gabexate mesilate, [ethyl-4-(6-guanidinohexanoyloxy) benzoate] methane-sulfonate ($C_{16}H_{23}N_3O_4 \cdot CH_3SO_3H$), is a preparation of the protease inhibitor developed by Fujii et al.[4] which has strong inhibitory effects on trypsin, kallikrein, plasmin, thrombin and C_1-esterase. Since gabexate mesilate has both antifibrinolytic and anticoagulatory effects, it is expected to be effective in the treatment of DIC, which causes abnormalities in both coagulation and the fibrinolytic system. In addition, gabexate mesilate has been found to inhibit the aggregation of endotoxin-induced experimental DIC in rats[5], and at a dose ranging from 10 and 50 mg/kg administered to the patients clinically, its effectiveness against DIC has been reported[6].

Although endotoxin induces diaphragm muscle dysfunction[7], it is still unclear if endotoxin-induced muscle dysfunction is accompanied by abnormalities of coagulation and fibrinolytic mechanisms. We hypothesized that gabexate mesilate (FOY), a synthetic serine protease inhibitor, may prevent diaphragm muscle contractile deterioration after endotoxin administration. In the present study, to evaluate the effects of FOY on diaphragm muscle contraction after endotoxin administration, we measured muscle contractile properties (twitch kinetics, force-frequency curves and fatigability) after 4 hours of continuous FOY injection following endotoxin administration, and compared the data with those of saline, endotoxin and thrombin injection groups.

Methods

Animal preparation

Experiments were performed on a total of 24 Wistar rats weighing 250-320 g (Charles River Japan, Kanagawa, Japan), divided into 4 groups: (I) a saline group ($n=6$) as control, (II) an endotoxin group (20 mg/kg, iv, $n=6$), (III) an endotoxin+FOY group (endotoxin 20 mg/kg+FOY 20 mg/kg, $n=6$), and (IV) a thrombin group (25 U/kg, $n=6$). Gabexate mesilate (FOY) was kindly provided by Ono Pharmaceutical Company. The control group was given a continuous injection of 0.5 ml/hour saline via the tail vein for 4 hours. The endotoxin group was given a bolus of 20 mg/kg of Escherichia Coli endotoxin (Sigma, E3131) with 0.5 ml saline. The endotoxin+FOY group was given a bolus of 20 mg/kg of Escherichia Coli endotoxin (Sigma, E3131), following the administration of 20 mg/kg/hour of FOY by a syringe pump (Top-5100) for continuous infusion via the tail vein for 4 hours. The thrombin group was given 25 U/kg/hour of thrombin (Sigma, T6759) using a syringe pump (Top-5100) for continuous infusion via the tail vein for 4 hours. The diaphragm muscle contractile properties of all animals in each group were measured after 4 hours of the treatments mentioned above. The time of measurements (at 4 hours) has been determined by a previous study, which showed that induction of diaphragm muscle contraction was worst around 4 hours after endotoxin injection[7]. Before the start of the experiment, we obtained written approval from the Ethics Committee of the Tohoku University Animal Facility.

Measurements of diaphragm muscle contraction

The measurements of diaphragm muscle contractility were performed according to the

previous report[8]. Namely, we dissected the diaphragm muscle en block under light anesthesia (Diethyl Ether), and made two muscle strips (3-4 mm wide) from the right and left hemidiaphragms. These were mounted in two 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 was as follows: Na^+, 153.8 mEq/l; K^+, 5.0 mEq/l; Ca^{2+}, 5.0 mEq/l; Mg^{2+}, 2.0 mEq/l; Cl^-, 145.0 mEq/l; HCO_{3-}, 15.0 mEq/l; HPO_4^{2-}, 1.9 mEq/l; SO_4^{2-}, 2.0 mEq/l; 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., about 1.5 times the current required to elicit maximal twitch tension, 200-250 mA, pulses of 0.2 msec duration) by a constant current stimulus isolation unit (SS-302J, Nihon Kohden) driven by a stimulator (SEN-3201, Nihon Kohden). 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 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 predetermined Lo was maintained in the following measurements.

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

Twitch contraction was elicited by single pulse stimulation (0.2 msec), and the trace of the twitch contraction was recorded at high speed (10 cm/sec). Twitch kinetics were assessed by measuring (1) twitch tension (TT, peak tension of twitch contraction, kg/cm^2), (2) contraction time (CT, the time required to develop peak tension, msec), and (3) half relaxation time (1/2RT, the time required for the tension to fall by 50% from peak tension) from the recorded curve during a single muscle contraction. For analysis of contractile velocity, TT/CT (slope during contraction time) and TT/(1/2RT) (slope during half relaxation time) were calculated from the trace of the twitch contraction line.

Muscle fatigability was then assessed by examining the rate of tension over 5 min of rhythmic contraction. Rhythmic contraction (fatigue run) was induced by applying trains of 20 Hz stimuli (train duration, 0.3 sec; rest duration, 0.7 sec) at a rate of 60 trains/minute. Muscle fatigability was expressed as a percentage of the final remaining tension from the initial tension (%). Therefore, a larger percentage of muscle fatigability indicates greater fatigue resistance than a smaller one.

Immediately after the fatigue run, we repeated the measurements of the force-frequency curve as data of post-fatigue runs. After completion of this protocol, muscle strips were removed from the organ bath and weighed by an electronic scale (A & D Co.).

Data Analysis

For the calculation of muscle tensions, the

cross-sectional area of the muscle strip was calculated by dividing muscle mass by the product of strip muscle length and muscle density (1.06 g/cm³)[9], and tension was presented as force per unit cross-sectional area (kg/cm²). The mean values for each frequency of the force-frequency curves, twitch kinetics and fatigability were compared by Student's t-test. All data are presented as means±SE. Data with a p value of less than 0.05 were considered to be statistically significant.

Results

Effects of endotoxin and thrombin on the force-frequency curves

Figure 1 shows the mean changes of the force-frequency curves in the control, endotoxin and thrombin groups at 4 hours after saline or endotoxin injection. In the endotoxin group, the force-frequency curves were significantly decreased (a peak of 1.31±0.09 kg/cm², $p<0.001$) compared to that of the control group (a peak of 2.15±0.12 kg/cm²). The mean force-frequency curve of the thrombin group showed a decrease intermediate between the curve of the control group and that of the endotoxin group (a peak of 1.66±0.11 kg/cm², $p<0.01$ compared with control). From these findings, it seems that the thrombin injection (at a dose of 25 U/kg/hour) induced coagulation systemically and also caused diaphragm muscle contractile deterioration, however, the decrement of force-frequency curves was still less than that of endotoxin (at a dose of 20 mg/kg).

Effects of FOY on force-frequency curves

Figure 2 shows the mean changes of force-frequency curves in control, endotoxin, and endotoxin+FOY groups at 4 hours after saline or endotoxin injection. The force-frequency curves of the endotoxin group were redrawn in the same manners in Fig. 1, and those significantly decreased (1.31±0.09 kg/cm², $p<0.001$) compared with that of the control group (a peak of 2.15±0.12 kg/cm²). In the endo-

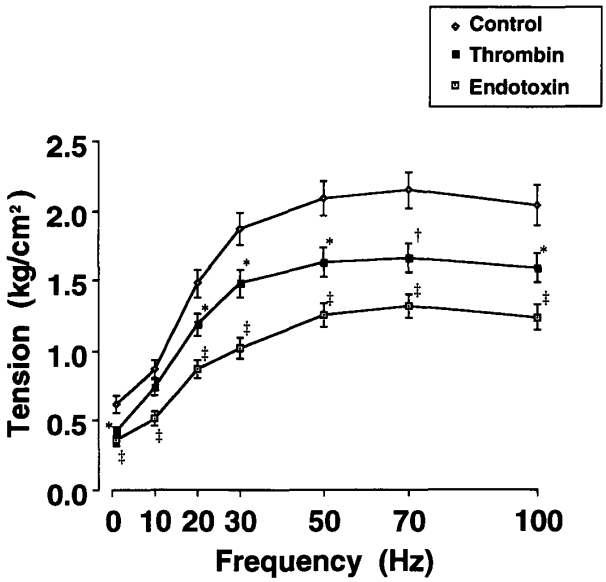

Fig. 1. Force-frequency curves of control, thrombin and endotoxin groups at 4 hr after each injection. Symbols indicate significant differences at given frequencies compared with control (*$p<0.05$, †$p<0.01$, ‡$p<0.001$).

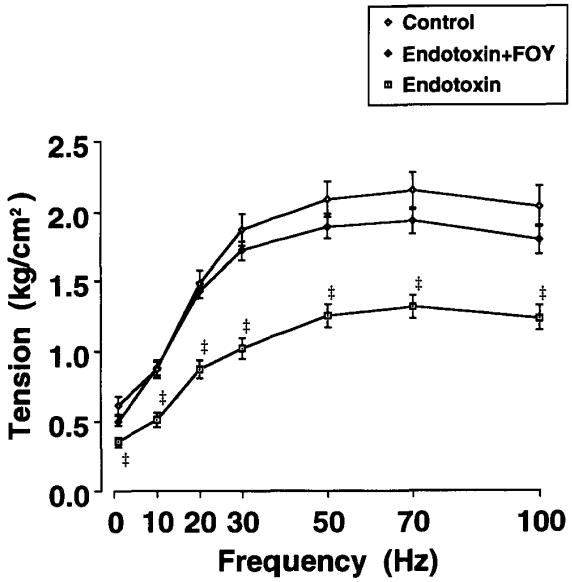

Fig. 2. Force-frequency curves of control, endotoxin+FOY and endotoxin groups at 4 hr after each injection. Symbols indicate significant differences at given frequencies compared with control (‡$p<0.001$).

toxin+FOY group, the force-frequency curves (a peak of 1.93 ± 0.09 kg/cm^2, $p < 0.001$) did not show significant changes from that of the control; in other words, FOY significantly prevented the decrement of endotoxin induced diaphragm muscle deterioration.

Changes of twitch kinetics

Figure 3 shows the mean data of twitch tension, contraction time and half relaxation time of each group. In terms of twitch tension (A), the endotoxin and thrombin groups showed significant decreases from that of the control ($p < 0.001$ and $p < 0.01$, respectively). However, the endotoxin+FOY group did not show significant differences. These tendencies were the same as the results of the force-frequency curves. As for the contraction time (B), both the endotoxin and thrombin groups did not show significant changes, but the endotoxin+FOY group showed a significant increase compared with control ($p < 0.05$). In the half relaxation time (C), the endotoxin, thrombin and endotoxin+FOY groups showed significant increases compared with the control ($p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively). Both the contraction and half relaxation times increased in the endotoxin+FOY group, indicating that diaphragm muscle contraction changed to slower contraction than control.

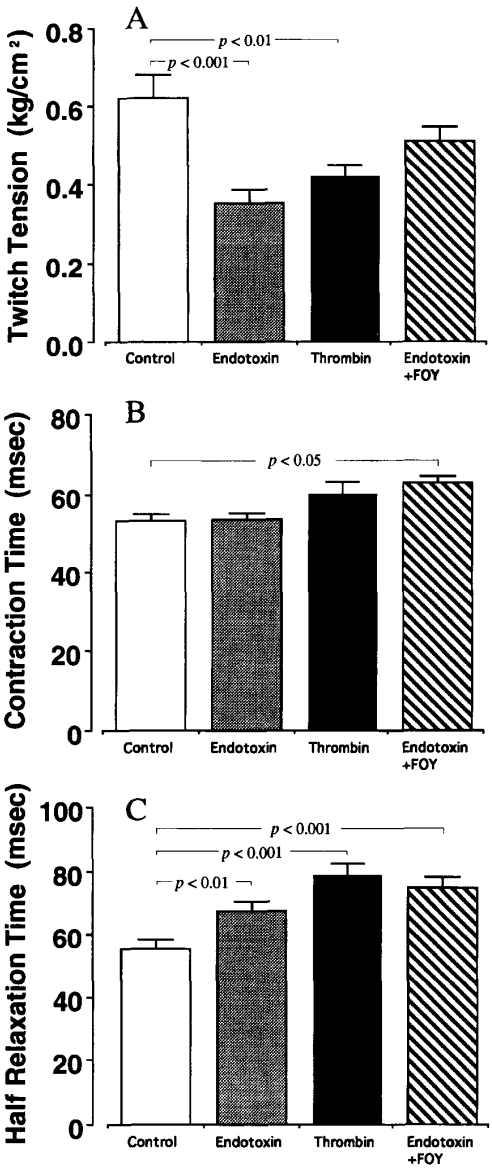

Fig. 3. Mean changes of twitch tensions (A), contraction time (B) and half relaxation time (C) fatigability in the control, endotoxin, thrombin and endotoxin+FOY groups. Differences are compared with control.

Table 1 summarized the slopes during contraction and half relaxation times of twitch contractile trace in each group for analysis of contractile velocity. In the endotoxin+FOY group, both TT/CT and TT/(1/2RT) in the trace of the twitch contraction showed 8.1 and 6.8 kg/cm^2/sec, respectively, compared to those of both endotoxin and thrombin groups. These

Table 1. Summary of slopes during contraction and half relaxation times of twitch contractile trace in each group.

	TT/CT (kg/cm^2/sec)	TT/(1/2RT) (kg/cm^2/sec)
Control	11.6	11.1
Endotoxin	6.6	5.2
Thrombin	6.9	5.3
Endotoxin+FOY	8.1	6.8

TT: twitch tension, CO: contraction time, 1/2RT: half relaxation time

results indicate that FOY increased contractile velocity and recovered in a direction to control from both endotoxin and thrombin groups.

Changes of fatigability and post-fatigue force-frequency curves

Figure 4 shows the fatigability after 5 minutes of repetitive stimulation in the experimental groups. The endotoxin group and the endotoxin+FOY group showed a significantly higher ratio of final tension/initial tension (21.5± 1.3%, $p<0.01$ and 24.3±2.1%, $p<0.01$, respectively) compared with the control (15.3± 0.6%). The thrombin group showed no significant changes in the ratio of final tension/initial tension (16.4±0.9%). This indicates that the endotoxin+FOY group became fatigue resistant at 20 Hz.

Figure 5 shows the force-frequency curves after the fatigue run in the control, endotoxin, thrombin and endotoxin+FOY groups. In the endotoxin+FOY group, the tensions of the force-frequency curves were significantly higher than those of the other groups. The symbols in the figure indicate significant differences from the endotoxin group. The findings indicate that the tensions remained greater in the range of not only 20 Hz but also at other frequencies in the endotoxin+FOY group.

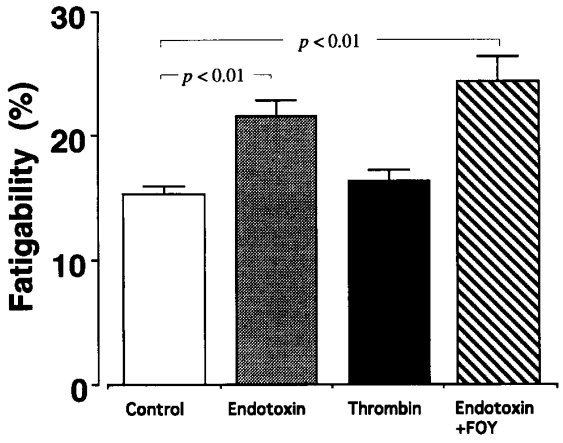

Fig. 4. Mean changes of fatigability in the control, endotoxin, thrombin and endotoxin+FOY groups. Differences are compared with control.

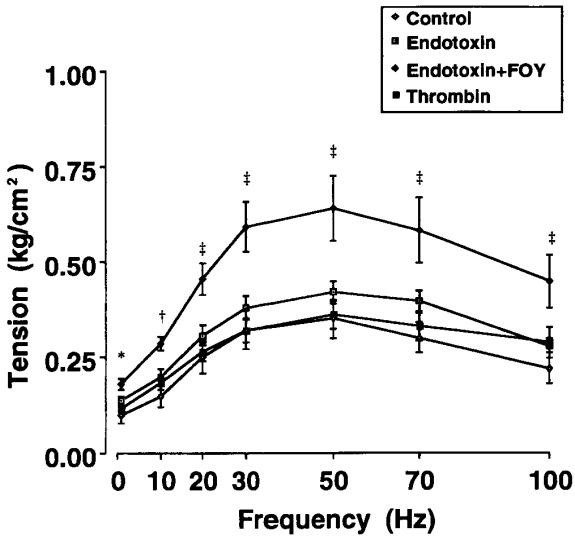

Fig. 5. Force-frequency curves of control, endotoxin, endotoxin+FOY and thrombin groups after fatigue runs. Symbols indicate significant differences at given frequencies compared with the endotoxin group (*$p<0.05$, †$p<0.01$, ‡$p<0.001$).

Discussion

In the present study, we found that FOY (20 mg/kg/hour) prevented the decrement of the force-frequency curves induced by endotoxin administration, and that groups treated with FOY showed greater fatigue resistance than the control group in diaphragm muscle contraction. Although both contraction and half relaxation times increased in the endotoxin+FOY group, FOY recovered contractile velocity in a direction to control compared to both endotoxin and thrombin groups. Furthermore, thrombin (25 U/kg/hour) induced a decrease intermediate between those of the control and endotoxin groups. Because it is known that endotoxin induces (1) DIC, (2) oxygen-derived free radical production, and (3) TNF-α induction, we propose that FOY simply or combinatively inhibits

these three potential mechanisms.

In terms of clinical findings, it has been reported that in five patients with septicemia, markedly increased levels of plasminogen activator (PA)-inhibitor (14.5±15.5 U/ml), as compared with those of control subjects (1.3±0.7 U/ml), were observed in plasma[10]. Okajima et al. have reported that leukocytes play a critical role in the activation of intravascular coagulation in patients with septicemia[11]. These data suggest that the appearance of this fast-acting PA-inhibitor is very sensitive to endotoxin stimulation. The marked increase in the level of PA-inhibitor in blood may contribute to the pathogenesis of DIC in septicemia. In addition, endotoxin-stimulated endothelial cells may trigger blood coagulation by producing procoagulant (thromboplastin-like) activity[12], and deficient fibrinolysis in DIC has been found to contribute to the precipitation of fibrin within blood vessels *in vitro*[13]. These reports thus confirm that septicemia induces DIC consistently, in other words, it may be said that endotoxin induces DIC.

Even though we used only one dose of thrombin (25 U/kg/hour), the fact that the force-frequency curves in the thrombin group showed a decrease intermediate between that of the control and endotoxin groups is an interesting finding, which suggests that an acceleration of coagulation may induce limitation of blood flow perfusion throughout the diaphragm muscle, which partially contributes to endotoxin-induced diaphragm deterioration. However, the anticoagulatory effects of FOY inhibited DIC, avoiding the limitation of blood flow perfusion and thus preventing the decrease of force-frequency curves by endotoxin. Although we did not measure FDP (fibrin/fibrinogen degradation products) for diagnosing DIC in the present study, it has been reported that FOY inhibited endotoxin-induced experimental DIC in rats[5]. In view of these reports and our results, we suggest that DIC contributes to endotoxin-induced diaphragm muscle deterioration and is inhibited by FOY.

Secondly, it is an interesting finding that FOY caused the diaphragm muscle to become fatigue resistant at a frequency of 20 Hz, and to maintain greater tension at all frequencies after fatigue runs. Because diaphragm muscle fatigue has been reported as a key phenomenon in the induction of diaphragm muscle failure[14], it is suggested that FOY functions to prevent diaphragm muscle fatigue. In general, in normal skeletal muscles, slow muscle fiber (type I) is oxidative and fatigue resistant and fast fiber (type II) is glycogenic and susceptible to fatigue[9]. Because slow muscle fiber requires more oxygen for energy than fast fiber, endotoxin produces oxygen-derived free radicals predominantly in slow muscle fibers rather than fast fibers, as observed by NADPH diaphorase staining[15]. Therefore, the produced oxygen-derived free radicals may easily injury slow muscle fibers and cause diaphragm muscle deterioration. Consequently, we suggest that FOY prevents injury of slow muscle fibers, and results in contractile properties of slow muscle fiber. This character of fatigue resistance induced by FOY may be very beneficial for the treatment of diaphragm muscle failure in septicemia.

In addition, although both contraction and half relaxation times increased in the endotoxin+FOY group, FOY improved contractile velocity in a direction to control compared to both endotoxin and thrombin groups. We speculate that FOY has a preventive effect of cellular injury not only in the slow twitch but also in the fast twitch muscle fibers.

Thirdly, we propose a mechanism by which

FOY blocks TNF-α induction in vascular epithelial cells and/or diaphragm muscle tissue. Recent studies have begun to establish a molecular basis for the observed link between the inflammatory response and the development of a hypercoagulable state, a state which often leads to DIC. It is now clear that both the procoagulant and anticoagulant properties of endothelial cells are altered by inflammatory mediators including endotoxin[16], interleukin-1[17], and TNF-α[18]. It has been reported that IL-1β and TNF-α reduce the synthesis of heparan sulfate, which is a proteoglycan and present throughout the endothelial cell surface (extracellular), resulting in diminished heparin-like anticoagulant activity of endothelial cells[19]. Heparin is a classic proteoglycan and fond stored in granules of mast cells (intracellular). It was also reported that TNF-α acts directly on cultured human vascular endothelium to induce a tissue factor-like procoagulant activity (PCA)[20] and that it induces the internalization and subsequent degradation of thrombomodulin molecules[21]. Thus, these evidences suggest that TNF-α may work to accelerate greater coagulation in DIC. On the other hand, we have recently reported that endotoxin induces deterioration in diaphragm muscle contraction[22] and that TNF-α mRNA expression is induced by endotoxin in the diaphragm[8]. Thus, TNF-α alone or in combination with other cytokines, is an important cytokine which contributes to diaphragm muscle deterioration after endotoxin administration. Therefore, we suggest that FOY reduces TNF-α induction, which leads to not only vascular coagulation but also diaphragm muscle deterioration.

In addition, although endotoxin directly activates the intrinsic pathway of coagulation[23], there is no direct evidence for thrombin generation in septicemia patients with leukopenia. Furthermore, the intrinsic pathway was not activated in normal human subjects who developed intravascular coagulation after administration of TNF-α[24]. Thus, it is suggested that activation of the extrinsic pathway by a tissue factor generated by monocytes and endothelial cells may be principally responsible for the activation of intravascular coagulation in septicemia.

In conclusion, an acceleration of coagulation may occur in endotoxin induced septicemia and contribute to diaphragm muscle contractile deterioration. As with the endotoxin administered animal model, we also speculate that an induction of an increase of intravascular coagulation occurs in patients with septicemia clinically. Because FOY has strong anticoagulant effects and inhibits TNF-α induction, it is suggested that FOY (gabexate mesilate), a synthetic serine proteinase inhibitor, may be useful for the treatment of respiratory muscle failure in patients with septicemia.

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