Antithrombin reduces endotoxin-induced hypotension by enhancing pulmonary sensory neuron activation in rats

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Summary
We recently demonstrated that activation of the pulmonary sensory neurons plays a critical role in prevention of endotoxin-induced shock by releasing calcitonin gene-related peptide (CGRP) in rats. CGRP increased the endothelial production of prostacyclin (PGI₂) in the lungs, thereby preventing endotoxin-induced shock response by inhibiting tumor necrosis factor-α (TNF-α) production. Since antithrombin (AT) enhances sensory neuron activation, we hypothesized that AT might reduce endotoxin-induced hypotension by enhancing the activation of pulmonary sensory neurons in rats. We examined this possibility using a rat model of endotoxin shock. AT-induced effects including reduction of hypotension (n=5) and inhibition of induction of iNOS (n=4 or 5) and TNF-α (n=5) in the lungs of endotoxin-treated animals were completely reversed by pretreatment with capsazepine (CPZ) (n=4 or 5), a vanilloid receptor antagonist, or CGRP(8–37), a CGRP receptor antagonist (n=4 or 5). AT enhanced endotoxin-induced increases in lung tissue levels of CGRP (n=4), but this effect of AT was not seen in animals pre-treated with CPZ (n=4). CGRP produced therapeutic effects (n=5) similar to those induced by AT, and such therapeutic effects were completely abrogated by pretreatment with indomethacin (n=4). AT increased CGRP release from cultured dorsal root ganglion neurons only in the presence of anandamide (n=5), and AT-induced increase in CGRP release was not observed in the presence KT5720, an inhibitor of protein kinase A (n=5). AT markedly increased intracellular levels of cAMP in the presence of anandamide (n=5). These results strongly suggested that AT might reduce endotoxin-induced hypotension in rats by enhancing activation of sensory neurons via activation of protein kinase A.

Keywords
Antithrombin, capsaicin-sensitive sensory neurons, calcitonin gene-related peptide, septic shock, protein kinase A

Introduction
Endotoxin released from the bacterial cell wall is critically involved in the development of septic shock seen in Gram-negative sepsis by promoting excessive monocytic production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) (1–3). TNF-α plays a key role in the propagation of inflammatory responses by activating neutrophils and inducing the inducible form of nitric oxide synthase (iNOS) (4, 5). Excessive nitric oxide produced by iNOS plays a critical role in the pathophysiology of septic shock (6, 7). Among the organs of rats administered endotoxin, the lung was demonstrated to be the main source of production of large amounts of nitric oxide by increasing the expression of iNOS (8, 9).

Antithrombin (AT) is an important natural anticoagulant that inhibits serine proteases generated during activation of the coagulation cascade (10). In addition to the inherent anticoagulant effect, AT has been shown to attenuate inflammatory responses by promoting endothelial production of prostacyclin (PGI₂), which is capable of inhibiting leukocyte activation (11). We previously reported that AT reduced endotoxin-induced hypotension in rats, when given before or after endotoxin administration, by inhibiting induction of iNOS in the lung through inhibition of TNF-α production (12). This effect might depend on an AT-induced increase in the endothelial production of PGI₂, which is capable of inhibiting TNF-α production (13). Consistent with our observation is a previous report by Taylor et al. (14) demonstrating that AT prevented any decrease in...
systemic arterial pressure in baboons challenged with *Escherichia coli*.

Capsaicin-sensitive sensory neurons are nociceptive neurons that are activated by a wide variety of noxious physical and chemical stimuli (15). Since ablation of the sensory fibers can result in a marked increase in the severity of inflammation (16), sensory neurons have been shown to play a role in the maintenance of tissue integrity by regulating local inflammatory responses. On activation, sensory neurons release calcitonin gene-related peptide (CGRP) that can increase the endothelial production of PGI\(_2\) *in vitro* (17). Since various noxious stimuli that activate sensory neurons to release CGRP are capable of inducing tissue damage (18), the CGRP-induced increase in the endothelial production of PGI\(_2\) might contribute to attenuation of local inflammatory responses, thereby reducing tissue damage.

Plasma levels of CGRP were shown to be elevated in endotoxin-treated rats (19) and in patients with sepsis (20). Since CGRP has potent vasodilator activity (21), it has long been considered as a critical etiologic factor involved in the development of septic shock (22). However, CGRP was shown to inhibit endotoxin-induced TNF-\(\alpha\) production in cultured mouse peritoneal macrophages (23) and in human circulating blood cells (24). Furthermore, CGRP is capable of inhibiting activation of NF-\(\kappa\)B, an important transcriptional factor for TNF-\(\alpha\) production in thymocytes (25). Thus, although CGRP has vasodilator activity, it is possible that the increase in the plasma level of CGRP in sepsis is a compensatory mechanism by which septic shock is attenuated. Consistent with this hypothesis, we previously reported that pulmonary capsaicin-sensitive sensory neurons were activated by endotoxin administration, leading to the reduction of shock responses through an increase in endothelial production of PGI\(_2\) (26). Furthermore, we recently reported that AT reduces ischemia/reperfusion-induced liver injury by increasing endothelial production of PGI\(_2\) through activation of sensory neurons (27).

Taken together, we hypothesized that AT might reduce endotoxin-induced hypotension via enhancement of activation of the sensory neurons. We examined this possibility using a rat model of septic shock in the present study.

Materials and methods

**Materials**

AT was kindly provided by Mitsubishi Pharma Corporation (Osaka, Japan). AT was purified from heat-treated pooled human plasma by absorption on fixed heparin according to a modification of the technique of Miller-Anderson et al. (28). The AT concentrated used in the experiment revealed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Further characterization of the AT concentrate demonstrated that the heparin concentration was <0.01 U/mL and that it was free of pyrogen. CGRP(8–37) and CGRP were purchased from Peptide Institute Inc. (Osaka, Japan); capsaospine (CPZ), indomethacin (IM) were from Sigma Chemical Co. (St. Louis, MO, USA); endotoxin (lipopolysaccharide, from *Escherichia coli*, serotype 055:B5) was from Difco (Detroit, MI, USA). Anandamide and KT5720 were from Alexis (Basel, Switzerland). All reagents used were of analytical grade.

**Animal model of endotoxin shock and measurement of mean arterial blood pressure**

The study protocol was approved by the Animal Care and Use Committee of the Graduate School of Medical Sciences, Kumamoto University, and the care and handling of the animals were in accordance with the guidelines of the National Institutes of Health. Specific pathogen-free male Wistar rats weighing 220 g to 280 g were obtained from Kyudo (Kumamoto, Japan). Mean arterial blood pressure (MAP) was measured as described previously (12). MAP equals the diastolic pressure plus one-third of the pulse pressure, the difference between the systolic and diastolic pressure. AT (250 U/kg) was administered intravenously 30 min prior to the administration of endotoxin (11). Saline, instead of AT, was administered intravenously 30 min before the administered endotoxin for controls. CPZ (15 mg/kg) was administered subcutaneously 60 min before the administration of endotoxin. CGRP(8–37) (0.1 mg/kg) was administered intravenously 60 min prior to endotoxin administration. CGRP (10 ng/kg/min) was administered continuously from 30 min prior to endotoxin administration up to 180 min after endotoxin administration. IM (5 mg/kg) was administered subcutaneously 60 min before endotoxin administration.

**Measurement of plasma levels of NO\(_2\)/NO\(_3\)**

NO\(_2\) and NO\(_3\) are the primary oxidized products of NO; therefore, total concentration of NO\(_2\)/NO\(_3\) in plasma was used as an indicator of NO production *in vivo* (29).

**Measurement of lung level of NOS activity**

The lungs were removed after perfusion via the right cardiac ventricle and frozen in liquid nitrogen. These lung samples were homogenized on ice in HEPES buffer (pH 7.5, 30 mM). The homogenate was sonicated and centrifuged at 12,500 x g for 15 min at 4°C. Conversion of \[^{[3]}\text{H}\]-L-arginine in the supernatant to \[^{[3]}\text{H}\]-L-citrulline was measured as described by Szabo et al. (8).

**Isolation of RNA and reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA from rat lungs was prepared by the acid-guanidinium-phenol-chloroform extraction procedure using TRIzol reagent (Invitrogen, Groningen, Netherlands) (30). Hybridization was performed with digoxigenin-labled rat iNOS antisense RNA as probes.

**Measurement of lung levels of TNF-\(\alpha\)**

Lung samples were obtained from rats, weighted, and homogenized on ice in 0.1 M phosphate buffer (pH 7.4) containing 0.05% sodium azide. After sonication and centrifugation (4,500 x g for 15 min) of the lung homogenate, the levels of TNF-\(\alpha\) in the supernatant were determined using an enzyme-linked immuno sorbent assay kit for rat TNF-\(\alpha\) (Genzyme, Cambridge, MA, USA).

**Measurement of lung tissue levels of CGRP**

Lung tissue levels of CGRP were determined before and after endotoxin administration in rats as described previously (26). The concentration of CGRP was assayed using a specific ELISA kit for rat CGRP (SPI-Bio, Massy, France).
Isolation and culture of dorsal root ganglion (DRG) neurons and measurement of CGRP in culture

DRG neurons from the lumbar, cervical and thoracic region were dissected from rats as described previously (27, 31). Cells were incubated with anandamide (10 µM), AT (5 U/ml), or anandamide in combination with AT for 30 min. CGRP levels were determined using a rat CGRP enzyme immunometric assay kit (SPI-Bio). KT5720 (10 µM), an inhibitor of protein kinase A (32), was added 30 min before anandamide or anandamide in combination with AT stimulation.

Determination of cAMP in DRG neurons

After collection of supernatants, plates were placed on ice, media were removed, and cells were washed by ice-cold phosphate buffered saline. Thereafter, ice cold 65% ethanol was added to each well and placed on ice. Ethanol was collected and dried under nitrogen gas. Intracellular levels of cAMP were determined with an enzyme immunoassay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer’s instructions.

Statistical analysis

Data are presented as means ± SD. Statistical analysis of changes in MAP from baseline in each group was performed using a repeated measures analysis of variance (MANOVA) followed by Scheffe’s post hoc test. Analysis of effects of AT and CGRP on endotoxin-induced decrease in MAP was performed at each time point after endotoxin administration using analysis of variance followed by Scheffe’s post hoc test. To analyze changes in CGRP levels in lung tissues, the t-test was used at each time point after endotoxin administration to avoid multiple testing. A level of p < 0.05 was considered statistically significant.

Results

Effect of pretreatment with CPZ and CGRP(8–37) on AT-induced reduction of hypotension in rats administered endotoxin

Mean arterial blood pressure (MAP) began to decrease from 90 to 120 min after endotoxin administration, and this hypotension was sustained for up to 180 min (p<0.05, MANOVA). AT significantly reduced the decrease in MAP after endotoxin administration in rats (Fig. 1A,B). To determine whether the preventive effect of AT on hypotension is mediated by activation of sensory neurons, we examined the effect of pretreatment with CPZ, a vanilloid receptor antagonist, on AT-induced inhibition of hypotension. Although CPZ itself did not affect the changes in MAP, pretreatment with CPZ completely reversed the preventive effect of AT (Fig. 1A). Furthermore, to determine whether CGRP is involved in the preventive effect of AT on hypotension, we examined the effect of CGRP(8–37), an antagonist of CGRP, on the inhibitory effect of AT on the decrease in MAP. Intravenous administration of CGRP(8–37) completely abrogated the preventive effect of AT on hypotension (Fig. 1B). CGRP(8–37) itself did not affect changes in MAP (Fig. 1B).

Figure 1: Effect of pretreatment with CPZ (A) and CGRP(8–37) (B) on AT-induced reduction of hypotension, and effects of CGRP and/or IM on changes in MAP (C) in endotoxin-treated rats. Changes in MAP were determined in rats administered with endotoxin (5 mg/kg). A) Rats were intravenously administered with AT (250 U/kg, ◆) or saline (●) 30 min before the endotoxin administration. CPZ (15 mg/kg) was administered s.c. 60 min prior to the administration of endotoxin (❑). B) Rats were administered with AT (◆) or saline (●) as described above. CGRP(8–37) (0.1 mg/kg) was administered intravenously 60 min before the endotoxin administration (❑). CGRP(8–37) and AT were administered 60 and 30 min before the endotoxin administration, respectively (△). C) Rats were continuously administered with CGRP (10 ng/kg/min, ◆) or saline (●) from 30 min prior to endotoxin administration up to 180 min after endotoxin administration. IM (5 mg/kg) was administered subcutaneously 60 min before the endotoxin administration (❑). IM was administered 60 min before the endotoxin administration, and CGRP was continuously administered as described above (△). Data are expressed as means ± SD of five animals. *, p < 0.05 vs. endotoxin plus saline.
Effect of CGRP on decreases in MAP in rats administered endotoxin

To examine whether CGRP reduces endotoxin-induced hypotension by increasing endothelial production of PGI₂, we analyzed the effect of CGRP and/or IM, a non-selective inhibitor of cyclooxygenase, on the decrease in MAP in animals administered endotoxin. As shown in Figure 1C, although intravenous administration of CGRP induced a slight and transient decrease in MAP, it reduced hypotension in animals administered endotoxin. The preventive effect of CGRP on hypotension was completely reversed by pretreatment with IM (Fig. 1C).

Effect of pretreatment with CPZ and CGRP(8–37) on AT-induced inhibition of increases in plasma levels of NO₂⁻/NO₃⁻, lung tissue levels of iNOS activity, and lung expression of iNOS mRNA in animals administered endotoxin

Excessive amounts of NO produced by iNOS in the lung play an important role in endotoxin-induced hypotension (6, 8). Plasma levels of NO₂⁻/NO₃⁻ were increased after administration of endotoxin with concomitant increases in lung levels of iNOS and iNOS mRNA, peaking at 180 min after endotoxin administration (12). Although AT significantly inhibited these increases at 180 min after endotoxin administration, such inhibitory effects of AT were not observed in animals pretreated with either CPZ or CGRP(8–37) (Figs. 2 and 3).
Effect of CGRP and/or IM on increases in plasma levels of NO\textsubscript{2}/NO\textsubscript{3}, lung tissue levels of iNOS activity, and lung expression of iNOS mRNA in rats administered endotoxin

Intravenous administration of CGRP significantly inhibited increases in plasma levels of NO\textsubscript{2}/NO\textsubscript{3}, lung tissue levels of iNOS activity, and lung expression of iNOS mRNA at 180 min after endotoxin administration in rats (Figs. 2 and 3). Administration of CGRP did not affect plasma levels of NO\textsubscript{2}/NO\textsubscript{3}, lung tissue levels of iNOS activity, and lung expression of iNOS mRNA at 180 min after saline administration in rats (data not shown). Administration of IM did not affect these changes induced by endotoxin administration (Figs. 2 and 3). However, pretreatment with IM completely reversed CGRP-induced inhibitory effects on these variables (Figs. 2 and 3).

Effect of pretreatment with CPZ and CGRP(8–37) on AT-induced inhibition of increases in lung tissue levels of TNF-α and TNF-α mRNA in rats administered endotoxin

Since TNF-α is one of the important proinflammatory cytokines that induce iNOS mainly in the lungs of rats given endotoxin (5), we examined the effect of pretreatment with CPZ and CGRP(8–37) on AT-induced inhibition of lung tissue levels of TNF-α and TNF-α mRNA in rats. Lung tissue levels of TNF-α and TNF-α mRNA were increased after endotoxin administration, peaking at 90 min and 60 min, respectively (33). Inhibition of increases in these variables by AT in animals given endotoxin was completely reversed by pretreatment with either CPZ or CGRP(8–37) (Figs. 4 and 5).

Effects of CGRP and/or IM on increases in lung tissue levels of TNF-α and TNF-α mRNA in rats administered endotoxin

Intravenous administration of CGRP inhibited increases in lung tissue levels of TNF-α and TNF-α mRNA at 180 min after endotoxin administration (Figs. 4 and 5). Administration of IM did not
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affect these changes induced by endotoxin administration, but the inhibitory effects induced by CGRP were not observed in animals pretreated with IM (Figs. 4 and 5).

**Effects of AT and/or CPZ on changes in lung tissue levels of CGRP in rats administered endotoxin**

To determine whether AT increases the release of CGRP in the lungs of rats administered endotoxin, we examined the effect of AT on increases in lung tissue levels of CGRP in rats administered endotoxin. Lung tissue levels of CGRP were increased after endotoxin administration, peaking after 60–90 min (Fig. 6A). Lung tissue levels of CGRP in animals treated with AT were significantly higher than those in animals treated with saline at 60 min after endotoxin administration (Fig. 6B). Pretreatment with CPZ significantly inhibited endotoxin-induced increases in lung tissue levels of CGRP at 60 min after endotoxin administration and it also completely reversed AT-induced increases in lung tissue levels of CGRP at 60 min after endotoxin administration (Fig. 6B). These results suggested that AT might accelerate the release of CGRP by enhancing sensory neuron activation in the lungs of rats administered endotoxin.

**Effects of AT and/or anandamide on CGRP release from DRG neurons isolated from rats in vitro**

To determine whether AT directly promotes CGRP release from the sensory neurons, we analyzed the effect of AT on CGRP release from DRG neurons isolated from rats. Although AT (5 µM) itself did not increase CGRP release from DRG neurons, it significantly enhanced CGRP release in the presence of anandamide (10 µM) at 30 min after incubation (Fig. 7). Since anandamide increases CGRP release from sensory neurons by activating protein kinase A (34) and AT enhanced anandamide-induced increase in CGRP release, it is possible that AT increases CGRP release from DRG neurons by enhancing protein kinase A activation. As shown in Figure 7, AT did not increase CGRP release from DRG neurons in the presence of anandamide when DRG neurons were pretreated with KT5720.

**Figure 6**: Changes in lung levels of CGRP in rats treated with saline or endotoxin (ET) (A), and effects of AT and/or CPZ on increases in lung levels of CGRP at 60 min after ET administration in rats (B). A) Lung levels of CGRP were determined at the indicated time points just before and after saline (control, open bars) or ET (closed bars) administration in rats. Data are expressed as means ± SD of four animals. *, p < 0.05 vs. control. B) Lung levels of CGRP were determined 60 minutes after ET administration. AT and CPZ were administered as in Figure 1. Control animals were received saline instead of ET. Data are expressed as means ± SD of four animals. *, p < 0.01 vs. control; §, p < 0.05 vs. ET plus saline.

**Figure 7**: Effects of AT and/or anandamide on CGRP release from DRG neurons. DRG neurons were incubated with anandamide (10 µM) or AT (5 µM) in the presence or absence of KT5720 (10 µM) for 30 min. These cells were also incubated with anandamide (10 µM) in combination with AT (5 µM) in the presence or absence of KT5720 (10 µM) for 30 min. Supernatants were sampled, and CGRP levels were measured by enzyme immunoassay. Each value represents the mean ± SD deprived from five animals. *, p<0.01 vs. media; †, p<0.01 vs. anandamide; ‡, p<0.01 vs. AT + anandamide.
Effects of AT and/or anandamide on intracellular cAMP levels in DRG neurons in vitro

To examine whether AT enhances anandamide-induced protein kinase A activation by increasing cAMP levels in sensory neurons, we investigated the effect of AT on cAMP levels in DRG neurons in the presence or absence of anandamide. As shown in Figure 8, AT significantly increased cAMP levels in DRG neurons, and this increase was more marked in the presence of anandamide.

Discussion

In the present study, we demonstrated that effects of AT on hypotension, increases in plasma levels of NO$_2$/NO$_3$, and other changes in the lungs leading to iNOS induction were completely abrogated by pretreatment with CPZ, a vanilloid receptor antagonist, and CGRP(8–37), a CGRP receptor antagonist. These observations strongly suggested that activation of capsaicin-sensitive sensory neurons might be critically involved in the AT-induced reduction of hypotension in endotoxin-treated rats. We previously reported that activation of sensory neurons in the lungs contributed to reduction of shock responses in rats given endotoxin (26). Thus, it is reasonable to assume that AT reduces endotoxin-induced shock responses by enhancing pulmonary sensory neuron activation in rats. Consistent with this assumption are observations in the present study demonstrating that AT accelerated CGRP release in the lungs of rats given endotoxin. Furthermore, CGRP-induced effects in endotoxin-treated rats were similar to those induced by AT as shown in the present study, supporting the hypothesis that AT might reduce endotoxin-induced hypotension by increasing CGRP release from sensory neurons (i.e. activation of sensory neurons).

Although AT itself did not increase CGRP release from DRG neurons isolated from adult rats in vitro, it significantly enhanced CGRP release from DRG neurons in the presence of anandamide, an endogenous agonist of vanilloid receptor-1 activation produced by macrophages (34). These observations are consistent with the assumption described above that AT enhances the sensory neuron activation in animals given endotoxin. AT-induced increase in CGRP release from DRG neurons in the presence of anandamide was inhibited by KT5720, an inhibitor of protein kinase A. In addition, AT increased cAMP levels in DRG neurons, and the AT-induced increase was more marked in the presence of anandamide. Since anandamide increases CGRP release from sensory neurons by activating protein kinase A through increases in intracellular cAMP levels (35), AT might enhance anandamide-induced increases in cAMP levels in DRG neurons, thereby enhancing protein kinase A activation. Although the mechanism(s) by which AT increases cAMP levels in DRG neurons is not known at present, AT has also been reported to increase cAMP levels in cultured endothelial cells (36). Mechanism(s) underlying this effect of AT should be elucidated by further investigations in the near future.

We previously reported that AT significantly increased the plasma levels of 6-keto-PGF$_{1α}$, a stable metabolite of PGI$_2$, in rats given endotoxin (11, 37), and pretreatment of rats with IM completely abrogated the therapeutic effects of AT on endotoxin-induced hypotension (12). These observations strongly suggested that the preventive effect of AT on endotoxin-induced hypotension could be mediated by PGI$_2$, released from endothelial cells in rats (12). Hoffmann et al. (38) demonstrated that inhibition of endotoxin-induced endothelial adhesion of leukocytes by IM was completely abrogated by pretreatment with IM in a skin-fold preparation of Syrian hamsters. Since TNF-α enhances endothelial adhesion of leukocytes by increasing the expression of endothelial leukocyte adhesion molecules (39), AT-induced increase in endothelial production of PGI$_2$ might also be critical for
inhibition of endothelial adhesion of leukocytes in their report.

Effects of CGRP on hypotension, increases in plasma levels of NO$_2$/NO$_3$ and other events in the lungs leading to iNOS induction in rats administered endotoxin were completely reversed by pretreatment with IM, suggesting that inhibitory effects of CGRP on endotoxin-induced hypotension in this rat model of septic shock might be mediated by prostaglandins. Consistent with this hypothesis is our previous report demonstrating that plasma levels of 6-keto-PGF$_{1\alpha}$ were increased as a consequence of pulmonary sensory neuron activation in rats given endotoxin (26).

Taken together, these observations strongly suggested that AT might reduce endotoxin-induced hypotension by increasing the endothelial production of PGI$_2$ via enhancement of activation of capsaicin-sensitive sensory neurons. Consistent with this observation is our recent report showing that AT reduces reperfusion-induced liver injury in rats by increasing endothelial production of PGI$_2$ through activation of sensory neurons (27).

Since sensory nerve endings are located in the perivascular areas of various tissues (40), the question arises as to how intravenously administered AT gains access to the nerve endings to stimulate the sensory neurons. Koj et al. (41) demonstrated that extravasation of AT was observed in rabbits at the site of local inflammation where vascular permeability was significantly increased. We previously reported that pulmonary vascular permeability was markedly increased in rats given endotoxin (42). These observations suggest that intravenously administered AT might interact with sensory nerve endings at the site of inflammation in the lungs of rats given endotoxin in the present study. This possibility should be examined further in future investigations.

We showed the possible mechanisms by which AT reduces endotoxin-induced shock responses in rats based on observations in the present study (Fig. 9).

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References


