In SIRS and sepsis, various proinflammatory and anti-inflammatory cytokines are produced in large quantities, and this either directly or indirectly induces tissue injury and finally organ dysfunction [8]. Neutrophils are intensely activated by multiple stimuli, such as bacterial toxins and endogenous proinflammatory mediators, and play key roles in inducing tissue injury and the resulting organ dysfunctions through the release of various cytotoxic molecules, including reactive oxygen intermediates and granular enzymes. Among these, neutrophil elastase (NE), a serine proteinase, is thought to be one of the major cytotoxic molecules because of its abundance and powerful degrading activity against a wide variety of substrates [9]. In addition, NE has recently become known for its proinflammatory functions [10]. Since NE is harmful even to the host, there exist multiple defensive mechanisms to neutralize free NE. In blood, alpha-1 antitrypsin (AT) and alpha-2 macroglobulin are abundant, and intravascularly released NE is immediately neutralized. In contrast, although there exist NE inhibitors in the extravascular space, such as AT, secretory leukocyte protease inhibitor (SLPI) and elafin, the concentrations of these endogenous protease inhibitors of high molecular weight may be insufficient to adequately neutralize extravascularly released NE, especially when a large amount of NE is released in patients with SIRS and sepsis [11].

Neutrophils exist mostly within the circulation and either float in or are loosely attached to the peripheral vasculature under physiological conditions, but they easily migrate out of capillaries in response to various stimuli. NE can be released either intravascularly or extravascularly, but the place where NE is released is critical in the induction of tissue injury and organ dysfunction. However, thus far, there has been no method or marker to indicate where NE is released, and a plasma marker for extravascularly released NE is eagerly awaited as it would enable prediction of the degree of NE-induced tissue injury, especially in patients with SIRS. We hypothesized that plasma levels of elastase digests of cross-linked fibrin (e-XDP) correlate with the amount of extravascularly released NE, especially in the lungs, and thus may be useful in predicting the degree of lung injury. In the present study, we determined plasma levels of NE–AT complex and e-XDP in critically ill patients, and examined their significance in ALI and ARDS.

2. Methods

2.1. Study population

We conducted a single-center retrospective observational study at the university hospital of the School of Medicine, Keio University. The study population included 136 patients, who were admitted to our intensive care unit, followed up for more than 7 days, and whose blood was drawn on the day of admission. The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. There were 94 men and 42 women (mean ± SEM age, 60.9 ± 1.4 years). Sixty-four had cardiovascular diseases, 32 had gastrointestinal diseases, 10 had respiratory diseases, seven had neurological diseases, and 23 had other diseases. Patients receiving postoperative care were included in each category. Based on the definition by the American–European consensus conference, all the patients were divided into the following three groups: (1) non-ALI: the PaO_2/FIO_2 ratio on the day of inclusion in the study was >300 or an apparent cause for hypoxia was identified; (2) mild ALI: $200 < PaO_2/FIO_2 \leq 300$ with bilateral infiltration on chest X-ray; and (3) ARDS: $PaO_2/FIO_2 \leq 200$ and bilateral infiltration on chest X-ray. One hundred twelve patients were categorized as non-ALI, 16 as mild ALI, and eight as ARDS. To analyze the outcome, the mild ALI and ARDS groups were combined because of the small patient numbers in these two groups. Plasma was used for the analysis of NE–AT and e-XDP. However, NE–AT and e-XDP in four and three patients were not determined because of insufficient sample volume, respectively.

The diagnosis of SIRS and sepsis was made based on the diagnostic criteria adopted by the combined committee of American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) [2]. Acute Physiology and Chronic Health Evaluation (APACHE) II score, Sequential Organ Failure Assessment (SOFA) score and multiple organ dysfunction score (MODS) were determined on the day of inclusion in the study [12,13].

2.2. Determination of neutrophil elastase-related molecules

The NE–AT complex and elastase digests of e-XDP in plasma were determined with a latex agglutination method specific for each molecule [14,15]. The detection limits were 14.3 ng/ml for NE–AT and 0.1 U/ml for e-XDP.

2.3. Statistical analysis

We used the Mann–Whitney *U* test to compare two or three independent groups and the Kruskal–Wallis test to compare more than three groups. To examine the correlations between two parameters, Spearman's rank correlation was used. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Patient characteristics

Of the 136 patients, 112 were placed in the non-ALI category, 16 in the ALI category, and eight in the ARDS category. Ninety-seven patients (71.3%) fulfilled the criteria of SIRS and 80 patients (58.8%) fulfilled the criteria of sepsis on the day of inclusion in the study. On the day of inclusion, the mean APACHE II score of the patients was 9.6 ± 0.5 (range 0–31), the mean SOFA score was 5.1 ± 0.3 (range 0–16), and the mean MODS score was 3.0 ± 0.3 (range 0–14). Mortality at discharge was 8.1%.

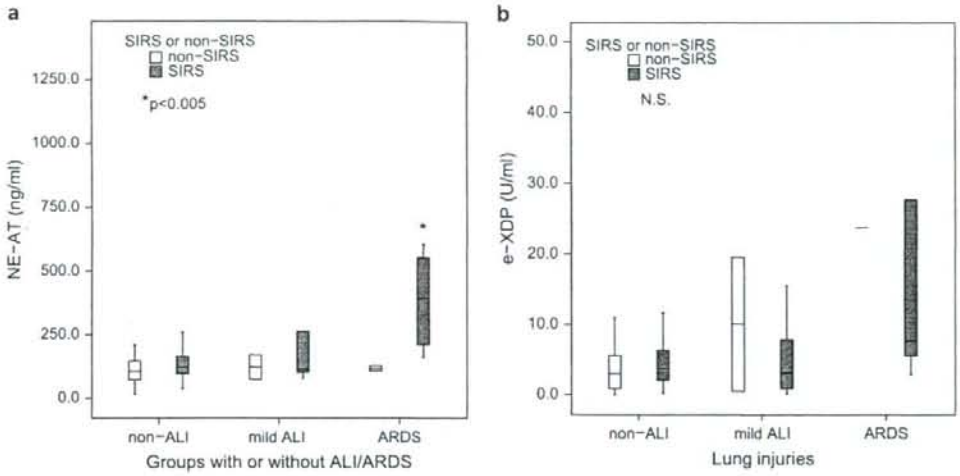

Fig. 1. Box plots showing median plasma levels of (a) NE–AT and (b) e-XDP in patients with or without SIRS in the non-ALI, mild ALI, and ARDS groups. Boxes show interquartile ranges and I bars represent highest and lowest values. *$p < 0.005$. NE–AT, neutrophil elastase–alpha-1 antitrypsin complex; e-XDP, elastase digests of cross-linked fibrin; and SIRS, systemic inflammatory response syndrome.

3.2. Plasma levels of NE–AT and e-XDP in non-ALI, mild ALI, and ARDS

Fig. 1a,b shows plasma NE–AT and e-XDP levels in the non-ALI, mild ALI, and ARDS groups with or without SIRS. NE–AT was detectable in all the patients, but e-XDP was undetectable in four patients. Among the three groups, plasma NE–AT and e-XDP levels were significantly higher in the ARDS group than in the non-ALI group ($p < 0.005$, $p < 0.05$, data not shown). When the presence of SIRS was considered, NE–AT levels were still significantly higher in the patients with SIRS and ARDS than in the SIRS and non-ALI groups (Fig. 1a). In contrast, there was no statistically significant difference in e-XDP levels among the groups (Fig. 1b). NE–AT levels were significantly higher in the patients with SIRS than in those without SIRS (115.0 ± 10.1 vs. 176.2 ± 17.7, $p < 0.05$), but there was no difference in e-XDP levels between the groups (5.3 ± 1.0 vs. 12.4 ± 3.3, $p = 0.15$). NE–AT levels were also significantly higher in septic patients than those in nonseptic patients (122.1 ± 8.2 *vs.* 210.7 ± 28.3, $p < 0.01$). Although there was a trend for higher e-XDP levels in sepsis, there was no statistically significant difference (5.4 ± 0.8 vs. 17.4 ± 5.6, $p = 0.06$). There was a weak correlation between NE–AT and e-XDP levels ($r = 0.178$, $p < 0.05$).

3.3. Correlations of NE–AT and e-XDP with clinical indices

We then divided patients into two subgroups with or without ALI/ARDS, namely non-ALI and ALI/ARDS groups, and examined the relationships of NE–AT and e-XDP with various clinical indices. In the ALI/ARDS group, plasma NE–AT levels significantly correlated with the decrease between the initial and lowest PaO_2/FIO_2 (P/F) ratio, and the duration of ALI/ARDS during hospital stay (Fig. 2a,b). When only patients with mild ALI were analyzed, the correlations with the decrease in P/F ratio and the duration of ALI/ARDS were still significant ($r = 0.40$, $p = 0.034$; $r = 0.43$, $p = 0.019$). In contrast, plasma e-XDP did not correlate with these parameters (Fig. 2c,d). NE–AT also correlated well with APACHE II, SOFA, and MODS scores on the first day in the ALI/ARDS group (Fig. 3a,b), but e-XDP did not (Fig. 3c,d). In the non-ALI group, neither NE–AT nor e-XDP correlated with any of the above parameters, except that a weak correlation was observed between NE–AT and the decrease in P/F ratio and SOFA score.

3.4. NE–AT and e-XDP for subsequent development of lung injury and fatal outcome at the 28th day

We next examined whether plasma NE–AT and e-XDP levels were predictive of patients' subsequent development of lung injury and fatal outcome at the 28th day in the patients with or without ALI and ARDS. In the ALI/ARDS group, plasma NE–AT levels were significantly higher in the subgroup with a decrease of more than 20% between the initial and lowest P/F ratios than in those with a decrease of equal to or less than 20% (Fig. 4a). In contrast, there was no difference in e-XDP level between the two subgroups (Fig. 4b). Similar results were observed when patients were divided into two subgroups with and without a decrease of more than 10% in the P/F ratio (data not shown).

We then examined plasma NE–AT and e-XDP levels and fatal outcome at the 28th day. There was a trend for a higher median NE–AT level in deceased patients than in alive patients, although the difference was not statistically significant

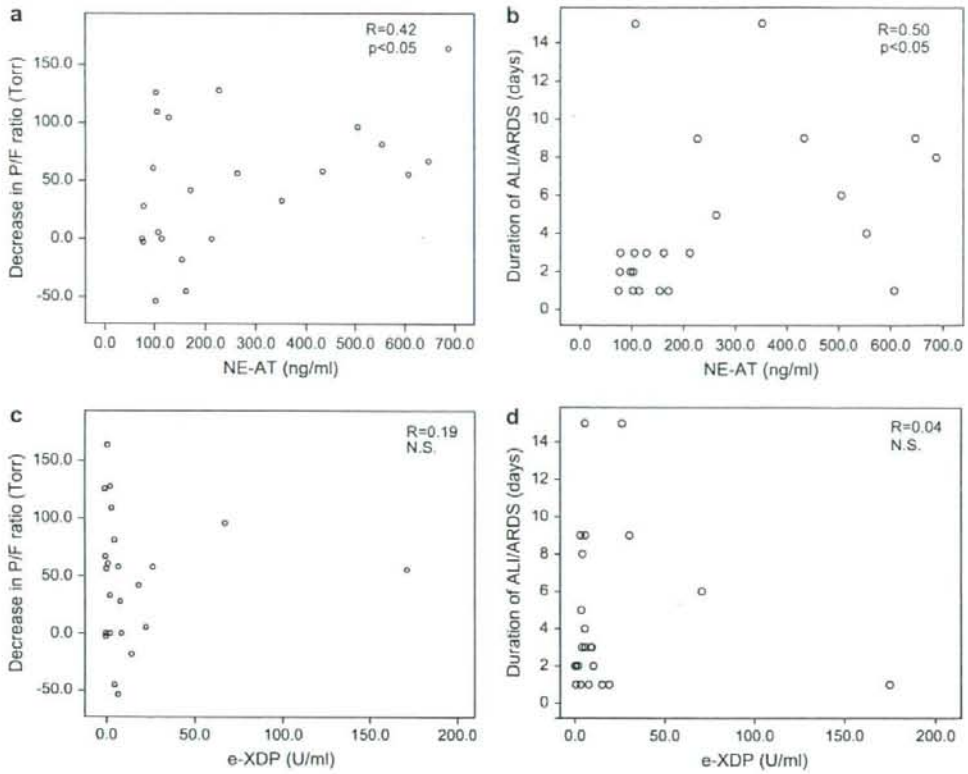

Fig. 2. Comparison of plasma NE–AT and e-XDP with the decrease in P/F ratio and duration of ALI/ARDS. (a) NE–AT *vs.* decrease in P/F ratio. (b) NE–AT *vs.* duration of ALI/ARDS. (c) e-XDP *vs.* decrease in P/F ratio. (d) e-XDP *vs.* duration of ALI/ARDS. NE–AT, neutrophil elastase–alpha-1 antitrypsin complex; e-XDP, elastase digests of cross-linked fibrin; and P/F ratio, PaO_2/FIO_2.

($p = 0.20$, data not shown). There was no difference in e-XDP between the two subgroups ($p = 0.44$, data not shown).

4. Discussion

In the present study, we examined plasma NE–AT and e-XDP levels in critically ill patients to elucidate the significance of NE in the initiation and progression of ALI and ARDS in the presence or absence of SIRS. We found significantly increased levels of plasma NE–AT in the patients with ARDS, especially when the definition of SIRS was met. Among the ALI/ARDS groups, the plasma NE–AT levels, but not the e-XDP levels, correlated significantly with the decrease between the initial and lowest P/F ratios and the duration of ALI/ARDS during the hospital stay. Furthermore, the levels of NE–AT, but not e-XDP, significantly increased in the subgroup with a decrease of more than 20% between the initial and lowest P/F ratios than in those with a decrease of equal to or less than 20%. In contrast, the above correlations and differences between the subgroups were not observed in the non-ALI patients. From these results, we speculated that NE–AT, but not e-XDP, may be predictive of progressive respiratory failure in the early stage of ALI and ARDS. In addition, the combined criteria of ALI/ARDS and SIRS may be useful for predicting neutrophil activation *in vivo*, and thus applicable for selecting the target for an anti-neutrophil strategy in critically ill patients.

High levels of plasma NE–AT have been reported previously. Rocker et al. examined plasma NE–AT in 50 patients with respiratory failure and showed that higher levels of NE–AT occurred in pre-ARDS and ARDS, as diagnosed by Petty's definition [15]. Moreover, they found that NE–AT was significantly correlated with the P/F ratio, BAL protein, and differential neutrophil counts. Donnelly et al. prospectively examined plasma NE–AT levels in patients with multiple trauma and found that NE–AT increased in patients who developed ARDS as defined by Murray's expanded definition [16]. In the present study, we examined critically ill patients with various etiologies and classified them into non-ALI, mild ALI, and ARDS with or without SIRS, following the definition by the American–European consensus conference. We found that plasma NE–AT increased in patients who fulfilled the criteria of SIRS and ARDS. Furthermore, NE–AT was predictive of progressive lung injury among patients with ALI and ARDS. Our results support the idea that NE makes a significant contribution to the initiation and development of ALI and ARDS during the early phase of critical illness, especially when SIRS is complicated.

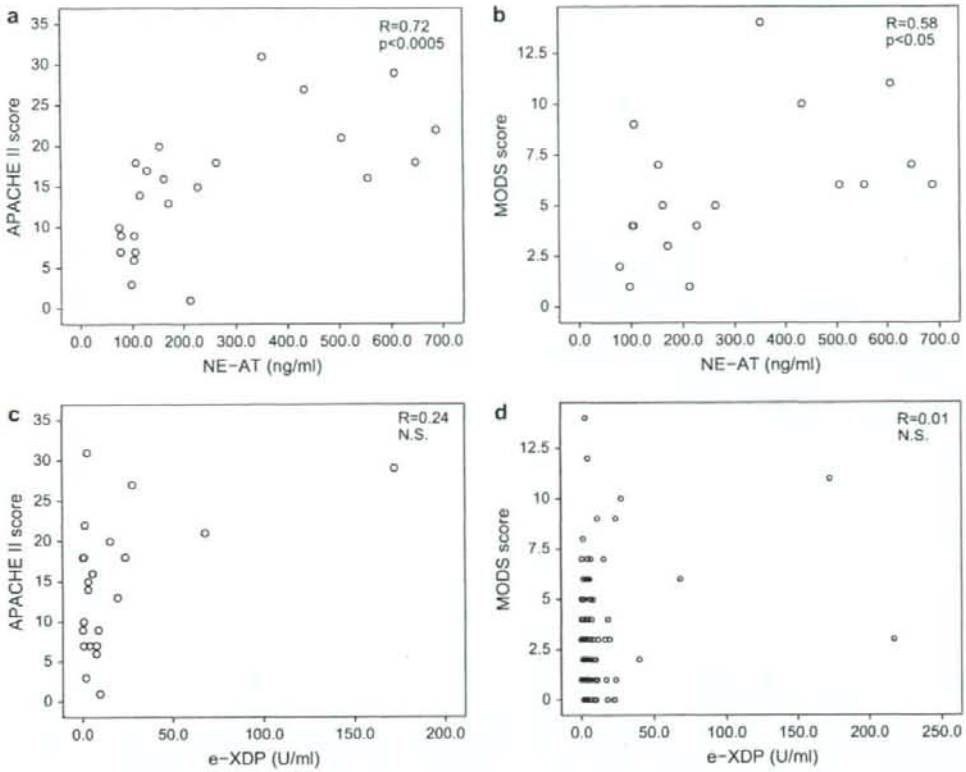

Fig. 3. Comparison of plasma NE–AT and e-XDP with the decrease in P/F ratio and duration of ALI/ARDS. (a) NE–AT *vs.* APACHE II score. (b) NE–AT *vs.* MODS score. (c) e-XDP *vs.* APACHE II score. (d) e-XDP *vs.* MODS score. NE–AT, neutrophil elastase–alpha-1 antitrypsin complex; e-XDP, elastase digests of cross-linked fibrin; APACHE II, acute physiology and chronic health evaluation; and MODS, multiple organ dysfunction score.

NE is a potent serine proteinase and could harm vital organs and tissues in addition to killing of invading microorganisms. Acting as protection against the harmful effects of NE, there are several endogenous NE inhibitors, including AT, in the circulation and they can neutralize NE soon after NE is released. In contrast, in SIRS and sepsis, neutrophils are activated, migrate out from the vasculature, firmly attach to lung endothelial and epithelial cells, and can directly injure them by releasing NE to the neutrophil–stromal cell interface, where no or only a very low concentration of natural NE inhibitors exist. In the present study, we expected that the amount of e-XDP produced would correlate with that of NE released

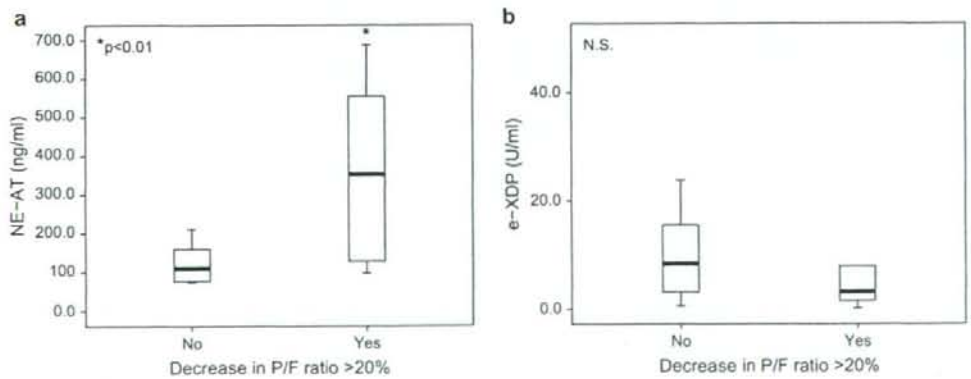

Fig. 4. Box plots showing median plasma levels of (a) NE–AT and (b) e-XDP in two groups of patients, one with a decrease of more than 20% in P/F ratio and another group with a decrease of equal to or less than 20%. Boxes show interquartile ranges and I bars represent highest and lowest values. $*p < 0.01$. NE–AT, neutrophil elastase–alpha-1 antitrypsin complex; e-XDP, elastase digests of cross-linked fibrin; and P/F ratio, PaO_2/FIO_2.

extravascularly, especially in the lungs, and therefore could predict the degree of lung injury. However, NE–AT was superior to e-XDP in predicting lung injury in our study. Since there could be several parameters other than free NE that regulate the amount of e-XDP produced, such as the concentration of cross-lined fibrin, extravascularly released NE may not be the major determinant of plasma e-XDP levels.

Although NE–AT seems to play pivotal roles in inducing lung injury, the international clinical trial of the NE antagonist sivelestat sodium for ALI and ARDS (the STRIVE study) failed to demonstrate a useful effect of this agent. In contrast, a Japanese clinical trial showed a significant improvement in lung oxygenation and the number of days not in the intensive care unit in patients with SIRS and ALI. In the international STRIVE study, 78.6% of patients fulfilled the criteria of ARDS [17], whereas only 39.0% of the patients enrolled into the Japanese phase III study fulfilled the criteria of ARDS, and patients with more than three organ failure were excluded [18,19]. In view of our present results showing that plasma NE–AT was predictive of upcoming lung injury in the mild ALI group as well as in the ARDS group, patients with mild ALI may be a better target for NE antagonists, including sivelestat sodium. It is quite probable that NE antagonism may not be effective enough in improving survival. However, since there are no established drugs for ALI/ARDS and sivelestat is available commercially in Japan and Korea, the effectiveness of sivelestat for mild ALI and SIRS needs to be validated in these countries.

Acknowledgements

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Neural Mechanisms of Sevoflurane-induced Respiratory Depression in Newborn Rats

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Background: **Sevoflurane-induced respiratory depression has been reported to be due to the action on medullary respiratory and phrenic motor neurons. These results were obtained from extracellular recordings of the neurons. Here, the authors made intracellular recordings of respiratory neurons and analyzed their membrane properties during sevoflurane application. Furthermore, they clarified the role of γ-aminobutyric acid type A receptors in sevoflurane-induced respiratory depression.**

Methods: **In the isolated brainstem–spinal cord of newborn rat, the authors recorded the C4 nerve burst as an index of inspiratory activity. The preparation was superfused with a solution containing sevoflurane alone or sevoflurane plus the γ-aminobutyric acid type A receptor antagonist picrotoxin or bicuculline. Neuronal activities were also recorded using patch clamp techniques.**

Results: **Sevoflurane decreased C4 burst rate and amplitude. Separate perfusion of sevoflurane to the medulla and to the spinal cord decreased C4 burst rate and amplitude, respectively. Both picrotoxin and bicuculline attenuated the reduction of C4 burst rate. Sevoflurane reduced both intraburst firing frequency and membrane resistance of respiratory neurons except for inspiratory neurons.**

Conclusion: **Under the influence of sevoflurane, the region containing inspiratory neurons, *i.e.*, the pre-Bötzinger complex, may determine the inspiratory rhythm, because reduced C4 bursts were still synchronized with the bursts of inspiratory neurons within the pre-Bötzinger complex. In contrast, the sevoflurane-induced decrease in C4 burst amplitude is mediated through the inhibition of phrenic motor neurons. γ-Aminobutyric acid type A receptors may be involved in the sevoflurane-induced respiratory depression within the medulla, but not within the spinal cord.**

SEVOFLURANE is a volatile anesthetic that is widely used in clinical practice; it depresses respiration as a side effect at anesthetic doses.[1,2] *In vivo* studies using extracellular recordings of respiratory neurons in decerebrate, vagotomized, and peripherally deafferented canines have suggested that this effect may be due to the anesthetic's action on medullary premotor neurons mediated by γ-aminobutyric acid type A ($GABA_A$) transmission.[3–6]

Based on findings in *in vitro* preparations of neonatal rodents, the ventrolateral medullary region of the brainstem is thought to be a critically important site for respiratory rhythm generation.[7–10] Specifically, the pre-Bötzinger complex and the more rostral parafacial respiratory group presumably constitute a dual respiratory center.[7,11,12] Among the two candidates, it is hypothesized that the pre-Bötzinger complex generates the inspiratory rhythm and the parafacial respiratory group generates the expiratory rhythm, although these groups are functionally coupled.[11] Medullary respiratory neurons in the brainstem–spinal cord preparation have been classified into inspiratory (which are further divided into three subtypes, I–III), preinspiratory, and expiratory neurons on the basis of the temporal correlation of their firing pattern with inspiratory-related C4 ventral root activity.[7] Considering its respiratory function, the medulla is probably one of the targets of the respiratory depression induced by sevoflurane. The spinal cord, containing phrenic motor neurons, may be another target because sevoflurane has been reported to depress the excitability of nonrespiratory spinal motor neurons[13] and the activity of hypoglossal motor neurons,[14] which are primarily active in the inspiratory phase in the *en bloc* preparation.[9]

Using the isolated brainstem–spinal cord preparation of neonatal rat, Otsuka[15] recorded the neuronal activities of respiratory neurons to investigate the effect of halothane on fictive breathing. In his study, neurons were recorded extracellularly but not intracellularly. Recently, several *in vitro* studies have made intracellular recordings of respiratory neurons using the perforated patch clamp technique to examine the effect on the respiratory neural network of other agents generally used by anesthesiologists, such as propofol[16,17] and opioids.[10] Intracellular recordings have made possible the analysis of membrane properties and synaptic input, which cannot be examined through extracellular recordings. Therefore, *in vitro* perforated patch clamp recordings using the isolated brainstem–spinal cord preparation of neonatal rat seem to be suitable for detailed investigation of the intracellular mechanisms of sevoflurane-induced respiratory depression.

After reviewing the previous data,[3–6,15] we hypothesized that the effect of sevoflurane may differ between the types of respiratory neurons. In the current study, we analyzed the response of medullary respiratory and phrenic motor

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neurons to sevoflurane, and the role of $GABA_A$ receptors in sevoflurane-induced respiratory depression.

Materials and Methods

Preparation

All procedures were conducted in accordance with the guidelines of the Keio University Laboratory Animal Care and Use Committee (Tokyo, Japan), Kitasato Institute Animal Care and Use Committee (Tokyo, Japan), and Teikyo University Laboratory Animal Care and Use Committee (Tokyo, Japan). Data were obtained from 64 neonatal Wistar rats (aged 0–4 days). Generation of the isolated brainstem–spinal cord preparation has been described in detail elsewhere.[18] In brief, the rats were anesthetized with diethyl ether, and after the paw withdrawal reflex disappeared, the brainstem caudal to the caudal cerebellar artery and cervical spinal cord were isolated in a chamber filled with oxygenated artificial cerebrospinal fluid (ACSF). The cerebellum and pons were ablated. Each preparation was placed ventral side up in a recording chamber (volume, 2 ml) and superfused (flow, 4 ml/min) with control ACSF equilibrated with a control gas mixture (5% CO_2 in oxygen, pH7.4). Its temperature was maintained at 25°–26°C. The composition of the ACSF was 126 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, and 30 mM glucose. C4 ventral root activity was recorded using a glass suction electrode, amplified with a conventional alternating current amplifier (AVH 11; Nihon Kohden, Tokyo, Japan), and integrated (time constant, 100 ms). We measured C4 burst rate as an index of the inspiratory rate[19] and the integrated amplitude as an index of the tidal volume.[20] All amplitude data were normalized to the values obtained during 10–15 min of the anesthetic-free control state, which was assigned a value of 100%.

Drug Administration

Sevoflurane was applied according to the method described by Matute *et al.*[13] Sevoflurane (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) was mixed with the control gas mixture (5% CO_2 in oxygen, pH 7.4) by a vaporizer (Sevotec 3; Ohmeda, Steeton, West Yorkshire, United Kingdom) at known volume percentages (1, 3, and 5%). The ACSF was gassed with this mixture for 30 min. In the preliminary experiment, in which the same perfusion system was used without the brainstem–spinal cord, we measured the concentration of sevoflurane in the ACSF in the recording chamber using gas chromatography (GC-2014; Shimadzu, Kyoto, Japan). The concentration of sevoflurane in the ACSF was estimated from the vaporizer readings (r^2 = 0.972; slope, 0.25 ± 0.01; y-intercept, −0.03 ± 0.06; fig. 1). The sevoflurane percentages of 1, 3, and 5% in gas corresponded to approximately 0.23, 0.74, and 1.25 mM, respectively. Sevoflurane EC_{50} equivalent to 1 minimum alveolar concentration (MAC) in clinical anesthesia corresponds to an aqueous concentration of 0.35 mM sevoflurane.[21] In the current study, we present the estimated aqueous concentration and MAC (in parentheses).

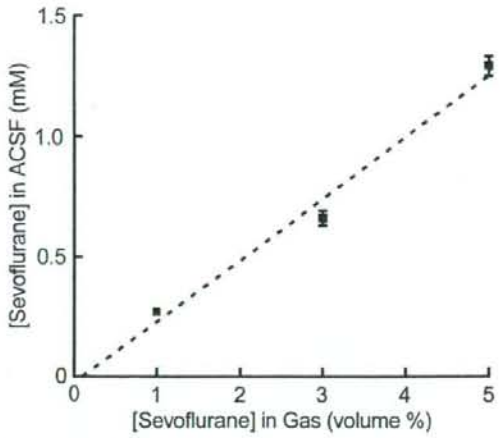

Fig. 1. Simple regression of vaporizer readings (volume %) and molar concentrations of sevoflurane in artificial cerebrospinal fluid (ACSF). Three observations were performed at each vaporizer reading (1, 3, and 5%).

The $GABA_A$ receptor antagonist picrotoxin or bicuculline methiodide (both Sigma Chemical Co., St. Louis, MO) was dissolved in ACSF containing 1.25 mM (3.57 MAC) sevoflurane at 10 μM.

Experiment 1: Effect of Sevoflurane on C4 Respiratory Activity

We analyzed the effect of sevoflurane on C4 burst rate and amplitude. Twenty preparations were randomly allocated to one of four groups to for exposure to sevoflurane at 0, 0.23, 0.74, or 1.25 mM (0, 0.77, 2.11, or 3.57 MAC); each group contained five preparations. Preparation were superfused after a control period of 10–15 min with a solution containing sevoflurane at the specified concentration for 30 min, followed by a washout period using control ACSF for 30 min.

Experiment 2: Separate Perfusion of Sevoflurane to the Spinal Cord or to the Medulla

We used a separate perfusion system[16,19,22,23] to permit selective medullary or spinal application of drugs. The chamber was partitioned at the spinomedullary junction to permit the selective application of drugs. The partition was made using two thin acrylic plates placed parallel to each other with woven nylon packed between them. Each partitioned section had a capacity of 2 ml and was superfused continuously at a flow rate of 4 ml/min. Five preparations each were randomly allocated either to a medulla perfusion group or a spinal cord perfusion group. After C4 activity stabilized, the super-

fusate was replaced with ACSF containing 0.74 mM (2.11 MAC) of sevoflurane for 30 min, followed by a washout period using the control ACSF for 30 min.

Experiment 3: Effect of Sevoflurane on C4 Respiratory Activity in the Presence of a $GABA_A$ Receptor Antagonist

Effects of 10 μM picrotoxin were studied on sevoflurane-induced reduction on C4 burst rate and amplitude. Experiments were performed in five preparations. After a control period of 10–15 min, the superfusate was replaced with ACSF containing 1.25 mM (3.57 MAC) sevoflurane in the presence of 10 μM picrotoxin for 30 min, followed by a washout period using the control ACSF for 30 min. To assess the effect of picrotoxin, these values were compared with those obtained using the same concentration of sevoflurane alone. Using 10 μM bicuculline in place of picrotoxin, the same experiments were repeated in another five preparations.

Experiment 4: Effect of Sevoflurane on Respiratory Neurons

We analyzed the effect of sevoflurane on the intracellular activity of respiratory neurons using the perforated patch clamp technique.[18] Respiratory neurons were identified and classified on the basis of their firing patterns and the temporal correlation of their activity with the respiratory cycle of C4 respiratory activity.[7] Inspiratory neurons discharged action potentials during the inspiratory phase (C4 burst activity phase) and were further categorized into three subtypes.[7,10] Type I inspiratory neurons are depolarized by periods of summated excitatory postsynaptic potentials before the onset of and after termination of C4 bursts (*i.e.*, the preinspiratory and postinspiratory phases). Type II inspiratory neurons show excitatory postsynaptic potentials during only the inspiratory phase. Type III inspiratory neurons are hyperpolarized by periods of summated inhibitory postsynaptic potentials in the preinspiratory and postinspiratory phases. Preinspiratory neurons were characterized by preinspiratory and postinspiratory action potential discharges and hyperpolarization during the inspiratory phase; they send excitatory and inhibitory inputs to type I and III inspiratory neurons, respectively.[7,10] Expiratory neurons are first characterized by tonic discharge of action potentials in the interval between the inspiratory phases and are categorized into two subtypes based on the following criterion.[7] In Exp-i neurons, inspiratory-related action potential discharge is blocked due to summating hyperpolarizing inhibitory postsynaptic potentials. In contrast, Exp-p-i neurons are subjected to a longer period of hyperpolarization covering the preinspiratory, inspiratory, and postinspiratory phases. Inspiratory neurons were searched for in the pre-Bötzinger complex, which covered presumably from the rostrocaudal extension root of the most rostral root of the hypoglossal nerve.[12] Preinspiratory neurons were searched for in the main area of the parafacial respiratory group, which extended presumably from the caudal cerebellar artery to approximately 0.25 mm caudal to the caudal end of the facial motor nucleus.[12] Expiratory neurons were searched for in the Bötzinger complex, which was the area between pre-Bötzinger complex and the caudal end of the vagal nerve root.[12]

Membrane potentials were recorded under current clamp mode with a single-electrode voltage clamp amplifier (CEZ 3100; Nihon Kohden). A glass pipette (GC100-TF-10; Clark, Reading, United Kingdom) was horizontally pulled (PA-91; Narishige, Tokyo, Japan) to a tip size of approximately 2 μm, with very gradual tapering. Electrode resistance ranged from 5 to 20 MΩ when the pipette was filled with a solution containing 140 mM K-gluconate, 3 mM KCl, 10 mM EGTA, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and nystatin (100 μg/ml). pH was maintained at 7.2–7.3 using potassium hydroxide. The micropipette was inserted into the rostral ventrolateral medulla using a manual hydraulic micromanipulator. During tracking for the neurons, positive pressure (10–20 cm H_2O) was applied inside the pipette. After a gigaohm seal was obtained, the recorded membrane potential became negative and stabilized in approximately 10 min. After we made the control recording, the control ACSF was changed to ACSF containing 1.25 mM (3.57 MAC) sevoflurane for 3–6 min, followed by a 10- to 20-min washout period using control ACSF. In this experiment, the brief application of sevoflurane at high concentration enabled us to obtain high-quality intracellular neuronal recordings because the effect then appears more quickly.[16]

To assess the intracellular activity of respiratory neurons, we focused on three parameters. *Intraburst firing frequency* of inspiratory neurons was calculated as the mean firing frequency during the inspiratory phase, whereas that of preinspiratory or expiratory neurons is represented by the mean firing frequency during the expiratory phase.[16] *Membrane potential* was presented without correcting for the liquid junction potential. *Membrane resistance* was determined from the change in voltage in response to direct current pulses (300 ms, −50 pA) during the silent phase of the respiratory cycle.

Perforated patch recording of phrenic motor neurons has been described elsewhere.[24] The spinal cord of the preparation was carefully transected with a vibratome between C4 and C5 ventral roots and was bent so that the cut edge, rostral to the section, was placed horizontally just below the surface of the ACSF. Phrenic motor neurons were identified on the basis of their firing pattern and location. They discharged action potentials during the inspiratory phase (C4 burst activity phase) and were located in C4 ventral horn.[25] To search for them, the micropipette was inserted into C4 ventral horn. The methods of perforated patch clamp techniques were

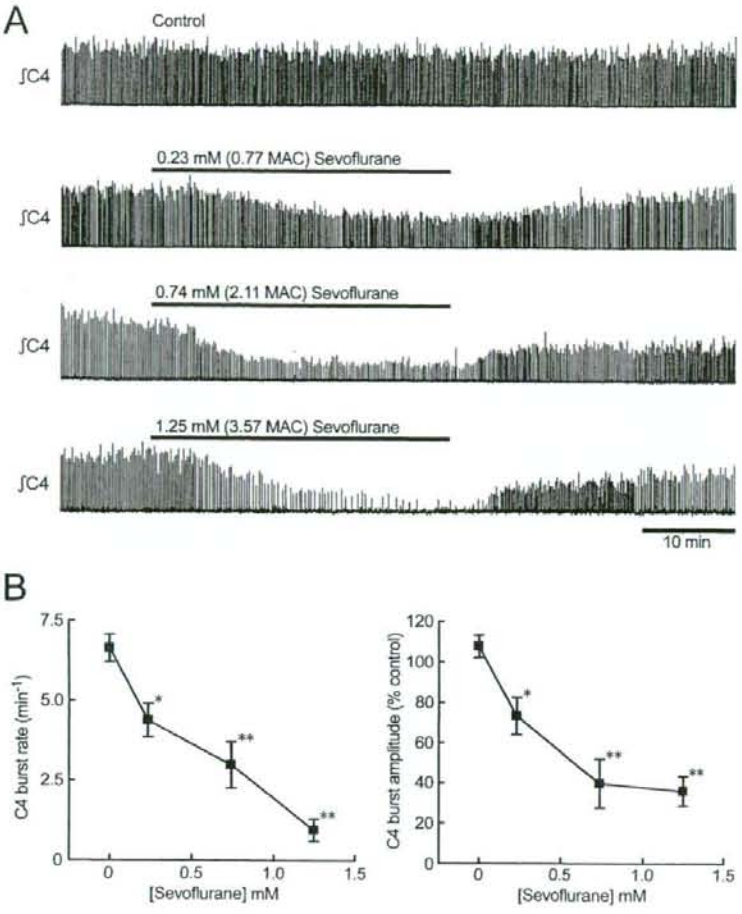

Fig. 2. (*A*) A representative recording of the integrated C4 activity (∫C4) before, during, and after superfusion with 0, 0.23, 0.74, and 1.25 mM (0, 0.77, 2.11, and 3.57 minimum alveolar concentration [MAC], respectively) sevoflurane solution. The *horizontal bars* indicate the duration of superfusion with sevoflurane. (*B*) Concentration-dependent effect of sevoflurane on C4 burst rate (*left*) and amplitude (*right*). Higher concentrations of sevoflurane tended to produce a greater decrease in C4 burst rate and amplitude. * $P < 0.05$ *versus* control. ** $P < 0.01$ *versus* control.

identical throughout to those described for medullary respiratory neurons.

Data Analysis

All signals were recorded on a thermal array recorder (WS-682G; Hihon Kohden) and fed into a personal computer after analog-digital conversion (Power Lab/4sp; AD-Instruments, Castle Hill, Australia) for subsequent analysis (Chart version 5; ADInstruments). Analysis of the respiratory parameters was performed off-line. Respiratory parameters obtained before the superfusion of the ACSF-containing drugs were defined as control values.

All variables were evaluated using a Kolmogorov-Smirnov test, which revealed that it was appropriate to make parametric assumptions about our data. In figure 1, the molar concentration of sevoflurane in the ACSF was practically estimated from the vaporizer readings by using simple regression. In experiment 1, changes in C4 burst rate and amplitude were compared using a one-way analysis of variance, followed by a Dunnett test. In experiments 2, 3 and 4, the data before and during the application of the drugs were analyzed using a paired t test. The significance of the difference between two groups was assessed using an unpaired t test. All statistical analyses were conducted using Graph-Pad Prism 3.0 software (Graph-Pad Software Inc., San Diego, CA). All values were reported as mean ± SE, and all P values less than 0.05 were considered significant.

Results

Effect of Sevoflurane on C4 Respiratory Activity

Representative recordings of integrated C4 activity before and during superfusion with sevoflurane-containing ACSF are shown in figure 2A. C4 burst rate and amplitude were both decreased by sevoflurane in a concentration-dependent manner (fig. 2B). C4 burst rate during the application of 0, 0.23, 0.74, or 1.25 mM (0, 0.77, 2.11, or 3.57 MAC) sevoflurane was 6.65 ± 0.43, 4.37 ± 0.52, 2.98 ± 0.72, and 0.954 ± 0.347 min^{-1} (98.2 ± 3.1, 71.6 ± 6.9, 42.4 ± 7.6, and 15.3 ± 4.7% of the control rate), respectively. C4 burst amplitude during the application of 0, 0.23,

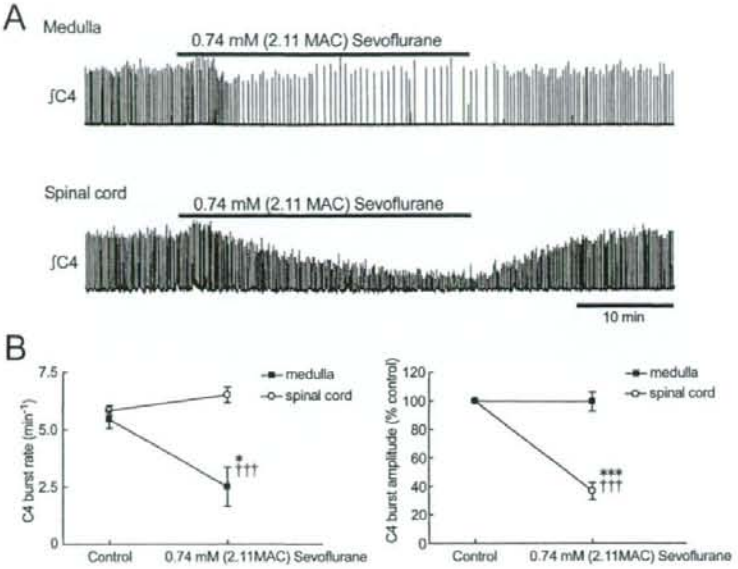

Fig. 3. (*A*) A representative recording of the integrated C4 activity (∫C4) before, during, and after separate superfusion with sevoflurane solution (*upper trace*: medulla; *lower trace*: spinal cord). The *horizontal bars* indicate the duration of superfusion with sevoflurane. (*B*) The effect of separate perfusion of 0.74 mM (2.11 minimum alveolar concentration [MAC]) sevoflurane on C4 burst rate (*left*) and amplitude (*right*). C4 burst amplitude decreased significantly upon application of sevoflurane to the spinal cord. On the other hand, C4 burst rate decreased significantly when sevoflurane was applied to the medulla. * $P < 0.05$ *versus* control. *** $P < 0.005$ *versus* control. ††† $P < 0.005$, medulla perfusion group *versus* spinal cord perfusion group.

0.74, or 1.25 mM (0, 0.77, 2.11, or 3.57 MAC) sevoflurane changed to 107.8 ± 5.4, 73.2 ± 9.1, 39.7 ± 12.1, and 35.9 ± 7.3% of the control amplitude, respectively.

Separate Perfusion of Sevoflurane to the Spinal Cord and to the Medulla

Representative recordings of C4 activity before and during application of 0.74 mM (2.11 MAC) sevoflurane to the medulla or the spinal cord are shown in figure 3A. During the administration of sevoflurane to the medulla, C4 burst rate decreased, but C4 burst amplitude did not change significantly. In contrast, when applied to the spinal cord, the application of 0.74 mM (2.11 MAC) sevoflurane decreased C4 burst amplitude only (figs. 3A and B). C4 burst rate and amplitude during the application of 0.74 mM (2.11 MAC) sevoflurane to the medulla were 2.51 ± 0.85 min^{-1} (46.1 ± 14.3% of the control rate) and 99.5 ± 6.6% of the control rate, respectively. C4 burst rate and amplitude during the application of 0.74 mM (2.11 MAC) sevoflurane to the spinal cord were 6.51 ± 0.33 min^{-1} (111.8 ± 5.8% of the control rate) and 36.6 ± 5.9% of the control amplitude, respectively.

Effect of Sevoflurane in the Presence of a $GABA_A$ Antagonist on C4 Respiratory Activity

Representative recordings of C4 activity before and during superfusion with ACSF containing 1.25 mM (3.57 MAC) sevoflurane in the presence of 10 μM picrotoxin or bicuculline are shown in figure 4A. A recording of the application of 1.25 mM (3.57 MAC) sevoflurane alone also is provided. Sevoflurane still reduced C4 burst amplitude in the presence of picrotoxin. However, the reduction of C4 burst rate was attenuated significantly compared with the application of 1.25 mM (3.57 MAC) sevoflurane alone ($P < 0.005$) (figs. 4A and B). C4 burst rate and amplitude in response to 1.29 mM (3.57 MAC) sevoflurane with 10 μM picrotoxin were 7.49 ± 0.86 min^{-1} (117.3 ± 10.1% of the control rate) and 38.6 ± 8.6% of the control amplitude, respectively. Application of sevoflurane with bicuculline still reduced C4 burst amplitude. Bicuculline, like picrotoxin, significantly attenuated only the reduction of C4 burst rate ($P < 0.005$ *vs.* application of 1.29 mM [3.57 MAC] sevoflurane alone; figs. 4A and C). C4 burst rate and amplitude during the application of 1.29 mM (3.57 MAC) sevoflurane with 10 μM bicuculline were 3.36 ± 0.42 min^{-1} (64.7 ± 5.0% of the control rate) and 52.6 ± 10.6% of the control amplitude, respectively.

Effect of Sevoflurane on Respiratory Neurons

Twenty-three medullary respiratory and phrenic motor neurons were recorded intracellularly to examine the effects of 1.25 mM (3.57 MAC) sevoflurane. From eight inspiratory neurons, presumably located in the pre-Bötzinger complex, three cells were classified as type I, and the remaining five neurons were classified as type II. In both types of inspiratory neurons, 1.25 mM (3.57 MAC) sevoflurane decreased the rate of inspiratory-related rhythmic drive potentials and concomitant action potential discharge (fig. 5). However, intraburst firing frequency, resting membrane potential, and membrane resistance remained unchanged ($P > 0.05$) during sevoflurane-induced respiratory depression (table 1).

In six preinspiratory neurons presumably located in the main area of the parafacial respiratory group, the depolarization of both preinspiratory and postinspira-

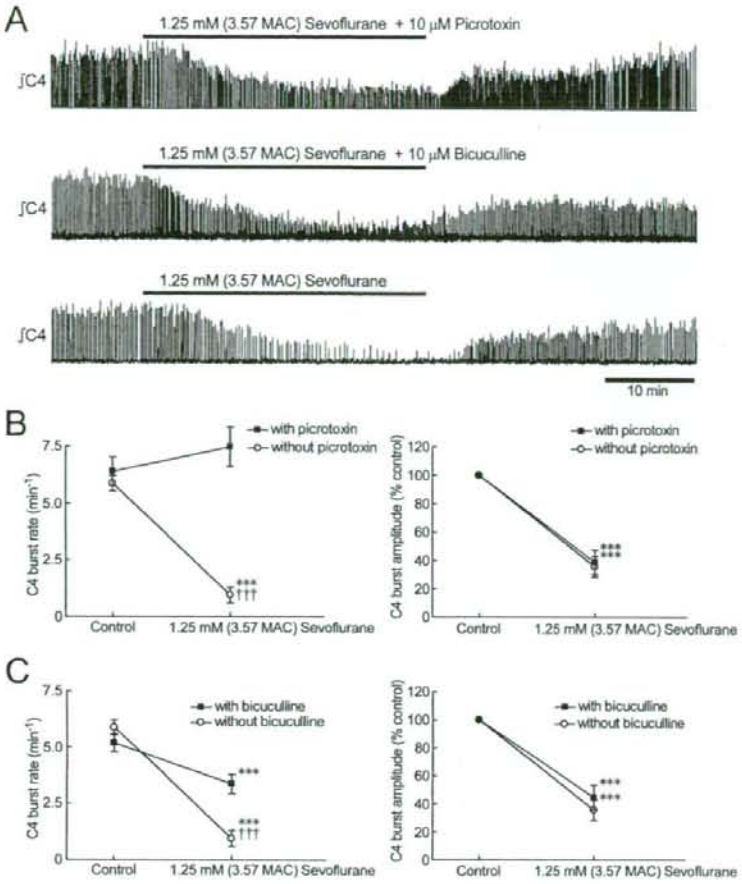

Fig. 4. (*A*) A representative recording of the integrated C4 activity (∫C4) before, during, and after superfusion with 1.25 mM (3.57 minimum alveolar concentration [MAC]) sevoflurane plus 10 μM picrotoxin (*upper trace*) or plus 10 μM bicuculline (*middle trace*). A recording of the superfusion with 1.25 mM (3.57 MAC) sevoflurane alone is also provided (*lower trace*). The *horizontal bars* indicate the duration of superfusion with drugs. (*B*) The effect of sevoflurane on C4 burst rate (*left*) and amplitude (*right*) with and without picrotoxin. C4 burst amplitude decreased significantly in both groups. In the sevoflurane plus picrotoxin group, C4 burst rate did not change significantly in comparison to the control rate, *i.e.*, the depressant effect on C4 burst rate was attenuated. (*C*) The effect of sevoflurane on C4 burst rate (*left*) and amplitude (*right*) with and without bicuculline. The amplitude decreased significantly in both groups. The respiratory rate decreased significantly in both groups but the depressant effect was weaker in the sevoflurane plus bicuculline group. *** $P < 0.005$ *versus* control. ††† $P < 0.005$, application of sevoflurane plus picrotoxin or bicuculline *versus* application of sevoflurane alone.

tory phases became smaller, and seemed to be phase locked during superfusion with 1.25 mM (3.57 MAC) sevoflurane, although C4 burst was maintained (fig. 5). In four of the six preinspiratory neurons, the inhibitory postsynaptic potential decreased, whereas that of the remaining two preinspiratory neurons did not change. A 1.25 mM (3.57 MAC) amount of sevoflurane decreased the intraburst firing frequency and membrane resistance significantly (4% of control value [$P < 0.05$] and 76% of control value [$P < 0.01$], respectively), although it did not change the resting membrane potential (table 1).

In five Exp-i neurons (no Exp-p-i neurons were recorded), presumably located in the Bötzinger complex, tonic discharge disappeared and the inhibitory postsynaptic potential decreased during superfusion of 1.25 mM (3.57 MAC) sevoflurane, although C4 burst was maintained (fig. 5). In these cells, 1.25 mM (3.57 MAC) sevoflurane significantly decreased the intraburst firing frequency and membrane resistance (29% of control value and 78% of control value [$P < 0.05$], respectively), although it did not change the resting membrane potential significantly (table 1).

In six phrenic motor neurons from C4 ventral horn, 1.25 mM (3.57 MAC) sevoflurane decreased the rate of inspiratory-related rhythmic drive potentials and concomitant action potential discharge (fig. 5). Sevoflurane significantly decreased the intraburst firing frequency and membrane resistance, and the cells hyperpolarized (78% of control value [$P < 0.01$], −5 mV from control value [$P < 0.01$], and 81% of control value [$P < 0.05$], respectively; table 1).

Discussion

The current investigation shows that sevoflurane decreases inspiratory-related C4 burst rate and intraburst firing frequency of preinspiratory and expiratory neurons, but not inspiratory neurons in the medulla. Furthermore, the agent decreased C4 burst amplitude (*i.e.*, tidal volume) by inhibiting the activity of phrenic motor neurons in the spinal cord. Our results suggest that the effect of GABAergic transmission contributes to the mechanism of this decreased C4 burst rate.

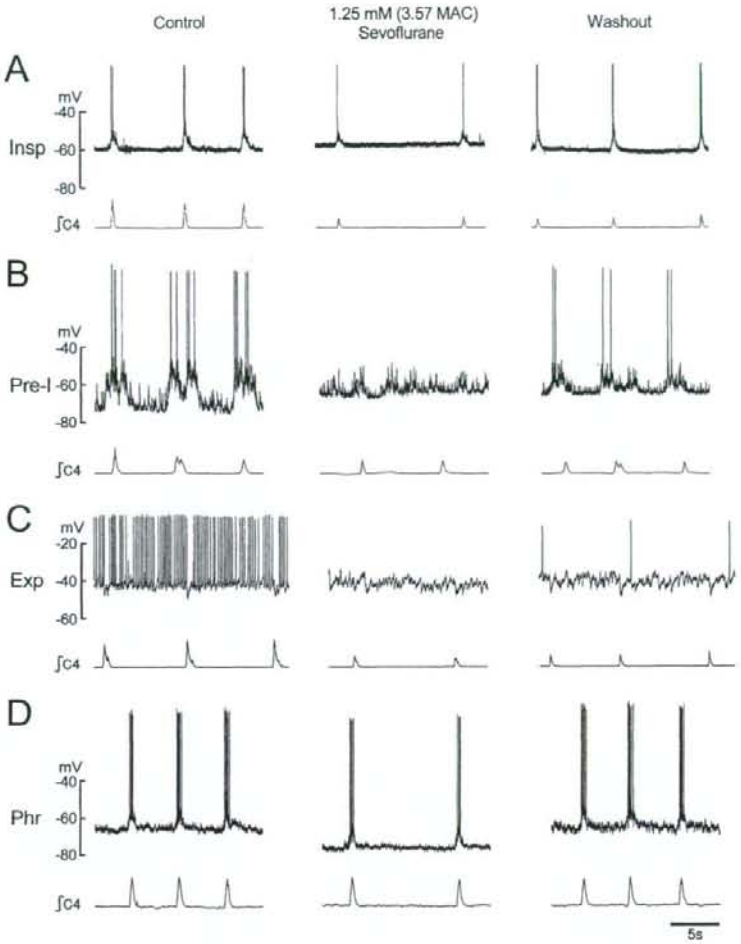

Fig. 5. The effect of approximately 1.25 mM (3.57 minimum alveolar concentration [MAC]) sevoflurane on the activities of inspiratory (Insp; *A*), preinspiratory (Pre-I; *B*), expiratory (Exp; *C*), and phrenic motor neurons (Phr; *D*). Simultaneous recordings of the integrated C4 activity (∫C4) are also provided. After the control recording, the control artificial cerebrospinal fluid was changed to artificial cerebrospinal fluid containing approximately 1.25 mM (3.57 MAC) sevoflurane for approximately 3–6 min, followed by a 10- to 20-min washout period using control artificial cerebrospinal fluid. In inspiratory neurons, sevoflurane did not affect the intraburst firing frequency or the resting membrane potential. The depolarizing cycle rate reduced by sevoflurane was synchronized with C4 burst rate. In preinspiratory and expiratory neurons, sevoflurane induced a decrease in the intraburst firing frequencies but did not affect the resting membrane potentials. In preinspiratory neurons, the depolarization of both preinspiratory and postinspiratory phases became smaller and seemed to be phase locked during superfusion of sevoflurane, although C4 burst was maintained. In phrenic motor neurons, sevoflurane induced hyperpolarization of the resting membrane potential and decreased the intraburst firing frequency.

Effect of Sevoflurane on C4 Burst Rate and Respiratory Neurons

Sevoflurane reduced C4 burst rate in a dose-dependent fashion, and this effect appeared when it was applied exclusively to the medulla. Halothane, isoflurane, and enflurane also have been shown to reduce C4 burst rate in this *en bloc* brainstem model.[15] Therefore, in this preparation, volatile anesthetics may have general rate-reducing properties. Contrary, in human studies,[1,2] sevoflurane at anesthetic doses increased the respiratory rate *via* a chemoreflex loop. In one *in vivo* study using decerebrate, vagotomized, and peripherally deafferented dogs,[3] sevoflurane also increased the respiratory rate. The discrepancy between the current results and the results of these *in vivo* and human studies may be explained as follows. The pons has a strong modulatory effect on respiratory rate, and its ablation increases C4 burst rate.[26] In addition, it was found[27] that the noradrenergic A_5 area in the pons exerts an inhibitory effect on the respiratory rate. Adenosine 5′-triphosphate-induced inward currents of the locus ceruleus neurons in the pons are reduced by sevoflurane,[28] suggesting that neural excitation is reduced. From these studies, it seems that sevoflurane may increase the respiratory rate by inducing disinhibition of the pontine respiratory neural circuits. Therefore, the finding that the sevoflurane decreased the C4 burst rate might be due to the absence of the pons.

Inspiratory medullary neurons exhibited a constant intraburst firing frequency, resting membrane potential, and membrane resistance during application of sevoflurane, despite the decrease of their burst rate that occurred in a 1:1 fashion with slowing of C4 burst rate. In *in vivo* canine experiments, however, sevoflurane reduced the discharge frequency of premotor inspiratory neurons.[5,6] The lack of agreement between these experiments and the current experiments may be due to differences in the subtypes of inspiratory neurons. Inspiratory neurons are categorized into several subtypes based on their location and function. The inspiratory

Table 1. Effect of Sevoflurane on Respiratory Neurons

	n	Control	1.25 mM (3.57 MAC) Sevoflurane
Inspiratory neurons			
Intraburst firing frequency, spike/s	7	5.2 ± 1.5	4.9 ± 1.5
Resting membrane potential, mV	7	−58.7 ± 8.6	−58.0 ± 8.4
Membrane resistance, MΩ	4	372.2 ± 53.4	341.5 ± 76.1
Preinspiratory neurons			
Intraburst firing frequency, spike/s	6	0.7 ± 0.2	0.03 ± 0.02*
Resting membrane potential, mV	6	−51.6 ± 4.8	−51.7 ± 3.0
Membrane resistance, MΩ	6	406.2 ± 68.1	309.0 ± 61.0†
Expiratory neurons			
Intraburst firing frequency, spike/s	5	2.4 ± 0.5	0.7 ± 0.4*
Resting membrane potential, mV	5	−42.5 ± 2.4	−41.5 ± 3.1
Membrane resistance, MΩ	4	417.0 ± 33.4	328.0 ± 21.2*
Phrenic motor neurons			
Intraburst firing frequency, spike/s	6	22.5 ± 6.1	17.5 ± 6.5†
Resting membrane potential, mV	6	−62.1 ± 5.7	−68.0 ± 6.6†
Membrane resistance, MΩ	3	612.0 ± 103.8	489.8 ± 97.5*

* $P < 0.05$ and † $P < 0.01$ indicate statistically significant differences from control values.

MAC = minimum alveolar concentration.

neurons in the current study were presumably located in the pre-Bötzinger complex, which is considered to be an inspiratory rhythm-generating region.[11] The inspiratory neurons recorded by Stucke *et al.*[5,6] were located in the caudal ventral respiratory group and are categorized as bulbospinal premotor neurons. In the same preparations as used here, halothane at high (but not low) concentration only slightly reduced the intraburst firing frequency of inspiratory neurons, *i.e.*, these neurons resisted the effect of halothane.[15] Therefore, inspiratory neurons in the pre-Bötzinger complex seem to resist the depressing effect of volatile anesthetics in brainstem–spinal cord preparations. Sevoflurane at concentrations higher than those used in clinical use may reduce the intraburst firing frequency of these inspiratory neurons.

Preinspiratory neurons showed a reduced intraburst firing frequency (the firing disappeared in most of these neurons although the respiratory rhythm of the C4 activity was maintained) as well as membrane resistance during application of sevoflurane. Preinspiratory neurons located in the parafacial respiratory group that may overlap the retrotrapezoid nucleus[11,29,30] receive inhibitory synaptic connections from inspiratory neurons, whereas they send excitatory and inhibitory synaptic input to inspiratory neurons.[7] It has been hypothesized[30] that the parafacial respiratory group (composed of preinspiratory neurons) is critical for respiratory rhythm generation. This is not in accord with the hypothesis that the pre-Bötzinger complex (which is composed of inspiratory neurons) is the primary site of respiratory rhythm generation.[9,11] For a unifying hypothesis, the functionally inspiratory pre-Bötzinger complex and the functionally expiratory parafacial respiratory group may constitute a dual respiratory center.[31] Recently, it was reported that the region containing the parafacial respiratory group (rostral to the pre-Bötzinger complex) seem to control expiratory abdominal muscles[12,32,33] and to contribute the longevity of inspiratory rhythm.[12] Several agents in general use by anesthesiologists, such as propofol[16,17] and opioids,[10] affected these two generators differently. Halothane as well as sevoflurane also reduced the intraburst firing frequency of the preinspiratory neurons in the brainstem–spinal cord preparation.[15] From these results, we suggest that inhibition of preinspiratory neurons by volatile anesthetics may result in the inhibition of active expiratory rhythm and that the inspiratory rhythm of pre-Bötzinger complex may be maintained during application of volatile anesthetics. Ruangkittisakul *et al.*[12] reported that transverse slices containing only a minor amount of brainstem tissue rostral to the pre-Bötzinger complex slices and *en bloc* medullas without major aspect of the parafacial respiratory group show stable inspiratory rhythm at physiologic calcium and potassium concentrations. During application of sevoflurane, the pre-Bötzinger complex containing inspiratory neurons may determine the respiratory rhythm, because C4 burst was preserved after the firing of preinspiratory neurons disappeared and the rhythm was always synchronized with the burst of inspiratory neurons.

Sevoflurane decreased intraburst firing frequency and membrane resistance of medullary expiratory neurons. The area in which we recorded the activity of the expiratory neurons corresponds likely to that of the Bötzinger complex in adult mammals, where expiratory neurons function as interneurons[11] and are found at high densities.[8] In the *in vivo* canine experiment, sevoflurane also reduced the firing of premotor expiratory neurons located in the caudal ventral medulla.[3,4] In the same preparation as ours, halothane also reduced the intraburst firing frequency of expiratory neurons.[15] Expiratory-related neurons, unlike inspiratory neurons, thus seem to be inhibited by volatile anesthetics across differences in location and function.

Blockade of $GABA_A$ receptors attenuated the sevoflurane-induced reduction in C4 burst rate. Because separate perfusion of sevoflurane to the medulla decreased the C4 burst rate, sevoflurane should act on $GABA_A$ergic transmission in the medulla. A previous study reported that sevoflurane seemed to enhance inhibitory synaptic transmission by promoting the function of the α_1 and α_2 subunits of the $GABA_A$ receptor, resulting in the activation of chloride channels.[34] Our finding that the membrane resistance of preinspiratory and expiratory neu-

rons is decreased by sevoflurane may be explained by the activation of these $GABA_A$ receptors. In addition, both preinspiratory and expiratory neurons are subject to $GABA_A$ergic inhibition, which causes the inhibitory postsynaptic potential during the inspiratory phase.[35,36] In fact, the current study suggests that activation of $GABA_A$ receptors on preinspiratory and expiratory neurons by sevoflurane inhibits the expiratory component of respiratory rhythm. However, the resting membrane potential of preinspiratory neurons and expiratory neurons did not change even when $GABA_A$ receptors may have been activated. Therefore, we cannot explain the unchanged resting membrane potential by pointing to $GABA_A$eric transmission only.

On the other hand, neurons in the pre-Bötzinger complex contained intense neurokinin-1 receptor immunoreactivity, and some of these neurons are presumed to be essential for inspiratory rhythm generation.[37] GABAergic synapses onto neurokinin-1 receptor–immunoreactive neurons in the pre-Bötzinger complex have been identified morphologically.[38] Mechanism of sevoflurane-induced depression of C4 burst rate may include the activation of $GABA_A$ receptors in the pre-Bötzinger complex. However, the electrophysiologic properties of inspiratory neurons did not change significantly. One such mechanism could be presynaptic inhibition within the "emerging" interneuronal network in the pre-Bötzinger complex as indicated by a lack of postsynaptic effects on putative inspiratory neurons in the pre-Bötzinger complex.[11,33]

Effect of Sevoflurane on C4 Burst Amplitude and Phrenic Motor Neurons

Sevoflurane reduced C4 burst amplitude in a dose-dependent fashion. This result agrees with human studies[1,2] in which sevoflurane at anesthetic doses decreased the tidal volume, and also with an *in vivo* canine study[3] in which sevoflurane decreased phrenic nerve activity. Because the reduced C4 burst amplitude appeared only when applied to the spinal cord, this effect of sevoflurane seems to target spinal respiratory neural circuits. This is supported by the current findings that sevoflurane does not affect the activity of the inspiratory neurons presumably located in the pre-Bötzinger complex, which has been thought to be an inspiratory rhythm generator.[39] Therefore, the amplitude depression seems to result from the effect of sevoflurane on the spinal cord.

In accord with this view, sevoflurane hyperpolarized phrenic motor neurons and decreased both their intraburst firing frequency and membrane resistance. The finding that blockade of $GABA_A$ receptors by picrotoxin or bicuculline did not attenuate the reduction of C4 burst amplitude suggests that sevoflurane does not reduce C4 burst amplitude by acting on $GABA_A$ergic transmission in the spinal cord. It has been reported that sevoflurane has multiple molecular targets at the spinal cord level. Matute and Lopez-Garcia[40] have shown that the depolarizations in motor neurons induced by α-amino-3-hydroxy-5-methylisoxazole-4-propionate and *N*-methyl-D-aspartate were reduced by sevoflurane. Two-pore-domain acid-sensitive K^+-1 channels, which have sensitivity to sevoflurane,[14] are reported to be densely expressed in the spinal cord.[41] Moreover, Grasshoff and Antkowiak[42] found that blocking glycine receptors reversed the depression of spontaneous firing elicited by sevoflurane on the spinal cord. Among these molecular targets, the current study suggests that the two-pore-domain acid-sensitive K^+-1 channel is involved. In slices containing the pre-Bötzinger complex, hypoglossal motor neurons generate endogenous respiratory-related motor output.[9] Recently, these neurons have been reported to express the two-pore-domain acid-sensitive K^+-1 channels and to be sensitive to sevoflurane.[14] Considering our data and these previous studies, it seems that in phrenic motor neurons, activation of the two-pore-domain acid-sensitive K^+-1 channel by sevoflurane may induce an increase in K^+ conductance (*i.e.*, K^+ leakage), resulting in membrane hyperpolarization and a reduction in intraburst firing frequency. However, further studies will be required to confirm the acid sensitivity of phrenic motor neurons during application of sevoflurane.

In summary, during application of sevoflurane, the pre-Bötzinger complex containing inspiratory neurons may determine the respiratory rhythm. Contrary, sevoflurane-induced depression of C4 burst amplitude seems to occur at the level of phrenic motor neurons. Our findings also suggest that $GABA_A$ receptors in the pre-Bötzinger complex plays an essential role in sevoflurane-induced depression of C4 burst rate, and that $GABA_A$ergic transmission in the spinal cord is not involved in sevoflurane-induced depression of C4 burst amplitude.

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Cardioprotective Effects of Nicorandil in Patients Undergoing On-Pump Coronary Artery Bypass Surgery

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Objective: The purpose of this study was to assess the cardioprotective effects of nicorandil in patients undergoing coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass (CPB).

Design: A prospective, double-blind, randomized clinical study.

Setting: A university hospital.

Participants: Thirty-two patients undergoing elective CABG surgery.

Interventions: Patients were randomized into 2 groups: the nicorandil and placebo groups. In the nicorandil group, intravenous nicorandil infusion was started after the induction of anesthesia by a loading dose of 0.1 mg/kg, followed by a continuous infusion of 0.1 mg/kg/h until 2 hours after CPB, then decreased to 0.05 mg/kg/h, and discontinued at the end of surgery. The placebo group received the same volume of saline. Arterial blood was sampled, and serum troponin T (TnT) and CK-MB were measured at the following 4 stages: after the induction of anesthesia (baseline), 2 hours after CPB, the first postoperative day (POD), and the third POD.

Measurements and Main Results: TnT concentrations were similar at baseline and increased with a peak on the first POD in both groups. In the nicorandil group, TnT concentration returned to the baseline value at the third POD, and the time course of TnT showed significantly lower levels ($p = 0.012$). CK-MB concentrations were similar at baseline, increased and peaked at 2 hours after CPB, and returned to the baseline on the third POD in both groups. There were no significant differences between the groups with respect to the changes in CK-MB concentrations.

Conclusions: The nicorandil group showed lower concentrations of TnT, suggesting that intraoperative administration of nicorandil may provide a degree of myocardial protection in CABG surgery.

KEY WORDS: nicorandil, cardioprotective effect, coronary artery bypass graft surgery

SHORT EPISODES OF transient ischemia have been shown to protect the myocardium against future prolonged ischemia, a phenomenon known as ischemic preconditioning. Ischemic preconditioning was first reported by Murry et al.[1] They showed in dogs that brief episodes of ischemia limited the size of the infarct resulting from a subsequent coronary occlusion. Clinical studies have also confirmed that angina occurring shortly before the onset of infarction has a beneficial effect on long-term prognosis after infarction.[2] Wu et al[3] showed that intraoperative ischemic preconditioning reduced postoperative ischemic reperfusion arrhythmias in patients with coronary artery bypass graft (CABG) surgery. Protein kinase C and adenosine triphosphate–sensitive potassium (K_{ATP}) channels are known to be involved in myocardial preconditioning.[4-6] Literature suggests the involvement of a mitochondrial potassium channel in mediating the beneficial effects of ischemic preconditioning or pharmacologically induced preconditioning.[7] Nicorandil is a hybrid drug that combines characteristics of nitrates and K_{ATP} channel activators, and the beneficial effects of nicorandil for myocardial protection via mitochondrial potassium channel activation have been shown experimentally.[8] Nicorandil also has been reported to improve prognosis and reduce cardiac events in cases of stable angina pectoris.[9,10] Recently, Kawamura et al[11] showed that nicorandil inhibits nuclear factor-κB activation, adhesion molecule expression, and cytokine production in patients with CABG. However, because the clinical use of intravenous nicorandil has been restricted to only Japan and Korea, few studies of perioperative usage of the drug have been reported.[12-14] This study was designed to assess the effects of nicorandil on perioperative myocardial damage in patients undergoing CABG surgery in a randomized, double-blind manner. Cardioprotection was evaluated by the measurement of the serum cardiac isozymes troponin T (TnT) and CK-MB.

METHODS

After approval from the institutional ethics committee, informed consent was obtained from all patients. Thirty-two patients undergoing elective CABG surgery were enrolled in the study. Exclusion criteria were as follows: redo operation, angina attack 1 week before the operation, severe hepatic or renal dysfunction, or nicorandil administration before operation.

Patients were premedicated with ranitidine, 150 mg, diazepam, 5 mg orally, and intramuscular meperidine, 35 mg. Anesthesia was induced with fentanyl (3-5 μg/kg), midazolam (0.05-0.15 mg/kg), and vecuronium (0.1-0.2 mg) and maintained with fentanyl (total dose of 15-45 μg/kg), midazolam (continuous infusion of 0.05-0.1 mg/kg/h), and vecuronium. Volatile anesthetics such as isoflurane and sevoflurane were not used. A radial or brachial artery catheter was inserted under local anesthesia before the induction of anesthesia. After the induction of anesthesia, a central venous catheter (Arrow International Inc, Reading, PA) and a pulmonary artery catheter (Edwards Swan-Ganz CCO/SvO_2/VIP Thermodilution Catheter; Baxter Edwards Corporation, Irvine, CA) were inserted through the right internal jugular vein. Using a Vigilance monitor (Baxter Healthcare Corporation, Edwards Critical Care Division, Irvine, CA), cardiac output and mixed venous oxygen saturation were continuously measured. Five-lead electrocardiography, pulse oximetry, capnography, urine output, and nasopharyngeal and bladder temperatures were monitored.

Cardiopulmonary bypass (CPB) was conducted by using a centrifugal pump (HPM-15; Nikkiso Co, Tokyo, Japan), a membrane oxygenator (HPO-20H-C; Senko Medical Inc, Tokyo, Japan), and an arterial filter (Auto Vento-SV; Pall Biomedical Products, Co, Glen Cove, NY).

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Nonpulsatile flow was maintained at a flow rate of between 2.2 and 2.6 L/min/m^2. Moderate hemodilution (hematocrit 18%-24%) and moderate hypothermia (bladder temperature 29°-32°C) were maintained during aortic cross-clamping. Myocardial preservation was performed by antegrade cold blood cardioplegia (Miotector; Mochida Pharma, Tokyo, Japan: Na^+ 120 mEq/L, K^+ 16.0 mEq/L, Mg^{2+} 32 mEq/L, Ca^{2+} 2.4 mEq/L, HCO_3^- 10 mEq/L, Cl^- 160.4 mEq/L). Anticoagulation was maintained with unfractionated heparin, 300 U/kg, for CPB. The celite activated coagulation time was measured every 30 minutes, and if the activated coagulation time was less than 480 seconds, additional heparin (100 U/kg) was administered during CPB. No antifibrinolytic drug was used during surgery. After the termination of CPB, protamine sulfate (1mg per heparin 100 U) was given to all patients.

Patients were randomly assigned in a double-blinded manner to 1 of 2 groups: the nicorandil group (n = 16) or the placebo group (n = 16). An independent person performed randomization by using a computer-generated random-number table and prepared the solution containing either 2 mg/mL of nicorandil or normal saline. The clinical dose of nicorandil is between 0.04 and 0.2 mg/kg/h, and Chinnan et al[15] showed that the administration of 0.1-mg/kg boluses of nicorandil did not cause significant hemodynamic changes or precipitate arrhythmias. The nicorandil group received a loading dose of 0.1 mg/kg of nicorandil via a central venous catheter after the induction of anesthesia followed by a continuous infusion of 0.1 mg/kg/h. The infusion rate was decreased to 0.05 mg/kg/h at 2 hours after the end of CPB and was discontinued at the end of surgery. The placebo group received the same volume of saline solution.

In all patients in both groups, lidocaine, 1 mg/kg/h, was infused continuously after the induction of anesthesia, and nitroglycerin infusion was started at 0.2 μg/kg/min and increased up to 1.0 μg/kg/min to maintain a mean systolic perfusion pressure of 50 to 70 mmHg during CPB. If the patient's radial artery was used for the bypass graft, diltiazem was used at a dose of 0.5 μg/kg/h. Approximately 15 minutes before the anticipated weaning time, dobutamine was initiated at doses of 5 μg/kg/min and then infusions of dobutamine, dopamine and nitroglycerin were titrated to wean from CPB. When the cardiac index was satisfactory (≥ 2.5 L/min/m^2) but a low blood pressure persisted, norepinephrine was infused to maintain a systolic arterial pressure greater than or equal to 90 mmHg. Red blood cells were transfused to maintain a hematocrit greater than 24%, preferentially by reinfusion of salvaged autologous blood.

After completion of the surgery, the patients were transferred to the intensive care unit. They were sedated with propofol (1-2 mg/kg/h) until the next morning and then were evaluated for tracheal extubation according to the criteria including hemodynamic stability, adequate pulmonary function, adequate urine output, and minimal chest tube output. ST changes on electrocardiogram, sudden symptomatic arrhythmias including heart block or ventricular tachyarrhythmia, and shock were evaluated.

Arterial blood was sampled, and measurements of serum TnT and CK-MB were made at the following 4 stages: (1) after the induction of anesthesia (baseline), (2) 2 hours after CPB, (3) the first postoperative day, and (4) the third postoperative day. Blood was drawn into the EDTA spits and centrifuged at 3,000 rpm for 5 minutes. The serum was separated and stored at −20° until assayed. TnT serum concentration was determined with the Electrochemilumi immunoassay method (Mitsubishi Chemical, Tokyo, Japan), and the serum concentration of CK-MB was determined with the Chemilumi immunoassay method (Mitsubishi Chemical). Reference ranges for TnT and CK-MB were less than 0.1 ng/mL and less than 6.0 ng/mL, respectively.

The number of patients was based on a power analysis from 17 cases of pilot data (mean difference was 0.244 and the common within-group standard deviation was 0.194), showing that a sample size of 15.7 per group would be adequate with a power of 80% (α = 0.05, 1 tailed). In this study, 32 patients undergoing elective CABG surgery were enrolled. Data were expressed as mean ± standard deviation. Statistical comparisons of the demographic and perioperative data were performed by a Student t test or Fisher exact test. The changes in serum TnT and CK-MB and hemodynamic data were analyzed by using 2-way analysis of variance with repeated measurements. A p value < 0.05 was considered significant.

RESULTS

In the 32 patients, reoperations were performed for bleeding in 2 patients of the placebo group, and they were excluded from the study. Table 1 shows the patient demographic data. There were no differences in terms of age; height; body weight; sex distribution; New York Heart Association class; preoperative left ventricular ejection fraction; or the number of patients with previous myocardial infarction, hypertension, diabetes mellitus, hyperlipidemia, or smoking history. Nor were differences between the groups observed with respect to the number of diseased coronary vessels and distal anastomoses; surgical and anesthetic time; cross-clamping and CPB time; or concomitant drugs such as dobutamine, dopamine, norepinephrine, milrinone, or diltiazem (Table 2). Table 3 shows the hemodynamic data during the study period; there was no difference between the 2 groups in regard to heart rate, mean blood pressure, and cardiac index. There were no significant differences in postoperative extubation time or duration of intensive care unit stay.

Table 1. Demographic Data

Variable	Placebo Group (n = 14)	Nicorandil Group (n = 16)	p Value
Age (y)	66.4 ± 8.7	63.3 ± 10.0	0.39
Height (cm)	162.7 ± 8.3	166.4 ± 4.3	0.15
Weight (kg)	64.9 ± 14.7	65.2 ± 8.4	0.95
Sex (M/F)	11/3	16/0	0.09
NYHA class	2.07 ± 0.8	2.13 ± 0.8	0.86
Left ventricular ejection fraction	55.5 ± 15.5	57.7 ± 13.2	0.69
No. diseased coronary vessels	2.88 ± 0.33	2.93 ± 0.27	0.62
Previous myocardial infarction	7	8	1.00
History of diabetes mellitus	8	10	0.77
History of hypertension	7	9	0.73
History of hyperlipidemia	7	8	1.00
Smoker	7	5	0.30

NOTE. Values are mean ± SD or n. There were no significant differences between the groups.

Table 2. Intraoperative and Postoperative Data

Variable	Placebo Group (n = 14)	Nicorandil Group (n = 16)	p Value
No. of distal anastomoses	4.39 ± 1.26	4.00 ± 1.12	0.38
Surgical time (min)	407.4 ± 52.1	393.3 ± 51.3	0.48
Anesthetic time (min)	518.7 ± 57.4	520.3 ± 57.6	0.94
Aortic cross-clamping time (min)	102.2 ± 16.4	99.5 ± 26.7	0.75
CPB time (min)	157.9 ± 30.8	146.6 ± 27.0	0.31
Concomitant drugs			
Dopamine	8	6	0.46
Dobutamine	13	16	0.47
Norepinephrine	2	2	1.00
Milrinone	1	1	1.00
Diltiazem	9	11	0.99
Postoperative extubation time	16.9 ± 1.6	22.1 ± 22.5	0.42
Duration of intensive care unit stay	40.8 ± 0.5	46.0 ± 23.0	0.42

NOTE. Values are mean ± SD or n. There were no significant differences between the groups.

Serum TnT concentrations were similar and within the reference range at baseline in both groups. TnT levels increased in both groups with a peak at the first postoperative day. In the nicorandil group, TnT concentration returned to the baseline value at the third postoperative day, and the time course of TnT showed significantly lower levels in the nicorandil group than in the placebo group ($p = 0.012$) (Fig 1). There were no differences between the groups in serum CK-MB concentrations at baseline. CK-MB levels increased in both groups and peaked at 2 hours after CPB, with a return within the reference range at the third postoperative day. There were no significant differences between the groups with respect to the changes in serum CK-MB concentrations ($p = 0.47$) (Fig 2).

With respect to ischemic electrocardiogram changes after CABG surgery, ST depression was noted at the first postoperative day in 1 patient in the nicorandil group, but coronary angiography revealed no evidence of myocardial ischemia. Four patients in the nicorandil group developed atrial fibrillation, whereas in the placebo group 3 patients developed atrial fibrillation and 1 patient developed ventricular premature beats at the first postoperative day. At the thirteenth postoperative day, 1 placebo patient experienced short runs of ventricular tachycardia.

Coronary angiography and a cardiac computed tomography scan were performed 2 to 4 weeks after operation and revealed 1 nicorandil patient and 3 placebo patients with anastomotic stenosis and 1 placebo patient with both anastomotic stenosis and thrombotic graft embolization. One of the patients with anastomotic stenosis in the placebo group had undergone coronary stent implantation. Two-year survival after CABG surgery was confirmed in all patients in the 2 groups.

DISCUSSION

In the present study, CABG surgery induced transient increases in serum concentrations of CK-MB and TnT in both the nicorandil and the placebo groups. Patients in the nicorandil group had lower TnT levels after CABG surgery. However, no significant differences were observed between the groups in CK-MB values, although these values were lower in the nicorandil group at the first and the third postoperative day.

In addition to nicorandil, there are several drugs that have myoprotective properties. Kersten et al[16] showed that isoflurane protects the myocardium from irreversible ischemic injury and that this isoflurane-induced protection is characterized by an early phase of preconditioning via activation of K_{ATP} channels extending at least 30 minutes after the volatile anesthetic has been discontinued.[17] However, the authors did not use inhalation anesthetic agents such as isoflurane or sevoflurane. In contrast to the early preconditioning, the late phase of ischemic preconditioning develops 12 to 24 hours after the brief suble-

Table 3. Hemodynamic Data

	Before Induction of Anesthesia	CPB Off	2 Hours After CPB	1st Postoperative Day	3rd Postoperative Day	p Value
Heart rate (beats/min)						
Placebo	61 ± 13	79 ± 18	83 ± 18	89 ± 10	86 ± 12	0.41
Nicorandil	68 ± 23	78 ± 15	83 ± 16	96 ± 7	89 ± 7	
Systolic arterial pressure (mmHg)						
Placebo	138 ± 18	103 ± 11	110 ± 12	124 ± 10	128 ± 8	0.37
Nicorandil	147 ± 28	102 ± 14	106 ± 14	115 ± 8	128 ± 17	
Cardiac index (L/min/m²)						
Placebo	2.12 ± 0.45	3.23 ± 0.83	3.05 ± 0.66			0.92
Nicorandil	2.16 ± 0.87	3.26 ± 1.12	3.21 ± 1.03			

NOTE. Values are mean ± SD. There were no differences between the 2 groups in regard to heart rate, systolic arterial pressure, and cardiac index.

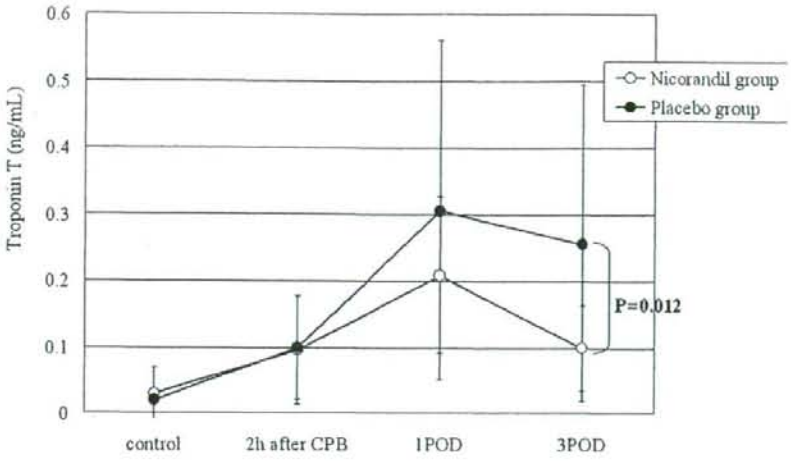

Fig 1. The time course of TnT concentration. The difference in regard to the changes in serum TnT between the nicorandil group and the placebo group was statistically significant ($p = 0.012$). (Values are expressed as mean ± standard deviation; group by time course difference by repeated-measures analysis of variance).

thal ischemia and lasts 3 to 4 days. Recent studies have shown that nitric oxide plays a major role in initiating the late phase of ischemic preconditioning and that a similar cardioprotective effect can be reproduced by pretreatment, 24 hours earlier, with nitric oxide donors in the absence of ischemia.[18] Banerjee et al[19] showed that nitroglycerin induces late preconditioning and a second period of protection reoccurs after 24 hours. In this study, nitroglycerin was used in all patients. However, nicorandil is a potent cardioprotective agent that has a K_{ATP} channel agonist with nitrate-like properties.

Because the clinical application of intravenous nicorandil is currently restricted to Japan and Korea, few reports have addressed the perioperative use of this drug.[12-14] Ito et al[12] examined the protective potential of nicorandil in patients undergoing off-pump CABG surgery, finding that concentrations of heart-type fatty acid–binding proteins in the nicorandil group were significantly lower than in the control group, suggesting that nicorandil exerts myoprotective properties. In their study, the concentration of TnT and CK-MB in the nicorandil group were lower than those in the control group, but the difference did not reach statistical significance. Hayashi et al[13] examined the myocardial protective effects of nicorandil on patients undergoing on-pump CABG surgery, finding that patients treated with nicorandil during CPB showed lower plasma levels of fatty acid–binding proteins and CK-MB. They concluded that nicorandil administration during CPB provides enhanced myocardial protective effects against ischemia-reperfusion. A study by Kaneko et al[14] showed that nicorandil exhibits dose-dependent prophylactic action against myocardial ischemia in patients at high risk for developing ischemic heart disease associated with major abdominal surgery. Kashimoto et al[20] showed that nicorandil reduces the frequency of cardiac events in patients undergoing noncardiac surgery both during and after the operation. Although there are certain differences in results between these studies and the present study, the results as a whole suggest that nicorandil may exert perioperative myocardial protective effects for patients undergoing cardiac and noncardiac surgery. In the present study, nitroglycerin was admin-

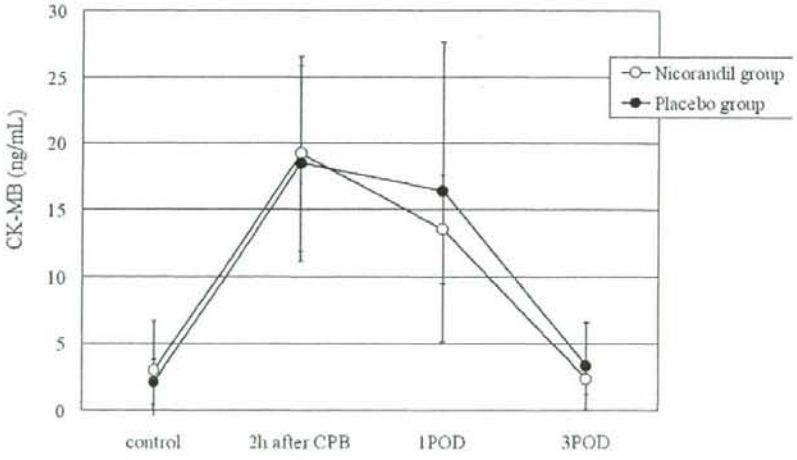

Fig 2. The time course of CK-MB concentration. There were no significant differences between the 2 groups with respect to the changes in serum CK-MB.

istered in all patients, and there were no differences in the use of diltiazem between the 2 groups. They may serve a cardioprotective effect, even in the placebo group, and may account for the fact that no significant differences were observed in the serum level of CK-MB.

There is a need to heighten awareness of the distressing nature of nicorandil-induced anal and oral ulceration. Although nicorandil-associated ulceration has been reported with a dose as low as 10 mg per day, most of the ulceration tends to occur at high doses of nicorandil of 40 mg per day or greater.[21,22] Reduction of the nicorandil dose is known to promote ulcer healing and prevent further recurrence.[23]

Both TnT and CK-MB have been shown to correlate well with the severity of myocardial damage, and the utility of these biochemical markers to diagnose myocardial infarction has been well established.[24,25] According to the consensus document of the Joint European Society of Cardiology and American College of Cardiology Committee for the redefinition of myocardial infarction, criteria for the diagnosis of myocardial infarction include pathologic evidence and changes in biochemical markers reflecting myocardial necrosis; the detection of myocardial troponin T or I is defined as the most sensitive and specific marker for myocardial infarction.[26]

Myocardial troponin is a myofibrillar regulatory protein that serves as a more specific indicator of irreversible cellular injury when compared with conventional cytosolic enzymes.[27] Troponin is released from the myocardium into the bloodstream 4 to 6 hours after the myofibril disintegration. Abnormal levels of troponin persist for 7 to 10 days, reflecting the course of myocardial injury. In general, myocardial troponin is not detected in the blood because of its high sensitivity to the myocardium. Elevated levels of troponin in the blood are generally grounds for suspecting myocardial damage. Although 0.1 ng/mL of TnT is often used as a cutoff level, even levels as low as 0.01 to 0.09 ng/mL may suggest trace myocardial damage.[28] Ohtani et al[29] evaluated the association between the serum TnT elevation and the angioscopically evaluated morphology of the culprit lesion in patients with non–ST-segment elevation acute coronary syndrome. They concluded that lower elevations (0.01-0.1 ng/mL) of TnT are frequently associated with intracoronary thrombus and represent myocardial necrosis. In this study, the TnT level increased above 0.1 ng/mL, suggesting the existence of transient myocardial damage. TnT levels peaked at the first postoperative day after CABG surgery, whereas CK-MB levels peaked at 2 hours after weaning from CPB. Wu et al[30] compared changes in CK-MB levels in patients undergoing CABG surgery and showed that CK-MB levels peaked at 6 hours after weaning from CPB. The timing and the value of CK-MB in their study are consistent with the present results. Inconsistencies in results among individual studies may be explained by the different time course of the serum levels of the troponin and CK-MB.

This study has certain limitations. The sample size was rather small; this may partly explain the reason why no difference was observed in the incidence of cardiac events or postoperative myocardial infarction. Therefore, there were no differences between the groups in the incidence of cardiac event or postoperative myocardial infarction. Benoit et al[31] and Januzzi et al[32] reported that troponin is a superior marker for assessing intraoperative myocardial infarction and cardiovascular complications after cardiac surgery. They proposed setting the cutoff value for TnT at 1.58 ng/mL as a predictor for postoperative cardiovascular events. TnT levels in all of the present patients were 1.58 ng/mL or less throughout the study, and none of the patients developed serious cardiac events. Although there was no statistical difference in sexes between the 2 groups, the number of female patients tended to be smaller in the nicorandil group. Larger studies may be needed to detect the effect of demographic data (ex, sex) on the perioperative myocardial damage and postoperative cardiac events.

There were significant differences in time course of the TnT changes between the nicorandil group and the placebo group, although transient subclinical myocardial damage may have occurred. The authors recommend administering nicorandil in patients undergoing CABG surgery when the possibility of severe myocardial damage is suspected before the procedure (ie, for patients with a history of extensive myocardial infarction and those with poor preoperative ventricular function).

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