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Title page

Non-invasive mapping of reactive oxygen species by *in vivo* electron spin resonance spectroscopy in indomethacin-induced gastric ulcers in rats

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Running title page

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d) Abbreviations

ANOVA, analysis of variance

COX, cyclooxygenase

DMSO, dimethylsulfoxide

DMTU, dimethylthiourea

ESR, electron spin resonance

H&E, hematoxylin and eosin

MRI, magnetic resonance imaging

NSAID, non-steroidal anti-inflammatory drug

ROS, reactive oxygen species

SOD, superoxide dismutase

TBARs, thiobarbituric acid-reactive substances

WIR, water immersion restraint

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Abstract

Reactive oxygen species (ROS) are thought to be involved in the gastric ulcer formation induced by indomethacin, a typical non-steroidal anti-inflammatory drug. However, the location and the time course of ROS generation remain unknown. To assess the sites of ROS generation, we applied the non-invasive measurement of ROS to indomethacin-treated rats. By giving orally a membrane-permeable or impermeable probe, the spectra were collected as a function of time by *in vivo* 300-MHz electron spin resonance (ESR) spectroscopy. The ESR signal decay rates of membrane-permeable probes, hydroxy-TEMPO and methoxycarbonyl-PROXYL in the gastric mucosal region were significantly enhanced 1 h after indomethacin treatment and the both caused the protection of ulcer formation, but membrane-impermeable probes, carboxy- and trimethylammonium-TEMPO, which did not exhibit the enhanced signal decay, also had no effect on ulcer formation. The enhanced signal decay in the gastric mucosa was suppressed by co-administration of the antioxidants tiron or dimethylthiourea with the nitroxyl probe. The results suggest that the enhanced signal decay rates in the gastric ulcers observed by *in vivo* ESR are associated with protective effects. The enhanced signal decay caused by ROS generation in stomach contributing to the ulcer formation induced by indomethacin is also suggested to occur at the gastric mucus layer or the interface or the intracellular compartment of epithelial cells. Overall, these results show the potentials of non-invasive assessment of ROS production and the sites of damage by

in vivo ESR using nitroxyl probes directed to specific sub-cellular regions.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) have defervescence, analgesia and anti-inflammatory effects, and their clinical applications to rheumatism, osteoporosis and osteoarthritis have been increasing. Indomethacin ([1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl] acetic acid) is one of the most widely used NSAIDs in the world because the anti-inflammatory effect of indomethacin on carrageenin-induced edema is approximately 30 times higher than that of aspirin (2-acetoxybenzoic acid) (Winter et al., 1963). NSAIDs cause therapeutic effects by the suppression of prostaglandins biosynthesis via the inhibition of cyclooxygenase (COX) gene expression. This class of agents is also known to cause significant gastrointestinal damage (Vane, 1971). It is known that the COX enzymes have isozymes of three types (Chandrasekharan et al., 2002) and the inhibition of COX-1 in gastric mucosa had been thought to be related with the gastric ulcer formation induced by NSAIDs (Wallace et al., 1998) since COX-1 is involved in maintaining the integrity of gastric mucosa, the increment of mucosal blood flow and mucus secretion (Morris and Wallace, 1981; Kitahora and Guth, 1987). However, ulcer formation did not occur in COX-1 knockout mice (Langenbach et al., 1995) and in mice pretreated with SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H- pyrazole), which is a COX-1 selective inhibitor, in spite of significant suppression of prostaglandin biosynthesis (Wallace et al., 2000). These findings suggest other factors are involved in

the mechanism of NSAIDs-induced gastric ulcer formation in addition of inhibition of COX-1.

Reactive oxygen species (ROS) are reportedly associated with the pathogenesis in indomethacin-induced gastric ulcer (Takeuchi et al., 1991; Yoshikawa et al., 1993). The formation of gastric ulcer and the increase of thiobarbituric acid-reactive substances (TBARs) in gastric mucosa induced by indomethacin was significantly suppressed by the intravenous infusion of superoxide dismutase (SOD) or dimethylsulfoxide (DMSO) (Takeuchi et al., 1991) and the subcutaneous treatment of the mixture of Cu, Zn-SOD and catalase (Yoshikawa et al., 1993). In these studies, the stable end products of ROS generation were monitored. However, individual free radical reactions or reactive oxygen species were not monitored.

The *in vivo* electron spin resonance (ESR)/spin probe technique is suitable for the examination of free radical reactions *in vivo* in experimental diseases, as demonstrated by us (Utsumi et al., 1990; Sano et al., 1998; Phumala et al., 1999; Han et al., 2001; Utsumi et al., 2002; Kasazaki et al., 2003a; Kasazaki et al., 2003b; Matsumoto et al., 2003; Yamato et al., 2003; Takeshita et al., 2004; Yasukawa et al., 2004; Sonta et al., 2005) and others (Miura et al., 1997; Kuppusamy et al., 1998; Leonard et al., 2002). In this technique, the signal decay of the nitroxyl probes mediated by ROS is monitored by ESR spectroscopy. Recently, we reported, for the first time, the generation of ROS in the stomachs of rats with NH_4OH-induced (Kasazaki et al., 2003a; Kasazaki et al., 2003b) and water immersion restraint (WIR)-induced (Yasukawa et al.,

2004) gastric lesions using this technique. In NH_4OH-induced gastric damage model, lesion formation induced by NH_4OH is acute, occurring within 30 min, and is associated with the enhancement of vascular permeability, neutrophil infiltration into the mucosa, and ROS generation in stomach. This rapidly developing pathology makes it difficult to ascertain whether ROS generation is a cause for and/or a result of ulcer formation. In WIR-induced gastric lesion model, which requires approximately 6 h to produce lesions, the relationships between the enhancement of signal decay and the neutrophil infiltration as well as lesion formation in the WIR model were different from that observed with NH_4OH. However, ROS generation in gastric region of WIR-treated rats was similar in extent to that observed in NH_4OH-treated rats. These studies demonstrated the usefulness of *in vivo* ESR/spin probe technique for non-invasive and real-time evaluation of *in vivo* free radical reaction in rats with gastric ulcers.

The availability of nitroxyl probes with functional groups, which make them compartmentalize in specific cellular/sub-cellular regions, makes *in vivo* ESR technique uniquely capable of providing unambiguous information pertaining to the sites of ROS generation non-invasively (Utsumi et al., 2002). The selection of nitroxyl probes with varying membrane permeability enables us to evaluate the site-specific ROS generation in various disease models. The *in vitro* cellular ESR measurement of three nitroxyl probes having different membrane permeability, carboxy-PROXYL, methoxycarbonyl-PROXYL and carbamoyl-PROXYL with liposomes revealed that carboxy-PROXYL, methoxycarbonyl-PROXYL and carbamoyl-PROXYL locate in

aqueous phase, in lipidic phase and at the interface between aqueous and lipidic phase, respectively (Yamato et al., 2003).

In this study, generation sites of ROS in rats with indomethacin-induced gastric ulcer were directly investigated using *in vivo* 300-MHz ESR spectroscopy and nitroxyl probes with different membrane permeability.

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Materials and Methods

Chemicals

4-Hydroxy-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl (hydroxy-TEMPO), 4-oxo-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl (oxo-TEMPO), 4-carboxy-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl (carboxy-TEMPO), 3-carbamoyl-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl (carbamoyl-PROXYL), dimethylthiourea (DMTU) and urethane were purchased from Aldrich Chemical Co. (Milwaukee,WI). 4-Trimethylammonium-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl (trimethylammonium-TEMPO, so-called CAT-1) was purchased from Molecular Probes, Inc (Eugene, OR, USA). 3-Methoxycarbonyl-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl (methoxycarbonyl-PROXYL) was synthesized as described previously (Sano et al., 1997). [1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl] acetic acid (indomethacin) and 4,5-dihydroxy-1,3-benzene-disulfonic acid (tiron) were obtained from Sigma Chemical Co. (St. Louis, MO). D-Mannitol (mannitol) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were commercially available reagent grade quality.

Animal Treatment

Male Sprague-Dawley rats (5 weeks old; 120-160 g body weight) were purchased from Seac Yoshitomi Co. (Fukuoka, Japan), and were acclimatized for one

week before experimentation. Diet (MF, Oriental Yeast Co. Tokyo, Japan) and water were provided *ad libitum.* The animals were fasted for 24 h with free access to water until 1 h before the experiment. Gastric mucosal ulcers were induced by the oral administration of indomethacin (5, 15, or 30 mg/kg). Indomethacin was dissolved in 5 % sodium hydrogen carbonate ($NaHCO_3$) solution.

In order to estimate the effect of antioxidants, either DMTU (0.33 mmol/kg at a time), tiron (0.33 mmol /kg at a time), mannitol (0.33 mmol /kg at a time) or nitroxyl radicals (0.18 mmol/kg at a time), hydroxy-, oxo-, carboxy- or trimethylammonium-TEMPO or carbamoyl- or methoxycarbonyl-PROXYL were orally administered 5 min before, 1 h, and 2 h after indomethacin treatment.

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Graduate School of Pharmaceutical Sciences, Kyushu University, and were conducted according to the Guidelines for Animal Experiments of the Graduate School of Pharmaceutical Sciences, Kyushu University.

ESR Measurement

Rats anesthetized by intramuscular injection of urethane (2 g/kg) were orally given hydroxy-, oxo-, carboxy- or trimethylammonium-TEMPO or carbamoyl- or methoxycarbonyl-PROXYL (1.0 ml of 10 mM) to individual rats, and the ESR spectra were observed *in vivo* in the gastric region with a 300-MHz ESR spectrometer (JES-CM-3L, JEOL, Japan). The microwave power was 1.19 mW. The amplitude of the

100-kHz field modulation was 0.125 mT. The external magnetic field was swept at a scan rate of 6.0 mT/min. The signal decay rate was calculated as described previously (Kasazaki et al., 2003a).

In order to determine the causes of the enhanced signal decay, DMTU (0.33 mmol/kg), tiron (0.33 mmol/kg) or mannitol (0.33 mmol/kg) was orally administered with the administration of the nitroxyl probe. In each experiment, control rats were treated with distilled water as a vehicle.

Evaluation of macroscopic and histologic gastric mucosal injury

The extent of gastric mucosal injury in rats treated with indomethacin was investigated macroscopically. The stomachs were removed, inflated with 10 ml of 1% formaldehyde, and opened along the greater curvature. The ulcer area (mm^2 per glandular stomach) was determined under a dissecting microscope with a square grid micrometer.

Histologic injury of indomethacin-treated gastric mucosa with hydroxy-TEMPO pretreatment was evaluated by hematoxylin and eosin (H&E) stain. A hydroxy-TEMPO solution (0.18 mmol/kg at a time) was orally administered 5 min before, 1 h, and 2 h after indomethacin treatment. Three hours after indomethacin treatment, the stomach was collected. All specimens were fixed overnight in 10 % neutral-buffered formalin and embedded in paraffin. Sections with thickness of 3 μm were cut, mounted on glass slides, dried overnight and then stained with H&E.

MRI Measurement

A rat anesthetized by intramuscular injection of urethane (2 g/kg) was turned up on the plate, where two syringes (3 mm inner diameter) containing distilled water were fixed as positional and contrast markers on both sides of the rat. The MR imaging was performed using a 0.2-T MRI system (MRP-20, Hitachi Medical Co., Tokyo, Japan) both before and after the oral administration of distilled water (1.0 ml). The pulse sequence of conventional spin echo proton density-weighted with TR(msec)/TE(msec) = 1600/40 was acquired. All images were acquired using a 200 × 200 mm field of view (FOV), two signal average, coronal plane and 3.0 mm thickness.

Statistical Analysis

Statistical analyses were carried out using Stat View 5.0. The data were analyzed by Student's *t*-test, by one way analysis of variance (ANOVA) with the Dunnett's test or the Tukey-Kramer's test as a post hoc test, or by two-way ANOVA with the Tukey-Kramer's test as a post hoc test. All the results are expressed as the mean ± S.D. $P < 0.05$ was considered statistically significant.

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Results

Longitudinal linear ulcers were produced in the gastric mucosa with oral administration of indomethacin to rats, and the typical picture of ulcer formation, which was induced by 30 mg/kg of indomethacin, is shown in Fig. 1A. The ulcer area was increased with its dose up to 30 mg/kg and that in the 30 mg/kg group was 18.2 $\pm$ 4.6 (mm^2) as shown in Fig. 1B. The ulcer area in the 5 mg/kg indomethacin group was only 2.0 $\pm$ 1.6 (mm^2) although the dose is reportedly high enough to inhibit the COX-1 and COX-2 expression (Wallace et al., 2000). The studies using the dose 20 mg/kg (Langenbach et al., 1995), 30 mg/kg (Tanaka et al., 2002), or 48 mg/kg of indomethacin (Swarnakar et al., 2005) were reported as the indomethacin-induced gastric ulcer model. Thus, the dose 30 mg/kg of indomethacin was used to the following experiments. The ulcer area in the mucosa gradually increased with time up to 3 h as shown in Fig. 1C.

The ulcer formation induced by indomethacin can be inhibited by antioxidants. Earlier studies showed that the intravenous infusion of SOD (15000 U/kg/h) and DMSO (30 mg/kg/h) suppressed the lesion area by 79.3% and 72.6%, respectively (Takeuchi et al., 1991). The continuous infusion via the tail vain of SOD (25000 U/kg/h) and catalase (25000 U/kg/h) prevented the gastric damage by approximately 60% and 50%, respectively (Vaananen et al., 1991). The subcutaneous administration of a combination of Cu, Zn-SOD (50000 U/kg) and catalase (90000 U/kg) and the intraperitoneal injection of DMSO (550 mg/kg) suppressed the area of erosions by 50% and 55%,

respectively (Yoshikawa et al., 1993). However, our previous studies demonstrate that the location of ROS generation observed by *in vivo* ESR/spin probe technique is not blood vessel but gastric cavity of rats with NH_4OH-induced and WIR-induced gastric lesions (Kasazaki et al., 2003a; Yasukawa et al., 2004). To examine correlation between the *in vivo* sites of ROS generation evaluated by this technique and the ulcer formation, it is important that the antioxidants are administered in the gastric cavity directly and the protective effects are examined. The suppressive effect of orally administered three antioxidants, both membrane-permeable and impermeable, on the ulcer formation was investigated. When tiron, which is a membrane-permeable superoxide scavenger, was orally administered 5 min before, 1 h, and 2 h after indomethacin treatment, the gastric ulcer area was suppressed to 35% of that in the vehicle group (Fig. 2). The oral administration of DMTU, which is a membrane-permeable hydroxyl radical scavenger, also depressed the ulcer area to 12% of that in the vehicle group (Fig. 2). However, mannitol, which is a membrane-impermeable hydroxyl radical scavenger, showed little inhibitory effect on the ulcer formation (Fig. 2). In our previous reports, the ulcer formation induced by NH_4OH and WIR was suppressed by the oral administration of mannitol by 80% and 50%, respectively (Kasazaki et al., 2003a; Yasukawa et al., 2004). Results from Fig. 2 and results from earlier studies (Kasazaki et al., 2003a; Yasukawa et al., 2004) suggest that the ulcers induced by indomethacin are quite different from the lesions caused by NH_4OH or WIR. These findings suggest that only membrane-permeable antioxidants have suppressive effects on the gastric ulcer

formation induced by indomethacin, whereas membrane impermeable antioxidants appear to be effective in other models of gastric ulcer induction.

In vivo ROS generation in the stomach of rats with NH_4OH-induced (Kasazaki et al., 2003a; Kasazaki et al., 2003b) and WIR-induced (Yasukawa et al., 2004) gastric lesions were directly detected using a carbamoyl-PROXYL probe and *in vivo* ESR spectroscopy. In this study, various nitroxyl probes having different Po/w values (Table 1) were orally administered to indomethacin-treated rat and *in vivo* ESR measurements were carried out. In order to confirm that indomethacin itself does not promote the excretion from stomach, the MR images at upper abdomen of rats intragastrically injected the water with and without indomethacin were obtained 4 and 14 min after oral administration of water using a clinical 0.2-T MRI system. The oral administration of water gave a clear MR image at the gastric domain, and the intensity of the MR image did hardly changed for 15 min as reported previously (Fig. 3, B and C)(Yasukawa et al., 2004). The presence of indomethacin in injected water had no influence on MR image at the gastric domain, and the intensity of the MR image continued to keep for 15 min (Fig. 3, E and F), suggesting that indomethacin itself does not promote the excretion from stomach during the *in vivo* ESR measurement.

The signal decay rate was calculated from the slope of the signal decay curve, as described previously (Kasazaki et al., 2003b). Because of the protective effect of membrane-permeable antioxidants on the indomethacin-induced gastric mucosal ulcer (Fig. 2), the hydroxy-TEMPO probe with the higher Po/w value (Po/w: 3.6 (Takeshita et

al., 1999)) may display greater differences in signal decay rates between indomethacin-treated and sham groups. The hydroxy-TEMPO probe was orally administered to indomethacin-treated rats, and the ESR spectra were obtained in the gastric region. The semi-logarithmic plot of ESR signal intensity of hydroxy-TEMPO against time showed a linear decrease (Fig. 4A). The signal decay rate, calculated from the slope of the semi-logarithmic plot, at 1 h after indomethacin treatment was enhanced as the dose of indomethacin, and the dose-response of signal decay was similar to that of ulcer area (Fig. 4B). The signal decay rate in the 30 mg/kg of indomethacin was significantly enhanced in the 1-h indomethacin group ($*p < 0.05$) compared to the sham group, and the enhancing ratio of hydroxy-TEMPO was 3.1 as shown in Fig. 4C. The signal decay was moderately decreased at 3 h compared to that observed at 1 h in spite of the development of ulcer formation (Fig. 4C). This result indicates that the enhanced signal decay of hydroxy-TEMPO occurs at the early phase of the gastric ulcer formation induced by indomethacin.

To identify the cause of the enhanced signal decay of the hydroxy-TEMPO probe in indomethacin-treated rats, selected antioxidants were administered to $NaHCO_3$-treated and indomethacin-treated rats, and the decay rates in the stomach were determined. It was verified by *in vitro* ESR that no direct chemical reactions occur between hydroxy-TEMPO and the various antioxidants. The simultaneous administration of tiron or DMTU with the nitroxyl probe suppressed the enhanced signal decay significantly. However, the oral administration of mannitol with the

nitroxyl probe had little effect on the enhanced signal decay (Fig. 5). Tiron and DMTU, which inhibited the ulcer formation induced by indomethacin, had the suppressive effect on the enhanced signal decay, while mannitol, which did not prevent the ulcer formation, had no effect on the enhanced signal decay. These results identify a strong association between the gastric ulcer formation and signal decay rates, which reflects ROS generation.

Since the localization of the nitroxyl probe appears to play an important role, several nitroxyl spin probes with different partition coefficients were tested *in vivo* ESR monitoring the rate of signal loss. When the methoxycarbonyl-PROXYL probe, which is also a membrane-permeable (Po/w: 8.7 (Sano et al., 2000)), was orally administered to indomethacin-treated rat, the signal decay rate was significantly enhanced ($*p < 0.05$) compared to the sham group, and the enhancing ratio was 11.7 (Fig. 6). When the oxo-TEMPO probe, which is moderately membrane-permeable (Po/w: 2.0 (Takeshita et al., 1999)), was used to indomethacin-treated rat, the signal decay rate showed a slight enhancement, and the enhancing ratio was 1.75 (Fig. 6). On the other hand, the signal decay rate of a carboxy-TEMPO probe, which is a membrane-impermeable one (Po/w: 0.019 (Eriksson et al., 1986)), showed minimal enhancement, and the enhancing ratio was 1.3. The signal decay rate of a trimethylammonium-TEMPO probe, which has the lowest membrane permeability of the probes used in the experiments (Po/w: 0.0033 (Takeshita et al., 1999)), did not cause the enhanced signal decay (Fig. 6). These data indicate that the enhanced signal decay increases as partition coefficient of the probe

increases. Carbamoyl-PROXYL with a Po/w value of 0.68 (Takeshita et al., 1999) (Table 1) localizes at the interface between aqueous and lipidic phase (Yamato et al., 2003). The ESR signal decay rates of carbamoyl-PROXYL in the group of $NaHCO_3$ and in the group of 1-h indomethacin were 0.0094 $\pm$ 0.0037 and 0.0166 $\pm$ 0.0067 (mean $\pm$ S.D.), respectively (Fig. 6). The carbamoyl-PROXYL signal decay in the 1-h indomethacin group was 1.8 times higher than that of the sham group, which was much smaller compared with that in the 6-h WIR group (Yasukawa et al., 2004) and in the 0.5-h NH_4OH group (Kasazaki et al., 2003a; Kasazaki et al., 2003b).

To assess the relationship between signal decay rates and protection of the ulcer, nitroxyl probes with different partition coefficients were orally administered to rats 5 min before, 1 h, and 2 h after indomethacin treatment, and the ulcer formation 3 h after indomethacin treatment was evaluated. The oral administration of hydroxy-TEMPO suppressed the ulcer area by 94.5% compared to the vehicle-treated group and the suppression was significant ($*p < 0.05$). When methoxycarbonyl-PROXYL was used, 75.6% compared to the ulcer area was inhibited ($*p < 0.05$). Oral administration of oxo-TEMPO significantly suppressed the gastric ulcer area by 77.5% of that in the vehicle-treated group (Fig. 7). On the other hand, the oral administration of membrane-impermeable probes, carboxy- and trimethylammonium-TEMPO, had no effect on the ulcer formation (Fig. 7). These findings suggest that indomethacin treatment induces ROS generation in the hydrophobic environment of gastric mucosa, such as the mucus layer or the interface or

the intracellular compartment of epithelial cells.

The histological evaluation of indomethacin-treated gastric mucosa using H&E stain was carried out in order to investigate where in gastric mucosal layer the damage occurs and how hydroxy-TEMPO suppress the gastric mucosal injury. The H&E stain of gastric mucosa in the 1-h indomethacin group showed foci of necrosis with hemorrhage, but inflammatory infiltration including neutrophils was not present (Fig. 8B), while in the sham group no damage of gastric mucosal layer was recognized (Fig. 8A). The H&E stain of the hydroxy-TEMPO group showed no evidence of the occurrence of necrosis with hemorrhage (Fig. 8C). These data suggest that the ROS generation contributing to the ulcer formation occurs at the mucus layer or the interface or the intracellular compartment of epithelial cells. Further nitroxyl probes with high Po/w value such as the hydroxy-TEMPO probe directly protect the disruption of mucosal layer by permeating into the mucus layer and the epithelial cells and scavenging the generated ROS.

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Discussion

Nitroxyl radical probes are a unique class of compounds useful for the *in vivo* monitoring of ROS. The following features in this class of compounds provide them with this important capability:

1) Housing an unpaired electron, these agents can be used to probe deep in tissue by low frequency ESR making it possible for the non-invasive detection *in vivo*.

2) These agents are stable free radicals which can participate in radical-radical recombination reactions or radical scavenging reactions with ROS and can provide antioxidant defense and consequently lose their ESR signal.

3) These probes can be derivatized with appropriate substituents to direct them to specific cellular compartments and assess their protective effects as well as monitor the scavenging of ROS non-invasively by ESR.

Therefore, nitroxyl probes, being probes in non-invasive imaging as well as effective antioxidants, offer unique capabilities for monitoring ROS production in intact living objects.

In this study, the use of an *in vivo* 300-MHz ESR spectrometer and nitroxyl probes with different membrane permeability provided us, for the first time, the non-invasive and real-time information on the location of ROS generation in stomach of rats with indomethacin-induced gastric ulcer. The association of the disappearance of the nitroxyl probe and their protective effects in probes with high Po/w values in the

stomach of rats treated with indomethacin suggests that ROS generation contributing to the ulcer formation occurs at the mucus layer or the interface or the intracellular compartment of epithelial cells.

The enhanced signal decay of administered nitroxyl probe increased as the Po/w value increased and the enhanced signal decay was suppressed by the administration of the hydrophobic antioxidants with the nitroxyl probe. The administered antioxidants or nitroxyl probes should localize in the gastric mucus layer and permeate the mucus layer depending on their Po/w values. It is characteristic that the surface of gastric mucosa is covered with a meshy macromolecule, mucin, maintaining hydrophobic and neutral atmosphere. Thus, the permeability of nitroxyl probes into the gastric mucosa might be different from that into the liposomal membrane.

The signal decay rates of hydroxy-TEMPO and carbamoyl-PROXYL (280 mM, 100 μl/g) injected intravenously to a normal mouse at the upper abdomen were reported to be 0.71 ± 0.30 (/min) and 0.09 ± 0.02 (/min), respectively (values were expressed as mean $\pm$ S.D.) (Utsumi et al., 1990). Hydroxy-TEMPO can easily permeate the membrane of cells and rapidly lose its paramagnetism by bioreduction such as enzymes in electron transport system (Quintanilha and Packer, 1977) and ascorbic acid (Perkins et al., 1980), while carbamoyl-PROXYL minimally permeates the cell membrane and is minimally reduced. On the other hand, in this study, the signal decay rates of all tested probes administered orally to $NaHCO_3$-treated rats at the gastric

region were similar. The mucus is composed of proteins 64%, carbohydrates 15% and lipids 18%, and mucin bounds to lipids of 20%, according to the analysis of gastric mucus components in pigs, (Sarosiek et al., 1988) and its secretion can not be altered by indomethacin (Nicoloff, 1968; Narumi and Kanno, 1972). The existence of mucus layer would make the surface of epithelial cells neutral and hydrophobic in spite of indomethacin treatment. However most of carboxy-TEMPO molecules in gastric liquid at pH 2 are the uncharged form because previously determined pKa value of carboxy-TEMPO is 4.0, the increment of charged form following the rise of pH in gastric mucus layer might impede the distribution to epithelial cell layers.

The finding that the enhanced signal decay was significantly suppressed by the co-administration of tiron (77% in 0.05 mmol group) or DMTU (78% in 0.025 mmol group) with the hydroxy-TEMPO probe suggests that the reactive oxygen species are generated in the stomach of indomethacin-treated rats. Another point to be noted from the fact that tiron and DMTU impact the signal decay rates is that the nitroxyl probes and antioxidants compete for the same reactive species. However, tiron, which is known as a superoxide scavenger, reacts with not only superoxide ($1\times10^{7}\,M^{-1}\cdot min^{-1}$) but also hydroxyl radical ($1\times10^{9}\,M^{-1}\cdot min^{-1}$) (Bors et al., 1979) as well as act as a chelator of metal such as Fe^{3+} or Cu^{2+} (Krishna et al., 1992). Therefore, the suppressive effect of tiron on the enhanced signal decay of hydroxy-TEMPO might be due to the scavenging of several primary species such as hydroxyl radical, superoxide etc or secondary species produced in lipid peroxidation chain reactions.

A hydrophobic nitroxyl probe, hydroxy-TEMPO protected the gastric ulcer formation induced by indomethacin supported by previous report that the oral administration of hydroxy-TEMPO (0.1 g/kg at a time) 5 min before as well as 1 h and 2 h after subcutaneous treatment of indomethacin (30 mg/kg) completely prevented the ulcer formation (Rachmilewitz et al., 1994). In this study, not only hydroxy-TEMPO but also other nitroxyl probes with different Po/w values were evaluated and the evidence that carboxy- and trimethylammonium-TEMPO with relative low Po/w values had no effect on the ulcer formation, for the first time, shows that the sites of accumulation of antioxidants is critical in protecting against gastric ulcers.

The signal decay rate of nitroxyl radicals could be influenced by not only their membrane permeability but also the reactivity of nitroxyl radicals with ROS. Using the hydroxyl radical generating system *in vitro* ($Fe(II)/H_2O_2$), the reactivity of nitroxyl probes with hydroxyl radical was reported previously (Kasazaki et al., 2003a).

In conclusion, *in vivo* ESR study with nitroxyl spin probes provides useful information as to the sites of ROS generation and the choice of protective agents to inhibit damage associated with indomethacin-induced gastric ulcers.

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Footnotes

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Legends for figures

Fig. 1. The gross appearance of gastric mucosa of a rat 3 h after indomethacin treatment (30 mg/kg) (A), the dose-dependent change of gastric mucosal ulcer formation with various doses of indomethacin (5, 15, or 30 mg/kg) (B), and the time-course of gastric mucosal ulcer formation with 30 mg/kg of indomethacin (C). The gastric ulcers were produced by the oral administration of indomethacin (5, 15, or 30 mg/kg). The stomach was removed and the ulcer area was evaluated under a dissecting microscope with a square grid micrometer. Each value represents mean ± S.D. of 5-7 rats indicated in the parentheses. N.D. indicates the value below detectable. *$p < 0.05$, **$p < 0.01$ as determined by the Dunnett's test when compared with the $NaHCO_3$-treated group.

Fig. 2. Effect of tiron, DMTU and mannitol on the ulcer formation induced by indomethacin. Tiron (0.33 mmol/kg), DMTU (0.33 mmol/kg) or mannitol (0.33 mmol/kg) were orally administered 5 min before, 1 h, and 2 h after indomethacin treatment (30 mg/kg). Three hour after indomethacin treatment, the area of mucosal ulcers was measured under a dissecting microscope with a square grid micrometer. Each value represents mean ± S.D. of 5-8 rats indicated in the parentheses. **$p < 0.01$ as determined by the Dunnett's test when compared with the vehicle-treated group, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ as determined by the Dunnett's test when compared with the

mannitol-treated group.

Fig. 3. Coronal MR images of a non-treated rat before (A), 4 min (B) and 14 min (C) and an indomethacin-treated rat before (D), 4 min (E) and 14 min (F) after oral administration of water. In D, E and F, a rat was orally treated with 30 mg/kg of indomethacin. After 20 min, a rat was anesthetized by urethane (2 g/kg) and turned up on the plate, in which two syringes (3 mm diameter) containing water (380 μl) were fixed on both sides of the rat. One hour after indomethacin treatment, the MR imaging was performed using a 0.2-T MRI system before the oral administration of water (1.0 ml). Within 20 sec after the administration, the MR imaging was started, and two images were sequentially obtained.

Fig. 4. Typical signal decay curve for hydroxy-TEMPO in the gastric region after oral administration (A), the dose-dependent change of signal decay rate and ulcer formation with various doses of indomethacin (5, 15, or 30 mg/kg) (B), and the time-course of signal decay rate in indomethacin-treated rats (C). Thirty minutes after anesthesia in sham group (○) or 1 h after indomethacin (30 mg/kg) group (●), hydroxy-TEMPO (10 mM, 1 ml) was orally administered, and then its ESR spectra were measured in the gastric region with *in vivo* 300-MHz ESR spectroscopy. The logarithm of peak height at the center field *h(0)* was plotted against time after administration. The decay rates were calculated from the slope of Fig. 4A in the sham or the indomethacin group. Each value

represents mean ± S.D. of 6-7 rats indicated in the parentheses. In Fig. 4B, $^{**}p < 0.01$ as determined by Dunnett's test when compared with the signal decay rate of the sham group, and $^{\#\#}p < 0.01$ as determined by Dunnett's test when compared with the ulcer formation of the sham group. In Fig. 4C, $^{*}p < 0.05$ as determined by two-way ANOVA with the Tukey-Kramer's test.

Fig. 5. Effect of antioxidants, tiron, DMTU and mannitol on the enhanced signal decay in rats treated with indomethacin. One hour after indomethacin treatment (30 mg/kg), 500 μl of tiron (0.33 mmol/kg), DMTU (0.33 mmol/kg) or mannitol (0.33 mmol/kg) was mixed with 500 μl of 20 mM hydroxy-TEMPO, and then the mixed solution was orally administered. Immediately after administration of the nitroxyl probe, *in vivo* 300-MHz ESR measurement was performed. Each value represents mean ± S.D. of 5-7 rats indicated in the parentheses. $^{**}p < 0.01$ as determined by Student's *t*-test when compared with the sham group, and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as determined by the Dunnett's test when compared with the indomethacin group treated with vehicle.

Fig. 6. The comparison of signal decay rate among nitroxyl probes with different partition coefficients in indomethacin-treated rats. One hour after indomethacin treatment (30 mg/kg), the hydroxy-, oxo-, carboxy- or trimethylammonium-TEMPO or methoxycarbonyl- or carbamoyl-PROXYL (10 mM, 1 ml) were orally administered, and then their ESR spectra were measured in the gastric region with *in vivo* 300-MHz

ESR spectroscopy. Each value represents the mean ± S.D. of 6-18 rats indicated in the parentheses. *$p < 0.05$ as determined by two-way ANOVA with the Tukey-Kramer's test.

Fig. 7. The comparison of suppressing effect on the ulcer formation among nitroxyl probes with different partition coefficients in indomethacin-treated rats. Hydroxy-, oxo-, carboxy- or trimethylammonium-TEMPO or methoxycarbonyl- or carbamoyl-PROXYL (0.18 mmol/kg at a time) were orally administered 5 min before, 1 h, and 2 h after indomethacin treatment. The area of gastric mucosal ulcers was measured 3 h after indomethacin treatment (30 mg/kg). Each value represents the mean ± S.D. of 4-7 rats indicated in the parentheses. *$p < 0.05$ as determined by two-way ANOVA with the Tukey-Kramer's test.

Fig. 8. Hematoxylin and eosin (H&E) staining of stomachs from the sham group (A), the indomethacin group (B) and the hydroxy-TEMPO group (C). Three hours after indomethacin treatment (30 mg/kg), the stomach was removed, and slides with 3 μm thick serial sections of paraffin-embedded tissues were stained with H&E stain. Hydroxy-TEMPO (0.18 mmol/kg at a time) was orally administered 5 min before, 1 h, and 2 h after indomethacin treatment. The arrow in (B) indicates the hemorrhage caused by indomethacin treatment.

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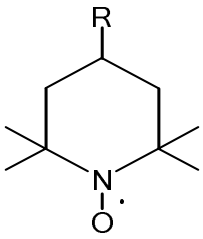
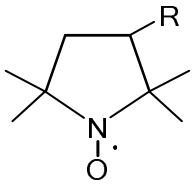
Table

TABLE 1

Structures and *n*-octanol/water partition coeffients (Po/w) of typical nitroxyl probes

Basic structure	R	probe	Po/w
	-OH	hydroxy-TEMPO	3.6
	=O	oxo-TEMPO	2.0
	$-COO^-$	carboxy-TEMPO	0.019
	$-N^+(CH_3)_3$	trimethylammonium-TEMPO	0.0033
	$-CONH_2$	carbamoyl-PROXYL	0.68
	$-COO^-$	carboxy-PROXYL	0.0047
	$-COOCH_3$	methoxycarbonyl-PROXYL	8.7

The partition coefficients (Po/w) were determined by the ESR spectra of nitroxyl probes in *n*-octanol and in phosphate-buffered saline as reported previously (Eriksson et al., 1986; Takeshita et al., 1999; Sano et al., 2000).

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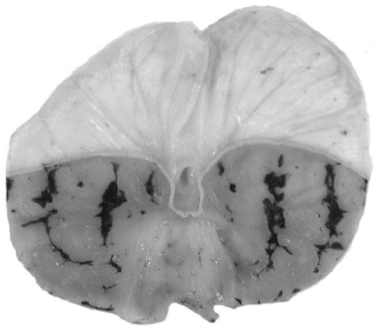
Fig. 1

(A)



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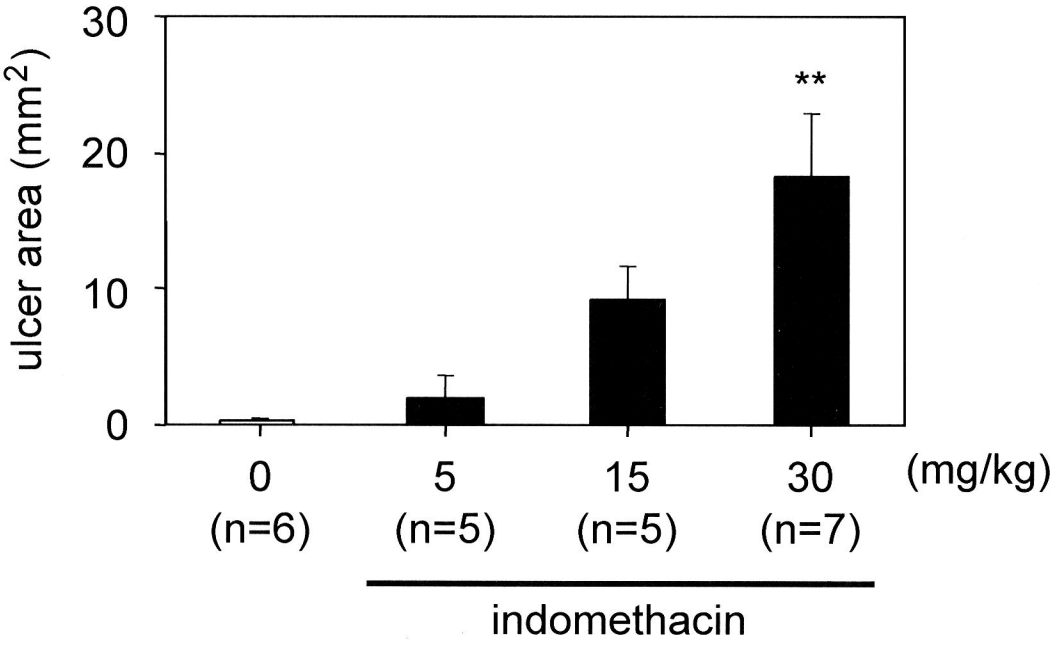

(C)

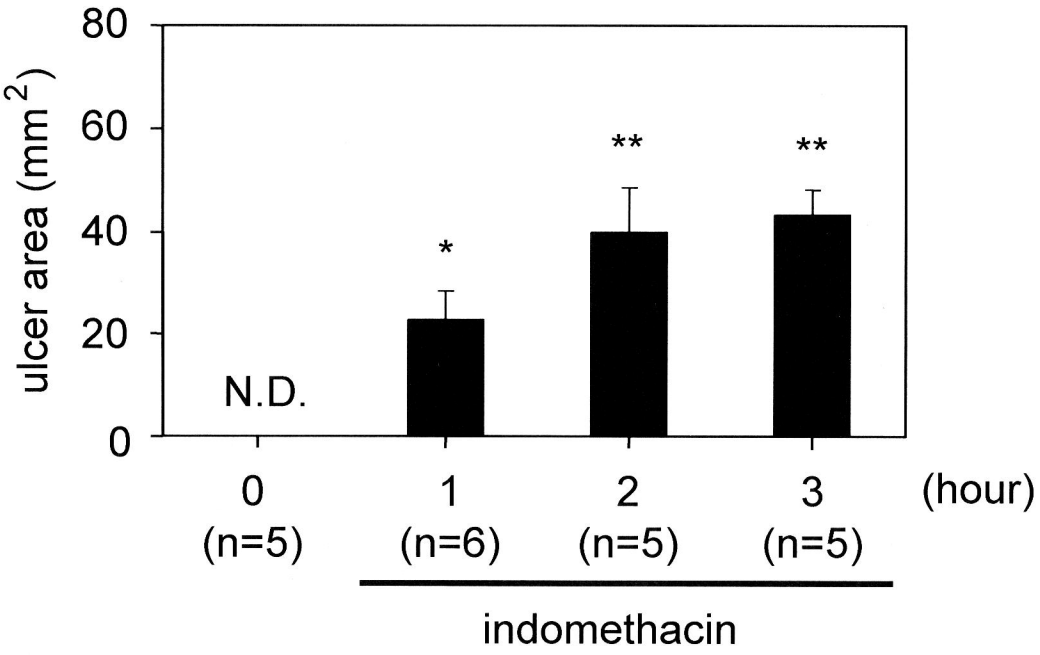

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Fig. 2

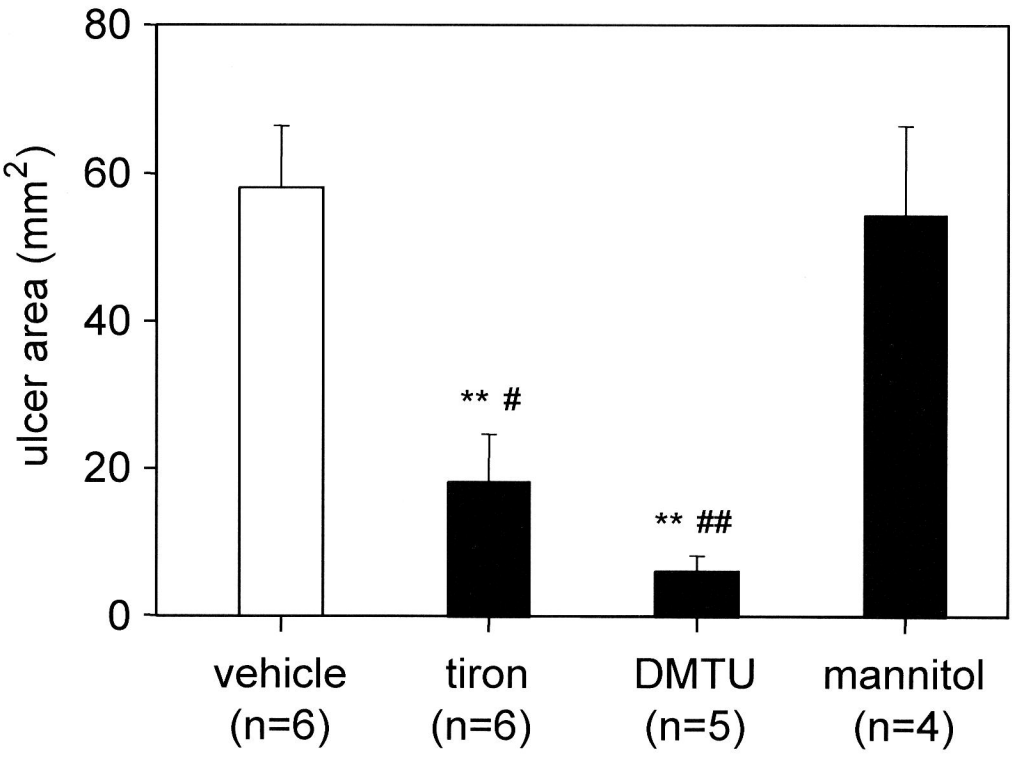

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Fig. 3

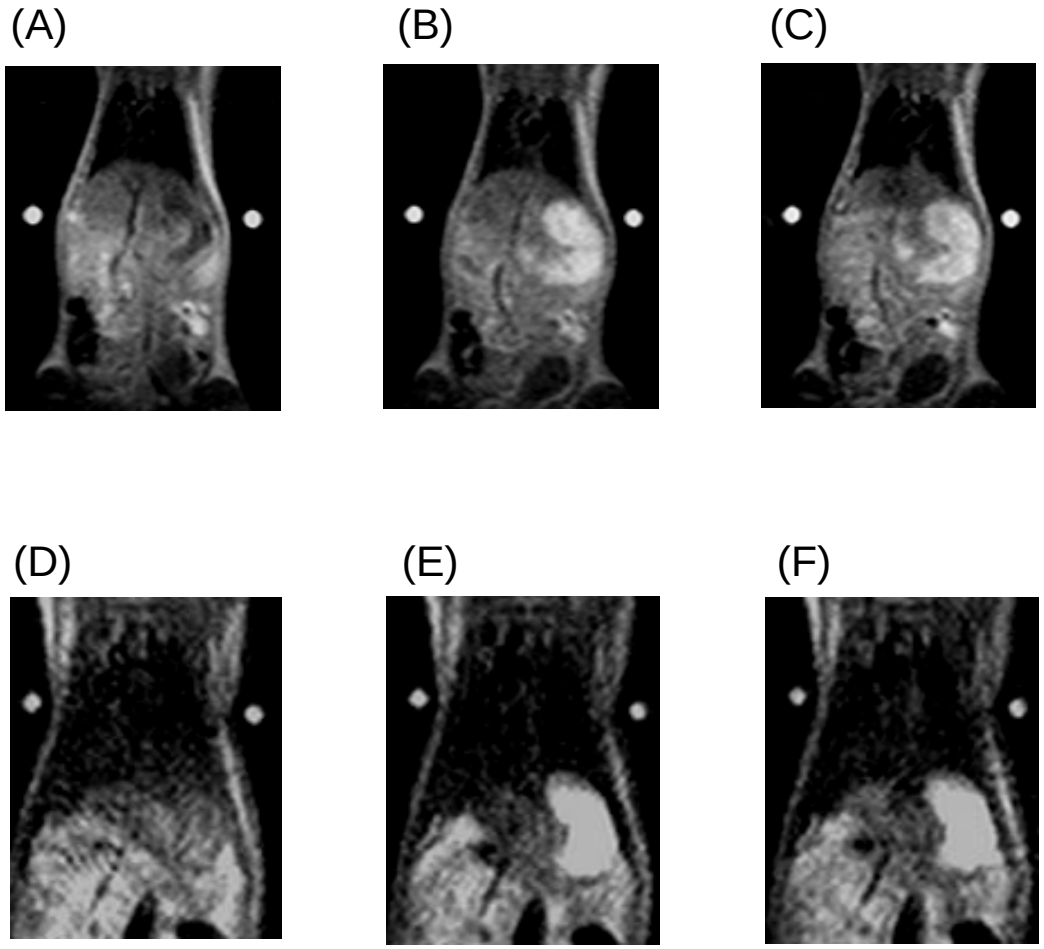

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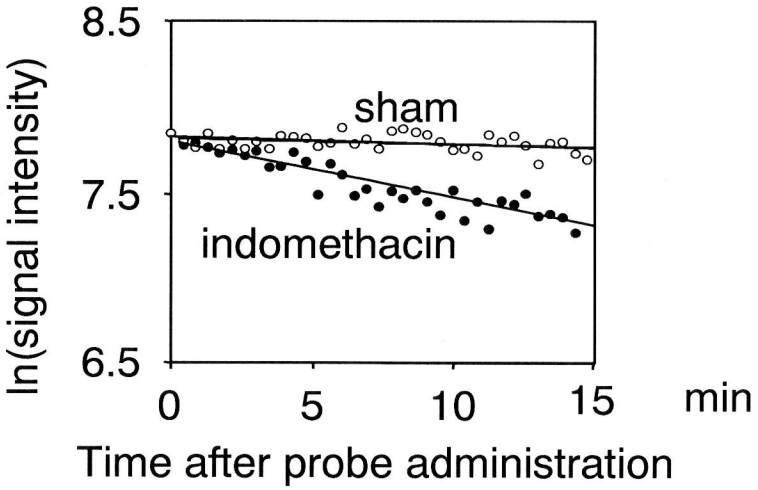
Fig. 4

(A)



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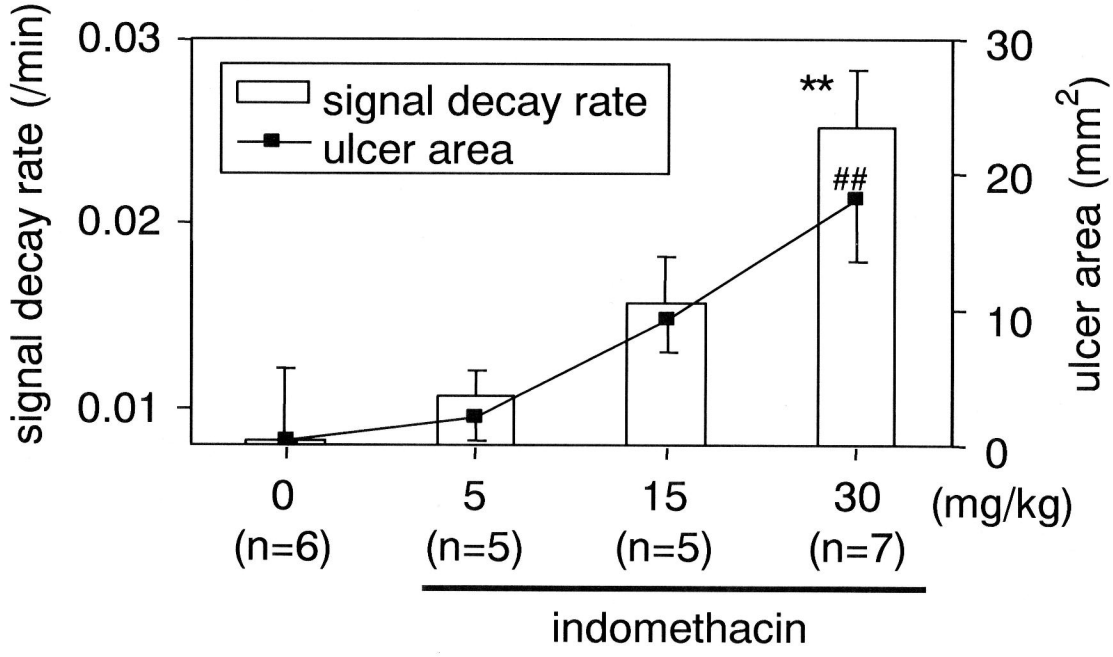

(C)

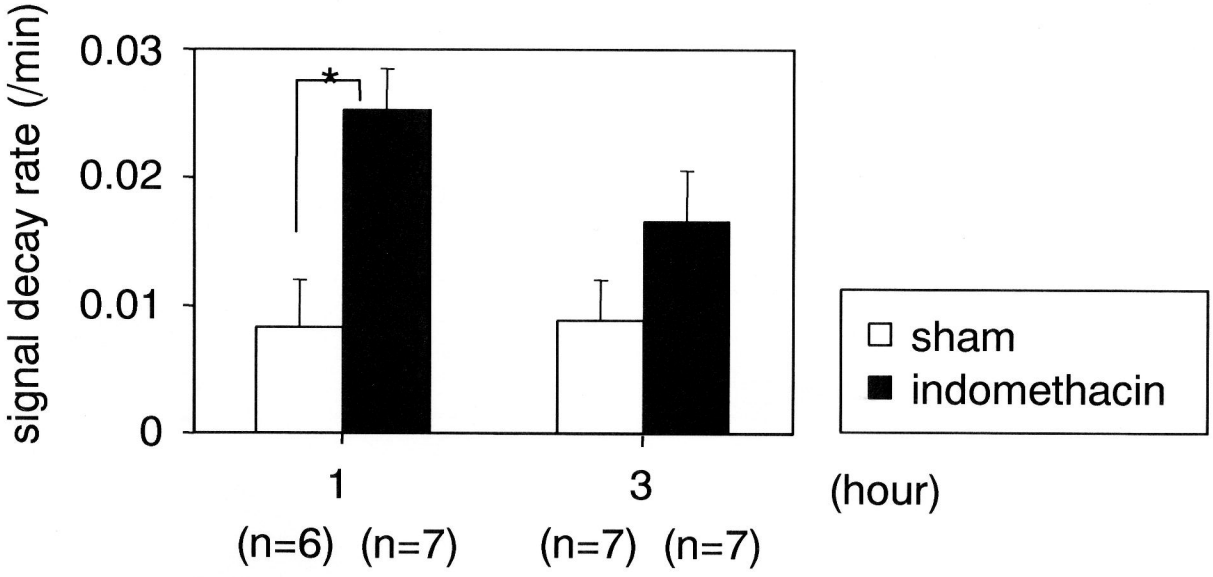

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Fig. 5

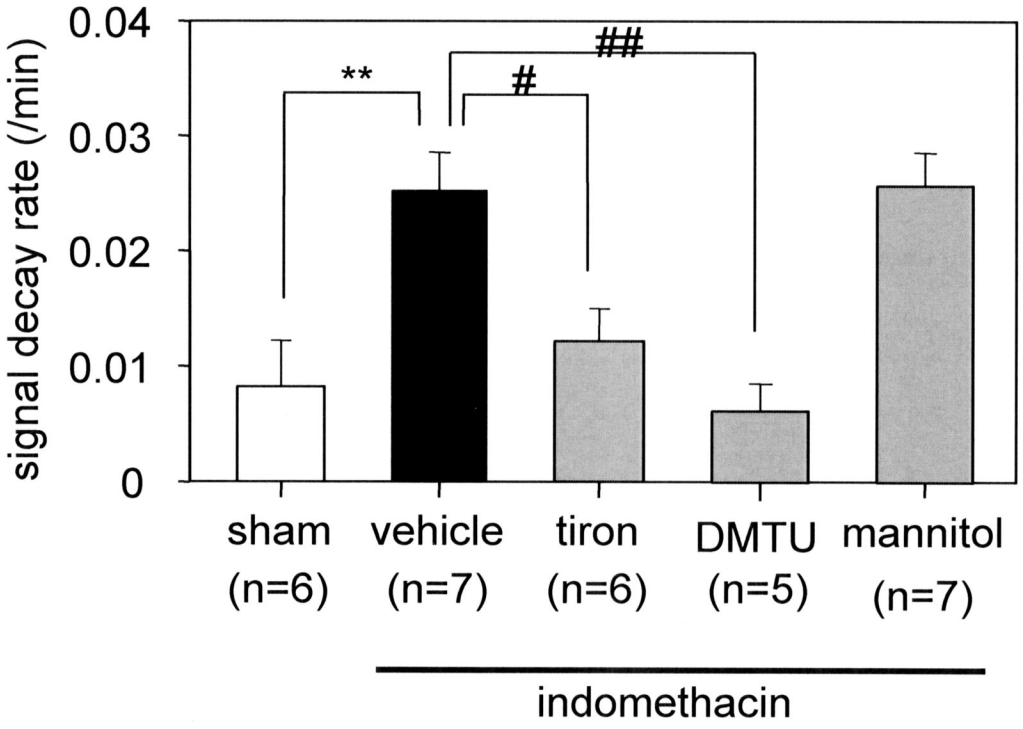

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Fig. 6

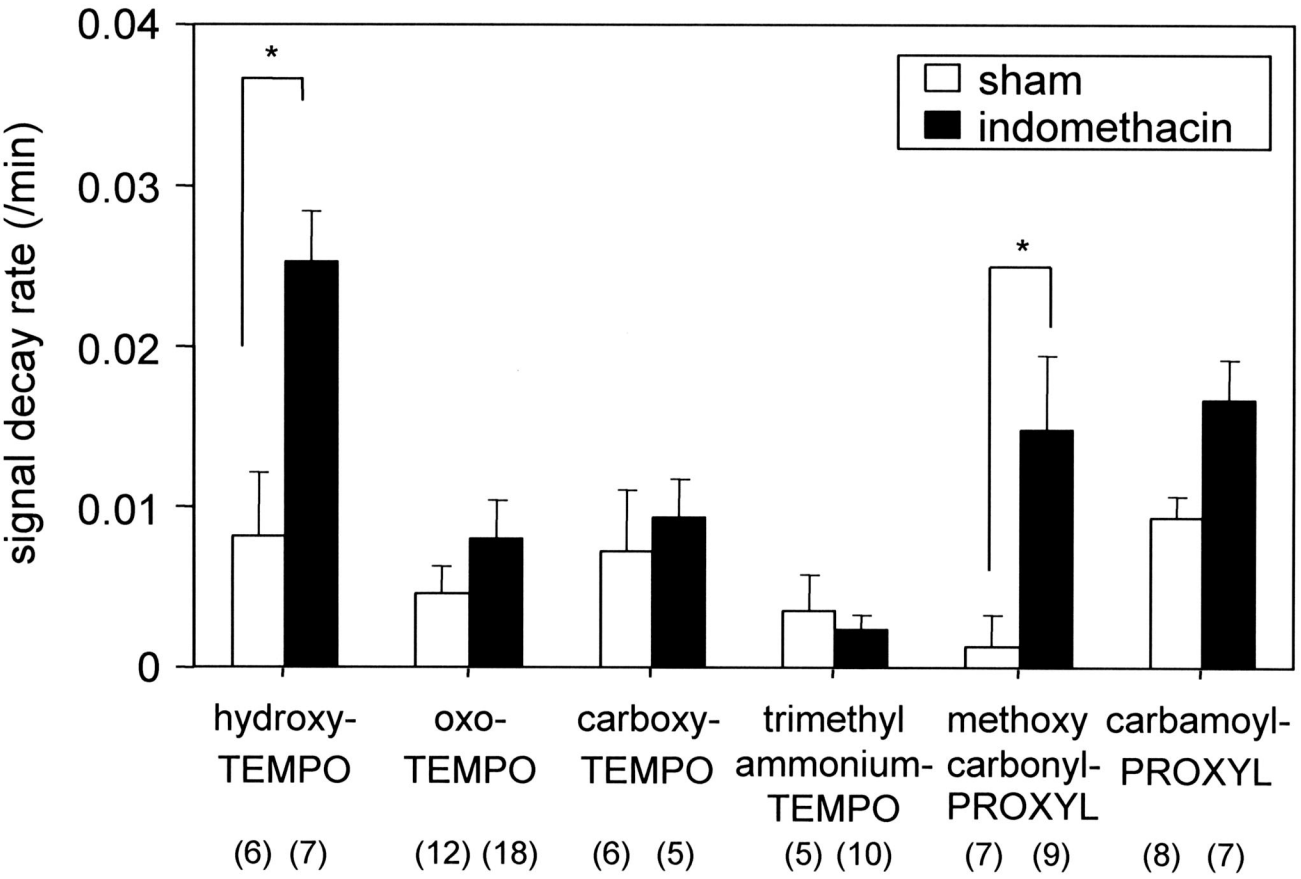

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Fig. 7

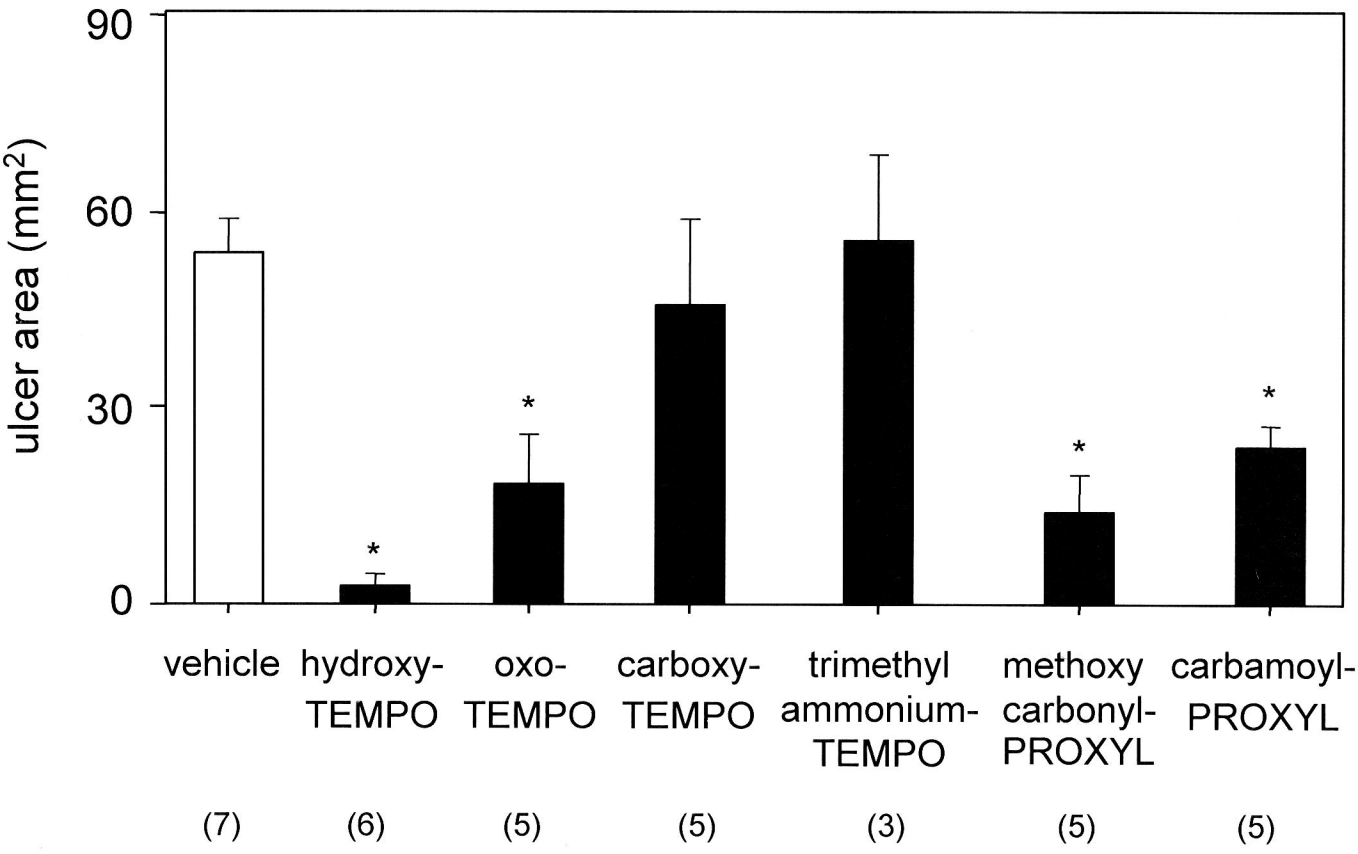

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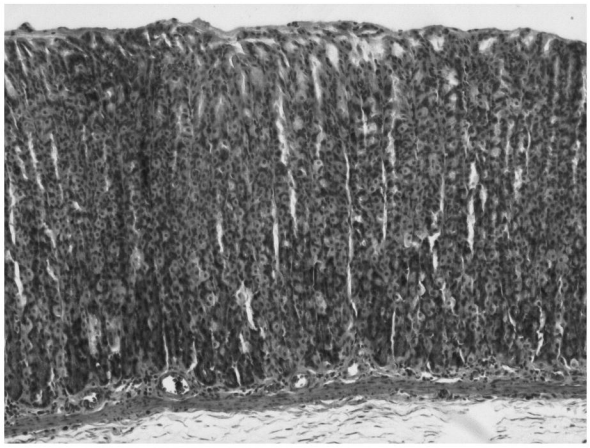
Fig. 8

(A)



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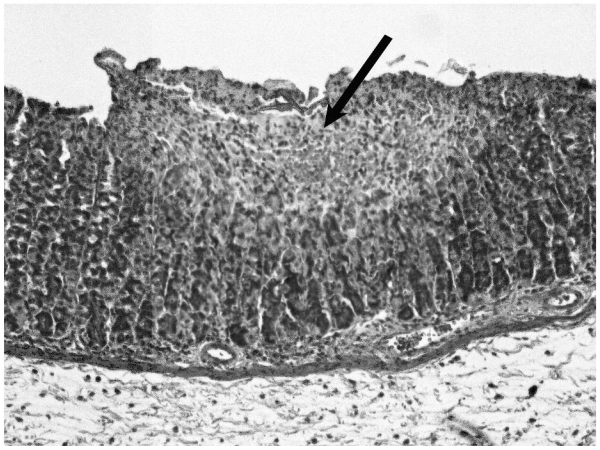

(C)