Free Radical Research, Volume 38 Number 2 (February 2004), pp. 147–155

Taylor & Francis
healthsciences

Non-invasive Analysis of Reactive Oxygen Species Generated in Rats with Water Immersion Restraint-induced Gastric Lesions Using *In Vivo* Electron Spin Resonance Spectroscopy

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Accepted by Professor E. Niki

(Received 8 August 2003; In revised form 9 October 2003)

Reactive oxygen species (ROS) are reportedly associated with gastric ulcer. We previously reported the use of an *in vivo* 300-MHz electron spin resonance (ESR) spectroscopy/nitroxyl probe technique to detect $^{\cdot}OH$ generation in the stomachs of rats with gastric ulcers induced by NH_4OH. However, this is an acute ulcer model, and the relationship between *in vivo* ROS generation and lesion formation remains to be clarified. To address this question, the same technique was applied to a sub-acute water immersion restraint (WIR) model. A nitroxyl probe that was less membrane-permeable was orally administered to WIR-treated rats, and the spectra in the gastric region were obtained by *in vivo* ESR spectroscopy. The signal intensity of the orally administered probe was clearly changed in the WIR group, but no change occurred in the control group. Both enhanced signal decay and neutrophil infiltration into mucosa were observed 2 h after WIR with little formation of any mucosal lesions. The enhanced signal decay was caused by $^{\cdot}OH$ generation, based on the finding that the decay was suppressed by mannitol, desferrioxamine and catalase. Intravenous treatment with either anti-neutrophil antibody or allopurinol also suppressed the enhanced signal decay, and allopurinol depressed neutrophil infiltration into the mucosa. In rats treated with WIR for 6 h, lesion formation was suppressed by 50% with all antioxidants used in this experiment except anti-neutrophil antibody. These findings suggest that $^{\cdot}OH$, which is generated in the stomach via the hypoxanthine/xanthine oxidase system upon neutrophil infiltrated into the mucosa, induces mucosal lesion formation, but that it accounts for only half the cause of lesion formation.

Keywords: Hydroxyl radical; Free radicals; Oxidative injury; ESR; Gastric ulcer

Abbreviations: ANS, anti neutrophil serum; DFO, desferrioxamine; ESR, electron spin resonance; MRI, magnetic resonance imaging; NS, normal serum; NSAIDs, non-steroidal anti inflammatory drugs; PROXYL, 2,2,5,5-tetramethylpyrrolidine-1-oxyl; ROS, reactive oxygen species; WIR, water immersion restraint

INTRODUCTION

Reactive oxygen species (ROS) are reportedly associated with pathogenesis in gastric ulcer induced by stress,[1,2] *Helicobacter pylori* (*H. pylori*),[3] non-steroidal anti inflammatory drugs (NSAIDs),[4] ethanol[5] and ischemia–reperfusion injury.[6] The involvement of ROS in gastric ulcer is clinically demonstrated by the increase in luminol- or lucigenin-dependent chemiluminescence[3] and the remarked infiltration of polymorphonuclear cells[3,7] in the gastric mucosa of *H. pylori*-positive patients. Intraperitoneal injection of allopurinol, a selective xanthine oxidase inhibitor, into rats with indomethacin-induced gastric lesions significantly suppressed lesion formation and lipid peroxidation in the mucosa.[4] Oral administration of allopurinol also reduced the incremental increase in the ulcer index of rats with NH_4OH-induced or water immersion restraint (WIR)-induced gastric lesions.[8,9] All of these reports demonstrated an ROS-associated pathology in gastric ulcer formation, but the way in which ROS are related to the initiation and

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/1071576036001641196

progression of gastric mucosal lesions induced by different causes is still unknown. It is important to clarify when, where, what type and how ROS are produced *in vivo* and to determine how ROS cause and contribute to the initiation and progression of mucosal injury.

The *in vivo* electron spin resonance (ESR)/nitroxyl probe technique is suitable for the examination of free radical reactions *in vivo* under varied physiological conditions and in experimental diseases, as demonstrated by us[10–21] and others.[22–24] We recently reported, for the first time, the generation of $^{\cdot}OH$ in the stomachs of rats with NH_4OH-induced gastric lesions.[16,17] The pathologic events involving the lesion area, the vascular permeability, neutrophil infiltration into the mucosa, and the $^{\cdot}OH$ generation all had the same time-course, and all of these events reached maximal levels at 30 min and then decreased. An acute model, as used in the earlier study, makes it difficult to clarify whether ROS generation is the cause of initiation and/or progression of lesion formation or is the result of lesion formation. To evaluate the relationship between ROS generation and gastric lesion formation, an investigation using a model in which gastric lesion occurs over a longer time is needed. The WIR gastric ulcer model requires approximately 6 h, and lesions form with good reproducibility.[9,25,26] An investigation using the sub-acute WIR model and the *in vivo* ESR/nitroxyl probe technique should provide more precise information on the relationship between ROS generation and gastric lesion formation.

In this study, type, location and origin of ROS generation were directly analyzed using *in vivo* 300-MHz ESR spectroscopy and a sub-acute WIR model in rats. The relationships among the pathologic events, ROS generation, neutrophil infiltration and gastric mucosal lesion formation are discussed.

MATERIALS AND METHODS

Chemicals

3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) and urethane were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Catalase and 3,3′,5,5′-tetramethylbenzidine were obtained from Sigma Chemical Co. (St. Louis, MO). Mannitol and allopurinol were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Desferrioxamine (DFO) was from Novartis Pharma (Tokyo, Japan). Rabbit normal serum (NS) and rabbit anti-neutrophil serum (ANS, >80% agglutinability against rat neutrophils at 1:20 dilution) were from Inter-cell Technologies, Inc. (Hopewell, NJ). All other chemicals were the commercially available reagent grade. Allopurinol solution was prepared by dissolving it in distilled water adjusted to pH 11 with 0.1N NaOH.

Animal Treatment

Male Sprague–Dawley rats (5-weeks-old; 120–150 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 lesions were induced by restraining each rat in a plastic case (height, 10 cm; diameter, 6 cm) and immersing it in a water bath (25 ± 2°C) up to the xiphoid process.

To measure the area of the mucosal lesions, the stomach was removed, inflated with 10 ml of 1% formaldehyde, and opened along the greater curvature. The lesion area (mm^2 per glandular stomach) was determined under a dissecting microscope with a square grid micrometer.

In order to estimate the effect of antioxidants, either mannitol (0.1 mmol/rat at a time) or carbamoyl-PROXYL (0.2 mmol/rat at a time) was orally administered just before and during WIR at intervals of 2 h, and either DFO (0.09 mmol/kg at a time), catalase (80,000 U/kg at a time) or carbamoyl-PROXYL (0.4 mmol/kg at a time) was intravenously injected just before and 3 h after WIR.

Neutrophil-depleted rats were prepared by intraperitoneal administration of a 4-fold dilution of ANS (500 μl) or NS 24 h before WIR treatment, and neutrophils were counted using Turk's stain and Wright-Giemsa stain. The number of neutrophils in ANS-treated rats was 21% of the number in NS-treated rats.

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 given carbamoyl-PROXYL orally (1.5 ml of 3 mM) or intravenously (1 ml/kg of 200 mM). Within 30 s after the administration, ESR observation was started 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 0.1 mT. The external magnetic field was swept at

a scan rate of 1.25 mT/min. The signal decay rate was calculated as described previously.[16,17]

In order to determine the causes of the enhanced signal decay, either mannitol (0.05 or 0.5 mmol/rat) or catalase (100 or 1000 U/rat) was orally administered with the administration of the spin probe, or DFO (0.015 or 0.045 mmol/rat) was intravenously injected just before the spin probe administration. In each experiment, control rats were treated with distilled water as a vehicle. The involvement of the hypoxanthine/xanthine oxidase system in the enhanced signal decay was evaluated with intravenous injections of allopurinol (in NaOH, pH 11; 0.3 mmol/kg at a time) immediately before and 3 h after WIR treatment (total 0.6 mmol/kg). Control rats were treated with distilled water adjusted to pH 11.

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 a carbamoyl-PROXYL solution (3 mM, 380 μl) 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 carbamoyl-PROXYL (1.5 ml of 3 mM). The pulse sequence of conventional spin echo proton density-weighted with TR (ms)/TE (ms) = 1600/40 was acquired. All images were acquired using a $200 \times 200\,mm^2$ field of view (FOV), two signal average, coronal plane and 2.5 mm thickness.

Assay of MPO Activity

Gastric mucosal myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation. The stomach was isolated from a rat, and the gastric mucosa was scraped off using two glass slides. The mucosa was homogenized with 0.1 M phosphate buffer, pH 5.4, (1:20, w/v) in a Teflon Potter Elvehjem homogenizer, sonicated (10 s × 3), subjected to three freeze–thaw cycles, and centrifuged (4000 rpm, 10 min) at 4°C. The supernatant (0.2 ml) was incubated with 0.1 ml of 0.03% H_2O_2 in the presence of 2.5 mM 3,3′,5,5′-tetramethylbenzidine (0.1 ml) for 15 min. After incubation, 1.5 ml of 0.2 M sodium acetate buffer (pH 3.0) was added, and MPO activity was assayed at 655 nm. The protein content of the sample was measured according to the method of Lowry *et al.*[27]

Mannitol (0.2 mmol/kg at a time), DFO (0.09 mmol/kg at a time), carbamoyl-PROXYL (0.4 mmol/kg at a time) or allopurinol (0.3 mmol/kg at a time) was intravenously administered just before and 3 h after WIR treatment.

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 test or the Tukey–Kramer test as a *post hoc* test, or by two-way ANOVA with the Tukey–Kramer test. All the results are expressed as the mean ± S.D.

RESULTS

The distinct formation of gastric mucosal lesions occurred 6 h after WIR and continued for 24 h; no significant mucosal lesions were observed within the first 2 h after WIR (Fig. 1). A lag-time in lesion formation in the WIR mucosal model has been reported elsewhere.[9,25]

Free radical reactions in the stomach and blood vessels were evaluated in a previous study[16] using an *in vivo* ESR/nitroxyl probe technique with oral and intravenous administration of carbamoyl-PROXYL, respectively. In this study, the signal intensity of the orally administered probe decreased very slowly, while that of the intravenously administered probe disappeared within 15 min, in agreement with the previous study.[16] The pH values of the gastric juices of rats before and after oral administration of a carbamoyl-PROXYL solution (3 mM, 1.5 ml) were 1.6 and 2.1, respectively.

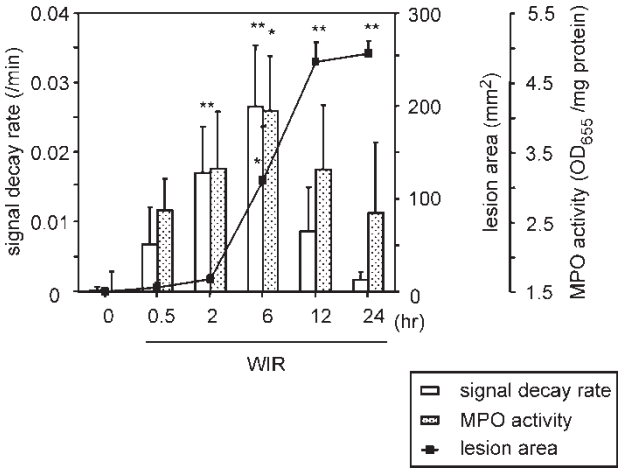

FIGURE 1 Time-course of gastric mucosal lesion formation, signal decay rate and MPO activity in WIR-treated rats. The WIR model was produced by restraint in a plastic case and immersion in a water bath up to the rat's xiphoid process for 0.5, 2, 6, 12 or 24 h. Under urethane anesthesia, the ESR spectra of orally administered carbamoyl-PROXYL were measured in the gastric region using 300-MHz ESR spectroscopy. Immediately after *in vivo* ESR measurement, the stomach was removed and the lesion area was evaluated under a dissecting microscope with a square grid micrometer. MPO activity in gastric mucosa was evaluated by the decomposition of hydrogen peroxide in the presence of 3,3′,5,5′-tetramethylbenzidine. Each value represents mean ± S.D. of 3–6 rats. $^*p < 0.05$ and $^{**}p < 0.01$ as determined by the Dunnett test when compared with the non-stressed group in each experiment.

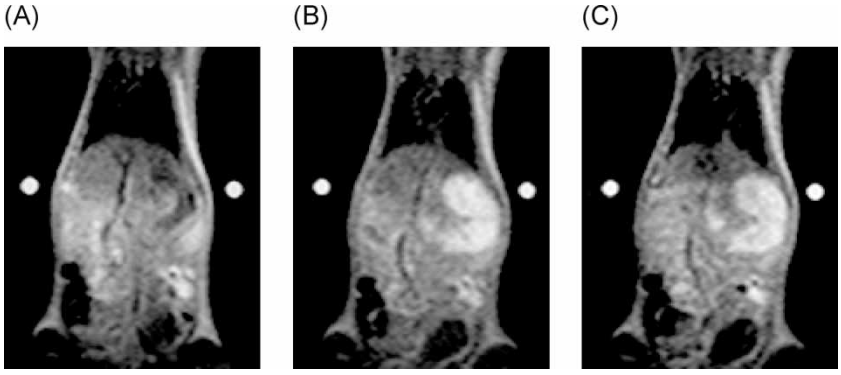

FIGURE 2 Coronal MR images of a rat before (A), 4 min (B) and 14 min (C) after oral administration of carbamoyl-PROXYL. A rat anesthetized by urethane (2 g/kg) was turned up on the plate, in which two syringes (3 mm diameter) containing carbamoyl-PROXYL (3 mM, 380 μl) were fixed on both sides of the rat. The MR imaging was performed using a 0.2-T MRI system before the oral administration of carbamoyl-PROXYL (1.5 ml of 3 mM). Within 20 s after the administration, the MR imaging was started, and two images were sequentially obtained.

To confirm the retention of the administered probe in stomach, the MR images at upper abdomen of rat were obtained at various time after oral administration of the probe using a clinical 0.2-T MRI system. Before the oral administration of the probe, only faint MR image was observed at the gastric domain (Fig. 2A). The oral administration of nitroxyl probe gave a clear MR image at the gastric domain, and the intensity of the MR image did hardly changed for 15 min (Fig. 2B,C), suggesting that most of the spin probe retain in stomach during *in vivo* ESR measurement.

The signal decay of the orally administered probe was distinctly increased in rats treated with WIR (Fig. 3), but no difference was observed in the signal decay of the intravenously injected probe in the non-stressed and WIR-treated groups (data not shown). The decay rate was calculated from the decline of the signal decay curve, as described previously.[16,17]

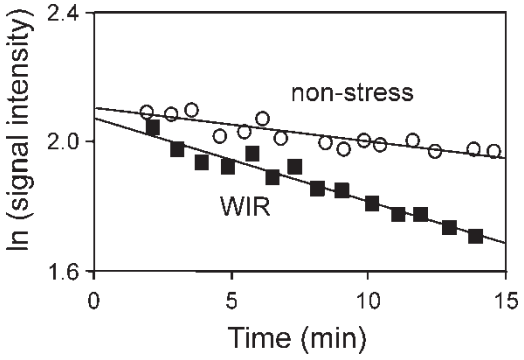

FIGURE 3 Typical signal decay curve for carbamoyl-PROXYL in the gastric region after oral administration (3 mM, 1.5 ml). Thirty minutes after anesthesia, rats were orally given carbamoyl-PROXYL. Within 30 s after administration, in non-stressed group (○) or 6-h WIR group (■), the carbamoyl-PROXYL ESR spectra were measured in the gastric region with 300-MHz ESR spectroscopy. The logarithm of peak height $h(0)$ was plotted against time after administration.

The signal decay rate was enhanced in the WIR-treated group, and the enhancement depended on the time of WIR (Fig. 1). The increment of signal decay was significant in the 2-h WIR group, reached a maximum at 6 h, and then decreased to the control level gradually. Few lesions formed within 2 h of WIR, although the enhancement of signal decay was significant (Fig. 1). The lesion area was maximal in the 24-h WIR group, yet the signal decay was barely enhanced. These facts indicate that the enhancement of signal decay occurred prior to lesion formation and that the lesion did not induce the enhanced signal decay.

In a previous paper, we demonstrated that neutrophils participate in the enhanced signal decay in NH_4OH-induced lesion formation.[16] Thus, neutrophil infiltration into the mucosa was determined in this study by the MPO activity in the gastric mucosa of WIR-treated rats. The time-course of MPO activity was similar to that of the enhanced signal decay (Fig. 1), indicating that neutrophil infiltration into the mucosa contributes to the enhancement of signal decay in the WIR-treated rat stomach.

To identify the cause of the enhanced signal decay of the carbamoyl-PROXYL probe in WIR-treated rats, selected antioxidants were administered to non-stressed and WIR-treated rats, and the decay rates in the stomach were determined. The simultaneous administration of mannitol, a water-soluble $^{\cdot}OH$ scavenger, with the spin probe completely suppressed the enhanced signal decay, suggesting that $^{\cdot}OH$ generated in the stomach contributed to the enhancement of the signal decay in WIR-treated rats (Fig. 4A). Intravenous injection of DFO 5 min before the ESR measurement also completely suppressed the enhanced signal decay in a dose-dependent manner (Fig. 4B). Administration of catalase with the probe tended to suppress the enhancement of signal decay in a dose-dependent

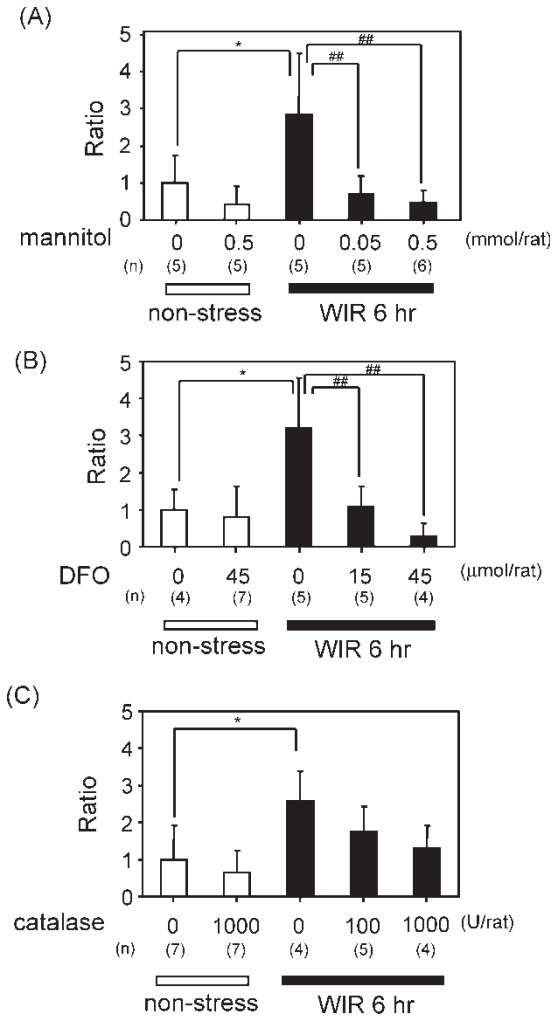

FIGURE 4 Effect of antioxidants, mannitol (A), DFO (B) and catalase (C), on the enhanced signal decay in rats treated with 6-h WIR One milliliter of mannitol (0.05 or 0.5 mmol/rat) or catalase (100 or 1000 U/rat) was mixed with 0.5 ml of 9 mM carbamoyl-PROXYL, and then the mixed solution was orally administered. Two hundred microliters of DFO (0.015 or 0.045 mmol/rat) was intravenously injected 5 min before administration of the spin probe. Immediately after administration of the spin probe, *in vivo* ESR measurement was performed. Each value represents mean ± S.D. of 4–7 rats. $^{*}p < 0.05$ as determined by Student's *t* test when compared with non-stressed group, and $^{\#\#}p < 0.01$ as determined by the Dunnett test when compared with the WIR group treated with vehicle.

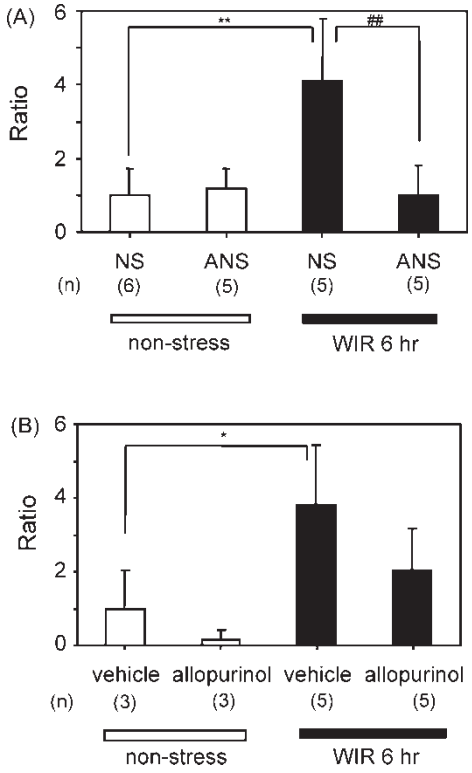

FIGURE 5 Effect of ANS (A) and allopurinol (B) on the enhanced signal decay. ANS or NS was intraperitoneally administered to rats 24 h before WIR treatment. Allopurinol (0.3 mmol/kg at a time) or vehicle was intravenously injected 5 min before and 3 h after WIR treatment (total 0.6 mmol/kg). The *in vivo* ESR measurement was performed in the gastric region after oral administration of carbamoyl-PROXYL (3 mM, 1.5 ml). Each value represents the mean ± S.D. of 3–6 rats. $^{*}p < 0.05$ and $^{**}p < 0.01$ as determined by two-way ANOVA with the Tukey–Kramer test when compared with the non-stressed group pretreated with NS or treated with vehicle. $^{\#\#}p < 0.01$ as determined by two-way ANOVA with the Tukey–Kramer test when compared with the WIR group pretreated with ANS.

manner, but not significantly (Fig. 4C). These results suggest that the enhancement of the *in vivo* signal decay of carbamoyl-PROXYL arises from $^{\cdot}OH$ generation, probably via a Fenton-like reaction in the rat stomach. It should, however, be noted that the spin-probe technique used in this paper is not sufficient to determine *in vivo* $^{\cdot}OH$ generation. It might be preferred for the determination of ROS generation to combine with the other techniques such as salicylic acid and spin-trapping methods, although these methods may not yet give distinct evidence for *in vivo* $^{\cdot}OH$ generation. Indeed, we recently reported that there is another mechanism for the enhancement of *in vivo* signal decay in transient MCAO model in which the enhanced signal decay was suppressed by SOD but not by $^{\cdot}OH$ scavengers.[21] Thus, we performed *in vitro* experiment in the previous report that the signal of carbamoyl-PROXYL was decayed with almost the same rate in Fe(II)/H_2O_2 system and that the signal decay was suppressed by trapping $^{\cdot}OH$ with DMPO.[16] The signal decay of carbamoyl-PROXYL in Fe(II)/H_2O_2 system was also confirmed using gastric juice (data not shown). These facts indicate that the enhanced signal decay of carbamoyl-PROXYL and its suppression by $^{\cdot}OH$ scavenger were strongly related with *in vivo* $^{\cdot}OH$ generation.

In Fig. 1, the $^{\cdot}OH$ generation shows the same time-course as neutrophil infiltration into the mucosa of WIR-treated rats. Neutrophil depletion, which was accomplished with anti-neutrophil antibody, completely suppressed the enhanced signal decay of the probe in the stomachs of WIR-treated rats (Fig. 5A), confirming that the origin of $^{\cdot}OH$ in the stomach was neutrophils, as in NH_4OH-induced gastric lesions. The hypoxanthine/xanthine oxidase

system is reportedly involved in mucosal lesion formation in WIR-treated rats.[9] To examine the involvement of the hypoxanthine/xanthine oxidase system in ROS generation, allopurinol (0.3 mmol/kg) was intravenously injected just before and 3 h after WIR. Allopurinol suppressed the enhanced signal decay, but not significantly (Fig. 5B). All of the results with *in vivo* ESR spectroscopy suggest that the hypoxanthine/xanthine oxidase system contributes to ·OH generation through neutrophil infiltration, and that infiltrating neutrophils probably produces ·OH via Fenton-like reaction in the WIR-treated rat stomach. This conclusion is also supported by the observation that neutrophil infiltration was significantly suppressed by the intravenous injection of allopurinol (0.3 mmol/kg) just before and 3 h after WIR (Fig. 6A). Neither mannitol nor DFO suppressed neutrophil infiltration, although these compounds did completely suppress ·OH generation in the stomachs of WIR-treated rats (Fig. 6B).

To confirm the relationship between lesion formation in WIR-treated rats and ·OH generation in the stomach, carbamoyl-PROXYL and the antioxidants that were used for the *in vivo* ESR measurements were orally or intravenously administered to rats, and the mucosal lesion area was measured 6 h after WIR treatment. Oral administration of mannitol immediately before and at 2 h-intervals during the WIR treatment resulted in a lesion area that was 51% of the area in the vehicle-only group (Fig. 7A). Repetitive administration of

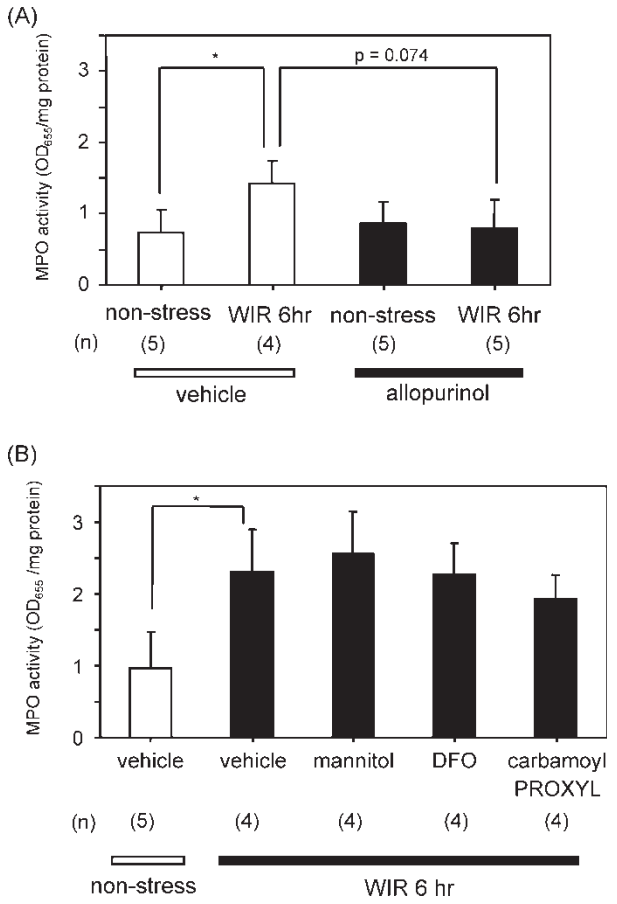

FIGURE 6 Effect of allopurinol (A) and antioxidants (B) on MPO activity. Allopurinol (0.3 mmol/kg at a time), mannitol (0.2 mmol/kg at a time), DFO (0.09 mmol/kg at a time) and carbamoyl-PROXYL (0.4 mmol/kg at a time) were intravenously injected just before and 3 h after WIR treatment. Gastric mucosa was collected 6 h after WIR and the sample was prepared as described in the "Material and methods" section. And then, the MPO activity was assayed at OD 655 nm in the presence of 3,3′,5,5′-tetramethylbendizine. Each value represents the mean ± S.D. of 4–5 rats. *$p < 0.05$ as determined by one-way ANOVA with the Tukey–Kramer test (A) and with the Dunnett test (B), respectively, when compared with the non-stressed group treated with vehicle.

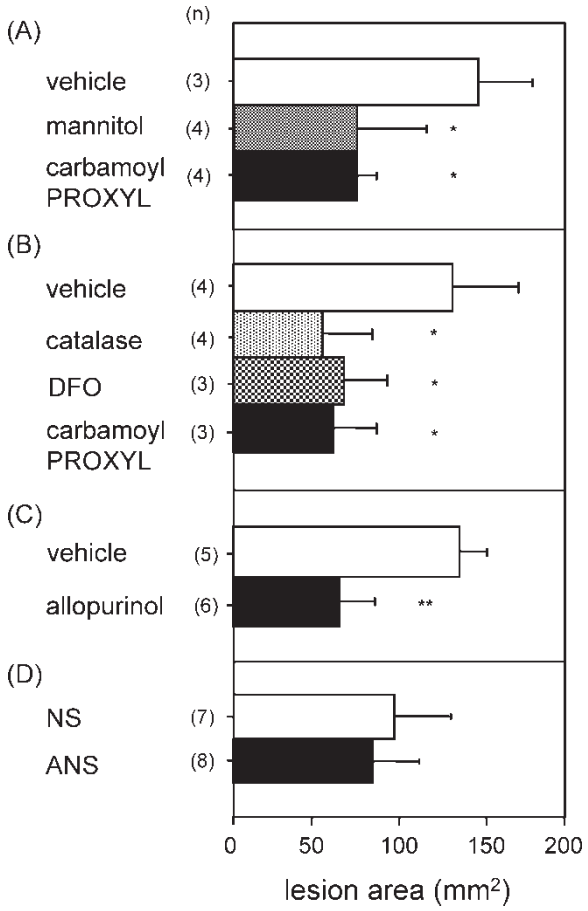

FIGURE 7 Effects of oral administration of mannitol and carbamoyl-PROXYL (A), intravenous injection of catalase (B), and of allopurinol (C), and intraperitoneal injection of ANS (D) on gastric lesion formation in rats treated with 6-h WIR. Mannitol (0.1 mmol/rat at a time) or carbamoyl-PROXYL (0.2 mmol/rat at a time) was orally administered immediately before WIR treatment and during 6 h of WIR treatment at intervals of 2 h (total 0.3 and 0.6 mmol/rat, respectively). DFO (0.09 mmol/kg), allopurinol (0.3 mmol/kg) and vehicle were intravenously injected 5 min before and 3 h after WIR treatment. ANS or NS was intraperitoneally administered to rats 24 h before WIR treatment. The area of gastric mucosal lesions was measured 6 h after WIR treatment. Each value represents the mean ± S.D. of 3–8 rats. *$p < 0.05$ as determined by one-way ANOVA with the Dunnett test or Student's t test when compared with the WIR group treated with vehicle.

carbamoyl-PROXYL also reduced the lesion area to 50% of that in the vehicle-only group (Fig. 7A). It is noteworthy that oral administration of mannitol or carbamoyl-PROXYL 4 h after WIR treatment showed little suppression of lesion formation (data not shown), suggesting the presence of a therapeutic window. Intravenous administration of DFO, catalase, or carbamoyl-PROXYL immediately before and 3 h after WIR treatment significantly suppressed the lesion area to half of that in the vehicle-only group (Fig. 7B). Intravenous injection of allopurinol (0.3 mmol/kg) before and 3 h after WIR suppressed the gastric lesion area to 63% of that in the vehicle-only group (Fig. 7C); however, anti-neutrophil antibody treatment showed no inhibitory effect on mucosal lesion formation. The results suggest that $^{\cdot}OH$ generation does indeed induce lesion formation in WIR-treated rats, but that $^{\cdot}OH$ generation accounts for only about half of the lesion formation. As the experiments using antioxidants demonstrated suppression of the lesion area by only about half, a cause other than $^{\cdot}OH$ generation may also contribute partially to lesion formation in WIR-treated rats.

DISCUSSION

Using *in vivo* 300-MHz ESR spectroscopy, we recently reported $^{\cdot}OH$ generation in the stomachs of rats with NH_4OH-induced gastric lesions and its relation to gastric lesion formation.[16,17] Mucosal lesion formation induced by NH_4OH is acute, occurring within 30 min, and is simultaneous with the enhancement of vascular permeability, neutrophil infiltration into the mucosa, and $^{\cdot}OH$ generation in the stomach. This simultaneous pathology makes it difficult to clarify whether ROS generation is a cause for and/or a result of lesion formation. A sub-acute gastric lesion model, which requires more time to produce lesions, should help to clarify the relationship between ROS generation and gastric lesion formation.

In the present paper, the WIR model, which requires at least 6 h to form mucosal lesions,[9,25] was used to investigate the relationship between ROS generation and lesion formation. This study provides the first direct evidence that $^{\cdot}OH$ is generated from neutrophils in the stomach of WIR-treated rats prior to the progression of lesion formation, and that the hypoxanthine/xanthine oxidase system induces neutrophil infiltration into the gastric mucosa.

To determine the ROS generation in the stomach, we proposed using an *in vivo* ESR/nitroxyl probe technique, and we have successfully used this technique in several different experimental disease models, such as those of hyperoxia,[10] ischemia–reperfusion,[10] streptozotocin-induced diabetes,[11,12] iron-overload,[13] lung injury by diesel exhaust particles,[14] liver damage induced by CCl_4,[15] and NH_4OH-induced gastric lesion.[16,17] Other groups have also observed the loss of paramagnetism in animal models of cancers,[22] X-ray irradiation[23] and ischemia–reperfusion-induced heart injury.[24] In these experiments, the enhanced signal decay of the probe, which is suppressed by antioxidants such as catalase, SOD, and mannitol,[11–17,22] has been used as an index of ROS generation. In the present study, *in vivo* $^{\cdot}OH$ formation in the stomach of WIR-treated rats was confirmed by the enhanced ESR signal decay of carbamoyl-PROXYL orally administered to WIR-treated rats. The enhanced signal decay was suppressed by oral administration of mannitol or catalase or by intravenous injection of DFO, suggesting that the enhanced signal decay is due to $^{\cdot}OH$ generation in the stomach, probably via Fenton-like reaction. Furthermore, pretreatment with anti-neutrophil antibody also suppressed the enhanced signal decay to control levels, indicating that neutrophils contribute to $^{\cdot}OH$ generation in the stomach of WIR-treated rats. The increase in MPO activity was depressed by allopurinol, as was the enhanced signal decay. These results agree well with previous results for NH_4OH-induced gastric lesion[16,17] and the *in vitro* observation that the paramagnetism of the nitroxyl radical is diminished very quickly by direct reaction with $^{\cdot}OH$ ($3–4 \times 10^{11}\,M^{-1}\,min^{-1}$).[16,28] These findings suggest that ROS generation in the stomach of WIR-treated rats is caused by the same mechanism as in the NH_4OH model,[16] that is, $^{\cdot}OH$ generation in the stomach is derived from neutrophils infiltrating into the mucosa through the hypoxanthine/xanthine oxidase system.

However, the relationships among the enhancement of signal decay, neutrophil infiltration, and lesion formation in the WIR model were different from those in the NH_4OH model. The time-course of the enhancement of signal decay was similar to that of the MPO activity in the gastric mucosa, while the enhanced signal decay occurred prior to lesion formation in WIR-treated rats. Evidence that $^{\cdot}OH$ generation derived from neutrophils occurs in the stomach before lesion formation was obtained by using a sub-acute WIR model.

The combination of the other technique such as spin-trapping method with the spin-probe method might be preferred to confirm $^{\cdot}OH$ generation in stomach, because the spin-trapping method has higher specificity than the spin-probe method. However, the reaction of hypochlorous acid with DMPO produced the DMPO-OH adduct independently of the $^{\cdot}OH$ generation.[29] Hypochlorous acid produced by MPO in gastric mucosa of rats treated with WIR might disturb the spin-trapping method with DMPO. Administration of catalase with the probe tended to suppress the enhancement of

signal decay in a dose-dependent manner, but not significantly (Fig. 4C). The consumption of hydrogen peroxide by MPO might weaken the effect of calatase on the enhanced signal decay.

The possibility that the enhanced signal decay occurs independent of ROS generation must be considered. The signal decay of the probe would be enhanced if some of the orally administered carbamoyl-PROXYL were rapidly excreted from the stomach during the ESR measurement. To exclude this possibility, we routinely confirmed that most of the administered liquid remained in the stomach with post-experimental *in vivo* ESR measurements. The MR images again confirmed a very slow clearance of the administered probe from the stomach (Fig. 2). The signal decay would be affected by direct interaction of the nitroxyl probe with hemoglobin in the stomach, but the enhanced signal decay occurred prior to lesion formation. These observations indicate that the enhanced signal decay is not due to the elimination of the spin probe from the stomach or to interactions with hemoglobin.

The finding that each antioxidant and route of administration tested, except for the anti-neutrophil antibody, was able to suppress lesion formation by only 50% has two possible explanations. First, the antioxidants might not have been effective because of the time-lag between ROS generation and lesion formation. A second possibility is that other ulcerative factors may be associated with lesion formation independent of ROS generation. Studies of the pathogenesis of stress ulceration have long focused on the disruption of the gastric mucosal layer by back-diffusing hydrogen ions.[30,31] The ischemic insult of stressed gastric mucosa is reportedly followed by a reduction in gastric mucosal blood flow.[32] With an unrestricted influx of hydrogen ions, mucosal ischemia may lead to changes in mucosal permeability and depletion of mucosal adenosine triphosphate energy stores, which could directly disrupt the gastric mucosa.[30,31,33]

A significant incremental increase in MPO activity was observed 6 h after WIR treatment, and the depletion of neutrophils significantly suppressed the enhanced signal decay with little effect on lesion formation. Neutrophil depletion could cause abnormalities of the immune system, including alterations of cytokine production,[34] which could produce the effects in the stomach in the WIR model that lead to ineffective lesion formation.

It is interesting that no enhanced signal decay was observed with intravenous injection of the probe, but it is not clear why the reduction of the nitroxyl probe signal inside the vessel was not altered by the WIR treatment, even though lesion formation was significantly suppressed by the intravenous injection of antioxidants. One possibility is that the concentration of the carbamoyl-PROXYL probe in the gastric microcirculation is not sufficient for detection of the ESR signal. The region sampled with an *in vivo* ESR spectrometer includes not only the stomach, but also the liver. The volume of gastric capillaries, and hence the amount of carbamoyl-PROXYL, would be much smaller than the volume of hepatic vessels. In addition, metabolic enzymes are abundant in the liver. Visualization of the signal decay using an ESR imaging system might be more effective for selectively observing *in vivo* free radical reactions at the specific target sites.

All of the facts presented in this paper strongly indicate that WIR-induced mucosal lesions are formed as follows: (1) ROS generated from the hypoxanthine/xanthine oxidase system induces neutrophil infiltration into the mucosa; (2) the resultant neutrophils produce $^{\cdot}OH$ in the stomach; and (3) $^{\cdot}OH$ production in the stomach induces mucosal lesion formation, but its contribution is limited to only about half of the lesion formation.

Therefore, neutrophils infiltrating into the gastric mucosa take part in the enhanced signal decay through $^{\cdot}OH$ generation.

Acknowledgements

This work was supported by Grants-in-Aid of Research for Developmental Scientific Research, and for General Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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