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Mechanisms Underlying Gastric Antiulcerative Activity of Nitroxides in Rats

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Reactive oxygen-derived species and redox-active metals are implicated in mediation of the pathogenesis of gastric mucosal damage and ulceration. Therefore, common strategies of intervention employ metal chelators, antioxidative enzymes, and low-molecular-weight antioxidants (LMWA). The aim of the present study was to elaborate the mechanism(s) responsible for the protection provided by nitroxide radicals in the experimental model of gastric ulceration.

Fasted male rats were treated ig with 1 ml 96% ethanol, with or without ig pretreatment with nitroxide or hydroxylamine. In several experiments, rats were injected ip or iv with iron(III) or iron(II) prior to ethanol administration. Rats were sacrificed 10 min after ethanol administration, the stomach was removed, washed and lesion area measured. Pretreatment with iron(III) complexed to nitrilotriacetate or citrate, aggravated the extent of the gastric injury. Conversely, iron(II) inhibited the formation of lesions. The nitroxides were rapidly reduced to their respective hydroxylamines and demonstrated antiulcerative activity for rats treated with iron. However, injecting the hydroxylamine resulted in a similar tissue distribution of nitroxide/hydroxylamine but did not provide protection.

The results show that: (a) the nitroxide radicals, rather than their respective non-radical reduced form, are the active species responsible for protection; (b) nitroxides protect by dismutating $O_2^{\bullet-}$ and possibly indirectly increasing the NO level; (c) unlike classical LMWA which are reducing agents, nitroxides inhibit gastric damage by acting as mild oxidants, oxidizing reduced metals and pre-empting the Fenton reaction; and (d) the nitroxides act catalytically as recycling antioxidants.

Keywords: Free radicals, hydroxylamine, EPR, oxygen reactive species, inflammatory bowel diseases, antioxidants

Abbreviations: EPR, electron paramagnetic resonance; b.w., body weight; ig, intragastric; IBD, inflammatory bowel diseases; LMWA, low-molecular-weight antioxidants; NTA, nitrilotriacetate; ROS, reactive oxygen-derived species; SOD, superoxide dismutase; TPL, 4-OH-2,2,6,6-tetramethyl-piperidine-1-N-oxyl; TPO, 2,2,6,6-tetramethyl-piperidine-1-N-oxyl; TPL-H, 4-OH-2,2,6,6-tetramethyl-N-hydroxypiperidine

INTRODUCTION

Reactive oxygen-derived radicals and non-radical species (ROS) are involved in the pathogenesis of gastric mucosal injury. Catalytically active iron has been implicated in potentiation of gastric

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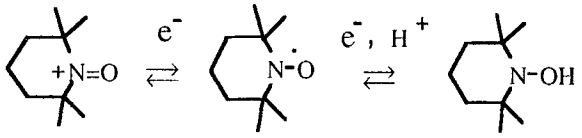
ulceration,[1–4] whereas copper was reported to confer protection.[3,5–8] Previous attempts at intervention employed common antioxidants such as chelating agents to neutralize redox-active iron;[9–12] LMWA to scavenge free radicals;[8,9,12–15] catalase to remove H_2O_2;[8–10] or inhibitors of xanthine oxidase to attenuate formation of ROS.[1,8,10,13–15] Conflicting evidence has been reported regarding the effect of SOD which was found to protect gastric mucosal cells in several cases[9–12,15] and ineffective treatment in others.[13,16]

More recent studies demonstrated a unique activity of stable nitroxide radicals in ameliorating oxidative damage in several experimental models,[17,18] including gastric and colonic injury in laboratory animals.[19,20] The study of the mode of action of nitroxides is important both for a better understanding of the pathophysiological processes of injury and also for the improvement of nitroxides' use as potential intervention to decrease the extent of gastrointestinal injury.

The chemistry of nitroxides has been extensively studied previously. Through electron transfer reactions nitroxides readily switch back and forth between their respective reduced (hydroxylamine) or oxidized (oxo-ammonium cation) forms. Consequently, all three forms as shown for 2,2,6,6-tetramethyl-piperidine-1-N-oxyl (TPO) in Scheme 1, can be present in the tissue:



Nitroxides in the tissue are rapidly reduced through enzyme-associated mechanisms.[21] This bioreduction, which predominantly takes place intracellularly in the mitochondria, yields almost exclusively the respective hydroxylamines.[21] Also the oxidation of hydroxylamine to nitroxide can occur at high rates.[22] Consequently, a distribution between nitroxide and hydroxylamine might be achieved soon, regardless of which of them is administered to the animal. The rapid metabolic exchange restores their levels in the tissue but also limits the distinction between the protective activities of the two forms.

The present study concentrates on the elucidation of the mechanism(s) underlying the antioxidative activity of nitroxides in the experimental model of gastric ulceration in rats.

MATERIALS AND METHODS

Chemicals

Ferrous ammonium sulfate, ferrous sulfate and ferric chloride were obtained from BDH Chemicals. Ferric ammonium sulfate and sodium citrate were from Baker Chemical Co. Copper sulfate was from Mallinckrodtt. Nitrilotriacetate (NTA) was from Sigma. The solutions of iron and copper were freshly prepared immediately before each experiment. The nitroxides 2,2,6,6-tetramethyl-piperidine-1-oxyl (TPO) and 4-OH-2,2,6,6-tetramethyl-piperidine-1-oxyl (TPL) were purchased from Aldrich Chemicals. The hydroxylamine 4-OH-2,2,6,6-tetramethyl-N-hydroxypiperidine (TPL-H) was prepared by bubbling HCl gas through an ethanolic solution of TPL. A colorless precipitate of TPL-H hydrochloride, which appeared, was collected, dried and kept in the cold. The solution of TPL-H was freshly prepared right before use.

Electron Paramagnetic Resonance (EPR) Measurements

To determine the residual nitroxide in the tissue, gastric mucosa samples were excised from the rat stomach immediately following sacrifice, weighed and frozen. Before measurement, a known volume of buffer was added to thaw the sample, vortexed thoroughly, and a liquid sample of 100–250 μl was drawn into a gas-permeable, teflon capillary of 0.8 mm inner diameter. The capillary was inserted into a quartz tube open at both ends and then placed within the EPR cavity. The EPR spectra were recorded on a JEOL

spectrometer, operating at X-band, 9.45 GHz, 100 kHz modulation frequency, 1 G modulation amplitude and 4 mW microwave power. The concentration of the nitroxide in the samples tested was quantitated using a TPL solution of a known concentration as a calibration standard. For determination of the total concentration of nitroxide and hydroxylamine, 1 mM $K_3Fe(CN)_6$ was added to oxidize reduced nitroxide.

Animals and Experimental Design

Male rats (Sprague-Dawley Strain), weighing 150–200 g were fasted overnight and allowed free access to water. Unless otherwise stated, the standard protocol included treatment with specified doses of nitroxide or hydroxylamine in saline, given ig 5 min before induction of damage by ig administration of 1 ml 96% ethanol. Control animals received equal volumes of vehicle alone before ethanol. Ten minutes after ethanol administration, rats were euthanized by cervical dislocation; the stomach was removed, washed with ice-cold 0.15 M NaCl, and the extent of macroscopic hemorrhagic erosions in the glandular mucosa was assessed.

Determination of Mucosal Damage

Mucosal damage was measured macroscopically and scored by multiplying lesion length (mm) and width (mm). For each rat, the total score was determined by summing up the ulcer scores (mm^2). All measurements are performed by two blinded observers using a stereo microscope. The variability between the two observers was 7%.

Effect of Metals on Gastric Mucosal Lesions

Rats were treated ip or iv with copper(II), iron(III) or iron(II) at various doses, 10–60 min prior to or at time of ethanol administration. Unless iron(III) ions are coordinated to a suitable ligand, they rapidly hydrolyze at physiological pH, forming aggregates of polynuclear iron which might precipitate. Therefore, iron(III) was injected ip or iv as nitrilotriacetate (NTA) or citrate complex in saline. NTA is widely used as a ligand for loading animals with iron because it binds the metal avidly enough to maintain its solubility, yet the chelate readily dissociates to deliver iron to cellular ligands or binding sites. Iron(II), which is more soluble, was given iv as a solution of ferrous ammonium sulfate. In control experiments, the rats were treated with the vehicle alone. Following the administration of iron, rats were treated ig with 1 ml of 96% ethanol, to induce injury. Ten min later, rats were euthanized and the gastric mucosal lesions were blindly assessed by independent observers.

Statistical Analysis

Statistical comparisons of lesion area were made using the Mann Whitney *U* test. A probability of $p < 0.05$ was considered as statistically significant. The data are presented as a mean ± SEM of (n) rats per each experimental group.

RESULTS

Effect of Metals on Gastric Mucosal Injury

Iron(III) increased the severity of the gastric mucosal injury, as judged by the lesions area, when given 30 min, but not 60 min, before administration of the irritant (Figure 1). Similar results were observed when iron(III) was given as ferric citrate rather than ferric NTA (data not shown). In control experiments, where rats were treated with iron alone without ethanol, no gastric lesions were observed (data not shown).

A similar aggravation of gastric injury by iron(III) was observed when rats were treated with 50% ethanol. In these experiments, rats were euthanized 30 min after ethanol administration. Treatment with iron(III) 175 μmol/kg b.w., 60 min prior to the administration of 50% ethanol increased significantly the gastric injury (Figure 2).

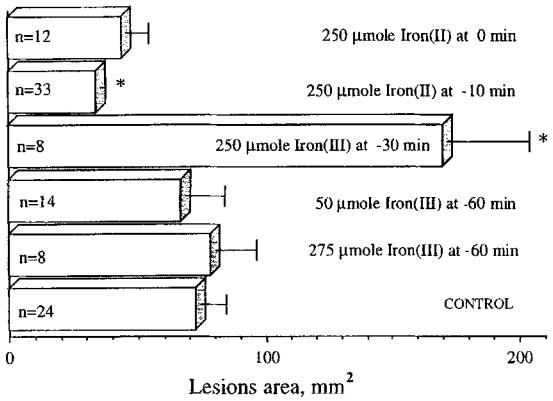


FIGURE 1 Effect of iron on gastric mucosal damage induced by 96% ethanol: Rats were treated iv with various doses of iron(II) or iron(III) either 60, 30, 10, or 0 min before ig administration of 1 ml of 96% ethanol. The rats were euthanized 10 min after ethanol administration. The area of macroscopically visible gastric lesions was measured. n = number of rats in each experimental group. Results are presented as mean ± SEM. *Significantly different from values measured for control rats treated with ethanol alone ($p < 0.05$).

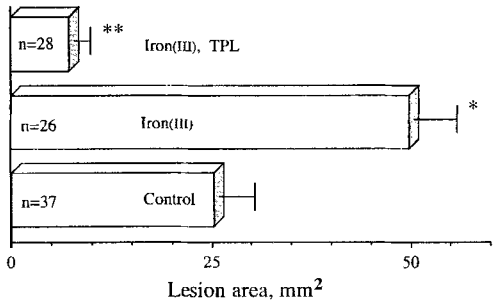


FIGURE 2 Effect of iron(III) and TPL on gastric lesions induced by 50% ethanol: Rats were treated iv with iron (III) 175 µmol/kg b.w. followed 60 min later by ig administration of 1 ml of 50% ethanol. Nitroxide-treated rats received TPL at various doses 5 min before ethanol treatment (all doses of 290, 58, or 14 µmol/kg b.w., had a similar effect and they were pooled together). The rats were euthanized 30 min after ethanol administration. The area of macroscopically visible gastric lesions was measured. n = number of rats in each experimental group. Results are presented as mean ± SEM. *Significantly different from ethanol-treated rats ($p < 0.05$). **Significantly different from rats treated with iron and ethanol ($p < 0.05$).

Effect of Iron(II) and Copper

Generally the toxic effects of iron involve the reduced form of the metal which can react with H_2O_2 to yield deleterious species. To check the effect on gastric injury, iron(II) 52–57 µmol/kg b.w., was injected iv as $Fe(NH_4)_2(SO_4)_2$ solution 10 min before ig administration of 96% ethanol. Figure 1 shows that administration of iron(II) did not increase the severity of injury when administered 10 min prior to ethanol but rather resulted in a significant decrease of the lesion area. Such a protective effect was not observed when iron(II) was given immediately before the administration of ethanol (Figure 1).

Since copper was shown to demonstrate anti-ulcer activity,[5] in the present study rats were treated with Cu(II) 50 µmol/kg b.w. given ip as $Cu(NTA)_2$, or iv as $CuSO_4$ before ethanol administration. Copper had no significant effect on the extent of gastric damage and did not provide protection whether given 10 min or immediately before ethanol administration (data not shown).

Effect of TPL

The protective effect of TPL on gastric injury induced in rats by 96% ethanol has been previously demonstrated and reported.[19] To examine the protective effect of nitroxides in iron-treated rats, various doses of TPL were administered 5 min prior to administration of 50% ethanol. TPL in a dose of 14 µmol/kg b.w. abolished the iron-induced sensitization. In fact, the lesion area was significantly smaller than that observed in rats that were not pre-treated with iron. Higher TPL doses of 57 and 290 µmol/kg b.w. did not further increase the extent of the protection. The mean gastric lesion area in rats treated with TPL is shown in Figure 2.

Effect of TPO

TPO is significantly more lipophilic than TPL as reflected by its higher 2-octanol/water partition coefficient. To evaluate its efficacy, rats were given iv various doses of TPO 5 min before ig administration of 1 ml ethanol 96%. A dose-dependent protective effect of TPO was observed (Figure 3).

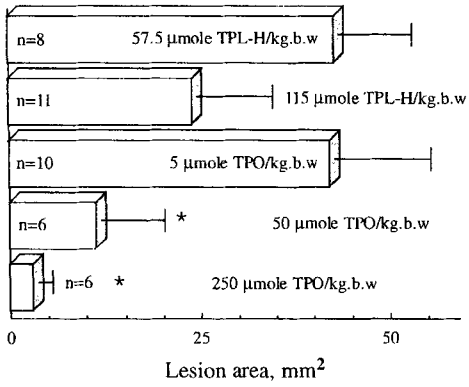


FIGURE 3 Effect of TPO and TPL-H on ethanol-induced gastric mucosal damage: TPO or TPL-H were injected iv to rats, 5 min before ig administration of 1 ml of 96% ethanol. Control rats were treated with ethanol only. The rats were euthanized 10 min later and the area of macroscopically visible gastric lesions was measured. n = number of rats in each experimental group. Results are presented as mean ± SEM. *Significantly different from lesions area of ethanol-treated rats, seen in Figure 1 ($p < 0.05$).

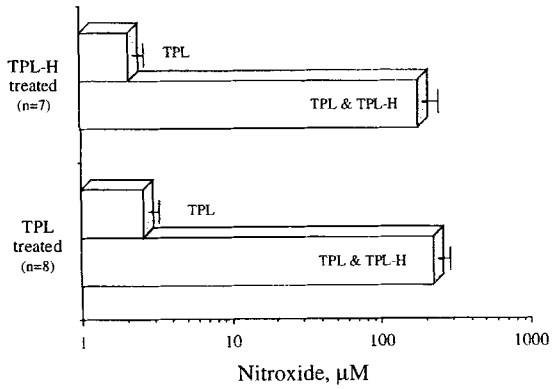


FIGURE 4 The exchange TPL ⇌ TPL-H in the tissue: TPO or TPL-H was injected iv at 290 µmol/kg b.w. to rats, 10 min before ig administration of 1 ml of 96% ethanol. The rats were euthanized 10 min later, mucosal tissue samples were excised, and the level of TPL or TPL and TPL-H in the tissue were determined by EPR spectrometry as detailed in Methods.

Effect of TPL-H

To examine the potential protective effect of the hydroxylamine, freshly prepared solution of TPL-H in saline was given iv, 57.5 and 115 µmol/kg b.w., 5 min before ethanol administration. Despite the high dose of hydroxylamine, no protective effect was observed (Figure 3).

The Exchange Nitroxide ⇌ Hydroxylamine in the Mucosal Tissue

To evaluate the conversion among the two forms, rats were treated iv with TPL (290 µmol/kg b.w.) 10 min prior to ig administration of 96% ethanol. Ten minutes later rats were killed and samples of gastric mucosa were excised, weighed and frozen. Before measurement, each sample was thawed by adding 100–250 µl buffer, vortexed and scanned for its EPR signal. Subsequently, ferricyanide was added and the EPR signal was measured again. From the intensity of the EPR signal, the tissue concentrations of TPL alone as well as TPL and TPL-H were calculated considering the respective dilutions. The nitroxide/hydroxylamine distribution in the gastric mucosa was about the same (1 : 10) when measured 10, 15, or 20 min after administration of TPL (data not shown). When TPL-H was given to the rats instead of TPL, similar distributions between the radical and its reduced form were observed (Figure 4).

DISCUSSION

The Mechanism(s) Underlying the Nitroxide Antiulcerative Activity

The reduced (non-radical) form of nitroxides has been previously found to be an effective antioxidant in several experimental models,[23,24] such as beating cultured cardiomyocytes exposed to H_2O_2 and in rats subjected to closed head injury.[25] In the present study, the nitroxide but not the hydroxylamine provided protection from gastric ulceration (Figure 3). Comparison of the effective molar doses, which is most appropriate when comparing pharmacological effects of compounds differing by their molecular masses, show that TPL and TPO, which confer similar

protection,[19] are far more effective than the hydroxylamine. The failure of the reduced form to protect, indicates that the mode of nitroxides' antiulcerative activity greatly differs from that of common LMWA, which act as a reducing agent. Moreover, the nitroxide/hydroxylamine distribution in the gastric mucosa, which is rapidly established irrespective of the sequence of their administration (Figure 4), combined with the difference between the biological activity of TPL and TPL-H, suggest that the former exerts its protective effect during an early and narrow time-window. Several mechanisms may explain the antiulcerative effect of nitroxides.

SOD-mimic Activity

The catalytic dismutation of $O_2^{\bullet -}$ by nitroxides and its kinetics were thoroughly studied[26,27] whereas the dose-dependent ethanol-induced formation of superoxide radicals in mucosal cells has been previously shown and correlated with the extent of damage.[28] These findings agree with the antiulcerative effects of SOD and of metal-containing SOD-mimics and account for the protective effect of nitroxide metal-free SOD-mimics. It was previously found that infusion of SOD abolished mucosal injury induced by local infusion of the NO donor nitroprusside.[12] Since $O_2^{\bullet -}$ radicals effectively react with NO, their facilitated dismutation by SOD or SOD-mimics not only results in an increase of the steady state concentration of NO but also preempts the formation of secondary deleterious species such as peroxynitrite. This mechanism might also underlie the antiulcerative activity of copper and copper chelates.[3,5–7,28–30] Low levels of NO were found to confer gastric protection whereas higher levels were pro-ulcerogenic.[12] Therefore, the elevation of $[NO]_{steady\ state}$ indirectly induced by nitroxides might have opposing effects on gastric injury. An alternative explanation for the link between roles played by the metal and the protective activity of nitroxides is to assume that, through a rapid oxidation of redox-active transition metals, nitroxides block their pro-oxidant effect.

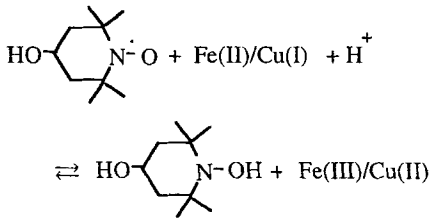
Nitroxides Preempt the Fenton Reaction

Copper, which is more soluble and labile, is generally more effective than iron in potentiating oxidative injury mediated by free radicals. On the other hand, copper and its chelates have demonstrated anti-inflammatory and anti-ulcerogenic activities as well as other protective effects against oxidative stress.[5] Such protection has been previously attributed to (a) an SOD-mimic activity of copper compounds or (b) a facilitation of *de novo* synthesis of cellular SOD or other metalloproteins instrumental in antioxidative action and modulation of biochemical responses to stress.[5]

Transition metals like iron and copper play a complex role in oxidative injury since they can act either as pro-oxidants or antioxidants, depending on the ligand, the kind of oxidative insult, and the experimental model tested. Generally the pro-oxidant effect requires a reduction of the metal ion which in turn can form, through the Fenton reaction, secondary reactive species such as OH and hypervalent metal. Chelatable iron, abundant in the gastrointestinal (GI) tract due to digestion of iron-containing proteins and limited absorption,[31,32] facilitates gastric and intestinal injurious processes as in gastric ulceration, colon cancer and inflammatory bowel disease.[9,33–38] The explanation for the protective effect of iron(II) is not straightforward. Generally iron and particularly iron(II) demonstrate pro-oxidant activity, however, under certain circumstances iron can exert an opposing effect. It has been previously proposed that expanding the intracellular free iron pool may activate multiple molecular mechanisms to reconstitute ferritin content, which in turn can limit the pro-oxidant challenge of iron.[39,40] In the present study iron(II) conferred a protective effect following 10 min pretreatment but not when given together with the irritant (Figure 1). Yet, 10 min seems to be

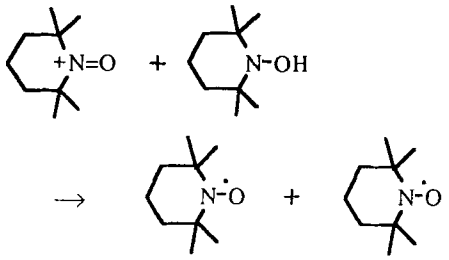
too short a period for the manifestation of the protection due to the stress response.

The present results suggest, therefore, that the protective activity of nitroxides depends indeed on their mild oxidant character. Previous kinetic studies demonstrated that nitroxides rapidly oxidize iron(II).[41] Very recently their reaction with Cu(I) was demonstrated.[42] It is very likely, therefore, that the reaction:



preempts the Fenton reaction and underlies the protective activity of the nitroxide.

Both mechanisms involve a recycling of the anti-oxidant. The SOD-mimic activity is a genuine catalytic process in which the nitroxide and its oxidized form exchange among themselves[26,27] without depletion of the nitroxide. The oxidation of reduced metals yields the hydroxylamine, which can be readily oxidized through cellular metabolism. Moreover, under flux of $O_2^{\bullet -}$, the oxo-ammonium cation, which is formed, reacts with the hydroxylamine to yield two nitroxide radicals (comproportionation reaction):



Through this pathway the nitroxide level can be replenished and maintained time-invariant in the tissue.

In conclusion, the results of the present study show that nitroxide radicals, rather than their respective non-radical reduced form, are the active species responsible for the provision of gastric protection. Nitroxides act catalytically as recycling anti-oxidants and protect by dismutating $O_2^{\bullet -}$ and possibly indirectly by increasing the NO level. Unlike classical LMWA which are reducing agents, nitroxides inhibit gastric damage by acting as mild oxidants, oxidizing reduced metals and preempting the Fenton reaction.

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