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EPR Studies of Nitric Oxide Interactions of Alkoxyl and Peroxyl Radicals in *In Vitro* and *Ex Vivo* Model Systems

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ABSTRACT

A model compound of lipid peroxidation, *tert*-butyl hydroperoxide (tBOOH), was used *in vitro* to investigate (i) the generation of tBOOH-derived free radicals by hematin or rat enterocytes and (ii) the modulation of cell-generated free radical production by a nitric oxide (NO) donor, or when these cells were primed to produce NO. In hematin-catalyzed decomposition of tBOOH, NO from nitrosoglutathione, or *S*-nitroso-*N*-acetylpenicillamine suppressed the generation of peroxyl radicals (measured by direct electron paramagnetic resonance) and *tert*-butyl-alkoxyl, methoxyl, and methyl radicals (measured by electron paramagnetic resonance spin trapping). Similarly, co-incubation of *S*-nitroso-*N*-acetylpenicillamine or nitrosoglutathione with tBOOH caused significant decreases in tBOOH-derived free radical generation catalyzed by enterocytes. Epithelial cells are the known source of the inducible form of NO synthase in the intestine of rats challenged with lipopolysaccharide (LPS). Enterocytes isolated from LPS-treated rats produced decreased levels of tBOOH-derived radicals. These decreases in free radical production were further decreased when these cells were treated with LPS *in vitro*. These findings demonstrated that exogenously added or endogenously produced NO could modulate the extent of tBOOH-derived free radical generation in enterocytes. These decreases in free radical production could, at least in part, describe the protective role of NO from hydroperoxide-induced injury. Antioxid. Redox Signal. 3, 177–187.

INTRODUCTION

As a free radical, nitric oxide (NO) reacts with reactive free radical species with diffusion-controlled rates, *i.e.*, 1–3 $\times$ 10^9 M^{-1} s^{-1} for peroxyl radicals (26) and 10 $\times$ 10^{10} M^{-1} s^{-1} for the hydroxyl radical (28). In the latter reaction, nitrous acid is formed rendering the protective role of NO in systems generating hydroxyl radical. Alkoxyl radicals, a known class of strong oxidants with a redox potential ($E^{\circ\prime}$) of 1.6 V (4), can react rapidly with NO forming inert nitrogen-containing products thus expected also to incur protection. However, NO reaction with (alkyl)peroxyl radicals forms (organic) peroxynitrite, which has redox potentials [$E^{\circ\prime}$ = 1.3 and 1.6 V, at pH 7.0 (20)] being higher than those of the initial peroxyl radicals [$E^{\circ\prime}$ = 1.0 V (4)]. These large differences in redox potentials render the prediction that NO may exhibit enhanced cytotoxicity and injury in biological systems producing peroxyl radicals. Hence, the role of NO in systems generating reactive organic radicals would depend on relative concentrations of alkoxyl and peroxyl radicals.

Recent studies demonstrate that alkoxyl radicals are the initial primary radicals during *tert*-butyl hydroperoxide (tBOOH) decompositon by cytochome *c* (2) and hematin (33). Alkoxyl

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radicals can also be produced from a self-reaction of peroxyl radicals (15). Thus, alkoxyl radicals may likely accumulate to reach a steady-state concentration during metabolism of tBOOH by purified enzymes (2), as well as enzymes present in cells. It is thus surmised that the role of NO in cells generating alkoxyl radicals may likely be a protective one. Several investigations have shown that NO from NO donors exhibited protective properties against hydroperoxide-induced injury in Chinese hamster V79 cells (34), endothelial cells (30), erythroleukemia K562 cells (13), and intestinal epithelial cells (5). In the presence of an NO donor, the depletion of *tert*-butylalkoxyl radical has consistently been correlated with the decreases in hydroperoxide-induced cytotoxicity (5, 13).

To study specifically NO interactions with peroxyl and alkoxyl radicals and correlate with cell viability, there is a need for the use of electron paramagnetic resonance (EPR) spectroscopy to demonstrate the concomitant decreases of reactive organic radicals in more complex systems. We could not exclude the possibility that the decreases of these free radicals could result from NO reaction with oxoferryl species or active irons present in cells (as discussed by Dr. Kagan in this forum). In this article, attention was focused on the decreases of tBOOH-derived free radicals in the presence of an NO donor, as well as under the conditions when cells are also capable of producing NO. Our previous studies showed that high levels of NO were produced in rat intestine in response to *in vivo* endotoxin (7, 8), and we now extend our studies to using tissues and cells isolated from endotoxic rats. tBOOH is an organic radical generator and has been used as a model for lipid hydroperoxides. The use of tBOOH is of relevance because intestinal cells are constantly exposed to oxidized fats and lipid hydroperoxides, and these compounds have been shown to exhibit toxicity *in vivo*.

MATERIALS AND METHODS

Materials

tert-Butyl hydroperoxide (tBOOH), hematin, 4-hydroxy-3,3,5,5-tetramethylpyrroline *N*-oxide (4-hydroxy-TEMPO), *N*-acetyl-DL-penicillamine, RPMI 1640, *E. coli* lipopolysaccharide (LPS), and trypan blue were purchased from Sigma (St. Louis, MO, U.S.A.). 5,5′-Dimethylpyrroline *N*-oxide (DMPO) was purchased from Oklahoma Medical Research Foundation Spin Trap Source (Oklahoma City, OK, U.S.A.). Nitrosoglutathione (GSNO), *N*,*N*′-bis(3-aminopropyl)-1,4-butanediamine tetrahydrochloride (Spermine), (*Z*)-1-{[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino}diazenium-1,2-diolate (SpNONOate), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) was obtained from Alexis (San Diego, CA, U.S.A.).

Animal preparation

Male Sprague–Dawley rats (300–350 g; from Hilltop Laboratory Animals, Inc., Scottdale, PA, U.S.A.) were housed in a controlled environment, exposed to 12-h light/12-h dark cycle, and provided with standard rodent chow (Purina Mills, St. Louis, MO, U.S.A.) and water *ad libitum* for at least 1 week before the experimental procedures were initiated. All experiments were performed in accordance with guidelines for the care and use of laboratory animals approved by the Louisiana State Medical Center New Orleans Animal Care Committee.

Primary enterocyte preparation

Three intestinal segments were flushed with ice-cold saline and inverted using plastic tubing (1.77 mm × 2.8 mm PE 260). Each segment was filled with Krebs buffer (4.5% NaCl, 5.8% KCl, and 10.6% KH_2PO_4, without Ca^{2+} and Mg^{2+} containing 0.5% bovine serum albumin, 5 m*M* glucose, and 20 m*M* HEPES, pH 7.4). The segments were placed in a plastic flask containing 40 ml of Krebs with protease (1–2 mg/ml) and EDTA (1 m*M*). The flask was incubated in a shaking 37°C water bath for 20 min. The segments were then transferred to another flask that contained 40 ml of Krebs, and incubated for a further 10 min. Dissociated cells were filtered and rinsed with ice-cold buffer. Cell pellets were obtained after centrifugation at 200 *g* for 5 min. Isolated enterocytes with viability of <80% were not used. For EPR studies, enterocytes were resuspended in chelexed

phosphate-buffered saline (PBS) containing 10 m*M* glucose and 0.1 m*M* diethylenetriaminepentaacetic acid (DTPA). PBS was chelexed by stirring 10 g of washed Chelex 100 resin (Bio-Rad, Hercules, CA, U.S.A.) in 100 ml of PBS overnight.

Ex vivo free radical generation

Rats were injected intravenously with a bolus dose of *E. coli* LPS (1 mg/kg). Saline injection was used as control. Treated animals were killed 4 or 18 h later. The small intestine was removed, and enterocytes were then prepared. In some experiments, rat blood was collected and kept frozen until EPR measurements of nitrosyl hemoglobin formation. In data shown in Figs. 5 and 7, enterocytes from control or LPS-treated rats (5×10^6 cells/ml) were added with DMPO (100 m*M*) and tBOOH (5 m*M*). EPR measurements were then performed at room temperature. In data shown in Fig. 6, enterocytes from LPS-treated rats were incubated with 1 m*M* L-arginine with or without 100 μg/ml LPS for 3 h in PBS at 37°C (enterocytes showed significant increased cell death when incubated for >3 h *in vitro*). The incubation mixtures were subsequently added with tBOOH (5 m*M*) followed by EPR measurements. In experiments for tBOOH-derived radical generation in rat intestinal tissues (Figs. 8 and 9), the vena cava of an anesthetized rat (control or LPS-treated) was cut. The small intestine was removed and flushed with cold saline. The intestine was cut along the mesenteric border and kept in 95% O_2/5% CO_2-bubbled Tyrode's salt solution. In some experiments, intestinal mucosa was scraped using a glass slide.

EPR measurements

EPR spectra were recorded at room temperature with an ER200D spectrometer operated at 9.72 GHz with a 100-kHz modulation frequency. After an addition of tBOOH, DMPO, and hematin or enterocytes (from control or LPS-treated rats), the incubation mixture was pipetted into an EPR aqueous flat cell, which was then centered in a TE_{011} cavity. For EPR measurements of tissue or mucosa, an EPR tissue flat cell was used for room temperature measurement. An aliquot of tBOOH and DMPO was added to the mucosa or the mucosal side of the whole intestine. The cover slip was mounted onto the tissue flat cell, and the EPR spectrum was then recorded.

RESULTS

NO reaction with molecular oxygen affecting nitroxide line width

NO reacts with most molecules containing unpaired electrons; therefore, initial experiments were performed to test whether NO could react with a nitroxide free radical that is a product in EPR spin-trapping experiments. We used 4-hydroxy-TEMPO as a nitroxide model of a radical adduct. The first peak of TEMPO signals is shown in Fig. 1. As shown in Fig. 1B, NO from SpNONOate did not alter the total signal intensity of TEMPO, indicating that NO does not react with nitroxides. How-

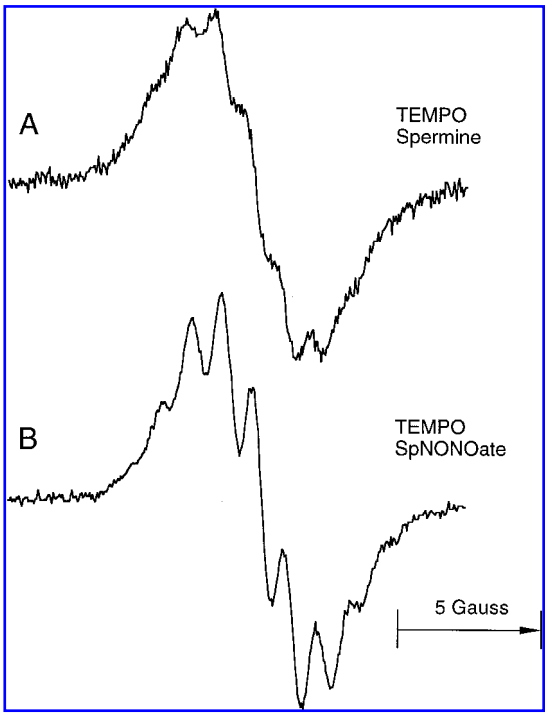

FIG. 1. The effects NO from SpNONOate on the hydrogen superhyperfine lines of the first peak of nitroxide 4-hydroxy-TEMPO. (**A**) 1 m*M* TEMPO added with 1 m*M* Spermine in 0.1 *M* phosphate buffer, pH 7.0, (**B**) 1 m*M* TEMPO added with 1 m*M* SpNONOate. Spectrometer conditions were as follows: modulation amplitude, 0.5 G; microwave power, 20 mW; time constant, 0.5 s; scan range, 15 G; scan time, 410 s.

ever, there were decreases of the line widths of the TEMPO hydrogen superhyperfine lines in Fig. 1B in comparison with those in Fig. 1A. This indicates that there was depletion of molecular oxygen in the mixture containing NO. The decreased line width was a result of NO reaction with molecular oxygen (which is paramagnetic), thus removing line-broadening effects of the TEMPO hydrogen interaction with the nitroxide. The lack of suppression of TEMPO signal assures the notion that NO does not react with the nitroxide radical adducts that are formed after spin-trapping reactions. Consistently, we found that NO released from 2 m*M* SpNONOate (measured as nitrite + nitrate or EPR-detectable PTIO/NO adduct intensity) was not altered by 100 μM TEMPO (data not shown).

Peroxyl radical and NO interactions in hematin system

Reactions of peroxyl radicals and NO were studied by direct EPR spectroscopy using an established hematin + tBOOH model system (16), where the alkylperoxyl radicals are characterized as the signal at $g \sim 2.014$. About 70% and 30% of this signal from the hematin + tBOOH reaction is attributed to *tert*-butylperoxyl and methylperoxyl radicals, respectively (33). The reactions of alkylperoxyl radicals and NO were monitored by measuring the decreases of the $g \sim 2.014$ signal in the presence of an NO donor. Figure 2 shows that the $g \sim 2.014$ signal intensity was decreased when GSNO or SNAP was added to the reaction mixture. The suppression was dependent on the NO donor concentrations. SNAP, when used at 4 m*M*, produced additional nitroxide signals (Fig. 2B). These SNAP-dependent nitroxides were also dependent on the presence of tBOOH and hematin (data not shown). EPR spectra obtained with low-modulation amplitude revealed three radical adducts (Fig. 3), two of which were characterized by computer simulations as radical adducts of SNAP, *i.e.*, SNAP/methyl radical [a^N = 16.3 G, and $a^H{}_\beta$ = 13.9 G (3H), marked with asterisks] and SNAP/sulfonyl radical (a^N = 14.0 G, marked with closed circles). In this case, SNAP acts as a nitroso spin trap. Methyl radical is a decomposition product of *tert*-butylalkoxyl radical formed during the hematin + tBOOH reaction. Methyl radical was trapped by excessive SNAP. Sulfonyl radical could be produced during the decomposition of SNAP. The hyperfine coupling constants of assigned adducts are in agreement with those previously reported (23). The nitroxide with hyperfine coupling of a^N = 15.5 G with 1.2 G low-field g shift (marked with open circles in Fig. 3) is an unknown SNAP-dependent nitroxide.

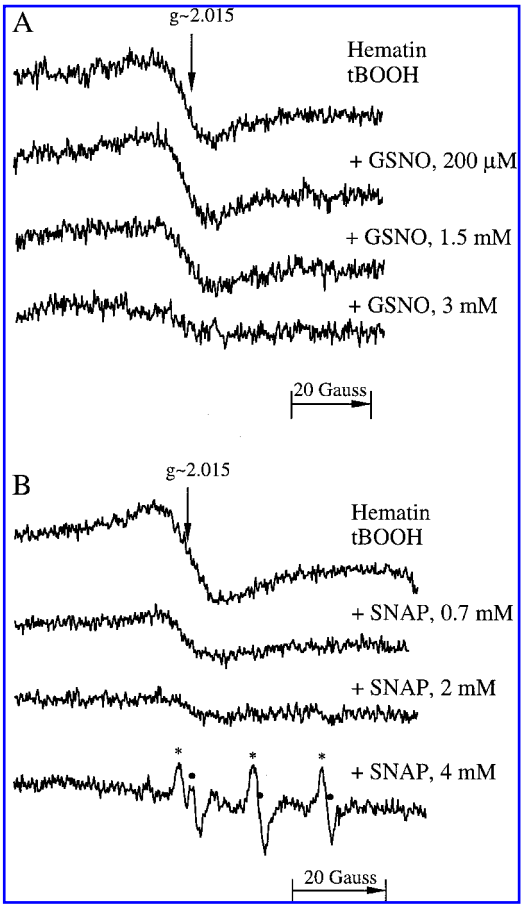

FIG. 2. An EPR spectrum of peroxyl radical at $g \sim 2.014$ measured at room temperature in a reaction containing 100 μM hematin and 50 m*M* tBOOH in 0.1 *M* borate buffer, pH 8.0. (**A**) Complete system or added with 0.2 m*M* GSNO, 1.5 m*M* GSNO, and 3.0 m*M* GSNO. (**B**) Complete system or added with 0.7 m*M* SNAP, 2 m*M* SNAP, and 4 m*M* SNAP. Spectrometer conditions were as follows: modulation amplitude, 2.5 G; microwave power, 20 mW; time constant, 0.5 s; scan range, 100 G; scan time, 410 s.

In addition to peroxyl radical studied by direct EPR, tBOOH metabolism catalyzed by hematin was also studied by the spin-trapping technique using DMPO (Fig. 4). For nearly 20

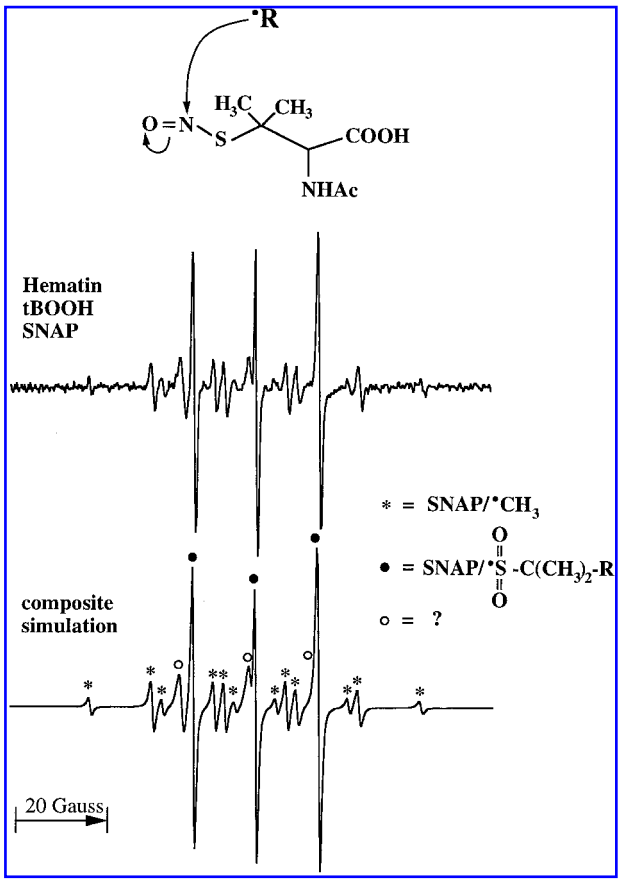

FIG. 3. SNAP reaction with a free radical (R˙) to produce three nitroxide signals. SNAP/methyl radical [asterisks; a^N = 16.3 G, and a^H_β = 13.9 G (3H)], SNAP/sulfonyl radical (closed circles; a^N = 14.0 G), and an unknown radical (open circles; a^N = 15.5 G with 1.2 G low-field g shift). This reaction was obtained from 100 μM hematin, 50 mM tBOOH, and 10 mM SNAP in 0.1 M borate buffer, pH 8.0. Ac, acetyl.

years (23), DMPO adducts of tBOOH-derived peroxyl, alkoxyl, and methyl radicals have been extensively investigated, whereby the assignment of DMPO/*tert*-butylperoxyl adduct was based on superoxide dismutase insensitivity, and the similarity of hyperfine parameters to those of DMPO/superoxide adduct. A recent report has shown that the previously assigned DMPO/*tert*-butylperoxyl adduct is indeed the DMPO/methoxyl adduct (10). Methoxyl radical may come from recombination of two methylperoxyl radicals and/or the decomposition of DMPO/methylperoxyl adduct. In Fig. 4, a spin-trapping experiment of hematin + tBOOH reaction was carried out, and accordingly, we now assigned the DMPO adducts of methyl radical (closed circles), *tert*-butylalkoxyl radical (asterisks), and methoxyl radical (open circles). In summarizing the reaction scheme of the hematin + tBOOH reaction, *tert*-butylalkoxyl radical is the primary radical (33). A secondary reaction of *tert*-butylalkoxyl radical produces methyl radical, which then undergoes a series of reactions to form methoxyl radical (10). Thus, spin-trapping experiments offered insights into the fates of alkoxyl radicals, *i.e.*, *tert*-butylalkoxyl, and methoxyl radicals, which are highly reactive. The effect of an NO donor on radical adduct formation is shown in Fig. 4. An addition of SNAP (Fig. 4) or GSNO (data not shown) suppressed radical adduct formation by 50–70%.

Reactions of exogenously added NO and tBOOH-derived radicals produced by primary enterocytes

In our study using primary enterocytes (Fig. 5), cell-mediated metabolism of tBOOH resulted

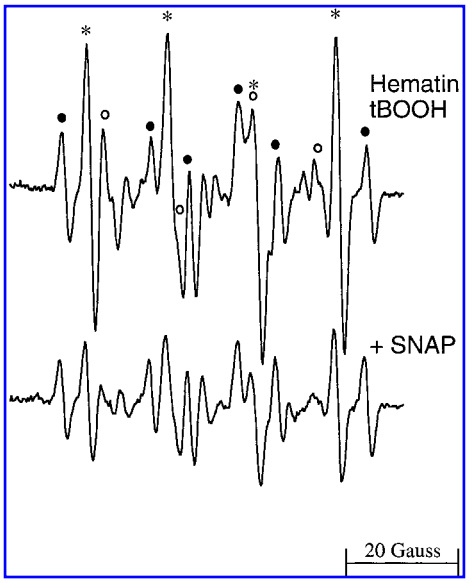

FIG. 4. Spin trapping of tBOOH-derived free radicals from hematin catalysis. The incubation in 0.1 M borate buffer, pH 8.0, was obtained from 100 mM DMPO, 100 μM hematin, 50 mM tBOOH, with or without 3 mM SNAP. Computer simulations showed three radical adducts of methyl radical (some marked with closed circles), *tert*-butylalkoxyl radical (marked with asterisks), and methoxyl radical (some marked with open circles). Spectrometer conditions were as follows: modulation amplitude, 0.63 G; microwave power, 20 mW; time constant, 0.5 s; scan range, 100 G; scan time, 410 s.

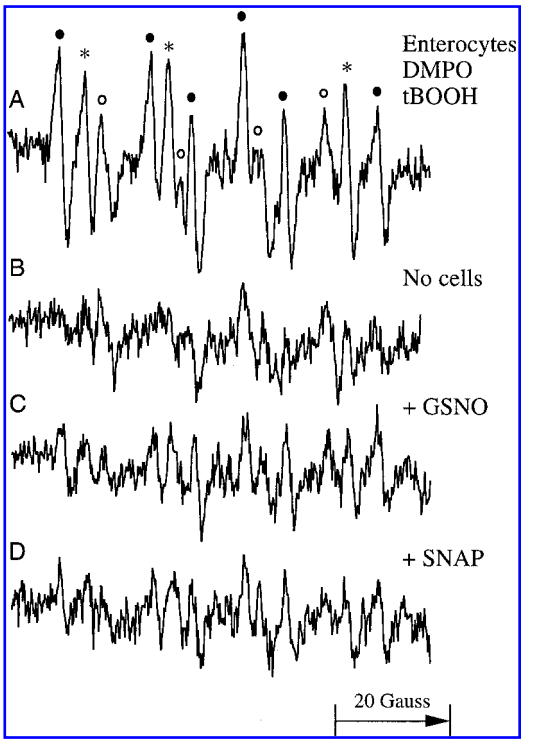

FIG. 5. tBOOH-derived radical formation catalyzed by rat enterocytes (5×10^6 cells/ml) in PBS, pH 7.4, containing 0.1 m*M* DTPA and 5 m*M* glucose. (**A**) A complete incubation containing enterocytes, 5 m*M* tBOOH, and 60 m*M* DMPO, (**B**) Same incubation as in A, but without cells. (**C**) Same incubation as in A, but added with GSNO (0.2 m*M*). (**D**) Same incubation as in A, but added with SNAP (0.5 m*M*). Spectrometer conditions were the same as those in the legend of Fig. 4.

in the generation of radical adducts similar to those previously observed in the hematin system. DMPO radical adducts of methyl radical (closed circles), *tert*-butylalkoxyl radical (asterisks), and methoxyl radical (open circles) were obtained in an incubation containing enterocytes and tBOOH (Fig. 5A). Incubation without cells (Fig. 5B) or with heat-denatured cells (data not shown) produced weak signals of radical adducts. This indicates that redox active enzymes present in enterocytes were responsible for tBOOH metabolism. Similar to the hematin + tBOOH reaction, an addition of GSNO or SNAP to an enterocytes + tBOOH mixture caused significant decreases in radical adduct formation. Note that, in all our cell work, the concentrations of tBOOH and NO donors were much less than those used in the hematin + tBOOH reaction. Our previous studies showed that these decreases of radical adducts shown in Fig. 5 were accompanied by protection against tBOOH-induced cytotoxicity (5).

Endogenously produced NO attenuates tBOOH-derived free radical formation in enterocytes

When rats are primed with endotoxin *in vivo*, isolated epithelial cells, such as hepatocytes (29) or epithelial intestinal cells (31, 32), produce increased NO concentrations *in vitro* compared with cells isolated from untreated rats. Addition of LPS *in vitro* further increases NO production. The priming and sensitivity to LPS to produce NO were therefore tested. In our studies (Fig. 6), rats were pretreated with endotoxin, and enterocytes were subsequently isolated 4 h later. These cells were added with L-arginine, with or without LPS, and further in-

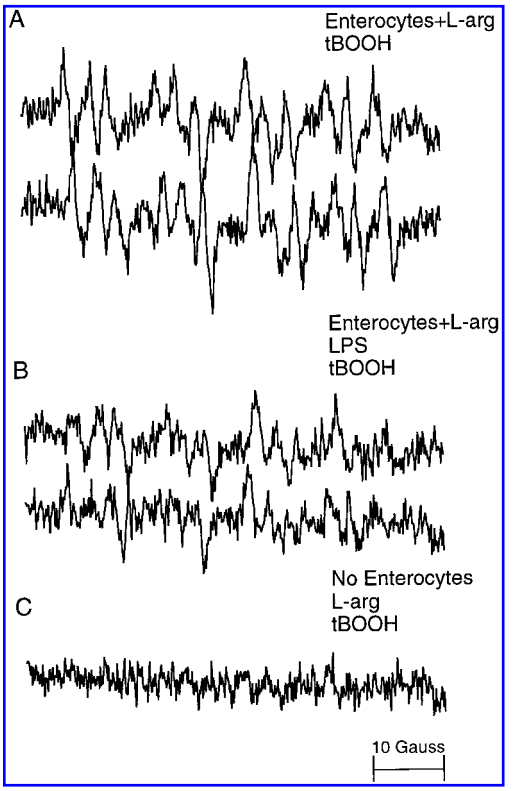

FIG. 6. tBOOH free radical production by *in vivo* LPS-primed enterocytes treated with LPS *in vitro*. Rats were treated with 1 mg/kg LPS for 4 h prior to enterocyte preparation. Isolated cells (5×10^6 cells/ml) were incubated for 3 h at 37°C with 1 m*M* L-arginine with or without 100 μg/ml LPS. Cells were then added with 5 m*M* tBOOH and 100 m*M* DMPO, and the EPR spectrum was recorded. Spectrometer conditions were the same as those in the legend of Fig. 4.

cubation was carried out. tBOOH was then added to the incubation mixture, and an EPR spectrum was recorded. Enterocytes isolated from LPS-treated rats were capable of catalyzing tBOOH to form DMPO radical adducts (Fig. 6A). In comparison with tBOOH metabolism from cells isolated from untreated rat (Fig. 5A), DMPO adducts in Fig. 6A exhibited weaker signal intensity. Each spectrum shown in Fig. 6 was obtained from each LPS-treated rat. An addition of L-arginine to enterocytes did not alter the signal intensity of adducts shown in Fig. 6A (data not shown). When *in vivo* LPS-primed enterocytes were incubated with L-arginine, and also LPS *in vitro* (Fig. 6B), the extent of radical adduct formation was decreased even further when compared with the incubations without LPS (Fig. 6A). Therefore, LPS addition to *in vivo* LPS-primed cells (to produce increased NO) caused suppression of free radical generation. Preliminary experiments showed that LPS (100 μg/ml) did not inhibit hydroxyl radical adduct formation in the Fenton reaction; thus, LPS does not inhibit nitroxide signals. Without enterocytes, tBOOH was not metabolized to form free radical adducts (Fig. 6C). Thus, the formation of methyl, *tert*-butylalkoxyl, and methoxyl radicals is depleted when enterocytes are manipulated experimentally to the conditions known to produce increased NO.

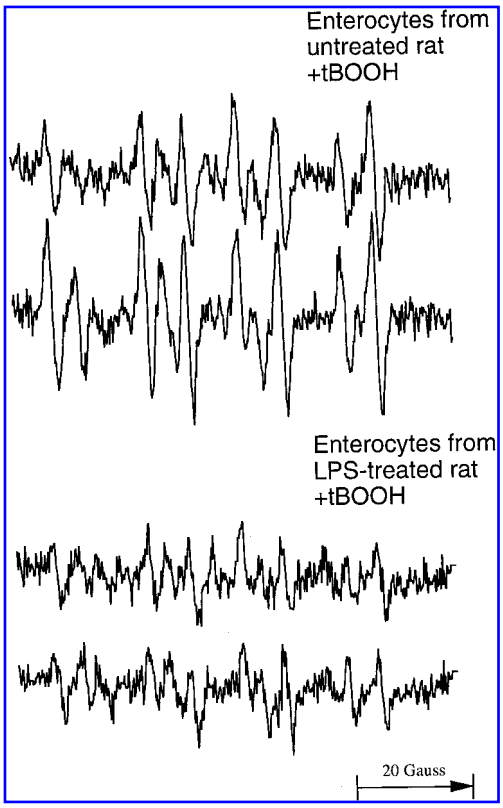

FIG. 7. ***In vitro*** **tBOOH metabolism by enterocytes isolated from control or LPS-treated rats.** Rats were injected intravenously with LPS (1 mg/kg) 4 h prior to enterocyte preparation. Enterocytes (5×10^6 cells/ml) were added with 5 m*M* tBOOH and 100 m*M* DMPO, and the EPR spectrum was recorded. Spectrometer conditions were the same as those in the legend of Fig. 4.

Endotoxin treatment in vivo attenuates tBOOH-derived free radical formation in enterocytes and intestinal mucosa in vitro

Rats were treated with endotoxin *in vivo*, and enterocytes were subsequently prepared 4 h after LPS injection. Under our experimental conditions, we did not find any significant differences in cell viability of enterocytes from control or LPS-treated rats. *In vivo* LPS-primed or control enterocytes were added with tBOOH and DMPO, and an EPR spectrum was recorded. Consistent with data shown in Figs. 5A and 6A, enterocytes isolated from LPS-treated rats metabolized tBOOH to free radicals in lesser concentrations than did those cells from untreated rats (Fig. 7).

DMPO spin-trapping experiments of tBOOH in enterocytes did not provide us information about peroxyl radicals that may be formed in secondary reactions. We therefore carried out further experiments using direct EPR utilizing a tissue flat cell (Fig. 8). tBOOH was added onto the mucosal side of rat ileum. Subtraction of a background spectrum from ileum alone (Fig. 8B) resulted in authentic signals of peroxyl radical ($g \sim 2.013$) and ascorbate semidione radical as a doublet with 1.9 G splitting (Fig. 8A and C). The latter radical was not present in the control ileum (Fig. 8B) and thus may have resulted from reaction of tissue ascorbic acid with tBOOH-derived radicals. Peroxyl radical signal (Fig. 8C) was attributable to *tert*-butylperoxyl or methylperoxyl radicals, whereas, the latter species may form in lesser amounts. Thus, similar to the hematin system, rat intestine was capable of metabolizing tBOOH to its peroxyl radicals, likely produced from secondary reactions of alkoxyl radicals.

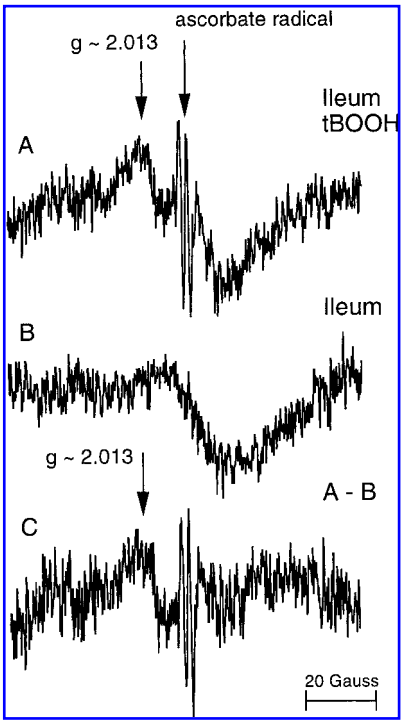

FIG. 8. Peroxyl radical ($g \sim 2.013$) formation in the rat ileum. tBOOH (3 μmol) was added onto the mucosal side of the ileum (150 mg). The tissue was then mounted onto a tissue flat cell for room temperature EPR measurement. Spectrometer conditions were the same as those in the legend of Fig. 2.

To study the role of *in vivo* LPS on radical production in intestinal tissues, spin-trapping experiments were carried out using intestinal mucosa instead of purified enterocyte suspensions (Fig. 9). Intestinal mucosa includes epithelial cells, lymphocytes, and red blood cells. LPS-treated rat produced systemic NO production as determined by nitrosyl hemoglobin in whole blood (Fig. 9B), as well as nitrosylated non-heme proteins in intestinal tissues (7,8). Mucosa (in a fixed 0.5–ml volume) from the jejunum of control or LPS-treated rats was added with an aliquot of tBOOH and DMPO. After thorough mixing, the mixture was placed in the EPR tissue flat cell. EPR spectra showed signals of the doublet ascorbate semidione radical and DMPO radical adducts of methyl and *tert*-butylalkoxyl radicals (Fig. 9C and D). Mucosal tissue from LPS-treated rats metabolized tBOOH to free radicals to a much lesser extent than did the mucosal tissue from untreated rats. Consistent with spin-trapping using enterocytes (Fig. 7), cells or tissues from LPS-treated rats (prepared under the conditions known to produce NO) produced decreased levels of tBOOH-derived radicals.

DISCUSSION

In this article, EPR spectroscopy is used as a unique tool to study specifically the interactions of NO and reactive organic radicals that are known to mediate cytotoxicity in cells. Our previous work using enterocytes has shown that the depletion of tBOOH-derived radicals by an NO donor is correlated with the protection (5). Herein, the investigations were extended to cells endogenously producing NO (Figs. 6, 7, and 9). Cells of the intestine were chosen in our research project because the in-

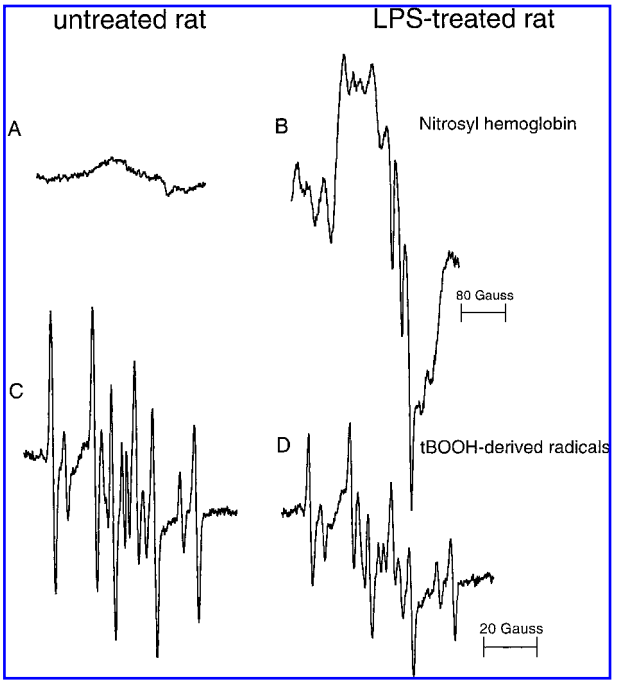

FIG. 9. Increased *in vivo* NO production induced by endotoxin caused decreases of tBOOH-derived radical formation *in vitro*. EPR spectra A and B were obtained from blood of untreated or LPS (1 mg/kg)-treated rats that were killed 18 h later. Blood samples were measured at 77°K. EPR spectra **C** and **D** were tBOOH-derived radical adduct formation obtained from an addition of 3 μmol of tBOOH and 5 μl of DMPO into scraped mucosa (in a fixed 0.5-ml volume). The mucosa was then mounted onto a tissue flat cell for EPR measurement at room temperature. Spectrometer conditions were the same as those in the legend of Fig. 4.

testinal epithelium has been recognized as the first line of host defense against various oxidants from the ingested foods and bacteria/pathogen. Intestinal epithelial cells have been used *in vitro* to determine cell cytotoxicity upon exposure of tBOOH (21), H_2O_2 (24), and superoxide radical from phorbol ester-stimulated neutrophils (1). The interactions of cell-generated oxygen-derived radicals with NO are a normal occurrence, because there is an extensive localization of constitutive NO synthase in the epithelium (25), and epithelial cells produce high levels of NO during chronic intestinal inflammation (11, 27).

We demonstrated by direct EPR that peroxyl radicals were produced during decomposition of tBOOH by hematin (Fig. 2) or intestinal tissue (Fig. 8). The pathway of peroxyl radical generation by intestinal tissues may follow similarly that of the hematin system, *i.e.*, *tert*-butylperoxyl and methylperoxyl radicals are produced secondarily from the *tert*-butylalkoxyl radical. Our data showing the depletion of peroxyl radicals in the presence of an NO donor in the hematin system indicate that NO is capable of reacting with secondary peroxyl radicals. It is also possible that NO directly reacts with hematin, thus inhibiting tBOOH metabolism to produce the initial *tert*-butylalkoxyl radical. However, the reaction rates of NO and ferric and ferrous heme proteins [$\sim 10^5$–10^7 M^{-1} s^{-1} (17)] are much slower than those of NO with peroxyl radicals [1–3 $\times$ 10^9 M^{-1} s^{-1} (26)]. In our hematin reactions, estimated ratios for hematin and tBOOH-derived radical concentrations are only 20–100. Thus, the NO reaction with peroxyl radicals could contribute significantly to the decreases of the detected $g \sim 2.014$ signal by NO.

Our spin-trapping experiments in hematin, enterocytes, and intestinal tissues revealed the presence of *tert*-butylalkoxyl, methoxyl, and methyl radicals (Figs. 4–9), but the absence of peroxyl radicals. This may be because DMPO does not form persistent adducts of peroxyl radicals. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide forms more persistent adducts of sulfite radical than does DMPO (6), making it a good candidate for the spin-trapping of peroxyl radicals. Recent investigations using cytochrome *c* (2), hematin, and DTPA-Fe^{2+} (33) demonstrate that one-electron reduction of tBOOH produces the primary *tert*-butylalkoxyl radical. *tert*-Butylalkoxyl radical can also be produced from a self-reaction of peroxyl radicals. Methoxyl radical is produced from methyl radical that is the product of *tert*-butylalkoxyl radical decomposition. Thus, these series of reactions support the notion that these reactive alkoxyl radicals can accumulate in high enough concentrations enabling them to induce cytotoxicity in cells. This situation may likely be applicable to enterocytes. Alkoxyl radicals may be produced by enterocytes in significant concentrations, and the suppression of these radicals by NO could explain the protection. The suppression of *tert*-butylalkoxyl and methoxyl radicals was observed in cells treated with an NO donor (Fig. 5), as well as when cells were prepared under the conditions known to produce NO (Figs. 6, 7, and 9). A mechanism of radical suppression can be described for each experimental protocol. When an NO donor was added at the same time as tBOOH to the enterocyte suspension (Fig. 5), it is likely that NO reacts directly with the primary *tert*-butylalkoxyl radical, and/or the secondary methoxyl, and methyl radicals prior to being spin-trapped by DMPO. When cells were prepared from LPS-treated rats (or primed to produce NO) and then treated with tBOOH (Figs. 6, 7, and 9), it is likely that NO had reacted with active enzymes in cells (to form nitrosyl complexes), thus inhibiting subsequent tBOOH metabolism. We unfortunately did not measure nitrosyl complexes in these cells. However, it has been reported that nitrosyl complex formation in cells pretreated with NO has been associated with the protection against subsequent treatment of H_2O_2 (19), tBOOH (13), and toxic levels of SNAP (18, 19). Only a few micromolar NO is sufficient to elicit protection against tBOOH in cells (5, 34); thus, LPS-primed enterocytes used in our experiments could achieve these concentrations, perhaps eliciting the protection against tBOOH. Our data demonstrating decreases in production of reactive free radicals are in agreement with *in vivo* findings that NO may be protective during acute conditions (with significantly elevated oxygen radical production), such as splanchnic ischemia–reperfusion (22), or at an early time point following endotoxin treatment (3).

Two unusual properties of SNAP were dis-

covered during the course of this study. SNAP is also a spin trap and also a free radical generator of sulfonyl radical (Fig. 3). Therefore, in addition to the expected protective effects of NO released by SNAP, SNAP may also be protective by trapping free radicals, *i.e.*, methyl and sulfonyl radicals. GSNO does not behave the same way as SNAP. This may be due to their differences in chemical structures. The nitrosothiol (O=N-S-) group for GSNO is bonded to a primary carbon (O=N-S-CH_2R), but for SNAP, it is bonded to a tertiary carbon [O=N-S-C(CH_3)$_2$R]. The latter structure seems to facilitate spin-trapping reaction, *i.e.*, a reduction of nitroso to nitroxide. Also, the carbon adjacent to the nitroso group of most nitroso spin traps, such as *tert*-nitrosobutane and nitrosobenzene, is a tertiary carbon.

In summary, the molecular targets of NO presented in this study include *tert*-butylperoxyl, methylperoxyl, *tert*-butylalkoxyl, methoxyl, and methyl radicals during hematin and enterocyte metabolism of tBOOH. The alkoxyl radicals are likely the major and key oxidant species in these reactions. The concentrations of all of these radicals were depleted in the presence of an NO donor or under the conditions when *in vivo* primed cells produce NO. These *ex vivo* findings indicate that cell-generated NO capable of decreasing the generation of alkoxyl radicals may elicit the protection against tBOOH. To study NO interactions with reactive organic radicals, the uniqueness of EPR applications to cells and tissue preparations is also demonstrated in this article.

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ABBREVIATIONS

DMPO, 5,5′-dimethylpyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; EPR, electron paramagnetic resonance; GSNO, nitrosoglutathione; LPS, lipopolysaccharide; NO, nitric oxide; PBS, phosphate-buffered saline; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; Spermine, *N*,*N*′-bis(3-aminopropyl)-1,4-butanediamine tetrahydrochloride; SpNONOate, (*Z*)-1-{[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino}diazenium-1,2-diolate; tBOOH, *tert*-butyl hydroperoxide; TEMPO, 3,3,5,5-tetramethylpyrroline *N*-oxide.

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