Protein Radical Formation During Lactoperoxidase-Mediated Oxidation of the Suicide Substrate Glutathione

Immunochemical Detection of A Lactoperoxidase Radical-Derived DMPO Nitrone Adduct

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The abbreviations used are: ABTS, 2,2′-azino-bis-[3-ethylbenzothiazoline]-6-sulfonic acid; CEE, cysteine ethyl ester; CME, cysteine methyl ester; DHLA, dihydrolipoic acid (or 6,8-thioctic acid, reduced); DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DTT, dithiothreitol; ESR, electron spin resonance; ELISA, enzyme-linked immuno-sorbent assay; EPO, esinophil peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; HRP, horseradish peroxidase; LPO, lactoperoxidase; MCE, 2-mercaptoethanol; MSC, mercaptosuccinic acid; MMI, methylmercaptoimidazole; MPO, myeloperoxidase; NAC, N-acetylcysteine; NBS, N-Bromosuccinimide; TPO, thyroid peroxidase.

Running title: Lactoperoxidase-Derived Radical
ABSTRACT

A novel polyclonal anti-DMPO antiserum, which specifically recognizes protein radical-derived DMPO nitrone adducts, has been developed. In this study, we have employed this new approach, which combines the specificity of spin trapping and the sensitivity of antigen-antibody interactions, to investigate protein radical formation from lactoperoxidase (LPO). When LPO reacted with glutathione (GSH) in the presence of DMPO, we detected an LPO radical-derived DMPO nitrone adduct using enzyme-linked immuno-sorbent assay (ELISA) and Western blot. The formation of this nitrone adduct depended on the concentrations of GSH, LPO and DMPO as well as pH values, and GSH could not be replaced by H$_2$O$_2$. The level of this nitrone adduct was decreased significantly by azide, catalase, ascorbate, iodide, thiocyanate, phenol or nitrite.

However, its formation was unaffected by chemical modification of free cysteine, tyrosine and tryptophan residues on LPO. Electron spin resonance (ESR) spectra showed that a glutathial radical was formed from the LPO/GSH/DMPO system, but no protein radical adduct could be detected by ESR. Its formation was decreased by azide, catalase, ascorbate, iodide or thiocyanate, while phenol or nitrite increased it. GSH caused marked changes in the spectrum of compound II of LPO, indicating that GSH binds to the heme of compound II, while phenol or nitrite prevented these changes and reduced compound II back to native enzyme. GSH also dose-dependently inhibited peroxidase activity of LPO by measuring 2,2'−azino-bis-[3-ethylbenzothiazoline]-6-sulfonic acid (ABTS) oxidation. Taken together, these results demonstrate that the GSH-dependent LPO radical formation is mediated by the glutathiy radical, possibly via the
reaction of the glutathiyl radical with the heme of compound II to form a heme-centered radical trapped by DMPO.

INTRODUCTION

Oxidation of thiols by peroxidases is of interest because peroxidases have been shown to catalyze thiol oxidation without \( \text{H}_2\text{O}_2 \) addition (1). More importantly, thiols play important roles in protecting biological systems from oxidative damage (2-4), e.g., glutathione (GSH)\(^1\) is the major intracellular low-molecular-weight thiol; dihydrolipoic acid (DHLA) is an endogenous dithiol with potent antioxidant properties (5,6); and other thiols such as penicillamine and \(N\)-acetylcysteine (NAC) are used pharmacologically (7,8). Thiols such as cysteine and GSH have been reported to be substrates for horseradish peroxidase (HRP) (9-12). Cysteamine and cysteine esters, but not cysteine and GSH, have been found to be good substrates for myeloperoxidase (MPO) (13,14). A recent study has shown that various aliphatic and aromatic thiols are oxidized by MPO (15).

Together with MPO, esinophil peroxidase (EPO) and thyroid peroxidase (TPO), lactoperoxidase (LPO) belongs to the mammalian peroxidase superfamily II in which the prosthetic heme group is covalently attached to the protein, as distinguished from the peroxidase superfamily I (enzymes from plants, fungi and bacteria). LPO possesses antibacterial properties and is found in several biological fluids such as milk, tears and saliva (16-18). \textit{In vivo}, LPO is thought to catalyze the oxidation of thiocyanate, iodide and bromide; \textit{in vitro}, it is able to oxidize many other substrates (19-21). Inactivation of
Thus far, there is no evidence to suggest protein radical formation during oxidation of thiols catalyzed by peroxidases. Rather, HRP and LPO oxidize thiol substrates by one-electron oxidation to form thiyl radical metabolites (9,22,25-28). However, protein radicals are now well recognized as normal intermediates for an increasing number of enzymes and some peroxidase-like hemoproteins. Examples of such radical intermediates include the thiyl radical of ribonucleotide reductase (29-31), the tyrosyl radicals of prostaglandin H synthase (32), cytochrome c (33), and horse heart myoglobin (34), the tyrosyl and thiyl radicals of cytochrome c peroxidase (35) and hemoglobin (36,37). GSH, a cysteine-containing tripeptide, is ubiquitously distributed in nature and is the most abundant low-molecular-weight thiol in mammalian cells. Therefore it is of great importance to further investigate the role of GSH in peroxidase-mediated oxidation reactions.

Recently a novel rabbit polyclonal antiserum against the nitrone spin trap DMPO has been developed and validated in the horse heart metmyoglobin/H2O2 system, where DMPO is known to trap the tyrosyl-103 radical (38). This new approach, which combines the specificity of spin trapping and the sensitivity of antigen-antibody interactions, has been employed to detect the amino acid radicals formed on oxyhemoglobin and methemoglobin in the presence of H2O2 (37). The anti-DMPO serum specifically recognizes protein radical-derived DMPO nitrone adducts, which are stable end products of the DMPO/protein radical adduct intermediates (see Schemes in the above two references). In contrast, ESR spin-trapping techniques using DMPO as a spin trap detects...
the transient DMPO/protein radical adducts. Unfortunately, only a few protein radicals such as tyrosyl radicals of myoglobin (34) and thiyl radicals of human myoglobin (39,40), hemoglobin (36) and cytochrome c oxidase (35) have been trapped by DMPO and detected by ESR, and even in these cases high concentrations of proteins had to be used. Therefore, in the detection of protein-derived radicals in biological systems, the sensitive immunochemical detection of protein radical-derived DMPO nitrone adducts is not only complementary to the ESR DMPO-spin-trapping technique, but a usually superior replacement (37).

In the present study, we investigated immunochemically protein radical formation in the interaction of LPO with GSH. For the first time, we detected the GSH-dependent LPO radical-derived DMPO nitrone adduct by ELISA and Western blot assays, demonstrating the formation of a protein radical on LPO, which has not been detected by ESR using DMPO as a spin trap agent. Thus immunochemical detection of protein radical-derived DMPO nitrone adducts has been used for the first time to explore the free radical-mediated protein radical formation that have not been successfully detected by ESR.

**MATERIALS AND METHODS**

*Chemicals* – LPO (80-150 units/mg protein, $A_{412}/A_{280} = 0.88-0.95$), sodium iodide, sodium thiocyanate, sodium nitrite, sodium azide, potassium cyanide, $N$-Bromosuccinimide (NBS), ascorbic acid, GSH, cysteine, NAC, DHLA, cysteamine, homocysteine, captopril, penicillamine, mercaptosuccinic acid (MSC), MMI, cysteine methyl ester (CME), cysteine ethyl ester (CEE), sodium sulfide, and oxidized glutathione
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(GSSG) were from Sigma Chemical Company (St. Louis, MO). 2-Mercaptoethanol (MCE) was obtained from Bio-Rad Laboratories (Hercules, CA). The concentration of LPO was optically determined at $\lambda = 412$ nm ($\varepsilon = 112 \text{ mM}^{-1}\text{cm}^{-1}$) (21,41). Hydrogen peroxide was from Fisher Scientific (Fair Lawn, NJ). Beef liver catalase was obtained from Roche Molecular Biochemicals (Indianapolis, IN). 2,2′-azino-bis-[3-ethylbenzothiazoline]-6-sulfonic acid (ABTS) was from Calbiochem-Novabiochem Corp. (La Jolla, CA). DMPO (high purity, $\geq 99\%$) was purchased from Alexis Biochemicals (San Diego, CA). Ultrapure phenol was from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). All other chemicals used were of analytical grade.

Chemical modification of free cysteine, tyrosine and tryptophan residues on LPO –

Free cysteine residues on LPO was blocked by mixing native LPO with NEM at a molar ratio of 1:20. The mixture was incubated for 30 min at room temperature and then passed through a prepacked Sephadex G-25 column (PD-10, Amersham Pharmacia Biotech, Piscataway, NJ) to remove excess NEM. Chemical modification of tyrosine residues on LPO by iodination was initialized by adding two $N$-chloro-benzenesulfonamide immobilized beads (IODO-BEADS, Pierce Chemical Co., Rockford, IL) to the reaction mixture containing LPO and NaI at a molar ratio of 1:20. The reaction was allowed to proceed with shaking at 25 °C for 15 min. To stop the reaction, the solution was passed through a PD-10 column to remove excess NaI. Chemical modification of tryptophan residues on LPO with NBS was performed as described in a previous report (42). Briefly, NBS was incubated with LPO in 50 mM sodium acetate buffer (pH 5.0) at 25 °C for 15 min at a molar ratio of 1:25. To
terminate the reaction, the incubation solution was passed through a PD-10 column to remove excess NBS.

Chemical reactions – Typically, 0.2 µM of native or modified LPO was reacted with 2 mM GSH in the presence of 100 mM DMPO with or without 100 µM H₂O₂. After 1 h of incubation at 37 °C, the reaction was stopped by adding 5 mM ascorbic acid, then the LPO radical-derived DMPO nitrone adduct was quantified by ELISA or Western blot as described below.

Antiserum anti-DMPO – A rabbit polyclonal anti-DMPO nitrone adduct antiserum was obtained and first applied in our laboratory (38). In the present study, we used this antiserum to evaluate immunochemically the LPO radical-derived DMPO nitrone adduct in the mixture containing LPO and GSH in the presence of DMPO.

Enzyme-linked immunosorbent assay (ELISA) – The LPO radical-derived DMPO nitrone adduct was determined using a standard ELISA in 96 well plates (Greiner Laborteknik, Frickenhausen, Germany). One hundred µl of the adduct solution (1.5 µg of protein) in 200 µl of distilled water was incubated for 90 min at 37 °C. The plates were washed once with 1X TBS washing buffer (0.05% Tween-20, 0.05% casein, and 0.05% BSA, pH 7.4) and blocked with coating buffer (0.1 M bicarbonate buffer, pH 9.6, containing 2.5% casein and 2.5% BSA) for 90 min. Thereafter, the rabbit anti-DMPO serum (1:5,000) in washing buffer was added and incubated for 60 min. After four washes, the secondary antibody, anti-rabbit IgG-alkaline phosphatase (Pierce Chemical
Co., Rockford, IL), 1:5,000 in washing buffer, was added and incubated for 60 min. After four more washes, the antigen-antibody complexes were developed by using a chemiluminescence system (CDP-Star, Roche Molecular Biochemicals, IN), and the light emitted was recorded in arbitrary units using Xfluor Software (Tecan US, Research Triangle Park, NC)

**Western blot assay** – After the reaction of LPO and GSH or H$_2$O$_2$ in the presence or absence of DMPO, the samples were dialyzed twice against 0.1 M phosphate buffer, pH 7.4 for 3 h. The proteins (1.0 µg/lane) were separated on reducing 4-12% Bis-Tris NuPAGE (Invitrogen, Carlsbad, CA), and electroblotted onto a nitrocellulose membrane. The membrane was blocked with a 2.5% BSA/2.5% casein solution in 1X TBS buffer (pH 7.4) for 90 min. After 4 washes with washing buffer (1X TBS containing 0.05% Tween-20, 0.05% BSA, and 0.05% casein, pH 7.4), the membrane was incubated for 90 min with the primary antibody (rabbit anti-DMPO serum, 1:5,000 in washing buffer). After that, the membrane was washed four times and incubated for 60 min with the secondary antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase) at a dilution of 1:5,000 in washing buffer. After another four washes, antibody-antigen complexes were detected using enhanced chemiluminescence (Nitro Block II, Tropix, Bradford, MA and CDP-Star II) and the nitrocellulose membrane was exposed to x-ray.

**SDS-PAGE assay** – Cross-linking experiments were carried out at 25 or 37 °C for 5 or 60 min. After the reactions of LPO with H$_2$O$_2$ or GSH were terminated by adding catalase for the systems containing H$_2$O$_2$ or ascorbic acid for the system
containing GSH, the proteins were separated on reducing 4-12% Bis-Tris NuPAGE (Invitrogen, Carlsbad, CA) and then stained with Coomassie Blue.

*ESR spin-trapping measurements* – ESR spectra were obtained with a Bruker ElexSys E500 spectrometer equipped with a super high Q cavity operating at 9.78 GHz and room temperature. The ESR spectrometer settings were as follows: modulation frequency, 100 kHz; modulation amplitude, 2.0 G; microwave power, 20 mW; receiver gain, $2 \times 10^5$; time constant, 164 ms; conversion time, 82 ms; magnetic field scan, 80 G.

*Oxygen consumption experiments* – Oxygen consumption measurements were made with a Clark-type oxygen electrode fitted to a 1.8 ml Gilson sample cell and monitored by a model 53 oxygen monitor (Yellow Spring Instruments, OH). The reagents were added in the following order: first, a phosphate buffer solution (pH 7.4, 100mM) containing 2 or 10 mM GSH in the presence or absence of either 0.01 mg/ml catalase or 100 mM DMPO was placed in the sample cell. Then, after establishing a one-minute baseline measurement, reactions were initiated by adding 8 µM LPO to the reaction chamber. Oxygen concentration in the samples as a function of time was recorded by a PC interfaced to the oxygen monitor with a Data Translation DT2801 data acquisition board. All the experiments were done at room temperature with gentle stirring.

*Effect of GSH on the spectrum of compound II of lactoperoxidase in the presence and absence of phenol and nitrite* – Spectrometric measurements were performed at room temperature with a Beckman DU®640 spectrometer. Compound II of LPO is predominant whenever LPO is exposed to a small excess of H$_2$O$_2$ (21,43-45). In the present work, we
prepared compound II by adding H₂O₂ to the ferric enzyme at a molar ratio of 2:1. The reaction was started by adding 2 mM GSH with or without 1 mM phenol or nitrite to compound II, which had been formed by the addition of 4 µM H₂O₂ to 2 µM LPO. The spectra were recorded with time by scanning from 350 nm to 700 nm.

Effect of GSH on the peroxidase activity of lactoperoxidase – Peroxidase activity was measured using ABTS (41,46) or guaiacol as an electron donor (22,47). For ABTS oxidation, 0.2 µM LPO was preincubated with various concentrations of GSH for 60 min in 96 well plates (Corning Incorporated, Corning, NY). After 3 washes with a 100 mM phosphate buffer (pH 7.4) to remove excessive GSH, 300 µl of the ABTS assay solution containing ABTS (0.5 mM) and H₂O₂ (100 µM) in the phosphate buffer (100 mM, pH 7.4) was added to each well. Spectral absorption was recorded with time at 415 nm. For guaiacol oxidation, after 0.2 µM LPO was preincubated with various concentrations of GSH for 60 min in 96 well plates, the plates were washed 3 times using 100 mM phosphate buffer (pH 7.4) to remove excessive GSH, and 300 µl of the guaiacol assay solution containing guaiacol (5 mM) and H₂O₂ (50 µM) in the phosphate buffer (100 mM, pH 7.4) was added to each well. Spectral absorption was monitored with time at 470 nm.

RESULTS

Protein radical formation from lactoperoxidase by GSH in the presence of DMPO by ELISA and Western blot analyses – When 0.2 µM LPO and GSH were incubated for one hour in the presence of 100 mM DMPO, an LPO radical-derived DMPO nitrone adduct
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was generated and detected by ELISA assay (Fig. 1A). GSH at a concentration higher than 0.5 mM increased the formation of this nitrone adduct in a dose-dependent manner with or without exogenously added H₂O₂, until a saturation point reached. When GSH, LPO or DMPO was omitted, no protein radical-derived nitrone adducts were detected. No LPO radical-derived nitrone adduct was detected when GSH was replaced either by its oxidized form GSSG or by H₂O₂ (data not shown). The amount of the nitrone adduct formed increased with incubation time and approached saturation after 1 h of incubation (Fig. 1B).

When Western blot assay was used instead of ELISA, we again detected the LPO radical-derived DMPO nitrone adduct generated from the interaction of LPO with GSH (Fig. 1D). Similarly, the levels of this nitronate adduct increased with increasing GSH concentration (Lanes 6 and 7 in Fig. 1D), but H₂O₂ could not mediate its formation (Lane 3 in Fig. 1D). The amount of LPO remained the same under the different conditions (Fig. 1C).

As shown in Fig. 2A, H₂O₂ at a concentration lower than 50 µM did not show any effects on the formation of the LPO radical-derived DMPO nitrone adduct no matter what concentration of GSH was used. However, when the concentration of H₂O₂ was increased to 50 µM, H₂O₂ significantly increased its formation in the system containing the higher concentration of GSH (10 mM) while significantly decreasing its formation in the system containing the lower concentration of GSH (0.5 mM). Exogenous H₂O₂ still had no effect on the system containing 2 mM GSH (data not shown). Fig. 2B and 2C showed that the formation of the LPO radical-derived nitrone adduct depended on the concentrations of LPO and DMPO, respectively. In
addition, its formation increased as the pH increased from 5.0 to 8.0, then decreased with a further increase in pH (Fig. 2D), which coincides with the deprotonation of GSH (pK\textsubscript{a, SH} = 8.8) (13).

Twelve thiol compounds were also examined for their ability to mediate the protein radical formation from LPO in the absence of H\textsubscript{2}O\textsubscript{2}. As shown in Fig. 3, at the same concentration of 1 mM, it was DHLA and DTT, which have two sulfhydryl groups, that showed the strongest ability to mediate the LPO radical formation while MCE, CEE and CME showed the weakest ability, followed by MSC. However, no LPO radical formation was detected in the presence of some other thiol compounds such as MMI and the sulfide compound Na\textsubscript{2}S (data not shown).

**Effects of inhibitors on lactoperoxidase radical formation** – To elucidate the mechanism of the GSH-dependent LPO radical formation, the effects of various inhibitors were examined. As shown in Fig. 4, LPO radical formation was strongly inhibited in the presence of 1 mM sodium azide, suggesting that the heme of LPO is required for the radical formation. Its formation was also inhibited by 70 % in the presence of 50 \(\mu\text{g/ml}\) of catalase, and completely inhibited by 100 \(\mu\text{g/ml}\) of catalase (data not shown), revealing a critical role for trace amounts of H\textsubscript{2}O\textsubscript{2} in the protein radical formation. Additionally, its formation was markedly decreased by ascorbate at 0.5 mM and completely inhibited when ascorbate was at a concentration of greater than 2 mM (data not shown). As little as 10 \(\mu\text{M}\) I\textsuperscript{−} and SCN\textsuperscript{−}, which are excellent two-electron donors for LPO compound I (48), inhibited the LPO radical formation by 88% and 89%, respectively. Phenol and nitrite at 1 mM, which are excellent one-electron donors for...
LPO compound I and II (41,49), inhibited the LPO radical formation by 94 % and 93 %, respectively.

Spin trapping of glutathiy radical from the reaction of lactoperoxidase with GSH in the presence of DMPO – Although GSH is reported to be a relatively poor substrate for LPO (13,18,26), it is a demonstrated one-electron donor for compound I and II (15,26). To elucidate the relationship between the formation of the LPO radical and the glutathiy radical (GS'), the glutathiy radical was trapped by DMPO and monitored by ESR. When 0.2 µM LPO reacted with 2 mM GSH in the presence of 100 mM DMPO (control in Fig. 5), an ESR spectrum typical of the DMPO/’SG adduct was obtained (inset of Fig. 5). This ESR spectrum was simulated using hyperfine coupling constants (\(a_N = 15.13\) G and \(a_H = 16.14\) G) similar to those in earlier reports (27,28,50). The glutathiy radical formation increased with the increase in GSH and LPO concentrations (data not shown). However, its formation significantly decreased by sodium azide, catalase, ascorbate, \(\Gamma\) or SCN\(^-\). Catalase at 100 µg/ml and ascorbate at 0.5 mM completely inhibited its formation.

Phenol and nitrite at 1 mM significantly increased the formation of DMPO/’SG by about 8-fold and 2-fold, respectively. This increase agrees with the previous reports that phenol and nitrite enhance glutathiy radical formation via the reaction of GSH with phenoxy radical or \(^-\)NO\(_2\) formed by HRP or LPO, a reaction that more efficient than the reaction of GSH with HRP or LPO alone (51,52).

Since the radical-driven oxidation of GSH is known to proceed via glutathiy radical and to be accompanied by oxygen reduction and consequent generation of superoxide
anion radical and \( \text{H}_2\text{O}_2 \) (26,27), we measured oxygen consumption in the LPO/GSH system. Oxygen consumption was undetectable when 0.2 \( \mu \text{M} \) LPO or 2 mM GSH alone, or both were present in 100 mM phosphate buffer, pH 7.4 (data not shown), in consistent with the previous reports that no oxygen consumption is detected in the reaction between 0.4 mM GSH and less than 100 nM EPO, LPO or HRP (18). When the concentration of LPO was increased to 8 \( \mu \text{M} \) in the presence of 2 mM GSH, oxygen was consumed (Fig. 6H). Oxygen consumption was greatly accelerated when GSH concentration was increased to 10 mM (Fig. 6D).

Catalase (0.01 mg/ml), whether added before or after LPO, inhibited oxygen consumption (Fig. 6E and G). 100 mM DMPO also markedly inhibited oxygen consumption (Fig. 6F and I) when added either before or after LPO. These results are similar to those in previous reports, in which GSH was oxidized by HRP or prostaglandin H synthase (27,53). In addition, 1 mM azide, 10 \( \mu \text{M} \) iodide or thiocyanate, or 0.5 mM ascorbate completely inhibited oxygen consumption when added prior to LPO (data not shown). Phenol at a low concentration of 20 \( \mu \text{M} \) greatly accelerated oxygen consumption (Fig. 6A), and the increased oxygen consumption was reduced by the presence of either 100 mM DMPO (Fig. 6B) or 0.01 mg/ml catalase (Fig. 6C).

**Investigation of involvement of amino acid residues in the formation of LPO radical-derived nitrone adduct** – Hemoprotein peroxidases commonly react with \( \text{H}_2\text{O}_2 \) to form a ferryl/porphyrin radical cation compound I intermediate, which often converts rapidly to a ferryl/protein radical species (54,55). The amino acid residues that are ultimately oxidized to free radicals have been demonstrated to be
cysteine, tyrosine and tryptophan (34-36). To determine the roles of cysteine, tyrosine and tryptophan residues on LPO in the protein radical formation, we modified these three residues by NEM, iodide and NBS, respectively. As shown in Fig. 7A, there was no significant difference in ELISA signal intensity between native and modified LPO, suggesting that the site of the protein radical is not on free cysteine, tyrosine, or tryptophan residues of LPO. H$_2$O$_2$- or GSH-mediated cross-links of LPO were also investigated via SDS-PAGE assay. The reaction of LPO with H$_2$O$_2$ resulted in the partial formation of dimers and trimers of LPO (Lanes 3 and 4 in Fig. 7B), supporting the previous report (56). However, GSH at 0.5-10 mM, where GSH mediated the LPO radical formation (Fig. 1A), did not lead to the formation of oligomers of LPO, suggesting that the site of the GSH-dependent protein radical is not on the amino acids since it is thought that the formation of dimers and trimers of LPO is mediated via amino acid-derived radical reactions (56).

Effect of GSH on the spectrum of compound II of lactoperoxidase – As Nakamura et al. have reported that thiol compounds such as MMI, cysteine and DTT reacted with compound II of LPO (57), we investigated the effect of GSH on the spectrum of compound II in the presence or absence of phenol and nitrite. LPO reacts with H$_2$O$_2$ to form compound I, which is very unstable and spontaneously decays to its one-electron reduction product, compound II (41,48). Curve b in Fig. 8A showed the spectrum of compound II formed upon addition of 4 µM H$_2$O$_2$ to 2 µM LPO, whose Soret band (431 nm) and two distinct peaks at 536 and 566 nm in the visible region are consistent with
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those of compound II reported previously (48). Compound II was stable for about 10 min, and then slowly converted back to its native ferric form (data not shown).

The addition of 2 mM GSH dramatically changed the spectrum of compound II of LPO with time (curves c and d in Fig. 8A). The Soret peak shifted from 431 to 426 nm concomitant with the appearance of a new peak at 641 nm, characteristic of chlorines (58). These results indicate that GSH or its product bound to the heme of compound II. The formation of the peak at 641 nm was accelerated by increasing GSH concentration (data not shown). However, at concentrations lower than 0.1 mM GSH only hastened the conversion of compound II to the native ferric enzyme (data not shown).

As little as 10 µM phenol or nitrite alone rapidly reduced compound II back to its native ferric form in 15 seconds (data not shown). As shown in Fig. 8B, the presence of 1 mM phenol prevented the appearance of the new spectrum in the interaction of compound II with GSH. The phenol instead stimulated the formation of an intermediate (curve c), whose Soret band at 423 nm and two distinct peaks at 550 and 589 nm in the visible region are the same as those of compound III reported previously (43,44). With time, compound III returned to the native ferric enzyme (curve d), as indicated by an isosbestic point at 422 nm. In contrast, 1 mM nitrite rapidly reduced compound II in the presence of GSH to its native ferric state without formation of compound III (Fig. 8C), as indicated by an isosbestic point at 422 nm, similar to previous reports (41,44).

Effect of GSH on the peroxidase activity of lactoperoxidase – The one-electron oxidation of ABTS to ABTS\(^{+}\) catalyzed by peroxidases has been used as an index of peroxidase activity (41,46). We examined the effect of varying concentrations of GSH on
the rate of ABTS oxidation catalyzed by LPO (Fig. 9). GSH dose-dependently inhibited ABTS oxidation, and its inhibitory effect approached saturation at 0.5 mM. Similarly, GSH dose-dependently inhibited LPO-catalyzed guaiacol oxidation (data not shown). DMPO at 100 mM could not protect LPO against inactivating by GSH in the range from 0.01 to 10 mM (data not shown).

**DISCUSSION**

The formation of various protein radicals mediated by H$_2$O$_2$ has been detected by direct ESR or ESR spin-trapping techniques in recent studies (33-36,39,40). However, in the present work, the formation of the DMPO-trapped LPO-derived protein radical is unique because it does not occur in the presence of H$_2$O$_2$, but instead requires GSH, as detected by ELISA and Western blot assays (Fig. 1A and 1D).

Despite GSH-mediating LPO-derived protein radical formation independent of H$_2$O$_2$, H$_2$O$_2$ was generated during the reaction of LPO with GSH. It is well known that the highly reactive thiyl radical intermediates can react with thiyl anion to produce the disulphide radical anion, which is responsible for oxygen reduction and consequent hydrogen peroxide generation (9,59-63) (Equations 1-4). Indeed, in our present work, we found that oxidation of GSH by LPO led to the formation of the glutathiyl radical (Fig. 5) and we also detected oxygen consumption (Fig. 6). The glutathiyl radical formed, we believe, is not from the direct reduction reaction of ferric LPO by GSH because (1) the glutathiyl radical formation was catalase-dependent and catalase at a concentration of 100 µg/ml completely inhibited the glutathiyl radical formation (Fig. 5); (2) we did not observe the appearance of the spectrum of ferrous LPO...
under anaerobic conditions (data not shown); (3) thermodynamically, GSH itself cannot reduce ferric LPO to ferrous LPO, as seen from the reduction potentials for RS'/RS' (0.92 V) (64) and Ferric/Ferrous LPO (-0.191 V) (65); and (4) to our knowledge, no references have thus far reported the direct reduction of ferric LPO by GSH.

\[
\text{GS}^+ + \text{GS}^- \longrightarrow \text{GSSG}^{2-} \quad (1)
\]

\[
\text{GSSG}^{2-} + \text{O}_2 \longrightarrow \text{GSSG} + \text{O}_2^- \quad (2)
\]

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (3)
\]

\[
\text{O}_2^- + \text{GSH} + \text{H}^+ \longrightarrow \text{H}_2\text{O}_2 + \text{GS}^+ \quad (4)
\]

The influence of pH on the overall thiol oxidation has also been demonstrated in previous studies where both thiol oxidation and oxygen consumption have been shown to increase with pH, presumably as a consequence of the increasing fraction of thiolate in the reaction medium (12,13). Thiyl radicals react $10^4$ to $10^5$ times faster with thiolates than with protonated thiols to produce the disulphide radical anion (66). Presumably this increased rate accounts for the result in Fig. 2D showing that the formation of the LPO radical-derived DMPO nitrone adduct also increased when the pH was increased from 5.0 to 8.0, which coincides with the deprotonation of GSH ($pK_a,_{\text{SH}} = 8.8$) (13). Therefore, the generation of H$_2$O$_2$ indirectly by GSH could explain the requirement for GSH.

However, generation of H$_2$O$_2$ is not the only role of GSH in the LPO radical formation since exogenous H$_2$O$_2$ could not substitute for the presence of GSH. Hence, GSH must play another role in its formation. Nevertheless, small amounts of H$_2$O$_2$ were beneficial, which again suggests that trace amounts of peroxides are necessary for the
reaction. Another piece of supporting evidence for this is that 50 µg/ml catalase inhibited the protein radical formation by 70 % (Fig. 4), and completely inhibited its formation when the concentration of catalase was increased to 100 µg/ml (data not shown).

In addition to its role in generating H$_2$O$_2$, the glutathiyyl radical directly mediated the protein radical formation from LPO. First of all, 0.5 mM ascorbate markedly decreased the protein radical formation (Fig. 4) and completely inhibited its formation at a concentration of higher than 2 mM (data not shown). In addition, ascorbate at 0.5 mM completely inhibited the formation of the DMPO/SG adduct (Fig. 5) and oxygen consumption (data not shown) while the ascorbate radical appeared concurrently (data not shown), consistent with a previous report (50). Therefore, the inhibitory effects of ascorbate on both LPO radical and glutathiyyl radical formation may be mainly attributed to its reacting with the glutathiyyl radical to form an ascorbate radical and GSH and consequently blocking the pathway by which GSH mediated the LPO radical formation.

Moreover, iodide and thiocyanate ions at a very low concentration of 10 µM strongly inhibited both LPO-derived protein radical and glutathiyyl radical formation (Figs. 4 and 5). Although iodide is a well-known good electron donor in the two-electron reduction of compound I of LPO with a rate constant of about $1.2 \times 10^8$ M$^{-1}$s$^{-1}$ (48), its competition with GSH for compound I can not explain its inhibitory effects on both protein radical and glutathiyyl radical formation because the oxidation of GSH by LPO is greatly accelerated by a small amount of iodide (26). However, the following pathways may explain its inhibitory effects: Iodide has been reported to be oxidized by compound I of LPO to an iodinium cation, which is assumed to be present as an enzyme-bound form (26,67,68). This iodinium cation then reacts with GSH (Equations 4-6). Through these
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reactions iodide acts as an effective mediator of the peroxidase-catalyzed two-electron oxidation of GSH, therefore, in this case no free radicals such as glutathiyil and superoxide radicals are formed, and O₂ is not consumed (26).

\[
\text{Compound I} + \text{I}^- \rightarrow \text{Compound I}^{2-} - \text{I}^+ \quad (4)
\]

\[
\text{Compound I}^{2-} - \text{I}^+ + \text{GSH} + \text{H}^+ \rightarrow \text{E} + \text{H}_2\text{O} + \text{GSI} \quad (5)
\]

\[
\text{GSI} + \text{GSH} \rightarrow \text{GSSG} + \text{I}^- + \text{H}^+ \quad (6)
\]

As for thiocyanate, it is thought to be a physiological substrate for mammalian peroxidases (69,70). Normal plasma levels of thiocyanate have been shown to be 20-120 µM (71,72), and in secreted fluids, which contain LPO, the levels are even higher. With thiocyanate, the reaction of compound I of LPO is extremely fast \( (2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}) \). The primary oxidation product in the LPO/SCN⁻/H₂O₂ system (73-75), HOSCN, is a weak oxidizing agent that may react primarily with thiol compounds such as GSH, converting them to sulfonyl thiocyanate or disulfide (76,77). In our study, we found that thiocyanate at very low concentrations did strongly inhibit both LPO radical and glutathiyil radical formation (Figs. 4 and 5) and completely inhibited oxygen consumption (data not shown). Taken together, these data further suggest that the glutathiyil radical not only generated \( \text{H}_2\text{O}_2 \) but also directly mediated the LPO radical formation.

Hemoprotein peroxidases commonly react with \( \text{H}_2\text{O}_2 \) to form a ferryl/porphyrin radical cation compound I intermediate, which often converts rapidly to a ferryl/protein radical species (54,55). The amino acid residues that are ultimately oxidized to free radicals have been demonstrated to be cysteine, tyrosine and tryptophan (34-36). An LPO protein radical trapped by DBNBS has been detected by ESR in the interaction of LPO
and H₂O₂ (56). However, in the present work, no LPO-derived radical was detected by ESR using DMPO as a spin trap agent during the reaction of LPO with H₂O₂ (data not shown), nor was a LPO radical-derived DMPO nitrore adduct detected by ELISA (data not shown) or Western blot using a polyclonal anti-DMPO antiserum (Lane 3 in Fig. 1D). These results demonstrate that the protein radical formed from the reaction of LPO with GSH is different from that formed from the reaction of LPO with H₂O₂ and trapped by DBNBS. In other words, the LPO-derived protein radical trapped by DMPO in this work is mediated by GSH, not by the H₂O₂ that is present in the system. No significant difference in ELISA signal intensity was found between native LPO and LPO whose free cysteine, tyrosine and tryptophan residues were modified by NEM, iodide and NBS, respectively, suggesting that the site of the protein radical is not on any of these residues of LPO (Fig. 7A). Moreover, the cross-linking experiment by SDS-PAGE assay in Fig. 7B also showed that no oligomers were formed from the reaction of LPO with GSH. This result further supports the conclusion that the radical site is not on the amino acid residues since it is thought that the formation of dimers and trimers of LPO is mediated via amino acid-derived radical reactions (56).

In our study, we found that GSH caused a shift of the Soret peak of compound II from 431 to 426 nm concomitant with the appearance of a new peak at 641 nm (Fig. 8A), indicating that changes in the environment of the heme may be caused by GSH or glutathiol radical attack. Previous studies have shown that GSH is oxidized to the glutathiol radical by prostaglandin H synthase, and subsequently the glutathiol radical attacks the double bond of styrene to form carbon-centered radical trapped by DMPO.
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(78,79). In addition, the mechanisms for addition of azide and cyanide to the heme of HRP have been proposed to be mediated by azidyl (80) and cyanyl radical (81), respectively. In the present work, we detected LPO radical-derived DMPO nitrone adduct by both ELISA and Western blot and the glutathiyl radical by ESR in the interaction of LPO with GSH. Therefore, based on both previous reports and present data, we propose a similar mechanism for the formation of the LPO radical-derived nitrone adduct as indicated in Scheme 1. That is, compound I of LPO reacts with GSH to form compound II and glutathiyl radical which, in turn, attacks the heme of compound II to form a heme-centered radical which is trapped by DMPO. As a result, LPO is inactivated by GSH (Fig. 9).

Surprisingly, phenol failed to increase the LPO radical formation, even though the presence of 1 mM phenol increased the signal intensity of the DMPO/glutathiyl radical adduct by about 8-fold (Fig. 5). Phenols are known to be good substrates for peroxidases (82,83). LPO, MPO and HRP invariably catalyze one-electron oxidation of phenols to phenoxy radical intermediates (49,84,85). Some phenoxy radicals can then be reduced by GSH to form glutathiyl radical (“thiol pumping”) (86-88). However, 1 mM phenol inhibited the formation of the LPO radical-derived DMPO nitrone adduct by 94 % (Fig. 4), indicating that in this reaction system, the main role of phenol was not to function as a thiol pump. Phenols have been reported to be able to reduce compound II of LPO to its ferric form at a rate constant of $1.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (49). We also found that at a very low concentration of 10 µM, phenol alone rapidly resulted in the reduction of compound II (data not shown) and 1 mM phenol prevented the spectral changes of LPO compound II caused by GSH (Fig. 8B). Thus, we suggest that the inhibitory effect of phenol on the
protein radical formation may be mainly attributed to its strong ability to reduce compound II of LPO to the native enzyme. Nitrite behaved similarly to phenol in inhibiting the LPO radical formation but enhancing the glutathiol radical formation (Figs. 4 and 5). Like phenol, nitrite has also been reported to reduce compound II of LPO to its native ferric enzyme with a rate constant of $3.5 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ (41). In the present work, we found that nitrite alone or in the presence of GSH rapidly caused the reduction of compound II (Fig. 8C). These results indirectly suggest the requirement of compound II for the LPO-derived protein radical formation, which supports the mechanism proposed above.

In summary, this work has for the first time reported the formation of the protein radical-derived nitrone adduct during the reaction of LPO with GSH in the presence of DMPO, which was detected by ELISA and Western blot assays, but could not be detected by ESR spin-trapping. The role of the glutathiol radical thus formed is not only to indirectly generate $\text{H}_2\text{O}_2$, which is necessary for the LPO radical formation, but also to directly mediate the protein radical formation by reacting with the heme of LPO compound II to form a hem-centered radical which was trapped by DMPO.

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LEGENDS

FIG. 1. Immunochemical detection of the lactoperoxidase radical-derived nitrone adduct generated from lactoperoxidase by GSH in the presence of DMPO. (A), GSH-dependent formation of the LPO radical-derived DMPO nitrone adduct by ELISA. 0.2 µM of LPO reacted with varying concentrations of GSH in the presence of 100 mM DMPO with (closed circles) or without (closed squares) 100 µM H₂O₂. After 1 h of incubation at 37 °C, the reaction was stopped by addition of 5 mM ascorbic acid, then the LPO radical-derived nitrone adduct was quantified by ELISA as described in Materials and Methods. (B), Time-dependent formation of the LPO radical-derived nitrone adduct by GSH. 0.2 µM of LPO was incubated with 2 mM GSH in the presence of 100 mM DMPO at 37 °C. At various time points, the reactions were terminated by adding 5 mM ascorbic acid, then the LPO radical-derived DMPO nitrone adducts were quantified by ELISA as described in Materials and Methods. Data are represented as mean ± S.D. from three independent determinations using the fresh preparations of LPO and GSH. (C-D), Identification of the LPO radical-derived DMPO nitrone adduct. 100 µg/ml of LPO reacted with 100 µM H₂O₂ or indicated concentrations of GSH in the presence or absence of 100 mM DMPO at 37 °C for 1 h, and then the samples were dialyzed twice against 0.1 M phosphate buffer, pH 7.4 for 6 h. After separated by SDS-PAGE, the proteins (1.0 µg/lane) were stained by (C) coomassie blue stain or (D) transferred to nitrocellulose membranes and analyzed by Western blot as described in Materials and Methods.

FIG. 2. Concentration- and pH-dependent effects of hydrogen peroxide, lactoperoxidase, and DMPO on the formation of the lactoperoxidase radical-derived
**DMPO nitrone adducts.** (A), Dose-dependent effect of hydrogen peroxide on the formation of the LPO radical-derived DMPO nitrone adduct from the reaction mixture containing 0.2 µM LPO and 0.5 (closed squares) or 10 mM (closed circles) GSH in the presence of 100 mM DMPO. *p < 0.05, significantly different vs. controls (without H₂O₂). (B), Dose-dependent effect of lactoperoxidase on the formation of the LPO radical-derived DMPO nitrone adduct from the reaction mixture containing varying concentrations of LPO and 2 mM GSH in the presence of 100 mM DMPO. (C), Dose-dependent effect of DMPO on the formation of the LPO radical-derived nitrone adduct from the reaction mixture containing 0.2 µM LPO and 2 mM GSH in the presence of various concentrations of DMPO. (D), pH-dependent effect on the formation of the LPO radical-derived DMPO nitrone adduct from the reaction mixture containing 0.2 µM LPO, 2 mM GSH and 100 mM DMPO incubated in 200 mM acetate buffer (pH 5), 200 mM phosphate buffers (pH 6-8), or 50 mM glycine/sodium hydroxide buffer (pH 9). After 1 h of incubation at 37 °C, these reaction mixtures were stopped by adding 5 mM ascorbic acid, then the LPO radical-derived nitrone adducts were quantified by ELISA as described in Materials and Methods. Data are represented as mean ± S.D. from three independent determinations using the fresh preparations of LPO and GSH.

**FIG. 3. Formation of the lactoperoxidase radical-derived DMPO nitrone adduct by various thiol compounds.** The reaction mixture containing 0.2 µM LPO, 1 mM various thiol compounds and 100 mM DMPO was incubated at 37 °C for 1 h. The reaction was stopped by adding 5 mM ascorbic acid, then the nitrone adduct formed was quantified by ELISA as described in Materials and Methods. Data are represented as mean ± S.D. from
three independent determinations using the fresh preparations of LPO and thiol compounds.

FIG. 4. **Effects of inhibitors on the formation of the lactoperoxidase radical-derived DMPO nitrone adduct.** The reaction mixture containing 0.2 µM LPO, 2 mM GSH and 100 mM DMPO with or without 1 mM NaN₃, 50 µg/ml catalase, 0.5 mM ascorbate, indicated concentrations of iodide (or thiocyanate), 1 mM phenol, or 1 mM nitrite was incubated at 37 °C for 1 h. The reactions were stopped by adding 5 mM ascorbic acid, and then the nitrone adducts formed were quantified by ELISA as described in Materials and Methods. Data are represented as mean ± S.D. from three independent determinations.

FIG. 5. **DMPO spin trapping of the glutathiy radical formed from the reaction of lactoperoxidase with GSH.** The reaction mixture containing 0.2 µM LPO, 2 mM GSH and 100 mM DMPO with or without various indicated compounds was incubated at room temperature for 1 min, and then transferred to the ESR cavity for ESR measurements. Instrumental conditions were described in Materials and Methods.

FIG. 6. **Effects of DMPO and catalase on oxygen consumption during the reaction of GSH and lactoperoxidase with or without phenol.** (A), Complete system with 8 µM LPO, 10 mM GSH, and 20 µM phenol. (B), As in A, but 100 mM DMPO was added 2 min after LPO initiation. (C), As in A, but 0.01 mg/ml catalase was added 2 min after LPO initiation. (D), As in A, but phenol was omitted. (E), As in D, but 0.01 mg/ml
catalase was added 4 min after LPO initiation. (F), As in D, but 100 mM DMPO was added 3 min after LPO initiation. (G), As in D, but 0.01 mg/ml catalase was added 1 min before LPO initiation. (H), As in D, but 2 mM GSH replaced 10 mM GSH. (I), As in D, but 100 mM DMPO was added 1 min before LPO initiation. The arrow indicates the time at which LPO, DMPO, or catalase was added to the reaction system.

FIG. 7. Involvement of amino acid residues in the formation of the LPO radical-derived DMPO nitrone adduct by GSH. (A), ELISA assay for the protein radical-derived DMPO nitrone adduct formed from the reactions of native and modified LPO with GSH. After chemical modification of free cysteine, tyrosine and tryptophan residues on LPO as described in Materials and Methods, 0.2 µM of native or modified LPO was reacted with 2 mM GSH in the presence of 100 mM DMPO. After 1 h of incubation at 37 °C, the reaction was stopped by adding 5 mM ascorbic acid, and then the LPO radical-derived nitrone adduct was quantified by ELISA. *p > 0.05, not significantly different vs. control (native LPO). (B), SDS-PAGE assay for cross-linking of LPO. After the reactions of LPO with H₂O₂ or GSH were terminated by adding catalase for the systems containing H₂O₂ or ascorbic acid for the system containing GSH, the proteins were separated on reducing 4-12% Bis-Tris NuPAGE (Invitrogen, Carlsbad, CA) and then stained with Coomasie Blue. Lane 1, molecular marker; Lane 2, LPO alone incubated at 37 °C for 60 min; Lane 3, LPO (10 µM) + H₂O₂ (12.5 µM) incubated at 25 °C for 5 min; Lane 4, LPO (10 µM) + H₂O₂ (12.5 µM) incubated at 37 °C for 60 min; Lane 5, LPO (10 µM) + GSH (0.5 mM) incubated at 37 °C for 60 min; Lane 6, LPO (10 µM) +
GSH (1 mM) incubated at 37 °C for 60 min; Lane 7, LPO (10 µM) + GSH (2 mM) incubated at 37 °C for 60 min; Lane 8, LPO (10 µM) + GSH (5 mM) incubated at 37 °C for 60 min; Lane 9, LPO (10 µM) + GSH (10 mM) incubated at 37 °C for 60 min.

FIG. 8. Effects of phenol and nitrite on spectral changes of compound II of lactoperoxidase by GSH. (A), Spectra changes of LPO compound II by GSH. The reaction was started by adding 2 mM GSH to compound II (curve b), which had been formed by the addition of 4 µM H₂O₂ to 2 µM LPO (curve a). The spectra were recorded 10 min (curve c) and 60 min (curve d) after GSH addition by scanning from 350 nm to 700 nm. (B), Effect of phenol on the spectral changes of LPO compound II caused by GSH. The reaction was started by adding 1 mM phenol and 2 mM GSH to compound II (curve b), which had been formed by the addition of 4 µM H₂O₂ to 2 µM LPO (curve a). The spectra were recorded 2 min (curve c) and 20 min (curve d) after the addition of phenol and GSH by scanning from 350 nm to 700 nm. (C), Effect of nitrite on the spectral changes of LPO compound II caused by GSH. The reaction was started by adding 1 mM nitrite and 2 mM GSH to compound II (curve b), which had been formed by the addition of 4 µM H₂O₂ to 2 µM LPO (curve a). The spectra were recorded 2 min (curve c) after the addition of nitrite and GSH by scanning from 350 nm to 700 nm.

FIG. 9. Dose-dependent effect of GSH on the peroxidase activity of lactoperoxidase. 0.2 µM LPO was preincubated with varying concentrations of GSH for 60 min in 96 well plates. After 3 washes using 100 mM phosphate buffer (pH 7.4) to remove excessive GSH, a 300µl ABTS assay solution containing ABTS (0.5 mM) and H₂O₂ (100 µM) in
the phosphate buffer (100 mM, pH 7.4) was added to each well. Formation of the ABTS radical cation was recorded with time at 415 nm.
Figure 1
Figure 2

A. Concentration of \( \text{H}_2\text{O}_2 \) (µM) vs. Antibody binding (Relative light units)

B. Concentration of Lactoperoxidase (µM) vs. Antibody binding (Relative light units)

C. Concentration of DMPO (mM) vs. Antibody binding (Relative light units)

D. pH vs. Antibody binding (Relative light units)
Figure 3
Figure 4

Antibody binding (Relative light units)

Control
+ 1 mM NaN₃
+ 50 µg/ml Catalase
+ 0.5 mM Ascorbate
+ 2 µM I⁻
+ 10 µM I⁻
+ 2 µM SCN⁻
+ 10 µM SCN⁻
+ 1 mM Phenol
+ 1 mM NO₂⁻
Control + 1 mM NaN₃ + 0.5 mM Ascorbate + 2 µM I⁻ + 50 µg/ml Catalase + 100 µg/ml Catalase + 0.1 mM Ascorbate + 0.5 mM Ascorbate + 2 µM I⁻ + 10 µM SCN⁻ + 10 µM SCN⁻ + 1 mM Phenol + 1 mM NO₂⁻

Figure 5
Figure 6
Figure 7
Figure 8
Figure 9

ABTS$^+$ formation (A at 415 nm)

Time (sec)

GSH

(0 mM)

(0.01 mM)

(0.05 mM)

(0.1 mM)

(0.5 mM)

(2 mM)

(10 mM)
Scheme 1 Proposed mechanisms for the formation of the GSH-dependent LPO-derived protein radical
Protein radical formation during lactoperoxidase-mediated oxidation of the suicide substrate glutathione: Immunochemical detection of A lactoperoxidase radical-derived DMPO nitrone adduct

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