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Ceruloplasmin Induces Polymorphonuclear Leukocyte Priming in Localized Aggressive Periodontitis

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Abstract

Background—Polymorphonuclear leukocytes (PMNs) from subjects with localized aggressive periodontitis (LAgP) present multiple functional abnormalities associated with a phenotypically primed PMN phenotype. Local inflammation is characterized by hypoxia, which leads to increased production of superoxide (O_2^-) by PMNs. Ceruloplasmin (CP) is also induced by hypoxia and inflammation. The aim of this study was to investigate the role of CP in O_2^- generation in PMNs from healthy subjects and patients with LAgP.

Methods—PMNs were isolated from healthy subjects and those with LAgP ($N = 36$). Superoxide was measured by cytochrome-C reduction at 550 nm. Intracellular CP expression was analyzed by real-time polymerase chain reaction and Western blotting. Serum levels of CP were measured by enzyme-linked immunosorbent assay. Intracellular iron ion conversion was spectrophotometrically determined by measuring the absorbance of σ -phenanthroline at 510 nm.

Results— O_2^- generation was significantly higher in LAgP PMNs before and after stimulation with formyl-methionylleucyl-phenylalanine (100 nM). CP expression in PMNs and CP levels in serum were significantly higher in subjects with LAgP compared to the PMNs and serum samples from matched healthy donors ($P < 0.05$). LAgP PMNs also had significantly higher levels of Fe^{3+} and lower levels of Fe^{2+} compared to healthy PMNs ($P < 0.05$), suggesting increased iron conversion. Exogenous CP treatment of healthy PMNs resulted in significant increases in O_2^- generation and iron ion conversion similar to LAgP PMNs.

Conclusion—LAgP PMNs are primed to express higher levels of CP, leading to hypoxia-mediated O_2^- generation in PMNs and increased oxidative stress and neutrophil-mediated tissue injury in LAgP.

Keywords

Ceruloplasmin; hypoxia; iron ion; priming; superoxide

Localized aggressive periodontitis (LAgP) is characterized by alveolar bone destruction with interproximal attachment loss around incisors and first molars.¹ The peripheral blood polymorphonuclear leukocytes (PMNs; neutrophils) from patients with LAgP present multiple

functional abnormalities associated with intracellular signaling defects.²⁻⁴ It was suggested that the pathogenesis of LAgP is associated with a “constitutively hyperactive (primed)” PMN phenotype, leading to neutrophil-mediated tissue damage.⁵ Combined with reduced chemotactic migration and motility,^{6,7} a primed PMN releases greater quantities of superoxide and granule contents in response to secondary stimuli, which, in turn, leads to oxidative and proteolytic damage in tissues. PMNs undergo an “oxidative burst” that results in the release of large amounts of reactive oxygen species (ROS) designed to kill invading bacteria.^{8,9} The oxidative killing is regulated by activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is an assembly of cytosolic and membrane components in the cell membrane.¹⁰⁻¹⁴ In primed PMNs, various cytosolic components of the NADPH oxidase (particularly the cytosolic subunit p47^{phox}) pretranslocate to the membrane.¹⁴ When the PMNs are exposed to further stimulation, the cells overrespond, producing a more rapid and greater response compared to the same stimulus by normal PMNs.^{12,13,15} Thus, the inactivation of NADPH oxidase and scavenging of extracellular superoxide by the enzyme superoxide dismutase (SOD), which occurs at the site of inflammation to limit ROS secretion and collateral tissue damage, does not take place in a timely manner in primed PMN-mediated inflammatory conditions.¹⁵ However, the exact mechanism of PMN priming in LAgP is not clear.

Iron ions are a critical component for NADPH oxidase activation and the production of superoxide,¹⁶ as well as for a variety of important biologic processes, including cell growth and differentiation; electron transfer reactions; and oxygen transport, activation, and detoxification.¹⁶⁻¹⁹ Iron is directly involved in cytotoxic immune defense mechanisms, where it catalyzes the formation of the hydroxyl radical (OH⁻) via Fenton and Fenton-like reactions.²⁰ Iron metabolism is regulated by transferrin and ceruloplasmin (CP).²¹⁻²³ CP is a multicopper-binding plasma protein containing ferroxidase activity necessary for ferric ion saturation of transferrin.^{24,25} CP catalyzes the conversion of ferrous to ferric ion and facilitates the net flux of iron between cells and tissues.^{25,26} Alterations in serum iron are often accompanied by changes in serum CP.²² CP acts as an anti-inflammatory reagent, limiting oxidative stress by its ability to scavenge superoxide anion radicals.^{27,28} Local inflammation is characterized by hypoxia, which stimulates the expression of hypoxia inducible factor (HIF)-1 α , which regulates the tissue response to hypoxia.^{29,30} CP is a downstream target for HIF-1 α ^{22,23} and may play a central role in excessive superoxide generation in phenotypically hyperactive and primed peripheral blood PMNs in LAgP when exposed to local inflammation-induced hypoxia. To test this hypothesis, we evaluated CP expression and regulation in human PMNs from healthy donors and patients diagnosed with LAgP.

MATERIALS AND METHODS

Subject Recruitment and PMN Isolation

The study was approved by the Institutional Review Board of Boston University (September 14, 2006 to July 16, 2008). All subjects provided written consent prior to blood donation. Normal PMNs were obtained from systemically healthy individuals (n = 36; age range: 21 to 39 years; average age, 30.7 $\pm$ 4.9 years; nine males and 27 females), and LAgP PMNs were obtained from subjects diagnosed with LAgP (n = 36; age range: 16 to 41 years; average age, 29.0 $\pm$ 7.7 years; 10 males and 26 females) using the criteria established by the American Academy of Periodontology nomenclature workshop of 1996.¹ Eighty-nine percent of subjects with LAgP (32 of 36) were of African American descent, and the remaining 11% were evenly distributed among white, Asian, and Hispanic races. Control subjects were age-, gender-, and race-matched when possible; control subjects were free of systemic disease, determined by history, and free of periodontal diseases and other oral diseases, determined by examination. Fifty milliliters of peripheral venous blood was collected into heparinized vacutainer tubes,

and PMNs were isolated using a discontinuous gradient system as previously reported.³¹ Contaminating erythrocytes were lysed with isotonic NH₄Cl buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 120 mM EDTA; pH 7.4). Cell preparations were routinely >99% PMNs with >95% viability as determined by trypan blue exclusion.

Superoxide Generation

Superoxide generation was monitored spectrophotometrically at 37°C as SOD-inhibitable reduction of cytochrome-C at 550 nm.³² Assays were carried out in 96-well microtiter plates with flat-bottomed wells.[§] Control wells contained all of the components of the assay mixture plus SOD^{||} (20 U/ml) to assess cytochrome-C reduction by agents other than superoxide. PMNs (0.5×10^5 cells/well) were suspended in phosphate buffered saline (20 µl/well), stimulated by the addition of formyl-methionyl-leucyl-phenylalanine[¶] (fMLP; 100 nM), and absorbance at 550 nm was recorded in a microplate reader.[#]

Measurements of CP mRNA and Protein in PMNs and Serum of Patients With LAGP

Total RNA was extracted from PMNs (3.0×10^6 cells) with phenol/guanidine isothiocyanate^{**} and subjected to real-time quantitative reverse transcription-polymerase chain reaction analysis using a sequence detection system.^{††} An assay for CP^{‡‡} was used, and the mRNA levels were adjusted to constitutive β-actin.^{§§}

Western blotting was performed as previously described.³³ Briefly, PMNs (3.0×10^6 cells) were lysed rapidly in urea buffer (1% [weight/volume; w/v] sodium dodecyl sulfate, 6.2 M urea, 10% glycerol, 5 mM dithiothreitol, 1% 2-mercaptoethanol, 0.01% [w/v] bromophenol blue, 1% [volume/volume; v/v] protease inhibitor cocktail,^{|||} and 1 mM phenylmethylsulfonyl-fluoride) and boiled for 5 minutes. Aliquots were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20 mg/lane) on 7.5% (v/v) polyacrylamide slab gels and transferred electrophoretically to polyvinylidene fluoride membranes^{¶¶} at 100 V for 1 hour at 4°C. Membranes were blocked for 1 hour at room temperature with 5% skim milk in tris-buffered saline (TBS) (pH 7.6) and incubated with primary antibody (mouse anti-human ceruloplasmin;^{##} 1:500 dilution). The membranes were subsequently washed with TBS with 0.01% Tween 20 and then incubated with secondary antibody (goat anti-mouse immunoglobulin G-horseradish peroxidase [HRP] conjugate;^{***} 1:2,000 dilution) in TBS-T with 5% skim milk for 1 hour at room temperature. HRP activity visualized as color change was quantified using an electrochemiluminescence detection system^{†††} followed by autoradiography. The blots were restained with an anti-β-actin antibody^{‡‡‡} (1:2,000 dilution) to confirm that equal amounts of protein were present in each lane. The band densities were measured using an imaging densitometer^{§§§} and normalized.

[§]Linbro type, Flow Laboratories, McLean, VA.

^{||}Sigma-Aldrich, St. Louis, MO.

[¶]Sigma-Aldrich.

[#]VMax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA.

^{**}TRIzol, Invitrogen, Carlsbad, CA.

^{††}ABI Prism 7100, Applied Biosystems, Foster City, CA.

^{‡‡}TaqMan Gene Expression Assay, Applied Biosystems.

^{§§}Applied Biosystems.

^{|||}Sigma-Aldrich.

^{¶¶}Millipore, Bedford, MA.

^{##}BD Biosciences, Rockville, MD.

^{***}Cell Signaling Technology, Danvers, MA.

^{†††}Thermo Fisher Scientific (Pierce), Rockford, IL.

^{‡‡‡}Sigma-Aldrich.

^{§§§}Bio-Rad, Hercules, CA.

CP concentrations in blood serum were measured by enzyme-linked immunosorbent assay (ELISA),^{||||} according to the manufacturer's protocol. The reaction was stopped with 2 M H₂SO₄, and the absorbance was measured at 450 nm.

Iron Conversion

Intracellular iron ions (ferric and ferrous ions) were released from PMNs by sonication in a hypotrophic solution, and intracellular and serum iron ions were detected. Ferrous ion was detected spectrophotometrically by measuring the absorbance of σ -phenanthroline^{|||} (7.5 mM) at 510 nm. Ferric iron was calculated by measuring ferrous ion after ferric ion was reduced to ferrous ion with hydroxylamine hydrochloride^{###} (0.33% w/w). Assays were carried out in 96-well plates. Control wells contained all of the components of the assay mixture, except for σ -phenanthroline, to assess σ -phenanthroline-induced absorbance at 510 nm.

Statistical Analysis

Experimental results are presented as mean $\pm$ SEM. The Student *t* test or analysis of variance (ANOVA) with post hoc analyses (Bonferroni correction for multiple comparisons) was used for analysis of relative density and fold induction. *P* < 0.05 was considered statistically significant.

RESULTS

Superoxide Generation in Untreated PMNs

To confirm that the LAgP PMNs presented a primed phenotype, superoxide generation was evaluated and compared to normal PMNs. Figure 1 demonstrates that the baseline superoxide release was increased prior to secondary challenge in peripheral blood PMNs from subjects with LAgP compared to cells from healthy individuals. In response to treatment with fMLP (100 nM), superoxide generation was significantly enhanced compared to healthy PMNs (*P* < 0.01).

CP mRNA and Protein Expression Levels in Serum and PMNs

CP expression in healthy and LAgP PMNs was assessed at the mRNA and protein levels. Compared to healthy PMNs, LAgP PMNs expressed significantly more CP mRNA (Fig. 2A). In healthy PMNs, CP protein levels were minimal without any stimulation. However, compared to healthy PMNs, LAgP PMNs had significantly higher levels of CP expression (Fig. 2B), and the difference was statistically significant as assessed by densitometric normalization to constitutive actin level (Fig. 2C; *P* < 0.05). Furthermore, CP protein levels in serum samples from subjects with LAgP were significantly higher than those from healthy individuals (Fig. 2D), suggesting that CP mRNA and intracellular and extracellular CP protein are increased in LAgP.

Iron Ion Content of Unstimulated PMNs and Serum

Intracellular ferric and ferrous ions were analyzed to investigate whether iron conversion was altered in LAgP PMNs in response to CP expression and activity. Ferric ion (Fe³⁺), which catalyzes the superoxide generation in PMNs, was significantly elevated in LAgP PMNs compared to normal PMNs (*P* < 0.01), in which >90% of the intracellular iron is in the ferrous ion (Fe²⁺) form (Fig. 3A). However, iron ions were not different in sera of healthy individuals and patients with LAgP (Fig. 3B), suggesting that the systemic iron content is not changed by

^{||||} GenWay, San Diego, CA.

^{|||} Sigma-Aldrich.

^{###} Sigma-Aldrich.

increased CP in serum, but that increases in intracellular ferric ion are associated with increased PMN oxidative stress.

CP Increases Iron Ion Conversion and Enhances Superoxide Generation

To test the hypothesis that elevated CP is responsible for increased ferric ion content and, thus, for increased superoxide generation by LAgP PMNs, normal cells from healthy individuals were treated with CP (10 nM) for 8 hours, and intracellular iron ion conversion was analyzed. CP treatment increased ferric ion and decreased ferrous ion in healthy PMNs (Fig. 4A) and resulted in a significant increase in superoxide generation in response to 100 nM fMLP ($P < 0.05$; Fig. 4B). Healthy PMNs were also exposed to exogenous ferric ion (500 nM) to demonstrate that it induced the increased superoxide generation and not CP by an alternate pathway. Ferric ion-treated PMNs released significantly greater amounts of superoxide ($P < 0.05$; Fig. 4B).

DISCUSSION

The aim of this study was to investigate the role of CP in the priming of LAgP PMNs and the release of excess superoxide anion, leading to increased oxidative stress. Several signaling alterations have been suggested to play a role in the priming of LAgP PMNs, and studies²⁻⁴ identified major differences compared to healthy PMNs. However, the exact mechanism of release of increased levels of superoxide by LAgP PMNs in the presence or absence of a secondary stimulus remains unclear. Intracellular iron conversion has been associated with the oxidative burst in PMNs,^{34,35} and this process is regulated by CP, which contains ferroxidase activity necessary for ferric ion saturation of transferrin.^{24,25} The experiments presented here demonstrate that CP expression and activity were significantly higher in PMNs and sera from subjects diagnosed with LAgP compared to healthy controls. This increase was consistent with a high content of ferric ion in LAgP PMNs. Direct stimulation of healthy cells with CP or ferric ion resulted in increased superoxide generation, further confirming the role of CP in LAgP PMN priming.

CP is a 132-kDa protein with multiple copper-binding domains, which is abundant in serum and increases during inflammation.³⁶ Oxygen tension is lower in inflamed tissues, and HIF-1 α is activated. CP is a primary target gene for HIF-1 α ,²² and CP serum levels are increased in inflammation.³⁷ CP regulates iron ion conversion and uptake into cells via transferrin after iron conversion.^{22,38} An increase in ferric ion, in turn, regulates superoxide formation.³⁹ Based on these reports in the literature, we hypothesized that CP plays an important role as a regulatory element leading to increased superoxide production in primed LAgP PMNs. The results support this hypothesis. CP was increased in LAgP PMNs and serum. In parallel with CP expression, the ferric ion content in LAgP PMNs was also significantly higher compared to healthy PMNs, whereas the serum levels of ferric and ferrous ion were similar. These results suggest that LAgP PMNs release more superoxide as a result of a constitutively higher expression of CP, and they react to hypoxia in local inflamed gingival tissues with excessive production of ROS. The role of hypoxia and HIF-1 α in LAgP is under investigation, and future studies will identify the specific pathways through which LAgP PMNs react to hypoxic conditions. However, it is clear that LAgP PMNs synthesize more CP than healthy PMNs and probably respond to elevated serum CP by prematurely converting intracellular ferrous ion to ferric ion and, when stimulated with secondary agonists, generate more superoxide.

The role of CP in inflammation is not fully understood. Although CP can act as an anti-inflammatory agent, limiting oxidative stress because of its ability to scavenge superoxide anion radicals,^{27,28,40} CP can also work as a proinflammatory molecule.⁴¹ The source of elevated CP in sera from patients with LAgP is also not clear. Although our data suggest that CP can be synthesized and released by PMNs, the liver is the major source of CP in blood.²⁶

Thus, further investigation is necessary to elucidate whether CP is an acute-phase reactant from the liver that can be used as a marker of disease. Conversely, if PMNs are the source of CP in LAgP serum, taken together with our findings that CP can stimulate PMN function, it is possible that primed PMNs in LAgP may be further activated by CP release by an autocrine regulatory mechanism.⁴²

Priming of human neutrophils is a complex phenomenon, which refers to the preactivated state of the cells in the peripheral blood and/or tissues.^{12,15} Upon stimulation with secondary agonists, primed cells exhibit a more rapid and increased response of several functions, including superoxide generation and granule release, which may have pathologic consequences when uncontrolled.^{12,15} Various diseases or pathologic conditions have been associated with primed PMNs, and it was shown that priming can be elicited through genetic determinants, bacterial factors, or metabolic agents.^{14,43-45} In PMNs, the activators of cell function can also prime the cells at subthreshold concentrations.^{12,15} For example, tumor necrosis factor- α , interleukin (IL)-1 β and -8, or lipopolysaccharide (LPS) can activate at higher doses or prime the cells to secondary challenge at lower doses.⁴⁵⁻⁴⁷ PMNs in patients with LAgP are primed and, although the exact mechanism of this phenomenon is not fully understood, genetic factors are hypothesized to play a major role.^{5,43} LAgP PMNs generate significantly higher levels of superoxide before stimulation and release excessive amounts of superoxide when challenged with agonists.^{43,48} As in other pathologic conditions in which PMNs are primed, uncontrolled activity of PMNs results in local tissue damage referred to as "PMN-mediated tissue injury."⁵ However, it is not clear how the systemically primed PMNs can result in local tissue damage. The mechanism of PMN priming leading to superoxide generation is regulated by the NADPH oxidase complex, mainly through the phosphorylation and translocation of various components of the oxidase (e.g., p47^{phox}) from the cytosol to the membrane.¹⁴ One of the membrane-bound components of NADPH oxidase complex is gp91^{phox},⁴⁹ which binds iron ion and regulates superoxide generation.⁵⁰ Our results suggest that CP may play a role in priming the PMNs in LAgP, providing the iron source for oxidase activation. Local hypoxia as a result of inflammation results in increased activation of HIF-1 α , which, in turn, activates CP to mediate iron ion conversion from ferrous ion to ferric ion.^{22,23} CP-mediated conversion of ferrous ion to ferric ion increases the intracellular ferric ion content,^{25,26} leading to increased binding of gp91^{phox}.⁵⁰ Thus, PMNs can produce a faster and greater response to secondary challenges and present a "primed" phenotype.^{12,15} Our results showed that LAgP PMNs have increased CP and ferric ion associated with increased superoxide generation, suggesting that local hypoxia leads the genetically primed PMNs to generate excessive levels of superoxide in response to factors originating from the periodontal pathogens (e.g., LPS). LAgP is a complex disease with multiple cellular and microbial factors that have the potential to prime PMNs. Although our results suggest that CP may be a major regulator of PMN priming, the exact mechanism leading to this elevation remains obscure and may be genetically determined.⁴³ In other acquired disease systems, the work by our laboratory and other investigators suggested that other molecular switches, such as p47^{phox}¹⁴, or CD11b/18,¹⁵ could contribute to PMN priming.

CONCLUSIONS

These findings suggest that increased basal superoxide generation in LAgP PMNs is due to an increase in CP-mediated iron ion conversion accounting, at least in part, for the increased tissue damage in local inflamed tissues of the periodontium in LAgP. These findings identify a potential new target for pharmacologic intervention.

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Figure 1.

Resting LAgP PMNs generate more superoxide than healthy PMNs. PMNs were isolated from healthy subjects or patients with LAgP. Superoxide generation was induced by fMLP (100 nM) on resting healthy or LAgP PMNs and assayed using a cytochrome-C reduction assay. Values are mean $\pm$ SEM ($n = 26$). These data show that resting LAgP PMNs generate more superoxide by fMLP stimulation and increase the baseline superoxide generation. * $P < 0.05$, ANOVA. fMLP(-) = no stimulation; fMLP(+) = fMLP stimulation.

Figure 2.

CP mRNA and protein expression in PMNs and serum of subjects with LAgP. PMNs were isolated from healthy subjects and patients with LAgP. **A**) CP mRNA expression was determined by real-time quantitative polymerase chain reaction. The values represent CP mRNA expression levels normalized by β -actin mRNA expression relative to healthy controls (mean $\pm$ SEM; $n = 14$). **B**) CP (upper panel) and actin (lower panel) protein expression were determined by Western blotting. **C**) The densities of CP and actin bands were measured with an image-analysis system. The values represent density levels normalized by actin bands relative to healthy control (mean $\pm$ SEM; $n = 13$). **D**) Sera were collected from peripheral blood from healthy subjects or patients with LAgP, and the concentration of CP in sera was

determined by ELISA (mean $\pm$ SEM; n = 12). These data show that CP mRNA and intracellular and extracellular CP protein are increased in LAgP. $^{\dagger}P < 0.01$, Student t test; $^{\ddagger}P < 0.05$, Student t test.

Figure 3.

PMN intracellular and serum iron ion content in healthy subjects and patients with LAgP. **A)** PMNs were isolated from healthy subjects and patients with LAgP. Intracellular iron ions (ferrous ion and ferric ion) were released from PMNs and analyzed with the σ -phenanthroline method (mean $\pm$ SEM; $n = 5$). **B)** Sera were collected from peripheral blood of healthy subjects or patients with LAgP, and iron ions (ferrous ion and ferric ion) in sera were analyzed with the σ -phenanthroline method (mean $\pm$ SEM; $n = 12$). These data suggest that systemic iron content is not changed by increased CP in serum but that intracellular ferric ion increases are associated with increased PMN oxidative stress. $^{\dagger}P < 0.01$, Student t test; $^{\ddagger}P < 0.05$, Student t test.

Figure 4.

CP increases iron ion conversion and superoxide generation. **A)** Healthy PMNs were treated with or without CP (10 nM) for 8 hours. After 8 hours, intracellular iron ions (ferrous and ferric) were released from PMNs and analyzed with σ -phenanthroline methods (mean $\pm$ SEM; n = 4). **B)** Healthy PMNs were treated by ferric ion (0.5 mM) or CP (10 nM) for 8 hours. Superoxide generation induced by fMLP (100 nM) was assayed by cytochrome-C reduction. The values represent superoxide generation levels (mean $\pm$ SEM) relative to vehicle (n = 4). These data suggest that CP increases superoxide generation via iron ion conversion. [†]P < 0.01, Student t test; [§]P < 0.05, ANOVA with Bonferroni correction.