Direct assessment of the antioxidant property of salivary histatin

Tomoko Komatsu,1,2 Kyo Kobayashi,2 Eva Helmerhorst,3 Frank Oppenheim3 and Masaichi Chang-il Lee2*

1Division of Dentistry for the Special Patient, Department of Critical Care Medicine and Dentistry and Yokosuka-Shonan Disaster Oral Health Research Center & Oxidative Stress/ESR Laboratories, Kanagawa Dental University Graduate School of Dental Medicine, 82 Inaoka-cho, Yokosuka, Kanagawa 238-8380, Japan
2Department of Molecular and Cell Biology, Boston University Henry M. Goldman School of Dental Medicine, Albany street, Boston, MA 02118, USA

(Received 1 June, 2019; Accepted 17 August, 2019)

Histatin, a salivary protein, affects oral homeostasis through preservation of tooth integrity and protection against caries and fungal infections. However, the effects of histatin in the generation of oxidative stress induced by reactive oxygen species and in the oral cavity remain unclear. In this study, the effects of histatin on direct reactive oxygen species scavenging activity were examined using electron spin resonance. We demonstrated, for the first time, that histatin exhibits antioxidant activity against hydroxyl radicals generated by Fenton’s reaction by metal chelation or binding. The direct antioxidant effects of histatin, along with its antimicrobial activity, may be important in the oral protection of salivary proteins.

Key Words: saliva, histatin, oxidative stress, reactive oxygen species, antioxidant

Salivary proteins play crucial roles in oral health as well as several lifestyle-related diseases through multiple host defense functions. These include homeostatic processes, lubrication, antimicrobial activity, and tooth demineralization/mineralization. These salivary elements may function as active indicators of both local and systemic disorders. Hyposalivation may also be a risk factor for acute respiratory infection. Because a number of salivary proteins have been identified, and their primary structures determined, it has become possible to explore their structure/function relationships. Saliva (oral fluid) is a biofluid with a perceived role in the protection of oral cavity surfaces against chemical, mechanical, and microbial attacks. Involvement in this protective role is a complex mixture of proteins and peptides derived from salivary glands, gingival exudate, and cellular debris. Of these components, approximately 30% are low molecular weight proteins, commonly referred to as salivary peptides, which are assigned to four main classes: cystatins, histatins, statherin, and proline-rich proteins. These salivary peptides impact oral cavity homeostasis through preservation of tooth integrity, protection from dental caries, and avoidance of fungal infections.

Reactive oxygen species (ROS) is a collective term for radical species of O2-, HO-, nitric oxide, and non-radical oxygen derivatives. ROS production is a normal part of cellular metabolism. However, ROS overproduction disrupts tissue redox balance, inducing oxidative damage to DNA, lipids, and proteins. Increased ROS level or reduced antioxidant function, including ROS overproduction or impaired ROS removal, is referred to as oxidative stress, and may lead to several conditions. ROS is toxic to cells via enzyme inactivation, protein denaturation, DNA destruction, and lipid peroxidation. These events increase reactive aldehyde levels and lead to cell membrane damage.

Oxidative stress is implicated in various lifestyle-related diseases, including atherosclerosis, myocardial infarction, cerebrovascular disease, diabetes mellitus, cancer, and osteoporosis. Furthermore, ROS causes loss of salivary antioxidant capacity, leading to the development of oral cancer in tobacco chokers and smokers. Antioxidant systems including antioxidant enzymes and antioxidants play a protective role by scavenging ROS. Histatin belongs to a family of slightly basic 3–4 kDa peptides containing multiple histidine residues. These peptides are secreted by parotid, submandibular, and sublingual glands, and were first characterized in the early 1970s as peptides that enhance the glycolytic activity of microorganisms. It was later reported that they have bactericidal and fungicidal properties. Structure-function studies on these proteins have identified distinct domains with specific functional properties. They display antifungal activity against a broad range of pathogens, including Candida albicans, Cryptococcus neoformans, and Aspergillus fumigatus, and have antibacterial properties based on their killing and growth-inhibitory activity against various species of oral bacteria. The main human histatins are histatin 1, 3, and 5. Like other phosphorylated salivary proteins, histatin 1 is involved in the maintenance of tooth enamel mineral and pellicle formation. Among the histatins, histatin 3 displays the highest antifungal activity, and antifungal domains have been located in its N-terminal and middle regions. A segment spanning residues 4–15, designated P-113, has been evaluated for therapeutic efficacy in vivo oral candidiasis. Recently, it has been reported that histatins 1 and 3, but not histatin 5, exhibit wound closure activities in vitro. The inactivity of histatin 5, comprising the 24 N-terminal residues of histatin 3, indicated that the C-terminal 8 residues in histatin 3 are essential for this activity. Because the last 7 of these 8 residues are homologous with the C terminus of histatin 1, this segment is possibly responsible for the wound-healing properties of histatins 1 and 3. Histatins also show affinity for mineral surfaces, reduce calcium phosphate precipitation, and maintain tooth integrity.

The identification of functional regions within salivary proteins is critical to the development of artifical saliva. However, questions remain regarding the roles of salivary proteins, especially histatin, in ROS generation and oxidative stress in the oral cavity. Few studies have investigated the antioxidant effects of salivary proteins by measuring SOD level or lipid peroxidation. While the effect of copper-mediated oxidation of histatin 8 on the generation of HO-, as evaluated by electron spin resonance (ESR), has been reported, the direct effects of histatin on ROS generation have not been investigated. In the current study, we used ESR to investigate the effects of histatin on ROS scavenging effects. Our results provide the first direct evidence of the antioxidant properties of histatin.
**Materials and Methods**

**Reagents.** Xanthine oxidase (XO) [grade III: from buttermilk, chromatographically purified suspension in 2.3 M (NH₄)₂SO₄, 10 mM sodium phosphate buffer (pH 7.8), containing 1 mM EDTA and 1 mM sodium salicylate], xanthine, and superoxide dismutase were obtained from Sigma (St. Louis, MO). Hydrogen peroxide (H₂O₂) and FeSO₄ were obtained from Wako Chemical (Osaka, Japan). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Labotec (Tokyo, Japan).

**Histatin.** Synthetic histatin 1, 3, and 5 were obtained from the American Peptide Company (Sunnyvale, CA) and from Quality Controlled Biochemicals (Hopkinton, MA). Human parotid secretion (HPS) was collected from five healthy volunteers ranging in age from 25 to 38 years. Informed consent was obtained according to approved protocols of the Institutional Review Board at Boston University Medical Center. HPS was collected with a Curby cup device positioned over the orifice of the Stensen’s duct. HPS flow was stimulated with hard sour candies and the secretion was collected in graduated cylindrical tubes placed on ice. A 25-μl aliquot of PS was plated on blood agar (Hardy Diagnostics) to verify the sterility of the collected PS secretion (absence of water-soluble contamination). Dialyzed and lyophilized PS proteins were dissolved in buffer A consisting of 50 mM Tris/HCl and 50 mM NaCl, (pH = 8), applied to a MonoQ HR16/10 column (Amersham Biosciences, Uppsala, Sweden), and eluted at a flow rate of 2 ml/min with buffer B containing 50 mM Tris/HCl and 1 M NaCl (pH = 8) using the following gradient steps: 0–38 min: 0–13% buffer B; 38–233 min: 13–22% buffer B; 233–250 min: 22–40% buffer B. The purity of the synthetic histatins was verified by cationic, anionic PAGE, and reversed-phase analysis.

**Determination of protein concentrations.** Sample protein concentrations were measured using a micro-bicinchoninic acid (BCA) protein assay (Pierce Chemical, Co., Rockford, IL), with bovine serum albumin used as a protein standard.

**In vitro ESR measurement.** HO• was generated by the Fenton reaction (H₂O₂ plus FeSO₄ or CuSO₄) as described previously.

The reaction mixtures comprised H₂O₂ (20 μM) and FeSO₄ (20 μM) or CuSO₄ (20 μM) in 0.1 M phosphate-buffered saline (pH 7.2) containing 50 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as spin trap, with or without salivary protein pretreatment, respectively. Mixtures were transferred to a cell and the DMPO-OH spin adduct was measured using ESR.

For generation of HO• by UV irradiation of H₂O₂, a reaction mixture containing 10 mM DMPO and H₂O₂ (20 mM) in 0.1 M phosphate-buffered saline (pH 7.2), with or without salivary protein pretreatment, was used. Mixtures were transferred to a cell and illuminated at 365 nm, 40 mW using a PAN UV lamp. After 20 s, the DMPO-OH spin adduct was measured with ESR.

O₂•− was generated using the xanthine-XO system, as described previously, followed by UV irradiation of H₂O₂ (20 mM) in 0.1 M phosphate-buffered saline (pH 7.2) containing 50 mM DMPO with or without salivary protein pretreatment, respectively. The mixtures were transferred to a cell and the DMPO-OOH spin adduct was measured with ESR.

ESR observations were performed with a JES-RE 3X, X-band spectrometer (JEOL, Tokyo, Japan) connected to a WIN-RAD ESR Data Analyzer (Radical Research, Tokyo, Japan) at the following instrument settings: microwave power, 8.00 mW; magnetic field, 334.8 ± 5 mT; field modulation width, 0.079 mT; receiver gain, 400; sweep time, 1 min; and time constant, 0.03 s. Hyperfine coupling constants were calculated based on resonance frequency, measured with a microwave frequency counter, and on resonance field, measured with a JEOL ES-FC5 field measurement unit. ESR spectra were used to quantify the detected spin adducts for manganese oxide standards. After recording ESR spectra, their signal intensities, expressed as relative height, were normalized against the signal intensity of the manganese oxide standard. All experiments were repeated a minimum of four times.

**Statistical analysis.** Statistical analyses were performed using Dunnett (OMS, Saitama, Japan). Data were tested for normality. Results are presented as mean ± SD. Two-way analysis of variance was used to compare the averages of three or four concentration levels. P values <0.05 were considered statistically significant.

**Results**

**Effects of histatin 1, 3, and 5 on HO• generation by the Fenton system.** The effects of histatin 1, 3, and 5 on HO• generated from the Fenton reaction were investigated by ESR spin trapping with DMPO. As reported previously, after adding H₂O₂ to FeSO₄, a characteristic DMPO-OH spin adduct spectrum with hyperfine splitting giving rise to four resolved peaks was observed (Fig. 1A, control). Results indicate that the DMPO-OH signal was significantly reduced in a dose-dependent manner, except for the pretreatment of a final concentration of 8 μM of histatin 1 (Fig. 1A and B).

The effects of histatin 1, 3, and 5 on HO• generated using CuSO₄, instead of FeSO₄, were also investigated. Though the peaks were smaller than those generated using FeSO₄, a characteristic DMPO-OH spin adduct spectrum with hyperfine splitting giving rise to four resolved peaks was observed following addition of H₂O₂ to CuSO₄ (Fig. 2A, control). With histatin 1, 3, and 5 (8, 16, and 32 μM) pretreated with CuSO₄ and subsequent addition of H₂O₂, the DMPO-OH signal was significantly reduced in comparison to the control (Fig. 2A and B).

**Effects of histatin 1, 3, and 5 on HO• generation by ultraviolet irradiation of H₂O₂.** The effects of histatin 1, 3, and 5 on HO• generated from UV irradiation of H₂O₂ were investigated by ESR spin trapping with DMPO. As reported previously, following UV irradiation of H₂O₂, a characteristic DMPO-OH spin adduct spectrum with hyperfine splitting giving rise to four resolved peaks was observed (Fig. 3A, control). When H₂O₂ was pretreated with histatin 1, 3, and 5 (8, 16, and 32 μM) and followed by UV irradiation, the DMPO-OH signal was not significantly reduced (Fig. 3A and B).

**Effects of histatin 1, 3, and 5 on O₂•− generation.** The effects of histatin 1, 3, and 5 on XO-mediated O₂•− generation were determined using ESR spin trapping with DMPO. As reported previously, after addition of xanthine to XO, a characteristic DMPO-OOH adduct spectrum with hyperfine splitting giving rise to 12 resolved peaks was observed (Fig. 4A). These signals were quenched by 150 U/ml superoxide dismutase, confirming that they were derived from O₂•− (data not shown). With histatin 1, 3, and 5 pretreatment of XO and subsequent addition of xanthine, however, alteration of the DMPO-OOH signal was not observed at any histatin concentration (Fig. 4A and B).

**Discussion**

Histatin a human salivary protein, has antifungal activity and is susceptible to enzymatic degradation when released into the oral cavity. Histatin 5 exhibits an antifungal effect by decreasing cell metabolism in Candida albicans. Histatin is unique to saliva and is rich in basic amino acid residues, which bind to the cell membranes of yeast and fungi, destroying their membrane structure. While mitochondrial-derived ROS is known to be involved in this antifungal action, the direct effect of histatin on ROS production has not been reported. In this study, we used ESR to evaluate the effect of histatin on ROS generation, showing that reduced HO• generation, but did not decrease O₂•− generation, from xanthine-XO (Fig. 1–4).

First, we confirmed that histatin 1, 3, and 5 suppressed HO• generation by the Fenton reaction in a dose-dependent manner.
Pathophysiological Fenton responses due to iron overload are reported by the ability of iron chelators, such as Desferal, to reduce high-intensity signals from DMPO-OH spin adducts, which are suggestive of \( \text{HO}^\cdot \) formation.\(^{(46,47)}\) The production of \( \text{HO}^\cdot \) from Fenton’s reaction in the living body is important in various ROS-induced diseases, including oral diseases.\(^{(16,47,48)}\)

All histatins are enriched in histidine, an amino acid capable of complexing with divalent metal ions. Histatin is also activated by complexation of its three N-terminal amino acids (\( \text{NH}_2\text{-Asp-Ser-His} \)) with \( \text{Cu}^{2+} \) ions through modification of the ATCUN motif as metal binding site.\(^{(49,50)}\) Therefore, in order to investigate the effects on \( \text{Cu}^{2+} \) ion-mediated ROS production, we examined the effects of histatin on \( \text{HO}^\cdot \) produced from \( \text{Cu}^{2+} \) and \( \text{H}_2\text{O}_2 \). Interestingly, histatin significantly inhibited \( \text{HO}^\cdot \) generation (Fig. 2). Furthermore, in Fenton’s reaction with \( \text{CuSO}_4 \), histatin 1, 3, and 5 suppressed the production of \( \text{HO}^\cdot \), even at low concentrations (Fig. 2). These results suggest that histatin may have two physiological effects: antifungal action activated by \( \text{Cu}^{2+} \), and antioxidant effects by scavenging \( \text{Cu}^{2+} \)-related ROS.

In biological systems, homeostatic balance is maintained...
between ROS production and removal. This occurs even in the oral cavity, and disruption of this balance due to increased production of ROS increases the risk of oral disease.\textsuperscript{(51,52)} Additionally, oxidative stress due to balance modulation of ROS and antioxidants increased production of ROS related to oral diseases such as periodontitis, and to systemic diseases such as diabetes and cardiovascular disease.\textsuperscript{(48,53)} ROS is one of the most effective pathogenic mechanisms of chronic inflammation caused by bacteria, and undoubtedly leads to bone resorption.\textsuperscript{(54,55)} Neutrophils obtained from the peripheral blood of acute apical periodontitis (AAP) patients show increased production of ROS, particularly in response to treatment of chronic periapical granuloma.\textsuperscript{(56)} In addition, antioxidant salivary vitamins are known to be effective against oxidative stress caused by oral diseases such as oral lichen planus.\textsuperscript{(57)} We confirmed that histatin suppressed the production of HO\textsuperscript{•} from the Fenton reaction using FeSO\textsubscript{4} or CuSO\textsubscript{4} with H\textsubscript{2}O\textsubscript{2}.

Fig. 3. Effects of histatins 1, 3, and 5 (8, 16, and 32 \textmu M) or control on HO\textsuperscript{•} generation from ultraviolet (UV) irradiation and H\textsubscript{2}O\textsubscript{2}. ESR spin trapping measurement of HO\textsuperscript{•} generation from UV irradiation and H\textsubscript{2}O\textsubscript{2} in 0.1 M PBS, 50 mM DMPO as spin trap in the absence of histatins (control), or with histatin 1, 3, and 5 pretreatment at 32 \textmu M, respectively. (B) The dose-response of histatins and control on HO\textsuperscript{•} generation from UV irradiation and H\textsubscript{2}O\textsubscript{2} is represented.

Fig. 4. Effects of histatin 1, 3, and 5 (8, 16, and 32 \textmu M) on O\textsubscript{2}•\textsuperscript{−} generation from xanthine oxidase (XO) and xanthine. (A) Electron spin resonance spin trapping measurement of O\textsubscript{2}•\textsuperscript{−} generation from XO (0.1 U/ml) and xanthine (362 \textmu M) in 0.1 M PBS, 440 mM DMPO as spin trap in the absence of histatins (control), or with histatin 1, 3, and 5 pretreatment at a final concentration of 32 \textmu M, respectively. (B) Dose-response of histatin 1, 3, and 5 or control on O\textsubscript{2}•\textsuperscript{−} generation from XO and xanthine.
Desferal, an iron chelator, reduced the high intensity signal of the T. Komatsu

References

1 Nishioka H, Nishi K, Kyokane K. Human saliva inactivates mutagenicity of carcinogens. Mutat Res 1981; 85: 325–333.
2 Burbelo PD, Bayat A, Lebovitz EE, Iadarola MJ. New technologies for the study of the complex oral diseases. Oral Dis 2012; 18: 121–126.
3 Iwabuchi H, Fujibayashi T, Yamane Y, Inui H, Nakao K. Relationship between hypersalivation and acute respiratory infection in dental outpatients. Gerontology 2012; 58: 205–211.
4 Schenkels LC, Veerman EC, Nieuw Amerongen AV. Biochemical composition of human saliva in relation to other mucosal fluids. Crit Rev Oral Biol Med 1995; 6: 161–175.
5 Amado FM, Vitorino RM, Domingues PM, Lobo MJ, Duarte JA. Analysis of the human saliva proteome. Expert Rev Proteomics 2005; 2: 521–539.
6 Cabras T, Pisano E, Boi R, et al. Age-dependent modifications of the human salivary secretory protein complex. J Proteome Res 2009; 8: 4126–4134.
7 Helmerhorst EJ, Oppenheim FG. Saliva: a dynamic proteome. J Dent Res 2007; 86: 680–693.
8 Lendenmann U, Grogan J, Oppenheim FG. Saliva and dental pellicle—a review. Adv Dent Res 2000; 14: 22–28.
9 Schipper RG, Silletti E, Vingerhoeds MH. Saliva as research material: biochemical, physicochemical and practical aspects. Arch Oral Biol 2007; 52: 1114–1135.
10 Bceeley JA. Fascinating families of proteins: electrophoresis of human saliva. Biochem Soc Trans 1993; 21: 133–138.
11 Vitorino R, Lobo MJ, Duarte JR, Ferrer-Correa AJ, Domingues PM, Amado FM. The role of salivary peptides in dental caries. Bisdmed Chromatogr 2005; 19: 214–222.
12 Oppenheim FG, Salih E, Siqueira WL, Zhang W, Helmerhorst EJ. Salivary proteome and its genetic polymorphisms. Ann N Y Acad Sci 2007; 1098: 22–50.
13 Rudney JD, Staikov RK, Johnson JD. Potential biomarkers of human salivary function: a modified proteomic approach. Arch Oral Biol 2009; 54: 91–100.

14 Sun X, Salih E, Oppenheim FG, Helmerhorst EJ. Kinetics of histatin proteolysis in whole saliva and the effect on bioactive domains with metal-binding, antiinflam, and wound-healing properties. FASEB J 2009; 23: 2691–2701.
15 Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994; 344: 721–724.
16 Halliwell B. Biochemistry of oxidative stress. Biochem Soc Trans 2007; 35 (Pt 5): 1147–1150.
17 Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology 2011; 283: 65–87.
18 Sies H. Oxidative stress: a concept in redox biology and medicine. Redox Biol 2015; 4: 180–183.
19 Beevi SS, Ramsead AM, Geetha A. Evaluation of oxidative stress and nitric oxide levels in patients with oral cavity cancer. Jpn J Clin Oncol 2004; 34: 379–385.
20 Nagler R, Dayan D. The dual role of saliva in oral carcinogenesis. Oncology 2006; 71: 10–17.
21 Kobayashi K, Yoshino F, Takahashi SS, et al. Direct assessments of the antioxidant effects of propofol medium chain triglyceride/long chain triglyceride on the brain of stroke-prone spontaneously hypertensive rats using electron spin resonance spectroscopy. Anesthesiology 2008; 109: 426–435.
22 Kobayashi K, Maehata Y, Kawamura Y, et al. Direct assessments of the antioxidant effects of the novel collagen peptide on reactive oxygen species using electron spin resonance spectroscopy. J Pharmacol Sci 2011; 116: 97–106.
23 Patel BP, Rawal UM, Shah PM, et al. Study of tobacco habits and alterations in enzymatic antioxidant system in oral cancer. Oncology 2005; 68: 511–519.
24 Edgerton M, Koshlukova SE. Salivary histatin 5 and its similarities to the other antimicrobial proteins in human saliva. Adv Dent Res 2000; 14: 16–21.
25 De Smet K, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol Lett 2005; 27: 1337–1347.
26 Holtbrook IB, Molan PC. A further study of the factors enhancing glycolysis
