Original Article

Proteomic Analysis of Lipopolysaccharide-treated Submandibular Gland in Rat

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Abstract

We investigated changes in the protein profile of submandibular gland (SMG) with inflammation induced by exposure to lipopolysaccharide (LPS) with the aim of identifying potential molecular markers of injured gland. Lipopolysaccharide ($2.5\,\mu$g) was directly administered into rat SMG unilaterally by retrograde ductal injection. At 12 hr after treatment, the gland was excised and the proteins identified by two-dimensional difference gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Many proteins in the LPS-treated gland showed a marked change compared to those in the contralateral gland. Of particular note were increases in ubiquitin, a highly-conserved small regulatory protein and in calgranulin B, which has an immunological function in inflammation. Proteins related to apoptosis and stress also showed change in the inflamed gland. The results of this study suggest that the ubiquitin system of protein modification is involved in LPS-induced inflammation in salivary gland, and that a number of specific proteins might be applicable as molecular markers in the monitoring of inflamed or injured gland.

Key words: Proteome—Submandibular gland—Lipopolysaccharide—
Inflamation—Ubiquitin

Introduction

The primary function of the salivary glands, which are typical exocrine glands, is to secrete saliva, a fluid composed of electrolytes, water and a variety of proteins[1,3]. Oral homeostasis is determined to a considerable extent by saliva production: not only must adequate amounts be produced, but a large number of specific proteins must also be secreted for oral function to be properly maintained. Salivary hypofunction increases risk for caries and periodontal disease and compromises oral health[18]. Submandibular gland (SMG), one of the three major salivary glands, produces nerve growth factor, epidermal growth factor, transforming growth factor-β, renin and kallikrein. These proteins regulate immune/

inflammatory responses in the mucosal tissue and participate in regeneration and healing of wound tissue[24]. Submandibular gland also produces antimicrobial proteins such as secretory immunoglobulin A antibody, histatin, cystatin, mucin and defensin. Secretion of these proteins into the saliva results in antibacterial, antifungal and antiviral activities[4,8,14,20].

In the field of dentistry, research on salivary gland and saliva is aimed at assessing risk for diseases and infections originating in the mouth and gland[17,19,27]. An increasing number of recent studies have focused on the diagnostic value of specific constituents of saliva as potential analytic markers of disease and disorders[2,5,26].

Inflammation is the response of tissue to injury or infection. In both acute and chronic inflammation, many inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α, and peptides and proteins are found in cellular infiltrates and are believed to be involved in cellular activation and systemic response[28]. However, little is known about the important issue of inflammatory response in salivary gland[7,29].

The main purposes of this study were to determine the potential of specific proteins, peptides and constituents to serve as diagnostic markers in inflamed-salivary gland and obtain useful information for the monitoring of saliva from inflamed gland. We investigated change in the SMG protein profile with bacterial lipopolysaccharide (LPS)-induced inflammation. We directly administered LPS to rat SMG by retrograde ductal injection and investigated the proteins in the inflamed gland using two-dimensional difference gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

Materials and Methods

1. Materials

Sodium dodecyl sulfate (SDS), dithiothreitol (DTT), trichloroacetic acid (TCA), deoxycholate sodium, urea, thiourea and iodoacetamide were purchased from Wako Pure Chemical Industries (Osaka, Japan); LPS obtained from *Escherichia coli* 0111:B4 and pharmalyte (pH 3–10) from Sigma-Aldrich (St. Louis, MO, USA); immobilized pH gradient (IPG) strip gel and SYPRO Ruby protein gel stain from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and Invitrogen (Carlsbad, CA, USA), respectively. All other reagents used were of high analytical grade.

2. Animals

Male Wistar rats weighing 220–230 g each were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The three rats used in this study were housed in an air-conditioned room (temperature: $23 \pm 2°C$; humidity: $55 \pm 10\%$) under a 12-hr light/dark cycle (lights on between 6:00 am and 6:00 pm) and maintained on commercial laboratory chow and water for at least one week before being used. The rats were treated in accordance with the Guidelines for the Treatment of Experimental Animals approved by The Japanese Pharmacological Society and Tokyo Dental College.

3. LPS treatment and sample preparation

The rats were anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*). A 50-μl-aliquot of LPS, which had been dissolved in physiological saline to 50 μg/ml, was administered by retrograde ductal injection into the SMG using a polyethylene tube[22]. Lipopolysaccharide treatment was performed on the left ductal side. An equivalent volume of physiological saline was injected into the right side of each rat as a control. The rats were sacrificed at 12 hr after treatment under ether anesthesia. The SMG was rapidly removed, frozen with liquid nitrogen and homogenized in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate sodium, 0.1% SDS and a protease inhibitor cocktail (Roche, Mannheim, Germany) with a polytron homogenizer. The homogenate was centrifuged at 13,000 $\times$g for 15 min. All preparative steps were performed at 4°C. The resulting supernatant was

stored at $-80°C$ until used. The protein concentration of the supernatant was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). A 50-μl-aliquot of extracted sample (4 mg protein/ml) was mixed with 8 μl of 100% TCA, kept for 1 hr under ice-cold conditions and centrifuged at $20,000 \times g$ for 20 min at 4°C. The resulting pellet was treated with 1 ml cold ether and re-centrifuged under the same condition. Treatment with ether was repeated three times and the dry sample obtained subjected to 2D-DIGE.

4. Electrophoresis and protein visualization

The 2D-DIGE was performed according to a previous method with some modifications[12]. The dry proteins extracted were dissolved with 50 μl rehydration solution containing 8.5 M urea, 0.2% SDS, 0.2% Triton X-100, 1% DTT and 2% pharmalyte (pH 3–10) and applied to an 18-cm IPG (pH 3–10) strip gel which had previously been treated with 5 ml swelling solution containing 6 M urea, 2 M thiourea, 0.2% DTT, 1% pharmalyte (pH 3–10), 2.5 mM acetic acid, 0.0025% orange G and 2% Triton X-100. Isoelectric focusing (IEF) was carried out at 20°C on a CoolPhoreStar IPG-IEF Type-P (Anatech Co., Ltd., Tokyo, Japan) with the following gradient voltage program: 500 V for 2 hr, 700 V for 1 hr, 1,000 V for 1 hr, 1,500 V for 1 hr, 2,000 V for 1 hr, 2,500 V for 1 hr, 3,000 V for 1 hr and 3,500 V for 10 hr. Prior to SDS-polyacrylamide gel electrophoresis (PAGE), the IPG strip gel was equilibrated in reducing buffer containing 50 mM Tris-HCl (pH 6.8), 6 M urea, 0.5% DTT, 2% SDS, 24% glycerol and 0.0025% bromophenol blue (BPB) for 30 min. The gel was further equilibrated in alkylating buffer containing 50 mM Tris-HCl (pH 6.8), 4.5% iodoacetamide, 2% SDS, 30% glycerol and 0.0025% BPB for 30 min. SDS-PAGE as the second dimension was carried out with a 12% running gel on the CoolPhoreStar SDS-PAGE Dual-200 (Anatech) at a constant 20 mA/gel for 20 min and then at a constant 40 mA/gel until the BPB reached the bottom of the gel. After electrophoresis, the gel was washed with 10% acetic acid and 50% methanol for 30 min, stained with SYPRO Ruby protein gel stain for 2 hr and re-washed with 7% acetic acid and 10% methanol for 30 min. The fluorescence intensity of each protein spot in the gel was digitally recorded with the FluoroPhoreStar 3000 (Anatech) and the gels of the LPS-treated and control glands matched with the Progenesis PG220 software (Shimadzu Co., Ltd., Kyoto, Japan). This procedure was carried out at three times with different rat samples. The result was expressed as the ratio of the volume of the protein spot in the LPS-treated gland divided by the volume of the matched protein spot in the control gland.

5. Protein identification

Each protein spot showing a significant change in intensity compared to that in the control sample was excised from the gel using the FluoroPhoreStar 3000, dehydrated in 50% acetonitrile and 50% ammonium bicarbonate and further dried in 100% acetonitrile. Proteins were digested with an enzyme solution containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 12.5 μg/ml trypsin. Aliquots of the purified samples were spotted on matrix crystals of α-cyano-4-hydroxy-cinnamic acid on a stainless-steel target and air dried. Mass determinations were performed on the AXIMA-TOF[2] Mass Spectrometer (Shimadzu). The proteins were identified by the method of peptide mass fingerprinting, using Mascot Search on the Web (Matrix Science, Ltd., London, UK). This procedure was repeated at least once with spots from different gels. The following search parameter was applied: appropriate isoelectric point and molecular weight/mass range depending on the region of the gel. The criteria used to identify proteins included comparison of the theoretical and observed molecular weights and isoelectric points, probability-based scores and number of matched peptides.
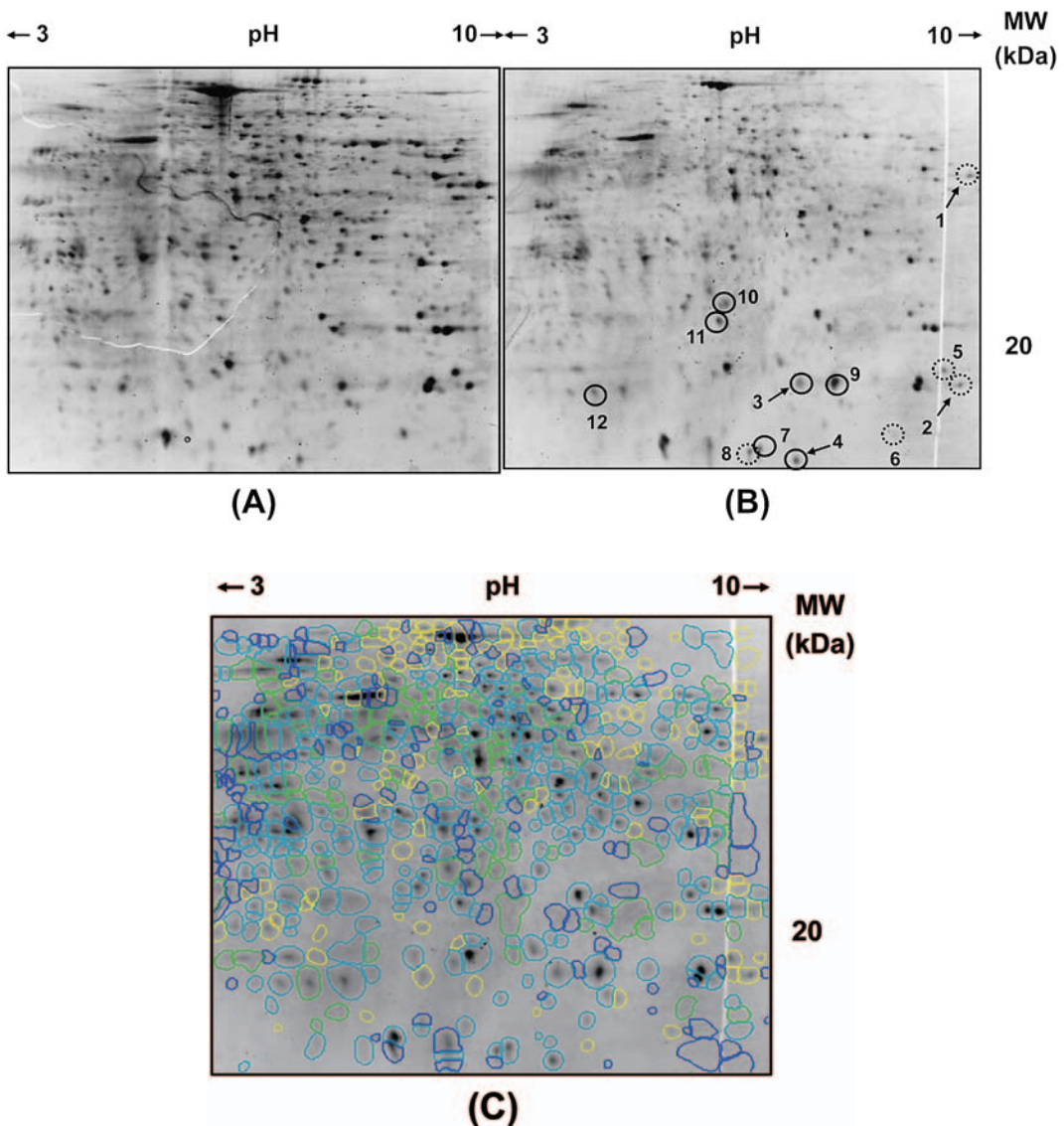
Results

Many proteins in the SMG showed a

Fig. 1 2D-DIGE images of SMG proteins

Proteomic analysis was performed at 12 hr after treatment with LPS, as described in "Materials and Methods". (A): gel of control gland. (B): gel of LPS-treated gland. Closed and dashed-circle spots indicate increased and decreased proteins, respectively, compared with intensities of control spots. Main protein spots identified by MALDI-TOF MS are numbered as follows: 1. malate dehydrogenase; 2. hemoglobin subunit alpha-1/2; 3. calgranulin B; 4. ubiquitin; 5. ena/VASP-like protein; 6. cysteine-rich protein 1; 7. gamma-synuclein; 8. cell division cycle and apoptosis regular 1 isoform 2; 9. 3' histone mRNA exonuclease 1; 10. stress induced-phosphoprotein 1; 11. superoxide dismutase (Cu-Zn); and 12. proto-oncogene tyrosine-protein kinase Src. Details for major spots are listed in Table 1. (C): Matching result of protein spots in control and LPS-treated gland. Analysis was carried out using the Progenesis PG220 software. Yellow field: more than 2-fold decrease in protein; green field: more than 2-fold increase in protein; light blue field: less than 2-fold increase or decrease in protein; dark blue field: unmatched protein. Figures are representative of data obtained from 3 similar experiments.

Table 1　Main SMG proteins showing change at 12 hr after LPS treatment

Spot No.	Protein	Spot intensity	Mass/PI	Score	Matched peptides
1	Malate dehydrogenase	−2.31	36117/8.93	63	18
2	Hemoglobin subunit alpha-1/2	−2.49	15490/7.82	59	5
3	Calgranulin B	2.22	13307/7.05	77	7
4	Ubiquitin	1.76	8560/6.56	55	4

Spot intensity was expressed as ratio of LPS-treated volume/control volume. Values of normal mass and calculated isoelectric point (PI) were obtained from Mascot search results. Each protein score was significant ($p < 0.05$).

marked change at 12 hr after treatment with LPS compared to those in the contralateral gland (Figs. 1A and B). Matching result analysis using the Progenesis PG220 software for 2D-DIGE revealed that protein spots of relatively low molecular weight increased and that spots of relatively high molecular weight decreased (Fig. 1C).

Protein spots showing a more than 1.5-fold change in intensity compared to those in the control samples were further excised and analyzed by MALDI-TOF MS, which yielded sufficient confirmation of protein identity. The main protein spots identified were mitochondrial malate dehydrogenase, hemoglobin subunit alpha-1/2, calgranulin B, ubiquitin, cysteine-rich protein 1, cell division cycle and apoptosis regular 1 isoform 2, gamma-synuclein, stress induced-phosphoprotein 1 and superoxide dismutase (Cu-Zn) (Fig. 1B). The spot intensities of malate dehydrogenase and hemoglobin subunit alpha-1/2 showed an approximately 2.3- and 2.5-fold decrease, respectively, compared to the control levels. On the other hand, the intensities of ubiquitin and calgranulin B increased by approximately 1.8- and 2.2-fold, respectively (Table 1).

Discussion

In this study, we directly administered LPS into rat SMG unilaterally by retrograde ductal injection and investigated protein response to inflammation. In the LPS-treated SMG, proteins of relatively low molecular weight showed an increase, whereas proteins of high molecular weight showed a decrease compared with those in the contralateral gland of the same rat. Of particular note is that there was an increase in ubiquitin and calgranulin B, which are involved in cellular response to inflammation, and a decrease in mitochondrial malate dehydrogenase, an enzyme involved in both the citric acid cycle and gluconeogenesis. Proteins related to stress or apoptosis also showed a change in LPS-treated SMG. LPS treatment resulted in an increase in stress-induced phosphoprotein 1 (STIP1) and a decrease in cell division cycle and apoptosis regulator 1 (CCAR1).

Ubiquitin is a small, highly-conserved regulatory protein that is ubiquitously expressed in eukaryotic cells, and ubiquitination refers to the post-translational modification of a protein. Furthermore, it is proposed that the ubiquitination system functions in a wide variety of cellular processes, including apoptosis, immune response, inflammation, stress response and viral infection[15,16]. Calgranulin B is a small calcium-binding protein with several immunological functions related to inflammation and cancer, and is overexpressed in many autoimmune inflammatory diseases such as rheumatoid arthritis, juvenile idiopathic arthritis and chronic inflammatory bowel disorder[10,11]. Malate dehydrogenase was downregulated under mitochondrial oxidative stress[25]. STIP1, which is known as heat shock protein (HSP)-organizing protein, acts

primarily as an adaptor that directs HSP 90 to HSP 70-client protein complexes, but recent evidence suggests that STIP1 can also modulate the chaperone activities of HSPs[21]. CCAR1 acts as a regulator of apoptosis signaling, as well as cell proliferation[23]. It has been further reported that LPS induced an elevation in levels of antimicrobial β-defensin in rat parotid gland and in IL-1β, an inflammation cytokine, in mouse SMG[7,29]. This strongly suggests that the changes in proteins observed in our present study were induced by local inflammatory response, and that these proteins might therefore be applicable as useful target molecules in monitoring of saliva from inflamed or injured salivary gland.

Salivary gland secretes proteins such as amylase and mucin, which aid digestion and protect the oral cavity[1,3]. In addition to these proteins, many other types of protein and cytokine involved in immune/inflammatory responses, antibacterial and antifungal activities and wound healing such as histatin, cystatin, defensin and IL-1β are also secreted into the saliva[4,8,14,20,24]. Saliva has a physiological function in the maintenance of oral health and oral tissue repair. Salivary secretion, however, is affected by oral and systemic diseases such as cancers, cirrhosis and diabetes[6,18]. In a preliminary experiment, stimulation of LPS-treated SMG with pilocarpine, an agonist of muscarinic receptor, induced a decrease in salivary secretion (data not shown). This suggests the potential of saliva to serve as an important source of useful information analogous to that of blood or urine in the inspection of wounded gland or systemic disease[9,13].

In conclusion, the results of this study suggest that the ubiquitin system of protein modification is involved in the inflammatory response in salivary gland, and that a number of specific proteins could serve as molecular markers in the monitoring of inflamed or injured gland. We plan to further investigate the role of the ubiquitin system in salivary gland during cellular inflammation. Additionally, we also plan to investigate protein levels in saliva in rats and patients with oral inflammation or infection. We believe that such data would provide valuable and practical information for diagnostic and therapeutic purposes in treating patients with oral disorders.

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