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# Activation of Protease-Activated Receptor-2 (PAR-2) Triggers Mucin Secretion in the Rat Sublingual Gland

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**Protease-activated receptor-2 (PAR-2) is distributed throughout the gastrointestinal systems. The present study investigated the role for PAR-2 in the rat salivary glands. PAR-2 mRNA was detected in the sublingual, submaxillary, and parotid glands by a reverse-transcriptase polymerase chain reaction. In the isolated sublingual gland that exhibited the strongest signal for PAR-2, Ser-Leu-Ile-Gly-Arg-Leu-$NH_2$, a PAR-2-activating peptide, and trypsin, a PAR-2-activating enzyme, but not thrombin that can activate PARs 1, 3, and 4, triggered secretion of *N*-acetylneuraminic acid, an indicator of mucin, that was a unique major sialic acid detectable after hydrolysis of the sublingual mucin with 0.1 N HCl. The PAR-2-mediated secretion of mucin was attenuated by genistein, a tyrosine kinase inhibitor, but not by inhibitors of protein kinase C and phosphatidyl inositol 3′-kinase. Thus, PAR-2 is expressed by the three distinct salivary glands in the rat, and sublingual PAR-2 appears to play a role in triggering mucin secretion, at least in part, via activation of tyrosine kinase.** 

***Key Words:*** **protease (proteinase)-activated receptor; trypsin; mucin; sublingual gland.**

Protease-activated receptor-2 (PAR-2) (1) belongs to a growing family of G protein-coupled receptors activated by extracellular proteases, which currently consists of four members (2, 3). Trypsin or mast cell tryptase activates PAR-2 by proteolytic unmasking of the N-terminal extracellular cryptic receptor-activating sequences, while thrombin activates PARs 1, 3, and 4 in the same manner. Synthetic peptides as short as about 6 amino acids based on the receptor-activating motif of PAR-2 (i.e., SLIGRL or SLIGRL-$NH_2$ for murine PAR-2), when applied exogenously, are capable of activating PAR-2 nonenzymatically. Structure-activity studies on PARs-activating peptides have demonstrated that the murine PAR-2-derived peptide SLIGRL-$NH_2$ specifically activates PAR-2 without activating other PARs (4–6).

The widespread distribution of PAR-2 in tissues strongly implies its critical roles in a variety of biological events (3). PAR-2 as well as PAR-1 may be involved in an inflammatory process (7–11). PAR-2 also appears to play multiple roles in gastrointestinal systems including the pancreas (2, 12–14). Most recently, we found that PAR-2-activating peptides, administered *in vivo,* triggered salivation in the mouse and rats (15). Thus, PAR-2 may be a key molecule in regulation of digestive functions. The aim of our study was to ascertain and characterize the role of PAR-2 in the salivary glands. Here, we describe that mRNA for PAR-2 was detected in the three distinct salivary glands isolated from the rat, and that PAR-2 activation triggered *in vitro* secretion of *N*-acetylneuraminic acid (Neu5Ac), an indicator of rat salivary mucin, by the sublingual gland that exhibited the strongest signal for PAR-2 mRNA.

## MATERIALS AND METHODS

*Animals.* Male Wistar rats (7–10 weeks old, Japan SLC. Ltd.) were used according to the notification issued from Japan's Prime Minister's Office, No. 6, 1980.

*Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of mRNAs for PAR-1 and PAR-2 in the sublingual, submaximally and parotid glands isolated from rats.* The sublingual, submaxillary and parotid glands were excised from the rat under urethane (1.5 g/kg) anesthesia. Total RNA was isolated from each gland using the TRIzol Reagent (Life Technologies. Inc., U.S.A.), and then mRNA was purified from the total RNA with the Oligotex-dT30 (Super) mRNA purification kit (Takara Shuzo, Japan). Essentially as described previously (16), mRNA was reverse-transcribed at 42°C for 50 min, and amplified by polymerase chain reaction (RT-PCR) using the RNA LA PCR kit (AMV) ver. 1.1 (Takara Shuzo, Japan). The PCR primers for amplification of PAR-1 sequences were 5′-CCCGCTCATTTTTTCTCAGGA-3′ and 5′-

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GCCAATCGGTCGCGGAGAAGT-3′, leading to amplification of 394-bp fragments. The primers targeted to PAR-2 were 5′-CACCAGTAA-AGGGAGAAGTCT-3′ and 5′-GGGCAGCACGTCGTGACAGGT-3′, yielding amplification of 598-bp fragments. The primers for β-actin were 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-GTCCTTAA-TGTCACGCACGATTTC-3′, amplifying 537-bp fragments. Amplification was allowed to proceed for 30 cycles, beginning with a 30-s denaturation period at 94°C followed by a 30-s reannealing time at 55°C and a 1-min primer extension period at 72°C. The PCR products were separated by 2% agarose gel electrophoresis and visualized by the ethidium bromide staining procedure.

*Determination of N-acetylneuraminic acid as an indicator of mucin secreted from the isolated rat sublingual gland in response to PAR-2 activators.* The sublingual gland is rich in mucous cells that secret mucin, a glycoprotein, upon stimulation. The salivary mucin is abundant in sialic acids in the carbohydrate chains, and the determination of sialic acids is useful to monitor secretion of salivary mucin (17). In the present study, we thus determined *N*-acetylneuraminic acid (Neu5Ac) as an indicator of mucin secreted from the sublingual gland, which was one of the major components among sialic acids that rat sublingual gland mucin contained in our preliminary experiments.

The bilateral sublingual glands were excised from the rat, and freed of fat and connective tissue in an ice-cold Krebs–Henseleit buffer of the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; $KH_2PO_4$, 1.2; glucose, 10. Each whole gland was placed in a tube containing 1.5 ml of the same buffer that was continuously gassed with 95% $O_2$/5% $CO_2$ and maintained at 37°C. After a 30-min preincubation period, the medium was changed to the agonist-containing reaction buffer that had been gassed and maintained at 37°C. After a 10-min incubation, the reaction was stopped by removing the gland from the medium. The reaction medium was concentrated by ultrafiltration (Ultrafree-MC, cut off MW 30,000, Millipore, U.S.A.) for subsequent assay of Neu5Ac secreted. The gland after the reaction was homogenized in 2 ml of the ice-cold buffer and the supernatant after centrifugation (30,000 g, 4°C) was collected for assay of residual Neu5Ac in the tissue. The agonists tested in the present study were trypsin, a PAR-2-activating enzyme, SLIGRL-$NH_2$, a specific PAR-2-activating peptide, LSIGRL-$NH_2$, a PAR-2-inactive control peptide, thrombin, an activating enzyme for PARs 1, 3, and 4. The effect of peptides was examined in the presence of 10 μM amastatin, an inhibitor of aminopeptidase that is primarily responsible for peptide degradation.

Essentially as described previously (18), an aliquot (10 μl) of the above samples was treated with 10 μl of 2 N acetic acid or 0.1 N HCl for 3 h at 80°C to release sialic acids from mucin by hydrolysis. For derivatization of the released sialic acids with DMB (1,2-diamino-4,5-methylenedioxy-benzene, Dojindo Laboratories, Japan), the hydrolyzed sample (20 μl) was mixed with a reagent (200 μl) containing 7 mM DMB, 0.75 M β-mercaptoethanol and 18 mM sodium hydrosulfite in 1.4 M acetic acid, and heated at 50°C for 2.5 h. The DMB-derivatized sialic acids were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) with a Cosmocil 5C18-AR column (150 × 6 mm i.d., Nacalai Tesque, Japan). Separation was performed using a linear gradient (from 7/14/79 to 11/14/75 of acetonitrile/methanol/water) over 40 min, at a flow rate of 0.9 ml/min. Detection was performed fluorometrically at 488 nm with a 373-nm light. The amount of Neu5Ac that was secreted into the reaction medium (S) and remained in the tissue (R) was calculated from a calibration curve obtained using authentic *N*-acetylneuraminic acid. Data are represented as % secretion of Neu5Ac that was calculated as follows: S/(S + R) × 100.

*Evaluation of effects of inhibitors on the Neu5Ac secretion by the rat sublingual gland in response to PAR-2 activation.* To investigate the intracellular mechanisms responsible for PAR-2-mediated secretory response in the sublingual gland, we evaluated effects of GF109203X, genistein and wortmannin, inhibitors of protein kinase C, tyrosine kinase and phosphatidyl inositol 3′-kinase, respectively, that are involved in PARs-mediated signal transduction mechanisms in other tissues (2, 12, 19).

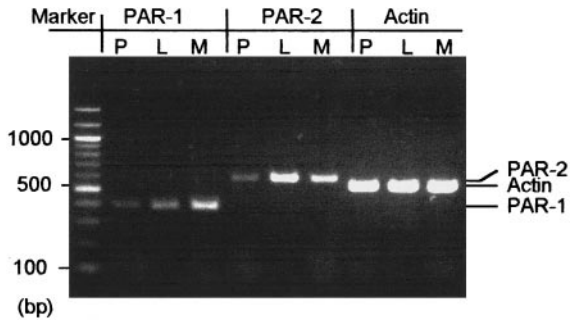


**FIG. 1.** Detection of mRNAs for PAR-2 and PAR-1 in the rat salivary glands. P, L, and M: the parotid, sublingual, and submaxillary glands. The expected positions of the PCR products obtained with the primers for PARs 1 and 2, and for actin are indicated on the right side.

In the preliminary experiments, the content of Neu5Ac in the sublingual gland exhibited a great variation among individual rats, whereas the contents in the right and left glands were almost the same in each rat. In addition, secretory responses of the bilateral glands to PAR-2 activation were also very close each other. Therefore, one of the two sublingual glands isolated from each rat was stimulated with the PAR-2 agonist SLIGRL-$NH_2$ in the presence of inhibitors, and the other was designated to the control that was challenged with the agonist only. After the 30-min preincubation period as described above, the sublingual gland was exposed to the medium containing an inhibitor or its vehicle (control), but not agonists, for 10 min, and then stimulated for 10 min by addition of SLIGRL-$NH_2$ at 100 μM in the presence of 10 μM amastatin.

*Peptides and other main chemicals employed.* Peptides used were prepared by solid-phase synthesis, and peptide composition and purity (>98%) were ascertained by HPLC analysis, amino acid analysis and mass spectrometry. Trypsin from porcine pancreas and human thrombin were purchased from Sigma (U.S.A.), and amastatin was from Peptide Institute Inc. (Japan). GF109203X and genistein were obtained from Research Biochemicals Internationals (U.S.A.), and wortmannin was supplied from Wako Pure Chem. (Japan).

*Statistics.* Data are expressed as means ± SEM. Statistical analysis was performed using the Dunnett's multiple comparison test or the paired *t* test, and an associated probability (*P* value) of less than 5% was considered significant.

## RESULTS AND DISCUSSION

RT-PCR analysis of mRNA harvested from the rat parotid, sublingual and submaxillary glands yielded signals for PCR products of the predicted size for PAR-2 (598 bp; lanes P, L and M under PAR-2, respectively, in Fig. 1). The order of the intensity of signals for PAR-2 in the three glands was roughly "sublingual gland > submaxillary gland > parotid gland," although the RT-PCR analysis employed was not quantitative. Signals for PAR-1 (394 bp) were also detected in the three glands (lanes P, L and M under PAR-1, in Fig. 1).

To clarify the role for PAR-2 in the rat sublingual gland that was the most abundant in PAR-2 mRNA among the three salivary glands on the basis of RT-

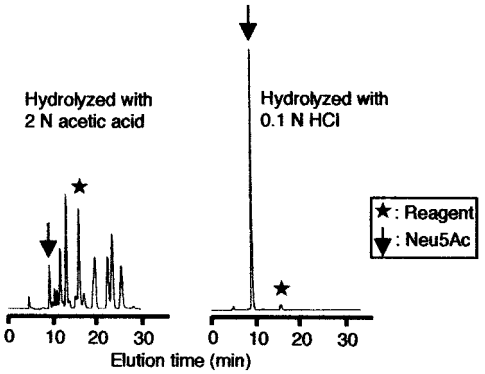


**FIG. 2.** RP-HPLC profile of DMB-derivatized sialic acids after acid hydrolysis in the reaction medium following 10-min incubation of the sublingual gland with the PAR-2-activating peptide SLIGRL-$NH_2$ at 100 $\mu$M. An on-line fluorescence detection was employed to monitor the profile. The samples were hydrolyzed at 80°C for 3 h with 2 N acetic acid (left) or 0.1 N HCl (right).

PCR analysis, we then tried to examine whether PAR-2 agonists could trigger secretion of mucin by the isolated rat sublingual gland *in vitro.* Salivary glands are abundant in sialomucin that is secreted upon stimulation, but the salivary mucin exhibits a great multiplicity in the sialic acid composition depending on species (18). To evaluate an appropriate sialic acid as an indicator of mucin, we analyzed sialic acids in the rat sublingual mucin. Figure 2 illustrates typical HPLC profile of DMB-derivatized sialic acids after hydrolysis by two distinct procedures in the reaction medium after 10 min incubation of the sublingual gland with 100 $\mu$M SLIGRL-$NH_2$, a PAR-2 agonist. RP-HPLC analysis of the sample hydrolyzed with 2 N acetic acid indicated the presence of Neu5Ac and also other multiple sialic acid derivatives. But we could not find the peak derived from N-glycolylneuraminic acid (elution time: 7.5 min). Furthermore, the RP-HPLC of the corresponding sample that was exposed to hydrolysis with 0.1 N HCl exhibited only one large peak for Neu5Ac, implying that the multiple peaks other than a peak for Neu5Ac seen after hydrolysis with 2 N acetic acid were for di- or tri-acetylneuraminic acids as described previously (18), which could be further hydrolyzed with 0.1 N HCl into Neu5Ac. Similar characteristics were also confirmed in the samples from the homogenized sublingual tissues after or without 10-min incubation with the agonist. Taken together, we decided to monitor mucin secretion by determining Neu5Ac in the samples hydrolyzed with 0.1 N HCl.

Figure 3 depicts effects of agonists for PARs on secretion of Neu5Ac by the isolated rat sublingual gland. Trypsin, a PAR-2-activating enzyme, at 1.3 $\mu$M, and SLIGRL-$NH_2$, a PAR-2-activating peptide, at 100 $\mu$M, triggered secretion of Neu5Ac. On the other hand, neither LSIGRL-$NH_2$, a PAR-2-inactive control peptide at a corresponding dose, nor thrombin, an enzyme activating PARs 1, 3, and 4, at 0.1 $\mu$M, induced secretory response. These findings strongly suggest that PAR-2, but not PARs 1, 3, and 4, plays a role in sublingual mucin secretion in the rat, being consistent with the *in vivo* salivation produced by PAR-2 agonists in the rat and mouse (15). The effective concentration (1.3 $\mu$M) of trypsin in the present study was relatively high, but still comparable with that in several PAR-2-mediated systems (13, 14, 20).

Given an absence of trypsin in the salivary glands and of a conventional circulating 'hormonal' ligand, a question to pose is: under what circumstances might sublingual PAR-2 become activated, so as to subserve physiological and/or pathophysiological roles? PAR-2 is involved in the setting of an inflammatory response (8–11), and can be induced in response to inflammatory stimuli (21), implying pro- or anti-inflammatory roles for PAR-2. Tryptase, a mast cell protease that is secreted upon inflammatory stimuli, is capable of activating PAR-2 (22). Taken together, it is hypothesized that, in an inflammatory process, sublingual PAR-2 may become activated in response to tryptase released from mast cells present in the sublingual gland or other tissues, resulting in secretion of mucin that, in turn, could play a role for the maintenance of the integrity of oral defense systems (17). Thus, our finding may predict a novel protective role for PAR-2 in inflammation. Nevertheless, the possibility can not be excluded that an unknown enzyme present in the salivary glands or in the circulating blood might activate sublingual PAR-2 to trigger mucin secretion under certain conditions.

To investigate intracellular signal transduction mechanisms responsible for the PAR-2-mediated mucin secretion in the rat sublingual gland, we evaluated

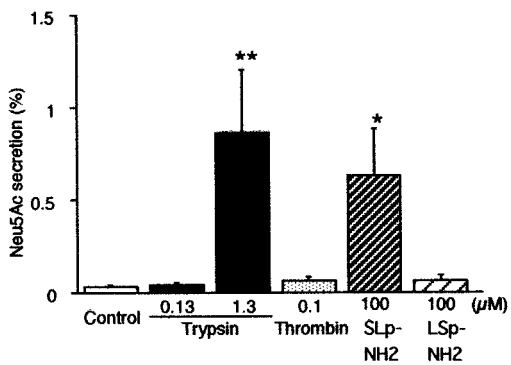


**FIG. 3.** Effects of agonists for PARs on Neu5Ac secretion in the isolated rat sublingual gland. The gland in the reaction medium was incubated at 37°C for 10 min immediately after addition of trypsin, a PAR-2-activating enzyme, thrombin, an agonist enzyme for PARs 1, 3, and 4, SLIGRL-$NH_2$ (SLp-$NH_2$), a PAR-2-activating peptide, or LSIGRL-$NH_2$ (LSp-$NH_2$), a PAR-2-inactive control peptide, in the presence of 10 $\mu$M amastatin. Data indicate the mean with SEM from 11 control and 4–6 challenged tissues. $*P < 0.05$, $**P < 0.01$ vs. the control (Dunnett's test).

**TABLE 1**

Effects of Inhibitors of Protein Kinase C, Tyrosine Kinase, and Phosphatidyl Inositol 3′-Kinase on the PAR-2-Activator SLIGRL-$NH_2$-Induced Neu5Ac Secretion from the Rat Sublingual Gland

| | *n* | % Secretion of Neu5Ac (Mean ± S.E.M.) | Significance |
|---|---|---|---|
| Control | 6 | 0.546 ± 0.129 | |
| GF109203X | 6 | 0.500 ± 0.143 | N.S. |
| Control | 8 | 0.417 ± 0.099 | |
| Genistein | 8 | 0.297 ± 0.067 | $P < 0.05$ |
| Control | 4 | 0.427 ± 0.150 | |
| Wortmannin | 4 | 0.393 ± 0.139 | N.S. |

*Note.* A sublingual gland and contralateral one from each rat were stimulated with SLIGRL-$NH_2$ at 100 $\mu$M in the absence (control) and presence of GF109203X at 1 $\mu$M, genistein at 15 $\mu$M or wortmannin at 0.1 $\mu$M, respectively. Difference from the control was statistically evaluated by paired *t*-test. Neu5Ac, *N*-acetylneuraminic acid; N.S., not significant.

effects of inhibitors of three kinases, tyrosine kinase (TK), protein kinase C (PKC) and phosphatidyl inositol 3′-kinase (PI3K), that can be activated following activation of PARs in some cells or tissues (2, 12, 19). The TK inhibitor genistein at 15 $\mu$M (12) significantly reduced the secretion of Neu5Ac, a mucin indicator, from the sublingual gland in response to SLIGRL-$NH_2$, a PAR-2-activating peptide, at 100 $\mu$M (Table 1). On the other hand, the PKC inhibitor GF109203X at 1 $\mu$M (12) and the PI3K inhibitor wortmannin at 0.1 $\mu$M (12, 19) failed to significantly attenuate the PAR-2-mediated Neu5Ac secretion (Table 1). The three inhibitors at the corresponding concentrations did not alter basal Neu5Ac secretion in the sublingual glands (data not shown). It is also of note that all three inhibitors at the same concentrations significantly reduced the duodenal smooth muscle contraction mediated by PAR-1 that is known to activate TK, PKC and PI3K (19), in our preliminary experiments. Thus, our data strongly suggest that the activation of TK is, at least in part, involved in sublingual mucin secretion due to PAR-2 activation, although PKC and PI3K appear to play, if any, only a minor role in this system. PAR-1 is generally considered to couple to several distinct G proteins such as $G_q$, $G_i$, or $G_o$ (23), leading to activation of phospholipase C (PLC) followed by diacylglycerol formation/PKC activation, and also to activation of other pathways including TK and PI3K (2, 12, 19). On the other hand, little is known about mechanisms of signal transduction that couple to PAR-2, although activation of the PLC/PKC pathway and of TK may occur following PAR-2 activation (2, 12, 23). In general, there are two major signal transduction pathways responsible for protein secretion in the salivary glands; one is the activation of the cyclic AMP/protein kinase A system, and the other is the activation of the PLC/PKC system, which occur following stimulation of $\beta$-adrenoceptors and muscarinic receptors, respectively. The role of tyrosine phosphorylation in the mediation of exocytosis in the salivary glands still largely remains to be investigated (24). Our results provide novel evidence for the potential involvement of TK in regulation of salivary exocytosis.

In conclusion, PAR-2 that is expressed by the three distinct salivary glands in the rat mediates sublingual mucin secretion, at least in part, via activation of TK, which, in turn, might play a role for the maintenance of the oral defense system.

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