ROLE OF ENDOTHELIN-1-DEPENDENT UP-REGULATION OF LEPTIN IN ORAL MUCOSAL REPAIR

Leptin, a multifunctional hormone that regulates food intake and energy expenditure, has emerged recently as an important modulator of inflammatory cascades associated with wound healing. In this study, we applied the animal model of buccal mucosal ulcer to investigate the role of endothelin-1 (ET-1) and leptin in soft oral tissue repair. Using groups of rats with experimentally induced buccal mucosal ulcers we show that ulcer onset was characterized by a marked increase in the mucosal level of ET-1 and leptin. However, while the ET-1 level gradually declined with healing, the mucosal level of leptin increased reaching maximum expression on the 4th day of healing. Therapeutic administration of phosphoramidon, an inhibitor of ECE-1 activity, not only led to a 53.2% drop in the ET-1, but also produced a dose-dependent reduction (up to 50.9%) in the mucosal level of leptin and up to 42.3% decline in the rate of ulcer healing. A marked drop (54.2%) in the mucosal level of leptin and the reduction (46.8%) in the rate of ulcer healing was also attained in the presence of ET\textsubscript{A} receptor antagonist BQ610 administration, but not the ET\textsubscript{B} receptor antagonist BQ788. Moreover, administration of ERK inhibitor, PD98059 in the presence of ET\textsubscript{B} receptor antagonist, but not the ET\textsubscript{A} receptor antagonist, caused the reduction the mucosal leptin level as well as a decline in the rate of ulcer healing. Our findings are the first to implicate the requirement for both ET-1 and leptin in orderly progression of the events of soft oral tissue repair. We also show that ET-1 is a key factor in up-regulation of leptin production associated with oral mucosal ulcer healing, and that the effect of ET-1 on leptin production is a consequence of ET\textsubscript{A} receptor activation and subsequent signaling through MAPK/ERK.

Key words: oral mucosa; ulcer healing; leptin; ET-1; ET\textsubscript{A} receptor.

INTRODUCTION

Leptin, a product of the \textit{ob} gene, is a pleiotropic 167 amino acid peptide secreted predominantly by adipocytes and recognized for its role in the
maintenance of body weight homeostasis by regulating food and energy expenditure, angiogenesis, modulation of β-cell insulin secretion, and regulation of immune responses (1 - 3). The biological activities of leptin are mediated through the interaction with its specific membrane receptor, OB-R, which exists in several variant forms sharing the same extracellular domain but differing in the length of transmembrane coding regions (1). While the appetite-regulating effect of leptin through binding to corresponding leptin receptor in hypothalamus is well recognized, a number of recent data indicate that the peptide is also involved in modulation of pancreatic acinar cell secretory function, wound healing, regulation of the extent of mucosal inflammatory responses to bacterial infection, and the processes of mucosal defense and repair (4 - 8). Furthermore, leptin and leptin receptors have been identified in gastric and intestinal mucosa, as well as oral mucosa and the acinar cells of salivary glands, and leptin released locally within mucosal tissue has been implicated in the interaction with proinflammatory cytokines to control local inflammations (9 - 12).

Indeed, the increased mucosal level of leptin accompany acute gastric mucosal injury as well as characterizes mucosal inflammatory responses to bacterial infection, and the exogenous leptin has been demonstrated to exert protective effect against gastric injury induced by ischemia-reperfusion as well as accelerates healing of experimentally induced gastric ulcers (5 - 7, 13, 14). Interestingly, studies with other tissues indicate that the release of adipose-derived hormones such as leptin, adiponectin and resistin is regulated by vascular factors such as ET-1, a potent vasoconstrictor recognized for its role in normal tissue repair (15 - 17). Moreover, it has been suggested that ET-1 may play a role in mediation of local leptin release (15).

The endothelins are family of cysteine-rich peptides consisting of 21-amino acid and containing two intramolecular disulfide bridges that exert their vasoactive and mitogenic actions through G protein-coupled receptors (18, 19). At the present, the existence of three active isoforms of ET (-1, -2, and -3), and two distinct receptors, ET_A and ET_B, is well documented (17). The active form of ET-1 arises from biologically inactive big-ET-1 through the action of a specific protease that removes 18 amino acids from its carboxyl terminal (20). This membrane-bound metallopeptidase, characterized by its sensitivity to phosphoramidon, is known as endothelin-converting enzyme-1 (ECE-1) (20 - 22).

A growing body of evidence indicate that ET-1 is a major player in numerous disease states including congestive heart failure, pulmonary fibrosis, obesity and diabetes, as well as a key mediator of tissue repair and normal wound healing (16, 17, 23, 24). Moreover, the increase in ECE-1 expression has been singled out as a primary factor responsible for the enhanced ET-1 levels observed in local and systemic inflammations as well as normal wound repair (21 - 24). Recently, we have established the presence of ECE-1 in oral mucosa and demonstrated that the increase in ECE-1 expression and the enhancement in ET-1 generation correlate with the onset of oral mucosal ulceration (22, 25). In this study, using buccal
mucosal ulcer model developed in the rat, we examined the role of ET-1 in the mucosal leptin production during soft oral tissue repair.

MATERIALS AND METHODS

Animals

The study was conducted with Sprague-Dawley rats weighing 180 - 200 g, in compliance with the institutional Animal Care and Use Committee. The animals were deprived of food and water 2 h before the procedure. All experiments were carried out with groups consisting five animals per treatment. Under ether anesthesia, the buccal surfaces of the animals were exposed for 20s to contact with glacial acetic acid, using a plastic tube of 4 mm in diameter. This produced an immediate mucosal necrosis within the affected area followed 2 days later (designated as ulceration day 0) by the development of chronic ulcer with a well-defined crater, which normally healed within 10 days (26, 27). The animals were killed at different intervals of ulcer healing for up to 10 days, and the buccal mucosa from the ulcer area together with its 2 mm margin was excised and used for biochemical measurements. The rate of ulcer healing was assessed by measuring the ulcer crater area by planimetry (26). In the experiments on the effect of ECE-1 inhibitor, phosphoramidon (Sigma), ET_A receptor antagonist, BQ610 (Sigma) and ET_B receptor antagonist, BQ788 (Sigma), the animals on the second day following the acetic acid injury (ulceration day, 0) were subjected to intragastric administration of phosphoramidon at 0 - 30 mg/kg, or BQ610 at 0 - 20 mg/kg, or BQ788 at 0 - 20 mg/kg, or vehicle consisting 5 % gum arabic in saline, and maintained on the twice daily regimen of the agents or vehicle for 4 days. To assess the effect of ERK1/2 inhibitor, PD98059 (Sigma) on ET_A and ET_B receptor antagonist-induced changes in the rate of ulcer healing and buccal mucosal leptin level, the animals on the second day following acetic acid injury (ulceration day, 0) were intragastrically administered with the indicated dose of ET_A(BQ610) or ET_B(BQ788) receptor antagonist followed 30 min later by ERK1/2 inhibitor, PD98059 at 0 - 30 mg/kg, and the twice daily regimen of the agents or the vehicle was maintained for 4 days. The rats in each group were killed 16 h after the last dose.

ET-1 and leptin quantification

ET-1 assays were carried out on the individual specimens of buccal mucosal tissue following homogenization with 4 volumes of 1 M acetic acid (28, 29). The homogenates were heated for 5 min at 100°C, centrifuged, and the resulting supernatants applied to a Sep-Pack C-18 cartridges. After initial washing with 0.1% trifluoroacetic acid, the adsorbed ET-1 was eluted with methanol-water-trifluoroacetic acid (90:10:0.1, v/v/v). The eluates were dried, reconstituted in the assay buffer, and subjected to ET-1 quantification using double-antibody sandwich technique in accordance with the manufacturer’s (Alexis) instructions. For leptin measurements, the specimens of buccal mucosa were homogenized at 4°C in phosphate-buffered saline, pH 7.4 (30), centrifuged for 10 min at 800 g, and the resulting supernatant was used for leptin quantification. Leptin assays were carried out using mouse leptin enzyme-linked immunometric assay as instructed by the manufacturer (Calbiochem). The protein content of samples was measured with the BCA protein assay kit (Pierce).

Data analysis

All experiments were carried out using duplicate sampling and the results are expressed as means ± SD. Analysis of variance (ANOVA) was used to determine significance and the significance level was set at P < 0.05. The tests were performed using Soft Stat STATISTICA, software.
RESULTS

The intricacy of ET-1 and leptin involvement in the process of soft oral tissue repair was investigated using an acetic acid-induced buccal mucosal ulcer model developed in the rat. The results of macroscopic examination revealed that the ulcer crater at the onset of the experiments (day, 0) averaged 12.4 mm² which decreased to 4.6 mm² by the forth day and to 0.3 mm² by the eight day, and virtually healed by the tenth day (Fig. 1). Compared with the values for normal buccal mucosa, the ulcer onset (day, 0) was characterized by a 4.1-fold increase in the mucosal level of ET-1 and a 3.2-fold elevation in leptin. However, while the mucosal expression of ET-1 showed a gradual decline with healing and at the end of ten days reached the level of only about 1.4-fold higher than that of normal, the level of leptin increased further by 35.8% during the initial four days of healing and then showed a slow decline (Fig. 1).

As the formation of ET-1 from its inactive precursor, big ET-1, is controlled by a specific metalloprotease, ECE-1 (20, 22), we next examined the changes in buccal mucosal ulcer healing pattern in the presence of phosphoramidon, a potent inhibitor of ECE-1 activity. As shown in Fig. 2, administration of phosphoramidon, commenced on the second day following the acetic acid injury (ulceration day 0) and continued with two daily doses for 4 days, resulted in a concentration-dependent reduction (up to 53.2%) in the mucosal level of ET-1.

Fig. 1. Buccal mucosal levels of ET-1 and leptin (Lp) during the course of buccal mucosal ulcer healing. The day of ulcer onset is referred to as day, 0. *P < 0.05 compared with that of normal mucosa. **P < 0.05 compared with that of ulceration day, 0.
and was accompanied by a 42.3% decline in the rate of ulcer healing. Moreover, the effect of phosphoramidon was also reflected in a marked reduction in the mucosal production of leptin, which at 30 mg/kg phosphoramidon showed a 50.9% decrease.

As the biological effects of ET-1 are mediated by two distinct ET<sub>A</sub> and ET<sub>B</sub> receptors (18, 19), we further investigated the role of ET-1 in leptin production during buccal mucosal ulcer healing in the presence of ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists. The results revealed that administration of the selective ET<sub>A</sub> receptor antagonist BQ610, commenced on the second day following the acetic acid injury (ulceration day 0) and continued with two daily doses for 4 days, led to a concentration-dependent reduction (up to 54.2% at 20 mg/kg) in the mucosal level of leptin. At the same time, the rate of ulcer healing declined by a 46.8% (Fig. 3A). However, only negligible changes in the mucosal level of leptin and the rate of ulcer healing occurred in the presence of treatment with the specific ET<sub>B</sub> receptor antagonist BQ788 (Fig. 3B).

Finally, we employed a specific inhibitor of ERK1/2, PD98059, in conjunction with ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists to assess whether blockade of the ERK1/2 pathway affects the ET-1-induced up-regulation in the mucosal leptin production and the rate of ulcer healing. The results of assays revealed that administration of PD9859 in the presence of ET<sub>A</sub> receptor antagonist, BQ610, neither produced

![Fig. 2. Effect of endothelin-converting enzyme-1 (ECE-1) inhibitor, phosphoramidon, on buccal mucosal levels of ET-1 and leptin (Lp), and the rate of ulcer healing. The administration of phosphoramidon (twice daily) was commenced on the ulceration day 0, and the assays were carried out after 4 days of treatment. *P < 0.05 compared with that of control.]
discernible effect in the mucosal leptin level nor affected the rate of healing (Fig. 4A). However, PD98059 administration in the presence of ET$_B$ receptor antagonist, BQ788, led to a concentration-dependent reduction in the mucosal level as well as resulted in a decline in the rate of ulcer healing (Fig. 4B).

**DISCUSSION**

Among the diseases affecting soft oral tissue integrity and causing considerable discomfort in eating in speaking are painful mucosal ulcerations that are often referred to as canker sores or recurrent aphthous stomatitis (31 - 33). The ulcers affect about 25% of the general population, exhibit tendency to recurrence, and based on clinical evidence appear to be linked with immunologic abnormalities and trauma (34, 35). This is supported by the studies with an animal model of buccal mucosal ulceration which revealed that oral mucosal responses associated with the ulcer onset are manifested by the increase in proinflammatory cytokine production, excessive nitric oxide and prostaglandin generation, rise in epithelial cell apoptosis, and a marked up-regulation in ET-1 level (22, 26, 28). Interestingly, ET-1 is also recognized for its involvement in numerous other disease states including pulmonary fibrosis, obesity, diabetes, and gastric ulcers (16, 21, 36). Moreover, a growing body of evidence implicates ET-1 as a key
mediator of inflammatory responses associated with tissue repair and normal wound healing (21, 24). There are also reports indicating that ET-1 directly impacts the release of adipose-derived hormones such as adiponectin, resistin, and leptin (15, 16).

Indeed, leptin and leptin receptors have been identified in oral mucosa (10), and leptin released locally within mucosal tissue has been suggested to play a role in controlling the extent of local inflammatory responses to bacterial insults as well as the processes of mucosal repair (11 - 14, 37). Therefore, in the study presented herein, we used buccal mucosal ulcer model developed in the rat to examine the role of ET-1 in the mucosal leptin production during soft oral tissue repair. The results obtained revealed that the ulcer onset was characterized by a marked increase in the mucosal level of ET-1 as well as leptin. However, while the ET-1 level gradually declined with ulcer healing, the mucosal expression of leptin first showed further increase during the initial four days of healing and then displayed tendency to a slow decline. Moreover, therapeutic administration of phosphoramidon, a potent inhibitor of ECE-1 activity responsible for the conversion of biologically inactive big ET-1 to a 21 amino acid bioactive ET-1 (21, 22), not only resulted in a decline in the mucosal level of ET-1 but also

Fig. 4. Effect of extracellular signal-regulated kinase (ERK1/2) inhibitor PD98059 (PD) on buccal mucosal leptin (Lp) production in the presence of ET\textsubscript{A} (A) and ET\textsubscript{B} (B) receptor antagonists during buccal mucosal ulcer healing. Starting on the ulceration day 0, the animals were administered twice daily with the indicated concentrations of PD98059 and ET\textsubscript{A}(BQ610) or ET\textsubscript{B}(BQ788) receptor antagonists at 10 mg/kg, and the assays were carried out after 4 days of treatment. *P < 0.05 compared with that of control. **P < 0.05 compared with that of 10 mg/kg BQ610 or BQ788.
produced a significant reduction in the level of leptin as well as caused up to 42.3% decline in the rate of ulcer healing. These findings thus provide an important indication as to the intimate involvement of ET-1 in the mediation of up-regulation in the mucosal leptin production required for orderly progression of soft oral tissue repair.

The above contention is supported by the literature data indicating that injury and wound healing responses lead to increase in ET-1 generation through ECE-1 mRNA stabilization (21, 23). Indeed, the enhanced local mucosal release of ET-1 and leptin, has been observed during acute gastric mucosal injury as well as in patients with \textit{H. pylori}-induced gastritis (7, 13, 14, 24, 38). Moreover, there are data showing that leptin and ET-1 production are both activated by insulin and inhibited by PPAR\textgamma agonists, and that activation of ERK and PI3K are crucial targets in mediation of leptin as well as ET-1 signaling events (15, 37, 39, 40). Furthermore, it has been demonstrated that leptin-deficient (\textit{ob}/\textit{ob}) mice show the enhanced susceptibility to bacterial- and TNF-\textalpha-induced mortality (11, 41).

As the biological actions of ET-1 are mediated through binding to two distinct endothelin receptors, ET\textsubscript{A} and ET\textsubscript{B}, which differ in their affinity for ET-1 as well as in the signaling pathways activation (17, 19, 21), we further investigated which of the two receptor subtypes might be involved in the observed up-regulation in buccal mucosal leptin production with the ulcer onset. The results demonstrated that administration of the selective ET\textsubscript{A} receptor antagonist BQ610, commenced on the day of ulcer onset and continued with two daily doses for 4 days, led to the reduction in the mucosal level of leptin, and the effect was accompanied by a marked decline (46.8%) in the rate of ulcer healing. Only negligible changes in the mucosal level of leptin and the rate of ulcer healing were, however, observed with the administration of the selective ET\textsubscript{B} receptor antagonist BQ788. These data, thus, allowed us to conclude that up-regulation in the mucosal leptin level associated with oral mucosal response to injury during buccal mucosal ulcer healing is a consequence of ET\textsubscript{A} receptor activation by ET-1. This interpretation of our findings is in line with the reports indicating that therapeutic treatment with bosentan, a mixed ET\textsubscript{A,B} receptor antagonist exhibiting higher affinity for the ET\textsubscript{A} receptor, exacerbates the severity of gastrointestinal injury (40), and the data obtained with several cell systems demonstrating ET\textsubscript{A} receptor activation in response to ET-1 (19, 43).

Moreover, since several recent studies indicated that the signaling events associated with ET-1 induction of ET\textsubscript{A,B} receptor activation occur with the involvement of MAPK/ERK (16, 17), we also employed a specific inhibitor of ERK1/2, PD98059, in conjunction with ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists. The data revealed that administration of PD98059 in the presence of ET\textsubscript{B} receptor antagonist, but not the ET\textsubscript{A} receptor antagonist, caused the reduction in buccal mucosal leptin level as well as a decline in the rate of ulcer healing. Hence, the signaling pathway leading to up-regulation in buccal mucosal leptin production involves ET-1 engagement of ET\textsubscript{A} receptor and subsequent signaling through
ERK-dependent mechanism. This is in keeping with the obtained results that demonstrated that buccal mucosal leptin production could be affected either by the ET$_A$ receptor antagonist or the inhibitor of ERK1/2, but not the ET$_B$ receptor antagonist. Therefore, the induction by ET-1 in the mucosal leptin production associated with healing of oral mucosal injury occurs via ET-1 activation of ET$_A$ receptor and subsequent involvement of ERK-dependent mechanism.

In conclusion, our findings are the first to implicate the requirement for both ET-1 and leptin in orderly progression of the events of soft oral tissue repair. We also show that the induction by ET-1 in up-regulation of leptin production associated with oral mucosal ulcer healing is a consequence of ET$_A$ receptor activation.

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