INTRODUCTION

Leptin, a pleiotropic 16 kDa peptide hormone product of the ob gene, is secreted predominantly by adipose tissue and well recognized for its role in the maintenance of body mass and energy expenditure (1-3). Moreover, advances in understanding the functional role of leptin in the processes affecting various tissues throughout the body, have brought to the forefront the importance of the local release of this cytokine to the processes of mucosal defense and repair (4-7). Consistent with its pluripotent nature, leptin and leptin receptors have been identified in oral mucosa, saliva, and the acinar cells of salivary glands (8). Leptin released locally within the mucosal tissue has been implicated in the interaction with proinflammatory cytokines, regulation of NO production, and the modulation of arachidonic acid generation by impacting the events of cytosolic phospholipase A2 (cPLA2) activation (6, 7, 9-11). The increase in leptin level characterizes oral mucosal responses to injury, and the exogenous leptin is known to accelerate wound repair, and protects the acinar cells of salivary glands against cytotoxic effects of ethanol (7, 11, 12).

The oral mucosal responses to ethanol cytotoxicity are manifested by the elevation in proinflammatory cytokine production, enhancement in epithelial cell apoptosis, disturbances in epidermal growth factor and nitric oxide signaling pathways, and the impairment in prostaglandin generation, (13-15). Moreover, salivary glands of alcoholics and animals exposed to ethanol display the evidence of fatty infiltration, acinar cell swelling, and change in cytoplasm vacuolation accompanied by cellular degeneration and even atrophy (16, 17). Interestingly, recent evidence indicates that the critical event responsible for rapid changes in prostaglandin production is the selective channeling of arachidonic acid substrate, released from membrane glycerophospholipids by the action of cPLA2 enzyme, to the site of cyclooxygenase-2 (COX-2) action for prostaglandin synthesis (18, 19). The activity of cPLA2 is tightly regulated by post-translational mechanism involving MAPK/ERK-dependent enzyme protein phosphorylation and calcium influx that facilitate the enzyme translocation from cytosol to phospholipid-rich membrane (20, 21). There are also reports suggesting that cPLA2 is a downstream effector of ERK in leptin signaling through Src, and that leptin-induced responses mediated by ERK involve epidermal growth factor receptor (EGFR) transactivation (22-24).

Review of the pertinent literature indicates that the signaling cross-talk involving EGFR transactivation is implicated in the regulation of a wide variety of cell functions of significance to oral mucosal repair and integrity maintenance, including cellular proliferation, differentiation, survival, and migration to the site of injury (24, 25). In general, the signals triggered by EGFR...
transactivation with the involvement of Src are short of duration and result in transient activation of ERK, which does not undergo nuclear translocation (26), and hence could be utilized for a direct activation through phosphorylation a number of cytosolic proteins of significance to cellular survival, including cPLA₂. Moreover, there are indications that Src-dependent transactivation pathway is involved in the induction of matrix metalloproteinase-9 (MMP-9), which promotes selective release of membrane-anchored EGFR ligands that subsequently activate EGFR to initiate ERK phosphorylation (27, 28).

Recently, using rat sublingual salivary gland acinar cells, we demonstrated that leptin protection of the acinar cells against ethanol cytotoxicity involves Src kinase-mediated cPLA₂ activation (11). In this study, we provide evidence that transactivation of EGFR is involved in the signaling cascade that leads to cPLA₂ activation and up-regulation in PGE₂ generation, and thus mediate leptin protection of salivary gland acinar cells against ethanol cytotoxicity.

MATERIALS AND METHODS

Sublingual gland cell preparation

The acinar cells of sublingual gland were collected from freshly dissected rat salivary glands by passage of the trimmed glandular tissue through a 50 mesh metal grid (29). The minced tissue was then suspended in five volumes of ice-cold Dulbecco's modified (Gibco) Eagle's minimal essential medium (DMEM), supplemented with fungizone (50 µg/ml), penicillin (50 U/ml), streptomycin (50 µg/ml), and 10% fetal calf serum, and dispersed into single cells and cell clusters by trituration with a glass homogenizer, and settled by centrifugation. After three consecutive rinses with DMEM, the cells were resuspended in the medium to a concentration of 2 x 10⁷ cell/ml. The viability of cell preparations before and during the experimentation, assessed by Trypan blue dye exclusion assay, was greater than 98%.

Ethanol-induced cytotoxicity

Aliquots of the acinar cells suspension (1 ml) were transferred to DMEM in culture dishes and incubated for 2 h at 37°C under 95% O₂/5% CO₂ atmosphere in the absence and the presence of 3% of ethanol (11). In the experiments evaluating the effect of leptin (mouse recombinant, Sigma), janus kinase (JAK) inhibitor AG490 (Calbiochem), Src kinase inhibitor PP2 (Calbiochem), EGFr and EGFR kinase inhibitor AG1478 (Sigma), ERK1/2 inhibitor PD98059 (Calbiochem), PKC inhibitor Ro318220, and a broad spectrum matrix metalloproteinase inhibitor (MMP), GM6001 (Calbiochem), the cells were first treated for 30 min with the indicated dose of the agent of interest, incubated for 2 h in the presence of 3% ethanol, and following centrifugation the supernatant was analyzed for the released [³H]arachidonic acid by scintillation spectrometry.

MMP-9 assay

The acinar cell suspensions from the control and experimental treatments were centrifuged at 300g for 5 min and the conditioned medium supernatant collected. The assays of matrix metalloproteinase-9 (MMP-9) were carried out using a MMP-9 ELISA kit (Calbiochem) and 100 µl aliquots of spent culture medium, according to manufacturer’s instruction.

Measurement of EGFR tyrosine kinase transactivation

The measurements of EGFR transactivation was conducted with PhosphoDetect Elisa kit (Calbiochem). The acinar cells from the experimental treatments were washed with phosphate-buffered saline, treated with the receptor extraction buffer and centrifuged at 1500 g for 10 min at 4°C. The supernatant was incubated at room temperature for 2 h with anti-EGFR antibody followed by 30 min incubation with protein A agarose and centrifuged (30). The pellet was suspended in kinase reaction buffer and 100 µl aliquots were used for EGFR phosphotyrosine assays following the manufacturer’s instructions.

Western blotting procedures

The acinar cells from the control and experimental treatments were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in ice-cold lysis buffer (11). Following brief sonication, the cell lysates were centrifuged at 12,000g for 10 min, and the supernatants were subjected to protein determination using BCA protein assay kit (Pierce). The samples were then resuspended in loading buffer, boiled for 5 min, and subjected to SDS-PAGE using 50 µg...
protein/lane (11). The separated proteins were transferred onto nitrocellulose membranes, blocked with 5% skim milk, and incubated with the antibodies against the phosphorylated proteins at 4 °C for 16 h (11). After 1 h incubation with the horseradish peroxidase-conjugated secondary antibody, the phosphorylated proteins were revealed using an enhanced chemiluminescence detection kit (Pierce). Membranes were stripped by incubation in 1M Tris-HCl (pH 6.8), 10% SDS, and 10 mM dithiotreitol for 30 min at 55 °C, and reprobed with antibodies against the proteins of interest. Immunoblotting was performed using specific antibodies directed against EGFR and phospho-EGFR (Tyr1173).

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 $P < 0.05$.

RESULTS

Building on our recent finding that leptin protection of salivary gland acinar cells against ethanol cytotoxicity stems from its ability to impact the events of cPLA2 activation for the increase in arachidonic acid release for prostaglandin synthesis (11), we investigated further the factors affecting signaling pathways that modulate cPLA2 activity and arachidonic acid release. Using rat sublingual gland acinar cells exposed to incubation with ethanol in conjunction with lactate dehydrogenase cytotoxicity, we demonstrated that preincubation with leptin at the previously determined optimal concentration of 1 µg/ml (11), resulted in a nearly complete protection against ethanol-induced cytotoxicity (Fig. 1). At the same time, the acinar cell assays of the content of endogenous leptin gave the mean value of 32.1 pg/mg protein.

Thus using pharmacological concentration of leptin at 1 µg/ml, we found that the protective effect of this pleiotropic hormone against the ethanol-induced salivary gland acinar cell toxicity was subject to suppression by AG490, a specific inhibitor of JAK (Fig. 1A), PP2, a selective inhibitor of tyrosine kinase Src (Fig. 1B), as well as an inhibitor EGFR kinase, AG1478 (Fig. 1C). Further results revealed that all three agents evoked also the inhibition in leptin-induced up-regulation in arachidonic acid release and the acinar cell capacity for PGE2 generation (Fig. 2). These findings point to the involvement of EGFR transactivation in the protective mechanism of leptin action against ethanol cytotoxicity in salivary glands.

Since cPLA2 activation for the rapid release of arachidonic acid involves MAPK/ERK-dependent enzyme protein phosphorylation on Ser505 that plays a crucial role in Ca2+-dependent translocation of the enzyme from cytosol to phospholipid-rich membrane (20, 21), we next assessed the acinar cell cPLA2 activation by leptin by measuring cPLA2 enzymatic activity following various treatments. As summarized in Fig. 3, the cytotoxic effect of ethanol was reflected in a 30% drop in the acinar cell cPLA2 activity, while preincubation with leptin countered the ethanol effect and evoked a 1.6-fold increase in the cPLA2 activity. Moreover, the leptin-induced up-regulation in the acinar cell cPLA2 activity was subject to suppression by ERK1/2 inhibitor, PD98059 as well as the inhibitors of JAK (AG490), Src (PP2), and EGFR (AG1478) kinases.

To characterize further the involvement of EGFR transactivation in leptin-induced signaling leading to up-regulation in salivary gland acinar cell cPLA2 activation we monitored the requirements for EGFR protein tyrosine kinase (PTK) activation. As shown in Fig. 4, we observed that leptin at its optimal concentration (1µg/ml) for the suppression of the cytotoxic effect of ethanol elicited a 1.9-fold stimulation in the acinar cell EGFR PTK activity, and the effect was not only subject to the suppression by EGFR kinase inhibitor AG1478, but also JAK kinase inhibitor, AG490 and Src kinase inhibitor,
However, the leptin-induced up-regulation in EGFR PTK activity was not affected by the preincubation with MAPK/ERK inhibitor, PD98059. These data together with the results on cPLA₂ activation and PGE₂ generation by sublingual salivary gland acinar cells in the presence of ethanol (Et). The [³H]arachidonic acid-labeled cells, preincubated with the indicated concentrations of AG490, PP2, and AG1478, were treated with Lp at 1µg/ml and incubated for 2 h in the presence of 3% Et. Values represent the means ± SD of five experiments. *P < 0.05 compared with that of control. **P < 0.05 compared with that of Et alone. ***P < 0.05 compared with that of Lp+Et.

As there are reports that membrane-anchored EGFR ligands in response to external stimuli are cleaved and processed by matrix metalloproteinases to promote receptor engagement (24-26, 28), we next assessed the role of metalloproteinases in leptin-induced EGFR transactivation in the acinar cells. We found that preincubation of the acinar cells with a broad-spectrum metalloproteinase inhibitor, GM6001 led to a concentration-dependent suppression in the protective effect of leptin against ethanol cytotoxicity (Fig. 5). Moreover, the GM6001 caused also the suppression (up to 29.3%) in leptin-induced EGFR PTK activity and blocked the leptin-induced EGFR phosphorylation, but had no effect on EGFR phosphorylation induced by EGF ligand (Fig. 6). These findings thus point to the involvement of matrix metalloproteinases in the event of leptin-induced EGFR transactivation that results in a signaling cascade leading to cPLA₂ activation and up-regulation in PGE₂ generation.
Recent literature data indicate that Src kinase-dependent EGFR transactivation occurs with the involvement of matrix metalloproteinase, MMP-9 (27, 28). Therefore, we further evaluated the effect of leptin on the acinar cell MMP-9 production. As shown in Fig. 7, leptin at its optimal concentration for the suppression of the cytotoxic effect of ethanol evoked a 2.1-fold increase in the acinar cell MMP-9 secretion, and the observed effect was subject to suppression by pretreatment with Src kinase inhibitor, PP2 as well as JAK kinase inhibitor, AG490 and metalloprotease inhibitor, GM6001. We have also observed that preincubation with EGFR kinase inhibitor, AG1478 and PKC inhibitor, Ro318220 did not produce any discernible impact on the leptin-induced MMP-9 secretion (Fig. 7). Thus, the induced up-regulation in the acinar cell MMP-9 secretion associated with the countering effect of leptin against ethanol cytotoxicity occurs upstream of EGFR, with the involvement of JAK and Src, and does not appear to show the requirement for PKC participation.

**DISCUSSION**

Investigations into the functional role of leptin released locally within peripheral tissues and secreted into saliva by the acinar cells of salivary glands, have brought to the forefront the importance of this peptide hormone to the processes of mucosal defense and repair along alimentary tract, including that of oral cavity (4-8). The cytokine released locally within the oral cavity has emerged as an important integrator of intracellular signaling pathways that are of significance to the maintenance of soft oral tissue homeostasis. Indeed, the increase in leptin level characterizes oral mucosal responses to injury, and the exogenous leptin is known to accelerate wound repair and protects the acinar cells of salivary gland against cytotoxic effect of ethanol (7, 11, 12, 31). As the diminished secretion of saliva and oral mucosal inflammatory changes are well-recognized consequences of alcohol abuse on the health of oral cavity (14-17, 32), we focused our attention on the understanding the way leptin protects the acinar cells of salivary glands against ethanol cytotoxicity by...
examining functional and signaling cross-talk between responses induced by leptin and those involving EGFR transactivation.

The available data indicate that the prominent manifestation of oral mucosal and the acinar cell ethanol cytotoxicity is the impairment in prostaglandin generation, and that the critical event responsible for rapid changes in prostaglandin production is the release of arachidonic acid from membrane phospholipids by the action of cPLA2 enzyme (9, 11, 13). The literature evidence, furthermore, implies that cPLA2 is a downstream effector of ERK in leptin signaling, and we have shown recently that leptin-induced cPLA2 activation for the increase in arachidonic acid release for prostaglandin generation requires MAPK/ERK participation (11, 22-24). Hence, using rat sublingual salivary gland acinar cells exposed to ethanol at the concentration range that impairs the cell capacity for mucin synthesis and prostaglandin generation (13, 33), we examined the mechanism of leptin-induced cPLA2 activation for the increase in PGE2 generation and the role of EGFR transactivation in this process.

The results of our findings revealed that the protective effect of leptin against ethanol cytotoxicity was associated with the increased EGFR protein tyrosine kinase and cPLA2 activation, as characterized by a marked increase in arachidonic acid release, and PGE2 generation. Furthermore, a significant loss in the induced up-regulation in PGE2 generation and the countering capacity of leptin on the ethanol-induced toxicity was attained with JAK kinase inhibitor AG490, Src kinase inhibitor PP2 and EGFR inhibitor AG1478, as well as MAPK/ERK inhibitor PD98059. These findings, together with the demonstrated dependence of cPLA2 activity on the enzyme protein phosphorylation (20, 21), attest to the involvement of EGFR transactivation in the processes of leptin-induced cPLA2 activation in the acinar cell and to the effect of leptin on prostaglandin synthesis (18-20).

In concordance with the prevailing evidence for the involvement of Src kinase in the signaling pathways associated with EGFR transactivation (26-28), the results of our findings suggest that leptin-induced EGFR transactivation is dependent on JAK and Src kinase, and that ERK activation is dependent on EGFR transactivation. We found that leptin-induced increase in the acinar cell EGFR PTK activity was not affected by MAPK/ERK inhibitor, PD98059. Hence, ERK activation by leptin for the increase in cPLA2 activity and PGE2 generation is clearly dependent on the event of EGFR tyrosine kinase activation. This is also in keeping with the reported association of Src kinase with EGFR and its activation by phosphorylation at the tyrosine residues (26, 34, 35). Moreover, our results lend further support to a prevalent concept that Src is an upstream effector of EGFR transactivation (26, 27, 34-36).

Recent studies indicate that Src kinase-dependent events involving cross talk between EGFR and Src that result in transient ERK activation occur with the involvement of matrix metalloproteinases (24-28). Indeed, the Src kinase-dependent EGFR transactivation has been demonstrated as an essential requirement for MMP-9 (type IV collagenase) activation in human tracheal smooth muscle cells, MMP-1 activation was observed in association with EGFR-induced esophageal keratinocytes migration, and the requirement for matrix metalloproteinase was shown to be an essential element of EGFR-mediated signaling induced in neuronal cell by gonadotropin-releasing hormone receptor activation (26, 27, 37). In our study, reported herein, we employed a broad-spectrum metalloproteinase inhibitor, GM6001, capable of membrane-anchored HB-EGF cleavage inhibition (24, 27, 28). We found that GM6001 not only evoked suppression in the protective effect of leptin against ethanol cytotoxicity and the inhibition in leptin-induced EGFR PTK activity, but also blocked the leptin-induced EGFR phosphorylation. However, the GM6001 had no effect on the acinar cell EGFR phosphorylation induced by the exogenous EGF ligand. These findings thus point to the involvement of matrix metalloproteinases in the event of leptin-induced EGFR transactivation that results in a signaling cascade leading to cPLA2 activation and up-regulation in PGE2 generation.

Moreover, since MMP-9 as well as proteases of ADAM class (a disintegrin and metalloprotease family) are known to respond to external stimuli by cleavage of membrane-anchored growth factors, including ligands of EGFR to promote receptor engagement (26, 27), we also evaluated the effect of leptin on the acinar cell MMP-9 production. Interestingly, we observed that protection by leptin against cytotoxic effects of ethanol was associated with the increase in the acinar cell MMP-9 production, and that the induced MMP-9 secretion was subject to the suppression by Src inhibitor PP2, as well as JAK inhibitor AG490 and metalloproteinase inhibitor GM6001. However, the leptin-induced production of MMP-9 was not affected by EGFR inhibitor AG1478 or PKC inhibitor Ro318220. These data suggest that the induced up-regulation by leptin in the acinar cell MMP-9 secretion occurs upstream of EGFR, with the involvement of JAK and Src, and does not require PKC participation.

Taken together, our findings point to the involvement of MMP-9 in the event of leptin-induced EGFR transactivation that results in the signaling cascade leading to cPLA2 activation and up-regulation in the acinar cell PGE2 generation. Hence, leptin protection of salivary gland acinar cells against ethanol cytotoxicity stems from the ability of this pluripotent cytokine to affect rapid and selective release of arachidonic acid at the site of COX-2 action to counter the detrimental effect of ethanol on prostaglandin generation.

Conflict of interests: None declared.

This article is dedicated to Prof. Stanislaw J. Konturek, M.D., on the occasion of his 77th birthday.

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