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DIRECT PROTECTIVE ACTION OF EPIDERMAL GROWTH FACTOR ON ISOLATED GASTRIC MUCOSAL SURFACE EPITHELIAL CELLS

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Epidermal growth factor (EGF) protects gastric mucosa against acute injury produced by a variety of damaging agents, but the mechanism of its protective action is not clear. Since the surface epithelial cells (SEC) are important component of gastric mucosal defence, we studied whether EGF may directly protect isolated gastric SEC against ethanol injury *in vitro*, in condition independent of systemic factors and whether endogenous prostaglandins may play a role in EGF's protective action. The isolated SEC from rat gastric mucosa were preincubated in medium only, or medium containing 0.0001—10.0 μg/ml of h-rEGF for 15 minutes, and incubated with 8% ethanol for 1 hour. In another study the above experiment was repeated but cells were pretreated with 10^{-4} or 10^{-5} M indomethacin before EGF treatment. The cell viability was assessed by fast green exclusion test. Incubation of SEC with 8% ethanol significantly reduced SEC cell viability to 50 ± 2%: EGF 0.1 or 1.0 μg/ml significantly reduced ethanol induced damage (cell viability 59 ± 3 and 62 ± 3% respectively). Pretreatment with 10^{-4} M indomethacin (the dose which does not affect SEC viability but inhibit PGE_2 and PGI_2 generation), significantly reduced protective action of EGF against 8% ethanol injury. EGF 1.0 and 10.0 μg/ml alone without ethanol increased PGE_2 and 6 keto $PGF_{1\alpha}$ generation by SEC. These studies demonstrated: 1) EGF is able to protect gastric surface epithelial cells directly without mediation by systemic factors. 2) EGF induced protection of SEC may in part be mediated by prostaglandins.

K e y w o r d s : *Epidermal growth factor, gastric mucosal surface epithelial cells, ethanol induced mucosal injury, cell viability, prostaglandins, indomethacin.*

INTRODUCTION

Epidermal growth factor (EGF) have been shown to exert mucosal protective and ulcer healing action (1, 2, 3, 4). The mechanisms of the protective action of EGF are not entirely clear, but may be related to its acid

inhibitory action, to stimulation of mucus secretion and/or cell proliferation. Some of these protective action of EGF *in vivo* may be mediated by endogenous prostaglandins as evidenced by experiments indicating that protective action of EGF is abolished, at least in part, by indomethacin (5, 6, 7). However, this subject remains controversial.

The surface epithelial cells secreting mucus, bicarbonates and prostaglandins, constitute the first line of gastric mucosal defense and they are the major target of acute injury by necrotizing and ulcerogenic agents.

The aims of this study were to determine whether exogenous EGF is able to protect isolated gastric surface epithelial cells against ethanol injury, directly without systemic mediation by neural, hormonal or vascular factors and whether protection if any is mediated by endogenous prostaglandins.

MATERIAL AND METHODS

Preparation of Cell Suspension

Nonfasted male Sprague-Dawley rats weighing 130—160 g were used to isolate gastric surface epithelial cells. After removal of the stomachs, surface epithelial cells were isolated according to method of Matuoka et al (8) and modified by Arakawa et al (9). The inverted stomachs were placed in shaking bath in 20 ml of oxygenated phosphate buffer, (pH 7.4) containing 0.2% pronase E at 37°C for 45 minutes. After a 15 minute incubation, the stomachs were transferred to the new solution to remove mucus and dead cells. Thirty minutes later, the stomachs were vortexed for 2 seconds to obtain surface epithelial cells. After completion of 45 minutes incubation, the solution rich in surface epithelial cells was filtrated twice through 80 μm nylon mesh. The detached cells were collected by centrifugation at 280 xg for 5 minutes at 4°C. After 3 times centrifugation and resuspension, cell preparation rich in surface epithelial cells was ready for the studies.

Experimental Design

1) The effect of EGF on surface epithelial cell injury by ethanol

Suspension of surface epithelial cells was preincubated in KH oxygenated medium only or medium containing either 0.0001, 0.001, 0.01, 0.1, 10.0 μg/ml human recombined-EGF (Chiron Co.) for 15 minutes at 37°C and incubated with 8% ethanol for 30 minutes *(Fig. 1)*. The concentration of ethanol (8%) was chosen for this study because a dose response curve with 1, 2, 4, 8 and 10% ethanol demonstrated that 8% ethanol reduces cell viability to approximately 50% within 30 minutes.

2) Effect of indomethacin on EGF afforded cell protection

To block the endogenous prostaglandins generation, surface epithelial cells suspension was pretreated with 10^{-5} or 10^{-4} M of indomethacin.

Cell Viability

To assess cell viability, fast green exclusion test according to the method described by Weisenthal et al (10) and adopted to gastric mucosal cells by Tarnawski et al (11) was used. This method is based on the fact that viable cells are able to exclude the dye, while dead cells lose this ability. Sixty μl of 2% fast green (Sigma Chemical Co.) in 0.15 M NaCl solution were added to 0.2 ml cell suspension. After shaking gently, cell suspension was poured into the chamber of Cytospin centrifuge (Shandon Southern Instruments Inc., Sewickley, PA) and centrifuged at 1250 rpm for 5 minutes. The resultant slides were then counterstained with hematoxylin and eosin. With this method, viable cells (i.e., cell excluding fast green) were stained pink with eosin, while dead cells were unable to exclude the dye and therefore had nuclei and/or cytoplasm stained green. Two slides were prepared from each sample. Coded slides were assessed for viability by 2 investigators unaware of the code. At least 5 field and 1000 cells were counted. Viability are expressed as a percentage of viable surface epithelial cells per all evaluated cells.

Prostaglandin Assay

Standard 0.5 ml aliquots of isolated surface epithelial cell suspensions were incubated in oxygenated KH medium containing 0, 0.01, 0.1, 1.0, 10.0 μg/ml of EGF for 15 minutes at 37°C. At the end of incubation, 10 μg of indomethacin was added to each sample to stop further synthesis of prostaglandins. The samples were centrifuged at 7000 xg for 30 seconds and supernatants stored in a freezer at −80°C.

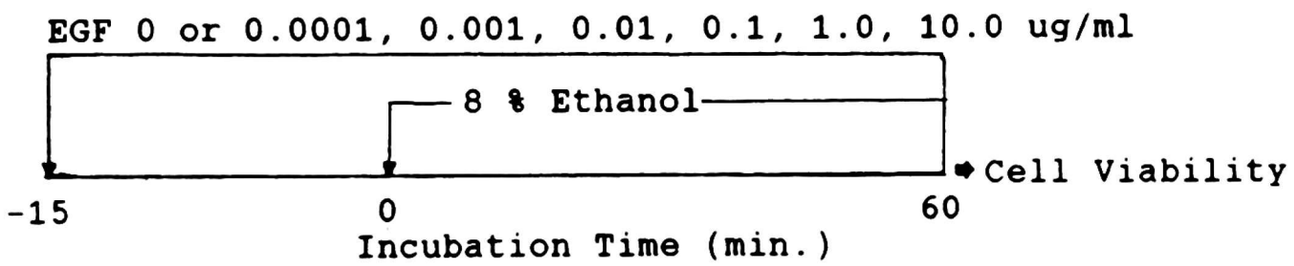
The 50 μl aliquots of the incubation medium were used for assay of PGE_2 and 6-keto-$PGF_{1\alpha}$ using ^{125}I-PGE_2 and ^{125}I-6-keto-$PGF_{1\alpha}$ RIA kits (New England Nuclear, Boston, Mass). Radioactivity was counted by a gamma counter *(Fig. 1)*.

Statistical Analysis

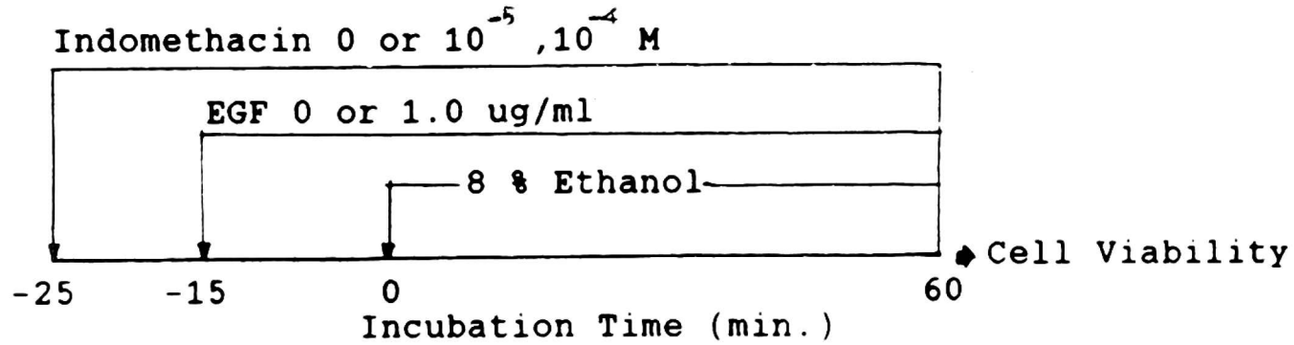
Statistical significance was evaluated by one way analysis of variance test (12). P values less than 0.05 were considered to be significant.

Cell Viability

1) The Effect of EGF on Ethanol-Induced Injury of the Surface Epithelial Cells



2) Preincubation with Indomethacin



Prostaglandin Generation

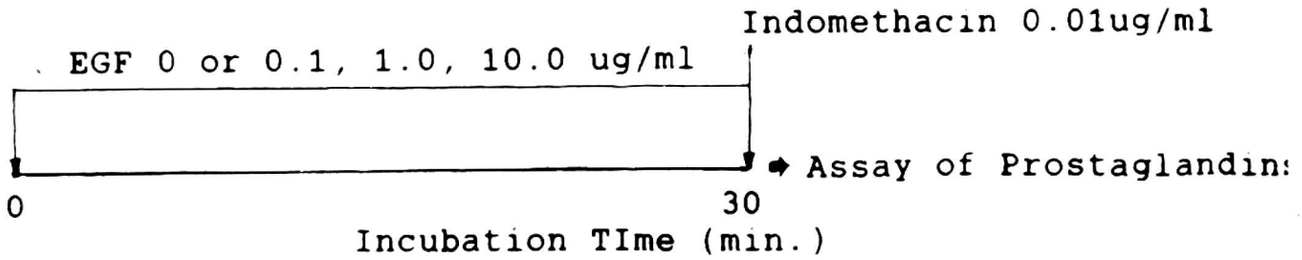


Fig. 1. Experimental design.

RESULTS

Cell Viability

Surface epithelial cells constituted 82.5±3.5% (Mean ±SE) of the total cell count. Viability of the cells at the base line was 88.5±4.3%.

Viability of the cells in the control group (incubated with medium only) was 85.6±3.0%. EGF itself did not influence cell viability (viability

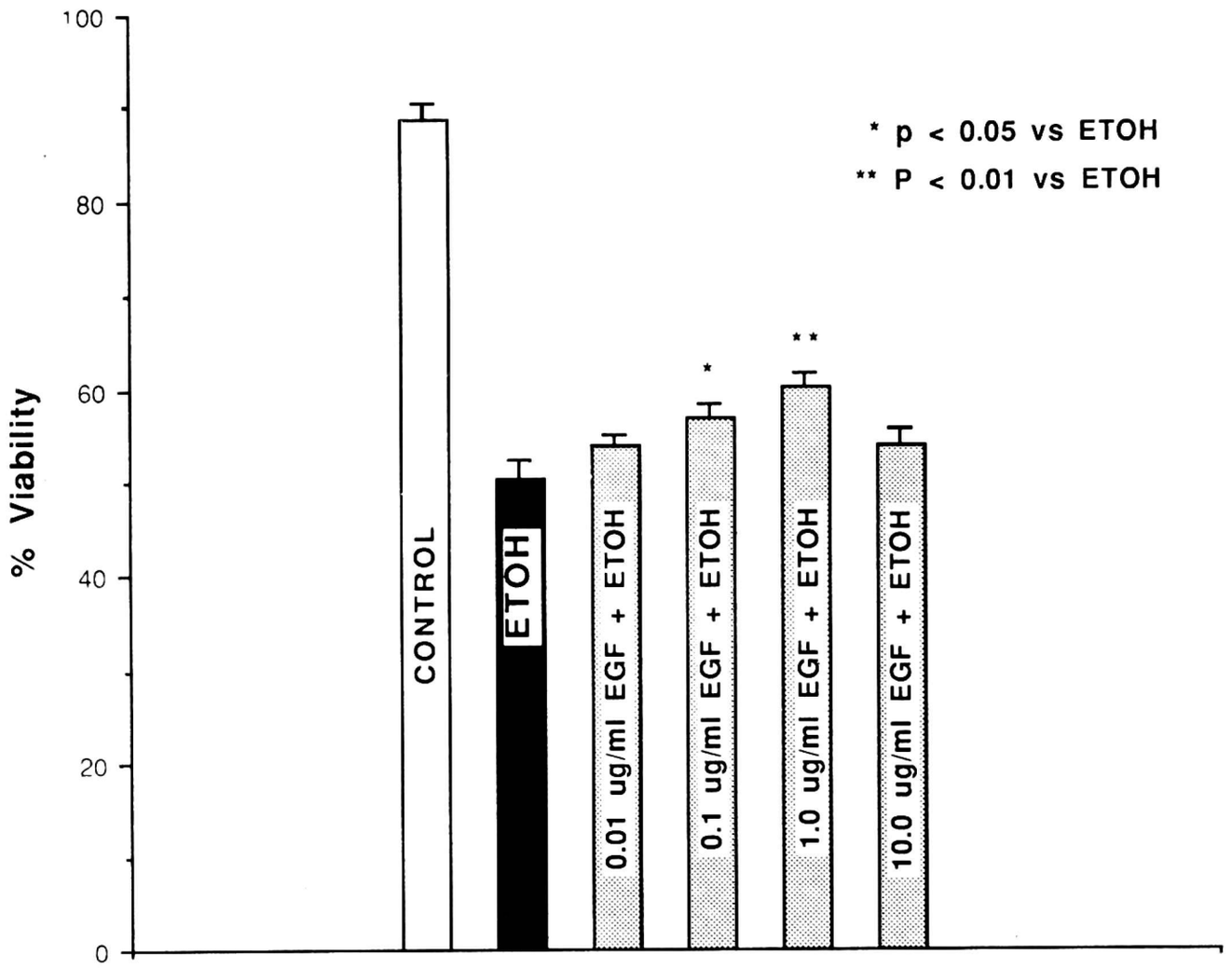


Fig. 2. Viability of the surface epithelial cells after incubation with 8% ethanol. Cell viability was significantly reduced by incubation with 8% ethanol for 60 min. Pre-incubation with 0.1 and 1.0 μg/ml of EGF significantly reduced ethanol-induced reduction in cell viability.

86.5±3.8%). After exposure to ethanol, the cell viability was significantly reduced to 50.4±1.9%. EGF in concentration 0.0001, 0.001, 0.01 and 10.0 μg/ml did not affect the viability of cells exposed to ethanol, while EGF 0.1 and 1.0 μg/ml significantly reduced ethanol induced decrease in cell viability *(Fig. 2)*.

Effect of Indomethacin Pretreatment on Protective Effect of EGF

The concentration of indomethacin used in this experiment was not damaging to the cell when given without ethanol (viability 81.8±4.5%) nor did it have influence on injury produced by ethanol (viability 52.7±2.5%). The pretreatment of 10^{-4} M indomethacin significantly reduced the protective action of EGF against ethanol induced damage *(Fig. 3)*.

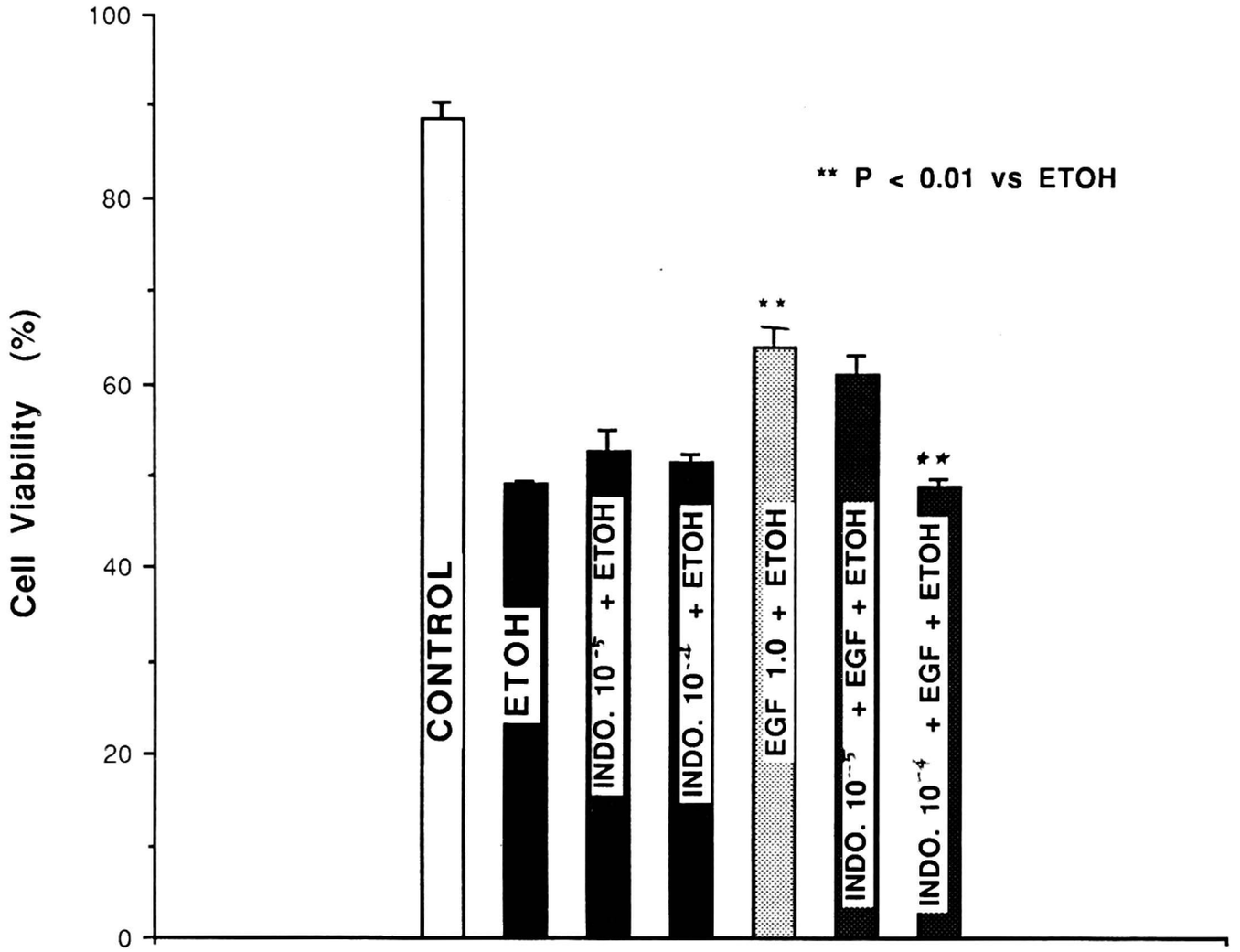


Fig. 3. Pretreatment of 10^{-4} M indomethacin reversed protective action of EGF against ethanol-induced reduction in cell viability.

Effect of EGF on PGE_2 and 6-Keto $PGF_{1\alpha}$ Generation by the Surface Epithelial Cells

EGF in concentration of 1.0 and 10.0 μg/ml significantly stimulated generation of both PGE_2 and 6-keto-$PGF_{1\alpha}$ (Fig. 4).

DISCUSSION

Our present study demonstrated that EGF is able to protect isolated gastric mucosal surface epithelial cells against ethanol induced injury as reflected by increased cell viability. According to our knowledge, it is the first demonstration of protective action of EGF on gastric mucosal cells *in vitro*. Furthermore, our experiments demonstrated that indomethacin (in a dose which is not injurious to cell nor affect ethanol injury) significantly reversed protective action of EGF against ethanol injury.

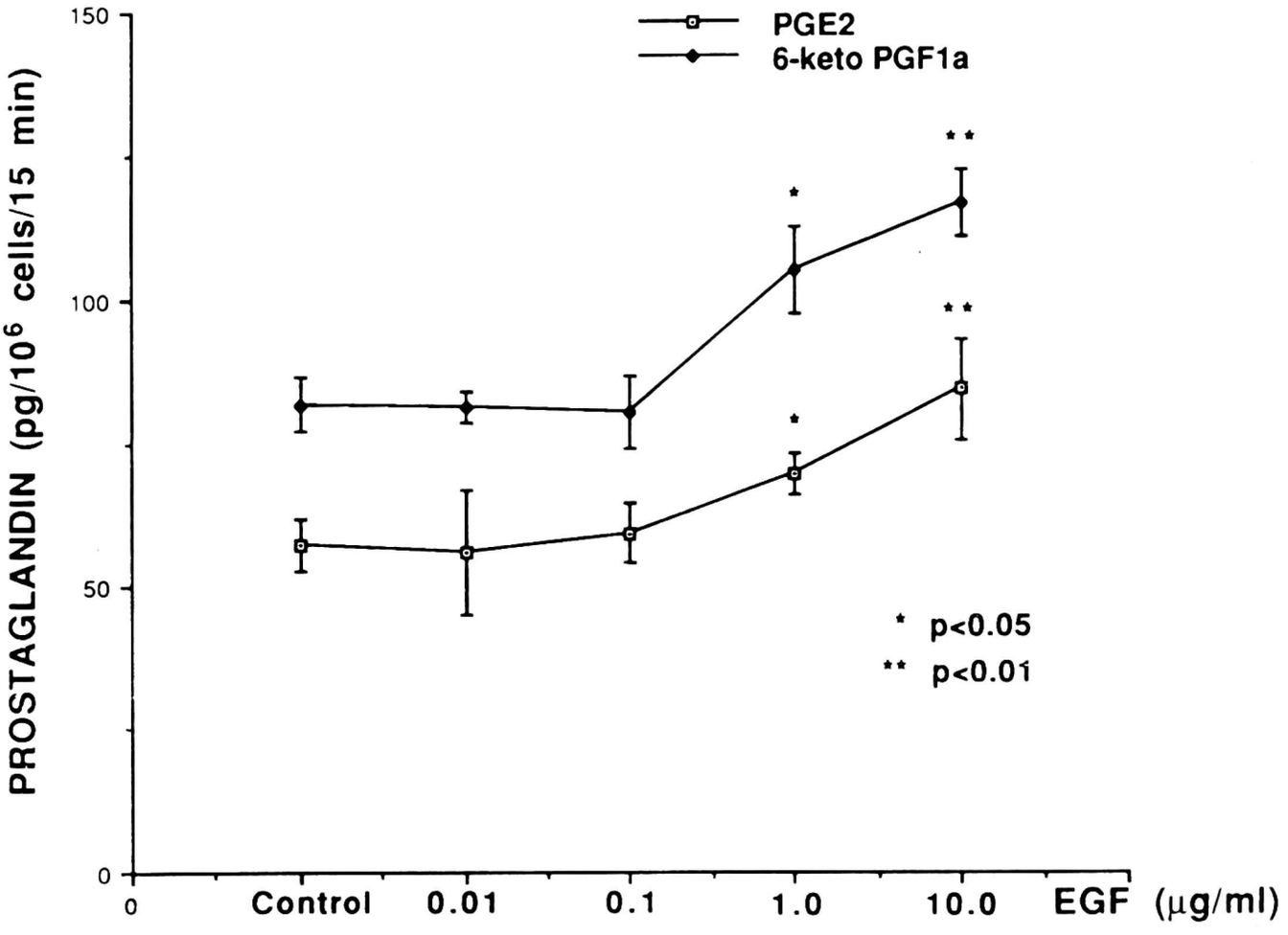


Fig. 4. Effect of EGF on prostaglandins generation by the surface epithelial cells. 1.0 and 10.0 μg/ml of EGF significantly increased both PGE_2 and 6-keto $PGI_{1\alpha}$ generation.

Numerous investigators demonstrated that EGF can protect gastric mucosa against a variety of injurious agents including aspirin (1), HCl (5), HCl and ethanol (6), ethanol (13, 14), cysteamine (2, 15) and water immersion stress (7). However, the mechanism of protective action remains unclear. It has been suggested that the protective action of EGF against necrotizing agents is linked to early effects exerted by EGF (2, 15). These early effects appear from minutes to hours after EGF binding to its receptor and include increased aminoacid, uridine, putrescine and glucose uptake.

In our previous study, we have demonstrated the protective effect of EGF on gastric mucosal injury produced by ethanol (14). That study suggested that EGF exerts protective action by maintaining mucus, blood flow and increasing cell proliferation. However, all these effects involve systemic actions. Our present data demonstrated that EGF can protect gastric surface epithelial cells in condition independent of systemic factors. EGF's protective action was dose dependent with doses of 0.1, 1.0 μg/ml being the most effective. Dose of 10 μg/ml did not offer more protective action than 1 μg/ml. The similar finding

was reported in regard to the protective action of prostaglandins on the surface epithelial cells and chief cells against ethanol injury (16, 17). The possible explanation of this phenomenon is a down regulation of EGF receptor by a huge EGF dose.

Numerous investigations reported on the interaction between EGF and prostaglandins. Konturek and coworkers reported that the protective effect of EGF against aspirin induced erosions was not mediated by prostaglandin generation (1). However, more recently the same authors reported that indomethacin pretreatment abolished the effect of EGF against water--immersion restraint stress induced ulcers (7). Amagase et al demonstrated, using indomethacin, that the vasoconstricting action of h-EGF is mediated by prostaglandins (6). Itoh et al reported that protective effect of EGF on the gastric mucosa, is independent of prostaglandin (5). It is possible that EGF may have 2 components of protective action; one mediated by prostaglandins, and second independent of prostaglandins. In our present study, we investigated the role of prostaglandin in protective action of EGF. We used indomethacin (10^{-4} M), which inhibits synthesis of prostaglandins and but by itself does not damage the cells (18). Our finding-increased PGE_2 and 6-keto $PGF_{1\alpha}$ generation by the surface epithelial cells incubated with EGF-partly supports the contention that protective action of EGF may be mediated by prostaglandins generation by the surface epithelial cells. Several investigators reported that EGF has the capacity of stimulation of prostaglandins generation. Hiraishi et al found that EGF stimulates synthesis of prostaglandins in cultured rat gastric mucosal cells (18). Chiba et al demonstrated that 10^{-7} M of EGF increases prostaglandin secretion from isolated perfused rat stomach, and this effect reaches maximum 15 minutes after administration of EGF (19). Hatt et al reported that EGF ($10^{-11} - 10^{-7}$ M) stimulates dose dependently PGE_2 production by isolated parietal cells (20). There have been many reports indicating that EGF stimulates prostaglandin generation also in the other tissue e. g. thyroid cells (21, 22), renal cells (23) and muscle cells (24).

Arakawa et al demonstrated that PGE_2 directly protects surface epithelial cells against ethanol damage (16). Therefore, our findings, that EGF stimulates prostaglandin generation by surface epithelial cells, may indicate prostaglandin mediation of EGF protective action.

However, the concentration of 10 μg/ml of EGF stimulated prostaglandins generation but did not protect surface epithelial cells against ethanol injury. Conversely the concentration of 0.1 μg/ml of EGF was effective to protect surface epithelial cells, while did not stimulate significantly generation of prostaglandins. These findings indicated that the optimal concentration of EGF to protect surface epithelial cells is different from that to stimulate prostaglandins generation. This discrepancy suggests that EGF may also exert protective action effects independent of prostaglandins.

In summary, in this study we demonstrated that EGF can directly protect gastric surface epithelial cells without mediation by systemic factors. This action may be, in part, mediated by endogenous prostaglandins.

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