Effect of 16,16 dimethyl prostaglandin E₂ on aspirin induced damage to rat gastric epithelial cells in tissue culture

A TERANO, T MACH, J STACHURA,
A TARNAWSKI, AND K J IVEY

From The Department of Medicine, Long Beach Veterans Administration Medical Center, Long Beach, and University of California, Irvine, California, USA

SUMMARY Prostaglandins (PGs) protect gastric mucosa against damage produced by acetylsalicylic acid (ASA). Whether this effect of prostaglandins is truly cytoprotective and whether cAMP plays an important role in this effect is uncertain. We studied the effect of: (1) 16,16 dimethyl prostaglandin E₂ (dmPGE₂), isobutylmethyl xanthine (IMX), and dibutyryl cAMP (DBcAMP) on ASA-induced damage to monolayer cultures of rat gastric mucosa composed primarily of mucus cells; (2) dmPGE₂ on ASA absorption into the cultured cells. Cell damage was quantitated by ⁵¹Cr-release and trypan blue staining. Ten millimoles ASA significantly increased ⁵¹Cr-release (indicating cell damage) at pH 5.0, but not at pH 7.4. DmPGE₂ significantly reduced ASA-induced increase of ⁵¹Cr-release. Isobutylmethyl xanthine did not change the rate of ⁵¹Cr-release caused by ASA plus dmPGE₂. Dibutyryl cAMP did not significantly alter ⁵¹Cr-release caused by ASA. A dose response study of ASA damage showed close correlation between ⁵¹Cr-release and trypan blue staining (r=0.93). Dimethyl prostaglandin E₂ did not affect ¹⁴C-ASA incorporation by the cells at either pH 7.4 or pH 5.0. We conclude that: (1) dmPGE₂ exerts a cytoprotective effect on cultured rat gastric cells; (2) cAMP does not play an important role in such cytoprotection; (3) this protection is not because of interference with ASA absorption by prostaglandin.

The mechanism by which prostaglandins protect the gastric mucosa *in vivo* against a variety of ulcerogenic agents such as aspirin, ethanol, or taurocholate is unknown.¹⁻⁹ Whether prostaglandins exert a truly 'cyto'-protective effect on gastric mucosal epithelial cells remains controversial;^{4 10} further, there is debate as to whether cyclic AMP (cAMP) is a mediator of the assumed cytoprotective effect of prostaglandins.^{7 11 12}

The present study was undertaken to determine: (1) if prostaglandins protect the gastric epithelial cells against acetylsalicylic acid (ASA) induced injury in cultured rat gastric epithelial cells; (2) the role of cAMP in any protective effect of prostaglandins *in vitro*; (3) if this protection is due to

interference with absorption of aspirin into gastric cells *in vitro*.

Methods

MATERIALS

Animals

Five to ten day old rats (Sprague-Dawley, Sasco, Neb). Medium 1: Coon's modified Ham's F-12 medium (KC Biological Inc, Lenexa, KS) containing 0.1 g/dl collagenase (120 U/mg, GIBCO, Grand Island, NY) and 0.05 g/dl hyaluronidase (type I-S, 270 U/mg, Sigma Chemical Co, St Louis, MO), 100 U/ml of penicillin, 100 µg/ml of streptomycin and 100 µg/ml of gentamicin (all antibiotics were obtained from Sigma). Medium 2: F-12 medium supplemented with heat inactivated (56°C for 30 minutes) 10 g/dl fetal bovine serum (GIBCO), 15 mM Hepes buffer (Sigma), fibronectin (2 µg/ml, Sigma), and antibiotics as described above. Acetyl

Address for correspondence: Kevin J Ivey, MD, Gastroenterology Section, Veterans Administration Medical Center, 5901 E 7th Street, Long Beach, CA 90822, USA.

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salicylic acid, Trypan blue, dibutyl cyclic AMP, and 3-isobutyl-1-methyl xanthine were purchased from Sigma. ^{51}Cr (sodium chromate, 200–500 Ci/g chromium) and ^{14}C -acetylsalicylic acid (^{14}C -ASA; ^{14}C label on the salicylic acid moiety) were obtained from New England Nuclear, Boston, MA. 16,16 dimethyl prostaglandin E_2 (dmPGE $_2$) was a generous gift from Dr J E Pike, Upjohn Co, Kalamazoo, MI. Tissue culture plates were purchased from Falcon, Oxnard, CA.

Cell culture

The method has been described in detail elsewhere.¹³ The intact corpus (oxyntic glandular mucosa) area was excised from the antrum and fore-stomach of the rat stomach and the full wall thickness minced into 2–3 mm³ pieces. The minced tissues were incubated in medium 1 at 37°C for 60 minutes, in an atmosphere of 5% CO $_2$ and 95% O $_2$. The tissues were pipetted several times and filtered through a nylon mesh. The filtrate was washed by centrifugation at 600 rpm for 5 minutes in Hank's balanced salt solution. The cultures were maintained at 37°C with 5% CO $_2$ in the air in a humidified atmosphere. Cells from three day old cultures, which were at the confluent state, were studied.

Dye exclusion test

Trypan blue exclusion test was performed according to a modification of the method of Phillips.¹⁴ The culture media were discarded and the monolayers were washed three times with Hank's balanced salt solution. The cultures were incubated in Hank's balanced salt solution containing test agents for 60 minutes in the culture condition. After incubation, Hank's balanced salt solution was discarded and the cells were washed with Hank's balanced salt solution. The washed monolayers were kept in 1 ml of Hank's balanced salt solution until the test was performed. Trypan blue 0.1 ml (0.4 g/dl), was added directly to the Hank's balanced salt solution and mixed. Within 10 minutes the number of stained or non-stained cells was counted in an inverted microscope at 200 $\times$ magnification. Cell viability was expressed as follows:

$$\text{Viability (\%)} = \frac{\text{non-stained cells}}{\text{stained cells} + \text{non-stained cells}} \times 100$$

All experiments were performed by counting approximately 300 cells in each culture.

Isotope (^{51}Cr)-release assay

The media were discarded and the monolayers were washed three times with 1 ml Hank's balanced salt solution (4°C) to remove floating cells in culture.

The cells were then incubated in Hank's balanced salt solution containing 10 $\mu\text{Ci/ml}$ of ^{51}Cr for two hours in the culture condition. The labelled cells were washed five times with Hank's balanced salt solution to remove excess isotope and then 1 ml Hank's balanced salt solution (37°C) containing test drugs as described under 'experimental design' was added to the monolayer. After incubation for experimental periods in the culture condition, Hank's balanced salt solution (1) was decanted and the monolayers were washed with 1 ml Hank's balanced salt solution (4°C) (2). Hank's balanced salt solution of (1) and (2) were combined and centrifuged at 2000 rpm for 15 minutes. The pellets were combined with washed monolayers and dissolved in 1M NaOH for 24 hours. ^{51}Cr radioactivity of the cells and of the supernatant was counted with a well type Automatic Gamma Counting System (Searle Analytical Inc, Des Plaines, IL).

Calculation

The percentage of ^{51}Cr released per sample was expressed as:

$$\text{Release (\%)} = \frac{\text{cpm of supernatant}}{\text{cpm of supernatant} + \text{cpm of cells}} \times 100$$

or specific release (%) due to drug action = (percent of release in the presence of test drug) – (percent of release in the control).

^{14}C -ASA absorption into rat gastric cultured cells

In order to study the effect of pH and dmPGE $_2$ on ASA absorption into the gastric cells, uptake of ^{14}C -ASA into the cultured cells was determined.

On day 2 of culture, the cultured cells were incubated in medium 2 containing 0.1 $\mu\text{Ci/ml}$ of ^{14}C -ASA in both groups at pH 5.0 and 7.4. Before incubation of the cells, dmPGE $_2$ (1.0 μM) or saline was added to the medium. After incubation for two hours, the medium was discarded and the monolayers were washed with Hank's balanced salt solution three times. After dissolving the cells with 1M NaOH, the radioactivity of ^{14}C -ASA in the cells was measured in a liquid scintillation counter (Packard Instr Co, Downers Grove, IL). Total protein of the cells was measured according to the method of Bradford.¹⁵ The result was expressed as cpm/mg protein.

EXPERIMENTAL DESIGN

ASA-induced damage to gastric mucosal cells

(a) Time course of ^{51}Cr -release: prelabelled cells were incubated in Hank's balanced salt solution containing saline (controls at pH 7.4 and 5.0) or 10

mM ASA (pH 5.0) for periods up to three hours. (b) Effect of ASA at various pH concentrations on ^{51}Cr -release: prelabelled cells with ^{51}Cr were incubated in Hank's balanced salt solution containing 10 mM ASA or saline at various pH concentrations (7.4, 6.0, 5.0, 4.0) for two hours. (c) Dose response study of ASA and correlation between specific ^{51}Cr -release and dye exclusion test: saline at pH 5.0 (control) and ASA 1, 5, 10, and 20 mM at pH 5.0 was added to the culture for two hours. Subsequent specific ^{51}Cr -release and cell viability (dye exclusion test) were determined and compared.

Effect of dmPGE_2 on ASA-induced cell damage

Prelabelled cells for ^{51}Cr -release study and non-labelled cells for the dye exclusion test were incubated in Hank's balanced salt solution containing the following agents at pH 5.0: (a) 10 mM ASA, (b) 10 mM ASA plus dmPGE_2 (0.1, 1.0, 10.0 μM), (c) saline (control), (d) saline plus dmPGE_2 (1.0 μM).

Effect of DBcAMP or IMX on cell viability

In order to investigate the role of cAMP in prostaglandin cytoprotection, DBcAMP (1.0 mM) or dmPGE_2 (1.0 μM) plus IMX (0.1 mM) was added to the medium containing 10 mM ASA. These cells were processed for ^{51}Cr -release assay or dye exclusion.

Results

CELL CULTURE

The cultured cells reached confluency on day 2 or 3. Over 90% of the cells were identified as mucus producing epithelial cells; mitoses were seen, the mitotic index on day 2 being 2.0% as previously reported.¹³

EFFECT OF ASA ON ^{51}Cr -RELEASE AND CELL VIABILITY

The time course of ^{51}Cr -release by cultured cells treated with 10 mM ASA (at pH 5.0) or saline (at pH 7.4 or 5.0) is shown in Fig. 1. In the control group at pH 7.4, only a slight increase of ^{51}Cr -release was observed up to three hours, whereas at pH 5.0 ^{51}Cr -release was increased highly significantly at two and three hours compared with pH 7.4. In the group treated with 10 mM ASA at pH 5.0, this value was significantly increased compared with its control group at pH 5.0 at all time periods. Figure 2 shows the effect of pH on ^{51}Cr -release caused by 10 mM ASA or saline (control). At neutral values (pH 7.4), there was no significant difference in ^{51}Cr -release between 10 mM ASA and

Fig. 1 Time course of effect of ASA (10 mM) on ^{51}Cr -release. Each point represents mean $\pm$ SEM of four culture (* $p < 0.05$, ** $p < 0.01$ control (pH 5.0) values compared with control at pH 7.4; *** $p < 0.01$ ASA values compared with control at pH 5.0).

Fig. 2 Effect of pH on ^{51}Cr -release caused by 10 mM ASA (two hour incubation). Each point represents mean $\pm$ SEM of four culture (* $p < 0.05$, ** $p < 0.01$ compared with control).

control, while, at pH 5.0, 10 mM ASA significantly increased this value compared to control.

Figure 3 illustrates that results of specific release of ^{51}Cr and dye exclusion test caused by different concentrations of ASA were strongly positively correlated ($r=0.926$, $p<0.001$).

EFFECT OF DMPGE₂ ON SPECIFIC ^{51}Cr -RELEASE OF ASA-TREATED GASTRIC CULTURED CELLS

Figure 4 shows a dose response study of the effect of dmPGE₂ (0.1–10.0 μM) on specific ^{51}Cr -release of 10 mM ASA-treated rat gastric cultured cells. Addition of dmPGE₂ (1.0 and 10.0 μM) significantly diminished the increase of specific ^{51}Cr -release caused by 10 mM ASA (pH 5.0), whereas 0.1 μM dmPGE₂ did not. Dimethyl prostaglandin E₂ did not significantly change the effect of pH 5.0 on ^{51}Cr -release.

A similar degree of protection against 10 mM ASA-induced damage with 1.0 μM dmPGE₂ was seen using the dye exclusion test (p<0.01, Fig. 5).

EFFECT OF DBCAMP AND IMX ON CELL VIABILITY

Dibutyl cAMP (1.0 mM) did not significantly change ASA-induced decrease of viability (trypan blue exclusion test) or increase of ^{51}Cr -release (Figs 5 and 6). Isobutylmethyl xanthine did not cause any significant change in the effect of dmPGE₂ on ASA-induced decrease of viability (dye exclusion test) or increase cAMP level compared with dmPGE₂ alone in rat gastric cultured cells.¹³ These results show that cAMP does not play a major role in prostaglandin cytoprotection *in vitro*.

Fig. 3 Correlation between viability (trypan blue exclusion test) and ^{51}Cr -release caused by ASA at pH 5.0—dose response study. Each parenthesis indicates concentration (0, 1, 5, 10, 20 mM) of ASA. Each point represents mean $\pm$ SEM of six to eight cultures.

Fig. 4 Effect of 16,16 dimethyl prostaglandin E₂ (dmPGE₂, 0.1–10.0 μM) on specific ^{51}Cr -release caused by 10 mM ASA at pH 5.0. Each bar represents mean $\pm$ SEM of 11–12 cultures. Asterisks indicate statistically significant difference between ASA treated group with and without dmPGE₂ (* $p<0.01$).

EFFECT OF DMPGE₂ ON ^{14}C -ASA ABSORPTION INTO CULTURED CELLS

Figure 7 shows the results of ^{14}C -ASA absorption by rat gastric cultured cells at different pH levels (7.4 and 5.0) and after dmPGE₂ treatment. ^{14}C -ASA absorption was significantly enhanced at pH 5.0 compared with pH 7.4. Addition of dmPGE₂ (1.0 μM), however, did not significantly change ^{14}C -ASA absorption into the cells over corresponding control values for either pH 7.4 or 5.0.

Discussion

In this study we have shown that dmPGE₂ can significantly reduce ASA-induced damage of rat gastric epithelial cells in tissue culture, under conditions independent of systemic factor such as blood flow.

Robert and colleagues reported that prostaglandins can protect gastric mucosa against various kinds of damaging agents—for example, aspirin, alcohol, or taurocholate.^{1,2} The mechanism of this action of prostaglandins, however, has not been clarified.³

Fig. 5 Effect of ASA (10 mM), 16,16 dimethyl prostaglandin E₂ (dmPGE₂, 1.0 μM), isobutyl methyl xanthine (IMX, 0.1 mM), and dibutyl cyclic AMP (DBcAMP, 1.0 mM) on cell viability determined by trypan blue exclusion test. Each bar represents mean ± SEM of eight cultures. Probability values are expressed diagrammatically at the top of the figure.

Whether prostaglandins exert a direct protective effect on gastric mucosa independent of systemic influences especially of blood flow⁵⁻⁸ has been questioned. Chaudhury and Jacobson⁷ reported prostaglandin cytoprotection in isolated canine gastric mucosa and Schiessel *et al* showed it in isolated frog mucosa.⁹ Controversy, however, exists as to whether prostaglandins are truly 'cyto'-protective against damaging agents to gastric epithelial cells *in vivo*. In a preliminary report, Lacy and Ito⁴ claimed that prostaglandins did not prevent histological damage of the surface epithelium produced *in vivo* by ethanol. Blum's group¹⁰ could not show protection by PGE₂ against damage produced by taurocholate in isolated gastric cells.

Fig. 6 Effect of ASA (10 mM), 16,16 dimethyl prostaglandin E₂ (dmPGE₂, 1.0 μM), isobutyl methyl xanthine (IMX, 0.1 mM), and dibutyl cyclic AMP (DBcAMP, 1.0 mM) in various combinations on specific ⁵¹Cr-release at pH 5.0. Each point represents mean ± SEM of 11-12 cultures. Asterisks indicate statistically significant change from ASA treated group (*p < 0.01).

We tested the effect of dmPGE₂ on ASA-induced damage to rat gastric mucosal cells in an *in vitro* tissue culture composed predominantly of mucus-producing epithelial cells.¹³ In the present study, ASA considerably decreased cell viability at pH 5.0, but not at pH 7.4. Moreover, absorption of ASA into the gastric cells was significantly increased at pH 5.0 compared with that at pH 7.4. These results were compatible with other studies performed *in vitro*.¹⁶

As parameters of cellular damage, we used the dye exclusion test and ⁵¹Cr-release. Although the dye exclusion test has been widely used for estimation of cell viability and is a simple method, it also has some disadvantages; it is subjective and time consuming. On the other hand, the quantitative measurement of cell damage, using the release of radioactive isotopes¹⁷⁻²¹ provides an objective and accurate method which permits the handling of a large number of samples. Our results showed a strongly positive correlation between

Fig. 7 Effect of 16,16 dimethyl prostaglandin E_2 (dmPGE₂, 1.0 μM) on ASA absorption into cultured rat gastric cells at different pH levels. Each bar represents mean $\pm$ SEM of six cultures. None of the differences between the groups treated and not treated with dmPGE₂ were statistically significant.

^{51}Cr -release and dye exclusion.

The data obtained from the dye exclusion test and ^{51}Cr -release clearly showed that dmPGE₂ significantly diminishes ASA-induced cellular damage in the culture. We conclude that dmPGE₂ can protect gastric mucosal epithelial cells against ASA-induced damage independently of systemic influences – that is, it does exert a ‘cyto’-protective effect. The effects of other prostaglandins, such as PGI₂, on cultured cells are under investigation in our laboratory. Dimethyl prostaglandin E_2 did not completely prevent ASA damage to tissue cultures of gastric epithelial cells. This could explain results of *in vivo* studies showing histological damage to gastric surface epithelial cells after drugs such as alcohol despite pretreatment with this prostaglandin.⁴ Prostaglandins may also act by other mechanisms *in vivo* much as effects on circulation, cell turnover and migration.

Whether cAMP is mediator for this prostaglandin cytoprotection has been controversial.^{7 8 11 12} As prostaglandins have been shown to increase cAMP concentration in nonparietal cells,^{13 22 24} and cAMP stimulates mucus production¹³ and ion transport in gastric mucosa⁷ (both postulated mechanisms of cytoprotection), it was reasonable to assume that the mechanism of cytoprotection involved cAMP.

In this study we tested the effect of DBcAMP, an analogue of cAMP, on ASA-induced cellular damage measured both by ^{51}Cr -release and dye exclusion. The data obtained have shown that DBcAMP did not significantly increase the viability of ASA-treated cells. Further, IMX, a phosphodiesterase inhibitor, did not change the effect of dmPGE₂ on ASA-induced cellular damage, though it significantly increases cellular cAMP levels caused by dmPGE₂.¹³ Thus, it does not appear that prostaglandins exert their cytoprotective effect by increasing intracellular cAMP concentration in gastric epithelial cells. These results are consistent with reports of cytoprotective properties of prostaglandins in human gastric mucosal tissue independent of their effect on adenyl cyclase activity.¹²

The effect of dmPGE₂ on ASA absorption into gastric cells was studied to test the hypothesis that prostaglandin cytoprotection may be because of interference with absorption of the damaging agent.²⁵ In our study, ASA absorption into the cultured cells was not significantly different between the groups treated with dmPGE₂ (1.0 μM) or saline (control), whereas this concentration of dmPGE₂ exerted a cytoprotective effect on these cells. This confirms results of Guth and Paulsen²⁶ who reported that dmPGE₂, at doses that prevented aspirin induced lesions, did not affect aspirin absorption by the gastric mucosa.

In summary, dmPGE₂ diminishes ASA-induced damage to rat gastric epithelial cells cultured *in vitro*. Thus, dmPGE₂ can exert a true cytoprotective effect on gastric mucosal epithelial cells independently of systemic factors such as blood flow.

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