Effect of 16,16 dimethyl prostaglandin E$_2$ on aspirin induced damage to rat gastric epithelial cells in tissue culture

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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 E$_2$ (dmPGE$_2$), isobutylmethyl xanthine (IMX), and dibutyryl cAMP (DBcAMP) on ASA-induced damage to monolayer cultures of rat gastric mucosa composed primarily of mucus cells; (2) dmPGE$_2$ on ASA absorption into the cultured cells. Cell damage was quantitated by $^{51}$Cr-release and trypan blue staining. Ten millimoles ASA significantly increased $^{51}$Cr-release (indicating cell damage) at pH 5-0, but not at pH 7-4. DmPGE$_2$ significantly reduced ASA-induced increase of $^{51}$Cr-release. Isobutylmethyl xanthine did not change the rate of $^{51}$Cr-release caused by ASA plus dmPGE$_2$. Dibutyryl cAMP did not significantly alter $^{51}$Cr-release caused by ASA. A dose response study of ASA damage showed close correlation between $^{51}$Cr-release and trypan blue staining ($r=0.93$). Dimethyl prostaglandin E$_2$ did not affect $^{14}$C-ASA incorporation by the cells at either pH 7-4 or pH 5-0. We conclude that: (1) dmPGE$_2$ 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. Whether prostaglandins exert a truly 'cyto'-protective effect on gastric mucosal epithelial cells remains controversial. Whether prostaglandins exert a truly 'cyto'-protective effect on gastric mucosal epithelial cells remains controversial. Further, there is debate as to whether cyclic AMP (cAMP) is a mediator of the assumed cytoprotective effect of prostaglandins. 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 vivo; (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 $\mu$g/ml of streptomycin and 100 $\mu$g/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 $\mu$g/ml, Sigma), and antibiotics as described above. Acetyl
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. 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 mm$^3$ 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. 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× magnification. Cell viability was expressed as follows:

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

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 μCi/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:

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

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 μCi/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. 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 51Cr-release: prelabelled cells with 51Cr 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 51Cr-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 51Cr-release and cell viability (dye exclusion test) were determined and compared.

Effect of dmPGE2 on ASA-induced cell damage
Prelabelled cells for 51Cr-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 dmPGE2 (0·1, 1·0, 10·0 μM), (c) saline (control), (d) saline plus dmPGE2 (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 dmPGE2 (1·0 μM) plus IMX (0·1 mM) was added to the medium containing 10 mM ASA. These cells were processed for 51Cr-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 51Cr-RELEASE AND CELL VIABILITY
The time course of 51Cr-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 51Cr-release was observed up to three hours, whereas at pH 5·0 51Cr-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 51Cr-release caused by 10 mM ASA or saline (control). At neutral values (pH 7·4), there was no significant difference in 51Cr-release between 10 mM ASA and

Fig. 1  Time course of effect of ASA (10 mM) on 51Cr-release. Each point represents mean ± 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 51Cr-release caused by 10 mM ASA (two hour incubation). Each point represents mean ± 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 51Cr and dye exclusion test caused by different concentrations of ASA were strongly positively correlated (r=0.926, p<0.001).

**EFFECT OF DM-PGE2 ON SPECIFIC 51CR-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 51Cr-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 51Cr-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 51Cr-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**

Dibutyryl cAMP (1-0 mM) did not significantly change ASA-induced decrease of viability (trypan blue exclusion test) or increase of 51Cr-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.

**EFFECT OF DM-PGE2 ON 14C-ASA ABSORPTION INTO CULTURED CELLS**

Figure 7 shows the results of 14C-ASA absorption by rat gastric cultured cells at different pH levels (7.4 and 5-0) and after dmPGE2 treatment. 14C-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 14C-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.12 The mechanism of this action of prostaglandins, however, has not been clarified.3
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Fig. 5  Effect of ASA (10 mM), 16,16 dimethyl prostaglandin E₂ (dmPGE₂, 1-0 μM), isobutyl methyl xanthine (IMX, 0-1 mM), and dibutyryl 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.

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. Out results showed a strongly positive correlation between
Whether cAMP is mediator for this prostaglandin cytoprotection has been controversial. As prostaglandins have been shown to increase cAMP concentration in nonparietal cells, and cAMP stimulates mucus production and ion transport in gastric mucosa (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 dmPGE$_2$ on ASA-induced cellular damage, though it significantly increases cellular cAMP levels caused by dmPGE$_2$. 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.

The effect of dmPGE$_2$ 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. In our study, ASA absorption into the cultured cells was not significantly different between the groups treated with dmPGE$_2$ (1-0 $\mu$M) or saline (control), whereas this concentration of dmPGE$_2$ exerted a cytoprotective effect on these cells. This confirms results of Guth and Paulsen who reported that dmPGE$_2$ at doses that prevented aspirin induced lesions, did not affect aspirin absorption by the gastric mucosa.

In summary, dmPGE$_2$ diminishes ASA-induced damage to rat gastric epithelial cells cultured in vitro. Thus, dmPGE$_2$ can exert a true cytoprotective effect on gastric mucosal epithelial cells independently of systemic factors such as blood flow.

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