British Journal of Pharmacology (2000) 130, 811–818 

www.nature.com/bjp

# Opposite effects of flurbiprofen and the nitroxybutyl ester of flurbiprofen on apoptosis in cultured guinea-pig gastric mucous cells

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**1** The nitric oxide (NO)-donating nitroxybutyl ester of flurbiprofen (NO-flurbiprofen), shows reduced gastro-intestinal toxicity relative to flurbiprofen. NO may exert either pro- or anti-apoptotic effects, while non-steroidal anti-inflammatory drugs may induce apoptosis. The aim of the present work was therefore to compare the effects of flurbiprofen and NO-flurbiprofen on apoptosis in guinea-pig gastric mucous cells.

**2** Apoptotic activity was assessed by assay of caspase activity and from the fragmentation and condensation of nuclei.

**3** Incubation with flurbiprofen for 24 h produced a concentration-dependent induction of apoptosis in cells attached to the culture plate (caspase 3-like activity increased by 257% at 500 $\mu$M), while NO-flurbiprofen inhibited basal apoptosis (caspase 3-like activity decreased by 71% at 500 $\mu$M).

**4** Caspase activity and nuclear fragmentation were substantially increased in cells that had spontaneously detached from the culture plate. NO-flurbiprofen inhibited caspase activity (55% at 500 $\mu$M) but not nuclear fragmentation in these detached cells.

**5** NO flurbiprofen inhibited the activation of apoptosis by 25 $\mu$M $C_6$-ceramide in cells attached to the culture plate.

**6** Inhibition of caspase activity by NO-flurbiprofen was detectable after 6 h of incubation with intact cells, but by contrast with the NO-donor S-nitrosyl-N-acetyl-penicillamine, was not demonstrable with cell homogenates.

**7** Activation of caspase 3-like activity by flurbiprofen was slow (>6 h incubation needed) and was inhibited by cycloheximide.

**8** The presence of a nitroxybutyl ester moiety on flurbiprofen prevents the pro-apoptotic activity of the parent compound and may contribute to the reduced gastro-intestinal toxicity of NO-flurbiprofen.

*British Journal of Pharmacology* (2000) **130,** 811–818

**Keywords:** Nitric oxide; apoptosis; gastric epithelial cell; non-steroidal anti-inflammatory drug; flurbiprofen; nitroxybutyl flurbiprofen; stomach

**Abbreviations:** $C_6$-ceramide, N-Hexanoyl-D-sphingosine; NO-flurbiprofen, flurbiprofen nitroxybutyl ester; NO, nitric oxide; NSAIDS, non-steroidal anti-inflammatory drugs; SNAP, S-nitroso-N-acetyl-penicillamine; TNFα, tumour necrosis factor α

## Introduction

Apoptosis in the gastro-intestinal tract occurs close to the proliferative zone and at the surface of the epithelium (Hall *et al*., 1994), and plays an important role under normal circumstances in regulating mucosal cell number. Under pathological conditions apoptosis in the stomach is stimulated by infection with *Helicobacter pylori* (Mannick *et al*., 1996; Moss *et al*., 1996), and by administration of non-steroidal anti-inflammatory drugs (NSAIDs) (Zhu *et al*., 1998), which could contribute to the gastric atrophy seen with these agents (Taha *et al*., 1992).

NSAIDs damage the gastric mucosa: possible mechanisms being a direct topical action on epithelial cells, inhibition of prostaglandin biosynthesis and a reduction of gastric blood flow associated with a tumour necrosis factor α (TNFα)-mediated recruitment of neutrophils to the capillary endothelium (Santucci *et al*., 1994; 1995). One approach to reducing the gastro-intestinal toxicity of NSAIDS has been the development of NO-donating NSAIDs by the addition of a nitroxybutyl moiety. These agents show a significantly reduced gastro-intestinal toxicity without reduction of their anti-inflammatory effect (Wallace *et al*., 1994). Release of NO may inhibit neutrophil adherence, promote mucosal blood flow and counteract the pro-apoptotic effects of TNFα on endothelial cells by inhibition of caspase activity (Wallace *et al*., 1994; Fiorucci *et al*., 1999a,b).

The effects of NO on apoptosis vary with cell-type. Thus NO stimulates apoptosis in macrophages, pancreatic islets, and thymocytes (Brune *et al*., 1998), and in a wide variety of cell lines including one, RGM1, of rat gastric mucosal origin (Honda *et al*., 1999). Furthermore, recent work with transformed cell lines (Lu *et al*., 1995; Chan *et al*., 1998; Simmons *et al*., 1999; Zhu *et al*., 1999) has indicated that NSAIDs can induce apoptosis, but effects on gastric epithelial cells, with which they have topical contact, and in which they may accumulate (Wallace, 1997), have not been determined. The purpose of this investigation was therefore to use a primary culture of guinea-pig gastric mucosal cells, which consists of at least 90% of gastric mucous epithelial cells (Byrne & Hanson, 1998; Teshima *et al*., 1998), to determine whether the NO-donating NSAID nitroxybutyl-flurbiprofen (NO-flurbiprofen) would affect apoptotic activity and to compare the results with those obtained with the parent

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compound flurbiprofen. Two late manifestations of apoptosis namely nuclear fragmentation and condensation and increased activity of the effector proteolytic enzyme, caspase 3 (Raff, 1998), were used as indicators of programmed cell death.

## Methods

### *Animals*

Male Dunkin-Hartley guinea-pigs of 200–300 g body weight were obtained from Charles River, Margate, Kent U.K. and were fed on SDS Economy guinea-pig diet supplied by Lillico, Betchworth, Surrey, U.K.

### *Materials*

RPMI 1640 medium, foetal calf serum, antibiotics and amphotericin B were from Life Technologies, Paisley U.K. N-Hexanoyl-D-sphingosine ($C_6$-ceramide) and caspase substrates and inhibitors were obtained from Alexis, Nottingham, U.K. Pronase E (70,000 PU units per g) was purchased from Merck, Lutterworth, U.K. NO-flurbiprofen was from NicOx SA, Sophia-Antipolis, France. Other reagents were from Sigma, Poole, U.K.

### *Isolation and culture of gastric mucous cells*

The method has been described in detail previously (Byrne & Hanson, 1998). Gastric mucosa was minced with fine scissors, incubated with 45 ml of RPMI 1640 containing 2 g $l^{-1}$ bovine serum albumin (isolation medium) and 0.5 mg $ml^{-1}$ pronase for 20 min at 37°C, and then, after centrifugation, with 45 ml isolation medium containing 0.4 mg $ml^{-1}$ of collagenase for 20 min. Cells were filtered through 150 $\mu$m nylon mesh, washed in culture medium (RPMI 1640 containing 10% foetal calf serum, 100 u $ml^{-1}$ penicillin, 100 $\mu$g $ml^{-1}$ streptomycin and 2.5 $\mu$g $ml^{-1}$ of amphotericin B) and cultured on six-well culture plates. The culture medium was renewed after 24 h, and again at 48 h, at which time the experiments were initiated by the inclusion of agents in the culture medium. All measurements were made after a further 24 h. Agents were dissolved in dimethylsulphoxide, the final concentration of which was 0.25% (v $v^{-1}$) in all wells, including control wells.

### *Detection of apoptosis by staining with Hoechst 33258*

Cells in suspension, and those removed from the plate by exposure to trypsin/EDTA, were subjected to centrifugation for 20 s at 12,000 $\times g$. Cells were resuspended in 4% (w $v^{-1}$) paraformaldehyde in 138 mM NaCl, 2.7 mM KCl, 10 mM phosphate (PBS) for 10 min at room temperature, subjected to centrifugation at 12,000 $\times g$ for 10 s, resuspended in ethanol: water (4 :1) and stored at 4°C. Cells were transferred to glass slides by use of a Shandon cytocentrifuge, and nuclei were stained with 8 $\mu$g $ml^{-1}$ of Hoechst 33258. Slides were coded, and then the proportion of apoptotic nuclei was determined by counting using a fluorescent microscope with apoptosis defined as the presence of two or more condensed bodies per nucleus.

### *Preparation of homogenates*

The incubation medium was removed from the culture plate into 2 ml microfuge tubes. The plate was placed on ice and 1 ml of PBS was added to each well. The cells were removed from the plate with a cell-scraper and transferred to microfuge tubes. All microfuge tubes were subjected to centrifugation for 20 s at 12,000 $\times g$. Pellets were rinsed with 1.0 ml of ice cold PBS and then were resuspended, by pipetting up and down 10 times, in 110 $\mu$l of homogenization buffer (mM): HEPES 100, NaCl 140, EDTA 1, (pH 7.4), containing 0.5 mM phenylmethylsulphonyl fluoride, 5 $\mu$g $ml^{-1}$ aprotinin, 5 $\mu$g $ml^{-1}$ pepstatin and 10 $\mu$g $ml^{-1}$ leupeptin. Extracts were then frozen in liquid nitrogen and stored at −70°C. Before assay homogenates were thawed and refrozen twice, before a final thaw and centrifugation at 12,000 $\times g$ for 20 min at 4°C.

### *Caspase assay*

Twenty five $\mu$l of supernatant was incubated at 37°C with 175 $\mu$l of assay buffer consisting of 100 mM HEPES (pH 7.4) containing protease inhibitors at the same concentration as above, and caspase substrate at a final concentration of 15 $\mu$M. The substrates Ac-Tyr-Val-Ala-Asp-7-amido-4-methylcoumarin and Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin were used for caspase 1-like and caspase 3-like activity respectively. Formation of fluorescent product was measured on a Victor multilabel counter.

### *Acid phosphatase activity*

Enzyme activity was determined by the breakdown of p-nitrophenyl phosphate at pH 4.8 as described by Wong & Tepperman (1994).

### *Protein concentration determination*

Protein was measured by using bicinchoninic acid reagent with bovine serum albumin as standard (Redinbaugh & Turley, 1986).

### *Statistical analysis*

Results are presented as means ± s.e.mean, with $n$ equal to the number of cell preparations, and unless stated otherwise were subjected to analysis of variance, to remove variation between cell cultures, followed by either a Newman-Keuls or a Dunnett's multiple comparison test. Data on percentage of apoptotic cells were arcsine-transformed before analysis.

## Results

### *Effect of detachment from the culture plate and of $C_6$-ceramide on apoptotic activity in gastric epithelial cells*

Caspase 3-like activity was increased in cells which had detached from the culture plate, and was also increased by incubation for 24 h with $C_6$-ceramide but only in cells which remained attached (Figure 1a). Analogous findings on the effects of $C_6$-ceramide and detachment were obtained if nuclear fragmentation and condensation was used as the measure of apoptotic activity (Figure 1b).

### *Effect of flurbiprofen and NO-flurbiprofen on apoptotic activity in gastric epithelial cells*

Incubation with flurbiprofen (250 and 500 $\mu$M) for 24 h increased caspase 3-like activity in cells attached to the culture plate, but did not further enhance the already elevated activity

in detached cells (Figure 2a). NO-flurbiprofen exerted a dose-related inhibition of caspase 3-like activity in both attached cells, and in those that had detached from the culture plate (Figure 2b). Inhibition of caspase 3-like activity by 100 $\mu$M NO-flurbiprofen in attached cells was $58\pm9$ and $69\pm11\%$ in the presence and absence of the guanylate cyclase inhibitor 1*H*-[1,2,4] Oxadiazole [4,3-a] quinoxaline-1-one (ODQ, 10 $\mu$M) ($n=3$, no significant difference by paired *t*-test). For attached cells, the effects of flurbiprofen and NO-flurbiprofen (500 $\mu$M) on the percentage of cells showing condensation and fragmentation of DNA were qualitatively similar to those on caspase activity (Figure 3a). In detached cells flurbiprofen (500 $\mu$M) increased the proportion of apoptotic cells despite the absence of any change in caspase activity which was however already substantially elevated (Figure 3b). NO-flurbiprofen did not decrease the proportion of detached cells which were apoptotic (Figure 3b) despite decreasing caspase activity (Figure 2), which, however, at $545\pm147$ pmol min$^{-1}$ mg protein$^{-1}$ was still above ($P<0.05$, paired *t*-test, $n=5$) the control value for attached cells of $182\pm26$ pmol min$^{-1}$ mg protein$^{-1}$.

### *Effect of flurbiprofen and NO-flurbiprofen on cellular protein and release of enzyme activity from gastric epithelial cells*

Attached cellular protein ($\mu$g well$^{-1}$, $n=4$) was: $216\pm28$, $236\pm24$ and $249\pm20$ for control cultures and those incubated for 24 h with flurbiprofen (500 $\mu$M) and NO-flurbiprofen (500 $\mu$M) respectively; there being no significant effect of the added agents by analysis of variance. The cellular protein present in suspension was (per cent of total cellular protein per well, $n=4$): $10\pm3$, $17\pm6$ and $12\pm3$ for control cultures and those incubated for 24 h with flurbiprofen (500 $\mu$M) and NO-flurbiprofen (500 $\mu$M) respectively. Again there was no significant effect of agents when the data were subjected to analysis of variance. Release of acid phosphatase activity into the culture medium, which was used previously as an indicator

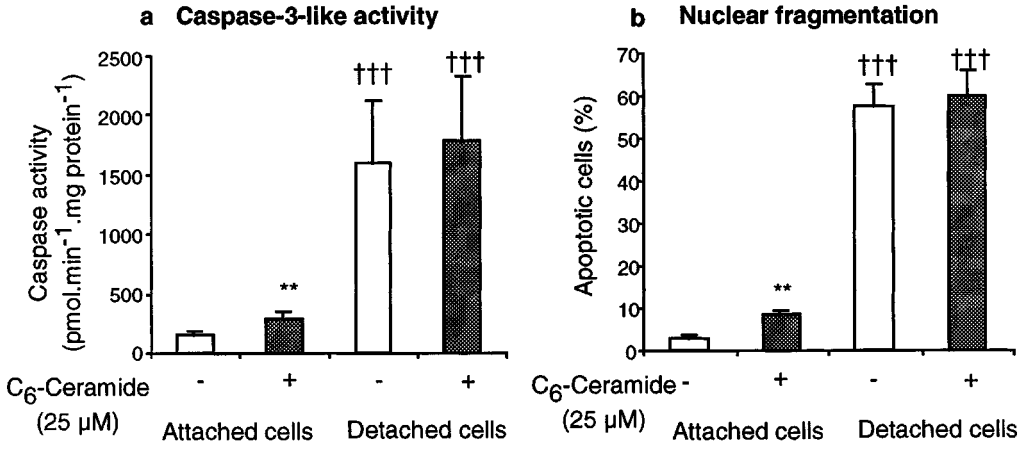


**Figure 1** Incubation of gastric epithelial cells for 24 h with $C_6$-ceramide, or detachment of cells from the culture plate increase (a) caspase 3-like activity and (b) nuclear condensation and fragmentation. Results are means$\pm$s.e.mean from six separate cell preparations, and have been analysed by analysis of variance followed by a Newman-Keuls multiple comparison test. $^{**}P<0.01$ for difference from appropriate control without ceramide, and $\dagger\dagger\dagger P<0.001$ for effect of detachment from the plate.

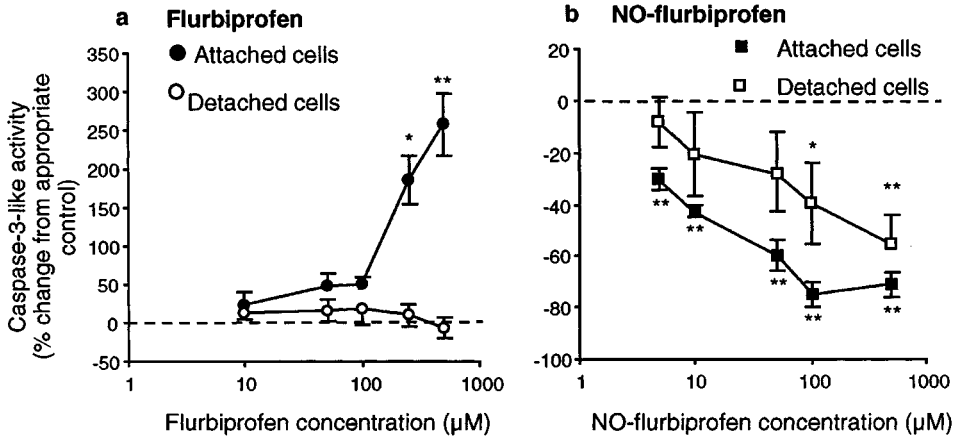


**Figure 2** Flurbiprofen activates caspase 3-like activity while NO-flurbiprofen inhibits it. Gastric epithelial cells were incubated for 24 h with different concentrations of (a) flurbiprofen and (b) NO-flurbiprofen. Results in (a) are means$\pm$s.e.mean from six separate cell cultures and are expressed as the per cent change from activities in the absence of agent which were: $147\pm36$ and $1779\pm775$ pmol min$^{-1}$ mg protein$^{-1}$ for attached and detached cells respectively. Results in (b) are means$\pm$s.e.mean from five separate cell cultures and are expressed as the per cent change from activities in the absence of agent which were: $182\pm26$ and $1439\pm353$ pmol min$^{-1}$ mg protein$^{-1}$ for attached and detached cells respectively. $^{*}P<0.05$, $^{**}P<0.01$ for difference from appropriate control by analysis of variance and Dunnett's test.

of damage to gastric mucosal cells (Wong & Tepperman, 1994), was (per cent of total activity, $n=4$) $1.33\pm0.24$, $1.35\pm0.60$ and $1.67\pm0.44$ for control, flurbiprofen (500 $\mu$M) and NO-flurbiprofen (500 $\mu$M) respectively (no significant effect of treatments by analysis of variance).

### *Selectivity of caspase activation*

Caspase 1-like activity, measured with the substrate Ac-Tyr-Val-Ala-Asp-7-amido-4-methylcoumarin, was much lower ($P<0.05$) than caspase 3-like activity under control conditions (respectively $10.4\pm4.7$ (5) and $147\pm37$ (6) pmol min$^{-1}$ mg protein$^{-1}$. Furthermore treatment for 24 h with respectively flurbiprofen (500 $\mu$M), NO-flurbiprofen (100 $\mu$M) or $C_6$-ceramide (25 $\mu$M), or detachment from the culture plate, gave the following results for caspase 1-like activity: $7.1\pm2$ (6), $12.2\pm2.3$ (4), $14.7\pm2.5$ (6) and $5.2\pm1.5$ (6). These treatments did not therefore produce changes in caspase 1-like activity of the magnitude seen with the caspase 3 substrate. The effect of preincubating homogenates for 10 min with the inhibitor Ac-Asp-Glu-Val-Asp-CHO (10 nM) on subsequent caspase 3-like activity was investigated. Inhibition of activity (%) on samples obtained after incubation for 24 h under control conditions, with flurbiprofen (250 $\mu$M) or after detachment from the culture plate was $90.5\pm1.4$ (3), $96.3\pm1.3$ (5) and $96.2\pm1.2$ (5) respectively.

### *Effect of NO-flurbiprofen on apoptotic activity in gastric epithelial cells incubated with $C_6$-ceramide*

Both caspase 3-like activity and the proportion of apoptotic cells was reduced by 500 $\mu$M NO-flurbiprofen (Figure 4) in attached cells incubated with $C_6$-ceramide (25 $\mu$M). NO-flurbiprofen did not affect either the proportion of apoptotic cells, or caspase 3-like activity, in cells which became detached in the presence of 25 $\mu$M $C_6$-ceramide (Figure 4).

### *Time course of changes in caspase 3-like activity*

All treatments decreased caspase 3-like activity in attached cells after 1 h relative to control. The reason for this is unclear, but it should be emphasized that the vehicle, dimethyl sulphoxide, was present in all wells. After 6 h NO-flurbiprofen (500 $\mu$M) decreased activity relative to control while $C_6$-ceramide increased it whether or not flurbiprofen (500 $\mu$M) was present. Flurbiprofen (500 $\mu$M) alone had no effect. The same situation pertained after 24 h of incubation except that

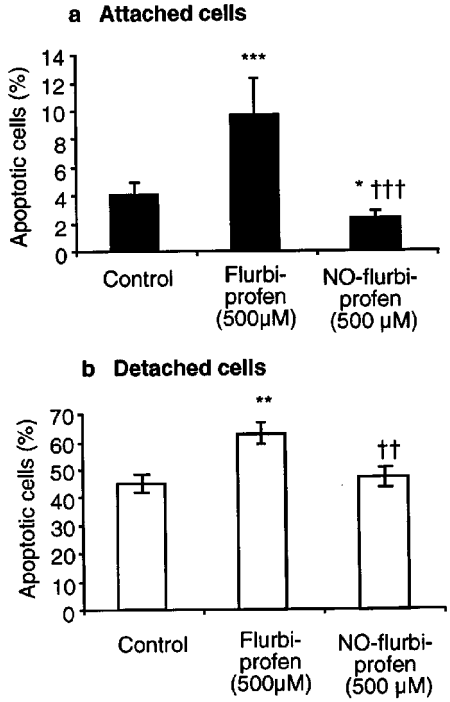


**Figure 3** Effect of incubation with flurbiprofen or NO-flurbiprofen on nuclear condensation and fragmentation in gastric epithelial cells, which after 24 h were attached (a) or detached (b) from the culture plate. Results are means$\pm$s.e.mean from five separate cell cultures and have been analysed by analysis of variance followed by a Newman-Keuls multiple comparison test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ for difference from control, and ††$P<0.01$, †††$P<0.001$ for difference between flurbiprofen and NO-flurbiprofen.

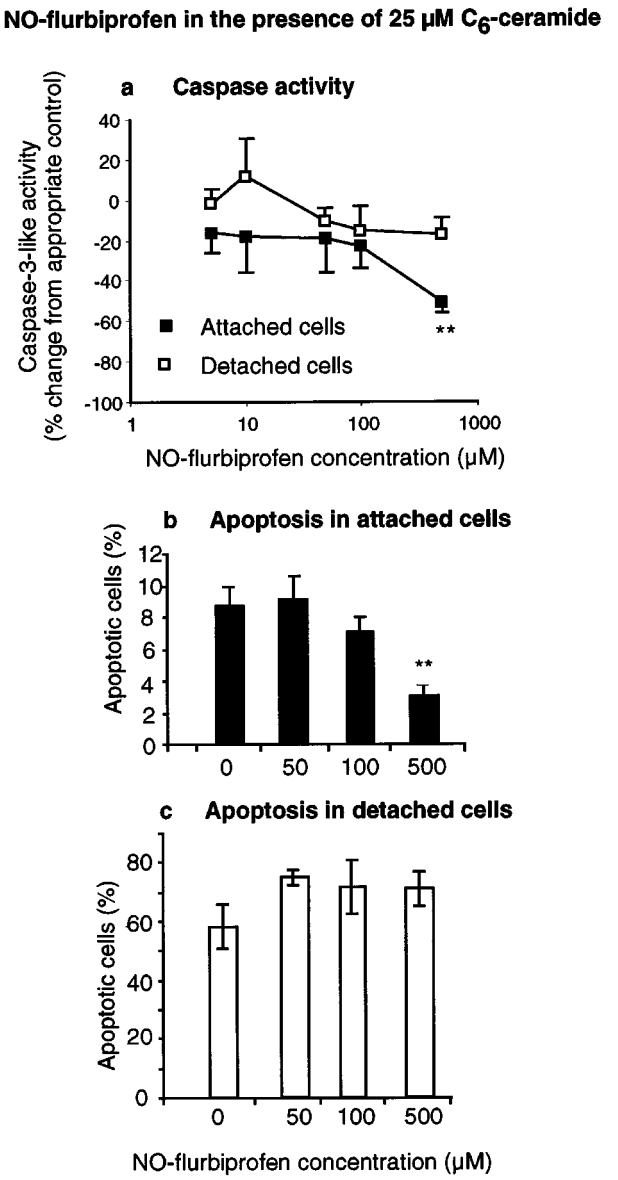


**Figure 4** Incubation for 24 h with NO-flurbiprofen inhibits (a) caspase activity and (b, c) nuclear condensation and fragmentation in attached gastric epithelial cells in the presence of 25 $\mu$M $C_6$-ceramide. Results in (a) are means$\pm$s.e.mean from five separate cell cultures and are expressed as the per cent change from activities in the absence of agent which were: $311\pm67$ and $2165\pm725$ pmol min$^{-1}$ mg protein$^{-1}$ for attached and detached cells respectively. Results in (b) and (c) are means$\pm$s.e.mean from three separate cell cultures. **$P<0.01$ for difference from appropriate control by analysis of variance and Dunnett's test.

there was now a marked stimulation of caspase activity with flurbiprofen (500 $\mu$M) alone. There were insufficient detached cells for measurement to be made at either 1 or 6 h (Figure 5).

### *Effect of cycloheximide on the stimulation of caspase activity by flurbiprofen*

Treatment for 24 h with cycloheximide (1 $\mu$M) reduced basal caspase activity in attached cells, while the higher concentration (5 $\mu$M) was without effect (Figure 6). One hundred $\mu$M cycloheximide produced variable, and in some instances highly elevated caspase activity $1100 \pm 522$ pmol min$^{-1}$ mg protein$^{-1}$ (4). The stimulatory effect of flurbiprofen (500 $\mu$M) was reduced by cycloheximide (1 $\mu$M) below that found under control conditions or with cycloheximide (5 $\mu$M).

### *Effect of S-nitroso-N-acetyl-penicillamine (SNAP) on the stimulation of caspase activity by flurbiprofen*

Flurbiprofen (500 $\mu$M) did not stimulate caspase activity in attached cells in the presence of the NO-donor SNAP. Results (pmol min$^{-1}$ mg protein$^{-1}$, $n=3$) were: control, $236 \pm 33$; SNAP (1 mM), $45 \pm 7.0$; and SNAP (1 mM) plus flurbiprofen (500 $\mu$M), $58 \pm 4.8$, with the two results in the presence of SNAP being not different from one another and lower ($P<0.01$) than the control (Newman-Keuls test).

### *Action of S-nitroso-N-acetyl-penicillamine (SNAP) and NO-flurbiprofen on caspase activity in homogenates and in intact cells: effects of dithiothreitol in the assay*

Homogenates from control attached cells were preincubated at room temperature for 0, 1, or 3 h before being assayed over the subsequent 40 min for caspase 3-like activity. Enzyme activity was relatively stable under control conditions and this situation was unaltered if NO-flurbiprofen (100 $\mu$M) was included during the preincubation period (Figure 7). However, incubations with SNAP (100 $\mu$M) produced a progressive decline in caspase activity, which was largely reversed by the inclusion of dithiothreitol (20 mM) during the assay period. If homogenates were taken from cells ($n=3$ separate experiments), which had been preincubated with 1 mM SNAP for 24 h, then the inhibitory effect of SNAP on caspase 3-like activity was respectively $76 \pm 4\%$ and $75 \pm 5\%$ with and without inclusion of dithiothreitol (20 mM) in the assay. Similarly the inhibitory effect of incubation with NO-flurbiprofen (100 $\mu$M) for 24 h on caspase activity was ($n=4$ separate experiments): respectively $64 \pm 9\%$ and $68 \pm 9\%$ assayed in the presence and absence of dithiothreitol. Inclusion of dithiothreitol (20 mM) in the assay did not affect basal caspase 3-like activity in the above experiments.

## Discussion

The main finding of this work is that NO-flurbiprofen inhibited apoptosis in guinea-pig gastric epithelial cells, while the parent compound, flurbiprofen promoted apoptosis. Apoptosis was assessed by the condensation and fragmentation of nuclei and by changes in caspase activity. Two pieces of evidence suggest that the hydrolysis of the substrate Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin represented the activity of caspase 3 and similar proteases and not some non-selective peptidase. Firstly, activity was profoundly inhibited

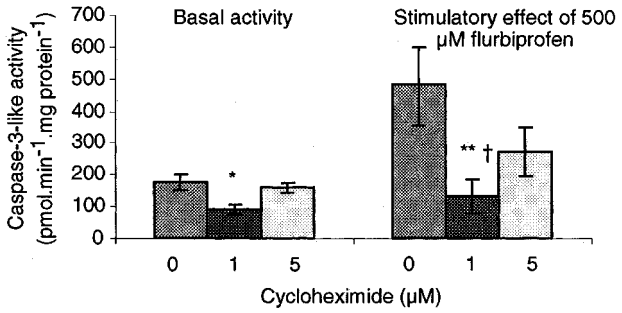


**Figure 6** Cycloheximide inhibits the stimulation of caspase activity by flurbiprofen. Results are means$\pm$s.e.mean from four separate cell preparations and have been analysed by analysis of variance and a Newman-Keuls test. Left-hand set: $*P<0.05$ for a significant difference of basal activity at 1 $\mu$M cycloheximide from activity at 0 or 5 mM. Right-hand set: $**P<0.01$ for difference in stimulation of caspase activity by 500 $\mu$M flurbiprofen at 0 and 1 $\mu$M cycloheximide, and $\dagger P<0.05$ for difference between 1 and 5 $\mu$M cycloheximide.

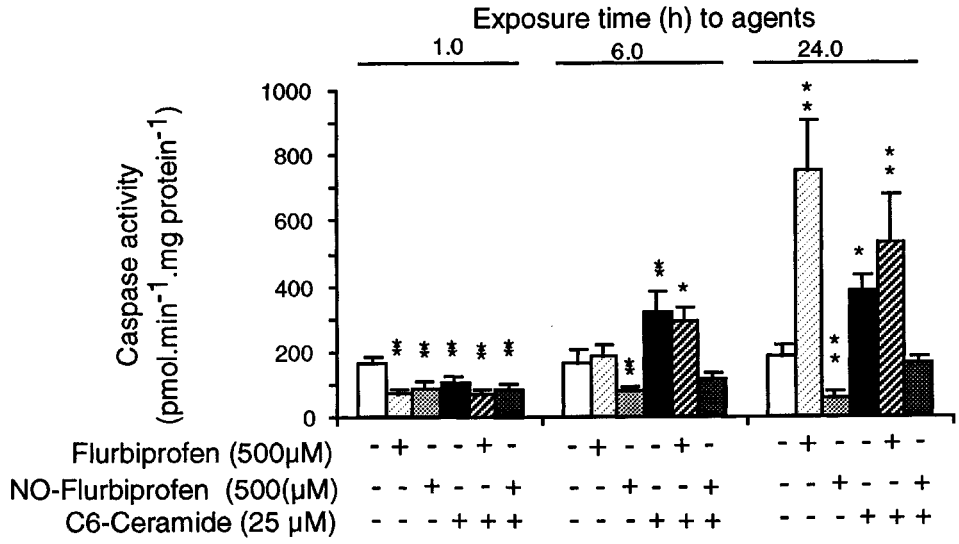


**Figure 5** Effects of $C_6$-ceramide and NO-flurbiprofen precede those of flurbiprofen on caspase activity in gastric epithelial cells attached to the culture plate. Results are means$\pm$s.e.mean from five separate cell cultures. $*P<0.05$, and $**P<0.01$ for difference from the control result for each time period by analysis of variance and a Newman-Keuls multiple comparison test.

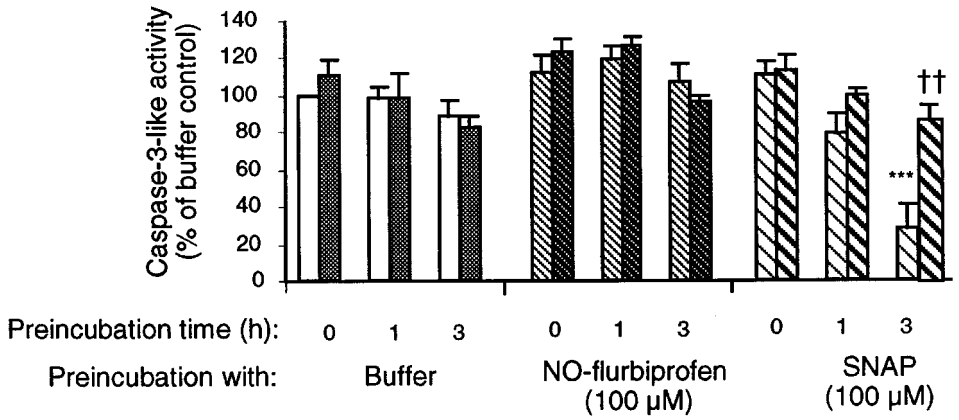


**Figure 7** Pre-incubation of 12,000 $\times g$ cell supernatants of gastric epithelial cells with S-nitroso-N-acetyl-penicillamine (SNAP), but not NO-flurbiprofen, causes a time-dependent inhibition of caspase activity. Results are means ± s.e.mean from four separate cell cultures. The left-hand and right-hand bars of each pair represent activity measured in the absence and presence of 20 mM dithiothreitol respectively. Results have been analysed by analysis of variance followed by a Newman-Keuls multiple comparison test. The difference highlighted *** is $P<0.001$ for the comparison between samples incubated with buffer and SNAP for 3 h, and †† is $P<0.01$ for the effect of dithiothreitol in samples preincubated with SNAP for 3 h.

by a low concentration of the caspase 3 inhibitor Ac-Asp-Glu-Val-Asp-CHO, and secondly substitution of three amino acids to generate a substrate for caspase 1 gave very much lower enzyme activities. Generally there was good agreement between the two indices of apoptotic activity. Thus both caspase activity and nuclear fragmentation were elevated by flurbiprofen and $C_6$-ceramide in attached cells and by detachment of cells from the culture plate under control conditions, while NO-flurbiprofen decreased both parameters in attached cells. However, detached cells treated with flurbiprofen exhibited an increase in apoptosis without a significant change in an already elevated caspase activity, and NO-flurbiprofen did not inhibit apoptosis in detached cells despite decreasing caspase activity although not to the basal value. In these two instances caspase activity would not seem to have been limiting apoptotic activity.

NO donors have been shown to inhibit caspase activity by S-nitrosylation of the cysteine residue at the active site (Dimmeler *et al*., 1997; Li *et al*., 1997) and this is thought to be the mechanism by which such agents inhibit apoptosis in hepatocytes preventing both caspase activity and activation (Kim *et al*., 1997; Li *et al*., 1999). Such a mechanism would be compatible with NO-flurbiprofen seeming a more effective inhibitor of caspase activity in control cells compared with those activated by $C_6$-ceramide, or by detachment from the culture plate. Thus the NO, or other reactive species, would be essentially titrated against the caspase activity which would be higher in activated cells. An involvement of cyclic GMP in the inhibitory effect of NO donors on caspase activity has been suggested (Kim *et al*., 1997; Brune *et al*., 1998; Fiorucci *et al*., 1999a). However this mechanism seems unlikely in the present case since the guanylate cyclase inhibitor ODQ did not prevent inhibition of caspase activity by NO-flurbiprofen.

The NO donor SNAP was shown in this work to inhibit caspase activity in homogenates and the reversal by 20 mM dithiothreitol strongly implicates a mechanism involving S-nitrosylation (Dimmeler *et al*., 1997; Li *et al*., 1997). However, no such reversal by dithiothreitol was seen if intact cells were pretreated with 1 mM SNAP. A possible explanation is that in intact cells NO prevents the activation of caspase 3 by effects on proximal caspases (Li *et al*., 1999) rather than inhibiting activated caspase 3. NO-flurbiprofen produced no inhibition of caspase-3-like activity in homogenates, despite inhibiting caspase activity in intact cells within 6 h of addition. Cirino *et al*. (1996) noted that NO-flurbiprofen released very little NO *in vitro*, and a possible explanation for the above results is that this compound requires metabolic activation within intact cells. Indeed metabolism of NO-flurbiprofen by an intestinal cytochrome P-450 isoenzyme has been suggested (Bertrand *et al*., 1998). Alternatively a mercapto group (-SH)-containing compound might be required for release of NO from NO-flurbiprofen (Feelish, 1991). It would therefore be of interest to determine formation of NO, nitrite, nitrate and S-nitrosothiols from NO-flurbiprofen both in the presence and absence of intact cells, but such an investigation is beyond the scope of the present work.

NO-donating NSAIDs inhibit apoptosis induced by TNFα in endothelial cells (Fiorucci *et al*., 1999b), and this mechanism is probably involved in the mucosal damage and stimulation of apoptosis caused by administration of NSAIDs to rats *in vivo* not being seen with NO-releasing NSAIDs (Fiorucci *et al*., 1999a,b). We have shown in the present study that a NO-releasing NSAID can also prevent apoptosis in gastric epithelial cells. Furthermore, this mechanism is operative in cells stimulated with $C_6$-ceramide. Ceramide has recently been implicated in the mechanism by which *Helicobacter pylori* induce apoptosis (Masamune *et al*., 1999), and there is also evidence that stimulation of apoptosis by TNFα may involve generation of ceramide (Mathias *et al*., 1998).

Flurbiprofen increased apoptosis in gastric epithelial cells at concentrations above 100 μM. There was no evidence of gross cytotoxicity, such as major loss of cellular protein or increased enzyme leakage, and it can be presumed that the drug was interacting with a specific site. The dependence of protein synthesis could reflect either flurbiprofen directly influencing the synthesis of a particular protein, or that its action on apoptosis depended on a protein which turned over rapidly. The above finding resembles the activation of apoptosis by high concentrations of NSAIDs in transformed cells, which, as here, was slow to take effect and dependent on protein synthesis (Lu *et al*., 1995; Chan *et al*., 1998; Simmons *et al*., 1999; Zhu *et al*., 1999). This appears to be the first demonstration that such a pathway exists in gastrointestinal cells in primary culture. However, further investigation will be required to establish whether these cells respond in this way to all NSAIDs. Uncoupling of mitochondrial oxidative phosphorylation by NSAIDs, which is abolished on modification of the carboxyl group as in

NO-flurbiprofen, may contribute to their gastro-intestinal toxicity (Mahmud *et al*., 1996). However such mechanism would not seem to be promoting apoptosis in the gastric epithelial cells since uncoupling by reducing ATP will tip the balance away from apoptosis towards necrosis (Leist *et al*., 1999), and a mitochondrially-mediated activation of caspase activity should be more rapid and independent of protein synthesis. Metabolism of NO-flurbiprofen within gastric epithelial cells seems likely (see above). The pro-apoptotic effect of any flurbiprofen that is released would however be masked by inhibition of caspase activity by NO or similar reactive species. Indeed the pro-apoptotic effect of flurbiprofen is not seen if cells are also incubated with the NO donor SNAP.

NO-releasing NSAIDs have been shown to have a reduced gastric toxicity compared with the parent compound (Wallace *et al*., 1994; Wallace, 1997) and in animal models this has been ascribed to maintenance of the gastric microcirculation through the prevention of the effects of TNF$\alpha$ on neutrophil recruitment to endothelial cells and increased apoptosis in these cells (Fiorucci *et al*., 1999a,b). The present work suggests that any apoptotic effect of NSAID, either direct, or mediated by TNF$\alpha$, on gastric epithelial cells, will not occur with the corresponding NO-NSAID and that such agents would be less likely than the parent compound to promote apoptosis and gastric atrophy in human subjects.

This work was supported by a grant from INTAS.

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*(Received November 23, 1999*
*Revised March 13, 2000*
*Accepted March 23, 2000)*