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Induction of apoptotic DNA fragmentation by nonsteroidal anti-inflammatory drugs in cultured rat gastric mucosal cells

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to cause apoptosis in several cell lines including transformed chicken embryo fibroblasts and human colon cancer cells. We herein report the apoptotic effect of NSAIDs in a non-transformed cell line derived from the rat gastric mucosa, RGM1 (rat gastric mucosa cell first). 1-[*p*-Chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid (indomethacin) and sodium 2-(2,6-dichloroanilino)phenylacetate (sodium diclofenac), potent and non-selective inhibitors of cyclooxygenase, were found to induce DNA fragmentation in RGM1 cells in a time- and concentration-dependent manner. The expression of mRNA for cyclooxygenase-2 was hardly detected in the intact cells but was clearly enhanced when the cells were incubated with the two NSAIDs. In contrast, the expression of mRNA for cyclooxygenase-1 was constitutive and was never affected by NSAIDs. The effect of [3,4-di(4-methoxyphenyl)-5-isoxazolyl] acetic acid (mofezolac), a potent and highly preferential inhibitor of cyclooxygenase-1, and *N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulphonamide (NS-398), a selective inhibitor of cyclooxygenase-2, on DNA fragmentation and cyclooxygenase-2 mRNA expression was weak compared to the effect of indomethacin or sodium diclofenac. The DNA fragmentation induced by sodium diclofenac was hardly affected by the exogenous addition of 16,16-dimethyl prostaglandin E_2 but was inhibited by caspase inhibitors such as Ac-YVAD-CHO and Ac-DEVD-CHO. The present data provide the first evidence that NSAIDs, such as indomethacin and sodium diclofenac, cause apoptotic DNA fragmentation in cultured gastric mucosal cells, and also indicate the involvement of caspases rather than the inhibition of cellular prostaglandin synthesis in the apoptotic process.

Keywords: Apoptosis; DNA fragmentation; Nonsteroidal anti-inflammatory drug; Cyclooxygenase; Prostaglandin; Caspase

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been clinically used for their anti-inflammatory, antipyretic, and analgesic properties. Unfortunately, their use is often accompanied by a high incidence of gastrointestinal side-effects. The molecular basis for the gastrointestinal toxicity of NSAIDs is widely believed to be their inhibitory activity against cyclooxygenase, which causes them to block the production of prostaglandins and their therapeutic actions (Vane, 1971; Wallace, 1994).

Two cyclooxygenase isoforms (cyclooxygenase-1 and -2) have been identified (Merlie et al., 1988; Funk et al., 1991; Kujubu et al., 1991; O'Neill and Ford-Hutchinson, 1993). Most NSAIDs inhibit both isoforms, although they vary in their relative potency against the two cyclooxygenase isoforms (Meade et al., 1993; Mitchell et al., 1994; Riendeau et al., 1997). Cyclooxygenase-1 is constitutively expressed, and the prostaglandins produced by this isoform have thus been thought to play a 'housekeeping' function involving the cytoprotection of the gastric mucosa. In contrast, cyclooxygenase-2 is an inducible enzyme that is normally absent in intact cells but is expressed in response to growth factors, tumor promoters, and cytokines (O'Banion et al., 1991; Xie et al., 1991; Hla and Neilson, 1992). Thus, the ability of NSAIDs to inhibit cyclooxygenase-2 may well explain their therapeutic utility. In contrast, the inhibition of cyclooxygenase-1 may explain their unwanted side-effects.

We previously reported the pharmacological properties of [3,4-di(4-methoxyphenyl)-5-isoxazolyl] acetic acid

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(mofezolac: Kusuhara et al., 1997a,b). This NSAID is a potent and highly preferential inhibitor of cyclooxygenase-1, and also exhibits potent analgesic activity especially in animal models of acute nociception. Mofezolac has been found to have relatively few gastrointestinal side-effects despite its potent activity against cyclooxygenase-1 (Ono et al., 1990). In addition, a recent report concerning cyclooxygenase-1 gene disruption in mice failed to point out any absolute involvement of the prostaglandins produced by cyclooxygenase-1 in gastric cytoprotection (Langenbach et al., 1995). The gastrointestinal toxicity of NSAIDs therefore remains to be elucidated regarding the involvement of other mechanisms.

Several NSAIDs have been shown to reduce the number and size of carcinogen-induced colon tumors (Pollard and Luckert, 1981; Reddy et al., 1993; Rao et al., 1995). Furthermore, NSAIDs such as 1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid (indomethacin) and sodium 2-(2,6-dichloroanilino)phenylacetate (sodium diclofenac) have been reported to induce apoptosis in certain cell lines, including Rous sarcoma virus-infected chicken embryo fibroblasts and human colon cancer cells (Lu et al., 1995; Hanif et al., 1996). The induction of apoptosis has thus been thought to be crucially involved in the cytotoxic effect of NSAIDs. NSAID-induced apoptosis has also been observed in a cancer cell line which produces undetectable amounts of prostaglandins, thus implicating the induction of apoptosis by a prostaglandin-independent pathway in such a cell line (Hanif et al., 1996).

In the present report, we describe the effects of NSAIDs on the induction of apoptotic DNA fragmentation in a non-transformed cell line derived from the rat gastric mucosa, RGM1 (rat gastric mucosa cell first).

2. Materials and methods

2.1. Reagents

Mofezolac ([3,4-di(4-methoxyphenyl)-5-isoxazolyl] acetic acid), sodium 2-(2,6-dichloroanilino) phenylacetate

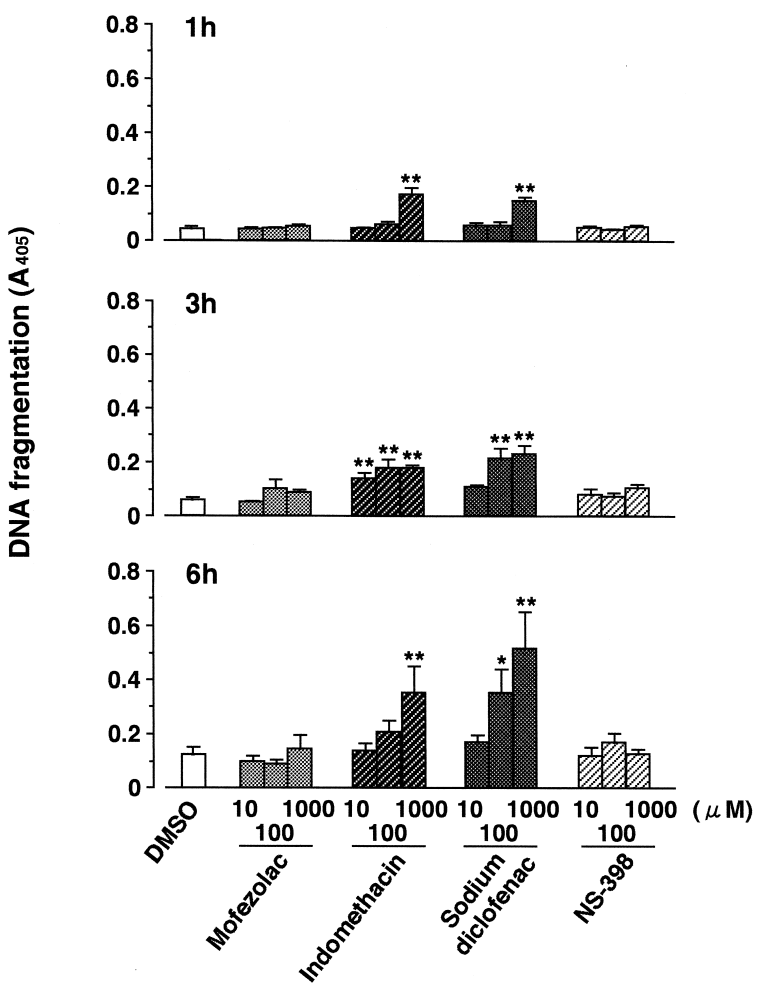

Fig. 1. The effect of nonsteroidal anti-inflammatory drugs on apoptotic DNA fragmentation in RGM1 cells. The cells were incubated with the indicated concentrations of drugs for 1, 3, and 6 h. The cytoplasmic histone-associated DNA fragments were determined by using a commercially obtained enzyme-linked immunosorbent assay kit. The results are the means ± S.E. from five independent determinations: $* P < 0.05$; $** P < 0.01$ vs. controls treated with only dimethyl sulfoxide (Dunnett's method, two-way layout).

(sodium diclofenac), and NS-398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulphonamide) were all synthesized at Yoshitomi Pharmaceutical Industries (Fukuoka, Japan). 1-[*p*-Chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid (indomethacin) was purchased from commercial sources. These drugs were dissolved and diluted in dimethyl sulfoxide (DMSO). Caspase-1 and -3 inhibitors were purchased from Takara Shuzo (Otsu, Shiga, Japan) and dissolved in sterilized water. The amino acid sequences of the inhibitors of caspase-1 and -3 are Ac-YVAD-CHO and Ac-DEVD-CHO, respectively. 16,16-Dimethyl prostaglandin E_2 was purchased from Sigma (St. Louis, MO).

2.2. Cell culture

The rat gastric mucosal cell line, RGM1, was obtained from the Riken cell bank (Tsukuba, Japan). This cell line is a diploid and non-transformed epithelial line isolated from the gastric mucosa of a normal Wistar rat (Kinoshita et al., 1995; Kobayashi et al., 1996; Miyazaki et al., 1996). The cells were seeded at a density of 1×10^5 per milliliter of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium supplemented with 20% fetal calf serum, and grown to confluence in 6-, 24-, and 96-well culture plates, depending on the experimental purposes. The cells were then washed, immersed in DMEM without serum, and subjected to each assay. The reagents were added to the medium at the onset of culture and thereafter were maintained until the termination of incubation. The final concentration of DMSO in the medium was 0.1%. Control cells treated without drugs were given DMSO. The cell culture was performed at 37°C in 5% CO_2/95% air.

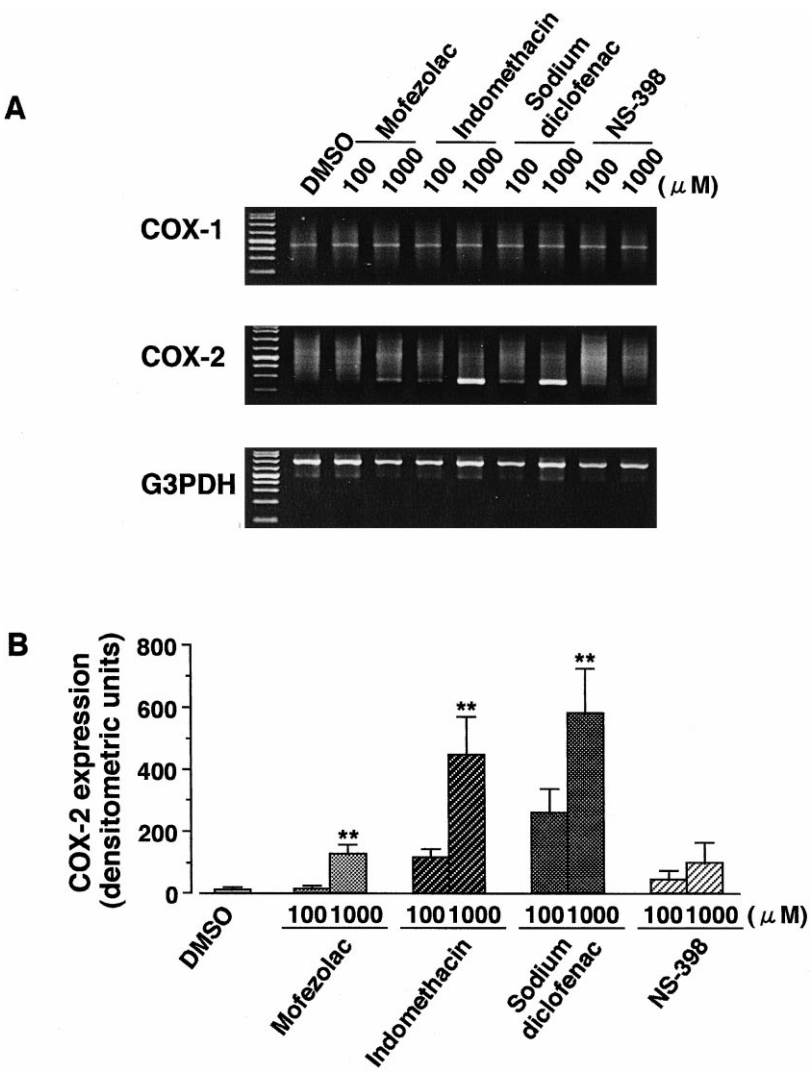

Fig. 2. The effect of nonsteroidal anti-inflammatory drugs on the expression of mRNA for cyclooxygenase (COX)-2 in RGM1 cells. The cells were incubated for 6 h with the indicated concentrations of drugs. The total RNA was then isolated and RT-PCR was performed with oligonucleotide primers specific for the nucleotide sequences of cyclooxygenase-1, cyclooxygenase-2, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) genes. (A) Typical patterns of the PCR products after agarose gel electrophoresis. The molecular standards are 2000, 1500, 1000, 700, 525, 500, 400, 300, 200, 100, and 50 base pairs in size. (B) A densitometric analysis of the PCR products. The results are the means ± S.E. from five independent determinations: $**P < 0.01$ vs. controls treated with only dimethyl sulfoxide (Dunnett's method, two-way layout).

2.3. Detection of apoptotic DNA fragmentation

RGM1 cells were grown in 96-well culture plates. Apoptotic DNA fragmentation was determined by using a commercially obtained enzyme-linked immunosorbent assay (ELISA) kit from Boehringer Mannheim (Mannheim, Germany). This assay is based on a quantitative sandwich-enzyme-immunoassay directed against cytoplasmic histone-associated DNA fragments (Bonfoco et al., 1995; Slomiany et al., 1997).

2.4. Measurement of mRNA expression by a semi-quantitative reverse transcription-linked polymerase chain reaction (RT-PCR)

Total RNA was isolated from the RGM1 cells grown in six-well culture plates by the guanidinium thiocyanate procedure (Chomczynski and Sacchi, 1987). The amount of mRNA for cyclooxygenase-1, cyclooxygenase-2, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was determined by using a RT-PCR kit from Takara Shuzo according to the protocol of the supplier. Briefly, cDNA was made from RNA samples by using antisense oligonucleotide primers and reverse transcriptase derived from avian myeloblastosis virus. Next, cDNA was amplified with additional sense oligonucleotide primers and Taq DNA polymerase in a Thermal Cycler Type MP (Takara Shuzo). RT-PCR was carried out under conditions in which the PCR product increased logarithmically depending on the amount of RNA and the number of cycles. One hundred nanograms of RNA was used in each RT reaction for cyclooxygenase-1 and cyclooxygenase-2, while 10 ng of RNA was used for G3PDH. The PCR was initiated in the thermal cycler programmed for 94°C, 30 s; 60°C, 30 s; and 72°C, 90 s. The PCR was performed for 25 cycles for cyclooxygenase-2 and G3PDH, and 30 cycles for cyclooxygenase-1. The primers for rat cyclooxygenase-1 and -2 were previously described (Beiche et al., 1996). The primers for rat G3PDH were purchased from Clontech (Palo Alto, CA). The PCR product was separated by electrophoresis through 3% NuSieve 3:1 agarose gel (FMC

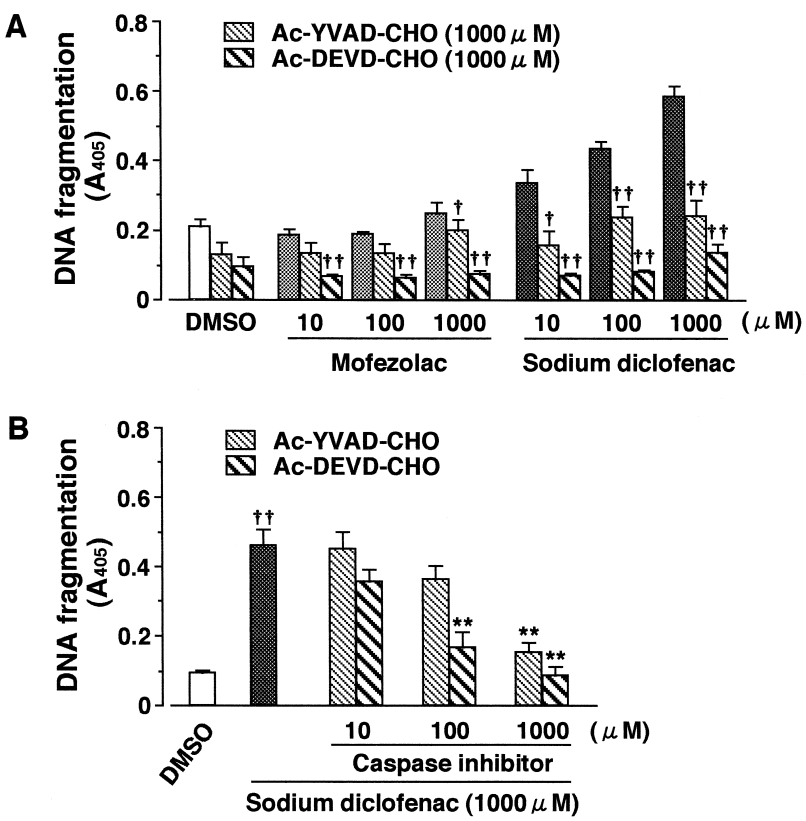

Fig. 3. The effect of caspase inhibitors on the DNA fragmentation induced by nonsteroidal anti-inflammatory drugs in RGM1 cells. (A) The cells were incubated for 6 h with the indicated concentrations of drugs in the presence of caspase inhibitors (Ac-YVAD-CHO or Ac-DEVD-CHO) at 1000 μM. The cytoplasmic histone-associated DNA fragments were determined by using a commercially obtained enzyme-linked immunosorbent assay kit. The results are the means ± S.E. from five independent determinations: †$P < 0.05$; ††$P < 0.01$ vs. cells untreated with caspase inhibitors (paired *t*-test). (B) The cells were incubated for 6 h with the indicated concentrations of caspase inhibitors in the presence of sodium diclofenac at 1000 μM. The cytoplasmic histone-associated DNA fragments were determined as described above. The results are the means ± S.E. from five independent determinations: ∗∗$P < 0.01$ vs. cells treated with only sodium diclofenac (Dunnett's method, two-way layout), ††$P < 0.01$ vs. controls treated with only dimethyl sulfoxide (paired *t*-test).

BioProducts, Rockland, ME) containing ethidium bromide, and visualized with a UV light source. The gel was photographed with Polaroid 665 film. The negative was scanned with a densitometer (CS-9300PC, Shimadzu, Kyoto, Japan), and the density of each band was determined as described earlier (Wang et al., 1991; Kusuhara et al., 1996).

2.5. Assays for cellular prostaglandin synthesis

The inhibitory activity of NSAIDs against cellular prostaglandin synthesis was determined in RGM1 cells and rat peritoneal cells. RGM1 cells grown in 24-well culture plates were preincubated with drugs for 30 min. Arachidonic acid (10 μM) was then added, and the cells were incubated for a further 30 min at 37°C as described by Mitchell et al. (1994). The formation of prostaglandin E_2 was measured by using a commercial ELISA kit.

Resident peritoneal cells were collected by lavage of the peritoneal cavity of unstimulated male Wistar rats (Japan SLC, Shizuoka, Japan) with heparinized Krebs solution. The cells (5×10^6) were treated for 20 min at 37°C with inactivated bacteria (*Bordetella pertussis*, Waco, Osaka, Japan) in the presence of drugs according to the method of Rosa and Persico (1979). The formation of prostaglandin E_2 was measured as described above.

2.6. Statistical analysis

The statistical significance of the data was analyzed by either the Dunnett method (two-way layout) or paired *t*-test. The values for the 50% inhibitory concentration (IC_{50}) and 95% confidence limit were calculated based on a linear regression analysis.

3. Results

3.1. Effect of NSAIDs on DNA fragmentation in RGM1 cells

The effect of mofezolac, indomethacin, sodium diclofenac, and NS-398 at concentrations from 10 to 1000 μM on DNA fragmentation in RGM1 cells is shown in Fig. 1. Of these drugs, sodium diclofenac and indomethacin both significantly induced DNA fragmentation. They caused DNA fragmentation within 1 h after the onset of incubation. During this period, these two drugs induced DNA fragmentation only at their highest concentrations; however, the same effect was also observed to a lesser degree at lower concentrations when the incubation was prolonged. The effect of indomethacin and sodium diclofenac was thus dependent on both the incubation time and their concentration. In contrast, mofezolac and NS-398 were found to have hardly any effect on DNA fragmentation.

3.2. Effect of NSAIDs on the expression of mRNA for cyclooxygenase-2 in RGM1 cells

The levels of expression of mRNA for cyclooxygenase-1 and -2 in RGM1 cells incubated for 6 h with the drugs are shown in Fig. 2. Expression of mRNA for cyclooxygenase-1, but not for cyclooxygenase-2, was detectable in the control cells. The mRNA expression for cyclooxygenase-2 was significantly induced in the presence of indomethacin and sodium diclofenac. These two drugs thus demonstrated a concentration-dependent effect. Mofezolac also showed a weak up-regulation of cyclooxygenase-2 mRNA expression. NS-398 hardly affected cyclooxygenase-2 mRNA expression. The expression of cyclooxygenase-1 mRNA was constitutive, and the various drug treatments had almost no effect.

3.3. Effect of caspase inhibitors on NSAID-induced DNA fragmentation

The effect of caspase inhibitors on the DNA fragmentation induced by mofezolac or sodium diclofenac is shown in Fig. 3. Cells were incubated for 6 h with the indicated amount of caspase inhibitors in the presence of the NSAIDs. Sodium diclofenac-induced DNA fragmentation was clearly inhibited by Ac-YVAD-CHO and Ac-DEVD-CHO. The inhibitory effect of Ac-DEVD-CHO was more potent than that of Ac-YVAD-CHO.

3.4. Effect of 16,16-dimethyl prostaglandin E_2 on NSAID-induced DNA fragmentation

The effect of 16,16-dimethyl prostaglandin E_2 on the sodium diclofenac-induced DNA fragmentation in RGM1 cells is shown in Fig. 4. Cells were incubated for 6 h with the indicated amount of 16,16-dimethyl prostaglandin E_2 in the presence of sodium diclofenac at 1000 μM. Exoge-

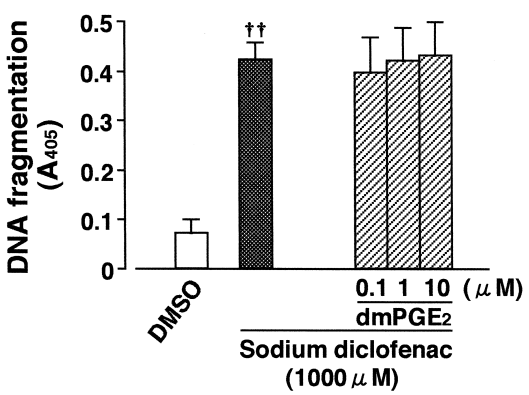

Fig. 4. The effect of 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$) on the DNA fragmentation induced by sodium diclofenac in RGM1 cells. The cells were incubated for 6 h with the indicated concentrations of 16,16-dimethyl prostaglandin E_2 in the presence of sodium diclofenac at 1000 μM. The cytoplasmic histone-associated DNA fragments were determined by using a commercially obtained enzyme-linked immunosorbent assay kit. The results are the means ± S.E. from five independent determinations: ††$P < 0.01$ vs. controls treated with only dimethyl sulfoxide (paired *t*-test).

Table 1
Inhibition of cellular prostaglandin E_2 synthesis by nonsteroidal anti-inflammatory drugs

Drug	IC_{50} [95% C.L.] (μM)
RGM1	
Mofezolac	0.057 [0.013–0.24]
Indomethacin	0.13 [0.030–0.57]
Sodium diclofenac	0.0097 [0.0023–0.052]
NS-398	11 [2.7–63]
Rat peritoneal cells	
Mofezolac	0.18 [0.093–0.36]
Indomethacin	0.15 [0.078–0.30]
Sodium diclofenac	0.30 [0.16–0.62]
NS-398	7.1 [3.7–14]

The values for the 50% inhibitory concentration (IC_{50}) and 95% confidence limit (C.L.) were calculated based on a linear regression analysis.

nous addition of 16,16-dimethyl prostaglandin E_2 did not affect the apoptotic process at the concentrations tested.

3.5. Effect of NSAIDs on cellular prostaglandin synthesis

The inhibitory effect of the drugs on cellular prostaglandin synthesis is summarized in Table 1. In RGM1 cells, sodium diclofenac was the most potent inhibitor of arachidonic acid-induced prostaglandin E_2 synthesis (IC_{50}: 9.7 nM), followed by mofezolac and indomethacin (IC_{50}: 57 nM and 130 nM, respectively). These three drugs showed a nearly equipotent inhibitory effect against prostaglandin E_2 synthesis in rat peritoneal cells stimulated with inactivated bacteria (IC_{50}: 150–300 nM). The inhibitory effect of NS-398 was less potent than that of the other drugs regarding both cell types (IC_{50}: 7.1–11 μM).

4. Discussion

In the present study, the effect of NSAIDs on the apoptotic DNA fragmentation was examined in cultured rat gastric mucosal cells. DNA fragmentation, a typical feature observable in cells undergoing apoptotic cell death (Carson and Ribeiro, 1993; Williams and Smith, 1993), was significantly induced in RGM1 cells by treatment with indomethacin and sodium diclofenac, which are potent and non-specific inhibitors of cyclooxygenase. The effect of these two NSAIDs was dependent on the incubation time and their concentration. In contrast, mofezolac, a potent and highly preferential inhibitor of cyclooxygenase-1 (Kusuhara et al., 1997a), and NS-398, a selective inhibitor of cyclooxygenase-2 (Futaki et al., 1994), hardly affected DNA fragmentation.

Indomethacin and sodium diclofenac showed potent inhibitory activity against prostaglandin E_2 synthesis in RGM1. mRNA for cyclooxygenase-1, but not for cyclooxygenase-2, was expressed in intact RGM1 cells. Accordingly, the inhibition of prostaglandin E_2 synthesis by the two drugs could be due to their potent inhibition of cyclooxygenase-1. Mofezolac also showed strong inhibitory activity against prostaglandin E_2 synthesis in RGM1. This activity was confirmed in an assay with rat peritoneal cells stimulated with inactivated bacteria. Taken together with the result which showed the weak activity of mofezolac on apoptotic DNA fragmentation, the inhibition of cyclooxygenase-1 does not seem to be closely involved in NSAID-induced apoptosis in RGM1 cells. In addition, exogenous 16,16-dimethyl prostaglandin E_2 did not inhibit the DNA fragmentation induced by sodium diclofenac in RGM1 cells. Based on these findings, NSAIDs may possibly cause apoptosis in gastric mucosal cells by means of a different mechanism than that which causes inhibition of prostaglandin synthesis.

Indomethacin and sodium diclofenac enhanced the expression of mRNA for cyclooxygenase-2 in RGM1 cells. Mofezolac weakly but significantly enhanced the expression of cyclooxygenase-2 mRNA. Since mofezolac failed to cause DNA fragmentation in the cells at the time cyclooxygenase-2 mRNA expression was determined, the up-regulation of cyclooxygenase-2 mRNA expression might precede the fragmentation of DNA. Overexpression of cyclooxygenase-2 mRNA also occurs in NSAID-induced apoptosis in Rous sarcoma virus-infected chicken embryo fibroblasts (Lu et al., 1995). In this report, expression of cyclooxygenase-2 mRNA is suggested to occur before cell death. Although there was no clear causal relationship between the two events in the present study, up-regulation of cyclooxygenase-2 expression is suggested to be a common phenomena in cells undergoing NSAID-induced apoptosis.

The DNA fragmentation in RGM1 cells induced by sodium diclofenac was efficiently inhibited by caspase inhibitors which have been shown to clearly inhibit apoptosis in several cell lines (Dubrez et al., 1996; Shimura et al., 1997). The inhibitory effect of Ac-DEVD-CHO was more potent than that of Ac-YVAD-CHO, thus indicating that caspase-3 is more involved than caspase-1 in the NSAID-induced apoptotic process in RGM1 cells. Caspase inhibitors also inhibited DNA fragmentation in the presence of mofezolac. Moreover, a time-dependent increase in DNA fragmentation was also observed in the cells incubated in serum-free medium without NSAIDs. This increase was also inhibited by caspase inhibitors. Caspases are thus suggested to be involved not only in NSAID-induced apoptosis but also in apoptosis caused by serum starvation in gastric mucosal cells. Thus, NSAIDs seem to induce apoptosis in gastric mucosal cells at least partly through mechanisms that are involved in the apoptotic process in other cell types.

Although mofezolac exhibited potent inhibitory activity against cyclooxygenase-1, this drug has been found to be less toxic to gastric mucosa than other NSAIDs including indomethacin (Ono et al., 1990). NS-398 is a selective inhibitor of cyclooxygenase-2 (Futaki et al., 1994; Riendeau et al., 1997). This drug has also been indicated to be

non-toxic toward gastric function (Futaki et al., 1993). In the present study, the apoptotic effect of mofezolac and NS-398 was shown to be less potent than that of indomethacin and sodium diclofenac. In our experiments, the drugs were used at relatively high concentrations but these are the concentrations to which the gastric mucosa would normally be exposed after oral administration of these drugs to rats. Thus, the induction of apoptosis in gastric mucosal cells by NSAIDs seems to be more closely related to their gastric cytotoxicity than to their inhibitory activity against cyclooxygenase-1.

In conclusion, in this study the induction of apoptotic DNA fragmentation by NSAIDs was demonstrated in cultured gastric mucosal cells. The apoptotic effect does not seem to be closely involved in the inhibition of cellular prostaglandin synthesis. Further studies on the molecular mechanisms by which NSAIDs induce apoptosis in gastric mucosal cells are thus still called for.

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