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European Journal of Pharmacology 369 (1999) 365–371

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Effect of combination of misoprostol and indomethacin on eicosanoid production in carrageenan-induced air pouch inflammation in rats

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Received 3 November 1998; revised 5 February 1999; accepted 9 February 1999

Abstract

The effect of single or combined administration of indomethacin and misoprostol on the exudate leukocyte count and thromboxane B_2, a stable metabolite of thromboxane A_2, and on the leukotriene B_4 level, as cyclooxygenase and lipoxygenase metabolites of arachidonic acid, was investigated in acute carrageenan-induced air pouch inflammation in rats. Administration of indomethacin (0.25 to 4 mg/kg) 1 h before carrageenan given by the orogastric route reduced the exudate leukocyte count and thromboxane B_2 level whereas it increased the exudate leukotriene B_4 level dose dependently. Administration of misoprostol, a synthetic prostaglandin E_1 analogue, (12.5 to 100 μg/kg) twice daily for two days before carrageenan given by the orogastric route increased the exudate leukocyte count. Combined misoprostol and indomethacin did not change the effect of indomethacin alone on exudate leukocyte count. Misoprostol, when used alone, decreased exudate thromboxane B_2 level significantly. However, misoprostol did not change the exudate leukotriene B_4 level, while its combination with indomethacin prevented the indomethacin-induced increase in exudate leukotriene B_4 level. In conclusion, although misoprostol can be combined with non-steroidal anti-inflammatory drugs in many chronic inflammatory situations, our results indicate that misoprostol may also be combined with indomethacin in acute inflammation without producing any change on the antiinflammatory efficacy of indomethacin in rats.

Keywords: Air pouch; Carrageenan; Thromboxane B_2; Leukotriene B_4; Misoprostol; Indomethacin

1. Introduction

Prostaglandins have distinct effects on inflammation. Prostaglandin E_2 and prostacyclin produce vasodilatation especially in arterioles, metaarterioles, precapillaries and venules (Greenberg and Sparks, 1969). Alone, they do not increase vascular permeability but contribute to the augmentation of vascular permeability in synergy with other mediators such as histamine and bradykinin (Williams, 1979). Prostaglandins can also cause pain (Ferreira, 1972) and fever (Feldberg and Saxena, 1971). As prostaglandins reproduce the cardinal signs of inflammation, it is generally believed that they are mediators of acute inflammation. However, prostaglandins exert anti-inflammatory effects by inhibiting the activation of inflammatory cells in chronic and immune-mediated inflammation (Cox and Karnovsky, 1973; Goodwin et al., 1978). It has been shown that exogenously applied prostaglandins have beneficial effects on adjuvant arthritis, which is accepted as an experimental model of rheumatoid arthritis (Zurier and Quagliata, 1971).

Misoprostol, a synthetic prostaglandin E_1 analogue, was developed for the prevention and treatment of non-steroidal anti-inflammatory drug (NSAID)-induced gastropathy (Graham et al., 1988; Roth et al., 1989). Since NSAID-induced gastropathy was generally accepted as a resulting from the suppression of cytoprotective prostaglandins, such as prostaglandin E_2 and prostaglandin I_2, this use of misoprostol is an example of substitution therapy. Although there are some controversial findings, the beneficial effects of NSAIDs in inflammation are also thought to be mediated by inhibition of cyclooxygenase, thereby preventing the production of prostaglandins. Studies on the efficacy of misoprostol alone or in combination with NSAIDs are limited. It has been found that the combination of misoprostol with acetyl salicyclic acid has beneficial effects in adjuvant arthritis (Tascilar et al., 1993).

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00093-X

The present study attempted to investigate the effect of indomethacin and misoprostol, singly or in combination, by measuring the exudate leukocyte count, exudate thromboxane B_2 and leukotriene B_4 levels in the carrageenan-induced air pouch model of acute inflammation.

2. Materials and methods

2.1. Animals

The experiments were performed on female Wistar albino rats weighing 130–200 g. The experiments were approved by Ankara University Animal Ethical Committee. The animals were kept at room temperature, fed their ordinary diet and allowed to drink water ad libitum.

2.2. Air pouch formation

The air pouch was formed by subcutaneous injection of 20 ml air into the back initially and further injections of 10 ml air every 3 days to sustain its patency (Sedgwick and Lees, 1986). Six days after the initial injection of air, 2 ml of 1% carrageenan solution in sterile 0.9% saline was injected into the cavity. Six hours after carrageenan injection, the animals were anesthetised with ether and the cavity was flushed out with 1 ml of heparinised saline (10 units per ml).

2.3. Collection and processing of exudates

The pouch cavity was opened and the exudate was harvested at 6 h after administration of carrageenan. The exudate was transferred to ice-cold tubes and BW 755C [3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride](10^{-5} M, final concentration), a cyclooxygenase and lipoxygenase inhibitor, was added immediately, in order to prevent in vitro production of these metabolites of arachidonic acid. Exudate volumes were measured using a graduated tube. Total leukocyte numbers were counted using the improved Neubauer technique. A cell-free exudate was obtained by centrifugation at 3000 g, 4°C for 15 min and stored at −44°C for the quantification of eicosanoids.

2.4. Enzyme immunoassay of thromboxane B_2 and leukotriene B_4

Thromboxane B_2 and leukotriene B_4 were measured by enzyme immunoassay (EIA) without prior extraction or purification (Pradelles et al., 1985). Exudates were diluted in EIA buffer for thromboxane B_2 (1:10 and 1:20) and leukotriene B_4 (1:5). The assay was performed on a total volume of 150 μl with the following components being added in 50 μl volumes; standards or biological samples, enzymatic tracer and antibody. After overnight incubation at 4°C, the plates were washed and 200 μl Ellman's reagent was added into each well. After 1–2 h, the absorbance of each well at 405 nm was measured. Standard curves from 7.8 to 1000 pg/ml and 15.6 to 2000 pg/ml were used in order to evaluate the concentrations of thromboxane B_2 and leukotriene B_4, respectively. The results were calculated in terms of percent of B/Bo.

2.5. Drugs and chemicals

Indomethacin (FAKO llaclari, Istanbul, Turkey) and misoprostol (Searle, Skokie, IL) were kindly provided by

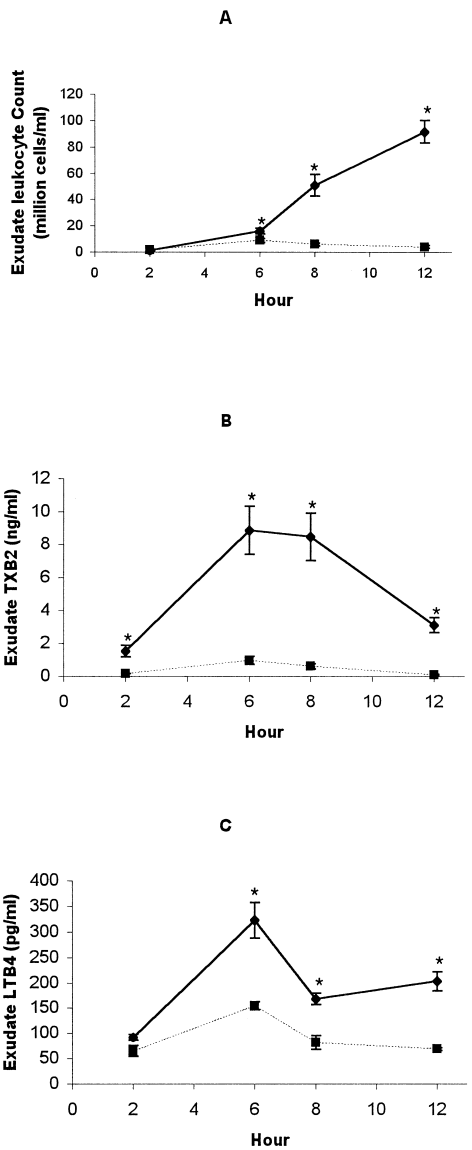

Fig. 1. Exudate (A) leukocyte count, (B) thromboxane B_2 and (C) leukotriene B_4 level 2, 6, 8 and 12 h after carrageenan injection. Solid lines indicate carrageenan experiments while dotted lines indicate control experiments. Control experiments were performed by injection of 2 ml sterile 0.9% saline into the air pouch. Results are expressed as means ± S.E.M. for 6–8 rats (carrageenan experiments) or 3–4 rats (control experiments). Comparison of the carrageenan group with the control group was made by Student's *t*-test. * Significantly different from control group ($P < 0.05$).

Table 1
Effect of indomethacin on exudate volume

Group	Exudate vol. (ml)
Saline control	1.12 ± 0.17
Carrageenan control	$2.45 \pm 0.08^{a,b}$
Indo 0.25 mg/kg	$2.48 \pm 0.12^{a,b}$
Indo 0.5 mg/kg	$2.28 \pm 0.03^{a,b}$
Indo 1 mg/kg	$2.36 \pm 0.06^{a,b}$
Indo 2 mg/kg	1.77 ± 0.07^{a}
Indo 4 mg/kg	1.96 ± 0.08^{a}

Indomethacin (Indo) was administered by the orogastric route 1 h before carrageenan injection. Exudate was harvested 6 h after carrageenan injection. Exudate volumes were measured using a graduated tube. Results are expressed as means ± S.E.M. for 6–8 rats. The different treatment protocols were compared using a one-way ANOVA following the Student–Neuman–Keuls, post-hoc test.
[a] Significantly different from saline control ($P < 0.05$).
[b] Significantly different from indo 2 and 4 mg/kg group ($P < 0.05$).

the manufacturers. Lambda carrageenan was purchased from Sigma (St. Louis, MO, USA). Indomethacin was dissolved in 0.5% carboxymethyl cellulose for daily use and administered 1 h before carrageenan administration by the orogastric route. Misoprostol was prepared from the lyophilized form and dissolved in absolute ethanol to obtain a stock solution (1 mg/ml), then stored at −44°C. Stock solution of misoprostol, 1 ml, was mixed with 1 ml of absolute ethanol, and 8 ml of phosphate buffer (pH 7.4) to obtain a concentration of 100 μg/ml of misoprostol for a weekly experimentation period. A daily dilution was freshly prepared in phosphate buffer (pH 7.4) just before use. It was administered twice daily for two days before carrageenan administration by the orogastric route. The final concentration of ethanol in the misoprostol solution was 2% and this concentration of ethanol did not affect either the exudate leukocyte count or thromboxane B_2 or leukotriene B_4 levels (data not shown). Standards and enzymatic tracers of thromboxane B_2 and leukotriene B_4, antibody to leukotriene B_4 and precoated EIA microtiter plates were purchased from Cayman Chemical (Ann Arbor, MI, USA). The antibody to thromboxane B_2 was kindly provided by Professor GianCarlo Folco (Institute of Pharmacological Sciences, University of Milan, Italy).

2.6. Statistics

Values were expressed as means ± S.E.M. Comparison of the various treatment protocols was made by one-way analysis of variance (ANOVA) following a Student–Neuman–Keuls, post-hoc test. The minimal level of significance was considered as $P < 0.05$. Dose dependence of the effect of indomethacin on exudate leukocyte count, exudate thromboxane B_2 and leukotriene B_4 level was assessed by evaluating the coefficient (r^2) of non-linear regression.

3. Results

3.1. Time profile of exudate leukocyte count, thromboxane B_2 and leukotriene B_4 levels in the carrageenan-induced air pouch model

Exudate was harvested at 2, 6, 8 and 12 h after carrageenan injection and exudate leukocyte count, thromboxane B_2 and leukotriene B_4 level were determined (Fig. 1A, B and C). Although exudate thromboxane B_2 and

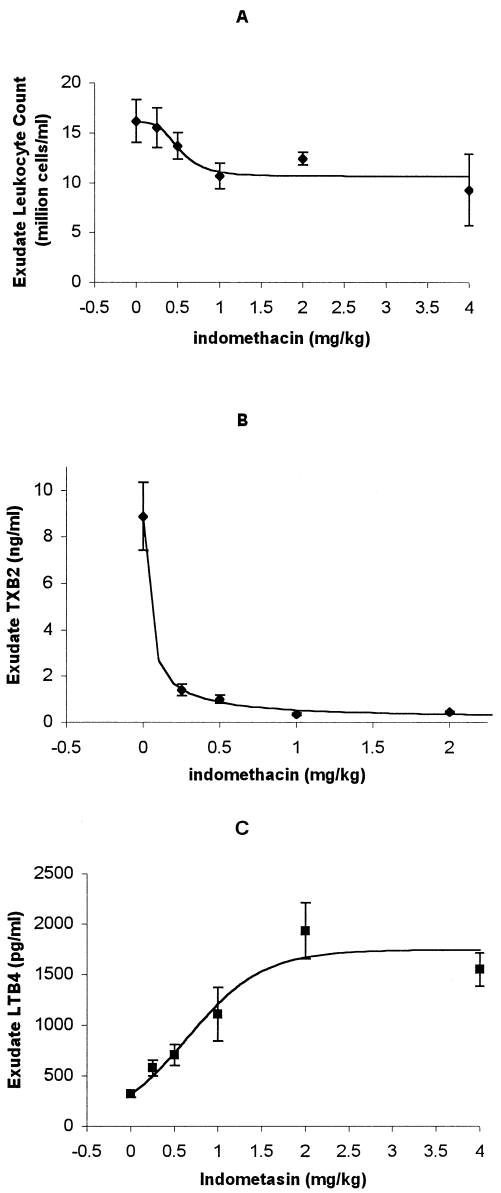

Fig. 2. Effects of indomethacin in doses ranging 0.25–4 mg/kg given 1 h before carrageenan injection by the orogastric route on exudate (A) leukocyte count, (B) thromboxane B_2 and (C) leukotriene B_4 level. Exudate was harvested 6 h after carrageenan injection. Solid curves are the best fit of logistic equations (r^2 for exudate leukocyte count, exudate thromboxane B_2 and exudate leukotriene B_4 are 0.86, 0.99 and 0.94 respectively). Dotted lines indicate control value from corresponding experiment. Results are expressed as means ± S.E.M. for 6–8 rats.

leukotriene B_4 levels reached maximum levels 6 h after carrageenan injection, the exudate leukocyte count continued to increase progressively up to the end of 12 h. Exudate was harvested in all subsequent experiments 6 h after carrageenan injection.

3.2. Effects of indomethacin on exudate volume, exudate leukocyte count, thromboxane B_2 and leukotriene B_4 levels

Higher doses of indomethacin, 2 and 4 mg/kg, significantly reduced exudate volume (Table 1). Indomethacin, from 0.25 to 4 mg/kg, decreased exudate leukocyte count and thromboxane B_2 level in a dose-dependent manner (Fig. 2A and B). However, the inhibition of thromboxane B_2 production was more marked than the decrease of the exudate leukocyte count. The 2-mg/kg dose of indomethacin induced 95% reduction of the production of thromboxane B_2, whereas the reduction of the exudate leukocyte count reached 30% with the same dose of indomethacin. In contrast, indomethacin increased the exudate leukotriene B_4 level in a dose dependent manner (Fig. 2C). The 2-mg/kg dose of indomethacin induced a 497% augmentation of production of leukotriene B_4.

3.3. Effects of misoprostol on exudate volume, exudate leukocyte count, thromboxane B_2 and leukotriene B_4 levels

Misoprostol, except for the lowest dose, did not significantly affect exudate volume compared to that of the carrageenan control (Table 2). Misoprostol, ranging from 12.5 to 100 μg/kg, increased the exudate leukocyte count (Fig. 3). This increase was significantly different from that in the control group at a dose of 25, 50 and 100 μg/kg. Although misoprostol reduced the exudate thromboxane B_2 levels for all doses except 25 μg/kg (Fig. 4), it did not significantly affect the exudate leukotriene B_4 levels (Fig. 5).

Table 2
Effect of misoprostol on exudate volume

Group	Exudate vol. (ml)
Saline control	1.12 ± 0.17
Carrageenan control	$2.45 \pm 0.08^{a,b}$
Miso 12.5 μg/kg	1.92 ± 0.05^{a}
Miso 25 μg/kg	2.15 ± 0.18^{a}
Miso 50 μg/kg	$2.27 \pm 0.09^{a,b}$
Miso 100 μg/kg	$2.34 \pm 0.08^{a,b}$

Misoprostol (Miso) was administered twice daily by the orogastric route for two days before carrageenan injection. Exudate was harvested 6 h after carrageenan injection. Exudate volumes were measured using a graduated tube. Results are expressed as means ± S.E.M. for 6–8 rats. The different treatment protocols were compared using a one-way ANOVA following the Student–Neuman–Keuls, post-hoc test.
[a] Significantly different from saline control ($P < 0.05$).
[b] Significantly different from miso 12.5 μg/kg group ($P < 0.05$).

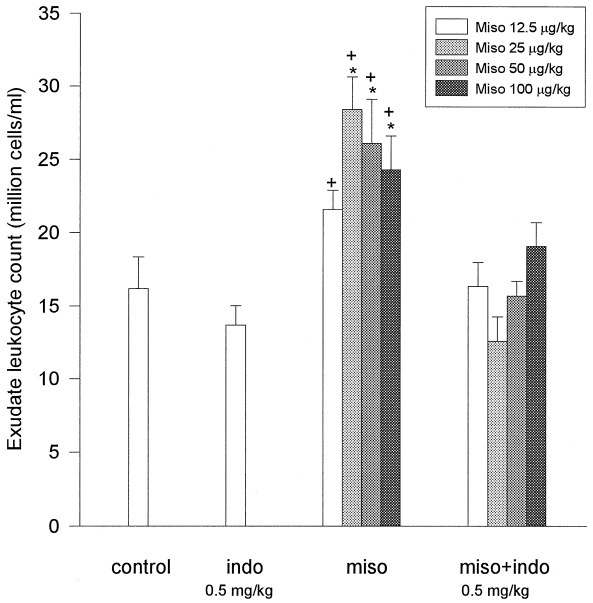

Fig. 3. Effects of combination of misoprostol in doses ranging from 12.5 to 100 μg/kg with a fixed dose of indomethacin (0.5 mg/kg) on exudate leukocyte count. Exudate was harvested 6 h after carrageenan injection. Misoprostol (miso) was administered by the orogastric route twice daily for two days before carrageenan injection. Indomethacin (indo) was also administered by the orogastric route 1 h before carrageenan injection. Results are expressed as means ± S.E.M. for 6–8 rats. The different treatment protocols were compared using a one-way ANOVA following the Student–Neuman–Keuls, post-hoc test. * Significantly different from control group ($P < 0.05$). + Significantly different from indo group ($P < 0.05$).

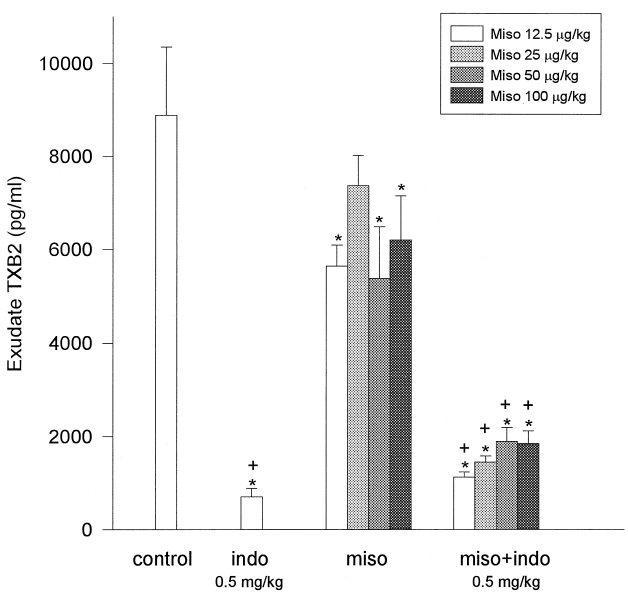

Fig. 4. Effects of combination of misoprostol in doses ranging from 12.5 to 100 μg/kg with a fixed dose of indomethacin (0.5 mg/kg) on exudate thromboxane B_2 level. Exudate was harvested 6 h after carrageenan injection. Misoprostol (miso) and indomethacin (indo) were administered as described for Fig. 3. Results are expressed as means ± S.E.M. for 6–8 rats. The different treatment protocols were compared using a one-way ANOVA following the Student–Neuman–Keuls, post-hoc test. * Significantly different from control group ($P < 0.05$). + Significantly different from miso groups ($P < 0.05$).

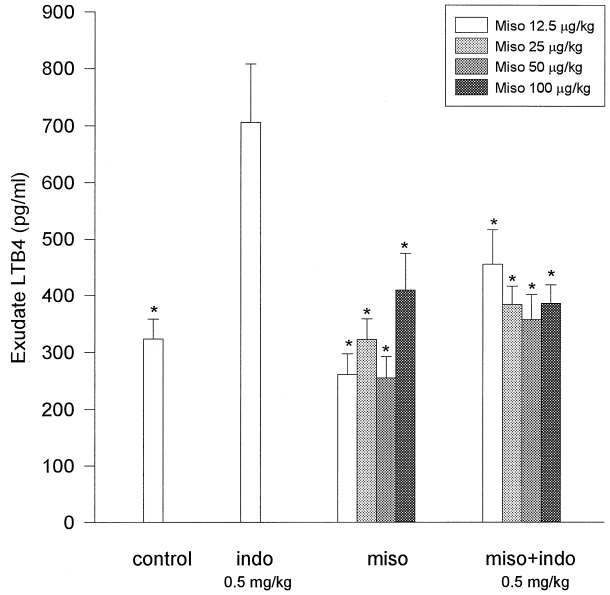

Fig. 5. Effects of combination of misoprostol in doses ranging from 12.5 to 100 μg/kg with a fixed dose of indomethacin (0.5 mg/kg) on exudate leukotriene B_4 level. Exudate was harvested 6 h after carrageenan injection. Misoprostol (miso) and indomethacin (indo) were administered as described for Fig. 3. Results are expressed as means ± S.E.M. for 6–8 rats. The different treatment protocols were compared using a one-way ANOVA following the Student–Neuman–Keuls, post-hoc test. * Significantly different from indo group ($P < 0.05$).

3.4. Effects of a combination of different doses of misoprostol with a fixed dose of indomethacin on exudate leukocyte count, thromboxane B_2 and leukotriene B_4 levels

The combination of misoprostol ranging from 12.5 to 100 μg/kg and a fixed dose of indomethacin (0.5 mg/kg) reduced the exudate leukocyte count when compared to the effect of misoprostol alone in a dose of 25, 50 and 100 μg/kg (Fig. 3). However, this decrease was not significantly different from that in the control or the indomethacin group. Although this combination treatment caused a significant reduction of exudate thromboxane B_2 levels when compared to the effect of misoprostol singly (Fig. 4), this effect was not significantly different from that in the indomethacin group. Combination treatment did not significantly change the exudate leukotriene B_4 levels compared to the misoprostol alone groups (Fig. 5). However, the addition of misoprostol to indomethacin significantly decreased the exudate leukotriene B_4 levels compared to those in the indomethacin alone group.

4. Discussion

There are many models of acute inflammation but the carrageenan-induced air pouch model has some advantages because the volume of exudate can be measured and exudate leukocyte counts obtained. This method also permits the assessment of inflammatory mediator release in the exudate.

Although the efficacy of NSAIDs in many acute inflammation models is well known, there are only few studies of their effectiveness in the carrageenan-induced air pouch model. However, it was shown that indomethacin, aspirin and flurbiprofen decrease exudate prostaglandin E_2 and thromboxane B_2 levels at low doses, but reduce the exudate leukocyte count at higher doses in the carrageenan-soaked polyester sponge model (Salmon et al., 1983). The results of the present study confirmed this finding. Leukocyte infiltration is reduced with doses of indomethacin higher than those required to prevent the synthesis of prostaglandins, suggesting that prostaglandins do not play a fundamental role in the infiltration process.

Indomethacin increased exudate leukotriene B_4 levels in a dose-dependent manner in our study. Although the mechanism is not clear, an increase in the production of lipoxygenase metabolites of arachidonic acid was found after inhibition of cyclooxygenase by NSAIDs (Robinson et al., 1986). It was claimed that lipoxygenase metabolites are responsible for the aspirin-induced asthma which occurred after ingestion of aspirin and other NSAIDs in sensitive patients (Israel et al., 1993) for NSAID-induced gastropathy (Hudson et al., 1993). Although, there is a minimal increase in exudate leukotriene B_4 levels after indomethacin in the carrageenan-soaked polyester sponge model (Salmon et al., 1983; Higgs et al., 1988), no reports were available concerning the effect of NSAIDs on the exudate leukotriene B_4 level in the carrageenan-induced air pouch model. Despite the enormous increase in exudate leukotriene B_4 level after indomethacin, the expected increase in exudate leukocyte count was not seen. Leukotriene B_4 is one of the powerful chemokinetic and chemotactic mediators (Bray, 1986), so that an increased exudate leukocyte count is to be expected after indomethacin administration. It was also reported that there was no correlation between the inhibition of leukotriene B_4 synthesis and the decrease in leukocyte accumulation after selective inhibition of 5-lipoxygenase in the carrageenan-soaked polyester sponge model (Higgs et al., 1988). This may be due to the fact that leukotriene B_4 is not a primary chemotactic mediator in the rat (Foster et al., 1986).

Misoprostol caused an increase in exudate leukocyte count in this study. Although prostaglandins have no effect on vascular permeability, they augment this effect of other mediators such as histamine and bradykinin primarily via vasodilatation (Williams, 1979). Misoprostol also inhibited exudate thromboxane B_2 release in our study. There are some examples of an inhibitory role of prostaglandins on mediator release. In this context, it was shown that exogenously applied prostaglandin E_2 inhibited both thromboxane B_2 and leukotriene B_4 release in human bronchial biopsy specimens (Schafer et al., 1996). It was also observed that prostaglandin E_2 inhibits in vivo histamine

release in the cheek pouch of immunized hamsters (Raud et al., 1988). Prostaglandin E_2 inhibits leukotriene B_4 release in the activated human neutrophils (Ham et al., 1983). In general, the prostaglandins have different effects on inflammatory mediator actions and mediator release. While vasodilating prostaglandins increase the effects of inflammatory mediators through their vasodilating properties, they inhibit the release of diverse inflammatory mediators including the cyclooxygenase and lipoxygenase metabolites of arachidonic acid. However, misoprostol did not inhibit the formation of leukotriene B_4 in our carrageenan-induced air pouch model of acute inflammation.

Combinations of different doses of misoprostol with a fixed dose of indomethacin reversed the effect of misoprostol at doses of 25–100 μg/kg on the exudate leukocyte count. As indomethacin is a cyclooxygenase inhibitor, it cannot be expected to inhibit the effect of exogenously applied misoprostol. This may be related to some effects of NSAIDs on inflammation, independent of the inhibition of cyclooxygenase. NSAIDs uncouple oxidative phosphorylation in mitochondria (Miyahara and Karler, 1965). They have also been found to inhibit superoxide generation by a cell-free NADPH oxidase system of neutrophils (Biemond et al., 1986) and the phospholipase C activity of mononuclear cells (Bomalaski et al., 1986). The above-mentioned effects of NSAIDs are independent of their ability to prevent synthesis of prostaglandins.

Administration of misoprostol either alone or combined with indomethacin did not influence the release of leukotriene B_4 in comparison to the control value. However, this combination prevented the indomethacin-induced increase in exudate leukotriene B_4 formation. It is rather interesting that, although misoprostol has no effect on the synthesis of leukotriene B_4, alone it prevents the indomethacin-induced increase of leukotriene B_4. In a similar way, inhalation of PGE_2 has been found to prevent the increased urinary leukotriene E_4 excretion due to the bronchial challenge with lysine acetylsalicylate in aspirin-sensitive asthma patients, while this application has not been found to influence the baseline urinary leukotriene E_4 excretion (Sestini et al., 1996), as was the case in the present study. The inhibitory effect of prostaglandin E_2 on arachidonic acid-induced release of peptidoleukotrienes but not their basal release in inflamed mucosa of human bronchial biopsy specimen was reported (Schafer et al., 1996). Although the mechanism is not clear, the inhibitory effect of prostaglandins on the synthesis of metabolites of 5-lipoxygenase was seen after inhibition of cyclooxygenase by NSAIDs in our present study and in studies by Sestini et al. (1996) and Schafer et al. (1996). Further studies will be necessary to explain this effect.

The combination of misoprostol with indomethacin did not change the exudate leukocyte count in comparison to the effect of indomethacin, although misoprostol use alone increased the exudate leukocyte count. Furthermore, this combination reversed the indomethacin-induced increase in the formation of exudate leukotriene B_4, one of the most powerful chemokinetic and chemotactic mediators. In conclusion, our results indicate that misoprostol may be combined with indomethacin in acute inflammation as well chronic inflammation, providing good tolerability without affecting the antiinflammatory efficacy of indomethacin.

Acknowledgements

This study was supported by a grant from the Turkish Scientific and Technical Research Council (SBAG-1516). The authors wish to thank Dr. GianCarlo Folco (Milan, Italy) and Dr. Ongun Onaran (Ankara, Turkey) for their helpful criticism of the manuscript.

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