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Prostaglandin H synthase-2 inhibitors interfere with prostaglandin H synthase-1 inhibition by nonsteroidal anti-inflammatory drugs

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Abstract

Ram seminal vesicle microsomes, a rich source of prostaglandin H synthase-1, were incubated with 100 nM of the prostaglandin H synthase-2 inhibitors *N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-398) and 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl) thiophene (DuP-697) prior to exposure to the prostaglandin H synthase inhibitors aspirin, indomethacin, ibuprofen or naproxen. Activity of the enzyme was measured by following the conversion of arachidonic acid to prostaglandin E_2 and prostaglandin F2α. Although prostaglandin H synthase-1 activity was not altered by these concentrations of the prostaglandin H synthase-2 inhibitors, it was found that exposure to these agents prior to aspirin or indomethacin (irreversible prostaglandin H synthase inhibitors) significantly attenuated the inhibition obtained by the latter inhibitors. On the other hand, the same concentrations of the prostaglandin H synthase-2 inhibitors did not interfere with prostaglandin H synthase-1 inhibition that was induced by naproxen or ibuprofen (competitive prostaglandin H synthase inhibitors). Attenuation of the indomethacin inhibition of prostaglandin H synthase-1 by prostaglandin H synthase-2 inhibitors was observed only when the microsomes were pre-exposed to DuP-697 or NS-398 in the absence, but not in the presence, of arachidonic acid. The effect of DuP-697 was found to be irreversible, however, washing away the agent reversed the action of NS-398. Similar phenomena have been reported by us in bovine aortic endothelial cells and in human dermal fibroblasts. Attenuation of the inhibition by aspirin and indomethacin, without altering the enzyme's basal activity or the inhibition induced by ibuprofen or naproxen may suggest the possibility that the prostaglandin H synthase-2 specific inhibitors DuP-697 and NS-398 affect prostaglandin H synthase-1 by interaction with a site different from the enzyme's catalytic site.

Keywords: DuP-697; NS-398; Aspirin; Indomethacin; Ibuprofen; Naproxen; Prostaglandin H synthase-1; Prostaglandin H synthase-2; Seminal vesicle microsome, ram

1. Introduction

Two distinct isoforms of prostaglandin H synthase are involved in the catalytic conversion of arachidonic acid to prostaglandin H2, the committed step in prostaglandin biosynthesis (Smith et al., 1996; Otto and Smith, 1995).

The expression of these two isoforms is regulated differently: prostaglandin H synthase-1 has been observed in a variety of prostanoid-producing cells to be constitutively expressed, while prostaglandin H synthase-2 is inducible in response to growth factors, cytokines and lipopolysaccharide (Inoue et al., 1995). Nonsteroidal anti-inflammatory drugs are believed to exert their antipyretic, anti-inflammatory and analgesic effects, as well as many of their adverse reactions, through prostaglandin H synthase inhibition (Vane. 1971). Currently available nonsteroidal anti-inflammatory drugs, at therapeutic concentrations preferentially inhibit prostaglandin H synthase-1 activity (Mitchell et al., 1994).

Several selective inhibitors of prostaglandin H synthase-2 were developed (Gans et al., 1990; Masferrer et al., 1994) such as: *N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-398) and 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl) thiophene (DuP-697). These two inhibitors specifically inhibit prostaglandin H syn-

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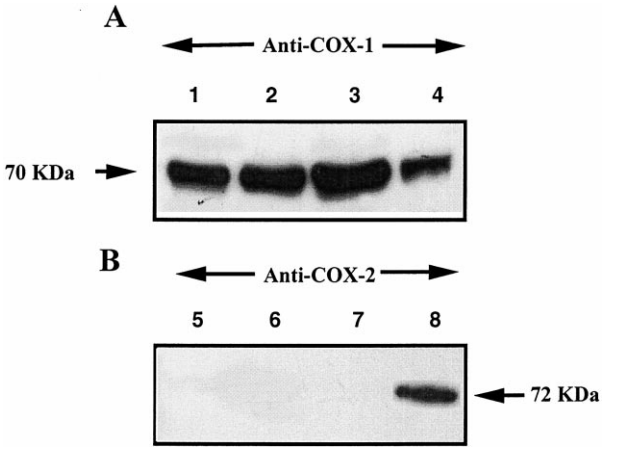

Fig. 1. Identification of prostaglandin H synthase isoenzymes in the ram seminal vesicle microsomes. Increasing amounts of microsomal samples (5, 10 and 20 μg (A), and 5, 10 and 25 μg (B) were primed either with prostaglandin H synthase-1 antibody (A) or with prostaglandin H synthase-2 antibody (B). Lanes 4 and 8 contained 0.5 μg of standard prostaglandin H synthase-1 and prostaglandin H synthase-2 protein, respectively. The Western blot was performed as described in Section 2.

thase-2 at low concentrations in a two-step process: an initial rapid and reversible interaction with both prostaglandin H synthase-1 and prostaglandin H synthase-2, however, only with prostaglandin H synthase-2 do they form a tightly bound complex (Gierse et al., 1996; Copeland et al., 1994). In a previous study, we showed that low concentrations of DuP-697 or NS-398 did not interfere with basal prostaglandin H synthase-1 activity, but attenuated the inhibition induced by aspirin and indomethacin (Rosenstock et al., 1997). These observations were made in intact bovine aortic endothelial cells at experimental conditions that allowed the expression of prostaglandin H synthase-1 only (Rosenstock et al., 1997). The same concentrations of the prostaglandin H synthase-2 inhibitors that were found to attenuate aspirin and indomethacin-induced inhibition failed to affect the inhibition induced by naproxen and ibuprofen (Rosenstock et al., 1999). Similar results were also obtained in human dermal fibroblasts, indicating that this phenomenon is not restricted to a specific cell type (Rosenstock et al., 1999). The goal of the present study was to characterize the effect of prostaglandin H synthase-2 inhibitors on prostaglandin H synthase-1 activity in a cell-free system. Microsomes prepared from ram seminal vesicles, a rich source of prostaglandin H synthase-1, were used. Our present findings confirm that although low concentrations of prostaglandin H synthase-2 inhibitors do not interfere with prostaglandin H synthase-1 activity, they markedly attenuate the inhibition induced by commonly used anti-inflammatory drugs such as aspirin and indomethacin. This may suggests that a site, different than the catalytic site of prostaglandin H synthase-1, is involved in the inhibitory mechanism of prostaglandin H synthase-1 activity.

2. Experimental procedures

2.1. Materials

Indomethacin and arachidonic acid (dissolved in ethanol) were purchased from Sigma (St. Louis, MO). NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide, dissolved in dimethylsulfoxide (DMSO) was from Calbiochem (La Jolla, CA). Aspirin was obtained from BDH Chemicals (Poole, England); ibuprofen (dissolved in DMSO) and naproxen were kindly donated by Teva Pharmaceuticals Industries (Israel), and DuP-697 (5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl) thiophene, dissolved in ethanol) was provided by the generosity of DuPont Merck Research Laboratories (Wilmington, DE). Ram seminal vesicle microsomes were purchased from Oxford Biomedical Research (Oxford, MI). [5,6,8,9,11, 12,14,15 ^{3}H(N)] Arachidonic acid, 185 Ci/mmol, was from NEN Life Science Products (Boston, MA, USA). Prostaglandin H synthase-1 Prostaglandin H synthase-2 antibodies were provided by the generosity of Dr. W.L. Smith, Michigan State University (East Lansing, MI, USA). Vehicle concentrations used in these experiments did not exceed 0.1%.

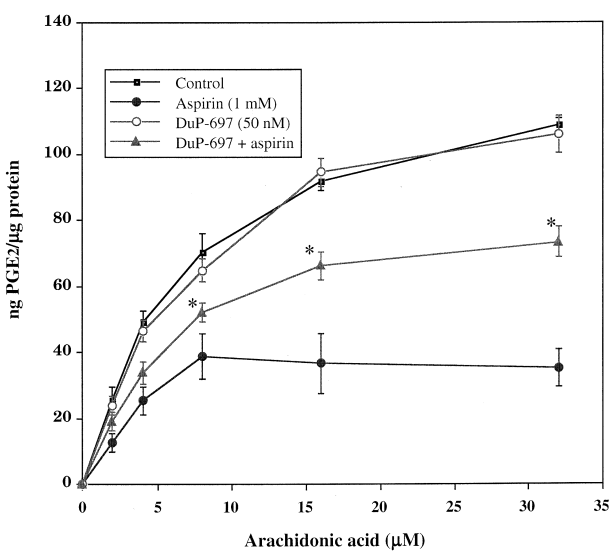

Fig. 2. Effect of DuP-697 on microsomal prostaglandin H synthase-1 inhibition by aspirin as a function of increasing arachidonic acid concentrations. Ram seminal vesicle microsomes were activated by the addition of a cofactor mixture for 5 min on ice. The mixture was preincubated with 50-nM DuP-697 for 10 min, followed by the addition of 1-mM aspirin for additional 10 min on ice. Different concentrations of AA were then added and incubated for 5 min at 37°C, and the reaction was terminated by the addition of 2 N HCl. Prostaglandin H synthase-1 activity was assessed by RIA of prostaglandin E_2, as described in Section 2. Data points and error bars represent the means ± S.E.M. of two experiments carried out in triplicates. * $P < 0.05$ vs. aspirin that was not pretreated with DuP-697.

2.2. Western blot analysis of prostaglandin H synthase-1 and prostaglandin H synthase-2 in ram seminal vesicle microsomes

Protein concentrations in the microsome samples were determined using the Bio-Rad protein assay (Bio-Rad, Munich, Germany) using bovine serum albumin as standard. Microsomes were mixed (1:1) with Laemmli reagent and boiled for 5 min. Samples (5–25 μg) were resolved by 10% lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose for protein immunoblotting. Nitrocellulose membranes (Bio-Rad) were blocked in Tris-buffered saline (TBS) containing 3% Tween-20 (TTBS) and 5% milk powder overnight followed by incubation with rabbit anti-human prostaglandin H synthase-1 or prostaglandin H synthase-2 (provided by Dr. W.L. Smith, Michigan State University, East Lansing, MI, USA) at 1:1000 dilution for 3 h. After two subsequent washes with 1% milk powder in TTBS, the membranes were incubated for 1.5 h with horseradish peroxidase conjugated anti-rabbit Immunoglubolin G (IgG) (Amersham Life Sciences, Piscataway, NJ), after which Luminescence was detected by enhanced chemiluminescence (Pierce, Rockford, USA). Density values of the different bands were obtained after scanning the blots with video densitometry with the UVP-GDF 5000 system (UVP, San Gabriel, California, USA).

2.3. Measurement of prostaglandin H synthase activity in ram seminal vesicle microsomes by radioimmunoassay (RIA)

Microsomal prostaglandin H synthase-1 assay was performed according to Noreen et al. (1998) with a few modifications. Briefly, 1 μg of ram seminal vesicle microsomal protein in 10-μl Tris buffer (0.1 M, pH 8.0) was activated by the addition of 50 μl of a cofactor mixture containing L-adrenaline (1.95 mM), reduced glutathione (0.49 mM) and hematin (1 μM), and maintained on ice for 5 min. In order to test the effect of the different prostaglandin H synthase inhibitors, 10 μl of each inhibitor were added to the reaction media and maintained on ice for 10 min. The enzyme reaction was initiated by the addition of 10 μl arachidonic acid (30 μM) followed by 5 min incubation at 37°C, and terminated by the addition of 10 μl 2 N HCl. The samples were frozen pending analysis of prostaglandin E_2 by single antibody RIA as described (Rosenstock et al., 1997).

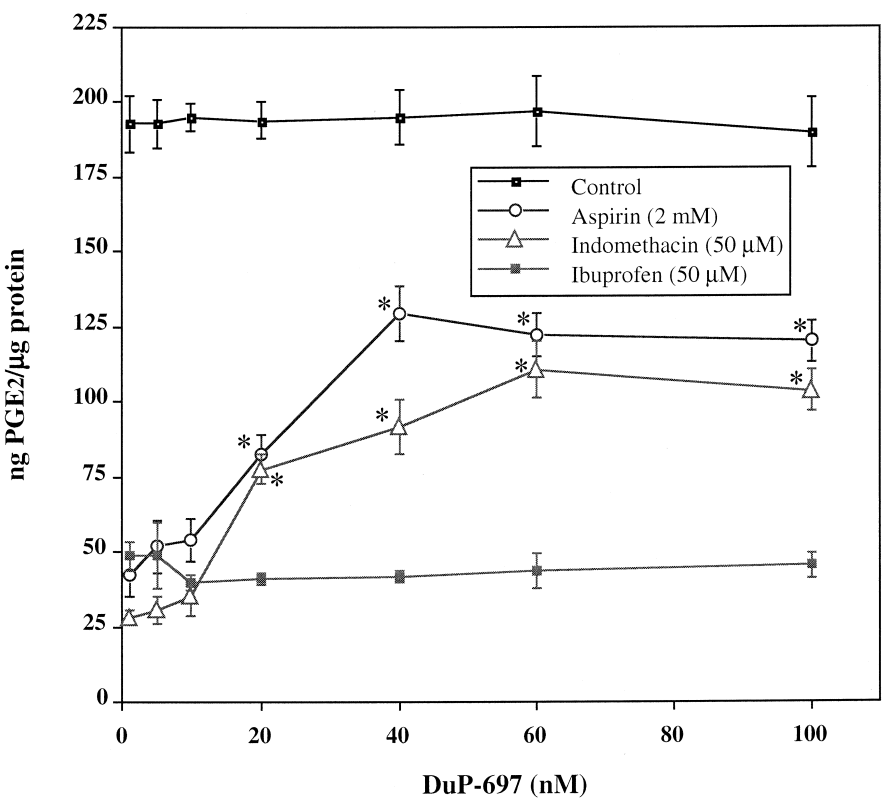

Fig. 3. Inhibition of microsomal prostaglandin H synthase-1 activity by aspirin, indomethacin and ibuprofen: effect of increasing concentrations of DuP-697. Ram seminal vesicle microsomes were activated as described in Fig. 2. Following the activation step, the microsomes were incubated for 10 min on ice with increasing concentrations of DuP-697. In order to test the effect of the different prostaglandin H synthase inhibitors (aspirin 2 mM, indomethacin 50 μM, ibuprofen 50 μM), each inhibitor was added to the reaction media for an additional 10 min on ice. Thirty-micromolar arachidonic acid was added to measure the activity of prostaglandin H synthase-1 as described in Fig. 2. Data represent the means ± S.E.M. of three experiments performed in triplicates. $^{*}P < 0.05$ vs. aspirin and indomethacin inhibition without the addition of DuP-697.

Prostaglandin E_2 was measured in unextracted samples by single antibody RIA with dextran-coated charcoal precipitation. RIA measurements were performed in duplicate for each sample. Rabbit antiserum to prostaglandin E was purchased from Sigma, and tritium-labeled prostaglandin E_2 (160 Ci/mmol) was obtained from The Radiochemical Center (Amersham, UK). The sensitivity of the assay was 0.15 ng/ml. Prostaglandin E_2 for standard curves was purchased from Sigma. The prostaglandin E antiserum cross-reacted with the following prostaglandins (at 50% displacement): prostaglandin E_1, 165%; prostaglandin E_2, 100%; prostaglandin A_1, 28%; prostaglandin A_2, 7%; prostaglandin B_1, 13%; prostaglandin B_2, 6%; prostaglandin $F_{1\alpha}$, 5%; prostaglandin $F_{2\alpha}$, 1.5%; other prostaglandins $< 1\%$. No cross-reactivity of the anti-PG sera was found with arachidonic acid, DuP-697 or NS-398 at the concentrations used in these experiments.

2.4. Measurement of microsomal prostaglandin H synthase-1 activity by thin-layer chromatography (TLC)

The reaction was carried out as above, except that arachidonic acid concentration was 20 μM containing 0.5 μCi [^{3}H]-arachidonic acid. The reaction was terminated by the addition of 50-μl sodium acetate buffer (1 M, pH 4.0) containing 1 μg each of arachidonic acid, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ as carriers. The samples were extracted with 2-ml diethyl ether, evaporated under N_2 and resuspended in 25-μl absolute ethanol. Thin layer chromatograph was performed on DC-Alufolien silica gel 60 F254 plates and developed with the upper phase of ethyl acetate–isooctane–acetic acid–water (110:50:20:100) [13]. Visualization of the major spots was done by short exposure to iodine vapor. The relevant bands of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were cut out and placed in vials for scintillation counting.

2.5. Data analysis

Results are means ± S.E.M. for each experiment. Statistical analysis of the results was performed using two-tailed Student's *t*-test, and $P \leq 0.05$ was considered significant.

3. Results

3.1. Identification of prostaglandin H synthase isoenzymes in ram seminal vesicle microsomes

In order to confirm that the results may be attributed to prostaglandin H synthase-1, Western blot analysis was

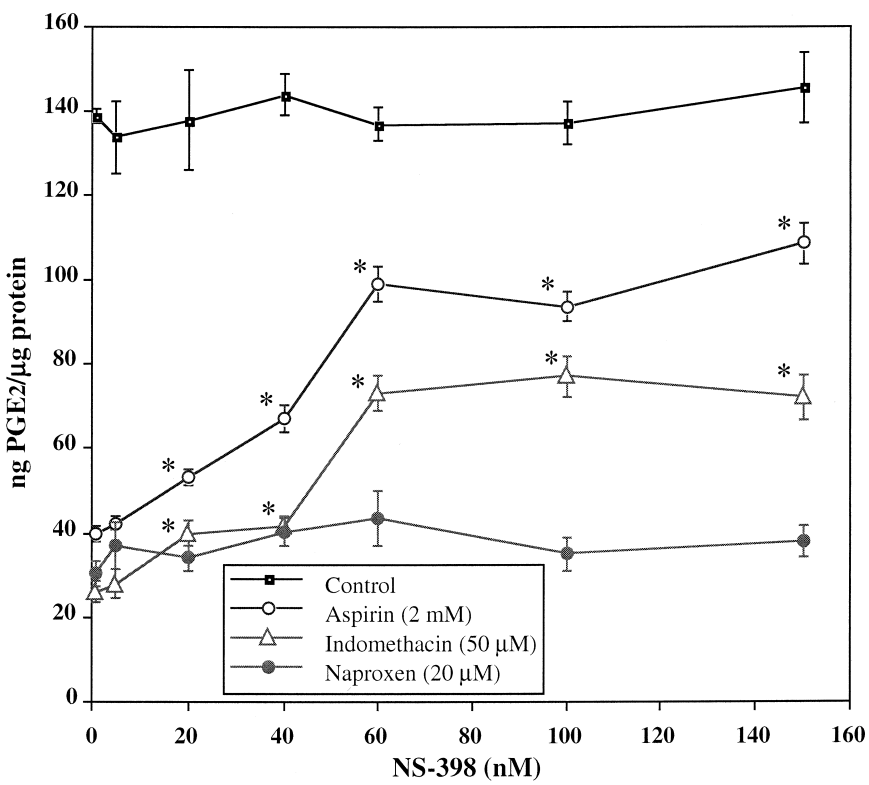

Fig. 4. Inhibition of microsomal prostaglandin H synthase-1 activity by aspirin, indomethacin and ibuprofen: effect of increasing concentrations of NS-398. Ram seminal vesicle microsomes were activated as described in Fig. 2. Following the activation step, the microsomes were incubated for 10 min on ice with increasing concentrations of NS-398. Each inhibitor (aspirin 2 mM, indomethacin 50 μM, naproxen 20 μM) was added and incubated with the microsomes as described in Fig. 3. Thirty-micromolar arachidonic acid were added and enzyme activity was measured as described. Data represent the means ± S.E.M. of three experiments performed in triplicates. * $P < 0.05$ vs. aspirin and indomethacin inhibition without the addition of NS-398.

performed on increasing amounts of microsomal samples (5–25 μg). Prostaglandin H synthase-1 and prostaglandin H synthase-2 standard proteins (0.5 μg) were used as positive controls for measurement of prostaglandin H synthase-1 and prostaglandin H synthase-2 antibody immunoreactivity, respectively. As depicted in Fig. 1, the predominant enzyme in these samples was the prostaglandin H synthase-1 isoenzyme (A), whereas prostaglandin H synthase-2 was undetectable even in 25-μg microsomes (B). This result confirms that ram seminal vesicle microsomes do not contain measurable amounts of prostaglandin H synthase-2.

3.2. Enzyme kinetics and linearity

The TLC experiments indicated that with 20–30 μM arachidonic acid, only 30% of the arachidonic acid was exhausted during the entire reaction. In addition, the time dependence of the reaction was found to be linear over 7–8 min.

3.3. Effect of DuP-697 on microsomal prostaglandin H synthase-1 activity and on aspirin-induced inhibition

The results presented in Fig. 2 show prostaglandin H synthase-1 activity at different substrate concentrations with and without inhibitors. It is demonstrated that incubation of ram seminal vesicle microsomes with 50-nM DuP-697, a specific prostaglandin H synthase-2 inhibitor, did not affect the activity of the enzyme. However, inhibition of prostaglandin H synthase-1 by 1 mM aspirin was significantly attenuated in the presence of the same DuP-697 concentration. This attenuation was observable over a wide range of arachidonic acid concentrations (2–32 μM).

3.4. Inhibition of prostaglandin H synthase-1 activity by aspirin, indomethacin, ibuprofen or naproxen in the presence of increasing concentrations of DuP-697 and NS-398

Fig. 3 shows that increasing concentrations of DuP-697 did not affect prostaglandin H synthase-1 activity, but progressively attenuated the inhibitory effects of both aspirin (2 mM) and indomethacin (50 μM) with IC_{50} values of about 30 nM. In contrast, the same range of DuP-697 concentrations did not affect the inhibition that was induced by ibuprofen (50 μM) or naproxen (not shown), both competitive prostaglandin H synthase inhibitors. An additional experiments, carried out with another specific prostaglandin H synthase-2 inhibitor, NS-398, yielded similar results (Fig. 4). Thus, NS-398 did not interfere with prostaglandin H synthase-1 activity or with naproxen-induced inhibition of prostaglandin H synthase-1, but decreased inhibition induced by aspirin or indomethacin in a concentration-dependent manner, with IC_{50} of about 30–50 nM.

3.5. Confirmation of the effects of DuP-697 and NS-398 by employing TLC measurements

In order to verify that the above observations were not the result of a possible artifact of the assay method, similar experiments were repeated with radioactive labeled arachidonic acid, and prostaglandin H synthase-1 products were measured after separation by TLC. As depicted in Fig. 5, DuP-697 and NS-398, at concentrations of 100 nM each, did not affect prostaglandin H synthase-1 activity (measured by prostaglandin E_2 or prostaglandin $F_{2\alpha}$ synthesis) or the inhibition induced by naproxen or ibuprofen. However, they significantly attenuated the inhibition induced by aspirin and indomethacin. These results confirm the observations made by the RIA measurements of prostaglandin E_2 synthesis (Figs. 2–4).

3.6. Reversibility of DuP-697 and NS-398 effects

In order to test the reversibility of the effects of these agents on the microsomal enzyme, a small volume of the sample was then prepared (0.07 ml) and pretreated with DuP-697 or NS-398 on ice then diluted into a larger volume (140-fold), precipitated by ultracentrifugation and resuspended for measurements of prostaglandin H syn-

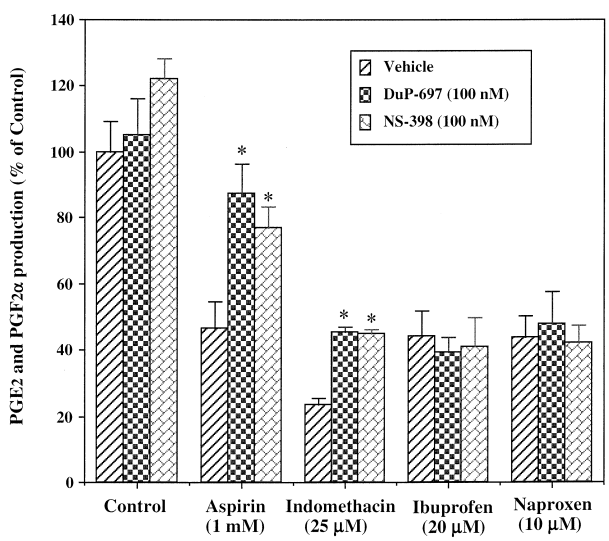

Fig. 5. Effects of DuP-697 and NS-398 on the inhibition of microsomal prostaglandin H synthase-1 activity by aspirin, indomethacin, ibuprofen and naproxen as measured by TLC. The reaction was carried out as described in Fig. 2 with changes detailed in Section 2.1 for TLC determination. The final concentrations of aspirin, indomethacin, ibuprofen and naproxen concentrations were 1 mM, 25, 20 and 10 μM, respectively. The effect of each inhibitor was measured in control microsomes (vehicle), and microsomes treated with 100 nM each of DuP-697 or NS-398. Prostaglandin production is expressed as the sum of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ areas under the curve (AUC) obtained from the TLC. The AUC in the absence of non steroidal anti-inflammatory drugs and prostaglandin H synthase-2 inhibitors was defined as 100%. The columns represent the percent of this control activity. Bars represent the means and S.E.M. of three to four independent experiments. * $P < 0.05$ vs. the appropriate vehicle.

thase-1 activity. After precipitation and resuspension, the final concentration of NS-398 or DuP-697 was less than 0.3 nM, at which no effect is expected. The results presented in Fig. 6 show that the ability of DuP-697 to interfere with indomethacin's inhibition was maintained, while the interference by NS-398 was fully reversed during the washing procedure. These results confirm our previous observations in intact cells, where the effect of DuP-697 was irreversible while that of NS-393 was reversible (Rosenstock et al., 1999).

3.7. Arachidonic acid interferes with the interaction of prostaglandin H synthase-2 inhibitors and indomethacin

In all the previous experiments, cells (Rosenstock et al., 1999) or microsomes were incubated with NS-393 or DuP-697 prior to the exposure to the NSAIDs, and finally arachidonic acid was added for measurement of prostaglandin H synthase activity. To evaluate a possible effect of arachidonic acid on the interaction between prostaglandin H synthase-1-putative site and the prostaglandin H synthase-2 inhibitors, the order of additions was reversed. Thus, the microsomes were first preincubated with arachidonic acid (20 μM) for 2 min on ice, then exposed to the prostaglandin H synthase-2 inhibitors, followed by exposure to indomethacin for additional 5 min on ice, before enzyme activity was measured at 37°C. As shown in Fig. 7, preincubation of the microsomes with arachidonic acid abolished the effects of DuP-697 and NS-398 on indomethacin-induced inhibition. This result suggests that the presence of arachidonic acid probably prevents the interaction of DuP-697 and NS-398 with their putative target site, which is responsible for attenuation of the inhibitory action of indomethacin on prostaglandin H synthase-1 activity.

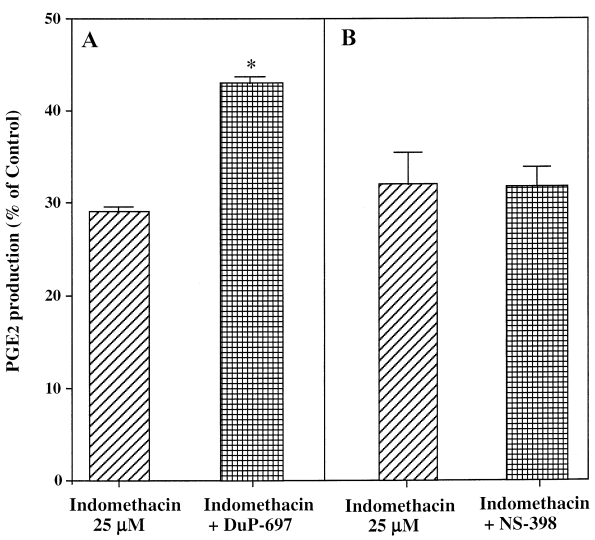

Fig. 6. Reversibility of DuP-697 and NS-393 effects on the inhibition induced by indomethacin. Microsomes were pretreated with 100 nM of DuP-697 or NS-393 in a volume of 0.07 ml for 10 min on ice, then diluted to 10 ml and centrifuged at 100,000× *g* for 20 min. The resulting pellet was resuspended in 0.4 ml for enzyme activity measurements. The final concentration of NS-393 or DuP-697 was therefore, < 0.3 nM. The diagonally lined columns represent enzyme activity after the addition of 25-μM indomethacin to untreated microsomes. The cross-hatched columns show the effect of indomethacin on microsomes that had been pretreated with DuP-697 (A) or NS-398 (B), washed and then exposed to indomethacin. The results represent means of two experiments performed in triplicates. Similar results were obtained with microsomes pretreated with 500 nM NS-393 or DuP-697 prior to washing. $^{*}P < 0.05$ vs. indomethacin.

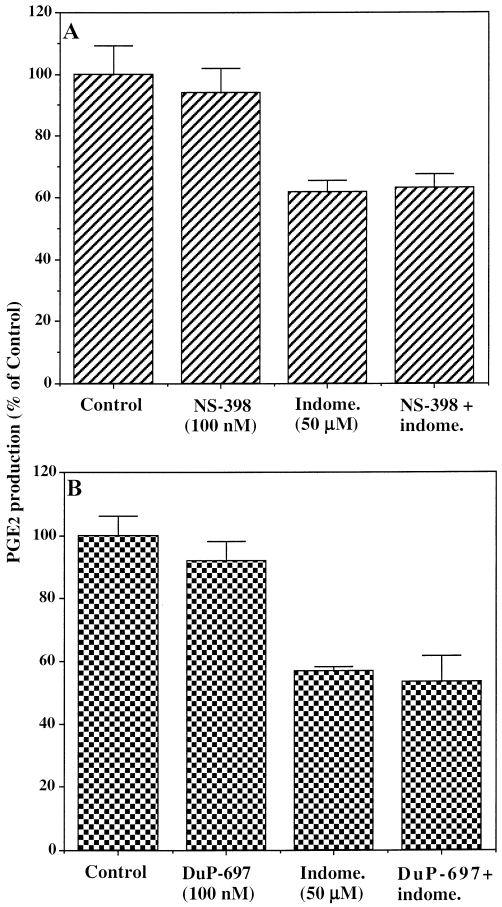

Fig. 7. Effect of prior exposure to arachidonic acid on the interaction between NS-398, DuP-697 and indomethacin. Microsomes were exposed to 20-μM arachidonic acid for 2 min on ice before 100 nM NS-398 (A) or DuP-697 (B) were added. Eight minutes later, 50-μM indomethacin was added for additional 10 min. The test tubes were transferred to 37°C and enzyme activity was measured as described in Section 2.1. The results represent means ± S.E.M. of three experiments done in triplicates.

4. Discussion

The present work shows that DuP-697 and NS-398, at concentrations that do not inhibit microsomal prostaglandin H synthase-1 activity, markedly attenuate the inhibition of prostaglandin H synthase-1 that is brought about by indomethacin and aspirin. The final concentration of arachidonic acid in the assay was actually 20–30 μM, as

indicated in Section 2.1 . We verified by TLC measurements that around 70% of the arachidonic acid remained in the medium at the end of the reaction.

The reduction in indomethacin's inhibition of prostaglandin H synthase-1 that is caused by DuP-697 is apparently irreversible, while that of NS-398 is effectively reversed by carefully washing off the microsomes. It is possible that NS-398 is readily washed out while DuP-697, which is more hydrophobic, is retained in the microsomal membranes. The same concentrations of DuP-697 and NS-398 that decreased the efficacy of indomethacin and aspirin did not interfere with the effects of competitive prostaglandin H synthase inhibitors, namely ibuprofen and naproxen. The present results confirm our previous observations made in intact cells (Rosenstock et al., 1999). Both in our present investigation on ram seminal vesicle microsomes and in our previous study on intact cells, the effects of DuP-697 and NS-393 were observed at concentrations smaller than 100 nM. By contrast, in agreement with others (Jouzeau et al., 1997) we have observed that prostaglandin H synthase-2 inhibitors inhibit prostaglandin H synthase-1 activity with IC_{50} values of 3.5 μM and > 25 μM for DuP-697 and NS-393, respectively (not shown). These concentrations of DuP-697 and NS-398 are two to three orders of magnitude higher than those required for the attenuation of prostaglandin H synthase-1 inhibition induced by aspirin and indomethacin. Two alternative explanations may be offered for the attenuation of the inhibition induced by aspirin and indomethacin. It is possible that in the absence of arachidonic acid NS-398 and DuP-697 compete with indomethacin and aspirin at the catalytic site. Due to this competition, the enzyme fraction that is irreversibly inhibited may decrease. On the other hand, prostaglandin H synthase-2 inhibitors failed to interfere with ibuprofen and naproxen inhibition since they are competitive reversible inhibitors. Alternatively, NS-398 and DuP-697 may alter the enzyme conformation via interaction with a site other than the catalytic site. The interaction of the prostaglandin H synthase-2 inhibitors with this site does not interfere with accessibility of the substrate, arachidonic acid, or a competitive inhibitor such as ibuprofen to the catalytic site. Nevertheless, it hinders the interaction of aspirin and indomethacin with the enzyme. It may be suggested that the prostaglandin H synthase-2 inhibitors interfere with the slow, time-dependent changes in the enzyme configuration. Occupation of this putative site by arachidonic acid (Fig. 7) prevents interaction of prostaglandin H synthase-2 inhibitors with this site.

Several observations in the literature may suggest that the additional site may actually reside on the enzyme itself. It was shown that (Kalgutkar et al., 1996) selective prostaglandin H synthase-2 inhibitors protected against tryptic cleavage of pure apo prostaglandin H synthase-1, but did not inhibit the protein's cyclooxygenase activity. This observation is compatible with more than a single inhibitor-binding site on prostaglandin H synthase-1. Possible cooperative interaction between two arachidonic acid sites was suggested by Swinney et al. (1997), who demonstrated a deviation from linearity of Eadie–Scatchard plots for prostaglandin H synthase-1 activity. Recently, Chen et al. (1999) suggested that this cooperativity may result from feedback activation by the intermediate hydroperoxide product prostaglandin G_2. However, this suggestion does not explain the activation of prostaglandin H synthase-1 by low concentrations of competitive prostaglandin H synthase inhibitors (Swinney et al., 1997).

Additional sites for interaction of inhibitors were also suggested by Tang et al. (1995) who found that two molecules of 4-(bromoacetoimido)-benzyl-indomethacin or 4-(bromoacetoamido)-(2,3-dimethyl)-anthranilic acid were incorporated into one monomer of prostaglandin H synthase-1. At present, it is not known whether the two monomers of prostaglandin H synthase can catalyze the reaction independently of each other or whether they are mechanically coupled. Kulmacz and Lands (1985) showed that only a single molecule of indomethacin, meclofenamic acid or flurbiprofen is required to inhibit the activity of two monomers of a purified cyclooxygenase homodimer. These observations imply a possible interaction between the two monomers. X-ray crystal structure of prostaglandin H synthase-1 and prostaglandin H synthase-2 have shown that the two monomers can be simultaneously occupied by fluribuprofen (Picot et al., 1994; Kurumbail et al., 1996). In addition, it was found that crystal structure of prostaglandin H synthase-2 is capable of simultaneously binding the inhibitor by its two monomers and that a 1:1 prostaglandin H synthase-2 monomer/inhibitor ratio is obtained with biaryl methyl sulfone inhibitors, which are similar in structure to DuP-697 (Chan et al., 1999; Riendeau et al., 1997). Nevertheless, one may suggest that DuP-697 and NS-398 could interact with one monomer, while the second is accessible to arachidonic acid procession.

Since our experiments were performed with intact cells (Rosenstock et al., 1999) and with crude ram seminal microsomes (present study), and not on purified enzyme, we cannot say whether these effects of prostaglandin H synthase-2 inhibitor reflect an intrinsic property of prostaglandin H synthase-1 or on another protein involved in the downstream synthesis of cyclooxygenase products. Further studies with purified prostaglandin H synthase-1 enzyme will confirm interaction of prostaglandin H synthase-2 inhibitors with prostaglandin H synthase-1 at the nanomolar concentration range. This study shows that although low concentrations of prostaglandin H synthase-2 inhibitors do not inhibit prostaglandin H synthase-1 activity, these agents interact with the enzyme. This interaction may interfere with the cellular regulation of prostaglandin H synthase-1. On the basis of the present observations, one may predict that in vivo treatment with prostaglandin H synthase-2 inhibitors may reduce the inhibitory potency of aspirin and indomethacin. Although simultaneous treatment with both classes of drugs is not expected to occur,

patients may switch from one class to another. Future clinical observations will support or refute this prediction.

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