EXPEDITED REVIEWS

Pharmacodynamic Interaction of Naproxen With Low-Dose Aspirin in Healthy Subjects

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Ce.S.I.; and ‡SS Annunziata Hospital, Chieti, Italy. Supported by grants from the Italian Ministry of University and Research (MIUR) to the Center of Excellence on Aging, “G. d’Annunzio” University; †“G. d’Annunzio” University Foundation, Chieti, Italy; ‡“G. d’Annunzio” University Foundation, Chieti, Italy. Published by Elsevier Inc. doi:10.1016/j.jacc.2005.01.045

OBJECTIVES
We investigated the occurrence of pharmacodynamic interaction between low-dose aspirin and naproxen.

BACKGROUND
The uncertainty of cardioprotection by naproxen has encouraged its combination with aspirin in patients with arthritis and cardiovascular disease.

METHODS
The incubation of washed platelets with naproxen for 5 min before the addition of aspirin reduced the irreversible inhibition of thromboxane (TX)B2 production by aspirin. The pharmacodynamic interaction between the two drugs was then investigated in four healthy volunteers who received aspirin (100 mg daily) for 6 days and then the combination of aspirin and naproxen for further 6 days: aspirin 2 h before naproxen (500 mg, twice-daily dosing). After 14 days of washout, naproxen was given 2 h before aspirin for further 6 days.

RESULTS
The inhibition of serum TXB2 production (index of platelet cyclooxygenase [COX]-1 activity) and platelet aggregation ex vivo and urinary 11-dehydro-TXB2 levels (index of TXB2 biosynthesis in vivo) by aspirin alone (99 ± 0.2%, 95 ± 0.6%, and 81 ± 4%, respectively) was not significantly altered by the co-administration of naproxen, given either 2 h after aspirin or in reverse order. In a second study, the concurrent administration of a single dose of aspirin and naproxen did not affect platelet TXB2 production and aggregation at 1 h after dosing, when aspirin alone causes maximal inhibitory effect. Moreover, the rapid recovery of platelet COX-1 activity and function supports the occurrence of a pharmacodynamic interaction between naproxen and aspirin.

CONCLUSIONS
Naproxen interfered with the inhibitory effect of aspirin on platelet COX-1 activity and function. This pharmacodynamic interaction might undermine the sustained inhibition of platelet COX-1 that is necessary for aspirin’s cardioprotective effects.

Aspirin and nonaspirin nonsteroidal anti-inflammatory drugs (NANSAIDs) inhibit the synthesis of platelet thromboxane (TX)A2 (the major product of arachidonic acid [AA] in platelets, serving as potent platelet agonists and vasoconstrictors) (1), but only the former has been shown to reduce the risk of myocardial infarction and stroke (2,3). It generally is accepted that the activity of platelet cyclooxygenase (COX)-1, the COX isoform expressed in platelets (4), has to be almost completely (>95%) and continuously inhibited ex vivo throughout the dosing intervals to translate into a detectable cardiovascular protection (1,3,5). This effect can be achieved by aspirin, which causes an irreversible inactivation of platelet COX-1 activity, through a selective acetylation of Ser529 of human COX-1 (6,7), which lasts for all platelet lifespan (i.e., 8 to 10 days), because the lack of transcription and only scant translation in anucleated platelets (8). In contrast, NANSAIDs, which are reversible inhibitors of platelet COX-1, generally cause an incomplete and intermittent inhibition of platelet TXA2, which may be inadequate to prevent cardiovascular events, as shown by the results of epidemiologic studies (3,9–12).

Among NANSAIDs, naproxen recently has gained interest because the results of one randomized clinical trial (13) and several observational studies have suggested its possible cardioprotective effect (12,14–16). These results are consistent with the demonstration that the chronic administration of a therapeutic anti-inflammatory dose of naproxen (500 mg, twice-daily dosing [BID]) to healthy subjects causes a persistent and almost-complete suppression of platelet TXB2 production throughout the 12-h dosing interval (17). However, in other observational studies, naproxen does not affect the risk of cardiovascular events (11,12,18,19). The conflicting results of epidemiologic studies reconcile in consideration of the dependence of getting an almost complete inhibition of platelet COX-1 activity ex vivo (<95%) at the end of the dosing interval by a reversible inhibitor of COX-1 to translate into cardiovascular protection (3). Thus, the use of naproxen in real-life...
situations, photographed by observational studies which is neither regular nor continuous nor necessarily at high doses may explain the variable risk reductions detected.

The uncertainty of a cardioprotective effect by NANAISDs has encouraged their combination with aspirin in patients with musculoskeletal disorders and vascular disease (20). However, it has been shown recently that the NANAISD ibuprofen interferes with the antiplatelet effect of low-dose aspirin when they are administered together (21,22). In fact, the stronger binding affinity of NANAISDs to Arg\textsuperscript{120} of the COX-1 channel (a common docking site for all nonsteroidal anti-inflammatory drugs [NSAIDs], including aspirin) may prevent the acetylation of Ser\textsuperscript{529} by aspirin (3,23). In contrast, no interaction is detected in the co-administration of aspirin with the NANAISDs acetaminophen and diclofenac or with the selective COX-2 inhibitor rofecoxib (21).

In the present study, we investigated the occurrence of a pharmacodynamic interaction between low-dose aspirin and naproxen. First, we assessed the effects of naproxen on the irreversible inhibition of platelet TXB\textsubscript{2} production by aspirin in vitro. Then, we studied in healthy subjects the potential interference of naproxen with aspirin inhibitory effects on platelet TXB\textsubscript{2} biosynthesis ex vivo and in vivo.

METHODS

Study subjects. The study protocol was approved by the ethical committee of “G. d’Annunzio” University of Chieti. Informed consent was obtained from the nine subjects enrolled. The subjects were between 23 and 30 years of age and within 30% of ideal body weight and had an unremarkable medical history, physical examination, and routine hematological and biochemical studies. Smokers and subjects with a bleeding disorder, an allergy to aspirin or any other NSAID, or a history of any gastrointestinal or cerebrovascular disease were excluded. Subjects abstained from the use of aspirin and other NSAIDs for at least two weeks before enrollment.

In vitro study. First, we characterized the nature of the interaction (i.e., reversible or irreversible) between aspirin or naproxen and platelet COX-1 by assessing the capacity of increasing concentrations of exogenous AA to overwhelm the inhibitory effect of TXB\textsubscript{2} production by aspirin or naproxen in washed human platelets. Fresh peripheral blood from healthy volunteers who had not taken NSAIDs for 14 days was collected in Vacutainer tubes without heparin and was mixed with 10% (vol/vol) anticoagulant solution (65 mmol/l citric acid/85 mmol/l sodium citrate/2% glucose, pH 7.4). Washed platelets (1.5 x 10\textsuperscript{8} cells/ml), prepared as previously described (23), were incubated with increasing concentrations of aspirin (0.01 to 100 μmol/l), naproxen (0.01 to 100 μmol/l), or dimethyl sulfoxide vehicle (1 μl) for 25 min, and then 0.5 or 10 μmol/l of AA (Sigma Chemical Co., St. Louis, Missouri) was added for an additional 30 min at 37°C. To determine whether naproxen inhibited the acetylation of COX-1 by aspirin, we incubated an increasing concentration of naproxen (0.01 to 10 μmol/l) with washed platelets (1.5 x 10^\textsuperscript{8} cells/ml) for 5 min before the addition of aspirin (10 or 100 μmol/l), and the incubation continued for additional 20 min at 37°C. The cells were washed twice with Hanks’ balanced salt solution supplemented with 25 mmol/l HEPES/10% anticoagulant solution to remove the reversible inhibitor. The platelets were then resuspended in 500 μl of Hanks’ balanced salt solution supplemented with 25 mmol/l HEPES, challenged with AA 10 μmol/l for 30 min at 37°C, and TXB\textsubscript{2} production was determined by radioimmunoassay (24). Under these experimental conditions, a detectable inhibition of platelet TXB\textsubscript{2} production was dependent on the chance of aspirin to acetylate COX-1.

Clinical study: Design, treatments, and assessment. The potential interactions between low-dose aspirin and naproxen co-administered to four healthy subjects on platelet TX biosynthesis in vivo and ex vivo and platelet aggregation induced by AA (1 mmol/l) ex vivo (25) were evaluated in two different studies (Fig. 1). In the first study, uncoated aspirin (100 mg daily, at 8 AM) to be swallowed whole was given for 6 consecutive days, and then the combination of aspirin and naproxen was administered for further 6 days: aspirin was given 2 h before naproxen (500 mg BID, at 10 AM and 10 PM). After a washout period of 14 days, the treatments were administered in reverse order, i.e., low-dose aspirin (100 mg daily at 10 AM) was taken 2 h after naproxen (500 mg BID, at 8 AM and 8 PM) for further 6 days. Blood samples were collected before and at 2, 5, 14, and 26 h after the first study drug on the 6th, 12th, 27th, and 32nd study day to assess the inhibition of serum TXB\textsubscript{2} (a capacity index of platelet COX-1 activity) (24) and lipopolysaccharide-induced prostaglandin (PG)E\textsubscript{2} production (a capacity index of monocyte COX-2 activity) (26). Three consecutive urinary samples (time of collection: 0 to 6 h, 6 to 12 h, and 12 to 24 h) were collected before treatment and on days 6, 12, 27, and 32 to evaluate the urinary excretion of 11-dehydro-TXB\textsubscript{2} (a major enzymatic metabolite of TXB\textsubscript{2} that is an index of TXA\textsubscript{2} biosynthesis in vivo) (27). In the second study, a single dose of aspirin (100 mg) and naproxen (500 mg) was administered concurrently to 5 healthy subjects, and peripheral blood samples

### Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>AA</td>
<td>arachidonic acid</td>
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<tr>
<td>BID</td>
<td>twice-daily dosing</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>IC\textsubscript{50}</td>
<td>concentrations required to inhibit 50% of enzyme activity</td>
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<tr>
<td>NANAISDs</td>
<td>nonaspirin nonsteroidal anti-inflammatory drugs</td>
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<tr>
<td>NSAIDs</td>
<td>nonsteroidal anti-inflammatory drugs</td>
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<td>PG</td>
<td>prostaglandin</td>
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were fitted, and IC$_{50}$ (concentrations required to inhibit 50% of enzyme activity) values were analyzed with PRISM (GraphPad, San Diego, California). The IC$_{50}$ values were reported as mean values, and 95% confidence intervals (CIs) were calculated.

**RESULTS**

Pharmacodynamic interaction between aspirin and naproxen in vitro. As shown in Figures 2A and 2B, aspirin and naproxen inhibited TXB$_2$ production by washed platelets in a concentration-dependent fashion. However, in contrast to aspirin, the potency of naproxen to inhibit platelet COX-1 activity decreased in the presence of the higher concentration of AA, which supports the reversible interaction of naproxen, but not aspirin, with COX-1. At 0.5 and 10 μmol/l of AA, aspirin inhibited COX-1 activity, with IC$_{50}$ values of 3.40 μmol/l (95% CI 2.70 to 4.30 μmol/l) and 4.50 μmol/l (95% CI 3.80 to 5.50 μmol/l), respectively, whereas the corresponding values for naproxen were 0.16 μmol/l (95% CI 0.10 to 0.20 μmol/l) and 0.75 μmol/l (95% CI 0.50 to 1.20 μmol/l), respectively. Experiments were then conducted to determine the ability of naproxen to affect platelet COX-1 acetylation by aspirin. Platelets were pretreated with naproxen for 5 min before the addition of 10 or 100 μmol/l of aspirin and incubated for an additional 20 min. The cells were then centrifuged and washed twice, as described previously, to remove any reversible clogging of COX-1 channel, and then challenged for TXB$_2$ production with 10 μmol/l of AA.

Under these experimental conditions, a detectable inhibition of platelet TXB$_2$ production was dependent on the chance of aspirin to acetylate COX-1. Aspirin alone at 10 and 100 μmol/l inhibited TXB$_2$ production by 73 ± 7% and 89 ± 2%, respectively. The preincubation of platelets with increasing concentrations of naproxen reduced the irreversible inhibition of TXB$_2$ production by aspirin (Fig. 2C). At aspirin concentrations of 100 μmol/l, the naproxen concentration–response curve was shifted to the right, suggesting that the pharmacodynamic interaction between the two drugs involved a competition at the enzyme active site (Fig. 2C). The irreversible inhibition of platelet COX-1 by aspirin was affected by naproxen concentrations lower than those blocking platelet COX-1 activity. In fact, it was detectable at 0.1 μmol/l of naproxen (Fig. 2C), which did not affect TXB$_2$ production by platelets challenged with 10 μmol/l of AA (Fig. 2B).

**Study with multiple daily doses.** As shown in Figures 3A and 3B, the administration of low-dose aspirin for six consecutive days caused an almost complete inhibition of platelet COX-1 activity and platelet aggregation (99 ± 0.2% and 95 ± 0.6%, respectively, mean ± SEM, n = 4) that persisted up to 26 h after the last dose and was not altered by the co-administration of naproxen (2 h after aspirin) or in reverse order (naproxen 2 h before aspirin) for further 6 days. The reduction of urinary levels of 11-
dehydro-TXB2 (81%/H11006 4%, 74%/H11006 6%, and 83%/H11006 6%, respectively) assessed in three consecutive urine collections (0 to 6 h, 6 to 12 h, and 12 to 24 h, respectively) obtained after chronic dosing with aspirin alone was not affected by the concomitant administration of naproxen given 2 h after aspirin (Fig. 3C). A comparable inhibition of the urinary enzymatic metabolite was found when naproxen was given 2 h before aspirin (Fig. 3C).

As shown in Figure 4, lipopolysaccharide-induced PGE2 production was not significantly affected by the chronic administration of low-dose aspirin, whereas it was profoundly inhibited by the concomitant chronic administration of naproxen given 2 h after aspirin (Fig. 3C). A comparable inhibition of the urinary enzymatic metabolite was found when naproxen was given 2 h before aspirin (Fig. 3C).

As shown in Figure 4, lipopolysaccharide-induced PGE2 production was not significantly affected by the chronic administration of low-dose aspirin, whereas it was profoundly inhibited by the concomitant chronic administration of naproxen given 2 h after aspirin (Fig. 3C). A comparable inhibition of the urinary enzymatic metabolite was found when naproxen was given 2 h before aspirin (Fig. 3C).

Study with single dose. Because the profound and long-lasting inhibition of platelet COX-1 activity caused by naproxen (17) might have shaded its possible antagonism on the irreversible antiplatelet effect of aspirin, we studied the
Time-dependent inhibition and recovery of platelet COX-1 activity and AA-induced platelet aggregation up to 14 days after the concurrent administration of single doses of aspirin (100 mg) and naproxen (500 mg). The co-administration of the two drugs caused a time-dependent inhibition of platelet COX-1 activity and function (Fig. 5). Interestingly, at 1 h after dosing, serum TXB₂ and platelet aggregation were not significantly affected (92 ± 5% and 99 ± 0.1% of pre-drug values, respectively), which suggests that naproxen concentrations lower than those inhibiting platelet COX-1 activity interfered with the irreversible inhibition of aspirin. The inhibition of serum TXB₂ production and platelet aggregation recovered in a time-dependent fashion. At 72 h after dosing, serum TXB₂ and platelet aggregation values (65 ± 12% and 80 ± 19% of pre-drug values, respectively) did not differ, in a statistically significant fashion, from those assessed before (100 ± 11% and 100 ± 0.6% of pre-drug values, respectively) or 14 days after treatment (86 ± 7% and 99 ± 0.7% of pre-drug values, respectively). The rapid recovery of COX-1 activity and function confirmed the occurrence of the pharmacodynamic interaction between naproxen and aspirin.

**DISCUSSION**

Several epidemiologic studies have been performed to establish the possible antithrombotic effect of naproxen, but the results did not present a clear picture (9–12,14–16,18,19). Probably, the administration of the drug not at high doses, not continuously and regularly, as occurs in real life photographed by the observational studies, may have contributed to the unsettled cardioprotective effect of naproxen. The final say on the risk reduction of myocardial infarction by naproxen will be made when prospective controlled trials of adequate size are performed. In the meantime, patients with musculoskeletal disorders treated with naproxen should receive low-dose aspirin as well if they require an antithrombotic treatment. However, a pharmacologic study has shown that the NSAID ibuprofen, but not diclofenac, acetaminophen, or rofecoxib, can antagonize the irreversible inhibition of platelet TXA₂ by aspirin when they are administered together (21).

In the present study, we have addressed whether naproxen might competitively inhibit the ability of aspirin to cause an irreversible inhibition of platelet COX-1 in vitro, ex vivo, and in vivo in healthy subjects. First, we characterized the interaction between aspirin or naproxen and platelet COX-1 in washed platelets in vitro. Aspirin caused an irreversible inhibition of platelet COX-1 activity. The inhibition of serum TXB₂ production and platelet aggregation recovered in a time-dependent fashion. At 72 h after dosing, serum TXB₂ and platelet aggregation values (65 ± 12% and 80 ± 19% of pre-drug values, respectively) did not differ, in a statistically significant fashion, from those assessed before (100 ± 11% and 100 ± 0.6% of pre-drug values, respectively) or 14 days after treatment (86 ± 7% and 99 ± 0.7% of pre-drug values, respectively). The rapid recovery of COX-1 activity and function confirmed the occurrence of the pharmacodynamic interaction between naproxen and aspirin.
substrate of COX-1) (Fig 2A). Differently, naproxen caused a reversible inhibition of COX-1 (Fig. 2B). Next, we studied whether the preincubation of naproxen had the ability to affect the irreversible inhibition of platelet COX-1 by aspirin. We pretreated platelets with naproxen for 5 min before the addition of aspirin, and we evaluated the production of TXB₂ in response to AA after having washed the cells to remove reversible blockage of COX-1. Under these experimental conditions, a detectable inhibition of platelet TXB₂ production was dependent on the chance of aspirin to acetylate COX-1. Naproxen, indeed, reduced the inhibition of platelet TXB₂ production by aspirin in a concentration-dependent fashion (Fig. 2C). This effect occurred even at lower drug concentrations than those inhibiting platelet COX-1 activity. The explanation is that in the first case naproxen competes with aspirin, which is a weak inhibitor of platelet COX-1, whereas in the second case the drug competes with AA, which binds the enzyme strongly. Similar results have been reported for ibuprofen and COX-2 inhibitors in vitro (23).

After having highlighted a pharmacodynamic interaction between aspirin and naproxen in vitro, we verified its occurrence ex vivo. The concurrent administration of a single dose of the two drugs to healthy subjects was associated with undetectable inhibition of serum TXB₂ production at 1 h after dosing. This finding is consistent with the interference of naproxen on the irreversible inhibition of platelet COX-1 by aspirin. In fact, it has been reported previously that the administration of aspirin inhibits platelet COX-1 activity (as assessed by the measurement of serum TXB₂ production) before the drug reaches the systemic circulation; this effect is maximal at 20 min after dosing, coinciding with the peak plasma concentrations of the active drug (28). Despite the short half-life of aspirin (approximately 20 min), the inhibition of platelet COX-1 activity persists up to 24 h as the result of irreversible enzyme inactivation (Fig. 2A) (18).

Similar to that found in vitro, naproxen interfered with aspirin acetylation at concentrations lower than those inhibiting platelet COX-1 activity. In fact, circulating plasma levels of the drug suited to inhibit the platelet enzyme are reached more than 1 h after dosing since time to reach peak concentration in the systemic circulation (T_max) of naproxen is 3 to 4 h. The finding of a rapid recovery of platelet TXB₂ production and platelet aggregation detected after the co-administration of a single dose of aspirin and naproxen corroborates the occurrence of the interaction between naproxen and aspirin (29). However, the chronic administration of naproxen 2 h after aspirin or in reverse sequence for 6 consecutive days did not affect the inhibition of TXB₂ biosynthesis ex vivo and in vivo and platelet aggregation ex vivo caused by aspirin alone. The most likely explanation of these results is that the pharmacodynamic interaction between the two drugs was shaded because of the capacity of high-dose naproxen to mimic the antiplatelet COX-1 effect of low-dose aspirin (17).

However, in our earlier work, we found that even within the context of a controlled and well-monitored study, the chronic administration of naproxen 500 mg BID gets into the functionally relevant range, i.e., >95% inhibition of platelet COX-1 activity ex vivo at the end of the dosing interval, in some but not all subjects (17). Such heterogeneity, as well as departure from the rigor of monitored drug intake, might explain the weak and irregular signal of cardioprotection from naproxen in epidemiologic analyses (19,22,30). We cannot assess whether naproxen heterogeneity occurred in the present study because of the background of aspirin; perhaps in those subjects in whom functional inhibition of COX-1 was not attained by naproxen, a partial contribution was afforded by aspirin. Another limitation is the small sample size, which allowed us to discern only large differences and did not enable us to assess the impact of other covariates such as gender and age.

**Conclusions.** Naproxen interfered with the irreversible inhibitory effect of aspirin on platelet COX-1 activity in vitro and ex vivo. This effect was undetectable during the continuous and regular administration of an anti-inflammatory dose of naproxen (500 mg BID) and low-dose aspirin because naproxen can mimic the inhibitory effect of aspirin on platelet TXA₂ generation. However, in the real world, naproxen combination with aspirin might undermine the sustained inhibition of platelet COX-1 necessary for cardioprotection from aspirin.

**Acknowledgments**

We thank the medical students of “G. d’Annunzio” University for their generous cooperation in the undertaking of this study.

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