ORIGINAL ARTICLE

Comparative effects of immediate-release and extended-release aspirin on basal and bradykinin-stimulated excretion of thromboxane and prostacyclin metabolites

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Abstract

A goal of aspirin therapy is to inhibit thromboxane production and platelet aggregation without inhibiting endothelial production of the vasodilator and anti-thrombotic prostacyclin. This study tested the hypothesis that extended-release aspirin (NHP-554C) would have increased selectivity for inhibition of basal and simulated thromboxane formation compared to immediate-release aspirin (ASA). Thirty-six healthy subjects were randomized to NHP-554C or ASA groups. Within each group, subjects were randomized to 5-day treatment with 81 mg/d, 162.5 mg/d and placebo in a crossover design in which treatment periods were separated by 2-week washout. On the fifth day of treatment, 81 mg/d and 162.5 mg/d ASA reduced basal urinary excretion of the stable thromboxane metabolite 11-dehydro-thromboxane B2 62.3% and 66.2% and basal excretion of the stable prostacyclin metabolite 2,3-dinor-6-keto-PGF1α 22.8% and 26.5%, respectively, compared to placebo. NHP-554C 81 mg/d and 162.5 mg/d reduced 11-dehydro-thromboxane B2 53% ($P = 0.03$ vs. ASA 81 mg/d) and 67.9% and 2,3-dinor-6-keto-PGF1α 13.4% and 18.5%, respectively. NHP-554C 81 mg/d did not significantly reduce basal excretion of the prostacyclin metabolite. Both doses of ASA and NHP significantly reduced excretion of both thromboxane and prostacyclin metabolites following intravenous bradykinin. During NHP-554C 162.5 mg/d, but not during ASA, bradykinin significantly increased urinary 2,3-dinor-6-keto-PGF1α. Nevertheless, 11-dehydro-thromboxane B2 and 2,3-dinor-6-keto-PGF1α responses to bradykinin were statistically similar during ASA and NHP-554C. In conclusion, at doses of 81 and 162.5 mg/d immediate- and extended-release aspirin selectively decrease basal thromboxane production. Both forms of aspirin decrease bradykinin-stimulated thromboxane and prostacyclin production, but some stimulated prostacyclin production remains during treatment with NHP-554C.

Abbreviations

AE, adverse event; ASA, aspirin; CRC, Clinical Research Center; GC/MS, gas chromatography/mass spectrometry; GC/NICI-MS, gas chromatography-negative-ion chemical ionization mass spectrometry; NHP, New Haven Pharmaceuticals; PGE2, prostaglandin E2.

Introduction

Aspirin reduces the risk of thrombotic events in patients with a history of myocardial infarction or stroke, as well as in individuals at risk for these events (Lewis et al. 1983; Steering Committee of the Physicians' Health Study Research Group 1989; Ridker et al. 1997; Baigent et al. 2009). Aspirin reduces thrombosis by acetylating prostaglandin G/H synthase at serine 529 and irreversibly inhibiting the enzyme (Funk et al. 1991). Inhibiting prostaglandin G/H synthase in platelets decreases the formation of thromboxane A_2, a potent platelet agonist and vasoconstrictor (Hamberg et al. 1975). This beneficial effect is offset by inhibition of endothelial prostaglandin G/H synthase which forms prostacyclin, an inhibitor of platelet aggregation and vasodilator (Moncada et al. 1976). In addition, inhibition of systemic prostaglandin G/H synthase can decrease the formation of prostaglandins that decrease gastric acid secretion and protect the gastric mucosa (Lee et al. 1994).

One strategy for increasing the selectivity of aspirin for its anti-thrombotic effects has been to develop controlled-release preparations of aspirin. Aspirin undergoes extensive first-pass metabolism in the liver to salicylate, a weak and reversible inhibitor of prostaglandin synthase (Ritter et al. 1989). Controlling the release of aspirin in the gastrointestinal track has been proposed to maximize exposure of platelets to aspirin in the preportal circulation, while minimizing systemic absorption and inhibition of endothelial or gastrointestinal prostaglandin G/H synthase (Pederson et al. 1994). Such a strategy could reduce thromboxane production while preserving prostacyclin production.

NHP-554C is a controlled release aspirin product made up of microparticles separately coated with a thin film based on ethylcellulose (Durlaza®; New Haven Pharmaceuticals, Inc., New Haven CT). The ethylcellulose film coating acts as a semi-permeable membrane that allows aspirin to diffuse progressively over the length of the gastrointestinal tract, resulting in prolonged absorption. This study tested the hypothesis that NHP-554C would have a selective inhibitory effect on basal and stimulated thromboxane production versus prostacyclin production compared to immediate-release aspirin.

Materials and Methods

Subjects

Healthy nonobese subjects between the ages of 18–55 years inclusive were studied. Subjects could smoke but were required to maintain their tobacco use constant throughout the study. Women of child-bearing potential were required to utilize prespecified means of contraception and to undergo a urine pregnancy test prior to each treatment period and on each study day. The use of non-steroidal anti-inflammatory agents or aspirin was excluded for 2 weeks prior to the study. All other medications other than oral contraceptives were excluded for at least 1 week prior to the study. Subjects with a history of peptic ulcer disease were excluded.

Protocol

The study has been carried out in accordance with the Declaration of Helsinki and approved by the Vanderbilt Institutional Review Board. Informed consent was obtained, and subjects reported to the Vanderbilt Clinical Research Center (CRC) after an overnight fast to provide a medical history, to undergo a physical examination and electrocardiocardiogram, and to provide blood and urine for screening laboratory.

Figure 1 illustrates the study protocol. Subjects who met inclusion and exclusion criteria were randomized in a one-to-one ratio to Group 1 or Group 2 using a permuted-block randomization algorithm after a 2-week washout of any medications. Subjects in Group 1 were randomized to receive rapid-release aspirin (ASA, 81 mg), ASA 162.5 mg, or identical-appearing placebo daily for 5 days. Subjects in Group 2 were randomized to receive NHP-554C 81 mg, NHP-554C 162.5 mg or identical-appearing placebo. Within each group, subjects were randomized to one of six possible treatment sequences. Each treatment period was separated by at least 2 weeks.

Subjects collected their urine for 24 h for measurement of sodium, creatinine, 11-dehydro-thromboxane B2, a major stable urinary metabolite of thromboxane A2, and 2,3-dinor-6-keto-PGF1α, the stable urinary metabolite of prostacyclin, 1 day prior to the first treatment period, and from the 4th to 5th day of each treatment period.

On the morning of the 5th day of each treatment period, subjects reported to the Vanderbilt CRC in the fasting state. Compliance was confirmed by pill count. An intravenous catheter was inserted in each arm - one for sampling and the other for infusion of bradykinin. Subjects were given 500 mL of D5W over 1 h to facilitate urine production. Beginning at time 0, they were given bradykinin intravenously in graded doses of 10, 25, 50, and 100 ng/kg per min for 15 min each. Blood pressure and heart rate were measured every two min using an automated oscillometric recording device (Dinamap, Critikon, Carlsbad, CA). Subjects were given an additional 500 mL D5W an hour following bradykinin infusion. Urine was collected prior to initiation of bradykinin and hourly thereafter for 4 h for measurement of urine 2,3-dinor-6-keto-PGF1α and 11-dehydro-thromboxane B2.
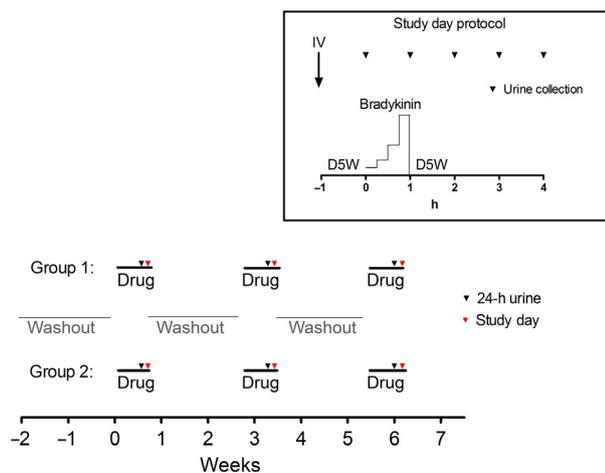
Figure 1. Study Protocol. Subjects in Group 1 were randomized to receive immediate-release aspirin 81 mg, 162.5 mg, or identical-appearing placebo daily for 5 days. Subjects in Group 2 were randomized to receive an extended-release formulation (NHP-554C) 81 mg, 162.5, mg or matching placebo. Subjects collected their urine for 24-h for measurement of the stable metabolites of thromboxane A2 and prostacyclin. On the fifth day of study medication, subjects underwent a bradykinin infusion study (inset). Each treatment period was separated by 2 weeks.

Safety laboratory, urinalysis, and electrocardiogram were repeated after the last study day.

Laboratory analyses

11-dehydro-thromboxane B2 was measured using gas chromatography-mass spectrometry (GC/MS) as previously described (Morrow and Minton 1993) and normalized to urine creatinine. Precision of the assay is $\pm 7\%$ and accuracy is 90%. The lower limit of sensitivity in urine is approximately 0.020 ng/mg creatinine. 2,3-dinor-6-keto-PGF1α, the major urinary metabolite of prostacyclin, was also measured using GC/MS (Daniel et al. 1994). The lower limit of detection in urine is approximately 0.015 ng/mg creatinine. In the first six subjects randomized to each group, serum thromboxane B2 was measured by gas chromatography-negative-ion chemical ionization mass spectrometry (GC/NICI-MS), based on a previously published method (FitzGerald et al. 1983). Urine creatinine concentrations were determined using a colorimetric assay kit from Enzo Life Sciences, Farmingdale, NY. Sodium and potassium were measured by flame photometry.

Statistical analyses

For univariate analyses, comparisons between study groups were made using the Wilcoxon rank sum test for continuous variables and the Pearson test for categorical variables. Within-subject comparisons were made using the Wilcoxon signed-rank test.

Using the method of Jones and Kenward, (Jones and Kenward 2003) we tested first order carryover effects and found no carryover difference for either urine 11-dehydro-thromboxane B2 or 2,3-dinor-6-keto-PGF1α concentration. To compare the effect of ASA and NHP-554C on bradykinin-stimulated urinary excretion of 11-dehydro-thromboxane B2 and 2,3-dinor-6-keto-PGF1α we used peak change in urine 11-dehydro-thromboxane B2 and 2,3-dinor-6-keto-PGF1α from time 0 as the dependent variable and fitted mixed-effect models with random subject effect and fixed effects of baseline measurement (before randomization), drug treatment, dose and interaction between treatment and dose. Period effects were also included in the models initially and were then removed after being found to be insignificant.

Data are presented as median and interquartile range or mean and standard error of the mean as indicated. A two-sided P value of <0.05 was considered statistically significant.

Results

Subject characteristics

Sixty-one subjects were screened to enroll 36 subjects who met inclusion and exclusion criteria. All thirty-six subjects completed three treatment periods and 24-h urine collections, with 18 subjects in each treatment group. Subject characteristics appear in Table 1. Baseline prerandomization 11-dehydro-thromboxzne B2 and 2,3-dinor-6-keto-PGF1α concentrations were significantly higher in the NHP-554C group compared to the ASA group. Heart rate was also higher among subjects in the NHP-554C group. There were no other differences in baseline characteristics between the treatment groups.

Effect of treatment on basal urinary thromboxane and prostacyclin metabolite excretion

During placebo treatment, as at baseline, 24-h excretion of 11-dehydro-thromboxane B2 and 2,3-dinor-6-keto-PGF1α were significantly higher in the NHP-554C group compared to the ASA group (Fig. 2). ASA 81 mg/d and 162.5 mg/d significantly and equivalently reduced 24-h urinary excretion of 11-dehydro-thromboxane B2. Both doses of NHP-554C also significantly reduced urine 11-dehydro-thromboxane B2, but the stable thromboxane metabolite was reduced to a significantly greater extent during 162.5 mg/d NHP-554C.

Table 1. Subject characteristics.

Parameter	ASA group	NHP-554C group
Age, years	27.4 (24.2, 33.6)	26.5 (24.1, 28.4)
Gender, M:F	10:8	5:13
Race/ethnicity, B:NHW:HW:multi	0:15:3:0	3: 13:1:1
BMI, kg/M^2	25.1 (23.8, 27.0)	23.9 (22.1, 24.8)
Smoking, never: current: former	16:0:2	18:0:0
Blood pressure mmHg		
Systolic	113.0 (105.2–118.5)	114.0 (102.0–121.0)
Diastolic	68.5 (64.2–73.5)	69.0 (62.0–74.0)
Heart Rate, beats per min	55.0 (52.2–62.0)	65.5 (62.8–74.2)[1]
Fasting plasma glucose (mg/dL)	79.5 (73.8, 86.0)	80.0 (74.5, 82.0)
Cholesterol (mg/dL)		
Total	170 (160, 178)	179 (158, 208)
Low density lipoprotein	102 (89, 110)	106 (90, 132)
High density lipoprotein	52 (42, 66)	56 (46, 62)
Triglycerides (mg/dL)	62 (55, 82)	83 (47, 104)
11-dehydro-thromboxane B2, ng/mg creatinine	0.180 (0.158–0.240)	0.214 (0.191–0.296) [1]
2,3-dinor-6-keto-PGF1α, ng/mg creatinine	0.100 (0.089–0.139)	0.139 (0.117–0.252) [1]

Data are presented as median (interquartile range).
[1] $P < 0.05$ versus immediate-acting aspirin (ASA) group.

ASA 81 mg/d and 162.5 mg/d significantly and equivalently reduced 24-h urinary excretion of 2,3-dinor-6-keto-PGF1α (Fig. 2). In contrast, treatment with 81 mg/d NHP-554C did not significantly reduce urine 2,3-dinor-6-keto-PGF1α compared to placebo, whereas treatment with 162.5 mg/d did.

Because urinary excretion of 11-dehydro-thromboxane B2 and 2,3-dinor-6-keto-PGF1α during placebo differed in the ASA and NHP-554C groups, we also measured the effect of treatment and dose on 24-h urine excretion of the thromboxane and prostacyclin metabolites, normalized to excretion during placebo. ASA 81 and 162.5 mg/d decreased 24-h urine 11-dehydro-thromboxane B2 62.3% and 66.2% compared to placebo and 24-h urine 2,3-dinor-6-keto-PGF1α 22.8% and 26.5% compared to placebo, respectively. NHP-554C 81 mg/d and 162.5 mg/d reduced 11–dehydro-thromboxane B2 53% ($P = 0.03$ vs. ASA 81 mg/d) and 67.9% versus placebo and 2,3-dinor-6-keto-PGF1α 13.4% and 18.5%, respectively.

To confirm that the effects of ASA and NHP-554C on 24-h urine 11-dehydro-thromboxane B2 resulted from inhibition of platelet thromboxane production, we also measured serum thromboxane B2 concentrations in the first six subjects enrolled in each group. As observed for 24-h urine 11-dehydro-thromboxane B2, serum thromboxane concentrations tended to be higher during placebo treatment in the NHP-554C group compared to the ASA group (Fig. 2, middle). ASA 81 and 162.5 mg/d decreased serum thromboxane B2 62.6% and 64.7%, respectively, compared to placebo. NHP-554C 81 mg/d and 162.5 mg/d reduced serum thromboxane B2 67.3% and 86.6%, respectively, compared to placebo.

Effect of treatment on bradykinin-stimulated 11-dehydro-thromboxane B2 and 2,3-dinor-6-keto-PGF1α excretion

Thirty-one subjects completed all three bradykinin infusion studies. Five subjects received only two bradykinin infusions. In two subjects, bradykinin infusion was canceled due to severe winter weather conditions (one during placebo and one during NHP-554C 81 mg). In two additional subjects, bradykinin was not available on the scheduled infusion days (one during placebo and one during ASA 81 mg) due to a delay in shipping. One subject was not able to complete the final bradykinin infusion (during NHP-554C 81 mg) due to a change in his work schedule.

Bradykinin significantly increased urinary excretion of 11-dehydro-thromboxane B2 during placebo in both ASA (from 0.255 ± 0.068 ng/mg Cr to 0.398 ± 0.289 ng/mg Cr at 3 h, $P = 0.009$) and NHP-554C (from 0.360 ± 0.180 to 0.530 ± 0.260 ng/mg Cr, $P = 0.009$) treatment groups (Fig. 3). ASA 81 mg/d and 162.5 mg/d significantly and equivalently reduced 11-dehydro-thromboxane B2 excretion after bradykinin; nevertheless, urinary excretion of the thromboxane metabolite increased significantly following bradykinin during both doses of ASA. NHP-554C 81 mg/d and 162.5 mg/d also significantly reduced 11-dehydro-thromboxane B2 excretion after bradykinin. During the bradykinin infusion study, 11-dehydro-thromboxane B2 excretion was significantly lower during NHP-554C 162.5 mg/d than during the 81 mg/d dose, but there was no significant increase in 11-dehydro-thromboxane B2 in response to bradykinin during either dose (Fig. 3C).

Bradykinin significantly increased urinary excretion of 2,3-dinor-6-keto-PGF1α during placebo in both ASA (from 0.109 ± 0.033 ng/mg Cr to 0.199 ± 0.115 ng/mg Cr at 2 h, $P < 0.001$) and NHP-554C (from 0.177 ± 0.066 ng/mg Cr to 0.330 ± 0.237 ng/mg Cr, $P < 0.001$) treatment groups (Fig. 3B and D). ASA 81 mg/d and 162.5 mg/d significantly and equivalently decreased 2,3-dinor-6-keto-PGF1α excretion after bradykinin, as did NHP-554C 81 mg/d and 162.5 mg/d. Bradykinin significantly increased urinary excretion of the prostacyclin metabolite during treatment with 162.5 mg/d NHP-554C.
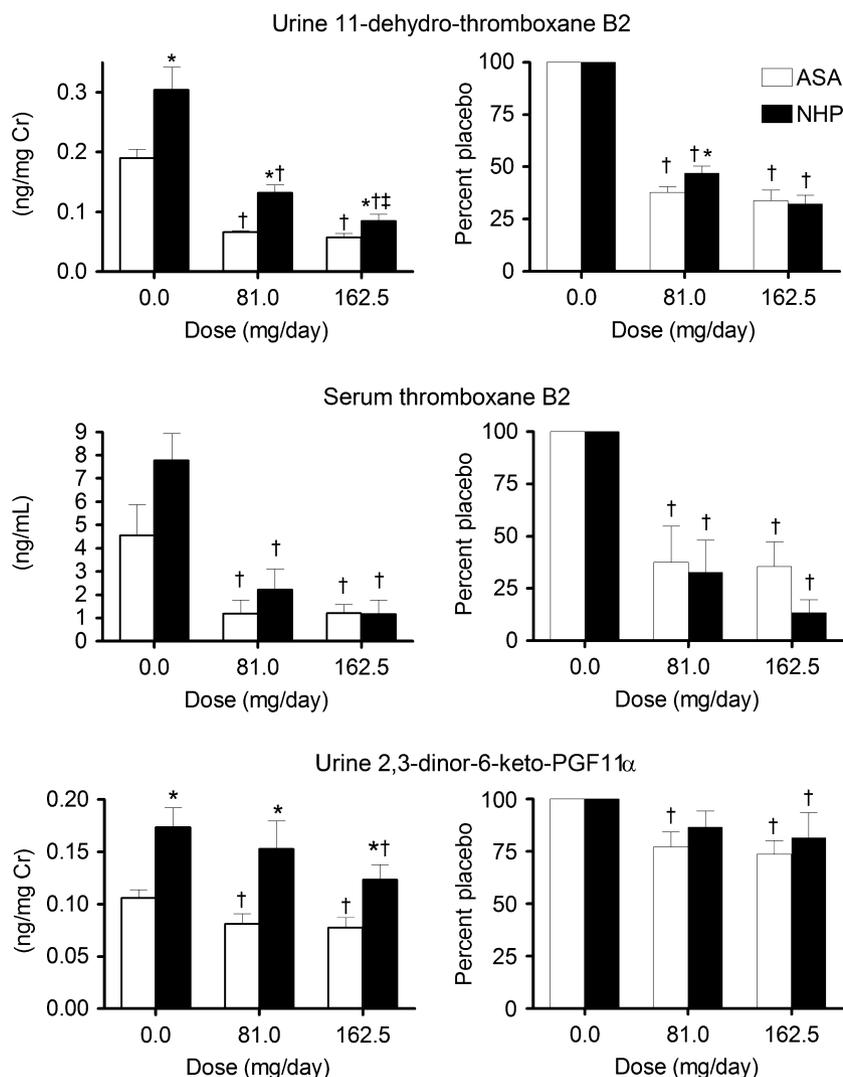
Figure 2. Effect of 5-day treatment with two doses of immediate-release aspirin (ASA) or NHP-554C on 24 h urinary excretion of (top) the stable metabolite of thromboxane, 11-dehydro-thromboxane B2, and (bottom) the stable metabolite of prostacyclin, 2,3-dinor-6-keto-PGF$_{1\alpha}$. In the first six subjects randomized to each treatment group, we also measured basal serum thromboxane B2 (middle). Data are presented as means ±standard error of the mean. *$P < 0.05$ versus ASA group, †$P < 0.05$ versus placebo, ‡$P < 0.05$ versus 81 mg/d NHP-554C.

We next compared the effect of ASA and NHP-554C on the maximal change in urine 11-dehydro-thromboxane B2 and 2,3-dinor-6-keto-PGF1α following bradykinin. In mixed effect models controlling for baseline concentrations, there were no significant differences in the effect of ASA and NHP-554C on the change in urine 11-dehydro-thromboxane B2 or the change in 2,3-dinor-6-keto-PGF1α concentrations at either the 81 mg/d or 162.5 mg/d doses. Inclusion of gender did not alter the model.

Safety

A total of ten adverse events (AEs) were reported by nine of 18 subjects in the ASA group, and seven AEs were reported in six of 18 subjects in the NHP-554C group. The most common AE was sinus or nasal congestion, occurring in four subjects in the ASA group and three subjects in the NHP-554C group. There were no serious adverse events.

One subject in the ASA group had a low albumin noted on safety laboratory testing. One subject in the NHP-554C had a mild transient increase in transaminases. There was no difference in hematocrit in the two study groups at the end of the study (39.8 ± 3.5% in the ASA group versus 38.5 ± 2.2% in the NHP-554C group).

Five subjects had transient nonspecific ST T wave changes noted on the electrocardiogram obtained after the last bradykinin infusion. Two subjects had been
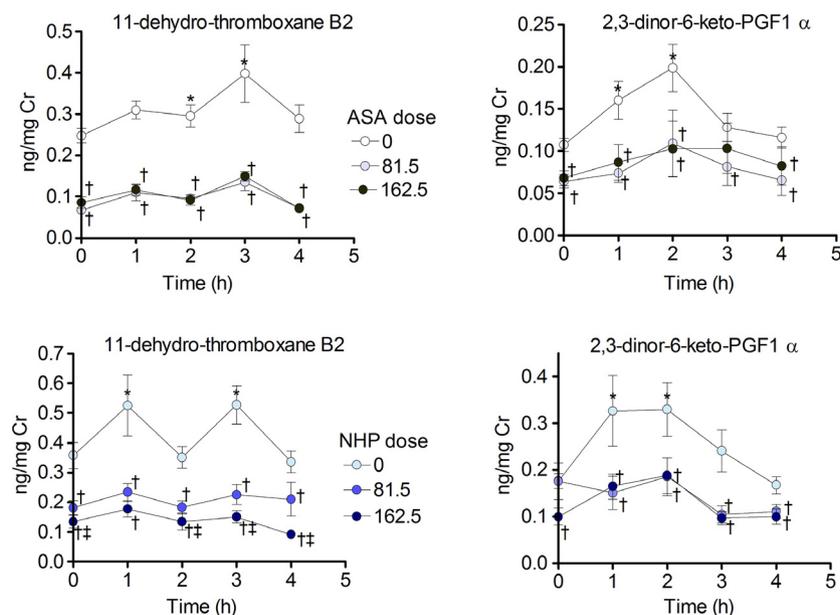
Figure 3. Effect of 5-day treatment with two doses of short-acting aspirin (ASA) or NHP-554C on urine 2,3-dinor-6-keto-PGF$_{1\alpha}$ and 11-dehydro-thromboxane B$_2$ before and after 1 h intravenous administration of bradykinin. Data are presented as means ±standard error of the mean. *$P < 0.05$ versus time 0, †$P < 0.05$ versus placebo, ‡$P < 0.05$ versus 81 mg/d.

taking placebo, one subject NHP-554C 81 mg, and two subjects NHP-554C 162.5 mg prior to bradykinin on these study days. The Data and Safety Monitoring Committee reviewed these ECGs and recommended continuing the study but requested that the investigators measure plasma troponin before and after bradykinin infusion. This was done in 11 subjects and there was no increase in plasma troponin before or after bradykinin.

Discussion

We compared the effect of two doses of immediate- (ASA) and extended- (NHP-554C) release aspirin on basal and bradykinin-stimulated excretion of stable metabolites of thromboxane and prostacyclin. We found that, whereas 81 mg/d and 162.5 mg/d ASA equivalently reduced basal thromboxane and prostacyclin metabolite excretion, 81 mg/d NHP-554C was significantly less effective than 162.5 mg/d in reducing thromboxane and did not significantly reduce basal prostacyclin synthesis. ASA and NHP-554C similarly decreased bradykinin-stimulated excretion of the thromboxane and prostacyclin metabolites, although stimulated prostacyclin excretion was somewhat preserved during 162.5 mg/d NHP-554C.

A goal of aspirin therapy is to inhibit platelet aggregation and the production of thromboxane without inhibiting the production of the vasodilator and anti-thrombotic prostacyclin. At the doses administered in this study, both immediate-release and extended-release aspirin selectively

decreased basal thromboxane production compared to prostacyclin production. Selectivity of low-dose (80 mg/d) aspirin for inhibition of thromboxane synthesis has been reported previously (Braden et al. 1991). Although NHP-554C 81 mg/d did not significantly reduce basal excretion of the stable prostacyclin metabolite and, at the 162.5 mg/d dose NHP-554C decreased prostacyclin synthesis 18.5% versus 26.5% after the same dose of ASA, the magnitude of the effect of the extended-release and immediate-release aspirin on prostacyclin synthesis were statistically and clinically similar.

To further assess the effect of immediate- and extended-release aspirin on vascular prostaglandin synthesis, we assessed the effect of ASA and NHP-554C on bradykinin-stimulated prostacyclin and thromboxane synthesis. Bradykinin stimulates prostaglandin synthesis by activating phospholipase and liberating arachidonic acid from membrane phospholipids (Hong and Levine 1976). Bradykinin stimulates the synthesis of prostacyclin from the endothelium of large vessels and prostaglandin E2 (PGE2) from the endothelium of the microcirculation (Gerritsen and Printz 1981; Gerritsen and Cheli 1983). Bradykinin stimulates platelet thromboxane formation, (Lefort et al. 1984) as well as the release of thromboxane from the vasculature through an endothelium-dependent mechanism (Sametz et al. 2000). Whereas both study drugs selectively inhibited *basal* thromboxane synthesis, immediate- and extended-release aspirin inhibited bradykinin-stimulated prostacyclin production as well as

thromboxane production. This observation is similar to the finding of Braden et al. (1991), who reported that treatment with low-dose aspirin spared basal prostacyclin synthesis but inhibited prostacyclin synthesis following percutaneous transluminal coronary angioplasty.

Our results differ from the results of Clarke et al. (1991) who found that 75 mg controlled-release aspirin had no effect on bradykinin-stimulated synthesis of prostacyclin, as measured by urinary 2,3-dinor-6-keto-PGF1α. Recently published pharmacokinetic data for NHP-554C (Patrick et al. 2015) may provide some insight into the discrepancy between the findings of Clarke and the present study. In the study of Clarke et al., the peak plasma aspirin concentration following a single dose of 75 mg controlled-release aspirin was 0.29 nmo/mL or 52.2 ng/mL (Clarke et al. 1991). In contrast, single doses of 81 mg and 162.5 mg NHP-554C produced maximum plasma aspirin concentrations of 106 ng/mL and 174 ng/mL, and 81 mg ASA yielded a maximum aspirin concentration of 504 ng/mL (Patrick et al. 2015). Although we did not measure aspirin concentrations in the current study, it is likely that they were even higher with repeated dosing and that these higher concentrations were sufficient to inhibit prostacyclin formation.

Unexpectedly, we noted transient asymptomatic focal ST TW changes on electrocardiograms obtained at the end of the study after the final bradykinin infusion in a few study participants. Because these changes were observed after bradykinin infusion during both placebo treatment and treatment with extended-release aspirin, the Data and Safety Monitoring Committee attributed the changes to bradykinin. Bradykinin has been reported to cause endothelium-dependent vasoconstriction following vasorelaxation in porcine arteries through a B2 receptor-dependent, thromboxane-independent mechanism (Miyamoto et al. 1999).

This study has a few limitations. By chance, subjects randomized to NPH-554C had higher basal urinary excretion of both 11-dehydro-thromboxane B2 and 2,3-dinor-6-keto-PGF1α and a higher heart rate. We controlled for differences in prostaglandin metabolite excretion by normalizing basal excretion during active drug to basal excretion during placebo and by measuring change from baseline in response to bradykinin. Because this study focused on the relative inhibition of thromboxane and prostacyclin inhibition, we measured urinary thromboxane metabolite, which reflects both platelet and vascular production. Measurement of serum thromboxane in a subset of subjects suggests that the reduction in platelet thromboxane production paralleled the reduction in urinary thromboxane metabolite. We studied healthy subjects in this mechanistic study to avoid adverse events due to bradykinin infusion; it is possible that the relative

effects of ASA and NHP-554C on thromboxane and prostacyclin synthesis could differ in patients with atherosclerosis.

In summary, immediate-release and extended-release aspirin selectively inhibit basal thromboxane production over prostacyclin production at doses of 81 and 162.5 mg/d. In the case of extended-release NHP-554C, there was a dose-dependent effect on thromboxane synthesis and only the higher dose reduced basal prostacyclin synthesis. While both forms of aspirin decrease stimulated production of thromboxane and prostacyclin, some increase in bradykinin-stimulated prostacyclin production was preserved during NHP-554C treatment. Whether this translates into beneficial clinical effects of NHP-554C remains to be tested.

Acknowledgements

Author Contributions

Brown and Gamboa participated in research design; Gamboa, Devin, Ramirez, and Brown conducted the experiments and contributed to writing of the manuscript; and Nian, Lee, Yu. performed data analysis.

Disclosures

None declared.

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