Published in final edited form as:
J Cardiovasc Pharmacol Ther. 2010 December ; 15(4): 344–348. doi:10.1177/1074248410375091.

A Randomized Trial of Aspirin at Clinically Relevant Doses and Nitric Oxide Formation in Humans

Charles H. Hennekens[1], **Wendy R. Schneider**[1], **Alex Pokov**[3], **Scott Hetzel**[4], **David DeMets**[4], **Victor Serebruany**[3], and **Henning Schröder**[5]

[1]Charles E. Schmidt College of Biomedical Science, Department of Clinical Science and Medical Education & Center of Excellence, Florida Atlantic University, Boca Raton, FL, USA

[2]Department of Preventive Medicine, Nova Southeastern University, Fort Lauderdale, FL, USA

Corresponding Author: Charles H. Hennekens, 2800 South Ocean Boulevard, Boca Raton, FL 33432, USA, profchhmd@prodigy.net.

Authors' Notes

Charles H. Hennekens, Principal Investigator, drafted the initial manuscript and coordinated revisions. Wendy R. Schneider, Project Coordinator, assisted in drafting and revising the manuscript. Alex Pokov, Co-Investigator, collected the data at the clinical site. Scott Hetzel, Project Statistician, analyzed the data. David DeMets, Co-Investigator, supervised all data analyses. Victor Serebruany, Co-Investigator, conducted all the laboratory testing. Henning Schröder, Co-Investigator, assisted in the trial design and interpretation. All coauthors provided input to and have approved the final version of the manuscript. Professor Hennekens reported that he is funded by the Charles E. Schmidt College of Biomedical Science, Department of Clinical Science, and Medical Education & Center of Excellence at Florida Atlantic University (FAU) as principal investigator on 2 investigator-initiated research grants funded to FAU by Bayer; serves as an independent scientist in an advisory role to investigators and sponsors as chair of the data and safety monitoring boards for Actelion, Amgen, Bristol-Myers Squibb, Dainippon Sumitomo, and Sanofi-Aventis, and as an independent scientist as a member of the data and safety monitoring boards for Bayer and the Canadian Institutes of Health Research; serves as an independent scientist in an advisory role to the US Food and Drug Administration, US National Institutes of Health, and UpToDate; serves as an independent scientist in an advisory role to legal counsel for General Electric and GlaxoSmithKline; serves as an independent scientist as a speaker for the Association of Research in Vision and Ophthalmology, National Association for Continuing Education, PriMed, the International Atherosclerosis Society, AstraZeneca, and Pfizer; receives royalties for authorship or editorship of 3 textbooks and as coinventor on patents for inflammatory markers and cardiovascular disease that are held by Brigham and Women's Hospital; and has an investment management relationship with the West-Bacon Group within SunTrust Investment Services, which has discretionary investment authority. Ms Schneider reported that she is funded by the Charles E. Schmidt College of Biomedical Science, Department of Clinical Science, and Medical Education & Center of Excellence at Florida Atlantic University (FAU) as project director on 2 investigator-initiated research grants funded to FAU by Bayer. Dr Pokov reported that he receives investigator-initiated research grant support from Bayer. Mr Hetzel reported that he has no competing financial interests. Professor DeMets reported that he is partially supported by a National Institutes of Health Grant to the University of Wisconsin for the Clinical Translational Science Award for statistical consultation and collaboration and administrative leadership, as a leader of the Data Management and Biostatistics Core (Core C) of the Wisconsin Alzheimer's Disease Research Center grant, by serving as a principal investigator on University of Wisconsin contracts with industry for statistical analysis center activity for multicenter trials, which are currently sponsored by Amgen, AstraZeneca, and Bristol-Myers Squibb; serves or has recently served as an independent biostatistician in an advisory role to investigators and sponsors as a member of the data and safety monitoring boards for Actelion, Amgen, Astellas, AstraZeneca, Biotronik, Boehringer-Ingelheim, CVRx, Genentech, GlaxoSmithKline, Novartis, Merck, Pfizer, Roche, Sanofi Aventis, Takeda, the Duke Clinical Research Institute, and the Population Health Research Institute of McMaster University, Canadian Institutes of Health Research, Harvard Clinical Research Institute, and Hamilton Clincal Research Institute; receives royalties from publishers of the 3 textbooks that he has coauthored and edited; has tax-sheltered retirement accounts in mutual funds with Fidelity and UBS; and has 2 small accounts of stock with Sun and Intel. Dr Serebruany reported that he receives investigator-initiated grant support from Bayer, as well as grant support and honoraria from Boehringer-Ingelheim and Bristol-Myers Squibb. Professor Schroeder reported that he receives investigator-initiated grant support from Bayer. The trial had an independent Data and Safety Monitoring Board (DSMB), which consisted of Peter Libby, MD, Marc A. Pfeffer, MD (Chair), and Bernard Rosner, PhD. The trial also had an independent Statistical Data Analysis Center at the Department of Biostatistics and Informatics at the University of Wisconsin with David DeMets, PhD, as Principal Investigator and Scott Hetzel, MS as Project Director.

Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

[3]HeartDrug Research, LLC, Towson, MD, USA

[4]Department of Biostatistics & Medical Informatics, University of Wisconsin, Madison, WI, USA

[5]Department of Pharmaceutics, College of Pharmacy, University of Minnesota, Minneapolis, MN, USA

Abstract

Background—We performed the first test in humans of whether aspirin at clinically relevant doses increases nitric oxide (NO) formation.

Methods—Seventy primary prevention patients with metabolic syndrome were randomly assigned to 81 mg, 162.5 mg, 325 mg, 650 mg, or 1300 mg aspirin daily for 12 weeks to test changes in heme oxygenase (HO-1), a downstream target of NO formation and asymmetrical dimethylarginine (ADMA), a competitive inhibitor of NO synthase.

Findings—For HO-1, the mean was 29.37 nanograms per milliliter at baseline and 57.45 at 12 weeks giving a mean ratio (MR) of 1.96 ($P < .001$) and 95% confidence interval (CI) from 1.91 to 2.00. There was no effect modification by dose or gender ($P = .341$). For ADMA, the mean was 1.70 micromoles per liter at baseline and 0.81 at 12 weeks, giving an MR of 0.48 ($P < .001$) and CI from 0.46 to 0.49. There was no effect modification by dose but a possible difference by gender ($P = .055$).

Interpretation—In high-risk primary prevention patients, aspirin significantly increases markers of NO formation. All doses produce similar increases in HO-1 and decreases in ADMA. The antiplatelet properties of aspirin to irreversibly inhibit platelet dependent cyclooxygenase are sufficient to explain benefits in patients with occlusive vascular diseases. Nonetheless, these data contribute to the formulation of the hypothesis that aspirin has additional beneficial effects mediated through NO formation. Further research, including direct randomized comparisons on atherosclerosis using noninvasive techniques as well as on occlusive vascular disease events, is necessary to test whether this hypothesis has clinical or public health relevance.

Keywords

atherosclerosis; cardiovascular disease; thrombosis; endothelium

Background

The most plausible biological mechanism of action of aspirin in the reduction of risks of occlusive vascular events in a very wide range of patients is derived from its ability in platelets to irreversibly acetylate the active site of cyclooxygenase required for the production of thromboxane A2, a powerful promoter of aggregation.[1] This antiplatelet property of aspirin to irreversibly inhibit platelet-dependent cyclooxygenase is sufficient to explain the statistically significant and clinically important beneficial effects on occlusive vascular diseases in secondary prevention[2] and on a first myocardial infarction in primary prevention.[3]

Nonetheless, basic research has suggested possible mechanisms by which aspirin may have additional beneficial effects mediated through nitric oxide (NO) formation.[4] Nitric oxide is

produced via 2-step oxidation of the amino acid L-arginine. Nitric oxide inhibits platelet aggregation, neutrophil adhesion to endothelial cells, and expression of inflammatory molecules. In high concentrations, NO inhibits the proliferation of smooth muscle cells.[5] More specifically, NO is released from vascular endothelium and plays a crucial role in the maintenance of vascular homeostasis. In basic research, endothelial NOS appeared to be a novel and functionally relevant target of aspirin, using the direct guanylyl cyclase activator 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) or rat fetal lung fibroblast cell line (RFL-6) reporter cells that are devoid of any NO synthase (NOS) activity.[6] In a cell culture model of oxidant injury, aspirin-dependent NO formation increased endothelial protection. In addition, in platelets, aspirin acetylates NO synthase.[7] Thus, in endothelial cells[8] and possibly platelets, aspirin is capable of activating the NO-cyclic guanosine monophosphate (cGMP) signalling pathway.[6,9] Finally, in the endothelium, NO and cGMP produce antioxidant protection and improved integrity. Heme oxygenase (HO-1) is a cytoprotective downstream target and is considered a surrogate for biologically active NO.[10] Asymmetric dimethyl arginine (ADMA) is a competitive inhibitor of NOS.[11,12] We conducted the first randomized trial in humans to test whether 5 clinically relevant doses of aspirin increase NO as measured by activation of HO-1. We also determined whether these 5 clinically relevant doses of aspirin decreased blood levels of ADMA.

Methods

In a randomized, double-blind trial, 70 primary prevention patients (37 men) aged 40 to 80 years inclusive, with metabolic syndrome not taking aspirin, were assigned to doses of 81 mg, 162.5 mg, 325 mg, 650 mg, or 1300 mg for 12 weeks (14 per arm). Metabolic syndrome was defined according to the National Cholesterol Education Program-III (NCEP-III), which is having at least 3 of the following 5 characteristics: waist measuring more than 40 inches in men or more than 35 inches in women, high-density lipoprotein (HDL) cholesterol levels lower than 40 milligrams per deciliter (mg/dL) in men or 50 mg/dL in women, triglyceride (TG) levels above 150 mg/dL, blood pressure greater than 130 millimeters of mercury (mm Hg) systolic or 85 mm Hg diastolic, and fasting blood sugar greater than 110 mg/dL.

At baseline and 12 weeks, antecubital venous blood samples were obtained for HO-1 and ADMA. The HO-1 assays were performed using enzyme-linked immunosorbent assay (ELISA; Millipore Corporation, Billerica, Massachusetts; R & D Systems, Minneapolis, Minnesota; Sigma-Aldrich Corp, St Louis, Missouri). Each sample was measured in triplicate, and the overall intra-assay coefficient of variation was 2.1% ± 0.3%, with a plasma recovery rate between 85% and 99%. The ADMA assays were performed by ELISA (ALPCO Diagnostics, Salem, New Hampshire). Each sample was measured in triplicate, and the overall intra-assay coefficient of variation was 7.2% ± 1.2%, with a plasma recovery rate between 85% and 99%.

Analysis of variance (ANOVA) was used to test for the significance of differences between doses at baseline and week 12, as well as for the paired differences between the 2 time points for HO-1 and ADMA. Because there were no significant differences by dose, we pooled all dose levels and used a paired Student *t* test to determine whether there were significant differences between baseline and 12 weeks for HO-1 and ADMA. We also used

paired Student *t* tests to determine whether there were significant modifications of the effects of aspirin by age (above or below 58 years), gender (men or women), and race (Caucasian, African American, or other). For HO-1 and ADMA, we calculated the ratios of the means, or mean ratios (MR), for week 12 to baseline. For each MR, we calculated 95% confidence intervals (CI) by computer simulation derived from the estimated distributions of each outcome. All significance tests were conducted using a 2-sided α level of .05.

Role of the Funding Source

This trial was funded as an investigator-initiated grant to Florida Atlantic University (FAU), with Charles H. Hennekens and Sir Richard Doll, Research Professor, as Principal Investigators by Bayer. The funding source, Bayer, had no role in the design, conduct, analysis, interpretation, preparation of the manuscript, or the decision to submit the manuscript for publication.

Findings

Despite the relatively small sample size, randomization achieved a fairly balanced distribution of baseline characteristics by treatment group. Among the notable baseline characteristics were mean age of about 57.7 and mean body mass index (BMI) of about 35.5 (Table 1).

Due to missing data, 60 and 59 participants had both baseline and week 12 measures for HO-1 and ADMA, respectively. There were no significant differences between any of the 5 clinically relevant doses of aspirin tested for HO-1 or ADMA (all *P* values > .20; Table 2).

For HO-1, there was a significant increase (28.08 ± 5.92, $P < .001$) from baseline (29.37 ± 2.13) to week 12 (57.45 ± 5.57). The MR of week 12 to baseline for HO-1 was significantly higher than unity (1.96, CI from 1.91 to 2.01, $P < .001$; Table 3). There were no significant modifications of the effects of aspirin on HO-1 by age, gender, or race.

For ADMA, there was a significant decrease (−0.89 ± 0.23, $P < .001$) from baseline (1.70 ± 0.22) to week 12 (0.81 ± 0.1), and the MR of week 12 to baseline for ADMA was significantly lower than unity (0.48, CI from 0.46 to 0.49, $P < .001$; Table 3). There were no significant modifications of the effects of aspirin on ADMA by age or race but a possible greater decrease in ADMA over time for men (−0.94 ± 0.23) compared to women (−0.83 ± 0.22; $P = .055$).

Conclusions

In high-risk primary prevention patients with metabolic syndrome, aspirin, at all 5 clinically relevant doses tested from 81 mg to 1300 mg daily, increases NO formation. The trial was designed to test whether aspirin increased NO formation and, if so, whether there was effect modification by dose. Aspirin produces significant increase in HO-1, a downstream target of NO formation, and significant decrease in ADMA, a competitive inhibitor of NO synthase. These data represent the first randomized evidence in humans and demonstrated beneficial effects across a wide range of usual doses of aspirin used in clinical practice.

Oxidative stress, a pervasive condition of increased amounts of reactive oxygen species, causes vascular endothelial dysfunction and plays an important role in the pathophysiology of occlusive vascular diseases. Basic research suggests that aspirin may provide direct protective effects on the vascular wall, potentially due to free radical scavenging properties and protection of endothelial cells from the deleterious effects of hydrogen peroxide. Antioxidant effects of aspirin leading to the suppression of lipid peroxidation and reduced vascular tone have also been demonstrated in vivo in experimental animals and humans. Although the mechanisms responsible for the observed aspirin-induced cytoprotection are largely unknown, there is a very similar profile of cytoprotection evoked by NO donors.

Downstream targets of NO that mediate tissue protection include the stress protein HO-1. HO-1 is both a target of and inducible by aspirin.[4,7,8] Increased HO-1 expression in the presence of aspirin has been shown to lead to vasoprotection via formation of anti-inflammatory and antioxidant products such as bilirubin and carbon monoxide. Novel lipid mediator aspirin-triggered lipoxin A4 induces HO-1 in endothelial cells. Moreover, aspirin-induced HO-1 induction was abrogated in the presence of the NOS blocker L-nitroarginine-L-monoethyl ester. These observations suggest that NO may be a signalling molecule in genomic actions of aspirin that may lead to stress gene activation and tissue protection.

These data are subject to several possible limitations. The sample size was small but, nonetheless, the design was randomized and the effect sizes for both HO-1 and ADMA were moderately large, resulting in statistically significant beneficial effects of aspirin. The study population of high-risk primary prevention patients had a mean age of about 57.7 and mean BMI of about 35.5. The hypothesis that aspirin increases NO in patients with established vascular disease has not yet been tested. In addition, the utilization rates of statins, other lipid lowering agents, ACE inhibitors, diuretics, β-blockers and calcium channel blockers were all relatively low. It is intriguing to speculate whether similar beneficial effects of aspirin on HO-1 and ADMA would be present in patients with higher rates of utilization of these drugs.

Despite these and other possible limitations, we believe that these data contribute to the formulation of the hypothesis that aspirin has additional beneficial effects mediated through NO formation in addition to its well-documented and well-accepted antiplatelet properties. Further research, including direct randomized comparisons on atherosclerosis, which may be performed using various noninvasive techniques as well as on clinical events due to occlusive vascular disease, is necessary to test whether this hypothesis has clinical or public health relevance.

Acknowledgments

Funding

The author(s) received no financial support for the research and/or authorship of this article.

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Table 1

Baseline Characteristics of Participants by Randomized Treatment Group

	Randomized Treatment Group				
Baseline Characteristics	**81 mg Aspirin**	**162 mg Aspirin**	**325 mg Aspirin**	**650 mg Aspirin**	**1300 mg Aspirin**
Mean age (years)	58.3	57.0	55.5	58.0	59.1
Mean height (inches)	67.4	65.2	67.9	68.5	68.1
Mean weight (pounds)	233.3	213.5	238.8	224.3	237.9
BMI (kg/m^2)*	36.1	35.3	36.4	33.6	36.1
Caucasian (%)	50.0	35.7	35.7	28.6	21.4
Statin (%)	35.7	28.6	14.3	28.6	28.6
Other lipid lowering agent (%))	14.3	14.3	7.1	0	28.6
Angiotensin converting Enzyme (ACE) inhibitor (%)	14.3	42.9	7.1	28.6	50.0
Diuretic (%)	14.3	21.4	21.4	21.4	21.4
β-blocker (%)	14.3	7.1	28.6	21.4	7.1
Calcium blocker (%)	7.1	21.4	14.3	14.3	21.4

BMI, weight in kilograms divided by height in meters squared.

Table 2

Lack of Statistically Significant Differences Between Each Clinically Relevant Dose of Aspirin on Markers of Nitric Oxide (NO) Formation

	Mean Ratios by Randomized Treatment Group[a]				
Marker of no Formation	**81 mg Aspirin**	**162 mg Aspirin**	**325 mg Aspirin**	**650 mg Aspirin**	**1300 mg Aspirin**
Heme oxygenase (HO-1)[b]	1.91	1.94	2.05	1.85	1.98
Asymmetrical dimethylarginine (ADMA)[c]	0.47	0.50	0.46	0.46	0.48

[a] All *P* values >.20.

[b] HO-1 was measured in nanograms per milliliter.

[c] ADMA was measured in micromoles per liter.

Table 3

Statistically Significant Differences Between Baseline and 12 Weeks for all 5 Clinically Relevant Doses of Aspirin on Markers of Nitric Oxide (NO) Formation

Marker of NO Formation	Baseline	12 Weeks	Mean Ratio (MR)	95% Confidence Interval (CI)	Significance Level (2-Sided *P* Value)
Heme oxygenase (HO-1)[a]	29.37	57.45	1.96	1.91–2.01	*P* < .001
Asymmetrical dimethylarginine (ADMA)[b]	1.70	0.81	0.48	0.46–0.49	*P* < .001

[a] HO-1 was measured in nanograms per milliliter.

[b] ADMA was measured in micromoles per liter.