

Effects of two different doses of acetylsalicylic acid on serum nitric oxide, asymmetric dimethylarginine, and homocysteine levels in healthy volunteers*

İdris MEHMETOĞLU, Sevil KURBAN

Aim: To examine the effects of ASA on serum nitric oxide (NO), asymmetric dimethylarginine (ADMA), and homocysteine levels in healthy volunteers.

Materials and methods: Totally, 26 apparently healthy volunteers were enrolled in the study. Of the participants, 13 (5F, 8M) received 100 mg of ASA daily and 13 (5F, 8M) received 150 mg of ASA daily for 2 months. Serum NO, ADMA, and homocysteine levels were measured before and 1 and 2 months after ASA treatment. Serum NO, ADMA, and homocysteine levels were measured before and 1 and 2 months after ASA treatment.

Results: ADMA levels of the group receiving 150 mg of ASA were significantly reduced after 2 months of treatment ($P < 0.05$). NO levels of both groups were slightly but not significantly increased and homocysteine levels of both groups were slightly reduced after ASA treatment compared to the baseline values.

Conclusion: Our findings indicate that ASA treatment reduces ADMA levels dose and time dependently, a beneficial effect that may contribute to the prevention of cardiovascular diseases.

Key words: Acetylsalicylic acid, nitric oxide, asymmetric dimethylarginine, homocysteine, healthy volunteers

Farklı iki doz asetilsalisilik asidin sağlıklı gönüllülerde serum nitrik oksit, asimetrik dimetilarjinin ve homosistein seviyelerine etkileri

Amaç: Bu çalışmanın amacı sağlıklı gönüllülerde ASA'nın serum nitrik oksit (NO), asimetrik dimetilarjinin (ADMA) ve homosistein seviyelerine etkisini belirlemektir.

Yöntem ve gereç: Çalışmada toplam 26 sağlıklı gönüllü yer aldı. İki ay süresince katılanlardan 13'ü (5K, 8E) günde 100 mg ve 13'ü (5K, 8E) günde 150 mg ASA aldı. ASA tedavisinden önce ve bir ve iki ay sonra serum NO, ADMA ve homosistein seviyeleri ASA tedavisinden önce ve bir ve iki ay sonra serum NO, ADMA ve homosistein seviyeleri ölçüldü.

Bulgular: 150 mg ASA kullanan grubun ADMA seviyeleri iki aylık tedavinin sonunda anlamlı olarak azalmıştı ($P < 0,05$). ASA tedavisinden sonra başlangıç değerleri ile karşılaştırıldığında her iki grubun NO seviyeleri hafif ama önemsiz artmış homosistein seviyeleri ise hafif azalmıştı.

Sonuç: Bulgularımız ASA tedavisinin ADMA seviyelerini doz ve zamana bağlı olarak azalttığını gösterdi. Bu faydalı etki kardiyovasküler hastalıkların önlenmesine katkıda bulunuyor olabilir.

Anahtar sözcükler: Asetilsalisilik asit, nitrik oksit, asimetrik dimetilarjinin, homosistein, sağlıklı gönüllüler

Received: 07.12.2010 – Accepted: 13.05.2011

Department of Biochemistry, Meram Faculty of Medicine, Selçuk University, Konya - TURKEY

Correspondence: Sevil KURBAN, Department of Biochemistry, Meram Faculty of Medicine, Selçuk University, Konya - TURKEY

E-mail: svlkrbn@yahoo.com

* This research was supported by the University of Selçuk Scientific Research Projects Department (BAP), Konya, Turkey. It was presented as a poster at the First European Joint Congress of EFCC and UEMS, 13-16 October, 2010, Lisbon, Portugal.

Introduction

Acetylsalicylic acid (ASA, aspirin) is a drug with anti-inflammatory, antithrombotic, and analgesic therapeutic properties (1). The beneficial effects of ASA are due mainly to its antiplatelet action, which prevents the formation of arterial platelet thrombi. However, other platelet inhibitory agents have not been found to be effective or as effective as ASA. Therefore, the discrepancy between the efficacy of these compounds and ASA suggests that the therapeutic efficacy of ASA may not be limited only to its platelet inhibitory effect (2).

Nitric oxide (NO), a critically important signaling molecule in the cardiovascular system (CVS) (3), is endowed with important antiatherosclerotic properties (4). NO is synthesized from L-arginine by NO synthase (NOS) in endothelial cells (3) and its effects are mediated by the second messenger cyclic guanosine monophosphate (cGMP) (3). An imbalance in the production or bioavailability of NO, or its downstream signaling molecule, cGMP, in the vasculature is implicated in the pathogenesis of endothelial and vascular smooth muscle dysfunction and is the background for hypertension, atherosclerosis, and other cardiovascular disorders (3). In fact, reduced NO bioavailability may be considered an important risk factor for atherothrombosis and acute cardiovascular events (3,4). Accumulating evidence suggests that ASA may have additional biological properties on the vasculature that contribute to increased NO formation and protect the endothelium from deleterious effects of oxidative stress (5,6) and the NO/cGMP system assumes a crucial function in mediating the cytoprotective action of ASA (7).

Asymmetric dimethylarginine (ADMA), a methyl derivate of the amino acid arginine, can inhibit NOS in vivo and in vitro (4). ADMA is actively metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (4). The activity of DDAH seems to be particularly susceptible to inhibition by oxidative stress (4,5). Increased plasma ADMA levels reduce NO synthesis, leading to endothelial dysfunction and increased cardiovascular risk and cognitive dysfunction (4,8-10). Elevated plasma ADMA levels have been reported in

connection with atherosclerotic diseases such as hypertension (8) and diabetes mellitus (DM) (11).

Another molecule associated with vascular disease and atherothrombosis is homocysteine. Homocysteine increases the damage to the CVS in different ways; most known forms of damage or injury are due to homocysteine mediated oxidative stress. Chief among these are changes in the intracellular redox potential, interference with the NO system, and activation of transcription factors with stimulation of gene expression (12,13).

Homocysteine has been shown to inhibit endothelial DDAH enzyme activity, causing the accumulation of ADMA and the inhibition of NO synthesis (9,10).

Both homocysteine (12,13) and ADMA (14) represent novel mediators of oxidative stress. If ADMA and homocysteine also could play a role in the pathogenesis of atherosclerosis and cardiovascular disease (CVD) it must be important to reduce concentrations of these molecules in preventing atherosclerosis.

ASA is one of the most potent antioxidant compounds known (15). However, in addition to its antithrombotic and anti-inflammatory effects (1) many details of the ASA action mechanism are not completely understood. Accordingly, the present study was performed to examine whether ASA in different doses (100 and 150 mg daily) affects serum ADMA, NO, and homocysteine levels in healthy volunteers.

Materials and methods

Patients

Totally, 26 apparently healthy volunteers were enrolled in the study. Of the participants, 13 (5F, 8M) received 100 mg of ASA daily (group I) and 13 (5F, 8M) received 150 mg of ASA daily (group II) for 2 months. Since it has been reported that a daily dosage of 75 to 150 mg is sufficient to reduce the rate of future cardiac events (16) and 75 to 325 mg is sufficient in acute treatment and secondary prevention of coronary diseases and CVD (6), and major bleeding has been demonstrated to depend on the dose of ASA (1), we used 100 and 150 mg of ASA in our study. The

ASA used (ecopirin®; Abdi İbrahim İlaç San, İstanbul, Turkey) was enteric coated. The age of the volunteers ranged from 40 to 53 (45.57 ± 3.98) years for group I and from 40 to 53 (48.54 ± 4.46) years for group II and the subjects were not on any medication for known risk factors for chronic diseases such as CVD, DM, or hypertension. The study protocol was approved by the Ethics Committee of Meram Medical School, University of Selçuk, Konya, Turkey. All volunteers were informed of the details of the study and written consent was received from each patient.

Measurement of biochemical parameters

Fasting blood samples of the subjects were drawn for biochemical analysis before and 1 and 2 months after ASA treatment. The blood samples were kept at room temperature for 30 min; then sera were separated from the cells by centrifugation at $4\text{ }^{\circ}\text{C}$ at $1469 \times g$ for 10 min. Serum samples were stored at $-80\text{ }^{\circ}\text{C}$ until the day of biochemical analysis. Then serum NO, ADMA, and homocysteine levels were measured.

Measurement of NO levels

Serum NO (nitrate plus nitrite) levels were measured by the Griess method with a commercially available kit (Nitrate/Nitrite colorimetric assay kit, Cayman Chemical Co., cat no: 780001). Briefly, after being passed through ultrafilters (Millipore, cat no: 42421), 40 mL of the serum was diluted with 240 mL of assay buffer and mixed with 10 mL of cofactor and 10 mL of nitrate reductase. After the plasma had been kept at room temperature for 3 h to convert nitrate to nitrite, total nitrite was measured as serum NO at 540 nm absorbance by reaction with Griess reagent (sulfanilamide and naphthalene-ethylene diamine dihydrochloride).

Measurement of ADMA levels

Measurement of ADMA was accomplished by high performance liquid chromatography (HPLC), using the method described by Chen et al. (17). In brief, to 1 mL serum was added 20 mg of 5-sulfosalicylic acid (5-SSA), and the mixture was left in an ice bath for 10 min. The precipitated protein was removed by centrifugation at $2000 \times g$ for 10 min. Ten microliters of the supernatant, which was filtered through a $0.2\text{ }\mu\text{m}$ filter, was mixed with 100 μL of derivatization reagent [prepared by dissolving 10 mg of o-phthalaldehyde in 0.5 mL of methanol, 2 mL of 0.4 M borate buffer

(pH 10.0), and 30 μL of 2-mercaptoethanol] and then injected into the chromatographic system. Separation of ADMA was achieved with a $150 \times 4\text{-mm}$ I.D. Novapak C18 column with a particle size of 5 μm (Waters Inc., Milford, MA, USA) using 50 mM sodium acetate (pH 6.8), methanol, and tetrahydrofuran as mobile phase (A, 82:17:1; B, 22:77:1) at a flow rate of 1.0 mL/min. The area of peak detected by fluorescent detector (excitation: 338 nm; emission: 425 nm) was used for quantification of ADMA levels in serum.

Measurement of homocysteine levels

Serum homocysteine levels were measured using a commercially available kit produced by Chromsystems for HPLC (Agilent 1100 series fluorescence detector).

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Version 16.0). Data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis of data within and between groups was performed with repeated measure analysis of variance. In the presence of significance within the groups individual paired comparisons were made using the Wilcoxon signed ranks test or paired t tests with Bonferroni multiple comparison adjustment. Differences were considered significant at a probability level of $P < 0.05$.

Results

Mean \pm SEM values of NO, ADMA, and homocysteine levels before and after treatment are given in the Table. NO of both groups were slightly but not significantly increased and homocysteine levels of both groups were slightly reduced 1 and 2 months after ASA treatment compared to the baseline values.

Moreover, ADMA levels of the group receiving 100 mg of ASA (group I) were slightly but not significantly reduced 1 and 2 months after treatment. However, ADMA levels of the group receiving 150 mg of ASA (group II) were significantly reduced after 2 months of the treatment ($P < 0.05$).

On the other hand, when the groups were compared with each other, there were no significant differences between their results measured before and after 1 or 2 months of ASA treatment.

Table. The measured parameters of the groups (mean \pm SEM).

Parameters	Groups	Before ASA treatment	One month after ASA treatment	Two months after ASA treatment
ADMA ($\mu\text{mol/L}$)	Group I	0.63 \pm 0.05	0.60 \pm 0.05	0.61 \pm 0.04
	Group II	0.61 \pm 0.06	0.59 \pm 0.06	0.50 \pm 0.05*
NO ($\mu\text{mol/L}$)	Group I	23.24 \pm 4.16	24.95 \pm 3.67	28.22 \pm 6.34
	Group II	21.99 \pm 3.10	22.90 \pm 2.95	29.63 \pm 5.67
Homocysteine ($\mu\text{mol/L}$)	Group I	20.24 \pm 6.24	20.01 \pm 5.86	17.62 \pm 4.59
	Group II	22.37 \pm 6.98	20.47 \pm 7.88	16.48 \pm 5.18

*P < 0.05 compared to the value before treatment

Discussion

The present study demonstrates that ASA treatment significantly decreased serum concentrations of ADMA in a dose- and time-dependent manner. We hypothesized that this reduction may have been due to increased degradation of ADMA by DDAH, because it has been shown that pharmacological prevention of the inhibition of DDAH activity can decrease the accumulation of ADMA (5,9,10).

Increased plasma ADMA levels mainly occur following inhibition of the enzyme responsible for ADMA catabolism, DDAH, by oxidative stress (4,5,18). Therefore, to reduce oxidative stress is expected to enhance DDAH activity and decrease plasma ADMA levels (9,10,19).

Probuco, a compound endowed with both antioxidant properties and lipid lowering effects, was found to significantly reduce plasma ADMA concentrations, increase NO levels, and improve endothelial function in rats with endothelial dysfunction induced by intravenous injection of LDL (20).

Since ASA has antioxidative properties, it can be argued that it preserves DDAH activity, which results in reduced ADMA level. Indeed, in a previous study, we found that LDL oxidation was significantly inhibited and total oxidant status (TOS) was decreased by ASA treatment, an effect that was time and dose dependent (21). Deng et al. (22) have reported that increased ADMA levels in rats treated

with LDL were decreased and DDAH activity was increased by ASA. Moreover, aging (5) and high glucose (23) induced ADMA accumulation, and increased reactive oxygen species (ROS) levels in human umbilical vein endothelial cells (HUVECs) were found to be reduced by ASA dose dependently. In another study, Hennekens et al. (24) found that, in patients with metabolic syndrome, ASA in doses from 81 mg to 1300 mg daily for 12 weeks produced significant decreases in the NOS inhibitor ADMA. These findings confirm our finding of reduced ADMA levels in the 150 mg ASA treated group, after 2 months.

Several mechanisms have been proposed to underlie the potential role of ASA as an antioxidant. For example, ASA at low concentrations protects endothelial cells from the deleterious effects of iron dependent oxygen radical formation (25). Furthermore, chronic treatment with ASA markedly reduced vascular production of superoxide anion (15). ASA was also reported to show free radical scavenging properties and protect endothelial cells from the deleterious effects of hydrogen peroxide and iron induced toxicity (26).

Taken together, all the above reports show that ASA has antioxidative properties that could reduce oxidative stress and enhance DDAH activity, which results in reduced ADMA levels as found in our study. On the other hand, ASA itself may activate DDAH activity, the mechanism of which is not known and needs to be investigated.

There is a regulatory feedback mechanism between DDAH, ADMA, NOS, and NO (4). Indeed, ADMA is the major endogenous inhibitor of NOS and increased plasma ADMA levels cause impaired NO synthesis (4,9,10). In our study, serum NO levels of the group receiving ASA were slightly but not significantly increased, whereas homocysteine levels were decreased. There are some conflicting reports about the effects of ASA on these parameters. For example, Bode Böger et al. (5) have reported that ADMA and ROS levels were significantly increased and NO levels decreased in HUVECs by aging. Yi et al. (23) have shown that ASA can protect endothelial cells from high glucose induced oxidative stress and senescence by decreasing ADMA accumulation and upregulating NO production via enhancing NOS activity. Taubert et al. (6) have demonstrated that therapeutically relevant concentrations of ASA elicit NO release from vascular endothelium.

Although the mechanisms of ASA induced endothelial protection are largely unknown, ASA has been reported to improve the endothelial functions by increasing NO level through the cyclooxygenase independent mechanism (6,7), because ASA was found to activate the NO-cGMP signaling pathway in endothelial cells. Thus, it has been suggested that NO might have a role as a downstream mediator in ASA dependent endothelial protection (7).

Zhao et al. (27) have shown that 75 mg daily ASA did not change plasma NO levels in healthy subjects in 2 weeks. This finding supports our finding of slightly but not significantly increased NO levels in our subjects. However, Girish et al. (28) treated 15

breast cancer patients and 15 age-matched controls with 150 mg daily of ASA and reported that there was an increase in serum NO levels in the patients and the normal volunteers. Moreover, Hennekens et al. (24) have shown that ASA in doses from 81 mg to 1300 mg daily for 12 weeks increased NO formation in patients with metabolic syndrome. These findings do not agree with ours. However, the subjects that participated in the above studies and the time period of the ASA treatment they used were different from those in our study. Therefore, we think that possibly higher doses of ASA for longer time periods are needed to obtain a significant increase in NO levels in healthy subjects.

Treatment of mitogen activated human peripheral blood mononuclear cells (PBMC) with ASA and salicylic acid was found to inhibit homocysteine formation dose dependently (29). Although pretreatment of PBMC with ASA concentrations of ≤ 1 mmol did not alter homocysteine concentrations, ASA concentrations of 3 and 5 mmol significantly reduced homocysteine concentrations (29). Moreover, people at high risk of dementia receiving low dose ASA (81 mg) for 12 weeks had decreased homocysteine concentrations but the changes were not significant (30).

In conclusion, our findings indicate that 150 mg/day ASA treatment significantly reduces ADMA levels related to antioxidant protection of ASA. Therefore, this effect of ASA may contribute to the prevention of CVD, a beneficial effect that is dose and time dependent.

References

- Lièvre M, Cucherat M. Aspirin in the secondary prevention of cardiovascular disease: an update of the APTC meta-analysis. *Fundam Clin Pharmacol* 2010; 24: 385-91.
- Mehta JL. Salutary effects of aspirin in coronary artery disease are not limited to its platelet inhibitory effects. *Clin Cardiol* 1998; 21: 879-84.
- Pepine CJ. The impact of nitric oxide in cardiovascular medicine: untapped potential utility. *Am J Med* 2009; 122: 10-15.
- De Gennaro Colonna V, Bianchi M, Pascale V, Ferrario P, Morelli F, Pascale W et al. Asymmetric dimethylarginine (ADMA): an endogenous inhibitor of nitric oxide synthase and a novel cardiovascular risk molecule. *Med Sci Monit* 2009; 15: RA91-101.
- Bode-Böger SM, Martens-Lobenhoffer J, Täger M, Schröder H, Scaleria F. Aspirin reduces endothelial cell senescence. *Biochem Biophys Res Commun* 2005; 334: 1226-32.
- Taubert D, Berkels R, Grosser N, Schröder H, Gründemann D, Schömig E. Aspirin induces nitric oxide release from vascular endothelium: a novel mechanism of action. *Br J Pharmacol* 2004; 143: 159-65.
- Grosser N, Schröder H. Aspirin protects endothelial cells from oxidant damage via the nitric oxide-cGMP pathway. *Arterioscler Thromb Vasc Biol* 2003; 23: 1345-51.
- Surdacki A, Nowicki M, Sandmann J, Tsikas D, Boeger RH, Bode-Boeger SM et al. Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension. *J Cardiovasc Pharmacol* 1999; 33: 652-8.

9. Stühlinger MC, Tsao PS, Her JH, Kimoto M, Balint RF, Cooke JP. Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. *Circulation* 2001; 104: 2569-75.
10. Selley ML. Homocysteine increases the production of asymmetric dimethylarginine in cultured neurons. *J Neurosci Res* 2004; 77: 90-3.
11. Abbasi F, Asagmi T, Cooke JP, Lamendola C, McLaughlin T, Reaven GM et al. Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus. *Am J Cardiol* 2001; 88: 1201-3.
12. Chamberlain KL. Homocysteine and cardiovascular disease: a review of current recommendations for screening and treatment. *J Am Acad Nurse Pract* 2005; 17: 90-5.
13. Stanger O, Herrmann W, Pietrzik K, Fowler B, Geisel J, Dierkes J et al. DACH-LIGA homocystein (German, Austrian and Swiss Homocysteine Society): consensus paper on the rational clinical use of homocysteine, folic acid and B-vitamins in cardiovascular and thrombotic diseases: guidelines and recommendations. *Clin Chem Lab Med* 2003; 41:1392-403.
14. Sydow K, Münzel T. ADMA and oxidative stress. *Atheroscler Suppl* 2003; 4: 41-51.
15. Wu R, Lamontagne D, de Champlain J. Antioxidative properties of acetylsalicylic Acid on vascular tissues from normotensive and spontaneously hypertensive rats. *Circulation* 2002; 105: 387-92.
16. Hofmann T. Risk management of coronary artery disease--pharmacological therapy. *Wien Med Wochenschr* 2004; 154: 266-81.
17. Chen BM, Xia LW, Zhao RQ. Determination of N(G),N(G)-dimethylarginine in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 1997; 692: 467-71.
18. Ito A, Tsao PS, Adimoolam S, Kimoto M, Ogawa T, Cooke JP. Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase. *Circulation* 1999; 99: 3092-5.
19. Saran R, Novak JE, Desai A, Abdulhayoglu E, Warren JS, Bustami R et al. Impact of vitamin E on plasma asymmetric dimethylarginine (ADMA) in chronic kidney disease (CKD): a pilot study. *Nephrol Dial Transplant* 2003; 18: 2415-20.
20. Jiang JL, Li NS, Li YJ, Deng HW. Probuocol preserves endothelial function by reduction of the endogenous nitric oxide synthase inhibitor level. *Br J Pharmacol* 2002; 135: 1175-82.
21. Kurban S, Mehmetoglu I. Effects of acetylsalicylic acid on serum paraoxonase activity, Ox-LDL, coenzyme Q10 and other oxidative stress markers in healthy volunteers. *Clin Biochem* 2010; 43: 287-90.
22. Deng S, Deng PY, Jiang JL, Ye F, Yu J, Yang TL et al. Aspirin protected against endothelial damage induced by LDL: role of endogenous NO synthase inhibitors in rats. *Acta Pharmacol Sin* 2004; 25: 1633-9.
23. Yi TN, Zhao HY, Zhang JS, Shan HY, Meng X, Zhang J. Effect of aspirin on high glucose-induced senescence of endothelial cells. *Chin Med J (Engl)* 2009; 122: 3055-61.
24. Hennekens CH, Schneider WR, Pokov A, Hetzel S, DeMets D, Schröder H, et al. Usual doses of aspirin markedly increase nitric oxide formation in humans. *Circulation* 2009; 120: 405.
25. Oberle S, Polte T, Abate A, Podhaisky HP, Schröder H. Aspirin increases ferritin synthesis in endothelial cells: a novel antioxidant pathway. *Circ Res* 1998; 82: 1016-20.
26. Podhaisky HP, Abate A, Polte T, Oberle S, Schröder H. Aspirin protects endothelial cells from oxidative stress—possible synergism with vitamin E. *FEBS Lett* 1997; 417: 349-51.
27. Zhao L, Gray L, Leonardi-Bee J, Weaver CS, Heptinstall S, Bath PM. Effect of aspirin, clopidogrel and dipyridamole on soluble markers of vascular function in normal volunteers and patients with prior ischaemic stroke. *Platelets* 2006; 17: 100-4.
28. Girish GV, Sinha N, Chakraborty K, Bhattacharya G, Kahn NN, Sinha AK. Restoration by aspirin of impaired plasma maspin level in human breast cancer. *Acta Oncol* 2006; 45: 184-7.
29. Schroecksnadel K, Frick B, Winkler C, Wirleitner B, Schennach H, Fuchs D. Aspirin downregulates homocysteine formation in stimulated human peripheral blood mononuclear cells. *Scand J Immunol* 2005; 62: 155-60.
30. Clarke R, Harrison G, Richards S. Vital Trial Collaborative Group. Effect of vitamins and aspirin on markers of platelet activation, oxidative stress and homocysteine in people at high risk of dementia. *J Intern Med* 2003; 254: 67-75.