Antioxidative effect of aspirin on vascular function of aged ovariectomized rats

Buket Demirci · Omer Demir · Turhan Dost ·
Mustafa Birincioglu

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Abstract This study investigated the vascular effects of nonsteroidal anti-inflammatory drugs (NSAIDs) in the very late stage of postmenopausal vascular aging and looked for a better choice of anti-inflammatory drug for women in reducing the cardiovascular risk by decreasing the oxidant status in this term. The rat aorta isolated from young and old rats that were treated with either aspirin (10 mg/kg/day) or indomethacin (INDO, 1 mg/kg/day) within last 10 weeks after 16-month ovariectomy (OVX) follow-up. Endothelium-dependant acetylcholine (Ach, 0.001–30 μ M) and independent sodium nitroprusside (SNP, 0.0001–3 μ M) relaxant; α -receptor phenylephrine (PE, 0.001–30 μ M) and voltage-dependant high potassium (KCl; 40 mM) contractile responses were assessed. Total oxidant and antioxidant status were measured from the serum samples. Aged OVX rat's both aortic endothelium and smooth muscle relaxation were significantly less than of younger ones, whereas their contractile functions tended to decrease. INDO did not treat the Ach, SNP responses, whereas it increased the PE and KCl contractility. Aspirin improved the relaxation function and antioxidant capacity and decreased the oxidant status. These data demonstrate that even if they are in the

very late stage of life and menopause, the analgesic choices could restore the well established endothelial dysfunction, vascular stiffness, and oxidant status.

Keywords Aging · Anti-inflammatory · Aspirin · Endothelium · Menopause · Oophorectomy · Vascular reactivity

Introduction

Age-related vascular pathologies such as endothelial dysfunction, vascular inflammation, calcification, and stiffness are the most important reasons for increased cardiovascular morbidity and mortality, and one of the leading causes of their underlying mechanism would be oxidative stress (Cau et al. 2012; Ungvari et al. 2010; Demirci et al. 2005). Vascular inflammation can be the source of enhanced reactive oxygen species (ROS) and this oxidative stress may result in development and progression of such cardiovascular diseases as atherosclerosis and hypertension (Wu et al. 2002). Increased ROS in aging inactivates nitric oxide (NO) which is released by endothelium for maintenance of vascular homeostasis (Cau et al. 2012; Taubert et al. 2004; Ungvari et al. 2010). Additionally, in this term, cardiovascular risk increases due to the lost benefit of estrogens (Miller et al. 2007; Stice et al. 2009). Estrogen deficiency further results in endothelial dysfunction and ROS production (Moncada and Higgs 2006).

Co-existing advanced age and menopause bring about some clinical problems in women as well. Age-

B. Demirci (✉) · T. Dost · M. Birincioglu
Department of Medical Pharmacology,
Adnan Menderes University,
Aydin 09100, Turkey
e-mail: drbuketdemirci@gmail.com

O. Demir
Department of Medical Pharmacology, Sifa University,
Izmir, Turkey

related diseases such as arthritis and osteoporosis cause women to take analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs) more often (Lapane et al. 2001). Whereas NSAIDs, namely ibuprofen and indomethacin (INDO) are the common cause of worsening in blood pressure (Sarafidis and Bakris 2008); especially INDO is used for the contractile treatment of patent ductus arteriosus (Lee et al. 2012), another NSAID low-dose aspirin (ASP) is used as a long-term major treatment for prevention of cardiovascular disease. Most of the effects of ASP are attributed to its antithrombotic activity which is exerted by inhibition of cyclooxygenase-1 (Taubert et al. 2004; Tauseef et al. 2008). The direct antilipolytic effect on adipocytes (Tauseef et al. 2008), the direct action of ASP on insulin secretion (Sethi et al. 2011; Tauseef et al. 2008), elimination of oxidative stress associated with inflammation (Wu et al. 2002), ameliorating effect of endothelium-dependant arterial relaxation by increasing vasodilator NO release (Taubert et al. 2004), prevention of atherosclerosis by inhibiting the vascular smooth muscle cell proliferation, and reducing the proinflammatory mediators (Tauseef et al. 2008) have been reported as well.

However, ASP neither affected the normotensive rats' endothelium-dependent relaxation nor it reversed established hypertension of spontaneously hypertensive rats (SHR; Wu et al. 2002). On the other hand, estrogens become more protective when getting closer to menopause, their replacement in the late-stage of life caused an increase in cardiovascular diseases (Miller et al. 2007; Rossouw et al. 2002; Stice et al. 2009). It has been widely accepted that timing of onset for estrogen replacement alters the progression of vascular inflammation and atherosclerosis formation (Novella et al. 2012). The aim of the study was to determine the safety and efficacy of NSAIDs on the endothelium and vascular functions, along with the (anti)oxidant status in highly advanced age and prolong menopausal term.

Materials and methods

Chemicals and equipments

Phenylephrine hydrochloride (PE), acetylcholine hydrochloride (Ach), sodium nitroprusside (SNP), and Krebs's chemical salts were purchased from Sigma Chemicals (Interlab, Izmir, Turkey). Aspirin and

indomethacin were obtained from the local pharmacy store. Total oxidant and antioxidant status (TOS/TAS) kits were supplied from Rel Assay Diagnostics (Gaziantep, Turkey; sensitivity, <10 pg/ml).

The measurement of isometric force was recorded by a force transducer (MAY FDT 05, Commat Ltd. Ankara, Turkey) and a data acquisition system (MP 150, Biopac Systems, Inc.). Multiwell plates for TOS/TAS were read at 450 nm wavelengths by a spectrophotometer (MultiskanGo, Thermo Fisher Scientific Inc, USA).

Animals

Wistar rats, 12–15 weeks old female, were obtained from Experimental Animal Center of Adnan Menderes University (ADU) and all experiments were performed according to the principles and guidelines of ADU Animal Ethical Committee's approval (HADYEK 2011/031). Anesthesia was induced by a single intraperitoneal injection of ketamine and xylazine (50 and 5 mg/kg, respectively). After shaving and cleaning, abdomen was dissected inferiorly to palpate the rib cage with 1.5 cm incision; ovaries and surrounding fatty tissues were extirpated (Kafkas et al. 2012). Incision was sutured and the animals were left to physiologic aging for an additional 16 months. Then OVX rats were randomized into one of the three subgroups, weighed, and they were followed for an additional 10 weeks as those untreated (OVX) and treated with aspirin (10 mg/kg/day; OVX+ASP) or indomethacin (1 mg/kg/day; OVX+INDO) with drinking water (Wu et al. 2002). On the other hand, to compare aging and menopausal progress, 14-week-old healthy rats (young) were used as young female controls.

Experimental design

Twenty-two-month-old rats were reweighed on the study day under anesthesia with ketamine and xylazine (50 and 5 mg/kg, respectively; OVX, 290.4 ± 10.0 ; OVX+ASP, 290.0 ± 2.2 ; OVX+INDO, 285.0 ± 6.3 ; whereas young, 235.9 ± 3.5). To decrease the number of animals used, two rings, approximately 3 mm, long were obtained from thoracic aorta. The rings were mounted in 20-ml organ baths containing Krebs Henseleit solution of the following composition (in millimolar): NaCl 118.1, KCl 4.56, CaCl_2 1.22, MgSO_4 1.22, KH_2PO_4 1.1, NaHCO_3 25, and D-glucose 10.1 at 37 °C and constantly oxygenated with carbogen and for 60–70 min under 2 g tension

(Demirci et al. 2008). Cumulative concentration response curves were obtained by adding increasing amount of PE (0.001–30 μM) or KCl (40 mM) concentrations to the bath. Relaxant responses were determined by using cumulative concentrations of Ach (0.001–30 μM) or SNP (0.0001–3 μM).

The blood samples obtained from heart were centrifuged (1,000 $\times g$ for 10 min) and their sera was separated and stored at -80°C for TOS/TAS measurements. TOS/TAS amounts were measured when the all frozen serum samples were gathered. Since the measurements of different oxidants/antioxidants molecules of serum concentrations separately are not practical, costly, and their each TOS or TAS effects are additive; total level of each status was assessed by using a commercially available colorimetric and automated method according to the manufacturer's instructions. Briefly, the TOS assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium and the measurement of the ferric ion by xylenol orange. Antioxidants in the sample reduce dark blue-green colored ABTS radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related with total antioxidant level of the sample (Erel 2004, 2005).

Data presentation and statistics

Concentration response curves were fitted by nonlinear regression with simplex algorithm and the rings, maximum responses (E_{max}) and vascular sensitivity (pD_2 ; $-\log\text{EC}_{50}$) values, were calculated. E_{max} values of PE and KCl were normalized to dry tissue weight of each ring. Relaxant responses were given as the percentages of PE precontraction. E_{max} , pD_2 , and TOS/TAS were assessed by using Mann–Whitney U test. Variance analysis was used to determine significance among the cumulative concentration curves. Data were presented as mean $\pm$ SEM; P values below 0.05 were considered significant.

Results

Contractile responses

Although it was not significant, in OVX rats' PE concentration response curve, there was a trend in deviating toward right and having a lower sensitivity and maximal tension. Whereas ASP did not influence these values, INDO has deviated the curve towards the left making it

significantly ($P<0.001$) different from OVX group as well as increasing the E_{max} and pD_2 values (both $P<0.001$, Fig. 1). KCl responses did not change between young and OVX groups. OVX rats' responses to KCl increased by INDO ($P<0.05$), but not ASP (Table 1).

Relaxant responses

Induction of OVX and aging have shifted the concentration response curve towards right ($P<0.001$, Fig. 2) resulted in decreased maximum relaxation and vascular sensitivity to Ach (both $P<0.001$, Table 1). ASP treatment has partially shifted the concentration response curve towards left, improved vascular sensitivity ($P<0.05$), and increased maximum relaxation ($P<0.001$) compared to OVX, whereas INDO had no effect on it (Table 1, Fig. 2).

Smooth muscle concentration relaxation curve to SNP was deteriorated by OVX and aging ($P<0.001$), its ring sensitivity decreased ($P<0.001$) without changing the E_{max} value compared to the young ones. ASP treatment has partially restored the curve to the left and increased the E_{max} value (both $P<0.05$; Table 1, Fig. 3).

TOS/TAS levels

While compared with the young female rats, aged OVX rats' TOS level tended to increase even in the presence of INDO treatment (6.11 ± 0.66 , 7.34 ± 0.93 , 7.77 ± 1.17 $\mu\text{mol H}_2\text{O}_2$ equivalent/L, respectively).

Fig. 1 Concentration–response curves for phenylephrine in intact endothelium aortic rings of control rats (young) and of ovariectomized rats (OVX). Some animals were treated with either aspirin (OVX+ASP, 10 mg/kg/day) or indomethacin (OVX+INDO, 1 mg/kg/day) for 10 weeks. Values are mean $\pm$ SEM of 12 or 18 rings. * $P<0.001$ versus OVX group

Table 1 Contractile responses to phenylephrine and KCl (in milligram per dry tissue weight) and relaxant responses to acetylcholine and sodium nitroprusside (percent phenylephrine precontraction) in intact endothelium aortic rings of control rats

	<i>n</i>	Phenylephrine		KCl	Acetylcholine		Sodium nitroprusside	
		$E_{\max}$	pD ₂		$E_{\max}$	pD ₂	$E_{\max}$	pD ₂
Young	12	666±97	6.56±0.07	321±40	89.4±2.53 [#]	7.60±0.11 [#]	96.73±2.54	7.79±0.04 [#]
OVX	16	499±53	6.52±0.06	369±41	56.4±2.84	7.36±0.11	94.2±1.22	7.03±0.10
OVX+ASP	18	595±49	6.78±0.05	480±39	77.1±1.19 [#]	6.99±0.12*	97.8±0.66*	7.23±0.08*
OVX+INDO	16	797±53 [#]	6.88±0.06 [#]	551±69*	58.7±2.61	7.15±0.08	96.2±1.25	7.08±0.07

Values are mean±SEM of 12 or 18 rings

* $P<0.05$, [#] $P<0.001$ versus OVX group

ASP treatment has lowered the TOS increment to $5.12 \pm 0.53 \mu\text{mol H}_2\text{O}_2$ equivalent/L and it was more significant than in OVX and INDO groups (both $P<0.05$).

Old OVX rats showed low antioxidant capacity than the young ones (1.09 ± 0.03 and 1.21 ± 0.04 mmol trolox equivalent/L, respectively; $P<0.05$). INDO did not increase TAS level significantly (1.18 ± 0.05) whereas ASP significantly improved the antioxidant capacity to 1.22 ± 0.03 level ($P<0.05$, compared to OVX).

Discussion

In this study, the possible benefits of the therapeutic strategies of NSAIDs that have the potential to promote

(young) and of ovariectomized rats (OVX). Some animals were treated with either aspirin (OVX+ASP 10 mg/kg/day) or indomethacin (OVX+INDO 1 mg/kg/day) for 10 weeks. $E_{\max}$ denotes maximum contraction and pD₂ represents $-\log\text{EC}_{50}$

cardiovascular health in the elderly and during postmenopausal term were investigated. With regard to this hypothesis, we demonstrated that (1) coexistence of aging and menopause has led to vessel stiffness, determined by both impaired vasocontraction and vasorelaxation functions; (2) physiologic aging and menopause were the conditions where increased oxidative stress was inevitable; (3) INDO has increased the PE and KCl contraction responses of aged OVX rats, whereas ASP normalized them to the young's value; (4) except for INDO, low-dose ASP treatment alone significantly improved the relaxation function, even if it is given in very late stage of aging; and (5) low-dose ASP treatment has decreased the oxidant status and increased the antioxidant capacity, whereas INDO failed to show any effect.

Fig. 2 Concentration–response curves for acetylcholine in intact endothelium aortic rings of control rats (young) and of ovariectomized rats (OVX). Some animals were treated with either aspirin (OVX+ASP, 10 mg/kg/day) or indomethacin (OVX+INDO, 1 mg/kg/day) for 10 weeks. Values are mean±SEM of 12 or 18 rings. * $P<0.001$ versus OVX group

Fig. 3 Concentration–response curves for sodium nitroprusside in intact endothelium aortic rings of control rats (young) and of ovariectomized rats (OVX). Some animals were treated with either aspirin (OVX+ASP, 10 mg/kg/day) or indomethacin (OVX+INDO, 1 mg/kg/day) for 10 weeks. Values are mean±SEM of 12 or 18 rings. * $P<0.05$, [#] $P<0.001$ versus OVX group

It is known that cardiovascular diseases in aging are mostly associated with increased oxidants. NO is crucial factor for healthy functioning of endothelium; excessive superoxide (O_2^-) in aging reacts with the vasodilator NO, decreases its bioavailability, and produces peroxynitrite causing damage in tissues. Also, decreased endothelial NO production in aging promotes apoptosis of endothelial cells (Ungvari et al. 2010). Similarly, the endothelial dysfunction of OVX rats depends on dramatic reduction in NO synthesis and leads to decreased vasodilatation; administration of estradiol increases endothelium-dependant relaxation by increasing the activity and expression of endothelial nitric oxide synthase, as well as reducing the effect of O_2^- generation (Moncada and Higgs 2006). Also decreased expression of soluble guanylyl cyclase and impaired NO signaling has been shown in old OVX rats as the underlying mechanisms (Stice et al. 2009). However, when an estrogen/progestine combination is given to aged postmenopausal women in a trial, the outcomes failed to confirm the animal studies and resulted in increasing risk for breast cancer, coronary heart disease, and stroke (Rossouw et al. 2002). Miller et al. demonstrated that estrogens can stimulate vascular injury responses in aged animals (Miller et al. 2007). It has been suggested that increased expression of inflammatory genes, such as TNF- α may be involved in some of deleterious effect of delayed E2 administration (Pechenino et al. 2011). The alterations of estrogen-mediated inflammatory biomarkers in women has been reported to correlate with the timing of initiation of estrogen treatment, type of drug, and the gene polymorphisms of ER (Dubey et al. 2005). On the other hand, adherence to hormone replacement therapy is less than optimal in the practice, especially in developing countries (Gold 2006). Even if estrogen was initiated in the early phase of menopause for protecting the bones and vessels, the rate of discontinuation can be 19–53 % by the end of first year (Read et al. 2010; Gold 2006; Légaré et al. 2003). It is clear that whether endothelial protection, decreasing the inflammation, and reducing oxidative stress in these vulnerable individuals are highly important for cardiovascular health.

In animal studies, two agents are widely used for understanding of vascular relaxation function of vessel. Ach stimulates NO release from endothelium, SNP works as NO donor for smooth muscle. In our study, both Ach and SNP responses were improved by low-dose ASP; therefore, this enhancement of vascular

function can be explained by NO pathway. The present findings of this study are in agreement with the previous researches. Grosser et al. demonstrated that ASP has activated NO-cGMP pathway in endothelial cells and protected them from hydrogen peroxide toxicity, but other NSAIDs had no protective effect (Grosser and Schroder 2003). ASP has induced concentration-dependant NO release from native porcine coronary arteries in vitro, whereas INDO and ibuprofen had no effect (Taubert et al. 2004). We have found that antioxidant action of ASP would contribute to the ameliorating effect of the endothelial cell injury observed in aged OVX rats. As a comparison, we did not see any efficacy of INDO neither on oxidant/antioxidant variables nor on relaxation. Wu et al. have shown that antioxidant power of ASP was dose-dependent and 10 mg/kg/day could reduce superoxide production by 19 % in SHR and resulted in vasorelaxation to Ach. Aortic vascular smooth muscle cell culture studies also suggested that ASP may account for the antioxidant effect on whole artery, when it was administered to young SHRs; it attenuated the development of hypertension (Wu et al. 2002). Napoli et al. reported NO-releasing aspirin, but not aspirin, reduced vascular smooth muscle cell proliferation in response to vascular injury in old rats (Napoli et al. 2002); although we have used the conventional form of aspirin, it was capable to achieve improvement of smooth muscle function. Similarly, it has been reported that ASP has ameliorating vasorelaxant effect to Ach in hypercholesterolemic rats (Tauseef et al. 2008).

Although Stice et al. have reported aged OVX rat contractility to PE increased in Brown Norway rat, the loss of estrogens and aging did not significantly affect the contraction to PE in this study similar to others (Wassmann et al. 2001). The rat strain might be the reason of the different findings. Changes in the extracellular matrix proposed one of the mechanisms of vascular stiffness. The aged males have been found more prone to develop the pathology; it can be seen in females as well and increases with age (Stice et al. 2009). We demonstrated that this vulnerable rings contractility highly increased after INDO treatment.

One of the limitations of this study was that we were not able to give high dose of INDO to the rats due to gastrointestinal bleeding (Polat et al. 2010). To make a better comparison in this experimental setup, we could not show another NSAID, INDOs' possible harmful effect on vascular relaxation. Although we used low-

dose INDO treatment (1 mg/kg/day) for 10 weeks, TOS level was tented to increase; KCl contractility, α -agonist contraction, and the vessel sensitivity were statistically induced, which might be deleterious if, for example, clinical (pre)hypertension is accompanying to menopause and aging. It is well known that INDO inhibits the formation of vasodilator prostanoids, such as prostacyclin (Moncada and Higgs 2006), to explain our results. However, in theory, ASP also has the same feature, but it is clear that this aspect of ASP (Taubert et al. 2004) does not influence the whole responses of vessel and ASP has additional prostaglandin-independent properties. One of remarkable findings of this study is that the vessels are more sensitive to even low-dose of INDO than ASP; especially when we consider that aged individuals might have a limited ability to compensate increased contractility.

Conclusion

We clearly demonstrated that ASP treatment alone has partially reversed the well-established endothelial dysfunction and smooth muscle stiffness with its antioxidant power, even if it is given in lower dose during very late stage of aging, whereas INDO did not create any improvement; moreover, it might be used in chronic usage with caution. Therefore, if the better anti-inflammatory and analgesic chose is the question of certain age for treatment of painful situation in women, ASP is a better option in providing additional cardiovascular benefit for vascular integrity both with antiplatelet function and antioxidant power.

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