Experimental Gerontology 76 (2016) 1–8

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Decreased bioavailability of nitric oxide in aorta from ovariectomized senescent mice. Role of cyclooxygenase

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ARTICLE INFO

Article history:
Received 26 October 2015
Received in revised form 23 December 2015
Accepted 12 January 2016
Available online 13 January 2016

Section Editor: Christiaan Leeuwenburgh

Keywords:
Senescence-accelerated mice
Aging
Experimental menopause
Vascular reactivity
Prostanoids
Nitric oxide
Estrogen

ABSTRACT

This study investigates the effects of aging and/or ovariectomy on vascular reactivity to thromboxane A_2 (TXA_2) receptor stimulation with U46619, and the modulation by nitric oxide (NO) and cyclooxygenase (COX) in aorta from female senescence-accelerated mice (SAMP8) and from senescence resistant mice (SAMR1). Five-month-old female SAMR1 and SAMP8 were divided into three groups: sham-operated, ovariectomized and ovariectomized plus estradiol. Twenty-eight days after surgery, thoracic aortic rings were mounted for isometric recording of tension and concentration–response curves for U46619 (10^{-10}–3×10^{-7} M) were performed in the absence and in the presence of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) and/or COX inhibitor indomethacin (10^{-5} M). Vascular superoxide production was detected by dihydroethidium staining on sections of thoracic aorta. NO bioavailability in response to U46619 was suppressed by estrogen withdrawn in young and senescent mice and was restored by the administration of estradiol. In the presence of indomethacin, contractions to U46619 decreased in all groups indicating an aging- and estrogen-dependent modulation of contractile prostanoids. The simultaneous incubation of L-NAME and indomethacin did not change the maximal responses and sensitivities to TXA_2 in any group in comparison with untreated aortic segments. The superoxide generation induced by TXA_2 was greater in aorta from SAMP8 than in SAMR1. Moreover, in ovariectomized groups superoxide production was further increased and treatment with 17β-estradiol reverted the effects of the ovariectomy. Inhibition of COX with indomethacin prevented the U46619-induced increase in superoxide formation. Our results indicate that NO bioavailability in response to TP receptor activation is both estrogen- and aging-dependent. TXA_2 induced contractions are partially mediated by COX activation. Both aging and ovariectomy enhanced COX-dependent component of the TXA_2-induced contraction. It is noteworthy that in the absence of estrogen, COX inhibition induces an increase of NO bioavailability. Therefore, in senescent female mice with an experimental menopause, TP-receptor stimulation is responsible for COX activation and enhanced superoxide generation, which may result in reduced NO bioavailability. These effects were reversed by estrogen administration.

1. Introduction

Vascular aging, characterized by endothelial dysfunction, is a major risk factor for developing cardiovascular disease (Lakatta and Levy, 2003). Estrogens possess vasculoprotective effects (Cano et al., 2007; Dantas and Sandberg, 2006) mostly mediated by an increase in endothelium-derived vasodilator factors, including nitric oxide (NO) (Mendelsohn, 2009) and prostacyclin (Sobrino et al., 2010). In women, the acceleration of the vascular dysfunction after menopause suggests that reduction in estrogen levels may be a triggering event that leads to increased vascular vulnerability (Moreau and Hildreth, 2014; Novella et al., 2012). However since menopause coincides with aging, whether the higher cardiovascular risk in postmenopausal women is a function of aging, a consequence of menopause, or both has been debated in the literature for many years (Bittner, 2009). Therefore, further understanding of the biological pathways involved in vascular aging in women during transition to menopause is needed.

Thromboxane A_2 (TXA_2), a vasoconstrictor prostanoid, plays an important role in the regulation of vascular tone in the normal systemic vasculature (Fulton and Stallone, 2002) through activation of the TXA_2 receptor (TP) (Li et al., 2008). In contrast, abnormal TXA_2-mediated

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http://dx.doi.org/10.1016/j.exger.2016.01.006
0531-5565/

activity is involved in the pathophysiology of vascular dysfunction during estrogen withdrawn (Dantas et al., 1999, 2004) and aging (Briones et al., 2005; Matz et al., 2000; Vanhoutte, 2009). Vascular dysfunction associated to aging involves cyclooxygenase (COX) activation and elevated TP receptor expression (Novella et al., 2013a; Tang and Vanhoutte, 2008). In humans, the production of COX-derived endothelium-derived contractile factors is a characteristic of the aged blood vessels that results in an earlier onset and acceleration of the endothelial dysfunction (Taddei et al., 1997a,b). Animal studies link the effect of both aging and ovariectomy, conditions present in menopausal women, proving that increased COX-dependent vasoconstriction is mediated by TP receptor activation (Davidge et al., 1996; Davidge and Zhang, 1998).

There are evidences that support a potential interaction between NO synthase (NOS) and COX systems (Goodwin et al., 1999; Mollace et al., 2005). On the one hand, NO has been shown to modulate COX activity and modify prostanoid production (Davidge et al., 1995; Miyamoto et al., 2007). Conversely, COX-derived prostanoids, such as TXA_2, modulate NOS activity by downregulation of phosphorylation and decreasing its activity (Ashton and Ware, 2004). With aging, enhanced vasoconstriction mediated by endothelium-derived TXA_2 induces a decrease in NO that amplifies the endothelial dysfunction (Feletou et al., 2009; Matz et al., 2000).

In addition, another mechanism underlying age-associated reductions in endothelium-dependent vasodilation and NO bioavailability is the development of vascular oxidative stress (Eskurza et al., 2004; Taddei et al., 2001). In fact, a great burden of oxidative stress is seen during aging (Hamilton et al., 2001) and menopause (Hildreth et al., 2014) which can contribute to vascular dysfunction.

Senescence-accelerated mice (SAM) model of vascular aging replicates most of the features and pathophysiological states founds in human aging, *i.e.* SAM model display impaired endothelial-dependent dilation with advancing age (Novella et al., 2013b). Our approach is to induce a surgical menopause in SAM to determine how aging and/or estrogen withdrawn affects vascular contraction to TXA_2 and how the interplay between vascular mediators such as NO and prostanoids affect this response.

2. Material and methods

2.1. Experimental animals

Female senescence-accelerated mouse-resistant 1 (SAMR1, $n = 42$) and senescence-accelerated mouse-prone 8 (SAMP8, $n = 42$) were obtained from the breeding stock at University of Valencia and housed according to institutional guidelines (22 °C constant room temperature, 12 h light/dark cycle, 60% humidity, standard mice chow and water *ad libitum*). All protocols were approved by the Institutional Ethics Committee at the University of Valencia, conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Both SAMR1 and SAMP8 were randomly separated at 5 months of age into three groups: 1) sham-operated (Sham); 2) ovariectomized (Ovx); and 3) ovariectomized chronically-treated with estrogen (OvE). Six-month old sham-operated SAMR1 was used as control group, as they present a regular life-spam.

2.2. Surgical procedures and hormonal treatment

Surgical procedures were performed as previously described (Novella et al., 2010). Briefly, ovariectomy was performed under isoflurane anesthesia and a single 1-cm dorsal midline incision was made in the skin and the ovaries were removed. At the moment of the surgery, half of the Ovx mice received estrogen replacement by subcutaneous implant of an osmotic pump (Model 2004, Alzet Osmotic Pumps®; Durect Corporation, Cupertino, CA, USA) containing 17β-estradiol in 50% DMSO at a dose of 10 μg/kg/day (OvE). Untreated Ovx mice were implanted with a pump filled with vehicle only (50% DMSO in saline). Sham-operated female mice were also included. To check the efficacy of ovariectomy and estrogen treatments, uterine weight and plasmatic 17β-estradiol concentration were evaluated. Four weeks after surgical procedures mice were euthanized and therefore all the experiments were performed in 6-month old female mice.

2.3. Determination of biochemical variables

Blood samples were withdrawn by cardiac puncture and centrifuged at 1200 ×*g* for 10 min. Plasma levels of glucose, creatinine, total bilirubin and 17β-estradiol were determined using an automatic analyzer (ADVIA Centaur® CP Immunoassay System, Siemens, Munich, Germany) at a commercial analytical service center.

2.4. Isolated mouse aorta preparation

For functional studies, thoracic aorta ($n = 8$ mice per group) was excised, placed immediately in ice cold Krebs–Henseleit solution and cleaned of surrounding tissue. Arteries were dissected into 4-mm rings, mounted between 2 stainless steel holders (100 μm inner diameter), and placed in 4 mL tissue baths containing modified Krebs–Henseleit solution (in mM: NaCl 115; KCl 4.6; KH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25; glucose 11.1; EDTA 0.01, pH 7.3–74) at 37 °C and aerated with 95% O_2/5% CO_2 for isometric force measurements (Grass FT03, Grass Instruments Division Astromed, Inc., West Warwick, RI, USA). Changes in isometric force were recorded by use of Chart *v.* 3.4/s software and a MacLab/8e data acquisition system (ADInstruments, East Sussex, UK). Once the optimal resting tension was reached (1 g), aortic rings were allowed to attain a steady level of tension during a 1-hour equilibration period before testing.

Following the equilibration period, arterial segments were exposed to the depolarizing agent KCl (60 mM) until the contraction reached a stable plateau (10 to 20 min). After washout and return to stable baseline, functional integrity of the endothelium was confirmed routinely by the presence of relaxation induced by acetylcholine (10^{-7}–10^{-6} M) during contraction obtained with serotonin (10^{-5} M). Only aortic segments in which acetylcholine reversed the serotonin induced tone by more than 85% were considered with functional endothelium and were used in this study.

Contractile responses were determined by cumulative concentration–response curves to the thromboxane A_2 (TXA_2) mimetic U46619 (10^{-10} to 3×10^{-7} M) in the absence (control) and in the presence of L-NAME (10^{-4} M) to inhibit NOS, indomethacin (10^{-5} M) to inhibit COX or the combination of L-NAME (10^{-4} M) plus indomethacin (10^{-5} M), to inhibit in conjunction NOS and COX. Inhibitors were added to the organ bath 15 min prior to the concentration–response curves to U46619 were performed. Only one concentration–response curve to U46619 was obtained in each artery ring. Control and treated rings obtained from the same animal were studied in parallel.

2.5. Quantification of oxidative stress

Vascular superoxide production was detected *in situ* by dihydroethidium (DHE) staining on unfixed frozen sections of thoracic aorta from another set of animals ($n = 6$ mice in each experimental group). Freshly prepared DHE (2 μM; Molecular Probes) was applied to 4-mm thoracic aorta segments in PBS and incubated for 30 min in a light protected humidified chamber at 37 °C and then viewed by fluorescent microscope. To study the effect of TXA_2 on superoxide production, U-46619 (10^{-8} M) was applied to the vascular segments 10 min after DHE addition. The role of cyclooxygenases on TXA_2-induced superoxide production was evaluated with the incubation for 15 min with indomethacin (10^{-5} M) before adding DHE. Control samples were only treated with DHE. After incubations, aortic segments were gently washed with PBS and mounted in Tissue-Tek® OCT compound (Sakura Finetek, USA), molded, snap frozen in dry ice, and stored at −80 °C.