Vascular Pharmacology 51 (2009) 140–146

Contents lists available at ScienceDirect

Vascular Pharmacology

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

Role of gender and estrogen receptors in the rat aorta endothelium-dependent relaxation to red wine polyphenols

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ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form 24 March 2009
Accepted 28 May 2009

Keywords:
Red wine polyphenols
Vascular relaxation
Nitric oxide
Estrogen receptors

ABSTRACT

Regular intake of moderate amounts of beverages rich in polyphenols such as red wine is associated with a protective effect on the vascular system, in part, by increasing the endothelial formation of nitric oxide (NO), a major vasoprotective factor. Since estrogens are potent inducers of NO formation and polyphenols have been shown to have phytoestrogen properties, we determined whether estrogen receptors mediate the stimulatory effect of red wine polyphenols (RWPs) on the endothelial formation of NO using isolated rat aortic rings and cultured endothelial cells.

RWPs caused endothelium-dependent relaxations, which were more pronounced in the aorta of female than male rats. Increased relaxations were also observed to acetylcholine but not to sodium nitroprusside. Relaxations to RWPs were abolished by nitro L-arginine and MnTMPyP, markedly reduced by polyethyleneglycol-catalase and wortmannin, and not affected by the estrogen antagonist ICI 182,780 in aortic rings from males and females. eNOS expression was higher in aortic sections of female than male rats. RWPs caused the phosphorylation of Akt and eNOS in endothelial cells, which was unaffected by ICI 182,780.

Thus, RWPs cause redox-sensitive PI3-kinase/Akt-dependent NO-mediated relaxations, which are more pronounced in the aorta of female than male rats; an effect most likely due to the increased expression level of eNOS rather than activation of estrogen receptors.

1. Introduction

Cardiovascular diseases, such as hypertension, coronary heart diseases, and atherosclerosis are associated with increased oxidative stress (Landmesser et al., 2002; Rajagopalan et al., 1996; Somers et al., 2000) and are more common in men than in pre-menopausal women (Forte et al., 1998). Studies on the regulation of blood pressure and vascular function between males and females suggest that female sex hormones are implicated, at least in part, in the protective effects of gender on the vasculature (Farhat et al., 1996; Orshal and Khalil, 2004; Sader and Celermajer, 2002). The incidence of cardiovascular diseases increases significantly after menopause, with less cardiovascular protection possibly due to estrogen deficiency. Although estrogens and phytoestrogens have antioxidant properties due to the presence of phenolic groups in their steroid structure, these compounds can also activate intracellular kinase cascades and gene transcription (Hu and Kong, 2004; Ruiz-Larrea et al., 1994; Williams et al., 2004) potentially accounting for their beneficial effects on the vasculature (Forte et al., 1998; Mendelsohn, 2002; Mendelsohn and Karas, 2005; Mikkola and Clarkson, 2002). It has been reported that estrogen treatment of human endothelial cells rapidly increases the formation of nitric oxide (NO) by estrogen receptor-dependent activation of endothelial nitric oxide synthase (eNOS) (Caulin-Glaser et al., 1997). The mechanism underlying this effect involves phosphorylation of eNOS via the redox sensitive PI3-kinase/Akt pathway (Haynes et al., 2003, 2000; Simoncini et al., 2000).

Several studies indicate an association between regular consumption of food and beverages rich in polyphenols and a reduced risk of coronary artery diseases (Gronbaek et al., 2000; McKay and Blumberg, 2007; Reed, 2002). The vascular protective effect has been attributable at least in part to an enhanced endothelial formation of vasoprotective factors such as NO (Andriambeloson et al., 1997; Fitzpatrick et al., 1993) and endothelium-derived hyperpolarizing factor (EDHF) (Ndiaye et al., 2004). Indeed, grape-derived polyphenols have been shown to strongly induce the endothelial formation of NO and NO-mediated endothelium-dependent relaxation of arterial rings (Andriambeloson et al., 1997; Fitzpatrick et al., 1993). These effects are mediated by the redox sensitive Src/PI3-kinase/Akt pathway, which leads to phosphorylation of eNOS at Ser 1177 thereby increasing eNOS activity (Anselm et al., 2007; Ndiaye et al., 2005). Since the PI3-kinase/Akt pathway mediates the stimulatory effect of estrogens on eNOS (Haynes et al., 2003, 2000) and polyphenols have been shown

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doi:10.1016/j.vph.2009.05.002

to have phytoestrogen properties (Anter et al., 2005), the aim of the present study was to determine the role of estrogen receptors in the RWPs-induced endothelium-dependent relaxations in aortic rings from female and male rats and formation of NO in cultured endothelial cells.

2. Methods

2.1. Preparation of red wine polyphenols

RWPs dry powder was obtained from French red wine (Corbières A.O.C), and was provided by Dr. M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France). Phenolic compounds were adsorbed on a preparative column; then alcohol desorbed; the alcoholic-eluent was gently evaporated; the concentrated residue was lyophilized and finely sprayed to obtain RWPs dry powder. One liter of red wine produced 2.9 g RWPs, which contained 471 mg/g of total phenolic compounds expressed as gallic acid.

The extract contained 8.6 mg/g catechin, 8.7 mg/g epicatechin, dimers (B1, 6.9 mg/g; B2, 8.0 mg/g; B3, 20.7 mg/g; and B4, 0.7 mg/g), anthocyanins (malvidin-3-glucoside, 11.7 mg/g; peonidin-3-glucoside, 0.66 mg/g; and cyanidin-3-glucoside, 0.06 mg/g), and phenolic acids (gallic acid, 5.0 mg/g; caffeic acid, 2.5 mg/g; and caftaric acid, 12.5 mg/g).

2.2. Animals and chemicals

Experiments were performed with 12 to 14 week-old male and female Wistar rats. N^{w}-nitro-*L*-arginine (LNA), wortmaninn, Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) and polyethylene glycol-catalase (PEG-catalase) were from Sigma, and 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) from Tocris Biosciences. Primary antibodies directed against p-eNOS (Ser 1177) and p-Akt (Ser 473), and the secondary antibody (peroxidase-labeled anti-rabbit IgG) were from Cell Signaling Technology. The antibodies directed against eNOS and β-tubulin were from BD Transduction laboratories.

2.3. Vascular reactivity studies

After anaesthesia with pentobarbital (50 mg/kg, intraperitoneally), the thoracic aorta was excised, cleaned of connective tissue and cut into rings (3–4 mm in length). In some preparations, the endothelium was removed by rubbing the intimal surface of rings with a pair of forceps. Rings with or without endothelium were suspended in organ baths containing oxygenated (95% O_2; 5% CO_2) Krebs bicarbonate solution (mmol/L: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and D-glucose 11, pH 7.4, 37 °C), for the determination of changes in isometric tension. Following equilibration for 45 min under a resting tension of 2 g, rings were contracted with phenylephrine (1 μM). After washout and a 30 min equilibration period, rings were again contracted with phenylephrine (1 μM) before addition of acetylcholine (Ach; 1 μM) to test the presence of a functional endothelium. After washout and a 30 min equilibration period, rings were again contracted with phenylephrine before a concentration–relaxation curve either to RWPs, Ach, sodium nitroprusside (SNP), or the water soluble 17-beta estradiol was constructed. Rings were exposed to an inhibitor or antagonist for 30 min before addition of phenylephrine (1 μM).

2.4. Cell culture

Porcine coronary artery segments were flushed with PBS without calcium to remove remaining blood. Thereafter, endothelial cells were isolated by collagenase treatment (type I, Worthington, 1 mg/ml for 12 min at 37 °C), and cultured in culture dishes containing medium MCDB 131 (Invitrogen) and 15% fetal calf serum supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 μg/ml), and L-glutamine (2 mM) (all from Cambrex), and grown for 48–72 h. All experiments were performed with confluent cultures of cells used at first passage. Cells were exposed to serum-free culture medium in the presence of 0.1% bovine serum albumin (QBiogene) for 6 h prior to treatment.

2.5. Western blot analysis

After treatment, cells were washed twice with PBS and then lysed in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na_3VO_4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen)). Total proteins (20 μg) were separated on 8% SDS-polyacrylamide (Sigma) gels at 80 V for 2 h. Separated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Amersham) at 100 V for 120 min. Membranes were blocked with blocking buffer containing 3% bovine serum albumin, Tris-buffered saline solution (Biorad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 h. Membranes were then incubated with the primary antibodies of either eNOS, p-eNOS (Ser 1177) or p-Akt (Ser 473) (dilution of 1:1000) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit IgG, dilution of 1:5000) at room temperature for 60 min. Detection of total eNOS or β-tubulin proteins were used for normalization and quantification of p-eNOS and p-Akt and of eNOS respectively. Prestained markers (Invitrogen) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

2.6. Immunohistochemical staining

Freshly isolated aorta was embedded in Tissue-Tek OCT compound, frozen in liquid nitrogen, sectioned at 7 μm and mounted on slides. Sections were post fixed for 30 min in 4% paraformaldehyde in PBS, washed twice 15 min in PBS and incubated for 1 h at room temperature in blocking solution (5% milk and 0.1% triton in PBS), to block non specific binding. Sections were incubated with the primary antibody, mouse monoclonal anti-eNOS, at dilution 1:100 overnight at 4 °C. After washing twice for 15 min in PBS, the secondary antibody (peroxidase-conjugated anti-mouse diluted at 1:200) was added and incubated for 2 h at room temperature in the dark. After washing twice for 15 min in PBS, vectashield, an anti-fading agent, was added. The slides were observed and analyzed using a confocal microscope.

2.7. Statistical analysis

Values are expressed as mean + SEM. Statistical analysis was performed with Student's *t*-test for paired data or ANOVA followed by Fischer's protected least significant difference test where appropriate. Values of $P<0.05$ were considered statistically significant.

3. Results

RWPs caused concentration-dependent relaxations in aortic rings with endothelium but not in those without endothelium, which were significantly more pronounced in female than male rats (Figs. 1A and 2). In addition, endothelium-dependent relaxations to acetylcholine were also greater in aortic rings from female than male rats whereas those to sodium nitroprusside, a donor of NO, were similar (Fig. 1B and C).

The RWPs-induced endothelium-dependent relaxations were abolished in the presence of L-nitro-arginine (LNA), a competitive inhibitor of NO synthase, in aortic rings from both female and male rats indicating that they are mediated exclusively by NO (Fig. 2C and D). Previous studies have indicated the grape-derived polyphenols