Acute responses to phytoestrogens in small arteries from men with coronary heart disease

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Cruz, Maria Natalia, Leonid Luksha, Henareth Logman, Lucilla Poston, Stefan Agewall, and Karolina Kubickiene. Acute responses to phytoestrogens in small arteries from men with coronary heart disease. Am J Physiol Heart Circ Physiol 290: H1969–H1975, 2006.—The aim of this study was to investigate acute vasodilator responses to phytoestrogens and selective estrogen receptor-α (ERα) agonist in isolated small arteries from men with established coronary heart disease (CHD) and with a history of myocardial infarction versus healthy male control subjects. As to methodology, small arteries obtained from subcutaneous fat biopsies and mounted on a wire myograph were preconstricted with norepinephrine, and dilator responses to increasing nanomolar-micromolar concentrations of the phytoestrogens resveratrol and genistein (predominantly ERα agonists) and to propyl-[1H]-pyrazole-1,3,5-triy-trisplenol (PPT, a selective ERα agonist) were determined. These were compared with responses to reference compound 17β-estradiol (17β-E2). Concentration-response curves were constructed before and after nitric oxide (NO) synthase inhibition with Nω-nitro-L-arginine methyl ester. As a result, relaxation induced by the investigated compounds was similar in men with CHD and control men, but in both groups PPT and genistein-induced relaxation was greater than that of resveratrol and 17β-E2. NO contributed to both phytoestrogens and PPT-induced relaxation but not to 17β-E2 responses in arteries from control men. This NO-mediated component of relaxation was absent in arteries from men with established CHD. In conclusion, phytoestrogens, at concentrations achievable by ingestion of phytoestrogen-rich food products, evoke dilation ex vivo of small peripheral arteries from normal men and those with established CHD. The contribution of NO to dilatory responses by these compounds is pertinent to arteries from control males, whereas other NO-independent dilatory mechanism(s) are involved in arteries from CHD.

vasodilation; propyl-[1H]-pyrazole-1,3,5-triy-trisplenol; nitric oxide

THE PROPOSED ROLE of estrogens in cardiovascular health may not be confined to women (17). In men, estrogens are synthesized in testes and adrenal glands by local tissue aromatization of androgenic precursors. In 1997, Sudhir et al. (44) described a 31-year-old man with a null mutation in the estrogen receptor (ER) gene who suffered from coronary heart disease (CHD) and demonstrated reduced endothelium-dependent dilatation to flow as assessed in the brachial artery. Impaired endothelium-dependent dilatation also occurs in men after suppression of endogenous synthesis of estrogens by aromatase inhibition (29), and associations in men between ERα and ERβ genetic polymorphisms, CHD, and blood pressure have been reported recently (42). In rodents, ERβ knockout male mice develop hypertension as they age (49). Thus the male cardiovascular system is a potentially important target for estrogens and nutritional and/or pharmaceutical supplementation by estrogenic compounds that might offer cardiovascular benefit.

Dietary phytoestrogens provide a suitable source of nutritional supplementation. The lower incidence of cardiovascular disease in East Asian compared with Western countries may in part reflect a much greater intake of soy-derived food products rich in the phytoestrogen genistein. In France, the cardioprotective effect of red wine, despite a diet rich in saturated fat (the “French paradox”) is attributed to trans-resveratrol (trans-3,5,4′-hydroxystilbene), another phytoestrogen (15). Several studies (19) have investigated vasodilator responses to phytoestrogens in women or in female animals; however, the contribution of nitric oxide (NO) in these responses remained uncertain. To our knowledge, little is known about the vascular responses of these substances in healthy men or men with established CHD.

Resistance-sized arteries play a key role in the maintenance of peripheral resistance and blood pressure, and ex vivo investigations on functional properties have given valuable insight into the disease process (39). Endothelial dysfunction, a known risk factor for subsequent CHD (6), is also, as might be anticipated, evident in subjects with established cardiovascular disease. Thus impaired endothelium-dependent dilatation in the forearm circulation has been reported previously in patients with established CHD in vivo with the use of venous occlusion plethysmography (6). We have also reported impaired flow-mediated dilatation in the brachial artery, accompanied by reduced ex vivo dilatory responses to intraluminal flow to the endothelium-dependent agonist bradykinin in subcutaneous resistance arteries from men with CHD (1).

In this study, we isolated small arteries from the subcutaneous circulation of men with CHD and a history of myocardial infarction (MI) and from control male subjects. In preconstricted arteries, we evaluated acute dilatory responses to the addition of the phytoestrogens genistein and resveratrol. Because phytoestrogens have a high affinity for the ERβ, we have also investigated responses to a recently developed, highly selective ERα agonist propyl-[1H]-pyrazole-1,3,5-triy-trisple-
nol (PPT) (21) that has been introduced as a useful tool for enhancing our understanding of how estrogens work through the two ER subtypes in the vasculature (3, 38). Dilation evoked by these agents was compared with that induced by the physiological ERα/β ligand 17β-estradiol (17β-E2), and the contribution of NO to vasodilatation after stimulation with estrogenic compounds was evaluated.

**METHODS**

**Patients population.** Fifteen male patients, aged between 38–71 yr with a history of acute MI (1–12 mo before study) were recruited from the Department of Cardiology at Karolinska University Hospital, Huddinge, Sweden. Acute MI was defined by the criteria of the European Society of Cardiology and the American College of Cardiology (45). Thus patients were diagnosed as having an acute MI if they had two values of serum troponin T >0.05 g/l together with either typical symptoms (chest pain >15 min; pulmonary edema in the absence of valvular heart disease; cardiogenic shock; and arrhythmia, such as ventricular fibrillation or ventricular tachycardia) or new Q waves in at least two of the twelve standard electrocardiographic leads or electrocardiogram changes indicating acute ischemia (ST elevation, ST depression, or T-wave inversion). Men with diabetes mellitus were excluded. All men were receiving routine pharmacological treatment for CHD (see Table 1), but it was minimized 5 days before the biopsy to avoid their direct pharmacological effects on vascular function. Nine control men, aged between 41 and 69 yr, free from medication and clinically healthy, were recruited as a control group from among hospital staff and acquaintances of patients. All subjects gave informed consent after written and oral information. The study was approved by the Ethics Committee of the Karolinska Institutet at Karolinska University Hospital, Huddinge, Sweden.

**Measurements.** With the use of established methods, venous blood was drawn after an overnight fast and 5 min of supine rest for determination of plasma glucose and plasma levels of cholesterol and triglycerides. Resting blood pressure was measured in the right arm after ~10 min supine rest. Body mass index (BMI) (BMI) was measured according to recommended principles. Smoking was assessed by a questionnaire.

**Isolated artery preparation.** Subcutaneous fat biopsies from the lower abdomen (~1.5 × 1.5 × 1.5 cm) were obtained after local anesthesia (1% prilocaine, Citanest). Arteries (internal diameter, ~400 μm) were carefully dissected from surrounding tissue and transferred to the experimental chamber of a wire myograph filled with physiological saline solution (PSS) and oxygenated with 5% CO2–95% O2 (4-channel Multi Myograph; model 610, Danish Myo Technology).

**Wire myography.** Each artery was mounted as an isometric preparation on stainless steel wires (diameter, 40 μm) attached to a force transducer and a micrometer, respectively, as described previously (16, 34). Commercially available software was used for calibrations and for data collection (Myodac, version 2.1, Danish Myo Technology). Arteries were subject to a standard run-up protocol (39) to confirm functional endothelium. Those that demonstrated <70% relaxation to bradykinin (3 μmol/l) after prior constriction to norepinephrine (NE, 1 μmol/l) were excluded.

Resveratrol, PPT, and 17β-E2 were initially dissolved in 95% ethanol, and genistein was dissolved in DMSO; the final concentration of either did not exceed 0.01% (vol/vol). Pilot studies demonstrated that an addition of vehicle at these concentrations did not induce vasodilatation in preconstricted arteries.

**Assessment of vascular function: dilatory responses to addition of estrogenic compounds and contribution of NO.** All arteries were initially preconstricted with NE (1 μM), and cumulative concentration-response curves to genistein, resveratrol, PPT, or 17β-E2 (10–30 μM/l added every 5 min) were obtained (Fig. 1). Each n represents the number of subjects studied, unless indicated otherwise. Only one agonist was tested in any given artery from any participant. With the establishment in pilot studies that two consecutive responses to the same agonist (with a washout period intervening) demonstrated similar dilator responses, the role of NO in the dilatory responses observed was determined as follows: after the first response and after a 15-min washout period, arteries were incubated with the NO synthase (NOS) inhibitor Nω-nitro-λ-arginine methyl ester (L-NAME, 0.1 mM, 30 min), and the second concentration-response curve was then carried out in the continued presence of L-NAME. Previous studies (33) in our laboratory have shown that a combination of NOS inhibitors Nω-nitro-λ-arginine (300 μM) and L-NAME produced a similar effect as L-NAME alone. Pilot studies carried out before the experiments demonstrated that incubation with L-NAME did not influence the basal tension or NE preconstriction.

**Chemicals and solutions.** The composition of the PSS contained (in mmol/l) 119 NaCl, 4.7 KCl, 2.5 CaCl2, 21.17 MgSO4, 25 NaHCO3, 1.18 KH2PO4, 0.026 EDTA, and 5.5 glucose, pH 7.4. NE and 17β-E2 were obtained from Sigma-Aldrich (Stockholm, Sweden). Resveratrol, PPT, and genistein were obtained from Tocris Cookson (Bristol, UK).

**Statistical analysis.** Force development (mN/mm of artery segment) was calculated with the use of Myodac software (Danish Myo Technology). All measurements were corrected for baseline force. Data were transferred to STATISTICA (version 6.0, StatSoft), in which all analyses were performed. Relaxation to vasodilators was calculated as the percent change of NE preconstriction. Data are expressed as means ± SE, unless indicated otherwise. Differences in responses between groups of arteries were determined by comparing concentration-response curves with the use of a two-way repeated-measures ANOVA, using substance concentration as a within-subject factor and group membership as a between-subject factor. The interaction effect between concentration and group membership tested the hypothesis that the concentration-response curves differ between the groups, and P < 0.05 was considered statistically significant. Baseline characteristics, initial artery diameters, and contractile responses to NE were analyzed by Student’s t-test.

**RESULTS**

Baseline characteristics of all individuals enrolled and the most frequently used drugs are shown in Table 1. There was no difference between baseline characteristics (i.e., age, BMI, lipid profile, and glucose levels) of all individuals.

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Table 1. Characteristics of patients with coronary heart disease and of healthy subjects

<table>
<thead>
<tr>
<th>Coronary Heart Disease</th>
<th>Control Subjects</th>
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<tbody>
<tr>
<td>n</td>
<td>15</td>
</tr>
<tr>
<td>Age, yr (median and range)</td>
<td>59 (38–71)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27±3</td>
</tr>
<tr>
<td>Serum total cholesterol, mmol/l</td>
<td>4.8±0.9</td>
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<tr>
<td>Serum triglycerides, mmol/l</td>
<td>1.5±0.5</td>
</tr>
<tr>
<td>Plasma glucose levels, mmol/l</td>
<td>5.2±0.6</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
</tr>
<tr>
<td>Previous smokers</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>Current smokers</td>
<td>3 (20%)</td>
</tr>
<tr>
<td>Never smoked</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>Pharmacological treatment</td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>15 (100%)</td>
</tr>
<tr>
<td>Betablockers</td>
<td>14 (93%)</td>
</tr>
<tr>
<td>Statins</td>
<td>13 (100%)</td>
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</tbody>
</table>

Values are means ± SD, unless otherwise is indicated; n, number of subjects.
Concentration-response curves PPT, genistein, resveratrol, and 17β-E2. Mean arterial diameter of the subcutaneous arteries used for experiments did not differ between CHD and the control group (405 ± 24 μm, n = 15, total number of arteries = 76 vs. 401 ± 25 μm, n = 9, total number of arteries = 49, respectively). Contractile responses to NE (1 μM/l) were similar in arteries from both groups [CHD: 4.5 ± 0.1 mN/mm, n = 15, total number of arteries = 76 vs. control: 5.0 ± 0.2 mN/mm, n = 9, total number of arteries = 49]. The four estrogenic compounds induced relaxation in a concentration-dependent manner in preconstricted arterial segments. All responses were rapid, attaining a plateau within 5 min. Genistein and PPT induced greater relaxations than 17β-E2 in both CHD patients’ arteries (P < 0.05, Fig. 1A) and those from controls (P < 0.05, Fig. 1B). In contrast, resveratrol-induced relaxation was similar to 17β-E2-induced relaxation in both groups (Fig. 1, A and B). However, there was no significant difference in the vasodilation generated by the different estrogenic substances in arteries from CHD versus control males.

Effect of NOS inhibition on vasorelaxation. Incubation with l-NAME had no effect on genistein-induced dilatation in arteries from CHD males (% dilatation at 100 nM/l: 28 ± 2% after L-NAME; at 3 μM/l: 38 ± 3 vs. 33 ± 5% after l-NAME, n = 5, Fig. 2A). In contrast, vasodilation to genistein in arteries from control males was reduced after NO inhibition.

Concentration-response curves PPT, genistein, resveratrol, and 17β-E2. Mean arterial diameter of the subcutaneous arteries used for experiments did not differ between CHD and the control group (405 ± 24 μm, n = 15, total number of arteries = 76 vs. 401 ± 25 μm, n = 9, total number of arteries = 49, respectively). Contractile responses to NE (1 μM/l) were similar in arteries from both groups [CHD: 4.5 ± 0.1 mN/mm, n = 15, total number of arteries = 76 vs. control: 5.0 ± 0.2 mN/mm, n = 9, total number of arteries = 49]. The four estrogenic compounds induced relaxation in a concentra-

Fig. 1. Concentration-response curves to propyl-1H-pyrazole-1,3,5-triy1-trisphenol (PPT; n = 6 patients), genistein (n = 6), resveratrol (n = 5), and 17β-estradiol (17β-E2; n = 7) in arteries from patients with coronary heart disease (CHD) (A) and concentration-response curves to PPT (n = 7), genistein (n = 6), resveratrol (n = 6) and 17β-E2 (n = 5) in arteries from healthy male volunteers (B). Data presented as means ± SE. *P < 0.05, genistein vs. 17β-E2; bP < 0.05, PPT vs. 17β-E2.

Fig. 2. Concentration-response curves to genistein (A) patients with CHD (n = 5) and control (B) male volunteers (n = 6) before and after endothelial nitric oxide synthase (eNOS) inhibition with Nω-nitro-l-arginine methyl ester (l-NAME). Data presented as means ± SE. *P < 0.05, before vs. after l-NAME.
Similarly, L-NAME had no influence on dilatation induced by PPT in arteries from CHD (e.g., % dilatation at 100 nM/l: 41 ± 13 vs. 40 ± 15% after L-NAME; at 3 μM/l: 64 ± 16 vs. 40 ± 18% after L-NAME, n = 4, Fig. 3A), but vasodilation to PPT in arteries from control males was reduced after incubation with L-NAME (% dilatation at 100 nM/l: 31 ± 10 vs. 3 ± 1% after L-NAME; at 3 μM/l: 54 ± 10 vs. 34 ± 14% after L-NAME, P < 0.05, n = 5, Fig. 3B).

Similar results were obtained with resveratrol: L-NAME did not influence vasodilation in CHD arteries (e.g., % dilatation at 100 nM/l: 21 ± 10 vs. 24 ± 10% after L-NAME; at 3 μM/l: 22 ± 10 vs. 28 ± 7% after L-NAME, n = 4, Fig. 4A) but reduced relaxation in control vessels (% dilatation at 100 nM/l: 21 ± 9 vs. 10 ± 4% after L-NAME; at 3 μM/l: 26 ± 9 vs. 11 ± 4% after L-NAME, n = 6, P < 0.05, Fig. 4B).

Vasodilatation to the reference compound 17β-E2 was similar before and after incubation with L-NAME in arteries from CHD (% dilatation at 100 nM/l: 28 ± 5 vs. 24 ± 8% after L-NAME; at 3 μM/l: 29 ± 4 vs. 29 ± 7% after L-NAME, n = 5, Fig. 5A) and arteries from control males (% dilatation at 100 nM/l: 17 ± 4 vs. 22 ± 7% after L-NAME; at 3 μM/l: 22 ± 5 vs. 25 ± 6% after L-NAME, n = 5, Fig. 5B).

**DISCUSSION**

This study has clearly shown that compounds with different selectivity for ER subtypes can evoke rapid dilatation of
isolated small arteries obtained from the subcutaneous circulation of male subjects. Furthermore, the estrogenic substances PPT, genistein, resveratrol, and the reference compound 17β-E2 achieved a similar degree of relaxation in arteries from men with established CHD and healthy controls. NO modulated the responses to phytoestrogens (genistein and resveratrol) and PPT, but not 17β-E2, only in the control men. The absence of this NO component of relaxation in CHD men supports several previous reports (1, 6, 24), indicating a reduction of basal and stimulated release of bioactive NO. However, because the total relaxation to these agents was similar between groups, it would appear that on upregulation of other NO-independent dilatory mechanisms must occur as a compensatory response. Overall, the results may offer potential for benefit pertaining to dietary supplementation with phytoestrogens in men.

The phytoestrogens tested evoked relaxation within the nanomolar/micromolar range. These concentrations are relevant to those achievable through diet or supplements. Whereas plasma concentrations of genistein are in the nanomolar range (<40 nM/l) in humans consuming soy-free diets, values rapidly rise to the micromolar range (7–8 μM/l) when soy is consumed in the diet (e.g., East Asian populations and vegetarians) or as supplements (e.g., postmenopausal women) (9). The 20–60 μM or higher concentrations of resveratrol found in red wine are reported to be dependent on storage conditions (15, 20). Oral administration of resveratrol in doses corresponding to reasonable wine intake is associated with concentrations of resveratrol in plasma similar to those shown to evoke vascular relaxation in this study (8). The vasodilatation induced by genistein and resveratrol in the present study may therefore have relevance for in vivo cardiovascular effect.

Previous studies (7, 12, 22, 30, 34) have identified both ER subtypes in vascular endothelium and smooth muscle in a wide range of blood vessels in different vascular beds and from different species. In this study, the dilatory actions of PPT, a selective ERα agonist, and genistein, a predominant ERβ agonist, were enhanced compared with those to 17β-E2, a mixed agonist, or to resveratrol. PPT has a 410-fold binding affinity preference for ERα versus ERβ (21). It has been shown that PPT failed to activate ERβ even at the highest supraphysiological concentrations (21), and it evoked greater relaxation than did 17β-E2 in small mesenteric arteries from male rats (38).

Genistein is similar in structure to 17β-E2 but has a higher affinity for ERβ and is considered to be the best natural ligand for ERβ (5). In isolated rat aorta, genistein has greater potency than 17β-E2, albeit at a concentration of 10 μM (37), and is also at least 10-fold more effective that 17β-E2 in prevention of endothelial damage by oxidized low-density lipoprotein (14). The observation that resveratrol was less potent than genistein in evoking relaxation suggests that this ligand might differ from other phytoestrogens in receptor binding affinity. Indeed, controversy exists as to whether resveratrol acts predominantly through ERβ (27) or has mixed agonist/antagonist actions on both receptors, depending on the cell type or vascular bed studied (10). Thus, from our data and previous reports, it could be suggested that occupancy of either receptor subtypes evokes vasodilatation and that ligands with higher selectivity for one ER subtype achieve a greater biological response than those with similar affinity for both (16, 35).

The underlying cellular mechanisms by which ER occupancy can lead to rapid NOS are increasingly recognized (23, 32). Subpopulations of both ERα and ERβ colocalize with the caveolae in endothelial cells, and agonist binding rapidly stimulates endothelial cells [endothelial NOS (eNOS)], thus implying a nongenomic response (12, 13, 36). The apparent lack of the NO component to 17β-E2-induced relaxation, in contrast to that obtained after selective stimulation of ERα by PPT and ERβ by genistein, in arteries from control men may therefore seem paradoxical (23, 32) and is a novel observation in respect to the human vasculature. This may indicate that the presence of ERβ apparently inhibits ERα-mediated NOS, suggesting interaction between ERs. A possible explanation may include the recognized “ying-yang” relationship between ERα
and ERβ reported in several cell lines, bone, and liver (35). When coexpressed, ERβ may act as a transdominant repressor on ERα and, in many instances, oppose or modulate the biological actions of ERα (35). Recently, our group (16) found supportive evidence in small arteries from wild-type male mice, in which the NO component was absent after stimulation with 17β-E2 but present in response to PPT, whereas in arteries from ERβ knockout mice, NO contributed to the rapid dilatation to 17β-E2. It might be also suggested that selective targeting of ER isoforms is more proficient to achieve a biological effect.

NO involvement in genistein-induced relaxation concurs with previous in vivo studies in the forearm vasculature of healthy men (46), as well as with other studies (28, 31, 37, 43) both in vivo and in vitro from different vascular beds in male animals. We have also previously shown that in healthy male subjects, flow-mediated dilatation of the brachial artery increases significantly after dealcoholized red wine intake; however, the underlying mechanisms behind this effect were unclear (2). To our knowledge, we are the first to report in human small arteries that the rapid dilatory response to resveratrol is NO mediated, although in endothelial cell culture, resveratrol increased eNOS expression and NOS (47) by rapidly activating MAPK signaling through both ERs (26) and led to NO-mediated relaxation of male rat aorta (12a, 13).

The dilatory responses after stimulation with estrogenic compounds in arteries from CHD patients versus controls were, however, characterized by a lack of NO contribution. Because NO has many known antiatherogenic properties (11), its absence in these responses may cause an individual to become prone to atherosclerosis. Compensatory mechanisms, however, were apparent, because the absolute vasodilator responses to these compounds were not different from controls. Although we have not investigated the compensatory mechanisms involved, these might include modulation of smooth muscle ion channels and intracellular calcium dynamics (3, 18, 38, 45a), AMP-activated signaling pathways (28, 31), and the involvement of endothelium-derived hyperpolarizing factor (40, 41, 48). In vivo, this would reduce peripheral vascular resistance, reduce blood pressure, and improve distribution of blood flow. Additional known properties of phytoestrogens, including reduction of reactive oxygen species generation, interference with LDL, inhibition of granulocyte and monocyte adhesion to endothelium, and platelet aggregation, may corroborate their acute dilatory action (15) in in vivo situations and provide cardiovascular protection.

In conclusion, this study has shown that phytoestrogens ex vivo can evoke acute dilatation of the resistance vasculature from males with CHD and in control male subjects at concentrations attainable in vivo with moderate red wine consumption and/or consumption of soy-derived products in the daily diet. The study also suggests that estrogenic compounds, which have a higher affinity for a specific ER subtype, may have significant implications for selective targeting by dietary supplements in men. The contribution of NO to acute dilatory responses by estrogenic compounds is pertinent to arteries from healthy men, whereas other NO-independent dilatory mechanism(s) are involved in arteries from men with CHD. Future research is needed to yield better insights into the processes that occur in the vascular wall, and prospective intervention studies on dietary phytoestrogens are encouraged.

GRANTS

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