Effects of aglycone genistein in a rat experimental model of postmenopausal metabolic syndrome

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Abstract

Genistein aglycone, a soy derived isoflavone, has been demonstrated to be effective in reducing cardiovascular risk in postmenopausal women. We therefore investigated its effects in an experimental model of postmenopausal metabolic syndrome. Female spontaneously hypertensive obese rats (SHROB, $n=40$), a genetic model of syndrome X, and age-matched Wistar Kyoto (WKY, $n=40$) rats were used. A group of SHROB ($n=20$) and WKY ($n=20$) animals were ovariectomized (OVX). Four weeks after surgery all animals were randomized to receive either genistein (54 mg/human equivalent dose/day for 4 weeks), or vehicle. Body weight, food intake, systolic blood pressure (SBP), heart rate, plasma glucose, insulin resistance (HOMA-IR), total plasma cholesterol and triglycerides, and uterine weights were studied. Furthermore, we investigated acetylcholine- and sodium nitroprusside-induced relaxation of aortic rings as well as NG-*L*-arginine (L-NMA: 10–100 mM) induced vasoconstriction in phenylephrine-precontracted aortic segments. Liver expression of

the peroxisome proliferator-activated receptor alpha (PPARA and gamma (PPARG was also assessed. OVX animals had a slight increase in SBP, body weight, insulin resistance, and plasma cholesterol. OVX-SHROB rats showed also impaired endothelial responses, blunted L-NMA induced contraction (L-NMA 100 mM, WKY = 2.2 ± 0.3 g/mg tissue; OVX-SHROB = 1.1 ± 0.4 g/mg tissue). Genistein treatment decreased SBP and plasma lipids, ameliorated endothelial dysfunction and insulin resistance, increased HDL cholesterol, and enhanced liver expression of PPARA and PPARG. Our data suggest that genistein is effective in ameliorating cardiovascular profiles in an experimental model of postmenopausal metabolic syndrome, attenuating the features of this disease. The effects of genistein are likely mediated by PPARA and PPARG receptors. This evidence would support the rationale for some pilot clinical trials using genistein in postmenopausal women affected by metabolic syndrome.

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Introduction

The metabolic syndrome consists of a combination of risk factors that include abdominal obesity, atherogenic dyslipidemia, hypertension, and insulin resistance. It increases the risk of cardiovascular disease and type 2 diabetes (Alexander & Clearfield 2006, Rosano *et al.* 2006, Sinagra & Conti 2007). The increased risk of cardiovascular disease is higher in women than in men. Menopause heralds a decline in circulating estrogen levels, which may increase cardiovascular risk through effects on adiposity, lipid metabolism, and prothrombotic state. It has been suggested that all these modifications may result in a menopausal metabolic syndrome, as many of the risk factors are more prevalent in postmenopausal women. The key elements involved in managing the metabolic syndrome are dietary and lifestyle

modification. Appropriate treatment may also include managing individual cardiac risk factors with the use of antihypertensive and lipid-modifying agents among others. Also, estrogen replacement improves insulin sensitivity and reduces the risk of diabetes (Schneider *et al.* 2006). Estrogens positively affect lipid profile, increase production of nitric oxide, decrease production of endothelin, and reduce intracellular calcium in arterial smooth muscle (Bracamonte & Miller 2001). However, the results of the heart and estrogen/progestin replacement study (HERS) trial (Hulley *et al.* 1998), the HERS II study (Grady *et al.* 2002), and the Women's Health Initiative (Rossouw *et al.* 2002) indicated that estrogens are not useful in decreasing the risk for cardiovascular accidents. However, more recently revisions of these trials suggested a potential beneficial effect of estrogens in early postmenopausal women. These observations have

prompted researchers to search for alternative strategies to manage all postmenopausal symptoms; genistein seems one of the most promising alternative therapies for postmenopausal women. Genistein is an isoflavone that has received a great deal of attention over the last few years because of its potential in the prevention of today's most prevalent chronic diseases, namely cardiovascular diseases, osteoporosis and hormone related cancers (McCarty 2006). Genistein *in vitro* relaxes rat arteries by a nitric oxide-dependent mechanism (Li *et al.* 2004) and enhances the dilator response to acetylcholine (ACh) of atherosclerotic arteries (Honoré *et al.* 1997). Genistein *in vivo* improves endothelial dysfunction induced by either oophorectomy (Squadrito *et al.* 2000) or genetic substrate in rats (Vera *et al.* 2007) and reduces infarct size in an experimental model of myocardial ischemia-reperfusion injury (Deodato *et al.* 1999). Furthermore, our group demonstrated, in double-blind randomized clinical trials, that genistein in healthy postmenopausal women increases plasma nitric oxide breakdown products, reduces endothelin-1 levels, improves endothelial-dependent vasodilation (Squadrito *et al.* 2002, 2003) reduces homocysteine and C-reactive protein levels (D'Anna *et al.* 2005) and shows a favorable effect on hot flushes (Crisafulli *et al.* 2004, D'Anna *et al.* 2007) and some cardiovascular risk markers in postmenopausal women (Crisafulli *et al.* 2005, Atteritano *et al.* 2007). In addition, genistein has been demonstrated to activate peroxisome proliferator-activated receptors PPARs (Dang *et al.* 2003) a subgroup of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are critical in regulating insulin sensitivity, adipogenesis, lipid metabolism, inflammation, and blood pressure. Synthetic peroxisome proliferator-activated receptors (PPARs) agonists, such as fibrates and thiazolidinediones are already used to treat metabolic disturbances such as hyperlipidemia and diabetes mellitus, both typical features of metabolic syndrome (Bragt & Popeijus 2008). The spontaneously hypertensive obese rats (SHROB) are a unique strain, with genetic obesity, hypertriglyceridemia, hyperinsulinemia, renal disease, and genetically determined hypertension. All these characteristics offer a useful animal model of metabolic syndrome (Ernsberger *et al.* 1999a). In addition, ovariectomy in the SHROB strain may also resemble the characteristic features of a metabolic syndrome occurring in menopause and it might be a useful tool to investigate new pharmacological treatments. However, the SHROB are a genetic model, thus the alterations observed in this strain following ovariectomy are more severe than those observed in human postmenopausal metabolic syndrome. Moreover, postmenopausal women, unless surgically ovariectomized (OVX), maintain the ovaries, thus the OVX-SHROB could be considered as a good, but not perfect model of the human condition. Since genistein has been addressed as a possible safe therapeutic alternative for the treatment of menopausal cardiovascular complications (Altavilla *et al.* 2004); it could be hypothesized also a role for this isoflavone in postmenopausal metabolic syndrome. To date, *in vivo* studies

underlying the role of genistein aglycone in established metabolic syndrome in a low estrogenic environment are still lacking. In an effort to clarify this issue, the aim of the present paper was to investigate whether genistein aglycone might exert beneficial effects on blood pressure, lipid, and glucidic profile in OVX-SHROB rats, and if the metabolic effects could be in part due to genistein's interaction with PPARs.

Methods

Animals

All procedures were evaluated and approved by the Ethics committee of the University of Messina and the study was conducted in accordance with Guide for the Care and Use of Laboratory Animals as published by the National Institutes of Health in 1985, and with The UFAW Handbook on the Care and Management of Laboratory Animals published by Blackwell Science. Female SHROB ($n=40$) and Wistar Kyoto (WKY; $n=40$) rats aged 6 months were purchased by Charles River, Italy. Of these animals 20 SHROB and 20 WKY were bilaterally OVX. Rats were maintained under standard conditions with access to low soy protein content (less than 8%) laboratory food (Mucedola, Italy) and tap water *ad libitum* for the duration of the experiment. Animals were housed one per cage and the day/night light cycle was 12 h. After 4 weeks of acclimation both OVX and intact rats were randomized, into eight groups of ten animals each, to receive genistein aglycone (Sigma) at the human equivalent dose of 54 mg/day (~ 4.8 mg/kg for a rat of 250 g) or its vehicle (5 g carboxymethylcellulose, 5 ml benzyl alcohol, 9 g NaCl, 4 ml Tween 80 in 1000 ml distilled water) for 4 weeks. Both genistein and vehicle were prepared daily and administered by oral gavages in a volume of 200 μ l. After the glucose tolerance test, animals were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg), blood was collected from the mesenteric artery and livers and uteri were removed for further analysis.

Evaluation of body weight, food intake, and glucose tolerance test

Body weight and food intake were monitored every week from the beginning to the end of the experiment. Body weight results were expressed compared with food intake, by using the following formula: food intake (g)/body weight (g) $\times 100$. Oral glucose tolerance tests were carried out at the end of the treatment period as described previously (Ernsberger *et al.* 1999b, Velliquette *et al.* 2002, Velliquette & Ernsberger 2003). All rats were fasted for 18 h and administered a 50% glucose solution in water by gavages at a dose of 6 g/kg body weight. Blood samples (0.4 ml) were collected under local anesthesia (lidocaine) applied from the tail into heparinized capillary tubes at 0, 30, 60, 90, 120, 180, and 240 min.

Uterine weight

At the end of the experiment the uteri of all animals were removed immediately after perfusion fixation and weighed.

Real time PCR for peroxisome proliferator-activated receptor alpha (PPARA and gamma PPARC mRNA)

Total RNA was extracted from liver by using a commercial kit (Trizol Reagent, Invitrogen) according to the manufacturer's instruction and spectrophotometrically quantified (Bio-photometer, Eppendorf, USA). Total RNA was then treated with DNaseI to digest residual DNA contamination, subsequently 5 µg total RNA were reverse transcribed using High capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) and random primers according to the manufacturer's instruction. Ten micrograms of total cDNA were used, to quantify the amount of PPARA and PPARC cDNA by real time polymerase chain reaction method (Real-Time PCR), and β-actin cDNA as endogenous control. Both reactions were carried out in the same microwell (biplex) with Taq Man Universal PCR master mix and Assays on Demand ready to use primers and probes with different reporter dye (Applied Biosystems).

The progression of PCRs was monitored by 7300 Real Time-PCR System (Applied Biosystems), and the relative quantification was determined by standard curve method for both target and endogenous reference. The results were expressed as an *n*-fold difference relative to endogenous control (relative expression levels).

Determination of plasma cholesterol, triglycerides, insulin, glucose, and leptin

Blood cholesterol was determined by an enzymatic cholesterol esterase/cholesterol oxidase using an automatic analysis technique on a chemical analyzer. For determination of high density lipoprotein (HDL) and low density lipoprotein (LDL) fractions of cholesterol, HDL was selectively precipitated from plasma using the method of [Vikari \(1976\)](#) by adding 0.1 ml of a 10% polyethylene glycol 6000 solution in glycine buffer (pH 10) to 0.1 ml plasma. After incubation at room temperature for 10 min and on ice for 20 min, samples were centrifuged at 3400 *g* for 20 min. Supernatants were assayed for cholesterol by the colorimetric enzymatic method. We defined HDL cholesterol as total cholesterol minus LDL cholesterol, determined after selective precipitation. Plasma triglycerides were measured by the use of a commercial kit (Sigma).

Glucose was measured in whole blood by colorimetric glucose oxidase assay (One-Touch; Lifescan, Milpitas, CA, USA). The remaining blood sample was centrifuged for 20 min at 5000 *g* at 4 °C, and the plasma frozen at −80 °C until assayed for insulin. An insulin RIA kit was used with rat insulin standards and antibodies directed against rat insulin (Linco, St Louis, IL, USA). Assays were conducted in duplicate and the intraassay coefficient of variation was less than 5%.

HOMA-IR was calculated using the following formula: glucose (mMol) × insulin (µUI/ml)/22.5.

Leptin plasma concentration was determined with an ELISA kit (Linco Research). The assay was performed in duplicate and the intraassay coefficient of variation and assay sensitivity was 4.1% and 0.04 ng/ml respectively.

Systolic blood pressure and heart rate

Systolic blood pressure (SBP) and heart rate (HR) were measured by a non invasive method using a tail cuff (Ugo Basile, Biological Research Apparatus, 21025, Comerio, Varese Italy) at the beginning of the experiment and every week during the 4 weeks of treatment.

Vessel reactivity

At the end of the treatment period heparinized rats were euthanized with an overdose of sodium pentobarbital (75 mg/kg per i.p.) as reported previously ([Squadrito et al. 2000](#)). Thoracic aortas were removed and placed in cold Krebs solution of the following composition: NaCl 118.4 mM, KCl 4.7 mM, MgSO₄ 1.2 mM, CaCl₂ 2.5 mM, KH₂PO₄ 1.2 mM, NaHCO₃ 25.0 mM, and glucose 11.7 mM. Then, aortas were cleaned of adherent connective and fat tissue and cut into rings ~2 mm in length. Rings were then placed under 1 g tension in an organ bath containing 10 ml Krebs solution at 37 °C and bubbled with 95% O₂ and 5% CO₂ (pH 7.4).

All experiments were carried out in the presence of indomethacin (10 µM) in order to exclude the involvement of eicosanoids and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs solution at 15–20 min intervals and basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (PE, 100 nM). When the contraction was stable, the functional integrity of endothelium was assessed by a relaxant response to ACh (100 nM). The tissue was then washed occasionally for 30 min. Endothelium response was evaluated with cumulative concentrations of ACh (10 nM–1 µM) in aortic rings precontracted with phenylephrine (PE; 100 nM). Endothelium independent response was investigated by analyzing the relaxant effects of sodium nitroprusside (SN 15–30 nM) in endothelium denuded aortic rings.

Relaxation of the rings was calculated as a percentage decrease of contractile force. Some rings were precontracted with phenylephrine and then incubated with *N*^G-arginine (L-NMA 10–100 µM) and the results were expressed as g of tension × mg of tissue.

Genistein dose calculation

To calculate genistein dose we converted the HDE for genistein into animal equivalents using the following formula:

$$(\text{human dose in mg/m}^2) = (\text{km}) \times (\text{dose in mg/kg})$$

where human m^2 for a woman of 60 kg (considered as a medium weight) is 1.62 and km is the conversion factor (for a rat of 250 g is 7.0) as stated previously (Freireich *et al.* 1966).

Statistical analysis

Data are expressed as means $\pm$ s.d. Comparison between the means of the two groups was performed using two-tail ANOVA followed by Bonferroni's test and considered significant at the $P < 0.05$ level.

Results

Body weight and food intake

The progression of body weight and food intake during the treatment period is shown in Fig. 1A and B. At baseline, the SHROB were overweight compared with WKY animals. Ovariectomy resulted in an augmented body weight of both OVX-WKY and OVX-SHROB groups. All animals gained weight during the treatment period. OVX-SHROB treated with genistein gained less weight over the course of the study (17 ± 6 g) than OVX-SHROB treated with vehicle (32 ± 8 g, $P < 0.001$). Intact WKY rats gained considerably less weight (11 ± 9 g).

Despite a decreased rate of weight gain in the OVX-SHROB treated with genistein, no corresponding change in daily food intake was observed, and there was no difference in food intake between groups.

Figure 1 Food intake/body weight changes in animals during the 4 weeks of treatment. Values are expressed as means $\pm$ s.d.

Uterine weights

To further confirm that genistein has no effect on reproductive tissues we examined uterine weight at the end of the experiment. Uterine weight decreases after ovariectomy as observed in OVX-SHROB compared with control animals and genistein administration did not modify uterine weights in either OVX-SHROB or OVX-WKY animals (Fig. 2).

PPARA and PPARG mRNA expression in the liver

To understand the mechanisms underlying the decreased weight gain observed in OVX-SHROB treated with genistein we evaluated PPARA (Fig. 3A) and PPARG (Fig. 3B) mRNA expression in liver samples. SHROB animals had a greater mRNA expression of both PPARs due to their metabolic derangement when compared with WKY ($P = 0.01$ for both PPARs). In the WKY ovariectomy caused a significant reduction of PPARA ($P < 0.01$), while for OVX-SHROB there was a significant reduction only in PPARG expression ($P < 0.01$). Genistein treatment significantly increased both PPARA (5.2 ± 0.6 $P < 0.001$ versus vehicle treated OVX-SHROB) and PPARG (4.7 ± 0.7 $P < 0.001$ versus vehicle treated OVX-SHROB) gene expression in OVX-SHROB animals. In SHROB, WKY, and OVX-WKY animals, genistein produced a less but still significant increase in both PPARs mRNA expression ($P < 0.05$).

Plasma leptin levels

Plasma leptin levels (Fig. 3C) were evaluated at the end of the experiment. Vehicle-treated WKY animals showed normal leptin levels (0.9 ± 0.5 ng/ml) compared with SHROB animals treated with vehicle (5.3 ± 0.49 ng/ml). Ovariectomy resulted in an increase in leptin levels in both groups of animals treated with vehicle (OVX-WKY = 2.6 ± 0.38 and OVX-SHROB = 6.2 ± 0.7 ng/ml). Genistein treatment significantly decreased plasma leptin in OVX animals of both strains (OVX-WKY = 1.8 ± 0.41 and OVX-SHROB = 4.7 ± 0.4 ng/ml).

Plasma cholesterol and triglycerides levels

Plasma total cholesterol and triglycerides levels were markedly augmented in SHROB, OVX-SHROB, and OVX-WKY

Figure 2 Uterine weight in animals after 4 weeks of treatment. Values are expressed as means $\pm$ s.d. # $P < 0.0001$ versus intact animals of both strains.

Figure 3 Effects of genistein on liver mRNA expression of (A) PPAR α (B) and PPAR γ genes and (C) leptin levels in animals after 4 weeks of treatment. Values are expressed as means $\pm$ s.d. # $P < 0.0001$ versus OVX-SHROB + vehicle; * $P < 0.05$ versus WKY + vehicle; § $P < 0.05$ versus OVX-WKY + vehicle; ¶ $P < 0.05$ versus SHROB + vehicle; ** $P = 0.01$ versus WKY + vehicle.

rats compared with WKY animals. At the end of the experiment, triglycerides, total HDL and LDL cholesterol levels were significantly reduced in genistein treated rats compared with the vehicle-treated animals of each strain (Table 1). In WKY animals slight modifications in both triglycerides and cholesterol levels were observed with a moderate increase in HDL cholesterol (p not significant).

Plasma glucose and insulin after glucose challenge

Oral glucose tolerance test was performed at the end of the experiment. Despite fasting normoglycemia, both SHROB and OVX-SHROB animals showed elevated blood glucose

levels following a glucose load at all time points tested, confirming that menopausal status worsened the MS features. Insulin levels in response to a glucose load reached peak values at 240 min in either SHROB or OVX-SHROB, while normally the peak is 60 min following glucose administration as observed in WKY animals (Fig. 4A and B). OVX-WKY reached insulin peak at 90 min. These data confirm that OVX-SHROB are extremely insulin resistant, as confirmed by HOMA-IR (Table 1), and mildly glucose intolerant compared with normal animals. Genistein administration significantly reduced glucose levels and insulin response (Fig. 4C) decreasing the time of plasma peak from 240 to 120 min in SHROB and OVX-SHROB ameliorating insulin resistance (Fig. 4A and B).

Table 1 Effects of genistein on HOMA-IR and plasma lipids. HOMA-IR triglycerides and cholesterol levels were evaluated after 28 days of genistein treatment. Total cholesterol was compared with LDL cholesterol, after selective precipitation of HDL by standard methods

Treatment	HOMA-IR	Triglycerides (mg/dl)	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)
WKY + vehicle	2.57 $\pm$ 0.4	25.72 $\pm$ 3.22	66 $\pm$ 10	44 $\pm$ 7	17 $\pm$ 5
WKY + genistein	1.50 $\pm$ 0.53	22.84 $\pm$ 2.25	62 $\pm$ 9	46 $\pm$ 5	11 $\pm$ 4
OVX-WKY + vehicle	6.8 $\pm$ 0.3	30.25 $\pm$ 3.14	70 $\pm$ 5	40 $\pm$ 2	25 $\pm$ 6
OVX-WKY + genistein	5.22 $\pm$ 0.8*	26.31 $\pm$ 2.54	65 $\pm$ 8	43 $\pm$ 6	20 $\pm$ 4
SHROB + vehicle	54.7 $\pm$ 2.1	38.22 $\pm$ 2.21	115 $\pm$ 8	53 $\pm$ 5	60 $\pm$ 4
SHROB + genistein	26 $\pm$ 1.2*	30.10 $\pm$ 3.15*	98 $\pm$ 6	54 $\pm$ 7*	20 $\pm$ 7*
OVX-SHROB + vehicle	73.3 $\pm$ 1.6†	45.35 $\pm$ 3.14†	128 $\pm$ 12	55 $\pm$ 6	64 $\pm$ 7†
OVX-SHROB + genistein	34.2 $\pm$ 1.1*	33.25 $\pm$ 2.54*	92 $\pm$ 10*	68 $\pm$ 8†	18 $\pm$ 8*

* $P < 0.0001$ versus vehicle; † $P = 0.004$ versus OVX-SHROB + vehicle; ‡ $P < 0.05$ versus SHROB + vehicle.

Figure 4 (A) and (B) Glucose and (C) insulin levels in study animals following oral glucose challenge after 4 weeks of treatment. Values are expressed as means $\pm$ s.d. # $P < 0.0001$ versus OVX-SHROB + vehicle.

SBP and HR

Elevated blood pressure is one of the features of MS; in fact, the SHROB rats are markedly hypertensive compared with WKY rats (Fig. 5). Ovariectomy caused an increase in blood pressure in both strains. Daily genistein supplementation caused a significant decrement in the SBP and heart rate throughout the study in SHROB ($P < 0.05$) and OVX-SHROB rats ($P < 0.001$). No significant change was observed in intact WKY, while a significant reduction was observed in OVX-WKY ($P < 0.05$).

Figure 5 (A) Systolic blood pressure and (B) heart rate changes in rats during the 4 weeks of treatment. Values are expressed as means $\pm$ s.d. * $P < 0.05$ versus WKY + vehicle; § $P < 0.05$ versus OVX-WKY + vehicle; # $P < 0.0001$ versus OVX-SHROB + vehicle.

Vessel reactivity

Endothelium-dependent response (ACh: ACh; 10 nM–10 μ M) and endothelium-independent relaxation (sodium nitroprusside: SN 15–30 nM) of thoracic aortas precontracted with phenylephrine (PE 100 nM) were studied at the end of treatment. SHROB animals suffering from metabolic syndrome show impaired vessel reactivity and this status was exacerbated by ovariectomy. Also WKY animals after ovariectomy showed some impairment in vessel reactivity.

The effect of sodium nitroprusside was investigated in endothelium denuded aortic rings. SHROB and OVX-SHROB rats had a marked reduced relaxant effect of ACh (Fig. 6) while the relaxant effect caused by sodium nitroprusside was unchanged (Fig. 6). Genistein supplementation did not modify the relaxant effects of either ACh or sodium nitroprusside in WKY rats (Fig. 6). By contrast, genistein treatment succeeded in improving the impairment in endothelium-dependent relaxation of SHROB, OVX-SHROB, and OVX-WKY rats (Fig. 6).

Addition of N^G -arginine (L-NMA 10–100 μ M) to the organ bath caused a significant contraction of the aortic rings precontracted with phenylephrine (Fig. 7). L-NMA induced vasoconstriction was significantly blunted in SHROB and OVX-SHROB rats when compared with WKY animals. Genistein supplementation markedly increased the constrictor response elicited by L-NMA in OVX-SHROB, OVX-WKY, and SHROB, but did not change L-NMA induced responses in WKY rats (Fig. 7).

Figure 6 Relaxant effect of both acetylcholine in thoracic aortas and of sodium nitroprusside in endothelium denuded aortic rings precontracted with phenylephrine (100 nM) obtained from all animals at the end of treatment. Values are expressed as means $\pm$ s.d. # $P < 0.0001$ versus OVX-SHROB + vehicle and SHROB + vehicle.

Figure 7 Effects of L-NMA in aortic rings (precontracted with phenylephrine 100 nM) obtained from all animals at the end of treatment. Values are expressed as means $\pm$ s.d. # $P < 0.0001$ versus OVX-SHROB + vehicle and SHROB + vehicle. * $P < 0.05$ versus OVX-WKY + vehicle.

Discussion

The SHROB rat carries a nonsense mutation in the leptin receptor (fa^K), propagated on a spontaneously hypertensive background (Ishizuka *et al.* 1998). The SHROB shows multiple metabolic phenotypes, including abdominal obesity, spontaneous hypertension, hyperinsulinemia, and hyperlipidemia without fasting hyperglycemia (Ernsberger *et al.* 1993, 1994, Koletsky *et al.* 1995). These features closely resemble those found in the human metabolic syndrome X. In addition, ovariectomy in the SHROB can generate the characteristic features of a metabolic syndrome occurring in menopause and it might be a useful tool to investigate new pharmacological treatments.

Other papers showed some positive effect of soy proteins and isoflavone intake (Davis *et al.* 2005, 2007) or soy-rich diets (Dyrskog *et al.* 2005) in other animal models of metabolic syndrome; however, the exact intake of genistein has not been evaluated, since genistein is mostly present as genistin (the glycoside) the plasma levels of genistein in this studies could largely differ depending on gut absorption and microflora (which is responsible for aglycone conversion), in light of these observations it is rather difficult to compare those results with the present paper.

The aim of our study was to study the effects of aglycone genistein in a well-established experimental model of metabolic syndrome (SHROB) and to evaluate the additional influence of a menopause status (OVX versus intact animals). Therefore, it was necessary to identify the contribution of menopause status 'per se' on the typical risk factors of metabolic syndrome. Since the SHR strain already has

some of the typical alterations of the metabolic syndrome (i.e., hypertension; insulin resistance; impaired vessel reactivity), the normotensive WKY strain was preferred as control.

In the present paper, we demonstrated *in vivo* for the first time positive effects of genistein aglycone in experimental postmenopausal metabolic syndrome, these results correlate very well with our previously reported observations in randomized clinical trials in healthy postmenopausal women (Crisafulli *et al.* 2005, Atteritano *et al.* 2007). We chose to use the dose already shown to be effective in clinical trials and we translated the human dose into animal equivalents (Crisafulli *et al.* 2005, Atteritano *et al.* 2007). Indeed, the dose of 54 mg/day of aglycone genistein provides the same plasma levels achieved by eastern population, as highlighted by McCarty (2006) in his recent review of the available literature. Since the effects of genistein are in part mediated by estrogen receptors (ERs) but in part independent, the use of a proper dose is of fundamental importance to obtain the expected results; in fact, at higher doses a stimulation of ESR1 could account for effects, and side effects, typical of estrogens, while at lower doses an insufficient stimulation of ESR2 may occur with few or absent beneficial effects. Additionally, to compare the age of rats with postmenopausal women we used 6 month old animals and then we started the experiment after 1 additional month in order to have an established menopausal status.

Our results suggest a marked effect of genistein in decreasing SBP as well as heart rate in the experimental model of postmenopausal metabolic syndrome, maintaining a high safety profile as demonstrated by the unchanged uterine weight, and confirming that genistein at this dose does not cause any ESR1 mediated effect on the reproductive tract. OVX SHROB rats developed a typical endothelial dysfunction resembling that observed in postmenopausal women with metabolic syndrome. SHROB animals show all the features of metabolic syndrome, nevertheless ovariectomy exacerbates the symptoms. More specifically OVX-SHROB had a markedly reduced relaxant response to ACh, an endothelium and NO-dependent relaxant agent and unchanged response to sodium nitroprusside, an endothelium and NO-independent relaxing compound. In addition, aortic rings showed a reduced contraction in response to L-NMA, an inhibitor of NO synthase. Genistein supplementation succeeded in reverting the impaired endothelial dysfunction observed in hypertensive OVX rats with metabolic syndrome. This isoflavone was able to restore the endothelium response likely through an increased production of basal endothelial NO release, as we previously demonstrated (Squadrito *et al.* 2000); in fact, genistein treatment increased the constrictor response elicited by L-NMA in OVX-SHROB. This strongly supports the idea that the isoflavone treatment was able to enhance the depressed NO pathway in particular conditions such as metabolic syndrome during menopause.

It has already been reported (Velliquette *et al.* 2002) that SHROB, despite fasting normoglycemia, are hyperinsulinemic and glucose intolerant compared with WKY animals.

In our study, the OVX SHROB confirmed these findings. In addition to and consistent with our previous observations in postmenopausal women taking 54 mg genistein daily (Atteritano *et al.* 2007), genistein administration at the HED of 54 mg lowered fasting serum glucose and ameliorated insulin resistance in OVX-SHROB rat; a model of postmenopausal metabolic syndrome. Furthermore, in intact WKY animals genistein did not significantly affect lipid profile, as previously reported in women (Squadrito *et al.* 2000); by contrast, we observed a significant decrease in total cholesterol and LDL cholesterol in OVX-SHROB. All these beneficial effects on lipid and glucose metabolism together with the observed decrease in body weight (but not in food intake) suggest a PPAR-stimulating effect of this soy isoflavone; in fact, it has been reported that genistein affects both PPAR α - and PPAR γ -directed gene expression (Dang *et al.* 2003, Mezei *et al.* 2003, Kim *et al.* 2004, Bragt & Popeijus 2008). This hypothesis was confirmed by the significantly augmented mRNA expression levels of PPAR α and PPAR γ in the liver of treated animals. Furthermore, a reduced mRNA expression of both PPARs due to ovariectomy as already reported by other authors (Paquette *et al.* 2008) was observed. Since PPARs are thought to play a prominent role to prevent dyslipidemia and maintain metabolic homeostasis, the great importance of their increased expression for the treatment of this pathological condition is clear. Moreover, genistein is known to act as an ER agonist in a low estrogenic environment and as antagonist in an estrogenic milieu, in this latter condition genistein acts as a ligand for PPAR γ determining antiestrogenic effects leading to adipogenesis (Dang *et al.* 2003). Activation of ERs by genistein could down-regulate PPAR γ transcriptional activity and vice versa. The balance between the activation of ERs and PPAR γ is concentration related and this may account for the different magnitude of the results we obtained, in fact, genistein was more effective in OVX SHROB animals than in intact rats. These data, together with the observation that pioglitazone and rosiglitazone are efficacious in the treatment of type 2 diabetes mellitus by maintaining plasma glucose and delaying the onset of long-term complications (Yki-Jarvinen 2004), with modest or even negative effects on blood lipid variables in patients with diabetes (Van Wijk *et al.* 2003), would suggest a potential role for genistein as an antidiabetic agent. In addition, in WKY rats we observed a slight amelioration of the study parameters, thus it could also be hypothesized that genistein could play a role in the treatment of premenopausal women at high risk of developing the metabolic syndrome after menopause, or in borderline conditions associated with a proper lifestyle, when is difficult for the physician to assess the real effectiveness of a 'strong' therapy.

In conclusion, the data obtained in this study suggest a role for genistein as a possible safe alternative for improving the cardiovascular and metabolic features related to menopausal metabolic syndrome. These observations need further preclinical and clinical evidence although there is a remarkable rationale that supports this idea.

Declaration of interest

The authors have nothing to declare.

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