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The soy isoflavone genistein induces a late but sustained activation of the endothelial nitric oxide-synthase system *in vitro*

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1 Cardiovascular diseases are known as the major causes of death or disability in western countries. Decreased bioavailability of endothelial derived nitric oxide (NO) is recognized as an important promoter in cardiovascular disease.

2 *In vivo* studies suggest that phytoestrogens, especially isoflavones from soy, enhance endothelium-dependent vasoreactivity.

3 We hypothesized that isoflavones may affect the expression of endothelial-type nitric oxide synthase (eNOS) and thereby NO formation *in vitro*.

4 Human EA.hy926 endothelial cells were treated with the soybean isoflavones biochanin A and formononetin and with their metabolites genistein and daidzein. eNOS promoter activity was examined by a luciferase reporter gene assay (20 h). Active eNOS was detected by quantifying conversion of L-arginine to L-citrulline and by measuring NO released from endothelial cells using the fluorescent probe DAF-2 (20–96 h).

5 eNOS promoter activity increased in response to isoflavone treatment (20 h). NO and L-citrulline production by EA.hy926 cells rose up to 1.7-fold of control levels after stimulation with genistein for 48–96 h. From these results, we conclude that the suggested positive effects of soy isoflavones on vascular reactivity may be indeed mediated *via* a long-term effect on the eNOS system.
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Abbreviations: ACE, angiotensin-converting enzyme; ADMA, asymmetrical dimethylarginine; DAF-2, 4,5-diaminofluorescein; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; HUVEC, human umbilical vein endothelial cell; PMA, phorbol-12-myristate-13-acetate

Introduction

Replacement of an animal protein-based diet by a soy protein-based diet reduces hyperlipoproteinemia and atherosclerosis (Meeker & Kesten, 1941; Kritchevsky, 1995). However, neither the components nor the mechanism(s) responsible for this effect are clearly defined. Besides studies showing the beneficial effects of soy protein on plasma lipid and lipoprotein concentrations (reviewed in Clarkson, 2002; Wagner *et al*., 2003; Yeung & Yu, 2003), evidence exists for lipoprotein-independent favorable effects implicating isoflavones as effective components. The latter include an antioxidant effect of soy isoflavones (Hwang *et al*., 2003; Jiang *et al*., 2003) as well as an influence of isoflavones on vascular function, such as vasodilation and arterial stiffness (for reviews, see Catania *et al*., 2002; Clarkson, 2002; van der Schouw *et al*., 2002; Steinberg *et al*., 2003; Teede *et al*., 2003).

A crucial vasodilator is endothelial nitric oxide (NO). NO is produced by the constitutively expressed enzyme endothelial nitric oxide synthase (eNOS) and is an essential factor of vascular homeostasis. Besides its activity as potent vasodilator, NO prevents intravasal coagulation and platelet aggregation, leukocyte adhesion to and immigration of inflammatory cells into the vessel wall as well as the release of chemokines. Furthermore, NO antagonizes the proliferation of smooth muscle cells. Reduced bioavailability of endothelium-derived NO and impaired reactivity of the vessel wall to the vasodilator acetylcholine are diagnostic markers in the development of atherosclerosis. Decreased levels of NO are detectable before alterations in the architecture of the vessel wall become manifested (Li & Förstermann, 2000).

Recent *in vivo* studies provided evidence that an application of soy isoflavones is able to restore impaired endothelial function probably by an increase in NO production (Honore *et al*., 1997; Catania *et al*., 2002; Squadrito *et al*., 2002; Cuevas *et al*., 2003).

Thus, it seems reasonable to hypothesize that isoflavones may mediate their antiatherogenic effect by directly influencing the eNOS system. Since the observed positive *in vivo* effects, which point to an influence of isoflavones on the eNOS system, were detected in response to isoflavone long-term exposure (4 weeks up to 6 months), the major aim of the study was to examine whether the soy isoflavones affect NO production after long incubation (20–96 h).

This hypothesis was addressed using the human endothelial cell line EA.hy926 (Edgell *et al*., 1983), which is a well-accepted human *in vitro* model expressing both eNOS and the estrogen

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receptor (ER, Kleinert *et al*., 1998; Bouis *et al*., 2001; Figtree *et al*., 2003; Haynes *et al*., 2003). Human eNOS gene promoter activity as well as NO and L-citrulline production were examined in response to soy isoflavones.

Methods

Cell culture

The human endothelial cell line EA.hy926 (kindly provided by Dr C.-J.S. Edgell, University of North Carolina, Chapel Hill, NC, U.S.A.) was grown in Dulbecco's modified Eagle's medium without phenol red supplemented with 584 mg ml^{-1} glutamine, 100 U ml^{-1} benzylpenicillin, 100 μg ml^{-1} streptomycin, HAT supplement (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) and 10% fetal bovine serum. To deplete estrogens, fetal bovine serum was incubated for 18 h at 4°C with charcoal, centrifuged and sterile filtered. For experiments, cells were seeded in six-well plates at a density of 0.4×10^6 cells per well. Stimulation of cells for the 4,5-diaminofluorescein (DAF-2) assay was performed exactly 3 days after seeding. In the L-arginine/L-citrulline conversion assay, cells were activated 4 days after seeding when they reached confluence. EA.hy926 cells stably transfected with a luciferase reporter gene construct as described below (kindly provided by Dr Paulus Wohlfahrt, Aventis, Germany) were cultivated as EA.hy926 cells, except medium supplementation with G418 400 μg ml^{-1}. Stably transfected cells were seeded into 24-well plates at a density of 0.5×10^5 cells per well. Experiments were performed exactly after 4 days when cells reached confluence. All cells were used up to passage 20–25 only. All substances were dissolved in DMSO and diluted into the culture medium 1 : 1000. Final DMSO concentration did not exceed 0.1%. Controls were treated with the respective vehicle.

Quantification of NO release

NO release by endothelial cells was measured using the DAF-2 fluorescence assay. The assay was performed as described previously (Leikert *et al*., 2001; Räthel *et al*., 2003).

[^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay

EA.hy926 endothelial cells were stimulated for 20 h up to 96 h as indicated. Cells were washed and equilibrated in HEPES buffer for 45 min. Then 0.32 μM [^{14}C]L-arginine (313 Ci mmol^{-1}) and, 10 min later, 1 μM of the calcium ionophore A23187 was added. After incubation for another 15 min, the reaction was stopped by lysing cells with ice-cold ethanol 96%. Cells were extracted with ethanol, then with ethanol/water (agitation). Extracts were unified and dried under vacuum. The extracts were resolved in water/methanol (1 : 1) and spotted on a thin-layer chromatography plate. [^{14}C]L-arginine was separated from [^{14}C]L-citrulline in the solvent system water : chloroform : methanol : ammonium hydroxide (1 : 0, 5 : 4, 5 : 2; v : v : v : v). The chromatography plates were dried and analyzed by a phosphoimager (Fujifilm BAS-1500).

Luciferase reporter gene assay

EA.hy926 endothelial cells, stably transfected with the plasmid p-eNOS-3500-Hu-Luc-neo (Li *et al*., 1998) containing 3500 bp of the human eNOS promoter driving a luciferase reporter gene, were stimulated for 20 h. Then cells were washed and lysed and the assay was performed following the manufacturer's instructions (Promega). Luminescence was detected with a luminometer (AutoLumat Plus, Berthold).

Chemicals and cell culture reagents

Chemicals: phorbol-12-myristate-13-acetate (PMA) was purchased from Calbiochem (San Diego, CA, U.S.A.). DAF-2 and the calcium ionophore A23187 were purchased from Alexis Biochemicals (Grünberg, Germany). G418 was bought from PAA Laboratories GmbH (Linz, Austria). [^{14}C]L-arginine (313 Ci mmol^{-1}) was purchased from NEN Life Science Products (Köln, Germany). 17-β-Estradiol was from Sigma (Taufkirchen, Germany). Genistein and daidzein were purchased from Acros Organics (Geel, Belgium). Formononetin and biochanin A were from Fluka (Deisenhofen, Germany). ICI 182,780 was delivered by Bio Trend (Köln, Germany). Glutamine was purchased from BioWhittaker Europe (Belgium), benzylpenicillin, and streptomycin from PAN Biotech (Germany). HAT supplement (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) was bought from Invitrogen GmbH (Karlsruhe, Germany). Fetal bovine serum was purchased from Life Technologies (Germany). The luciferase assay system was obtained from Promega (Madison, WI, U.S.A.).

Statistical analysis

Statistical analysis (one-way ANOVA with Bonferoni post test) was performed using GraphPad Prism version 3.0 (GraphPad software).

Results

Isoflavones increase the promoter activity of the human eNOS gene

To examine whether isoflavones affect the human eNOS promoter, genistein, daidzein, formononetin and biochanin A were employed to stably transfected EA.hy926 cells containing a 3.5 kb fragment of the human eNOS gene promoter driving a luciferase reporter gene. Cells were stimulated for 20 h with isoflavones 1 and 10 μM, concentrations detected to be most effective in dose–response studies, or with 17-β-estradiol at concentrations of 1, 3, 10 and 30 nM. After stimulation, cells were harvested, lysed and the luciferase activity determined. As can be seen in Figure 1a, all soy isoflavones were able to increase the luciferase activity in a dose-dependent manner up to 2.5-fold. Concentrations below 1 μM were ineffective (data not shown). Concentrations above 50 μM showed cytotoxicity (data not shown). In contrast, 17-β-estradiol had no effect on eNOS promoter activity at the investigated concentrations (1–30 nM) (Figure 1b).

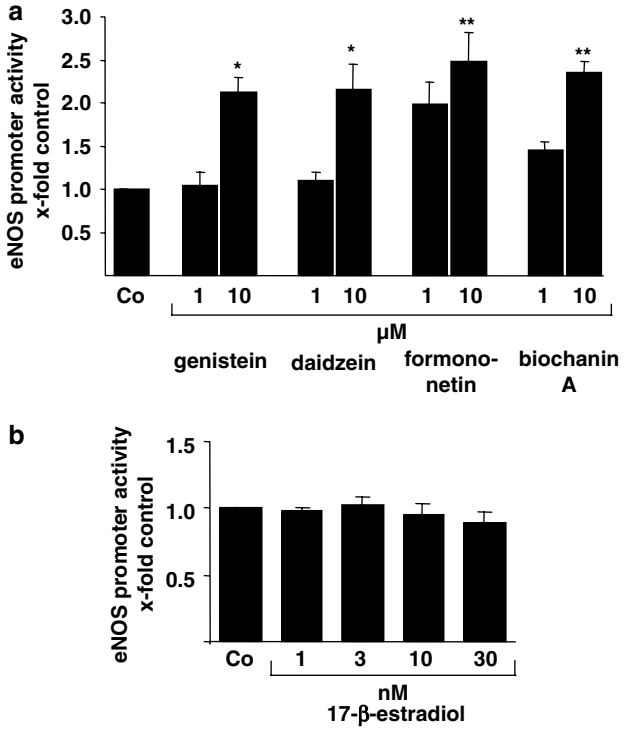

Figure 1 Soy isoflavones increase the promoter activity of the human eNOS gene. EA.hy926 cells containing a 3500 bp fragment of the human eNOS promoter driving a luciferase reporter gene were incubated with the soy isoflavones genistein, daidzein, biochanin A and formononetin at concentrations of 1 and 10 μM (a) or with 17-β-estradiol at concentrations of 1, 3, 10 and 30 nM, (b) for 20 h. Luciferase activity normalized by the activity of vehicle-treated control cells was taken as an indicator for eNOS promoter activity. Bars represent mean$\pm$s.e.m. of three independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (ANOVA/Bonferoni).

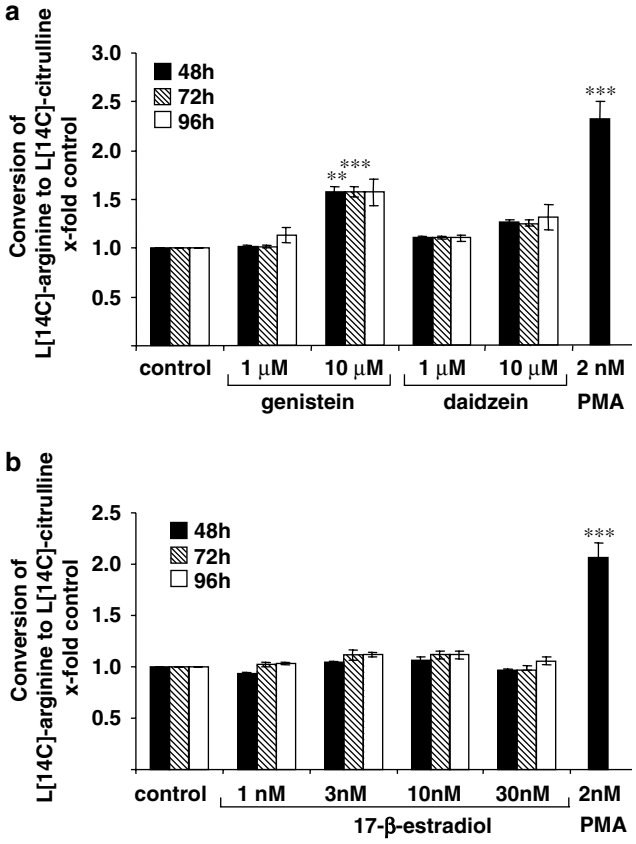

Figure 2 Genistein enhances L-citrulline production by human endothelial cells. The conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline is an indicator of eNOS protein activity. EA.hy926 cells were stimulated with isoflavones (a) or 17-β-estradiol (b) as indicated for 48–96 h. PMA (2 nM, 24 h) served as positive control. [^{14}C]L-arginine (0.32 μM) was added and the assay performed as described under Methods. Amino acids were separated on a thin-layer chromatography plate and quantified by autoradiography. L-citrulline production normalized by the activity of vehicle-treated control cells was taken as indicator for eNOS enzyme activity. Bars represent mean$\pm$s.e.m. of three independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (ANOVA/Bonferoni).

Genistein enhances L-citrulline production in human endothelial cells

To examine whether the increased promotor activity results in more active eNOS, we measured L-citrulline production of EA.hy926 endothelial cells in response to isoflavones by the L-arginine/L-citrulline conversion assay. EA.hy926 cells were incubated with isoflavones (1 and 10 μM) for 20 h up to 96 h or with 17-β-estradiol (1, 3, 10 and 30 nM) and then exposed to [^{14}C]L-arginine. Produced [^{14}C]L-citrulline was separated by thin-layer chromatography and detected by autoradiography. PMA (2 nM, 24 h) served as positive control. None of the soy isoflavones altered eNOS-dependent L-citrulline formation up to 20 h of incubation (data not shown). However, between 48 and 96 h genistein led to an increase in [^{14}C]L-citrulline production up to 1.7-fold of control levels (Figure 2a), whereas 17-β-estradiol, daidzein, formononetin and biochanin A exhibited no significant effect (Figure 2a, b and data not shown).

Genistein significantly increases NO production after 48–96 h in human endothelial cells

To confirm the above result, we tested whether soy isoflavones are also able to affect NO levels released from endothelial cells. NO was quantified in cell culture supernatants of EA.hy926 endothelial cells stimulated with the isoflavones genistein, daidzein, biochanin A and formononetin (1 and 10 μM, 20 h) using the fluorescent probe DAF-2 (Leikert *et al*., 2001; Räthel *et al*., 2003). Isoflavone as well as 17-β-estradiol were further applied for 48–96 h. None of the isoflavones significantly increased the amount of NO released from endothelial cells up to 20 h of incubation (Figure 3a). Genistein, however, increased NO output up to 1.7-fold after long-term incubation of 48–96 h. Although daidzein showed a clear tendency to increase endothelial NO output, this effect did not reach significance (Figure 3b). Biochanin A revealed no effect (data not shown). Formononetin, unfortunately, showed a strong autofluorescence impeding a clear judgement as to whether it increases NO production or not. 17-β-Estradiol tended to increase NO production. The effect, however, was significant only at a concentration of 10 nM (72 h) (Figure 3c).

Discussion

In the present study, we show that soy isoflavones (1–10 μM) are able to enhance human eNOS promoter activity more than

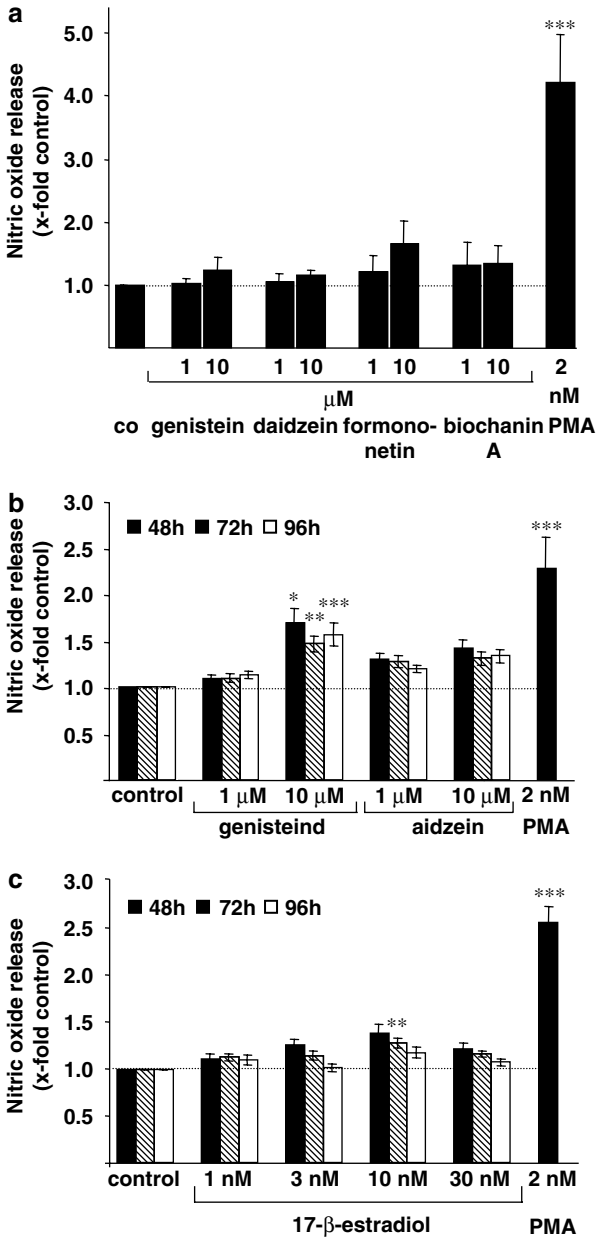

Figure 3 Genistein enhances NO output from endothelial cells. EA.hy926 cells were either treated with vehicle (co, control) or stimulated with the isoflavones as indicated for 20 h (a) or 48–96 h (b). For comparison, EA.hy926 cells were activated with 17-β-estradiol (48–96 h) (c). PMA (2 nM) was used as positive control. NO production was quantified using DAF-2 as described under Methods. The amount of released NO was normalized to that of the vehicle-treated control. Bars represent the mean$\pm$s.e.m. of three (20 h) or six (48–96 h) independent experiments performed in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (ANOVA/Bonferoni).

two-fold (20 h). Measuring NO formation and citrulline production in EA.hy926 human endothelial cells, we found that the genuine soy isoflavones, biochanin A and formononetin, were ineffective, whereas the metabolite genistein (10 μM) (Tolleson *et al*., 2002) increased eNOS activity up to 1.7-fold after prolonged incubation (48–96 h). Daidzein, as a metabolite of formononetin (Tolleson *et al*., 2002), tended to increase NO formation, although this observation did not reach significance. This outcome supports *in vivo* data pointing to an influence of isoflavones on the endothelial NO system (Honore *et al*., 1997; Catania *et al*., 2002; Squadrito *et al*., 2002; Cuevas *et al*., 2003). In contrast, 17-β-estradiol showed no (eNOS promoter activity) or little effect (NO production) in the same cell system.

The present study also supports data suggesting that mainly the isoflavone metabolites are mediating the protective effects attributed to soy isoflovones (Tikkanen & Adlercreutz, 2000).

Isoflavones belong to the group of phytoestrogens. They differ, however, strongly in their ability to bind to ERs: whereas genistein has a high affinity to the ERβ (87% of estrogen) and a low affinity to ERα (4% of estrogen), daidzein binds weakly to both ERα and ERβ (0.1 and 0.5% of estrogen, respectively; Kuiper *et al*., 1998). In the present study, genistein and daidzein showed the same activity on the human eNOS promoter. 17-β-Estradiol showed no effect at all. Thus, this effect seems to occur independently of the estrogenic activity of isoflavones. On the other hand, at the level of eNOS enzyme (L-citrulline and NO production), daidzein exhibited no significant effect, whereas genistein was active. This may implicate at least in part a possible ER-dependent activity of genistein. 17-β-Estradiol (10 nM) promoted NO production after 72 h of incubation, further supporting the possibility of an ER contribution to the isoflavone effect. The ER antagonist ICI 182,780, however, was not able to block the isoflavone-mediated effect on NO release and eNOS promoter activity (data not shown) counteracting this idea. A further argument against a role for ER are reports showing that under estrogen-deprived culture condition EA.hy926 cells express a 46 kDa membrane associated splice variant of the ERα. This truncated ERα exhibits only weak genomic activity, but mediates rapid intracellular signal responses (Russell *et al*., 2000; Figtree *et al*., 2003; Li *et al*., 2003). These reports fit well into our observation of an overall weak long-term 17-β-estradiol response in EA.hy926 cells. Alltogether, our data suggest, if at all, only a small contribution of the ER to the effects on eNOS observed for isoflavones.

Recently, the human estrogen-related receptor α1 (ERRα1) was shown to upregulate eNOS expression (Sumi & Ignarro, 2003). The ERRα1-mediated transactivation of the eNOS-dependent luciferase activity in BPAEC was not inhibited by ICI 182,780. Furthermore, effects on the protein levels were seen late, between 48 and 72 h. Thus, we cannot exclude that the isoflavone effects are mediated at least in part by ERRα1 reported to be expressed in endothelial cells (Sumi & Ignarro, 2003). Studies focusing on this hypothesis are under way.

In contrast to daidzein, genistein is also known as potent tyrosin kinase inhibitor (Akiyama & Ogawara, 1991). Human eNOS is reported to be tyrosin phosphorylated. eNOS tyrosin phosphorylation affects its activity, probably by influencing the docking of regulatory proteins (for a review, see Flemming & Busse, 2003). In addition, Hsp90 functioning as a scaffold for eNOS and Akt kinase, which activates eNOS by phosphorylating Ser 1177, seems to be tyrosin phosphorylated in response to agonists (Flemming & Busse, 2003). Moreover, the protein tyrosine kinase c-src was shown to be critically involved in eNOS activation and expression in several experimental settings (Davis *et al*., 2003; Haynes *et al*., 2003). Thus, tyrosine kinase inhibition as described for genistein is supposed to negatively regulate eNOS. In our human endothelial cells, however, genistein is the most active

isoflavone with respect to eNOS activation, suggesting that a tyrosine kinase inhibitory activity most likely does not contribute to the behavior of the tested isoflavones.

Several *in vivo* studies strongly suggest an impact of isoflavones on the eNOS system (Honore *et al*., 1997; Catania *et al*., 2002; Squadrito *et al*., 2002). In our human *in vitro* model, we were able to implicate a positive influence of isoflavones on NO output from endothelial cells. These data fit also well into a study detecting increased levels of NO oxidation products in the plasma of isoflavone-treated post menopausal woman (Squadrito *et al*., 2002). Scavenging of reactive oxygen species due to the antioxidative activity of soy isoflavones may further contribute to the increased bioavailability of physiologically active NO (Hwang *et al*., 2003; Jiang *et al*., 2003).

In conclusion, this study clearly demonstrates for the first time that soy-derived isoflavones do enhance eNOS promoter activity and NO output from endothelial cells after long-term administration *in vitro*. Thus, the vasorelaxing properties attributed to these compounds are likely to be the result of an effect on the eNOS system that is, if at all, only partly mediated *via* the ER.

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