Lipid Peroxidation and Nitric Oxide Inactivation in Postmenopausal Women

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Objective – To assess the effect of endogenous estrogens on the bioavailability of nitric oxide (·NO) and in the formation of lipid peroxidation products in pre- and postmenopausal women.

Methods – NOx and S-nitrosothiols were determined by gaseous phase chemiluminescence, nitrotyrosine was determined by ELISA, COx (cholesterol oxides) by gas chromatography, and cholesteryl linoleate hydroperoxides (CE\textsubscript{18:2}-OOH), trilinolein (TG\textsubscript{18:2}-OOH), and phospholipids (PC-OOH) by HPLC in samples of plasma.

Results – The concentrations of NOx, nitrotyrosine, COx, CE\textsubscript{18:2}-OOH, and PC-OOH were higher in the postmenopausal period (33.8±22.3 µM; 230±130 nM; 55±19 ng/µL; 17±8.7 nM; 2775±460 nM, respectively) as compared with those in the premenopausal period (21.1±7.3 µM; 114±41 nM; 31±13 ng/µL; 6±1.4 nM; 1635±373 nM). In contrast, the concentration of S-nitrosothiols was lower in the postmenopausal period (91±55 nM) as compared with that in the premenopausal period (237±197 nM).

Conclusion – In the postmenopausal period, an increase in nitrotyrosine and a reduction of S-nitrosothiol formation, as well as an increase of COx, CE\textsubscript{18:2}-OOH and PC-OOH formation occurs. Therefore, NO inactivation and the increase in lipid peroxidation may contribute to endothelial dysfunction and to the greater risk for atherosclerosis in postmenopausal women.

Keywords: menopause, nitric oxide, lipid peroxidation

Cardiovascular diseases are less prevalent in premenopausal women and in those receiving hormone replacement therapy as compared with postmenopausal women and men\textsuperscript{1}. This protective effect is attributed to estrogens, and one of the mechanisms of action may be related to the metabolism of plasma lipoproteins\textsuperscript{2}.

Estrogens reduce LDL-cholesterol and increase HDL-cholesterol\textsuperscript{3}. However, these changes in lipid profile only contribute to approximately 25% of the protective effect of estrogens\textsuperscript{4}. Other potential mechanisms of action of estrogens may include protection against LDL oxidation\textsuperscript{5}, reduction in lipoprotein(a), potentiation of fibrinolysis\textsuperscript{6}, and increase in insulin sensitivity. In the arteries, estrogens improve vasodilating function, decrease calcification, secretion of cell adhesion molecules (E-selectin, ICAM-1, and VCAM-1), and formation of atherosclerotic lesions\textsuperscript{7}.

Direct action of estrogens on the arterial wall has also been demonstrated. The administration of estrogens for prolonged periods inhibits the deposition of cholesterol in the arteries and thickening of the intima in monkeys and rabbits fed an atherogenic diet\textsuperscript{8}. However, the mechanisms determining the direct effects of estrogens on the arterial wall have not been completely elucidated. The hemodynamic effects may be partially measured by the activity of estrogens on the arterial wall and not completely elucidated. The hemodynamic effects may be partially measured by the activity of estrogens on the endothelial nitric oxide\textsuperscript{9}. Estrogens have been suggested to possibly improve endothelial function and decrease the risks of atherosclerosis in premenopausal women. Nitric oxide has its bioactivity reduced in postmenopausal women. However, it is not clear whether this occurs due to its lower production by nitric oxide synthase, or whether nitric oxide is inactivated by reaction with the superoxide radical also generated by endothelial cells, forming the peroxynitrite anion (ONOO\textsuperscript{-}), which is a strong oxidizing agent.

Peroxynitrite is decomposed into other reactive oxygen species (fig. 1)\textsuperscript{10} and reacts with tyrosine residues of proteins to form nitrotyrosine\textsuperscript{11}. Although little is known
molecule may also be oxidized with the consequent formation of lipid peroxidation. A cholesterol such as peroxynitrite. Lipid hydroperoxides (LOOH) are formed by free radicals and by nonradical reactive species, and cholesterol esters may occur in reactions promoted by lipid peroxidation, an increase in vascular permeability, and platelet adhesion.

Fig. 1 - Generation of peroxynitrite in the vascular compartment and its decomposition into other oxidizing species. Macrophages and neutrophils may simultaneously generate nitric oxide and superoxide, which interact to form peroxynitrite. Peroxynitrite may react with tyrosine residues present in proteins forming nitrotyrosine or decomposes into various reactive species of oxygen and nitrogen that promote lipid peroxidation, an increase in vascular permeability, and platelet adhesion.

about the effects of nitration of tyrosines on the in vivo function of proteins, peroxynitrite and its decomposition products induce peroxidation of the membrane lipids and may initiate lipid peroxidation of human LDL, causing endothelial lesions and an increase in vascular permeability. Several studies indicate that the endothelium-dependent deficiency in vascular relaxation is associated with inactivation of nitric oxide by formation of peroxynitrite.

Nitrogen dioxide (NO\textsubscript{2}) is one of the oxidizing products formed in peroxynitrite decomposition, which rapidly crosses cell membranes due to its high lipophilicity, initiating oxidation of the polyunsaturated fatty acids. Therefore, nitrogen dioxide may take out 1 hydrogen atom from the chains of polyunsaturated fatty acids, initiating the process of lipid peroxidation (fig. 2). Recently, nitrogen dioxide has also been suggested to form nitrated lipids.

Peroxidation changes in phospholipids, triacylglycerols, and cholesterol esters may occur in reactions promoted by free radicals and by nonradical reactive species, such as peroxynitrite. Lipid hydroperoxides (LOOH) are nonradical intermediates of lipid peroxidation. A cholesterol molecule may also be oxidized with the consequent formation of cholesterol oxides (oxysterols). Cholesterol oxidized in position 7 may reduce endothelium-dependent relaxation and nitric oxide production by endothelial cells. High concentrations of cholesterol oxides are found in atherosclerotic arteries and in lipoproteins of hypercholesterolemic patients. Some cholesterol oxides, mainly 7-cetocholesterol, have already been shown to have anti-estrogenic activity.

The objective of this study was to assess the effect of endogenous estrogens on the bioavailability of nitric oxide, by determining the concentration of its derivatives, such as NO\textsubscript{x} (nitrite and nitrate), S-nitrosothiols (which also have vasodilating action) and nitrotyrosine, and also the formation of lipid hydroperoxides and cholesterol oxides in the plasma of pre- and postmenopausal women.

Methods

This study was approved by the committees on ethics in research of the institutions involved.

Forty postmenopausal women, with ages ranging from 50 to 65 (57.1±5.6) years, and 24 premenopausal women, with ages ranging from 20 to 40 (28.1±5.4) years, who were in the middle of their menstrual cycle, were followed up for the minimum period of 1 year. The exclusion criteria were as follows: smoking, uncontrolled hypertension, diabetes, and obesity. After a 12- to 15-hour fasting period, blood was collected in tubes containing EDTA. Plasma was separated and stored in tubes containing antioxidant agents (butylated hydroxytoluene, BHT 20 µM) and protease inhibitors (aprotinin, 2mg/µL), phenylmethyl sulfonyl fluoride (PMSF, 1mM), and benzamidine (2mM).

The measurements of total plasma cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides were performed with commercial enzymatic methods (BioSytems). The plasma nitrate concentration was measured with the NO\textsubscript{a} analyzer (NOA\textsuperscript{TM280}, Sievers Inc., USA). Plasma was previously deproteinized in cold ethanol (1:2, v/v), was left resting for 30 minutes at 0°C, and centrifuged at 10,000 g for 10 minutes. A 10-µL aliquot of the floating material was injected into the NO\textsubscript{a} analyzer (NOA\textsuperscript{TM280}). The calibration curve was built with patterns of sodium nitrate. In the NOA\textsuperscript{TM280}, plasma nitrate was reduced to NO with vanadium (III) at 90°C, and the NO formed was detected by gaseous phase chemiluminescence after reaction with ozone.

Measurement of total S-nitrosothiols was performed according to Marley et al with modifications. Aliquots of 500 µL of previously prepared plasma were double injected into the purge vessel of the NO\textsubscript{a} analyzer (NOA\textsuperscript{TM280}) containing 16 mL of glacial acetic acid, 4 mL of KI 50 mg/mL, 1000 µL of decanol (antifoam agent), and 400 µL of CuSO\textsubscript{4} 200 mM (this solution was exchanged every 8 injections). The calibration curve was built based on S-nitroso-albumin synthesized according to Marley et al.

Nitrotyrosine concentration in plasma was determined with competitive ELISA. The plates were sensitized with 0.5 mg/µL of nitrated albumin (ALBNO, 100 µL, approximate
nitrotyrosine concentration ranging from 40 to 60 μM) diluted in 0.1 M carbonate-bicarbonate buffer in pH 9.4 at 37°C overnight. After washing with 200 µL of TBS buffer (TRIS-HCl 50 mM, NaCl 150 mM, pH 7.4) containing 0.05% Tween 20, the plates were incubated for 2 hours at 37°C with 200 µL/flask of TBS buffer containing 0.05% Tween 20 and 5% skim milk. After a new washing, the plates were incubated with 100 µL/flask of anti-nitrotyrosine polyclonal antibody (Upstate biotechnology, diluted 1:1000 in TBS containing 0.05% skim milk and 0.05% Tween 20) in addition to 100 µL of the sample (the plasma samples and nitrated albumin used in the calibration curve were diluted 1:4 in TBS containing 0.05% milk) for 2 hours at 37°C. The plates were washed and incubated again with 100 µL/flask of conjugate (antirabbit IgG produced in mice conjugated with peroxidase - Stressgen Biotechnologies Corp.) diluted 1:2000 in TBS containing 0.05% skim milk and 0.05% Tween 20 for 1 hour at 37°C. After washing, luminol (2.3 mM), p-iophenol (0.9 mM) (200 µL/flask), and hydrogen peroxide (3.9 mM) (50 µL/flask) were added. The chemiluminescence produced was immediately read, by using the plates of ELISA for chemiluminescence (LumiCount, Packard, Meriden, USA). The concentration of nitrotyrosine in the samples was estimated with the concentration calibration curve built with nitroso-albumin and expressed as equivalents of nitro-ALB. The samples and the calibration curve were tripled. The bovine nitrated albumin used as a pattern to build calibration curves (nitro-ALB; 2-4 mol of nitrotyrosine/mol of protein) was prepared by adding an alkaline solution of peroxynitrite to a final concentration of 1 mM to a bovine albumin solution (ALB - 1 mg/µL). The solution was then dialyzed overnight in PBS buffer (10 mM of phosphate-buffered sodium, 140 mM of sodium chloride, 2.7 mM of potassium chloride, and 0.02% sodium azide). The concentration of nitrotyrosine in nitro-ALB was determined by using the molar extinction coefficient 4300 M⁻¹ cm⁻¹ at 438 nm, pH 9.0.

The hydroperoxides derived from cholesterol linoleate (CH18:2-OOH), trilinolein (TG18:2-OOH), and phosphatidylcholine (PC-OOH) were extracted from plasma (500 µL) with acetonitrile (4 µL) containing 0.02% BHT in a 4:1 mixture of methanol and chloroform-methanol. The hydroperoxides were identified with high-performance liquid chromatography with a flame ionization detector was 300°C. The patterns used (Steraloids, Inc.) were as follows: 5-cholestene-3β,7α-diol (7α-CH); 5-cholestene-3β,7β-diol (7β-CH); 5-cholesten-3β,6α-epoxy-3β-ol (α-epoxy); 5-cholestan-3β,6β-epoxy-3β-ol (β-epoxy); 5-cholestan-3β,5α,6β-triol (CT); 5-cholestan-3β-ol-7-one (7-Keto); and 5-cholestan-3β,25-diol (25-OH). The estradiol concentration was determined with the IMx System Estradiol kit from Abbott Laboratories.

Data are presented as mean ± standard deviation. The statistical analysis was performed with the one-way ANOVA and ORIGIN 5.0 software. The correlations were performed with Sigma Stat 1.0 software, Pearson Product Moment method, “r” = value of the correlation coefficient, and “p” = descriptive level. A significance level of P < 0.05 was adopted.
Results

Figure 3 depicts the plasma concentrations of cholesterol, LDL, and HDL in pre- and postmenopausal women. Postmenopausal women (total cholesterol: 281±42 mg/dL; LDL-cholesterol: 184±37 mg/dL; HDL-cholesterol: 65±15 mg/dL) had a higher concentration of cholesterol as compared with premenopausal women (total cholesterol: 145±27 mg/dL; LDL-cholesterol: 83±24 mg/dL; HDL-cholesterol: 51±10 mg/dL).

Figures 4, 5, and 6 show the plasma concentrations of NOx (p=0.01), S-nitrosothiols (p<0.0001), and nitrotyrosine (p=0.0005), respectively, in the 2 groups analyzed. As compared with premenopausal women (21.1±7.3 µM; 114±41 nM; 237±197 nM), postmenopausal women (33.8±22.3 µM; 230±130 nM; 91±55 nM) had increased concentrations of NOx and nitrotyrosine and decreased concentrations of S-nitrosothiols, respectively.

Figure 7 shows the total concentration of cholesterol oxides in plasma, which was higher in postmenopausal women (55±19 ng/µL) as compared with that in premenopausal women (31±13 ng/µL, p=0.0003). Figure 8 shows the concentrations of all cholesterol oxides analyzed. Only µ-epoxy-cholesterol showed a significant increase (p=0.0003) in postmenopausal women (24±10 ng/µL) as compared with that in premenopausal women (12±8 ng/µL).

The concentrations of hydroperoxides of cholesterol esters (17±8.7 nM; 6±1.4 nM, fig. 9) and of phospholipids (2775±460 nM; 1635±373 nM, fig. 10) were greater (p=0.0007; p<0.0001, respectively) in the group of postmenopausal women as compared with those in the group of premenopausal women (respectively), indicating greater lipid peroxidation in that group. No difference in triglyceride hydroperoxides (11±2.9 nM; 12±4.9 nM) was observed between pre- and postmenopausal women, respectively (fig. 9).
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Estrogens increase cholesterol flow from dietary intake to the liver, due to the cholesterol incorporation into chylomicra and remnants of chylomicra. Subsequently, cholesterol flows to the cells of the peripheral tissues, as VLDL and LDL, and returns to the liver via the reverse transportation of cholesterol, as HDL. In liver cells, cholesterol is transformed into bile acids, which, in turn, are excreted in bile. Under the influence of estrogens, the following events may occur: (i) plasma triglycerides increase due to an increase in VLDL production; (ii) LDL concentration is reduced due to an increase in the expression of LDL receptors; (iii) HDL is increased by 2 mechanisms: greater secretion of apolipoprotein A-1, and lower removal of its lipids, because estrogens lead to a reduction in hepatic lipase activity (revised by Zhu et al).

In this study, we observed an increase in nitrate concentration associated with a higher cholesterol concentration and with lipid peroxidation in postmenopausal women. Recently, the localization of endothelial nitric oxide synthase (eNOS) in specialized invaginations of the plasma membrane, called caveolae, has been proposed as necessary to the maximal activity of that enzyme. Caveolae are highly rich in cholesterol. Caveolin mRNA (protein present in the caveolae and that interacts with eNOS) is up-regulated when the uptake of free cholesterol from LDL to the cell is increased. The regulation of the efflux of free cholesterol via the caveolar path is mediated by caveolin mRNA. Evidence indicates that the increase in caveolin regulation in cells exposed to high levels of LDL-cholesterol results directly from an increase in the transcription of the eNOS gene induced by the higher cellular content of cholesterol. In vitro studies have shown that, when endothelial cells are exposed to low concentrations of oxidized LDL (LDL-ox) for more than 24 hours, an increase in eNOS mRNA is observed, but in high concentrations of LDL-ox, this effect is reversed. These observations show the existence of a correlation between the production of nitric oxide, hypercholesterolemia, and lipid peroxidation. The increase in nitrate concentration may result from the increase in eNOS regulation, via caveolin, induced by the increase in the plasma, and consequently caveolar, concentration of cholesterol, and by the increase in LDL-ox concentration.

Barbacanne et al. found an increase in NO activity induced by estrogens, but observed no change in eNOS gene expression, or in the activity of this enzyme, although...
they detected a lower production of the superoxide radical. The decrease in the generation of endothelium-derived superoxide in response to estrogens could increase the bioactivity of nitric oxide by reducing its inactivation by forming peroxynitrite. Hayashi et al. observed that 17β-estradiol increases eNOS activity mediated by the receptor; high concentrations of 17β-estradiol tend to inhibit eNOS activity through a receptor-independent path. 17β-estradiol was also observed to decrease the release of nitrogen oxides (NOx) produced by nitric oxide synthase (iNOS) in macrophage culture. On the other hand, 17β-estradiol may act in eNOS by another mechanism, such as activation of the Ca²⁺/calmodulin system.

Holm et al. 35 reported that, in ovariectomized rabbits, the antiatherogenic effect of estrogen was eliminated by an endothelial lesion induced with a balloon-catheter (ie, it is endothelium-dependent) and that estrogens increased NOX formation in rabbit plasma, showing that the effect of estrogens in atherosclerosis might relate to greater bioavailability of nitric oxide. The effect of estrogens in reducing cell adhesion molecules and the consequent reduction in adhesion of mononuclear cells to the endothelium may also be mediated by an increase in nitric oxide.

Some studies have shown that the vasodilatation produced by 17β-estradiol is endothelium-independent. Gonzales and Kanagy 36 observed that N-octra nitroarginine, a nitric oxide synthase inhibitor, did not affect vasodilatation produced by estrogens in arteries of male rats, both with intact and denuded endothelium. The inhibition of guanylate cyclase did not affect relaxation. Those authors also observed that 17β-estradiol causes vasodilation by inhibiting Ca²⁺ influx without causing Ca²⁺ intracellular release. Therefore, the study showed that 17β-estradiol causes receptor-mediated vasorelaxation through inhibition of calcium influx, independent of nitric oxide production, of guanylate cyclase stimulation, and of potassium channel activation.

Several pathological conditions, including ischemia/reperfusion, inflammation, and deficiency in tetrahydrobiopterin, a cofactor for nitric oxide synthesis, may induce the simultaneous production of superoxide (O₂⁻) and NO radicals in several tissues, generating peroxynitrite, which has a much greater oxidizing potential than the nitric oxide (NO) and superoxide (O₂⁻) radicals alone. Peroxynitrite causes nitration (incorporation of a nitro group - NO₂) of aliphatic and aromatic residues. Tyrosine residues in proteins are key targets of nitration, mediated by peroxynitrite, and the presence of 3-nitrotyrosine in proteins represents a marker of the endogenous formation of peroxynitrite. The process of nitration involves radical mechanisms, in which an electron derived from peroxynitrite attacks the aromatic ring of tyrosine, leading to the formation of a tyrosyl radical, which rapidly combines with nitrogen dioxide (NO₂) to form 3-nitrotyrosine. In addition to peroxynitrite, another nitration path is the H₂O₂/NO₃⁻/myeloperoxidase system, which uses nitrite derived from nitric oxide for nitration. 17β-estradiol has been shown to block the induction of formation of peroxynitrite in cell culture.

Both nitrate and nitrotyrosine concentrations were increased in postmenopausal women. This implies that nitric oxide synthesis was not reduced in postmenopausal women, because nitrate is the major metabolite derived from nitric oxide. In addition, the greater formation of nitrotyrosine indicates possible NO inactivation by its reaction with the superoxide radical. Therefore, the product of this reaction, the peroxynitrite anion, or the nitrogen dioxide radical derived from its decomposition would cause nitration of the tyrosine residues of plasma proteins, generating the increase in plasma nitrotyrosine.

Peroxynitrite formation may be associated with estradiol in other pathological conditions. A higher incidence of breast cancer has been associated with exposure to estrogens 41-44. Estrogen metabolism is altered in most breast cancer tumors. These alterations lead primarily to the formation of catechols, which may generate superoxide 45. In breast cancer, NO synthase activity is increased, resulting in increased nitric oxide production, and a consequent increase in peroxynitrite and its decomposition products, which may be related to DNA lesions in breast cancer 45. On the other hand, the effect of the administration of estrogens on NO inactivation has not been clarified. Tamoxifen inhibits NO synthase activity and nitrotyrosine formation in the brain of rats 44. Therefore, further studies assessing the effect of hormone replacement therapy on NO inactivation are still required.

Nitrosothiols (RSNOs) may be formed by the reaction of NO with thiol groups 29 (present in cysteine, homocysteine, and glutathione residues), and its concentration depends on the synthesis of NO 46. Nitrosothiols act promoting vasodilatation and inhibition of platelet aggregation 45. Nitrosothiols are considered reservoirs of NO, and many of their effects may be explained by NO release. However, not all biological effects reported for RSNOs are attributed to the NO donor function of these compounds. Evidence exists that RSNOs by themselves may activate guanylate cyclase, producing vasodilation 47. Alteration in the vasodilating activity in postmenopausal women may result from the accelerated NO decomposition or its smaller conversion to RSNOs, or both. The lower concentration of RSNOs in postmenopausal women only confirms the reduced NO bioavailability in face of the high nitrotyrosine concentration. NO synthesized in postmenopausal women may be inactivated prior to the formation of S-nitrosothiols.

The formation of peroxynitrite directly related to the formation of nitrotyrosine may be related to the formation of lipid hydroperoxides by free radicals derived from peroxynitrite decomposition. Because nitric oxide is converted into peroxynitrite, it is not available for the formation of S-nitrosothiols in postmenopausal women (fig. 5).

Among the cholesterol oxides analyzed, only α-epoxycholesterol was significantly increased in postmenopausal women. That oxide does not inhibit nitric oxide synthesis and does not act on estrogen receptors, but has a cytotoxic effect on endothelial cells 47-48. Fielding et al. 29 reported that cholesterol oxides reduce caveolin mRNA, the
transportation of free cholesterol to cell surface, and free cholesterol efflux. Caveolae are probable intermediates of free cholesterol efflux via HDL. The presence of oxysterols in HDL reduces the capacity of that lipoprotein to stimulate cholesterol efflux. In vitro studies have shown that estradiol is more effective than alpha-tocopherol or beta-carotene in inhibiting the formation of cholesterol oxides.

Several studies have shown the in vitro and in vivo antioxidant action of estrogens. The addition of estrogens to a system of LDL oxidation mediated by copper showed an increase in the initiation time of LDL oxidation. Several studies have shown the effect of the administration of estrogens on human LDL oxidation. Therefore, the reduced concentration of estrogens could partially explain the increase in lipid peroxidation in postmenopausal women observed in this study.

Other factors could have influenced these results, such as age and the presence of hypercholesterolemia. Because the objective of this study was to compare pre- and postmenopausal women, normalization of the age factor in these 2 groups was not possible. Aging is an important determinant in vascular disease, and some studies have reported a reduction in nitric oxide bioactivity with aging, independent of the sex of the groups studied. However, Ohata et al. analyzing the endothelial function in old women (> 80 years), reported that the preservation of endothelial function was associated with estradiol and estriol concentrations. Sader et al. also reported that supplementation with estradiol is associated with an improvement in the endothelial function in healthy young men. Although aging reduces nitric oxide bioavailability, estradiol is also an important factor for endothelial function. The elevated concentration of cholesterol in postmenopausal women may have influenced the results obtained, independent of the plasma concentration of estradiol, although estradiol also affects lipid metabolism. Estradiol deficiency in postmenopausal women is not the only factor responsible for the elevation in plasma cholesterol; other factors, such as age, diet, and genetic factors may also influence these results. Wakatsuki et al. observed a strong correlation between estradiol and LDL-cholesterol concentrations in a similar study with pre- and postmenopausal women, suggesting that estrogen plasma concentrations are inversely related to LDL-cholesterol concentration.

Several studies have shown an increase in HDL-cholesterol and a reduction in LDL-cholesterol due to estrogen replacement. Some studies have clearly shown that estrogens reduce total plasma cholesterol and that hormone replacement therapy may be beneficial for primary prevention of coronary artery disease. However, this protective effect of estrogens has been questioned in some epidemiological studies. The HERS, ERA, and WHIHR studies have suggested that hormone replacement therapy has no cardioprotective effect in postmenopausal women with coronary artery disease and may even increase the risk of thromboembolism and cardiovascular events. This unexpected effect may have been due to mutations in thrombogenic factors in a subgroup of women. Therefore, the cardioprotective effect of estrogens still requires additional studies.

The increase in nitric oxide inactivation due to peroxynitrite formation, the consequent reduction in the formation of S-nitrosothiols, the increase in nitrotyrosine and in lipid peroxidation are factors that can induce a reduction in vasodilation and in the formation of atherosclerotic lesions in postmenopausal women. These results allow for new perspectives for future research concerning the efficacy of hormone replacement therapy on vasodilation and atherosclerosis.

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