Additive antioxidative effect of hormone replacement therapy combined with a statin

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Summary

In treating postmenopausal women both statins and estrogens have been shown to elicit favourable effects on lipids and lipoproteins. In addition direct beneficial effects of these substances on the vascular wall are discussed. However, progestin addition to estrogen replacement therapy, which is mandatory in women with an intact uterus, is thought to deteriorate at least partly estradiol-induced direct effects on the vasculature. Oxidation of LDL, which mainly takes place in the vessel wall, seems to be a crucial step in the development of atherosclerosis. Therefore, for the first time, the effect of a statin and an estrogen/progestin combination on the in vitro oxidation of human LDL was investigated comparing the monosubstances fluvastatin, 17ß-estradiol and norethisterone acetate (NETA) as well as the effect of the combination. LDL was isolated from human female serum and oxidation was initiated by copper(II)-chloride. The progression of LDL oxidation was monitored spectrometrically at 234 nm for 300 min. Fluvastatin significantly delayed the onset of LDL oxidation (controls = 85 min) by 21 min at 1 µM, by 99 min and by 210 min at 5 and 10 µM, respectively. 17ß-estradiol significantly reduced the onset by 73 min at 1 µM and by more than 300 min at 5 and 10 µM. NETA had no significant effect. The combination of 1 µM 17ß-estradiol and 1 µM fluvastatin with 1, 5 and 10 µM NETA showed an additive antioxidative effect of estradiol and fluvastatin and no deterioration by the addition of NETA even at high dosages.

It can be concluded that treatment of postmenopausal women with fluvastatin and a combination of 17ß-estradiol with NETA may have not only beneficial effects on lipid disorders but may also elicit a direct potent antiatherosclerotic action on the vasculature.

Key words: Estradiol; Norethisterone acetate; Fluvastatin; LDL-oxidation.

Introduction

Fluvastatin is a synthetic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor. This drug class has been shown to reduce intracellular cholesterol synthesis thereby reducing circulating lipid and lipoprotein levels. Several epidemiological studies were able to demonstrate that statins are potent compounds for the primary and secondary prevention of cardiovascular diseases [1]. The natural estrogen, 17ß-estradiol, is mainly used for the alleviation of menopausal symptoms but also has beneficial effects on the lipid profile and may reduce morbidity and mortality due to cardiovascular diseases [2].

However, both substance classes may elicit their beneficial effects on the development of cardiovascular diseases not only by reducing hypercholesterolemia but also by direct actions on the vascular wall [3, 4]. Thus, among other effects, fluvastatin and 17ß-estradiol enhance endothelial prostacyclin production and reduce endothelin production [4, 5]. Moreover both substances have already been shown to possess antioxidative potency [6, 7].

The concomitant administration of a statin and 17ß-estradiol may be required in hypercholesterolemic postmenopausal women suffering from menopausal symptoms. So far only a few in vivo studies have been conducted in this aspect, whereby the effect of the combination on the lipid profile was examined [8-10]. In vitro effects of the combination of a statin with an estrogen were conducted by our group with respect to prostacyclin and endothelin production by human vascular endothelial cells [11] In addition, in a previous study we investigated the effect of a fluvastatin/estradiol combination on the oxidation of LDL [12]. Since clinically progestin addition to estrogen replacement therapy is mandatory in women with an intact uterus to prevent endometrial hyperplasia, we extended our experiments by inclusion of a commonly used progestin, i.e. norethisterone acetate.

Material and Methods

Fluvastatin was donated by Novartis, Nuremberg, Germany, 17ß-estradiol and norethisterone acetate (NETA) were purchased from Sigma, Munich, Germany. The substances were dissolved in ethanol and tested alone at the concentrations 1 µM, 5 µM and 10 µM and in combinations of 1 µM estradiol and 1 µM fluvastatin with 1, 5 and 10 µM NETA.

LDL oxidation was investigated according to Esterbauer et al. [13] using pooled blood serum from healthy premenopausal women containing the antioxidants EDTA (1 mg/ml) and butylated hydroxytoluene (BHT) (4.4 µg/ml). The collected blood was stored at 4°C and LDL was obtained by ultracentrifugation within 4 h after the collection of blood samples. In brief: 1.638 g sodium bromide was added to 3 ml serum which was overlaid with saline. After ultracentrifugation of this solution at 105,000 g for 9 h (fixed angle rotor), the LDL-layer (density 1.02-1.05 g/ml) was aspirated using a syringe. Oxidation of LDL was started immediately after ultracentrifugation as follows: LDL-solution was separated from BHT and EDTA by gelfiltration (Sephadex G-25, column 10 x 1.5 cm, eluent: saline, fraction volumes: 500 µl). Samples of 150 µl of LDL (adjusted to 300 µg protein/ml) were mixed with 850 µl saline containing 10 µM $CuCl_2$ and the test substances. Fluvastatin, 17ß-estradiol and NETA, alone or in

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combination were added to this mixture. Control values were obtained by the addition of alcohol alone in the same concentration as in the test substances (final ethanol concentration in all samples approx. 1%).

The increase of conjugated diene formation, characteristic of the oxidation of LDL, was monitored spectrometrically at 234 nm. Tangents were drawn to the segments of the absorption curve corresponding to the lag phase and propagation phase of LDL oxidation. The length of the lag phase = lag time was determined as the intercept of the two tangents.

The antioxidative effect of the substances tested is expressed as the elongation of the lag time after subtracting the time for the oxidated LDL formation of the control. The lag time was measured for an observation time of 300 min. Each concentration of the substances alone and in combination was tested in duplicates from six different LDL pools.

Protein content of LDL was determined by a colorimetric protein assay (Bio-Rad, München, Germany). Statistical analysis was performed using the Student's t-test.

Results

The values for oxidated LDL formation without test substances showed an average lag time of 85.1 ± 13.6 min. The changes in the lag time in minutes caused by fluvastatin and 17ß-estradiol are depicted in Table 1. Fluvastatin increased the lag time prior to the onset of LDL oxidation by 21.4 ± 5.2 min ($p<0.05$) at 1 µM, by 99.3 ± 9.4 min at 5 µM ($p<0.01$) and by 209.8 ± 11.1 min at 10 µM ($p<0.01$). 17ß-estradiol elongated the onset of LDL oxidation at 1 µM by 73.2 ± 6.2 min ($p<0.01$) and at 5 and 10 µM by more than 300 min ($p<0.01$), which was the longest test time observed in our test. NETA had no significant effect on all concentrations tested.

The combinations of fluvastatin and 17ß-estradiol with NETA increased the lag time significantly over the effect of each substance alone; the values for 1 µM fluvastatin and 1 µM 17ß-estradiol combined with 1 µM NETA were 99.8 ± 4.9 min ($p<0.01$), for 5 µM and for 10 µM NETA addition 101.7 ± 6.3 min and 103.8 ± 5.3, respectively ($p<0.01$).

Table 1. — *Increase of the lag time of LDL transformed into oxidated LDL by copper(II)-chloride after addition of different concentrations of fluvastatin, 17ß-estradiol, norethisterone acetate (NETA) and combinations of these substances; values are expressed in minutes as means ± SD, duplicates from six different LDL pools.*

	Fluvastatin	17ß-Estradiol	NETA	Fluvastatin/Estradiol/NETA Combination
1 µM	23.2±5.0*	73.8±6.2**	<5	Fluvastatin 1 µM + Estradiol 1 µM + NETA 1 µM = 99.8±4.9**
5 µM	109.6±11.7.**	>300**	<5	Fluvastatin 1 µM + Estradiol 1 µM + NETA 5 µM = 101.7±6.3**
10 µM	220.5±12.4**	>300**	<5	Fluvastatin 1 µM + Estradiol 1 µM + NETA 10 µM = 103.8±5.3**

** $p<0.01$ compared to control value.

Discussion

An early step in the development of atherosclerosis is the oxidative modification of LDL which has penetrated into the vascular wall because of insufficient clearance [14]. Oxidated LDL is able to initiate the recruitment of monocytes, which after conversion to macrophages and after ingestion of oxidated LDL form the so-called foam cells [14]. Moreover oxidated LDL is able to provoke migration and proliferation of smooth muscle cells which is a crucial step in the progression of atherosclerosis [14]. In addition oxidated LDL negatively influences hemodynamics by shifting the balance towards vasoconstriction [15, 16].

Several antioxidative compounds like vitamin E and probucol have been shown to inhibit LDL oxidation and thus may have an anti-atherosclerotic potential [17,18]. The present study confirmed the previously observed antioxidative potential of fluvastatin and 17ß-estradiol. The potency of 17ß-estradiol is about five times greater than that of fluvastatin. The magnitude of the fluvastatin effect in our experiments is of a similar order as observed by Suzumura et al. [19] for 1 µM. However, at 10 µM the inhibitory potency of fluvastatin was greater under our experimental conditions. This difference may be attributed to methodical variations.

The progestin NETA exhibits, as already shown in previous experiments [20], no antioxidative capacity. In combination with fluvastatin and 17ß-estradiol no deteriorating effect by NETA was observed either.

In in vivo studies conducted so far, the combination of an estrogen/progestin therapy with simvastatin or pravastatin, respectively, was investigated on its effect on lipid metabolism [8-10]. These studies showed beneficial effects of the combination on lipid metabolism which were slightly superior to the effect of the single substances. The progestin used in these studies was medroxyprogesterone acetate; for NETA no studies are yet available. However, it seems unlikely that NETA antagonizes the beneficial effects of a statin/estrogen combination on the lipid profile.

It is noteworthy that the effective dosages of the substances tested were in the micromolar range. However, higher concentrations may be required in vitro in short-term tests in which the reaction threshold can only be achieved with supraphysiological dosages. Ex vivo experiments on the resistance of LDL oxidation after treatment of patients with fluvastatin or estrogens have already been done. For fluvastatin one study found no antioxidative effect ex vivo [21] which, however, may be attributed to the specific patient group investigated, i.e. renal transplant patients. In another study fluvastatin administration did not significantly increase the lag time compared to the control values but significantly reduced the diene production rate [22].

Most studies on estrogens have found a delay in the onset of LDL oxidation after treatment with hormone replacement therapy [23-27]. However, other studies failed to prove such an effect [28-30]. This discrepancy may be explained by different kinds of estrogen, duration and modes of administration.

The mechanism by which fluvastatin and estradiol elicit their antioxidative property is not clear yet. Most likely both agents act as radical scavengers, estradiol by virtue of its phenolic structure, whereas for fluvastatin the fluorophenyl indole moiety seems to be important for the antioxidant activity [19].

In conclusion, fluvastatin and 17ß-estradiol are antioxidants in vitro with respect to the oxidation of human LDL. Since the combination of these substances showed an additive effect on the inhibition of LDL oxdiation, the combined administration in postmenopausal women may improve further prevention of the development of atherosclerosis. Addition of the progestin NETA seems to have no negative effect on this beneficial antiatherosclerotic action of fluvastatin and estradiol.

References

[1] Hebert P. R., Gaziano J. M., Chan K. S., Hennekens C. H.: "Cholesterol lowering with statin drugs, risk of stroke, and total mortality". *JAMA*, 1997, *278*, 313.

[2] Bush T. L., Barrett-Connor E., Cowan L. D., Crique M. H., Wallace R. B., Suchindran C. M. *et al.*: "Cardiovascular mortality in noncontracceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study". *Circulation*, 1987, *75*, 1002.

[3] Vaughan C. J., Murphy M. B., Buckley B. M.: "Statins do more than just lower cholesterol". *Lancet*, 1996, *348*, 1079.

[4] Mendelsson M. E., Karas R. H.: "The protective effects of estrogen on the cardiovascular system". *New Engl J Med*, 1999, *340*, 1801.

[5] Seeger H., Mueck A. O., Lippert T. H.: "Fluvastatin increases prostacyclin and decreases endothelin production by human umbilical vein endothelial cells". *Int. J. Clin. Pharm. Therap.*, 2000, *38*, 270.

[6] Suzumara K., Yasuhara M., Tanaka K., Odawara A., Narita H., Suzuki T.: "An in vitro study of the hydroxyl radical scavenging-property of fluvastatin, an HMG-CoA reductase inhibitor". *Chem Pharm Bull*, 1999, *47*, 1010.

[7] Banka C. L.: "Non-genomic action of estrogens: estrogens as antioxidants". In: "Estrogen and the Vessel Wall". Rubanyi G. M., Kuffman R. (eds.). Harwood Academic Publishers, 1998, 91.

[8] Darling G. M., Johns J. A., McCloud P. I., Davis S. R.: "Estrogen and progestin compared with simvastatin for hypercholesterolemia in postmenopausal women". *N Engl J Med*, 1997, *337*, 595.

[9] Herrington D. M., Werbel B. L., Riley W. A., Pusser B. E., Morgan T. M.: "Individual and combined effects of progestin therapy and lovastatin on lipids and flow-mediated vasodilation in postmenopausal women with coronary artery disease". *J Am Coll Cardiol*, 1999, *33*, 2030.

[10] Averbuch M., Ayalon D., Eckstein N., Dotan I., Shapira I., Levo Y. *et al.*: "Hormone replacement therapy and simvastatin in the therapy of hypercholesterolemic postmenopausal women". *J Med*, 1998, *29*, 343.

[11] Seeger H., Lippert C., Wallwiener D., Mueck A. O.: "Effect of fluvastatin and estradiol on the production of prostacyclin and endothelin in human umbilical vein endothelial cells". *Pharm Pharmacol Lett*, 2000, *1*, 16.

[12] Mueck A. O., Seeger H., Wallwiener D.: "Fluvastatin combined with 17ß-estradiol: Effect on the oxidation of human low density lipoprotein". *Exp Clin Endocrinol Diabetes*, 2000, *108*, 316.

[13] Esterbauer H., Striegl G., Puhl H., Rotheneder M.: "Continuous monitoring of in vitro oxidation of human low density lipoprotein". *Free Rad Res Comms*, 1989, *6*, 67.

[14] Steinberg D.: "Oxidative modification of LDL and atherogenesis". *Circulation*, 1997, *95*, 1062.

[15] Galle J., Mülsch A., Busse R., Bassenge E.: "Effects of native and oxidized low density lipoproteins on formation and inactivation of endothelium-derived relaxing factor". *Arterioscl Thromb*, 1991, *11*, 198.

[16] Horio T., Kohno M., Yasunari K., Murakawa K., Yokokawa K., Ikeda M. *et al.*: "Stimulation of endothelin-1 release by low density and very low density lipoproteins in cultured human endothelial cells". *Atherosclerosis*, 1993, *101*, 185.

[17] Sasahara M., Raines E.W., Chait A., Carew T. E., Steinberg D., Wahl P. W. *et al.*: "Inhibition of hypercholesteremia-induced athersoclerosis in the nonhuman primate by probucol. I. Is the extent of atherosclerosis related to resistance of LDL to oxidation". *J Clin Invest*, 1994, *94*, 155.

[18] Stephens N. G., Parsons A., Schofield P. M., Kelly F., Cheeseman K., Mitchinson M. J. *et al.*: "Randomised controlled trial of vitamin E in patients with coronary artery disease: Cambridge Heart Antioxidant Study (CHAOS)". *Lancet*, 1996, *347*, 781.

[19] Suzumura K., Yasuhara M., Tanaka K., Suzuki T.: "Protective effect of fluvastatin sodium (XU-62-320), a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on oxidative modification of human low-density lipoprotein in vitro". *Biochem Pharmacol*, 1999, *57*, 697.

[20] Mueck A. O., Seeger H., Lippert T. H.: "Estradiol inhibits LDL oxidation: Do the progestins medroxyprogesterone acetate and norethisterone acetate influence this effect?". *Clin. Exp. Obstet. Gynecol.*, 1998, *25*, 26.

[21] Martinez-Castelao A., Grinyo J. M., Fiol C., Castineiras M. J., Hurtado I., Gil-Vernet S. *et al.*: "Fluvastatin and low-density lipoprotein oxidation in hypercholestermic renal transplant patients". *Kidney International*, 1999, *56* (Suppl. 71), S231.

[22] Leonhardt W., Kurktschiev T., Meissner D., Lattke P., Abletshauser C., Weidinger G. *et al.*: "Effects of fluvastatin therapy on lipids, antioxidants, oxidation of low density lipoproteins and trace metals". *Eur J Clin Pharmacol*, 1997, *53*, 65.

[23] Sack M. N., Rader D. J., Cannon R. O. III: "Oestrogen and inhibition of oxidation of low-density lipoproteins in postmenopausal women". *Lancet*, 1994, *343*, 269.

[24] Guetta V., Panza J. A., Waclawiw M. A., Cannon R. O. III: "Effect of combined 17 beta-estradiol and vitamin E on low-density lipoprotein oxidation in postmenopausal women". *Am J Cardiol*, 1995, *75*, 1274.

[25] Schröder J., Dören M., Schneider B., Oettel M.: "Are the antioxidative effects of 17 beta-estradiol modified by concomitant administration of a progestin?". *Maturitas*, 1996, *25*, 133.

[26] Wilcox J. G., Hwang J., Hodis H. N., Sevanian A., Stanczyk F. Z., Lobo R. A.: "Cardioprotective effects of individual conjugated equine estrogens through possible modulation of insulin resistance and oxidation of low-density lipoprotein". *Fertil Steril*, 1997, *67*, 57.

[27] Wakatsuki A., Ikenoue N., Sagara Y.: "Effects of estrogen on susceptibility to oxidation of low-density and high-density lipoprotein in postmenopausal women". *Maturitas*, 1998, *28*, 229.

[28] van der Mooren M. J., Demacker P. N., Blom H. J., de-Rijke Y. B., Rolland R.: "The effect of sequential three-monthly hormone replacement therapy on several cardiovascular risk estimators in postmenopausal women". *Fertil Steril*, 1997, *67*, 67.

[29] McManus J., McEneny J., Thompson W., Young I. S.: "The effect of hormone replacement therapy on the oxidation of low-density lipoprotein in postmenopausal women". *Atherosclerosis*, 1997, *135*, 73.

[30] Wen Y., Doyle M. C., Norris L. A., Sinnott M. M., Cooke T., Harrison R. F. *et al.*: "Combined oestrogen-progestogen replacement therapy does not inhibit low-density lipoprotein oxidation in postmenopausal women". *Br J Clin Pharmacol*, 1999, *47*, 315.

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