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Angiogenetic and Anti-Angiogenetic Effects of Estradiol and its Metabolites

A. O. Mueck, H. Seeger, C. Lippert, D. Wallwiener

Atherosclerotic plaques in later stages exhibit marked presence of new micro vessels. Thus angiogenesis may be important for the development of atherosclerotic plaques and long-term anti-angiogenetic therapy may present an effective new anti-atherosclerotic approach. 2-Methoxyestradiol, an endogenous estradiol metabolite, has already been shown to be an effective anti-angiogenetic substance. In the present study 14 endogenous estradiol metabolites were tested on their angiogenetic and anti-angiogenetic properties and compared to the effect of their parent substance, 17β-estradiol.

Endothelial cells from human umbilical veins were used for the experiments. 17β-estradiol showed a biphasic reaction on the proliferation of vascular endothelial cells. At low concentration it stimulated and at high concentrations it inhibited cell growth. The same pattern was observed for the hydroxylated A-ring metabolites. Methylation of these metabolites, however, completely abrogated the anti-proliferative effect at high concentrations, except for the metabolite 2-hydroxyestradiol. For the D-ring metabolites no marked changes were observed.

These results indicate that in addition to 2-methoxyestradiol other endogenous estradiol metabolites are potent anti-angiogenetic substances at high dosages. Since some of these metabolites are almost devoid of any estrogenic property, they may be useful for long-term anti-angiogenetic therapy in both men and women. This should be of interest to clinical pharmacological research since it points to potential new aspects in the treatment of cardiovascular diseases. *J Clin Basic Cardiol 2001; 4: 153–155.*

Key words: estradiol, estradiol metabolites, angiogenesis

The inhibition of neo-angiogenesis, *ie* growth of new micro vessels, is currently a topic of major concern, because tumour growth is mainly influenced by formation of new blood vessels [1]. Yet several clinical studies are under way investigating the effect of anti-angiogenetic substances in the treatment of various cancers. However, prevention of neo-angiogenesis may also be of clinical significance in cardiovascular diseases, since neo-vascularization has been observed in atherosclerotic plaques [2]. Furthermore animal experiments have demonstrated that the development of atherosclerotic plaques in later stages can be inhibited by anti-angiogenetic substances [3].

It is well recognized that the natural sex steroid 17β-estradiol is able to influence the vascular system in various ways [4]. The vascular endothelium appears to be a target organ for estradiol, especially since it possesses a highly expressed estradiol receptor system [5, 6]. At physiological concentrations estradiol has been shown to possess angiogenetic properties by inducing stimulation of cell attachment, migration and proliferation of endothelial cells [7], all stages of neo-angiogenesis.

In vivo estradiol is metabolised almost exclusively by oxidation at the A- and D-ring. In the course of metabolism estradiol is first converted reversibly to estrone which is subsequently metabolised irreversibly at the A-ring to 2-hydroxy- and 4-hydroxymetabolites and at the D-ring to 16α-hydroxyestrone and estriol. The endogenous estradiol metabolite 2-methoxyestradiol has already been shown to possess marked anti-proliferative, anti-angiogenetic and tumour-inhibiting effects [8].

The present study investigates the relationship between dosage and effect of 14 endogenous estradiol metabolites on the proliferation of endothelial cells from human umbilical veins in comparison with the effect of estradiol.

Material and Methods

17β-estradiol and the A-ring metabolites 2-hydroxyestrone, 2-methoxyestrone, 2-hydroxyestradiol, 2-methoxyestradiol, 2-hydroxyestriol, 2-methoxyestriol, 4-hydroxyestrone, 4-methoxyestrone, 4-hydroxyestradiol, 4-methoxyestradiol, and the D-ring metabolites estrone, estriol, estetrol and 16α-hydroxyestrone were purchased from Steraloids, USA. The steroids were dissolved in ethanol and tested at the concentrations 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol/L. The experiments were carried out with cells at passage 7.

Endothelial cells from human umbilical veins were purchased from Biowhittaker, Germany. The cells were cultured in MCDB 131, 10 % foetal calf serum (FCS), 5 % endothelial cell growth factor and heparin, 0.3 mg/ml glutamine, 1 % amphotericin B and 1 % penicillin/streptomycin. Before reaching confluence 900 cells per well were transferred into 96-well plates and cultured in standard medium. The cells were preincubated for 3 days. The steroids were dissolved in ethanol and added to the medium while the controls were treated with the same concentration of ethanol used in the steroid solutions *ie* 0.1 %. Medium and test substances were changed every 48 h.

Proliferation of the cells was measured after 7 days incubation using a crystal violet staining technique according to Kueng et al. [9], which is based on the staining of the cell nuclei. Statistical analysis was performed by ANOVA and Dunett's-test from duplicates of three different experiments.

Results

Table 1 shows a summary of the changes in cell numbers after treatment with the test substances, expressed in percentages of the control values. At the lowest concentration used *ie* 10^{-8} mol/L

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Table 1. Changes in cell number of endothelial cells from human umbilical veins after treatment with estradiol and its metabolites. The values are expressed in percent of cell counts compared to cell counts of the controls = 100 % (means ± SD, n= 6)

Compound	10^{-8} mol/L	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
Estradiol	137.6 ± 12.7**	127.9 ± 11.5**	108.8 ± 5.6**	87.8 ± 6.7**
A-ring metabolites				
2-Hydroxyestrone	110.7 ± 4.2**	100.3 ± 3.4	86.5 ± 9.3**	8.5 ± 2.2**
2-Methoxyestrone	124.5 ± 9.5**	116.9 ± 7.0**	99.1 ± 8.0	94.6 ± 6.0
2-Hydroxyestradiol	124.2 ± 12.5**	112.5 ± 11.3*	72.6 ± 0.2**	9.0 ± 2.7**
2-Methoxyestradiol	116.5 ± 7.9**	102.2 ± 10.3	46.9 ± 7.0**	6.4 ± 4.0**
2-Hydroxyestriol	113.0 ± 6.8**	108.8 ± 6.2**	100.0 ± 6.3	83.2 ± 6.1**
2-Methoxyestriol	128.8 ± 7.6**	124.4 ± 6.9**	113.4 ± 10.6	95.4 ± 7.0
4-Hydroxyestrone	129.2 ± 7.4**	115.4 ± 9.7**	86.2 ± 18.1	0.2 ± 0.6**
4-Methoxyestrone	124.4 ± 10.3**	113.5 ± 5.2**	108.3 ± 13.5	97.5 ± 7.7
4-Hydroxyestradiol	130.4 ± 7.5**	127.3 ± 7.0**	103.2 ± 18.2	1.6 ± 2.4**
4-Methoxyestradiol	118.2 ± 5.5**	114.6 ± 9.9**	105.2 ± 12.9	84.8 ± 9.3**
D-ring metabolites				
Estrone	117.0 ± 14.8*	120.1 ± 14.6**	103.2 ± 13.4	102.7 ± 9.7
Estriol	110.8 ± 10.0*	114.2 ± 4.2	99.6 ± 6.8	100.3 ± 3.0
Estetrol	115.0 ± 17.1*	104.9 ± 5.6	97.6 ± 9.3	93.8 ± 7.7
16α-Hydroxyestrone	92.8 ± 15.3	77.8 ± 5.2**	76.7 ± 9.6**	66.1 ± 6.9**

* $p < 0.05$, ** $p < 0.01$

the parent substance estradiol caused a significant increase in the proliferation by 37.6 %. This proliferation stimulating effect gradually diminishes with increasing concentration, even showing a reverse action at the highest concentration i.e. 10^{-5} mol/L inhibiting proliferation by 13.2 %.

A significant increase in the proliferation of the endothelial cells at 10^{-8} mol/L is also seen for the 10 A-ring metabolites tested, the catechol estrogens. Of these 4-hydroxyestradiol showed the strongest effect with a growth of 30.4 %, and 2-hydroxyestrone the weakest with 10.7 %.

For all 10 A-ring metabolites the stimulating effects decreased with increasing concentrations, even resulting in significant proliferation inhibiting effects for 7 metabolites at 10^{-5} mol/L. For 5 metabolites the reduction in cells was over 90 % of the original cell number measured. In the case of 2-methoxyestrone, 2-methoxyestriol and 4-methoxyestrone the cell numbers returned to the values of the controls, *ie* they had no effect on cell proliferation when high concentrations were administered. The lowest cell numbers measured *ie* strongest inhibitions were 1.6 % and 0.2 % for the 4-catechol estrogens 4-hydroxyestrone and 4-hydroxyestradiol.

No marked changes were seen for the D-ring metabolites. At the lowest concentration, small increases in proliferation were observed for estrone, estriol and estetrol. At 10^{-7} mol/L, the next highest concentration, the proliferation stimulating effect only remained for estrone. Higher concentrations showed no detectable effect on proliferation. 16α-hydroxyestrone takes up a special position in the D-ring metabolites since it causes an inhibition of proliferation compared with the control value at the higher concentrations tested i.e. from 10^{-7} to 10^{-5} M.

Discussion

The results show the parent substance 17β-estradiol exhibiting a biphasic reaction on the proliferation of human umbilical vein cells having a stimulating effect at low concentrations and an antiproliferative effect at high concentrations.

At low concentrations the A-ring metabolites, the catechol estrogens, respond similarly to the parent substance while substantial differences can be seen at the highest concentration *ie* 10^{-5} mol/L. Methylation of the catechol estrogens appears to be of great importance regarding their biological activity, an exception being the metabolite 2-hydroxyestradiol. Methylation completely abolishes the very strong antiproliferative effect on the endothelial cells *ie* at high concentrations the metabolites no longer have an effect on natural cell growth. Only 2-methoxyestradiol is still a strong antiproliferative substance at high concentrations. There are several research reports on the antiproliferative effect of 2-methoxyestradiol, both on vascular endothelial cells and on various tumour cells [10]. An earlier study examined the effect of various estradiol metabolites on capillary endothelial cells of the bovine brain [8]. The effectiveness was determined by metabolite concentrations, which were able to reduce control cell numbers by 50 %. Since figures for the test concentrations were not reported, a direct comparison with our results is not possible. A biphasic response depending on the concentration used was not described as seen in our study. The authors concluded that 2-methoxyestradiol had the greatest proliferation inhibiting effect of all metabolites tested. In our study high proliferation inhibiting effects were also found with other metabolites at 10^{-5} mol/L.

The D-ring metabolites showed no marked effects on the proliferation of vascular endothelial cells except for 16α-hydroxyestrone. Estrone, estriol and estetrol showed slight stimulation at the lowest concentration. Surprisingly no proliferation inducing effect was elicited by 16α-hydroxyestrone which is regarded as a powerful estrogen [12]. In fact the opposite effect, significant inhibition of proliferation was observed at the higher concentrations. This result is in contrast to several other experimental investigations, which show a proliferative action of 16α-hydroxyestrone in cell cultures of human breast cancer cells [13]. This might be due to major differences in the action of 16α-hydroxyestrone on the proliferation of cancer cells compared with healthy vascular endothelial cells.

The concentrations used in our study 10^{-8} to 10^{-5} mol/L are clearly in the pharmacological range. However, as seen from the *in vivo* experiments in mice even high doses of 2-methoxyestradiol, approx. 10^{-4} mol/kg, administered for several weeks did not show any toxic effects in the animals [8].

While the use of estradiol metabolites is already being considered for the inhibition of tumour growth, little research is being carried out regarding the effect of estradiol metabolites on cardiovascular disease [13]. We found that estradiol metabolites increase endothelial prostacyclin synthesis [14], decrease proliferation of smooth muscle cells from human coronary arteries [15] and exhibit potent capacity in delaying the oxidation of human LDL [16], and thus may be cardioprotective. These effects were, at least in part, more pronounced than those of their parent substance, 17β-estradiol.

The recent results of Moulton et al. [3], who found an anti-atherosclerotic effect of anti-angiogenetic substances in an animal experiment, open up new avenues for anti-angiogenetic compounds for the prevention of coronary artery diseases. Since 2-methoxyestradiol and other potent anti-angiogenetic estradiol metabolites possess only little estrogenic activity, these compounds may be of interest for therapeutic approaches in women as well as in men. Thus estradiol metabolites at high dosages might be an exciting option for clinical use in the future.

In summary it can be concluded that estrogen metabolites as well as the parent substance estradiol, are able to exert effects on the endothelium of the vascular system in vitro. However, the effects of the metabolites on the endothelium are substantially different from estradiol, which can be seen from the observation that the catechol estrogens exhibit much stronger proliferation inhibiting effects at high concentrations. This should be of interest to clinical pharmacological research since it points to potential new aspects in the treatment of cardiovascular diseases.

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