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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

Material and Methods

17β-estradiol and the A-ring metabolites 2-hydroxyestrone, 2-methoxyestrone, 2-hydroxyestradiol, 2-methoxyestradiol, 2-hydroxyestradiol, 2-methoxyestradiol, 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 Dunnett’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^{-5} 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)

<table>
<thead>
<tr>
<th>Compound</th>
<th>10^{-6} mol/L</th>
<th>10^{-7} mol/L</th>
<th>10^{-8} mol/L</th>
<th>10^{-5} mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>115.0 ± 17.1*</td>
<td>104.9 ± 5.6</td>
<td>97.6 ± 9.3</td>
<td>93.8 ± 7.7</td>
</tr>
<tr>
<td>A-ring metabolites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Hydroxyestrone</td>
<td>110.7 ± 4.2**</td>
<td>100.3 ± 3.4</td>
<td>86.5 ± 9.3**</td>
<td>8.5 ± 2.2**</td>
</tr>
<tr>
<td>2-Methoxyestrone</td>
<td>124.5 ± 9.5**</td>
<td>116.9 ± 7.0**</td>
<td>99.1 ± 8.0</td>
<td>94.6 ± 6.0</td>
</tr>
<tr>
<td>2-Hydroxyestradiol</td>
<td>124.2 ± 12.5**</td>
<td>112.5 ± 11.3*</td>
<td>72.6 ± 0.2**</td>
<td>9.0 ± 2.7**</td>
</tr>
<tr>
<td>2-Methoxyestradiol</td>
<td>116.5 ± 7.9**</td>
<td>102.2 ± 10.3</td>
<td>46.9 ± 7.0**</td>
<td>6.4 ± 4.0**</td>
</tr>
<tr>
<td>2-Hydroxyestradiol</td>
<td>112.3 ± 6.8**</td>
<td>108.8 ± 6.2**</td>
<td>100 ± 6.3</td>
<td>83.2 ± 6.1**</td>
</tr>
<tr>
<td>2-Methoxyestradiol</td>
<td>126.8 ± 7.6**</td>
<td>124.4 ± 6.9**</td>
<td>113.4 ± 10.6</td>
<td>95.4 ± 7.0</td>
</tr>
<tr>
<td>4-Hydroxyestrone</td>
<td>129.2 ± 7.4**</td>
<td>115.4 ± 9.7**</td>
<td>86.2 ± 18.1</td>
<td>0.2 ± 0.6**</td>
</tr>
<tr>
<td>4-Methoxyestrone</td>
<td>124.4 ± 10.3**</td>
<td>113.5 ± 5.2**</td>
<td>108.3 ± 13.5</td>
<td>97.5 ± 7.7</td>
</tr>
<tr>
<td>4-Hydroxyestradiol</td>
<td>130.4 ± 7.5**</td>
<td>127.3 ± 7.0**</td>
<td>103.2 ± 18.2</td>
<td>1.6 ± 2.4**</td>
</tr>
<tr>
<td>4-Methoxyestradiol</td>
<td>118.2 ± 5.5**</td>
<td>114.6 ± 9.9**</td>
<td>105.2 ± 12.9</td>
<td>84.8 ± 9.3**</td>
</tr>
<tr>
<td>D-ring metabolites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>117.0 ± 14.8*</td>
<td>120.1 ± 14.8**</td>
<td>103.2 ± 13.4</td>
<td>102.7 ± 9.7</td>
</tr>
<tr>
<td>Estriol</td>
<td>110.8 ± 10.0*</td>
<td>114.2 ± 4.2</td>
<td>99.6 ± 6.8</td>
<td>100.3 ± 3.0</td>
</tr>
<tr>
<td>Estetrol</td>
<td>115.0 ± 17.1*</td>
<td>104.9 ± 5.6</td>
<td>97.6 ± 9.3</td>
<td>93.8 ± 7.7</td>
</tr>
<tr>
<td>16α-Hydroxyestrone</td>
<td>92.8 ± 15.3</td>
<td>77.8 ± 5.2**</td>
<td>76.7 ± 9.6**</td>
<td>66.1 ± 6.9**</td>
</tr>
</tbody>
</table>

*p < 0.05, ** p < 0.01

Discussion

The results show the parent substance 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 i.e. 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 anti-proliferative effect on the endothelial cells at high concentrations the metabolites no longer have an effect on natural cell growth. Only 2-methoxyestradiol is still a strong anti-proliferative 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, estradiol 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-angiogenic substances in an animal experiment, open up new avenues for anti-angiogenic compounds for the prevention of coronary artery diseases. Since 2-methoxyestradiol and other potent anti-angiogenic 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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