Regulation of growth-regulated oncogene $\alpha$ expression by estrogen in human endothelial cells

LEI Zhu-Bin$^1$, LI Xiao-Yan, WANG Bao-Cheng, YANG Yan-Feng, YOU Nai-Zhen, SUN Jun
(Department of Cardiology, General Hospital of Jinan Military Region, Jinan 250031, China)

KEY WORDS endothelial cells; oncogenes; estrogens; atherosclerosis

ABSTRACT

AIM: To study the effect of estrogen on expression of growth-regulated oncogene $\alpha$ (GRO$\alpha$) in human umbilical vein endothelial cells (HUVEC) in vitro. METHODS: Expressions of CXC chemokine GRO$\alpha$ mRNA and protein were measured by Northern blotting assay and ELISA, respectively. The physiological significance of GRO$\alpha$ expression was tested by static cell adhesion assay.

RESULTS: Both the GRO$\alpha$ mRNA and protein levels decreased markedly after HUVEC were exposed to 17$\beta$-estradiol (E$_2$) 0.05 $\mu$mol/L. Moreover, the inhibition of the protein was depended on the concentration of 17$\beta$-estradiol. Tamoxifen (0.1 $\mu$mol/L), an estrogen receptor $\alpha$ antagonist, alone did not affect GRO$\alpha$ protein expression, but can reverse the E$_2$-induced inhibition of GRO$\alpha$ protein expression (by up to 50%) and the binding of U937 cells to E$_2$-treated HUVEC (by up to 40%). CONCLUSION: Estrogen might functionally down-regulates GRO$\alpha$ expression through estrogen receptor $\alpha$ on endothelial cells.

INTRODUCTION

Monocyte adhesion and emigration involve sequential and overlapping interactions of signal molecules and are fundamental in the initial pathogenesis and progression of atherosclerosis. The ingress, retention, and activation of monocytes in atherogenesis are the sequels of atherogenic responses that include the expression of the CXC chemokine growth-regulated oncogene $\alpha$ (GRO$\alpha$)$^{11}$. The endothelium is a known source for multiple cytokines that could regulate function of monocytes$^{2-3}$. The induction and immobilization of GRO$\alpha$ can induce monocyte adhesion to endothelium stimulated by minimally modified low-density lipoprotein (LDL), implying a role of GRO$\alpha$ in monocyte recruitment during atherogenesis$^{4}$. Chemokine GRO$\alpha$ binds endothelial cells via heparin sulfate proteoglycans, helps monocytes stick to endothelium, and contributes to roll into firm, shear-resistant arrest of monocytes in physiological flow$^{3, 4-6}$.

Estrogens prevent heart disease in women and have also been shown to retard atherogenesis in animal models. Recent evidence, however, demonstrated that endothelial cells may express estrogen receptors$^7$. Thus, the effects of sex steroids on GRO$\alpha$ expression of endothelial cells may involve some indirect mechanisms of monocyte adhesion to endothelium during atherogenesis.

We thus hypothesized that sex steroids may be involved in the regulation of monocytes retention on the endothelium by way of regulation of GRO$\alpha$ expression. In this study, we investigated the regulation of GRO$\alpha$ mRNA and protein production by estrogen in human endothelial cells.

MATERIALS AND METHODS

Materials A rabbit polyclonal antibody against GRO$\alpha$ and the secondary antibody complexed alkaline phosphatase for cell surface ELISA were from Chiron Corp (California, USA). FCS and DMEM were provided by Gibco-BRL (NY, USA). All other reagents, unless indicated, were from Sigma Chemical Co.

Cell culture Human umbilical vein endothelial cells (HUVEC) were harvested from human umbilical vein using 0.05% trypsin with 0.02% edetic acid and plated on 0.1% gelatin-coated dishes and incubated in DMEM containing 20% FCS, basic fibroblast growth factor 10 $\mu$g/L, benzylpenicillin 100 KU/L, and streptomycin sulfate 100 mg/L. Confluent HUVEC at passages 6 to 9 on gelatin-coated polyester sheets were

---

$^1$Correspondence to LEI Zhu-Bin.
Phn 86-531-798-6603. E-mail: leizhub@163.com.cn
Received 2001-03-23 Accepted 2001-08-30
used for the experiments.

**Northern blot analysis** As described previously, total RNA was extracted using TRIzol (Gibco-BRL, NY), size-fractioned by electrophoresis, transferred electrophoretically to Hybond-N membranes, and cross-linked to the membranes. Prehybridization was conducted for at least 4 h at 65 °C, and hybridizations were conducted with the addition of a 30-mer oligonucleotide DNA probe complementary to a specific sequence of GROα mRNA and radiolabeled with [γ-32P]ATP. The autoradiographic bands were quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, CA). The presence of similar amounts of total RNA in each lane was verified by visualization of ethidium bromide-stained 28 S and 18 S ribosomal RNA subunits.

**Assay of surface-associated GROα protein** HUVEC were grown in 96-well plates. After indicated time in the results, HUVEC were directly incubated with a 1:10,000 dilution of polyclonal antibody to GROα. After the primary antibody was removed, the cells were rinsed and incubated with the secondary antibody complexed alkaline phosphatase. The alkaline phosphatase was detected with 1 g/L pararosanilin-phosphate in 10% diethanolamine, 0.1% levamisole. Plates were left overnight at room temperature (about 20 °C) and read the next day at 450 nm using an automated microplate reader.

**Static cell adhesion assay** After HUVEC were plated in 96-well plates [\(3 \times 10^3/4 \times 10^3\)]/well] and incubated for 48 h, indicated management was added to each well and then U937 cells [\(2 \times 10^6 - 3 \times 10^6\)]/well] were added to every well. The plate was incubated for additional 30 min at 37 °C. Non-adherent U937 cells were removed by washing twice with PBS. Adherent U937 cells were counted in four high-powered microscopic fields (HPF) for each treatment.

**Statistical analysis** Statistical analysis was performed using t test. Data are expressed as \(x \pm s\), \(n\) was number of independent experiments.

**RESULTS**

**Regulation of GROα mRNA expression in HUVEC by estradiol** HUVEC were placed in serum-free medium 24 h before incubation with culture medium containing 17β-estradiol (E2) 0.05 μmol/L for indicated time. At the end of the incubation period, the culture media were removed, GROα mRNA was evaluated by Northern blot analysis of total RNA (20 μg per lane). The GROα mRNA level decreased markedly in response to 17β-estradiol 0.05 μmol/L. This decrease was evident as early as 1 h and became more pronounced by 8 h (Fig 1). Experiments on the time course of the estrogen effect on GROα mRNA levels were repeated on three occasions, and similar results were observed.

**Effect of E2 on surface-associated GROα protein levels in endothelial cells** We then assessed the effect of E2 on surface-associated GROα protein production in HUVEC. HUVEC were placed in serum-free medium 24 h before incubation with culture medium alone (control) or with culture medium containing E2 (1 - 1 x 10^6 μmol/L). GROα protein started to decrease in response to 8 h of treatment with E2 (1 x 10^6 μmol/L) compared with the control, but the difference was not significant. The decrease in the GROα protein in HUVEC was dependent on the concentration of E2 (1 - 1 x 10^6 μmol/L for 24 h) and the difference was significant (P < 0.05) at concentrations above 1 x 10^6 μmol/L with an IC50 of 1.48 x 10^{-7} mol/L (95% confidence limits; 3.63 x 10^{-9} - 4.57 x 10^{-6} mol/L) (Fig 2).

**Effect of tamoxifen on GROα protein expression in HUVEC and adhesion of U937 cells to HUVEC** We evaluated the effect of tamoxifen, an anti-estrogen, on surface-associated GROα protein production in HUVEC and adhesion of U937 cells to HUVEC. HUVEC were placed in serum-free medium 48 h before incubation with culture medium alone (control), with culture medium containing E2 (0.05 μmol/L), with tamoxifen (0.1 μmol/L), or with E2 (0.05 μmol/L) plus tamoxifen (0.1 μmol/L) for 8 h. At the end of the incubation period, the culture media were removed, and surface-associated GROα protein production was evaluated by ELISA analysis, or operated according to static cell
adhesion assay. Tamoxifen treatment alone for 8 h did not affect GROα protein expression and the number of U937 cells bound to E2-treated HUVEC. On the other hand, when used together with E2 (0.05 μmol/L), tamoxifen reversed the E2-treated inhibition of GROα protein expression (by up to 50%) or the number of U937 cells bound to E2-treated HUVEC (by up to 40%) (Fig 1).

Fig 2. Effect of E2 on surface-associated GROα protein production in HUVEC. n = 4. ± s. *P < 0.05, **P < 0.01 vs control.

Tab 1. Effect of tamoxifen on surface-associated GROα protein production in E2-treated HUVEC and adhesion of U937 cells to E2-treated HUVEC. IFP indicates high-powered microscopic field. ± s. n = 4. *P < 0.05, **P < 0.01 vs control. †P < 0.01 vs E2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GROα/μL L⁻¹</th>
<th>The number of U937 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.69 ± 0.07</td>
<td>75 ± 16</td>
</tr>
<tr>
<td>E2 (0.05 μmol/L)</td>
<td>0.39 ± 0.05†</td>
<td>40 ± 11</td>
</tr>
<tr>
<td>Tamoxifen (0.1 μmol/L)</td>
<td>0.66 ± 0.07*</td>
<td>70 ± 15*</td>
</tr>
<tr>
<td>Tamoxifen + E2</td>
<td>0.54 ± 0.06†</td>
<td>61 ± 13†</td>
</tr>
</tbody>
</table>

DISCUSSION

Estrogen may act at several steps in the atherogenic process to prevent cardiovascular disease. Previous studies have presented that estrogen inhibits both monocyte adhesion and transendothelial migration in hypercholesterolemic rabbits, and the estrogen-mediated reduction in monocyte adhesion is probably due to inhibition of the expression of vascular cell adhesion molecule-1 (VCAM-1) and CC chemokine MCP-1. However, CXC chemokine GROα helps monocytes to stick to endothelium, and it is not clear whether the associated reduction in the number of monocytes within the subendothelial space is also due to an estrogen-induced inhibition of the expression of CXC chemokine GROα.

Our study showed that estradiol at physiological levels significantly reduced GROα gene expression in a time-dependent fashion in HUVEC and this decrease in the expression of GROα protein production was also confirmed in a concentration-dependent fashion in HUVEC. These results indicate that estradiol most likely exerts its regulatory effect on GROα at the level of transcription. GROα bound to HUVEC helps monocytes stick to these endothelial cells and roll into firm, shear-resistant arrest of monocytes in physiological flow.

Our study demonstrated that decreased surface expression of GROα gave rise to a lessened adhesion of monocytes to HUVEC. Tamoxifen, an estrogen receptor α (ERα) antagonist, failed to inhibit the GROα expression when used alone. Moreover, when used together with E2, tamoxifen reversed the inhibition of GROα by E2. This suggests that the inhibitory effect of E2 on GROα is an estrogen receptor-mediated activity. However, it is possible that part of the effect of estradiol on HUVEC may be mediated by the newly discovered ERβ, as it has recently been demonstrated that after carotid arterial injury in a mouse model in which the ERα gene is disrupted, estradiol inhibited vascular smooth muscle cell proliferation.

In summary, we have found that estrogen acts to inhibit GROα expression in human HUVEC and this down-regulation may be mediated through ERα. The down-regulated expression of GROα may be functionally associated with a lessened adhesion of monocytes to HUVEC.

ACKNOWLEDGEMENTS The authors gratefully acknowledge academician PEI Gang (Chinese Academy of Sciences) for helpful advice. We also wish to thank the technical assistance of Dr ZHANG Zhe, WU Ya-Lan, WU Guo-Xiang, and LIN Yi-Ming of Shanghai Institute of Cell Biology (Chinese Academy of Sciences).

REFERENCES
3 Watanabe T, Hanoka S, Shinokamura T. Inflammatory and


雌激素功能性调节入内皮细胞的生长调节摄取基因α表达

雷著斌1，李晓燕，王宝成，杨艳峰，尤乃祯，孙军
（济南军区总医院心内科，济南 250031，中国）

关键词 内皮细胞；癌基因；雌激素类；动脉粥样硬化

目的：研究雌激素在体外调节人脐静脉内皮细胞（HUCVE）的生长调节癌基因α（GROα）。方法：以体外培养的HUCVE为模型，Northern法检测CXC亚族趋化因子GROαmRNA；ELISA方法检测细胞表面的GROα蛋白表达；静态细胞粘附实验测定细胞表面的GROα蛋白的生理意义。结果：17β-雌二醇（0.05 μmol/L）明显抑制HUCVE产生GROαmRNA和蛋白表达水平；而且17β-雌二醇抑制其蛋白表达水平显示剂量依赖性关系；雌激素受体α拮抗剂他莫昔芬（0.1 μmol/L）单独使用不影响其蛋白表达。但可显著逆转17β-雌二醇抑制的GROα蛋白表达，显著逆转17β-雌二醇抑制单核细胞因子细胞因子受体细胞黏附到HUCVE的作用。结论：通过内皮细胞上雌激素受体α、雌激素可能功能性调节入内皮细胞的GROα的表达。