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Effect of estradiol on chemokine receptor CXCR2 expression in rats: implications for atherosclerosis[1]

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ABSTRACT

AIM: To study the effect of 17β-estradiol on expression of chemokine receptor CXCR2 in monocytes *in vivo*. **METHODS:** Expressions of chemokine receptor CXCR2 mRNA and protein were measured by RT-PCR and flow cytometry, respectively. **RESULTS:** In both ovary-intact and ovariectomized (OVX) rats, CXCR2 protein and mRNA expression were significantly increased in rats fed with a high-cholesterol diet for 6 weeks. The cholesterol-induced increases in CXCR2 protein and mRNA expression were significantly attenuated in OVX rats injected with estradiol-17β (17β-E_2) (5 and 20 μg· kg^{-1}· d^{-1}). In normal diet rats, CXCR2 protein and mRNA expression were increased in OVX rats compared with ovary-intact rats, and this increase was prevented by 17β-E_2. **CONCLUSION:** Both basal and hypercholesterolemia-induced increases in chemokine receptor CXCR2 are modulated by physiological concentrations of estradiol.

INTRODUCTION

Women are protected by coronary artery disease until their sixth decade and beyond. Ovarian hormones are vasoactive substances that inhibit atheroma formation. The adhesion of monocytes to endothelial cells[1,2] is essential elements of an inflammatory response and occurs continuously throughout the entire atherogenic process. It has been presented that estradiol supplementation to ovariectomized (OVX) rats fed a cholesterol-enriched (0.5 %) diet inhibits the adhesion of monocytes to endothelial cells *in vivo* when compared with OVX rats not receiving estrogen supplementation[3]. It is therefore possible that estradiol inhibits the development of atherosclerotic lesions by regulating the inflammatory component of the atherogenic process. Recent studies suggest that CXC chemokines may be involved in the pathogenesis of atherogenesis. A potential role in atherogenesis of growth-regulated oncogene (GRO), IL-8, and their shared receptor, CXCR2, which is normally expressed in circulating monocytes[4,5], is receiving increasing attention[6]. We have found that estrogen acts to inhibit GROα expression in human endothelial cells and the inhibition may be mediated through estradiol receptor α[7]. The inhibition of GROα may be functionally associated with a lessened adhesion of monocytes to human endothelial cells. IL-8 is a powerful trigger for firm adhesion of monocytes to vascular endothelium[8]. Furthermore, mice lacking CXCR2 are less susceptible to atherosclerosis

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and have fewer monocytes in vascular lesions[9].

Based on these data, we therefore assessed whether feeding a cholesterol-enriched diet to rats may increase the production of CXCR2 in the fresh peripheral blood monocytes and whether supplementation with estradiol could inhibit its production.

MATERIALS AND METHODS

Chemicals and reagents FCS and RPMI-1640 were purchased from Gibco-BRL. Phycoerythrin-conjugated mouse antibody IgG1 (Ab_{IgG1}), and phycoerythrin-conjugated mouse anti-rat CXCR2 monoclonal antibody (Ab_{CXCR2}) were generous gifts from Dr ZHAO Z (Beijing, China). All other reagents, unless indicated, were from Sigma Chemical Co.

Animal procedures All animal procedures were approved by the local government authorities. Twelve-week-old (average weight, 222 g; range, 214-233 g) female Sprague-Dawley rats were used in all experiments. The animals were housed at 21 ºC with a 12-h light and 12-h dark cycle and were fed rat laboratory diet (Lillico, Betchworth, Surrey, UK). The animals were pair-fed. Water was available *ad libitum*. The rats were either OVX or sham-operated. Physiological plasma levels of estrogen in the ovariectomized rats were maintained as previous study[10]. After surgery, rats received vehicle (Veh), or 17β-estradiol 5 $\mu g \cdot kg^{-1} \cdot d^{-1}$, or 17β-estradiol 20 $\mu g \cdot kg^{-1} \cdot d^{-1}$ as a daily sc injection (17β-estradiol was dissolved in 95 % corn oil/ 5 % benzyl alcohol). Blood was taken at 6th week postsurgery, and was used for subsequent FACS or RT-PCR analysis.

Experimental protocols Rats were divided into 8 groups (n=8, each group): (1) ovary-intact animals fed with normal diet; (2) ovary-intact animals with sham operation fed with a 0.5 % cholesterol diet for 6 weeks; (3) OVX animals with Veh and fed with a 0.5 % cholesterol diet for 6 weeks; (4) OVX animals with Veh and fed with a normal diet for 6 weeks; (5) OVX animals received 17β-estradiol 5 $\mu g \cdot kg^{-1} \cdot d^{-1}$ and fed with normal diet for 6 weeks; (6) OVX animals received 17β-estradiol 5 $\mu g \cdot kg^{-1} \cdot d^{-1}$ and fed with a 0.5 % cholesterol diet for 6 weeks; (7) OVX animals received 17β-estradiol 20 $\mu g \cdot kg^{-1} \cdot d^{-1}$ and fed with normal diet for 6 weeks; and (8) OVX animals received 17β-estradiol 20 $\mu g \cdot kg^{-1} \cdot d^{-1}$ and fed with a 0.5 % cholesterol diet for 6 weeks.

Cell separation At the end of the feeding period the animals were euthanized, and blood samples were collected by cardiac puncture into vacuum tubes containing edetic acid to prevent coagulation. Mononuclear cells were isolated by Ficoll-Hypague method[11]. After adherence to plastic, cells were cultured at 37 º C in a 5 % CO_2 incubator for 2 h and the non-adherent cells were removed. The adhered cells were harvested (monocytes>90 %).

Reverse transcription-PCR (RT-PCR) analysis Total mRNA from 1×10^6 monocytes was isolated with TRIzol (Gibco). First strand cDNA was then synthesized from 2 μg total RNA using reverse transcriptase (Gibco). A parallel control for DNA contamination was carried out without adding reverse transcriptase in the first strand synthesis. Primers were synthesized according to motif: CGGAATTCAAATGGAAGATTTTA-ACATGGAG (CXCR2, sense), CCGCTCGAGTTAGA-GAGTAGTGGAAGTGTG (CXCR2, antisense), TCCATGACAACTTTGGCATCGTGG (GAPDH, sense), and GTTGCTGTTGAAGTCACAGGAGAC (GAPDH, antisense). PCR amplification of CXCR2 cDNA for 40 cycles was 94 º C denaturation (60 s), 58 º C annealing (60 s), and 72 º C extension (60 s). A 500-ng aliquot of total RNA was reverse-transcribed under similar conditions followed by PCR amplification of GAPDH cDNA for 30 cycles. PCR products were analyzed by 2 % agarose gel electroresis. The specificity of the amplification was confirmed by DNA sequencing. The concentration of the reverse transcribed cDNA in the PCR mixture was adjusted to assure a liner correlation between template and product. The housekeeping gene GAPDH was used as a control template for normalizing relative changes of CXCR2 mRNA in RT-PCR.

Fluorescence-activated cell sorting (FACS) Mononuclear cells were washed three times with cold buffer (PBS-1 % FCS-0.1 % sodium azide), 5×10^5 cells were resuspended at in 100 μL binding buffer supplemented with 1 μg phycoerythrin-conjugated anti-CXCR2 mAb, or 1 μg phycoerythrin-conjugated isotype anti-IgG_1 mAb control, and incubated on ice in the dark for 45 min. Subsequently, the cells were washed twice and analyzed by flow cytometry (Becton Dickson). Only monocytes were accounted according to their light scatter and size during flow cytometry. Mean fluorescence intensity (MFI) was determined by subtracting the MFI of cells stained with Ab_{IgG_1} control from MFI of cells stained with Ab_{CXCR2}. All flow cytometric analyses were performed on a FACScan flow cytometer using FACScan and Consort 30 software (Becton Dickinson).

Statistical analysis All data represent the mean±SD of at least three independent experiments. Student's *t*-test was used for the statistical analysis of the results. Values of $P<0.05$ were considered to be significant.

RESULTS

Effect of ovariectomy and cholesterol-enriched diet on CXCR2 expression in rats Compared with ovary-intact rats, ovariectomy increased CXCR2 mRNA expression by 2.1-fold in rats fed with normal diet. In ovary-intact animals fed with a cholesterol-enriched diet, CXCR2 mRNA expression was increased by 2.3-fold, whereas in the OVX animals fed with a cholesterol-enriched diet, CXCR2 mRNA expression was increased by 4.1-fold (Fig 1). The values for pooled protein expression paralleled those for mRNA expression (Fig 2).

Effect of estradiol supplementation on basal CXCR2 expression in rats fed with normal diet FACS analysis of pooled samples for CXCR2 protein expression from each of the 4 groups is shown in Fig 3A. MFI of FACS analysis performed on each of the 4 groups is shown in Fig 3B. Compared with ovary-intact rats, ovariectomy increased CXCR2 expression by 130 %. Estradiol 5 and 20 $\mu g \cdot kg^{-1} \cdot d^{-1}$ attenuated the increase in CXCR2 protein expression by 25 % and 50 %, respectively, and MFI of the higher dose was similar to that observed in ovary-intact animals.

Effect of estradiol supplementation on CXCR2 expression in rats fed with a cholesterol-enriched diet FACS analysis of CXCR2 protein expression of pooled samples from the 3 groups is shown in Fig 4A, and the MFI performed on each of the 3 groups is shown in Fig 4B. Estradiol 5 and 20 $\mu g \cdot kg^{-1} \cdot d^{-1}$ reduced CXCR2 protein expression by 31 % and 53 %, respectively.

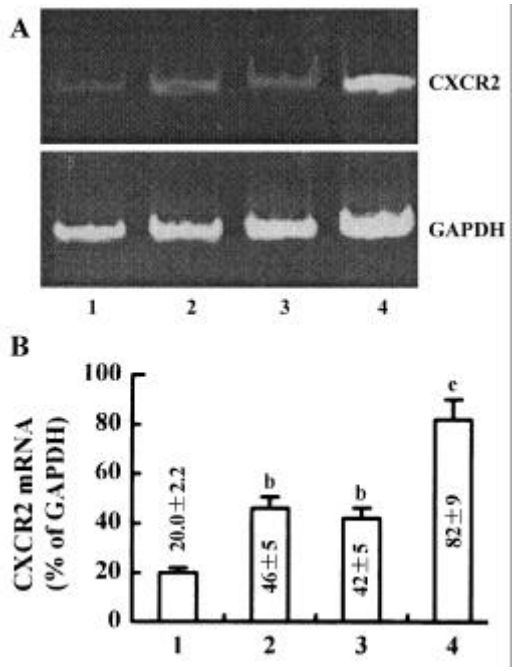

Fig 1. Effect of high-cholesterol diet and ovariectomy on CXCR2 mRNA expression. A) Agarose gel electrophoresis of amplified CXCR2 transcripts. Ovary-intact rats were fed with normal diet for 6 weeks (group 1, control); ovary-intact rats were fed with a high-cholesterol diet for 6 weeks (group 2); OVX rats fed with normal diet for 6 weeks (group 3); or fed with a high-cholesterol diet for 6 weeks (group 4). B) CXCR2 expression was determined by semi-quantitative RT-PCR, normalized to GAPDH. *n*=4. Mean±SD. $^bP<0.05$ *vs* control. $^cP<0.05$ *vs* group 3.

DISCUSSION

In previous studies, we have demonstrated that enhanced surface expression of CXCR2 gave rise to an enhanced migration and an improved adhesion of monocytes[12]. Preliminary evidence has shown that estrogen reduces monocyte adherence to vascular endothelium *in vivo*[3]. However, 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 CXCR2.

Other investigators have demonstrated that the chemokine GROα, which significantly contributes to monocyte arrest, is expressed on native atherosclerotic endothelium[14]. We have also shown that estradiol at physiological levels significantly reduced GROα expression in human umbilical vein endothelial cells[7]. In the present study, we demonstrated the effect of estradiol on the chemokine GROα receptor CXCR2. Consistent with the result of GROα, estradiol 5 and 20 $\mu g \cdot kg^{-1} \cdot d^{-1}$ attenuated the increase of CXCR2 protein expression. In animals fed with normal diet, CXCR2 mRNA expression was apparently undetectable, because a visible band for CXCR2 appeared only after 3 μg of total cellular RNA was used for 40 cycles of PCR amplification and visualization on 2 % agarose gel after ethidium bromide staining. These results indicate that estradiol most likely exerts its regulatory effect on CXCR2 at the level of transcription.

Potential mechanisms by which estradiol may inhibit CXCR2 expression must be considered. Estradiol acts as an antioxidant *in vivo*[14], and in its absence, an increase in lipoprotein oxidation in the artery wall could

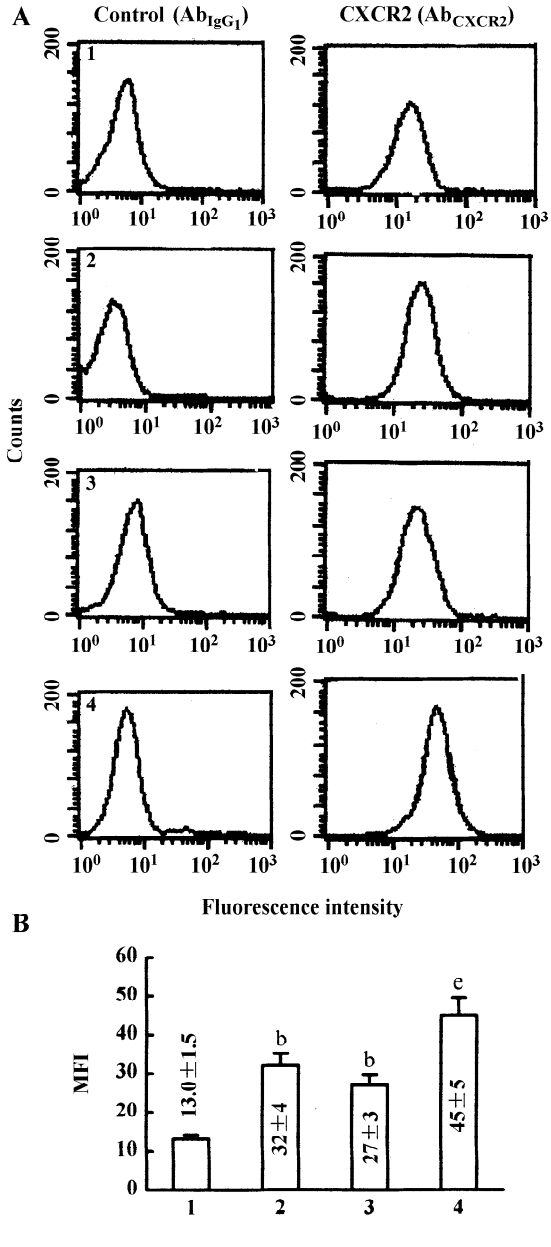

Fig 2. Effect of high-cholesterol diet and ovariectomy on CXCR2 protein expression. A) FACS histograms of rat monocytes stained with control PE-IgG_1 or PE-CXCR2. Ovary-intact rats were fed with normal diet for 6 weeks (group 1); ovary-intact rats were fed with a high-cholesterol diet for 6 weeks (group 2); OVX rats fed with normal diet for 6 weeks (group 3); or fed with a high-cholesterol diet for 6 weeks (group 4). B) Mean fluorescence intensity (MFI) of different groups. n=4. Mean±SD. $^bP<0.05$ *vs* control. $^eP<0.05$ *vs* group 3.

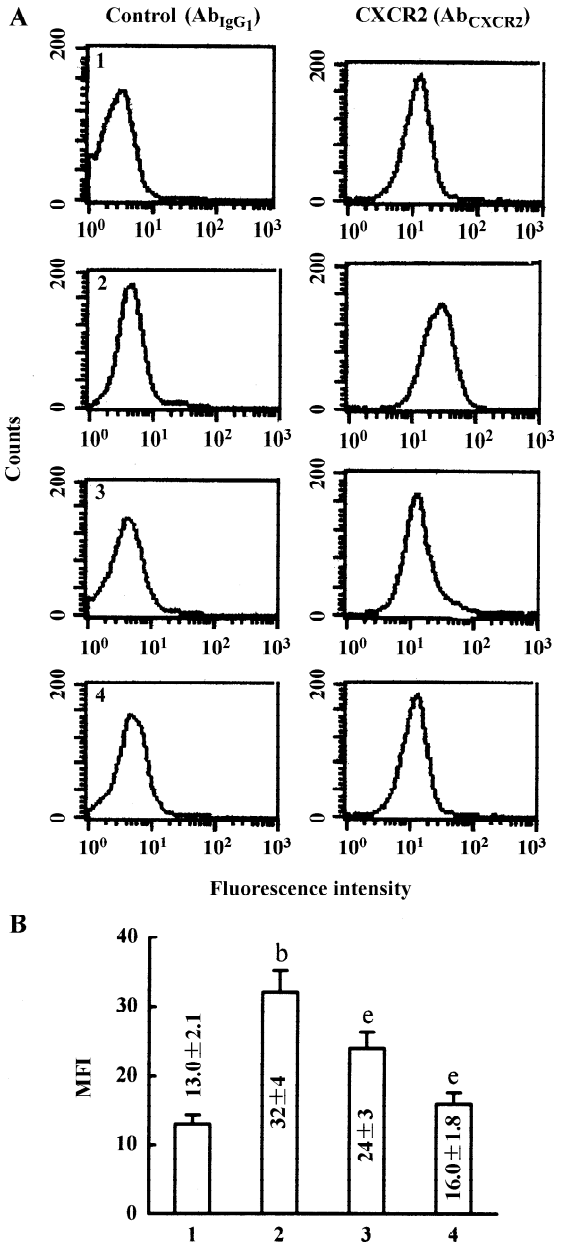

Fig 3. Effects of ovariectomy and 17b-estradiol supplementation on CXCR2 protein expression in rats fed with a normal diet for 6 weeks. A) FACS histograms of rat monocytes stained with control PE-IgG_1 or PE-CXCR2. Ovary-intact rats fed with normal diet (group 1, control); OVX rats supplemented with placebo and fed with normal diet (group 2); OVX, injected with 17b-estradiol 5 μg and fed with normal diet (group 3); or OVX rats injected with 17b-estradiol 20 μg and fed with normal diet (group 4). B) Mean fluorescence intensity (MFI) of different groups. n=4. Mean± SD. $^bP<0.05$ *vs* control. $^eP<0.05$ *vs* group 2.

account for the increase in CXCR2 expression in the OVX animals. This *scenario* is also possible in the animals fed with normal diet, as rat diet contains significant amounts of linoleic acid, which makes normal rat LDL especially susceptible to oxidation. In concurrent studies, 17β-estradiol reduced expressions of CXCR2 mRNA and protein in endothelial cells incubated with oxLDL, and the decrease of the CXCR2 protein was dependent on the concentration of 17β-estradiol.

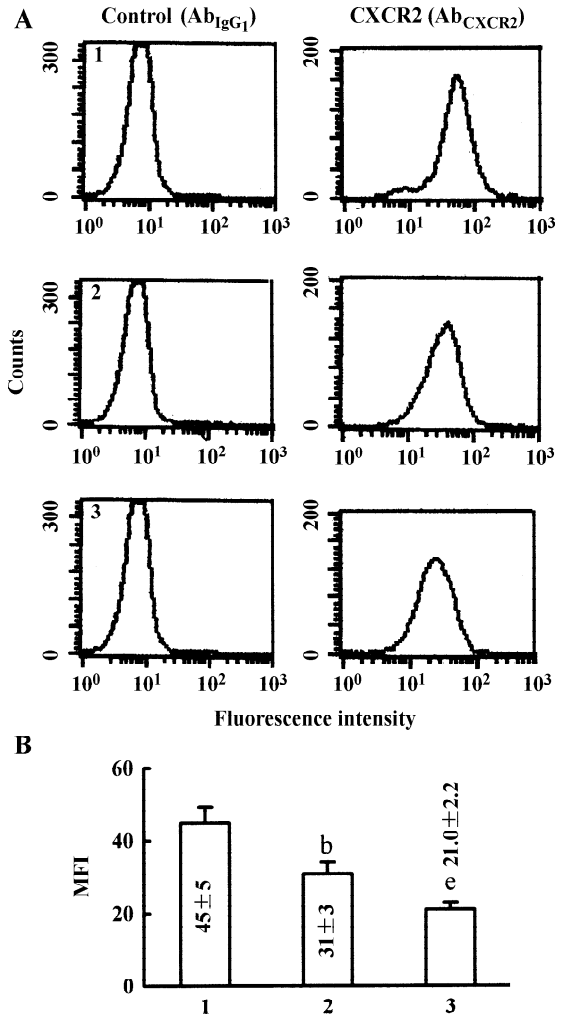

Fig 4. Effect of 17b-estradiol on CXCR2 protein expression in OVX rats fed with a high-cholesterol diet for 6 weeks. A) FACS histograms of rat monocytes stained with control PE-IgG_1 or PE-CXCR2. OVX rats fed with a high-cholesterol diet (group 1, control); OVX rats injected with 17b-estradiol 5 μg and fed with a high-cholesterol diet (group 2); or injected with 17b-estradiol 20 μg, and fed with a high-cholesterol diet (group 3). B) Mean fluorescence intensity (MFI) of different groups. *n*=4. Mean±SD. $^bP<0.05$ *vs* control. $^eP<0.05$ *vs* group 2.

In summary, our results demonstrate that estradiol at physiological concentrations inhibits CXCR2 expression *in vivo*. Estrogen causes the associated reduction in the number of monocytes within the subendothelial space due to an estrogen-induced inhibition of the expression of CXCR2. This finding therefore may be one potential mechanism by which estradiol retards atherogenesis.

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