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The Up-Regulation of SOCS3 Expression by Estrogen Simulation in Macrophages is not Through the Estrogen Receptor (ER) Signaling

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ABSTRACT

Immuno-inflammation hypothesis is the hot spot of atherosclerosis mechanism at present, while SOCS (Suppresor of Cytokine Sigaling) is the key inhibitor of cytokine and its immuno-inflammatory signaling. Recent researches suggest that SOCS3 is probably a latent atherosclerosis protective factor, while estrogen is shown to play a protective role to atherosclerosis. In this paper, we demonstrated that estrogen could increase SOCS3 expression in a dose dependent manner in macrophages cells. We further demonstrated that estrogen up-regulate SOCS3 expression is not through the traditional ER (estrogen receptor) pathway as detected by estrogen inhibitor-ICI182780. Our findings illustrate more details about the mechanism of estrogen-induced atherosclerosis protection.

INTRODUCTION

With the changes in people's lifestyle and social changes in environmental factors, the incidence of cardiovascular disease (CVD) increased year by year, especially the coronary heart disease (CHD) [1]. Atherosclerosis is one of the most common pathological process that leads to CVD. It is characterized by a formation of atherosclerotic plaques in arteries consisting of necrotic cores, calcified regions, accumulated modified lipids and various cells [2]. There are several hypotheses to explain the pathogenesis of atherosclerosis, such as lipid infiltration, thrombosis, smooth muscle cell clones, endothelial damage response and immunity inflammation [3-5]. Suppressor of cytokine signaling (SOCS) protein are inhibitors of cytokine signaling pathways that were highlighted in immunity inflammation hypothesis [6]. Cytokines, including interleukins, interferons (IFNs), growth factors et al. have essential roles in the development, differentiation and function of immune cells [6]. Most SOCS proteins are induced by cytokines and therefore act in a classical negative-feedback loop to inhibit cytokine signal transduction, thus regulating the immunity inflammation response [7]. SOCS3, a sub-family of SOCS, is involved in inflammatory response, because it regulated macrophage and dendritic cell activation and was essential for T-cell development and differentiation [7]. Furthermore, it has been reported that loss of SOCS3 expression in T cells significantly affects atherosclerotic development [8]. Epidemiological survey showed that estrogen might be a beneficial factor in atherosclerosis as the CHD incidence was much higher in men than that in women and postmenopausal women rapidly increased its CHD incidence [9,10]. Several groups have demonstrated the mechanism for the improvement of atherosclerosis by estrogen, including the regulation of lipid metabolism [11] and inhibition of smooth muscle proliferation and migration [12]. Whether SOCS mediated inflammation inhibition was involved in improvement of atherosclerosis by estrogen has not been studied yet. In breast cancer and hepatoma cells, SOCS3 expression were found to be correlated to estrogen level [13,14], and might be regulated by the interaction of estrogen response element with SOCS3 promoter [13]. Recent researches reported that macrophages play an important role in the development of atherosclerosis [15,16]. Therefore, we focus on the relationship of estrogen and the expression of SOCS3 in macrophages, which might be involved in inflammation regulation of atherosclerosis by estrogen.

MATERIALS AND METHODS

Cell cultures

RAW264.7 cells were cultured and maintained in DMEM medium (Hyclone) supplemented with 10% FCS (Hyclone), 100 U/

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mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine (supplements from Hyclone). Cells were trypsinized by 0.25% trypsin and refreshed every 2-3 days.

Cell treatment with 17β-estrodiol and ICI 182,780

RAW264.7 cells were cultured in 6 wells at a concentration of 5 × 104 / ml. Cells were washed with PBS twice and refreshed in 2 ml DEME medium with blank、1% DMSO、17 β -estrodiol 100 nM、10 nM、1 nM respectively. Cells were further incubated for 12h and 24h and cell viability was checked by MTT assay. Meanwhile, cells were harvested for real-time PCR analysis. For ICI 182,780 treatment, the same procedure was performed as above. For each well, we added 17 β -estrodiol 10 nM, 17 β -estrodiol 10 nM+ICI 182,780 100 nM and incubated for 12h for real-time PCR analysis.

Real-time quantitative PCR

Cells were lysed in 250 μL Trizol (Invitrogen, Carlsbad, USA) and maintained at -80°C. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands). The concentration and purity of RNA were measured using the NanoDrop ND-1000 spectrophotometer (Thermo, USA). Total RNA (2 μg) was converted into cDNA by the SuperScript First-Strand Synthesis System (Invitrogen) using random hexamers as primers. Real-time PCR was done using the ABI PRISM 7700 Sequence Detector (Life Technologies, USA). PCR was performed in a 20 μL reaction mix containing 10 μL SYBR Premix Ex Taq TM II; 0.8 μL forward and reverse primer (10 μM) and 6 μL cDNA. Samples were amplified by 95°C for 10 s, followed by 45 cycles at 95°C for 5s, 63.5°C for 15s and 72°C for 10s. To standardize the amount of sample RNA, we amplified the endogenous housekeeping gene GAPDH as a control. PCR products were electrophoresed on a 1.2% agarose gel to check the fragment size.

Western blot

Cell pellets were lysed in lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% NP40, and 0.25% sodium deoxycholate. The following protease and phosphatase inhibitors were added: 4 mM Na3VO4(450243), 1 mM Na4P2O7(P8010), 2 μg/mL aprotinin(A6103), 50 μg/mL leupeptin (L2884), 500 μg/mL trypsin inhibitor (T9378), 10 μM benzamidin (12072), 2.5 mM pnp benzoate (N8264), 1 mM AEBSF (A8456) and 50 μg/mL pepstatin A (P5318) (all from Sigma), 50 mM NaF (27860.231), 5 mM EDTA (20296.260) (both from VWR International, West Chester, PA). Cell debris was removed by centrifugation (5 min, 14000 rpm) and sample buffer was added. After boiling, the samples were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked with PBS containing 5% low-fat milk and 0.1% Tween 20. Antibodies against SOCS3 and GAPDH were from Cell Signaling Technology.

Statistical analysis

All experiments were repeated at least 3 times. Date was presented as Mean±SD (standard deviation). The significance between groups was determined using the Mann-Whitney U test. The results were considered significant when $P<0.05$.

RESULTS

To investigate whether estrogen could regulate the expression of SOCS3 in macrophages, we designed the real-time PCR primers for SOCS3 gene and GAPDH gene (a housekeep gene control). The sequences of these primers were shown in Table 1. After real-time PCR amplifications, we check the specificity of the PCR products by electrophoresis. In the gel it clear showed a band at around 366bp for SOCS3 gene **(Figure 1)**. Furthermore, from the dissolve curve we can see a singlet at around 87°, indicating the specificity of the SOCS3 primer **(Figure 1)**.

Table 1. Primer sequences of SOCS3 and GAPDH gene.

Gene	Sequences	
SOCS3	forwards	CAC AGC AAG TTT CCC GCC GCC
	reverse	GTG CAC CAG CTT GAG TAC ACA
GAPDH	forwards	TCA ACG GCA CAG TCA AGG
	reverse	ACT CCA CGA CAT ACT CAG C

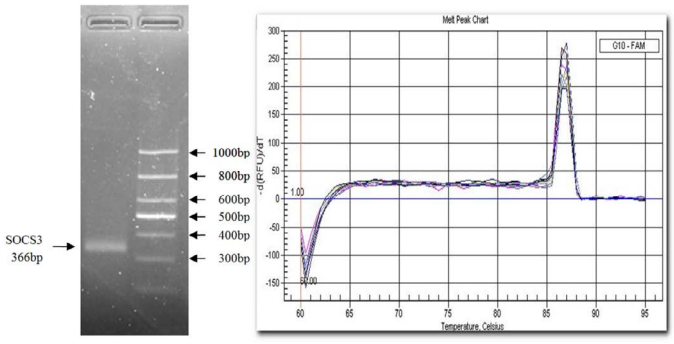

Figure 1. The electrophoresis result and the dissolve curve of SOCS3 gene by RT-PCR.

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For the housekeep GAPDH gene, we got a band around 498bp in the gel and a singlet of 87°C in the dissolve curve **(Figure 2)**.

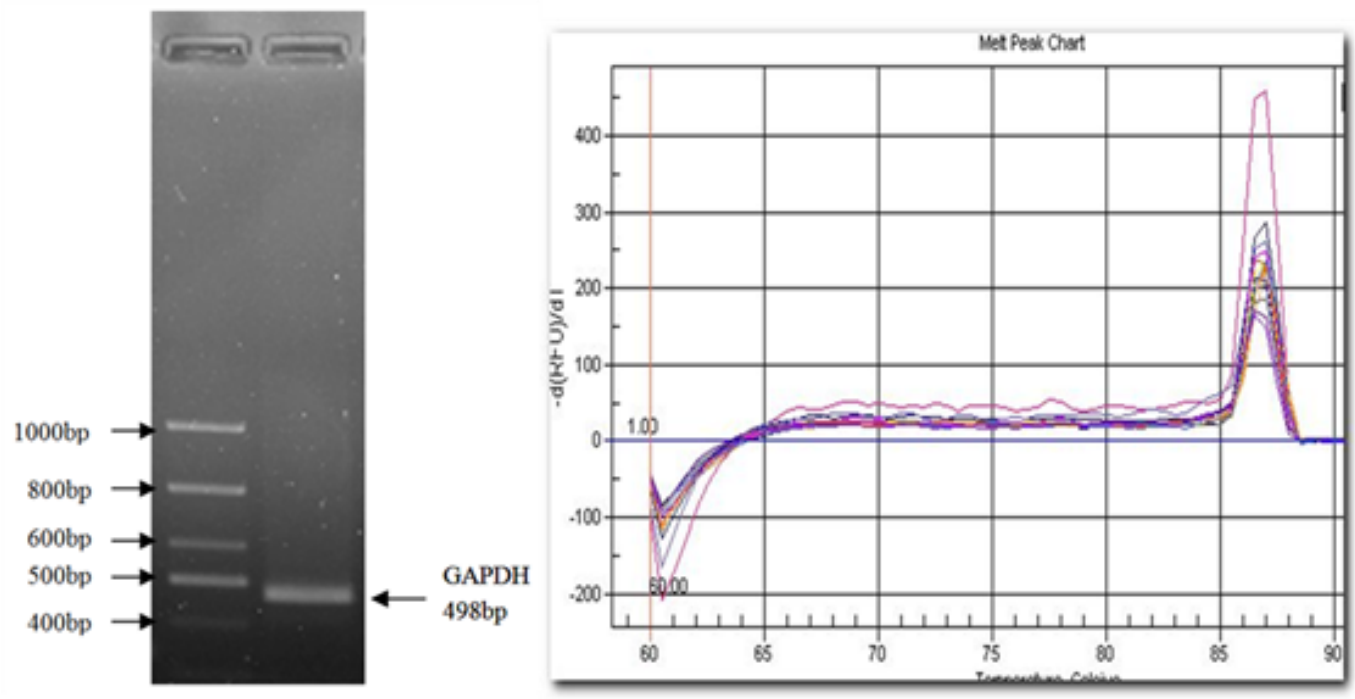

Figure 2. The electrophoresis result and the dissolve curve of GAPDH gene by RT-PCR.

Next, we analyzed the expression of SOCS3 gene in macrophages treated with different concentration of 17β-estrodiol using the $2^{-\Delta\Delta Ct}$ method [17]. The results showed that 17 β -estrodiol could up-regulate SOCS3 gene expression in a concentration dependent manner after 17 β -estrodiol treatment for 12h **(Figure 3)**. Similar results were obtained after 17 β -estrodiol treatment for 24h in macrophages **(Figure 4)**. Next, we investigated the expression of SOCS3 in macrophages treated with 10 nM 17 β -estrodiol by western blot. We used the BCA assay to determine the concentration of the protein samples. We illustrated the standard curve of the BCA

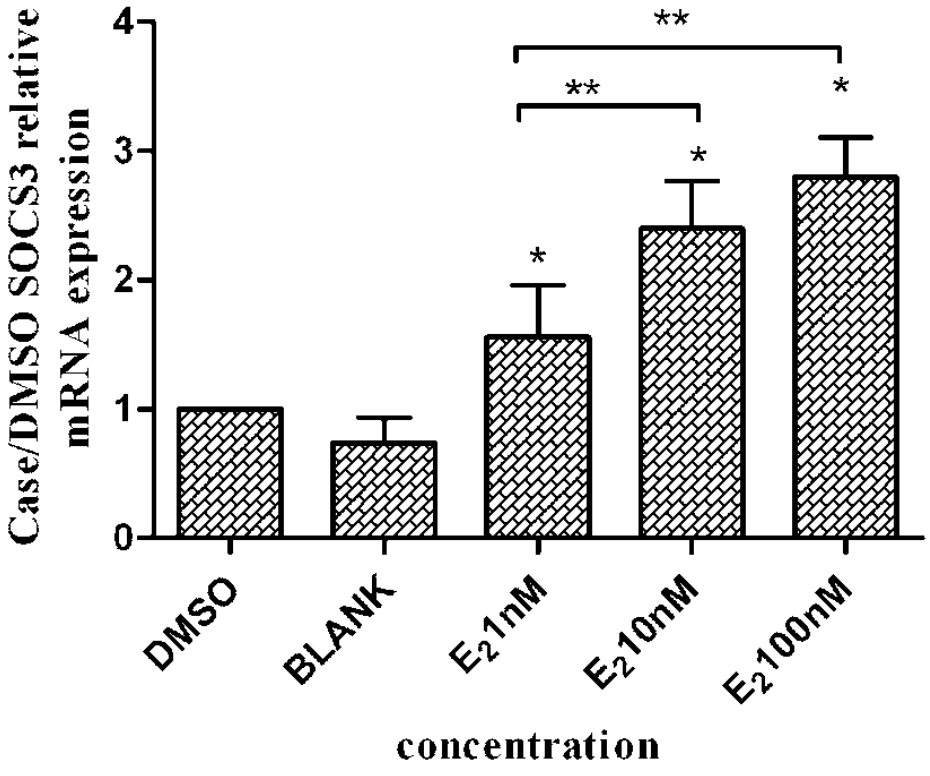

Figure 3. SOCS3 expression after 17 β-estrodiol treatment for 12h in macrophages. *P<0.05 VS DMSO; **P<0.01 VS 17 β-estrodiol 1 nM.

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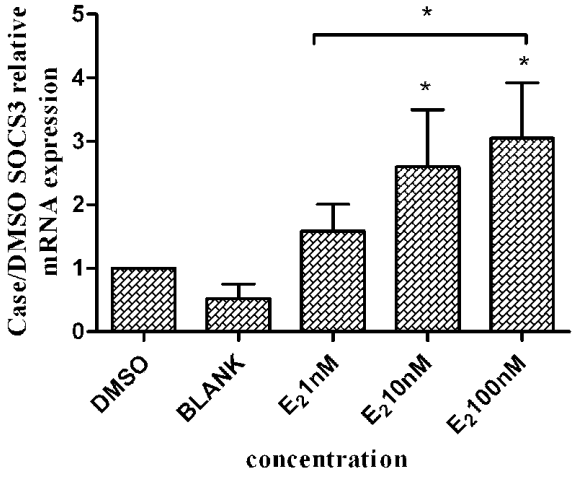

Figure 4. SOCS3 expression after 17 β -estrodiol treatment for 24h in macrophages. *P<0.05 VS DMSO.

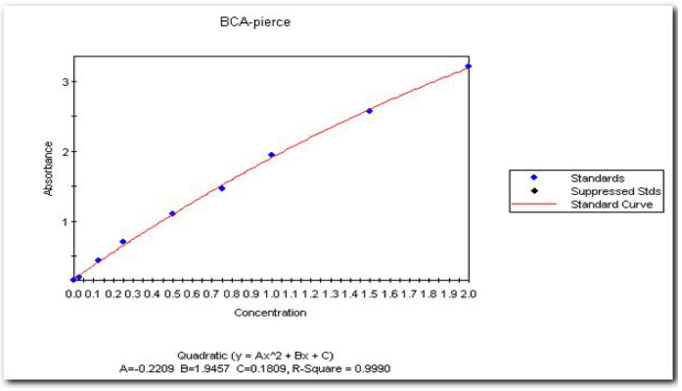

Figure 5. Standard curve of BCA measurement.

assay using the standard sample in the kit. The standard curve was described as $y=0.2209X^2+1.9457X+0.1809$ **(Figure 5)**. Base on the standard curve we calculated the corresponding concentration of each sample, which was shown in **Table 2**. We quantified the same protein loading for western blot and the results showed that SOCS3 expression increased in macrophages after treatment with 10 nM 17 β-estrodiol for 12h and 24h **(Figure 6A)**. **Figure 6B** shows the corresponding gray analysis for each sample, which clear indicates that SOCS3 expression was up-regulated after 17 β -estrodiol simulation.

Group	Blank	DMSO	17β-estrodiol 10nM	
			12h	24h
Protein mg/ml	1.621	1.821	1.876	1.952

Table 2. Protein concentration measured by BCA assay.

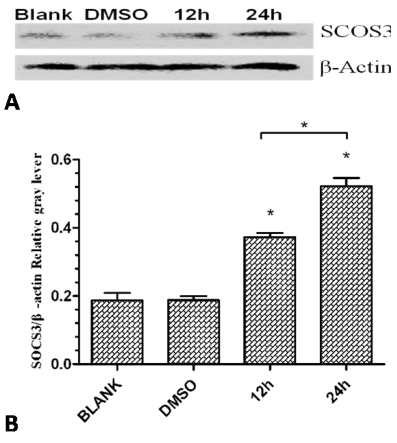

Figure 6. (A and B) SOCS3 expression in macrophages treated with 10 nM 17 β -estrodiol for 12h and 24h were analyzed by western blot. B

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indicates the ratio of SOCS3/ β -actin by gray analysis. *P<0.05 VS DMSO.

Next, we wonder whether the up-regulation of SOCS3 expression in macrophages by estrogen simulation was mediated by estrogen and estrogen receptor (ER) signaling. We used ICI182780 (a specific inhibitor of estrogen receptor) to block the ER signaling when simulating macrophages with estrogen. The real-time PCR results showed that despite inhibiting the estrogen receptor, 17 β-estrodiol could still up-regulate SOCS3 expression **(Figure 7)**, indicating that the up-regulation of SOCS3 by estrogen was through the ER independent signaling.

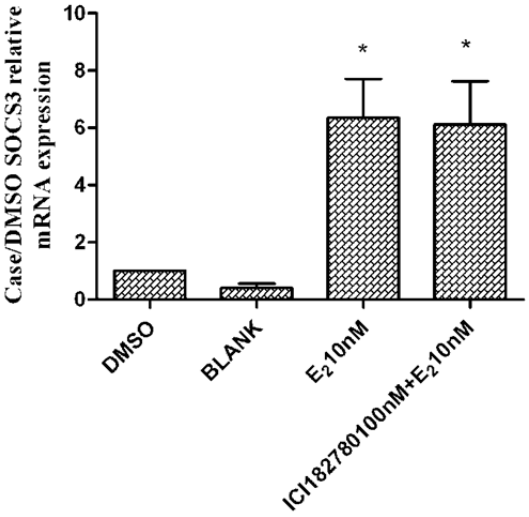

Figure 7. The expression of SOCS3 in macrophages after block the ER signaling by 100 nM ICI182780 while adding 10 nM 17 β -estrodiol for 12h. * P<0.05 VS DMSO.

DISCUSSION

Matthews et al. reported that estrogen could up-regulate SOCS3 expression in a time-dependent manner in breast cancer T47D cells [14]. They demonstrated that SOCS3 mRNA expression increased after 1h treatment with estrogen, and reached to a peak after 3h of treatment. 6h later, the SOCS3 expression return to the basal level. Leong et al. demonstrated that SOCS3 mRNA expression reaches to a peak at 2h after 100nM estrogen simulation in hepatoma HuH7 cells [13]. While in vivo ovariectomized mice experiments, short time estrogen treatment (10h and 48h) could not up-regulate SOCS3 expression in liver tissue. However, with long term estrogen treatment (3-5 weeks), SOCS3 mRNA expression in liver tissue was up-regulated [13]. In human embryonic kidney cells, Leung et al. reported that estrogen could not up-regulate SOCS3 mRNA expression [19], indicating that up-regulation of SOCS3 expression by estrogen simulation exists cellular and histological differences. We first reported in macrophages that estrogen simulation could up-regulate SOCS3 expression, we further pointed out that SOCS3 up-regulation was not mediated by estrogen receptor signaling. Normally estrogen interacts with estrogen receptor (ER α and ER β) and activate its downstream pathway. There are some novel receptors, such as G protein-coupled receptor 30, GPR30 (GPER1), that was reported to activate downstream signaling by estrogen simulation [20]. Elisabetta et al. reported the expression of ER α but not ER β expression in RAW264.7 cells [21], the same results was found in mice that only ERα was expressed in macrophages cells [22]. In human macrophages, not only the ER but also GPER are highly expressed [23], yet there is no data shows the expression of GPER in RAW264.7 cells. We will further investigate whether the up-regulation of SOCS3 was mediated by estrogen-GPER signaling. Moreover, to figure out whether the up-regulation of SOCS3 in macrophages induced by estrogen has a protection role in atherosclerosis and the molecular mechanism.

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