Supporting Information

17β-Estradiol Suppresses the Macrophage Foam Cells Formation Associated with SOCS3

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Material and Methods in the Supplementary data

1. Animal Treatment with CP-690,550

ApoE null mice (5 male and 5 female mice in each group) were treated with CP-690,550 (Sigma, MO, USA. 5mg/kg/day by intragastric administration) or vehicle for 4 weeks from 12th week after birth. Blood and aorta roots were harvested for lipid level or ORO staining and ABCA1 expression detection. Each sample was tested three times.

2. Detection of Serum Lipid and Glucose Levels

Serum was separated via centrifugation from blood at 3,000 rpm for 15 min. The total cholesterol (TC) and triglyceride (TG) levels were measured using assay kits (Dong’ou, Wenzhou, China) and glucose was measured using the ACCU-CHEK® Advantage Glucometer (Roche Diagnostics, Inc., Penzberg, Germany), according to the manufacturer’s instructions. Each sample was tested three times.

3. Immunohistochemistry and Histological Staining

The root of the aorta was dissected under a microscope and frozen in an embedding medium at the optimal cutting temperature for serial cryosectioning at 8 μm increments, covering 1.0 mm of the aortic root. The first section was collected as the three aortic valve cusps became visible in the lumen of the aorta. Oil red O staining was used to detect lipids in the plaque. Immunohistochemistry was performed with antibodies to identify Mac3 (1:100 dilution, Serotec, Oxford, UK), SOCS3 (1:50 dilution, Santa Cruz, CA, USA) and ABCA1 (1:100 dilution, Santa Cruz, CA, USA). Section images were captured digitally using an Olympus BX51 imaging system.
(Olympus, Tokyo, Japan) and were quantified using the Image-Pro Plus 6.0 software
(Olympus). The cross-sectional surface area of the lesion and the total cross-sectional
vessel area were also quantified, and each sample was tested three times, as
previously described [1].

4. RNA Extraction and Quantitative PCR

The mRNA expression of SOCS3 and ABCA1 in the main aorta of mice and of
SOCS3, ABCA1, ABCG1, liver X receptor α (LXRα) and CD36 in RAW264.7 cells
were determined using quantitative PCR. Total RNA was isolated from the main aorta
with Trizol reagent (Invitrogen, Carlsbad, CA). The Nanodrop 1000 (Thermo, CA,
USA) was used to quantify the total amount of RNA. The resulting RNA was reverse
transcribed and analyzed via quantitative PCR with the SYBR PrimeScript™ RT-PCR
Kit (TaKaRa, Dalian, China). All real time reactions were performed on the iQ5™
Multicolor Real time PCR detection system (Bio-Rad, CA, USA). A three-step PCR
of 5 s at 95°C, 20 s at 63°C and 10 s at 72°C was applied for 45 cycles. GAPDH was
used as a housekeeping gene. The primer sequences are shown in Table S2. Each
experiment was performed in triplicate with three replicates each. The data were
analyzed using the \(2^{-\Delta\Delta CT}\) method.

5. Western Blot Analysis

Proteins from the main aorta of mice and the RAW264.7 cells were extracted using
RIPA buffer according to the manufacturer’s instructions, and a protease inhibitor
cocktail (Roche Molecular Biochemicals, Mannheim, Germany) was added to all
samples. The BCA protein assay reagent kit (Pierce, IL, USA) was used to quantify
the total amount of protein. Equal amounts of the protein extracts were separated using a 10% SDS-PAGE gel, and then the proteins were transferred to a nitrocellulose membrane using a Bio-Rad transfer blotting system (Bio-Rad, CA, USA). The blots were incubated in 5% skim milk to block non-specific binding for 1 h at room temperature with slow shaking. Then the blots were incubated overnight at 4°C with anti-SOCS3 (1:500 dilution, Santa Cruz, CA, USA), anti-ABCA1 (1:500 dilution, Santa Cruz, CA, USA), anti-ABCG1 (1:5000 dilution, Epitomics, CA, USA), anti-CD36 (1:500 dilution, Santa Cruz, CA, USA), anti-LXRα (1:1000 dilution, Proteintech, IL, USA), anti-JAK2 (1:1000 dilution, Cell Signal Technology, MA, USA), anti-pJAK2 (1:1000 dilution, Cell Signal Technology, MA, USA), anti-STAT3 (1:1000 dilution, Cell Signal Technology, MA, USA), anti-pSTAT3 (1:2000 dilution, Cell Signal Technology, MA, USA) or anti-GAPDH (1:1000 dilution, Santa Cruz, CA, USA), respectively. A horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000 dilution, Abcam, MA, USA) and an enhanced chemiluminescent substrate (Pierce, IL, USA) were used for detection.

Reference


Figure S1: Protein and mRNA expression of SOCS3 with pcDNA3.0-SOCS3 plasmid and siRNA transfection in RAW264.7 cells. A. Protein and mRNA expression of SOCS3 with pcDNA3.0-SOCS3 plasmid after 12, 24, 36 and 48h. pcDNA-vector was used as negative control. B. Protein and mRNA expression of SOCS3 with SOCS3 siRNA transfection after 0.5, 12, 24, 36 and 48h. siRNA-GAPDH was used as positive control. IFN-γ was used to stimulating SOCS3 expression. GAPDH was detected as an internal reference. Data represent the means ± S.D. Each experiment was performed three times.
Figure S2

Figure S2: E2 increased ABCA1 mRNA expression depending on SOCS3 in vitro.

RAW264.7 cells were treated with IFN-γ (100μg/l, 12h) and/or E2 (100 nM, 12h) after transfection with SOCS3-pcDNA3.0 plasmid or SOCS3 specific siRNA. IFN-γ was used to activate JAK/STAT. GAPDH was detected as an internal reference. Data represent the means ± S.D. **p < 0.01. Each experiment was performed three times.
Figure S3

Figure S3: ABCG1 and CD36 mRNA expression after transfection with the SOCS3-pcDNA3.0 plasmid or SOCS3-specific siRNA. GAPDH was detected as an internal reference. The data represent the means ± S.D. Each experiment was performed three times.
Figure S4: Protein expression of ABCA1 with siRNA transfection in RAW264.7 cells after 12, 24, 36 and 48h. siRNA-GAPDH was used as positive control. The sequence 5’-GAGCUGUUCAGCACAAAGATTUCUUUGUGGCUGAAGACGCUCTT -3’ was used to suppress endogenous ABCA1 expression. GAPDH was detected as an internal reference. Data represent the means ± S.D. Each experiment was performed three times.
Figure S5: Promotion effect of E2 on cholesterol efflux was weakened by siRNA-ABCA1 transfection. RAW264.7 cells were treated with NBD-cholesterol for 6h, then transfected by ABCA1 siRNA for 12h. After RNAi, E2 and IFN-γ were used to treat cells and cholesterol efflux rate was detected as described in Methods. Data represent the means ± S.D. Each experiment was performed three times.
Figure S6: Lipid deposit and ABCA1 expression in plaque of ApoE null mice treated with/without CP-690,550. Magnification X 40 (ORO) and 100 (ABCA1), bar = 100 μm. The data represent the means ± S.D. Each sample was tested three times.
Table S1: Blood cholesterol level of ApoE null mice with/without CP-690,550 treatment for 4 weeks.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>BW</th>
<th>CHO</th>
<th>TG</th>
<th>GLU</th>
</tr>
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<tbody>
<tr>
<td>CON</td>
<td>10</td>
<td>20.2±3.22</td>
<td>730.3±26.17</td>
<td>166.2±33.12</td>
<td>6.3±0.81</td>
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<tr>
<td>CP-690,550</td>
<td>10</td>
<td>21.6±2.95</td>
<td>799.1±29.88*</td>
<td>170.2±24.22</td>
<td>6.4±0.62</td>
</tr>
</tbody>
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N: Number, BW: Body Weight (g), CHO: Serum Cholesterol (mg/dl), TG: Serum Triglyceride (mg/dl), GLU: Serum Glucose (mmol/l)

*p < 0.05 vs. ApoE null mice (CON) at 16w.