Leptin upregulates COX-2 and its downstream products in aortic endothelial cells

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Received November 15, 2016; Accepted June 22, 2017

DOI: 10.3892/etm.2017.5177

Abstract. The adipocyte-derived hormone leptin is associated with hypertension. The involvement of cyclooxygenase-2 (COX-2) and its downstream vasomotor products prostaglandin (PG) and thromboxane (TX)A2 in the mechanisms of action of leptin have remained elusive. The aim of the present study was to investigate the effects of leptin on the expression of COX-2 by rat aortic endothelial cells (RAECs) and the concentration of its products, represented by 6-keto PGF1α and TXB2, in the culture media. RAECs were isolated, cultured and identified by immunofluorescence staining. The RAECs were incubated with different concentrations of leptin (10−6, 10−5 and 10−4 M) for various durations (36 or 48 h). COX-2 mRNA and protein expression in the cells was detected by reverse-transcription quantitative PCR and western blot analysis, respectively. The vasodilator 6-keto PGF1α and the vasoconstrictor TXB2 were detected in the supernatant by ELISA. The cultured cells displayed specific factor VIII expression in the cytoplasm. Compared with the PBS-treated control group, leptin significantly increased the expression of COX-2 mRNA and protein in a time- and dose-dependent manner (P<0.01). Furthermore, the vasodilator 6-keto PGF1α was increased and the TXB2/6-keto PGF1α ratio decreased only with relatively high concentrations of leptin (10−5 or 10−4 M; P<0.01), but TXB2 levels were not affected (P>0.05). In conclusion, leptin significantly increased the expression of inflammatory marker COX-2 and its downstream product 6-keto PGF1α, while also decreasing the TXB2/6-keto PGF1α ratio in vitro. These observations suggested that COX-2 may have an important role in the effects of leptin on inflammation, such as the low-inflammatory disease hypertension. However, selective COX-2 inhibitors may increase the risk of hypertension due to inhibiting 6-keto PGF1α, the vasodilator product of COX-2.

Introduction

Leptin, almost exclusively synthesized and secreted by white adipocytes, is a protein hormone encoded by the obesity (OB) gene. Its main function is to target the hypothalamic arcuate nucleus, resulting in the suppression of appetite and increase of energy consumption so as to regulate the energy balance (1). Leptin receptors are widely expressed in cardiovascular tissues, including endothelial cells (ECs) (2). Therefore, leptin also targets arterial ECs, and is involved in the occurrence and development of hypertension (3), which it may achieve through causing EC dysfunction (4) and inflammation (5) as well as increasing the expression of endothelin-1 (6). In addition, leptin enhances endothelium-dependent vasorelaxation by upregulating the expression of neuronal nitric oxide synthase (nNOS) (7) and endothelium-derived hyperpolarizing factor (EDHF) (8). These opposite roles of leptin make it necessary to further study the expression of factors by ECs in association with hypertension regulated by leptin.

Cyclooxygenases (COXs) are rate-limiting enzymes, which catalyze free arachidonic acid and synthesize prostaglandin (PG)H2, which is further converted to prostacyclin (PGI2) and thromboxane (TX)A2. Among the COXs, COX-2 is an inducible enzyme whose expression is low in ECs under physiological conditions. The expression of COX-2 increases during pathological conditions of inflammation, such as hypertension, a lower-grade inflammatory disease. The downstream products of COX-2, PGI2 and TXA2, affect vasomotion and platelet aggregation (9). However, the expression of COX-2 and its downstream products involved in hypertension induced by leptin has remained to be clarified.

The purpose of the present study was to investigate the expression of COX-2 and its downstream products PGI2 and TXA2 (represented by 6-keto PGF1α and TXB2, respectively) induced by leptin in vitro, so as to further understand the
mechanisms of the involvement of leptin in hypertension, or to obtain information on the influence of leptin on systolic and/or diastolic function of blood vessels. The findings partially explained the mechanisms by which leptin mediates hypertension and put forward precautions from the aspect of medication use in hypertension.

**Materials and methods**

**Experimental animals.** A total of 8 male Wistar rats were purchased from the Experimental Animal Center of Sunyat-sen University (Guangzhou, China). All rats were between 4 and 5 weeks of age and fed normal chow in specific pathogen-free facilities. All animal protocols were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China).

**Isolation and culture of rat aortic endothelial cells (RAECs).** Wistar rats were anesthetized by an intraperitoneal injection of pentobarbital (300 mg/100 g; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) resulting in euthanasia. Aortic separation was performed as described previously (10). RAECs were isolated from aortic strips by the method of mixed enzyme digestion [0.1% type II collagenase (4 ml) and 0.1% trypsin (4 ml; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) digested for 20 min separately]. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin and 100 µg/ml endothelial cell growth supplement (Sigma-Aldrich; Merck KGaA) and passaged as described previously (10).

**Characterization of RAECs by immunofluorescence analysis.** When the cells reached 80% confluence, the medium was changed, the cells were washed twice with PBS, fixed with 4% methanol for 30 min, permeabilized with 0.1% Triton X-100 for 15 min and washed three times with PBS successively. After incubation with 1% bovine serum albumin (BSA) (Beyotime Institute of Biotechnology, Haimen, China) for 1 h. All above procedures were performed at room temperature. The cells were incubated overnight at 4°C with rabbit anti-rat factor VIII antibody (1:150 dilution; 65707; Cell Signalongn Technology, Inc., Danvers, MA, USA), followed by incubation with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G antibody (A0562; 1:100 dilution; Beyotime Institute of Biotechnology) for 2 h at room temperature. Finally, the cells were washed three times with PBS and stained with DAPI (Sigma-Aldrich; Merck KGaA). Cells were observed and images were captured under an inverted fluorescence microscope.

**Leptin treatment of RAECs.** RAECs at passage 3 were cultured in a 6-well plate at 5x10⁵ cells/well in complete culture medium for 12 h. Leptin (PS0596; R&D Systems, Inc., Minneapolis, MN, USA) in a stock solution in PBS was added to the complete culture medium to achieve final concentrations of 0, 10⁻¹⁰, 10⁻⁹ or 10⁻⁸ M, followed by incubation for 36 or 48 h. Each experimental condition was set up in triplicate.

**Reverse-transcription quantitative polymerase chain reaction (RT-qPCR).** Total RNA was obtained from RAECs by lysis with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min and extraction with chloroform, isopropanol and 75% ethanol. Complementary DNA was synthesized by using a PrimeScript RT Master mix kit (RR064A; Takara Bio Inc., Otsu, Japan). The primers used for PCR were as follows: Rat COX-2 forward, 5'-GCTTAAAGACCGCATCGAGGTTGGT-3' and reverse, 5'-GACTTGAAGAGTGGCCTGTGTGTTGTT-3'; rat 18S-ribosomal (r)RNA forward, 5'-GAAATTCCCAAGTAAGTGCGGCTCAT-3' and reverse, 5'-CGAGGCCCTCACCAAACCATT-3'. PCR reactions were performed using a SYBR Premix Ex Taq II kit (R82LR, Takara Bio, Inc.) in an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 62°C for 30 sec; 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. COX-2 mRNA was normalized to the 18S-rRNA and relatively quantified by standard curve analysis; the 2⁻ΔΔCq method was used for quantification, as described previously (11).

**Western blot analysis.** Protein from RAECs was lysed with RIPA lysis buffer (WB0101; Biotech Well, Shanghai, China). The protein concentration was determined using a BCA protein quantification kit (23225; Shanghai Yu Bo Biological Technology Co., Ltd., Shanghai, China). Protein (50 µg per lane) was separated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. After incubation with 3% BSA for 1 h at room temperature, 2 membranes (corresponding to 2 gels) were blotted with polyclonal rabbit anti-rat COX-2 antibody (1:1,000 dilution; 12282; Cell Signaling Technology, Inc.) and polyclonal rabbit anti-β-actin antibody (1:1,000 dilution; 4970; Cell Signaling Technology, Inc.) as a control overnight at 4°C. The membranes were washed with Tris-HCl-buffered saline and re-blotted with secondary antibody (HRP-conjugated goat anti-rabbit antibody; 1:5,000; sc-204; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 2 h. Bands were visualized using a chemiluminescent reagent (P0018; Beyotime Institute of Biotechnology). Densitometric analysis was performed with ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA).

**ELISA for assessment of 6-keto PGF₁₀ and TXB₂ protein secretion.** The concentrations of 6-keto PGF₁₀ and TXB₂ in the cell culture supernatant were detected by competitive ELISA (6-keto Prostaglandin Flau ELISA kit; 515211; Cayman Chemical Co., Ann Arbor, MI, USA); and TXB₂ ELISA kit (KGE011; R&D Systems, Inc.) according to the manufacturer's instructions. Optical density (OD) values of 6-keto PGF₁₀ and TXB₂ were read using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 405 or 450 nm, respectively. According to the OD values and the formulas included in the instructions of the ELISA kits, the percentage of sample bound vs. maximum binding were calculated for standard and test samples.

**Statistical analysis.** Values are expressed as the mean ± standard error of the mean and were analyzed by SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). The
statistical significance of differences among ≥3 groups was determined by one-way analysis of variance followed by Dunn's post hoc analysis. Student’s 2-tailed test was performed when only 2 groups were being compared. P<0.05 was considered to indicate a statistically significant difference.

Results

Isolation, culture and identification of RAECs. At 6 days of primary culture, RAECs presented as shuttle-like or polygonal structures (Fig. 1A), and subsequently grew and integrated into a monolayer. After culture for 10 days, RAECs had a cobblestone-like appearance (Fig. 1B). Green fluorescence was observed in the cytoplasm under a fluorescence microscope after immunofluorescence staining with anti-factor VIII antibody, indicating EC-specific factor VIII expression in the cytoplasm. Expression of factor VIII was present in ~90% of the cells (Fig. 1C-F).

Effects of leptin on COX-2 mRNA expression. RAECs treated with PBS were used as controls and they expressed low levels of COX-2 mRNA (Fig. 2). However, when RAECs were treated with different concentrations of leptin for different durations, the expression levels of COX-2 mRNA were increased. Although there was no statistical significance between the PBS group and that treated with the lowest concentration of leptin (10⁻¹⁰ M) at 36 h (P=0.14), all other groups of RAECs treated with different concentrations of leptin for different durations exhibited a significantly increased expression of COX-2 mRNA (P<0.01; P=0.003 for 10⁻⁹ or 10⁻⁸ M leptin vs. PBS at 36 h). Furthermore, these increases were dependent on the concentration of leptin and the incubation time (Fig. 2). Particularly after treatment with the high concentration of leptin (10⁻⁸ M) for 48 h, the expression of COX-2 mRNA was significantly increased by up to 1.65-fold compared with that in the PBS group (P<0.01; P=0.002, leptin 10⁻¹⁰ M vs. PBS; P<0.001, leptin 10⁻⁹ M vs. PBS; P=0.004, leptin 10⁻⁸ M vs. PBS at 48 h).
Effects of leptin on COX-2 protein expression. In agreement with the COX-2 mRNA results above, the expression of COX-2 protein was consistently decreased by RAECs treated with PBS (Fig. 3). Furthermore, the expression levels of COX-2 protein were concentration- and time-dependently increased by leptin. However, the increases at the protein level were not as significant as those at the mRNA level at 36 h, as there was no statistical significance between the PBS group and the $10^{-10}$ or $10^{-9}$ M leptin group ($P>0.05$); only at the high concentration, leptin ($10^{-8}$ M) significantly upregulated COX-2 protein, namely by up to 2.64-fold of that of the PBS group ($P<0.05$; $P=0.135$, leptin $10^{-10}$ M vs. PBS; $P=0.072$, leptin $10^{-9}$ M vs. PBS; $P=0.002$, leptin $10^{-8}$ M vs. PBS at 36 h). At 48 h, the expression of COX-2 protein was similar to that of COX-2 mRNA, as all three concentrations of leptin led to a significant upregulation of COX-2 protein expression ($P=0.007$, leptin $10^{-10}$ M vs. PBS; $P=0.004$ leptin $10^{-9}$ M vs. PBS; $P<0.001$, leptin $10^{-8}$ M vs. PBS at 48 h; Fig. 3).

Effects of leptin on 6-keto PGF$_{1\alpha}$ and TXB$_2$. To explore the effects of leptin on the secretion of COX-2 products 6-keto PGF$_{1\alpha}$ and TXB$_2$, these proteins were detected in the cell culture supernatant by ELISA (Table I). At 36 and 48 h, the low concentration of leptin ($10^{-10}$ M) had no significant effect on the levels of 6-keto PGF$_{1\alpha}$. However, the higher concentrations of leptin ($10^{-9}$ and $10^{-8}$ M), particularly at the longer incubation time (48 h), produced significantly elevated levels of 6-keto PGF$_{1\alpha}$ ($P<0.05$; $P<0.01$). No significant effect on TXB$_2$ was observed. However, the leptin concentrations of $10^{-9}$ or $10^{-8}$ M significantly reduced the TXB$_2$/6-keto PGF$_{1\alpha}$ ratio at 48 h ($P<0.01$; Table I). The purpose of calculating the ratio was to predict the effects of leptin on vascular systolic and diastolic function, as vasomotor function partly depends on this ratio.

Table I. Effects of leptin treatment at different concentrations and for different durations on 6-keto-PGF$_{1\alpha}$ and TXB$_2$ in the supernatant of cultured rat aortic endothelial cells.

<table>
<thead>
<tr>
<th>Time-point/group</th>
<th>6-Keto PGF$_{1\alpha}$ (pg/ml)</th>
<th>TXB$_2$ (ng/ml)</th>
<th>TXB$<em>2$/6-keto PGF$</em>{1\alpha}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>255.78±18.06</td>
<td>15.07±3.47</td>
<td>59.48±12.89</td>
</tr>
<tr>
<td>$10^{-10}$ M</td>
<td>259.11±30.22</td>
<td>16.03±1.45</td>
<td>62.76±12.38</td>
</tr>
<tr>
<td>$10^{-9}$ M</td>
<td>321.73±10.65$^a$</td>
<td>15.74±3.10</td>
<td>49.18±11.15</td>
</tr>
<tr>
<td>$10^{-8}$ M</td>
<td>420.19±17.54$^{b}$</td>
<td>16.8±2.39</td>
<td>39.99±5.60</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>257.48±11.42</td>
<td>16.85±2.03</td>
<td>65.48±7.79</td>
</tr>
<tr>
<td>$10^{-10}$ M</td>
<td>264.67±15.03</td>
<td>15.98±1.55</td>
<td>60.38±4.61</td>
</tr>
<tr>
<td>$10^{-9}$ M</td>
<td>444.69±20.07$^b$</td>
<td>14.35±3.30</td>
<td>32.38±7.79$^b$</td>
</tr>
<tr>
<td>$10^{-8}$ M</td>
<td>625.34±17.59$^{b}$</td>
<td>17.43±3.95</td>
<td>27.77±5.61$^{b}$</td>
</tr>
</tbody>
</table>

$^aP<0.05$ and $^bP<0.01$ compared with PBS at the same time-point. PG, prostaglandin; TX, thromboxane.
Leptin is an endocrine hormone; in addition to inhibiting food intake and increasing energy consumption, it has a broad role in regulating biological functions, including immune, inflammation and hematopoietic functions. Studies have found that leptin participates in the occurrence and development of hypertension by activating the sympathetic nerve (12) as well as increasing renal Na+–K+-adenosine triphosphatase activity (13) and oxidative stress (14) in vivo. In vitro, leptin was found to act on endothelial cells by causing cell dysfunction (4), inflammatory injury (5) and endothelin-1 expression (6) to presumably participate in hypertension (3).

COX-2 is mainly expressed in ECs, macrophages and fibroblasts. It is generally accepted that when ECs are in inflammatory or pathological states, they increase the expression of COX-2 and therefore the production of its downstream products PGI2 and TXA2, which regulate vascular tension and platelet aggregation. The synthesis and release of PGI2 are mainly from ECs. PGI2 has a role in vasodilation and anti-platelet aggregation. The stable metabolite of PGI2 is 6-keto PGF1α. TX is an arachidonic hormone, which may occur in two major forms: TXA2 and TXB2. In vivo, TXA2 is mainly synthesized and secreted by the platelet microsomes and ECs, and has a strong effect on promoting vascular contraction and platelet aggregation (9). However, the biological half-life of TXA2 is only 30 sec, and is quickly converted into the inactive and stable metabolite TXB2. Therefore, the levels of 6-keto PGF1α and TXB2 reflect the levels of PGI2 and TXA2; studying the effects of leptin on the expression of COX-2 and it downstream products may help to explain the pathogenesis of hypertension mediated by leptin. At the same time, it may also provide evidence for understanding the mechanisms of obesity-associated diseases.

In order to explore the effect of leptin on the expression of COX-2 and its downstream products PGI2 and TXA2, ECs were separated from rat aortas and cultured. Factor VIII as a marker of ECs was detected by immunofluorescence staining. The results suggested that the purity of RAECs reached ~90%. After treatment of the RAECs, the expression levels of COX-2 mRNA and protein were significantly as well as leptin increased by relatively high concentrations of leptin for the longer incubation time. Although the expression of TXB2 was not affected by leptin, the TXB2/6-keto PGF1α ratio was increased after incubation with leptin at high concentrations and the longer incubation time. Thus, leptin upregulated the expression levels of the inflammation marker COX-2 and increased the vasodilator PGI2, while decreasing the ratio of TXB2 (vasoconstrictor substance) to PGI2. These results implied that leptin is associated with inflammation, while it enhanced endothelium-dependent vasorelaxation.

Studies have indicated that endothelium-dependent vasorelaxation mainly includes three pathways (15): i) Release of nitric oxide by activation of endothelial (e)NOS in the aorta; ii) release of endothelium-derived hyperpolarization factor in arteries with low resistance; and iii) stimulation of COX-2 to produce PGI2. Regarding the first two pathways associated with leptin, it has been confirmed that leptin promotes the expression of eNOS via phosphatidylinositol 3-kinase (16) and increases the release of EDHF (8). The latter pathway associated with leptin has remained to be fully elucidated, although leptin has been found to increase the expression of COX-2 (16,17). Manuel-Apolinar et al (17) found that leptin is involved in the inflammatory response by increasing the expression of intercellular adhesion molecules and COX-2 on murine aorta tissue mediated by the long leptin receptor. Garonna et al (18) found that the pro-angiogenic actions of leptin required a functional endothelial p38 mitogen-activated protein kinase/Akt/COX-2 signaling axis. To the best of our knowledge, the effects of different doses of leptin on COX-2 and its downstream products 6-keto PGF1α and TXB2 have remained elusive. The results of the present study provided direct evidence to answer this question.

As selective COX-2 inhibitors, non-steroidal anti-inflammatory drugs, such as aspirin and celecoxib, are widely used, which have anti-inflammatory analgesic effects. At different stages of hypertension and atherosclerotic plaque formation, ECs may highly express COX-2. A selective COX-2 inhibitor was able to reduce inflammation and platelet aggregation, resulting in a decrease of the incidence of cardiovascular events and a protective effect on the cardiovascular system (19,20). However, when taking such medication at large dosages for a long time, patients present with various types of complications, the most common of which is digestive tract damage. Importantly, adverse effects on the cardiovascular system have been reported for prostanooid inhibition by COX-2 inhibitors: Certain studies have demonstrated that COX-2 inhibitors may increase the risk of cardiovascular events, such as myocardial infarction and stroke (21). Therefore, the effects of COX-2 inhibitors on the cardiovascular system require re-analysis; consistently with the results of the present study, COX-2 inhibitors may increase the risk of hypertension.

In conclusion, the present study investigated the effects of leptin on the expression of COX-2 and it downstream products 6-keto PGF1α and TXB2 from RAECs. Treatment with leptin, a mediator of hypertension, was identified to significantly upregulate the expression of COX-2, a mediator of inflammation, and the levels of its vasodilator product 6-keto PGF1α, while downregulating the ratio of the vasoconstrictor TXB2 to 6-keto PGF1α, suggesting that leptin may promote cardiovascular diseases by increasing the expression of COX-2. However, elective COX-2 inhibitors may not provide a benefit for leptin-mediated cardiovascular diseases, as they may rather increase the occurrence of hypertension due to inhibiting vasodilator 6-keto PGF1α, a downstream product of COX-2, as well.

Acknowledgements

The present study was funded by grants from the Science and Technology Plan Project of Guangzhou city in China (grant no. 201510010181), the Science and Technology Plan Project of Guangdong Province in China (grant nos. 2014A020212364 and 2013B021800282) and the Guangdong Natural Science Foundation in China (grant nos. 2015A030313467 and S20130015962).

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