Leptin activates the JAK/STAT pathway to promote angiogenesis in RF/6A cells in vitro

Le Zhang¹,², Rong Li³, Bing-Hui Wu⁴, Ting-Ting Liang⁵, Zhe Liu⁶, Wei Ju⁶, Yi Wang⁶, Yu-Ting Wen⁶, Ming-Cui Liu⁶, Jun-Hui Du⁶,⁷

¹Department of Ophthalmology, Northwest Woman’s and Children’s Hospital, Xi’an 710061, Shaanxi Province, China
²Department of Ophthalmology, Shaanxi Provincial People’s Hospital, Xi’an 710068, Shaanxi Province, China
³Department of Ophthalmology, the First Affiliated Hospital of Xi’an Medical University, Xi’an 710077, Shaanxi Province, China
⁴Department of Ophthalmology, Xi’an No.1 Hospital, Xi’an 710001, Shaanxi Province, China
⁵Medical College of Xi’an Medical University, Xi’an 710021, Shaanxi Province, China
⁶Department of Medical Interdisciplinary Research, Xi’an Ninth Hospital Affiliated to Medical College of Xi’an Jiaotong University, Xi’an 710054, Shaanxi Province, China
⁷Department of Ophthalmology, Xi’an Ninth Hospital Affiliated to Medical College of Xi’an Jiaotong University, Xi’an 710054, Shaanxi Province, China

Co-first authors: Le Zhang and Rong Li

Correspondence to: Jun-Hui Du. Department of Ophthalmology, Xi’an Ninth Hospital Affiliated to Medical College of Xi’an Jiaotong University, Xi’an 710054, Shaanxi Province, China. djh79918@163.com

Received: 2021-10-14 Accepted: 2022-01-26

Abstract

- **AIM:** To investigate the effect of leptin on the angiogenesis of RF/6A cells (monkey retinal choroidal endothelial cells) in vitro and test the cellular signaling in the mechanism.
- **METHODS:** RF/6A cells were cultured in vitro and randomly divided into four groups: normal control, with leptin at 50, 100, 200 ng/mL for cell counting kit-8 (CCK8). RF/6A cell proliferation and migration were examined by Transwell assays, while RF/6A cell tube formation by Matrigel assay. JAK2, p-JAK2, STAT3, and p-STAT3 protein expression was measured by Western blotting. Cells were then divided into the following treatment groups: control, 100 ng/mL leptin and AG-490 (100 ng/mL leptin+10 μmol/L AG-490) for examinations of RF/6A cellular behaviour again. Analysis of differences was carried out using one-way ANOVA and least significant difference (LSD).
- **RESULTS:** RF/6A cell proliferation, migration and cell tube formation were promoted significantly by leptin in a dose-dependent manner (P<0.05). Western blotting showed that leptin up-regulated p-JAK2 and p-STAT3 expression levels. Treatment with the JAK/STAT pathway inhibitor, AG-490, decreased leptin-induced p-JAK2 and p-STAT3 expression, and inhibited cell proliferation, migration and cell tube formation induced by leptin (P<0.05).
- **CONCLUSION:** Leptin can promote RF/6A cell angiogenesis in vitro via activation of the JAK2/STAT3 signaling pathway.
- **KEYWORDS:** leptin; JAK/STAT; angiogenesis; RF/6A cells; proliferation; migration; tube formation

DOI:10.18240/ijo.2022.04.05


INTRODUCTION

Diabetic retinopathy is the primary cause of adult blindness in the world. Obesity, which is due to weight gain caused by increased adipose tissue, is usually related to lifestyle-related cardiovascular and metabolic diseases, for instance, diabetes, hypertension, and hyperlipidemia which lead to a higher risk of developing vascular disease or atherosclerosis. Adipose tissue is a vital endocrine organ that secretes many bioactive substances, such as leptin, chemerin and apelin¹,³, collectively known as adipocytokines⁴. Evidence suggests that adipocytokines play a pathophysiological role in complications related to obesity and diabetes⁵. Leptin is an adipocytokine that acts directly on the hypothalamus, regulating energy intake and consumption⁶,⁷. Leptin receptors are expressed not only in the hypothalamus but also in various peripheral tissues⁸,⁹. Leptin concentrations in the peripheral blood of obese people is proportional to the degree of obesity, suggesting that leptin may play a pathophysiological role in obesity-related complications¹⁰. In addition, leptin is closely related to energy metabolism and insulin resistance¹¹. Serum leptin concentrations were found to be significantly higher in
hyperplastic diabetic retinopathy patients than non-proliferative retinopathy patients[12]. In proliferative diabetic retinopathy patients, intravitreous leptin concentrations were higher[13-14], suggesting that leptin may play a role in proliferative diabetic retinopathy. The most crucial pathological change of proliferative diabetic retinopathy is the formation of retinal neovascularization[15-16]. However, it remains unclear whether leptin is involved in the process. Thus, this study aimed to clarify the role and mechanism of action of leptin on retinal neovascularization in vitro.

MATERIALS AND METHODS

Cell Grouping and Processing RF/6A cells were purchased from the Cell Bank of Typical Cell Culture Preservation Committee, Chinese Academy of Sciences. Cell culture consumables were purchased from Biyuntian Biotechnology Company (China). RF/6A cells growing in good condition were digested by 0.25% trypsin, collected and centrifuged at 1000 rpm for 5 min, and then moistened with phosphate buffered saline (PBS) twice to remove the residual serum. According to different treatment, RF/6A cells were randomly divided into four groups: control, 50, 100, and 200 ng/mL leptin groups. In leptin treatment group, different concentrations of leptin (0, 50, 100, and 200 ng/mL) were added to the culture medium for culture. Recombinant human leptin was purchased from Peprotech (USA). In leptin+AG-490 treatment group, leptin (0, 50, 100, and 200 ng/mL) were added to the culture medium. AG-490 inhibitor was purchased from Abcam (UK), the STAT3 antibody was purchased from Wuhan Sanying Biotechnology Co., Ltd. (China) and the GAPDH antibody was purchased from Hangzhou Xianzhi Biological Co., Ltd. (China). After washing, membranes were incubated with the corresponding secondary HRP-conjugated antibodies at 37°C for 2h. Protein bands were analyzed by BandScan.

Statistical Analysis Data are analyzed using SPSS 20.0 software and given as the means±standard deviation. Comparison of the means between groups was analyzed by single-factor ANOVA, while the LSD method was used to compare two groups. P<0.05 was considered to be statistically significant.

RESULTS

Leptin Promotes Proliferation of RF/6A Cells The effects of different concentrations of leptin on cell proliferation were examined using the CCK8 assay. As shown in Figure 1, varying concentrations of leptin (20, 50, 100, and 200 ng/mL) led to a significant dose-dependent increase in RF/6A proliferation after 24h (P<0.05).

Leptin Promotes Migration of RF/6A Cells Migration of endothelial cells is essential during early angiogenesis. To determine the effect of leptin on angiogenesis, we used the Transwell assay to measure the cell migration ability of RF/6A cells. We found that leptin led to an obvious increase in the migration of endothelial cells after 24h significantly. The number of migrating cells in the control, 50, 100, and 200 ng/mL leptin groups were 43±4.5, 65±6.5, 83.5±7.2, and 112±7.6, respectively (F=145.8, P=0.001; Figure 2).

Leptin Promotes Tube Formation in RF/6A Cells The Matrigel cell tube formation assay revealed that the number of tubular structures that developed in control, 50, 100, and 200 ng/mL leptin groups was 38±6.2, 50.1±3.5, 67.7±5.2, and 84±5.6, respectively (F=68.7, P=0.001). These findings suggest that leptin can promote RF/6A cell tube formation (Figure 3).
Leptin activates JAK/STAT to promote angiogenesis

To determine the effect of leptin on the JAK/STAT signaling pathway, we used Western blot analyses to detect the protein expression levels of JAK2, p-JAK2, STAT3, and p-STAT3. It was found that incubation with leptin increased the phosphorylation levels of JAK2 and STAT3 were significantly increased in RF/6A cells significantly (P-JAK2: $F=47.33$, $P=0.001$; P-STAT3: $F=111.86$, $P=0.001$).

AG-490 is a tyrosine kinase inhibitor that inhibits STAT-3 activation by selectively blocking JAK2. Thus, JAK/STAT-3 activation is inhibited using AG-490 selectively. To verify the inhibitory effect of AG-490 on the JAK/STAT signaling pathway, cells were separated into control, 100 ng/mL leptin, and AG-490 (100 ng/mL leptin+10 μmol/L AG-490) groups. We found that AG-490 could inhibit leptin-induced JAK2 and STAT3 phosphorylation (P-JAK2: $F=63.29$, $P=0.001$; P-STAT3: $F=70.68$, $P=0.001$; Figure 4).

**Effect of Inhibition of the JAK/STAT Signaling Pathway on Cell Proliferation** The role of the JAK/STAT signaling pathway on leptin-induced cell proliferation was examined using AG-490, the selective JAK/STAT signaling pathway inhibitor. The effects of the following treatment groups: control, 100 ng/mL leptin, and AG-490 (100 ng/mL leptin+10 μmol/L AG-490) on cell proliferation were assessed using the CCK8 assay. We found that AG-490 could significantly inhibit the proliferation of RF/6A cells induced by 100 ng/mL leptin ($F=131.99, P=0.001$; Figure 5).

**Effect of Inhibition of the JAK/STAT Signaling Pathway on Cell Migration** To determine the role of the JAK/STAT signaling pathway on leptin-induced cell migration, the Transwell assay was used to assess the levels of migration in control, 100 ng/mL leptin, and AG-490 (100 ng/mL leptin+10 μmol/L AG-490)-treated cells. We found that inhibition of the JAK/STAT pathway by AG-490 significantly inhibited the migration of RF/6A cells induced by 100 ng/mL leptin ($F=150.31; P=0.001$; Figure 6).
Effect of Inhibition of the JAK/STAT Signaling Pathway on Cell Tube Formation

The Matrigel tube formation experiment was used to detect the formation of tubular structures in control, 100 ng/mL leptin- and AG-490- (100 ng/mL leptin+10 μmol/L AG-490) treated cells. We found that inhibition of the JAK/STAT pathway by AG-490 inhibited the number of tube formations in RF/6A cells induced by 100 ng/mL leptin, significantly ($F=80.50$, $P=0.001$; Figure 7).

DISCUSSION

In the final stages of varieties of ocular diseases, including diabetic retinopathy and retinal vein occlusion, retinal neovascularization often leads to catastrophic vision loss. In this study, it was demonstrated that leptin can stimulate RF/6A cells’ proliferation, migration, and tube formation in vitro. As an adipocytokine, leptin was found to be expressed nearly exclusively in adipose tissue in mammals [17]. It acts directly on the hypothalamus to regulates energy intake and expenditure. It has been shown that leptin expressed in retina, choroid, sclera, vitreous and tears [18-20]. Serum levels of leptin were closely related to obesity and obesity-associated microvascular complications [12]. Our findings are similar to previous studies [21-22]. Cell migration, proliferation, and tube formation are vital factors in the formation of new blood vessels [23-24]. Our data suggest that leptin could promote new blood vessels formation. Furthermore, our findings confirm that leptin could activate the JAK2/STAT3 signaling pathway in RF/6A cells, and that inhibition of the JAK2/STAT3 signaling pathway through AG-490 blocks leptin-induced endothelial cells’ proliferation, migration, and tube formation.

The JAK/STAT pathway was initially regarded as a transmitter of interferon signaling on cells [25-26]. The JAK family is comprised of non-receptor tyrosine protein kinases and includes JAK1, JAK2, JAK3, and Tyk2. The JAK family has a C-terminal tyrosine kinase domain, while the N-terminus plays a regulatory role when JAK couples with the receptor protein. The JAK/STAT signaling pathway relays signals from the membrane to the nucleus, via tyrosine phosphorylation, leading to the activation and nuclear translocation of STATs [25]. Activation of the upstream JAK2 kinase by cytokines or growth factors results in tyrosine phosphorylation and activation of STAT3, which in turn regulates the transcription of target genes and mediates diverse biological effects including cell proliferation, differentiation, and apoptosis [27]. The activation of STATs is transient in normal cells, lasting only a few minutes or hours. In tumor cells, abnormal and persistent activation of STAT3 is closely associated with the biological behavior of the tumor and its pathogenesis [25].

In addition to cell proliferation, differentiation and immune regulation [24-29], the JAK2/STAT3 signaling pathway also plays a role in the process of neovascularization [30-31] and the regulation of VEGF expression. It has been shown that JAK2/STAT3 can activate VEGF expression in some studies [32], while inhibition of the JAK2/STAT3 pathway inhibits VEGF expression [33]. Abnormal activation of STAT3 regulates the STAT3/VEGF signaling pathway and promotes VEGF expression [34-35]. VEGF expression can be down-regulated by inhibiting the JAK2/STAT3 pathway [36]. The well-established JAK/STAT/pathway inhibitor AG-490, also known as Tyrophostin AG-490, was used in our study to specifically block the phosphorylation and activation of JAK2 and STAT3 [37-38]. Consistent with previous studies, leptin can activate the JAK/STAT signaling pathway [39-42]. Thus, leptin may increase the expression of VEGF in retinal endothelial cells by activating the JAK2/STAT3 pathway, and then promotes neovascularization.

Due to the shortage of human primary retinal vascular endothelial cells, RF/6A cells have been widely used in experiments.
Leptin activates JAK/STAT to promote angiogenesis

of angiogenesis, cell differentiation and various drug and environmental treatments in the choroid and retina\cite{43,44}. In this experiment, the RF/6A cells were selected because they were easy to obtain, stable and already widely used. Although RF/6A cells have been used for many years, they may also be different from real human retinal vascular endothelial cells. Further research is required to determine whether leptin has the same effect on human retinal microvascular endothelial cells and retinal neovascularization in vivo.

In conclusion, our study shows that leptin can activate the JAK2/STAT3 signaling pathway, resulting in increased RF/6A cell proliferation, migration, and tube formation, which may participate in neovascularization. If it can be proved by more studies that leptin promotes the angiogenesis of retinal endothelial cells via activation of the JAK2/STAT3 signaling pathway, the inhibitor or antagonist of this pathway could prevent the neovascularization of diabetic retinopathy. Further research is required to demonstrate whether leptin has the same effect on retinal neovascularization in vivo.

ACKNOWLEDGEMENTS

Foundations: Supported by the Matching Funds of the National Natural Science Foundation of China (No. XYFYPT-2020-01); the Natural Science Foundation of Shaanxi Province (No.2020JM-685; No.2021JM-547); the Fundamental Research Funds for the Central Universities (No.1191329116); the Foundation of Xi’an Health Committee (No.2020MS07).

Conflicts of Interest: Zhang L, None; Li R, None; Wu BH, None; Liang TT, None; Liu Z, None; Ju W, None; Wang Y, None; Wen YT, None; Liu MC, None; Du JH, None.

REFERENCES


11 Ghadge AA, Khaire AA. Leptin as a predictive marker for metabolic syndrome. Cytokine 2019;121:154735.


35 Yun S, Yun CW, Lee JH, Kim S, Lee SH. Crypto enhances proliferation and survival of mesenchymal stem cells by up-regulating JAK2/STAT3 pathway in a GRP78-dependent manner. *Biomer Ther* (Seoul) 2018;26(5):464-473.


