Research Article

Pro-inflammatory role of leptin in peripheral blood mononuclear cells

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ABSTRACT

Background: Hyperleptinemia is a pathological condition that mediates activation of inflammatory cells and trigger events that promote atherosclerosis. In the present study, we investigated the signaling mechanisms (JAK-2/STAT-3 pathway) underlying the pro-inflammatory role of leptin in peripheral blood mononuclear cells (PBMCs). **Materials and Methods:** PBMCs were isolated from healthy volunteers and treated with leptin in the presence or absence of JAK-2 inhibitor, tyrphostin AG490. The mRNA and protein levels of TNF-α and inducible nitric oxide synthase (iNOS) were then analyzed by ELISA and reverse transcription-PCR, respectively. NO levels were measured using Griess reagent. **Results:** Leptin activated PBMCs and promoted the synthesis of TNF-α, iNOS, and NO. However, exposure of PBMCs to tyrphostin AG490 significantly reduced the protein levels of both TNF-α and iNOS and decreased the secretion of NO. **Conclusion:** Leptin augments inflammatory response in PBMCs through JAK-2/STAT-3 pathway.

KEY WORDS: iNOS, Leptin, NO, Peripheral blood mononuclear cells, TNF-α

INTRODUCTION

Leptin is a 16 KDa protein hormone that plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism.[1] Most of the biological effects of leptin is mediated by the long-form receptor, namely, Ob-Rb.[2,3] Recent evidences suggest that the proportional increase of blood leptin with adiposity plays an important role in the regulation of immunity.[4] In fact, several immune cells, including T-lymphocytes, monocytes, and macrophages, bear the leptin receptor and generally are activated by leptin.[5] Leptin is suggested to activate proinflammatory cells, promote T-helper 1 responses, and mediate the production of pro-inflammatory cytokines including TNF-α and other molecules such as nitric oxide (NO). However, the signaling cascade involved in this process is not clear. The inflammatory cells including peripheral blood mononuclear cells (PBMCs) play a key role in the development and progression of cardiovascular diseases, and the adipocytokine leptin is found to exert its pathologic and pro-inflammatory effect by activating these cells. Hence, in the present study, we investigated the signaling mechanism underlying the pro-inflammatory role of leptin in PBMCs.

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MATERIALS AND METHODS

PBMC Culture

Normal human peripheral blood samples (5 mL) were collected from the healthy volunteers, ranging between 25 and 30 years of age. Subjects were considered for the study only when they are healthy and not under any medication for at least 3 months before the study. Written consent was obtained from the subjects before blood collection. EDTA-coated blood were then transferred to Leucosep tubes filled with lymphocyte separation medium and centrifuged at 4000 rpm for 15 min at room temperature. The opaque interface layer was carefully transferred into sterile centrifuge tube containing RBC lysis buffer (pH 7.4) and centrifuged at 3000 rpm for 15 min to lyse RBC. The cells were then washed thrice with phosphate-buffered saline (PBS) (pH 7.4) and the cell pellet was suspended in RPMI 1640 medium and mixed gently

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by aspiration, washed twice and resuspended in the medium. Cell viability was then assessed using trypan blue exclusion test.

For each experiment, 1×10^6 cells were grown in RPMI 1640 medium with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin. PBMCs were then incubated with human recombinant leptin at 37ºC for required incubation period to analyze its effect on TNF-α and inducible NO synthase (iNOS) synthesis. To study the signaling mechanism, by which leptin induces the synthesis of TNF-α, inhibitor studies were performed, PBMCs were exposed to tyrphostin AG 490 (JAK-2 inhibitor) at 37ºC and then treated with human recombinant leptin. Experiments were performed in triplicates. PBMCs were been grouped as follows:

Group I – Control cells
Group II – Leptin-treated cells
Group III – Cells exposed to tyrphostin AG 490 and then treated with leptin

ELISA

After incubation period, the culture supernatants were carefully isolated and the TNF-α level in the supernatant was evaluated using Human TNF-α Sandwich ELISA kit.

Reverse Transcriptase-PCR

After the incubation period, cultured cells were harvested, washed thrice with PBS and RNA was isolated using Trizol reagent. 1 µg RNA was reverse transcribed in a 20 µL reaction using mMULV RT enzyme. Resulting cDNA was then amplified using PCR. The amplified products were separated on 2% agarose gel for 30 min at 100 volts.

NO Measurement

In aqueous solutions that contain no heme proteins, NO is oxidized to nitrite only, which can serve as an indirect marker for the presence of NO. Hence, from the supernatant of the cultured cells, NO was estimated using Griess reaction by the NO detection kit. Absorbance value was measured between 520 and 560 nm using the ELISA plate reader.

(i) **Western Blot:** The protein expression of iNOS from the cultured cells was assessed using western blot.
(ii) **Statistical Analysis:** The data obtained were analyzed using SPSS software version 17.0 and the results were presented as mean + SD. Student's *t*-test was done to compare values between two groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

TNF-α Protein Levels in Cell Culture Supernatant

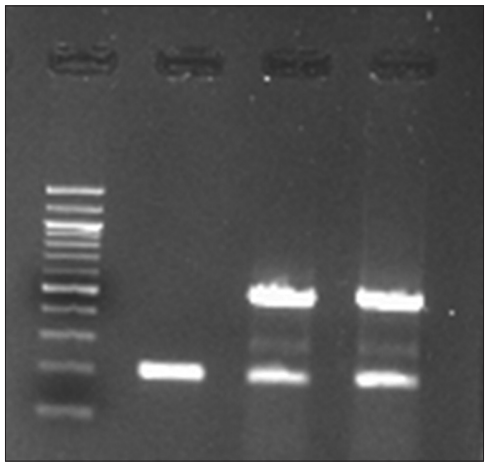
Table 1 shows the values of TNF-α level in the supernatant of cultured cells. Leptin-treated cells secreted elevated levels of TNF-α when compared to control cells. Fascinatingly, Group III cells showed a significant reduction in TNF-α protein level when compared to Group II cells.

TNF-α mRNA Levels in PBMCs

Figure 1 shows the gene expression of TNF-α in human PBMCs understudy. Lanes I, II, III, and IV correspond to 100 bp DNA ladder, Group I, Group II, and Group III cells, respectively. In the present study, the mRNA level of TNF-α is found to be increased in leptin-treated cells when compared to control cells. However, exposure of PBMCs to tyrphostin AG 490 did not affect the leptin-induced TNF-α gene expression.

Expression of NO and iNOS

Table 2 shows the values of NO obtained from the supernatant of the cultured cells understudy.



Lane I: 100 bp DNA ladder
Lane II: Group I cells (Control PBMCs)
Lane III: Group II cells (PBMCs treated with leptin [100 nM])
Lane IV: Group IV cells (PBMCs exposed to tyrphostin AG 490 [10 µM] and treated with leptin [100 nM])

Figure 1: Effect of tyrphostin AG 490 on TNF-α gene expression induced by leptin in PBMC

Table 1: Effect of leptin on TNF-α production in human PBMCs

Category	TNF-α (pg/ml)
Group I cells (Control PBMC)	15.5+0.8
Group II cells (PBMC treated with leptin)	72.5+1.9
Group IV cells (PBMC exposed to tyrphostin AG490 and treated with leptin)	68+1

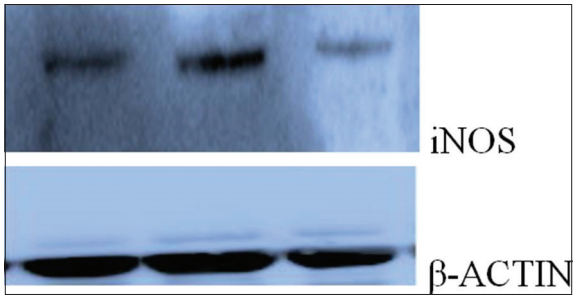
Table 2: Effect of leptin on nitric oxide production in human PBMCs

Category	NO (µM)
Group I cells (Control PBMC)	5.3+0.7
Group II cells (PBMC treated with leptin)	10.2+0.5
Group IV cells (PBMC exposed to tyrphostin AG490 and then treated with leptin)	6+0.3

Leptin-treated cells were found to possess a higher level of NO when compared to that of control cells (10.2 + 0.5 µM vs. 5.3 + 0.7 µM, $P = 0.004$). Group III cells showed a considerable reduction in NO level when compared to Group II cells (5.8 + 0.5 µM vs. 10.2 + 0.5 µM, $P = 0.002$).

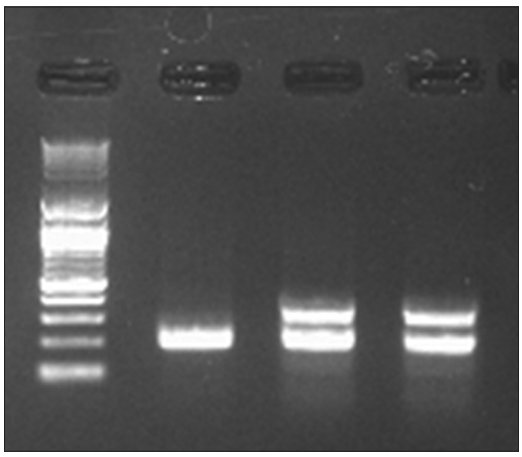
Figure 2 shows the protein expression of iNOS in human PBMCs. Lanes I, II, and III correspond to Group I, Group II, and Group III cells, respectively. Control cells were found to express basal level of iNOS, whereas in leptin-treated cells, iNOS protein expression level was found to be significantly increased ($P = 0.009$). The inhibition of the upstream molecule JAK2 with tyrphostin AG 490 showed a reduction in leptin-induced iNOS expression ($P = 0.04$).

Figure 3 illustrates the RT-PCR analysis of iNOS gene expression in human PBMCs activated by leptin (100 nM). Our results show that stimulation of PBMCs with leptin leads to a significant increase in iNOS gene expression. There was no difference in iNOS gene expression in Group II and Group IV cells when compared to Group II cells.



Lane I: Group I cells (Control PBMCs)
Lane II: Group II cells (PBMCs treated with leptin [100 nM])
Lane III: Group IV cells (PBMCs exposed to tyrphostin AG 490 [10 µM] and treated with leptin [100 nM])

Figure 2: Effect of tyrphostin AG 490 on iNOS expression induced by leptin in PBMCs iNOS gene expression in human PBMCs



Lane I: 100 bp DNA ladder
Lane II: Group I (Control PBMCs)
Lane III: Group II (PBMCs treated with leptin [100 nM])
Lane IV: Group IV (PBMCs exposed to tyrphostin AG 490 [10 µM] and then treated with leptin [100 nM])

Figure 3: Effect of tyrphostin AG 490 on iNOS gene expression induced by leptin in PBMCs

DISCUSSION

Atherosclerosis is one of the most important and common causes of death and disability in the world and acts as an important precursor of significant cardiovascular events.[6] It is considered to be a chronic inflammatory disease of blood vessels due to the intimal accumulation of lipids and inflammatory cells in medium- and large-sized arteries (also known as plaques).[7] Clinical studies have shown that this emerging biology of inflammation in atherosclerosis applies directly to human patients.[8,9] Elevation in markers of inflammation predicts outcome of patients with acute coronary syndromes, independently of myocardial damage. Monocytes are prominently involved in initiation, progression, and complication of the atherosclerotic lesions.[10] Emerging evidences show that hyperleptinemia plays a major role in augmenting inflammatory response in PBMCs, triggering atherogenesis, and predisposing the patients to CVD risk. In the present study, we investigated the signaling pathways involved in the immunomodulatory effects of leptin in PBMCs. Human PBMCs treated with leptin secreted higher levels of pro-inflammatory cytokine, TNF-α at mRNA, and protein levels when compared to control cells. This, in turn, suggests that, under normal physiological conditions, leptin does not trigger inflammation; however, under hyperleptinemic conditions, elevated leptin levels augment inflammation in PBMCs

Exposure of leptin-treated PBMCs to tyrphostin AG 490 (Group III cells) significantly reduced the protein levels of TNF-α in culture supernatants. This, in turn, suggests that leptin might augment inflammatory response in PBMCs by upregulating JAK-2/STAT-3 pathway. However, tyrphostin AG490 did not affect the gene expression of TNF-α in Group III cells. These findings suggest that tyrphostin AG 490 might inhibit leptin-induced TNF-α secretion only at post-transcriptional level.

Leptin maintains vascular tone by promoting the release of NO by various cells.[11,12] iNOS, a calcium-independent enzyme is majorly involved in the release of NO from PBMCs.[13] Under physiological conditions, iNOS produces basal level (in picomolar range) of NO which, in turn, maintains vascular tone through vasorelaxation and cytoprotection. However, under pathological conditions such as CVD, iNOS plays a deleterious cardiotoxic role due to its potential to secrete enormous amounts of NO (nanomolar

to micromole range) and promote its oxidation to peroxynitrites.[14-17] This, in turn, leads to detrimental cytotoxic events including hyperreactivity, platelet adhesion, and protein fragmentation.[18,19] Hence, in the present study, we analyzed if leptin could activate PBMCs and stimulate iNOS synthesis and NO release by these cells. Furthermore, we also investigated the potential involvement of JAK-2/STAT-3 pathway in leptin-mediated NO release in PBMCs using specific JAK-2 inhibitor.

Our results for the 1st time show that leptin treatment could augment the secretion of NO by PBMCs though the stimulation of the gene and protein expression of iNOS. Group III was found to express a lower level of iNOS protein when compared to that of Group II cells. NO level was also found to be lower in Group III cells than in Group II cells. There was no significant difference in the mRNA level of iNOS between Group II and Group III cells, suggesting that leptin might stimulate the synthesis of iNOS expression in human PBMCs and this could be effective at transcriptional level. The inhibitory effect of tyrphostin AG 490 over leptin-induced iNOS synthesis might take place at post-transcriptional level. Thus, the synthesis of NO during pathological conditions might, in turn, act as an important mediator in progression of CVD.

Taken together, our *in vitro* results suggest that hyperleptinemia can trigger inflammatory response in PBMCs and promote TNF-α and iNOS synthesis by activating JAK/STAT pathway. Thus, hyperleptinemia can be considered as a pro-inflammatory condition that augments event contributing to atherogenesis.

CONCLUSION

It is concluded from our present study that leptin augments inflammatory response in PBMCs through JAK-2/STAT-3 pathway.

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