

## Leptin - An Immunomodulator in Peripheral Blood Mononuclear Cells

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### Abstract:

**Background:** Leptin, a 16 KDa protein hormone is one of the most important adipocytokine that modulates metabolic processes by regulating energy intake and expenditure, including appetite and metabolism. However, under pathological conditions such as leptin resistance, leptin has been found to activate inflammatory cells including Peripheral Blood Mononuclear Cells (PBMCs) thereby causing damage to the heart. In the present study, we aimed at investigating if leptin could act as an immunomodulator in PBMCs and trigger the secretion of TNF- $\alpha$  and iNOS (inducible Nitric Oxide Synthase).

**Methods:** PBMCs from healthy volunteers were treated with leptin in the presence and the absence of LY294002 (PI3K inhibitor). The gene and the protein levels of TNF- $\alpha$  and iNOS and also the levels of nitric oxide in the cells under study were then estimated.

**Results and conclusion:** Our study suggest that leptin can activate human PBMCs and promote TNF- $\alpha$  and iNOS synthesis. The subsequent synthesis of TNF- $\alpha$  and NO during pathological conditions might act as an important mediator in the progression of CVD. The immunomodulatory role of leptin in PBMC in turn suggests that leptin might act as a novel therapeutic target for Cardiovascular Diseases.

**Keywords:** Leptin, Cardiovascular Disease, Inflammation, Peripheral Blood Mononuclear Cells, Leptin resistance

### I. Introduction

Leptin, a 16 KDa protein hormone is a novel and very promising molecule of research that may act as a mediator between obesity and Cardiovascular Diseases (CVD)<sup>[1]</sup>. It is one of the most important adipocytokine that modulates metabolic processes by regulating energy intake and expenditure, including appetite and metabolism<sup>[2]</sup>. Apart from metabolism, leptin has systemic effects including regulation of angiogenesis, wound healing, lipolysis, blood pressure homeostasis, reproduction, hematopoiesis and immune function<sup>[3]</sup>. Despite the weight reducing effects of leptin, obese individuals possess unusually high concentrations of circulating leptin which in turn is indicative of leptin resistance.

Leptin signaling pathway is a complex network that regulates a cellular pathway involved in a myriad of physiological and pathological scenarios. Leptin transmits signal by binding to its receptor Ob-R, which belongs to the class I cytokine receptor family. The long form, namely Ob-Rb is essential in mediating most of the biological effects of leptin and is highly expressed in the hypothalamus. It is also found to be expressed in several cell types relevant to CVD including Peripheral Blood Mononuclear Cells (PBMCs) thus providing evidence for the role of leptin in signaling for atherogenic events<sup>[4]</sup>.

Hyperleptinemia mediated activation of inflammatory cells represent a potentially ominous combination of mechanisms by which leptin may cause damage in the heart<sup>[5]</sup>. Leptin has been found to mediate proliferation, activation and production of proinflammatory cytokines from cultured monocytes<sup>[6,7]</sup>. It is also found to activate JAK-STAT, IRS-1-PI3K and MAPK signaling pathways in human PBMCs<sup>[7]</sup>. These signal transduction pathways provide possible mechanisms whereby leptin may modulate activation of PBMCs. It is fairly well known that leptin can stimulate the production of iNOS which in turn can lead to generation of NO<sup>[8]</sup>. In pathological conditions, the iNOS mRNA and protein that is expressed in response to leptin, inflammatory cytokines etc., generates substantially larger amounts of NO for long periods of time which might act as a causative factor in CVD. Thus, it has been of our interest to understand if leptin could act as an immunomodulator in PBMCs.

In the present study, we assessed the role of leptin on the secretion of TNF- $\alpha$  and (inducible Nitric Oxide Synthase) iNOS in PBMCs. This would be of relevance to establish the role of inflammatory markers in CVD and also to develop leptin based therapeutic strategy.

## II. Methodology

### 2.1 Human Recombinant Leptin:

78 mM stock concentration of human recombinant leptin (MP Biomedicals, India) was prepared by reconstituting 1 mg lyophilized leptin in 500  $\mu$ L of 15 mM sterile HCl. After the protein is completely dissolved, 300  $\mu$ L of 7.5 mM sterile NaOH was added. The aliquots were then stored at  $-20^{\circ}\text{C}$ .

### 2.2 LY 294002:

LY 294002 is a high selective inhibitor of PI3K. 10 mM stock concentration of LY 294002 (Cell signaling Technology, UK) was prepared by reconstituting 1.5 mg LY 294002 in 488  $\mu$ L DMSO. The aliquots were then stored at  $-20^{\circ}\text{C}$ .

### 2.3 Isolation of PBMCs:

Normal human peripheral blood samples (5 mL) were donated by healthy volunteers, ranging between 25 and 30 years of age. None of the subjects considered for the study were on any inflammatory drug either before or at the time of the study. Subjects were not allowed to take medication for at least 3 months before the study. Written consent was obtained from the subjects before blood collection. EDTA coated blood was then transferred to leucosep tubes (Griener Bio-one, Germany) filled with lymphocyte separation medium (MP Biomedicals, India) 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 in order 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 (Sigma Aldrich, USA) and mixed gently by aspiration, washed twice and resuspended in the medium. Cell viability was then assessed using trypan blue exclusion test. Cells suspended in RPMI 1640 medium were mixed with 0.4% trypan blue solution in 1:1 ratio and the viability of the cells were assessed. Cell viability assessed was found to be more than 95%.

For each experiment,  $1 \times 10^6$  cells were grown in RPMI 1640 medium with L-glutamine supplemented with 10% heat inactivated fetal bovine serum (Sigma Aldrich, USA), 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin. PBMCs were then incubated with 100nM Human Recombinant Leptin for 6 h at  $37^{\circ}\text{C}$  to analyze its effect on TNF- $\alpha$  and iNOS synthesis. To study the signaling mechanism by which leptin induces the synthesis of TNF- $\alpha$ , inhibitor studies were performed. PBMCs were exposed to LY 294002 (10  $\mu$ M) for 1 hour at  $37^{\circ}\text{C}$  and then treated with Human Recombinant Leptin (100 nM) for 6 hours. Experiments were performed in triplicates. PBMCs were grouped as follows:

- Group I – Control cells
- Group II – Leptin treated cells
- Group III – Cells exposed to LY 294002 and then treated with leptin

After incubation period, the culture supernatants were carefully isolated, centrifuged and filtered using 0.22  $\mu$ m filter to remove cell debris and the TNF- $\alpha$  level in the supernatant was evaluated using Human TNF- $\alpha$  Sandwich ELISA kit using the manufacturer's protocol.

### 2.4 Reverse Transcriptase-PCR:

After the incubation period, cultured cells were harvested, washed thrice with PBS and RNA was isolated using Trizol reagent (Sigma Aldrich, USA). RNA samples were quantified using Nanodrop (Thermoscientific, USA). Purity of RNA samples were assessed by determining the ratio of absorbance of samples at 260 nm and 280 nm. The purity of RNA obtained by 260/280 ratio was between 1.7-1.8.

1  $\mu$ g RNA was reverse transcribed in a 20  $\mu$ L reaction using mMULV RT enzyme (New England Biolabs, UK). Resulting cDNA was then amplified using PCR. PCR amplification was performed in 20  $\mu$ L reaction containing 10  $\mu$ L Taq DNA Polymerase Master Mix Red (Ampliqon, Denmark), 4  $\mu$ L cDNA and 100 nM primers specific for TNF- $\alpha$  and GAPDH. Amplification was performed using thermocycler starting with denaturation at  $95^{\circ}\text{C}$  for 2 minutes followed by 35 cycles of  $95^{\circ}\text{C}$  for 30 seconds,  $57^{\circ}\text{C}$  for 30 seconds and  $72^{\circ}\text{C}$  at 30 seconds for TNF- $\alpha$  and denaturation at  $94^{\circ}\text{C}$  for 5 minutes followed by 40 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $56^{\circ}\text{C}$  for 30 seconds and  $72^{\circ}\text{C}$  for 30 seconds for iNOS. A final extension step at  $72^{\circ}\text{C}$  for 10 minutes was performed and the samples were kept at  $4^{\circ}\text{C}$ . Amplification of TNF- $\alpha$ , iNOS and GAPDH yielded a PCR product of 513 bp, 290 bp and 206 bp respectively. The amplified products were separated on a 2% agarose gel for 30 minutes at 100 volts. The run included DNA molecular marker (100 bp ladder, New England Biolabs, UK).

The primers used are as follows:

TNF- $\alpha$  Sense : 5'-ATGAGCACTGAAAGCATGATC-3'

Antisense : 5'-AGAGAGGAGGTTGACCTTGGTCTGGTA-3'

iNOS Sense : 5'-CCTGAGCTCTTCTTCGAAATCC-3'

Antisense : 5'-AGGATGTTGTAGCGCTGGAC-3'

GAPDH Sense : 5'-GGAGTCAACGGATTTGGT-3'

Antisense : 5'-GTGATGGGATTTCCATTGAT-3'

### 2.5 Nitric Oxide Measurement:

In aqueous solutions that contain no heme proteins, NO is oxidized to nitrite only<sup>[9]</sup>, which can serve as an indirect marker for the presence of NO<sup>[10]</sup>. Hence, from the supernatant of the cultured cells, NO was estimated using Griess reaction by the Nitric Oxide (NO) detection kit (Intron Biotechnology Inc., Korea). To avoid the cell debris while collecting the supernatant from cultured cells, cells were harvested by centrifugation and the supernatant was filtered using 0.22  $\mu$ m filter. Absorbance value was measured between 520-560 nm using the ELISA plate reader (Molecular Devices, Versa Max, USA).

### 2.6 Western Blot:

The protein expression of iNOS from the cultured cells was assessed using western blot. After the incubation period, cultured cells were harvested, washed thrice with PBS and suspended in RIPA buffer containing protease inhibitor cocktail (Biobasic Inc., Canada). Protein from the cultured cells was isolated by sonication and estimated spectrophotometrically (UV-2450, SHIMADZU, Japan) by Lowry method using Bio-Rad DC protein assay (Bio-Rad Laboratories, USA). 50  $\mu$ g protein was run out in 8% SDS-PAGE. Proteins were then subsequently transferred for 1 hour at 100 V at 4°C to nitrocellulose membrane and blocked in blocking solution (1X Tris Based Saline (TBS) with Tween 20 containing 5% non-fat dry milk) for 1 hour at room temperature. Blots were incubated overnight at 4°C with rabbit polyclonal iNOS antibody (Cell Signaling Technology, UK) or rabbit polyclonal  $\beta$ -actin antibody (Cell Signaling Technology, UK) antibody in 1:1000 and 1:5000 dilution respectively. Antibodies were diluted in blocking solution. The antigen-antibody complexes were visualized using mouse anti-rabbit peroxidase conjugated IgG antibody (1:5000 dilution) (Bangalore Genei, Bangalore) with the Enhanced Chemiluminescence detection system (Amersham ECL prime western blotting detection reagent, GE Healthcare Biosciences, Sweden). The intensity of the bands was determined using Biorad Versadoc Imaging System.

### 2.7 Statistical Analysis:

The data obtained was analyzed using SPSS software version 17.0 and the results are presented as mean $\pm$ SD. Student's T test was used to compare continuous variables between two groups. A value of p<0.05 was considered statistically significant.

## III. Results

To analyze the immunomodulatory role leptin in PBMCs, these cells were isolated from normal subjects and treated with Human Recombinant Leptin (100 nM) for 6 hours at 37°C and the expression of TNF- $\alpha$  and iNOS was evaluated. Since PI3K/AkT pathway is the common signaling pathway suggested, we have treated PBMCs with leptin alone and leptin in combination with the LY294002 (PI3K inhibitor). PBMCs were exposed to 10  $\mu$ M LY294002 and then treated with human recombinant leptin.

In the present study, leptin treated cells were found to possess a higher level of TNF- $\alpha$  when compared to control cells. Group III showed a reduction in TNF- $\alpha$  protein level when compared to Group II cells (Table 1).

**Table 1:** effect of leptin on tnf- $\alpha$  production in human pbmcs:

CATEGORY	TNF- $\alpha$ (pg/mL)
Group I cells (Control PBMC)	15.5 $\pm$ 0.8
Group II cells (PBMC treated with Leptin)	72.5 $\pm$ 1.9
Group III cells (PBMC exposed to LY294002 and then treated with Leptin)	67.9 $\pm$ 1.4

Fig 1 shows the RT-PCR analysis of TNF- $\alpha$  gene expression in human PBMCs where Lane I, II, III and IV corresponds to 100 bp DNA ladder, Group I, Group II cells and Group III cells respectively. In the present study, TNF- $\alpha$  mRNA level was found to be highly elevated in leptin treated cells when compared to

control cells. The inhibition of the PI3K pathway with LY 294002 did not show any reduction in leptin induced TNF- $\alpha$  gene expression.

FIGURE 1: RT-PCR ANALYSIS SHOWING THE EFFECT OF LEPTIN ON TNF- $\alpha$  GENE EXPRESSION

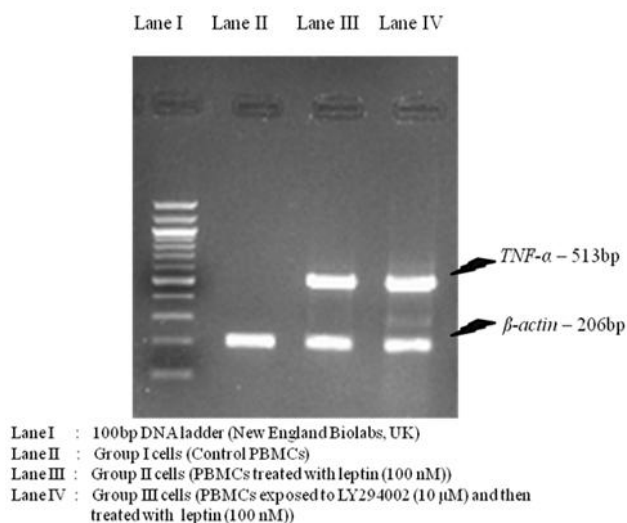


Table 2 shows the values of NO obtained from the supernatant of the cultured cells. Leptin treated cells were found to possess a higher level of NO when compared to that of control cells ( $10.2 \pm 0.5 \mu\text{M}$  versus  $5.3 \pm 0.7 \mu\text{M}$ ,  $p=0.004$ ). Group III cells showed a considerable reduction in NO level when compared to Group II cells ( $5.8 \pm 0.5 \mu\text{M}$  versus  $10.2 \pm 0.5 \mu\text{M}$ ,  $p=0.002$ ).

Table 2: effect of leptin on nitric oxide production in human pbmcs:

Category	No (Mm)
Group I Cells (Control Pbmcs)	$5.3 \pm 0.7$
Group Ii Cells (Pbmcs Treated With Leptin)	$10.2 \pm 0.5$
Group Iii Cells (Pbmcs Exposed To Ly294002 And Then Treated With Leptin)	$5.8 \pm 0.5$

Fig 2 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.

FIGURE 2: RT-PCR ANALYSIS SHOWING THE EFFECT OF LEPTIN ON iNOS GENE EXPRESSION

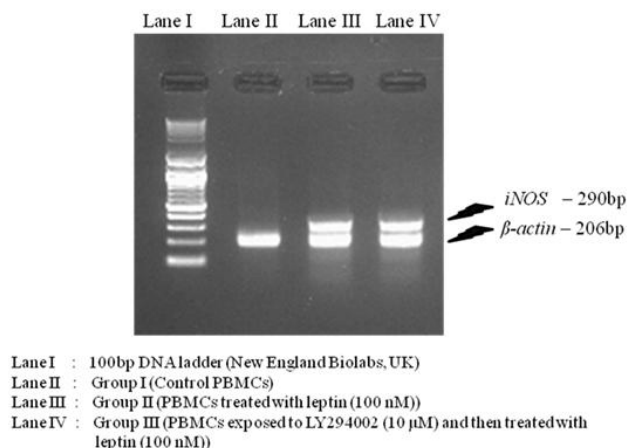
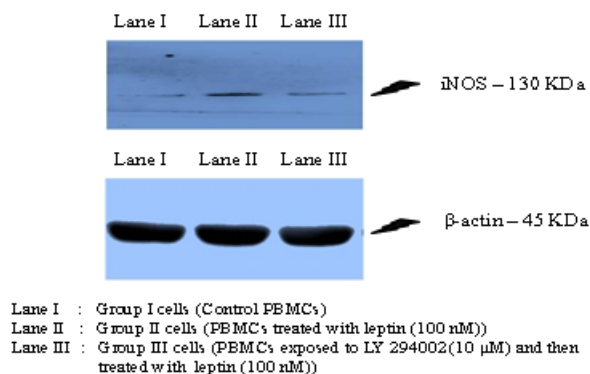


Fig 3 depicts the protein expression of iNOS in human PBMCs. Lanes I, II and III correspond to Group I, Group II and Group III cells respectively. Control cells were found to express basal level of iNOS whereas in leptin treated cells iNOS protein expression level was found to be significantly increased ( $p=0.009$ ). The inhibition of the upstream molecule PI3K with LY 294002 showed a reduction in leptin induced iNOS expression ( $p=0.02$ ).

**FIGURE 3: WESTERN BLOT ANALYSIS SHOWING THE EFFECT OF LEPTIN ON iNOS PROTEIN EXPRESSION**

**Figure 4.2 (a): Effect of LY294002 on iNOS expression induced by leptin in PBMC**



#### IV. Discussion

Atherosclerosis is increasingly viewed as an inflammatory disease, driven by lipoproteins, metabolic signals, hemodynamic stress, and the integrated activity of immune cells and inflammatory cytokines and it is intriguing that leptin and inflammatory pathways demonstrate reciprocal modulation and shared association with cardiovascular risk<sup>[11]</sup>. Several immune cells implicated in atherosclerosis, including PBMCs bear leptin receptor and generally are activated by leptin<sup>[12]</sup>. Hence, to analyze the influence of leptin over inflammation, we performed an *in vitro* analysis in which human PBMCs were treated with human recombinant leptin (100 nM) and its effect on the synthesis of pro-inflammatory cytokine, TNF- $\alpha$  was studied. In the present study, leptin treated cells were found to express a higher level of TNF- $\alpha$  both at gene and protein level when compared to that of the control cells (Table 4.12). Thus, leptin can activate PBMCs and stimulate the synthesis of TNF- $\alpha$  at transcriptional level. The TNF- $\alpha$  protein value was found to be at basal level in control cells whereas a prominent increase in its value was observed in leptin treated cells. This suggest that the normal physiological effect of leptin on the regulation of TNF- $\alpha$  expression is suppressive, but still, hyperleptinemic conditions might contribute to the rise in TNF- $\alpha$  thereby leading to the pathogenesis of CVD.

Exposure of PBMCs to LY294002 (Group III cells) showed a reduction in TNF- $\alpha$  protein expression when compared to cells activated with leptin which inturn suggest that LY294002 might inhibit leptin induced TNF- $\alpha$  secretion at post-transcriptional level.

Nitric oxide (NO) represents the key molecule for the depressor response induced by leptin<sup>[13]</sup>. It plays an important role in the maintenance of vascular tone. In PBMC, NO is mainly produced by iNOS, a Ca<sup>2+</sup>-independent enzyme that is regulated at transcriptional and posttranscriptional levels<sup>[14]</sup>. In pathophysiological conditions such as CVD, induction and activation of iNOS is reported to be toxic<sup>[15,16]</sup>. iNOS is reported to exert some deleterious effects due to the production of peroxynitrite from NO and superoxide<sup>[17-20]</sup>. An overall protective effect of iNOS (vasorelaxation and cytoprotection) results from the production of a ‘basal’ rate of NO (in picomolar range), whereas cytotoxicity (hyperreactivity, platelet adhesion and protein fragmentation) results from the production of higher rates (nanomolar to micromolar range) of the same (Depre et al., 1999). Hence, in the present study, we examined the potential of leptin (100 nM) to stimulate iNOS synthesis *in vitro* by activating PBMCs. To gain further insight into the signaling cascades activated by leptin, the potential involvement of PI3K/Akt transduction signal was analyzed using specific pharmacological inhibitor.

A significant increase in the NO level is observed in leptin treated cells when compared to control cells with an increase in iNOS gene and protein expression. Interestingly, for the first time, our results show that leptin might stimulate the synthesis of NO in PBMC through the activation of iNOS.

Also, the pathways by which iNOS could be synthesized by activation with leptin were studied. Group III cells were 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 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 LY294002 over leptin induced iNOS synthesis might take place at post-transcriptional level. Thus, synthesis of NO during pathological conditions might in turn act as an important mediator in progression of CVD.

### V. Conclusion

In vitro activation of human PBMCs with leptin (100 nM) suggest that leptin (100 nM) can activate human PBMCs, thereby enhancing TNF- $\alpha$  and iNOS synthesis and this effect takes place at a transcriptional level. The subsequent synthesis of TNF- $\alpha$  and NO during pathological conditions might act as an important mediator in the progression of CVD. The immunomodulatory role of leptin in PBMC in turn suggest that leptin might act as a novel therapeutic target for Cardiovascular Diseases.

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