EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN CARDIOVASCULAR DISEASE

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ABSTRACT

Hyperleptinemia may play an important role in obesity-associated cardiovascular diseases including atherosclerosis. Leptin exerts many potentially atherogenic effects such as induction of endothelial dysfunction, stimulation of inflammatory reaction, oxidative stress, decrease in paraoxonase activity, platelet aggregation, migration, hypertrophy and proliferation of vascular smooth muscle cells. Nitric oxide (NO) is a short-lived, gaseous free radical, synthesized from L-arginine by NO synthases (NOS). The inducible nitric oxide synthase (iNOS) is a main enzyme producing nitric oxide during inflammation and thus contributes to the initiation and development of inflammatory cardiovascular diseases such as atherosclerosis. The aim of the study was to elucidate the link between leptin and inflammation by in vitro activation of the PBMCS with leptin and to confirm the expression of iNOS by western blotting technique in CVD. The results show that the In vitro activation of PBMC with leptin showed bands of 130 kDa which confirmed the presence of iNOS. The in vivo expression of iNOS correlates with the leptin levels in normal and overweight BMI CVD patients respectively. This shows the elevated leptin levels was associated with elevated iNOS.

KEYWORDS: Obesity, inflammation, CVD, iNOS, leptin.

INTRODUCTION

Leptin, an adipocyte-derived nonglycosylated polypeptide hormone, promotes the onset of CVD in its ability to modulate the immune response. Leptin receptor has been found to be expressed in human peripheral blood mononuclear cells, which mediates the leptin effect on
proliferation and activation.\(^{[1]}\) Also, leptin stimulates the production of proinflammatory cytokines from cultures of monocytes and enhances the production of T helper 1 (Th1) type cytokines such as interferon (IFN)-\(\gamma\) and tumor necrosis factor (TNF)-\(\alpha\) from stimulated lymphocytes.

Leptin stimulation activates janus kinase-signal transducer and activator of transcription (JAK-STAT) and insulin receptor substrate -phosphatidylinositol 3-kinase (IRS-1-PI3K) signaling pathways. These signal transduction pathways provide possible mechanisms whereby leptin may the modulate activation of peripheral blood mononuclear cells (PBMC). Therefore, these support our hypothesis regarding leptin as a proinflammatory cytokine with a possible role of acting as a link between the nutritional status and immune response.

Inflammatory cytokines associated with atherosclerosis may be capable of stimulating the synthesis and activity of inducible nitric oxide synthase (iNOS), which could further influence the pathologic features associated with the disease. Although there is a certain amount of indirect evidence to support the presence of iNOS in atherosclerosis, there has been no definitive study to confirm this.\(^{[2]}\)

Though leptin plays a physiological role, leptin resistance may be patho physiological for metabolic and cardiovascular dysfunction under inflammatory conditions. While the role of leptin has been determined in the elevation of sympathetic nervous system activity, many of its action on cardiovascular events such as inflammation has not been carefully studied.

Currently, atherosclerosis is considered as an inflammation-mediated disease driven by complex interactions between leukocytes, especially lymphocytes and monocytes (collectively termed as peripheral blood mononuclear cells or PBMC), platelets and cells of the vessel wall. The demonstrated beneficial role of anti-inflammatory drugs in reducing the incidence of nonfatal events in many large clinical trials has demonstrated the major role of PBMC in the atherosclerotic process. Hence, a potential insight into the mechanistic and signaling pathways that leads to inflammatory events in PBMC becomes necessary for tailoring efficient therapeutic strategies for CVD.

Taken together, obesity has become an all too common condition in many of the world's industrialized societies today and is continuing to rise at an alarming rate. Although at first sight it might seem that this is simply the result of changes to our diet and a dramatic drop in
the level of physical activity in recent years, the reality is that scientific studies into the causes of obesity show that the condition results from a complex combination of factors. One such factor which has come under increasing examination over past ten years or so is genetics and in particular, the genes involved in the regulation of leptin within the body. Therefore, the present study was undertaken to assess the correlation between leptin & other adipokines and their association in control & CVD subjects

MATERIALS AND METHODS

**Blood collection:** The blood samples (5ml) were collected from control and CVD subjects of normal & overweight BMI. The blood was allowed to clot and retract, then the serum was separated by centrifugation at 3500 rpm for 10 minutes. The separated serum was transported in ice cold box and stored at -80°C until use.

**EXPRESSION OF INFLAMMATORY MARKERS INOS**

**Preparation of human recombinant leptin:**

Human recombinant leptin was purchased from MP biochemicals. To reconstitute lyophilized leptin, 15 mM sterile HCl (0.5 ml/ mg vial) was added to the vial. After the protein was completely dissolved, 7.5 mM sterile NaOH (0.3 ml/mg vial) was added to the vial.

**Isolation and culturing of PBMC**

5ml of venous blood was obtained from healthy volunteers. 3 ml of ficoll Hypaque was layered over the leucosep tube. The tube was then centrifuged at 3000 rpm for 30 seconds at room temperature.

5 ml of blood was then layered over the membrane in the leucosep tube and centrifuged at 4000 rpm for 15 minutes at 25°C. Three layers were separated. The intermediate layer rich in PBMC was taken carefully and transferred to another centrifuge tube containing 2 ml of 1X phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 10 minutes. Cells were then washed thrice with PBS and washed once with RPMI medium (supplemented with glutamine, 100 U/ml pencillin, 100µg /ml, Streptomycin, 10% of heat inactivated fetal bovine serum).

PBMCs thus obtained were counted using haemocytometer. 2x 10^6 cells/ ml medium were then cultured in the wells of a 24 well plate. 1000nM leptin was added to these cultures and the cells were incubated for 24 hours at 37°C in 5 % CO₂. Untreated cells were used as controls. After the incubation period, cells were harvested and washed once in Roswell Park
Memorial Institute medium (RPMI) medium and thrice in PBS. The cells were then suspended in Radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor cocktail and then subjected to ultrasonication and centrifuged at 12000rpm for 20 minutes at 4°C. The supernatant thus obtained was then collected and stored at -80°C until use.

**Estimation of protein**

Protein was estimated by Bradford’s Method (Kit Manual)[3-8]

**Expression of iNOS**

The protein expression of iNOS was assessed by western blot analysis.[9] For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 5% stacking gel and 10% resolving gel was used. After electrophoresis, the gel was placed over a nitrocellulose membrane. The gel and the membrane were packed by three cut-pieces of Whatman filter paper no.3. This set-up was covered on both sides with the absorbers and clipped. The whole set-up was immersed in a transfer buffer and a current of 100V was passed for 1 hour. The membrane was then removed from the set-up and the empty sites in the membrane was blocked by treating the membrane with blocking solution overnight. Later, the membrane was incubated and washed three times in T-TBS and reacted with primary antibody (1:250 dilution) against iNOS for 5 hours. The membrane was then washed four times with T-TBS and incubated with horseradish peroxidase labeled secondary antibody (1:2500) for 1 hour. After four washes with T-TBS, the immunoreactive bands were visualized using the DAB chromogen system.

**RESULTS**

**Western blotting**

![Figure 1: shows (a) SDS-PAGE of iNos (b) western blotting for iNOS.](image-url)
Lane 1 Molecular weight markers  
Lane 2 iNOS in PBMC  
Lane 3 LPS (Lipopolysaccharide (LPS) stimulates NO production in macrophages)

On Western blotting, bands of 130 kDa was identified in the PBMC and recognized by iNOS antibodies

![Western Blot Image]

**Figure 2: shows the expression of iNOS in different lane**

Lane 1 serum from Normal BMI control  
Lane 2&3 serum from Overweight BMI control  
Lane 4 serum from Normal BMI CVD  
Lane 5&6 serum from Overweight BMI CVD

**DISCUSSION**

The total protein in PBMC which was equivalent to protein concentration of Phytohaemagglutinin (control), a molecular tracer. It was confirmed by Bradford’s method. It is necessary to analysis protein quantitatively before the western blotting in order to determine its concentration.

In vitro activation of PBMC with leptin showed bands of 130 kDa which confirmed the presence of iNOS.

The presence of iNOS was confirmed by western blotting technique. The expression of iNOS was observed in the normal and overweight BMI CVD patients, but it was not observed in normal and overweight BMI control individuals.
The iNOS expression correlates with the leptin levels in normal and overweight BMI CVD patients. This shows the elevated leptin levels is associated with elevated iNOS.

CONCLUSION
The results of this study suggesting that In vitro activation of the PBMC with leptin confirmed that leptin activates the inflammatory process which may be the cause for CVD. In vitro and in vivo methods confirmed the expression of inflammatory marker iNOS by leptin.

REFERENCES
7. Spectror T. Refinement of the Coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 micrograms of protein, *Analytical Biochemistry.*, 1978; 86: 142-146