

PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATED FROM NORMAL WEIGHT AND OWEIGHT SUBJECTS DIFFERENTIALLY RESPOND TO LIPOPOLYSACCHARIDES STIMULATION

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ABSTRACT

Bacterial lipopolysaccharides (LPS) has been widely used both in *in vitro* and *in vivo* models studying inflammation and the mechanisms of anti-inflammatory action of variety of substances. Profiling of peripheral blood mononuclear cells (PBMCs) could serve as less invasive alternative to tissue biopsies for research purposes in human intervention studies. A model utilizing PBMCs would provide a more direct approach to study inflammation and related cell responses. The aim of the present work was to establish a working model of *ex vivo* stimulation with LPS of PBMCs isolated from human whole blood. Volunteers were divided into two groups: normal weight subjects with BMI<25 (NW) and overweight subjects with BMI≥25 (OW). Expression of two pro-inflammatory genes was measured in order to verify inflammatory response upon LPS stimulation.

LPS treatment effectively induced inflammatory response in PBMCs by increased mRNA levels of IL-1 β and IL-6 genes both in NW ($p<0.001$) and OW group ($p<0.05$). IL-1 β mRNA levels exceeded that of IL-6 both in control and LPS treated PBMCs of NW and OW subjects ($p<0.001$).

Our measurements did not establish significant difference between NW and OW group in regard to the initial levels of IL-1 β and IL-6. Moreover, OW PBMCs responded less prominently after LPS stimulation - mRNA levels of IL-6 in OW group were by 73% ($p<0.001$) and these of IL-1 β – by 55% ($p<0.05$) lower in the OW group. The presence of various unknown factors in plasma could possibly predetermine the subsequent difference in the response of PBMCs obtained from NW and OW individuals. In conclusion, our model effectively induced inflammatory response in freshly isolated and cultured PBMCs from NW and OW individuals. Gene induction in NW group was more prominent. Although IL-1 β mRNA levels were always higher than IL-6, the last responded more prominently to the treatment.

Key words: PBMCs, LPS, IL-6, IL-1 β

INTRODUCTION

Whole blood is an example of a complex tissue that contains different cell types and each of them expresses different patterns of gene transcription as related to its specific function. Profiling of peripheral blood mononuclear cells (PBMCs) could serve as less invasive alternative to tissue biopsies for research purposes in human intervention studies. Transcriptomics of PBMCs has become an innovative and reliable method to study dietary effects in humans (Stepien et al., 2014; Eady et al., 2005; van Erk et al., 2006; Bouwens et al., 2007).

Lipopolysaccharide (LPS) is a glycolipid, the most abundant component within the cell wall of Gram-negative bacteria. It can stimulate the release of inflammatory cytokines, including TNF- α , IL-6 and IL-1 β in various cell types (Jansky et al., 2003; Frost et al., 2002) leading to an acute inflammatory response towards pathogens (Sweet et al., 1996). Bacterial LPS has been widely used in models studying inflammation both in *in vitro* (Jansky et al., 2003; Ngkelo et al., 2012; Di Caprio

et al., 2015) and *in vivo* (Cai et al., 2014; Ma et al., 2015), as well as the mechanisms of anti-inflammatory action of variety of substances (Hougee et al., 2005; Ma et al., 2015).

The aim of the present work was to establish a working model of *ex vivo* induced inflammation in PBMCs isolated from human whole blood. The model is supposed to be further applied for examining anti-inflammatory properties of herbal extracts, fractions or isolated compounds, and even of drugs and food additives, as well as to assess changes in LPS-induced response as a result of nutrient/drug interventions.

MATERIALS AND METHODS

Volunteers

An approval from the local ethics committee was received prior to research analyses (Protocol №27(6)/21.02.2013). All the participants enrolled in the study signed informed consent prior to start of blood sampling.

Study involved 40 clinically healthy volunteers, aged between 20 and 60 years. They were divided in two groups according their Body Mass Index (BMI) – normal weight (NW) group (BMI<25), n=23 and overweight (OW) group (BMI≥25), n=17.

PBMCs collection

Whole blood samples were collected using lithium heparin vacutainer tubes. LeucoSep™ centrifuge separation tubes (by Greiner Bio One), enabling cell separation by means of density gradient centrifugation were used for PBMCs isolation. Centrifugation step was performed according to the manufacturer's instructions.

Cell cultivation and treatment

Cells were counted with standard trypan blue method and seeded at density of 1×10^6 cells/well in 6-well flasks. Cells were cultivated in RPMI medium containing 1M HEPES, heat-inactivated FBS, 200mM α -glutamine and penicillin/streptomycin solution to a final concentration of 100U/mL each. Culture medium suggested for inflammatory treatment contained LPS (*Escherichia coli*, 026:B6; Sigma-Aldrich) dissolved in saline solution (0.9% NaCl) to a final concentration of 100ng/mL in the treatment medium. Flasks were incubated in humidified chamber with 5% CO₂ atmosphere for 4 hours at 37°C. The treatments were performed in duplicates.

RNA isolation and cDNA synthesis

After the incubation period following the manufacturer's protocol total RNA was extracted from the cells using Trizol reagent (Tri Reagent®, Ambion®, Life Technologies). RNA was subsequently DNase treated (RiboPure™ – Blood Kit; Sigma-Aldrich) in order to purify the yielded material and increase its quality. First strand cDNA synthesis was performed with 0.8µg of total RNA as described previously (Kiselova-Kaneva et al., 2012).

Real-Time PCR

Quantitative gene expression analysis was performed using two-step real-time qPCR (Applied Biosystems® 7500 Real-Time PCR system). Each reaction was amplified in a reaction mix containing SYBR Green qPCR 1xMaster Mix with ROX (KAPA SYBR FAST qPCR Kit, Kapa Biosystems, USA) and 0.3µM of each primer. Primer sequences used for the Real-Time PCR are shown in Table 1. Samples were incubated for an initial denaturation at 95°C for 5min, followed by 40 cycles. Each cycle consisted of 95°C for 15sec, and 60°C for 1min. To confirm amplification of specific transcripts a dissociation step was included in the instrument protocol. The amount of mRNA of each cytokine was normalized according to the amount of mRNA encoding 60S ribosomal protein L37a (RPL-37A), used as endogenous control. Gene expression levels were

calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and expressed in relative units (RU). All measurements were performed at least in triplicate.

Statistical analysis

The results are presented as relative units (RU)±SEM. Unpaired Student's *t*-test was used to perform the statistical analysis. *p* values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In the present study we aimed to establish an effective and working model for *ex vivo* LPS stimulation of freshly isolated and subsequently cultured PBMCs. Volunteers were divided into two groups in order to compare the results in regard to BMI of the subjects. So as to verify the inflammatory response to LPS treatment the expression levels of two pro-inflammatory cytokines were measured - IL-1 β and IL-6. The main source for IL-1 α and β are mononuclear phagocytes, fibroblasts, keratinocytes, and T and B lymphocytes. They both can trigger the fever by activation of cyclooxygenase (COX) enzymes and therefore enhancing the prostaglandin E₂ synthesis (Warren, 1990) and can stimulate the T cell proliferation. Furthermore, they are reported to induce synthesis of C-reactive protein (CRP), an acute-phase protein produced during infections and inflammation, both *in vitro* and *in vivo* (Sheldon et al., 1993).

IL-6 is a glycoprotein produced by mononuclear phagocytes, T cells, and fibroblasts (Van Snick, 1990; Hirano, 1992; Hirano et al., 1990). IL-6 is considered to be a stress-induced cytokine with varying effects on a variety of tissues. As an inflammatory mediator IL-6 stimulates acute phase protein synthesis (Trayhurn and Wood, 2004). In the acute phase of inflammation IL-6 acts as a growth factor for mature B cells and stimulates their transformation into antibody-producing plasma cells. Up-regulation of IL-6 production is established also in variety of chronic inflammatory and autoimmune disorders (Hirano, 1992).

Variety of LPS concentrations and treatment durations have been reported to stimulate cytokine production in PBMCs – from 5 μ g/mL to up to 10ng/mL for a period of 24 hours (Jansky et al., 2003; Zhao et al., 2007; Schildberger et al., 2013). In these studies, IL-1 β and IL-6 protein concentration strongly increase 4 hours after LPS treatment, followed by gradual increase during the treatment period. IL-1 β and IL-6 mRNA levels established in PBMCs incubated for 4h in medium containing 100ng/mL LPS are presented in figure 1 and figure 2. LPS treatment effectively induced inflammatory response in PBMCs in both groups, as represented by increased mRNA levels of IL-1 β and IL-6 genes. In NW group 3.68 fold increase ($p<0.001$) of IL-1 β and approximately 14 fold increase ($p<0.001$) of IL-6 mRNA levels were established. Less prominent was the effect in OW group, where 2.8 ($p<0.05$) and 2.5 ($p<0.05$) fold increase was established for IL-1 β and IL-6, respectively. According to other published data from experiments involving PBMCs, isolated from healthy volunteers TNF- α and IL-1 β are the first cytokines to be produced at highest concentrations, followed by IL-6 (Jansky et al., 2003). Our results also represented that IL-1 β mRNA levels exceed these of IL-6 mRNA both in the control and the LPS treated PBMCs of NW and OW subjects (Table 2).

It is known that obesity is characterized by increased macrophage infiltration in adipose tissue (Wellen and Hotamisligil, 2003). Adipose tissue macrophages are suspected to be the major source of inflammatory mediators such as TNF- α and IL-6 that interfere with adipocyte function by increasing inflammatory profile and inhibiting insulin action (Sethi and Hotamisligil, 1999; Trayhurn, 2005; Suganami and Ogawa, 2010; Zeyda and Stulnig, 2007). However, our measurements did not establish significant difference between NW and OW group in regard to the initial levels of IL-1 β and IL-6. Moreover, OW PBMCs responded less prominently after LPS stimulation - mRNA levels of IL-6 in OW group were by 73% ($p<0.001$) and these of IL-1 β were by 55% ($p<0.05$) lower in the OW group. The presence of various unknown factors in plasma could

possibly predetermine the subsequent difference in the response of PBMCs obtained from NW and OW individuals.

CONCLUSION

According to the results we conclude that 4h treatment with LPS (*E. coli* 026:B6) at concentration of 100ng/mL effectively induces inflammatory response in freshly isolated and cultured at density 1×10^6 cells/well PBMCs from NW and OW individuals. However, gene induction in the NW group was more prominent, which should be taken into consideration when selecting study target groups and analyzing data. Although IL-1 β mRNA levels were higher than IL-6 mRNA levels in all measurements, the last responded more prominently to the treatment.

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Table 1. Primer sequences of the investigated genes used in the quantitative Real-Time PCR analysis.

Gene	Sequence 5'–3'
<u>RPL37A (Bioneer, USA)</u>	
Forward	ATTGAAATCAGCCAGCACGC
Reverse	AGGAACCACAGTGCCAGATCC
<u>IL-1β (Alpha DNA, Canada)</u>	
Forward	TCCCCAGCCCTTTTGTGTA
Reverse	TTAGAACCAAATGTGGCCGTG
<u>IL-6 (Bioneer, USA)</u>	
Forward	AAACAACCTGAACCTTCCAAAGA
Reverse	GCAAGTCTCCTCATTGAATCCA

Table 2. Relative IL-1 β mRNA levels

	Control	p value	LPS	p value
BMI < 25	61.55 \pm 18.22/14.06	p<0.001	14.93 \pm 3.25/2.67	p<0.001
BMI \geq 25	28.39 \pm 13.37/9.09	p<0.001	24.45 \pm 8.34/6.22	p<0.001

Data are presented as RU \pm SEM; IL-1 β mRNA levels were calculated and presented as relative to IL-6.

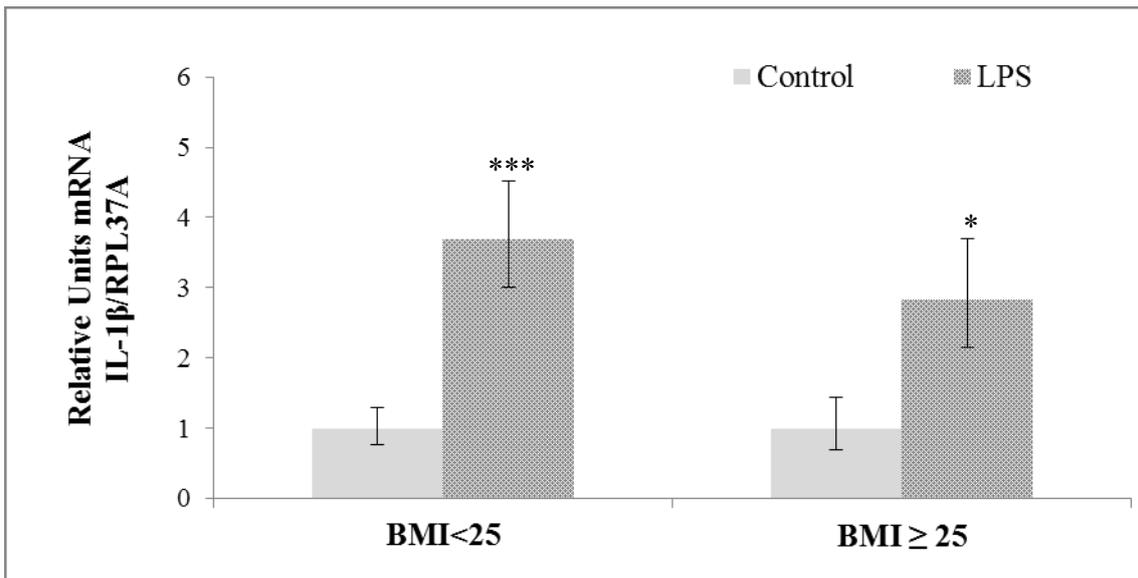


Figure 1. IL-1 β mRNA levels in PBMCs treated with 100ng/mL LPS.

Data are presented as RU \pm SEM; IL-1 β mRNA levels in LPS treated cells were calculated and presented as relative to untreated control. *p<0.05 versus untreated control; ***p<0.001 versus untreated control.

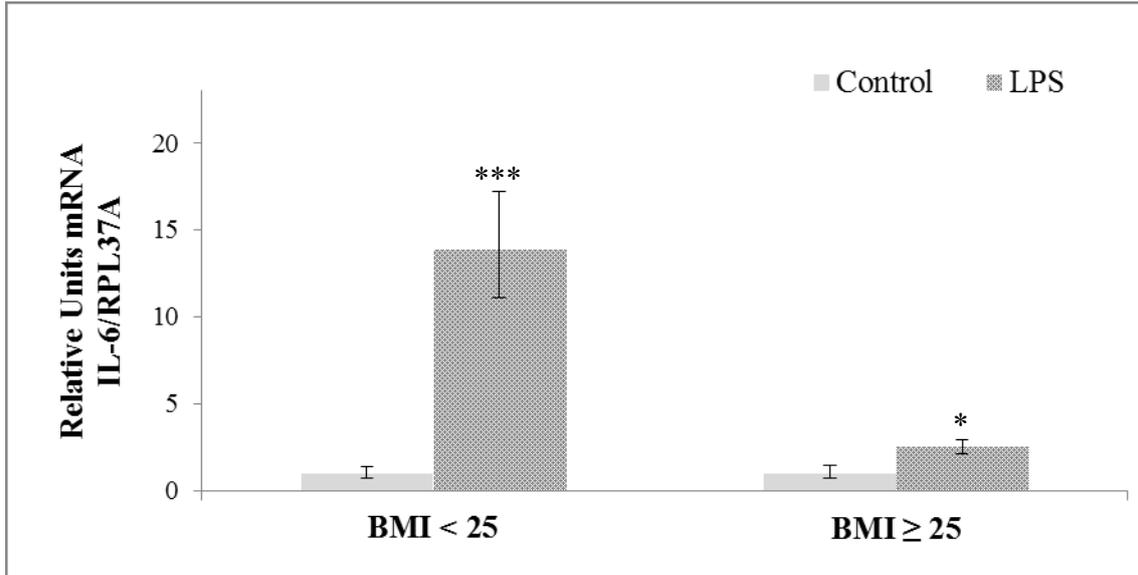


Figure 2. IL-6 mRNA levels in PBMCs treated with 100ng/mL LPS.

Data are presented as RU±SEM; IL-6 mRNA levels in LPS treated cells were calculated and presented as relative to untreated control. *p<0.05 versus untreated control; ***p<0.001 versus untreated control.

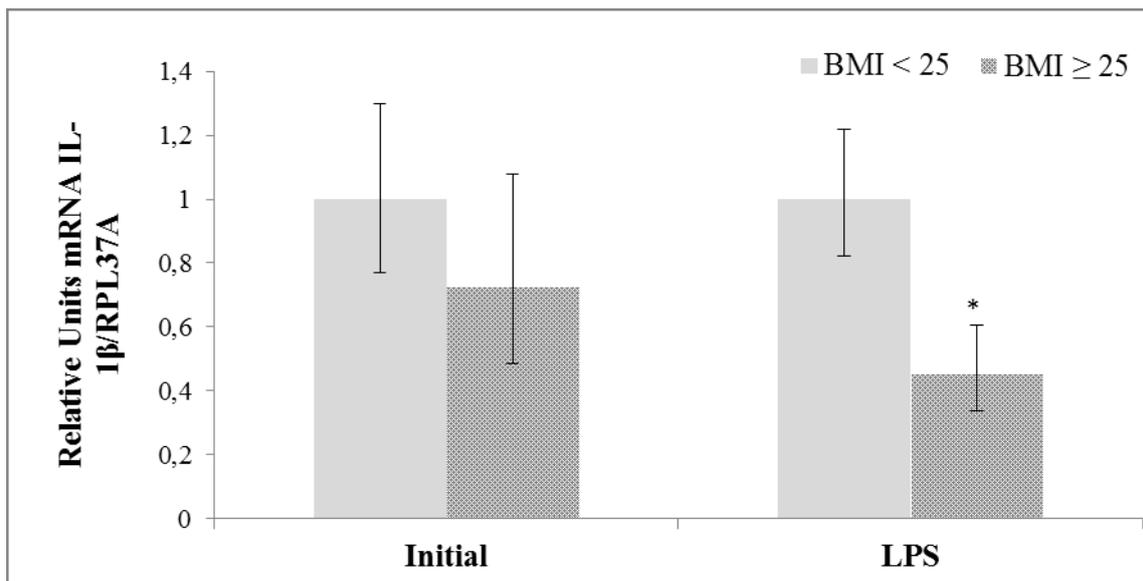


Figure 3. IL-1β mRNA levels in PBMCs from NW and OW individuals.

Data are presented as RU±SEM; IL-1β mRNA levels in OW group were calculated and presented as relative to NW. *p<0.05 versus BMI<25.

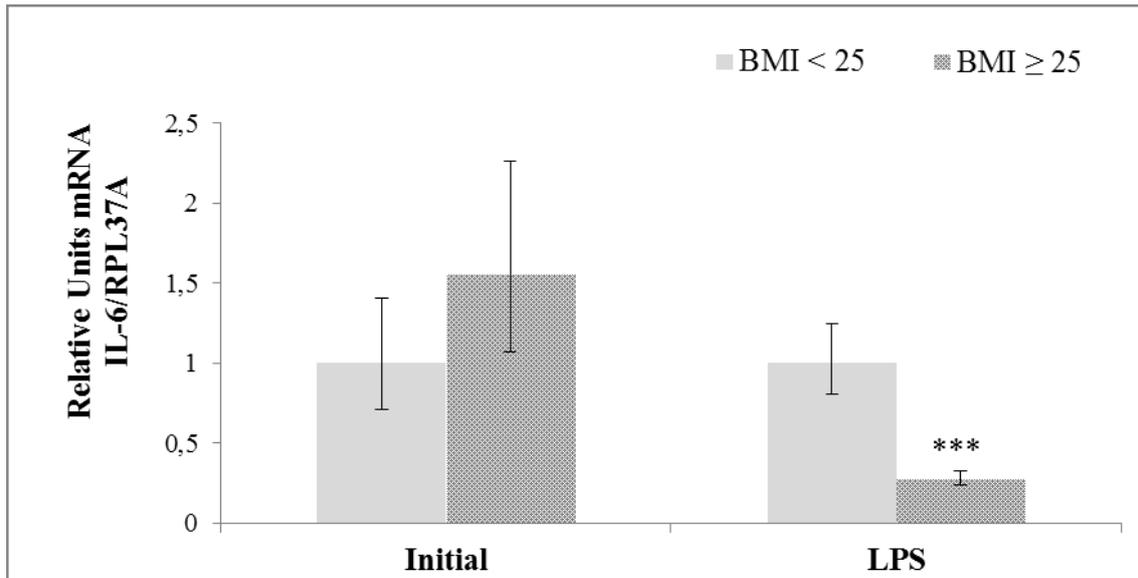


Figure 3. IL-6 mRNA levels in PBMCs from NW and OW individuals.

Data are presented as $RU \pm SEM$; IL-6 mRNA levels in OW group were calculated and presented as relative to NW. *** $p < 0.001$ versus BMI < 25.