LPS-INDUCED RESPONSE IN J774A.1 MACROPHAGE CELL CULTURE – INDICATIVE MARKERS FOR STIMULATED ANTIOXIDANT DEFENSE, INFLAMMATION AND PHAGOCYTOSIS

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ABSTRACT

INTRODUCTION: Treatment with bacterial lipopolysaccharides (LPS) is a convenient model used for stimulation of inflammatory response in cell cultures, which is also suggested to be associated with provoked antioxidant defense. Well defined markers are needed to verify pronounced cellular response in this model.

AIM: The aim of current study was to measure changes in expression levels of selected genes in order to identify indicative markers for verification of induced cellular response in a model of LPS-treated J744A.1 macrophages.

MATERIALS AND METHODS: In order to determine most appropriate LPS treatment concentration, an MTT test was performed. LPS was applied in different concentrations (50 - 300 ng/mL) and their effect on the cell viability of J744A.1 macrophages was measured.

RESULTS AND DISCUSSION: Analysis of the results of the MTT test showed a statistically significant ($p<0.001$) and equal effect of the three LPS concentrations (100, 200 and 300 ng/mL) applied. Cell viability was decreased with 20%. The effect of 100 ng/mL LPS treatment on the following genes was evaluated: antioxidant defense-related (GCLc, GPx1, GSS, GR and SOD2); inflammation-related (IL1β, IL6, MCP1, TNFα, IL1RN, NOS2, CRP, COX2); phagocytosis-related (NOX1 and MPO), and LPS/TLR4 signaling cascade-related (TLR4, IKK2, NRF1, NQO1). All of the studied genes were significantly induced upon LPS treatment for 24h determining it is sufficient to provoke pronounced cellular response. However, the following genes appear to be most affected by 24h LPS treatment: GCLc, COX2, NOS2, IL6, IL1β, CRP, NOX, TLR4 and IKK2.

CONCLUSION: In conclusion, studied genes may serve as suitable indicative markers for triggered cellular response were LPS stimulation of J774A.1 cells is about to be used as a model of oxidative and inflammatory provocation.

Keywords: *LPS, inflammation, phagocytosis, antioxidant defense, macrophages*

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INTRODUCTION

Monocytes and macrophages play a significant role in inflammatory and immune responses and modulating their functions may be a useful strategy for anti-inflammatory therapies. Lipopolysaccharide (LPS) is a major component of gram-negative bacterial cell walls and can elicit an acute inflammatory response, triggering the release of a

number of inflammatory cytokines. As a potent activator of monocytes/macrophages (1), bacterial LPS has been conventionally used to study *in vitro* and *in vivo* inflammatory response and its regulation in various cell types (2-4). Macrophages increase reactive oxygen and nitrogen species production after exposure to a number of different signals, including LPS (5), requiring provocation of antioxidant defense mechanisms.

AIM

Using models for induced inflammatory response researchers needs well-established markers to detect and validate the efficiency of the model. In this context, the aim of current study was to measure changes in expression levels of selected genes in order to identify indicative markers for verification of induced cellular inflammatory and associated oxidative stress response in a model of LPS-treated J744A.1 macrophages.

MATERIALS AND METHODS

1. Cell Culture

J774A.1 mouse macrophage cell line was obtained from American Type Culture Collection (ATCC). Cells were cultured in 75 cm^2 flasks at 37°C in a humidified chamber containing 5% CO_2 in DMEM (Sigma-Aldrich) with 4.5 g/L glucose, L-glutamine and supplemented with fetal bovine serum (FBS, Sigma-Aldrich) to a final concentration of 10%. Cells were sub-cultivated until 80% confluence was achieved.

2. Experimental Design

Cells were seeded in 6-well flasks with an initial density of $2x10^5$ cells/well, with a total volume of inoculum of 2 mL/well. After 24 hours of incubation, the cells were treated for 20h with LPS dissolved in the appropriate in cell culture media DMEM at selected concentrations (50, 100, 200, 300 ng/mL) for MTT test or for 24h with 100 ng/mL for gene expression analysis.

3. MTT Test

The cytotoxicity test was performed by the method of Mosmann (1983) with some modifications (6). At the end of the 20h treatment time, 100 μL MTT solution in PBS (pH=7.4) at a concentration of 2 mg/mL was added to each well, followed by incubation for 4 hours at dark. To measure the amount of formazan, the medium was removed and the crystals were dissolved with 1 mL of dimethyl sulfoxide (DMSO). Absorption was measured at λ=550 nm on a Synergy 2 multifunction reader (BioTek). Cell viability is presented as [%] from the nontreated control, where viability is considered 100%. Each test was performed three times on three separate days, and in each experiment the individual treatments were performed in triplicate.

4. Determination of Levels of Gene Expression

To determine the level of gene expression of selected genes in cell cultures, two-step quantitative real-time PCR was used. Gene expression values were calculated by the $2^{-\Delta\Delta Ct}$ method and were presented in relative units as compared to the untreated control at which the expression level of the analyzed gene was considered to be equal to 1. The results were presented as mean (n=3) of the relative units±standard error of mean (SEM). Expression of the following genes was studied: Glutamate Cysteine Ligase, catalytic subunit (GCLc) - F: AGGAGCTTCGGGACTGTATCC, R: GGGAAGTCCATTCATTCAAGGT; Glutathione peroxidase – 1 (GPx1) – F: CCCCACTGCGCTCATGA, R: GGCACACCGGAGACCAAA; Glutathione synthetase (GSS) – F: CCCAAGTGGTCCAGTCTATC, R: TCACCAGTGTTGTTCCCTG; Glutatione reductase (GR) – F: CACGGCTATGCAACATTCGC, R: TGTGTGGAGCGGTAAACTTTTT; Superoxide dismutase – 2 (SOD2) – F: AGACCTGCCTTACGACTATGG, R: GCGCGTTAATGTGTGGCTC; Cyclooxygenase – 2 (COX2) – F: TGAGCAACTATTCCAAACCAGC, R: GCACGTAGTCTTCGATCACTATC; Monocyte chemoattractant protein – 1 (MCP1) – F: GGCTCAGCCAGATGCAGTTAA, R: CCTACTCATTGGGATCATCTTG; Interleukin – 6 (IL6) – F: CTGCAAGAGACTTCC; R: GAAGTAGGGAAGGCC; Tumor necrosis factor alpha (TNFα) – F: CCCTCACACTCAGAT CATCTTCT, R: GCTACGACGTGGGCTACAG; C-reactive protein (CRP) – F: GTCTGCTACGGGGATTGTAGA, R: GCACCTTGGGTTTCC CATCAA; Interleukin – 1 beta (IL1β) – F: TTCAGGCAGGCACTA, R: CCACGGGAAAGACAC; Interleukin 1 receptor antagonist (IL1RN) – F: GCTCATTGCTGGGTACTTACAA, R: CCAGACTTGGCACAAGACAGG; Tool like receptor – 4 (TLR4) – F: AGGCACAT-

GCTCTAGCACTAA, R: AGGCTCCCCAGTT-TAACTCTG; Nitric oxide synthase – 2 (NOS2) – F: GGCAGCCTGTGAGACCTTTG, R: GCATTG-GAAGTGAAGCGTTTC; NADPH oxidase – 1 (NOX1) – F: AGAGGAGAGCCCTTATCCCAACC, R: TGTCCAGAATTTCTTGAGCCTTG; Myeloperoxidase (MPO) – F: GACATGCCCACCGAAT-GACAA; R: CAGGCAACCAGCGTACAAAG; Inhibitor of nuclear factor kappa-B kinase subunit beta (IKK2) – F: AAGTACACCGTGACCGTT-GAC, R – GCTGCCAGTTAGGGAGGAA; Nuclear respiratory factor 1 (NRF1) – F: AGCACG-GAGTGACCCAAAC, R: AGGATGTCCGTCAT-CATAAGA; NAD(P)H quinone dehydrogenase – 1 (NQO1) – F:TGAAGAAGAGAGGATGGGAGG, R: GATGACTCGGAAGGATACTGAAA. Actin beta (β-actin) – F: CAAGAAGGAAGGCTGGAAAAG, R: ACGGCCAGGTGATCACTATTG) served as an endogenous control.

5. Statistical Processing and Graphical Presentation of the Results

The values obtained were represented as the average of a minimum of three measurements ± SEM. The data were statistically treated with one-way ANOVA, at a confidence level $p<0.05$ and compared with Student's *t*-test. Data processing was performed using the statistical software product Graph Pad Prism (Ver. 5.0 Graph Pad Software, Inc.).

RESULTS AND DISCUSSION

In this study we aimed to establish well-recognized gene expression changes in selected genes in order to use them to verify the model of LPS-induced cellular response in J744A.1 macrophages. This model can be used to study possible protective effects of variety of pure compounds and/or plant extracts for the needs of functional foods and drug development.

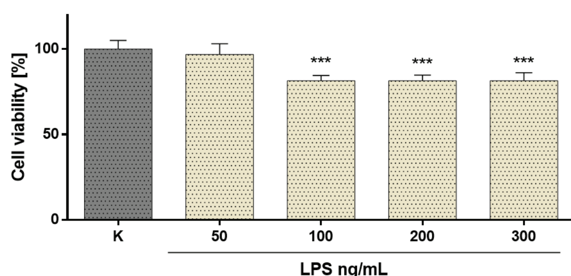

*Fig. 1. Cell viability of J744A.1 macrophages treated with LPS in a concentration range from 50 ng/mL to 300 ng/mL. Cells were treated for 20h before cell viability was measured. Cell viability is presented as [%] from the non-treated control, where it is considered 100%. *p<0.001 vs. non-treated control*

In order to determine most appropriate LPS treatment concentration, an MTT test was performed. LPS was applied in different concentrations (50 - 300 ng/mL) and their effect on the cell viability of J744A.1 macrophages was measured. The aim of this test was to select a concentration, contributing to significant cell death needed for subsequent experiments. At the same time, extremely cytotoxic concentrations should be avoided, as the cytotoxic effect may interfere with gene expression profiles that are likely to be related to cell death instead of cell defense.

Analysis of the results of the MTT test showed a statistically significant ($p <0.001$) and equal effect of the three LPS concentrations (100, 200 and 300 ng/mL) applied (Fig. 1). Cell viability was decreased with 20%. The lowest applied concentration (50 ng/L) had a nonsignificant effect.

Considering the MTT test results, we selected a concentration of 100 ng/mL LPS for the needs of the next experiment of gene expression measurement.

LPS treatment is known to trigger cellular response as revealed by the triggering of variety of cell response mechanisms. In this study we decided to study the effect of 100 ng/mL LPS treatment on the following genes: antioxidant defense-related (GCLc, GPx1, GSS, GR and SOD2); inflammation-related (IL1β, IL6, MCP1, TNFα, IL1RN, NOS2, CRP, COX2); phagocytosis-related (NOX1, MPO), and LPS/TLR4 signaling cascade-related (TLR4, IKK2, NRF1, NQO1).

All of the studied genes were significantly induced in J744A.1 macrophages upon LPS treatment. Among antioxidant-related genes changes in GCLc were most prominent (3.32-fold change, $p< 0.05$), followed by GPx1 (1.84-fold change, $p<0.01$), SOD2 (1.75, $p<0.01$), GR (1.75-fold change, $p<0.01$) and GSS (1.72-fold change, $p<0.01$) and (fig. 2a).

Elevated transcriptional levels of the antioxidant enzymes GCL and GSS in LPS-stimulated macrophages have been reported by other authors (2, 7). All of selected genes (GCLc, GPx1, GSS and GR) are related to glutathione (GSH) metabolism. GSH

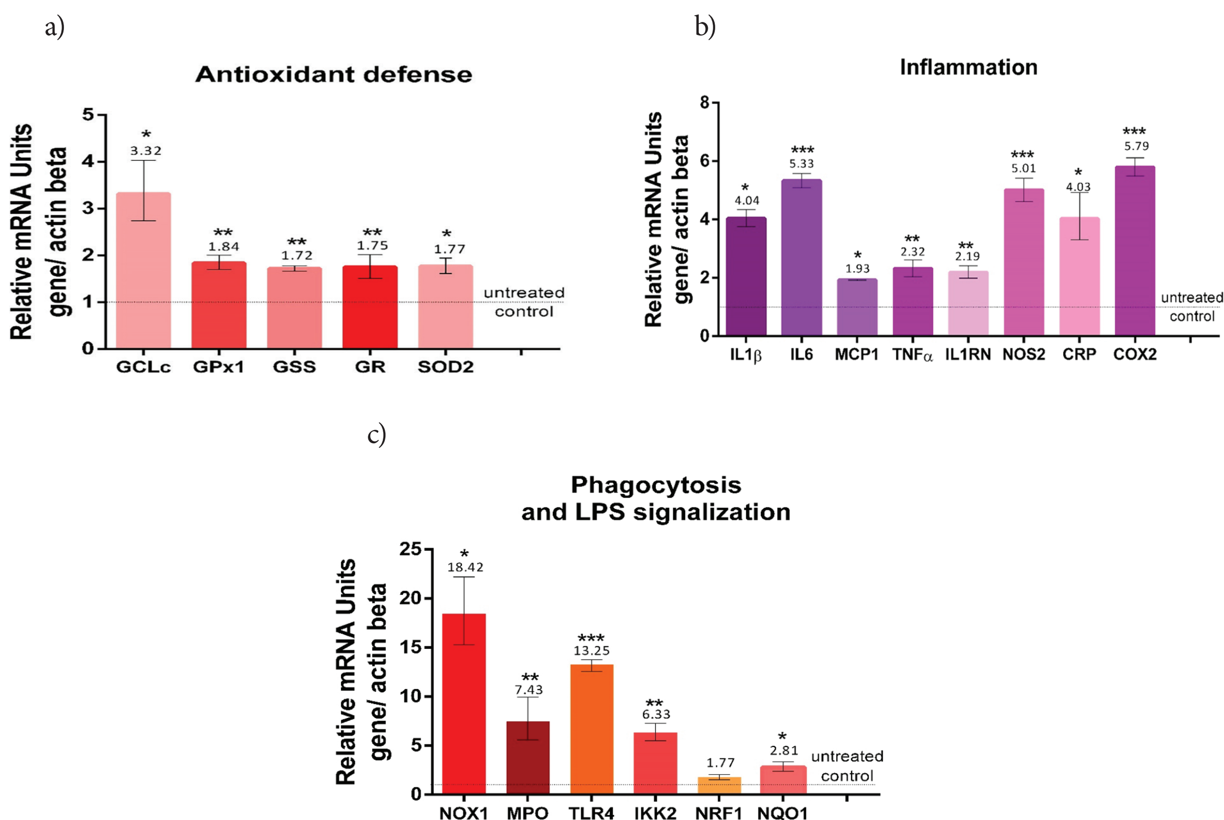

*Fig. 2. Fold change in gene expression levels upon treatment with 100 ng/mL LPS for 24h. a) antioxidant-related genes; b) inflammation-related genes; c) phagocytosis- and LPS/TLR signaling cascade-related genes. Gene expression levels are presented as relative units ±SEM as compared to the non-treated control, where it is considered to be equal to 1. *p significant vs. non-treated control*

is a tripeptide, essential for cellular antioxidant defense and both GCLc and GSS are involved in gamma-glutamyl cycle, where GSH is *de novo* synthesized. GPx1 and GR code for antioxidant enzymes, responsible for peroxide elimination with the participation of GSH as a cofactor and GSH recovery, respectively in the so called "glutathione cycle". GSH is an important variable that determines susceptibility to LPS-induced damage suppressing LPS-induced inflammatory response and reducing cell mortality (2,8). GCLc codes for the catalytic subunit of the rate limiting enzyme in *de novo* GSH synthesis. The most prominent GCLc up-regulation reveals that probably it is an important key point in GSH levels regulation during LPS-induced stress (9). As antioxidant enzyme, SOD2 is responsible for intracellular neutralization of superoxide anion radical, thus contributing to hydrogen peroxide production as an immediate product further neutralized by GPx1. Among inflammation related genes changes in COX2 (5.79-fold change, $p<0.001$); NOS2 (5.01-fold change, $p<0.001$), IL6 (5.33-fold change, $p<0.001$), IL1β (4.04-fold change, $p<0.05$) and CRP (4.03-fold change, $p<0.05$) were most prominent (fig. 2b). Up-regulation of TNFα (2.32-fold change, $p<0.01$), IL1RN (2.19-fold change, $p<0.01$) and MCP 1 (1.93-fold change, $p<0.05$) was lower but still significant as compared to a non-treated control (fig. 2b).. Inflammatory reactions have been associated with increased production of NO and inflammatory cytokines such as IL6 and TNFα (10). IL6 is an important mediator of fever and is a major cytokine in the acute phase of inflammation that initiates the innate immune system in response to infection (11), but also plays an important role in chronic inflammation (12). LPS treatment induces gene expression by increasing both cytosolic protein levels of cytokines (IL1β and IL6) and proinflammatory enzymes (NOS2) by activating NF-κB transcription factor (2,7,8). We detected significant cellular response at the following levels of

the inflammatory process: prostaglandin synthesis (COX2); NO synthesis (NOS2); inflammatory chemokine and cytokines synthesis (MCP1, TNFα, IL6 and IL1β), as well as acute-phase protein production (CRP). Upon proinflammatory activation, phagocytic cells, such as macrophages and monocytes, produce large amounts of reactive oxygen species (ROS), mainly in the form of a superoxide anion and subsequent radical formation in the process of „respiratory burst" (13). The process of high oxygen consumption and superoxide anion production that accompanies a respiratory burst is controlled by the NOX enzyme (13,14). Actually, namely NOX1 gene was most considerably elevated in this study (18.42-fold change, $p<0.05$). LPS triggered a 7.43-fold change ($p<0.01$) in MPO expression is also a strong indication for a response on the level of phagocytosis stimulation. MPO is a downstream enzyme in the reactions of "respiratory burst" where hypochlorous acid (a kind of reactive oxygen species) is formed.

The members of LPS-triggered signaling cascade - TLR4 and IKK2 (15,16) were significantly upregulated upon 24h 100 ng/mL LPS treatment. Apparently, TLR4 receptor and down-stream IKK2 protein were most sensitive among the other signalization related genes to this stimulation representing 13.25- ($p<0.001$) and 6.33-fold ($p<0.01$) change in their mRNA levels (fig. 2c).

CONCLUSION

LPS treatment of J774A.1 macrophages for 24h is sufficient to provoke pronounced cellular response as manifested with significant change in gene expression. All of studied genes may serve as suitable indicative markers for triggered cellular response in conditions of LPS stimulation of J774A.1 cells as they were significantly up-regulated in this model. However, the following genes appear to me most affected by 24h LPS treatment: GCLc, COX2, NOS2, IL6, IL1β, CRP, NOX, TLR4 and IKK2.

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