CHANGES IN INTERLEUKIN 6 AND MONOCYTE CHEMOATTRACTANT PROTEIN-1 EXPRESSION LEVELS IN 3T3-L1 CELLS AFTER EXPOSURE TO AGRIMONIA EUPATORIA L. EXTRACT AND SUBSEQUENT OXIDATIVE STIMULATION WITH TERT-BUTYL HYDROPEROXIDE REVEAL POSSIBLE ADAPTOGENIC POTENTIAL

Yoana Kiselova¹, Deyana Vankova¹, Oskan Tasinov¹, Inna Feklicheva², Irina Potoroko², Diana Ivanova¹

¹Department of Biochemistry, Molecular Medicine and Nutrigenomics, Faculty of Pharmacy, Medical University of Varna, Bulgaria
²School of Medical Biological Sciences, South Ural State University, Chelyabinsk, Russian Federation

ABSTRACT

INTRODUCTION: Synthesis and secretion of many inflammatory factors increase with oxidative stimulation and the activity of many transcription factors that regulate synthesis of inflammatory cytokines is influenced by the redox condition in the cell.

AIM: The aim of the current study was to investigate the effects of aqueous-alcoholic agrimony extract on interleukin 6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) gene expression levels in cultured preadipocytes under induced oxidative stress conditions.

MATERIALS AND METHODS: To determine the possible adaptogenic potential of agrimony extract treatment on gene expression, preadipocytes were incubated in a medium containing different amounts of agrimony extract. Tert-butyl hydroperoxide was used to provoke oxidative response in treated cells. Expression of selected genes was measured using two step quantitative PCR. Results were analyzed using the 2⁻ΔΔCt method.

RESULTS: Incubation of preadipocytes with 2.5% agrimony extract resulted in a significant decrease in mRNA levels of MCP-1. Significant increase in IL-6 transcription levels was detected in the cells incubated with 1.25% and 2.5% agrimony extract. Pre-incubation of the cells with agrimony extract prevented subsequent oxidative induced stimulation of MCP-1 gene expression. Oxidative provocation appeared to decrease the stimulatory activity of agrimony on IL-6 gene expression.

CONCLUSION: Agrimony extract appeared to have adaptogenic potential in 3T3-L1 preadipocytes, as revealed by inhibition of MCP-1 transcription in non-stimulated cells and prevents its activation by oxidative stimulation. Agrimony treatment results in elevated IL-6 expression and the effect is less prominent when followed by oxidative treatment.

Keywords: Agrimonia eupatoria, gene expression, MCP-1, IL-6, 3T3-L1, adaptogenic
INTRODUCTION

As a result of the increasing obesity and related metabolic disorders all over the world, many studies are focused on adipose tissue. Although there are differences between adipogenesis in mice and humans (1), a lot of studies are performed on a murine preadipocyte cell line 3T3-L1 as a useful model system. It is known that obesity is accompanied by macrophage adipose tissue infiltration (2), and macrophage-secreted inflammatory cytokines, such as interleukin 6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), affect the metabolism of adipocytes. Hence, it is important to study the behavior of this type of cells under the influence of various stimuli. A cause of systemic chronic inflammation in obesity, for example, can be oxidative stress (3). It is known that the synthesis and secretion of many inflammatory factors increases with oxidative stimulation and the activity of many transcription factors such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), ARE/EPR (Antioxidant Response Element/Electrophile Response Element) that regulate synthesis of inflammatory cytokines, such as MCP-1, IL-6, tumor necrosis factor α (TNF-α), C-reactive protein (CRP), etc., is influenced by the redox condition in the cell. Reactive oxygen species (ROS) may affect gene expression either by direct interaction and modification of the activity of various enzymes and signal molecules, or by altering the redox state in the cell.

IL-6 is considered to be a stress-induced cytokine with varying effects on different tissues. Increased IL-6 levels highly correlate with body mass, hence IL-6 is regarded as a hallmark of chronic inflammation accompanying obesity, preceding metabolic diseases, such as insulin resistance, type 2 diabetes mellitus, atherosclerosis, and cardiovascular disease (4,5). MCP-1 expression could be upregulated following different pro-inflammatory stimuli (6,7). The overproduction of MCP-1 is linked to a decrease in PPARγ expression, thus leading to enhanced insulin resistance of adipocytes (8,9).

Agrimonia eupatoria L. (agrimony) is a medicinal plant rich in tannins, flavonoids and phenolic acids and it is widely used by folk medicine in many countries. High correlation between polyphenolic content and total antioxidant activity has been demonstrated in aqueous-alcoholic agrimony extracts (10,11). It is considered that chronic intake of certain herbal products may affect human capacity to adapt to inflammatory stimuli from the environment, boosting this way the immune system to resist to infections or other unfavorable stress conditions. Possibly these plant effects are mediated by the effects that plant products have on gene expression levels, including the expression of different stress-induced cytokines and leading to the adaptation of the human organism to changes in the environment. In this respect, it could be assumed that different plant products have adaptogenic potential with regard to human metabolism adaptation. Few studies only (12) discuss plant product effects from this point of view. Studies of the adaptogenic potential of medicinal plants in cell cultures are almost absent, as well as those examining the molecular mechanisms of this adaptation.

AIM

The aim of the current study was to investigate the possible adaptogenic potential of aqueous-alcoholic agrimony extract on the IL-6 and MCP-1 gene expression levels in cultured preadipocytes under induced oxidative stress conditions.

MATERIALS AND METHODS

Plant Material and Extraction Procedure

Powdered dry plant material (150 mg) was extracted by three minutes of continuous vortexing at room temperature in 40% (v/v) ethanol/phosphate-buffered saline (PBS). The combined supernatants from three subsequent extractions were diluted to 15 ml with PBS. For cell treatment, further dilutions of the extract were prepared as follows: 25 μl and 50 μl of extract were dissolved to a final volume of 2 ml in phenol red free DMEM to a final content of the extract in the treatment medium of 1.25% and 2.5%, respectively. According to previous data on the same type of agrimony extract (13), we calculated that treatment media contained approximate polyphenols in final concentrations of 26.54 μM and 53.08 μM, respectively. Control ethanol solutions were prepared as follows: 25 μl and 50 μl 40% (v/v) of ethanol/PBS solution were dissolved to a final volume of 2 ml in phenol red free DMEM to a final content in the treatment medium of 1.25% and 2.5%, respectively.
Cell Culture

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC). Preadipocytes were cultured in 75 cm² flasks at 37°C in a humidified chamber with 5% CO₂ atmosphere. Nutrient medium comprised phenol red-containing Dulbecco’s modified Eagle’s medium (DMEM, Lonza) with 4.5 g/L glucose, L-glutamine and supplemented with fetal bovine serum (FBS, Sigma-Aldrich) to final concentration 10% and penicillin/streptomycin mixture to final concentration 100U/ml of each.

Experimental Procedure

Cells were collected and seeded in six well flasks at density 2x10⁵ cells/well. To determine the effect of agrimony extract (AE) treatment on gene expression in standard conditions, 3T3-L1 preadipocytes were incubated for 24 h in a medium containing different amounts of AE. Non-treated cells, incubated for the same time period in the nutrient medium, were used as a control group. In another experiment 50-100 μM tert-butyl hydroperoxide (t-ButOOH) was used to provoke oxidative response in treated for 24 hours cells to study the expression of the genes under investigation. Non-treated cells, incubated for the same time period in the nutrient medium were used as a control group. To study the possible adaptogenic potential of the herb in condition of oxidative stimulation, cells were incubated with AE for 24 h, then nutrient medium was removed and the cells were incubated for another 24 h in a medium containing t-ButOOH to a final concentration of 100μM. As a control group for gene expression analysis, cells incubated with only t-ButOOH were used.

Gene Expression Analysis

RNA extraction, copy DNA synthesis and quantitative Real-Time PCR gene expression analyses were performed as previously described (14). Primer sets were as follows: actin beta - forward AC-GGGAGGCTCATGACTATGG, probe FAM-AC-GAGCCGGTCCGATCCCTG-TAMRA, reverse CAAGAGGAGGTGACAAAG; MCP-1 – forward GGCTCAGCCAGATGCATTAA, probe FAM-CCCAGCTACCTGCTGCTACTCATAAATC TAMRA, reverse CCTACTCATGAGGGATCATCTTGCT; IL-6 forward GAGTTGGCATACTGGAATCTG, reverse GCAATGCATCATCAGTGTTCAT. Actin beta was used as an endoge-

ous control. MCP-1 gene expression was analyzed with Maxima Probe/ROX qPCR Master Mix (Fermentas) and IL-6 gene expression - with GreenMasterMix (Genaxxon). Gene expression levels are presented in relative units and were calculated according to the 2⁻ΔΔCt method (15).

Statistical Analysis

Statistical analyses were performed using Microsoft Excel Office 2013 software. Variation analyses for evaluation of quantitative variables were applied. Differences between the means of the two groups were analyzed by unpaired two-tailed Student’s t-tests. P value<0.05 was considered as statistically significant.

RESULTS

MCP-1 and IL-6 mRNA levels in the cells incubated with 50 μM t-ButOOH were not affected, whereas 75 μM (p<0.05) and 100 μM (p<0.01) of t-ButOOH significantly increased MCP-1 mRNA levels by 27% and 154%, respectively, as compared to untreated control cells (Fig. 1). While IL-6 mRNA levels were not affected significantly in cells incubated with 50 μM and 75 μM t-ButOOH, incubation with 100 μM t-ButOOH resulted in 100% (p<0.01) stimulation of the IL-6 gene expression vs. the untreated control cells (Fig. 1).

Incubation of 3T3-L1 cells with 2.5% agrimony extract resulted in a significant reduction in mRNA

Fig. 1. Expression levels of MCP-1 and IL-6 in 3T3-L1 preadipocytes treated with 50 μM, 75 μM and 100 μM final concentration of t-ButOOH in culture medium. *p < 0.05, **p < 0.01 vs. K (0 μM B); Legend: K - untreated cells (0 μM B), B - tert-Butyl hydroperoxide, t-ButOOH. Gene expression levels were normalized to non-treated K group, where gene expression levels were considered to be equal to 1.
levels of MCP-1 by 72% (p<0.01) (figure 2). Significant increase in IL-6 transcription levels by 517% and 4300% were detected in the cells incubated with 1.25% and 2.5% AE, respectively (Fig. 2). As Agrimonia eupatoria extract was obtained with 40% ethanol solution, we analyzed the effect of ethanol (E) separately. Ethanol as a second control, in contrast to the effect of the herbal extract, increased significantly MCP-1 transcription levels by 220% – 277% in cells incubated with AE 1.25% and AE 2.5% (Fig. 2). Similar effect was observed for IL-6 mRNA levels increased by 77% (p<0.01) by ethanol in cells treated with E 1.25% and E 2.5% (Fig. 2).

In order to assess the possible adaptogenic potential of AE in conditions of oxidative stimulation, we performed 24-hour pre-treatment of cells with AE, followed by oxidative stimulation with 100 μM t-ButOOH for another 24 h. Pre-incubation of the cells with agrimony extract prevented subsequent t-ButOOH-induced stimulation of MCP-1 gene expression (Fig. 3). Transcription levels of MCP-1 were reduced by AE pre-treatment by 1.25% and 2.5%, extracts by 30% (p<0.05) and 68% (p<0.01), respectively. Interestingly, oxidative provocation appeared to decrease the stimulatory activity of AE on IL-6 gene expression. In 1.25% and 2.5% AE of the pre-treated cells the levels of this inhibition were 63.7% and 85.3%, respectively (Fig. 3).

**DISCUSSION**

Nowadays it is known that transcriptional control of some inflammatory factors is redox sensitive (16). In the promoters of certain genes there are regulatory regions for binding the transcription factors, such as NF-kB and AP-1, and their activation is associated with ROS-dependent induction of proinflammatory factors (17). As mentioned above, application of the oxidative agent t-ButOOH showed stimulatory effects on proinflammatory MCP-1 and IL-6 gene expression in 3T3-L1 preadipocytes. Similar results for induction by an oxidant were reported in other studies where expression and secretion of IL-6 increased in 3T3-L1 adipocytes treated with H$_2$O$_2$ (18). The observation could be explained by the assumption that some oxidative stimuli can induce the NF-kB transcription factor activation and this is supported by the findings that oxidative stress is accompanying inflammatory response.
In our study a significant inhibitory effect of AE on MCP-1 transcription was found both in non-stimulated and oxidatively treated cells. The inhibitory effect on MCP-1 expression has been described for a number of different plant extracts (19). Similar effect was shown to be a result of the activation of NF-κB mediated signaling pathway (21,21). Quercetin, the most common polyphenol, for example, was found to inhibit activation of promoters containing AP-1 and NF-κB-binding consensus sequences (22). Inhibition of NF-κB by quercetin is likely to be due to antioxidant properties as well as to the ability of quercetin to inhibit certain protein kinases (23). Other authors, such as Lampronti and co-authors (24,25) established that some of the extracts can inhibit the interaction between transcription factors and DNA directly. Inhibition of the ability of the oxidizing agent to stimulate inflammatory response as indicated by elevated levels of MCP-1 mRNA by AE reveals a possible adaptogenic potential of the herb. Pre-treatment with AE provides phytochemicals to the cells, which have the ability to influence cellular metabolism in a way that the cells become less susceptible to oxidative environment, associated with inflammatory response. As a result, less prominent inflammatory response, as indicated by less affected MCP-1 gene expression was detected in this experiment. Pre-treatment of 3T3-L1 preadipocytes with AE reduced the inducing effect of the oxidative stimulus on MCP-1 expression. Similar results are reported by other authors (26). Possible explanation for this effect of AE is the manifestation of antioxidant properties of plant compounds, directly involved in reaction with the oxidizing agent, thus decreasing its stimulatory effect on NF-κB mediated transcription of MCP-1.

Administration of AE on 3T3-L1 cells, contrary to our expectations, significantly stimulated IL-6 expression and the effect was concentration dependent. In the literature, little data about the inhibitory effect of various plant extracts on IL-6 expression could be found and these studies are usually aimed at demonstration of the anti-inflammatory activity of plants (27). Both MCP-1 and IL-6 are regulated by NF-κB transcription factor. Since the mechanism of NF-κB mediated regulation is quite complex and probably depends on the cooperative action of more than one transcription factor (28), it is difficult to clari-

CONCLUSIONS

Agrimony extract appears to have adaptogenic potential in 3T3-L1 preadipocyte cell culture, as revealed by its effect on MCP-1 gene expression – inhibition of transcription in non-stimulated cells and subsequent prevention of activation by oxidative stimulation with t-ButOOH. Agrimony extract treatment results in elevated IL-6 expression and the effect is less prominent when followed by oxidative treatment.

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