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Uric Acid Effects on Glutathione Metabolism Estimated by Induction of Glutamate-Cysteine Ligase, Glutathione Reductase, and Glutathione Synthetase in Mouse J744A.1 Macrophage Cell Line

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Abstract

Introduction: Elevated plasma levels of uric acid (UA) are considered an independent risk factor for hypertension, diabetes, cardiovascular disease, endothelial and vascular damage, obesity, and metabolic syndrome. Even physiological concentrations of soluble UA have been proved to induce gene expression of macrophage-secreted inflammatory cytokines and stimulate production of reactive oxygen species in mature adipocytes. UA is also described as a powerful endogenous plasma antioxidant, which reveals a paradox of duality for this parameter.

Aim: The aim of this study was to investigate the effect of UA on expression of antioxidant defense related enzymes in cultured J744A.1 macrophage cell line.

Materials and methods: Mouse macrophage J744A.1 cells were treated with uric acid at increasing concentrations of 200 to 800 μM. Changes in expression levels of genes related to the metabolism of glutathione – glutamate-cysteine ligase, catalytic subunit (GCLc), glutathione peroxidase 1 (GPx1), glutathione reductase (GR) and glutathione synthetase (GS) were analyzed. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Results: When UA is applied in concentrations of 200 μM and 400 μM, cell viability did not change significantly. Higher, pathophysiological concentrations of 600 μM, 800 μM UA, and 1000 μM of UA caused significant decrease in cell viability to 95.81% ($p<0.01$), 76.22% ($p<0.001$), and 18.01% ($p<0.001$), respectively. UA treatment in concentrations of 200 μM, 400 μM, 500 μM, and 800 μM induced significant transcription levels of glutathione reductase – 8.14 ($p<0.05$), 7.15 ($p<0.01$), 22.07 ($p<0.001$), and 27.77 ($p<0.01$), respectively, and of glutathione synthetase – 13.71 ($p<0.01$), 13.05 ($p<0.05$), 18 ($p<0.01$), and 48.60 ($p<0.01$) folds, respectively. GCLc and GPx1 genes were transcriptionally activated by higher (500 μM and 800 μM) concentrations of UA. For these UA concentrations the measured levels of mRNA were 7.51 ($p<0.05$) and 12 fold ($p<0.05$) higher than the non-treated control for GCLc and 1.90 ($p<0.05$) and 1.93 ($p<0.01$) for GPx1. Significant difference in the GCLc expression was found between the 200 μM and 500 μM ($p<0.05$) and 800 μM ($p<0.01$) treated cells. mRNA levels were significantly different between 400 μM and 800 μM ($p<0.05$) for both GCLc and GR genes. Very strong correlation was found between GCLc and GR (0.974, $p=0.005$) and GS (0.935, $p=0.020$) expression and between GS and GR (0.886, $p=0.045$) expression levels.

Conclusions: It appears that 500 μM and pathophysiological concentrations (800 μM) of UA induce antioxidant cell response in J744A.1 macrophages proved by the indicative elevation GCL, GPx1, GR, and GS transcription. GR and GS can be stimulated even by lower concentrations (200 μM and 400 μM) indicating that glutathione metabolism in macrophages is tightly regulated in order to keep adequate GSH levels.

PENSOFT.

INTRODUCTION

Uric acid (UA) is a small, organic, heterocyclic compound which is the final catabolite of purines derived from RNA and DNA.[1] In general, it is considered that serum UA values between 3.5 and 7.2 mg/dL (0.21–0.43 mmol/L) in adult males and postmenopausal women and between 2.6 and 6.0 mg/dL (0.16-0.36 mmol/L) in premenopausal women are normal in many countries.[2] However, the normal UA levels could nonsignificantly vary depending on the type analyzer and kits used in a given laboratory. There are studies connecting UA levels with oxidative stress[3] and inflammation[4]. Many studies have demonstrated that elevated UA level in humans is an independent risk factor for hypertension[5], diabetes[6], cardiovascular disease[7], endothelial dysfunction[8], coronary artery calcification[9], vascular damage[10], obesity and metabolic syndrome.[11,12] Some reports reveal that physiological concentrations of uric acid could induce gene expression of macrophage-secreted inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), and growth factors, such as platelet-derived growth factor.[13-15] Some experimental studies report that UA stimulates the synthesis of proinflammatory cytokines such as IL-1 beta, IL-6 and TNF-alpha.[16]

Recent studies have indicated that UA acts as an endogenous danger signal and at the same time triggers NOD-like receptor protein 3-dependent inflammation. These effects have been found to have important implications for systemic inflammatory responses.[17] Also, obesity is accompanied by macrophage adipose tissue infiltration, and macrophage-secreted inflammatory cytokines, such as MCP-1, affect the metabolism of adipocytes.[18]

On the other hand, UA is described as a powerful endogenous plasma antioxidant which reveals a paradox of duality for this parameter. Uric acid cannot neutralize free radicals such as hydrogen peroxide, hydroxyl radicals, O_2^-, and peroxynitrite and exhibits fewer antioxidant properties against radicals such as CH_3 (methyl radical) and tert-butyl hydroxyperoxide.[19] It plays an antioxidant role only in a hydrophilic environment, hence its antioxidant activity is established to be predominantly in the blood plasma. Intracellularly, mainly in adipose tissue, its antioxidant action is limited, thus uric acid could interact with other radicals, and this could initiate a cascade of reactions to generate superoxide forms. Further, UA or subsequently formed radicals can activate NADPH oxidase-dependent signaling pathways, causing oxidative stress and further activation of inflammatory processes in adipose tissue.[20]

There is still a controversy whether high UA is a compensatory mechanism to overcome increased oxidative stress, which is an independent cause of cardiovascular disease[21] and is a component of the inflammatory process.

Glutathione (GSH) is highly abundant in all cell compartments and is the major soluble antioxidant. Glutamate cysteine ligase (GCL) is the first and rate-limiting enzyme in glutathione biosynthesis and its activity is of high importance for maintaining glutathione levels in the cells.[22] The second step in GSH synthesis is catalyzed by GSH synthetase (GS). The enzyme glutathione reductase (GR) is responsible for GSH regeneration – reduction of glutathione disulfide to the sulfhydryl form of glutathione. As an antioxidant enzyme, glutathione peroxidase (GPx) is involved in the reduction of organic hydroperoxides.

AIM

Our aim was to investigate the effect of UA on the metabolism of endogenous antioxidant glutathione by estimating UA induced changes in the expression of glutamate-cysteine ligase, glutathione peroxidase, glutathione reductase, and glutathione synthetase in J744A.1 mouse macrophage cell line.

MATERIALS AND METHODS

Cell line and subcultivation

J744A.1 mouse macrophage cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in 75 cm² flasks at 37°C in a humidified chamber with 5% CO_2 atmosphere. The complete nutrient medium was comprised of 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 a 10% final concentration and penicillin/streptomycin mixture to final concentrations of 100 U/mL for each of them.

Experimental procedure

J744A.1 mouse macrophage cells were collected and seeded in six well flasks at the density of 2.5×10^5 cells/well. The treatment solutions were prepared using phenol red free DMEM medium containing 4.5 g/L glucose without any supplementations. The uric acid was dissolved in the cell-growing medium without any supplements to reach the following concentrations: 200 μM, 400 μM, 500 μM,

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800 μM, and 1000 μM. All concentrations were applied in triplicate.

The viability of the treated cells was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (commonly abbreviated as MTT assay).[23,24] The assay is based on the ability of viable cells to reduce the yellow MTT to purple insoluble formazan. At each well 100 μL of MTT solution in phosphate buffer saline (pH=7.4) at concentration of 2 mg×mL^{-1} was added 20 hours after the start of UA treatment. After a 4-hour incubation, the medium was removed and 1 mL dimethyl sulfoxide was added to each well for cell lysis. After thorough mixing, 100 μL were transferred to 96 wells plates and the absorbance was measured at 550 nm wavelength using Synergy 2 plate reader (BioTek). The viability of the treated cells was presented as percentage of the viability of the non-treated cells, which was considered 100%. Results were presented as mean ±SD. All treatments were performed in triplicate.

Gene expression analysis

Total RNA isolation was performed with TRI reagent (Ambion) following the manufacturer's protocol. RNA (200 ng) was reversely transcribed with First Strand cDNA Synthesis Kit (Thermo Scientific) containing oligo (dT)18 primer and reverse transcriptase. cDNA synthesis was performed on Gene Amp PCR thermal cycler. Reaction conditions were done in final volumes of 10 μL according to the manufacturer's guidelines. cDNA was dissolved after synthesis by adding 30 μL of nuclease-free distilled water to each sample. Primers for the examined GCL, GPx, GR, GS genes for the quantitative real-time PCR (**Table 1**) were designed using real-time PCR Gene Expression Design Tool (http://eu.idtdna.com/Scitools/Applications/RealTimePCR/Default.aspx) and a commercially synthesized one (Alpha DNA, Canada). Beta-actin was used as endogenous control and primers were ordered from Sigma Aldrich. As a template for real-time PCR, 0.39 μL of cDNA was amplified in 5 μL final volume. Final primers' concentration was 300 nM.

Two-step real-time PCR analysis was performed to estimate gene expression levels using KAPA Sybr Fast qPCR

Table 1. Primers sequences

Actin beta	F 5'ACG GCC AGG TCA TCA CTA TTG 3'
	R 5'CAA GAA GGA AGG CTG GAA AAG 3'
GCLc	F 5'AATGGAGGCGATGTT 3'
	R 5' CAGAGGGTCGGATGG 3'
GPx1	F 5' CCCCACTGCGCTCATGA 3'
	R 5' GGCACACCGGAGACCAAA 3'
GR	F 5'CACGGCTATGCAACATTCGC 3'
	R 5'TGTGTGGAGCGGTAAACTTTT 3'
GS	F 5'CCCAAGTGGTCCAGTCTATC 3'
	R 5'TCACCAGTGTTGTTCCCTG 3'

Kit. The reaction parameters were the following: enzyme activation and denaturation at 95°C/3 min, amplification at 95°C/03 sec, annealing at 60°C/1 min, 45 cycles.

Gene expression levels were calculated using the 2$^{-\Delta\Delta Ct}$ method[25] and expressed as relative units (RU) compared to the untreated controls where the level of gene expression was considered to be equal to 1. Results were presented as mean ±SEM. Amplification products were examined for nonspecific amplification by including an additional denaturation step in the real-time thermal cycler protocol. All the measurements were performed in triplicate.

Changes in gene expression levels of glutamate-cysteine ligase (GCL), glutathione peroxidase (GPx1), glutathione reductase (GR) and glutathione synthetase (GS) genes were analyzed by real-time qPCR on ABI PRISM 7500 (Applied Biosystems).

Statistical analysis

Differences among all UA treated groups were analyzed with one-way ANOVA, Tukey's multiple comparisons test on GraphPad Prism 6 software. Pearson correlation analysis using GraphPad Prism 6 software was applied to evaluate the causal links between the tested parameters. *P* values less than 0.05 were considered significant.

RESULTS

Results obtained from measuring cell viability are summarized in **Fig. 1** as mean values and show the general decline of the cell viability of J744A.1 mouse macrophages after treatment with elevating concentrations of UA. When UA was applied at concentrations of 200 μM and 400 μM, cell viability did not change significantly. When cells were treated applying pathophysiological concentrations of UA of 600 μM, 800 μM, and 1000 μM, cell viability decreased significantly compared to an untreated control to 95.81% ($p<0.01$), 76.22% ($p<0.001$), and 18.01% ($p<0.001$), respectively.

Uric acid affected differently the expression of enzymes involved in the metabolism of glutathione (**Fig. 2**). After incubation with increasing concentrations of UA, the expression of GCL in the macrophage cell line elevated in a concentration-dependent manner. Application of UA in normal concentrations from 200 μM to 500 μM caused 2.11, 3.42, and 7.51 ($p<0.05$) fold change in GCLc expression. When applied in pathophysiological concentration of 800 μM, UA triggered 12-fold ($p<0.05$) elevation compared to the untreated controls. Expression levels in the 500 μM treated group differed significantly from the 200 μM treated group ($p<0.05$) and in the 800 μM treated group from 200 μM ($p<0.01$) and 400 μM ($p<0.05$). GPx1 mRNA levels were 1.44, 1.93, 1.90 ($p<0.05$) and 1.93 ($p<0.01$) fold higher in the 200 μM, 400 μM, 500 μM, and 800 μM treated groups, respectively. There was no significant difference between all the applied UA concentrations on GPx1 expression.

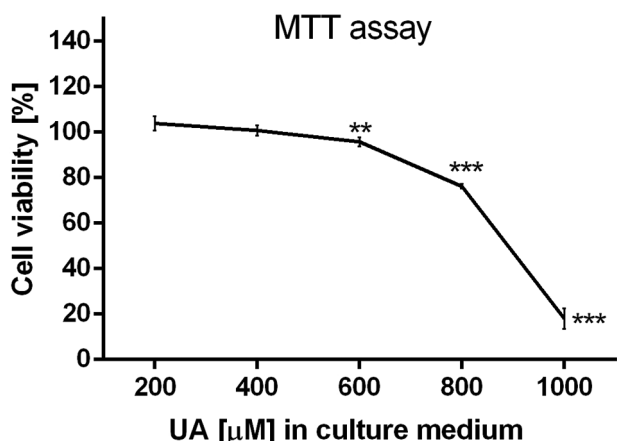Glutathione reductase expression levels were increased

Figure 1. Cell viability of J744A.1 mouse macrophages treated with increasing concentrations of uric acid in culture medium. Cell viability is presented as [%] of viability of non-treated cells, which is considered to be equal to 100%. ** $p<0.01$ – p value vs. control, ***$p<0.001$ – p value vs. control. Results are presented as mean±SD.

8.14 ($p<0.05$) and 7.15 ($p<0.01$) folds in 200 μM and 400 μM groups. Treatment with UA in 500 μM and in pathophysiological concentration of 800 μM triggered 22.07 ($p<0.001$) and 27.77 ($p<0.01$) fold change in the gene expression, respectively.

There was a significant difference between the samples treated with 400 μM and 800 μM of UA ($p<0.05$).

Application of 200 μM and 400 μM of UA increased significantly the expression levels of GS 13.71 ($p<0.01$) and 13.05 ($p<0.05$) folds, respectively. Treatment with the higher concentrations of 500 μM and 800 μM resulted in 18 fold ($p<0.01$) and 48.60 ($p<0.01$) elevation of GS mRNA levels. No significant difference between the effects of the applied concentrations was established.

Very strong correlation was established between GCLc and GR (0.974, $p=0.005$) and GS (0.935, $p=0.020$) expression and between GS and GR (0.886, $p=0.045$) expression levels (**Table 2**).
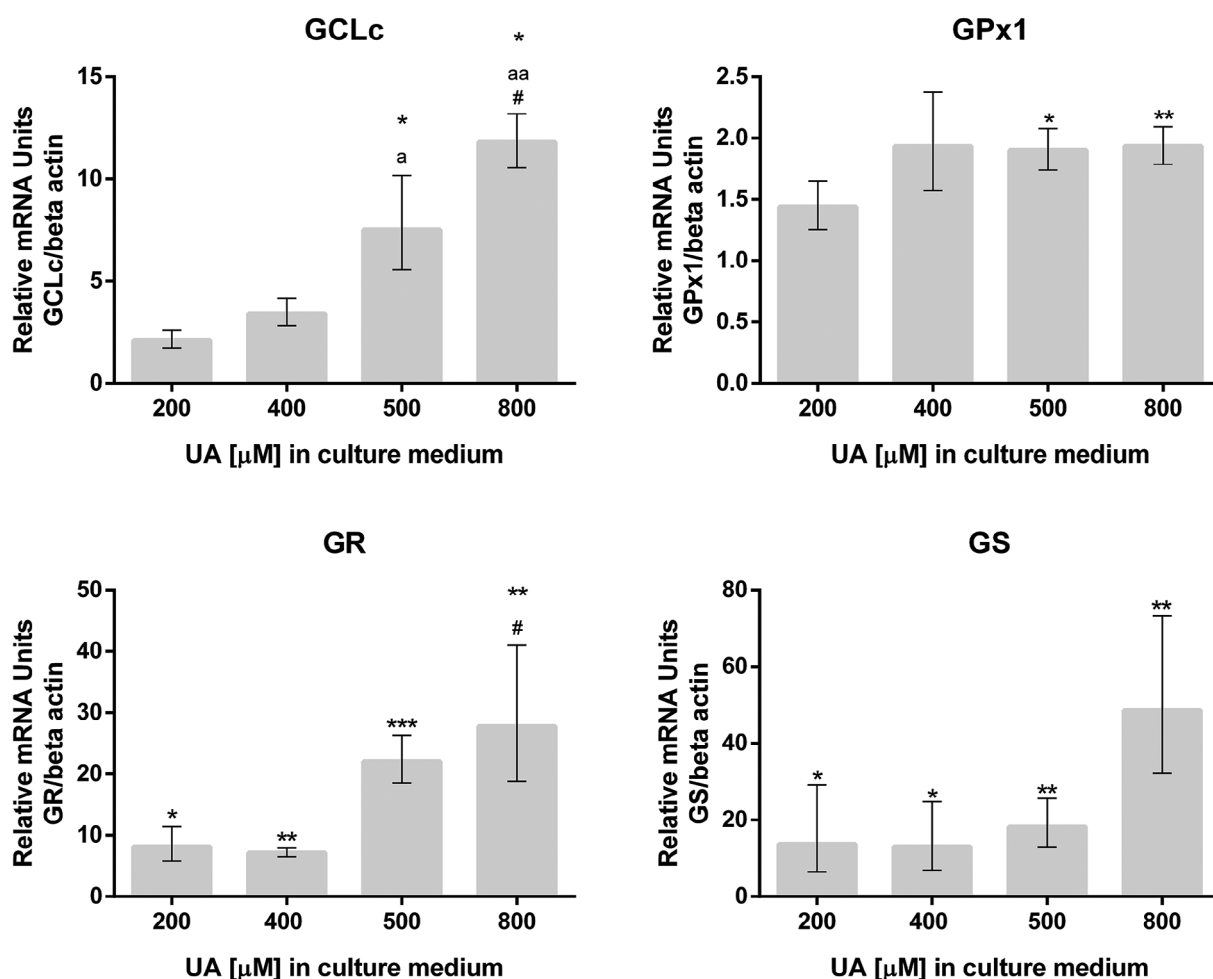

Figure 2. Relative mRNA units of studied genes. Gene expression is calculated with the delta-delta Ct method ($2^{-\Delta\Delta Ct}$) where the value of mRNA in the control, non-treated group is considered to be equal to 1. * $p<0.05$ – p value vs. control; ** $p<0.01$ – p value vs. control, ***$p<0.001$ – p value vs. control; **a** – $p<0.05$– p value vs. 200 μM; **aa** – $p<0.01$ – p value vs. 200 μM; # -$p<0.05$ – p value vs. 400 μM. Results are presented as mean±SEM.

Table 2. Correlation between relative mRNA levels of studied genes

Relative gene expression	GPx1	GR	GS
	r^2, *p*-value	r^2, *p*-value	r^2, *p*-value
GCLc	0.721, 0.169	0.974, 0.005	0.935, 0.020
GPx1		0.733, 0.158	0.654, 0.231
GR			0.886, 0.045

DISCUSSION

Cell cultures are a useful model for establishing molecular mechanisms underlying the cellular effects of biologically active compounds, such as metabolites, signaling molecules, drugs etc. In our study, a J744.A1 mouse macrophage cell line was used to illuminate some mechanisms of interrelationship between hyperuricemia and its possible effect on the expression of oxidative stress-related genes.

J744A.1 is a differentiated mouse macrophage cell line and cultured cells secrete inflammatory cytokines in the nutrition medium.[26] This process is also observed in vivo, e.g. it is known that obesity is accompanied by macrophage adipose tissue infiltration and macrophages secrete abundant inflammatory cytokines, which affect the metabolism of adipocytes. On the other hand, obesity is also characterized with increased UA levels.[11] Recent studies have demonstrated that elevated concentrations of UA could contribute to elevated production of inflammatory cytokines by macrophages. Some experimental studies have reported that UA stimulates the synthesis of proinflammatory cytokines such as IL-1 beta, IL-6, IL-8, and TNF-alpha.[16,22] Treatment of human umbilical vein endothelial cells (HUVECs) with UA triggered inflammatory response mediated by significant elevation of expression levels of IL-1β, IL-6 and ICAM.[27,28] A recent paper studied primary human monocytes which were differentiated into macrophages and subsequently exposed to different concentrations of uric acid. According to the study, UA had a direct proinflammatory effect on human macrophages manifested by increased macrophage phagocytic activity, the production of TNF-alpha and toll-like receptor 4 (TLR4), and downregulated the urate anion transporter 1 expression.[29] Uric acid is believed to contribute to the antioxidant potential in plasma but within the cell it is rather pro-oxidant.[20] Thus, serum UA levels were associated with the levels of derivative of reactive oxygen species (d-ROM).[30] In previous studies, it was demonstrated that UA significantly increased superoxide production in differentiated adipocytes.[4] UA effects on cellular redox balance also involve the glutathione system. Silva et al.[31] established significantly decreased GSH/GSSG ratio in vitro in human neutrophils upon UA treatment. As antioxidant molecule, glutathione participates in reactions of neutralization of reactive oxygen species (ROS). Maintenance of high levels of reduced glutathione (GSH) ensures adequate cellular response to impaired redox balance. Our experiments demonstrated increased mRNA levels for GCL and GPx1 genes after incubation of J744A.1 cells with 500 µM and 800 µM of UA. Expression levels of GS and GR were elevated in all treatment groups (200-800 µM UA) as compared to the non-treated group. These findings demonstrate that UA treatment provokes the antioxidant system in macrophages leading to increased expression of the enzymes involved in the so-called glutathione system.

Up-regulated GCL expression to ensure GSH elevation under conditions of inflammatory and oxidative stimulation seems reasonable, as GCL activity is considered especially important for maintaining GSH levels to provide relevant antioxidant defense. In our study, application of 500 µM and 800 µM of UA elevated significantly the GCL expression levels, thus providing a possible mechanism to ensure glutathione synthesis under conditions of oxidative stimulation by UA. GPx1 catalyzes the neutralization of hydrogen peroxide and organic peroxides and thus protects cells from oxidative stress. Established increase in GPx1 mRNA levels in our study is in accordance with the position of the enzyme on the first-line defense against oxidative stress. Its up-regulation is a part of adaptive mechanisms of the cells.

All the applied UA concentrations (200 µM to 800 µM), both normal and pathological, triggered significant increase in the GR and GS mRNA levels. This stimulation in the respective genes expression is possibly a part of the same mechanism to provide appropriate antioxidant defense by ensuring relevant GSH levels under conditions of oxidative stimulation by UA. Thus, we may assume that increased levels of all studied genes could be a result of the glutathione depletion triggered by UA application.

All of the studied enzymes work in an integrated way, which allows the cell to adapt to stressful conditions associated with impaired redox balance. The link between the up-regulation of glutathione-related genes and UA treatment may be redox and stress-sensitive transcription factors. For example, UA-induced cytokine expression is NF-κB-mediated.[32] Regulation of glutathione peroxidase and GCL is also NF-κB-dependent.[33]

In our study, we established a very strong positive correlation between mRNA levels of GCLc, GS, and GR on the one hand, and between GR and GS on the other. A possible reason could be that these three enzymes are related to maintenance of adequate reduced glutathione levels, whereas GPx1 is involved in its direct consumption. Maintenance of high GSH levels seems to be of high priority to the antioxidant defense system under UA treatment.

Limitations of the study

This study shows results from a cell culture experiment. Hyperuricemia is a condition characteristic for a multicellular organism and so the results may not obligatory

be related to real pathophysiological conditions. However, we still believe that this study reveals possible molecular mechanisms underlying UA mediated processes in macrophages that can be related to macrophage infiltration in adipose tissue in obesity. One of the limitations of our study is that we measured expression levels on transcriptional level only and the activity and quantity of the studied enzymes was not evaluated.

CONCLUSIONS

Treatment of J744A.1 macrophage cell line with 500 μM and pathophysiological concentrations (800 μM) of UA induces antioxidant cell response proved by the indicative elevation of GCL, GPx1, GR, and GS transcription. GR and GS can be stimulated even by lower concentrations (200 μM and 400 μM) indicating that glutathione metabolism in macrophages is tightly regulated in order to keep adequate GSH levels.

Acknowledgments

Competing Interests

The authors have declared that no competing interests exist.

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Влияние мочевой кислоты на метаболизм глутатиона, оценённое по индукции глутамат-цистеинлигазы, глутатионредуктазы и глутатионсинтетазы в мышиной линии макрофагов J744A.1

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Резюме

Введение: Повышенный уровень мочевой кислоты (МК) в плазме считается независимым фактором риска развития гипертензии, диабета, сердечно-сосудистых заболеваний, эндотелиального и сосудистого повреждения, ожирения и метаболического синдрома. Было доказано, что даже физиологические концентрации растворимой МК индуцируют экспрессию генов секретируемых макрофагами воспалительных цитокинов и стимулируют продукцию активных форм кислорода в зрелых адипоцитах. МК также описывается как мощный эндогенный антиоксидант плазмы, что обнаруживает парадокс двойственности этого параметра.

Цель: Целью данного исследования было изучение влияния МК на экспрессию ферментов, связанных с антиоксидантной защитой, в культивируемых линиях макрофагов J744A.1.

Материалы и методы: Клетки мышиных макрофагов J744A.1 обрабатывали мочевой кислотой в возрастающих концентрациях от 200 μM до 800 μM. Проанализированы изменения уровня экспрессии генов, связанных с метаболизмом глутатиона – глутамат-цистеинлигазы, каталитической субъединицы (GCLc), глутатионпероксидазы 1 (GPx1), глутатионредуктазы (GR) и глутатионсинтетазы (GS). Уровни экспрессии генов рассчитывали с использованием метода $2^{-\Delta\Delta Ct}$.

Результаты: При применении МК в концентрациях 200 μM и 400 μM жизнеспособность клеток существенно не менялась. Более высокие патофизиологические концентрации 600 μM, 800 μM УК и 1000 μM МК вызывали значительное снижение жизнеспособности клеток до 95.81% ($p<0.01$), 76.22% ($p<0.001$) и 18.01% ($p<0.001$) соответственно. Обработка МК в концентрациях 200 μM, 400 μM, 500 μM и 800 μM вызывала значительные уровни транскрипции глутатионредуктазы – 8.14 ($p<0.05$), 7.15 ($p<0.01$), 22.07 ($p<0.001$) и 27.77 ($p<0.01$) соответственно, а глутатионсинтетазы – 13.71 ($p<0.01$), 13.05 ($p<0.05$), 18 ($p<0.01$) и 48.60 ($p<0.01$) раз соответственно. Гены GCLc и GPx1 транскрипционно активировались более высокими (500 μM и 800 μM) концентрациями МК. Для этих концентраций МК измеренные уровни мРНК были в 7.51 ($p<0.05$) и в 12 раз ($p<0.05$) выше, чем в необработанном контроле для GCLc, и 1.90 ($p<0.05$) и 1.93 ($p<0.01$) для GPx1. Значительная разница в экспрессии GCLc была обнаружена между клетками, обработанными 200 μM и 500 μM ($p<0.05$) и 800 μM ($p<0.01$). Уровни мРНК значительно различались между 400 μM и 800 μM ($p<0.05$) как для генов GCLc, так и для генов GR. Очень сильная корреляция была обнаружена между экспрессией GCLc и GR (0.974, $p=0.005$) и GS (0.935, $p=0.020$), а также между уровнями экспрессии GS и GR (0.886, $p=0.045$).

Заключение: По-видимому, 500 μM и патофизиологические концентрации (800 μM) МК индуцируют антиоксидантный клеточный ответ в макрофагах J744A.1, что подтверждается характерным повышением транскрипции GCL, GPx1, GR и GS. GR и GS могут стимулироваться даже более низкими концентрациями (200 μM и 400 μM), что указывает на то, что метаболизм глутатиона в макрофагах жёстко регулируется для поддержания адекватных уровней GSH.

Ключевые слова

глутамат-цистеинлигаза, глутатионпероксидаза, глутатионредуктаза, глутатионсинтетаза, мочевая кислота

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