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ARTICLES

Regulation of Cyclooxygenase 2 mRNA Degradation by Rosiglitazone in C6 Glioma Cells in the Presence of Inflammation Inductors

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Abstract—It has recently become clear that regulation of mRNA stability plays an important role in the development of cell responses to stimulation of toll-like receptors during inflammation. This discovery motivated a search for low molecular weight substances modulating the stability of mRNA encoding proteins involved in inflammatory responses. In this work, regulation of the cyclooxygenase 2 (COX-2) expression by rosiglitazone – a promising drug for regulation of inflammation – was studied on rat glioma cell line C6. Inflammatory response was induced by lipopolysaccaride (LPS). The concentration of prostaglandin E_2 (PGE_2) was measured by ELISA, the level of COX-2 mRNA was determined by real-time PCR. It was shown that treatment with LPS caused a 6-fold increase in the PGE_2 synthesis, which correlated with an increase in the COX-2 mRNA expression. Rosiglitazone induced a 2-fold decrease of the LPS-stimulated PGE_2 release and reduced the levels of COX-2 transcripts. To explore the molecular mechanisms of the rosiglitazone effect, we estimated the stability of COX-2 mRNA. Cells were incubated in the presence of LPS for 1 h, and then de novo mRNA transcription was blocked with actinomycin D. The levels of mRNA were determined at various time points. Treatment with rosiglitazone was carried out for 30 min before the LPS addition. The COX-2 mRNA half-life in native cells was found to be 75 min. LPS stimulation slowed down the mRNA degradation so that its half-life time was 120 min, and the treatment with rosiglitazone restored this process back to the normal level. The results suggest that rosiglitazone regulates the stability of COX-2 mRNA. This opens up new perspectives for therapeutic applications of this drug.

Keywords: rosiglitazone, lipopolysaccharide, prostaglandin E_2, cyclooxygenase 2, mRNA decay

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INTRODUCTION

At the moment it is shown that the majority of neurodegenerative and metabolic diseases are accompanied by the development of inflammatory processes [1, 2]. Glial cells play an important role in the development of inflammatory response in brain [3]. Stimulation of glial cells with lipopolysaccharide (LPS) is used as a model of neuroinflammatory conditions in vitro [4–6].

Rosiglitazone (RG) is a thiazolidinedione-type drug, selective agonist of nuclear receptor PPARγ used in the treatment of type 2 diabetes. Recently a number of works has noticed RG as a promising tool for regulation of cellular inflammatory response, in particular in central neural system (CNS) [7]. The anti-inflammatory effect of RG has been shown in different cell types, including cells of the immune system [8, 9], where the inflammatory response plays a crucial role, and also in microglial cells in the Parkinson's disease model [10]. RG can decrease the level of cell production of tumor necrosis factor alpha (TNFα), interleukins 1β and 6 (IL-1β, IL-6) influencing the expression level of the corresponding genes [9]. The treatment of patients with RG leads to a decrease of expression of CD14 [8], a component of the LPS receptor complex causing inflammatory response in monocytes [11].

At the same time, RG increases the risk of heart attacks and other disruptions leading to death of patient, which is also bound with disruptions in the development of cellular inflammatory response [12, 13]. It is shown that the synthetic PPAR agonists mod-

Abbreviations: CNS, central neural system; COX-2, cyclooxygenase 2; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; RG, rosiglitazone.

ulate functions of CNS cells, in particular they regulate inflammatory response [14, 15].

Earlier in our laboratory RG was shown to strengthen the inflammatory response in primary astrocytes [16] increasing the gene expression of cyclooxygenase 2 (COX-2), a crucial enzyme of prostaglandin biosynthesis and a classic marker of inflammation [17]. Moreover, DNA chips showed that tumor cells amplify inflammation [18]. LPS is known to strengthen the COX-2 expression in rat glioma cells [19]. However, the RG action on the system of prostaglandin synthesis and COX-2 expression in these cells is not yet studied. Therefore, we investigated the regulation of the level of prostaglandin E_2 (PGE_2) produced by C6 cells and also possible regulatory mechanisms of COX-2 expression at transcriptional and post-transcriptional levels.

MATERIALS AND METHODS

Reagents. LPS (Sigma–Aldrich, USA), RG (Cayman, Germany), actinomycin D (Act D) (Sigma–Aldrich, USA), DNase I RNase-free (with $MnCl_2$, 1 U/μL) (Thermo Scientific, USA), streptomycin, penicillin, fetal calf serum, Hanks solution, DMEM (PanEco, Russia).

Cell culture and treatment. Rat C6 glial cells were plated into 75-cm^2 culture flasks. The cells were cultured in DMEM (10 mL per flask) containing 10% fatal calf serum and penicillin and streptomycin (100000 U/mL of each antibiotic) in CO_2 incubator (Heraeus BBD 6220, USA) with 5% CO_2 at 37°C. After 3 days of cultivation, culture medium was changed by the fresh one or cells were plated into 6-well plates at a density of 10^6 cells per well. Culture medium was changed by the fresh one (2 mL per well) 2 h before the experiment.

Determination of the PGE_2 release level. Cells were cultured in 6-well plates. After the experiment, the supernatant was collected and the PGE_2 concentration was detected using an enzyme-linked immunoassay Prostaglandin E2 Express EIA (Cayman Chemical, Germany). The concentration was determined by using a Synergy H4 plate reader (BioTek, USA).

Measurement of relative mRNA expression level. Total mRNA was isolated using PowerLyzer RNA Isolation kit (MO BIO, USA), then DNase treatment was performed according to the manufacturer's instructions. The concentration of RNA was measured using a spectrophotometer. cDNA was generated according to the manufacturer's instructions using oligo(dT)-primers. Real-time PCR was performed using Maxima SYBR Green/Fluorescein qPCR Master Mix (2×) (Thermo Scientific, USA) and the DTlite 4 amplificator (DNA-Technology, Russia). The sequences of PCR primers used in this study were as follows: COX-2: forward 5'-TGTACAAGCAGTGGCAAAGG-3', reverse 5'-TAGCATCTGGACGAGGCTTT-3'; β-actin: forward 5'-TCATCACTATCGGCAATGAGCGGT-3', reverse 5'-ACAGCACTGTGTTGGCATAGAGGT-3', the annealing temperature was 57°C. Expression of each gene was measured in 25-μL reactions using cDNA synthesized from 70 ng RNA per reaction well. The relative mRNA expression level was determined by ΔC_T method by the formula: $2^{-\Delta Ct}$ [20]. The β-actin gene was used as a constitutive gene for normalization.

Statistics. The data are presented as mean and standard error obtained from three independent experiments. Data were subjected to an ANOVA test with Bonferroni corrections. The difference was considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The influence of RG on the PGE_2 release and COX-2 expression in LPS-stimulated C6 cells. To characterize the anti-inflammatory action of RG on the level of prostaglandin synthesis, we investigated its influence on the PGE_2 release from cells C6. We used LPS (1 μg/mL) and RG (10 μM), the concentrations were previously shown to be not cytotoxic for these cells [20, 21]. Cells were incubated in the presence of RG for 30 min, then incubated without additional treatment or stimulated with LPS for 3 h. Untreated cells were used as control; the expression level of the COX-2 gene in control cells was taken as 1.

LPS was shown to cause a nearly 6-fold increase in the PGE_2 release in comparison with untreated cells. The incubation with RG alone increased the PGE_2 level approximately 3-fold, showing a correlation with the data obtained using mouse macrophages [23]. At the same time, pre-incubation of cells with RG reduced the PGE_2 release level about twofold (Fig. 1). This indicates the anti-inflammatory action of RG in this cell model.

The data obtained and the molecular mechanisms of RG action suggested before [16] allowed the assumption that the RG influence on PGE_2 synthesis occurred at the level of COX-2 mRNA expression regulation in LPS-stimulated cells. This was checked in a similar set of the experiments. The mRNA level was detected by RT-qPCR.

It was established that the LPS-stimulation for 3 h leads to a 3.5-fold increase in the expression of COX-2 mRNA in comparison with native cells (Fig. 2). The preincubation with RG leads to a decrease of COX-2 expression level in LPS-stimulated cells down to the level in control cells.

The data presented in Figs. 1 and 2 show that the negative effect of RG on the PGE_2 level in LPS-stimulated cells can be explained by the regulation of the COX-2 mRNA level. A possible mechanism of this regulation became a challenge for further research.

The influence of RG on COX-2 mRNA degradation in C6 cells. Lately, the regulation of gene expression at post-transcriptional level by changing the rate of mRNA degradation has been actively studied. The

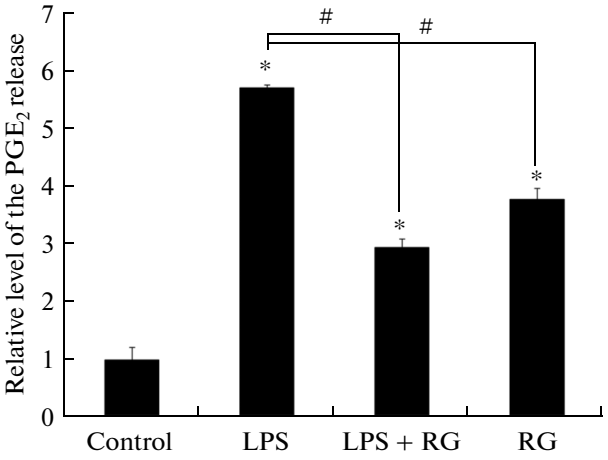

Fig. 1. The influence of rosiglitazone (RG) on the PGE_2 release in LPS-stimulated and untreated C6 cells. Cells were treated with RG (10 μM) for 30 min and then stimulated with LPS (1 μg/mL) for 3 h. *The value differs significantly from that of the non-stimulated cells (Control) at $p < 0.05$. #The difference is statistically significant at $p < 0.05$.

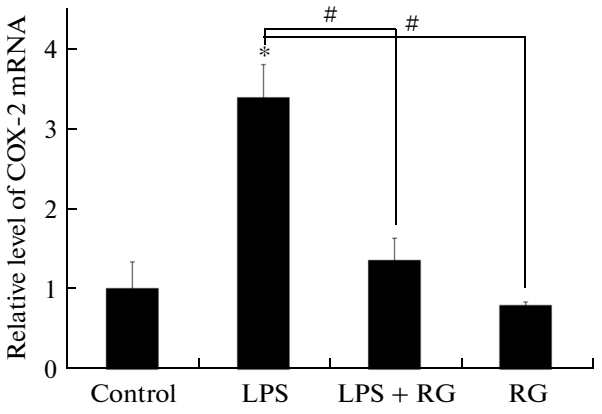

Fig. 2. The influence of rosiglitazone (RG) on the level of COX-2 mRNA in LPS-stimulated and untreated C6 cells. Cells were treated with RG (10 μM) for 30 min and then stimulated with LPS (1 μg/mL) for 3 h. The relative level of COX-2 mRNA was determined by real-time PCR, the data normalized to the β-actin mRNA level. *The value differs significantly from that of the non-stimulated cells (Control) at $p < 0.05$. #The difference is statistically significant at $p < 0.05$.

genes involved in inflammatory processes are of special interest, as the degradation rate of many of them is changed under the influence of different stimuli [24]. The same regulation is observed for COX-2 [25, 26]. Therefore, we supposed that the changes of the COX-2 expression level upon the LPS stimulation and RG action observed in the experiments can be explained by the influence on the rate of mRNA degradation. mRNA stability of COX-2 has not been studied in C6 cells. Therefore, we evaluated changes in the COX-2 mRNA level in the presence of actinomycin D, a transcription inhibitor, most frequently used in studies of the mRNA half-life. The addition of actinomycin D is known to cause no changes in the level of pre-existed mRNA allowing estimation of the mRNA half-life. [27]. Concentrations of actinomycin D in the range of 1–10 μg/mL are shown to block the synthesis of more than 80% of mRNA. We chosed the concentration of actinomycin D shown to be effective in studies of mRNA half-life in C6 cells and astrocytes [28, 29]. To estimate the mRNA stability in the presence of LPS, cells were stimulated with LPS (1 μg/mL) for 1 h and then de novo mRNA transcription was blocked with actinomycin D (5 μg/mL). The cells without LPS stimulation (untreated cells) were exposed to the similar treatment. The mRNA level was taken as 100% before actinomycin D treatment in untreated cells or after 1 h of the LPS stimulation and before actinomycin D treatment in cells of interest. The data are presented in Fig. 3.

The COX-2 mRNA half-life was found to be ~75 min in untreated cells and ~120 min in LPS-stimulated cells (Fig. 3). Thus, LPS treatment slows down the degradation of COX-2 mRNA. To estimate the RG influence on COX-2 mRNA stability, the mRNA half-lives in LPS-stimulated cells and cells pre-incubated with RG were compared. The pre-incubation with RG for 30 min prevented the mRNA stabilization caused

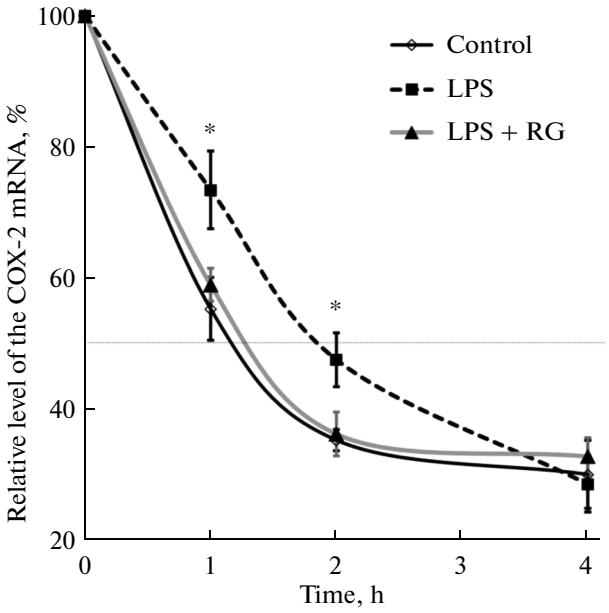

Fig. 3. The influence of rosiglitazone (RG) and LPS on the rate of COX-2 mRNA degradation in C6 cells. Cells were treated with RG (10 μM) for 30 min and then stimulated with LPS (1 μg/mL) for 1 h. Then the cells were incubated with actinomycin D (5 μg/mL) for 1, 2, or 4 h. Relative COX-2 mRNA level was determined by real-time PCR, the data are normalized to the β-actin mRNA level. *The value differs significantly from that of the non-stimulated cells (Control) at $p < 0.05$.

by LPS (Fig. 3). The mRNA half-life was approximately 80 min, corresponding to the half-life of the control cells. These data show that RG takes a part in the post-transcriptional regulation of COX-2 mRNA in LPS-stimulated cells.

The degradation rate of COX-2 mRNA is in the range indicated for other cells types. The data published show different values of the COX-2 mRNA half-life, which can be 0.5–2 h [24, 27–30].

Our data on slowing down the COX-2 mRNA degradation rate upon the LPS treatment correspond to those obtained previously on different cell types stimulated with pro-inflammatory agents (LPS, TNFα) [24, 30]. The COX-2 expression in C6 cells was shown to be regulated through similar mechanisms as it occurs in other cell types.

The possibility of increasing the COX-2 mRNA degradation rate by RG represents an interesting feature of this substance. Slowing down the mRNA degradation is typical of the majority of inflammatory markers, such as TNFα, IL-1, -6, and other [9, 23]. Recovering processes may slow down after the pro-inflammatory stimuli action and chronic diseases may develop [24]. Searching for low-molecular weight substances able to impact this process is necessary. Recently, the regulation of mRNA degradation by p38 MAP-kinases inhibitors was discussed [31, 32]. However, this class of inhibitors are not drugs. The possibility of using RG for modulation of this process demonstrated above opens new perspectives of medicinal application of this substance.

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