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Toll-like receptors control p38 and JNK MAPK signaling pathways in rat astrocytes differently, when cultured in normal or high glucose concentrations

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ABSTRACT

Astrocytes play a vital role in regulating central nervous system inflammation, energy metabolism and brain homeostasis. Unlike macrophages and microglia, which are cells of myeloid ancestry, astrocytes are of ectodermal origin. However, regulatory specificities of signaling pathways connecting inflammatory and metabolic processes are still largely unknown. We analyzed firstly cellular responses to toll-like receptor (TLR) agonists and secondly, modulation of the mRNA of the three isoforms of the transcription factors PPARs (peroxisome proliferator-activated receptors) in primary rat astrocytes exposed to normal glucose (5.5 mM) and high glucose (25 mM). Cell culturing of rat brain astrocytes for 2 days in high glucose did not alter cellular morphology, but i) enhanced the release of TNFα that was induced by TLR4 agonist LPS or TLR3 agonist PIC and the synthesis of prostaglandin E_2 (PGE_2), ii) changed the signaling pathways of TLR4/MAPK (increase in p38 MAPK, and decrease in JNK activities at early stages of TLR activation) and iii) modulated mRNA expression of PPARs. High glucose cultivation reduced PPARα and PPARβ mRNA levels, without altering PPARγ mRNA level and changed the sensitivity of expressions to agonists of TLR1/2 (PGN), TLR4 (LPS), TLR3 (PIC), and TLR5 (FGN). Differences between low and high glucose-adapted cells were obtained for agonists of TLR1/2 (PPARα, PPARβ), TLR4 (PPARβ), TLR3 (PPARα). In the TLR4/p38/PPARβ signaling pathway, there was a stimulatory connection in normal glucose but an inhibitory connection in high glucose. TLR4/JNK/activated PPARβ, TLR4/JNK/inhibited PPARγ both in cells adapted to normal or high glucose, but PPARα expression was not affected. As PPARs in astrocytes are involved in inflammatory processes in the form of the recently published PPAR triad, the changes in expression revealed here are most likely resulting in implications of high glucose in inflammatory processes. Our data underline the complexity of multiple regulatory interactions between inflammatory responses and energy metabolism in astrocytes.

1. Introduction

Astrocytes are glial cells with homeostatic, metabolic and defensive functions and play an important role in the development of inflammatory responses in brain (Sofroniew, 2014, 2015). Hyperglycemia is a condition of elevated glucose concentrations in body fluids with profound detrimental effects on the whole organism, including the brain. High glucose concentrations were demonstrated to cause neurotoxicity, a serious complication of long-term diabetes (Tomlinson and Gardiner, 2008). Patients with diabetes exhibit symptoms of neuroinflammation, cognitive impairment and neurodegenerative disorders (Barrett et al., 1995; Chen et al., 2011; Ristow, 2004). Moreover, hyperglycemia has long been known to induce, and to alter the inflammatory response. Although it was shown long ago that there is a

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FGL, flagellin; PIC, Poly:IC; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; JNK, c-Jun N-term Kinase; p38, p38 mitogen-activated protein kinases; PGE_2, prostaglandin E_2; PGN, peptidoglycan; PPAR, peroxisome proliferator-activated receptor; TLR, toll-like receptors; TNFα, tumor necrosis factor α

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connection between inflammatory and metabolic processes in brain (Ristow, 2004), only recently these connections come into the focus of investigations on signaling pathways levels and various experimental cellular models of hyperglycemia have been suggested (Gandhi et al., 2010; Quincozes-Santos et al., 2017; Wang et al., 2012). Both *in vivo* and *in vitro* models of hyperglycemia or high-glucose demonstrated impairment of astrocyte functions on the cellular level such as a slow-onset, poorly reversible decrement in gap junctional communication or oxidative stress induction (Gandhi et al., 2010; Hsieh et al., 2014; Quincozes-Santos et al., 2017).

As the innate immune system is the major component of inflammation, its role in glial cells is under intensive investigation (Sofroniew, 2015). The toll-like receptors (TLR) are important targets of the glial innate immune system, upon activation of these signaling pathways astrocytes produce pro- and anti-inflammatory cytokines and polyunsaturated fatty acid derivatives such as prostaglandins (Aleshin et al., 2009; Sofroniew, 2015). Mitogen activated protein kinases (MAPKs) are components of TLR-mediated signaling and their roles in astrocytes are intensively investigated (Chistyakov et al., 2014, 2015; Quincozes-Santos et al., 2017). Important to note is a special interest into TLR/MAPK signaling in astrocytes, because these cells are of ectodermal origin, unlike macrophages and microglia – conventionally studied cells that have myeloid ancestry (Filous and Silver, 2016). Therefore, it is obvious that not only inflammatory responses in astrocytes might have special features of regulation in comparison to cells of immune origin, but also the inflammatory and metabolic connections may be different.

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors crucial for cellular homeostasis and they may also serve as a marker of metabolic and inflammatory connectors. Three PPAR isoforms, PPARα, PPARβ, and PPARγ encoded by three different gene loci are expressed in all types of cells (Bensinger and Tontonoz, 2008), including astrocytes –system (CNS) (Sofroniew, 2014). Participation of PPARs in immune responses within the brain puts them among promising targets for treatment of various brain pathologies that involve neuroinflammation (Aleshin et al., 2013; Bensinger and Tontonoz, 2008; Bernardo and Minghetti, 2008; Heneka and Landreth, 2007). Recently the role of PPARs during inflammatory and metabolic adaptation of astrogliosis in various brain pathologies has been considered (Iglesias et al., 2017). All three PPAR subtypes have been demonstrated to be active participants of astrocyte-mediated processes and the obtained data suggest that the three types of transcription factor are coordinately regulated in astrocytes, forming a functional triad group in which alteration of a single component results in major group rearrangements (Aleshin et al., 2013). Particularly, PPARγ has positive influence while PPARα has negative influence on PPARβ expression and activity in primary astrocytes (Aleshin et al., 2009). We have shown that a number of molecular triggers modulate PPAR both mRNA and protein expression in astrocytes. These triggers include specific synthetic agonists of PPAR (Aleshin et al., 2009), endogenous agonists released in the course of phospholipase A_2 activity (Sergeeva et al., 2010), and mitogen activated protein kinases (MAPK) and the transcription factor NF-κβ (Chistyakov et al., 2014, 2015). In the present study that has been developed as a continuation of our previous research we use mRNA expression levels of PPARs as a marker of an intersection between inflammatory and metabolic signaling pathways and our aim was to find out if PPARs are differentially regulated in astrocytes exposed to normal versus high concentration of glucose.

The present study aimed at the investigation of connection between TLRs and p38 and JNK MAPK signaling pathways in rat astrocytes cultured in normal and high glucose concentrations. As a first step we established the *in vitro* model of cell incubation in media with 5.5 (normal glucose; NG) and 25 mM glucose (high glucose; HG) concentrations and subsequent challenge with various TLRs agonists.

A major objective was then to test the TLR/p38 MAPK and TLR/c-Jun N-term Kinase (JNK) signaling pathways in astrocytes at various glucose adaptations. We used the following markers, p38 and JNK inhibitors and PPARα, PPARβ, PPARγ mRNA expression to estimate the specificity of TLR/MAPK/mRNA PPARs signaling in astrocytes.

2. Materials and methods

2.1. Reagents

The cell culture medium Dulbecco's modified Eagle's medium (DMEM, cat.no FG 0435); fetal calf serum, penicillin, and streptomycin were obtained from Biochrom (Berlin, Germany). Flagellin (FGL, Bacillus subtilis) and peptidoglycan (PGN, Bacillus subtilis) were from Invivogen (San Diego, CA, USA). Lipopolysaccharide (Escherichia coli strain 0111:B4), Actinomycin D, Bay 11–7085, β-tubulin antibodies were purchased from Sigma Chemicals (Taufkirchen, Germany), Poly:IC (Catalog No. B5551) were from Apexbio (Houston, TX, USA). COX-2, JNK, pJNK, p38 and phospho-p38 antibody (Thr180/Tyr182) were from Cell Signaling Technology (Frankfurt, Germany). SB 203580, and SP600125 were ordered from ALEXIS Biochemicals (Grünberg, Germany). Confocal antibodies GFAP and isolectin B4 purchased from (Millipore, Bedford, USA), secondary antibodies anti-rabbit Alexa Fluor 488 and anti-chicken Alexa Fluor 546 purchased from (Thermo Scientific, Bremen, Germany).

2.2. Primary cell culture

Primary astrocyte cell cultures were obtained from newborn Wistar rats as described before (Chistyakov et al., 2014). All procedures were conducted in accordance with the present German regulations (Tierschutzgesetz). The study, as approved by the authorities of the State of Saxony-Anhalt, Germany, was performed according to institutional guidelines for animals, and all efforts were made to minimize the number of animals used. Animals were delivered by Charles River Laboratories (Sulzfeld, Germany). Brains from decapitated pups were rinsed with ice-cold Puck's solution (137.0 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.3 mM Na_2HPO_4, and 5.5 mM glucose, pH 7.4) and triturated against nylon meshes with the pores of 250 and 136 μm in consecutive order. Dissociated cells were plated into 75 cm^2 culture flasks at a density of 6×10^5 cells per ml. The cells were subsequently cultured in DMEM with 10% fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin (37 °C, 10% CO_2). After 5 days of cultivation in DMEM, culture medium was replaced with fresh medium and flasks were placed on a shaker at 200 RPM for 4 h to dissociate microglial cells. Microglia containing medium was discarded and astrocytes-enriched cultures were further grown for the following 4 days, medium was replaced every 2 days. Subsequently cells were washed with phosphate buffered saline and detached from the plastic with trypsin–EGTA solution (Biochrom) and plated into six-well plates and maintained for 2 days in DMEM with 5.5 mM glucose or 25 mM glucose concentration. After this, the medium was replaced by medium of the same composition (10% fetal calf serum in DMEM), and the cells were used for experiments. All experiments were repeated three times. Each experiment was performed on 3 independent primary astrocyte cell culture preparations. In each preparation cells were obtained from 3 to 5 pups. In these cultures, more than 95% of the cells were positive for the astrocyte marker glial fibrillary acidic protein, and only < 2% were positive for isolectin B4, a microglia-specific marker.

2.3. Measurement of the relative RNA expression level

Real-time PCR was performed as described previously (Chistyakov et al., 2015, 2014). In brief, total RNA was isolated using total RNA isolation kit RNeasy (Qiagen, Hilden, Germany). cDNA was generated from 1 μg of total RNA with iScript cDNA synthesis kit (Bio-Rad, Munich, Germany) according to the manufacturer's protocol. Real-time

PCR was performed on the iCycler (BioRad) using SYBR green PCR Master Mix (Bio-Rad), as described by the manufacturer. Amplification specificity of PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. The sequences of PCR primers used in the present study were as follows: β-actin: sense 5′-TCATCACTATCGG CAATGAGCGGT-3'; antisense 5′-ACAGCACTGTGTTGGCATAGAGGT-3′, 5′-AGCCCAGGATGCCCTTTAGT-3′; PPARα: sense, 5′-TGCGGACTACCA GTACTTAG-3′; antisense, 5′-CGACACTCGATGTTCAGTGC-3′; PPARβ: sense, 5′-CTCCTGCTCACTGACAGATG-3′; antisense, 5′-TCTCCTCCTGT GGCTGTTC-3′; PPARγ: sense, 5′-CCTGAAGCTCCAAGAATACC-3′; antisense, 5′-GATGCTTTATCCCCACAGAC-3′. The mRNA signals for PPARα, PPARβ and PPARγ were normalized to the β-actin mRNA signal and expressed relative to that in control cells treated with vehicle.

2.4. Western blot analysis

Astrocytes were lysed in modified radio immuno-precipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 1% NP-40 Sigma Chemicals, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). The protein concentration was determined by the standard Bradford assay. Samples containing 20 μg of protein in conventional Laemmli buffer were loaded on each lane of a 10% sodium dodecyl sulfate–polyacrylamide gel and subjected to a standard SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membrane with 0.2 μm pores. Membranes were blocked in 10% Rotiblock (Roth, Nürnberg, Germany) solution for 1 h and subsequently subjected to Phosphate-Buffered Saline with Tween 20 0.05% with a respective primary antibody: anti-p38 (1:1000), anti-pp38 (1:1000), anti-JNK or anti-pJNK (1:1000) or anti-COX-2 (1:2000) at 4 °C overnight. Analyzing the phosphorylation of MAPKs, ppMAPKs were first stained, and then total MAPKs were stained. Secondary species-specific antibodies (Dianova, Hamburg, Germany) were applied at the concentration of 1:10000 for 1 h at room temperature. Conjugates were visualized using SuperSignal™ West Femta Chemiluminescent Substrate (Thermo Scientific). For β-tubulin analysis, membranes were stripped at 21 °C for 20 min with Restore Western Blot Stripping Buffer (Pierce, Bonn, Germany). Membranes were re-probed with antibody against β-tubulin (1:10.000) from Sigma Chemicals and secondary anti-mouse IgG (Dianova, Hamburg, Germany) to control for protein loading. Protein bands were visualized by SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific). Densitometry was carried out on four different experiments. Band intensity was measured using a GS-800 calibrated densitometer signal and Quantity One software (Bio-Rad), and normalized to the intensity of the respective bands obtained for β-tubulin.

2.5. Immunofluorescence analysis

Astrocytes were plated onto glass-bottom Petri dishes at the quantity of 10^5 cells/glass and allowed to attach for 12 h. After media change cells were left for additional 24 h and used in the experiments as described elsewhere. Slides with cells fixed in 4% paraformaldehyde buffered with PBS were treated with Triton X-100 containing buffer and blocked with FBS and subsequently incubated overnight with primary antibodies against isolectin B4 (IB-4, 1:200) and glial fibrillary acidic protein (GFAP, 1:500). Alexa secondary antibodies from goat were used at following dilutions: Alexa 488 anti-rabbit 1:500, Alexa 633 anti-chicken 1:200. Negative control involved incubating astrocytes with only secondary antibodies. No significant staining was observed in the negative controls. Images were obtained with an Axiovert 100M (Zeiss, Göttingen, Germany), equipped with confocal microscopy software LSM 510. The images are representative of three independent experiments. Images were processed using ImageJ (1.51s) software (National Institute of Health, Bethesda, Maryland, USA).

2.6. Determination of TNFα and PGE_2 by enzyme-linked immunoassay

Astrocytes were grown in six-well plates. After the experiments, the supernatant was collected and the prostaglandin E_2 (PGE_2) concentration was detected using an enzyme-linked immunoassay (Cayman Chemical, Steinheim, Germany) and tumor necrosis factor α (TNFα) concentration was detected using an enzyme-linked immunoassay (Pierce, Bonn, Germany) according to the manufacturer's instructions. TNFα and PGE_2 concentrations were determined by using a Thermo Multiskan EX plate reader (Thermo Scientific, Bonn, Germany). The results were presented as picograms per ml of cell culture medium.

2.7. Experimental data analysis and statistics

Data are expressed as mean ± SEM. Normality of data sets was assessed using the Shapiro-Wilk test. Data were subjected to a one-way ANOVA followed by Bonferroni's *post hoc* test to determine statistical significance. $p < 0.05$ was considered statistically significant. All experiments were repeated at least three times.

3. Results

3.1. Cell adaptations to high glucose (HG) do not alter morphology, but modulate inflammatory implications

As a first step for elucidation of the cellular mechanisms of dependence of PPARs mRNA expression on metabolic adaptations, we established the *in vitro* model of cell incubation in media with 5.5 (normal glucose; NG) and 25 mM glucose (high glucose; HG) concentrations and subsequent challenge with various TLRs agonists. We used a protocol with 48 h duration of culturing in complete medium containing serum where only the glucose concentration was changed to analyze the influence of glucose on cell parameters, such as the morphology. GFAP was used as a marker for astrocytes, IB-4 was used to detect microglia and ethidium bromide (EtBr) was used to stain nuclei. The characteristics of the cell culture during cultivation with the increased concentration of glucose were no changed. Fig. 1a shows an example of the astrocytes cell culture, which has been exposed to high glucose medium. For characterization of inflammatory implications, we compared the influence of glucose concentration on astrocyte response to challenges with PIC – a TLR3 agonist and LPS – a TLR4 agonist. Responses were evaluated by determining the levels of pro-inflammatory markers, cytokine TNFα and prostaglandin E_2, in extracellular media after 4 h of TLR stimulation (Fig. 1 b and c). Both TLR3 and TLR4 agonists induced a nearly two-fold increase in levels of TNFα in astrocytes incubated in high glucose medium as compared to normal glucose medium (Fig. 1b). Similarly, an increase in inflammatory responses was obtained for another type of pro-inflammatory marker, namely PGE_2 (Fig. 1c). For estimation of changes in PPAR expression we compared mRNA levels of PPARs in naive, non-stimulated cells (Fig. 1d) and obtained that culturing of cells for 48 h in high glucose medium decreased the levels of PPARα and PPARβ, but did not influence the PPARγ mRNA levels (Fig. 1d). We also measure protein levels of cyclooxygenase 2 (COX-2) expression (Fig. 1e and f). Exposure to high glucose (25 mmol/L) increased the expression of COX-2 protein, both in naïve and LPS-stimulated cells (Fig. 1e and f).

Thus, although 48 h long incubation with high glucose did not affect cell morphology, TLR-mediated responses of pro-inflammatory markers and levels of PPAR expression were clearly altered.

3.2. Cell adaptations to high glucose modulate TLR-mediated p38 and JNK MAPK activities

Recently it has been shown that fluctuation in glucose levels in cultured astrocytes exacerbate glial injury and p38 signaling pathways were putative mechanisms of the glucose effects (Quincozes-Santos

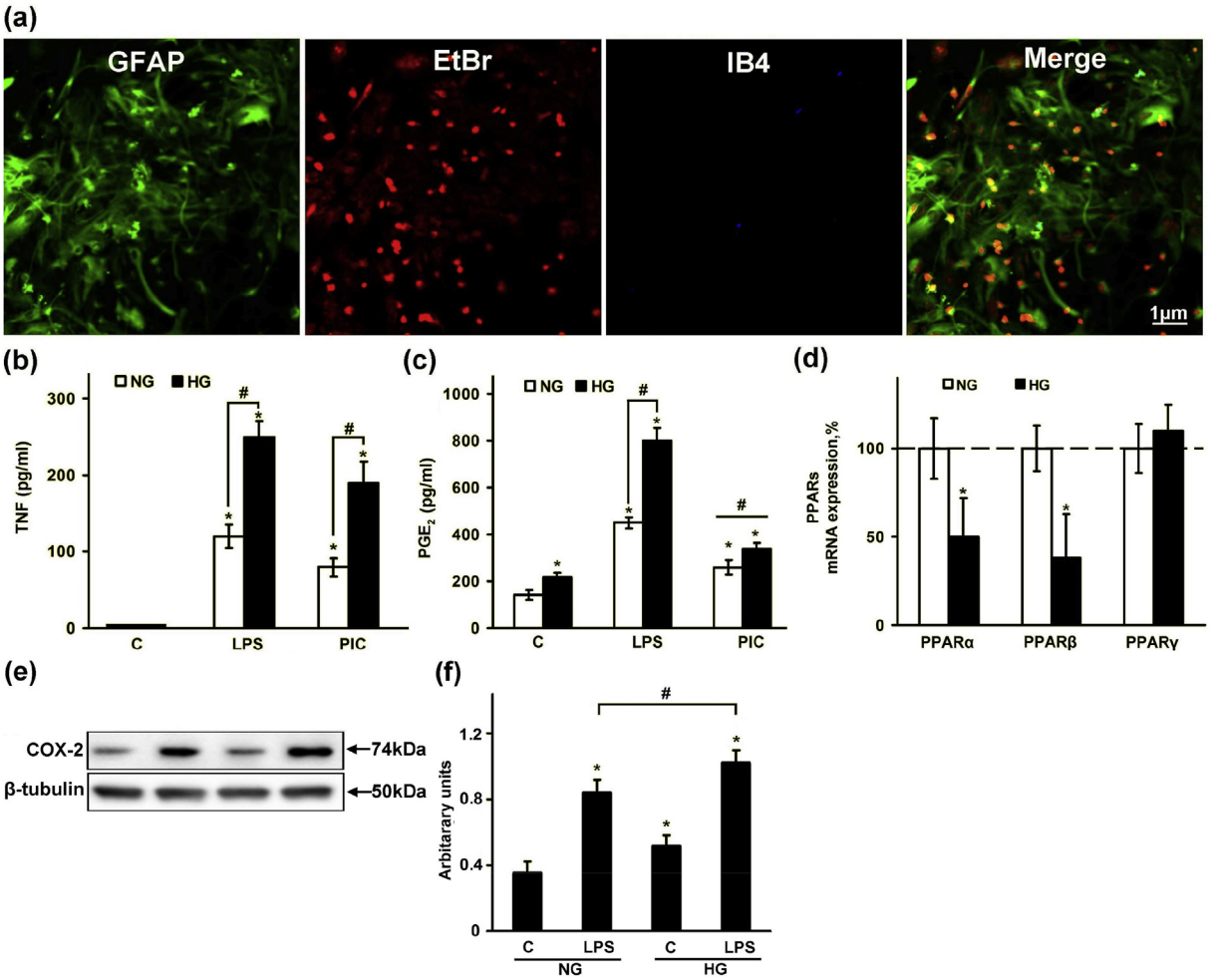

Fig. 1. Comparison of astrocyte cultures cultivated in high and normal glucose for 48 h. Astrocytes from newborn rats were cultured in normal glucose (5.5 mM; NG) or high glucose (25 mM; HG) concentration for 48 h. **(a)**. Immunocytochemistry of cultures of astrocytes in HG medium, stained for astrocytes (GFAP, green), nucleus (EtBr, red) and microglial cells (IB4, blue) with separate and merged images shown in panels (a). Scale bar = 100 μm. **(b), (c)** Comparison of influence of Toll-like receptor (TLR) agonists on release of tumor necrosis factorα (TNFα) (b) and PGE_2 (c). Cells were stimulated with the TLR agonists: lipopolysaccharide (LPS; 100 ng/ml, TLR4) and Poly:IC (PIC; 10 μg/ml, TLR3) for 4 h. PGE_2 and TNFα concentrations were measured by ELISA in supernatant samples of three cell cultures. (d) PPARs mRNA expression in naïve astrocytes, cultured in high and normal glucose**.** The levels of PPARα, PPARβ and PPARγ mRNA were determined by real-time RT-PCR. Values were normalized to β-actin mRNA level. COX-2 protein level was evaluated by western blotting and normalized to the loading control β-tubulin. The example in (e) is representative for three independent experiments. Results in (f) are expressed as arbitrary units normalized on the expression of the housekeeping protein β-tubulin. Values represent mean ± SEM from three independent experiments performed in triplicate. *$p < 0.05$ compared with the unstimulated cells, #$p < 0.05$ compared for indicated bars.

et al., 2017). We have shown that p38 and JNK MAPK are involved in LPS-mediated regulation of mRNA PPARs expression in astrocytes (Chistyakov et al., 2015). For characterization of the MAPK activity alterations in our experimental model with high glucose adaptation, we measured p38 MAPK and Jun N-terminal kinase (JNK) MAPK proteins expression and phosphorylation in astrocytes cultured in normal (5.5 mM) or high (25 mM) glucose for 48 h and subsequently stimulated with LPS (Fig. 2).

The levels of phospho-JNK, JNK, phospho-p38 and total p38 are demonstrated for the same samples in Fig. 2a. Levels of both MAPKs did not change in non-stimulated cells exposed to high or normal glucose (Fig. 2). Activation of TLR4 signaling pathways induced 4-fold upregulation of both p38 and JNK with peaks at 2 h (Fig. 2b). A comparable 4-fold increase in JNK activity and a similar time-course of responses were previously demonstrated for astrocytes treated with a much higher concentration of LPS, namely 2 μg/ml, as reported (Liao et al., 2013). We obtained that the LPS stimulation enhanced the p38 MAPK but reduced the JNK activity in cells cultured at high glucose at 2 h (Fig. 2b). Incubation for 4 h did not significantly affect the levels of phosphorylation of both p46 and p54 subunits for JNK. Thus, our data reveal that there is a clear influence of high glucose concentration during the early stage of challenge with LPS.

3.3. Modulation of PPARs mRNA expression by inhibition of p38 or JNK MAPK in LPS-stimulated astrocytes at normal (5.5 mM) and high (25 mM) glucose

Changes in MAPKs activities obtained in high glucose adaptation models must be manifest at the levels of sensitivity to specific MAPK inhibitors, as it was shown that PPAR mRNA expression is sensitive to various MAPKs (Chistyakov et al., 2014, 2015; Necela et al., 2008). Therefore, we compared the influence of inhibitors of p38 and JNK on the expression of PPARs mRNA (Fig. 3). We used SB203580 as an inhibitor of p38 MAPK and SP600125 as an inhibitor of JNK at concentrations which have been validated previously (Chistyakov et al., 2015). In those works, it was demonstrated that the concentrations used here are active and specific (Chistyakov et al., 2015). After the 48 h adaptation to glucose, astrocytes were pretreated with the inhibitor of

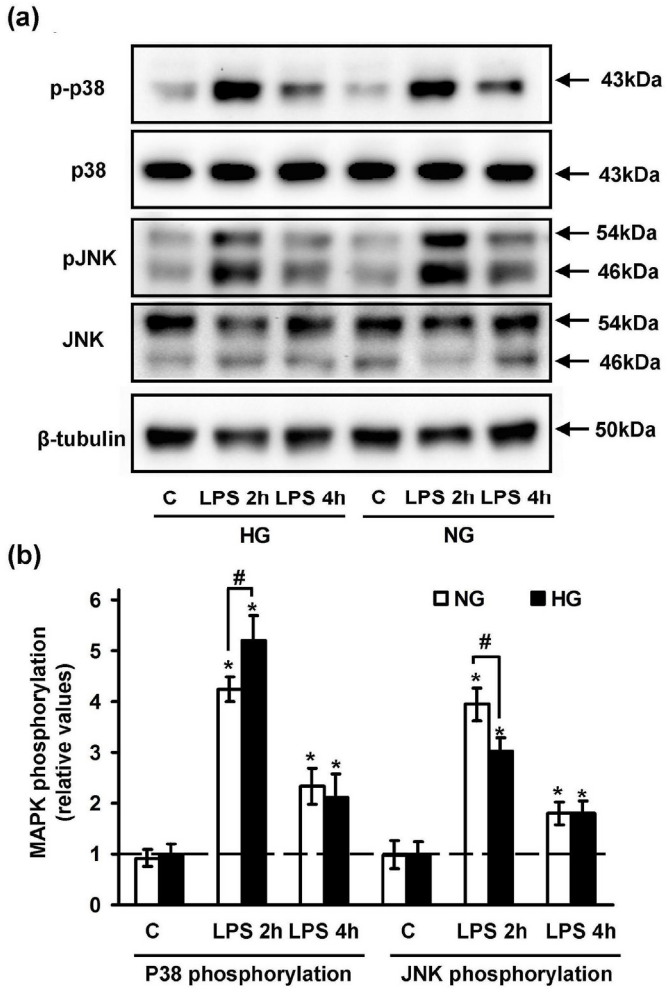

Fig. 2. Comparison of p38 and JNK MAPK activity in astrocytes cultured with glucose at 5.5 mM and 25 mM concentrations after stimulation with LPS. Astrocytes were exposed to glucose at 5.5 mM (NG) and 25 mM (HG) for 48 h. Then cells were stimulated for 2 h and 4 h with the TLR4 agonist lipopolysaccharide (LPS; 100 ng/ml). p38, p-p38, pJNK and JNK protein levels were evaluated by western blotting at indicated time (given in h) and normalized to the loading control β-tubulin. (a) Representative Western blots demonstrating phospho-p38 (p-p38), phospho-JNK (pJNK) and total p38 (p38) and total JNK (JNK) protein levels. The example is representative for three independent experiments. (b) Results are expressed as fold-changes, relative to untreated control astrocytes. Values represent mean ± SEM from three independent experiments. *$p < 0.05$, compared with the unstimulated cells, #$p < 0.05$ compared for indicated bars.

p38 or JNK for 0.5 h, and then further incubated without any additional stimulation or challenged with LPS for 4 h.

Levels of PPAR mRNA in conditions of normal (NG; white columns) and high glucose (HG; black columns) were measured by real-time PCR (Fig. 3). None of the tested substances, when added to naıve cells, influenced the levels of expression of PPAR genes either in high or in normal glucose, and these levels of expression were used for normalization of the data shown in Fig. 3. LPS reduced PPARα mRNA similarly in high and in normal glucose (Fig. 3a). Moreover, the inhibitors of p38 MAPK or JNK did not influence the reduction of PPARα mRNA expression level by LPS (Fig. 3a).

LPS reduced the PPARγ mRNA both in high and in normal glucose (Fig. 3c). JNK inhibition with SP600126 completely abolished the LPS-mediated decrease of PPARγ, while the p38 MAPK inhibitor had no influence on the LPS-mediated reduction of PPARγ mRNA expression in both groups of cells, cultured either in high or in normal glucose (Fig. 3c).

It is important to note that for PPARα and PPARγ there was no difference in sensitivity to MAPK inhibition between cells cultured in different concentrations of glucose.

Most striking were our results for PPARβ. LPS raised the PPARβ mRNA to significantly different levels for high and normal glucose (Fig. 3b). Moreover, both MAPK inhibitors had differential effects on PPARβ expression in a glucose concentration-dependent manner. The JNK inhibitor SP600125 completely abolished the LPS-induced PPARβ mRNA expression in normal glucose, and had significant, albeit weaker inhibition of LPS-induced PPARβ mRNA expression in high glucose (Fig. 3b). The p38 inhibitor abolished LPS-induced PPARβ mRNA expression in normal glucose, but greatly potentiated the expression level in high glucose. Thus, SB203580 acts as an enhancer of LPS in high glucose (Fig. 3b).

The role of MAPK in regulating changes in PPAR expression is multifaceted and glucose-concentration dependent, as presented in a scheme Fig. 3d that summarizes the results shown in Fig. 3a–c. Blue lines indicate the influence of MAPK in normal glucose, red lines that in high glucose. The arrowheads indicate increased expression of mRNA (corresponding to an inhibitory effect of the MAPK inhibitor), the hammerheads give a respective reductions (corresponding to a potentiating effect of the MAPK inhibitor).

3.4. Astrocytes with various glucose adaptations react differently to TLR agonists in terms of PPARs mRNA expression

Previously we have shown that agonists of plasma membrane TLRs induced PPARβ (Chistyakov et al., 2014) and reduced PPARγ and PPARα mRNA expression (Chistyakov et al., 2015) in primary astrocytes that were cultured in high glucose medium. Therefore, here we systematically compared the effects of different types of TLR agonists on cells, grown at high glucose (HG) and normal (NG) glucose concentration (Fig. 4). We tested the effects of lipopolysaccharide (LPS, 100 ng/ml; agonist of TLR4), peptidoglycan (PGN, 5 μg/ml; agonist of TLR1/2), flagellin (FGL, 5 μg/ml; agonist of TLR5), and Poly:IC (PIC; 10 μg/ml; agonist of TLR3) applications for 4 h (Fig. 4). Treatment of astrocytes with each of these agonists increased PPARβ mRNA expression with notable differences between normal and high glucose for TLR4 and TLR1/2 agonists (Fig. 4b).

TLR1/2, TLR4 and TLR5 suppressed the expression of PPARα and PPARγ mRNA (Fig. 4a, c) to the same level in normal and high glucose, except for the PGN influence on PPARα mRNA expression, where the effect in high glucose was significantly stronger than in normal glucose (Fig. 4a). More significant changes in high and normal glucose were obtained with PIC, an agonist of TLR3. PIC did not influence the PPARα and PPAR γ mRNA expression levels in normal glucose, while it significantly decreased the expression in high glucose (Fig. 4a, c). Such a difference in glucose sensitivity between plasma membrane receptor TLR (agonists of bacterial nature) and internal membranes (agonists of viral origin) signal transduction pathways may indicate different signaling mechanisms that regulate the inflammatory response in metabolic context.

4. Discussion

The relationships of metabolism and inflammation at the level of the organism are in the focus of numerous present studies. Obviously, the violation of these relationships is the basis of many diseases of the central nervous system. Astrocytes play a vital role in regulating central nervous system inflammation, energy metabolism and brain homeostasis. Unlike macrophages and microglia (cells that have myeloid ancestry), astrocytes are of ectodermal, neuroepithelial origin (Sofroniew, 2014). This origin seems to result in the fact that many responses to pro-inflammatory stimuli are regulated on these cells in a different way than on cells of immune origin (Aleshin et al., 2009;

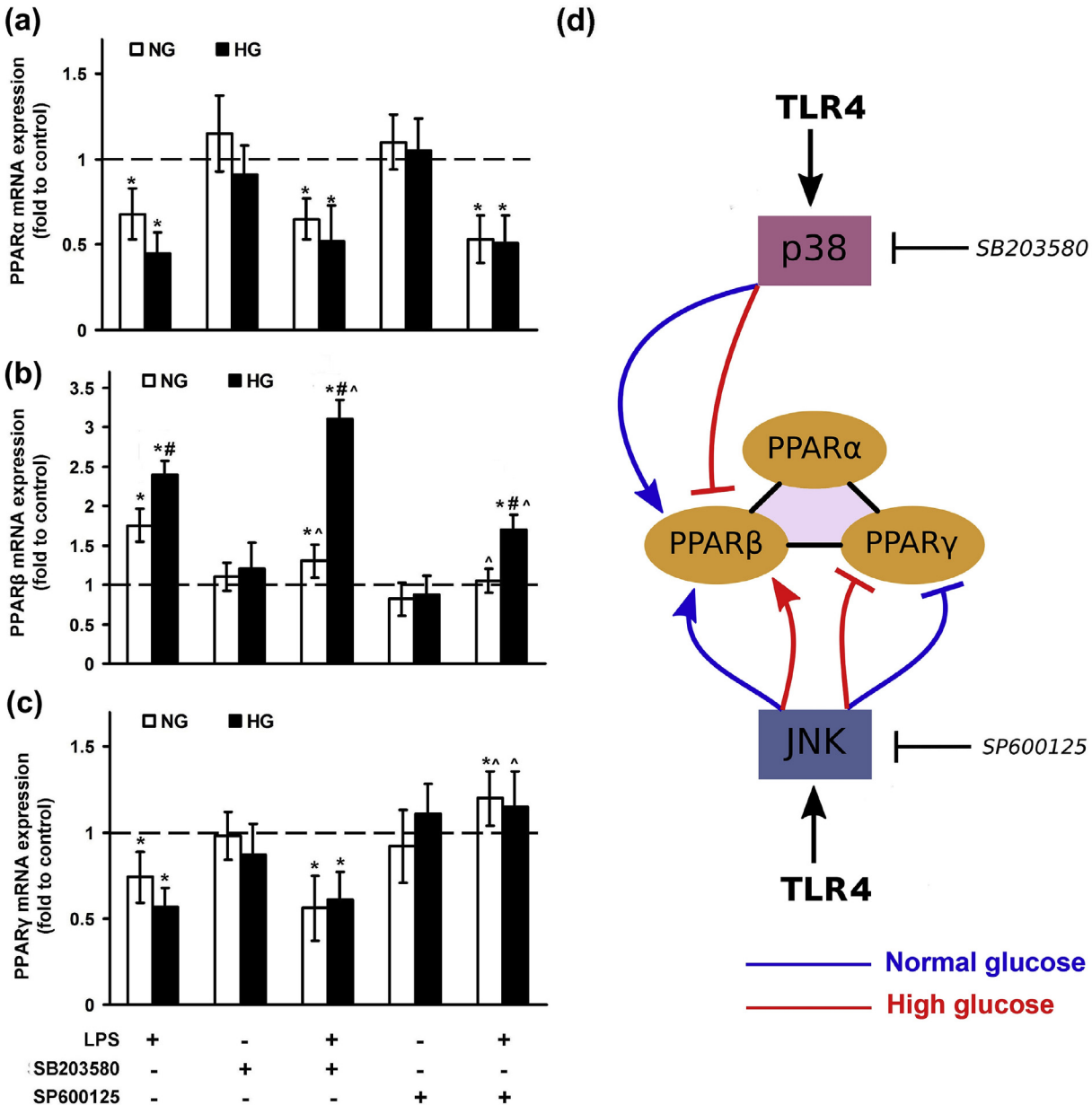

Fig. 3. High glucose changes the sensitivities of PPARs mRNA expression levels to inhibitors of p38 and c-Jun N-terminal Kinase (JNK), either alone or in combination with lipopolysaccharide (LPS). Astrocytes were cultured for 48 h in medium with normal (5.5 mM; NG; white columns) and high (25 mM; HG; black columns) glucose. Astrocytes were pretreated for 30 min with inhibitors of the MAPK signaling axis that include p38 (SB203580, 20 μM), JNK (SP600125, 10 μM) and subsequently kept for 4 h without any additional stimulation or with lipopolysaccharide (LPS, 100 ng/ml). The levels of PPARα (a), PPARβ (b) and PPARγ (c) mRNA were determined by real-time RT-PCR. Values were normalized to β-actin mRNA level. Results are provided as fold-changes, relative to unstimulated astrocytes (whose expression was taken as 1); *p < 0.05 compared with the unstimulated cells, #p < 0.05 compared within the same glucose treatment, ^p < 0.05 compared with astrocytes after LPS treatment. d) Scheme of LPS-induced PPARs mRNA expression in normal and high glucose. Red lines - high glucose, blue line - normal glucose, arrows - activation, hammer-inhibition.

Astakhova et al., 2018; Chistyakov et al., 2015). It is important to note that research at the cellular level is usually carried out under conditions of cell cultivation in high glucose, since cell cultures sense more clearly when there is an excess of nutrition. To avoid the possible influence of the dependence of the response to inflammatory stimuli on the adaptation of cells to cultivation under high glucose conditions, in this work we compared the ability of astrocytes to respond to the activation of TLR by agonists when cells are cultured in high (25 mM) and normal (5.5 mM) glucose. The data showed that cell culturing for 2 days in high glucose i) did not affect cellular morphology, but ii) enhanced the LPS (TLR4 agonist)- or PIC (TLR3 agonist)-induced release of TNFα and prostaglandin E_2 (PGE_2), iii) changed TLR4/MAPK signaling pathways (increase in p38 MAPK, and decrease in JNK activities at early stages of TLR activation), iv) modulated COX-2 expression at protein level. These data indicate that incubation in high glucose concentration switches astrocytes into a pro-inflammatory state and impairs inflammatory responses for TLR4 or TLR3 agonists. The agonists are either of bacterial (LPS) or viral (PIC) origins. These alterations were accompanied by increased activity of p38 MAPK and decreased activity of JNK. Noteworthy, the differences were transient and modulated initial stage of TLR signaling, the most significant change was seen at 2 h after TLR4 agonist stimulation and no differences between normal and high glucose cultured cells were detected at 4 h. The opposite effect of high glucose on JNK and p38 MAPK activities is especially relevant in those

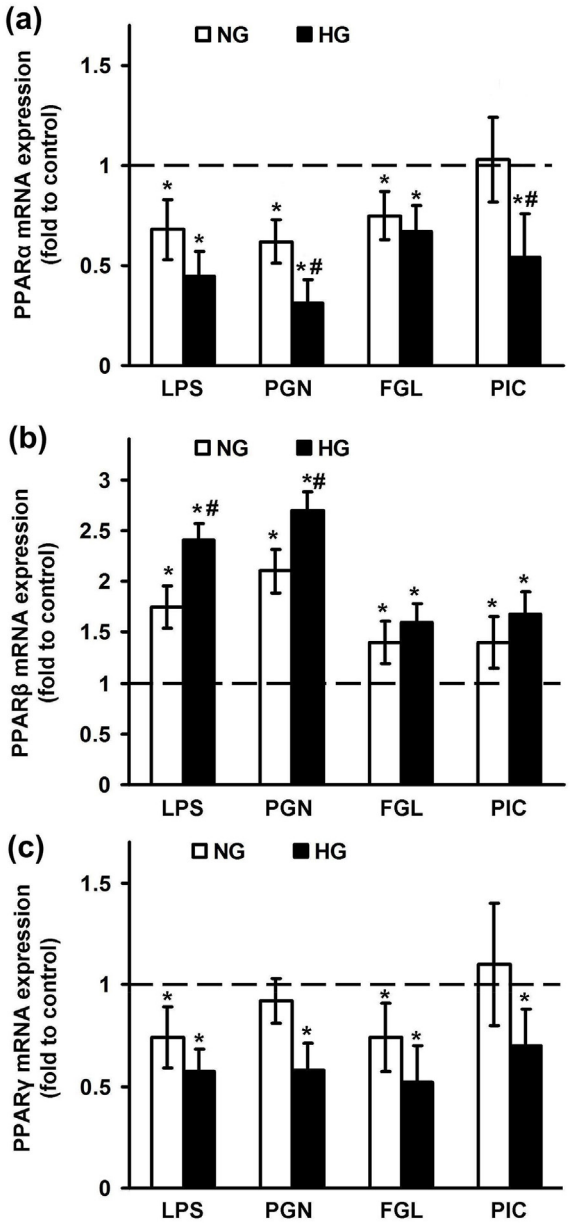

Fig. 4. Comparison of PPARs mRNA expression after stimulation of various TLR of astrocytes cultured with normal (NG) and high (HG) glucose. Astrocytes were cultured for 48 h in medium with normal (5 mM, white columns) and high (25 mM, black columns). Then cells were stimulated for 4 h with the TLR agonists: lipopolysaccharide (LPS; 100 ng/ml, TLR4), Poly:IC (PIC; 10 μg/ml, TLR3), peptidoglycan (PGN; 5 μg/ml, TLR1/2), and flagellin (FGL; 5 μg/ml, TLR5). The levels of PPARα (a), PPARβ (b) and PPARγ (c) mRNA were determined by real-time RT-PCR. Values were normalized to β-actin mRNA level. Results are provided as fold-changes, relative to unstimulated astrocytes (whose expression was taken as 1). Values represent mean ± SEM from three independent experiments performed in triplicate. *$p < 0.05$ compared with the unstimulated cells, #$p < 0.05$ compared within the same treatment in normal and high glucose.

pathways, where the ratio of the activities of the two kinases JNK and p38 MAPK is important for signaling.

The observed changes in the expression of COX-2 and the activity of p38 and JNK attracted our attention to the study of the expression of three types of PPAR at the mRNA level. We chose these genes for the following reasons. First, changes in PPARs mRNA levels in high glucose are well shown in other cell types and are considered to be a promising target for the inflammatory process manipulations. Indeed, it was shown that a decrease of PPARγ mRNA expression in high glucose was accompanied by increased release of pro-inflammatory cytokines, which was found for various cells, such as hepatocytes, adipose cells, kidney epithelial cells, endothelial cells, macrophages (Edvardsson et al., 2006; Fujiki et al., 2009; Liu et al., 2015, 2018; Panchapakesan et al., 2004; Pereira et al., 2016; Sartippour and Renier, 2000; Zhang et al., 2015). Return of PPARγ expression to a level corresponding to normal glucose upon manipulation with appropriate chemical substances correlated with a decreased release of cytokines (Liu et al., 2018). Noteworthy, substances and physiological states that induce PPARγ demonstrate anti-inflammatory features (Liu et al., 2015; Zhang et al., 2015), and alterations in expression of PPARγ mRNA accompany macrophage polarization and insulin resistance (Zhang et al., 2016). So far, there were no comparable data available concerning the PPARα and PPARβ expression. We observed a decrease in PPARα and PPARβ mRNA levels and did not detect changes in PPARγ mRNA in naive cells exposed to high glucose. Second, it is tempting to assume that the changes of PPARα, PPARβ, and PPARγ expression in different metabolic states result in various patterns of involvement of PPARs in TLR-mediated signaling pathways in astrocytes. This assumption is supported by our data on the change in COX-2 protein levels. Earlier, we showed that COX-2 directly depends on the mRNA level of PPARβ expression (Aleshin, 2009), i.e. COX-2 can serve as a reference gene for assessing the activity of PPARβ in astrocytes. Indeed, incubation in high glucose leads to an increase in the basal level of COX-2 and an increase in its expression during LPS stimulation in parallel with an increase in the mRNA PPARβ expression. Third, our data not only add new features of the molecular mechanism of regulation of PPARα and PPARβ mRNA expression in high and normal glucose, but also raise the interesting question how the regulation of expression of the three types of PPAR can be seen with the PPAR triad concept ((Aleshin et al., 2013)). The fundamental idea of the PPAR triad concept is that alterations in the ratio of basal levels of PPAR types are important in cellular responses to pro-inflammatory stimuli (Aleshin et al., 2013). Previously, we have shown a direct correlation between mRNA and protein levels for the three types of PPAR in astrocytes under various stimulation conditions (Aleshin et al., 2009; Chistyakov et al., 2014, 2015).

Regulation of the PPARs may be realized via the combination of various MAPKs. Indeed, expression of all PPARs mRNA in naive astrocytes does not depend on p38 or JNK MAPK signaling pathways, as addition of the respective MAPK inhibitors does not alter the levels of expression of all three PPAR genes, either in normal or high glucose concentration. Treatments with the TLR4 agonist with either p38 or JNK inhibitors does not influence the PPARα mRNA expression independently of the glucose concentrations, PPARγ mRNA expression is sensitive to JNK inhibitors, also independently of the glucose concentration, although we have obtained TLR4-mediated activation of JNK downregulated PPARγ transcription in high glucose. These variations in PPARs expression sensitivity may change the PPARs ratio and therefore change the patterns of gene expression.

PPARβ mRNA expression demonstrated the most interesting sensitivity to MAPK inhibition and alteration of glucose concentration. Indeed, although JNK induced PPARβ mRNA expression both in high and normal glucose, the effect of p38 differed according to the metabolic conditions. In high glucose, the activation of p38 MAPK reduced, but in normal glucose p38 activated PPARβ mRNA expression. This switch in PPARβ mRNA expression regulation in high glucose into the opposite direction is most striking among our results obtained here.

An alternative consideration concerning the effect of the SB203580 inhibitor should also be mentioned. We used a concentration of SB203580 inhibitor that is sufficient to inhibit p38 MAPK (Chistyakov et al., 2015), but the used concentration has also been reported to inhibit protein kinase B/Akt (PKB) (Lali et al., 2000; Webster et al., 2002). PKB is a regulator of cell glucose metabolism. Possibly treatment with high glucose induced PKB, which, in turn, blocked the LPS-induced activation of PPARβ. Therefore, treatment with SB203580 might

additionally boost the PPARβ mRNA levels. In normal glucose conditions, however, this boost would be absent due to the lower PKB activity in normal glucose (Campbell et al., 2003). This hypothesis needs further consideration.

There is no doubt that metabolic and inflammatory pathways interact at the cellular level, but it remains unclear which cellular models are suitable for studying these processes in brain cells. Here we present such a model of brain hyperglycemia – a culture of primary astrocytes exposed to high glucose concentrations (25 mM) for a prolonged period (48 h) and compared their behavior to cells cultured in media with normal glucose concentration. Previously published studies of the effect of high glucose on astrocytes are difficult to evaluate, how these studies relate to inflammatory pathways, because the protocols used in the literature (Gandhi et al., 2010; Wang et al., 2012) cannot be clearly interpreted. Both the long-term incubation of astrocytes (4 weeks) (Gandhi et al., 2010), as well as the short-term (24–48 h) incubation of astrocytes with 15 mM glucose in serum-free medium (Wang et al., 2012) altered the cell morphology. These protocols might induce additional responses of cellular adaptation in addition to serum deprivation. Important to note once more that our model of adaptation to high glucose concentration does not involve morphological changes in comparison with other previously suggested cellular models of adaptation (Gandhi et al., 2010; Wang et al., 2012). Taken together, changes in inflammatory markers indicate that astrocytes adaptation to high glucose concentrations for 48 h gives a suitable cellular model to investigate the connection between metabolic state and inflammatory pathways.

We conclude from the present data that astrocyte adaptation to high glucose concentration involves changes in TLR-mediated signaling pathways. It is difficult to compare other published data on molecular mechanisms of cellular responses to pro-inflammatory stimuli because most researchers do not consistently indicate the glucose concentration of the media used for cell cultures, unless the investigation is dedicated to analyze the influence of glucose concentrations. Especially our data of PPARβ regulation in conditions of high and normal glucose underline the necessity to revisit reports on the cellular signaling pathways involved in TLR-mediated responses in different metabolic conditions. Our data suggest that we have a suitable cellular model for investigation of interconnections between metabolic state and inflammatory pathways. In particular, the model will allow the characterization of TLR/MAPK/PPARs signaling pathways.

Conflict of interest disclosure

The authors have no conflicts of interest to declare.

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References

Aleshin, S., Grabeklis, S., Hanck, T., Sergeeva, M., Reiser, G., 2009. Peroxisome proliferator-activated receptor (PPAR)-γ positively controls and PPARα negatively controls cyclooxygenase-2 expression in rat brain astrocytes through a convergence on PPARβ/δ via mutual control of PPAR expression levels. Mol. Pharmacol. 76, 414–424.

Aleshin, S., Strokin, M., Sergeeva, M., Reiser, G., 2013. Peroxisome proliferator-activated receptor (PPAR)β/δ, a possible nexus of PPARα- and PPARγ- dependent molecular pathways in neurodegenerative diseases; review and novel hypotheses. Neurochem. Int. 63, 322–330.

Astakhova, A.A., Chistyakov, D.V., Sergeeva, M.G., Reiser, G., 2018. Regulation of the ARE-binding proteins, TTP (tristetraprolin) and HuR (human antigen R), in inflammatory response in astrocytes. Neurochem. Int. 118, 82–90.

Barrett, T.G., Bundey, S.E., Macleod, A.F., 1995. Neurodegeneration and diabetes: UK nationwide study of Wolfram (DIDMOAD) syndrome. Lancet 346, 1458–1463.

Bensinger, S.J., Tontonoz, P., 2008. Integration of metabolism and inflammation by lipid-activated nuclear receptors. Nature 454, 470–477.

Bernardo, A., Minghetti, L., 2008. Regulation of glial cell functions by PPAR-γ natural and synthetic agonists. PPAR Res. 2008, 864140.

Campbell, M., Allen, W.E., Silversides, J.A., Trimble, E.R., 2003. Glucose-induced phosphatidylinositol 3-kinase and mitogen-activated protein kinase-dependent upregulation of the platelet-derived growth factor-beta receptor potentiates vascular smooth muscle cell chemotaxis. Diabetes 52, 519–526.

Chen, J., Cui, X., Zacharek, A., Cui, Y., Roberts, C., Chopp, M., 2011. White matter damage and the effect of matrix metalloproteinases in type 2 diabetic mice after stroke. Stroke 42, 445–452.

Chistyakov, D.V., Aleshin, S., Sergeeva, M.G., Reiser, G., 2014. Regulation of peroxisome proliferator-activated receptor β/δ expression and activity levels by toll-like receptor agonists and MAP kinase inhibitors in rat astrocytes. J. Neurochem. 130, 563–574.

Chistyakov, D.V., Aleshin, S.E., Astakhova, A.A., Sergeeva, M.G., Reiser, G., 2015. Regulation of peroxisome proliferator-activated receptors (PPAR) α and -γ of rat brain astrocytes in the course of activation by toll-like receptor agonists. J. Neurochem. 134, 113–124.

Edvardsson, U., Ljungberg, A., Oscarsson, J., 2006. Insulin and oleic acid increase PPARgamma2 expression in cultured mouse hepatocytes. Biochem. Biophys. Res. Commun. 340, 111–117.

Filous, A.R., Silver, J., 2016. Targeting astrocytes in CNS injury and disease: a translational research approach. Prog. Neurobiol. 144, 173–187.

Fujiki, K., Kano, F., Shiota, K., Murata, M., 2009. Expression of the peroxisome proliferator activated receptor γ gene is repressed by DNA methylation in visceral adipose tissue of mouse models of diabetes. BMC Biol. 7, 38.

Gandhi, G.K., Ball, K.K., Cruz, N.F., Dienel, G.A., 2010. Hyperglycaemia and diabetes impair gap junctional communication among astrocytes. ASN Neuro 2, e00030.

Heneka, M.T., Landreth, G.E., 2007. PPARs in the brain. Biochim. Biophys. Acta 1771, 1031–1045.

Hsieh, H.L., Chi, P.L., Lin, C.C., Yang, C.C., Yang, C.M., 2014. Up-regulation of ROS-dependent matrix metalloproteinase-9 from high-glucose-challenged astrocytes contributes to the neuronal apoptosis. Mol. Neurobiol. 50, 520–533.

Iglesias, J., Morales, L., Barreto, G.E., 2017. Metabolic and inflammatory adaptation of reactive astrocytes: role of PPARs. Mol. Neurobiol. 54, 2518–2538.

Lali, F.V., Hunt, A.E., Turner, S.J., Foxwell, B.M., 2000. The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. J. Biol. Chem. 275, 7395–7402.

Liao, C.K., Jeng, C.J., Wang, H.S., Wang, S.H., Wu, J.C., 2013. Lipopolysaccharide induces degradation of connexin43 in rat astrocytes via the ubiquitin-proteasome proteolytic pathway. PLoS One 8, e79350.

Liu, J., Jiang, C., Ma, X., Wang, J., 2018. Notoginsenoside Fc attenuates high glucose-induced vascular endothelial cell injury via upregulation of PPAR-gamma in diabetic Sprague-Dawley rats. Vasc. Pharmacol. 109, 27–35.

Liu, W.X., Wang, T., Zhou, F., Wang, Y., Xing, J.W., Zhang, S., Gu, S.Z., Sang, L.X., Dai, C., Wang, H.L., 2015. Voluntary exercise prevents colonic inflammation in high-fat diet-induced obese mice by up-regulating PPAR-gamma activity. Biochem. Biophys. Res. Commun. 459, 475–480.

Necela, B.M., Su, W., Thompson, E.A., 2008. Toll-like receptor 4 mediates cross-talk between peroxisome proliferator-activated receptor gamma and nuclear factor-kappaB in macrophages. Immunology 125, 344–358.

Panchapakesan, U., Pollock, C.A., Chen, X.M., 2004. The effect of high glucose and PPAR-gamma agonists on PPAR-gamma expression and function in HK-2 cells. Am. J. Physiol. Renal. Physiol. 287, F528–F534.

Pereira, V.H., Marques, F., Lages, V., Pereira, F.G., Patchev, A., Almeida, O.F., Almeida-Palha, J., Sousa, N., Cerqueira, J.J., 2016. Glucose intolerance after chronic stress is related with downregulated PPAR-gamma in adipose tissue. Cardiovasc. Diabetol. 15, 114.

Quincozes-Santos, A., Bobermin, L.D., de Assis, A.M., Goncalves, C.A., Souza, D.O., 2017. Fluctuations in glucose levels induce glial toxicity with glutamatergic, oxidative and inflammatory implications. Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1863, 1–14.

Ristow, M., 2004. Neurodegenerative disorders associated with diabetes mellitus. J. Mol. Med. (Berl.) 82, 510–529.

Sartippour, M.R., Renier, G., 2000. Differential regulation of macrophage peroxisome proliferator-activated receptor expression by glucose : role of peroxisome proliferator-activated receptors in lipoprotein lipase gene expression. Arterioscler. Thromb. Vasc. Biol. 20, 104–110.

Sergeeva, M.G., Aleshin, S.E., Grabeklis, S., Reiser, G., 2010. PPAR activation has dichotomous control on the expression levels of cytosolic and secretory phospholipase A_2 in astrocytes; inhibition in naive, untreated cells and enhancement in LPS-stimulated cells. J. Neurochem. 115, 399–410.

Sofroniew, M.V., 2014. Multiple roles for astrocytes as effectors of cytokines and inflammatory mediators. The Neuroscientist 20, 160–172.

Sofroniew, M.V., 2015. Astrocyte barriers to neurotoxic inflammation. Nat. Rev. Neurosci. 16, 249–263.

Tomlinson, D.R., Gardiner, N.J., 2008. Glucose neurotoxicity. Nat. Rev. Neurosci. 9, 36–45.

Wang, J., Li, G., Wang, Z., Zhang, X., Yao, L., Wang, F., Liu, S., Yin, J., Ling, E.A., Wang, L., Hao, A., 2012. High glucose-induced expression of inflammatory cytokines and reactive oxygen species in cultured astrocytes. Neuroscience 202, 58–68.

Zhang, M., Zhou, Z., Wang, J., Li, S., 2016. MiR-130b promotes obesity associated adipose tissue inflammation and insulin resistance in diabetes mice through alleviating M2 macrophage polarization via repression of PPAR-gamma. Immunol. Lett. 180, 1–8.

Webster, C.R., Srinivasulu, U., Ananthanarayanan, M., Suchy, F.J., Anwer, M.S., 2002. Protein kinase B/Akt mediates cAMP- and cell swelling-stimulated Na+/taurocholate cotransport and Ntcp translocation. J. Biol. Chem. 277, 28578–28583.

Zhang, X., Zhou, M., Guo, Y., Song, Z., Liu, B., 2015. 1,25-Dihydroxyvitamin D(3) promotes high glucose-induced M1 macrophage switching to M2 via the VDR-PPARgamma signaling pathway. BioMed Res. Int. 157834 2015.