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IL-1β potently stabilizes IL-6 mRNA in human astrocytes

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ABSTRACT

Uncontrolled expression of IL-6 in the central nervous system is associated with neurodegenerative pathology and glioma development. Astrocytes are the predominant source of IL-6 in the central nervous system, and they are characteristically susceptible to synergistic IL-6 expression. Combined β-adrenergic and TNF-receptor triggering induces synergistic IL-6 expression in 1321N1 cells via a transcriptional enhancer mechanism. Here, we have investigated the molecular basis of the very potent "super"-synergistic IL-6 expression that is apparent after combined treatment of astrocytes with a β-adrenergic agonist, isoproterenol, and the inflammatory cytokines TNF-α and IL-1β. We found that IL-1β treatment strengthens the IL-6 synergy by inducing a distinct stabilization of IL-6 mRNA. Surprisingly, the mRNA-stabilizing effect seems to be dependent on protein kinase C (PKC), but not on the prototypical mRNA-stabilizing kinase p38. Moreover, although the mRNA-binding protein HuR basally stabilizes IL-6 mRNA, the mRNA-stabilizing effect of IL-1β is independent of HuR. Our data using pharmacological inhibitors suggest PKC is an important modulator of IL-6 expression in the central nervous system and this might have therapeutic implications.

1. Introduction

Control of the expression levels of cytokines is essential for retaining cellular homeostasis and the orchestration of inflammation. In the central nervous system (CNS), excessive expression of cytokines, such as interleukin-6 (IL-6), has been associated with the pathogenesis and progression of different CNS affections [1,2].

For instance, IL-6 has been demonstrated to play an important role in the development and malignant progression of brain glioma by promoting angiogenesis, cell proliferation and resistance to apoptosis and radiation [3–5]. Moreover, a positive correlation between IL-6 gene expression and shortened survival in glioblastoma patients has been shown [6]. During the last decade, more insight has been gained into the physiological consequences of IL-6 upregulation in gliomas; however, the molecular mechanisms leading to excessive IL-6 expression in astrocytes remain largely unclear.

Apart from being involved in tumor development, IL-6 dysregulation in the CNS has been associated with various neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease. The accompanying neuro-inflammatory reaction is believed to become an enhancer of neurodegeneration at the moment it escapes the normal control mechanisms to restrict the expression of inflammatory mediators, such as cytokines [7].

To guarantee their transient expression, many cytokines contain AU-rich elements (AREs) in their 3′ untranslated regions (3′UTR). The AREs allow rapid mRNA degradation by promoting the recruitment of mRNA-destabilizing proteins to the 3′UTR. The IL-6 3′UTR contains six AREs [8]. In several systems, IL-6 has been described to have a short half life, varying from 30 min [8–10] to 50 min [11]. Several extracellular stimuli have been described to have a stabilizing effect on IL-6 mRNA, such as IL-1β [9,12]; IL-17 [13,14]; TNF-α [11] and IL-6 itself [10]. Relatively little is known about the signaling pathways responsible for IL-6 mRNA stabilization. It has been found by multiple groups that the mitogen-activated protein kinase (MAPK) p38 stabilizes IL-6 mRNA [12,15–18]. Another kinase often

Abbreviations: ARE, AU-rich element; CNS, central nervous system; COX-2, cyclooxygenase 2; ELAV, embryonic lethal abnormal vision; HuR, human antigen R; IL-6, interleukin-6; IL-1β, interleukin-1β; iso, isoproterenol; MAPK, mitogen-activated protein kinase; miRNA, microRNA; PKC, protein kinase C; TNF, tumor necrosis factor; 3′UTR, 3′ untranslated region; veh, vehicle.

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associated with mRNA stabilization is protein kinase C (PKC). PKC activation has been implicated in mRNA stabilization of, among others, GAP-43 [19,20], COX-2 [21], IL-1β [22] and p21 [23]. Moreover, PKC and p38 target several RNA-binding proteins that bind 3′UTRs and stabilize or destabilize the mRNA. More specifically for IL-6, direct binding of both AUF-1 [8] and HuR (also known as ELAV-1 (embryonic lethal, abnormal vision, Drosophila-like 1)) [24,25] has already been described, with the former protein both stabilizing and destabilizing IL-6 mRNA, depending on its expression level, and the latter stabilizing IL-6 mRNA.

Astrocytes are the main source of IL-6 in the CNS [26]. In light of the pathological consequences of uncontrolled IL-6 expression in the CNS, we aimed to unravel the pathways leading to IL-6 production in a human astrocytoma cell line. The 1321N1 cells used in this study were originally isolated from primary cultures of a cerebral glioma multiforme [27], and are often used as an astrocyte model [28]. We have shown before that combined β-adrenergic and TNF-receptor triggering induces synergistic IL-6 expression in 1321N1 cells via a transcriptional enhancer mechanism, involving the cooperative recruitment of CREB, NF-κB and CBP to the IL-6 promoter [29]. Here, we describe that IL-1β can further enhance IL-6 production via a post-transcriptional mechanism involving mRNA stabilization. Surprisingly, this effect does not involve a prototypical p38- or HuR-dependent mechanism and, based on pharmacological inhibitor experiments, seems to be PKC-dependent.

2. Materials and methods

2.1. Reagents

Isoproterenol, GF109203X, Brefeldin A (BFA) and actinomycin D were purchased from Sigma–Aldrich (St. Louis, MO). IL-1β was from Invitrogen (Carlsbad, CA). Anti-P-p65-Ser536, anti-P-ERK, anti-P-p38, anti-P-JNK and anti-P-CREB-Ser133 were from Cell Signaling (Danvers, MA); anti-p65 and anti-PARP were from Santa Cruz (Santa Cruz, CA) and anti-tubulin was from Sigma–Aldrich (St. Louis, MO). siHuR SMART pool (M-003773-04) and siControl (D-001210-05) were from Dharmacon (Lafayette, CO). SB203580 was from Alexis Biochemicals (Buttler Pike, PA), Ro31-8220 was from Calbiochem (Gibbstown, MJ). Recombinant human tumor necrosis factor-α (TNF-α) was obtained from the Department of Molecular Biology of Ghent University (DMBR, Ghent, Belgium) (with specific activity of 3.37×10^5 U/μg).

2.2. Cell culture

The human astrocytoma cell line 1321N1 was a kind gift from Prof. Dr. Müller (University of Bonn). 1321N1 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. All cells were passaged using 0.05% (w/v) of trypsin in 0.4% (w/v) EDTA. Cells were starved overnight prior to inductions in DMEM supplemented with 1% FCS.

2.3. Human IL-6 ELISA

Human IL-6 levels were determined using a specific ELISA kit (Biosource, Camarillo, CA) with detection limits of 5 pg/ml, according to manufacturer's instructions.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted with the acid-guanidinium-thiocyanate-phenol chloroform method using Trizol Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed on 0.5 μg of total mRNA using MMLV (Promega, Madison, WI). For real time cDNA amplification we used the Biorad SYBR Green Mastermix (Biorad, Hercules, CA) and the following primers: h-IL-6 fw: GAC AGC CAC TCA CCT CTT CA, h-IL-6 rv AGT GCC TCT TTG CTG CTT TC, h-HPRT fw TGA CAC TGG CAA AAC AAT GCA, h-HPRT rv GGT CCT TTT CAC CAG CAA GCT, h-COX-2 fw GCC CTT CCT CCT GTG CC, h-COX-2 rv AAT CAG GAA GCT GCT TTT TAC CTT T, h-IL-8 fw CTC TCT TGG CAG CCT TCC TGA, h-IL-8 rv CAA TAA TTT CTG TGT TGG CGC. Fluorescence was monitored using the BioRad iCycler (BioRad, Hercules, CA). A serial dilution of a cDNA mix standard was used to determine the efficiency of the PCR reaction and to calculate relative mRNA inputs. Absolute values were normalized to the HPRT reference gene.

2.5. Reporter gene assays

The IL-6 promoter luciferase construct containing 1168 bp of the human IL-6 promoter (1168-IL-6-luc) has been described before [30]. The 3′UTR IL-6 luciferase vector (IL-6 3′UTR-luc, with the IL-6 3′UTR (1–403) cloned in the pGL3 promoter vector, under control of an SV40 promoter) was a kind gift of Prof. Kirkwood (University of Michigan) and has been described before [15]. FLAG-HuR was a kind gift of Dr. Doller (J.W. Goethe-Universität, Frankfurt am Main) and has been described before [31]. Cells were seeded in 24-well plates (50,000 cells/well) and transfected using the calcium phosphate method with 0.8 μg IL-6 construct and 0.2 μg neogal constructs per well or with 0.5 μg of IL-6 constructs, 0.5 μg expression vector plasmid DNA and 0.2 μg neogal-construct. 48 h following transfection, cells were starved overnight and induced for the indicated time period. Total lysates were subsequently incubated with luciferase or galactosidase reagent and luminescence was measured on the Viktor3 system (Perkin Elmer Life Sciences, Boston, MA). Luciferase output was normalized to β-galactosidase values transcribed from the cotransfected galactosidase vector.

2.6. Western immunoblotting

Total cellular extracts were made in SDS sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.5% β-mercapto-ethanol). Equal amounts of total lysates from each condition were resolved by 10% SDS–PAGE, transferred onto nitrocellulose membranes and analysed by Western blotting. Chemiluminescent detection was performed using fluorophore-coupled secondary antibodies (Rockland, Gilbertsville, PA) in combination with the Odyssey Imaging System (Licor, Lincoln, NA). Quantification was performed using ImageJ software and based on 3 independent experiments. Tubulin was used as a loading control.

Nuclear and cytosolic extracts were obtained as described earlier [32]. Briefly, cells were lysed in B1 buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA pH 7.5, 0.1 mM EGTA pH 7.5, 0.5 mM DTT, protease inhibitors and 0.65%, v/v, NP40). Nuclei were pulled down by centrifugation at 800 rpm for 15 min and nuclear pellets were lysed in B2 buffer by shaking for 15 min at 4 °C (20 mM HEPES pH 7.5, 1% NP40, 1 mM $MgCl_2$, 400 mM NaCl, 10 mM KCl, 20% glycerol, 0.5 mM EDTA pH 7.5, 0.1 mM EGTA pH 7.5, 0.5 mM DTT and protease inhibitors). Lysates were subsequently analyzed by Western blotting. Fractionation was verified using tubulin as a cytoplasmic and PARP as a nuclear control.

2.7. siRNA silencing of HuR

For siRNA experiments, cells were seeded in 6-well plates (200,000 cells/well) and transfected using the calcium phosphate

method with a final concentration of 40 nM siGENOME SMART pools. siControl transfected samples were used to assess for aspecific effects. 48 h after transfection and after overnight starvation, cells were induced for the indicated time periods. Subsequently, cells were lysed in SDS sample buffer or RNA was isolated, as described in Sections 2.4 and 2.6.

2.8. Immunofluorescent PKC translocation assay

Immunofluorescence assay of PKC translocation was adjusted from [33]. Briefly, cells were starved overnight, followed by induction for the indicated time periods. Cells were fixed and permeabilized in ice cold methanol for 5 min at −20 °C. Next, samples were blocked for 1 h at room temperature with PBS 3% BSA and then washed 3 times with PBST (PBS with 0.1% Tween). Nuclei were coloured with DAPI and the coverslips were mounted using Mowiol (Calbiochem, Gibbstown, MJ). Samples were analyzed using an Axiovert 200M Zeiss Microscope (Thornwood, NY).

2.9. Actinomycin D assays

For actinomycin D assays, cells were seeded in 6-well plates (250,000 cells/well). After overnight starvation, cells were pre-induced with the indicated stimuli and/or inhibitors. Subsequently media were aspirated and replaced by starvation medium containing 5 μg/ml of actinomycin D to block transcription. After incubation for the indicated time periods with actinomycin D, RNA was isolated, reverse transcription was performed and cDNA was amplified using qPCR with SYBR Green and specific primers for IL-6. Absolute mRNA values were normalized to a housekeeping gene (HPRT) and subsequently recalculated as percentages of mRNA values after preinduction with the respective inductantia without actinomycin D treatment (0 min, 100%).

2.10. Analysis of let-7a expression levels

Let-7a expression levels were determined using a specific Taqman microRNA assay (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions. Briefly, 1321N1 cells were seeded in 6-well plates (250,000 cells/well), starved overnight and induced for 6 h. RNA was isolated as described under 2.4, and 100 ng of non-denatured RNA was used for RT-PCR using specific stem-loop primers. cDNA was subjected to qPCR using specific Taqman probes according to manufacturer's instructions. For normalization, the following small RNA controls were used: RNU6B, RNU44, and RNU58B. Stability of these small RNA controls was assessed using the geNorm algorithm implemented in the qbasePLUS Software package (Biogazelle, Ghent, Belgium) [34].

2.11. Statistical analysis

Results are represented as mean values ± standard error of mean (SEM). Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's multiple comparison test, except in Figs. 2, 4 and 6Figs. 2i, 4d and 6b where a Student's *t*-test was used. Both tests were performed using Graphpad Prism 4 software (Graphpad Software Inc., San Diego, CA). Results were considered as significant when $P < 0.05$.

3. Results

3.1. IL-1β potently enhances the isoproterenol/TNF-α-induced IL-6 synergy

We have shown before that combined β-adrenergic and TNF receptor triggering elicits synergistic IL-6 expression in human astrocytoma 1321N1 cells at protein, mRNA and promoter level [29]. Here we show that addition of the pro-inflammatory cytokine IL-1β to the isoproterenol and TNF-α treatment protocol substantially strengthened this synergistic IL-6 production, inducing a "super"-synergy. This synergy is prominently present at the IL-6 protein level, as measured by ELISA (Fig. 1a), as well as at the mRNA level, as measured by RT-qPCR (Fig. 1b). Analysis of the ratio of IL-6 mRNA after triple (iso + TNF + IL) versus double (iso + TNF) induction indicates a 10.9 ± 1.32-fold enhancement.

3.2. IL-1β does not enhance isoproterenol/TNF-α-induced IL-6 promoter activation

The prototypical signaling cascades activated by isoproterenol, TNF-α and IL-1β were screened to investigate if (a) there was synergistic activation of one of the downstream kinases or transcription factors and (b) if there were factors that were exclusively activated by IL-1β, thereby explaining the synergy. The MAP kinases p38, JNK and ERK1/2, previously shown to be potently activated by TNF-α in 1321N1 cells [29], were also activated by IL-1β (Fig. 2a, b, c and f). However, triple induction did not lead to synergistic activation of any of the MAP kinases compared to double induction. Additionally, combined isoproterenol and TNF-α treatment activated CREB [29], and IL-1β also induced minor CREB phosphorylation, but again triple induction

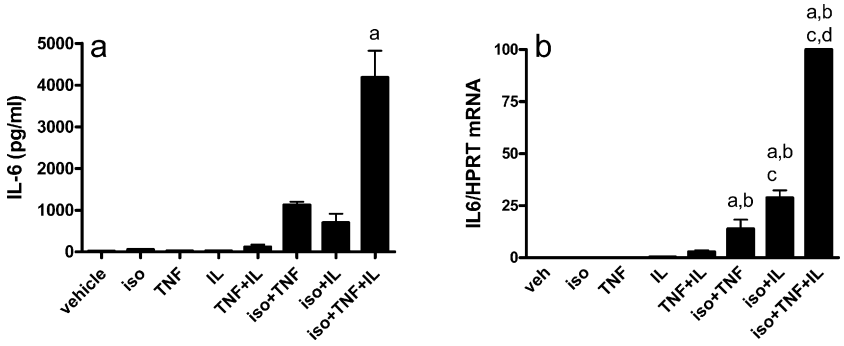

Fig. 1. Synergistic IL-6 induction at the protein and mRNA level, after combined induction with isoproterenol, TNF-α and IL-1β in 1321N1 cells. (a) IL-6 secretion is synergistically induced in 1321N1 cells by various combinations of isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml) and IL-1β (IL, 2 ng/ml). Cells were induced for 6 h, after which medium was collected and IL-6 concentrations in medium were determined by ELISA. Data shown are means of 3 independent experiments, each performed in triplicate. [a]$P < 0.05$ versus all other treatments (veh, iso, TNF, IL, TNF + IL, iso + TNF and iso + IL). (b) IL-6 mRNA expression is synergistically induced in 1321N1 cells by combinations of isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml) and IL-1β (IL, 2 ng/ml). Cells were induced for 6 h, and RT-qPCR analysis was performed. Data shown are the means of 4 independent experiments, with [a]$P < 0.05$ versus all individual inductions (veh, iso, TNF, IL); [b]$P < 0.05$ versus TNF + IL; [c]$P < 0.05$ versus iso + TNF; [d]$P < 0.05$ versus iso + IL.

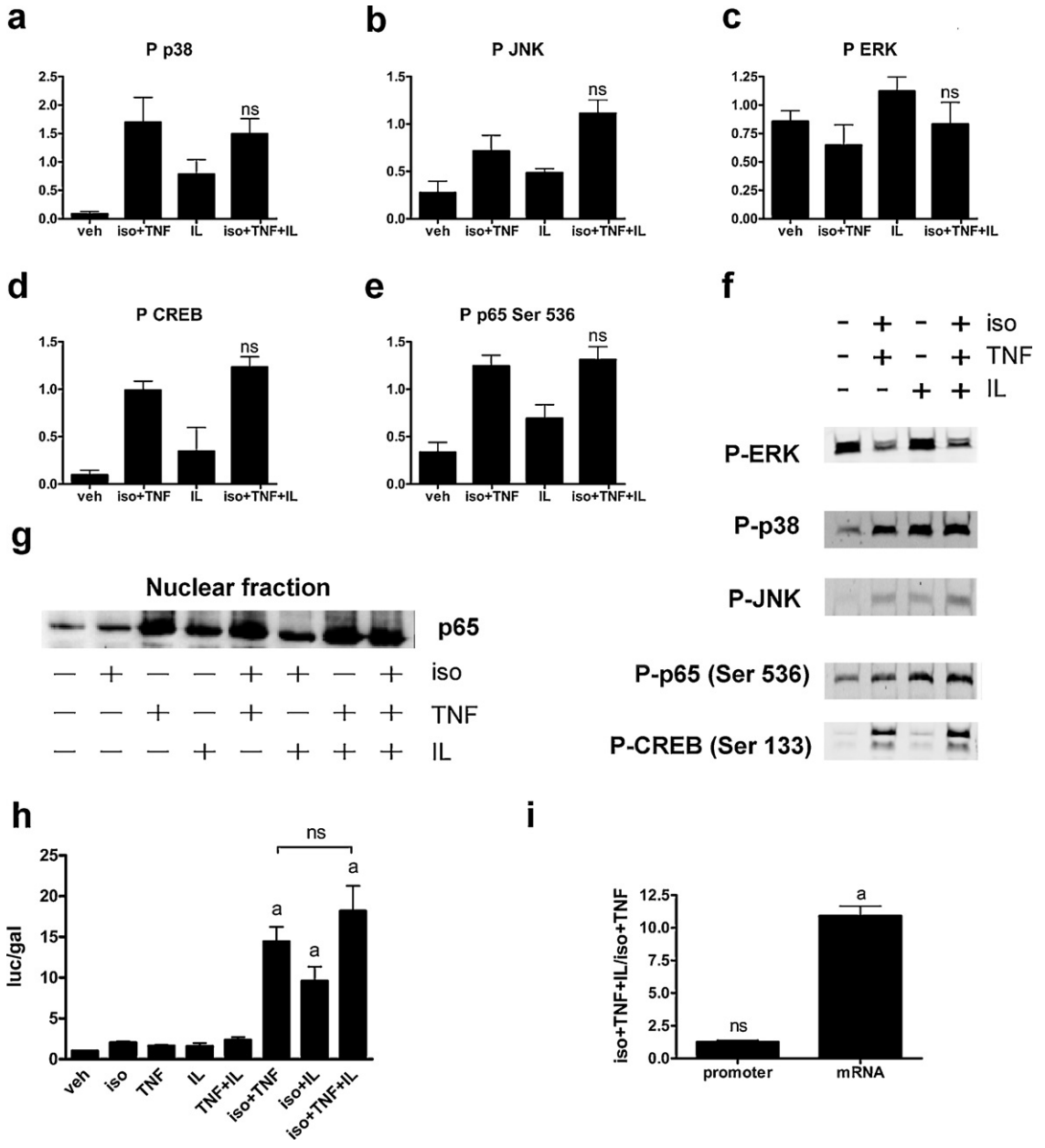

Fig. 2. Effect of combined induction with isoproterenol, TNF-α and IL-1β on downstream signaling cascades and IL-6 promoter activation. (a–e) After induction of 1321N1 cells for 30 min with vehicle (veh), isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml) and IL-1β (IL, 2 ng/ml) individually or combined, cells were lysed in SDS sample buffer and subjected to Western analysis. Activation of p38 (a), JNK (b), ERK (c), CREB (d) or p65 (e) pathways was assessed using phospho-specific antibodies and tubulin as a loading control. Three independent experiments were used for quantification of Western blots. $^{ns}P > 0.05$ versus iso + TNF. (f) A representative blot after treatment of 1321N1 cells for 30 min as described under (a–e). (g) Effect on nuclear translocation of p65. 1321N1 cells were induced with isoproterenol (10 μM), TNF-α (2000 IU/ml), IL-1β (2 ng/ml) or combinations thereof for 30 min. Subsequently nuclear extracts were subjected to Western blot analysis to determine p65 levels. Data shown are representative for 3 independent experiments. (h) IL-6 promoter activation after treatment with isoproterenol, TNF-α and IL-1β. 1321N1 cells were transiently transfected with an IL-6 promoter construct (1168-IL-6-luc). Reporter gene activation was measured with a luciferase reporter gene assay after induction for 6 h with isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml) and IL-1β (IL, 2 ng/ml), individually or combined. Data shown are means of 5 independent experiments. $^{a}P < 0.05$ versus all individual inductions (veh, iso, TNF, IL); $^{ns}P > 0.05$ for iso + TNF versus iso + TNF + IL. (i) Comparison of the ratio of triple (iso + TNF + IL) versus double (iso + TNF) induction at mRNA and promoter level. Mean ratios were calculated on the basis of 3–4 independent experiments, performed as described under Fig. 1b (mRNA level) and 2h (promoter level). $^{a}P < 0.05$ versus promoter; $^{ns}P > 0.05$ in one sample *t*-test with the theoretical mean of 1.

did not induce synergistic CREB activation as compared to double induction (Fig. 2d and f). Lastly, NF-κB activation, measured by translocation (Fig. 2g) and Serine 536 phosphorylation of p65 (Fig. 2e and f), was induced by both iso + TNF and IL-1β. Again triple induction did not lead to synergistic NF-κB activation compared to double induction at 30 min. Investigation of the main signaling cascades activated by isoproterenol, TNF-α and IL-1β, thus showed that IL-1β did not activate any 'extra' signaling cascades, i.e. that were not already activated by isoproterenol or TNF-α, nor did IL-1β induce synergistic activation of any of the investigated factors. In line with this, we found that, at the promoter level, as measured by a reporter gene assay, triple induction did not significantly strengthen promoter activation, as compared to double induction (Fig. 2h). The ratio of the triple versus double induction was reduced to 1.3 ± 0.28 at the promoter level (Fig. 2i). This ratio does not differ significantly from 1 ($P = 0.0959$), which indicates that the synergy has disappeared from mRNA to promoter level.

3.3. IL-1β stabilizes IL-6 mRNA

Because the strengthening effect of IL-1β was not conserved at the promoter level, a potential explanation for the synergy could be the stabilization of IL-6 mRNA by IL-1β. It has been shown that IL-1β stabilizes multiple mRNA species, including IL-6, in a variety of cell types [9,12,14,16,35,36]. A paradigm commonly used to study effects on mRNA stabilization is the blockade of new transcription by a transcriptional inhibitor, such as actinomycin D, followed by quantification of the mRNA levels at several time points after inhibition of transcription [37]. This allows measuring the degradation rate of a certain mRNA species. When we investigated the degradation rate of IL-6 mRNA after double versus triple induction, we observed a very clear stabilizing effect of IL-1β. Double induction with isoproterenol and TNF-α yielded IL-6 mRNA with a half-life of approximately 60 min. Adding IL-1β to the isoproterenol/TNF-α induction clearly stabilized the IL-6 mRNA by blocking virtually all IL-6 mRNA degradation (Fig. 3a). This effect was sustained for up to 6 h after addition of actinomycin D (Supplementary data, Fig. 1). We also investigated whether IL-1β induces mRNA stabilization on its own, and, although the IL-1β-mediated mRNA induction is relatively small, complicating establishment of the degradation rate, it was clear from our experiments that IL-1β by itself also stabilizes IL-6 mRNA (Supplementary data, Fig. 2). Furthermore, we analyzed the degradation rates of two other genes that are induced by isoproterenol, TNF-α and IL-1β in 1321N1 cells, namely COX-2 and IL-8 [29,38]. COX-2 showed a synergistic mRNA induction and an mRNA degradation pattern that was very comparable to the one observed for IL-6 (Fig. 3c and d). Triple induction stabilized COX-2 mRNA completely compared to double induction. IL-8, on the other hand, showed a slower, but progressive mRNA degradation pattern, with no significant stabilization by IL-1β at the earlier time points (60 and 120 min, Supplementary data, Fig. 3).

As the 3′UTR is the main target for mRNA-(de)stabilizing mechanisms, we also studied IL-6 mRNA stability using a reporter gene assay. In this system, the IL-6 3′UTR is cloned behind the luciferase coding sequence, thus reporting 3′UTR-mediated effects on mRNA stability via changes in the luciferase signal. Both the double induction with isoproterenol and TNF-α and the individual induction with IL-1β induced a small, but insignificant increase in the RLU values after 6, 16 and 24 h induction (Fig. 3b). Only the triple induction with isoproterenol, TNF-α and IL-1β elicited a significant increase in RLU values after 6, 16 and 24 h, indicating luciferase mRNA stabilization.

3.4. The effect of IL-1β seems to be mediated by PKC and not by p38

Two kinases that have been particularly implicated in mRNA-stabilizing effects are p38 and PKC [39,40]. Importantly, both can be activated by IL-1β and have been convincingly implicated in COX-2 mRNA stabilization [21,31,41–46]. Moreover, in multiple studies, IL-1β-induced mRNA stabilization of IL-6 was shown to be crucially dependent on p38 [12,16], whereas dependence on PKC has not yet been described. Therefore, we used pharmacological inhibitors of p38 and PKC to investigate if they are involved in the mRNA-stabilizing effect of IL-1β in our experimental system. When we blocked p38 with SB203580, IL-1β was still able to stabilize IL-6 mRNA in the actinomycin D assay (Fig. 4a), indicating that p38 activation is not necessary for IL-6 mRNA stabilization. The effectiveness of SB203580 was demonstrated by the complete inhibition of p38-mediated MSK phosphorylation after treatment with the inhibitor (Fig. 4b). On the other hand, when we blocked PKC with GF109203X, we observed complete inhibition of the

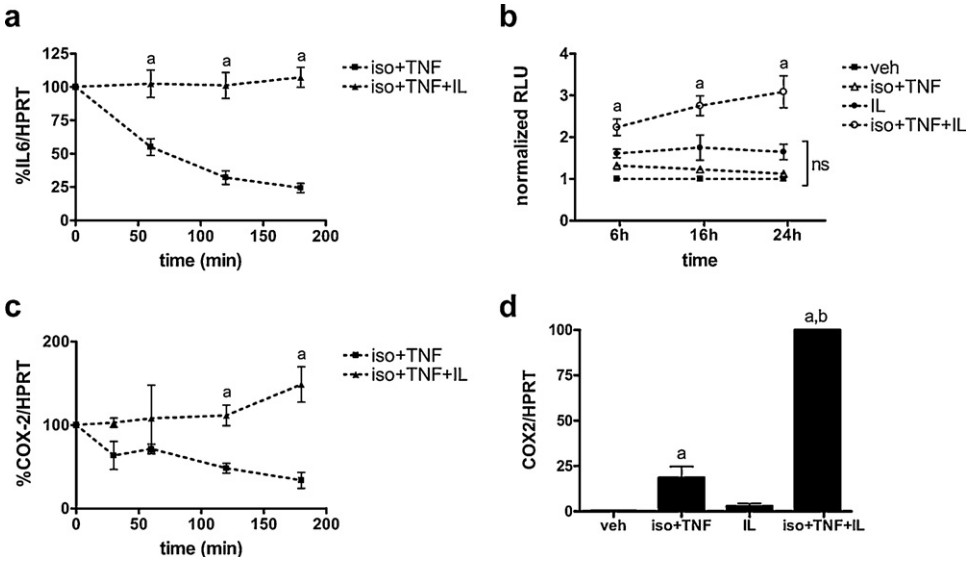

Fig. 3. Effect of isoproterenol, TNF-α and IL-1β treatment on IL-6 and COX-2 mRNA stability. (a) mRNA degradation rates were determined using actinomycin D. 1321N1 cells were pretreated for 6 h with isoproterenol (10 μM) + TNF-α (2000 IU/ml) (iso + TNF) or isoproterenol (10 μM) + TNF-α (2000 IU/ml) + IL-1β (2 ng/ml) (iso + TNF + IL). Subsequently, iso, TNF-α and IL-1β were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR analysis. Data shown are means of 8 independent experiments. $^{a}P < 0.05$ versus iso + TNF at that time point. (b) Kinetics of mRNA stability as measured by a reporter gene assay. 1321N1 cells were transiently transfected with an IL-6 3′UTR construct (IL-6 3′UTR-luc). Reporter gene expression was measured with a luciferase reporter gene assay after induction for 6, 16 and 24 h with isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml) and IL-1β (IL, 2 ng/ml), individually or combined. Data shown are mean values calculated from 4 independent experiments. $^{a}P < 0.05$ versus iso + TNF at that time point; $^{ns}P > 0.05$ between the inductions with iso + TNF, IL and veh at the corresponding time points. (c) mRNA degradation rates were determined using actinomycin D. 1321N1 cells were pretreated for 6 h with isoproterenol (10 μM) + TNF-α (2000 IU/ml) (iso + TNF) or isoproterenol (10 μM) + TNF-α (2000 IU/ml) + IL-1β (2 ng/ml) (iso + TNF + IL). Subsequently iso, TNF-α and IL-1β were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 30, 60, 120 and 180 min) and subjected to RT-qPCR analysis. Data shown are mean values calculated from 3 independent experiments. $^{a}P < 0.05$ versus iso + TNF at that time point. (d) COX-2 mRNA expression is synergistically induced in 1321N1 cells by combinations of isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml) and IL-1β (IL, 2 ng/ml). Triple (iso + TNF + IL) versus double (iso + TNF) induction clearly induces a "super"synergy. Cells were induced for 6 h, after which RT-qPCR analysis was performed. Data shown are mean values of 3 independent experiments. $^{a}P < 0.05$ versus veh; $^{b}P < 0.05$ versus iso + TNF.

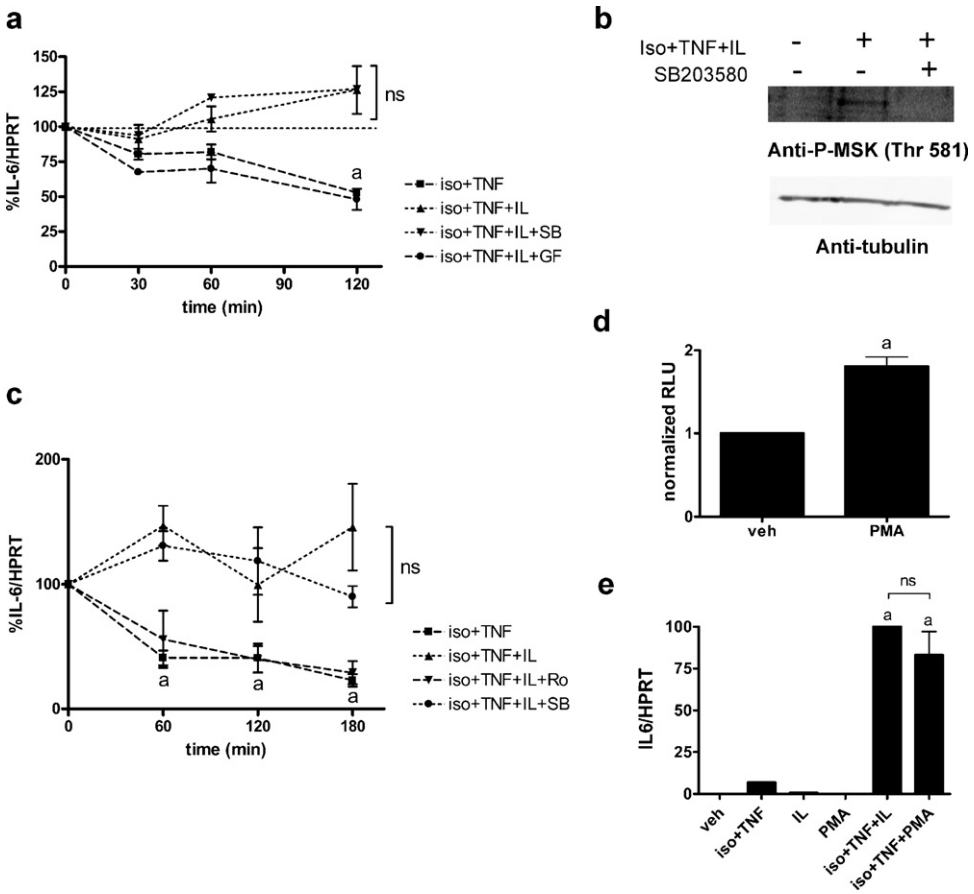

Fig. 4. Effect of pharmacological inhibitors of p38 and PKC on IL-1β-induced mRNA stability of IL-6 mRNA. (a) Effect of a p38 inhibitor (SB) and a PKC inhibitor (GF) on mRNA degradation rate of IL-6, determined using actinomycin D. 1321N1 cells were pretreated for 6 h with isoproterenol (10 μM) + TNF-α (2000 IU/ml) (iso + TNF) or isoproterenol (10 μM) + TNF-α (2000 IU/ml) + IL-1β (2 ng/ml) (iso + TNF + IL) with or without SB203580 (SB, 10 μM) or GF109203X (GF, 10 μM). Subsequently iso, TNF-α, IL-1β, SB and GF were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 30, 60 and 120 min) and subjected to RT-qPCR analysis. Data shown are mean values calculated from 3 independent experiments. $^{a}P < 0.05$ between iso + TNF + IL + GF versus iso + TNF + IL at that time point; $^{ns}P > 0.05$ between iso + TNF + IL versus iso + TNF + IL + SB at the corresponding time points. (b) Effectiveness of SB203580. 1321N1 cells were induced for 30 min using isoproterenol (10 μM), TNF-α (2000 IU/ml) and IL-1β (2 ng/ml) (iso + TNF + IL) with or without SB230580 (10 μM). Lysates were subjected to Western analysis. Activation of MSK was assessed using a phosphospecific antibody (anti-P-MSK (Thr 581)). (c) Effect of a p38 inhibitor (SB) and a PKC inhibitor (Ro) on the mRNA degradation rate of IL-6, determined using actinomycin D. 1321N1 cells were pretreated for 6 h with isoproterenol (10 μM) + TNF-α (2000 IU/ml) (iso + TNF) or isoproterenol (10 μM) + TNF-α (2000 IU/ml) + IL-1β (2 ng/ml) (iso + TNF + IL) in combination with SB203580 (SB, 10 μM) or Ro31-8220 (Ro, 5 μM). Subsequently, iso, TNF-α, IL-1β, SB and Ro were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR. Data shown are mean values calculated from 3 independent experiments. $^{a}P < 0.05$ iso + TNF + IL + Ro versus iso + TNF + IL at that time point; $^{ns}P > 0.05$ between iso + TNF + IL versus iso + TNF + IL + SB at the corresponding time points. (d) PMA mimics IL-1β-induced stabilization of the IL-6 3′UTR reporter gene. 1321N1 cells were transiently transfected with an IL-6 3′ UTR construct (luc-IL-6 3′UTR). Reporter gene expression was measured with a luciferase reporter gene assay after induction for 6 h with vehicle (veh) or PMA (1 μg/ml). Data shown are mean values calculated from at least 5 independent experiments. $^{a}P = 0.0021$ in a two-tailed *t*-test versus veh. (e) PMA mimics IL-1β-induced 'super'synergistic IL-6 mRNA expression. IL-6 mRNA expression is synergistically induced in 1321N1 cells by combinations of isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml), IL-1β (IL, 2 ng/ml) and PMA (1 μg/ml). Triple induction with iso + TNF + PMA mimics the triple induction with iso + TNF + IL-1β. Cells were induced for 6 h, and RT-qPCR analysis was performed. Data shown are mean values of 3 independent experiments. $^{a}P < 0.05$ versus iso + TNF; $^{ns}P > 0.05$ for iso + TNF + PMA versus iso + TNF + IL.

stabilizing effect of IL-1β (Fig. 4a). This suggests that IL-1β stabilizes IL-6 mRNA via PKC. To further substantiate the finding that PKC was involved, we used a second PKC inhibitor, namely Ro31-8220. Again we observed complete inhibition of the IL-1β-mediated IL-6 mRNA stabilization (Fig. 4c), whereas parallel inhibition by SB203580 had no effect. Additional evidence for the involvement of PKC came from the ability of PMA, a PKC activator, to mimic IL-1β-induced stabilization of the IL-6 3′UTR reporter gene (Fig. 4d). Moreover, like IL-1β, PMA also induced 'super'-synergistic IL-6 mRNA production, when added to isoproterenol and TNF-α, imitating the super-synergy induced by IL-1β (Fig. 4e).

3.5. Identification of the PKC isoform involved

PKC is a family of kinases composed of 10 isoforms [47]. The family is subdivided into 3 classes, based on their Ca^{2+}, diacylglycerol and phospholipid dependency, namely, the classical (α, βI, βII, γ), novel (η, θ, δ, ε) and atypical PKCs (ι, ζ). In order to identify the PKC isoform responsible for the IL-1β-induced IL-6 mRNA stabilization, we set up a large screening experiment using different previously validated PKC-GFP constructs [31,48–52]. Our aim was to assess which PKC isoform was activated after triple induction, as manifested by PKC translocation to the membrane, and sometimes also to the nucleus. However, at various time points ranging from 1 min to 60 min, we did not observe translocation of any of the 10 isoforms after triple induction, even though PMA, used as a positive control, induced clear membrane translocation of the classical and novel isoforms. An exemplary image of PKC distribution after triple induction or PMA treatment is shown in Supplementary data, Fig. 4 for the various PKC isoforms at the 30 min time point. Similar results were obtained at earlier and

later time points (1 min, 5 min, 10 min, 15 min, 30 min, 60 min, 6 h; data not shown).

3.6. The IL-1β-induced IL-6 mRNA stabilization is a late, indirect effect, independent of protein secretion

Since we could block the IL-1β-induced IL-6 mRNA stabilization by PKC inhibitors on the one hand, but did not observe rapid PKC activation after triple induction on the other hand, we hypothesized that the mRNA-stabilizing effect of IL-1β was possibly indirect and delayed. In line with this, the full-blown IL-1β-mediated mRNA-stabilizing effect was not apparent when we reduced the preinduction time to 1 or 3 h in our actinomycin D experiments (Fig. 5a). Similarly, IL-1β did not induce COX2 mRNA stabilization after 1 or 3 h preincubation with isoproterenol, TNF-α and IL-1β combined (Fig. 5b). These data suggest that although IL-1β stabilizes IL-6 mRNA, it does so via triggering a late-onset, possibly indirect, mechanism. To investigate if a secreted factor was responsible for the effect of IL-1β, we blocked secretion of proteins using brefeldin A (BFA). Pretreatment with 5 μg/ml BFA did not inhibit IL-1β-induced IL-6 mRNA stabilization (Fig. 5c). The effectiveness of BFA was demonstrated by visualizing the Golgi network, using the Golgi-marker GM130 (Fig. 5d). Treatment of 1321N1 cells with BFA resulted in the transformation of the discrete perinuclear, vesicular structure to a largely dispersed, dotted structure, reflecting the desintegration of the Golgi network. In addition, we could not mimic the IL-6 mRNA-stabilizing effect by using conditioned medium fractions, suggesting the mRNA-stabilizing effect is not dependent on an IL-1β-induced secreted factor (data not shown). We also investigated the role of novel protein synthesis in the stabilizing effect of IL-1β, using the translational elongation inhibitor cycloheximide. However, as described before by several other authors, translational inhibition led to "superinduction" of IL-6 mRNA [35,37] (data not shown). This effect has been attributed to the freezing of the translation machinery on the IL-6 mRNA, thereby protecting it from degradation, and thus rules out the possibility of using translational inhibitors to study whether protein synthesis is

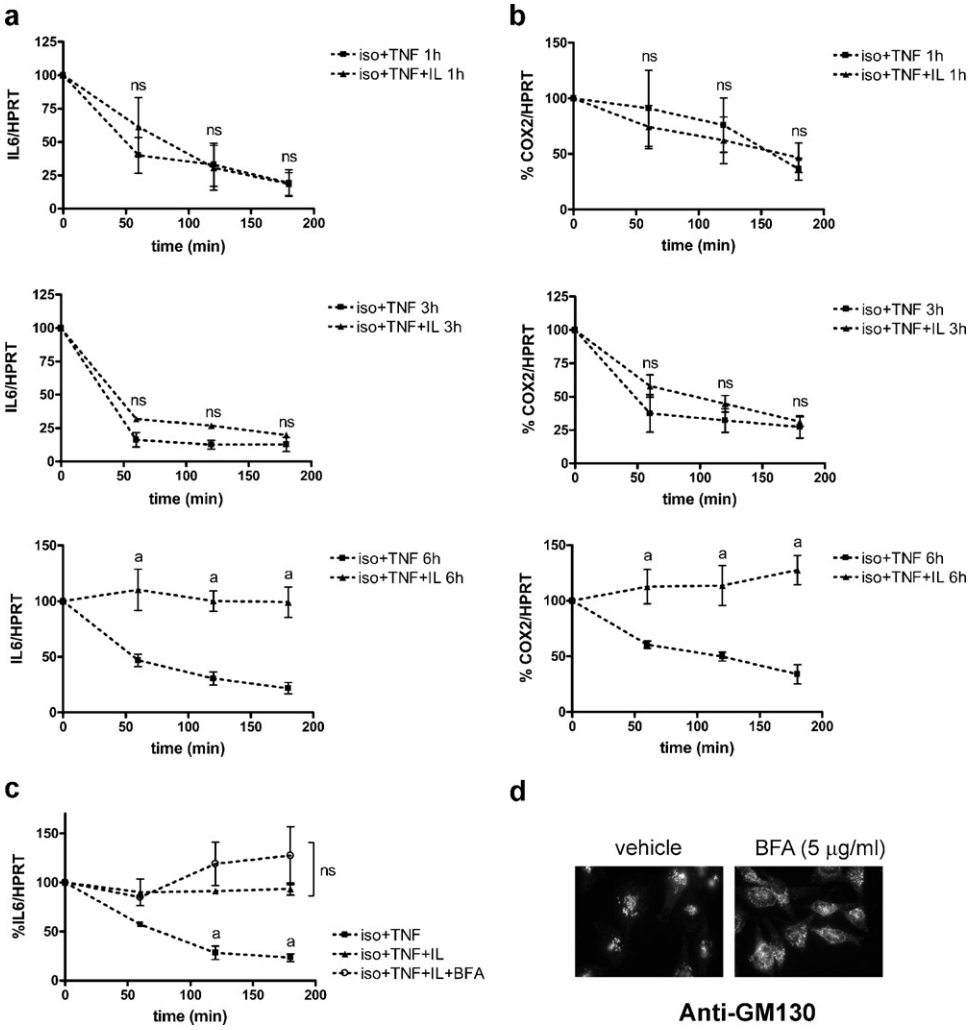

Fig. 5. Effect of kinetic preinduction on IL-6 mRNA degradation rate. (a and b) 1321N1 cells were preinduced with isoproterenol (10 μM) and TNF-α (2000 IU/ml) (iso + TNF) or isoproterenol (10 μM), TNF-α (2000 IU/ml) and IL-1β (2 ng/ml) (iso + TNF + IL) for 1, 3 or 6 h. Subsequently iso, TNF-α and IL-1β were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR. Data are means of 3–4 independent experiments. $^{ns}P > 0.05$ between iso + TNF versus iso + TNF + IL at that time point; $^{a}P < 0.05$ versus iso + TNF at that time point. (c) Effect of blocking protein secretion using brefeldin A (BFA) on IL-6 mRNA degradation rate. 1321N1 cells were preinduced for 30 min with or without BFA (5 μg/ml) and subsequently induced with isoproterenol (10 μM) and TNF-α (2000 IU/ml) (iso + TNF) or isoproterenol (10 μM), TNF-α (2000 IU/ml) and IL-1β (2 ng/ml) (iso + TNF + IL) for 6 h. Iso, TNF-α, IL-1β and BFA were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR. Data shown are means of 2 independent experiments. $^{ns}P > 0.05$ between iso + TNF + IL versus iso + TNF + IL + BFA for the corresponding time points; $^{a}P < 0.05$ versus iso + TNF + IL at that time point. (d) Effectiveness of brefeldin A. Cells were treated with BFA (5 μg/ml) for 6.5 h and fixed with formaldehyde. Immunofluorescence staining was performed using an antibody for a Golgi marker (anti-GM130) to visualize the Golgi compartment.

required for IL-1β induced IL-6 mRNA stabilization [35]. However, when 1321N1 cells are treated with IL-1β in the presence of actinomycin D (instead of the here employed treatment with IL-1β before actinomycin D treatment), IL-6 mRNA is no longer stabilized, indicating the importance of transcription for the mRNA stabilizing effect of IL-1β (data not shown).

3.7. IL-1β does not stabilize IL-6 mRNA via a prototypical HuR-dependent pathway

One of the main mRNA-stabilizing proteins, is the ELAV protein HuR, which upon stimulation with mRNA-stabilizing stimuli translocates from the nucleus to the cytoplasm where it binds and stabilizes the 3′UTRs of several mRNAs [53]. HuR has previously been shown to stabilize IL-6 [24,25] and COX-2 mRNA [21,31,54]. Moreover, HuR is a major effector of PKC-mediated mRNA stabilization [21,31,54] and can be induced by IL-1β [55,56]. Therefore, we sought to determine if HuR was involved in the IL-6 mRNA-stabilizing effect of IL-1β.

First, we analyzed the effect of overexpressing HuR in the IL-6 3′UTR luciferase assay. Although HuR overexpression did clearly enhance the basal luciferase signal (Fig. 6a), there was no effect on the magnitude of the triple induction (Fig. 6b). This suggests that HuR is able to stabilize IL-6 mRNA via its 3′UTR, which corresponds to findings in other studies, but nevertheless the effect of IL-1β does not seem to be mediated via HuR. In line with this, we found that the nuclear/cytoplasmic distribution of endogenous HuR after triple induction at different time points (0–6 h) did not change (Fig. 6c). In 1321N1 cells, HuR is already present in the cytoplasm in small amounts, possibly due to the fact that they are tumor-

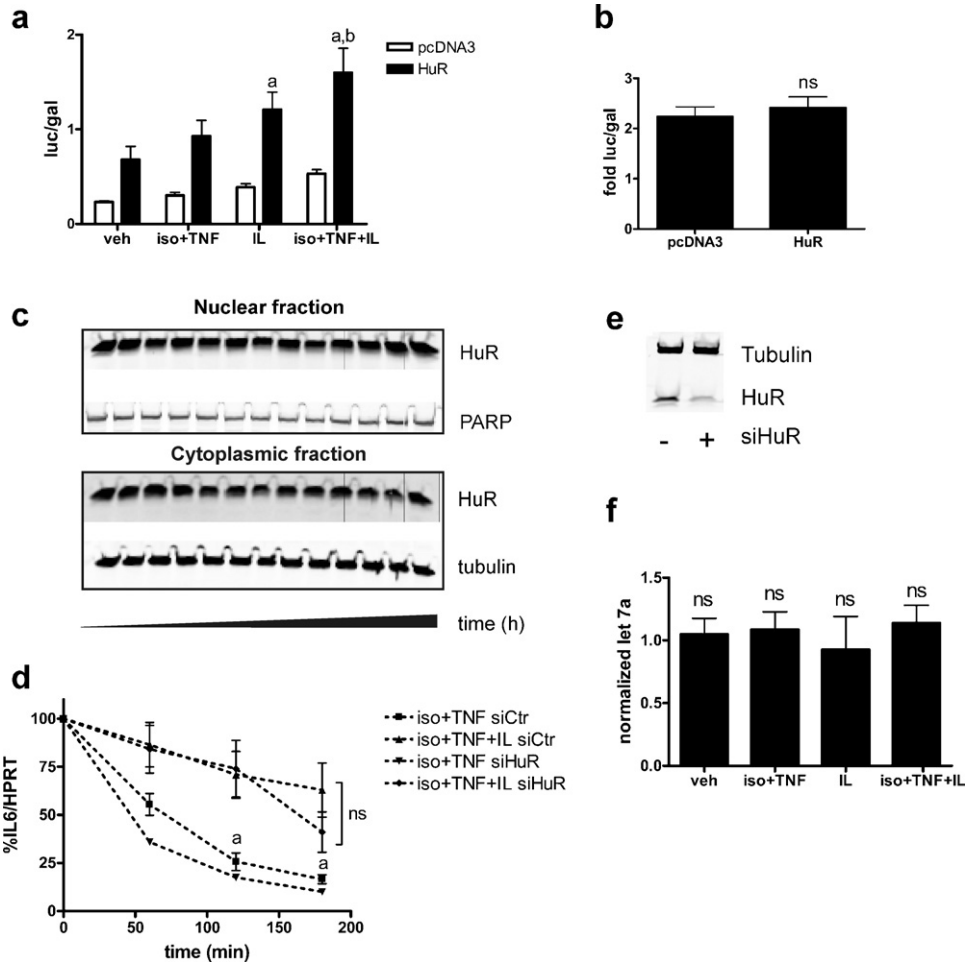

Fig. 6. Involvement of the mRNA stabilizing protein HuR and let-7a in IL-1β-induced IL-6 mRNA stabilization. (a) Effect of overexpression of HuR on an IL-6 3′UTR reporter gene assay. 1321N1 cells were transiently transfected with an IL-6 3′ UTR construct (luc-IL-6 3′UTR) combined with an empty vector (pcDNA3) or a HuR expression vector (HuR). Reporter gene expression was measured with a luciferase reporter gene assay after induction for 6 h with isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml) and IL-1β (IL, 2 ng/ml), individually or combined. Data are means of 3 independent experiments. $^{a}P < 0.05$ versus the corresponding pcDNA3 condition; $^{b}P < 0.05$ versus the corresponding veh condition. (b) The fold enhancement after triple induction with isoproterenol, TNF-α and IL-1β is not affected by HuR overexpression. 1321N1 cells were transfected, induced and subjected to reporter gene analysis as described under (a). Values were obtained by normalizing to non-induced condition (vehicle). $^{ns}P > 0.05$ versus pcDNA3. (c) Nuclear–cytoplasmic shuttling of HuR after induction for increasing time points. 1321N1 cells were treated with isoproterenol (10 μM), TNF-α (2000 IU/ml) and IL-1β (2 ng/ml) for 0–6 h with 30 min intervals. Nuclear and cytoplasmic fractions were isolated and subjected to Western analysis. Data shown are representative for 3 independent experiments. (d) Effect of knockdown of HuR using siRNA on IL-6 mRNA degradation rate, determined using actinomycin D. 1321N1 cells were transiently transfected with siControl (siCtr, 40 nM) or siHuR (40 nM). 48 h after transfection, cells were pretreated for 6 h with isoproterenol (10 μM) + TNF-α (2000 IU/ml) (iso + TNF) or isoproterenol (10 μM) + TNF-α (2000 IU/ml) + IL-1β (2 ng/ml) (iso + TNF + IL). Subsequently iso, TNF-α and IL-1β were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR. Data are means of 3 independent experiments. $^{a}P < 0.05$ between iso + TNF siCtr versus iso + TNF + IL siCtr for that time point; $^{ns}P > 0.05$ between iso + TNF + IL siCtr versus iso + TNF + IL siHuR for the corresponding time points. (e) Control of knockdown of HuR. 1321N1 cells were transiently transfected with siControl (40 nM) or siHuR (40 nM). 48 h after transfection, cells were lysed in SDS sample buffer and subjected to Western analysis. (f) Analysis of let-7a expression levels. 1321N1 cells were induced for 6 h with combinations of isoproterenol (iso, 10 μM), TNF-α (TNF, 2000 IU/ml) and IL-1β (IL, 2 ng/ml). RNA was isolated and let-7a expression levels were determined using a specific Taqman microRNA assay. Data shown are the mean of 4 independent experiments. $^{ns}P > 0.05$ between all treatments.

derived cells [57]. Since HuR has also been shown to stabilize mRNAs in response to IL-1β in the absence of cytoplasmic translocation, but via direct binding to the 3′UTR [56], we next employed an siRNA approach to finally exclude the involvement of HuR. Knockdown of HuR using siRNA did not inhibit IL-1β mediated IL-6 mRNA stabilization (Fig. 6d), even though there was substantial knockdown of HuR protein as assessed by Western blot (Fig. 6e) indicating HuR was not involved in IL-1β-mediated IL-6 mRNA stabilization.

3.8. Let-7a microRNA is not involved in the IL-1β-induced IL-6 mRNA stabilization

Because a prototypical p38- or HuR-dependent mechanism did not seem to be involved in IL-6 mRNA stabilization in our experimental system, we additionally investigated whether microRNAs (miRNAs) played a role in IL-6 mRNA stabilization [58]. miRNAs are particularly abundant in the brain and are suggested to have a pivotal role in several aspects of CNS functioning [59]. Recently, the let-7a miRNA was shown to bind the IL-6 3′UTR [60]. The let-7 miRNA family is comprised of 12 members. Importantly, it is was shown that NF-κB activation led to downregulation of let-7a and this subsequently leads to upregulation of IL-6, resulting in a positive feedback loop [60]. Since IL-1β has been shown to cause long-term, persistent NF-κB activation in 1321N1 cells [28], we hypothesized that IL-1β might downregulate let-7a in 1321N1 cells. However, when we analyzed let-7a levels after treatment with isoproterenol, TNF-α and IL-1β, alone or in combinations, we did not observe significant differences in the expression level of this miRNA, indicating that let-7a is not involved in the IL-6 mRNA-stabilizing effect of IL-1β in 1321N1 cells (Fig. 6f).

4. Discussion

Here, we have reported that IL-1β potently enhances TNF-α/isoproterenol-induced IL-6 expression in astrocytes. Susceptibility to synergy is a hallmark of IL-6 expression by astrocytes [26]. IL-6 has paradoxical effects in the CNS, combining pro-inflammatory and neurotrophic traits [61], and dose and timing of IL-6 expression are regarded as crucial determinants in shifting the balance between beneficial and detrimental effects. Therefore, it is important to understand the molecular mechanisms underlying synergistic IL-6 expression in astrocytes.

In 1321N1 cells, IL-1β did not enhance isoproterenol- and TNF-induced IL-6 expression via a transcriptional mechanism, but instead very potently enhanced IL-6 mRNA stability. IL-1β has been previously demonstrated to stabilize multiple mRNA species, such as COX-2 [42,43,62–64], IL-8 [56] and NGF [65,66]. Importantly, it has already been reported that IL-1β stabilizes IL-6 mRNA in osteoblasts [16,35], fibroblasts [9], myofibroblasts [14], astrocytes [36] and fibroblast-like synoviocytes [12], although relatively little was known about the molecular mechanisms underlying this stabilization. Moreover, other studies found no stabilization of IL-6 mRNA after IL-1β induction, but instead found that TNF-α [11] or cAMP [67] were the stabilizing agents in this instance, indicating the cell type specificity of IL-1β-mediated mRNA stabilization.

Two kinases in particular emerge as possible candidates for mediating IL-1β-induced IL-6 mRNA stability, namely p38 and PKC. p38 has been identified as an essential kinase in regulating IL-1β-mediated mRNA stabilization in multiple studies [12,15–17,41–44,56], of which several have specifically investigated IL-1β-mediated COX-2 and IL-6 mRNA stabilization. On the other hand, PKC has been implicated in the stabilization of a variety of other mRNAs such as, p21, GAP-43 and IL-1β itself [19,20,22,23]. Importantly, PKC has been convincingly implicated in COX-2 mRNA stabilization in several recent studies [21,31,44,54,68] and, indirectly, in IL-6 mRNA stabilization in one older study [69]. In astrocytes, PKC was previously shown to be important for IL-6 induction after IL-1β [70]. Moreover, IL-1β has been shown to activate several PKC isoforms, such as PKC-ζ [71,72], PKC-γ [45], PKC-δ [46] and PKC-β [73], in some of these studies in the context of mRNA stabilization.

To the best of our knowledge we are the first to show that the mRNA-stabilizing effect of IL-1β on IL-6 mRNA can also be p38-independent and might instead be mediated via PKC. However, the conclusion that the mRNA-stabilizing effect of IL-1β is late, and thus probably indirect, substantially complicated our attempts to identify the involved PKC isoform. Since PKC activation can be a very fast process, sometimes disappearing already after a few minutes [74], and since the time frame in which PKC could be activated in our experimental system ranged from approximately 4 to 6 h, it unfortunately became virtually impossible to identify the responsible PKC isoform. Moreover, the existence of 10 different PKC isoforms complicates a traditional siRNA-based approach. Although the fact that PMA mimicked the effect of IL-1β indicates that the involved isoform belongs to the novel or classical subfamily, 8 isoforms are still left. The pharmacological PKC inhibitors that we used are not very suitable for distinguishing between the different PKC isoforms, since they both interact at the homologous ATP-binding site, and therefore they do not allow clear distinction between the different isoforms [75]. Lastly, although the involvement of PKC in mRNA stabilization is well established [76], and the pharmacological inhibitors we used have been intensively employed previously to investigate PKC involvement in a variety of processes, we cannot exclude that aspecific inhibition of another kinase might occur, causing inhibition of IL-1β-mediated IL-6 mRNA stabilization. For example, both Rsk-2 and p70 S6 kinases can be inhibited by both GF103209X and Ro31-8220 [77]. The involvement of these kinases in mRNA stabilization processes has however, to our knowledge, not yet been documented. A well characterized downstream target of PKC in mRNA stabilization processes is the ARE-binding protein HuR [76]. Moreover, the importance of the PKC-HuR axis in COX-2 mRNA stabilization is well established [21,31]. Nevertheless, the mRNA-stabilizing protein HuR, which has formerly been shown to bind the 3′UTR of IL-6 [24], did not mediate IL-1β-induced mRNA stabilization of IL-6 in 1321N1 cells, although a constitutive stabilization of IL-6 mRNA did occur.

Whereas the actinomycin D experiments pointed to a very potent mRNA-stabilizing effect, IL-1β had only a modest, although highly reproducible and significant effect in the IL-6 3′UTR assay. This could mean that other elements in the IL-6 mRNA, such as introns or the 5′UTR, are involved in IL-6 mRNA stabilization after IL-1β treatment in astrocytes. On the other hand, in the IL-6 3′UTR assay, luciferase mRNA levels were assessed indirectly, via measurement of enzymatic activity of the translated luciferase protein. It could be that the mRNA-stabilizing effect in this assay is blurred by additional (post)translational mechanisms targeting the luciferase protein and we also cannot rule out that IL-1β affects the half-life of the luciferase protein.

Interestingly, in a recent paper, treatment of 1321N1 cells with β-adrenergic agonists inhibited their proliferation [78]. In view of the known proliferative effects of IL-6 on brain glioma [4,5], this seems contradictory to the results we present here. This contradiction can however be explained by, on the one hand, the differences in timing of the treatment with β-adrenergic receptor agonists (20 h by Toll et al. and 6 h in this paper); and on the other hand, the fact that IL-1β or TNF-α might possibly overrule the effects isoproterenol has on 1321N1 proliferation.

The exact molecular cascade leading to IL-6 mRNA stabilization in our model system remains unclear. Nevertheless, we can hypothesize on several possible mechanisms, although we did not explore all of them. First of all, it was recently demonstrated that APOBEC-1 complementation factor (ACF) controls IL-6 mRNA stability in liver [79]. ACF is present in brain tissue [80], so could be a potentially interesting target. Another recently identified novel mechanism involving IL-6 mRNA stabilization is the TLR-inducible RNase Zc3h12a [81]. Zc3h12a was shown to destabilize IL-6 mRNA after TLR-triggering, thus providing an essential mechanism for controlling immune reactions *in vivo*. Interestingly, Zc3h12a is also expressed in brain and was recently shown to be involved in glial differentiation of NT2 cells [82]. Thirdly, alternative polyadenylation of IL-6 mRNA, i.e. the use of an upstream polyadenylation signal, resulting in a shorter mRNA, could possibly contribute to changes in mRNA stability. This mechanism has been convincingly described for COX-2 [83] and HuR [84]. The human IL-6 gene contains an alternative polyadenylation signal less than 100 bp upstream from the canonical polyadenylation site [85], and alternative polyadenylation would lead to the exclusion of several AU-rich regions in the alternative mRNA species. The possibility that alternative polyadenylation of the human IL-6 cDNA influences mRNA metabolism is intriguing, but remains uninvestigated up to now. Finally, AUF-1, another RNA binding protein, has been shown to bind IL-6 mRNA and to influence its stability [8]. We have investigated if AUF-1 is involved in IL-1β-induced IL-6 mRNA stabilization by overexpressing the different AUF-1 isoforms (p37, p40, p42 and p45) in the IL-6 3′UTR reporter gene assay, but our preliminary data did not suggest a role for AUF-1 in this system (data not shown).

In conclusion, IL-1β stabilizes IL-6 and COX-2 mRNA via a novel, not yet completely characterized mechanism. This mRNA stabilization translates into a very potent synergistic IL-6 protein expression. Based on pharmacological inhibitor data, the stabilization process seems to depend on PKC, but not on p38, contrary to the earlier described modes of IL-6 mRNA stabilization [12,15–17]. Moreover, the prototypical mRNA-stabilizing protein HuR is not involved. Finally, the effect is late and probably indirect, although it seems to be independent of protein secretion.

Further elucidation of the molecular mechanism underlying this novel mode of IL-1β-induced mRNA stabilization is required, but in any case the very potent enhancement of IL-6 levels by combining two classical pro-inflammatory stimuli, TNF-α and IL-1β with a β-adrenergic agonist, isoproterenol, is bound to have profound physiological effects *in vivo*, specifically in the context of gliomas and neurodegenerative diseases. Moreover, the finding that PKC inhibitors are powerful tools to reduce astrocytic IL-6 expression might have interesting therapeutic implications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.01.019.

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