Peroxisome proliferator-activated receptor-γ agonists inhibit the replication of respiratory syncytial virus (RSV) in human lung epithelial cells

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Abstract

We have previously shown that peroxisome proliferator-activated receptor-γ (PPARγ) agonists inhibited the inflammatory response of RSV-infected human lung epithelial cells. In this study, we supply evidence that specific PPARγ agonists (15d-PGJ2, ciglitazone, troglitazone, Fmoc-Leu) efficiently blocked the RSV-induced cytolysis and development of syncytia in tissue culture (A549, HEp-2). All PPARγ agonists under study markedly inhibited the cell surface expression of the viral G and F protein on RSV-infected A549 cells. This was paralleled by a reduced cellular amount of N protein-encoding mRNA determined by real-time RT-PCR. Concomitantly, a reduced release of infectious progeny virus into the cell supernatants of human lung epithelial cells (A549, normal human bronchial epithelial cells (NHBE)) was observed. Similar results were obtained regardless whether PPARγ agonists were added prior to RSV infection or thereafter, suggesting that the agonists inhibited viral gene expression and not the primary adhesion or fusion process.

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Introduction

Respiratory syncytial virus (RSV) is the leading cause of serious respiratory tract infections in infants and young children throughout the world (Hall and McCarthy, 2000). It accounts for approximately 125,000 pediatric hospitalizations and up to 450 deaths of children each year in the United States (Shay et al., 2001). It has been recognized as a major cause of morbidity and mortality in immunocompromised patients, the elderly, and children with underlying lung–heart diseases (McDonald et al., 1982; Falsey et al., 1995; Glezen et al., 2000). Despite nearly five decades of intensive RSV research, there exist neither an effective active vaccination nor a promising anti-inflammatory therapy (Kimpen, 2001). The prophylactic administration of a humanized monoclonal anti-RSV antibody is highly effective but costly and currently only recommended for high-risk children up to 2 years of age, i.e., premature infants and children with bronchopulmonary dysplasia or congenital heart disease (IMPACT-RSV Study Group, 1998). Moreover, the value of ribavirin, the only licensed anti-RSV preparation, has been increasingly questioned in the past (American Academy of Pediatrics Committee on Infectious Diseases, 1996). During the last several years, a variety of RSV antiviral substances has been described (Torrence, 2000; Brooks et al., 2004). Different promising inhibitors of the F protein were generated (Cianci et al., 2004; Douglas et al., 2005), but due to the lack of a proofreading mechanism of the RNA polymerase, the selection of drug-resistant RSV variants is a major problem (Douglas et al., 2005; Zhao and Sullender, 2005). Due to their property to inhibit the virus–host infection process, these drugs would be primarily useful as a prophylactic agent. Quite recently, a successful inhibition of respiratory syncytial virus replication obtained by nasally administered siRNA was reported (Bitko et al., 2005). Considerable therapeutic value was observed when used as a prophylactic as well as a treatment drug after infection. However, currently neither of these substances is approved for the treatment of RSV infection.

Therefore, it remains an important task to develop novel therapeutical targets. A better understanding of the cellular signaling transduction pathways which are intimately involved in the RSV replication process is of major importance. It is...
known that the infection of the human airway epithelial cells, the target for RSV infection, results in a prominent activation of the transcription factor NF-κB leading to the expression of a wide spectrum of immune response genes (Tian et al., 2002). Quite recently, Reimers et al. reported that the viral M2-1 protein mediates the activation of NF-κB by direct protein–protein interaction (Reimers et al., 2005). However, the activation of NF-κB is dependent on RSV replication and not vice versa (Fiedler et al., 1996). In contrast, when NF-κB is activated either by TNF-α or by IL-1β prior to RSV infection, this transcription factor is capable of signaling an innate antiviral response that is independent of IFN and the JAK/STAT pathway (Bose et al., 2003). In addition, the blocking of STAT-1α or Map kinase ERK-1/2 activity attenuates RSV gene transcription and infection (Kong et al., 2003, 2004). Interestingly, the non-structural RSV proteins NS1 and NS2 are discussed as viral evasion factors. They inhibit the expression of Stat2 and block thereby the antiviral α/β interferon response of the target cell (Lo et al., 2005). Nevertheless, despite of these first insights, a detailed knowledge of the activated cellular signal transduction pathways involved in the RSV replication process in infected human lung epithelial cell is still lacking.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors which form a subfamily of the nuclear receptor gene family consisting of three isotypes: PPARα, PPARβ, and PPARγ (Lee et al., 2003). They regulate the transcription of distinct genes through heterodimerization with the retinoid X receptors (RXR). They were first identified as regulators of the lipid and glucose metabolism (Evans et al., 1988), but during the last decade, data accumulated showing that PPARs may also be involved in the complex regulation of immune and inflammatory processes (Daynes and Jones, 2002). The activation of PPARα and PPARγ correlated with the inhibition of inflammatory cell responses in a variety of cell types and the lung (Clark, 2002; Standiford et al., 2005). In addition, it has been shown that some viruses may exploit these ligand-activated transcription factors of the nuclear hormone receptor family for their own growth advantage. For example, the hepatitis B virus uses the nuclear hormone receptor RXRα and the peroxisome proliferator-activated receptor α (PPARα) for its own replication cycle in liver cells (Tang and McLachan, 2001). Also, the HIV controls, at least partly, its replication by the interaction of the virus encoded Nef protein with the PPARγ of the host cell (Otake et al., 2004). Therefore, the objective of the present study was to assess whether PPAR agonists might influence the replication of RSV in human lung epithelial cells. We analyzed the following specific PPARγ agonists in our study: the natural ligand 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), the synthetic agonist Fmoc-Leu, as well as the recently FDA approved antidiabetic thiazolidinedione derivatives ciglitazone and troglitazone, respectively. Besides the different specific PPARγ agonists, we also included the hypolipidemic drug bezafibrate, as a representative PPARα ligand.

In this study, we supply evidence that the synthetic PPARγ agonists Fmoc-Leu, ciglitazone, troglitazone, and the natural PPARγ ligand 15d-PGJ2 profoundly protected epithelial monolayers (HEp-2, A549) from cytopathic effects up to 48 h post-infection. After 3 days post-infection, only the synthetic PPARγ agonists were still able to protect the RSV-infected cell monolayer from detrimental major cytopathic cell damage. The RSV-infected human lung A549 epithelial cells cultured in the presence of the PPARγ ligands under study expressed a reduced amount of the cell surface bound viral G and F protein. Concomitantly, a downregulated viral N protein mRNA level was observed. This coincided with a markedly reduced release of infectious progeny virus from RSV-infected lung epithelial cells (A549, NHBE). In addition, the potential use of these PPARγ agonists in the light of RSV-infection is discussed.

Results

Expression profile of PPAR isoforms in A549 cells

Since A549 cells are a well-accepted in vitro RSV infection model, we used these cells to analyze which of the PPAR isotypes are expressed in these human airway epithelial cells at the protein level. The cells were permeabilized and stained intracellularly with isoform-specific polyclonal Abs. The immunofluorescence signals were determined by FACS analysis. As shown in Fig. 1, A549 cells expressed high amounts of all three isoforms of PPAR in a constitutive manner. When the primary unlabeled Abs were omitted, the cells showed no remaining immunofluorescence signal verifying that the second labeled Abs did not stain the cells in an unspecific manner (data not shown). Additionally, prior to cell staining, an excess of blocking peptides specific for the used PPARα- and PPARγ-specific Abs was added to the primary Abs. These pretreated PPAR-specific Abs did not stain A549 cells anymore proving binding specificity of the used Abs (Figs. 1A and B).

Increased cell viability of RSV-infected epithelial cell monolayer exposed to PPARγ agonists

To assess whether activation of PPARα and PPARγ might have some antiviral activity, we infected and cultured confluent A549 cell monolayer in the presence of specific PPAR agonists. The following PPARγ-specific ligands were used in our study: the naturally occurring compound 15d-PGJ2, a metabolite of prostaglandin D2, the synthetic antidiabetic thiazolidinedione derivatives ciglitazone and troglitazone, and a N-protected leucine analog designated as Fmoc-Leu (Rocchi et al., 2001). Two molecules of Fmoc-Leu interact with one PPARγ molecule in a highly specific manner. In addition, the PPARα-specific agonist bezafibrate was included in this study to determine whether activation of PPARα might also interfere with RSV infection in airway epithelial cells.

The cells were preincubated with the PPAR agonists for 30 min, infected with RSV and then cultured for 72 h still in the presence of the agonists. Thereafter, the cytopathic effect of RSV was assessed by light microscopy (Fig. 2). As can be seen from Fig. 2b, when compared with the medium control (Fig. 2a), the previously confluent cell monolayer was totally destroyed by RSV infection. The infected monolayer showed
an extensive loss of adherent cells. However, RSV-infected monolayers preincubated with ciglitazone, troglitazone, and Fmoc-Leu, respectively, were totally protected from RSV-induced cell damage (Figs. 2c–e). The natural PPARγ ligand 15d-PGJ2 showed a slight increase of adherent A549 cells when compared to the RSV-infected monolayer cultured in medium alone (Figs. 2f and h). In contrast, the PPARα agonist bezafibrate and the vector control DMSO had no protective impact on the RSV-induced cell damage (Figs. 2g and h).

Since HEp-2 cells are well known for their extensive syncytia formation following RSV infection, we also analyzed the cytopathic changes in RSV-infected HEp-2 monolayers...
cultured in the presence of PPAR agonists. Since the formation of syncytia proceeded faster and more efficiently in HEp-2 monolayer than in A549 cells, we evaluated the cell monolayers by light microscopy already 48 h post-RSV infection. Similar to A549 cells, pretreatment of the monolayers with ciglitazone, troglitazone, and Fmoc-Leu (Figs. 3c–e) totally abolished the RSV-induced syncytium formation (Fig. 3b). Furthermore, when analyzed 48 h post-infection, the natural PPARγ ligand 15d-PGJ2 protected the HEp-2 monolayer from syncytium formation (Fig. 3f). Again, the PPARα agonist bezafibrate and the vector control DMSO did not influence the RSV-induced cell fusion (Figs. 3g and h).

Similar results were obtained in A549 and HEp-2 cell monolayers, when PPARγ ligands were added post-RSV infection (data not shown). Taken together, all PPARγ ligands under study fully protected the RSV-infected airway epithelial cell monolayer from RSV-induced cell damage at least up to 48 h post-infection.

**Inhibitory effect of PPARγ agonists on viral protein and mRNA expression**

To analyze whether the PPARγ ligands might have a general inhibitory effect on the cellular protein synthesis, we determined the Toll-like receptor 2 (TLR2) expression pattern and the release of dehydrogenases from RSV-infected cells in the presence or absence of the agonists. As shown in Fig. 4A, the infection with RSV led to a significant upregulation of TLR2 on A549 cells. However, neither of the PPARγ ligands under study modulated the RSV-induced TLR2 expression pattern. Moreover, the enzyme activity of secreted dehydrogenases determined in the harvested cell supernatants of RSV-infected A549 cells was independent of the presence of PPARγ ligands (Fig. 4B). In summary, these data suggest that the PPARγ agonists neither inhibit the cellular protein synthesis nor have any general inhibitory effect on cell viability and/or cell metabolism which might account for the reduced viral cytopathic effect.

The viral attachment (G) protein and fusion (F) protein, glycoproteins incorporated in the viral envelope, are responsible for the initial attachment and fusion of the viral envelope with the target cell membrane. Moreover, the cytopathic effect in tissue culture is mediated by the epithelial cell surface expression of the viral F protein. We, therefore, analyzed whether the observed protective effect of PPARγ ligands on RSV-infected human lung epithelial cells might be due to a reduced expression of these viral envelope proteins. The cells were pretreated with PPARγ agonists, infected with RSV, and then cultured for 36 h. As shown in Fig. 5, the expression of both viral G and F protein was markedly reduced when cells were cultured in the presence of ciglitazone, 15d-PGJ2, and Fmoc-Leu, respectively. Similar to ciglitazone, troglitazone (20 μM) markedly reduced the expression of both proteins (data not shown). Again, the PPARα agonist bezafibrate (100 μM) did not modulate the expression level of viral proteins on RSV-infected cells (data not shown). To exclude that the PPARγ agonists primarily interfere with the viral adhesion and fusion process or may simply kill the virus, we analyzed the F protein expression on RSV-infected A549 cells that were either preincubated with the agonists or exposed to the agonists subsequent to the RSV infection. Our data show that the cell surface F protein expression was even downregulated by PPARγ agonists which were added to the infected cells 8 h post-infection (Fig. 6).

Next, we asked whether the diminished viral protein expression might be associated with a reduced viral mRNA level. For that purpose, we analyzed the cellular amount of N protein-specific mRNA by real-time RT-PCR. Our data show that A549 cells expressed a high amount of N protein-encoding mRNA 36 h post-infection (Fig. 7). However, when cells were cultured in the presence of PPARγ agonists, the cellular amount

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*Fig. 3. Reduced cytopathic effects in RSV-infected HEp-2 cells cultured in the presence of PPAR agonists. Confluent monolayers were preincubated with medium (a, b), ciglitazone (20 μM) (c), troglitazone (20 μM) (d), Fmoc-Leu (100 μM) (e), 15d-PGJ2 (20 μM) (f), bezafibrate (100 μM) (g), and the vector control DMSO (0.2% (v/v)) (h) for 30 min. Thereafter, cells were infected with RSV (b–h) and cultured for further 48 h in the presence of the agonists. Photomicrographs are shown illustrating cytopathological changes in HEp-2 cells 48 h post-infection. Images were taken at a magnification of ×100.*
of N protein-encoding mRNA was significantly downregulated. All PPAR\(\gamma\) agonists under study reduced dose dependently the cellular N protein mRNA level (Figs. 7A and B). Also, a reduced G protein mRNA level was demonstrated by RT-PCR (data not shown).

**PPAR\(\gamma\) agonists diminish the replication of RSV in A549 and NHBE cells**

To investigate whether the PPAR\(\gamma\) agonist-mediated inhibition of viral protein and gene expression results in a diminished replication of RSV, we analyzed the expression levels of RSV proteins G and F in A549 cells cultured with PPAR\(\gamma\) ligands using flow cytometry. The results showed a significant reduction in the expression of both proteins compared to RSV-infected control cells (Fig. 5). The findings were consistent with the decreased viral protein synthesis and viability observed in the previous experiments.

**Fig. 4.** PPAR\(\gamma\) agonists do not affect cell protein synthesis and viability. (A) The RSV-induced expression of Toll-like receptor 2 (TLR2) is not downregulated by PPAR\(\gamma\) agonists. Cells were pretreated either with ciglitazone (20 \(\mu\)M), troglitazone (20 \(\mu\)M), 15d-PGJ\(_2\) (20 \(\mu\)M), Fmoc-Leu (100 \(\mu\)M), bezafibrate (100 \(\mu\)M), the vehicle DMSO (0.2% (v/v)), or medium, respectively for 30 min, then infected with RSV, washed, and incubated with freshly supplied agonists for 48 h. Results are means ± SEM (\(n = 3\)). (B) PPAR\(\gamma\) agonists did not reduce cell viability. Cells were incubated with the abovementioned agonists for 48 h. Cell viability/proliferation after treatment with PPAR agonists was assessed by WST-1 assay. Data are shown as means ± SD of a representative experiment (\(n = 3\)) performed in quadruplicate.

**Fig. 5.** Reduced expression of RSV G and F protein on human A549 cells cultured with PPAR\(\gamma\) ligands. Flow cytometric analysis of cell surface bound viral G protein (left column) and F protein (right column) expression. Cells were pretreated with the agonists for 30 min, RSV-infected, and cultured still in the presence of the agonists for another 36 h. Expression of viral G and F protein on RSV-infected A549 cells cultured in medium alone (thin line) or pretreated with ciglitazone (20 \(\mu\)M) (a, b), 15d-PGJ\(_2\) (20 \(\mu\)M) (c, d), and Fmoc-Leu (100 \(\mu\)M) (e, f) (thick line), respectively. Background fluorescence signals of stained noninfected cells are shown by dotted lines. The results are representative of multiple independent experiments (\(n = 3\)).
production of infectious progeny virus, we determined the amount of infectious particles in the cell supernatants harvested 48 h post-infection. The cells were pretreated with different concentrations of the PPARγ agonists as well as the PPARα agonist bezafibrate. Our data show that all used PPARγ agonists significantly reduced the amount of infectious RSV particles in the cell supernatants in a dose-dependent manner (Figs. 8A and B). Especially ciglitazone and Fmoc-Leu showed the most prominent inhibitory effect on viral replication, i.e., the virus titer decreased by nearly 1000-fold. Furthermore, cells immediately exposed to PPARγ agonists post-RSV infection showed a comparable diminished release of infectious progeny virus (data not shown). In contrast, neither the PPARα agonist bezafibrate nor the vector control DMSO (0.05–0.3% (v/v)) did affect the viral replication rate (Fig. 8B).

The A549 line originated from type II alveolar adenocarcinoma cells. Although this cell line is a well-accepted in vitro model for analyzing RSV infection in human lung epithelial cells, we next verified whether the PPARγ agonists ciglitazone, Fmoc-Leu, and 15d-PGJ2 might also interfere with RSV replication in primary human bronchial epithelial cells. Similar to a more clinical setting, we first infected NHBE cells and cultured them thereafter for 48 h in the presence of the PPARγ agonists. Our data show that ciglitazone, troglitazone, and the endogenous ligand 15d-PGJ2 inhibited the replication of RSV in
NHBE cells in a comparable manner. The addition of Fmoc-Leu reduced the release of progeny virus most efficiently by nearly 1000-fold (Fig. 9A). Moreover, similar to A549 cells, the reduced production of infectious virus particles was paralleled by a downregulated N protein mRNA level, suggesting that PPARγ agonists might also interfere with RSV gene expression in NHBE cells (Fig. 9B). Similar to A549 cells, the PPARα agonist bezafibrate and DMSO had no effect on the release of infectious progeny virus and the cellular mRNA level encoding RSV N protein (Figs. 9A and B).

Discussion

In the present study, we supply evidence that the four PPARγ agonists ciglitazone, troglitazone, 15d-PGJ2, and Fmoc-Leu, respectively, suppress the replication of RSV in an in vitro RSV infection model. Human lung epithelial cells of the cell line A549 as well as primary bronchial epithelial cells showed a significantly reduced release of infectious progeny virus. In contrast, activation of PPARα by means of the specific agonist bezafibrate had no impact on RSV replication.

We determined a constitutive expression of PPARα, β, and γ in human lung A549 cells by FACS analysis. With regard to the expression of PPARγ, our data are in accordance with data previously published (Michael et al., 1997; Wang et al., 2001; Pawliczak et al., 2002). However, Wang and Pawliczak argued against a prominent expression of PPARα in human NIH-A549, A549, and NHBE cells. In contrast, rabbit corneal epithelium expresses PPARα and β but not γ mRNA (Bonazzi et al., 2000). Whether our contrasting PPARα expression pattern is due to the different antibodies used, the different sensitivities of the detection systems, i.e., FACS analysis vs. immunoblot technique, or the analyzed cell type or cell charge remains to be determined.

To analyze the inhibitory effect of PPARγ agonists on RSV production in more detail, we determined the viral protein and mRNA expression pattern. The reduced release of infectious progeny virus was paralleled by a downregulation of the viral F and G protein expressed on the cell surface of the infected A549 epithelial cells. These data suggest that the observed reduced release of progeny virus might be a direct consequence of an impaired viral protein synthesis and is obviously not due to an altered budding process from the infected cell. It is known that the F protein by interacting with the small GTPase RhoA is solely responsible for the cytopathic effect primarily observed in cell culture (Pastey et al., 1999). Therefore, the fact that all four PPARγ agonists significantly reduced the expression of the viral F protein is fully sufficient to account for the increased viability and reduced cytopathic effect observed in A549, HEp-2, and NHBE cell monolayers. When assayed 48 h post-infection the natural ligand 15d-PGJ2 inhibited the release of infectious progeny virus and fully protected RSV-infected HEp-2 cell monolayer from cell damage. However, when RSV-infected A549 cell monolayers were assayed 72 h post-infection, the natural ligand 15d-PGJ2 protected the monolayers to a lesser degree than the synthetic PPARγ agonists ciglitazone, troglitazone, and Fmoc-Leu, respectively, which cannot be explained at the present moment. The reason for that is not known. However, little is known about the biological activity and turnover of 15d-PGJ2 in vivo as well as in vitro lung epithelial cell cultures up to an incubation time of 72 h. It is assumed that 15d-PGJ2 secreted into the microenvironment of the inflamed tissue is effectively metabolized by macrophages and other resident cells. In line with this assumption is the observation made by Bell-Parikh et al. (2003) that the concentrations of 15d-PGJ2, measured in the joint fluids of patients suffering from rheumatoid arthritis, are too low to activate PPARγ. We, therefore, hypothesize that the loss of protection observed in RSV-infected lung epithelial cell monolayers cultured up to 72 h post-infection might be due to a more effective metabolism/inactivation of 15d-PGJ2 compared to the other PPARγ agonists under study. Detailed time kinetic studies have to address this point in future.

We observed a significant inhibition of progeny virus release when PPARγ agonists were used either as a prophylactic drug in A549 cells or as therapeutic drug in A549 and NHBE cells, i.e., pre- and post-infection. By analyzing the viral protein expression, we determined that the PPARγ agonists still supply an antiviral effect when added 8 h post-infection. Thus, the analyzed PPARγ agonists did not primarily interfere with the viral adhesion and fusion process. Our data concerning the reduced N protein mRNA expression level suggest that the lifecycle of RSV

Fig. 9. PPARγ agonists reduced the release of progeny virus and the cellular amount of RSV N protein mRNA in NHBE cells. The cells were infected with RSV (MOI = 3) for 2 h, washed, and cultured for 48 h in the presence of the PPAR ligands ciglitazone (20 μM), troglitazone (20 μM), 15d-PGJ2 (10 μM), Fmoc-Leu (100 μM), bezafibrate (100 μM), and the vehicle control DMSO (0.2% (v/v)), respectively. (A) Supernatants were harvested and immediately analyzed by plaque assay on HEp-2 cells. Results are means ± SEM (n = 4); *P < 0.01, significant vs. nonpretreated RSV-infected cells. (B) Cells were harvested, and the cellular amount of mRNA encoding for viral N protein was quantitatively determined by real-time RT-PCR. Results are means from two independent experiments.
might be, at least partly, inhibited at the transcriptional level. The detailed molecular mechanisms leading to the reduced viral gene expression pattern are still not known. However, a general inhibitory effect on the cellular protein and/or mRNA synthesis rate can be ruled out since the PPARγ agonists did not interfere either with cell viability or the RSV-induced upregulation of TLR2.

Recently, the direct activation of PPARγ in A549 cells either by 15d-PGJ₂ (1–5 μM) or ciglitazone (5–25 μM) was demonstrated by Wang et al. (2001). Therefore, we did not reproduce these data. However, in agreement with other published results analyzing the anti-inflammatory and antiviral capacities of thiazolidinediones and 15d-PGJ₂, we also used with respect to their PPARγ-binding constants relatively high concentrations of the agonists to influence RSV production (Hayes et al., 2002; Kwak et al., 2002). One may argue that natural and synthetic PPARγ agonists also exert part of their anti-inflammatory activity by interfering with a variety of other signal transduction pathways in a PPARγ-independent manner. Especially, the modulation of the Janus/STAT and MAP kinase signaling pathways has been reported (Takeda et al., 2001; Chen et al., 2003). In this regard, it was recently published that activation of ERK1 and 2 is a prerequisite for a successful replication of RSV (Kong et al., 2004). Therefore, PPARγ agonist-mediated modulation of MAP kinase activity might contribute to the observed antiviral effect. In addition, whether the RSV-induced inhibition of the antiviral cellular α/β-interferon response, mediated by the NS1 and NS2 proteins, might be counter-regulated by the PPARγ agonists should also be addressed in future experiments (Bossert et al., 2003; Spann et al., 2004; Lo et al., 2005).

The fact that PPARγ agonists are able to modulate the activity of other transcription factors is well demonstrated (Straus et al., 2000; Pérez-Sala et al., 2003). Recently, we observed that all PPARγ agonists under study profoundly reduced the binding activity of NF-κB (p65/p50) in RSV-infected cells (Arnold and König, in press). Quite recently, Bailey and Ghosh (2005) published data that PPARγ agonists, which induce sumoylation of PPARγ, prevent the degradation of the NF-κB-bound coreceptor complex thereby excluding its exchange by a coactivator complex. This leads to a transrepression of the NF-κB-dependent promoters. However, no data are available which support the notion that a reduced NF-κB activity interferes with the replication of RSV (Fiedler et al., 1996). Nevertheless, the reduced NF-κB binding activity correlated with a diminished release of proinflammatory cytokines (IL-1α, IL-6, TNF-α) and chemokines (CXCL8 and CCL5) from RSV-infected lung epithelial cells (Arnold and König, in press). Furthermore, a reduced cell surface expression of ICAM-1 and MHC-I was observed (our own unpublished data). Currently, there exist no satisfactory anti-inflammatory treatment for RSV infection. Corticosteroids have been shown to have little to no effect in the overall outcome of RSV infection (Bonville et al., 2001). In contrast, several studies have clearly demonstrated that PPARγ agonists have additional anti-inflammatory effects compared to corticosteroids. In this regard, Patel et al. (2003) reported that PPARγ agonists, but not dexamethasone, inhibited the release of G-CSF from human airway smooth muscle cells. Furthermore, the accumulation of huge amounts of PMN in the RSV-infected lung is a hallmark of severe lower respiratory tract disease (Everard et al., 1994). Quite recently, it was published that the neutrophil infiltration of the lung was significantly reduced by the application of PPARγ agonists during bleomycin-induced mouse lung injury (Genovese et al., 2005). Moreover, it has been shown in a murine LPS-induced airway inflammation model that the thiazolidinedione compound rosiglitazone, in contrast to dexamethasone, reduced the neutrophil number in the lung tissue when administered after the LPS insult (Birrell et al., 2004). A direct anti-inflammatory effect of PPARγ agonists on neutrophil and eosinophil cell function was also reported (Woerly et al., 2003; Imamoto et al., 2004).

In summary, proinflammatory immune effector mechanisms as well as direct cytopathic effects contribute to the overall outcome of RSV-induced lower respiratory tract disease. Our data presented herein, together with our previously published anti-inflammatory capacity of PPARγ agonists on the native immune response of the RSV-infected lung epithelial cell, suggest that PPARγ agonists might have a beneficial effect in the course of an acquired RSV infection.

Materials and methods

Compounds

The PPAR agonists ciglitazone, troglitazone, bezafibrate, Fmoc-Leu (Merck Biosciences, Schwalbach, Germany), and 15-deoxy-Δ12,14-prostaglandin J₂ (15d-PGJ₂) (Biomol, Hamburg, Germany) were stored in DMSO (Sigma, Deisenhofen, Germany) at −80 °C. At the day of experiment, compounds were freshly diluted in basal medium (DMEM or BGM) and added to the cells with a final DMSO concentration of 0.05–0.3% (v/v).

Cell culture

Human A549 pulmonary type II epithelial cells (passages 4–20) and HEp-2 cells (American Type Culture Collection (ATCC), Rockville, MD, USA) were cultured in Dulbecco’s modified eagle medium (DMEM) (4500 mg/l D-glucose) supplemented with 2 mM glutamine, 5% (v/v) inactivated fetal calf serum (FCS), streptomycin (100 μg/ml), and penicillin (100 IU/ml). Normal human bronchial epithelial cells (NHBE) (Cambrex/Clonetics, Verviers, Belgium) were cultured in complete BGM medium (5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 μg/ml transferrin, 6.5 ng/ml triiodothyronine, 0.5 μg/ml epinephrine, 0.5 ng/ml human epidermal growth factor, 0.1 ng/ml retinoid acid, 50 μg/ml gentamicin, and 52 μg/ml bovine pituitary extract). NHBE cells were cultured and expanded according to the instructions of the manufacturer. To support cell attachment and growth of NHBE cells, tissue culture flasks and plastic plates (Greiner, Frickenhausen, Germany) were precoated with fibronectin (10 μg/ml) (Sigma) for 30 min at 37 °C. Cells of passages 3–7 were
seeded on 24-well plates and cultured until confluency in complete medium. All cell cultures and prepared virus stocks were free of mycoplasmic contamination routinely verified by a commercially available mycoplasma detection kit (Roche Diagnostics, Mannheim, Germany).

**Virus growth and preparation**

The Long strain of RSV (ATCC) was propagated and titrated in HEp-2 cells. The Long strain was used, as this strain is capable of causing severe disease in human infants. For virus propagation, confluent monolayers were infected with RSV (MOI = 0.1) for 3 h in DMEM without FCS. The monolayers were washed, overlayed with DMEM (0.5% FCS), and incubated at 37 °C in 5% CO2 atmosphere until cytopathic effect reached ~80%. Thereafter, the supernatants were harvested, and cellular debris was removed by centrifugation (5000 × g, 10 min). RSV was concentrated by polyethylene glycol precipitation (10%) and purified by means of discontinuous sucrose gradient centrifugation (Ueba, 1978). To stabilize the purified virus particles, they were resolved in 20% sucrose/NT-buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5) and stored at −80 °C. No contaminating cytokines, including IL-1α, TNF-α, IL-6, IL-8, RANTES, GM-CSF, and interferona/β, were detected in these sucrose-purified viral preparations. Also, virus stocks and media were free of lipopolysaccharide routinely assayed by the Limulus hemocyanin agglutination assay (Sigma).

**RSV titration**

For virus titration prepared RSV stock solutions and harvested supernatants were serially tenfold diluted onto confluent HEp-2 monolayers cultured in 96-well flat-bottomed plates. The virus titer was quantified as previously described (Arnold et al., 2004). Briefly, infected cells were cultured for 48 h under methyl cellulose. Then monolayers were fixed with paraformaldehyde (2%) and stained with mouse anti-P protein mAb (clone: 3C4, kindly supplied by Dr. H. Werchau, Department of Medical Virology, Ruhr-University, Bochum, Germany). Binding of the primary Ab was detected by a second noninfected A549 cells was analyzed by flow cytometry. By colorimetric staining, counted microscopically and expressed as log10 plaque forming unit (pfu)/ml. The stock titer of the used virus pool was 10⁸ pfu/ml.

**Cell experiments**

Epithelial cells (A549 and NHBE cells) were seeded in 24-well (1 × 10⁵ cells/well) culture plates and cultured overnight in DMEM and BEGM, respectively. If not stated otherwise, confluent monolayers were washed with basal medium and pretreated with PPAR agonists for 30 min prior to RSV adsorption. The cells were treated with varying doses of ciglitazone (5–50 μM), troglitazone (5–50 μM), 15d-PGJ₂ (5–20 μM), Fmoc-Leu (50–300 μM), bezafibrate (50–300 μM), and solvent (DMSO; 0.05–0.3% (v/v)), respectively, in a volume of 1 ml. Thereafter, the medium was reduced to a volume of 200 μl, and cells were infected with RSV for 2 h still in the presence of the used PPAR agonists. The virus stock was diluted in culture medium to a defined multiplicity of infection (MOI) of 3. An equivalent amount of 20% sucrose/NT-buffer added to noninfected cells served as mock control. Then, cells were washed and incubated with fresh medium (DMEM (2% FCS) or supplemented BEGM in case of NHBE cells) in the presence of freshly supplied agonists for another 36 h–72 h. In addition, RSV-infected cells were incubated with PPAR agonists post-RSV infection. Thereafter, cells were detached and harvested as previously described (Arnold and König, 2005). As was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide (MTT) assay, designated as WST-1 assay (Roche), the added agonists induced no significant cell death (>3%) at the concentrations used (data not shown).

**Quantification of cell proliferation and cell viability**

Confluent A549 cell monolayers (5 × 10⁴ cells/well) placed in 96-well flat-bottomed plates were incubated with the agonists for 48 h. For control, cells were incubated with the vehicle DMSO alone. Cell viability and cell proliferation was determined by analyzing the cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan by mitochondrial dehydrogenases (Roche). A reduction in the number of viable cells results in a decrease in the overall activity of mitochondrial dehydrogenases in the cell supernatant. This reduced enzyme activity leads to a reduced amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture. Briefly, 10 μl of a ready to use solution was added to 100 μl (1:10 final dilution) for the last 1 h of incubation. The plate was directly read at 450 nm against a reference wavelength of 620 nm on a SpectraFluorPlus Reader (Tecan, Crailsheim, Germany).

**FACS staining**

The constitutive intracellular expression of PPARs in noninfected A549 cells was analyzed by flow cytometry. By using the Fix and Perm kit for intracellular staining (BD Biosciences (BD), Heidelberg, Germany), the cells were fixed and permeabilized according to the manufacturer’s instructions. Thereafter, the cells were washed two times in permeabilization washing buffer and analyzed with the primary unlabeled polyclonal rabbit anti-PPARα-Ab, rabbit anti-PPARγ-Ab (Cayman Chemicals, Ann Arbor, MI, USA), rabbit anti-PPARβ-Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and unspecific rabbit IgG (Sigma), respectively, for 1 h at 4 °C. Then, primary bound Abs were detected by secondary staining with PE-labeled AffiniPure F(ab')₂ fragment of goat anti-rabbit IgG (H + L) (Dianova, Hamburg, Germany) for 1 h at 4 °C. To determine binding specificity of the used primary anti-PPARα and anti-PPARγ Abs, we blocked antibody/protein complex formation by using PPARα- and PPARγ-specific blocking
peptides (Cayman Chemicals). As recommended by the manufacturer, prior to cell staining, the blocking peptides (0.5 mg/ml) were mixed with Abs in a 1:1 (v/v) ratio and incubated for 60 min. To determine RSV protein synthesis in RSV-infected A549 cells, the cells were analyzed for cell surface expression of viral G and F protein (Arnold and König, 2005). Binding of monoclonal mouse anti-G protein and anti-F protein Ab (Biotrend, Köln, Germany) was visualized by a secondary staining with Cy3-labeled AffiniPure goat anti-mouse IgG (H + L) (Dianova). The expression of TLR2 was determined with a PE-labeled monoclonal mouse anti-TLR2 Ab (eBioscience, San Diego, USA). The corresponding unlabeled and labeled mouse IgG subtypes served as isotype controls (BD). After cell washing, the cell bound fluorescence signal was determined by FACSCalibur (BD), and data were analyzed by means of CellQuest software (BD). The mean fluorescence intensity (MFI) of 10,000 cells was determined and corrected by subtraction of background fluorescence of the isotype control.

RNA extraction and real-time RT-PCR

The total cellular RNA from noninfected and RSV-infected epithelial cells (5 × 10⁵) was extracted using the QiAmp 96 viral RNA Kit on the Biorobot 3000 System from Qiagen (Düsseldorf, Germany). Reverse transcription (RT)-PCR was performed with M-MLV RT-buffer components (Invitrogen, Karlsruhe, Germany). Prepared total RNA (2 μg) was added to 50 μl PCR reaction mix consisting of 50 mM Tris–HCl buffer (pH 8.3), 3 mM MgCl₂, 75 mM KCl, 10 mM DTT, 0.5 mM dNTPs, 2 ng/μl oligo d(T)(12–18), 1 μM RNAase inhibitor (Applied Biosystems, Foster City, CA, USA), and 4 μM M-MLV. The synthesis of cDNA was performed at 37 °C for 60 min. The cDNA was stored at −20 °C.

The amount of RSV N protein mRNA was quantified by Taqman real-time RT-PCR as previously reported (Dewhurst-Maridor et al., 2004). Briefly, using the Gene Amp 5700 Sequence Detector (Applied Biosystems) the RNA (100 ng) was reverse transcribed to 2.5 μl cDNA and amplified in a volume of 25 μl by means of TaqMan Universal PCR Master Mix (Applied Biosystems) containing specific primers for RSV N protein and a fluorescently labeled probe synthesized by Metabion (Planegg-Martinsried, Germany): forward, 5′-CTCAATTTCTC-CACTTCTCCAGTGT-3′; reverse, 5′-CTTGGATCTCCT- GGTTGATCCTCTGT-3′; probe, 5′-carboxy-fluorescein (FAM)-TCCCATTATGCTAGGCCAGCA-6-carboxy-tetramethyl- rhodamine-(TMRA)-3′. The cDNA was denatured at 95 °C for 10 min and amplified by 40 cycles (95 °C, 15 s and 60 °C, 1 min). Concentrations of primers and probes were optimized to obtain a reaction yield at the lowest threshold cycle and a high cycle to cycle increase of the resulting fluorescent signal (ΔΔCT). The primers were used at a concentration of 300 nM together with the probe diluted to 200 nM. The primer/probe set for the housekeeping gene human GAPDH was supplied as a predeveloped kit by Qiagen. The reaction components were mixed and the amplification profile settled according to the instructions of the manufacturer. Negative controls were carried out with water instead of cDNA. The cDNA prepared from RSV-infected cells and diluted up to 10⁴ served as a positive control validating that the efficiencies of the RSV N protein and GAPDH PCR were approximately equal. Expression of GAPDH gene was not significantly altered during the time of incubation with RSV, drugs and vehicle. Therefore, the relative mRNA expression of each gene was normalized to the level of GAPDH in the same RNA preparation, i.e., the comparative Ct method was used to analyze the relative quantities of RSV N protein mRNA in cell samples. The relative RNA amount was calculated by using the following equation: 2^−ΔΔCT were ΔΔCT = ΔCTq − ΔCt,b. CTq is defined as the Ct value for RSV N protein minus the Ct value for GAPDH for a given sample, q is the unknown sample, and b is the calibrator (noninfected sample).

Statistics

If not stated otherwise, data were expressed as the mean ± SEM. For statistical significance, analysis of data was performed using Student’s t test (two-sided). A value of P < 0.05 was considered significant.

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