PPARγ1 attenuates cytosol to membrane translocation of PKCα to desensitize monocytes/macrophages

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Introduction

Monocyte/macrophage desensitization is characteristic for late-phase immune responses (Liew et al., 2005). Confined proinflammatory cytokine expression and mediator synthesis is important to avoid pathological settings, such as sepsis or atherosclerosis (Hotchkiss and Karl, 2003; Hansson, 2005). Down-regulating proinflammatory cytokine expression (TNF-α, interleukin [IL]-1β, and IFNγ) or proinflammatory mediator release (nitric oxide and reactive oxygen species [ROS]) concomitantly switches the proinflammatory phenotype toward an antiinflammatory one. The latter is characterized by the synthesis of antiinflammatory cytokines, such as TGF-β or IL-10, and is often accompanied by cellular desensitization upon secondary proinflammatory stimulation (Docke et al., 1997; Kalechman et al., 2002). Therefore, the identification of molecular mechanisms contributing to cellular desensitization attracted growing interest (Docke et al., 1997; von Knethen and Brune, 2002).

One factor attenuating proinflammatory gene expression is peroxisome proliferator–activated receptor γ (PPARγ). PPARγ is a nuclear hormone receptor that, upon agonist binding, transactivates gene expression as a heterodimer bound to retinoic acid receptor-α (Abdelrahman et al., 2005). Its role in blocking proinflammatory gene expression comprises several options, mainly antagonizing signaling cascades. Specifically, PPARγ negatively regulates transcription factors by scavenging transcriptional coactivators, such as the cAMP-response element–binding protein or the steroid receptor coactivator-1 (Yang et al., 2000). However, a direct association with the transcription factors NF-κB, NF of activated T cells, signal transducer, and activator of transcription or NF-E2–related factor 2 (Ikeda et al., 2000; Wang et al., 2001, 2004; Chung et al., 2003) blocks their recruitment to responsive elements in promoter structures of target genes. Recently, it has been shown that PPARγ is targeted to nuclear receptor corepressor–histone deacetylase-3 complexes in response to ligand-dependent SUMOylation (Pascual et al., 2005), protecting these complexes from proteosomal degradation. Normally, histone deacetylase-3 removes a corepressor complex, provoking expression of proinflammatory genes. Additionally, PPARγ represses activation of a mitogen-activated protein kinase, which keeps downstream transcription factors

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Abbreviations used in this paper: 15d-PGJ2, 15-deoxy-D12,14-prostaglandin J2; AF, activating function; CHX, cycloheximide; DAG, diacylglycerol; DBD, DNA-binding domain; DGKa, DAg kinase α; EMSA, electrophoretic mobility shift assay; HEK, human embryonic kidney; IL, interleukin; LBD, ligand-binding domain; MCS, multicloning site; NFKB, nuclear factor-κB; PPARγ, peroxisome proliferator–activated receptor γ; ROS, reactive oxygen species.
unphosphorylated and, consequently, inactive (Desreumaux et al., 2001). Moreover, PPARγ influences the cell cycle by upregulating p21 expression, which is an established cell cycle inhibitor (Han et al., 2004), or down-regulating phosphatase PPA2, which is known to adjust E2F/DP DNA-binding activity, which is necessary for the G1 to S-phase transition (Altiok et al., 1997). In response to proinflammatory stimulation, PPARγ-dependent gene transcription also contributes to cellular desensitization. PPARγ agonists inhibit diacylglycerol (DAG)–PKC signaling by inducing DAG kinase-α (DGKα) expression (Verrier et al., 2004). This enzyme lowers the amount of DAG, which is an established PKC activator. Normally, DAG is released from membrane lipids and activates classical PKCs (Liu and Heckman, 1998). Based on gene induction of DGKα as the underlying mechanism, this type of desensitization demands at least 6–15 h. Thus, it appears that PPARγ transrepresses proinflammatory gene expression, often in a DNA-unbound state, by provoking direct protein–protein interactions.

We provide evidence for a new PPARγ-dependent mechanism in blocking PKCα signaling. Depletion of PKCα is attenuated by PPARγ1 activation in RAW 264.7 cells or human primary monocyte–derived macrophages. Cytosolic localization of PPARγ1 interferes with PKCα cytosol to membrane translocation, which is a prerequisite for its activation-dependent depletion. Translocation is restored in cells transfected with a dominant-negative PPARγ1 mutant. Coimmunoprecipitation studies and a mammalian two-hybrid system revealed a direct PPARγ1–PKCα interaction as the underlying mechanism. PPARγ1 deletion constructs support the idea that ligand-dependent PPARγ activation is necessary for PKCα binding, which is mediated by the helix 1 of the PPARγ1 hinge domain. Our data suggest a new mechanism for how activation of PPARγ1 blocks PKCα translocation, thereby achieving cellular desensitization.

**Results**

**PPARγ agonists inhibit PKCα depletion**

Recent data demonstrate that monocyte/macrophage desensitization in response to phagocytosis of apoptotic cells is achieved by attenuating PKCα signaling, which blocks NADPH oxidase–dependent formation of ROS (Johann et al., 2006). Therefore, we were interested in identifying molecular mechanisms interfering with PKCα depletion. A potential candidate known to affect the pro- versus antiinflammatory phenotype in monocyes/macrophages is PPARγ. Because controversial data exist concerning its expression in monocyctic and macrophage cell lines, as well as in primary human monocytes and macrophages, we performed a first set of experiments determining PPARγ expression in the monocyctic cell lines and primary cells under investigation. As shown in Fig. 1 A, PPARγ is constitutively expressed in murine RAW 264.7 macrophages. In contrast, in THP-1 cells, PPARγ is only fractionally expressed, but differentiation toward macrophages with 100 nM PMA for 24 h provoked up-regulation of PPARγ (Fig. 1 A, lane 2 vs. 3). A similar expression pattern is observed in primary monocytes and macrophages, respectively. PPARγ is only marginally expressed in monocytes, but induced upon differentiation toward macrophages (Fig. 1 B). To identify the expressed PPARγ isoform 1 or 2, we performed a Western blot using human PPARγ1-transfected human embryonic kidney (HEK) cells as a positive control. Taking into consideration that murine and human PPARγ1 are identical in size (475 aa), we conclude that PPARγ1 is expressed in RAW 264.7 macrophages, differentiated THP-1 cells, and primary macrophages (unpublished data). Based on these results, we choose RAW 264.7 cells, differentiated human THP-1 cells, and primary monocyte–derived macrophages as experimental cell models.

To analyze the role of PPARγ in macrophages in affecting PKCα activation, we pretreated RAW 264.7 macrophages for 1 h with the PPARγ agonists ciglitazone and rosiglitazone, followed by the addition of 100 nM PMA, which is a DAG homologue and established activator of PKCα. As expected, PKCα depletion was observed in control cells in response to 100 nM PMA (Fig. 2 A, lane 2). Depletion of PKCα was attenuated in cells prestimulated with a PPARγ agonist, such as ciglitazone (Fig. 2 A, lanes 3 and 4) or rosiglitazone (Fig. 2 A, lanes 5 and 6), in a concentration-dependent manner. However, 1 μM PMA-mediated PKCα depletion was not blocked (unpublished data). From these data, we conclude that PPARγ agonists attenuate activation-dependent PKCα depletion, in part controlled by the magnitude of the PKCα–activating stimulus. In PPARγ activating function (AF) 2 mutant overexpressing RAW 264.7 macrophages (Johann et al., 2006), pretreatment with 10 μM rosiglitazone or 10 μM ciglitazone did not inhibit PKCα depletion in response to PMA (Fig. 2 B).

Because a 1-h prestimulation period is short for gene expression and protein synthesis, we hypothesized that preserved PKCα expression did not require protein synthesis. To prove this assumption, we added the established translation inhibitor cyclohexamide (CHX) 1 h before PPARγ agonist stimulation.
The physiological significance of these results obtained in murine RAW 264.7 macrophages was verified in primary human monocyte–derived macrophages isolated from peripheral blood. Similar to RAW 264.7 cells, in primary macrophages, pretreatment with ciglitazone and rosiglitazone preserved PKCα expression upon PMA addition (Fig. 2 D).

Antiinflammatory consequences of PPARγ1–PKCα interaction

To elucidate whether the PPARγ1–PKCα interaction shows an impact on PKCα signaling in inflammatory gene expression in macrophages, we analyzed two proinflammatory markers of macrophage activation, i.e., NF-κB DNA binding and TNF-α expression in response to PMA in RAW 264.7 macrophages. To determine activation of the proinflammatory transcription factor NF-κB, we performed a set of electrophoretic mobility shift assays (EMSAs), demonstrating the DNA-binding capability of the transcription factor. As shown in Fig. 3 A, 100 nM PMA supplied for 3 h significantly induced NF-κB activation (Fig. 3 A, second lane) compared with the untreated control (Fig. 3 A, first lane). To elucidate the composition of the transcription factor complex, we used antibodies against the p50 (Fig. 3 B, left) and p65 subunits (Fig. 3 B, right) of NF-κB. As shown in Fig. 3 B (left), the lower and the upper NF-κB shifts contained the p50 subunit. Therefore, the two bands were significantly reduced when an α-p50 antibody was included in the binding reaction and a new band, the p50 supershift, occurred. Only the upper NF-κB shift included the p65 subunit, as indicated by the addition of the α-p65 antibody, which provoked the reduction of the upper NF-κB shift, but did not alter the lower NF-κB shift (Fig. 3 B, right). As expected, a new band was detectable (the p65 supershift). Thus, we conclude that the lower NF-κB shift is formed by a p50 homodimer, whereas the upper NF-κB shift consists of a p50/p65 heterodimer.

To identify whether activation of NF-κB complexes is influenced by PPARγ activation, we treated RAW 264.7 cells with the natural PPARγ agonist 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2; Kobayashi et al., 2005; Rogler, 2006). Taking into consideration that 15d-PGJ2 may also act PPARγ independently on NF-κB activation (Straus et al., 2000), we included the PPARγ antagonist GW9662 in this experiment (Leesnitzer et al., 2002). This allowed us to discover to what extent 15d-PGJ2 affected PMA-mediated NF-κB activation PPARγ dependently. As depicted in Fig. 3 C, pretreatment of RAW 264.7 cells with 10 μM 15d-PGJ2 for 1 h reduced the p50/p65 heterodimer formation in response to PMA (Fig. 3 C, second lane) compared with PMA-treated controls (Fig. 3 C, first lane). Preincubation of the cells 1 h with 10 μM GW9662 completely eliminated the influence of 15d-PGJ2 on NF-κB activation (Fig. 3 C, right lane). To show that these results are not restricted to our cell line model, we performed a similar EMSA using nuclear extracts isolated from primary human macrophages. In primary cells, 10 μM of the natural PPARγ agonist 15d-PGJ2 inhibits 100 nM PMA-mediated NF-κB activation (Fig. 3 D, middle lane), which is restored after 10 μM GW9662 pretreatment for 1 h (Fig. 3 D, right lane). However, in human macrophages, only one NF-κB shift in response to PMA, which is formed by a p50/p65 heterodimer (unpublished data), is observed. From these results, we reasoned that PPARγ activation reduced the NF-κB DNA-binding ability in response to PMA by ~50% compared with PMA-treated controls. To determine whether reduced NF-κB activation modulates expression of proinflammatory cytokines, we finally examined TNF-α expression of RAW 264.7 macrophages in response to PMA. TNF-α expression was determined...
For supershift analysis, a p50 antibody (left, second lane) or a p65 antibody (right, second lane) was included. NF-κB activation without antibody addition (left and right, first lane) is shown. [C] 15d-PGJ2 inhibited PMA-mediated NF-κB activation. RAW 264.7 cells were pretreated with 10 μM of the endogenous PPARγ ligand 15d-PGJ2 for 1 h, followed by the addition of 100 nM PMA for 3 h (middle lane). To ensure a PPARγ-dependent effect, one sample was prestimulated before 15d-PGJ2 addition with 10 μM of the PPARγ antagonist GW9662 for 1 h (right lane). One sample remained PMA treated only as a control (left lane). Cells were harvested, nuclear protein extracts were isolated, and NF-κB EMSA was performed as described in the Materials and methods. [D] PMA-mediated NF-κB activation is inhibited in human primary macrophages in response to 15d-PGJ2. Primary monocyte-derived macrophages were treated as described in C. Cells were harvested and processed, and NF-κB EMSA was performed as described in Materials and methods. [E] Inhibition of PMA-mediated PKCα activation by PPARγ reduces proinflammatory TNF-α expression. RAW 264.7 cells were treated for 1 h with 10 μM rosiglitazone or remained as controls. Afterward, cells were incubated with 100 nM PMA for 6 h, and TNF-α expression in the cell supernatant was analyzed using the CBA system. All experiments were performed at least three times. Data are the means ± the SD of the individual experiments (*, P < 0.05) or representative of three similar experiments.
PKCα translocation in response to 100 nM PMA. Based on the aforementioned Western blot results, RAW 264.7 cells express isoform 1, which is partially located in the cytosol.

To verify the impact of PPARγ activation on PKCα translocation, we used HEK293 cells. Cells were transiently transfected with a PPARγ wild-type–encoding vector, tagged with DsRed-monomer or a DsRed-monomer–tagged PPARγ1 AF2 mutant–encoding vector in combination with a PKCα-EGFP–encoding vector. The PPARγ1 AF2 mutant contains two amino acid exchanges (L468A/E471A), thus preventing ligand binding and concomitant PPARγ1 activation (Gurnell et al., 2000). To follow PKCα translocation, 100 nM PMA was added to rosiglitazone-pretreated and control cells. Changes in PKCα localization were documented 1 h after rosiglitazone stimulation and 50 min after 100 nM PMA addition. PMA provokes PKCα-EGFP translocation to the cell membrane in DsRed-tagged PPARγ1 wild type, as well as DsRed-tagged PPARγ1 AF2 mutant–expressing cells, as expected (Fig. 5 A, second row, second panel vs. fourth row, second panel). Localization of PPARγ does not change (Fig. 5 A, first row, third panel vs. second row, third panel; and third row, third panel vs. fourth row, third panel). In cells transfected with the DsRed-tagged PPARγ1 wild-type construct, rosiglitazone pretreatment inhibited PKCα-EGFP translocation to the cell membrane in response to PMA (Fig. 5 B, second row, second panel), whereas in cells transfected with the DsRed-tagged PPARγ2 AF2 mutant, rosiglitazone preincubation does not prevent PKCα-EGFP translocation (Fig. 5 B, fourth row, second panel). However, PPARγ localization remains unaltered in all analyzed samples (Fig. 5 A and B, first through fourth row, third panel). As shown in Fig. 5 C, preincubation of the cells with the PPARγ antagonist GW9662 (10 μM) for 1 h, completely abolished the PPARγ-dependent inhibition of PKCα translocation in response to PMA (bottom row, second panel). Inline pretreatment of the cells with the PPARγ agonist WY14643 (10 μM) for 1 h did not inhibit PMA-mediated PKCα translocation (Fig. 5 D, bottom row, second panel), which further approved a PPARγ-dependent effect. In corroboration with Fig. 5 (A and B), PPARγ localization was unaffected in response to GW9662 or WY14643 and PMA treatment (Fig. 5, C and D, first and second row, third panel).

Based on these findings, we went on to analyze whether PPARγ inhibits PKCα translocation by a direct protein–protein interaction.

### PPARγ Directly Binds to PKCα

To elucidate whether PPARγ inhibits PKCα translocation by a direct PPARγ–PKCα interaction, we performed a set of coinmunoprecipitation experiments. Immunoprecipitation of PKCα from lysates of differentiated THP-1 cells, which had been stimulated for 1 h with rosiglitazone or left untreated, was conducted. As shown in Fig. 6 A, immunoprecipitation of PKCα resulted in coinmunoprecipitation of PPARγ1 in THP-1 cells that had been challenged with a PPARγ agonist (Fig. 6 A, lane 2). In the flowthrough, PPARγ1 was only detected when agonist stimulation was omitted (Fig. 6 A, lane 1). After PPARγ1 activation, PPARγ1 was almost completely retarded in the immunoprecipitation column.

To verify a PPARγ1-dependent mechanism, we transfected COS-7 cells with PPARγ1 wild-type or AF2-encoding plasmids and a PKCα-EGFP expression plasmid. Immunoprecipitation was performed using μMacs anti-EGFP beads. In cells transfected with the PPARγ1 AF2 mutant, little if any PPARγ1 coinmunoprecipitated with PKCα-EGFP in response to 10 μM rosiglitazone (Fig. 6 B, lane 4). In cells transfected with the PPARγ1 wild-type plasmid, rosiglitazone treatment allowed to coinmunoprecipitate PPARγ1 with PKCα-EGFP (Fig. 6 B, lane 2), pointing to the importance of agonist activation to promote PKCα binding.

To provide further evidence for a direct PPARγ1–PKCα interaction, we used the mammalian two-hybrid system. In COS-7 cells transiently transfected by electroporation with a combination of pCMV-AD-PPARγ1, pCMV-BD-PKCα, and the Gal4 reporter vector pFR-luc, addition of rosiglitazone or ciglitazone provoked induction of luciferase expression as determined by a luciferase assay. As shown in Fig. 7, addition of both PPARγ agonists induce luciferase expression roughly threefold compared with untreated controls. A PPARγ-dependent effect was verified because addition of the PPARγ agonist...
WY14643 left basal luciferase activity unaltered. With this two-hybrid model, direct binding of target (PPARγ₁) to bait protein (PKCα) is required to induce luciferase expression. Therefore, our data suggest that PPARγ₁ directly binds PKCα upon agonist activation. This interaction inhibits PKCα translocation to the cell membrane, and thus, PKCα activation.

**Identification of PPARγ₁ domains involved in PKCα binding**

To identify PPARγ₁ domains that promote binding to PKCα, we first generated a set of point mutations, each substituting one aa in helix 4 of the ligand-binding domain (LBD), taking into consideration that this region is important in binding transcriptional coactivators (Nolte et al., 1998; Westin et al., 1998), and therefore might be responsible for binding to PKCα as well. We generated six clones, with L309, N310, G312, V313, L316A, or K317 being individually substituted by an alanine (Fig. 8 A). In addition, we generated the construct PPARγ₁ AAaa309-319, with helix 4 (aa309-319) being completely removed (Fig. 8 A). To prove the functionality of these constructs, we first verified their expression by Western blotting. As a control, the DsRed-PPARγ₁ wild-type–encoding vector was included in the experiment.
translocation in rosiglitazone-pretreated cells. From these data, α (Fig. 8 C, right), showing no PMA-mediated PKC and PKC α γ as indicated. (B) COS-7 cells

Because of a single aa exchange, or the 12 aa deletion, the molecular mass of proteins originating from the constructs remained unaltered compared with DsRed-PPARγ wild type when transfected into HEK293 cells (unpublished data).

To finally analyze the impact of the various mutations and the deletion on PKCo translocation, HEK293 cells were transiently cotransfected with the mutated/deleted PPARγ constructs tagged with DsRed-monomer, in combination with a PKCo-EGFP. The localization was documented in HEK293 cells, were verified using an anti-red fluorescent protein antibody (Fig. 9 B). Taking into account that the DBD of PPARγ is completely removed in the DsRed-PPARγ γ1 deletion constructs, as described in Materials and methods. Afterward, cells were cotransfected with the mutated/deleted PPARγ constructs in combination with a PPRE-reporter plasmid into HEK293 cells. As expected, adding 10 μM rosiglitazone for 6 h to cells transfected with the PPARγ deletion constructs did not alter basal transactivation. In contrast, the DsRed PPARγ γ1 wild-type–encoding plasmid provoked a twofold induction of luciferase expression, whereas the DsRed PPARγ AF2 dominant-negative mutant blocked transactivation even below basal values, mediated by endogenous PPARγ in HEK293 cells (unpublished data).

To elucidate the role of these deletions on PKCo translocation, HEK293 cells were transiently cotransfected with the shortened DsRed-monomer–tagged PPARγ constructs in

Figure 7. PPARγ directly binds to PKCo. COS-7 cells were transiently transfected with a combination of a target (PPARγ1), a bait (PKCo), and a reporter construct, as described in Materials and methods. Afterward, cells were treated with 10 μM ciglitazone, 10 μM rosiglitazone, and remained as controls. 6 h later, cells were harvested and lysed for a reporter analysis as described in Materials and methods. Experiments were performed at least three times in duplicate. *, P < 0.05. Data are the means ± the SD.

...we conclude that helix 4 of the LBD is not involved in PPARγ binding to PKCo.

Based on these results, we decided to generate three PPARγ deletion constructs (DsRed-PPARγ1 aa32-198, DsRed-PPARγ1 Delta32-250, and DsRed-PPARγ1 Delta51-406) with the belief that ligand binding is necessary for PPARγ–PKCo interactions. As shown in Fig. 9 A, all deletions lack the DNA-binding domain (DBD) of PPARγ. Furthermore, to characterize the role of the hinge domain in PKCo binding, it was eliminated to variable extents. In the DsRed-PPARγ γ1 deletion construct, the first 26 aa of the hinge domain were deleted, and in the DsRed-PPARγ γ1 Delta32-250 construct, 78 aa of the hinge domain were deleted. The hinge domain was completely removed in the DsRed-PPARγ γ1 Delta51-406 construct. In this construct, a part of the LBD/AF2 domain was deleted as well (aa288-406). All constructs lack a part of the AF1 domain.

Expression of the cloned constructs was verified by Western blotting. As controls, the DsRed-PPARγ wild-type– and AF2 mutant–encoding vectors were included in the experiment. Estimated molecular mass of deletion construct proteins, transfected into HEK293 cells, were verified using an anti–red fluorescent protein antibody (Fig. 9 B). Taking into account that the DBD was removed, DNA binding and concomitant transactivation by corresponding PPARγ deletion constructs should be abolished. Therefore, we performed a set of reporter experiments, cotransfecting DsRed-PPARγ deletion constructs in combination with a PPRE-reporter plasmid into HEK293 cells. As expected, adding 10 μM rosiglitazone for 6 h to cells transfected with the PPARγ deletion constructs did not alter basal transactivation. In contrast, the DsRed PPARγ wild-type–encoding plasmid provoked a twofold induction of luciferase expression, whereas the DsRed PPARγ AF2 dominant-negative mutant blocked transactivation even below basal values, mediated by endogenous PPARγ in HEK293 cells (unpublished data).
combination with a PKCα-EGFP-encoding vector. To follow PKCα translocation, 100 nM PMA was added to 100 μM rosiglitazone-pretreated cells. PKCα localization was documented in untreated cells (Fig. 9 C, first row), cells treated for 50 min with PMA (Fig. 9 C, second row), for 1 h with rosiglitazone (Fig. 9 C, third row), or preincubated for 1 h with rosiglitazone followed by the addition of 100 nM PMA for 50 min (fourth row) or remained as controls (first row). Experiments were performed three times, and representative data are shown.

From these data, we conclude that for PKCα, binding a part of the hinge domain of PPARγ1 is indispensable. To further narrow the involved region of PPARγ1, we finally created the construct DsRed-PPARγ1 Δaa309-319 (Fig. 10 A), containing a deletion of helix 1 (aa206-224) of PPARγ1, which is located in the hinge domain (aa173-288). Helix 1 has already been identified to mediate the protein–protein interaction of PPARγ with ERK5 (Akaike et al., 2004). Expression of the construct results as expected in protein, demonstrating a slightly reduced protein mass (Fig. 10 B, lane 2) because of the aa206-224 deletion compared with the DsRed-PPARγ1 wild type (Fig. 10 B, lane 1).

We transiently cotransfected HEK cells with the PPARγ1 Δaa206-224 construct tagged with DsRed-monomer in combination with a PKCα-EGFP-encoding vector. In cells expressing the DsRed-tagged PPARγ1 Δaa309-319 (Fig. 10 C), PKCα translocated to the cell membrane in response to 100 nM PMA.
We conclude that PPARγ1 binds to PKCα via the helix 1, which is located in the hinge domain of PPARγ1.

Discussion

Recently, we demonstrated that monocyte/macrophage desensitization at least partially attenuates PKCα signaling (von Knethen et al., 2005; Johann et al., 2006). We provide evidence that PPARγ agonists block PKCα translocation to the cell membrane and concomitant protein depletion, which normally occurs after cell activation. In monocytic cell lines, PPARγ expression has been previously described (McIntyre et al., 2003; Musiek et al., 2005; von Knethen et al., 2005), and it was verified using primary human monocyte–derived macrophages. These data corroborate the work of Tontonoz et al. (1998) and Chimenti et al. (1998), showing PPARγ expression in differentiated macrophages. However, even if PPARγ is expressed, PPARγ agonists are known to mediate PPARγ-dependent and -independent effects (Nesjean and Boutin, 2002). To this end, 15d-PGJ2 has been described to directly modify H-ras, provoking a constitutively active enzyme (Oliva et al., 2003) or inhibiting IκB kinase, and thus suppressing NF-κB signaling (Straus et al., 2000). Our approach, using cells expressing PPARγ1 wild type or the PPARγ1 agonist-binding mutant AF2, substantiates the need of PPARγ activation in our system. Only in cells expressing PPARγ1 wild type was translocation of PKCα blocked by PPARγ activation. The PPARγ1 AF2 mutant did not prevent PMA-mediated PKCα translocation. These data support the notion of a PPARγ-dependent mechanism.

PPARγ-mediated inhibition of classical PKCs has been previously described (Verrier et al., 2004). In their case, PKCβ translocation was blocked by PPARγ agonists via DGKα up-regulation. DGKα metabolizes DAG, which is an established activator of classical and novel PKC isoforms. Therefore, its induction/activation will remove the potential PKC activator, causing desensitization as seen in our experiments. However, in
our experiments, a role of DGKe up-regulation must be excluded because the protein-synthesis inhibitor CHX did not restore PKCa translocation. In line with this, our PPARγ1 Δaa32-198 construct, where the PPARγ1 DBD was removed, still inhibits PKCa translocation. Further support for our hypothesis, suggesting a direct PPARγ1–PKCa interaction in preventing PKCa translocation, came from previous studies (Johann et al., 2006). In this case, PPARγ was activated in response to apoptotic cells, attenuating PKCa translocation and concomitant ROS production. In this study, the role of PPARγ was verified using a PPARγ d/n cell line. In these cells, pretreatment with apoptotic cells left PMA-mediated PKCa translocation and subsequent ROS production unaltered. A premise for this assumption is that PPARγ is expressed at least partially in the cytosol. Generally, the nuclear hormone receptor PPARγ is described to be exclusively localized in the nucleus (Akiyama et al., 2002; Feige et al., 2005). In support of our hypothesis, suggesting cytoplasmatic localization as well, we noticed a minor amount of PPARγ1 to remain in the cytosol. This is based on results using DsRed-PPARγ1-transfected cells, as well as immunohistochemical detection of endogenous PPARγ1 located in the cytosol of RAW 264.7 macrophages besides its major nuclear localization. It should be noted that cytoplasmatic distribution of PPARγ is in line with the work of Abella et al. (2005). In their study, an approach similar to our experiments was used, with EGFP-tagged PPARγ used to characterize intracellular distribution of PPARγ. Results indicated that PPARγ is not exclusively located in the nucleus. Furthermore, localization of PPARγ in the cytoplasm in the promonocytic cell lines HL-60 and K-562 has been observed, especially in response to the PPARγ agonist troglitazone (Liu et al., 2005). This work was done using immunohistochemical detection of endogenous PPARγ. Therefore, side effects, such as unphysiological high expression or a modified protein behavior as a result of a tag or label (Feige et al., 2005), can be excluded. In addition, Burgermeister et al. (2006) recently provided evidence that PPARγ is actively exported from the nucleus into the cytosol in a MEK1-dependent manner, further supporting our observed PPARγ localization pattern. Furthermore, Patel et al. (2005) described cytoplasmatic localization of a different PPAR isoform, PPARα, when coexpressed with CAP350, which is a putative centrosome-associated protein of unknown function. Therefore, we propose that members of the PPAR family may localize in the cytoplasm, possibly after activation, when bound to cytoplasmatic proteins such as PKCa. Immunoprecipitation of PKCa from lysates of differentiated THP-1 cells coimmunoprecipitated PPARγ. Remarkably, PPARγ coimmunoprecipitation was only seen once PPARγ1 became activated. The requirement of PPARγ1 activation was verified using an agonist-binding mutant of PPARγ1, which did not block PKCa translocation in response to PMA stimulation. A direct PPARγ1–PKCa interaction was further supported by a mammalian two-hybrid system with PPARγ1 as the target and PKCa as the bait construct, provoking luciferase reporter gene expression when target and bait proteins interact. To avoid autocrine activation of the reporter system, PPARγ has to be cloned as a target protein linked to the NF-κB transactivation domain, not allowing this hybrid protein to bind to the promoter of the reporter. However, DNA binding of PPARγ1 to PPREs, and concomitant scavenging the NF-κB-AD-PPARγ1 hybrid protein from the two-hybrid assay, cannot be excluded.

Based on the well-established role of helix 4 of the PPARγ LBD in mediating protein–protein interaction of PPARγ with coactivators, such as CBP and SRC-2, or repressors, such as the nuclear receptor corepressor and the silencing mediator for retinoic acid receptor and thyroid-hormone receptor (Nolte et al., 1998; Westin et al., 1998; Perissi et al., 1999; Perissi and Rosenfeld, 2005), we first generated 6 PPARγ constructs in which only
1 aa was exchanged and 1 construct in which helix 4 was completely removed. Unexpectedly, these constructs did not alter rosiglitazone-dependent inhibition of PKCα translocation.

Taking into account that PPARγ binding to other factors, such as adipocyte-type fatty acid–binding protein or extracellular signal-related kinase 5, which do not belong to the family of transcriptional coactivators, can be mediated by other PPARγ domains, such as A/B/C and D/E/F (Adida and Spener, 2006) or the hinge domain (domain D; Akaike et al., 2004), we created three PPARγ deletion constructs. All of them lack the entire DBD (domain C). In addition, different parts of the A/B and D domains have been removed, and one construct contained the C-terminal third of the E/F domains only. Based on our collective results, we provide evidence that a part of the hinge domain probably confers the PPARγ1–PKCα interaction, which is present in the PPARγ1 Δaa32-198 construct but absent in the Δaa32-250 construct, when PPARγ1 is activated by an agonist, thus requiring the LBD/AF2 domains. One known region of PPARγ1 located in aa198-250 is the hinge helix 1 (aa 206–224). Therefore, we cloned a PPARγ1 construct with helix 1 deleted (DsRed- PPARγ1 Δaa206-224). In cells transfected with this construct, PKCα translocated even after rosiglitazone pretreatment in response to PMA. From these results, we conclude that PPARγ1 binds to PKCα via the hinge helix 1 domain, after PPARγ1 has been activated by a ligand.

The proposed mechanism of PPARγ1–PKCα binding proceeds fast. 1 h of prestimulation with PPARγ agonist is sufficient to inhibit PKC translocation in response to 100 nM PMA. However, PKCα translocation by 1 μM PMA was not blocked. These results support the assumption that the capacity of cytoplasmatic PPARγ to bind PKCα correlates with the strength of PKCα activation. Likely, very strong activation signals, such as 1 μM PMA, exceed the inhibitory impact of PPARγ. Thus, the role of PPARγ in blocking PKCα signaling might be only transient, allowing PKCα activation by a more stringent activator. This makes the mechanism more interesting for the development of new therapy strategies. Prolonged periods of PPARγ activation, which provoke transcriptional control to target members of the NADPH oxidase system, have already been described (p22phox, p47 phox, and gp91 phox; Inoue et al., 2001; von Knethen and Brune, 2002; Hwang et al., 2005). Consequently, in these cells PPARγ contributes to an anti-inflammatory phenotype by blocking NADPH oxidase-dependent ROS production.

An involvement of PPARγ in attenuating inflammatory reactions to improve the clinical picture of sepsis has previously been shown (for review see Zingarelli and Cook, 2005). In line with this, our results add to this data. In our system, PMA-mediated NF-κB activation was inhibited in response to PPARγ agonist pretreatment to 50% in RAW 264.7 cells, as well as primary human macrophages. In accordance, PMA-induced TNF-α expression was PPARγ1-dependent reduced to 70%. It has been observed that PPARγ1 activation inhibits multiple organ failure in an animal model (Abdelrahman et al., 2005), although the underlying mechanism remains unclear. The option to adjust a pro- versus antiinflammatory monocyte/macroage phenotype will provide new possibilities for the development of therapies to control systemic inflammation. Our data add a new antiinflammatory role for PPARγ based on the ability to scavenge PKCα in the cytosol, thus, blocking membrane translocation and downstream signaling.

Materials and methods

Monocyte isolation

We analyzed human cells from peripheral blood of healthy donors. For monocyte enrichment, we isolated PBMCs from donors using Ficoll-Hypaque gradients (PAA Laboratories). Cells were left to adhere on culture dishes (Primaria 3072; Becton Dickinson) for 60 min at 37° C. Nonadherent cells were removed. Afterward, cells were differentiated to macrophages by culturing them in complete RPMI containing 10% AB-positive human serum. Flow cytometry confirmed that the monocyte-like population was 90–95% pure (CD14+ vs. CD14+).

Cell culture

We cultivated RAW 264.7 and THP-1 in RPMI 1640 (PAA Laboratories). HEK293 and COS-7 cells were cultured in DMEM high glucose (PAA Laboratories). Both media were supplemented with 100 U/ml penicillin (PAA Laboratories), 100 μg/ml streptomycin (PAA Laboratories), and 10% heat-inactivated fetal calf serum (PAA Laboratories). Ciglitazone (Biomol), Rosiglitazone (Biomol), and ChX (Sigma-Aldrich) were dissolved in DMSO. Appropriate vehicle controls were performed.

Immunofluorescence staining

To determine intracellular PPARγ localization, we seeded RAW 264.7 macrophages directly on a slide. After 24 h, cells were treated as indicated and fixed on the slides by 1-h incubation in 4% paraformaldehyde at 4°C. Thereafter, cells were permeabilized in PBS containing 0.2% Triton X-100 for 15 min. After a washing step in PBS, cells were incubated for 2 h with a 1:250 dilution of a rabbit anti-PPARγ antibody (Calbiochem) at 4°C. After three 5-min washing steps with PBS, cells were incubated with a secondary goat anti-rabbit antibody (1:250) labeled with Alexa Fluor 546 (Invitrogen) for 2 h at 4°C. Cells were incubated for 2 h with a 1:250 dilution of a mouse anti-PKCα antibody (BD Biosciences) at 4°C. After three 5-min washing steps with PBS, cells were incubated with a secondary goat anti-mouse antibody (1:250) labeled with Alexa Fluor 488 (Invitrogen) for 2 h at 4°C. Again, cells were washed three times with PBS and counterstained with DAPI (1 μg/ml in PBS for 15 min). After a final 5-min washing step in PBS, cells were covered with Vectorshield mounting medium (Linaris) and a coverslip. PPARγ and PKCα localization were determined using an AxioScope fluorescence microscope with the ApoScope upgrade (Carl Zeiss MicroImaging, Inc.; lens 63×/0.6 NA; ocular 10×) at room temperature, documented by a charge-coupled device camera (Carl Zeiss MicroImaging, Inc.) and AxioVision Software (Carl Zeiss MicroImaging, Inc.).

Vector construction, transient transfection, fluorescence microscopy, and reporter analysis

To examine cellular PPARγ localization, we subcloned human PPARγ1 into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.) using the infusions ligation kit (CLONTECH Laboratories, Inc.). To allow integration of the PPARγ1 fragment, the vector was cut with the DsRed coding sequence and then ligated into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.). To allow integration of the PPARγ1 fragment, the vector was cut with the DsRed coding sequence and then ligated into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.) using the infusion ligation kit (CLONTECH Laboratories, Inc.). To allow integration of the PPARγ1 fragment, the vector was cut with the DsRed coding sequence and then ligated into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.). To allow integration of the PPARγ1 fragment, the vector was cut with the DsRed coding sequence and then ligated into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.). To allow integration of the PPARγ1 fragment, the vector was cut with the DsRed coding sequence and then ligated into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.). To allow integration of the PPARγ1 fragment, the vector was cut with the DsRed coding sequence and then ligated into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.). To allow integration of the PPARγ1 fragment, the vector was cut with the DsRed coding sequence and then ligated into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.). To allow integration of the PPARγ1 fragment, the vector was cut with the DsRed coding sequence and then ligated into the DsRed-monomer–encoding vector pDsRed-Monomer-C1 (CLONTECH Laboratories, Inc.).
The extension phase was performed at 68°C for 20 min. After this incubation, the reaction was precipitated with CaPO4-precipitation with combinations of pDsRed-Monomer-C1 PPARα G312A, 50 μg/ml in PBS for 15 min). After washing with PBS and counterstained with DAPI (1:1,000; MBL). After fixation in 4% paraformaldehyde at 4°C, cells were treated as indicated. Afterward, cells were fixed on the slides by 1-h incubation and the remaining proteins were eluted using 50 μl of lysis buffer.

To follow PKCα distribution, HEK293 cells were transiently transfected by electroporation (450 V). Western blot analysis was performed for the pFR-luciferase reporter vector (Stratagene). Afterward, cells were incubated for 24 h, and then stimulated for 6 h with 10 μM rosiglitazone, or 10 μM WY14643, or they remained as controls.

For reporter analysis, HEK293 cells were transiently transfected by electroporation (450 V) with the BD Cytometric Bead Array TNF-α Flex Set (BD Biosciences) according to the supplier’s instructions using a FACSCanto flowcytometer. Interpreta-

**Mammalian two-hybrid assay**

To use PPAR-1 and PKCα in the mammalian two-hybrid system (Stratagene), PPAR-1 was cloned into the BamHI-HindIII site of the pCMV-MD MCS, and PKCα was cloned into the BamHI-HindIII site of the pCMV-MD MCS. PPAR-1 was amplified from the pcDNA3-PPAR-1 wild-type vector and PKCα from the vector pPKCα-EGFP. The following primers were used: pCMV-PPAR-1 5′-GGCCGAAATTCGCGCACTGTGTTAAGTCGGTCAAACTTCTC-3′ and 5′-CCTGCTGCTACATTTCCCTTCACGCCTGCGACGGCAGG-3′ and 5′-CAGCGGCCA AAGCCCTGATACGCGCCTGCGACGGCAGG-3′ and 5′-CAGCGGCCA AAGCCCTGATACGCGCCTGCGACGGCAGG-3′ and 5′-CAGCGGCCA AAGCCCTGATACGCGCCTGCGACGGCAGG-3′ and 5′-CAGCGGCCA AAGCCCTGATACGCGCCTGCGACGGCAGG-3′.

**Quantification of TNF-α expression**

Supernatants from RAW 264.7 macrophages treated as indicated were subjected to the BD Cytometric Bead Array TNF-α Flex Set (BD Biosciences) according to the supplier’s instructions using a FACSCanto flowcytometer. Interpretation of the results was performed with the FCAP Array software (Soft Flow, Inc./BD Biosciences).

**EMSA**

Nuclear extracts were prepared as previously described (von Knethen and Brune, 2001). An established EMSA method, with slight modifications, was used (Camandola et al., 1996). Nuclear protein (20 μg) was incubated for 30 min at room temperature with 2 μg poly(dI-dC) from GE Healthcare, 2 μl buffer D (20 mM Heps/KOH, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, and 1 mM DTT) to the assay cell lysis buffer and then diluted 10-fold in assay buffer. To assure cell lysis, 5 μl were applied on a 16-gauge needle, followed by a brief 10 s sonication (Sonifier; Branson, duty cycle 100%, output control 60%). Cell debris was removed by centrifugation (10,000 g for 5 min), and 1 μg of protein was used for immunoprecipitation. Sample volume was adjusted with lysis buffer to 1 ml. 2 μg anti-PKR antibody (BD Biosciences) was added and incubated at 4°C overnight. Thereafter, 50 μl μM ACS protein A microbeads (Millenyi Biotec) were added and incubated for 2 h with rotation. The beads were washed 4 times with lysis buffer. For analysis, the column was rinsed 4 times with 200 μl lysis buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris HCl, pH 8.0), followed by 2 washes with low ionic buffer (20 mM TrisHCl, pH 7.5). Afterward, the column was removed from the magnetic field and the remaining proteins were eluted using 50 μl of lysis buffer.
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