Acute Antiinflammatory Properties of Statins Involve Peroxisome Proliferator–Activated Receptor-α via Inhibition of the Protein Kinase C Signaling Pathway

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Abstract—Statins are inhibitors of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase used in the prevention of cardiovascular disease (CVD). In addition to their cholesterol-lowering activities, statins exert pleiotropic antiinflammatory effects, which might contribute to their beneficial effects not only on CVD but also on lipid-unrelated immune and inflammatory diseases, such as rheumatoid arthritis, asthma, stroke, and transplant rejection. However, the molecular mechanisms involved in these antiinflammatory properties of statins are unresolved. Here we show that the peroxisome proliferator–activated receptor (PPAR) α mediates antiinflammatory effects of simvastatin in vivo in models of acute inflammation. The inhibitory effects of statins on lipopolysaccharide-induced inflammatory response genes were abolished in PPARα-deficient macrophages and neutrophils. Moreover, simvastatin inhibited PPARα phosphorylation by lipopolysaccharide-activated protein kinase C (PKC) α. A constitutive active form of PKCα inhibited nuclear factor κB transrepression by PPARα whereas simvastatin enhanced transrepression activity of wild-type PPARα, but not of PPARα mutated in its PKC phosphorylation sites. These data indicate that the acute antiinflammatory effect of simvastatin occurs via PPARα by a mechanism involving inhibition of PKCα inactivation of PPARα transrepression activity. (Circ Res. 2006;98:361-369.)

Key Words: inflammation ■ macrophages ■ neutrophils ■ nuclear receptors ■ statins ■ PKC

Statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, are widely prescribed for the treatment of hypercholesterolemia.¹ In addition to plasma lipid-modulating action, statins exert pleiotropic antiinflammatory effects, which might contribute to their beneficial effects on cardiovascular disease (CVD).² Emerging evidences also suggest beneficial therapeutic activities of statins in immune and inflammatory diseases such as multiple sclerosis, Alzheimer’s disease, ischemic stroke, transplant rejection, rheumatoid arthritis, and asthma.³–⁶ Several clinical observations indicate that these effects cannot be attributed to their cholesterol-lowering activities only.⁷ Statin therapy decreases plasma concentrations of inflammatory markers, such as C-reactive protein (CRP), within 1 week after treatment initiation, before any lipid changes are observed.⁸ Statin treatment reduces the incidence of ischemic stroke for which plasma cholesterol levels are not considered a risk factor.⁹ Moreover, statins also exert antiinflammatory actions in animal models, which are resistant to their hypolipidemic actions.¹⁰ In models of acute and chronic inflammation, statins inhibit endothelial adhesion and transendothelial migration of leukocytes to sites of inflammation,¹⁰ acting both on endothelial cells and leukocytes. Statins modulate macrophage functions by inhibiting the activation of inflammatory response genes, such as interleukin (IL)-1β and IL-6, tumor necrosis factor (TNF) α, metalloproteinase (MMP)-2, and MMP-9, and inducible nitric oxide synthase (iNOS).¹¹ These antiinflammatory actions of statins are attributed to their ability to modulate signal transduction pathways activating proinflammatory transcription factors, such as nuclear factor (NF) κB.¹² PPARα is a nuclear receptor that regulates gene expression by binding with its heterodimeric partner the retinoid-X-receptor (RXR) to PPAR-responsive elements (PPREs). PPARα not only regulates lipid metabolism¹³ but also exerts pronounced antiinflammatory activities.¹⁴ Clinical trials have shown that fibrates decrease inflammation and have beneficial effects on CVD and stroke.¹⁵ In animals, PPARα deficiency induces a prolonged inflammatory response in a
mouse ear-swelling model. PPARα exerts antiinflammatory activities by negatively interfering with proinflammatory signaling pathways including NFκB. This molecular action is exemplified by the inhibition of inflammatory induction of genes, such as vascular cell adhesion molecule-1, MMP-9, IL-6, and TNFα.14

These similarities between the antiinflammatory effects of statins and PPARα led us to investigate whether PPARα could mediate antiinflammatory effects of statins in vivo in models of acute inflammation and in vitro in macrophages and neutrophils.

Materials and Methods

Inflammation Tests

Subcutaneous dorsal pouches and carrageenan footpad edema were induced in C57BL6 wild-type and PPARα-null mice as described.16,17 Simvastatin at indicated doses or vehicle (CMC 0.5%) was given by gavage to mice 1 hour before inflammatory challenges (see the online data supplement available at http://circres.ahajournals.org).

Cell Culture

Lipopolysaccharide (LPS)-elicited neutrophils from air pouches and thioglycollate-elicited peritoneal macrophages were isolated as described.18 Cells were treated with the indicated reagents (see the online data supplement).

RNA Analysis

RNA extraction was performed using TRIzol reagent followed by reverse transcription (Invitrogen Life Technologies, Cergy-Pontoise, France). cDNA was quantified by real-time PCR on a MX4000 apparatus (Stratagene) using specific primers (see the online data supplement).

Kinase Assays and Immunoblot

After treatment, cells were washed with PBS and suspended in protein kinase C (PKC) lysis buffer, sonicated (Rbracell Hiddock 72442), and centrifuged at 4°C (3000 rpm, 15 minutes). Cell extracts (10 μg) or cell extract–immunoprecipitated PKCa (200 μg) were incubated in kinase reaction buffer, histone H1 (1 μg), or purified PPARα protein (400 ng) as substrates and (γ-32P)ATP (5 μCi) (2000 Ci/mmol). Kinase reactions were performed as described previously.19 Immunoblots were performed using the Aurora detection system (ICN Pharmaceuticals, Orsay, France) (see the online data supplement).

Transient Transfections and Metabolic Labeling

COS-7 cells were transfected by lipofection with reporter and expression plasmids as indicated and incubated overnight with DMEM supplemented with 2% Ultrasen. Cells were collected and luciferase and β-galactosidase assays performed. For 35S-methionine labeling, cells were cultured in methionine-free minimum essential medium for 1 hour before supplementation with 35S-methionine (100 μCi) for an additional 3 hours. For 32P-phosphate labeling, cells were deprived in phosphate-free minimum essential medium for 2 hours before supplementing the medium with 32P-phosphate (500 μCi) for 5 hours, followed by PPARα immunoprecipitation (see the online data supplement).

Statistical Analysis

Statistical significance was determined using nonparametric Mann–Whitney or multivariate ANOVA tests followed by Scheffe post hoc or the unpaired t tests (transient transfections). Values of P<0.05 were considered as significant.

Results

PPARα Mediates the Acute Antiinflammatory Action of Simvastatin In Vivo

To investigate whether PPARα plays a role in inflammatory response modulation by statins in vivo, the influence of simvastatin was tested in wild-type and PPARα-null mice using 2 models of acute inflammation in which statins display antiinflammatory activity.16,17 Doses were chosen in accordance with these previous studies.16,17 The acute antiinflammatory action of simvastatin (10 to 50 mg/kg) administered orally 1 hour before LPS was first measured by the number of neutrophils recruited in air pouches by LPS.19 Simvastatin treatment decreased neutrophil recruitment in a dose-dependent manner (Figure 1A). Administration of a single dose of atorvastatin (30 mg/kg) exerted similar effects on neutrophil recruitment (not shown). Interestingly, the decrease of LPS-induced neutrophil recruitment by simvastatin was only observed in wild-type, but not in PPARα-null mice (Figure 1B). Similarly, in the carrageenan-induced footpad inflammation mouse model,17 a single dose of simvastatin given 1 hour before carrageenan injection blocked swelling only in wild-type, but not in PPARα-null mice (Figure 1C). These effects occurred independently of alterations in plasma lipid levels, because plasma cholesterol levels did not change after simvastatin treatment in either model (not shown). Thus, PPARα mediates the lipid-independent acute antiinflammatory activity of simvastatin in mice.

PPARα Mediates the Inhibition of LPS-Induced Inflammatory Response Genes by Simvastatin in Primary Macrophages and Neutrophils

Because neutrophils are a major cell type mediating the inflammatory response in these in vivo models, the expression of PPARα was analyzed in LPS-elicited neutrophils recovered from air pouches and compared with other cell types. PPARα mRNA levels were highest in neutrophils, whereas macrophages express similar levels as primary endothelial cells, a cell type in which the antiinflammatory effects of PPARα have been well documented20 (Figure 2A).

Subsequently, the role of PPARα in the modulation of inflammatory response gene expression (iNOS, TNFα, or IL-6) by statins was investigated in macrophages and neutrophils isolated from wild-type and PPARα-null mice. Pretreatment (2 hours) of macrophages isolated from wild-type mice with a single dose of simvastatin, sufficient to inhibit HMG-CoA reductase activity,18 significantly decreased LPS-induced iNOS and IL-6 mRNA levels (Figure 2B and 2C). By contrast, simvastatin was without effect in macrophages isolated from PPARα-null mice. Similarly, pretreatment (2 hours) with simvastatin also significantly decreased LPS-induced iNOS and TNFα mRNA levels in neutrophils isolated from wild-type but not from PPARα-null mice (Figure 2D and 2E). Simvastatin induced a dose-dependent decrease in iNOS mRNA and protein levels after LPS induction only in wild-type but not in PPARα-null macrophages (Figure 3A and 3B). Atorvastatin and fluvastatin pretreatment also decreased LPS-induced iNOS expression in a PPARα-dependent manner (Figure 3C and 3D). These data indicate

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that the antiinflammatory effect of statins on LPS-induced inflammatory response genes, such as iNOS, in macrophages and neutrophils is PPARα dependent.

Previous studies have shown that statins induce the expression of apolipoprotein A-I in human hepatoma HepG2 cells by modulating PPRE-dependent transcriptional activity of PPARα. To determine whether statins also regulate PPRE-dependent PPARα target genes in macrophages, the effects of simvastatin on the induction of CPT1 mRNA levels, a gene induced by PPARα in human primary macrophages, by the PPARα agonist GW9578 were investigated. As expected, GW9578 treatment (12 hours) increased CPT1 mRNA levels, but simvastatin treatment did not influence this induction (Figure 4A). By contrast, both simvastatin and GW9578 treatment decreased iNOS mRNA levels, and coincubation with both compounds resulted in a more pronounced inhibi-
tion of iNOS mRNA levels (Figure 4B). These results indicate that, unlike in hepatocytes, simvastatin selectively interferes in macrophages with PPARα inhibition of inflammatory response genes, likely by modulating PPARα-dependent transrepression activity.

Figure 3. PPARα mediates the inhibition of LPS-induced iNOS mRNA and protein expression by statins in macrophages. A, Dose-response effect of simvastatin on iNOS mRNA levels. Peritoneal macrophages from wild-type (PPARα+/−) or PPARα-null (PPARα−/−) mice were pretreated with the indicated concentrations of simvastatin for 2 hours and then treated with LPS (10 μg/mL) for 2 hours. iNOS mRNA levels were analyzed using real-time quantitative PCR. B, Dose-response effect of simvastatin on iNOS protein expression. Peritoneal macrophages from wild-type (PPARα+/−) and PPARα-null (PPARα−/−) mice were pretreated with the indicated concentrations of simvastatin for 2 hours and then treated with LPS (10 μg/mL) for 10 hours. iNOS and actin protein levels were measured using immunoblot analysis. C and D, Peritoneal macrophages from wild-type (PPARα+/−) and PPARα-null (PPARα−/−) mice were pretreated with atorvastatin (ATV, 10 μmol/L) (C) or fluvastatin (FLU, 10 μmol/L) (D) or vehicle for 2 hours. Cells were then treated with LPS (10 μg/mL) for 2 hours. iNOS mRNA levels were analyzed using real-time quantitative PCR. C indicates vehicle.

The PKC Signaling Pathway Is Involved in LPS-Induced iNOS Expression and Is Inhibited by Simvastatin

Because the effect of simvastatin on LPS-induced iNOS expression occurs rapidly and requires PPARα, it was hypothesized that simvastatin exerts its effects via posttranslational modulation of PPARα activity. To determine which signaling pathway mediates LPS-induced iNOS expression in macrophages and neutrophils, the effects of different protein kinase inhibitors, which inhibit the PKC or MAPK signaling pathways, were tested. Incubation of macrophages with either the PKC inhibitor Gö6976, which selectively inhibits the Ca2+-dependent PKCα and PKCβ isoforms, or the PKC inhibitor Ro318220, which inhibits all Ca2+-dependent PKC isoforms, prevented LPS-induced iNOS expression, whereas a MEK inhibitor U0126 was without effect (Figure 5A).

Similarly, Gö6976 inhibited LPS-induced iNOS expression in neutrophils, whereas U0126 was without effect (Figure 5B).

To determine whether simvastatin modulates the Ca2+-dependent PKC signaling pathway in macrophages and neutrophils, its effect on the activity of PKCα was investigated. PKCα was immunoprecipitated from LPS-activated macrophages or neutrophils and in vitro phosphorylation experiments using purified histone H1 protein as substrate were performed. LPS treatment induced PKCα activity in macrophages, whereas LPS-elicited neutrophils already displayed high basal PKCα activity. Interestingly, pretreatment (2 hours) with simvastatin decreased PKCα activity both in macrophages and neutrophils (Figure 5C and 5D). In addi-
tion, simvastatin treatment also decreased LPS-induced PKCβII activity both in macrophages and neutrophils (not shown). Thus, inhibition of the Ca²⁺-dependent PKC signaling pathway by simvastatin could be involved in the effects of simvastatin on LPS-induced iNOS expression both in macrophages and neutrophils.

To determine whether the Ca²⁺-dependent PKC signaling pathway is involved in the PPARα-dependent inhibition of LPS-induced iNOS expression, the effect of Gö6976 on LPS-induced iNOS expression was investigated in macrophages isolated from wild-type and PPARα-null mice. Pretreatment (2 hours) of wild-type macrophages with Gö6976 resulted in a decrease of LPS-induced iNOS mRNA levels (Figure 6). By contrast, Gö6976 was without effect in macrophages isolated from PPARα-null mice. These results suggest a role for PPARα in the control of LPS-induced iNOS expression by the Ca²⁺-dependent PKC signaling pathway.

**Simvastatin Decreases PPARα Phosphorylation by LPS-Activated PKCα**

To determine whether PKCα modulates PPARα phosphorylation in macrophages and whether statins influence this phosphorylation, in vitro phosphorylation experiments using purified PPARα protein as substrate were performed using extracts from LPS-stimulated macrophages pretreated with simvastatin. Incubation with LPS induced the activity of kinases that phosphorylate PPARα in vitro. This effect was prevented by both simvastatin and Gö6976 (Figure 7A), whereas mPKCl, an inhibitor of all PKC isoforms, inhibited both basal and LPS-stimulated PPARα phosphorylation. Furthermore, PPARα immunoprecipitated from LPS-activated macrophages was able to phosphorylate PPARα and this effect was inhibited by simvastatin pretreatment (Figure 7B).

To confirm that simvastatin prevents PKCα-induced PPARα phosphorylation in cells, metabolic labeling experiments were performed in PPARα and PKCα-transfected COS cells. PPARα induced the phosphorylation of PKCα, an effect that was inhibited by simvastatin (Figure 7C). Thus, LPS-induced PPARα phosphorylation occurs, at least partly, via PKCα in macrophages and simvastatin inhibits this phosphorylation.

**Simvastatin Increases PPARα Transrepression Activity on NFκB via Its PKC Phosphorylation Sites**

We have recently shown that the PKC signaling pathway modulates the transrepression activity of PPARα in hepatocytes via the PKC phosphorylation sites S179 and S230. Because the antiinflammatory action of PPARα is, at least partly, mediated by the repression of NFκB transcriptional activity via direct interaction with NFκB-p65 protein, it was investigated whether phosphorylation of PPARα on its PKC sites modulates its transrepression activity on NFκB-p65. To eliminate confounding effects of NFκB-activating pathways, PPARα activity was directly tested on nuclear-activated NFκB by using a chimeric protein composed of the yeast GAL4 DNA binding domain fused to p65 and a reporter vector driven by a GAL4 response element. Cells were

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**Figure 5.** The PKC signaling pathway is implicated in the control of LPS-induced iNOS expression in macrophages and neutrophils and is inhibited by simvastatin. A, Peritoneal macrophages were pretreated with the MEK inhibitor U0126 (25 μmol/L), the PKC inhibitors Gö6976 (Gö) (0.1 μmol/L), Ro318220 (Ro, 2 μmol/L), or vehicle for 2 hours and then treated with LPS (10 μg/mL) for 2 hours. B, LPS-elicited neutrophils recovered from air pouches were pretreated with the MEK inhibitor U0126 (25 μmol/L), the PKC inhibitor Gö6976 (Gö) (0.1 μmol/L), or vehicle for 2 hours and then treated with LPS (10 μg/mL) for 2 hours. iNOS mRNA levels were measured using real-time quantitative PCR. C, Peritoneal macrophages were pretreated with simvastatin (Sim) (25 μmol/L) or vehicle for 2 hours and then treated with or without LPS (10 μg/mL) for 15 minutes. D, LPS-elicited neutrophils recovered from air pouches were pretreated with simvastatin (Sim) (5 μmol/L) or vehicle for 2 hours and then treated with or without LPS (10 μg/mL) for 15 minutes. Kinase assay: PKCα was immunoprecipitated from cell extracts using an anti-PKCα antibody. PKCα activity was assayed using purified histone H1 protein as substrate. Phosphorylated proteins were visualized by autoradiography. Expression of PKCα was determined by immunoblot analysis. C indicates vehicle.
cotransfected with increasing concentrations of PPARα wild type or PPARα mutated in its PKC phosphorylation sites, PPARα(S179A-S230A), a nonphosphorylatable, nonphosphomimetic mutant. At all concentrations tested, PPARα(S179A-S230A) induced a more pronounced inhibition of p65-driven reporter activity compared with wild-type PPARα (Figure 8A), suggesting that PKC phosphorylation of PPARα inhibits PPARα transrepression activity on NFκB.

To demonstrate a role for PKCα in the transrepression activity of PPARα on NFκB-p65, a constitutive active form of PKCα (CA-PKCα) was tested on p65 transrepression by wild-type PPARα or PPARα(S179A-S230A). To obtain optimal sensitivity, wild-type PPARα was transfected at a concentration ratio exerting clear, basal repression of p65-driven transcriptional activity. Under these conditions, cotransfection of CA-PKCα prevented repression of p65-driven transcriptional activity by wild-type PPARα. By contrast, CA-PKCα did not repress the activity of PPARα(S179A-S230A) (Figure 8B).

Finally, to determine whether statin treatment modulates PPARα transrepression activity on NFκB via its PKC phosphorylation sites, the effect of simvastatin was tested on p65 transrepression by wild-type PPARα or PPARα(S179A-S230A). In this experiment, PPARα wild type was transfected at a concentration ratio that does not yet influence p65-driven transcriptional activity. Under these conditions, incubation with simvastatin induced a significantly more pronounced inhibition of p65-driven transcriptional activity in the presence of wild-type PPARα, an effect that was not observed with PPARα(S179A-S230A) (Figure 8C). These results indicate that inhibition of PKCα by simvastatin enhances PPARα transrepression activity on NFκB.

Discussion

Clinical trials and in vitro studies have shown that statins and PPARα agonists share antiinflammatory properties by regulating inflammatory-response genes.14,25 In the present study, using 2 well-characterized animal models of acute inflammation, we demonstrate that simvastatin requires PPARα expression to exert its antiinflammatory effects in vivo. The in
vivo antiinflammatory effects of simvastatin on footpad swelling and neutrophil recruitment in air pouch–bearing mice already occur within 1 hour after a single oral administration, indicating that the PPARα-dependent antiinflammatory effects of simvastatin occur rapidly. The observed effects, therefore, cannot be explained by the plasma lipid-lowering activities of the drug. Indeed, as previously shown, simvastatin treatment did not change lipid levels in mice (not shown). Thus, simvastatin exerts direct antiinflammatory effects via PPARα, independent of its plasma cholesterol-lowering activities.

The involvement of PPARα in the antiinflammatory effects of statins is further evidenced in vitro in experiments with primary macrophages and neutrophils, 2 cell types mediating acute inflammatory responses. PPARα activators act on a variety of vascular cells such as endothelial cells (ECs), vascular smooth muscle cells (VSMCs), monocytes/macrophages, and T cells, which all express PPARα. Although PPARα mRNA is expressed at low levels in peritoneal macrophages, it clearly plays a functional role in the antiinflammatory effects of simvastatin in vitro and in vivo, as evidenced by the lack of simvastatin effects on PPARα-deficiency. These results are consistent with a recent study showing that PPARα prevents macrophage foam cell formation in the peritoneal cavity. In addition, we show that neutrophils express high levels of PPARα mRNA. In these cells, PPARα also mediates the antiinflammatory effects of simvastatin, thus identifying a novel cell type in which PPARα exerts antiinflammatory activities.

To our knowledge, this is the first demonstration of the existence of a cross-talk between statins and PPARα in the regulation of lipid-independent inflammatory responses. These results thus extend previous studies on liver and lipid metabolism, indicating that both hypolipidemic and antiinflammatory effects of statins could involve PPARα.

We provide molecular evidence that statins modulate Ca2+-dependent PKCα signaling pathway in macrophages and neutrophils resulting in PPARα-dependent inhibition of LPS-induced inflammatory response genes, such as iNOS. These results are of particular interest because PKCα plays a role in the inflammatory response. In vivo overexpression of PKCα in the epidermis results in severe neutrophil-mediated inflammation. In vitro in macrophages, PKCα regulates LPS-induced iNOS, TNFα, and IL-1β expression. Our results suggest the implication of other Ca2+-dependent PKC isoforms, such as PKCβII, in the PPARα-dependent antiinflammatory effect of statins, because simvastatin inhibits also LPS-induced PKCβII activity in macrophages and neutrophils (not shown) and because PKCβII also phosphorylates PPARα on its PKC phosphorylation sites S179-S230 in vitro. The mechanism underlying the activation of PKCα by LPS and the inhibitory effect of simvastatin on LPS-induced PKCα activation in macrophages and neutrophils is presently unclear. Our results suggest that LPS induces PKCα translocation to the cell membrane in macrophages, but not in neutrophils, and that simvastatin may block this effect (not shown). It will be of interest to determine whether PKCα...
activators (PLC, PKD1) or repressors (DAGK or PPI phosphatases) are regulated by simvastatin in these cells.

We previously demonstrated that classical PKCs phosphorylate PPARα in vitro. Here, we show that LPS induction of PKCα in macrophages results in increased PPARα phosphorylation in vitro and that PKCα overexpression increased PPARα phosphorylation in cells. Moreover, simvastatin inhibited PKCα-induced PPARα phosphorylation. PPARα is a phosphoprotein phosphorylated by different kinases, such as extracellular signal-regulated kinase, p38, and PKA. Previously identified PPARα-phosphorylating kinases include PKC, NFκB, and mitogen-activated protein kinase.

We also observed that simvastatin enhances PPARα transactivation activity acting via its PKC phosphorylation sites (S179-S230) as well as a CA-PKCα, which activated PKCα inhibits the transactivation properties of PPARα on NFκB-p65. By contrast, simvastatin enhances PPARα trans-repression activity acting via its PKC phosphorylation sites (S179-S230), suggesting that simvastatin stimulates PPARα trans-repression activity via inhibition of PPARα inactivation by PKCα. Whereas in liver cells, the PKC signaling pathway also regulates the ligand-dependent PPARα transactivation properties, as demonstrated by enhanced CPT1 induction, in macrophages, simvastatin treatment did not modify PPARα-induced CPT1 expression, even in the presence of a PPARα agonist. Inhibition of the Ca2+-dependent PKC signaling pathway by simvastatin thus only influences the transrepression properties of PPARα in macrophages. We propose that activation of PKCα by inflammatory stimuli, such as LPS, leads to the phosphorylation and subsequent deactivation of PPARα. Studies performed on PPARα activation by PKCα, leading to enhanced PPARα transrepressive activity on NFκB (Figure 8D).

The effects of statins on inflammation could also involve NFκB-independent mechanisms, e.g., via modulation of CD62L and CD11b adhesion molecule expression in monocytes. However, we did not observe any effect of statins on the expression of these adhesion molecules in neutrophils (not shown). Nonetheless, our results do not exclude that other PPARα- and PKC-independent mechanisms contribute also to the antiinflammatory effects of statins because statins regulate other signaling pathways such as phosphatidylinositol 3-kinase and mitogen-activated protein kinase.

Macrophages and neutrophils are mediators of the early inflammatory response that play a major role in the inflammation and tissue damage associated with both infectious and noninfectious diseases, such as sepsis, acute coronary syndrome, rheumatoid arthritis, and ischemic stroke. Results from basic research and clinical trials indicate that the pleiotropic antiinflammatory effects of statins may result in clinical benefit in such inflammatory diseases. Our results demonstrating that statins exert their antiinflammatory effects through PPARα provide further evidence for the importance of such pleiotropic activities. Clinical studies with PPARα agonists have shown significant protective effects against CVD and stroke, effects that cannot be attributed to their cholesterol-lowering activities alone. Our results thus provide a potential clinically relevant mechanism for the pleiotropic effects of statins through PPARα.

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Material and methods

Inflammation tests

Air-pouch model: Subcutaneous dorsal pouches were induced in female C57BL6 wild-type and PPARα-null mice, 8 weeks of age (7-12/group) by injection of 5 ml of steril air followed 3 days later by reinjection of 3 ml of steril air. On day 6, LPS (0.2 µg/mouse) in 1 ml of CMC (0.5%) was injected into the air-pouches. Simvastatin at indicated doses in CMC (0.5%) or vehicle (CMC) was given by oral administration to air pouch-bearing mice 1h before LPS injection for an additional 4h. The animals were then killed by cervical dislocation and the pouches were flushed with cold PBS (2 ml). The lavage fluid was immediately cooled on ice, its volume determined, and neutrophils counted using a haemocytometer. Viability, as determined by trypan blue exclusion, was consistently greater than 95%. Neutrophil purity, as determined by Wright's-stained cytospin preparations, was greater than 90-95%.

Carrageenan footpad edema model: female C57BL6 wild-type and PPARα-null mice between 8 and 12 weeks of age were used (12-14/group). Footpad swelling was induced by a single subplantar injection of 0.05 ml of a sterile 1% solution of carrageenan in water. Simvastatin at indicated doses in CMC (0.5%) or vehicle (CMC) was given by oral administration to mice 1h before carrageenan injection for an additional 3h. Footpad swelling was then measured using a micrometer and compared with the preinjection volume of the same paw. Swelling was then expressed in percent relative to the level of carrageenan-treated controls (CMC) group.

Cell culture and treatments

Murine endothelial cells from adipose tissue were isolated as described. Murine thioglycollate-elicited peritoneal macrophages were isolated as described. Briefly, mouse peritoneal macrophages were collected by peritoneal lavage with saccharose (0.34 mol/L) from mice given a 3 ml intraperitoneal injection of 4% thioglycollate (Sigma Aldrich, St Quentin, France) in water for 3 days. Cells were then washed twice with serum-free RPMI (GIBCO BRL), plated and allowed to adhere to dishes for 2h. Plates were then washed 3 times with RPMI to remove nonadherent cells and incubated in RPMI containing fetal calf serum (FCS, 10%) over night.
Then, cells were incubated in RPMI containing 1% Nutridoma for 24h before treatment. Murine LPS-elicited neutrophils were isolated from air-pouches after 4h LPS treatment and incubated in RPMI containing FCS (0.5%) and treated with the indicated reagents. COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with FCS (10%) and antibiotics at 37°C. Cells were treated with the indicated reagents (GW9578 (GlaxoSmithKline), U0126 and mPKCI (Promega, Madison, USA), Gö6976 and Ro318220 (Calbiochem, San Diego, California), E.coli 026:B6 LPS (Sigma Aldrich, St Quentin, France)). For in vitro studies, simvastatin (Zocor, Merck laboratories) was converted to the active compound 5. Atorvastatin (Pfizer) and fluvastatin (Novartis pharma) were diluted in DMSO.

**RNA analysis**

RNA extraction was performed using TRizol reagent and reverse transcription was performed according to the manufacturer’s protocol (Invitrogen Life technologies, Cergy-Pontoise, France). RNA levels were measured by quantitative PCR using brilliant SYBR Green QPCR Master Mix on the MX4000 detection system (Stratagene). The amplifying primers were: murine PPARα (FOR: 5'-AGGCGGTGTCCTACGTGTCAG-3’ and REV: 5'-AGCCCTCTTCATCCCCAACG-3’), murine iNOS (FOR: 5'-TTGCCCTGGGAAGTTTCTCTTC-3’ and REV 5'-GGAGCCATTTTTGTTGACTCTTAGT-3’), murine TNFα (FOR, 5'-ATCCAGTTGTGTCGCGAG-3’ and REV, 5'-CGTCGTCGTAATGGCGCATC-3'), murine IL-6 (FOR, 5'-CCAGTTGCCTTCTTGGGACTG-3' and REV, 5'-CAGGTCTGTTGGAGTGTTATCC-3') and murine CPT1 (FOR: 5'-CATCATGACTGCATCGCTACTC-3’ and REV: 5'-CATCATGACTGCATCGCTACTC-3’ and REV: 5'-CAGTGCTGTCATGGCGTTGC-3'). Crossing threshold (Ct) values were determined for target genes and normalised to the Ct of cyclophilin using the following equation: relative values= $2^{-\Delta\Delta Ct}$ (target gene - Ct cyclophilin). Results are expressed as means +/- SD (n=3) relative to the level of LPS-treated controls. All experiments were repeated at least 3 times.

**Kinase assays and immunoprecipitation**
After treatment, cells were washed with PBS and suspended in PKC lysis buffer (50 mmol/L Tris pH 7.5, 3 mmol/L DDT, 5 mmol/L EDTA and 10 mmol/L EGTA) containing freshly added protease and phosphatase inhibitors (1 mmol/L β-Glycerophosphate, 1 mmol/L Na3VO₄, 1 mmol/L PMSF, 10 µg/ml aprotinin). Lysates were sonicated using a VibraCell hiddock 72442 (70%, 80 J/sec, 5 s) and then clarified by centrifugation at 4°C (3000 rpm, 15 min). Cell extracts (10 µg) or immunoprecipitated PKCα from cell extract (200 µg) were incubated for 30 min at 30 °C in 20 µl of kinase reaction buffer containing 1/3 Calcium Buffer (12 mmol/L CaCl₂, 50 mmol/L Tris), 1/3 Lipid activator (Sigma Aldrich, St Quentin, France), 1/3 DTT buffer (30 mmol/L DTT, 50 mmol/L Tris), 1 µg of Histone H1 or 400 ng of purified PPARα protein as substrates and 5 µCi of (γ-32P) ATP (2000 Ci/mmol). For histone H1 protein substrate, kinase reaction was stopped by addition of 10 µl of 3X Laemmli buffer and electrophoresed on 12.5% SDS-PAGE. For the purified PPARα protein substrate, kinase reaction was stopped by addition of ice cold RIPA with inhibitors and PPARα was immunoprecipitated overnight at 4°C using anti-PPARα polyclonal antibody as described previously. Immune complexes were incubated with 30 µl of protein-A sepharose beads for 1 h at 4 °C and then washed once with RIPA, RIPA/NaCl 1 mol/L, RIPA/TNE and TNE. Immunoprecipitates were resuspended in 10 µl of 3X Laemmli buffer and electrophoresed on 10% SDS-polyacrylamide gel electrophoresis. For equal loading of purified proteins, gels electrophoresis were stained with coomassie blue (data not shown). Gels were then dried and incorporation of (γ-32P) ATP was visualised by autoradiography. Signal intensity corresponding to the various bands was quantified using the Image 1D software.

**Immunoblot**

After treatment, cells were washed with PBS and suspended in PKC lysis buffer for PKCα detection or lysis buffer (25 mmol/L Hepes pH 7.5, 100 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.25 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L NaF and 0.1% NP40) for other protein detection containing freshly added protease and phosphatase inhibitors (1 mmol/L β-Glycerophosphate, 1 mmol/L Na3VO₄, 1 mmol/L PMSF, 10 µg/ml aprotinin). Lysates were clarified by centrifugation at 4°C (14000 rpm, 30 min). Cell extracts were resolved on 10% SDS-polyacrylamide gel and
transferred onto PVDF membrane. Immunoblots were performed using the Aurora detection system (ICN pharmaceuticals, Orsay, France) as previously described using anti-iNOS polyclonal antibody (BD Biosciences, Le Pont de Claix, France), anti-PKCα C20 and anti-actin I19 polyclonal antibodies (Santa-Cruz Biotechnology, Le Perray en Yvelines, France).

**Transient transfection assay**

COS-7 cells, grown to 50%-60% confluence in DMEM supplemented with 10% FCS, were transiently transfected by lipofection with reporter and expression plasmids as indicated in the figure legends. The GAL4-TkpGL3, pSG5-PPARαWT, pSG5-PPARα(S179A-S230A) 8, GAL4-p65 9, CA-PKCα 10 plasmids were obtained as described. The pCDNA-PKCαWT was a kind gift of Laurence Suaud. A β-galactosidase expression plasmid was co-transfected as a control for transfection efficiency. The total amount of transfected DNA was kept constant by adding empty vector. After 2h (COS-7 cells), cells were refed with DMEM supplemented with 2% Ultroser and incubated overnight. Cells were then treated as indicated in the figure legends. After the indicated time, cells were collected and subjected to luciferase and β-galactosidase assays. All experiments were repeated at least 3 times.

**References**


