Activation of Liver X Receptors Prevents Statin-induced Death of 3T3-L1 Preadipocytes*

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The biological functions of liver X receptors (LXRs) α and β have primarily been linked to pathways involved in fatty acid and cholesterol homeostasis. Here we report a novel role of LXR activation in protecting cells from statin-induced death. When 3T3-L1 preadipocytes were induced to differentiate by standard isobutylmethylxanthine/dexamethasone/insulin treatment in the presence of statins, they failed to differentiate and underwent massive apoptosis. The simultaneous addition of selective LXR agonists prevented the statin-induced apoptosis. By using mouse embryo fibroblasts from wild-type (LXR+/+) or a dominant negative version of LXR (LXR−/−), LXRα knock-out mice (LXRα+/−/LXRβ+/+), LXRβ knock-out mice (LXRα+/+/LXRβ−/−), and LXR double knock-out mice (LXRα−/−/LXRβ−/−) as well as 3T3-L1 cells transduced with retroviruses expressing either wild-type LXRα or a dominant negative version of LXRα, we demonstrate that the response to LXR agonists is LXR-dependent. Interestingly, LXR-mediated rescue of statin-induced apoptosis was not related to up-regulation of genes previously shown to be involved in the antiapoptotic action of LXR. Furthermore, forced expression of Bcl-2 did not prevent statin-induced apoptosis; nor did LXR action depend on protein kinase B, whose activation by insulin was impaired in statin-treated cells. Rather, LXR-dependent rescue of statin-induced apoptosis in 3T3-L1 preadipocytes required NF-κB activity, since expression of a dominant negative version of IκBα prevented LXR agonist-dependent rescue of statin-induced apoptosis. Thus, the results presented in this paper provide novel insight into the action of statins on and LXR-dependent inhibition of apoptosis.

Inhibitors of the rate-limiting enzyme of the mevalonate pathway, 3-hydroxy-3-methylglutaryl-CoA reductase, collectively known as statins, are well established effective agents used in the treatment of hypercholesterolemia (1, 2). Some beneficial effects of statins cannot solely be ascribed to the lowering of low density lipoprotein cholesterol, and these effects are collectively termed pleiotropic effects (3). Statins induce cell death in a number of different cell lines (4), and it appears that especially malignant cells are dependent on isoprenoids for survival (5–8). Although tumor cells derived from acute myelogenous leukemia undergo apoptosis when treated with statins, myeloid progenitor cells from normal bone marrow or cord blood do not (9, 10). Statins are well tolerated by humans and considered safe (11, 12); hence, the concept of using statins for cancer treatment is receiving considerable attention (13, 14).

The precise mechanism by which statins induce apoptosis is not yet elucidated, and it remains unclear which proteins and signal transduction cascades are involved. Inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase results in decreased farnesylation and geranylgeranylation of several proteins essential for cellular proliferation and survival, such as members of the Ras and Rho families, but mechanistic studies also show that the expression of the proto-oncogene, Bcl-2, is down-regulated in transformed cells undergoing apoptosis in response to statin exposure (8). Bcl-2 is a key regulator prolonging cell survival by blocking apoptosis (15, 16). Statins down-regulate Bcl-2 expression levels in colon cancer cells (17), neuroblasts (18), glioma cells (19), human hepatocytes (20), human breast cancer cells (21), and acute myelogenous leukemia cell lines (22). Forced expression of Bcl-2 is able to inhibit statin-induced apoptosis in colon cancer cells (17) and in NIH-3T3 fibroblasts (23), and the Bcl-2-related Bcl-xL, protects murine tubular cells from statin-induced apoptosis (24). Both Bcl-2 (25–28) and Bcl-xL (29) are up-regulated by activation of NF-κB in certain cell lines. NF-κB activation has been shown to protect against apoptosis in different cell lines (30–32), and forced expression of NF-κB has been reported to increase cell viability and suppress apoptosis (30–32). Interestingly, the reported effects of statins on different cell types comprise repression of NF-κB activation (33, 34) as well as activation of NF-κB (35), underlin-

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4 The abbreviations used are: NF-κB, nuclear factor-κB; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; IκB, inhibitory factor NF-κB; IKK, IκB kinase; LXR, liver X receptor; LXR-DN, dominant negative LXR; MEK, mouse embryo fibroblast; PKB, protein kinase B; RT, reverse transcription; qPCR, quantitative PCR; FBS, fetal bovine serum; PPARY, peroxisome proliferator-activated receptor γ; IGF, insulin-like growth factor.
LXR Activity Prevents Statin-induced Death of Preadipocytes

ing the cell type-dependent complexity of statin action. Other prosurvival pathways down-regulated by statins include PKB activation and nuclear translocation via a P2X7 purinergic receptor and mTOR-dependent signaling pathway (36, 37).

The nuclear receptor liver X receptor (LXR) exists in two isoforms, LXRα and LXRβ. Whereas LXRβ is ubiquitously expressed, LXRα is preferentially expressed in liver, adipose tissue, small intestine, and macrophages (38, 39). Both receptors are activated by oxysterols (40) and have primarily been linked to pathways involved in fatty acid and cholesterol homeostasis (41, 42). Activation of LXR induces expression of several ATP-binding cassette transporters, which mediate cholesterol efflux from cells (43). Whereas LXRβ is expressed in both preadipocytes and adipocytes, the expression of LXRα is strongly induced during adipocyte differentiation (44, 45). Recently, LXR has also been implicated in cell survival and apoptosis. Gene array analysis revealed that expression of a subset of both pro- and antiapoptotic genes is regulated in an LXR-dependent manner (46, 47). LXR-null macrophages undergo accelerated bacterially induced apoptosis (48), and activation of LXR prevents bacterially induced macrophage apoptosis (49). Finally, it was reported that inactivation of LXRβ leads to neuronal degeneration (50).

Although the beneficial effects of statins on lipid homeostasis are well documented, the effects of statins on glucose homeostasis and adipocyte function are less well understood, and conflicting results have been reported (51–55). Here we report that the lipophilic statin, simvastatin, induced apoptosis in differentiating 3T3-L1 preadipocytes and mouse embryonic fibroblasts (MEFs) when administered during the first 4 days of the differentiation program. The addition of mevalonate, but not cholesterol or farnesol, rescued survival and differentiation, and the addition of geranylgeraniol partly rescued differentiation. Insulin-dependent activation of PKB was impaired in statin-treated 3T3-L1 preadipocytes. We show that the addition of LXR agonists rescued survival and differentiation. Inhibition of PKB did not prevent LXR agonist-mediated rescue of cell survival but, as expected, abolished adipocyte differentiation. By using MEFs deficient for LXRα or LXRβ or both and by using retroviral expression of a dominant negative form of LXR, we demonstrated that LXR agonist-dependent rescue was indeed dependent on LXR activity. Interestingly, LXR-mediated rescue of statin-induced apoptosis was not related to up-regulation of the genes, AIM/Spα/Api6 and Bcl-xL, previously shown to be involved in the antiapoptotic action of LXR in macrophages (48, 49). Furthermore, forced expression of Bcl-2 did not prevent statin-induced apoptosis. Rather, activation of LXR prevented statin-induced apoptosis in 3T3-L1 preadipocytes in a manner dependent on NF-kB activity, possibly via an up-regulation of CARD14, known to activate IκB kinase to induce phosphorylation and degradation of IκBα (56). Thus, the results presented in this paper add novel facets to the action of statins on adipocyte differentiation and LXR-dependent inhibition of apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—3T3-L1 cells were cultured to confluence in DMEM supplemented with 10% calf serum. Two days postconfluent (designated day 0) cells were induced to differentiate with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 μM dexamethasone (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), and 1 μg/ml insulin (Roche Applied Science). Inhibitors and ligands were dissolved in Me2SO and added when differentiation was induced. Cells not treated with ligands or inhibitors received similar volumes of Me2SO. After 48 h, the media were replaced with DMEM supplemented with 10% FBS and 1 μg/ml insulin. The cells were subsequently refed every 48 h with DMEM supplemented with 10% fetal bovine serum. When present, 5 μM simvastatin (Calbiochem) was added every 48 h. Simvastatin was converted to the active form by dissolving it in absolute ethanol, followed by the addition of 1 M NaOH to a final concentration of 100 mM. The simvastatin solution was neutralized with 1 M HCl and diluted in vehicle (Me2SO) before use.

MEFs—MEFs were isolated from 13.5-day embryos from crosses between LXRα+/− /LXRβ+/− (57) or p53+/− /p53−/− (58) heterozygotes, respectively. The isolated MEFs were genotyped by PCR. MEFs were grown in AmnioMax basal medium (Invitrogen) supplemented with 7.5% FBS, 7.5% AmnioMax-C100 supplement, 2 mM glutamine, 62.5 μg/ml penicillin, and 100 μg/ml streptomycin (growth medium) in a humidified atmosphere of 5% CO2 at 37 °C. Medium was changed every second day. For differentiation, 2-day postconfluent cells (day 0) were treated with growth medium containing 1 μM dexamethasone (Sigma), 0.5 mM isobutylmethylxanthine, and 5 μg/ml insulin for 2 days. From day 2, medium contained 5 μg/ml insulin and was changed every second day.

Oil-Red-O Staining—Staining of lipid by Oil-Red-O was performed as described previously (59).

Microscopical Assessment of Apoptosis—Cells were grown on collagen-coated coverslips in 12-well plates. Cells were fixed in 4% paraformaldehyde in PBS for 15 min, washed in PBS, stained with 2.5 μg/μl Hoechst 33258 (Sigma B-2883) in PBS for 5 min, and washed three times for 5 min each in PBS. The cells were examined by fluorescence microscopy to assess chromatin condensation and by phase-contrast microscopy to assess apoptotic cell budding and shrinkage.

Whole Cell Extracts and Western Blot Analysis—Whole cell extracts, electrophoresis, blotting, visualization, and stripping of membranes were performed as described (59). Primary antibodies used were rabbit anti-phospho-PKB (Ser-473), rabbit anti-PKB (Cell Signaling Technology), rabbit anti-phospho-IKKα (Ser-181), rabbit anti-IKKβ/β, rabbit anti-IκBα, mouse anti-Bcl-2, mouse anti-PPARγ, and rabbit anti-TFIIB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies obtained from DAKO.

De Novo Cholesterol Synthesis—Cells were incubated with [1(2)-3H]acetate acid, sodium salt (0.2 μCi/ml medium) (Amersham Biosciences) for 4 h, harvested in water, and frozen. Cellular lipids were extracted from the cell suspensions by the method of Folch, Lees, and Sloane-Stanley (60), with minor modifications. The cell suspension was mixed with 20 volumes of chloroform/methanol (2:1, v/v) and 4 volumes of 0.9% NaCl, pH 2. The organic phase was evaporated under N2, and the
lipids were dissolved in n-hexane and separated by TLC on silica gel plates developed in hexane/diethyl ether/acid (80:20:1, by volume). The bands were detected with iodine vapor, cut into pieces, and assayed for radioactivity by scintillation counting.

Cellular Levels of Triacylglycerols—Cells grown in 6-well plates were harvested in 1 ml of water and frozen. The cells were sonicated, and the cellular levels of triacylglycerols were measured on an AXON Byer spectrophotometer using the TRINDER reaction kit from BioMérieux.

Plasmids—The retroviral expression vector pBABE-PPARγ2 was constructed by inserting an Smal fragment containing full-length mouse PPARγ2 cDNA into the SnaBI site of pBABE-puro. pBABE-LXRα and pBABE-LXRαDN were a kind gift of Dr. Peter Akerblad. pWZL-Bcl-2 was kindly provided by Dr. Camilla Krakstad, and pLZRS-IkBα-mut (61) was kindly provided by Dr. Paul Khavari. The pBABE-ADD1-DN was kindly provided by Dr. Bruce M. Spiegelman (62). The reporter construct containing three tandem copies of the κB site (p(κB)3-luc +; Stratagene, La Jolla, CA) has been described previously (63).

Transient Transfections—50% confluent 3T3-L1 cells in 12-well dishes were transiently transfected by the calcium phosphate method. Each well received 50 ng of p(κB)3-luc + reporter and 25 ng of CMV-β-galactosidase were used for normalization. 6 h after transfection, the medium was changed, and cells were incubated for 20 h with tumor necrosis factor-α or vehicle (Me2SO) as indicated.

Retrovirus Production and Transduction—Phoenix cells were transfected with viral DNA at 50% confluence. Two days post-transfection, the virus-containing media were collected by centrifugation and immediately used to infect 30–40% confluent 3T3-L1 cells by mixing viral supernatant 1:1 with DMEM supplemented with 10% calf serum. Polybrene (Sigma) was added to a final concentration of 7 μg/ml. After 24 h, the transduced cells were split and subjected to selection (3 μg/ml puromycin (InvivoGen)) (IkBα-mut– and vector-transduced 3T3-L1 cells were not subjected to selection). Approximately 3 days later, the selected clones were pooled and replated for differentiation.

RT-qPCR—Total RNA was prepared from cells using the RNAeasy minikit (Qiagen) according to the manufacturer’s instructions. One μg of total RNA was reverse transcribed into cDNA using SuperscriptII and random hexamer primers (Invitrogen). The concentration and quality of the purified total RNA were determined spectrophotometrically at A260 and by the A260/A280 ratio, respectively. mRNA expression levels were quantified using the ABI 7500 instrument and the SYBR green technology (Applied Biosystems, Foster City, CA). All primers were designed with the Primer Express® software, version 2.0, a program specifically provided for primer design using ABI qPCR instruments. 100 nM SYBR green assay primers were used, and for each primer pair, a dissociation curve analysis was carried out to ensure the specificity of the qPCR amplification. We calculated relative changes employing the comparative CT method using 18 S or TBP as the internal reference gene.

The primers (upstream and downstream) were as follows: TBP, 5′-ACCCCTTCAAAATGACTCTATG and 5′-ATGATGACTGCAAGAAATCGC; LXRα, 5′-CCGACAGAGCT-TCGTC and 5′-CCACGACACTGCAAC; LXRβ, 5′-TACTATCGTGTCATCTTTAGAG and 5′-GGCACAGCTCATTG; ABCA1, 5′-TGGTTGCTGTACGCAAGC and 5′-CCCCATTTACATAACATG; ABCG1, 5′-CGTGGACCTTTCCCC and 5′-GGTTAGTCCAAATTCGAGCC; NiP3, 5′-TTATCGAGATCCAAAGCTCTA and 5′-GGCAGATCTTTTTAAAACCATG; CIDE-A, 5′-CCGAGATCTGCGGATACAGA and 5′-AAGGTGATGCGGGCA; Aatk, 5′-CCGACATTGCGGCGGA; Bad, 5′-CTCCCAAAGTGAGTGGCG and 5′-GGCAGGAGTCTCTTGAAG; Bcl-2, 5′-CTGGGATCTTGTATGG and 5′-GGCAGAAGAAATTTATCCAAC;

RESULTS

LXR Agonists Rescue Statin-induced Cell Death—When 2 days postconfluent 3T3-L1 preadipocytes are treated with isobutylmethylxanthine, dexamethasone, and insulin, they synchronously reenter the cell cycle and undergo two sequential rounds of mitosis in a process known as mitotic clonal expansion and subsequently express genes that control the adipocyte phenotype (64). During this phase, statin treatment induced severe cell death (Fig. 1A). By contrast, statin was well tolerated if added 4 days after induction (Fig. 1A), when the cells are growth-arrested (64). The statin-induced cell death was prevented by the LXR agonist T0901317 but only when it was present from day 0 (Fig. 1B). The protective effect was not restricted to the Tularik compound T0901317, since the LXR agonist GW3965 also prevented statin-induced cell death (Fig. 1C). Hoechst staining of nuclei revealed that statin-induced cell death was associated with pronounced hypercondensation of nuclear chromatin (Fig. 1C). Furthermore, apoptotic cell budding and shrinkage was observed upon treatment with simvastatin (results not shown). This indicates that simvastatin induced an apoptotic type cell death in 3T3-L1 preadipocytes.

RT-qPCR analysis confirmed that T0901317 treatment induced known LXR target genes, including the ones involved in cholesterol transport, and demonstrated that they were upregulated also in the presence of simvastatin (Fig. 1E). The activation of LXR can stimulate the expression also of other cellular transport systems (65). To exclude the possibility that activa-
tion of LXR simply led to transport of simvastatin out of the cells, the effect of simvastatin on cholesterol synthesis was determined in the absence and presence of the LXR agonist T0901317. Although T0901317 tended to increase cholesterol synthesis in the absence of simvastatin, it failed to rescue cholesterol synthesis in the presence of simvastatin (Fig. 1D).

We considered next if treatment with LXR agonists worked by normalizing the expression of members of the SREBP (sterol regulatory element-binding protein) family, known to be affected by statin-induced cholesterol depletion in preadipocytes (66). Furthermore, expression of SREBP-1c also known as adipocyte determination and differentiation-dependent factor 1 (ADD1) is induced by LXR (67). Treatment with simvastatin markedly reduced the expression of SREBP-1c, and although the addition of T0901317 induced expression of SREBP-1c, it failed to restore the expression level to that of cells not treated with simvastatin. As expected, simvastatin treatment increased expression of SREBP-2, and the addition of T0901317 further increased the expression in the absence or presence of simvastatin (supplemental Fig. 1A). The expression data, therefore, did not support that T0901317-dependent survival was linked to a restoration of SREBP-1c or SREBP-2 expression. The

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**FIGURE 1.** LXR agonists prevent statin-induced cell death in 3T3-L1 cells. Two-day postconfluent 3T3-L1 cells were induced to differentiate by treatment with isobutylmethylxanthine, dexamethasone, and insulin, as described under “Experimental Procedures.” From day 4, medium consisted of DMEM with 10% FBS and was changed every second day. A, 5 μM simvastatin dissolved in Me2SO or Me2SO alone was added when differentiation was induced on day 0 or on day 2 or 4 and was present throughout the remaining part of the differentiation period. On day 8, the cells were stained with Oil-Red-O and photographed (×100 magnification). B, 5 μM simvastatin was included from the time of induction at day 0 and was present throughout the differentiation period. 1 μM T0901317 was included on day 0, 2, or 4 and was present throughout the remaining part of the differentiation period. On day 8, the cells were stained with Oil-Red-O and photographed (×100 magnification). C, 5 μM simvastatin dissolved in Me2SO or Me2SO alone was added from the time of induction at day 0 in the absence or presence of 1 μM T0901317 or 1 μM GW3965. All chemicals were present throughout the differentiation period. The cells were stained with Oil-Red-O or Hoechst on day 4. D, 5 μM simvastatin dissolved in Me2SO or Me2SO alone was added from the time of induction at day 0 in the absence or presence of 1 μM T0901317. The media contained U-14C-labeled acetic acid. After 4 h, the cells were harvested, and labeled cholesterol was extracted, isolated on TLC, and quantitated by scintillation counting. E, 5 μM simvastatin dissolved in Me2SO or Me2SO alone was added from the time of induction at day 0 in the absence or presence of 1 μM T0901317. On day 4, RNA was harvested, and the expression levels of LXRα, LXRβ, ABCG1 (ATP-binding cassette, subfamily G (WHITE), member 1) and ABCA1 (ATP-binding cassette, subfamily A (ABC1), member 1) were analyzed by real time RT-PCR. One representative experiment out of 3–5 independent experiments performed in duplicates or triplicates is shown (A–C). The bars represent mean ± S.D. of four experiments (D–E).
notion that SREBP-1 activity was not required for rescue of statin-induced cell death received further support from experiments where preadipocytes were transduced with a retrovirus expressing a dominant negative version of SREBP-1, ADD1-DN (62). This blunted the expression of the SREBP-1-responsive genes ACC1 and FAS in response to treatment with T0901317 (supplemental Fig. 1, B and C). However, administration of T0901317 still rescued the cells from statin-induced cell death (supplemental Fig. 1D).

As reported previously (62), expression of ADD1-DN inhibited adipocyte differentiation, as determined by Oil-Red-O staining. Of note, treatment with T0901317 also partially restored lipid accumulation in cells expressing the dominant negative form of SREBP-1 (supplemental Fig. 1D).

The fact that statin treatment did not induce apoptosis if administered after day 4 of the differentiation program, when PPARγ2 expression is induced and the cells begin to accumulate triacylglycerol lipid droplets, might imply that statin-dependent repression of PPARγ2 expression was involved in the induction of apoptosis (54). To investigate this possibility, we transduced 3T3-L1 cells with an empty retrovirus or a retrovirus expressing PPARγ2. Fig. 2A illustrates the overexpression of PPARγ2 in the transduced 3T3-L1 cells. The cells were subjected to the standard protocol for induction of adipocyte differentiation and treated with different combinations of the PPARγ agonist rosiglitazone, the LXR agonist T0901317, and simvastatin, as shown in Fig. 2B. Forced expression of PPARγ2 in 3T3-L1 cells led to the expected enhancement of adipocyte differentiation as determined by accumulation of triacylglycerol (Fig. 2C). However, in relation to statin-induced apoptosis, no differences between cells transduced with the empty retroviral vector or the retroviral vector expressing PPARγ2 were observed. In both cases, statin was able to induce cell death when administered from day 0, irrespective of whether rosiglitazone was added or not, and the simultaneous addition of T0901317 protected the cells from apoptosis and partly rescued differentiation (Fig. 2, B and C). Thus, the statin-induced apoptosis was independent of PPARγ2 expression.

Statin-induced Cell Death Is Prevented by Mevalonate and Geranylgeraniol—Statins are competitive inhibitors of the 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting step in the mevalonate biosynthetic pathway. This pathway gives rise to not only cholesterol but also ubiquinone, dolichol, and other isoprenoids, farnesol and geranylgeranol, which are
involved in regulation of cellular proliferation. As anticipated, mevalonate rescued statin-induced cell death and restored differentiation of statin-treated cells (Fig. 3A). By contrast, the addition of cholesterol or farnesol did not rescue statin-treated cells from cell death (Fig. 3B and C), whereas the addition of geranylgeraniol rescued the cells from statin-induced cell death but only partly restored adipocyte differentiation (Fig. 3D).

Insulin/IGF-1 stimulation plays a pivotal role in the survival of preadipocytes and induction of adipocyte differentiation. These processes have been shown to be intimately associated with prenylation of members of the Ras and RhoA families. Insulin stimulates the activity of farnesyl and geranylgeranyl transferases (68), and pharmacological or genetic inhibition of these transferases impairs insulin signaling and prevents adipocyte differentiation (69–71). Impaired prenylation also interferes with activation of ERK in 3T3-L1 preadipocytes and adipocytes (68, 69). In line with a previous study showing that simvastatin impaired insulin/IGF-1 signaling (72), we found that simvastatin almost completely prevented the increase in IGF-1-mediated PKB phosphorylation in 3T3-L1 cells (Fig. 4A). To know if ERK- and PKB-dependent signaling had a major role in LXR agonist-dependent rescue of statin-induced cell death, we treated 3T3-L1 preadipocytes with standard adipogenic inducers and different combinations of simvastatin and inhibitors of the ERK and the PKB signaling pathways. Inhibition of either the ERK or PKB activation did not interfere with the ability of T0901317 to protect the cells from apoptosis, but as expected, inhibition of PKB activation prevented adipocyte differentiation with accumulation of lipid (Fig. 4B). We conclude that rescue of statin-treated cells from death by LXR activation proceeds in the absence of active signaling via ERKs or PKB and that protection of preadipocytes against death can be dissociated from differentiation.

The Antiapoptotic Response to LXR Agonists is LXR-dependent, and Overexpressed LXR Can Protect Even in the Absence of Added Agonists—LXRβ is constitutively expressed in both preadipocytes and mature adipocytes (44), whereas LXRα is induced during adipogenesis and has much higher expression in adipocytes than in preadipocytes (45). Growth-arrested 3T3-L1 cells on day 4 expressed relatively high levels of LXRα and did not undergo extensive simvastatin-induced apoptosis even in the absence of added LXR agonists, suggesting that the endogenous level and/or activity of LXR was sufficient to protect them against statin-induced death (Fig. 1A). If this is true, we expected even the growth-arrested 3T3-L1 cells (day 4) to become sensitive to simvastatin if LXR expression/activity was experimentally attenuated. Conversely, 3T3-L1 cells undergoing mitotic clonal expansion should become resistant to statins if LXR expression was ectopically increased. To test this notion, 3T3-L1 cells were transduced with retroviruses expressing wild-type LXRα, a
LXR Activity Prevents Statin-induced Death of Preadipocytes

In order to decipher whether cell survival was LXR subtype-selective, we used primary MEFs from LXR\(\alpha\)^{\text{\textminus/\textminus}} and LXR\(\beta\)^{\text{\textminus/\textminus}} as well as LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} and wild-type embryos. The MEFs were treated with adipogenic inducers as described under “Experimental Procedures” and treated with simvastatin and/or the LXR agonist T0901317 from day 0, as shown in Fig. 6. As previously described, LXR\(\alpha\) was expressed at a low level in wild-type undifferentiated MEFs (73), but similar to 3T3-L1 cells, expression of LXR\(\alpha\) was induced upon induction of adipocyte differentiation (Fig. 6A). Expression of LXR\(\beta\) tended to be slightly higher in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} MEFs than wild-type MEFs (Fig. 6A). To examine the ability of LXR agonists to induce expression of a canonical LXR-responsive gene, day 0 MEFs were treated for 24 h with T0901317 or GW3965, and expression of ABCA1 was determined by RT-qPCR analysis. Fig. 6B shows that both LXR agonists induced ABCA1 expression more strongly in wild-type than in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} MEFs, suggesting that even the loss of the low level of LXR\(\alpha\) in wild-type cells could impact ABCA1 expression. No induction of ABCA1 was observed in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} or LXR\(\alpha\)^{\text{\textplus/\textplus}}/LXR\(\beta\)^{\text{\textminus/\textminus}} MEFs. In order to address whether LXR activity was dependent on cellular differentiation, we used either primary MEFs or 3T3-L1 cells that were retrovirally transduced with LXR\(\alpha\)^{\text{\textplus/\textplus}}, LXR\(\alpha\)^{\text{\textminus/\textminus}}, or LXR\(\alpha\)^{\text{\textplus/\textminus}} and then treated with simvastatin or the LXR agonist T0901317 from day 0, as shown in Fig. 6. As previously described, LXR\(\alpha\) was expressed at a low level in wild-type undifferentiated MEFs (73), but similar to 3T3-L1 cells, expression of LXR\(\alpha\) was induced upon induction of adipocyte differentiation (Fig. 6A). Expression of LXR\(\beta\) tended to be slightly higher in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} MEFs than wild-type MEFs (Fig. 6A). To examine the ability of LXR agonists to induce expression of a canonical LXR-responsive gene, day 0 MEFs were treated for 24 h with T0901317 or GW3965, and expression of ABCA1 was determined by RT-qPCR analysis. Fig. 6B shows that both LXR agonists induced ABCA1 expression more strongly in wild-type than in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} MEFs, suggesting that even the loss of the low level of LXR\(\alpha\) in wild-type cells could impact ABCA1 expression. No induction of ABCA1 was observed in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} or LXR\(\alpha\)^{\text{\textplus/\textminus}}/LXR\(\beta\)^{\text{\textminus/\textminus}} MEFs. 

dominant-negative LXR\(\alpha\) (LXR-DN), or an empty vector, and the transduced cells were then treated with simvastatin and/or LXR agonists from day 0 or day 4. 

Judging by the expression of known LXR-responsive gene ABCA1 (ATP-binding cassette, subfamily A, member 1), forced expression of LXR\(\alpha\) sensitized the cells to agonist treatment, whereas LXR-DN abolished the effects of LXR agonist treatment (Fig. 5A). Furthermore, forced expression of LXR\(\alpha\) rescued the cells from simvastatin-induced cell death, also in the absence of exogenous LXR ligands. As previously reported (45), the addition of LXR agonists to 3T3-L1 preadipocytes with forced expression of LXR prevented adipocyte differentiation, further demonstrating that LXR-dependent rescue of statin-induced apoptosis was independent of cellular differentiation. On the other hand, ablation of LXR activity by expression of LXR-DN sensitized the cells to simvastatin-induced cell death, which was not prevented by T0901317 or GW3965 (Fig. 5B). Similarly, growth-arrested 3T3-L1 cells became sensitive to simvastatin when LXR-DN was expressed, further corroborating the notion that cell survival was LXR-dependent (Fig. 5B). 

In order to decipher whether cell survival was LXR subtype-selective, we used primary MEFs from LXR\(\alpha\)^{\text{\textminus/\textminus}} and LXR\(\beta\)^{\text{\textminus/\textminus}} as well as LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} and wild-type embryos. The MEFs were treated with adipogenic inducers as described under “Experimental Procedures” and treated with simvastatin and/or the LXR agonist T0901317 from day 0, as shown in Fig. 6. As previously described, LXR\(\alpha\) was expressed at a low level in wild-type undifferentiated MEFs (73), but similar to 3T3-L1 cells, expression of LXR\(\alpha\) was induced upon induction of adipocyte differentiation (Fig. 6A). Expression of LXR\(\beta\) tended to be slightly higher in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} MEFs than wild-type MEFs (Fig. 6A). To examine the ability of LXR agonists to induce expression of a canonical LXR-responsive gene, day 0 MEFs were treated for 24 h with T0901317 or GW3965, and expression of ABCA1 was determined by RT-qPCR analysis. Fig. 6B shows that both LXR agonists induced ABCA1 expression more strongly in wild-type than in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} MEFs, suggesting that even the loss of the low level of LXR\(\alpha\) in wild-type cells could impact ABCA1 expression. No induction of ABCA1 was observed in LXR\(\alpha\)^{\text{\textminus/\textminus}}/LXR\(\beta\)^{\text{\textplus/\textplus}} or LXR\(\alpha\)^{\text{\textplus/\textminus}}/LXR\(\beta\)^{\text{\textminus/\textminus}} MEFs.
LXR Activity Prevents Statin-induced Death of Preadipocytes

**A**

**LXRα and LXRβ expression**

![Graph showing LXRα and LXRβ expression](image)

**B**

**ABCA1 expression**

![Graph showing ABCA1 expression](image)

**C**

![Graph showing ABCA1 expression](image)

**FIGURE 6. The response to LXR agonists is LXR-dependent in MEFs.** MEFs were isolated from LXRα<sup>−/−</sup>, LXRβ<sup>−/−</sup>, LXRα<sup>+/−</sup> /LXRβ<sup>−/−</sup>, and wild-type embryos and grown to confluence in AmnioMax basal medium supplemented with 7.5% FBS, 7.5% AmnioMax-C100 supplement, and 2 mM L-glutamine. A and C, MEFs were grown to 2 days postconfluence and induced to differentiate. 5 μM simvastatin dissolved in Me2SO or Me2SO alone was added when differentiation was induced on day 0 in the absence or presence of 1 μM T0901317 and was present throughout the remaining part of the differentiation period. RNA was harvested at the indicated time points, and the expression levels of LXRα and LXRβ were measured by RT-qPCR. On day 8, the cells were stained with Oil-Red-O and photographed (×100 magnification) (C). B, MEFs were grown to 2 days postconfluence and treated with the LXR agonist 1 μM T0901317, 1 μM GW3965, or Me2SO for 24 h. RNA was harvested, and the expression level of ABCA1 (ATP-binding cassette, subfamily A (ABC1), member 1) was measured by RT-qPCR. The bars represent mean ± S.D. of three experiments performed in triplicates (A–B). One representative experiment of three independent experiments performed in duplicates or triplicates is shown (C).

**LXRβ<sup>−/−</sup>** MEFs (Fig. 6B). Simvastatin induced cell death more strongly in LXRα<sup>−/−</sup> /LXRβ<sup>−/−</sup> than in wild-type, LXRα<sup>+/−</sup> /LXRβ<sup>−/−</sup>, or LXRα<sup>+/−</sup> /LXRβ<sup>+/−</sup> MEFs (Fig. 6C), suggesting that the complete loss of LXR expression sensitized the cells to statin-dependent cell death.

The LXR agonist T0901317 rescued statin-treated cells from apoptosis in LXRα<sup>−/−</sup> /LXRβ<sup>+/−</sup> MEFs as well as in wild-type MEFs (Fig. 6C). The rescue was abolished, however, in LXRα<sup>−/−</sup> /LXRβ<sup>−/−</sup> and LXRα<sup>+/−</sup> /LXRβ<sup>−/−</sup> MEFs (Fig. 6C). This result suggested that LXRβ was responsible for the antiapoptotic effect of LXR agonists in nondifferentiated cells. The very low LXRα expression in undifferentiated MEFs, although apparently able to provide some protection against simvastatin in the absence of added LXR agonist (see above), was probably insufficient to mediate the antiapoptotic effect of T0901317. Since forced expression of LXRα rescued undifferentiated cells from statin-induced cells death (Fig. 5), our results collectively indicate that both LXRα and LXRβ are able to partially protect against statin-induced death in the absence of added LXR agonist and are required to mediate the pronounced protection afforded by added LXR agonist.

**Regulation of Expression of Genes Involved in Apoptosis and Survival by Simvastatin and the LXR Agonist T0901317**—Since LXR is a well established transcription factor, an attractive possibility would be that activation of LXR reverses a statin-dependent up-regulation of proapoptotic genes or down-regulation of antiapoptotic genes. In fact, the expression of several genes involved in apoptosis or survival has been reported to be regulated in response to administration of LXR agonists (47). Based on these reports and an Affymetrix<sup>TM</sup>-based expression analysis of 3T3-L1 preadipocytes treated with simvastatin and LXR agonists (not shown), we selected a subset of genes for further analysis by RT-qPCR. Treatment of cells with T0901317 reduced the expression of CIDE-A, CIDE-B, and Nip3, as well as Aatk and TIA1, but to a similar degree in the absence and presence of simvastatin (Fig. 7, row J). Certain other genes involved in apoptosis, such as p53 and Bax, have been reported to be up-regulated by statins in different cell lines, and expression of p53 and Bax was also up-regulated by simvastatin in 3T3-L1 cells (Fig. 7, row 2). Concomitant treatment with the LXR agonist T0901317 suppressed the up-regulation of p53 but not Bax (Fig. 7, row 2). The effects were selective, since expression of several additional genes involved in apoptosis or survival, such as Bcl2l2, Mcl-1, and Bad, was unaffected by either simvastatin or T0901317 treatment (Fig. 7, row 2).

LXR-dependent gene expression was recently shown to be important for macrophage survival (48, 49). AIM (apoptosis...
inhibitor of macrophages), also known as *SPα* or *Api6*, is regulated in an LXR-dependent manner and protects macrophages from bacteria-induced apoptosis (48, 49). In 3T3-L1 cells, surprisingly, *AIM/SPα/Api6* was up-regulated by simvastatin treatment in the absence but not in the presence of T0901317 (Fig. 7, row 3). Thus, expression of *AIM/SPα/Api6* was not up-regulated by LXR activation in 3T3-L1 cells. The expression of Brca4, an antiapoptotic form of Bcl-x, also known to be up-regulated by LXR agonist in macrophages, was not up-regulated by T0901317 in 3T3-L1 cells (Fig. 7, row 3). Collectively, these results illustrate the cell-specific regulation of LXR-responsive genes and clearly demonstrate that the mechanism for LXR-mediated prevention of bacteria-induced apoptosis in macrophages is not operative in the context of LXR-dependent rescue of statin-mediated apoptosis in 3T3-L1 preadipocytes.

The RT-qPCR analysis further revealed that expression of a major gene involved in the prevention of apoptotic cell death, Bcl-2, was up-regulated by T0901317 and further enhanced in the presence of simvastatin (Fig. 7, row 3). In addition, expression of *CARD14* (caspase recruitment domain-containing 14)
was up-regulated by T0901317, both in the absence and presence of simvastatin (Fig. 7, row 3).

Statin-induced Cell Death Is Independent of p53 and Cannot Be Rescued by Bcl-2 Overexpression—Based on the above results (Fig. 7), we studied whether the prevention of simvastatin-enhanced p53 expression or the enhancement of Bcl-2 induction by LXR agonist was relevant for cell survival. p53 is a key regulator of cell death, and p53 deficiency or p53 inhibition protects certain cell types from a wide variety of acute apoptosis-inducing agents (74). The finding that T0901317 treatment prevented both simvastatin-induced cell death and p53 induction indicated that simvastatin-induced cell death might be connected with induction of p53. To examine this possibility, we investigated whether simvastatin was able to induce cell death in p53-deficient MEFs induced to undergo adipocyte differentiation. MEFs isolated from p53−/− embryos were treated with simvastatin in the absence and presence of T0901317. The lack of p53 did not affect simvastatin-induced cell death or the ability of T0901317 to counteract the effects of simvastatin (Fig. 8A). Therefore, although p53 expression is regulated in response to simvastatin and T0901317 administration, p53 is dispensable for statin-induced cell death.

Since forced expression of Bcl-2 has been reported to prevent statin-induced apoptosis in colon cancer cells (17) and in NIH-3T3 fibroblasts (23), the significant up-regulation of Bcl-2 expression by T0901317 in the presence of simvastatin was intriguing and suggested that statin-induced cell death might be counteracted by up-regulation of Bcl-2 expression. To test this, we stably transduced 3T3-L1 preadipocytes with an empty vector or a vector expressing Bcl-2. Western blotting verified a strong overexpression of Bcl-2 that was far in excess of the levels observed in cells transduced with the empty vector and was similar in cells treated with vehicle, simvastatin, and/or T0901317 (Fig. 8B). Surprisingly, forced expression did not diminish statin-induced cell death, indicating that up-regulation of Bcl-2 expression was insignificant for T0901317-mediated rescue of cell death (Fig. 8C).

LXR-mediated Protection against Simvastatin-induced Apoptosis Requires NF-κB Activity—Although NF-κB has been implicated in activation of apoptosis in several cell lines (75–77), activation of NF-κB also plays a key role in the protection against apoptosis in many cell types and cell lines (30–32), including 3T3-L1 cells (78). In this context, it was of interest that the expression of CARD14 was strongly enhanced in cells treated with simvastatin and T0901317 (Fig. 7, row 3). CARD14 has been shown to associate with the CARD domain of BCL10, forming a signaling protein complex activating NF-κB through the IKK complex (56). Hence, we hypothesized that NF-κB activity might be involved in the LXR agonist-dependent rescue of statin-induced cell death. This hypothesis was further supported by the finding that T0901317 augmented the phosphorylation of IKKβ in 3T3-L1 cells in the absence or presence of simvastatin (Fig. 9A). To investigate the importance of NF-κB-dependent signaling, we stably transduced 3T3-L1 cells with a vector expressing a dominant negative (nonphosphorylatable) form of IκBα. Overexpression of the mutated IκBα was verified by Western blotting (Fig. 9B). The inability of the stably transduced cells to activate NF-κB-dependent transactivation was confirmed in transient transfection experiments, demonstrating that tumor necrosis factor-α was unable to induce a κB-responsive reporter gene in cells overexpressing the mutant form of IκBα (Fig. 9C).

The vector-transduced cells and cells with forced expression of the mutant IκBα were subsequently induced to differentiate in the presence or absence of simvastatin, and the effect on cell
LXR Activity Prevents Statin-induced Death of Preadipocytes

**FIGURE 9.** LXR-mediated protection against simvastatin-induced apoptosis requires NF-κB activation. A, 3T3-L1 cells were grown to 2 days postconfluence and induced to differentiate. 5 μM simvastatin dissolved in MeSO or MeSO alone was added when differentiation was induced on day 0 in the absence or presence of 1 μM T0901317. Whole cell extracts were prepared on day 4 after induction and analyzed for the presence of phosphorylated IKKa and total IKKa/β by Western blotting. Autoradiographies were analyzed by densitometric scanning, and the levels of phosphorylated IKKa relative to total IKKa/β were determined. The error bars represent S.D. (n = 3). B–D, 3T3-L1 cells were retrovirally transduced by a nondegradable IkB mutant or an empty vector and grown to confluence. B, whole cell extracts were prepared and analyzed for the presence of IkB by Western blotting. An antibody recognizing TFII B (transcription factor IIB) was used as control for equal loading. C/EBPβ activation.

It is possible that the statin-dependent down-regulation of IGF-1/insulin signaling (81), fibroblastic cells capable of undergoing adipocyte differentiation, namely the preadipocyte cell line 3T3-L1 and mouse embryo fibroblasts. Adipocytes are important players in the control of whole body lipid and glucose homeostasis, and the effects of statins prescribed for the treatment of hypercholesterolemia, often associated with obesity, are therefore not only of interest from a basic scientific point of view but also in a more clinical perspective. It should, however, be pointed out that the concentration of simvastatin used in this article (5 μM) and the concentrations of statins used in most in vitro studies are higher than the 1 μM plasma concentration achieved in vivo (79, 80). It is generally observed that statins affect negatively the differentiation of adipocytes (51–55). Various mechanisms have been suggested, but upon close inspection of many reports, it appears that the statins most potently affected adipogenesis during the first 4 days of the differentiation program, as shown explicitly in the present study. Regarding the molecular mechanism, it has been reported that pitavastatin, another lipophilic statin, prevented adipocyte differentiation of 3T3-L1 preadipocytes by inhibiting PPARY expression but not expression of C/EBPa, and interestingly pitavastatin also prevented the normal down-regulation of Pref-1 expression (54). Here we show that forced expression of PPARY2 did not rescue adipocyte differentiation in the presence of simvastatin. Since elevated and sustained expression of Pref-1 is associated with an inhibition of IGF-1/insulin signaling (81),
LXR Activity Prevents Statin-induced Death of Preadipocytes

Astatation process elicited pronounced 3T3-L1 cell death with nuclear morphology of apoptosis. Statin-induced cell death was, as expected, prevented by the addition of mevalonate but not by the addition of cholesterol or farnesol. The addition of geranylgeraniol rescued the cells from statin-induced death but only partly restored differentiation and lipid accumulation. The rescue of cell death and partial rescue of differentiation by geranylgeraniol point to the involvement of impaired geranylgeranylation of members of the Rho family of G-proteins. This conclusion is in line with the finding that geranylgeranyl-pyrophosphate/geranylgeraniol, but not cholesterol or farnesyl-pyrophosphate/farnesol, was able to rescue statin-induced cell death of cortical neurons (83) and myotubes (84).

Members of the Rho family of G-proteins are crucially involved in IGF-1/insulin signaling, survival, and adipocyte differentiation (85–88); hence, it is not surprising that perturbation of geranylgeranylation impacts seriously on preadipocytes induced to undergo adipocyte differentiation. It is possible that treatment with differentiation inducers per se causes a condition of stress in the cells and that this aggravates the effects of statins. Along this line, it was reported that simvastatin did not affect 3T3-L1 fibroblasts, which were not induced to undergo differentiation (55).

Surprisingly, we discovered that statin-induced cell death was prevented by the simultaneous addition of LXR agonists. The effect was not just observed for the classical T0901317 LXR agonist but could also be reproduced using the more selective LXR agonist GW3965. Rescue from statin-induced cell death was not dependent on the activity of SREBP-1. By using a combination of forced expression of wild-type and dominant negative LXR in 3T3-L1 cells with the use of primary MEFs isolated from LXRα−/− and LXRβ−/− as well as LXRα−/−/LXRβ−/− and wild-type embryos, we provided evidence that the rescue of statin-induced cell death was dependent on the expression and/or activity of either LXRα or LXRβ. Thus, our data did not indicate subtype-specific effects of LXR in relation to the rescue of statin-induced cell death. This finding distinguishes LXR-dependent rescue of statin-induced cell death in 3T3-L1 cells from the LXR-dependent rescue of bacterially induced apoptosis of macrophages, which is orchestrated via an LXRα-selective up-regulation of AIM1/Spα/Api6 (48). Furthermore, in the case of LXR-dependent rescue of statin-induced cell death in 3T3-L1 cells, we did not observe any up-regulation of AIM1/Spα/Api6 expression by administration of an LXR agonist; in contrast, we observed a marked up-regulation of AIM1/Spα/Api6 expression by simvastatin.

Since LXR signaling is induced by insulin (89), we speculated whether LXR activation could override the statin-induced abrogation of IGF-1/insulin survival signals impinging on alternative pathways for PKB and/or ERK-dependent signaling. However, LXR-mediated rescue of statin-induced cell death proceeded unabated in the presence of inhibitors of PI3-K/PKB and ERK activation, suggesting that LXR-dependent rescue did not require signaling along these pathways. The expression of p53 was up-regulated by simvastatin, but this up-regulation was prevented by the addition of T0901317, suggesting that p53-mediated processes might be involved in statin-induced cell death. However, the addition of simvastatin to p53-deficient MEFs induced to undergo adipocyte differentiation elicited the same degree of cell death as was observed in wild-type MEFs.

Statins are reported to down-regulate expression of Bcl-2 and/or Bcl-xL in several different cell lines (17–20, 22, 24). However, in 3T3-L1 cells, simvastatin did not affect expression of either Bcl-2 or Bcl-xL. Expression of Bcl-2 was, however, increased by T0901317, especially in the presence of simvastatin. Forced expression of Bcl-2 inhibits statin-induced apoptosis in colon cancer cells (17), whereas Bcl-xL overexpression protects from apoptosis induced by statins in murine tubular cells (24). Thus, up-regulation of Bcl-2 might contribute to the LXR-mediated rescue of simvastatin-induced cell death also in 3T3-L1 cells. Unexpectedly, forced overexpression of Bcl-2 did not prevent statin-induced cell death.

A surprising clue to a possible mechanism involved in LXR-mediated rescue came from the observation that T0901317 increased the expression of CARD14, a protein reported to be involved in IKK activation, leading to increased NF-κB activity (56). In keeping with this, we observed that T0901317 administration increased IKKβ phosphorylation and, furthermore, that inhibition of NF-κB activity by overexpression of a non-degradable form of IκBα completely prevented LXR-dependent rescue of statin-induced cell death. The finding that LXR/LXR agonist-dependent rescue relied on NF-κB activity was unexpected, since it was well established that LXR/LXR agonists normally down-regulate NF-κB activity or prevent NF-κB activation (90). However, it is conceivable that the perturbation of signaling by members of the Rho family of G-proteins caused by impaired prenylation changes the normal repressive mode of LXR in a manner analogous to that observed for the statin-induced switch between an activating and a repressive state of PPARα (91). In conclusion, in this paper, we describe a novel mechanism for LXR-dependent rescue of cell death that does not depend on regulation of the previously described mediator of LXR-dependent rescue of apoptosis or regulation of the normally involved pro- or antiapoptotic genes. Rather, the mechanism involves a novel and puzzling positive collaboration between LXR- and NF-κB-dependent pathways. Considering the common use of statins for treatment of hypercholesterolemia, the emerging use of statins combined with nuclear receptor agonists for prevention of coronary heart diseases and the possibilities to employ statins for cancer treatment, further studies of the interplay between statins and LXR agonists appear warranted.

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