0026-895X/00/061389-09$3.00/0
MOLECULAR PHARMACOLOGY
Vol. 58, No. 6
156/867384
Mol Pharmacol 58:1389–1397, 2000
Printed in U.S.A.

Inhibition of Protein Isoprenylation Impairs Rho-Regulated Early Cellular Response to Genotoxic Stress

RENATE GNAD, KLAUS AKTORIES, BERND KAINA, and GERHARD FRITZ

Division of Applied Toxicology, Institute of Toxicology, University of Mainz, Mainz, Germany (R.G., B.K., G.F.); and Institute of Pharmacology and Toxicology, University of Freiburg, Freiburg, Germany (K.A.)

Received March 17, 2000; accepted September 7, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Activation of c-Jun N-terminal kinases (JNKs) and nuclear factor-κB (NF-κB) are early cellular responses to genotoxic stress involved in the regulation of gene expression. Pretreatment of cells with the hydroxymethyl glutaryl-CoA reductase inhibitor lovastatin blocked stimulation of JNK1 activity by UV irradiation and by treatment with the alkylating compound methyl methanesulfonate but did not affect activation of extracellular signal-regulated kinase 2 by UV light. Lovastatin also attenuated UV-induced degradation of the NF-κB inhibitor IκBα. The effects of lovastatin on UV-triggered stimulation of JNK1 as well as on IκBα degradation were reverted by cotreatment with geranylgeranylpyrophosphate but not with farnesylpyrophosphate. Both a geranylgeranyltransferase type I inhibitor and a farnesyltransferase inhibitor blocked JNK1 stimulation by UV irradiation without impairing signaling to NF-κB. This indicates that different types of isoprenylated proteins impair UV-induced signaling to JNK1 and NF-κB, respectively. Since lovastatin caused a rapid decrease in the level of membrane-bound Rho GTPases, we hypothesize that Rho signaling is inhibited by lovastatin. In line with this hypothesis, Rho-inactivating toxin B from *Clostridium difficile* abolished both JNK1 activation and IκBα degradation evoked by UV irradiation. In summary, lovastatin-mediated inhibition of protein isoprenylation abrogates cellular stress responses involving JNK- and NF-κB-regulated pathways, which seems to be caused by inactivation of Rho GTPases.

Ras and Ras-homologous (Rho) GTPases are involved in the regulation of a variety of cellular functions, such as transformation (Marshall, 1984; Khosravi-Far et al., 1995; Qiu et al., 1995) and genotoxic stress-induced signaling leading to the activation of MAP kinases (e.g., ERKs, JNKs, and p38 MAP kinases) (Coso et al., 1995; Minden et al., 1995; Canman and Kastan, 1996). MAP kinases trigger the phosphorylation and activation of transcription factors that regulate the expression of genes whose products influence cell proliferation, differentiation, survival, and apoptosis (Canman and Kastan, 1996). Besides the activation of MAP kinase-related pathways, a further response of cells to genotoxic stress resulting in stimulation of gene expression is the activation of NF-κB (Baldwin, 1996; Bender et al., 1998). Recently, it has been shown that the activity of NF-κB is also affected by Rho proteins (Perona et al., 1997). As inferred mainly from knock-out studies, NF-κB protects cells from apoptosis induced by TNFα and genotoxic agents (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996, 1999). A prerequisite for activation of NF-κB by TNFα is IκB kinase complex-dependent phosphorylation of the inhibitory molecule IκBα (Baldwin, 1996). Upon phosphorylation on Ser-32, NF-κB is released for nuclear translocation, and free IκBα is proteasomally degraded (Baldwin, 1996). In contrast, in cases of exposure of cells to UV-light, IκBα degradation and activation of NF-κB occur independently of the IκB kinase complex and phosphorylation of IκBα on Ser-32 (Bender et al., 1998; Li and Karin, 1998). This indicates that different mechanisms are involved in the regulation of NF-κB by DNA-damaging treatments and TNFα, respectively.

An essential requirement for correct intracellular localization, and consequently also for physiological function, of low-molecular-weight GTPases is their modification by isoprenylation (Glomset et al., 1990; Cox and Der, 1992). The C-terminal CAAX box is the signal structure determining whether these proteins become geranylgeranylated or farnesylated by the corresponding transferases (Schafer and Rine, 1992; Brown and Goldstein, 1993; Newman and Magee, 1993). Ras proteins are mainly farnesylated (Schafer and

This work was supported by the Deutsche Forschungsgemeinschaft (Grant Fr-1241/1–3).

ABBREVIATIONS: Rho, Ras-homologous; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor κB; TNFα, tumor necrosis factor α; IκBα, inhibitor κBα; HMG, hydroxymethyl glutaryl; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase type I inhibitor; FCS, fetal calf serum; CHO, Chinese hamster ovary; AP-1, activator protein 1; MMS, methyl methanesulfonate; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; LT, lethal toxin from *C. sordellii*; ToxB, toxin B from *C. difficile*; GGTase, geranylgeranyltransferase.

Rine, 1992; Newman and Magee, 1993), whereas Rho GTPases, in general, are geranylgeranylated. Based on this, it is tempting to hypothesize that pharmacological inhibitors of isoprenylation affect the physiological function of small GTPases and, therefore, affect genotoxic stress-induced signaling and gene expression. However, this aspect has not been analyzed until now. One class of pharmaceuticals that may interfere with protein isoprenylation is HMG-CoA reductase inhibitors such as lovastatin (Goldstein and Brown, 1990). The major application of this group of clinically extremely relevant and potent therapeutics is the therapy of hypercholesterolemia, a main risk factor for heart attack and stroke (Shepherd et al., 1995; Blauw et al., 1997; Lipid Study Group, 1998). Recently, it was reported that lovastatin causes up-regulation of endothelial nitric-oxide synthase (Endres et al., 1998). This effect is thought to contribute to the stroke-preventing activity of lovastatin (Endres et al., 1998). Considering that long-term intake of statins is required to achieve optimal protective effect against heart attack and stroke, a rise in undesirable side effects based on inhibition of isoprenylation of regulatory GTPases is imaginable. To the best of our knowledge, putative effects of HMG-CoA reductase inhibitors on signal mechanisms regulated by small GTP-binding proteins have not been investigated so far.

Therefore, in the present study, we addressed the question of whether or not inhibition of protein isoprenylation by lovastatin has an influence on signal pathways that are rapidly activated by genotoxic stress and regulate gene expression. Here, we present evidence that lovastatin impairs genotoxic stress-induced signaling to both JNK1 and NF-κB. Furthermore, the data indicate that this effect of lovastatin is caused by inhibition of Rho.

Experimental Procedures

Materials. Cytochalasin D was obtained from Sigma (Deisenhofen, Germany). The HMG-CoA reductase inhibitor lovastatin, farnesyltransferase inhibitor (FTI), geranylgeranyltransferase type I inhibitor (GGTI), and p53 antibody were purchased from Calbiochem (Bad Soden, Germany). All other antibodies used in this study were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GST-Jun (1–166) was generously provided by P. Angel (DKF2, Heidelberg, Germany). NF-κB-specific reporter gene was a gift of U. Rapp (Institute of Medical Radiation and Cell Research, Würzburg, Germany). N17Cdc42 expression constructs and N19RhoA cDNA were provided by A. Hall (Medical Research Council, Laboratory for Molecular and Cell Biology, London, UK).

Cell Culture. Chinese hamster ovary (CHO) and HeLa cells were routinely grown in Dulbecco's modifed Eagle's medium containing 5% fetal calf serum (FCS). Primary mouse fibroblast cell line BK4 was grown in Dulbecco's modifed Eagle's medium supplemented with 10% FCS. Cell rounding was analyzed by microscopy upon formaldehyde/Triton X-100 fixation of the cells as described (Barth et al., 1998). Breakdown of actin cytoskeleton was confirmed by fluorescein isothiocyanate-phalloidin staining (Barth et al., 1998). Lovastatin was not preactivated by alkaline hydrolysis, because in initial experiments it was observed to be as effective [with respect to the induction of morphological changes (Koch et al., 1997)] as the preactivated compound.

Kinase Assays. JNK1 activity was determined by the immune complex kinase assay system. After immunoprecipitation using a JNK1-specific antibody (Santa Cruz), phosphorylation reaction was performed at 30°C for 30 min in a total volume of 40 μl of reaction buffer [25 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, 25 μM ATP, and 1 μCi of [γ-^{32}P]ATP]. As a substrate for JNK reaction, GST-Jun 1/166 (a gift of P. Angel, Heidelberg, Germany) was used. Reaction products were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography of the dried gel. For quantification, autoradiograms were analyzed densitometrically. For determination of ERK2 activation, Western blot-based analysis was performed as described (Fritz and Kaina, 1999).

Western Blot Analysis. Protein from total cell extracts (30 μg) was separated onto 10 to 12.5% SDS polyacrylamide gels. After wet-blotting to nitrocellulose, protein expression was analyzed using the corresponding antibodies (1:100 to 1:1000). After incubation with peroxidase-coupled anti-rabbit (anti-mouse) antibody, proteins were visualized by chemiluminescence using Renaissance enhanced luminol reagent (DuPont NEN, Boston, MA).

Reporter Gene Analysis. To determine the effect of lovastatin on NF-κB-regulated gene expression, reporter gene analyses, using NF-κB-specific minimal promoter (3×NF-κB-luciferase) (Baumann et al., 1998), were performed. After transfection of CHO cells with the NF-κB-Luciferase construct (24 h), cells were pretreated with lovastatin for 8 h. After pretreatment period, cells were exposed to UV light. A further 16 h later, cells were harvested for determination of luciferase activity using the luciferase assay (Promega, Madison, WI). To analyze the effect of dominant negative Rho forms on stimulation of NF-κB-specific gene expression by UV light, expression vectors encoding dominant negative Cdc42 (N17Cdc42) RhoA (N19RhoA) and Rac (N17Rac) (Fritz and Kaina, 1997) were cotransfected.

Gel Retardation Analysis. ^{32}P-labeling of oligonucleotides was performed by use of T4 kinase. A cell extract was prepared as described previously (van Dam et al., 1993). Binding reactions were performed by incubation of 5 μg of protein with ~5 fmol of ^{32}P-labeled oligonucleotide for 30 min at room temperature [binding buffer: 10 mM HEPES, pH 7.9, 60 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 μg/ml BSA, 10% glycerol, 0.5 μg poly(dI-dC)]. Subsequently, reaction mixtures were separated onto 5% native polyacrylamide gels. After electrophoresis, gels were dried and subjected to autoradiography. For analysis of the DNA-binding activity of AP-1, an AP-1-specific oligonucleotide originating from the mouse collagenase promoter (5′-AGTGGTGACTCATCACT-3′) was used.

Analysis of GTP-Binding Activity (GTP Overlay). GTP-binding activity of a membrane fraction was analyzed upon separation of membrane proteins by SDS-polyacrylamide gel electrophoresis (15% gel). After blotting to nitrocellulose, proteins were renatured by overnight incubation in buffer containing 25 mM Tris/192 mM glycine. After 20 min of preincubation in binding buffer [50 mM Tris (pH 7.5), 0.3% Tween 20, 5 mM $MgCl_2$, 1 mM EGTA], [α-^{32}P]GTP (1 μCi/ml) was added. After a further incubation period of 90 min at room temperature, filters were washed three times for 30 min with binding buffer. Subsequently, the level of [^{32}P]GTP binding was visualized by autoradiography.

Fig. 1. Lovastatin blocks genotoxic stress-induced activation of JNK1. After pretreatment of logarithmically growing CHO cells with either 50 μM lovastatin (Lov) (A) or 5 μg/ml cytochalasin D (CytD) (B), cells were exposed to UV light (60 J/m^2) and MMS (2 mM), respectively. After treatment (30 min), cells were harvested for determination of JNK1 activity as described under *Experimental Procedures*. Shown are the autoradiographic results.

Results

CHO cells were pretreated with the HMG-CoA reductase inhibitor lovastatin for 24 h. Afterward, they were exposed to UV light (UV-C) or to the monofunctional alkylating agent methyl methanesulfonate (MMS). Thirty to 60 min after exposure, JNK1 activity was determined by the immune complex kinase assay. As shown in Fig. 1A, pretreatment of cells with lovastatin completely blocked both the UV- and the MMS-induced stimulation of JNK1 activity. Pre-exposure of cells to cytochalasin D did not abolish JNK1 activation by genotoxic agents (Fig. 1B), indicating that the inhibitory effect of lovastatin on genotoxic stress-induced JNK1 activation is independent of cytoskeletal changes, which are also known to be evoked by lovastatin. This view is supported by the finding that pretreatment with lovastatin for only 4 h, which is too short a period to cause cell rounding (Fig. 2A), is already sufficient to completely impair stimulation of JNK1 activity (Fig. 2B). Lovastatin did not inhibit UV-induced activation of ERK2 (Fig. 2C). This shows the specificity of lovastatin on JNK-related signal pathways. Besides attenuating activation of JNK1, lovastatin also inhibited agent-induced increase in c-Jun protein expression and AP-1-binding activity (Fig. 3). After a pretreatment period of 4 h, lovastatin exerts partial inhibitory effects (data not shown).

Next we analyzed whether lovastatin interferes with other types of early cellular responses to genotoxic stress, in particular with UV-induced signaling to p53/p21 and to NF-κB (Fritsche et al., 1993; Bender et al., 1998). To investigate the effect of lovastatin on p53/p21, we made use of a primary mouse fibroblast cell line expressing wild-type p53. Pretreatment of these cells with lovastatin for 2 h did not inhibit the UV-induced rise in p53 protein level (Fig. 4A). Moreover, p21 protein induction was also not affected by lovastatin pretreatment (Fig. 4B). Under identical experimental conditions, an increase in c-Jun protein expression was blocked (Fig. 4B). After an extended period of time of lovastatin pretreatment (16 h), the UV-stimulated increase in p53 protein level was no longer detectable (Fig. 4C). In contrast, the UV-induced increase in the amount of p21 protein remained unchanged (Fig. 4C). To analyze the effect of lovastatin on stress-induced signaling to NF-κB, we monitored its influence on IκBα degradation upon UV irradiation. As shown in Fig. 4D, the UV-induced decrease in IκBα protein level was completely blocked after 24 h of lovastatin pretreatment. After a pretreatment period of 4 h, a significant ($P < .05$) inhibitory effect of lovastatin on IκBα degradation by UV light was already observed (Fig. 4E). Cytochalasin D pretreatment did not impair UV-induced degradation of IκBα (data not shown), indicating that lovastatin affects NF-κB signaling independent of cytoskeletal changes. Overall, the HMG-CoA reductase inhibitor lovastatin impaired genotoxic stress-induced signaling to JNK1 and NF-κB without affecting mechanisms leading to the activation of ERK2 and p21. Also, notably, a lower concentration of lovastatin (e.g., <10

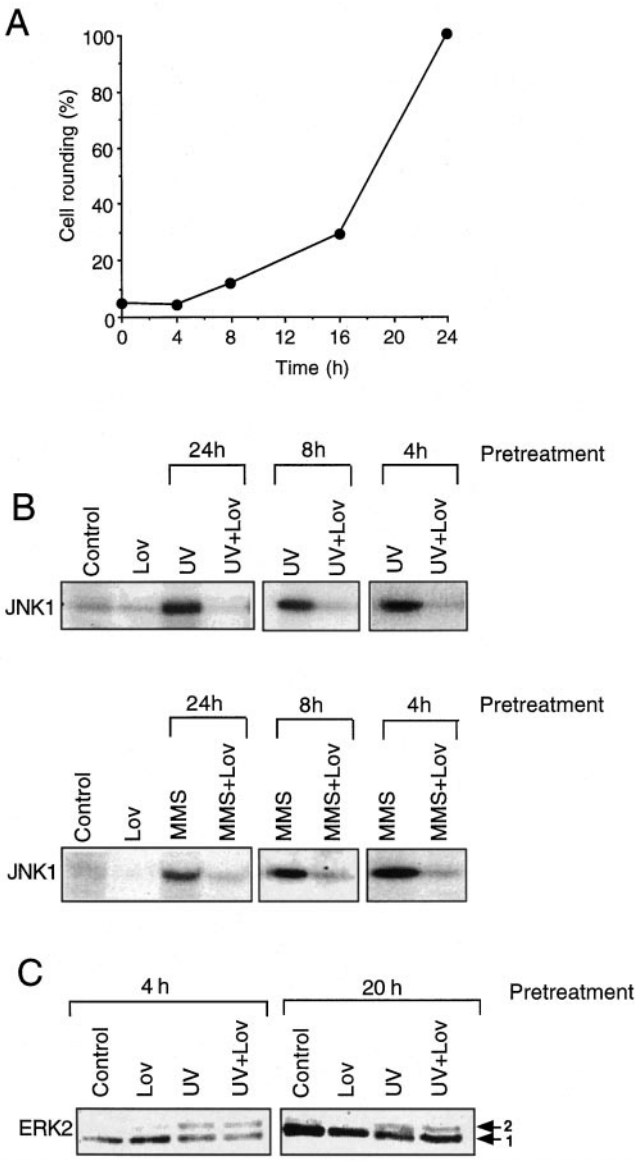

Fig. 2. Lovastatin (Lov)-mediated inhibition of JNK1 activation by genotoxic stress is independent of cytoskeletal changes. A, logarithmically growing CHO cells were exposed to lovastatin (50 μM). After different times, cells were fixed, and the number of rounded cells was counted as described under *Experimental Procedures*. B, 4, 8, and 24 h after lovastatin pretreatment, cells were irradiated with UV light (60 J/m^2) or treated with MMS (2 mM). After a further incubation period of 30 min, cells were harvested for determination of JNK1 activity as described. Shown are the autoradiographic results. C, serum-starved (24 h, 0.5% FCS) HeLa cells were pretreated for different periods of time with lovastatin (50 μM). Afterward, cells were exposed to UV light (60 J/m^2); 10 min later, cells were harvested, and ERK2 activation was determined by Western blot analysis as described under *Experimental Procedures*. 1, nonphosphorylated; 2, phosphorylated (active) form.

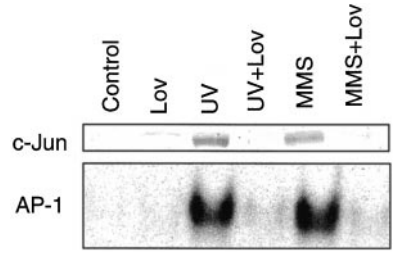

Fig. 3. Lovastatin (Lov) impairs the UV- and MMS-induced increase in c-Jun protein level and DNA-binding activity of AP-1. Logarithmically growing CHO cells were pretreated with lovastatin (50 μM) for 20 h. Subsequently, cells were exposed to UV (60 J/m^2) or treated with MMS (1 mM). After a further incubation period of 4 h, cells were harvested, and the amount of c-Jun protein was determined by Western blot analysis. DNA-binding activity of AP-1 was analyzed by gel retardation analysis as described under *Experimental Procedures*.

μM) effectively inhibited UV-inducible signaling. Thus, 4 to 5 μM lovastatin is sufficient to cause 50% inhibition of the UV-stimulated JNK1 activation and IκBα degradation, respectively (Fig. 5A). Furthermore, 5 μM lovastatin largely abrogated the stimulation of NF-κB-regulated gene expression by UV exposure (Fig. 5B).

Basically, inhibitors of HMG-CoA reductase cause depletion of precursor molecules that are required for both protein geranylgeranlyation and protein farnesylation (Goldstein and Brown, 1990). To identify the type of protein isoprenylation that is important for signaling to JNK1 and NF-κB, lovastatin pretreatment was performed in the presence of either FPP or GGPP, respectively. As shown in Fig. 6A, cotreatment with GGPP restored JNK1 activity upon UV irradiation to >80% of the level that is observed in nonpretreated control cells. In contrast, addition of FPP had no effect on the lovastatin-mediated inhibition of JNK1 activation by UV light (Fig. 6A). This indicates that blockage of UV-triggered JNK activation by lovastatin is caused mainly by inhibition of protein geranylgeranylation. To further address this question, we investigated the effect of pharmacological inhibitors of protein geranylgeranylation (e.g., GGTI) and farnesylation (e.g., FTI) on UV-induced JNK1 activity. Pretreatment of cells for 4 h with both GGTI and FTI exerted no inhibitory effect (Fig. 6B), whereas pretreatment for 24 h inhibited the UV-induced increase in JNK1 activity by 80 to 90% (Fig. 6B). Obviously, protein geranylgeranylation as well as farnesylation is essential for JNK1 stimulation by UV. Because FPP did not reverse the lovastatin effect, we conclude that farnesylated proteins are necessary, but insufficient on their own, to mediate activation of JNK by UV light. With respect to UV-stimulated degradation of IκBα, we found that FPP did not abolish the inhibitory effect of lovastatin on IκBα degradation by UV light, whereas GGPP did

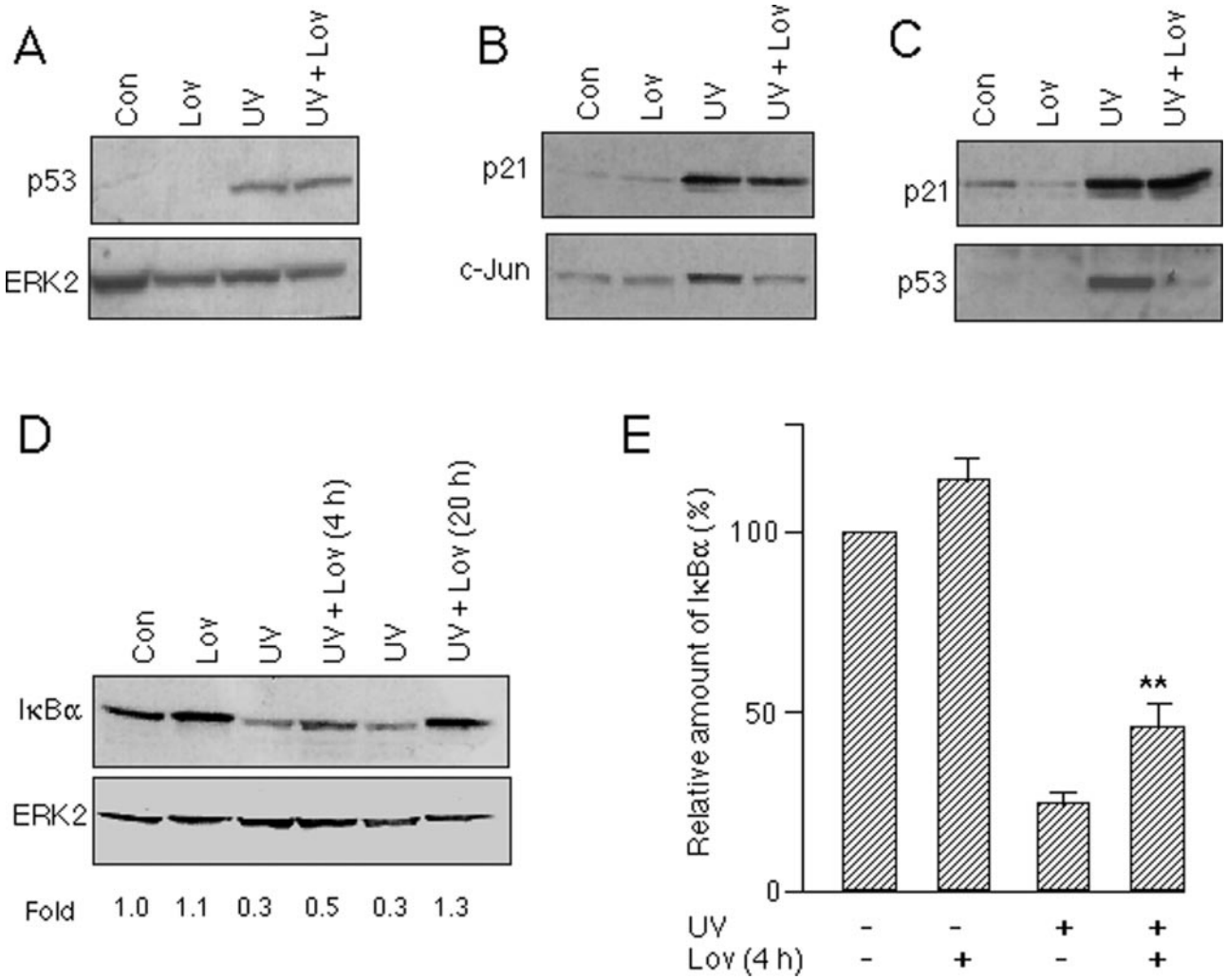

Fig. 4. Effect of lovastatin on the UV-induced increase in p53/p21 and degradation of IκBα. A and B, logarithmically growing primary mouse fibroblast cells were left untreated (Con) or were pretreated (Lov) for 2 h with lovastatin (50 μM). Afterward, cells were irradiated (60 J/m^2) and further incubated in the presence of lovastatin; 6 h (for analysis of p53) and 10 h (for p21 analysis) later, cells were harvested, and the expression of p53 and p21 protein, respectively, was analyzed by Western blot analysis. For the control, filters were rehybridized with ERK2 and c-Jun specific antibodies. C, cells were left untreated (Con) or were pretreated for 16 h with lovastatin (Lov) as described under A. Subsequently, cells were irradiated and analyzed for p53/p21 protein expression 6 to 10 h later. D, after the pretreatment period of 4 and 20 h with lovastatin (50 μM), cells were irradiated (80 J/m^2) and harvested a further 4 h later for determination of the amount of IκBα by Western blot analysis. Shown is one representative experiment. The relative amount of IκBα (IκBα/ERK2) in the control was set to 1.0. Con, nonpretreated; Lov, lovastatin pretreatment as described before. E, Mean values $\pm$ S.D. from three independent experiments performed as described under D with a lovastatin pretreatment period of 4 h. The relative amount of IκBα in untreated control was set to 100%. **, significant effect ($P < .05$).

(Fig. 7A). Thus, as with JNK1 activation, inhibition of protein geranylgeranylation by lovastatin is the main underlying cause for abrogation of IκBα degradation induced by UV light. In contrast to JNK1, inhibition of protein isoprenylation by FTI and GGTI did not prevent the decrease in IκBα protein level after UV exposure (Fig. 7B). This indicates that different types of isoprenylated proteins that are targeted by lovastatin are involved in the regulation of UV-induced signaling, leading to JNK1 activation or IκBα degradation.

Ras-related GTPases of the Rho family are modified by isoprenylation and are reported to be involved in the regulation of both JNKs/stress-activated protein kinases and NF-κB (Coso et al., 1995; Minden et al., 1995; Perona et al., 1997). Therefore, we considered Rho GTPases as putative candidates whose function might be inhibited by lovastatin. This hypothesis is supported by the finding that lovastatin treatment resulted in a rapid, time-dependent decline in the level of membrane-bound RhoA, Rac, and Cdc42 protein (Fig. 8A). The overall level of Rho GTPases in whole cell extracts was not changed by lovastatin (data not shown). Compared with the untreated control, the amount of these GTPases was largely (>50%) reduced already 8 h after addition of lovastatin (Fig. 8B). In contrast, the overall level of [^{32}P]GTP-binding proteins remained largely unchanged within the first 8 h after lovastatin treatment (Fig. 8B). This indicates that lovastatin treatment preferentially results in a rapid drop of Rho GTPases from the membrane fraction. After 24 h of treatment with GGTI, a decrease in the level of membrane-bound Rho proteins was not observed (data not shown).

To further prove the essential role of Rho proteins in UV-induced signaling, we made use of *Clostridium difficile* ToxB, which specifically inactivates Rho GTPases (e.g., RhoA, Rac, Cdc42) (Just et al., 1994, 1995) by glucosylation. As shown in Fig. 9, ToxB completely blocked UV-induced stimulation of JNK1 activity (Fig. 9A) and also impaired the decrease in IκBα protein level (Fig. 9B). Statistical analysis revealed that the blocking effect of ToxB on UV-induced IκBα degradation is highly significant [relative amount of IκBα protein after UV irradiation: 0.35 ± 0.10 (n = 3); relative amount of IκBα protein after UV irradiation plus ToxB pretreatment: 1.10 ± 0.15 (n = 3)]. Thus, Rho GTPases are essentially required for genotoxic stress-induced signaling to both JNK1 and NF-κB. To identify the types of Rho GTPase primarily involved, we used two additional bacterial toxins that specifically inhibit different subtypes of Rho proteins. Inactivation of Rac by lethal toxin from *Clostridium sordellii* (LT) (Just et al., 1996) impaired activation of JNK1 by UV light (Fig. 9A) without affecting IκBα degradation (Fig. 9B). On this basis, we suggest Rac activity to be crucial for signaling to JNK1 but not to NF-κB. Inactivation of Rho subtypes (RhoA, -B, and -C) of Rho GTPases by *Clostridium botulinum,* exoenzyme C3 (Aktories et al., 1988; Kikuchi et al., 1988; Chardin et al., 1989) partially inhibited activation of JNK1 by UV light (Fig. 9A) and, similar to LT, did not affect IκBα degradation (Fig. 9B). This indicates that Rho (e.g., RhoA, -B, and -C)-regulated mechanisms contribute to JNK1 activation by UV irradiation without affecting NF-κB-related pathways. Of all Rho-inactivating toxins tested, only ToxB impaired UV-induced signaling to NF-κB. ToxB distinguishes from LT and C3 toxin in that it has Cdc42 as an additional substrate. This raises the question of whether or not inactivation of Cdc42 is sufficient to block NF-κB activation by UV irradiation. To this end, we analyzed the effect of dominant negative Cdc42 (N17Cdc42) on stimulation of NF-κB-regulated gene expression by reporter gene analysis. As shown in Fig. 9C, neither dominant negative Cdc42, Rac (N17Rac), nor RhoA (N19RhoA) inhibit the UV-driven NF-κB-specific gene expression. This indicates that down-modulation of a single Rho-regulated pathway alone is not sufficient to block NF-κB activation by UV irradiation.

Discussion

In the present study, we provide evidence that the HMG-CoA reductase inhibitor lovastatin abrogates stimulation of c-Jun-N-terminal kinase 1 (stress-activated protein kinase 1) activity by genotoxic stresses such as UV irradiation and MMS exposure. Cytochalasin D, which causes depolymerization of the actin cytoskeleton in a manner similar to lovastatin, failed to inhibit JNK1 activation by UV light, showing

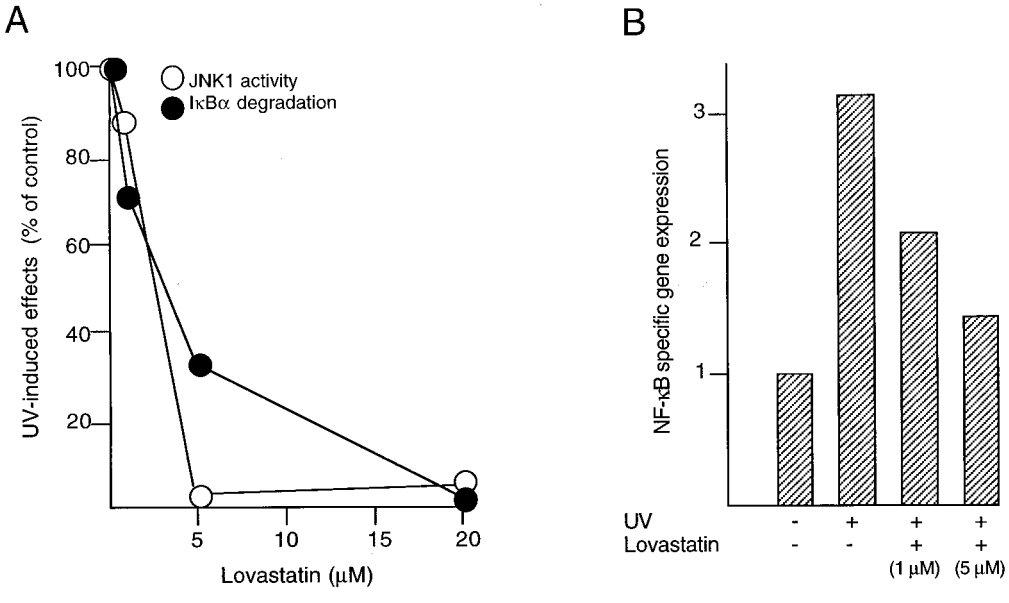

Fig. 5. Lovastatin-mediated inhibition of JNK1 and NF-κB is dose dependent. A, logarithmically growing cells were pretreated with different concentrations of lovastatin (1, 5, and 20 μM) for 20 h. Afterward, cells were irradiated (80 J/m^2); 30 min and 4 h later, JNK1 activity and IκBα degradation, respectively, were determined as described before. Data shown are mean values from two independent experiments. The relative JNK1 activity (IκBα degradation) without lovastatin pretreatment was set to 100%. B, 24 h after transfection with the NF-κB-specific reporter gene construct (3xNF-κB-Luc), cells were pretreated with 1 and 5 μM lovastatin for 8 h. Afterward, cells were irradiated (80 J/m^2), and a further 16 h later, they were harvested for determination of luciferase activity. Data shown are mean values from at least two independent experiments, each performed in duplicate.

that the inhibitory effect of lovastatin on genotoxic stress-induced activation of JNK1 is independent of cytoskeletal changes, which are also evoked by lovastatin (Schmidt et al., 1982; Fenton et al., 1992; Bifulco et al., 1993; Koch et al., 1997). Inhibition of UV-induced JNK1 activation by lovastatin is accompanied by blockage of c-Jun protein expression and AP-1-binding activity. This is in contrast to the phosphatidylinositol-3 kinase inhibitor wortmannin, which blocks genotoxic stress-induced activation of JNK1 without affecting c-Jun protein expression and AP-1 binding (Fritz and Kaina, 1999). Because lovastatin does not interfere with signaling to ERK2 and p21, a nonspecific inhibitory effect of this compound on genotoxic stress-inducible mechanisms can be ruled out. Moreover, bearing in mind that activation of ERKs by UV light is regulated mainly via the Ras/Raf pathway (Radler-Pohl et al., 1993; Canman and Kastan, 1996), the data also indicate that Ras/Raf-regulated mechanisms are not significantly affected by lovastatin. Another cellular response to genotoxic stress that we found to be impaired by lovastatin, too, is UV-induced signaling to NF-κB. The IC_{50} concentration of lovastatin for inhibition of UV-mediated activation of JNK1 as well as IκBα degradation is 4 to 5 μM. This indicates that the observed inhibitory effects of lovastatin on stress-inducible signal pathways might also be relevant under in vivo conditions, in particularly in the liver, where the highest concentration of lovastatin is achieved.

Although lovastatin was able to block UV-induced signaling to both JNK1 and NF-κB, different mechanisms are very likely to be involved. This hypothesis is based on the observations that 1) 4 h of lovastatin treatment were sufficient to completely block JNK1 activation by UV light; under these conditions UV-induced signaling to NF-κB was only partially attenuated and 2) inhibitors of both FTI and GGTI were able to impair JNK1 activation, although none of them inhibited signaling to NF-κB. The data indicate that different types of isoprenylated proteins are involved in the activation of JNK1 and NF-κB by genotoxic stress. Surprisingly, the inhibitory effect of lovastatin on NF-κB signaling was reversed by GGPP but was not affected by the inhibitor of GGTase type I. This indicates that the activity of GGTases other than GGTase type I is crucial for the modification of regulatory proteins triggering NF-κB activation by UV irradiation. Alternatively, compensatory mechanisms by other types of GGTases might come into play. This would also explain our observation that, upon pretreatment of cells for 24 h with the GGTase type I inhibitor, clear changes in the level of membrane-bound Rho proteins were not observed.

Rho family GTPases are reported as central players in the regulation of JNKs (Coso et al., 1995; Minden et al., 1995) and are also known to interfere with NF-κB regulation (Perona et al., 1997). Therefore, it was reasonable to assume that Rho proteins might be important cellular targets whose activity is inhibited by lovastatin. If this assumption is true, lovastatin should be expected to reduce the fraction of physiologically active Rho proteins that are located in the membrane fraction. Indeed, lovastatin caused a rapid decline in the amount of membrane-bound Rho proteins. Eight hours after exposure to lovastatin, RhoA, Rac, and Cdc42 protein

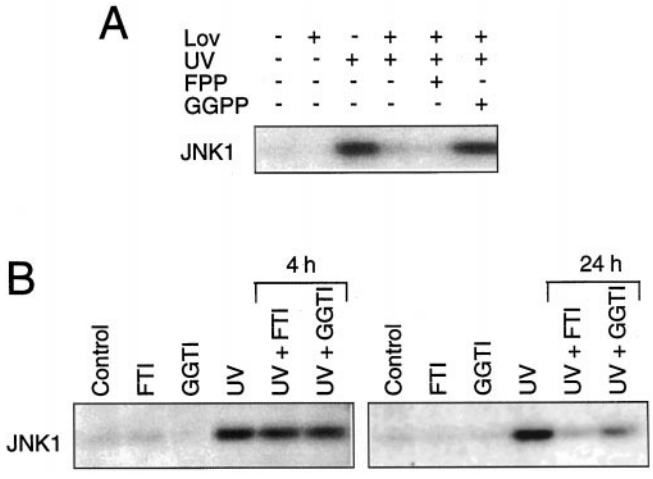

Fig. 6. Both geranylgeranylated and farnesylated proteins are involved in the lovastatin-mediated inhibition of UV-induced activation of JNK1. A, logarithmically growing CHO cells were pretreated with lovastatin (Lov) for 4 h in the presence (+) or absence (−) of FPP (50 μM) and GGPP (50 μM), respectively; 30 min after UV irradiation (60 J/m^2), cells were analyzed for JNK1 activity as described. Shown is the autoradiography of one representative experiment (of three independent experiments, each giving basically identical results). B, CHO cells were left untreated (Control) or were pretreated with either GGTI (10 μM) or FTI (50 μM) for 4 h and 24 h, respectively. After a preincubation period, cells were irradiated and harvested for determination of JNK1 activity as described under A. Shown is the autoradiography of one representative experiment (of at least two independent experiments, giving basically the same results).

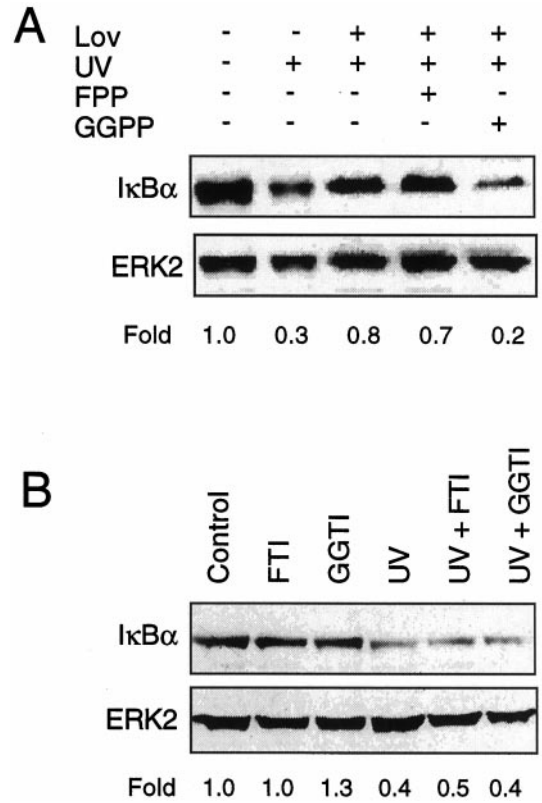

Fig. 7. Involvement of protein geranylgeranylation and farnesylation in UV-induced signaling to NF-κB. A, cells were pretreated with lovastatin (Lov) for 16 h in the presence (+) or absence (−) of FPP (50 μM) and GGPP (50 μM), respectively. After pretreatment, cells were irradiated with UV light (80 J/m^2). After a further incubation period of 4 h, cells were harvested for the determination of IκBα protein level by Western blot analysis. Autoradiograms were analyzed densitometrically, and the relative amount of IκBα (IκBα/ERK2) in the control was set to 1.0. B, CHO cells were pretreated or not (Control) with either GGTI (10 μM) or FTI (50 μM) for 24 h. After the preincubation period, cells were exposed to UV light and harvested a further 4 h later for determination of the IκBα protein level. Quantification was performed as described under A.

levels were already decreased by >50%, whereas the overall level of [^{32}P]GTP-binding proteins was only slightly reduced (<20%). Obviously, lovastatin causes a rapid, preferential decline of Rho-type GTPases from the membrane fraction. A lovastatin-induced decrease in membrane localization of RhoA was recently reported by Koch et al. (1997). Changes in the overall level of Rho GTPases by lovastatin treatment were not observed, as analyzed in whole cell extracts. Yet, an additional Rho form, which can be assumed to represent the nonisoprenylated form, became detectable (data not shown).

Another line of evidence showing that Rho proteins are crucial for UV-induced signaling to both JNK1 and NF-κB is based on the use of Rho-inactivating clostridial toxins. Upon inactivation of all Rho species (e.g., RhoA-like species, Rac and Cdc42) by *C. difficile* ToxB (Just et al., 1994, 1995), a stimulatory effect of UV irradiation on JNK1 activation and IκBα degradation was no longer detectable. This shows that Rho proteins are essentially required for genotoxic stress-induced signaling to JNK1 and NF-κB. From transient transfection of constitutively activated Rho species (Coso et al., 1995; Minden et al., 1995) and from the use of Rho-activating necrotizing factor from *E. coli* (Lerm et al., 1999), it is known that activation of Rho results in stimulation of JNK activity. However, we would like to point out that it was still quite unclear what subtype of Rho GTPase might be of particular physiological relevance to trigger JNK activation upon UV irradiation. Furthermore, participation of Rho proteins in genotoxic stress-induced activation of NF-κB has not been described so far. To identify the type of Rho GTPase that is physiologically most relevant for signaling to JNK1 and NF-κB we made use of bacterial toxins specifically inactivating particular Rho isoforms. Inactivation of Rac signaling by use of LT from *C. sordellii* (Just et al., 1996) completely abrogated activation of JNK1 by UV light without affecting IκBα degradation. This strongly indicates that Rac is the most physiologically relevant Rho GTPase triggering UV-induced signaling to JNK1. Inhibition of RhoA-like GTPases (e.g., RhoA, -B, and -C) by *C. botulinum* exoenzyme C3 (Aktories et al., 1988; Kikuchi et al., 1988; Chardin et al., 1989) partially inhibited UV-stimulated JNK1 activity and did not affect IκBα degradation. Obviously, although to a lesser extent than Rac, activation of RhoA-like GTPases also influences the level of JNK1 activity upon UV exposure. However, neither Rac nor RhoA-like GTPases seem to be decisive for signaling to NF-κB. Of all toxins tested, only ToxB was able to block UV-induced IκBα degradation. ToxB is distinguished from LT (Rac specific) and C3 exoenzyme (RhoA-like specific) only in additionally inactivating Cdc42. Therefore, it is tempting to speculate that Cdc42 is important for the activation of NF-κB by UV light. However, dominant negative Cdc42 (N17Cdc42) failed to inhibit UV-induced activation of NF-κB-regulated gene expression. The same is true for N17Rac and N19RhoA, which we included as further controls. These data are in line with a recent report (Perona et al., 1997). Therefore, we suggest that down-modulation of Cdc42-regulated signaling alone is not sufficient for inhibition of NF-κB activation by UV irradiation. Maybe, inactivation of several Rho species is required for blockage of UV-induced signaling to NF-κB. This interesting point will be the subject of forthcoming studies. When discussing Rho GTPases as putative relevant targets for lovastatin-mediated attenuation of genotoxic stress-induced activation of NF-κB, it should not be concealed that the evidence available for the participation of Rho proteins in the regulation of NF-κB is much more tenuous than it is for JNK signaling. Therefore, we cannot completely rule out that mechanisms independent of Rho GTPases are also involved in the inhibitory effect of lovastatin on NF-κB signaling. In particular, this is true for a participation of regulatory prenylated proteins other than Rho GTPases. Alternative possibilities, such as an interference of cytoskeleton or effects unrelated to prenylation, are unlikely, because cytochalasin D fails to block the UV-induced degradation of IκBα and the inhibitory effect of lovastatin is neutralized by supplementation with GGPP.

In summary, we have shown that the clinically very relevant group of statines, which are established to prolong life

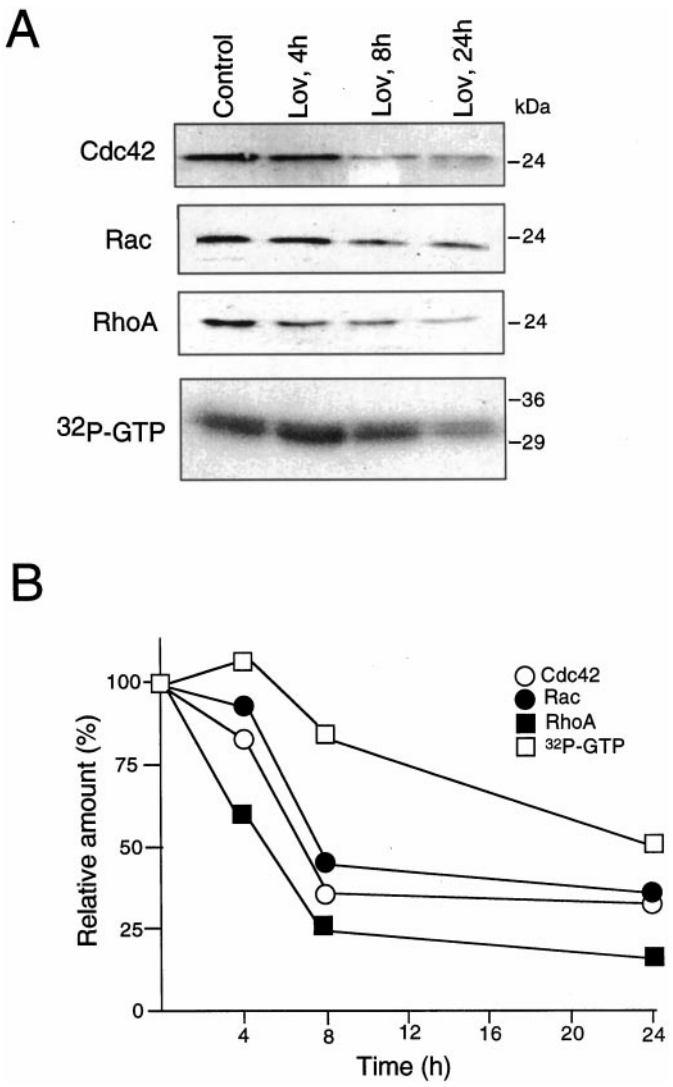

Fig. 8. Lovastatin causes a rapid decrease in the level of membrane-bound Rho GTPases. A, after exposure of CHO cells for different periods of time with lovastatin (Lov, 50 μM), membrane fractions were prepared, and the amount of Rho GTPases (Cdc42, Rac, and RhoA) was analyzed by Western blotting. Additionally, the overall level of GTP-binding proteins was determined using a [^{32}P]GTP overlay assay as described under *Experimental Procedures*. B, quantitative densitometric analysis of the data shown under A. The relative amount of a given Rho GTPase ([^{32}P]GTP binding level) in the untreated control was set to 100%.

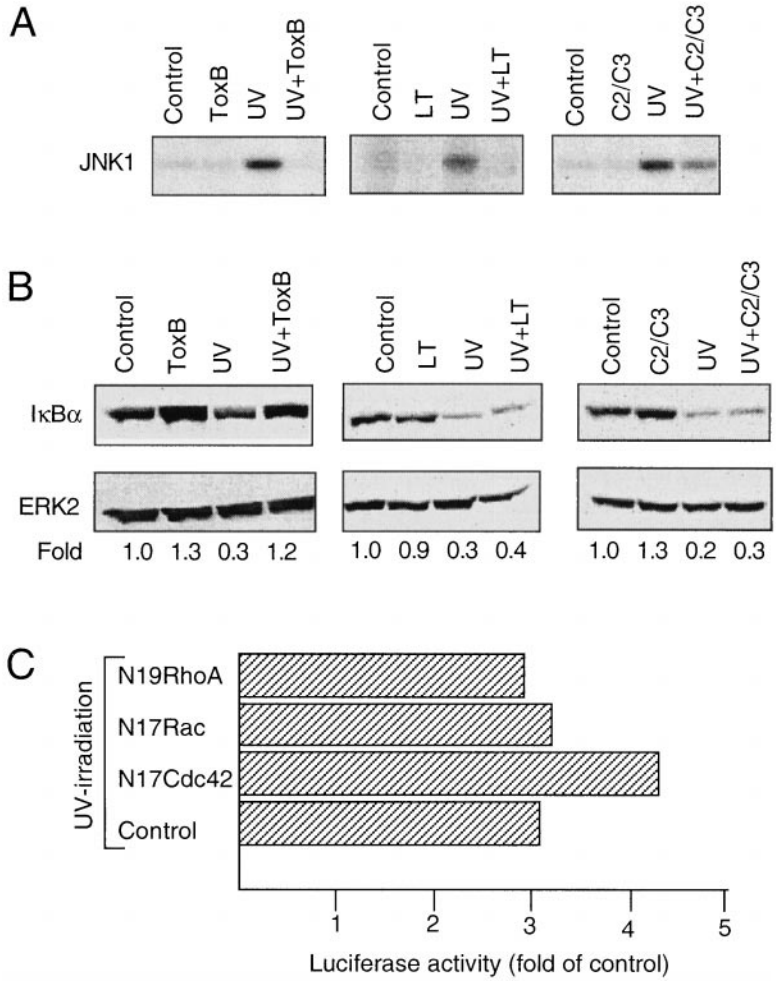

Fig. 9. Rho-inactivating clostridial toxins affect UV-induced signaling to JNK1 and NF-κB differently. A, logarithmically growing CHO cells were exposed to Rho-inactivating clostridial ToxB (50 ng/ml), LT (200 ng/ml), or C3 exoenzyme (C2/C3; 500 ng/ml) (Barth et al., 1998). ToxB causes inactivation of Rho, Rac, and Cdc42 (Just et al., 1995), LT affects Rac and Ras (Just et al., 1996), whereas C2/C3 toxin specifically acts on RhoA-like GTPases (Barth et al., 1998). After a pretreatment period (2 h with ToxB, 10 h with LT, 8 h with C2/C3), cells were UV irradiated (60 J/m^2) and analyzed for JNK1 activity 30 min later. B, Cells were exposed to clostridial toxins as described under A; 2 h after UV irradiation (80 J/m^2), cells were harvested, and the amount of IκBα was determined by Western blot analysis. The relative amount of IκBα was determined after densitometric analysis as described. C, cells were transfected with NF-κB-specific reporter gene construct without (Control) or together with expression vectors encoding dominant negative Cdc42 (N17Cdc42), Rac (N17Rac), and RhoA (N19RhoA), respectively; 24 h after transfection, cells were irradiated (80 J/m^2). After a further incubation period of 24 h, cells were harvested for determination of luciferase activity. Data shown are mean values from two independent experiments. The relative luciferase activity in the corresponding nonirradiated controls was set to 1.0.

span in cases of coronary insufficiencies, exerts dramatic inhibitory effects on stress-inducible mechanisms involving JNKs and NF-κB. Both factors are central regulators of cellular responses to genotoxic and nongenotoxic stress, thereby affecting cellular survival, apoptosis, and inflammation (Baldwin, 1996; Canman and Kastan, 1996; Wang et al., 1996). With the data available, we suggest that members of the Rho family of small GTPases, which are highly important for genotoxic stress-induced signaling to JNK1 and NF-κB, are inhibited by lovastatin. The identification of Rho-regulated stress-inducible signal pathways as cellular targets of HMG-CoA reductase inhibitors provides a novel molecular basis for understanding the mechanisms underlying the clinical effectiveness of this group of therapeutic drugs. Furthermore, it also points to putative undesirable side effects of statins, brought about by an altered cellular response to extracellular stress. This might have implications for cytostatic drug-based cancer therapy and anti-inflammatory therapies.

References

Aktories K, Rösener S, Blaschke U and Chhatwal GS (1988) Botulinum ADP-ribosyltransferase C3: Purification of the enzyme and characterization of the ADP-ribosylation reaction in platelet membranes. *Eur J Biochem* **172:**445–450.

Baldwin AS Jr (1996) The NF-kB and IkB proteins: New discoveries and insights. *Annu Rev Immunol* **14:**649–681.

Barth H, Hofmann F, Olenik C, Just I and Aktories K (1998) The N-terminal part of the enzyme component (C2I) of the binary *Clostridium botulinum* C2 toxin interacts with the binding component C2II and functions as a carrier system for a Rho ADP-ribosylating C3-like fusion toxin. *Infect Immun* **66:**1364–1369.

Baumann B, Kistler B, Kirillov A, Bergman Y and Wirth T (1998) The mutant plasmacytoma cell line S107 allows the identification of distinct pathways leading to NF-kB activation. *J Biol Chem* **273:**11448–11455.

Beg AA and Baltimore D (1996) An essential role for NF-kB in preventing TNF-a-induced cell death. *Science (Wash DC)* **274:**782–784.

Bender K, Göttlicher M, Whiteside S, Rahmsdorf HJ and Herrlich P (1998) Sequential DNA damage-independent and -dependent activation of NF-kB by UV. *EMBO J* **17:**5170–5181.

Bifulco M, Laezza C, Aloj SM and Garbi C (1993) Mevalonate controls cytoskeleton organization and cell morphology in thyroid epithelial cells. *J Cell Physiol* **155:** 340–348.

Blauw GJ, Lagaay AM, Smelt AH and Westendorp RG (1997) Stroke, statins, and cholesterol: A meta-analysis of randomized, placebo-controlled, double-blind trials with HMG-CoA reductase inhibitors. *Stroke* **28:**946–950.

Brown MS and Goldstein JL (1993) Protein prenylation: Mad bet for Rab. *Nature (Lond)* **366:**14–15.

Canman CE and Kastan MB (1996) Three paths to stress relief. *Nature (Lond)* **384:**213–214.

Chardin P, Boquet P, Madaule P, Popoff M, Rubin EJ and Gill DM (1989) The mammalian G protein RhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilament in Vero cells. *EMBO J* **8:**1087–1092.

Coso AA, Chiariello M, Yu J-C, Teramoto H, Crespo P, Xu N, Miki T and Gutkind JS (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81:**1137–1146.

Cox AD and Der CJ (1992) Protein prenylation: More than just glue? *Curr Opin Cell Biol* **4:**1008–1016.

Endres M, Laufs U, Huang Z, Nakamura T, Huang P, Moskowitz MA and Liao JK (1998) Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* **95:**8880–8885.

Fenton RG, Kung HF, Longo DL, and Smith MR (1992) Regulation of intracellular actin polymerization by prenylated cellular proteins. *J Cell Biol* **117:**347–356.

Fritsche M, Haessler C and Brandner G (1993) Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene* **8:**307–318.

Fritz G and Kaina B (1997) rhoB encoding a UV-inducible Ras-related small GTP-binding protein is regulated by GTPases of the Rho family and independent of JNK, ERK, and p38 MAP kinase. *J Biol Chem* **272:**30637–30644.

Fritz G and Kaina B (1999) Activation of c-Jun N-terminal kinase 1 by UV irradiation is inhibited by wortmannin without affecting c-jun expression. *Mol Cell Biol* **19:**1768–1774.

Glomset JA, Gelb MH and Farnsworth CC (1990) Prenyl proteins in eukaryotic cells: A new type of membrane anchor. *Trends Biochem Sci* **15:**139–142.

Goldstein JL and Brown MS (1990) Regulation of the mevalonate pathway. *Nature (Lond)* **343:**425–430.

Just I, Fritz G, Aktories K, Giry M, Popoff MR, Boquet P, Hegenbarth S and Von Eichel-Streiber C (1994) *Clostridium difficile* toxin B acts on the GTP-binding protein Rho. *J Biol Chem* **269:**10706–10712.

Just I, Selzer J, Hofman F, Green GA and Aktories K (1996) Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. *J Biol Chem* **271:** 10149–10153.

Just I, Selzer J, Wilm M, Von Eichel-Streiber C, Mann M and Aktories K (1995) Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature (Lond)* **375:**500–503.

Khosravi-Far R, Solski PA, Clark GF, Kinch MS and Der CJ (1995) Activation of Rac1, RhoA and mitogen-activated protein kinases is required for Ras transformation. *Mol Cell Biol* **15:**6443–6453.

Kikuchi A, Yamamoto K, Fujita T and Takai Y (1988) ADP-ribosylation of the bovine brain rho protein by botulinum toxin type C1. *J Biol Chem* **263:**16303–16308.

Koch G, Benz C, Schmidt G, Olenik C and Aktories K (1997) Role of Rho protein in lovastatin-induced breakdown of actin cytoskeleton. *J Pharmacol Exp Ther* **283:** 901–909.

Lerm M, Selzer J, Hoffmeyer A, Rapp UR, Aktories K and Schmidt G (1999) Deamidation of Cdc42 and Rac by *Escherichia coli* cytotoxic necrotizing factor 1: Activation of c-Jun N-terminal kinase in HeLa cells. *Infect Immun* **67:**496–503.

Li N and Karin M (1998) Ionizing radiation and short wavelength UV activate NF-kappaB through two distinct mechanisms. *Proc Natl Acad Sci USA* **95:**13012–13017.

Lipid Study Group (1998) Prevention of cardiovascular events and death with pravastatin in patients with coronary heart desease and a broad range of initial cholesterol levels. *N Engl J Med* **339:**1349–1357.

Marshall C (1984) Functions of ras oncogenes. *Nature (Lond)* **310:**448.

Minden A, Lin A, Claret F-X, Abo A and Karin M (1995) Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81:**1147–1157.

Newman CM and Magee AI (1993) Posttranslational processing of the ras superfamily of small GTP-binding proteins. *Biochim Biophys Acta* **1155:**79–96.

Perona R, Montaner S, Saniger L, Sanchez-Perez I, Bravo R and Lacal JC (1997) Activation of the nuclear factor kappaB by Rho, Cdc42 and Rac1 proteins. *Genes Dev* **11:**463–475.

Qiu R-G, Chen J, Kirn D, McCormick F and Symons M (1995) An essential role for Rac in Ras transformation. *Nature (Lond)* **374:**457–459.

Radler-Pohl A, Sachsenmaier C, Gebel S, Auer H-P, Bruder JT, Rapp U, Angel P, Rahmsdorf HJ and Herrlich P (1993) UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase. *EMBO J* **12:**1005–1012.

Schafer WR and Rine J (1992) Protein prenylation: Genes, enzymes, targets, and functions. *Annu Rev Genet* **26:**209–237.

Schmidt RA, Glomset JA, Wight TN, Habenicht AJ and Ross R (1982) A study of the influence of mevalonic acid and its metabolites on the morphology of swiss 3T3 cells. *J Cell Biol* **95:**144–153.

Shepherd J, Cobbe SM. and Ford IEA (1995) Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia: West of Scotland Coronary Prevention Study group. *N Engl J Med* **333:**1301–1307.

Van Antwerp DJ, Martin SJ, Kafri T, Green DR and Verma IM (1996) Suppression of TNF-a-induced apoptosis by NF-kB. *Science (Wash DC)* **274:**787–789.

van Dam H, Duyndam M, Rottier R, Bosch A, de Vries-Smits L, Herrlich P, Zantema A, Angel P and van-der-Eb AJ (1993) Heterodimer formation of c-Jun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. *EMBO J* **12:**479–483.

Wang C-Y, Cusack JCJ, Liu R and Baldwin ASJ (1999) Control of inducible chemoresistance: Enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kB. *Nat Med* **5:**412–417.

Wang CY, Mayo MW and Baldwin AS Jr (1996) TNF and cancer therapy-induced apoptosis: Potentiation by inhibition of NF-kB. *Science (Wash DC)* **274:**784–786.

Send reprint requests to: Gerhard Fritz, Division of Applied Toxicology, Institute of Toxicology, Obere Zahlbacher Strasse 67, D-55131 Mainz, Germany. E-mail: fritz@mail.uni-mainz.de