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Simvastatin Attenuates Expression of Cytokine-inducible Nitric-oxide Synthase in Embryonic Cardiac Myoblasts*

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Cardiac stem cells or myoblasts are vulnerable to inflammatory stimulation in hearts with infarction or ischemic injury. Widely used for the prevention and treatment of atherosclerotic heart disease, the cholesterol-lowering drugs statins may exert anti-inflammatory effects. In this study, we examined the impact of inhibition of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase with simvastatin on the expression of inducible nitric-oxide synthase (iNOS) in embryonic cardiac myoblasts stimulated with the proinflammatory cytokines, interleukin-1 or tumor necrosis factor. Treatment with simvastatin significantly reduced the levels of iNOS mRNA and protein in cytokine-treated rat H9c2 cardiac embryonic myoblasts. Addition of the HMG-CoA reductase product, L-mevalonate, and the by-product of cholesterol synthesis, geranylgeranyl pyrophosphate, could reverse the statin inhibitory effect on iNOS expression. Simvastatin treatment lowered the Rho GTPase activities, whereas the Rho-associated kinase inhibitor Y27632 partially blocked the statin inhibitory effect on nitrite production in the cytokine-treated H9c2 cells. Treatment with simvastatin led to inactivation of NF-κB by elevation of the NF-κB inhibitor IκB and reduction of the NF-κB nuclear contents in the cytokine-stimulated H9c2 cells. Hence, treatment with simvastatin can attenuate iNOS expression and NO synthesis in cytokine-stimulated embryonic cardiac myoblasts. The statin inhibitory effect may occur through isoprenoid-mediated intracellular signal transduction, which involves several key signal proteins, such as Rho kinase and IκB/NF-κB. These data suggest that statin therapy may protect the cardiac myocyte progenitors against the cytotoxicity of cytokine-induced high output of NO production in infarcted or ischemic hearts with inflammation.

Transplantation or mobilization of stem cells with growth factors has been emerging as a potentially novel therapy for regenerative medicine. However, one of the major challenges to successful stem cell therapy is the difficulty of stem cell survival and differentiation in the harsh microenvironment of diseased tissues or organs, such as infarcted or ischemic hearts with inflammation (1). Nitric oxide (NO),[1] a gaseous signaling molecule, plays an important role in the regulation of myocardial metabolism and function (2). Through NO synthases (NOS), cardiovascular cells can transiently synthesize increased levels of NO which, in turn, regulate the heart contractility (3). Exposure to NO at high levels may cause dysfunction of mitochondria (4, 5) and trigger apoptosis (6). Proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), can induce expression of inducible NOS (iNOS) that generates large quantities of NO, resulting in cardiac cell degeneration and apoptosis (7, 8). The cytokine-induced expression of iNOS can occur in both ischemic and nonischemic heart failure, septic cardiomyopathy, cardiac allograft rejection, and myocarditis. Suppression of the cytokine-inducible, NO-synthesizing enzyme may reduce generation of cytotoxic reactive nitrogen radicals, leading to increased survival of grafted or preexisting stem cells in the diseased hearts with inflammation.

3-Hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase acts as the rate-limiting enzyme for endogenous cholesterol synthesis. The HMG-CoA reductase inhibitors, statins or vastatins, are widely used cholesterol-lowering medicines for both primary and secondary prevention of coronary heart disease. Statins can effectively diminish endogenous cholesterol synthesis and reduce the plasma low density lipoprotein cholesterol levels in patients with hypercholesterolemia (9, 10). Recent studies suggest that by blocking the mevalonate pathway, statins exert pleiotropic effects on vascular cell function which may not be related directly to cholesterol synthesis (11, 12). Accumulating evidence indicates the involvement of the intermediates or by-products, *e.g.* isoprenoids, from the mevalonate-cholesterol synthesis pathway in the regulation of intracellular signal transduction and activation of transcriptional factors critical for inflammatory proteins and iNOS gene expression in heart failure (13). However, there are controversial reports on the statin regulation of iNOS expression in different cell types. For instance, Pahan *et al.* (14, 15) showed that lovastatin

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[1] The abbreviations used are: NO, nitric oxide; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, hydroxymethylglutaryl coenzyme A; IκB, inhibitor κB; IL-1, interleukin-1; iNOS, inducible NOS; NOS, nitric-oxide synthase; eNOS, endothelial NOS; TNF, tumor necrosis factor.

inhibits iNOS and cytokine expression in rat macrophages, astrocytes, and microglia by reducing Ras farnesylation and inactivating NF-κB. By contrast, Hattori *et al.* (16) reported that statins augment cytokine-mediated induction of NO synthesis in vascular smooth muscle cells through altering the synthesis of tetrahydrobiopterin, a key cofactor for iNOS, with little influence on NF-κB activities. Recent work has shown that statin treatment may confer cardioprotection in the isolated perfused hearts during ischemia-reperfusion, which is in part the result of a reduction in myocyte apoptosis (17).

However, the molecular mechanism underlying the statin protective effect is unknown. Because stem cells participate in postischemic heart repair, it is of great interest to determine whether statin treatment inhibits cytokine-induced expression of iNOS in premature, undifferentiated cardiac myoblasts. In this study, we employed an *in vitro* system to determine whether inhibition of HMG-CoA reductase by simvastatin alters expression of iNOS in embryonic, undifferentiated cardiac myoblasts exposed to proinflammatory cytokines. Our data showed that simvastatin treatment significantly reduced expression of iNOS in cardiac myoblasts stimulated with cytokines. The statin inhibitory effect might occur through a mechanism in which isoprenoids, an intermediate or similar byproducts from cholesterol synthesis, regulate activation of several key intracellular signaling proteins, such as Rho A kinase and NF-κB. Taken together, our data strongly support the notion that statins have regulatory effects on iNOS expression by undifferentiated myoblasts during the development of cardiac failure and inflammation.

EXPERIMENTAL PROCEDURES

Materials—All reagents were from Sigma unless otherwise specified. Human recombinant IL-1α and TNF-α were from R&D Systems, Inc. (Minneapolis). L-Mevalonate was obtained from Sigma. Geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were from Biomol Research Laboratories, Inc. (Plymouth, PA). Rho inhibitor Y27632 was from Calbiochem. Simvastatin obtained from Merck Sharp and Dohme was activated to its active form by alkaline hydrolysis before use. Briefly, 4 mg of simvastatin prodrug was dissolved in an 8-ml solution of 0.1 N NaOH and 0.154 mol/liter NaCl and then incubated at 50 °C for 2 h. The pH was brought to 7.0 by HCl. The final concentration of the stock solution was adjusted to 4 mg/ml and stored at −20 °C.

Cell Cultures—H9c2 cells purchased from American Type Culture Collection (ATCC, Rockville, MD) are spontaneously immortalized ventricular myoblasts from the rat embryo, with preservation of several electrical and biochemical characteristics found in adult cardiomyocytes. They were cultured in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum under 95% air and 5% CO_2 at 37 °C. At subconfluence (70–80%), H9c2 myoblasts cultured in Petri dishes or 24-well plates were preexposed to simvastatin and then stimulated with the proinflammatory cytokines, IL-1 and TNF-α, in the presence or absence of isoprenoids, the intermediates or by-product from cholesterol synthesis. After certain time intervals, 100 μl/well culture medium was collected for determination of nitrite production.

Assay for NO Production—The activity of iNOS was determined in the culture medium by assaying nitrites, taken as an index of NO production, using Griess reagent (1% sulfanilic acid and 0.1% *N*-(1-naphthyl)ethylenediamine HCl in 5% phosphoric acid) as reported previously (4, 18, 19). Equal volumes of medium and Griess reagent were mixed, and the purple products were quantified spectrophotometrically at 550 nm. Nitrite concentrations were determined from a linear standard curve constructed with known concentrations of sodium nitrite (0–40 μmol/liter nitrite).

Immunoblotting—Total proteins were isolated from H9c2 cells in an ice-cold lysis buffer containing 10 mmol/liter Tris, pH 7.4, 1% SDS, and 1× protease inhibitor (1 mmol/liter sodium orthovanadate). Proteins (15 μg/lane) were separated under reducing conditions (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol blue, 2% β-mercaptoethanol) by electrophoresis onto 5–10% SDS-polyacrylamide gel and electroblotted to nitrocellulose membranes (Osmonics, Westborough, MA). The membranes were reversibly stained with Ponceau red (Sigma) to verify equal protein loading and/or transfer. After blocking in Tris-buffered saline (0.2 M Tris and 8% NaCl) containing 5% nonfat powdered milk and 0.1% Tween 20 for 1 h at room temperature, the membranes were incubated overnight at 4 °C with the following primary antibodies to 1) iNOS (Transduction Laboratories, Lexington, KY); 2) NF-κB p65/Rel (Santa Cruz Biotechnology, Santa Cruz, CA); 3) Ser^{32}-phosphorylated inhibitor IκBα (Santa Cruz Biotechnology). The blots were incubated with horseradish peroxidase-coupled secondary antibodies, washed, and developed using a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce). The intensity of each immunoreactive protein band was quantified by densitometric analysis. Cytokine-stimulated RAW 264.7 cells (Transduction Laboratories) were used as positive controls.

RNA Isolation and Reverse transcription-PCR—Total cellular RNA was isolated by a single extraction using an acid guanidinium thiocyanate-phenol-chloroform method with modification as reported elsewhere (17). Semiquantitative multiplex reverse transcription-PCR was performed with a set of specific primers for iNOS. As the housekeeping controls, the 18 S rRNA was also analyzed by reverse transcription-PCR. The PCR products were visualized and quantified using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy).

Electrophoretic Mobility Gel Shift Assay—Nuclear proteins were extracted from stimulated or unstimulated H9c2 cells. Cells were harvested and homogenized with an Ultra-Turrax T25-tissue homogenizer (Janke and Kunkel) in a low salt solution (0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged for 30 s at 2,000 rpm. The supernatant was incubated for 5 min on ice and then centrifuged for 5 min at 5,000 rpm. The nuclei were resuspended in a high salt solution (25% glycerol; 20 mM HEPES, pH 7.9; 420 mM NaCl; 1.2 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride; 2 mM benzamidine; 5 g/ml each aprotinin, leupeptin, and pepstatin). Protein concentrations were determined by Bio-Rad Laboratories Protein Assay. Double-stranded synthetic oligonucleotide NF-κB motif (5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-CCT GGG AAA GTC CCC TCA ACT-3′) was labeled with [γ-^{32}P]ATP. Binding reactions containing 10 μg of crude nuclear extract were performed using the electrophoretic mobility gel shift assay core system (Promega, Madison WI) according to the manufacturer's protocol. For supershift experiments, 1–2 μg of mouse monoclonal anti-NF-κB antibody was added into the reaction.

Rho Kinase Assay—Cell pellets were homogenized in the lysis buffer (50 mM NaCl, 50 mmol/liter Tris-HCl, pH 8.0, 0.1% Triton X-100, 0.5 mM EDTA, 1 mM EGTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 2 mM NaF, 2 mM sodium orthovanadate, 5 mM β-mercaptoethanol). After the cell lysates were centrifuged at 30,000 × *g* for 30 min, supernatants were collected for assessing Rho kinase activities using the Rho kinase assay kit (Cyclex MBL International Corporation, Woburn, MA). In brief, the supernatants of lysed cells were measured for protein concentrations and justified to the same protein concentration and then added (10 μl/well) into 96-well plates precoated with a substrate corresponding to the C terminus of the recombinant myosin-binding subunit of myosin phosphate (MSB), which contains a threonine residue that may be phosphorylated by Rho kinase. Subsequently, 90 μl of kinase reaction buffer (containing 0.1 mM ATP diluted in kinase buffer)/well was added to the kinase reaction, incubated for 60 min at room temperature, washed five times in washing buffer provided in the kit, and incubated with 100 μl of horseradish peroxidase-conjugated monoclonal anti-phospho-specific MSB antibody, which specifically detects only the phosphorylated form of threonine 697 on MSB (provided in the kit). The colored products were developed by incubating with 100 μl of the horseradish peroxidase substrate tetramethylbenzidine at room temperature for 10 min. The reaction was stopped by adding 100 μl of stop solution containing 0.5 M H_2SO_4, The colored products were quantified by spectrophotometry at 450 nm. All samples were assayed in duplicate, and each experiment was repeated three times. Purified Rho kinase was used as a positive control, and cell lysates from the cultures treated with the Y27632 were the negative controls.

Statistical Analysis—Two-group comparisons were performed by Student's *t* test for unpaired values. Comparisons of means of multiple groups were performed by analysis of variance, and the existence of individual differences, in case of significant *F* values at analysis of variance, were tested by Scheffé's multiple contrasts.

RESULTS

Simvastatin Inhibits iNOS Expression and Nitrite Production in Cardiac Myoblasts Induced by Proinflammatory Cytokines—Exposure to 20 ng/ml IL-1α or 20 ng/ml TNF-α signifi-

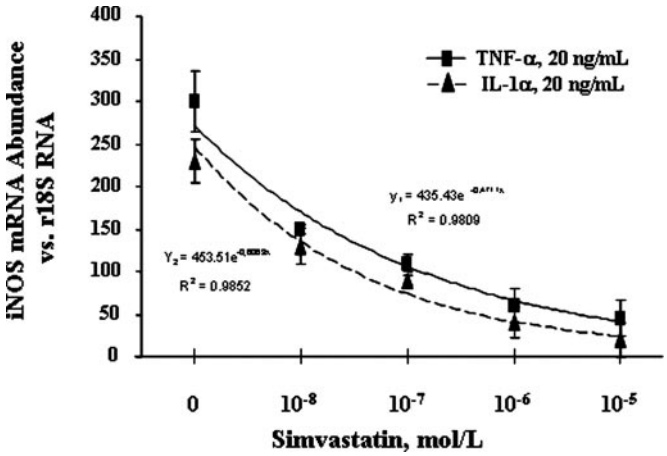

FIG. 1. **Simvastatin treatment reduces iNOS mRNA levels in TNF-α- and IL-1α-stimulated cardiac myoblasts.** Reverse transcription-PCR for iNOS mRNA and 18 S rRNA was performed using total RNA isolated from H9c2 cardiac myoblasts exposed to 10^{-8} to 10^{-5} mol/liter simvastatin in the presence of 20 ng/ml TNF-α (*closed squares*) or 20 ng/ml IL-1α (*closed triangles*) for 24 h. PCR products were semi-quantified by densitometry after electrophoresis on agarose gels stained with ethidium bromide. The steady-state level of iNOS mRNA was normalized to the level of the 18 S rRNA against unstimulated controls. Data show the mean ± S.D. from triplicate experiments.

cantly increased the levels of iNOS mRNA in H9c2 cardiac myoblasts (Fig. 1) compared with those in normal, untreated H9c2 cells. To determine whether statins affect iNOS expression in embryonic cardiac cells, we added simvastatin into the cell cultures simultaneously with the cytokines. We observed that in a concentration-dependent fashion, simvastatin markedly diminished expression of iNOS mRNA in H9c2 cells stimulated with the proinflammatory cytokines (Fig. 1). Under the same concentrations, both IL-1α- and TNF-α-stimulated cells showed a similar response to simvastatin in terms of iNOS mRNA expression. In the presence of simvastatin, IL-1α-treated cells showed a dose-dependent decline in iNOS mRNA to the same or similar degrees as that in TNF-α-treated cells (Fig. 1). Thus, simvastatin reduced steady-state levels of iNOS mRNA in H9c2 cardiac myoblasts stimulated with the proinflammatory cytokines.

Further analysis of iNOS protein expression by immunoblotting with antibodies against iNOS confirmed the presence of high levels of iNOS expression in H9c2 cardiac myoblasts stimulated with IL-1 α and TNF-α. Intense protein bands immunoreactive to anti-iNOS antibody corresponding to a molecular mass of 130 kDa were detected in the cytokine-stimulated H9c2 cells (Fig. 2, *A* and *B*). At the same concentrations, both of the proinflammatory cytokines exerted stimulatory effects on iNOS protein expression in the cells. Interestingly, consistent with its effect on iNOS mRNA expression, simvastatin (10^{-8} to 10^{-5} mol/liter) reduced expression of iNOS protein in H9c2 cells stimulated with IL-1α (Fig. 2, *A* and *C*) as well as TNF-α (Fig. 2, *B* and *D*). Thus, treatment with simvastatin inhibited expression of both iNOS mRNA and protein in cardiac myoblasts induced by proinflammatory cytokines. To verify the statin inhibitory effect on iNOS gene expression, we examined the accumulation of nitrite, a stable NO end product reflecting the NOS activities, in the cultures treated with or without IL-1α and simvastatin. Under the base-line condition without cytokine stimulation, H9c2 cells generated nitrite at the rates of ~0.8 μmol/10^5 cells/24 h (Fig. 3). Stimulation with 20 ng/ml IL-1α increased nitrite production markedly. The rates of nitrite production were more than 3.5 μmol/10^5 cells/24 h in the cytokine-stimulated cells for the period of incubation from 24 to 48 h ($p < 0.05$). Simvastatin (up to 10^{-6} mol/liter) significantly diminished cytokine-induced nitrite production in H9c2 cells (Fig. 3). The range of simvastatin concentrations leading to inhibition of nitrite production in cytokine-stimulated cells is comparable with the plasma levels of the drug in patients treated (20). Thus, at the pharmacological doses, simvastatin markedly reduced production of nitrite by H9c2 cells stimulated with the proinflammatory cytokines.

L-Mevalonate Mediates the Simvastatin Inhibitory Effect on iNOS Expression in Cardiac Myoblasts—Generated by HMG-CoA reductase, L-mevalonate serves as a key intermediate of cholesterol synthesis from acetyl-CoA. To determine whether the statin inhibition of iNOS gene expression occurred through blocking the HMG-CoA reductase activity or reduction in L-mevalonate synthesis, we added exogenous L-mevalonate into the cultures of H9c2 cells with 20 ng/ml IL-1α in the presence or absence of 10^{-6} mol/liter simvastatin. We observed that the addition of L-mevalonate in excess amounts significantly increased the nitrite concentrations in the cultures of H9c2 cells exposed to a combination of IL-1α and simvastatin (Fig. 3). Examination of iNOS protein by immunoblotting revealed that L-mevalonate diminished the inhibitory effect of simvastatin by increasing iNOS protein expression in H9c2 cells treated with simvastatin plus IL-1α (Fig. 4, *A* and *C*) or TNF-α (Fig. 4, *B* and *D*). In the absence of simvastatin, L-mevalonate had no or little effect on cytokine-induced iNOS expression in H9c2 cells (Fig. 4), suggesting that L-mevalonate selectively blocked the simvastatin inhibitory effect on cytokine induction of iNOS in cardiac myoblasts. Because there was no major difference in induction of iNOS expression between IL-1α- and TNF-α-treated cells, the following experiments with simvastatin were performed mainly on the cells stimulated with IL-1α.

The Isoprenoid Intermediate GGPP, but Not FPP, Blocks the Simvastatin Inhibitory Effect on iNOS Expression—In addition to L-mevalonate, several downstream intermediates or by-products from the cholesterol biosynthetic pathway (21) may contribute to the inhibitory effect of simvastatin on expression of iNOS protein and activities. To exhaust the endogenous intermediates and by-products from cholesterol synthesis, we pretreated H9c2 cells with 10^{-8} to 10^{-7} mol/liter simvastatin up to 24 h and then stimulated the cells with 20 ng/ml IL-1α in the presence or absence of the isoprenoid intermediates, such as GGPP and FPP. Immunoblotting showed that the addition of 10^{-7} to 10^{-5} mol/liter GGPP reversed the simvastatin-mediated suppression of the cytokine-induced expression of iNOS protein (Fig. 5, *A* and *B*) and nitrite production (Fig. 5*C*) at almost the same levels as those in the L-mevalonate-treated cells. In contrast, however, treatment with FPP at the same concentrations as GGPP did not alter nitrite accumulation in the cells pretreated with simvastatin and then IL-1α (Fig. 5*C*). These results suggested that GGPP but not FPP can reverse the statin-induced inhibition of iNOS expression in the cardiac myoblasts stimulated with IL-1α.

Rho Kinase Contributes to the Statin Inhibitory Effect on iNOS Expression in Cardiac Myoblasts Treated with IL-1α—The Rho family of small GTP-binding proteins consists of three subfamilies, Rho, Rac, and Cdc42, which play important roles in signal transduction and cell cycle regulation (22, 23). The intermediate derivative of L-mevalonate, GGPP, acts as a lipid attachment to the Rho proteins, whereas FPP mainly targets Ras. Therefore, we investigated whether Rho kinase mediates the simvastatin effect on iNOS expression. The Rho kinase inhibitor Y27632 (24) specifically inactivates p160ROCK, a key subunit of this kinase, known to regulate NO synthesis (11). We tested whether Y27632 could mimic the regulatory effect of simvastatin on iNOS expression in IL-1-treated H9c2 cells. We observed that inhibition of Rho kinase with Y27632 (10^{-6} to

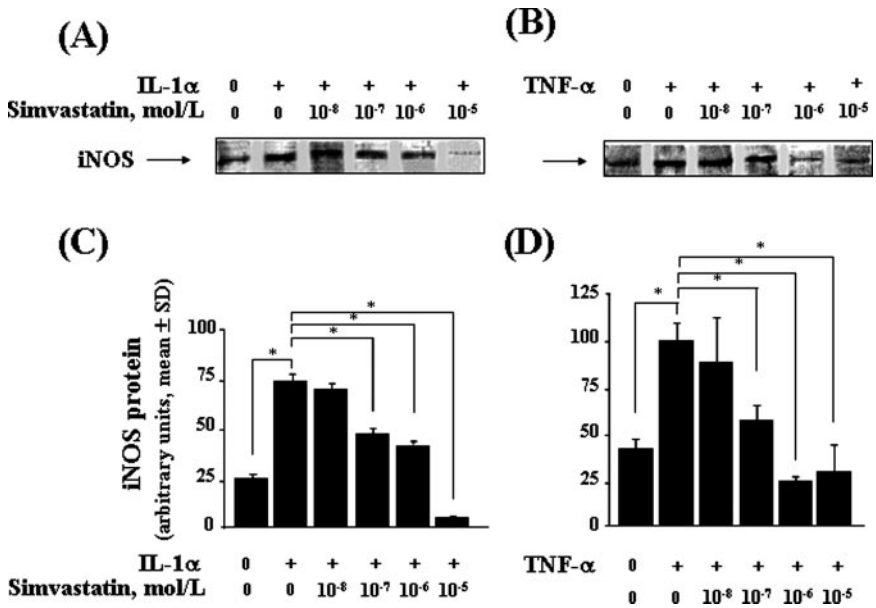

FIG. 2. **Simvastatin reduces iNOS protein expression in TNF-α- or IL-1α-stimulated cardiac myoblasts.** Immunoblotting with anti-iNOS antibody was conducted with total proteins extracted from H9c2 cells exposed to 10^{-8} to 10^{-5} mol/liter simvastatin in the presence of 20 ng/ml IL-1α (*A* and *C*) or 20 ng/ml TNF-α (*B* and *D*) for 24 h. Protein bands probed by antibody are quantified by densitometry (*C* and *D*). Data represent the means ± S.D. from three separate experiments. * $p < 0.05$.

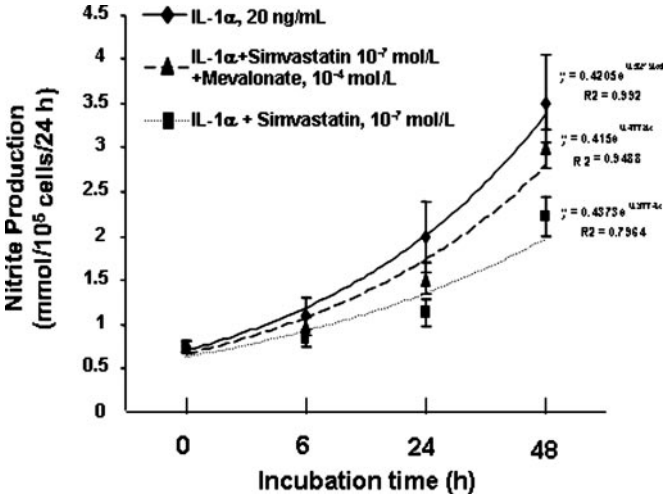

FIG. 3. **Time course of nitrite production in cardiac myoblasts treated with IL-1α alone or in combination with simvastatin or L-mevalonate.** The logarithmic regression curves show the time-dependent nitrite production in H9c2 cardiac myoblasts exposed to 20 ng/ml IL-1α in the presence (*closed squares*) and absence (*closed diamonds*) of 10^{-6} mol/liter simvastatin, or pretreated with simvastatin in combination with 10^{-4} mol/liter L-mevalonate (*closed triangles*) for 30 min prior to the addition of IL-1α. Data represent the means ± S.D. from three separate experiments. * $p < 0.05$.

10^{-5} mol/liter) did not alter cytokine-induced iNOS protein expression in H9c2 cells (Fig. 6*A*). However, addition of the Rho kinase inhibitor into the cell cultures almost abolished the inhibitory effect of simvastatin on iNOS protein expression (Fig. 6*A*) as well as nitrite production (Fig. 6*B*). The reversal of the statin-induced reduction in iNOS expression by Y27632 occurred to the same extent as seen in the cells treated with L-mevalonate (Fig. 6*B*). To elucidate further that Rho kinase A participates in the statin suppression of iNOS expression, we examined the Rho kinase A bioactivities in H9c2 cells treated with both IL-1α and simvastatin with purified Rho kinase as the positive controls and the Y27632-treated cell lysates as the negative controls. We found that simvastatin dose-dependently reduced the Rho kinase activities (Fig. 6*C*). Thus, the statin-mediated Rho kinase inactivation was concurrent with down-regulation of iNOS expression, albeit in a fashion that was opposite that in Y27632-treated cells.

Simvastatin Inactivates NF-κB through Elevation of Intracellular IκB in Cardiac Myoblasts Stimulated with Cytokines—The nuclear transcription factor NF-κB binds to the iNOS gene promoter critical for iNOS gene transcription (25). We examined the effect of simvastatin on IL-1α-induced activation of NF-κB in H9c2 cells by using the gel shift assay with a ^{32}P end-labeled NF-κB oligonucleotide. The nuclear proteins extracted from serum-starved H9c2 cells showed a stronger NF-κB activity after stimulation by IL-1α for 15 min (Fig. 7*A*). The specificity of the NF-κB·DNA protein complex formation was verified by competition with unlabeled, cold oligonucleotides and by the addition of anti-NF-κB antibody which supershifted the bands of NF-κB·DNA complexes (Fig. 7*A*). In addition, there was no significant binding with the oligonucleotide probe alone or by omitting protein substrate or using the nuclear protein extract from the unstimulated H9c2 cells. In the subsequent experiments, we treated the cells with 1 μM simvastatin before IL-1α stimulation. We found a time-dependent decline in NF-κB·DNA binding (Fig. 7*A*, *lanes 7–10*). Simvastatin treatment for 24 h markedly inhibited IL-1α-induced activation of NF-κB (Fig. 7*A*, *lane 11*) ($p < 0.001$ *versus* IL-1α-stimulated cells). Inclusion of antibody directed against NF-κB in the binding reactions resulted in a further reduction in the IL-1α-induced mobility of the complex (Fig. 7*A*, *lane 12*). The reduction in the nuclear NF-κB activity did not appear to be caused by overall inhibition of expression of this transcription factor by the statin because immunoblotting analysis of total cellular NF-κB levels showed no significant difference between simvastatin-treated or untreated cells with cytokine stimulation (Fig. 7, *B* and *C*). We further delineated the potential mechanism underlying the simvastatin inhibitory effect on NF-κB activation by performing immunoblotting analysis on the same nuclear protein extracts from IL-1α-stimulated H9c2 cells with an antibody specific for the p65 subunit of NF-κB. We observed a time-dependent decrease in the p65 NF-κB protein nuclear translocation with pretreatment with simvastatin (Fig. 7, *D* and *F*). The statin-suppressed NF-κB nuclear translocation was clearly related to the HMG-CoA reductase activity because addition of L-mevalonate could elevate the nuclear p65/Rel protein levels in the statin-treated, cytokine-stimulated cells (Fig. 7, *E* and *G*). In addition, the Rho kinase inhibitor Y27632 showed no major effect on nuclear NF-κB translocation. Although 10^{-4} mol/liter Y27632 reversed the statin inhibitory effect on iNOS activities, this Rho kinase inhibitor

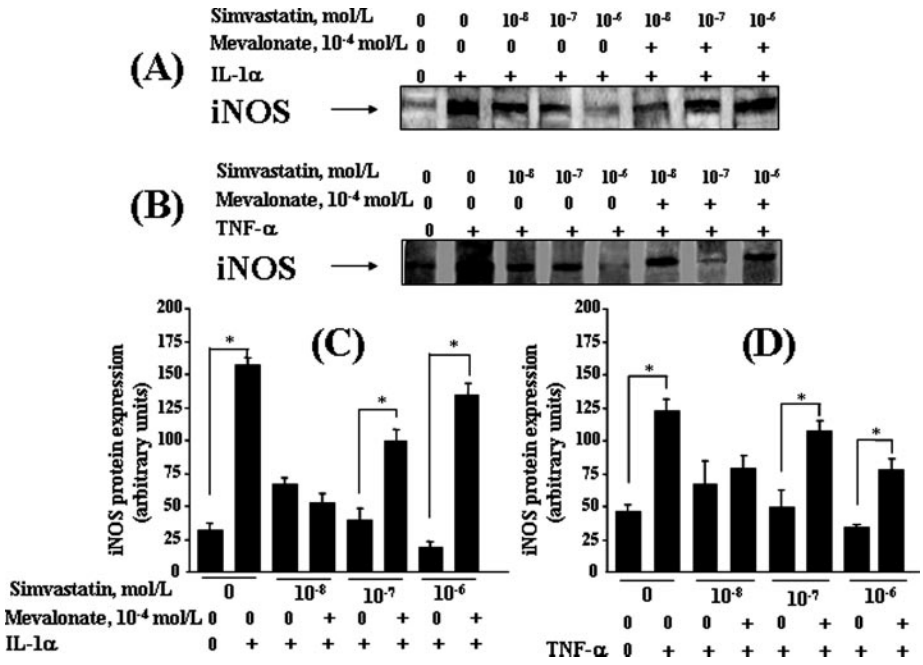

FIG. 4. **L-Mevalonate blocks the simvastatin inhibitory effect on TNF-α- and IL-1α-induced iNOS protein expression in cardiac myoblasts.** Immunoblotting with anti-iNOS antibody was performed using total proteins extracted from H9c2 cardiac myoblasts treated with 10^{-8} to 10^{-5} mol/liter simvastatin alone or in combination with 10^{-4} mol/liter L-mevalonate for 30 min prior to the addition of 20 ng/ml IL-1α (*A* and *C*) or 20 ng/ml TNF-α (*B* and *D*). Immunoreactive iNOS bands were quantified by densitometry. Data represent the mean ± S.D. from three separate experiments. * $p < 0.05$.

did not influence the nuclear p65 translocation because there was no difference in the nuclear p65 concentration between the cells treated with and without Y27632 in the presence of IL-1α (Fig. 7, *E* and *G*). These observations suggest a discrepancy between L-mevalonate and the Rho inhibitor in regulating the statin modulation of iNOS expression and activation in the cytokine-stimulated cells.

Simvastatin Increases the Phosphorylated IκBα in the Cytosol without Subsequent Degradation—Under physiological conditions, NF-κB is sequestered as an inactive form in the cytosol through noncovalent interactions with inhibitory proteins, such as IκBα, β, and ϵ. Each IκB isoform contains, in its N-terminal region, a pair of serine residues. In the case of IκBα, these serine residues (amino acids 32 and 36) become phosphorylated by a serine-specific kinase after stimulation. Phosphorylation does not disassociate IκBα from NF-κB but renders IκBα a substrate for ubiquitination and then degradation by the 26 S proteasome. To provide further insight into the regulatory role of simvastatin, by immunoblotting with a specific antibody to IκBα, we examined the IL-1α-induced degradation of IκBα phosphorylated at Ser^{32} in the presence or absence of simvastatin. After a 15-min treatment with 20 ng/ml IL-1α, we detected a decrease in the cellular content of Ser^{32}-phosphorylated IκBα in H9c2 cells. However, in the cytokine-stimulated cells, pretreatment with 10^{-8} to 10^{-6} mol/liter simvastatin increased the levels of Ser^{32}-phosphorylated IκBα in a dose-dependent manner (Fig. 8). The highest levels of the phosphorylated IκBα were detected in H9c2 cells exposed to the statin at 10^{-7} mol/liter for 24 h (Fig. 8). The increased accumulation of phosphorylated IκBα in the simvastatin-treated cells implicates a prolonged life span or a lower rate of degradation of phosphorylated IκB protein that inactivates NF-κB.

DISCUSSION

The embryonic H9c2 cardiac myoblast model has been used widely to study cardiac stem cell development and differentiation. Highly resembling premature cardiac myogenic cells, H9c2 cells show well characterized cardiac properties in electrophysiology as well as in cellular receptor and signal transduction (26, 27). It is notable that the embryonic H9c2 cardiac myoblasts can constitutively express iNOS as well as eNOS at low levels under the normal cell culture. The constitutively expressed, moderate NOS activities may reflect the role of NO in intracellular signaling of undifferentiated, premature myoblasts. Bloch *et al.* (28, 29) have reported that both iNOS and eNOS exist in E9.5 rat and murine embryos, correlated with high expression of soluble guanylyl cyclase as well as a high cyclic GMP content. The NO production mediated by the NOS isoforms present constitutively in the cardiac myogenic cells contributes to cardiomyogenesis because continuous incubation of embryoid bodies with the NOS inhibitors results in a pronounced differentiation arrest in the premature cardiomyocytes, and coapplication of NO-donors reverses the inhibitory effect.

However, the high output, persistent NO production by overexpression of iNOS induced by proinflammatory cytokines may have deteriorating effects on cardiac cells (8, 13). In this study, we showed that the H9c2 embryonic cardiac myoblasts are sensitive to stimulation of proinflammatory cytokines, such as IL-1α and TNFα. The high levels of iNOS expression and NO production in this premature, delicate myocyte progenitor cell line suggest a vulnerability of cardiac stem cells to the proinflammatory environment of a heart with acute infarction or ischemic injury. Because the high output of NO production causes cardiac cell dysfunction and even apoptotic cell death, inhibition of iNOS expression may have beneficial impacts on stem cell survival and differentiation. Increased resistance to proinflammatory or proapoptotic insults may represent a key factor that leads to successful cardiac stem cell therapy. Our current data from the *in vitro* studies provide evidence that the cholesterol-lowering drugs, statins, can regulate iNOS expression.

The statin regulatory effect on iNOS expression appears through specific inhibition of HMG-CoA reductase, reversibly blocked by excess amounts of L-mevalonate. The statin effect seems, however, independent of the statin-mediated reduction in cholesterol synthesis because addition of exogenous cholesterol does not prevent the statin effect. The concentrations for simvastatin achieving its inhibitory effect are as low as 10^{-8} mol/liter. The dose range of simvastatin used in this study is about 10^{-8} to 10^{-6} mol/liter, very close to the expected plasma levels of simvastatin in patients (20). This suggests that statins, at therapeutic doses, may block iNOS expression during inflammation.

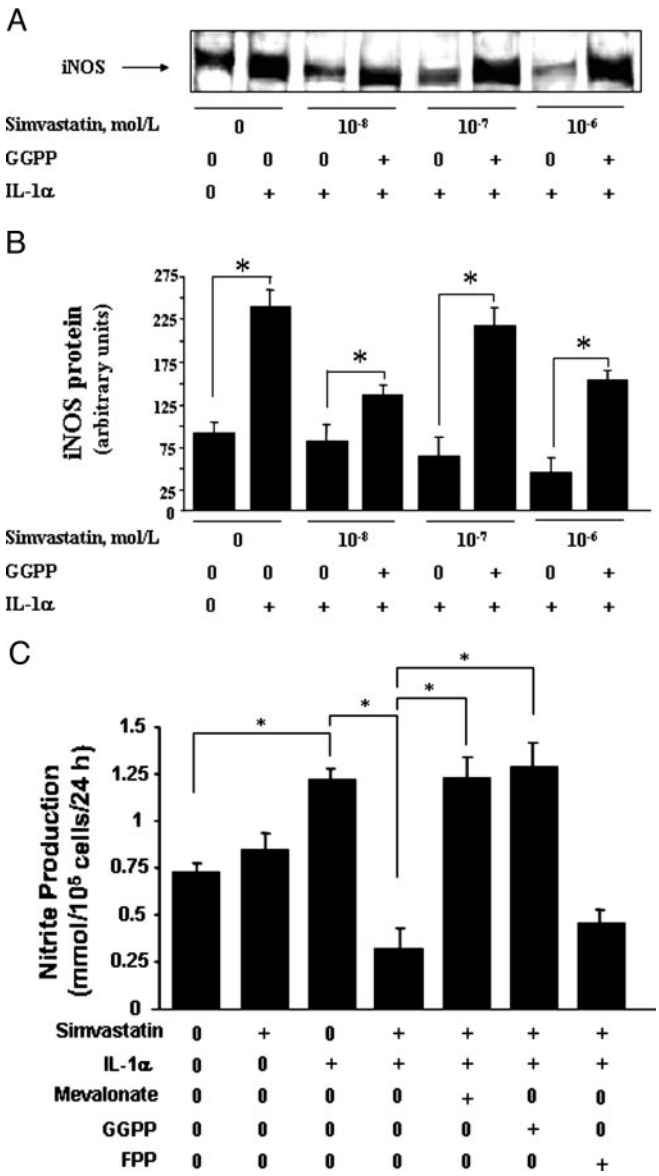

FIG. 5. **GGPP attenuates the simvastatin inhibitory effect on IL-1α-induced iNOS expression and nitrite production in cardiac myoblasts.** *A* and *B*, immunoblotting with anti-iNOS antibody was conducted with total proteins extracted from H9c2 cells treated with increasing concentrations of simvastatin (10^{-8} to 10^{-6} mol/liter) alone or in combination with 10^{-6} mol/liter GGPP for 30 min, followed by stimulation with 20 ng/ml IL-1α for 24 h. Immunoreactive iNOS bands were quantified by densitometry. Data represent the means ± S.D. from three separate experiments. *C*, nitrite concentrations were determined by the Griess reaction in the media of H9c2 cells under the same treatment shown in *A* and *B*. Data show the means ± S.D. from three experiments. * $p < 0.05$.

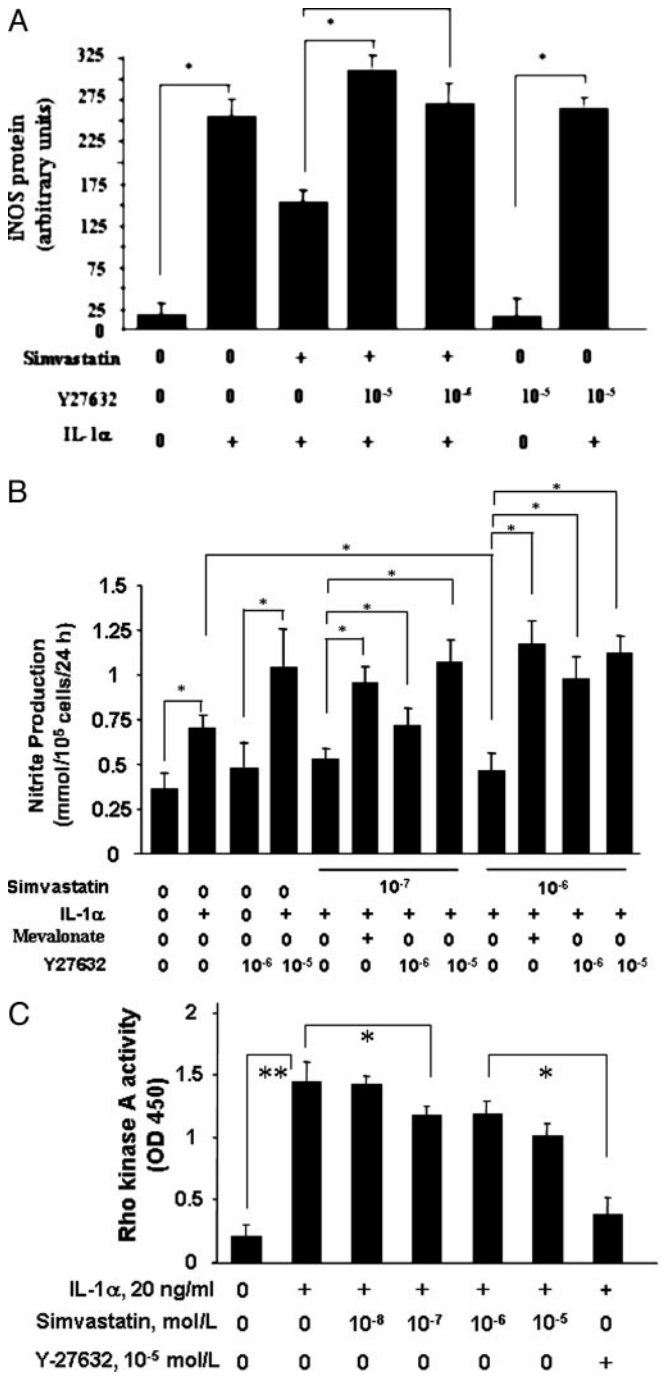

FIG. 6. **Rho-associated kinase mediates simvastatin suppressive effect on iNOS expression in IL-1α-stimulated cardiac myoblasts.** *A*, immunoblotting with anti-iNOS antibody in H9c2 cardiac myoblasts pretreated with 10^{-6} to 10^{-5} mol/liter Y27632 alone or in combination with 10^{-6} mol/liter simvastatin for 30 min followed by the addition of 20 ng/ml IL-1α for 24 h. Immunoreactive iNOS bands were quantified by densitometry. Each *bar* represents the means ± S.D. from three separate experiments. * $p < 0.05$. *B*, nitrite production determined by the Griess reaction with the media of H9c2 cell cultures treated under the same conditions shown in *A*. Each *bar* represents the means ± S.D. from five separate experiments. * $p < 0.05$. *C*, Rho kinase assays. After treatment with cytokine and simvastatin or Y27632, cells were lysed, centrifuged, and resulting supernatants collected for enzymatic assays. Rho kinase-mediated protein phosphorylation was quantified by spectrophotometry at 450 nm. Data are presented as the means ± S.D. ($n = 5$). * $p < 0.05$; ** $p < 0.01$.

In this study, we exploited the mechanism by which statins regulate iNOS expression in embryonic cardiac myoblasts. We observed that simvastatin acts multilaterally at different phases of iNOS expression and on the levels of mRNA, protein, and enzymatic activities. Recently, the isoprenoid intermediates, including GGPP and FPP, have been implicated in contributing to the statin regulatory effects on expression of NOS expression. This is largely because the addition of GGPP can significantly diminish the statin inhibitory effect. It has been demonstrated previously that in addition to reducing hepatic cholesterol biosynthesis by inhibiting HMG-CoA reductase, statins may display biological activities associated with depletion of L-mevalonate as well as biologically active isoprenoid intermediates. Some of the isoprenoids (*e.g.* GGPP) play an important role in the covalent attachment to cell membranes, subcellular localization, and intracellular trafficking of membrane-associated proteins, including regulating endothelial cell

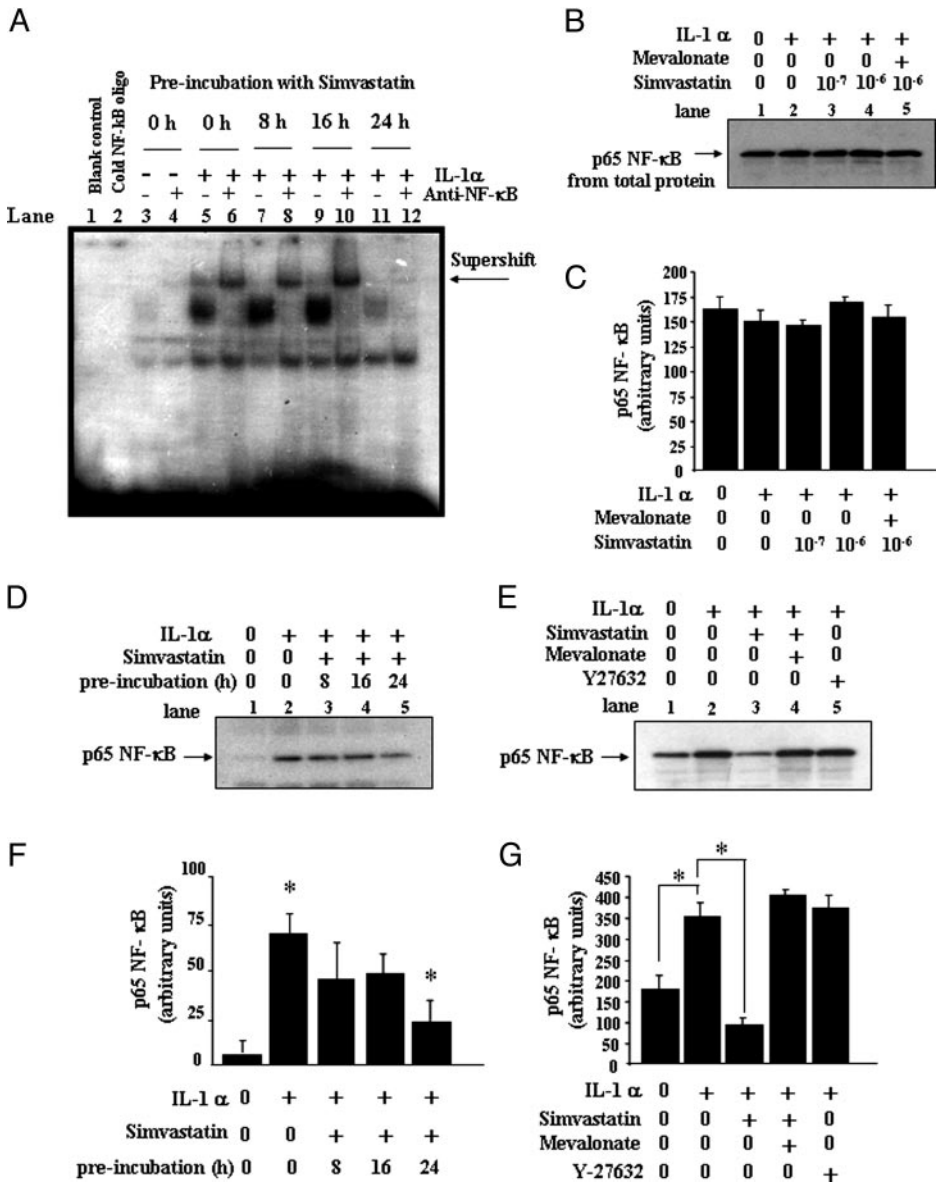

FIG. 7. **Simvastatin inactivates NF-κB and prevents its p65/RelA subunit from nuclear translocation in cytokine-stimulated cardiac myoblasts.** *A*, an electrophoretic mobility gel shift assay was performed by mixing a ^{32}P-labeled oligonucleotide coding for the consensus sequence of NF-κB-binding promoter with the nuclear proteins from H9c2 cells preincubated with 10^{-6} mol/liter simvastatin for 8, 18, or 24 h followed by incubation with 20 ng/ml IL-1α for 15 min. Specificity of the NF-κB·DNA complex formation was determined by competition with unlabeled, cold oligonucleotide and by supershift with anti-NF-κB antibody. The *autoradiogram* is a representative of three separate gel shift experiments for NF-κB. *B–G*, immunoblotting with antibody against the p65/RelA subunit of NF-κB in the total cellular (*B* and C) or nuclear proteins (*D* and G) extracted from H9c2 cells stimulated with IL-1α after a 24-h preincubation with 10^{-6} mol/liter simvastatin in the presence or absence of L-mevalonate and Y27632 (*C*). Immunoreactive p65 bands were quantified by densitometry (*E*, *F*, and *G*). Data represent the means ± S.D. from three separate experiments. * $p < 0.05$.

function (30). In this study we observed that the addition of GGPP, but not FPP, prevented the inhibitory effect of simvastatin on cytokine-induced iNOS expression and activity, indicating that the inhibition of post-translational geranylgeranylation, but not of farnesylation, can be related to the inhibitory effect of simvastatin on cytokine-induced iNOS expression in premature myoblasts.

The Rho proteins (23) are a group of small GTP-binding molecules involved in the control of NOS protein expression and turnover in cardiovascular cells (11, 31, 32). The members of the Rho family, including RhoA, RhoB, Rac, and Cdc42 proteins, are normally geranylgeranylated, whereas Ras proteins are predominantly farnesylated. Our current data from the studies with the Rho kinase inhibitor point to the involvement of Rho proteins in simvastatin-associated iNOS inhibition in premature cardiac myoblasts. Rho kinase activation has been implicated in reducing iNOS activities. In this study, we found that inhibition of Rho kinase with Y27632 enhances NO production in cytokine-stimulated H9c2 cells. Furthermore, with enzymatic assays, we demonstrated that statin treatment can reduce the Rho kinase activity, suggesting that the Rho signaling pathway may participate in statin regulation of iNOS expression. Our observation confirms previous work by other laboratories showing that lipid-soluble statins (*e.g.* lovastatin, simvastatin, and atorvastatin) inhibit Rho kinase activity (33). Although Rho kinase inactivation may contribute to regulation of iNOS expression by statins, the impact of the Rho kinase inhibition on iNOS expression remains uncertain because treatment of cytokine-stimulated cells with the Rho kinase inhibitor Y27632 could not mimic the statin effect. In addition, from the data generated in this study, we found that simvastatin treatment did not appear to cause superinduction but suppression of iNOS expression by IL-1 in H9c2 cells, in opposition to a recent report showing the statin potentialization of iNOS expression in cytokine-treated vascular smooth muscle cells (33). The different statin effects between the embryonic cardiac myoblasts and mature smooth muscle cells may reflect the fact that different signal transduction pathways may operate between those cells.

Pretreatment with lovastatin, an inhibitor of protein prenylation, resulted in superinduction of iNOS. This superinduction can be reversed by geranylgeraniol, but not by farnesol, suggesting that inhibition of geranylgeranylation, not farnesylation, is responsible for enhanced iNOS expression. The results demonstrate that a farnesylated protein(s) mediates IL-1β induction of iNOS, whereas a geranylgeranylated protein(s) re-

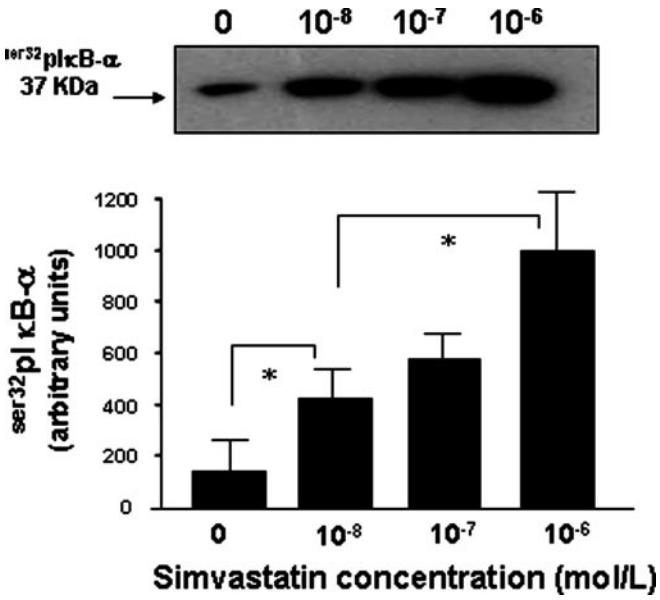

FIG. 8. **Simvastatin elevates the phosphorylated IκBα intracellular pool without subsequent degradation in IL-1α-stimulated cardiac myoblasts.** Immunoblotting with antibody against Ser^{32}-phosphorylated IκBα in total proteins extracted from IL-1α-induced H9c2 cells after 24 h preincubation with 10^{-8} to 10^{-6} mol/liter simvastatin (*A*). Immunoreactive bands were quantified by densitometry (*B*). Each *bar* represents the means ± S.D. from three separate experiments. * $p < 0.05$.

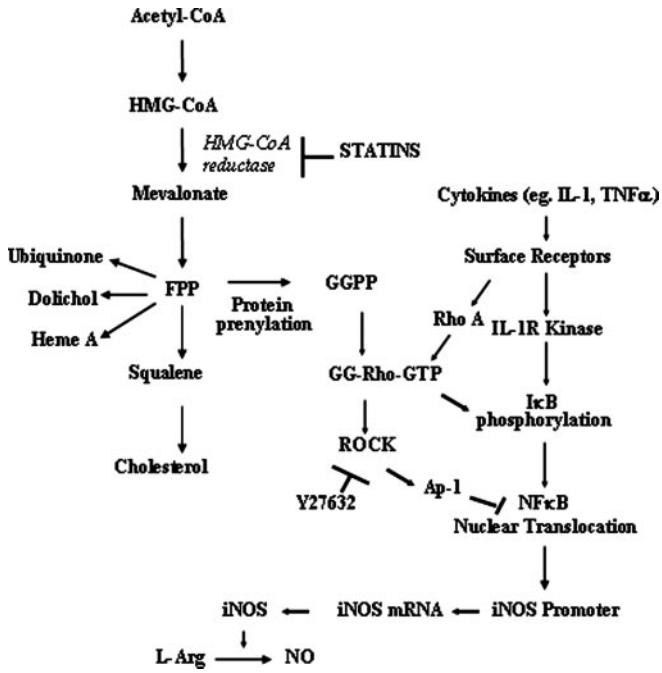

FIG. 9. **Schematic presentation of the potential signal pathways underlying statin inhibition of iNOS expression in embryonic myoblasts induced by cytokines.** *GG-Rho-GTP*, geranylgeranyl-Rho-guanosine triphosphate; *IL-1R*, IL-1 receptor; *L-Arg*, L-arginine; *ROCK*, Rho-associated protein kinase.

presses this induction. Also, by modulating Ras farnesyl protein transferase, lovastatin blocks lipopolysaccharide-induced iNOS expression in rat primary astrocytes in a manner reversible by L-mevalonate and FPP (14). However, on the contrary, treatment with cerivastatin and fluvastatin was recently reported to enhance cytokine induction of NO synthesis in rat vascular smooth muscle cells (16). An increase in IL-1β-induced nitrite production and apoptosis in adult cardiac myocytes may occur after treatment with very high concentrations (10^{-5} and 10^{-4} mol/liter) of fluvastatin through inhibition of Rho-associated kinase (34, 35). In addition, there have been reports showing that statin treatment may induce apoptosis of vascular cells (35–37). However, in our system, we did not observe any appreciable cell death via apoptosis in the statin-treated cardiac myoblasts, regardless of the presence or absence of the proinflammatory cytokines. Recent studies (38, 39) have also demonstrated that statin treatment can increase the numbers of circulating endothelial cell progenitors, suggesting that statins are cytoprotective rather than cytotoxic to undifferentiated stem cells.

The cytokine induction of iNOS expression involves multiple transcription factors, in particular the nuclear factor NF-κB. Upon activation by cytokines (*e.g.* IL-1 and TNF-α), NF-κB translocates from the cytosolic compartment to the nucleus, where it binds to the iNOS gene promotor and ultimately triggers iNOS gene transcription. This process is mediated by kinase-mediated protein phosphorylation and requires disassociation between NF-κB and its native inhibitor IκB. One of the statins, mevastatin, has been reported to inactivate NF-κB and reduce NF-κB-dependent expression of the endothelial adhesion proteins, vascular cellular adhesion molecules (VCAM), and E-selectin (40). Consistently, the data from our current study clearly show that simvastatin treatment can reduce NF-κB nuclear translocation and elevate the cytosolic content of phosphorylated IκBα. Rho-associated kinases have been reported to mediate the regulatory effect of statins on iNOS expression in airway epithelial cells (41). Rho-associated kinases may control the iNOS gene promoter activities via NF-κB; however, the Rho-associated kinase inhibitor Y27632 shows effects that are different from those of statins in terms of iNOS gene activation. Thus, it is likely that statins may regulate cytokine-induced iNOS expression through different signal pathways that involve both Y27632-sensitive and -insensitive molecular signaling, in particular the NF-κB/IκBα pathway (Fig. 9).

There is still a dispute as to whether NO protects or damages the myocardium during inflammation or whether statin suppression of iNOS expression is beneficial or harmful to the heart (2). We have recently established that simvastatin reduces reperfusion injury in the isolated perfused working rat heart by preventing eNOS from inactivation and by suppressing ischemia-related iNOS induction, which correlates with a reduction in cardiomyocyte apoptosis (17). Our current finding that simvastatin decreases cytokine-induced iNOS expression in cultured premature cardiac myoblasts illustrates a causal relationship between increased NO and depressed cardiac contractility in heart failure. Further clarification of the mechanism underlying the statin effect on NO production and survival of cardiac stem cells may provide valuable information that helps design therapies for patients with myocardial infarction or ischemic heart failure.

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