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The Role of Rho-Associated Kinase in Differential Regulation by Statins of Interleukin-1β- and Lipopolysaccharide-Mediated NF-κB Activation and Inducible Nitric Oxide Synthase Gene Expression in Vascular Smooth Muscle Cells

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Running title: Negative regulator of Rho-associated kinase for nuclear factor κB activation in vascular smooth muscle cells

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ABBREVIATIONS:

EMSA, electrophoretic mobility shift assay; eNOS, endothelial nitric oxide synthase; α-HFPA, α-hydroxyfarnesylphosphonic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IFN-γ, interferon-γ; IκB, inhibitor of NF-κB; IKK, IκB kinase; IL-1β,

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interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MYPT, myosin phosphatase target subunit; NF-κB, nuclear factor κB; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; ROCK, Rho-associated kinase; RT-PCR, reverse-transcription polymerase chain reaction; VSMCs, vascular smooth muscle cells.

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ABSTRACT

An optimal level of NO has protective effects in atherosclerosis, while large amounts contribute to septic shock. To study how statins, the potent inhibitors of cholesterol synthesis, regulate NO in the vascular wall, we determined their effects on interleukin-1β (IL-1β)- and lipopolysaccharide (LPS)-induced NO production in aortic vascular smooth muscle cells (VSMCs). Compared to the large amounts of NO and iNOS protein expression induced by LPS, the responses of IL-1β were modest. Various statins were found to inhibit LPS-induced iNOS expression and NO production, while they potentiated IL-1β responses. In addition, fluvastatin increased IL-1β-induced p65 nuclear translocation and NF-κB activity, while it inhibited those induced by LPS. To address the role of small G proteins in statin's actions, farnesyl transferase inhibitors (α-hydroxyfarnesylphosphonic acid and L-744382), Rac inhibitor (NSC23766), and Rho-associated kinase (ROCK) inhibitor (Y-27632) were utilized. We found that Y-27632 potentiated IL-1β-induced iNOS expression, p65 nuclear translocation, IKK and NF-κB activation, while it had minimal effects on LPS-induced responses. In contrast, farnesyl transferase inhibitors blocked iNOS protein expression induced by LPS and IL-1β, while NSC23766 had no effect. Further studies showed that LPS downregulated Rho and ROCK activity, while IL-1β increased them, suggesting a negative role of Rho and ROCK signaling, which is regulated in contrary manners by IL-1β and LPS, in IKK/NF-κB activation. Through abrogating this negative signaling, statins

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differentially regulate iNOS expression induced by LPS and IL-1β in VSMCs. These differential actions of statins on iNOS gene regulation might provide an additional explanation for the pleiotropic beneficial effects of statins.

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Introduction

The prominent role which inflammation plays in atherosclerosis has begun to be understood, and thus therapeutic benefits of anti-inflammatory drugs for atherogenesis have garnered increased interest (Libby, 2002). Statins are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This action attributes to the cardiovascular benefits of statins in reduction of LDL-cholesterol. However, recent studies have indicated statins exert therapeutic effects beyond that of simply lowering plasma cholesterol (McFarlane et al., 2002; Werner et al., 2002). Statins have pleiotropic anti-inflammatory actions, such as stabilization of eNOS mRNA in vascular endothelial cells, and inhibition of vascular smooth muscle cells (VSMCs) proliferation (Laufs et al., 1998; Laufs and Liao, 2000). All these effects are related to statins' ability to interrupt the important signaling pathways mediated by small G proteins, whose biological activities rely on the posttranslational modification by isoprenylation (Goldstein and Brown, 1990).

Nitric oxide (NO) is formed by constitutive endothelial NO synthase (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). It has been demonstrated that iNOS is present in atherosclerotic lesions (Joly et al., 1992; Behr et al., 1999; Luoma and Yla-Herttuala, 1999). Although the significance of iNOS expression in the atherosclerotic vasculature is still a matter of debate, NO synthesized by iNOS in VSMCs may be a supportive substitute for the lost eNOS function in the endothelium with disease

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progression. It thus leads to prevention of the progression of atherosclerosis via its vasodilation, inhibition of VSMC proliferation, platelet adhesion, and LDL oxidation (Maxwell and Cooke, 1999). Supporting this concept is the protection by iNOS in the development of transplant arteriosclerosis (Fukumoto et al., 1997; Qian et al., 2001). Conversely, high amounts of iNOS-derived NO in the infection state induced by bacterial endotoxin, lipopolysaccharide (LPS), leads to tissue self-destruction and septic shock (Szabo and Billiar, 1999). Therefore, the optimal and timely regulation of iNOS expression in VSMCs is an important issue in pathophysiological conditions.

For iNOS gene expression, NF-κB is an essential transcription factor (Xie et al., 1994). In its inactive state, NF-κB is located in the cytoplasm where it is retained by the inhibitory protein, IκBs. IκB kinase (IKK) has been identified as a converged mediator essential for IκB phosphorylation and proteolytic degradation. This event allows NF-κB translocation to the nucleus where it binds DNA. Small GTPases, such as Rho (Perona et al., 1997; Montaner et al., 1998, 1999; Benitah et al., 2003; Anwer et al., 2004), Rac (Perona et al., 1997; Montaner et al., 1998; Kim et al., 1999), and Ras (Montaner et al., 1998; Norris and Baldwin, 1999) are critical elements involved in the regulation of NF-κB.

Until now, although more attention has been focused on the upregulation of eNOS function by statins (Laufs et al., 1998), their effects on iNOS still remain controversial and seem to be dependent on the cell type and the individual stimulus. In astrocytes,

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microglia, and macrophages, lovastatin was shown to block iNOS induction by LPS through an inhibitory step at the farnesylation of Ras and Rac (Pahan et al., 1997, 2000; Huang et al., 2003). In contrast, statins upregulate IL-1β-induced iNOS promoter activity in VSMCs (Chen et al., 2000; Muniyappa et al., 2000), airway epithelial cells (Kraynack et al., 2002), fibroblasts (Hausding et al., 2000), and cardiac myocytes (Ikeda et al., 2001). This stimulatory action might occur through inhibition of the geranylgeranylation of Rho family proteins and downstream Rho-associated kinase (ROCK) signaling (Chen et al., 2000; Muniyappa et al., 2000; Yamamoto et al., 2003).

Based on the distinct effects of statins on IL-1β- and LPS-mediated iNOS induction in various cell types, we clarified this event in VSMCs, and determined the underlying mechanism played by ROCK and NF-κB.

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Materials and Methods

Reagents. Antibodies for iNOS, ROCK, IKKα/β, p65, p50, RhoA and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Murine IFN-γ and IL-1β were purchased from R&D (Minneapolis, MN). The peptide of myosin phosphatase target subunit (714-1004), MYPT-1, was purchased from Upstate (Charlottesville, VA). An NF-κB Trans AM kit was purchased from Active Motif (Carlsbad, CA). Lovastatin and phenol-extracted LPS (L8274) from *Escherichia coli* were purchased from Sigma Aldrich (St. Louis, MO). Atorvastatin, fluvastatin, and pravastatin were respectively provided by Pfizer (New York, NY), Novartis (Basel, Switzerland), and Sankyo (Tokyo, Japan). Y-27632 was purchased from Tocris (Ellisville, MO). The farnesyl transferase inhibitors, α-hydroxyfarnesylphosphonic acid (α-HFPA) and L-744832, and the Rac inhibitor, NSC23766, were purchased from Calbiochem (La Jolla, CA). The other materials were as we described before (Huang et al., 2003; Ho et al., 2004).

Cell Culture. Rat aortic VSMCs were prepared from thoracic aortas of male Sprague-Dawley rats using the collagenase digestion method and cultured in Dulbecco's modified Eagle's medium (DMEM). For all experiments, rat aortic VSMCs from passages three to eight were used.

Nitrite measurement. Nitrite production was measured in the culture medium of rat VSMCs. Briefly cells were cultured in 12-well plates in 500 μl of culture medium

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until confluence. Cells were treated with vehicle (control group), LPS, IL-1β, and/or IFN-γ for the times indicated, and then the culture media were collected. Nitrite was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of culture medium. The optical density at 550 nm (OD550) was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the OD550 produced using standard solutions of sodium nitrite in the culture medium.

Immunoblotting. After stimulation, cells were rinsed twice with ice-cold PBS, and 100 μl of cell lysis buffer (20 mM Tris-HCl (pH 7.5), 125 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was then added to each plate. Protein was denatured in SDS, electrophoresed on a 10% SDS/polyacrylamide gel, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature. After incubation with the appropriate first antibodies, membranes were washed three times with TBST. The secondary antibody was incubated for 1 h. Following three washes with TBST, the protein bands were detected using the ECL reagent.

Reverse-transcription polymerase chain reaction (RT-PCR). VSMCs were homogenized in 1 ml of RNAzol B reagent (Gibco), and total RNA was extracted by an

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acid guanidinium thiocyanate-phenol-chloroform extraction. RT was performed using a StrataScript RT-PCR kit, and 10 μg of total RNA was reverse-transcribed to cDNA following the manufacturer's recommended procedures. RT-generated cDNA encoding iNOS and β-actin genes were amplified using PCR. The oligonucleotide primers used corresponded to iNOS (5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3' and 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3') and β-actin (5'-GAC TAC CTC ATG AAG ATC CT-3' and 5'-CCA CAT CTG CTG GAA GGT GG-3'). The PCR was performed in a final volume of 50 μl containing *Taq* DNA polymerase buffer, all four dNTPs, oligonucleotide primers, *Taq* DNA polymerase, and the RT products. After initial denaturing for 2 min at 94 °C, 35 cycles of amplification (94 °C for 45 s, 65 °C for 45 s, and 72 °C for 2 min) were performed, followed by a 10-min extension at 72 °C. PCR products were analyzed on 2% agarose gels. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

Transfection and reporter gene assay. Using Lipofectamine 2000 reagent, VSMCs were cotransfected with 1 μg pGL2-ELAM-Luc (κB-Luc) or iNOS promoter luciferase reporter plasmid, together with 1 μg of the β-galactosidase expression vector. The iNOS reporter containing binding sites for AP-1 and NF-κB was provided by Dr. D. K. Glass (UCSD, CA). After 24 h, cells were incubated with the indicated concentrations of agents for 6 h. Cell lysates containing equal amounts of protein (10~20 μg) were used for the luminescence measurement. Luciferase activity values

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were normalized to the transfection efficiency monitored by β-galactosidase expression, and presented as multiples of the control response, in the absence of stimulation.

Assay of NF-κB binding ability. After extracting the nuclear protein, the NF-κB binding ability was assayed by electrophoretic mobility shift assays (EMSAs) as we previously described (Huang et al., 2003), or using a TransAM NF-κB p65/NF-κB p50 Transcription factor assay kit under the manufacturer's recommended procedures.

Immunoprecipitation for *in vitro* IKK and ROCK kinase assay, and protein association. To determine activities and protein interaction of IKK and ROCK, anti-IKKα/β or anti-ROCK and protein A/G-agarose beads were added to the prepared cell extracts. Immunoprecipitation proceeded at 4 °C overnight. For kinase assay, the precipitated beads were added to the kinase reaction buffer containing 1 μg of GST-IκBα (for the IKK assay) or MYPT-1 (for the ROCK assay), 25 μM ATP, and 3 μCi of [γ-^{32}P]ATP. The phosphorylated IκB and MYPT-1 were visualized by autoradiography.

RhoA activation assay. After treatment, cell lysates were incubated with GST-RBD (20-30 μg) beads on ice for 60-90 min, followed by washing beads four times with 50 mM Tris buffer (pH 7.2) containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 ul/ml each of leupeptin and aprotinin, and 0.1 mM PMSF. Affinity precipitated active RhoA protein was determined by immunobloting. The amount of RBD-bound RhoA was normalized to the total amount of RhoA in cell lysates for the comparison of activity.

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Statistical evaluation. Values are expressed as the mean ± S.E.M. of at least three experiments. Analysis of variance was used to assess the statistical significance of the differences, and a *p* value of less than 0.05 was considered significant.

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Results

Statins Enhance IL-1β- But Reduce LPS-Induced iNOS Expression and NO Production. Before examining the effects of statins, we investigated the effects of LPS (10 μg/ml), IL-1β (10 ng/ml), and IFN-γ (10 ng/ml) on NO production in VSMCs. IFN-γ is a cytokine released from Th1 lymphocytes, which has been established to enhance LPS-induced iNOS gene expression in macrophages through STAT activation and binding of the iNOS gene promoter (Lowenstein et al., 1993). The role of IFN-γ in the chronic inflammation of atherosclerosis has been elucidated (Mallat and Tedgui, 2004). Figure 1A clearly shows that both LPS and IL-1β activated VSMCs to release nitrite, with a greater extent of nitrite production induced by LPS than by IL-1β. Although IFN-γ alone did not produce a detectable increase in nitrite, it markedly potentiated LPS's action on nitrite production, while it did not affect IL-1β's action.

Next we investigated the effects of four different statins on nitrite accumulation in LPS-stimulated VSMCs. We observed that these statins reduced the LPS-induced NO response to different extents. Examining the inhibitory efficacies achieved at the highest concentration tested and without cell toxicity (100 μM for pravastatin, and 30 μM for the other statins), the potency among the four statins was in the order of fluvastatin > lovastatin > atorvastatin > pravastatin (Fig. 1B). Similar inhibition was also observed in cells that were co-stimulated with IFN-γ and LPS (Fig. 1C). In

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agreement with the extent of NO reduction, iNOS protein induced by LPS ± IFN-γ was accordingly inhibited by lovastatin and fluvastatin (Fig. 1D). Unlike the inhibitory results under LPS treatment, nitrite production by IL-1β was significantly enhanced by statins, and this effect displayed concentration-dependency. The potency among the four statins was in the order of fluvastatin > lovastatin > atorvastatin >> pravastatin (Fig. 1B). Similar findings were observed when cells were co-treated with IL-1β and IFN-γ (Fig. 1C). Likewise, NO enhancement was reflected by the increased expression of iNOS protein (Fig. 1D). Since treatment of VSMCs with 30 μM fluvastatin showed the most-prominent effect on the regulation of LPS- and IL-1β-stimulated NO production, we thus chose fluvastatin as an example in the following experiments for further study.

Fluvastatin Differentially Regulates LPS- and IL-1β-Stimulated iNOS Gene Transcription and NF-κB Activation. We performed RT-PCR analysis to examine whether fluvastatin increases stable mRNA levels of iNOS in IL-1β-stimulated VSMCs, while reducing that in LPS-stimulated VSMCs. Results revealed that treatment for 6 h with LPS (10 μg/ml) and IL-1β (10 ng/ml) alone elevated iNOS mRNA levels, and their responses were respectively reduced and increased by the presence of fluvastatin (Fig. 2A). The iNOS mRNA signal was not detectable in non-stimulated VSMCs or in VSMCs treated only with fluvastatin. Supporting these findings, iNOS-promoter activity as assessed by the iNOS luciferase assay was correlated to the results of RT-

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PCR (Fig. 2B). These data prompted us to suggest that this differential regulation toward LPS- and IL-1β-stimulated iNOS induction was associated with iNOS gene transcription.

To further dissect the differential effects on iNOS gene regulation, analysis of NF-κB activity that has been reported to be a prerequisite of the induction of iNOS gene expression was carried out. An NF-κB reporter assay indicated that this differential regulatory effect of fluvastatin also occurred in this transcription factor (Fig. 3A). Extensive examination of the NF-κB binding ability by TransAM kits confirmed the ability of fluvastatin to reduce the LPS-stimulated NF-κB binding ability, while increasing the IL-1β-stimulated NF-κB binding ability. Moreover, distinct effects of fluvastatin toward both LPS and IL-1β action markedly occurred at 1 h of stimulation, and were sustained for at least 6 h (Fig. 3B). Results from EMSA supported these observations of NF-κB activation. In the presence of fluvastatin, the translocation and DNA binding activity of NF-κB were reduced in LPS-treated cells, while they were increased in IL-1β-treated cells (Fig. 3C).

The ROCK Inhibitor, Y-27632, Enhances IL-1β-Induced But Has No Effect on LPS-Induced iNOS Gene Expression. ROCK has been suggested to be a tonic inhibitor for IL-1β-stimulated iNOS expression. To assess whether ROCK indeed is the key step as a switching molecule to determine the distinct outcome regulated by fluvastatin in LPS and IL-1β signaling, we used Y-27632, a ROCK inhibitor. As shown

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in Figure 4A, Y-27632 (10 μg/ml) had no significant effect on NO production in LPS-stimulated VSMCs, whereas like fluvastatin, it enhanced the IL-1β-induced NO response. The latter effect of Y-27632 was comparable and non-additive to the effect of fluvastatin. These changes in NO production were accompanied by altered iNOS protein expression (Fig. 4B). Y-27632 itself did not induce iNOS expression, and had no effect on the expression of iNOS protein induced by LPS. In contrast, Y-27632 treatment with IL-1β increased iNOS expression. These results suggest that a ROCK-dependent signal pathway plays an opposing role in the signaling transduction mediated by LPS and IL-1β. Furthermore, this event controlled by ROCK might explain the differential outcome of statins on the responses to both stimuli. This is because statins are small G protein inhibitors, which can inhibit Rho-dependent ROCK.

In addition to ROCK, we further clarified the roles of other GTP-binding signaling molecules, for example Ras and Rac, in statin's actions. Figure 4C shows that farnesyl transferase inhibitors (α-HFPA and L-744382), which have been reported to disrupt membrane localization and activation of Ras, were able to inhibit IL-1β- and LPS-induced iNOS protein expressions. In contrast, the Rac inhibitor, NSC23766, had no significant effect. These results suggest the involvement of Ras rather than Rac in LPS- and IL-1β-elicited signaling cascades.

ROCK is a Negative Regulator of NF-κB Activation. To elucidate whether ROCK can regulate the NF-κB signaling pathway, we measured NF-κB activity by a

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reporter assay. As shown in Figure 5A, Y-27632 did not alter the κB-luciferase activity induced by LPS, while it increased κB-luciferase activity induced by IL-1β. Since the nuclear translocation of NF-κB subunit p65 is an essential step in NF-κB binding to cognate DNA elements and thus drives gene promoter activity, we analyzed the effects of Y-27632 and fluvastatin on this translocation event. Results indicated that similar to the effect on NF-κB activation, fluvastatin inhibited LPS-induced p65 translocation, while accelerating the response caused by IL-1β. Y-27632 treatment also led to an increased response of IL-1β, while it failed to change the LPS response (Fig. 5B).

Observing these changes in p65 translocation, we wondered how ROCK acts to induce this event. To address the signaling pathway upstream of p65 activation, we assayed IKK activity. Accumulating evidence has pointed to IKK possibly coordinating the complicated upstream signaling pathways triggered by LPS and IL-1β, which in turn causes NF-κB to be freely translocated to nuclei. We performed an *in vitro* kinase assay and found that Y-27632 enhanced IKK activation induced by IL-1β, but did not change LPS response (Fig. 5C).

Next we conducted immunoprecipitation to determine the existence or not of protein interaction between IKK and ROCK. Figure 5D reveals that in basal condition, ROCK can be associated with IKK, and this interaction is strikingly enhanced by the presence of Y-27632. IL-1β treatment did not have significant change in this event.

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ROCK Is Differentially Regulated by IL-1β and LPS. Based on the above results it is suggest that ROCK might be differentially regulated by LPS and IL-1β in VSMCs. To clarify this suggestion, ROCK activities in LPS- and IL-1β-stimulated VSMCs were determined. Using ROCK specific target MYPT-1 as an assay substrate, figure 6A shows that ROCK was constitutively activated in the basal state of VSMCs, and this activity was sensitive to inhibition by fluvastatin and Y-27632. Interestingly, we found that LPS caused inhibition while IL-1β caused stimulation of ROCK. In the presence of fluvastatin or Y-27632, the stimulation effect of IL-1β was markedly diminished, but LPS-mediated ROCK inhibition still appeared. Moreover, to link ROCK activity to RhoA, we used pull-down assay to determine active RhoA. As shown in Figure 6B, after 5 min treatment, LPS led to a reduction, while IL-1β led to an increase, of RhoA.

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Discussion

Stains are potent inhibitors of cholesterol synthesis and have numerous pleiotropic effects in cardiovascular diseases (Koh, 2000; Laufs and Liao, 2000; McFarlane et al., 2002, Werner et al., 2002). In this study, we demonstrate that stains decrease NO synthesis induced by LPS, but enhance NO synthesis induced by IL-1β in VSMCs. Since IKK-mediated NF-κB plays a crucial role in iNOS gene transcription (Xie et al., 1994), the present study for determining κB-luciferase activity, NF-κB binding ability, p65 translocation, and IKKα activity all indicated that changes in NF-κB activity account for the differential effects of statins on LPS- and IL-1β-induced iNOS expression.

Among the small GTP-binding proteins, Rho and its downstream target, ROCK, play important roles in controlling contractions, proliferation, migration, and gene regulation of VSMCs. Moreover, Rho-dependent ROCK signaling has been suggested to upregulate eNOS function in endothelial cells, while downregulate IL-1β-induced iNOS expression in VSMCs. In contrast to the increased eNOS mRNA stability induced by Rho/ROCK in endothelial cells (Laufs and Liao, 1998; Laufs et al., 1998), this signaling did not interfere with the half-life of iNOS mRNA in VSMCs (Chen et al., 2000), while it acted directly on the transcription machinery (Muniyappa et al., 2000). In this study, we demonstrated that statins (the general inhibitors of small GTP-binding

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proteins, including Rho) and Y-27632 (the specific ROCK inhibitor) could in parallel upregulate IL-1β-induced iNOS gene expression through negating this negative function of Rho/ROCK. In support of this point, lipophilic statins but not pravastatin were reported to inhibit Rho in VSMCs (Guijarro et al., 1998). Its hydrophilic nature makes it difficult for pravastatin to diffuse through the plasma membrane. Moreover, based on the results of Y-27632, we extend our knowledge of the inhibitory role of the Rho/ROCK signaling pathway on iNOS gene induction through its relation to IKK activation.

Depending on cell types and stimuli, Rho and/or ROCK signals might regulate NF-κB activity in distinct manner. Even though previous studies in epithelial cells (Kraynack et al., 2002; Benitah et al., 2003) and endothelial cells (Anwar et al., 2004) demonstrate that RhoA/ROCK pathway signals IKK and NF-κB activation, our current results suggest the existence of a negative cross-talk between Rho-ROCK pathway and IKK signaling in VSMCs. Supporting data include the enhancement effects of statins and Y-27632 on IL-1β-mediated IKK activity, p65 nuclear translocation, NF-κB activation, and/or iNOS gene expression. Moreover, it is interesting to note that under basal situation, ROCK is constitutively associated with IKK, and this interaction is markedly increased after Y-27632 treatment. This suggests that the interaction between ROCK and IKK might be dependent on the activated status of ROCK; namely inactive ROCK might be easily recruited by IKK. In addition, our data also indicated that

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although Y-27632 increases ROCK association with IKK, it is not able to alter basal activity of IKK unless IKK signaling is triggered by IL-1β. For these novel findings, thus far we need more data before understanding their interacting characteristics.

In a further examination of ROCK and RhoA activity under LPS and IL-1β stimulation, contrasting results were unexpectedly shown in the present study. LPS itself seems to inhibit the activities of ROCK and RhoA, but IL-1β increases them. These actions could explain why Y-27632 does not enhance LPS-stimulated NO response. This is because under condition in which ROCK activity has already been depressed by LPS, enhanced inhibitory action assumed to be induced by Y-27632 is masked. In contrast, Y-27632 reverses ROCK activation elicited by IL-1β, leading to upregulate IL-1β-stimulated iNOS expression. Taken together, we suggest that in addition to activating NF-κB through the identified MyD88/TRAF/NIK/IKK signaling pathway, IL-1β simultaneously induces the Rho/ROCK pathway to negatively balance IKK/NF-κB activity. Y-27632 and statin can reverse this existing inhibitor activity under IL-1β stimulation, thus unmasking NF-κB activity. Returning to the situation of LPS, we found that LPS did not enhance this negative pathway; in contrast, it virtually removed this constitutive negative control, thus higher NF-κB activity and greater iNOS response were detected compared to the case of IL-1β.

Similar to our previous finding in macrophages (Huang et al., 2003), statins inhibited LPS-mediated IKK and NF-κB activity in VSMCs. The ability of farnesyl

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transferase inhibitors to inhibit the LPS response suggests that Ras is an essential signaling player in LPS-mediated iNOS induction. For IKK-dependent NF-κB activation, Ras possibly transduces intermediate signaling events of PKC and ERK (Trushin et al., 1999; Chen and Lin, 2001). Like Ras, while Rac is also reported to be an upstream signaling player for NF-κB activation through formation of reactive oxygen species (Sanlioglu et al., 2001), our data using a Rac inhibitor (NSC23766) excluded its involvement in LPS- and IL-1β-mediated signal pathways for iNOS gene induction in VSMCs. Overall, the roles of some small GTP-binding proteins and ROCK in IL-1β and LPS-mediated iNOS expression in VSMCs, and their contribution to the distinct regulatory actions of statins deduced from this study are summarized in Figure 7.

The function of iNOS expression in the vascular wall is still controversial. Some studies support the hypothesis that the expression of iNOS plays antiatherogenic and vasculoprotective roles by exerting vasorelaxation, stimulation of endothelial cell growth, and inhibition of leukocyte adherence, platelet aggregation, LDL oxidation, VSMC proliferation, and migration (Yan et al., 1996; Fukumoto et al., 1997), while others propose opposite roles (Buttery et al., 1996; Chyu et al., 1999). These discrepancies may be due to different concentrations of NO used, because the overexpression of iNOS as in LPS-induced sepsis has cytotoxic effects that eventually damage the vascular wall (Szabo and Billiar, 1999). Alternatively, the biological

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significance of iNOS in the vascular system under different pathological conditions is regulated in a timely manner. Regulation of iNOS provides an additional explanation for the pleiotropic beneficial effects of statins; these results also suggest that alterative means of modulating iNOS should be useful for the treatment of cardiovascular disorders.

In summary, we show that HMG-CoA reductase inhibitors can differentially regulate iNOS expression in cultured VSMCs under LPS and IL-1β stimulation, and that this effect is associated with different involvements of ROCK in the actions of LPS and IL-1β. We clarified that ROCK is a crucial negative regulator of the IKK/NF-κB signaling pathway in VSMCs, and this negative control can be released by statins and ROCK inhibitor. The present study sheds new light on the beneficial effects of HMG-CoA reductase inhibitors in the prevention of cardiovascular disease, and highlights the important novel role of ROCK in LPS- and IL-1β-mediated NF-κB signaling pathways.

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Footnote

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Legends

Fig. 1. Distinct effects of statins on LPS- and IL-1β-stimulated nitrite production and iNOS expression in VSMCs. (A) VSMCs were treated with LPS (10 μg/ml), IL-1β (10 ng/ml), and/or IFN-γ (10 ng/ml) for 24 h. After incubation for 24 h, nitrite production was measured. (B, C) VSMCs were pretreated with statins at the indicated concentrations for 30 min, and then treated with LPS, IL-1β, and/or IFN-γ for 24 h. Nitrite levels are shown as a percentage of the control response without statin pretreatment. Data are presented as the mean ± S.E.M. of at least three independent experiments. * $p < 0.05$, significantly different from the response of stimuli in the absence of statins. (D) After agent incubation for 24 h, the iNOS protein level was determined by immunoblotting. The immunoreactivity of β-actin was used as an internal control. Each blot is representative of three separate experiments.

Fig. 2. Effects of statins on LPS- and IL-1β-induced iNOS gene expression. (A) VSMCs were pretreated with fluvastatin (30 μM) for 30 min, and then treated with LPS (10 μg/ml) or IL-1β (10 ng/ml) for 6 h. After incubation, RT-PCR for iNOS and β-actin mRNA expression was carried out. The data are representative of at least three independent experiments. (B) After transfection with the iNOS promoter plasmid, VSMCs were pretreated with fluvastatin for 30 min, and then treated with LPS or IL-1β

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for 6 h. Quantification of iNOS-luciferase activity was normalized by Lac Z expression. Data are presented as the mean ± S.E.M. of three independent experiments.* $p < 0.05$, significantly different from the response of LPS or IL-1β alone.

Fig. 3. Effects of fluvastatin on LPS- and IL-1β-induced NF-κB activation in VSMCs. (A) After transfection with the κB-reporter plasmid, VSMCs were pretreated with fluvastatin (30 μM) for 30 min, and then treated with LPS (10 μg/ml) or IL-1β (10 ng/ml) for 6 h. Quantification of κB-luciferase activity was normalized to Lac Z expression. (B) VSMCs were pretreated with fluvastatin for 30 min, and then treated with LPS or IL-1β for different intervals. Nuclear fractions were extracted and assayed for κB binding activity by TransAM kits. Data are presented as the mean ± S.E.M. of at least three independent experiments. * $p < 0.05$, significantly different from the response of LPS or IL-1β alone. (C) VSMCs were pretreated with fluvastatin for 30 min, and then treated with LPS or IL-1β for 1 h. Nuclear fractions were assayed by EMSA. To show the binding specificity, p65 and p50 antibodies were included in the binding mixture. The data are representative of at least three independent experiments.

Fig. 4. Effects of Y-27632, farnesyl transferase inhibitors and NSC23766 on LPS- and IL-1β-induced NO production and/or iNOS expression. (A) VSMCs were pretreated with fluvastatin (30 μM) or Y-27632 (10 μM) for 30 min, and then treated with LPS

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(10 μg/ml) or IL-1β (10 ng/ml) for 24 h. Nitrite production was measured. Data are presented as the mean ± S.E.M. of at least three independent experiments. * $p < 0.05$, significantly different from the LPS or IL-1β response alone. (B, C) As indicated, VSMCs were pretreated with fluvastatin (30 μM), Y-27632 (10 μM), α-HFPA (1 μM), L-744382 (10 μM), or NSC23766 (100 μM) for 30 min, followed by stimulation with LPS (10 μg/ml) or IL-1β (10 ng/ml) for 24 h. Then iNOS protein was determined by immunoblotting. Each blot is a representative of three separate experiments.

Fig. 5. Effects of Y-27632 and fluvastatin on LPS- and IL-1β-induced NF-κB activation, p65 nuclear translocation, and IKK activation. (A) As described in Fig. 3A, κB-luciferase activity was determined in VSMCs pretreated with fluvastatin (30 μM) or Y-27632 (10 μM) for 30 min, followed by stimulation with LPS or IL-1β. Data are presented as the mean ± S.E.M. from at least three independent experiments. * $p < 0.05$, significantly different from the response of stimuli in the absence of fluvastatin and Y-27632. (B) After 1 h of treatment with fluvastatin, Y-27632, LPS, and/or IL-1β, the nuclear fraction was prepared to determine the protein level of p65. Nuclear protein lamin-B was used as an internal control. (C) After 1 h of treatment as indicated, the IKK complex was immunoprecipitated followed by an *in vitro* kinase assay and IKK immunoblotting. (D) After 15 min of treatment as indicated, the IKK complex was

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immunoprecipitated followed by immunobloting with ROCK and IKKα. The data are representative of at least three independent experiments.

Fig. 6. Effects of Y-27632 and fluvastatin on LPS- and IL-1β-induced RhoA and ROCK activation. (A) After 10 min of treatment, ROCK activity was determined by immunoprecipitation and a kinase assay. Equal amounts of immunoprecipitate were used to determine the ROCK level as an internal control. (B) After 5 min treatment with LPS (10 μg/ml) or IL-1β (10 ng/ml), GST-RBD-bound RhoA, an index of active RhoA, was determined as described in the Methods. The amount of RBD-bound RhoA was normalized to the total amount of RhoA in cell lysates. The data are representative of at least three independent experiments.

Fig. 7. Mechanisms underlying the distinct regulation by statins of iNOS gene expression induced by LPS and IL-1β in VSMCs. Rho/ROCK signaling, a negative regulator of IKK activation and iNOS gene expression in VSMCs, was stimulated by IL-1β but was inhibited by LPS. In contrast, Ras is a transducer for LPS- and IL-1β-induced NF-κB activation and the iNOS expression. Through interruption of these small GTP-binding protein functions, statins exert differential net effects on NO production by LPS and IL-1β.

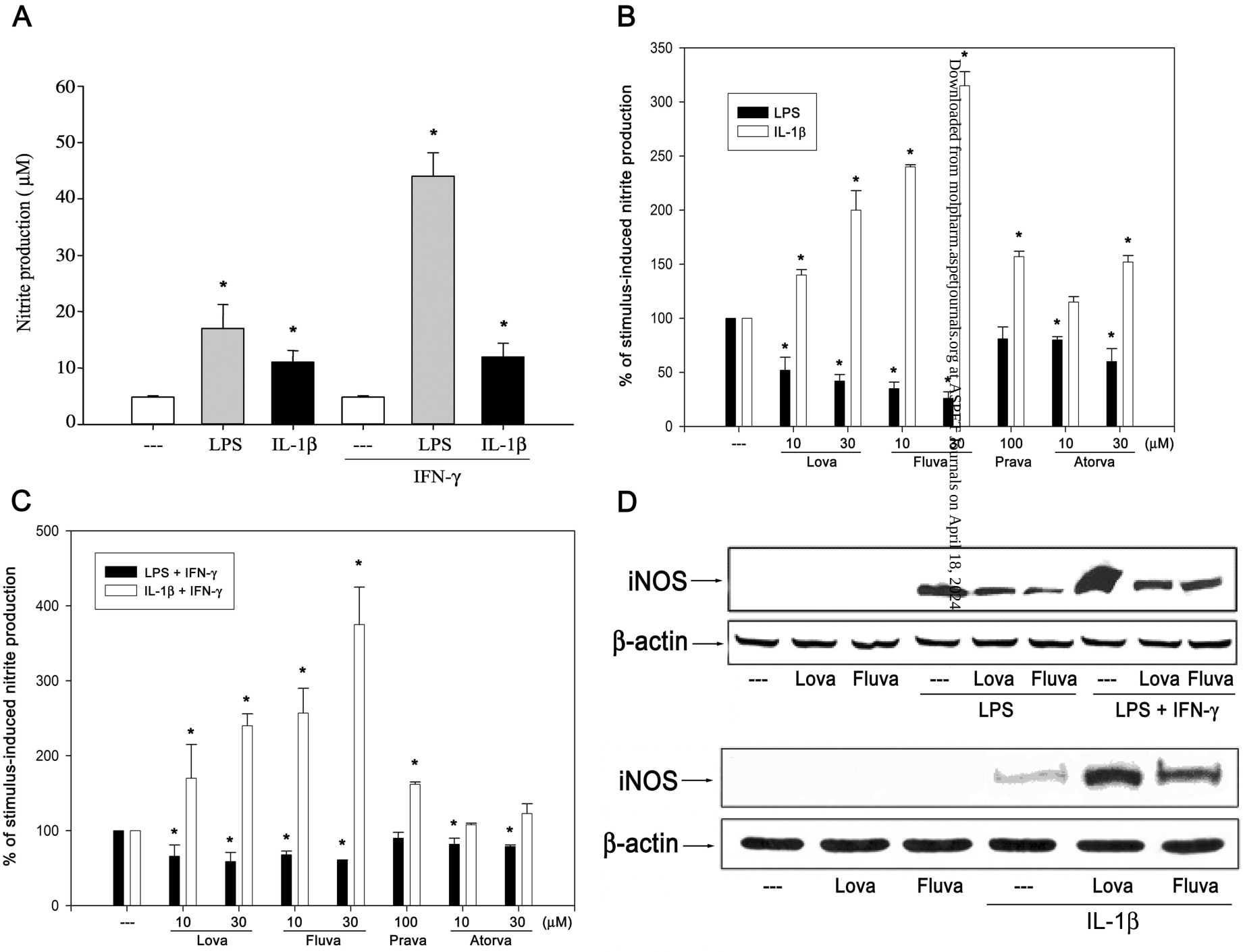

Fig. 1

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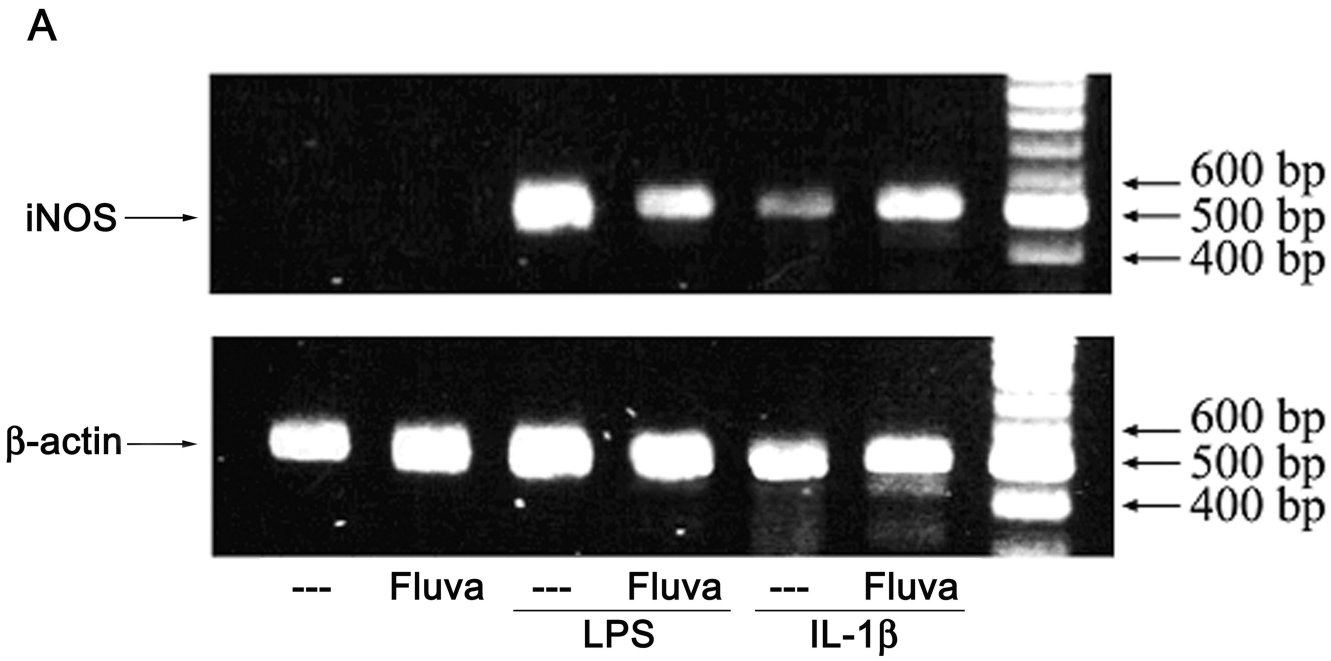

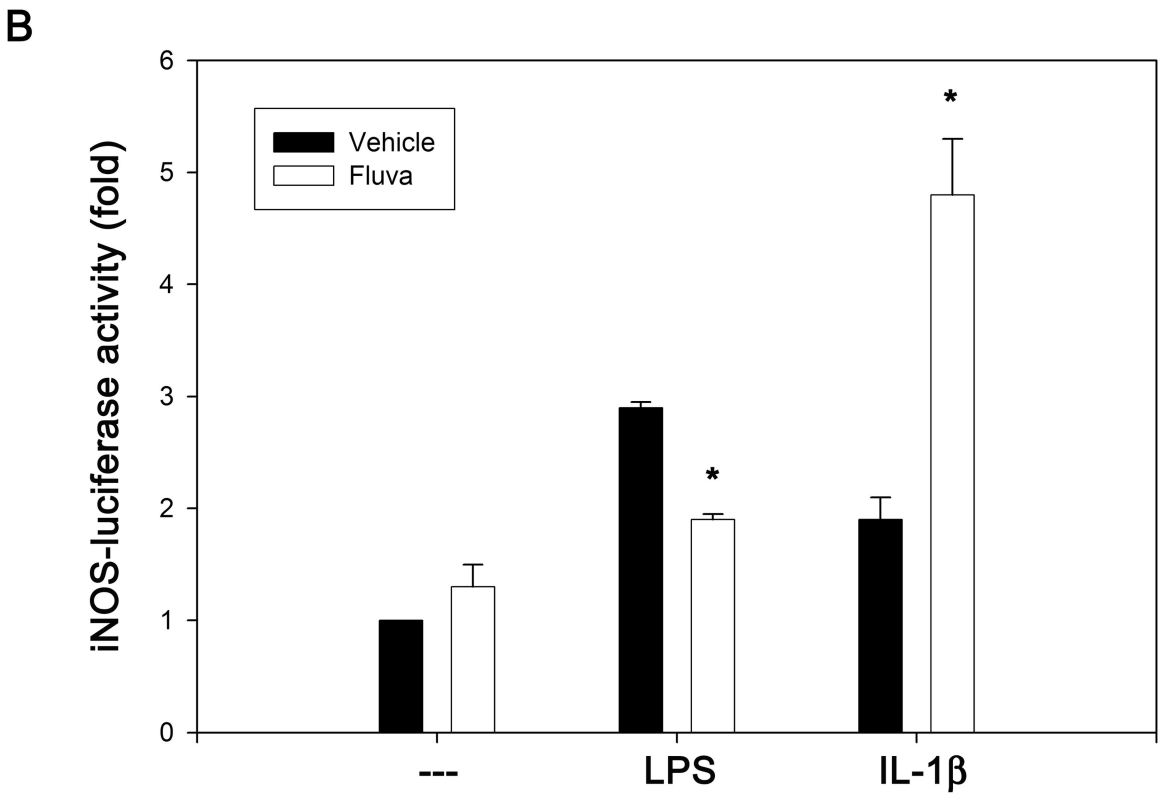

Fig. 2

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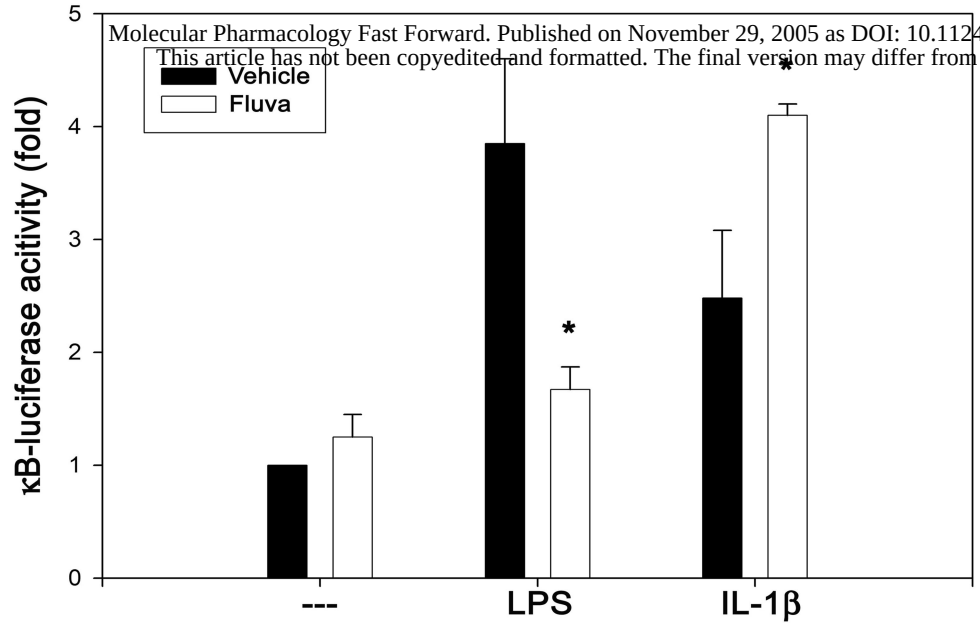

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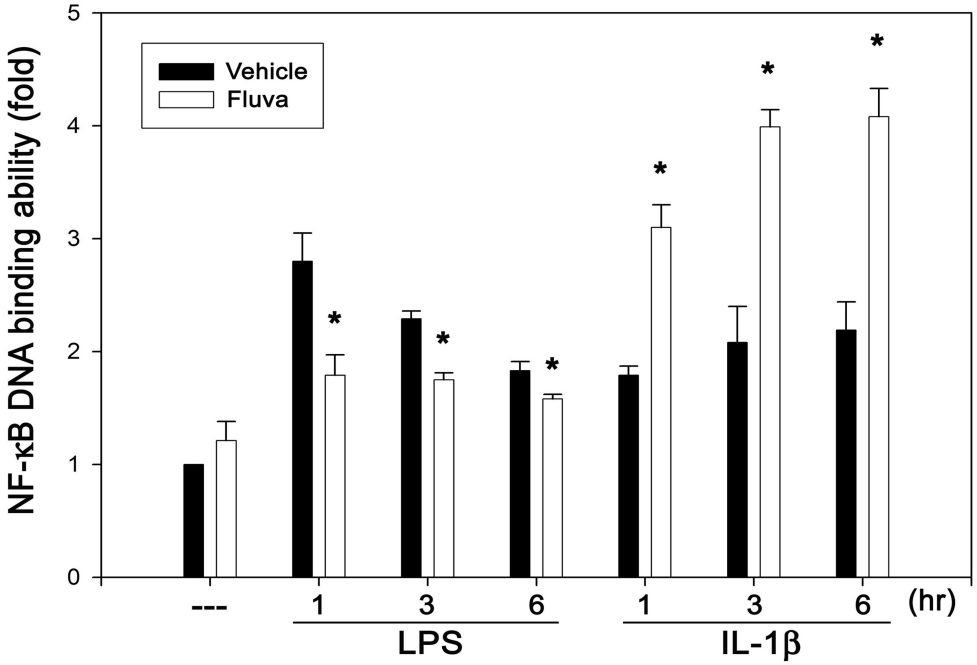

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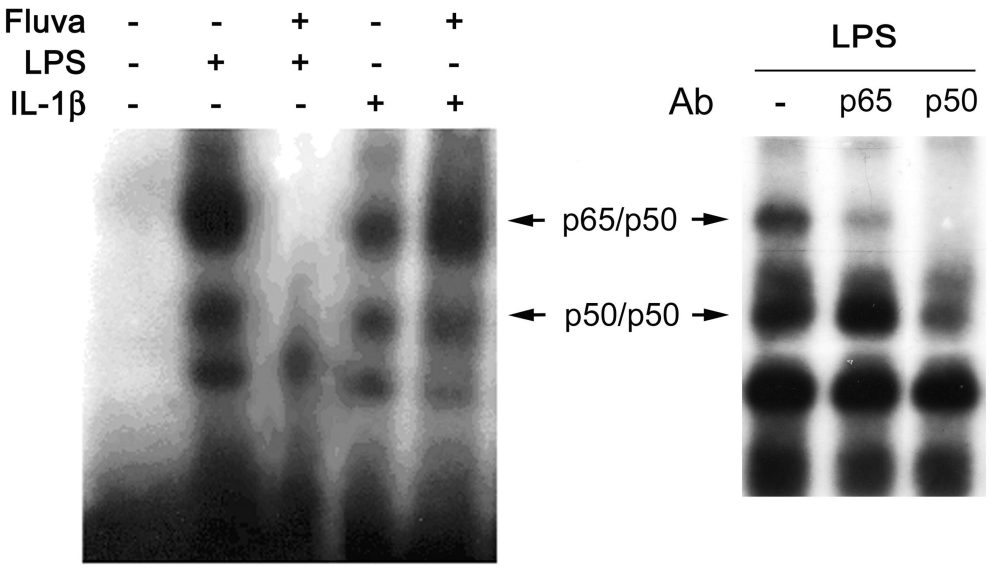

Fig. 3

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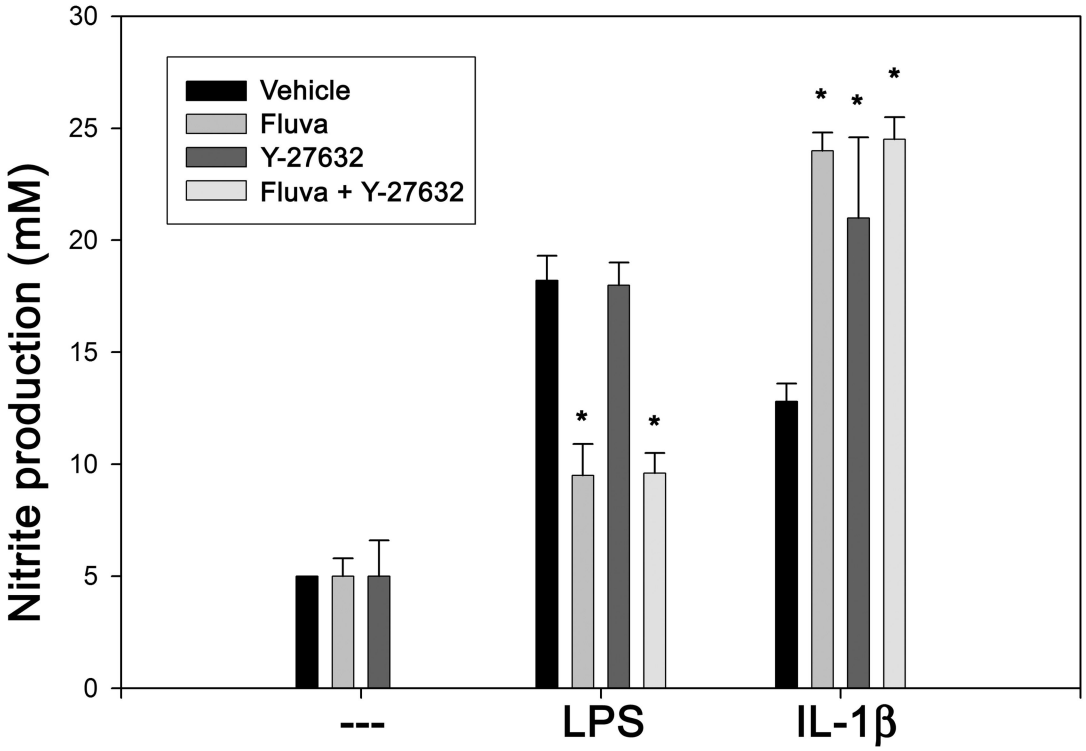

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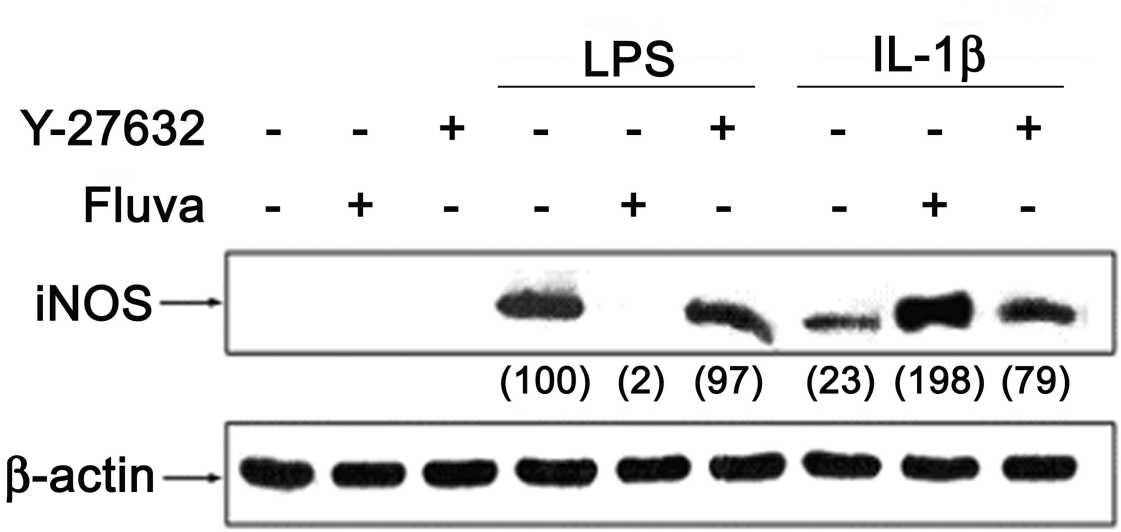

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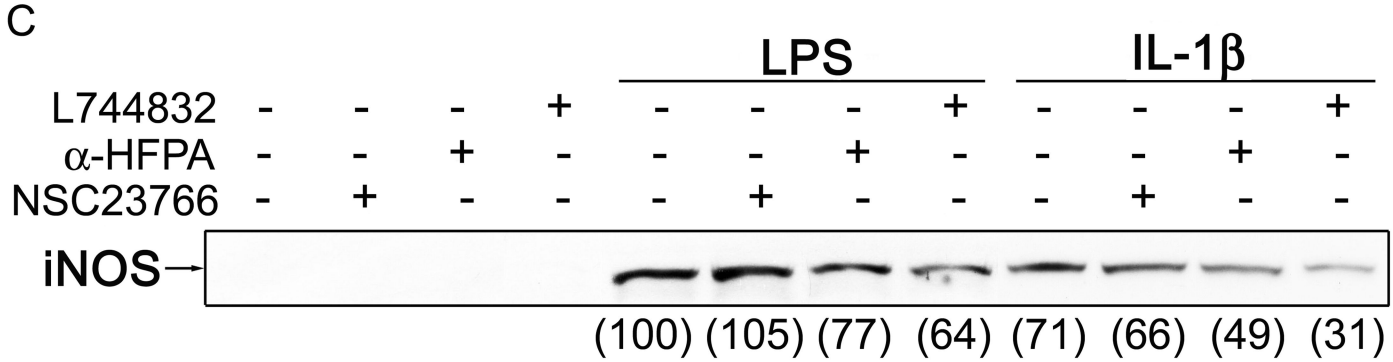

Fig. 4

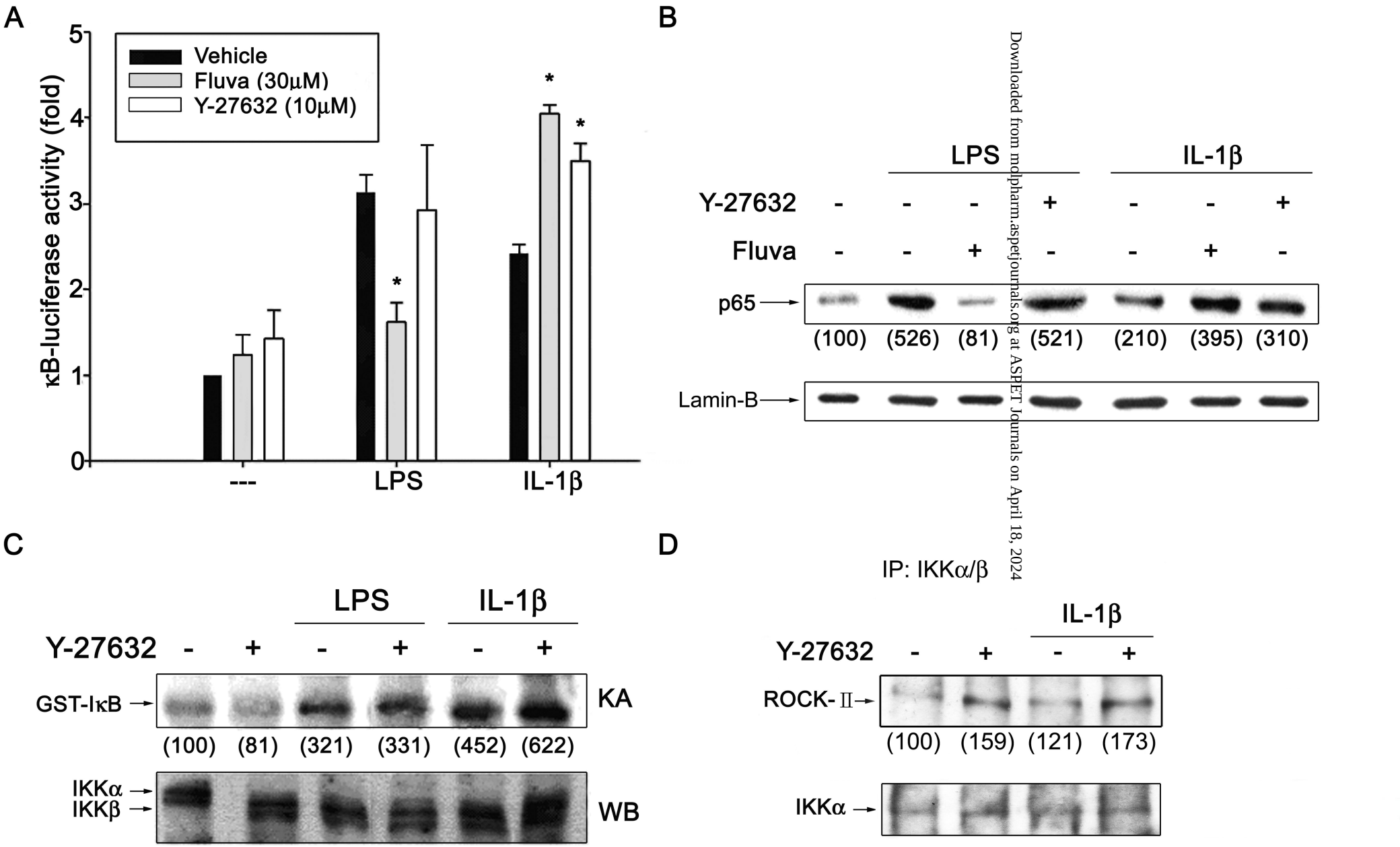

Fig. 5

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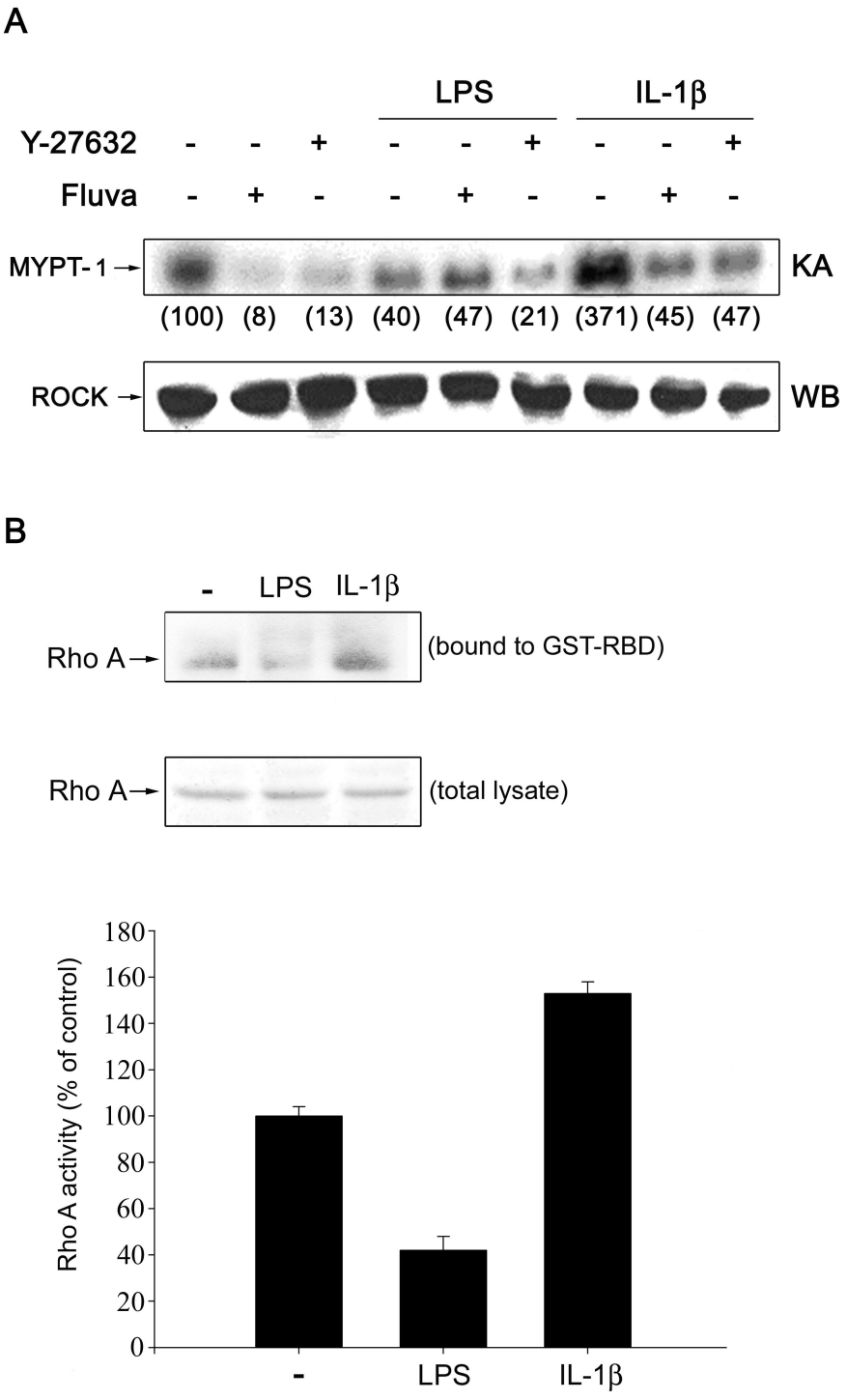

Fig. 6

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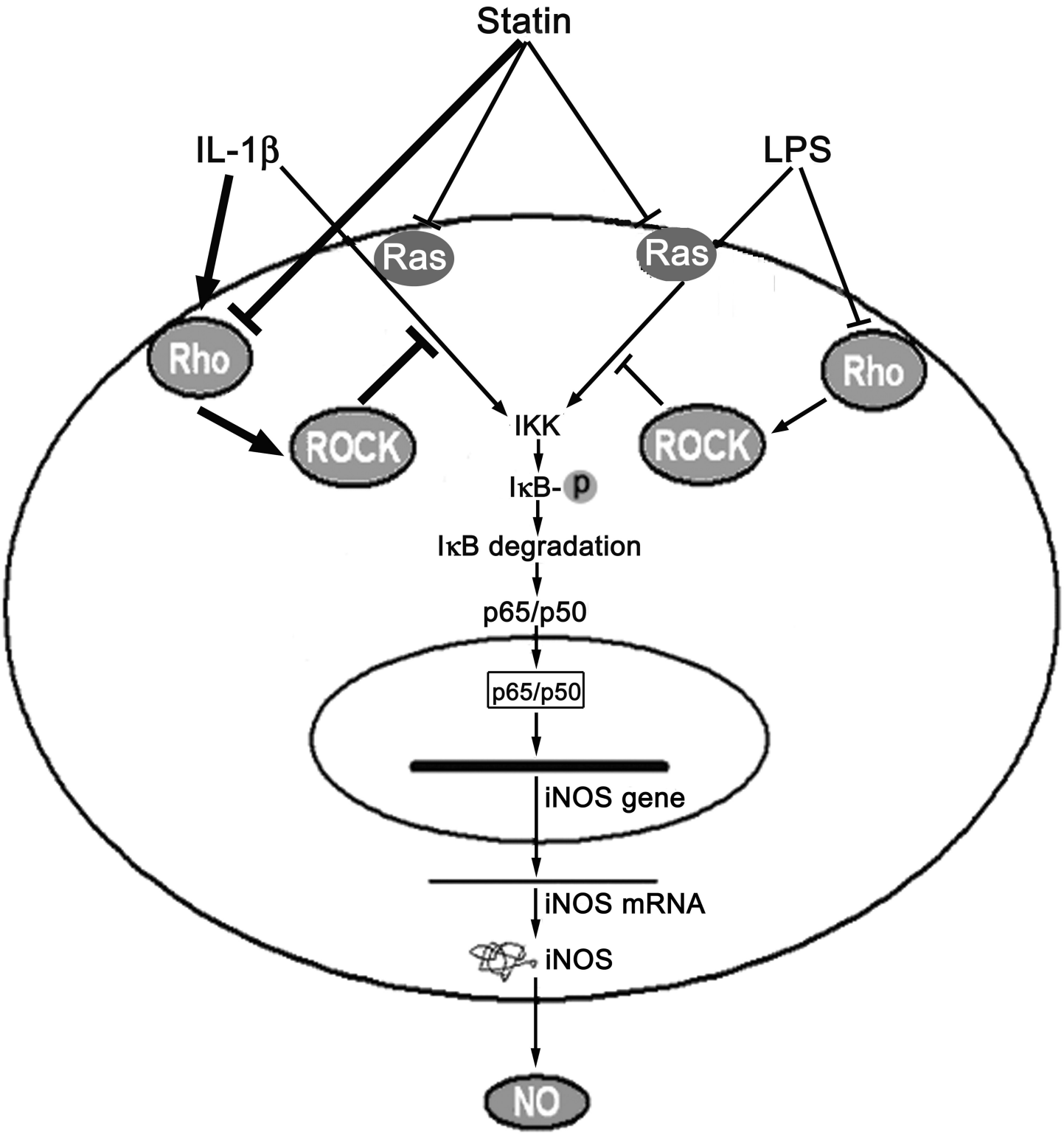

Fig. 7