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Lipopolysaccharide activates Akt in vascular smooth muscle cells resulting in induction of inducible nitric oxide synthase through nuclear factor-kappa B activation

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Abstract

Bacterial lipopolysaccharide and other immunostimulants induce gene expression of an isoform of nitric oxide synthase (iNOS) in vascular smooth muscle cells. This process is dependent on nuclear factor-kappa B (NF-κB) activation. The aim of this study was to investigate whether the NF-κB and Akt signaling pathways converge to induce iNOS in lipopolysaccharide-stimulated vascular smooth muscle cells. Treatment of vascular smooth muscle cells with lipopolysaccharide plus interferon-γ (LPS/IFN) caused activation of Akt and NF-κB. LPS/IFN caused activation of the iNOS promoter and transcription of iNOS mRNA/protein, resulting in NO production. A pharmacological inhibitor of the phosphoinositide 3-kinase (PI3K)-Akt pathway, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), inhibited phosphorylation of Akt and suppressed activation of NF-κB by attenuating the phosphorylation and degradation of IκB. LY294002 thereby inhibited LPS/IFN-induced iNOS expression and NO production. Another inhibitor of the PI3K-Akt pathway, wortmannin, also inhibited NO production in VSMC. LY294002 similarly inhibited interleukin-1β- or tumor necrosis factor-α-induced NO production. The data indicate that lipopolysaccharide or cytokine stimulation of vascular smooth muscle cells leads to activation of the PI3K-Akt pathway, which then activates the NF-κB pathway. Thus, the PI3K-Akt pathway controls the expression of iNOS in lipopolysaccharide- and cytokine-stimulated vascular smooth muscle cells.

Keywords: NF-κB; Akt; iNOS; Vascular smooth muscle

1. Introduction

Nuclear factor-kappa B (NF-κB) plays a critical role in regulating the induction of genes in states of immunity, stress responses, and inflammation (Karin and Ben-Neriah, 2000; Huxford et al., 1999; Baldwin, 1996). A wide variety of NF-κB-responsive genes have been identified. One of these genes encodes an inducible isoform of nitric oxide (NO) synthase (iNOS) and NF-κB has been shown to mediate induction of this gene in several NO-producing cell types. This based on studies examining iNOS gene promoter constructs and inhibitors of NF-κB (Sherman et al., 1993; Mulsch et al., 1993; Eberhardt et al., 1994; Xie et al., 1994). Classical NF-κB is a heterodimer composed of p50 and p65/RelA subunits, which exist in the cytoplasm as an inactive complex bound by IκB proteins (Karin and Ben-Neriah, 2000; Huxford et al., 1999; Baldwin, 1996). Generally, activation of NF-κB involves phosphorylation of IκB, which then marks IκB for ubiquitination and degradation. This permits NF-κB to translocate to the nucleus, where it activates gene transcription. A key regulatory step in NF-κB activation is activation of a high molecular weight IκB kinase (IKK) complex, containing kinases such as IKKα and IKKβ, which are thought to catalyse NF-κB activation.

Akt/protein kinase B (PKB) is a serine–threonine kinase that is best known for its ability to inhibit cell death pathways (Kandel and Hay, 1999; Brazil and Hemmings, 2001; Scheid and Woodgett, 2001). Activation of Akt by growth factors and cytokines generally occurs via the phosphoinositide 3-kinase (PI3K) pathway. Upon stimulation, PI3K phosphorylates specific phosphoinositide lipids, which accumulate in the plasma membrane, creating

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docking sites for Akt. At the plasma membrane Akt undergoes phosphorylation at two residues, leading to its activation.

Although NF-κB and Akt were initially thought to be components of distinct signaling pathways, several studies have demonstrated convergence of the NF-κB and Akt signaling pathways (Thomas et al., 2002; Madrid et al., 2001). Indeed, IκB kinase, the kinase involved in NF-κB activation, is a substrate of Akt, thus, activation of Akt stimulates NF-κB activity. These results place Akt upstream of NF-κB activation in the sequence of signaling events. Thus, we investigated whether modulation of Akt/PKB activity can affect NO synthesis through NF-κB activation in vascular smooth muscle cells.

2. Methods

2.1. Cell culture and RNA extraction

Vascular smooth muscle cells were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats (Chamley-Campbell et al., 1979). Cultures were fed twice weekly with Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 μg/ml piperacillin and 100 μg/ml streptocymin). Cells in passages 10 to 15 were used for the experiments. Total RNA was extracted from confluent vascular smooth muscle cells after passages 10–15 using a modified guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987).

2.2. Nitrite assay

Nitrite accumulation, an indicator of NO synthesis, was measured in the cell culture medium of confluent vascular smooth muscle cells (Gross and Levi, 1992). Nitrite was quantified colorimetrically after adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100-μl samples. Absorbance at 550 nm was determined using a microplate reader (Molecular Devices, Richmond, CA, USA). Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium.

2.3. iNOS mRNA analysis

Standard Northern blotting was used to investigate iNOS mRNA expression, as previously described (Hattori et al., 1999a,b). A probe was obtained by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers for iNOS (Hattori and Gross, 1993), after which it was labeled with [α-^{32}P]dCTP by random priming and used for Northern blot analysis of mRNA expression. After probing for iNOS expression, membranes were stripped and re-probed for GAPDH mRNA. Radioactivity of the blots was quantified using a BAS2000 image analyzer (Fuji Photo Film, Tokyo, Japan).

2.4. NF-κB activation

To study NF-κB activation, the cells were stably transfected with a *cis*-reporter plasmid containing a luciferase reporter gene linked to five repeats of NF-κB binding sites (pNFκB-Luc: Stratagene, La Jolla, CA, USA) (Hattori et al., 1999a,b). For this, the pNFκB-Luc plasmid was transfected together with a pSV2neo helper plasmid (Clontech, Palo Alto, CA, USA) into rat vascular smooth muscle cells using the FuGEN 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). The cells were cultured in the presence of G418 (Clontech) at a concentration of 500 μg/ml with medium replacement at 2- to 3-day intervals. Approximately 3 weeks later, G418-resistant clones were isolated using a cloning cylinder and analyzed individually for expression of luciferase activity. Several clones were selected for analysis of NF-κB activation. Luciferase activity was measured using a luciferase assay kit (Stratagene).

2.5. NOS promoter analysis

To study iNOS promoter function, rat vascular smooth muscle cells were stably transfected with a construct containing a 1.7-kb fragment of the mouse iNOS promoter which was cloned upstream of a reporter gene that encodes the secreted form of human placental alkaline phosphatase (SEAP) (Hattori et al., 1999a,b). SEAP activity, which is released into the cell culture medium, was measured using a sensitive chemiluminescent assay (Tropix).

2.6. Western blot

Monolayer vascular smooth muscle cells treated with lipopolysaccharide plus interferon-γ (LPS/IFN) in the presence and absence of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) for various intervals were lysed using cell lysis buffer (Cell Sinaling) with 1 mM pheylmethyl suphonyl fluoride. The protein concentration of each sample was measured using a Bio-Rad detergent-compatible protein assay. Subsequently, β-mercaptoethanol was added at a final concentration of 1%, and each sample was denatured by boiling for 3 min. Samples containing 15 μg of protein were resolved by electrophoresis on 12% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to polyvinyldienefluoride (PVDF) membranes (Bio-Rad) incubated with Phospho-Akt antibody, Akt antibody, Phospho-IκB-α antibody, IκB-α antibody, Phospho-ERK antibody, and ERK antibody (1:1000, Cell Signaling). The binding of each of these antibodies was detected using sheep anti-rabbit IgG horseradish peroxidase (1:5000) and the ECL Plus system (Amersham,

Buckinghamshire, UK). For rat iNOS, vascular smooth muscle cells treated with LPS/IFN plus different concentrations of LY294002 for 24 h were lysed using electrophoresis sample buffer and analysed by immunoblotting, as previously described.

2.7. Statistical analysis

Data are presented as means ± S.E.M. Multiple comparisons were evaluated by analysis of variance between groups (ANOVA) followed by Fisher's protected least significant difference test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. LY204992 or wortmannin inhibits lipopolysaccharide-induced NO production in vascular smooth muscle cells

We examined the role of the PI3K-Akt pathway in induction of NO as a result of immunostimulation in vascular smooth muscle cells using two different pharmacological inhibitors, LY294002 and wortmannin. These block the activation of PI3K via different mechanisms. The induction of NO by lipopolysaccharide plus interferon-γ (LPS/IFN) was measured by examining nitrite accumulation in the medium, in the presence or absence of LY294002 or wortmannin. LY294002 (2 ~ 10 μM) dose-dependently inhibited LPS/IFN-induced nitrite production in vascular smooth muscle cells with a half-maximal inhibitory concentration of ~ 5 μM. Similarly, wortmannin (25–200 nM) inhibited LPS/IFN-induced nitrite accumulation in a concentration-dependent manner with a half-maximal inhibitory concentration of ~ 150 nM.

3.2. LY204002 inhibits interleukin-1β- or TNF-α-induced NO production in vascular smooth muscle cells

We next examined the effect of LY294002 on interleukin-1β- or tumor necrosis factor-α (TNF-α)-induced NO production in vascular smooth muscle cells. The induction of nitrite production by interleukin-1β plus interferon-γ (IL-1/IFN) was significantly inhibited by LY294002. The induction of nitrite production by TNF-α plus interferon-γ (TNF-α/IFN) was also markedly inhibited by LY294002 (Fig. 1).

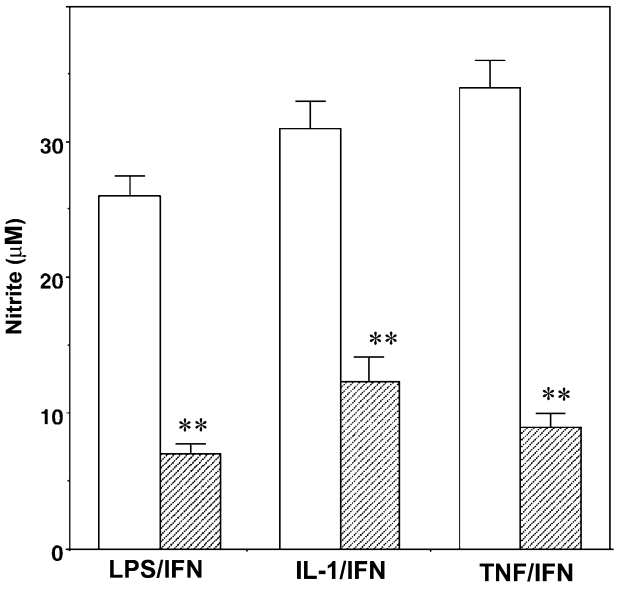

Fig. 1. Effect of LY294002 on nitrite production after stimulation of rat vascular smooth muscle cells with lipopolysaccharide (30 μg/ml), interleukin-1β (10 ng/ml), or TNF-α (10 ng/ml) in combination with interferon-γ (10 ng/ml) (LPS/IFN, IL-1/IFN, or TNF/IFN). LY294002 (5 μM, shaded bars) was added 1 h before stimulation. Nitrite accumulation was measured 24 h after stimulation. Data are presented as means ± S.E. ($n = 6$). $^{**}P < 0.01$ compared with control (no LY294002).

3.3. LY294002 inhibits lipopolysaccharide-induced NF-κB activation and iNOS promoter activity

Next, we evaluated the role of PI3K-Akt signaling in lipopolysaccharide-induced NF-κB activation and iNOS promoter activity. LPS/IFN substantially induced NF-κB activity, which was clearly inhibited by LY294002 (Fig. 2A). Similarly, the induction of iNOS promoter activity by LPS/IFN was inhibited by LY294002 in a dose-dependent manner (Fig. 2B).

3.4. LY294002 inhibits lipopolysaccharide-induced iNOS mRNA and protein

The role of the PI3K-Akt pathway in induction of iNOS mRNA and protein synthesis was determined by Northern and Western blot analysis. LPS/IFN induced maximal levels of iNOS mRNA at 16 h. The induction of iNOS mRNA expression by LPS/IFN was suppressed by LY294002 in a dose-dependent manner (Fig. 3A). We also examined the induction of iNOS protein by LPS/IFN after 24 h. Likewise, the level of iNOS protein induced by LPS/IFN was attenuated by LY294002 in a concentration-dependent manner (Fig. 3B).

3.5. LY294002 down-regulates lipopolysaccharide-induced Akt phosphorylation and inhibits IκB-α phosphorylation

To ascertain activation of PI3K-Akt in response to lipopolysaccharide, we examined the phosphorylation of Akt for 4 h after stimulation of VSMC with LPS/IFN. A time-dependent phosphorylation of Akt was observed, while nonphosphorylated Akt remained the same. Preincubation with LY294002 significantly down-regulated Akt phosphorylation (Fig. 4). We then examined whether LY294002 could affect the activation-induced proteolysis of IκB inhibitor protein, which traps NF-κB dimers in the cytosol.

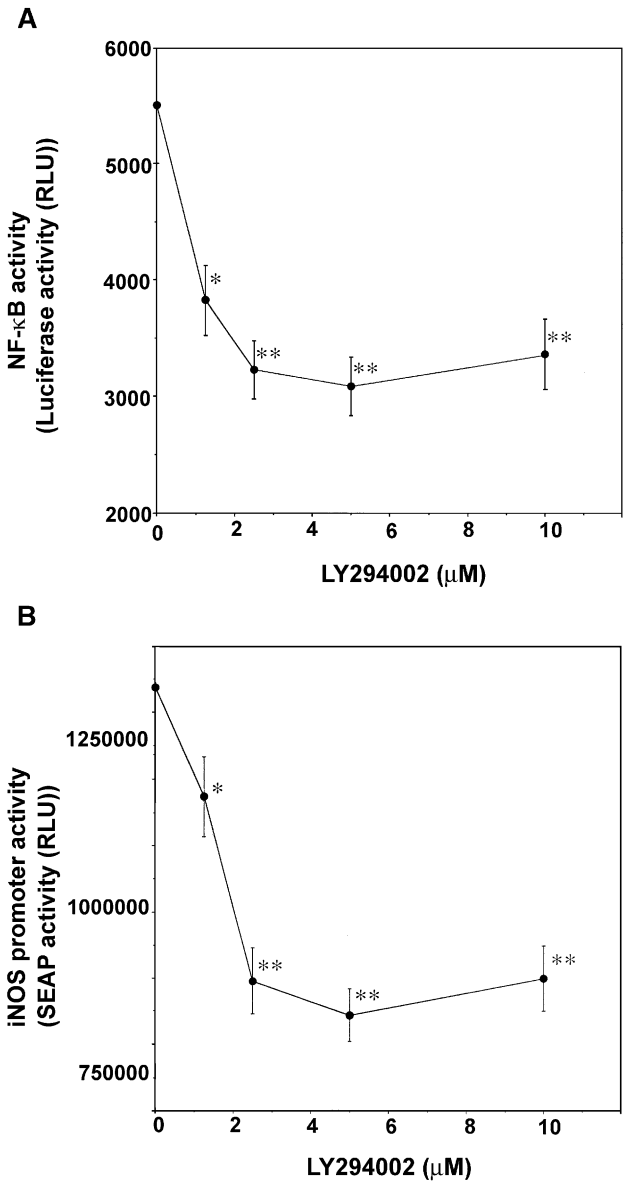

Fig. 2. (A) NF-κB-dependent transcriptional activity/luciferase reporter expression in stably transfected rat vascular smooth muscle cells. Cells were treated with lipopolysaccharide (30 μg/ml) in combination with interferon-γ (10 ng/ml) (LPS/IFN) in the presence of various concentrations of LY294002 for 3 h. Luciferase activity in the cells was measured. Values represent the means ± S.E. ($n = 3$). **$P < 0.01$ versus LPS/IFN alone. (B) iNOS promoter/SEAP reporter expression in stably transfected rat vascular smooth muscle cells. Cells were treated with LPS/IFN in the presence of various concentrations of LY294002 for 24 h. After this time, SEAP activity was quantified in the cell culture medium. Values represent the means ± S.E. ($n = 6$). **$P < 0.01$ versus LPS/IFN alone.

Incubation of vascular smooth muscle cells with LPS/IFN for 15 min to 1 h led to significant proteolysis of IκB-α. We next examined whether LY294002 could prevent LPS/IFN-stimulated proteolysis of IκB-α. As shown in Fig. 4, LPS/IFN-induced proteolysis was partially prevented by LY294002. We also examined the level of phosphorylated

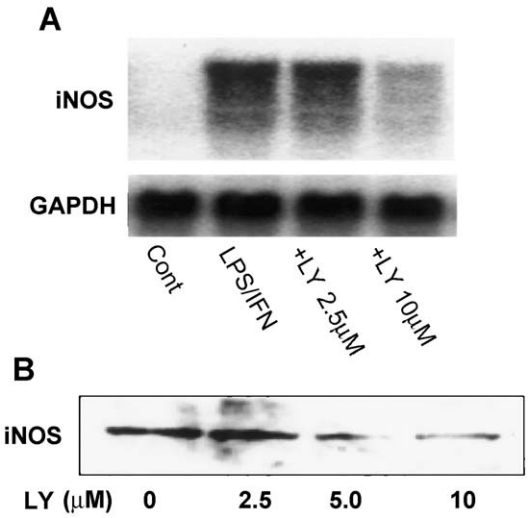

Fig. 3. (A) Effect of LY294002 on iNOS mRNA expression in rat vascular smooth muscle cells stimulated with lipopolysaccharide (30 μg/ml) in combination with interferon-γ (10 ng/ml) (LPS/IFN). Cells were incubated with LPS/IFN in the presence of the indicated concentrations of LY294002 for 16 h. Total RNA was isolated and Northern hybridized with a rat iNOS-specific probe. (B) Effect of LY294002 on iNOS protein expression in LPS/IFN-stimulated vascular smooth muscle cells. Cells were incubated with LPS/IFN in the presence of the indicated concentrations of LY294002 for 24 h, after which iNOS protein was detected by Western blotting as a band with a molecular mass of ~125 kDa.

IκB-α in order to estimate the activity of IκB kinase. Phosphorylation of IκB-α was inhibited in the presence of LY294002 (Fig. 4). However, time course of ERK phos-

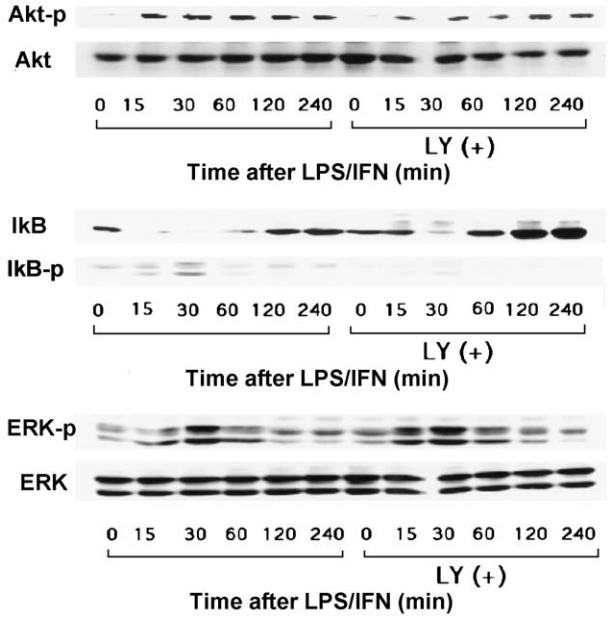

Fig. 4. Effect of LY294002 on phosphorylation of Akt, IκB-α, and ERK in rat vascular smooth muscle cells stimulated with lipopolysaccharide (30 μg/ml) in combination with interferon-γ (10 ng/ml) (LPS/IFN). Cells were treated with LY294002 (10 μM) and stimulated with LPS/IFN for the indicated time periods. The extracted cell lysates were subjected to Western blot analysis using Phospho-Akt antibody, Akt antibody, Phospho-IκB-α antibody, IκB-α antibody, Phospho ERK antibody, and ERK antibody.

phorylation was similar even in the absence and presence of LY29002 (Fig. 4).

4. Discussion

Inhibition of lipopolysaccharide-induced Akt activation led to down-regulation of NF-κB activation, resulting in decreased iNOS expression and NO production. NF-κB-activating stimuli such as interleukin-1β and TNF-α were also down-regulated by inhibition of Akt activation, leading to decreased NO production. LPS-mediated phosphorylation and activation of Akt were blocked by pharmacological inhibitors of Akt. Events necessary for the activation of NF-κB (such as IκB degradation, nuclear translocation, and increased NF-κB DNA binding) were all suppressed by inhibitors of Akt. These results place Akt upstream of NF-κB activation in the sequence of signaling events, whereby activation of Akt up-regulates iNOS promoter activity, leading to transcription and translation of iNOS and increased NO production.

The activation of NF-κB requires phosphorylation of IκB, which then targets IκB for ubiquitination and degradation. Inhibition of Akt, which was demonstrated as diminished Akt phosphorylation in the present experiment, caused decreased phosphorylation of IκB and attenuated the degradation of IκB in VSMC. This might inhibit translocation of NF-κB to the nucleus, where it normally activates gene transcription. A key regulatory step in this pathway of NF-κB activation is the activation of a high molecular weight IκB kinase (IKK) complex, containing kinases such as IKKα and IKKβ, which are thought to catalyse NF-κB activation. Given that IκB kinase is a substrate of Akt, activation of Akt might therefore stimulate NF-κB activation. Thus, increased NO production of might result from convergence of the Akt and NF-κB signalling pathways in LPS-stimulated VSMC.

Although induction of nuclear translocation of NF-κB has always been highly regarded as the principal mechanism by which NF-κB-dependent gene expression is activated, an alternate mechanism of NF-κB activation is emerging involving phosphorylation of the RelA/p65 transactivation subunit. The proinflammatory cytokines, TNF-α and interleukin-1β, have been shown to stimulate RelA/p65 phosphorylation and subsequent NF-κB transactivation through pathways that are distinct from those involved in induction of nuclear translocation (Sizemore et al., 1999; Wang and Baldwin, 1998; Bird et al., 1997). Recently, the generation of GSK3 and T2K (TBK1) knockout mice has highlighted the physiological importance of NF-κB transactivation, since cells generated from these animals are capable of inducing NF-κB nuclear translocation but are deficient in their ability to stimulate transactivation functions of NF-κB (Hoeflich et al., 2000; Bonnard et al., 2000). Thus, Akt might target the stimulation of the transcription function of NF-κB via the transactivation domain of RelA/p65. This possibility requires further investigation.

The current study demonstrates that lipopolysaccharide or cytokine stimulation of VSMC leads to activation of the PI3K-Akt pathway, which then activates the NF-κB pathway by phosphorylation of IκB kinase. Inhibition of the PI3K-Akt pathway limits activation of NF-κB, which regulates iNOS gene expression. Thus, the PI3K-Akt pathway modulates the expression of iNOS in lipopolysaccharide- and cytokine-stimulated vascular smooth muscle cells.

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