Thrombin Induces Nitric-oxide Synthase via G\textsubscript{\textalpha}12/13\textsuperscript{\textgamma}-coupled Protein Kinase C-dependent I\textkappaB\textalpha Phosphorylation and JNK-mediated I\textkappaB\textalpha Degradation*

Received for publication, January 16, 2003, and in revised form, February 24, 2003
Published, JBC Papers in Press, February 26, 2003, DOI 10.1074/jbc.M300471200

Keon Wook Kang, So Yeon Choi, Min Kyung Cho, Chang Ho Lee, and Sang Geon Kim§

From the National Research Laboratory, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, and §Department of Pharmacology and Institute of Biomedical Science, College of Medicine, Hanyang University, Seoul 133-791, Korea

An imbalance between thrombin and antithrombin III contributed to vascular hyporeactivity in sepsis, which can be attributed to excess NO production by inducible nitric-oxide synthase (iNOS). In view of the importance of the thrombin-activated coagulation pathway and excess NO as the culminating factors in vascular hyporeactivity, this study investigated the effects of thrombin on the induction of iNOS and NO production in macrophages. Thrombin induced iNOS protein in the Raw264.7 cells, which was inhibited by a thrombin inhibitor, LB30057. Thrombin increased NF-\kappaB DNA binding, whose band was supershifted with anti-p65 and anti-p50 antibodies. Thrombin elicited the phosphorylation and degradation of I\kappaB\alpha prior to the nuclear translocation of p65. The NF-\kappaB-mediated iNOS induction was stimulated by the overexpression of activated mutants of \textalpha12/13\textgamma (\textalpha12/13\textgammaQL). Protein kinase C depletion inhibited I\kappaB\alpha degradation, NF-\kappaB activation, and iNOS induction by thrombin or the iNOS induction by \textalpha12/13\textgammaQL. JNK, p38 kinase, and ERK were all activated by thrombin. JNK inhibition by the stable transfection with a dominant negative mutant of JNK1 (JNK1\(-/-\)) completely suppressed the NF-\kappaB-mediated iNOS induction by thrombin. Conversely, the inhibition of p38 kinase enhanced the expression of iNOS. In addition, JNK and p38 kinase oppositely controlled the NF-\kappaB-mediated iNOS induction by \textalpha12/13\textgammaQL. Hence, iNOS induction by thrombin was regulated by the opposed functions of JNK and p38 kinase downstream of \textalpha12/13\textgamma. In the JNK1\(-/-\) cells, thrombin did not increase either the NF-\kappaB binding activity or I\kappaB\alpha degradation despite I\kappaB\alpha phosphorylation. These results demonstrated that thrombin induces iNOS in macrophages via \textalpha12 and \textalpha13, which leads to NF-\kappaB activation involving the protein kinase C-dependent phosphorylation of I\kappaB\alpha and the JNK-dependent degradation of phosphorylated I\kappaB\alpha.

Septic shock is the detrimental consequence of the host response to a bacterial infection. Septic shock involves hypotension, disseminated intravascular coagulation, and acute organ dysfunction accompanying the inflammatory and coagulopathic responses. Hypotension and the signs of inadequate organ perfusion are the major manifestations of sepsis. The changes in the vascular reactivity do not depend on infectious pathogens but on disturbances in the coagulation and fibrinolytic cascade (1–5). The cascade of inflammatory and clotting reactions induces the development of disseminated intravascular coagulation, and microparticules cause the acute generation of thrombin (6). It has been shown that the level of the thrombin and antithrombin complexes is higher in patients with sepsis than in healthy control subjects (7). In sepsis, the level of antithrombin III (ATIII), an endogenous coagulation inhibitor, is reduced as a result of complex formation with multiple activated clotting factors (8, 9). The plasma ATIII level is low in septic patients as a consequence of ATIII consumption in severe sepsis (10). It is highly likely that the activation of prothrombin to thrombin by the coagulation pathway increases the level of unbound free thrombin in these patients.

Vascular hyporeactivity is attributable to excess nitric oxide (NO) production, a key gaseous molecule inducing the collapse of the cardiovascular system, by an inducible form of NOS (11). The inducible nitric-oxide synthase (iNOS) expression and NO production greatly affect the inflammatory processes (12, 13). Prolinflammatory cytokines such as the tumor necrosis factor-\alpha induce NO production (14, 15). Activated protein C, as a natural anticoagulant, regulates the coagulation system by inhibiting thrombin generation and attenuating the inflammatory responses induced by lipopolysaccharide (LPS). Protein C prevents LPS-induced hypotension by inhibiting excess NO production (16). ATIII inhibits nuclear factor-\kappaB (NF-\kappaB) activation in monocytes and endothelial cells (17). In addition, it has been shown that ATIII prevents LPS-induced hypotension by inhibiting NO induction in animals (18).

Macrophages, which are effector cells in eliminating microorganisms and other noxious elements, participate in many complex immunological and inflammatory processes. They produce the cytokines that recruit other inflammatory cells, which are responsible for the diverse effects of inflammation. Septic shock syndrome results from an excessive triggering of endogenous inflammatory mediators, which are released primarily by activated macrophages (19).

In view of the imbalance between thrombin and antithrombin in septic patients as a result of the exhaustion of antithrombin...
and excess NO as culminating factors in vascular hypore-
activity, this study investigated the effect of thrombin on the
production of NO in macrophages. In addition, the signaling
pathways responsible for the induction of iNOS by thrombin
were examined. This study reports for the first time that
thrombin induces iNOS through I-κB phosphorylation and
subsequent NF-κB activation via the protein kinase C (PKC)
and c-Jun N-terminal kinase (JNK) pathways in macrophages.
Thrombin exerts mitogenic proliferation through the receptors
coupled with the G12/13 proteins belonging to the G12
subfamily (20–22). We determined whether thrombin-induced
NF-κB activation occurred via a pathway involving G12/13. In
view of the uncertainty of the PKC linkage to the G12 sub-
family, we assessed whether PKC was associated with the G12
family in the activation of NF-κB by thrombin. The G12 pro-
tein is linked to the Rho-directed guanine nucleotide exchange
factor, p115, and the GTPase-activating protein, RasGAP1 (23,
24). The G12/13 proteins are implicated in the Rho-dependent
cytoskeletal shape change and JNK activation (20, 25, 26). This
study further determined that JNK was involved in the degra-
dation of the phosphorylated I-κB downstream of G12/13.

**EXPERIMENTAL PROCEDURES**

Reagents—LB30057 was the kind gift from LG Biotech Inc. (Daeduk,
Korea). [γ-32P]ATP (3000 mCi/mmol) was obtained from Amersham
Biosciences. Horseradish peroxidase-conjugated goat anti-rabbit IgG
and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium were
supplied from Invitrogen. Alkaline phosphatase-conjugated goat anti-
mouse IgG was purchased from Kirkegaard & Perry Laboratories
(Gaithersburg, MD). Anti-c-Rel (p65), anti-p50, and anti-I-κB antibodies
were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).
Anti-phospho-I-κB antibody was supplied from New England Biolabs
(Beverly, MA). Anti-iNOS antibody was obtained from Transduction
Laboratories (Lexington, KY) or Santa Cruz Biotechnology. Horserad-
ish peroxidase-conjugated and fluorescein isothiocyanate-conjugated
anti-rabbit IgG were obtained from Zymed Laboratories Inc. (San

**Fig. 1. Induction of iNOS by thrombin.** A, the time course of iNOS induction
and NO production. The macrophages were incubated with or without thrombin
(10 units/ml) or LPS (1 μg/ml), and the iNOS levels were immunochemically de-
termined. The amount of nitrite in the medium was monitored, as described un-
der “Experimental Procedures.” B, the effect of varying concentrations of thrombin
on iNOS induction. Both iNOS expression and NO production were measured in the
cells incubated with 1, 10, or 100 unit(s) of thrombin per ml for 18 h. C, the effect of
LB30057 on the induction of iNOS by thrombin. The cells were treated with
0.1–100 μM of LB30057 for 30 min and further incubated with thrombin (10
units/ml) for 18 h. Subsequently, the iNOS expression level was assessed. Each
lane was loaded with 30 μg of the cytosolic proteins. The data represent the mean ±
S.E. with 6 separate experiments.
Francisco, CA). PD98059 was obtained from Calbiochem. Thrombin and other reagents in the molecular studies were supplied from Sigma. The Limulus Amoebocyte Lysate test (i.e. an endotoxin test using the gel clot method) showed that the thrombin was endotoxin-free with the sensitivity limit of 0.066 enzyme units/ml. Activated mutants of G5123 (G5123aQL, wild types of G5123aQL5 and JNK1 dominant negative mutant (KmJNK1) were kindly provided from Dr. N. Dhanasekaran (Fels Institute for Cancer Research and Molecular Biology, Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA)).

**Cell Culture**—The Raw264.7 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Raw264.7 cells were plated at a density of 2×10⁵ cells/ml and grown to 80% confluence. The transfection efficiency was measured by mixing with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthyl-
e
dihamidochloride, and 2.5% phosphoric acid). Absorbance was measured at 540 nm after incubation for 10 min.

**Immunoblot Analysis**—SDS-PAGE and immunoblot analyses were performed according to the procedures published previously (27). Cells were lysed in the buffer containing 20 mM Tris/HCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. Cell lysates were centrifuged at 10,000×g for 10 min to remove debris. The proteins were fractionated using a 7.5% separating gel to assess the level of iNOS, whereas the 12% gel was used to detect phospho-regulated forms of the proteins. The activated JNK, p38 kinase, and ERK1/2 were measured using the respective antibody directed against the p65 or p50 protein. A competition study was carried out by adding a 20-fold excess of the unmodified NF-κB oligonucleotide. The arrowhead(s) indicate(s) the p65/p50 dimer bound with DNA (closed arrowhead) and the supershifted NF-κB DNA complex (open arrowhead). C, inhibition of NF-κB binding to the NF-κB consensus oligonucleotide by LB30057. LB30057 (10 μg/ml) inhibited the thrombin-inducible NF-κB binding to the NF-κB consensus oligonucleotide (3 h). The results were confirmed by repeated experiments.

**Gel Retardation Assay**—A double-stranded DNA probe for the consensuses of nuclear factor-κB (NF-κB, 5'-AGTTGAGGGGACTTTCCCAGGC-3') was used for gel shift analysis after end-labeling of the probe with γ-32P ATP and T4 polynucleotide kinase. The reaction mixture contained 2 μl of 5× binding buffer containing 20% glycerol, 5 mM MgCl₂, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly (dI-dC), and 50 mM Tris-Cl (pH 7.5), 8 μg of nuclear extracts, and sterile water in a total volume of 10 μl. Incubations were initiated by addition of 1-μl probe (10⁶ cpm) and continued for 20 min at room temperature. In some experiments, an aliquot of nuclear extracts (8 μg each) was incubated with 2 μg of highly specific anti-p65 and/or p50 antibody (NF-κB) at room temperature for 1 h, according to the method described previously (28). Samples were loaded onto 4% polyacrylamide gels at 100 V. The gels were removed, fixed, and dried, and then autoradiographed.

**Immunocytochemistry of p65**—Cells were grown on Lab-TEK chamber slides® (Nalge Nunc International Corp.) and incubated in serum-deprived medium for 24 h. Standard immunocytochemical method was used as described previously (29). For immunostaining, cells were fixed in 100% methanol for 30 min and washed three times with PBS. After blocking in 5% bovine serum albumin in PBS for 1 h at room temperature or overnight at 4 °C, cells were incubated for 1 h with polyclonal rabbit anti-p65 antibody (1:100) in PBS containing 0.5% bovine serum albumin. Cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:100) after serial washings with PBS. Counter-staining with propidium iodide verified the location and integrity of nuclei. Stained cells were washed and examined using a laser scanning confocal microscope (Leica TCS NT, Leica Microsystems, Wetzlar, Germany).

**Transient Transfection**—Cells were plated at a density of 0.5×10⁵ cells/well in a 6-well dish and transfected the following day. Briefly, cells were incubated with G5123a-W or G5123aQL plasmid (1 μg each of the plasmid DNA) and 3 μl of LipofectAMINE® reagent (Invitrogen) in 1 ml of antibiotic-free MEM for 3 h. Culture medium was changed with serum-free MEM with antibiotics, and cells were further incubated for 12 h to assess iNOS expression and for 1–3 h to monitor NF-κB activity. The transfection efficiency was ~50%, as determined by transfection
with the lacZ reporter gene. For phorbol 12-myristate 13-acetate (PMA) experiments, cells were treated with 1 μM PMA for 18 h and transfected with G232QL plasmid.

Stable Transfection with JNK1 Dominant Negative Mutant Plasmid—Cells were transfected using Transfectam® according to the manufacturer's instruction (Promega, Madison, WI). Raw264.7 cells were replated 24 h before transfection at a density of 2 × 10⁶ cells in a 10-cm² plastic dish. Transfectam® (20 μl) was mixed with 10 μg of a JNK1 dominant negative mutant (JNK1(-)) plasmid in 2.5 ml of minimal essential medium (MEM). Cells were transfected by addition of MEM

FIG. 3. Nuclear translocation of p65, phosphorylation of I-κBα, and degradation of I-κBα by thrombin. A, immunofluorescence subcellular localization of the p65 protein. The p65 protein was immunocytochemically detected using anti-p65 antibodies. Thrombin (10 units/ml) caused the p65 protein to migrate toward the nucleus or to translocate into the nucleus over a period ranging from 30 min to 3 h. The same fields were counter-stained with propidium iodide to locate the nuclei. B, phosphorylation of I-κBα and I-κBβ degradation. The phosphorylated I-κBα and I-κBβ protein levels were both immunocytochemically determined in the cells treated with thrombin between 15 min and 3 h. The data represent the mean ± S.E. with 3 separate experiments (significant as compared with control, *, p < 0.05; **, p < 0.01).
containing each plasmid and Transfectam\textsuperscript{\textregistered} and then incubated at 37 °C in a humidified atmosphere of 5% CO\textsubscript{2} for 6 h. After addition of 6.25 ml of MEM with 10% fetal bovine serum, cells were incubated for an additional 48 h, and geneticin was added to select the resistant colonies. In the present study, a mixture of stably transfected JNK1(−) clones were used. JNK1(−) cells had no inducible JNK activity, as monitored by phosphorylation of glutathione S-transferase-c-Jun (stimulation by 10 μg/ml bovine collagen/I) for 1 h. The expression of JNK1, but not JNK2, was decreased 43% by JNK1(−) transfection.

Statistical Methods—One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at \( p < 0.05 \) or \( p < 0.01 \).

RESULTS

Induction of iNOS by Thrombin—The effect of thrombin on NO production in macrophages was next assessed. Thrombin (10 units/ml, 12 h) increased the NO production by 4–5-fold. NO production was maintained for at least 48 h (Fig. 1A). Western blot analysis confirmed iNOS induction by thrombin at the time points examined (Fig. 1A). LPS (1 μg/ml), which was used as a positive control, induced iNOS with a concomitant increase in NO production (Fig. 1A). The extent of iNOS induction by thrombin was comparable with that by LPS. iNOS induction by thrombin was potentiated by the presence of serum (data not shown). In order to characterize the effects of thrombin on the iNOS expression per se, the subsequent experiments were conducted with cells starved of serum for 24 h.

The effect of varying thrombin concentrations on the iNOS expression was next examined. Although 1 unit/ml thrombin (i.e. equivalent to the plasma concentration observed in healthy control animals (30)) affected iNOS expression minimally, 10 units/ml thrombin or greater notably induced the protein (Fig. 1B). This was in agreement with the Western blot results.

Therefore, thrombin exhibited a threshold effect. The level of thrombin was increased severalfold by sepsis with a reciprocal decrease in the ATIII levels (7, 10). Hence, the concentration used in this study was considered appropriate for assessing the role of thrombin in sepsis.

LB30057 is a direct thrombin inhibitor with a \( K_{i} \) of 0.38 nM (31). Treatment of the Raw264.7 cells with 1 μM or greater LB30057 completely blocked the induction of iNOS by thrombin (18 h), which demonstrated that thrombin induces iNOS per se presumably through the thrombin receptor (Fig. 1C). Reverse transcriptase-PCR analysis confirmed that the iNOS induction by thrombin accompanied an increase in the mRNA level (data not shown).

Activation of NF-κB Transcription Factor—iNOS expression is controlled primarily by the transcription factor, NF-κB (32). In order to determine whether or not the iNOS induction by thrombin was mediated by NF-κB activation, the nuclear extracts prepared from the cells treated with thrombin for 0.5–12 h were probed with the radiolabeled NF-κB consensus oligonucleotide (Fig. 2A). NF-κB was activated by thrombin with a band intensity of a slow migrating p65/p50 complex being increased from 30 min to 12 h. The p50/p50 homodimer complex migrated slightly faster in the cells treated with thrombin. Supershift analysis was carried out using anti-p65 and anti-p50 antibodies to confirm whether or not the retarded band consisted of the p65 and p50 proteins. A 20-fold excess of the NF-κB probe abolished the band retardation (Fig. 2B). Either anti-p65 or the anti-p50 antibodies supershifted the retarded band. The addition of both anti-p65 and anti-p50 antibodies also caused a supershift with the reduction in the band intensity of the p65/p50 complex (Fig. 2B). These results suggest that the p65 and p50 proteins were the components actively

![Fig. 4. NF-κB-mediated induction of iNOS by activated Gα12Q12. A, induction of iNOS by thrombin in the cells transfected with the plasmid encoding the wild type Gα12 (Gα12,W) or Gα13 (Gα13,W), or by an activated mutant of Gα12 (Gα12Q12L) or Gα13 (Gα13Q12L) (1 μg DNA per well). The control cells were transfected with the pcDNA plasmid. The effect of Gα12Q12L (1 μg DNA per well, negative control) on iNOS expression was compared with that by the tumor necrosis factor-α (TNF-α, 5 ng/ml, 18 h). The cells transfected with the Gα12,W, Gα13,W, Gα12Q12L, or Gα13Q12L plasmid (0.25 μg DNA per well) were exposed to thrombin (10 units/ml) for 12 h. The cells transfected with the Gα12Q12L plasmid (0.25 μg of DNA per well) were used as a negative control. The iNOS protein and nitrite production levels were monitored 12 h after transfecting the Raw264.7 cells (3 h) with the plasmid encoding for Gα12Q12L plasmid. Each lane contained 8 μg of the nuclear extracts and 5 ng of the labeled NF-κB-binding oligonucleotide.](http://www.jbc.org/)

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
The effect of PMA pretreatment on NF-κB-mediated induction of iNOS by thrombin. A, inhibition of thrombin-inducible iNOS expression by PKC depletion. The iNOS protein and nitrite production were monitored 24 h after exposing the PMA-pretreated (18 h) cells to thrombin (10 units/ml). The data represent the mean ± S.E. of 4 separate experiments (significant compared with the control, **, *p < 0.01; significant compared with thrombin, ##, *p < 0.01). B, gel shift analysis of NF-κB binding to DNA. The NF-κB binding activity was assessed 6 h after exposing the PMA-pretreated (18 h) cells to thrombin. C, effect of PKC depletion on I-κBα phosphorylation and degradation by thrombin. The phosphorylated I-κBα and I-κBε proteins were immunocytochemically assessed 15–30 min after exposing the PMA-pretreated (18 h) cells to thrombin. D, inhibition of Gα12,13- or Gα15,16-inducible iNOS expression by PKC depletion. iNOS induction in the cells transfected with the Gα12,13-QL plasmid was compared with that in the Gα12,13-QL-transfected PKC-depleted cells. The iNOS level was assessed 18 h after transfecting the cells that had been pretreated with PMA for 18 h with the plasmid encoding for Gα12,13-QL. Results were confirmed by repeated experiments.

Because p65 was the major component of the NF-κB activated by thrombin, this study determined the translocation of p65 into the nucleus. The Raw264.7 cells were treated with thrombin for 30 min to 3 h, fixed, and permeabilized. Immunocytochemistry showed that the p65 protein was located mainly in the cytoplasm of the control cells (Fig. 3A). In contrast, the p65 protein moved into the nucleus after the thrombin treatment. The nuclear integrity was confirmed by propidium iodide staining of the identical cells (Fig. 3A). The proteolytic degradation of the I-κBα subunit preceded the translocation of NF-κB to the nucleus. These studies were extended to determine whether or not NF-κB activation by thrombin resulted from the degradation of I-κBα (Fig. 3B). I-κBα phosphorylation preceded I-κBα degradation. Thrombin (10 unit/ml) increased I-κBα phosphorylation at 15 min (Fig. 3B). The I-κBα level was subsequently decreased between 30 min and 1 h. Therefore, thrombin activates NF-κB through I-κBα degradation following its phosphorylation.

iNOS Induction and NF-κB Activation by Gα12,13— Previously, it was reported (20) that thrombin binding to its receptor stimulates the guanine nucleotide exchange of Gα12 subfamily proteins. This study was interested in whether or not the Gα12,13 subunits were responsible for NF-κB-mediated iNOS induction. Gα12,13-QL or Gα15,16-QL expression also notably increased NO production in the cells (Fig. 4A). In order to confirm that thrombin induced iNOS in the Raw264.7 cells through the Gα12,13 pathway, the cells were transfected with the either Gα12,13-W, Gα13,15-W, Gα13,16-QL, or Gα13,15-QL (Gα12 or Gα15 activated mutant) plasmid and then treated with thrombin. Thrombin induced iNOS in the cells transfected with the Gα12,13-W plasmid, which was comparable with that by either Gα13,14-QL or Gα15,16-QL. iNOS was not inducible in the cells transfected with the Gα15,16-QL (Gα15 activated mutant) plasmid, which was used as a negative control. This study next determined whether or not the activated mutants of Gα12,13 stimulated p65/p50 complex binding to the NF-κB consensus oligonucleotide. Either Gα13,15-QL or Gα15,16-QL increased the band intensity of the p65/p50 DNA complex (Fig. 4B).

PKC-mediated NF-κB Activation by Thrombin Downstream...
of \( \alpha_{1,2,3} \) — Thrombin induced cell differentiation via the PKC-dependent pathway (34). The potential role of PKC in the NF-\( \kappa \)B-mediated iNOS induction by thrombin was assessed in the PKC-depleted cells. The cells were treated with PMA for 18 h for PKC depletion, which was followed by exposure to thrombin (10 units/ml) for 24 h. Pretreatment of the cells with...
PMA completely inhibited both the iNOS induction and the increase in NO production by thrombin (Fig. 5A). PKC depletion also inhibited NF-κB activation, I-κBα degradation, and I-κBα degradation by thrombin (Fig. 5, B and C). These results show that PKC plays a role in the I-κBα phosphorylation by thrombin.

In the case of the pathways of the G₁₂ protein-coupled receptors, the PKC-dependent phosphorylation is linked to G₁₂ activation (35). As an approach to determine whether or not PKC controlled iNOS induction downstream of G₁₂, iNOS expression was monitored in the G₁₂ QL-transfected cells, which had been depleted of PKC. PKC depletion completely inhibited the iNOS induction by G₁₂ QL (Fig. 5D).

The Role of MAP Kinases in the iNOS Induction by Thrombin—The MAP kinases were involved in iNOS expression (36). Subsequently, we determined whether the MAP kinases including JNK, p38 kinase, and ERK1/2 controlled iNOS induction by thrombin. Thrombin activated all three MAP kinases in the Raw264.7 cells (Fig. 6A). JNK and ERK1/2 phosphorylation was distinct at 30 min to 1 h, which gradually returned toward the control levels (i.e. 3–6 h). p38 kinase was only weakly phosphorylated by thrombin. Cells stably transfected with the dominant negative mutants or chemical inhibitors were used to assess the role of each MAP kinase in the induction of iNOS. JNK inhibition by the stable transfection with a dominant negative mutant of JNK1 (JNK1(-)) completely suppressed iNOS induction by thrombin (Fig. 6B). In contrast, p38 kinase inhibition by SB203580 (10 μM) enhanced the enzyme expression (Fig. 6C). PD98059 (50 μM) failed to affect the iNOS expression level. Hence, the induction of iNOS by thrombin was regulated by the distinct and opposed functions of JNK and p38 kinase.

Coupling of G₁₂ to JNK—The activation of the G proteins stimulates the MAP kinases in a variety of cells (26, 37, 38). This study determined whether or not the MAP kinase pathways were connected with G₁₂ by using either JNK1(-) cells or chemical inhibitors. First, the iNOS protein was monitored in the JNK1(-) cells that were transiently transfected with the plasmid encoding for either G₁₂ QL or G₁₃ QL. The expression of the active mutant of G₁₂ failed to induce iNOS in the JNK1(-) cells (Fig. 7). SB203580 slightly enhanced iNOS induction in the cells expressing G₁₂ QL (Fig. 7). PD98059 did not alter the induction of iNOS by G₁₃ QL or G₁₃ QL. Therefore, JNK and p38 kinase oppositely function downstream of G₁₃.

JNK-dependent Degradation of Phosphorylated I-κBα—In order to assess whether or not the JNK pathway controls the NF-κB activation by thrombin, the nuclear extracts, which were prepared from the cells treated with thrombin for 3 h, were probed with the radiolabeled NF-κB consensus oligonucleotide. In the JNK1(-) cells, thrombin did not increase the NF-κB binding activity (Fig. 8A, left). The basal NF-κB binding to DNA was lower in the JNK1(-) cells than in the control cells (Fig. 8A, right). This is consistent with the observation that the nuclear translocation of p65 was blocked by the JNK1(-) stable transfection (Fig. 8B). However, I-κBα was not degraded by the presence of thrombin in the JNK1(-) cells (Fig. 8C). The phosphorylated I-κBα level in the JNK1(-) cells treated with thrombin was comparable with that of the control (Fig. 8C). These results support the notion that the JNK pathway is responsible for the degradation of phosphorylated I-κBα.
DISCUSSION

Overproduction of the proinflammatory cytokines in sepsis (e.g., tumor necrosis factor-α) is critically involved in activating the coagulation system, leading to vascular hyporeactivity and disseminated intravascular coagulation (39, 40). LPS and/or the cytokines induce iNOS expression in many cell types (41). NO and the major inflammatory mediators are produced mainly by activated macrophages. Excessive NO production by iNOS plays a crucial role in activating the immune system and in the proinflammatory effects during LPS-induced septic shock (42–44). In particular, NO induces the collapse of vascular reactivity and the pathologic alterations (13, 45). In clinical
situations, there is a discrepancy between the serum endotoxin level and the mortality of the patients with Gram-negative sepsis (46). The persistent NO production in septic patients may result from other unknown mediator(s) that are generated by LPS. The endogenous coagulation inhibitors reverse the vascular hyporeactivity induced by LPS (16, 18). Protein C and ATIII have been studied extensively as the protective modulators of septic shock (47, 48).

Thrombin, a serine protease of the trypsin family, is a key enzyme of the blood clotting system. It is the key coagulant molecule that is commonly involved in two independent (e.g., contact and extrinsic systems) activation pathways. The coagulation pathway involves a series of reactions, which culminate in the production of sufficient thrombin (6). Thrombin converts fibrinogen to fibrin and participates in regulating numerous physiological and pathological processes. Thrombomodulin serves as a thrombin receptor. When thrombin is bound to thrombomodulin, it loses its procoagulant activity, and its inactivation by ATIII is accelerated with an enhancement of protein C activation (6). The generation of excessive thrombin leads to thrombosis, which is the major cause of morbidity and mortality. Thrombin in close proximity to the active mediators also plays a role in the diverse cellular responses in the vascular and avascular tissues (49). It has been reported that thrombin potentiates both interferon-γ and tumor necrosis factor-α-induced NO production in C6 glioma cells (50). Thrombin stimulates the proliferation of smooth muscle cells and vascular disturbances through NF-κB activation (51). This study found for the first time that thrombin induces iNOS in the macrophages via members of the Gα13 family. The inflammatory cytokines exert their biological effects through the cyto-kine receptor superfamily. Hence, the regulatory mechanisms for iNOS induction by thrombin appear to differ from those by inflammatory cytokines. This study found a threshold effect for thrombin in iNOS induction. The concentration of 10 units/ml markedly increased iNOS expression, whereas 1 unit/ml thrombin has a minimal effect. The threshold effect may reflect the septic pathological situation, wherein the level of unbound activated thrombin is increased as a result of the conversion of prothrombin to thrombin and the reciprocal consumption of ATIII in sepsis.

NF-κB is a pleiotropic regulator of many genes (e.g., iNOS) involved in the immune and inflammatory responses (32). This study found that iNOS induction and excess NO production by thrombin are mediated primarily by NF-κB activation. NF-κB exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor (52). Thrombin was found to activate the p65/p50 NF-κB DNA binding complex and to induce the nuclear translocation of the p65 protein.

In this study, there is the role of the Gα12 family members downstream of thrombin signaling (35) in NF-κB-mediated iNOS induction was verified by experiments using an activated mutant of Gα12 or Gα13. It was shown that thrombin induces the stress fiber assembly via Gα12- or Gα13-coupled receptor activation (20). The Gα-coupled receptor activates the downstream signals in a PKC-dependent, fully PKC-independent, or partially PKC-dependent pathway (54, 55). Thrombin differentiates the normal lung fibroblasts to a myofibroblast phenotype via its receptor via a protein kinase C-dependent pathway (34). Macrophage activation by external stimuli causes the phosphorylation and degradation of IκBα. IκB kinase activation by LPS is dependent on PKC and ERK (56). This study found that PKC was involved in the NF-κB-mediated iNOS induction by thrombin via the phosphorylation and degradation of IκBα. PKC depletion prevented the induction of iNOS by the activated mutants of Gα12/13, which raised the notion that the PKC pathway functions downstream of Gα12/13 activation.

Thrombin activated ERK in the endothelial cells (57). While this report was being revised, it was reported that thrombin activated p38 kinase in the platelets, which led to NF-κB-dependent leukocyte recruitment (58). In this study, it was found that all three MAP kinases JNK, p38 kinase, and ERK1/2, were activated by thrombin. Among the MAP kinases, the JNK pathway was responsible for the induction of iNOS by thrombin, which was strongly supported by the lack of iNOS induction in the thrombin-treated JNK1(−) cells. The p38 kinase pathway oppositely regulated iNOS induction by thrombin. ERK1/2 activation was not responsible for iNOS induction, as evidenced by the results from the chemical inhibitor. The lack of iNOS induction by Gα12/13 in the JNK1(−) cells supports the concept that JNK serves as an essential pathway downstream of the Gα12/13 Proteins. Again, JNK and p38 kinase oppositely control the NF-κB-mediated iNOS induction by the activated mutants of Gα12/13. Thus, the induction of iNOS by thrombin was regulated by the opposed functions of JNK and p38 kinase downstream of Gα12/13.

The gel shift and immunoblot analyses revealed that the pathway involving JNK controlled NF-κB activation in response to thrombin. The increase in NF-κB DNA binding activity and the nuclear translocation of p65 were both completely abolished by the JNK1(−) transfection. The diminished NF-κB DNA binding activity in the JNK1(−) cells may be due in part to the decrease in the basal NF-κB activity. This study found for the first time that thrombin failed to degrade IκBα in the JNK1(−) cells despite its IκBα phosphorylation. These results strongly support the belief that the JNK pathway was responsible for degrading the phosphorylated IκBα. The time course in IκBα phosphorylation and degradation by thrombin paralleled that in JNK activation.

The ubiquitin-proteasome pathway controls the timed destruction of the phosphorylated IκBα in order to activate NF-κB (33, 59). Recently, it was shown that the activation of the stress-activated protein kinase, JNK, by the forced expression of the constitutively active mutants of JNKK2 and members of the Jun family leads to the accumulation of β-TrCP, which mediates the ubiquitination of the phosphorylated IκBα via the recruitment of a ubiquitin ligase complex (53). Therefore, it is highly likely that the accumulation of phosphorylated IκBα and the failure of IκBα degradation by thrombin in the JNK1(−) cells might result from the inhibition of the ubiquitin-proteasome pathway.

In summary, this study demonstrated that thrombin plays an important role in the vascular responsibility by inducing iNOS and NO production. In addition, thrombin activates the pathway coupled with Gα12/13 for enzyme induction. Gα12/13 activation then leads to the PKC-dependent phosphorylation of IκBα and the JNK-mediated IκBα degradation. The cellular signaling pathways, by which thrombin induces iNOS, may serve as the pharmacological targets for both preventing and treating vascular hyporeactivity in septic patients.

REFERENCES

1. Venet, C., Zeni, F., Viallon, A., Ross, A., Pain, P., Gery, P., Page, D., Vernesch, R., Bertrand, M., Bancou, F., and Bertrand, J. C. (2000) Intensive Care Med. 26, 538–544
2. Prins, J. M., Schultz, C., Speelman, P., and van Deventer, S. J. (1996) FEMS Immunol. Med. Microbiol. 16, 283–289
3. van der Poll, T. (2001) J. Endotoxin Res. 7, 301–304
4. Nieuwland, R., Berckmans, R. J., McGregor, S., Boing, A. N., Remijn, F. P., Westendorp, R. G., Hald, C. E., and Sturk, A. (2000) Blood 95, 930–935
5. Mavrommatis, A. C., Theodoridis, T., Orfanidou, A., Reusser, C., Christopoulos-Kokkinou, V., and Zakythinos, S. (2000) Crit. Care Med. 28, 451–457
6. Esmon, C. T. (2000) Biochim. Biophys. Acta 1477, 349–360
7. Mavrommatis, A. C., Theodoridis, T., Konomou, M., Kotanioud, A., El Ali, M., Christopoulos-Kokkinou, V., and Zakythinos, S. G. (2001) Intensive Care
Thrombin Induces Nitric-oxide Synthase via $G_\alpha_{12/13}$-coupled Protein Kinase C-dependent I-κBα Phosphorylation and JNK-mediated I-κBα Degradation

Keon Wook Kang, So Yeon Choi, Min Kyung Cho, Chang Ho Lee and Sang Geon Kim

J. Biol. Chem. 2003, 278:17368-17378.
doi: 10.1074/jbc.M300471200 originally published online February 26, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300471200

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 59 references, 22 of which can be accessed free at http://www.jbc.org/content/278/19/17368.full.html#ref-list-1