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Distinct Involvement of NF-κB and p38 Mitogen-Activated Protein Kinase Pathways in Serum Deprivation-Mediated Stimulation of Inducible Nitric Oxide Synthase and Its Inhibition by 4-Hydroxynonenal

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Abstract Cytokine-induced expression of inducible nitric oxide synthase (iNOS) and concomitant production of nitric oxide (NO) involve activation of mitogen-activated protein (MAP) kinases and are in most cases mediated by the transcription factor NF-κB. We investigated the role of p38 MAP kinase activation and IκB phosphorylation in iNOS expression in a novel iNOS-inducing model in mouse macrophages. Deprivation of serum from the culture medium of RAW 264.7 cells up-regulated iNOS and NO production, which were inhibited by 4-hydroxy-2-nonenal (HNE), a component of oxidatively modified low-density lipoprotein (oxLDL). Serum withdrawal induced phosphorylation of Akt, IκB, and p38 MAP kinase. Pretreatment with the potent PI3 kinase inhibitor wortmannin, the NF-κB inhibitor PDTC or the specific p38 MAP kinase inhibitor SB203580 each partially attenuated the induction of iNOS and NO production, demonstrating that both p38 activation and IκB phosphorylation are required for iNOS expression. SB203580, however, did not prevent the phosphorylation of Akt and IκB, suggesting that the p38 MAP kinase signal contributes to iNOS gene expression through an IκB-phosphorylation-independent pathway. HNE, which markedly inhibited iNOS expression and NO production, prevented the serum withdrawal-triggered IκB phosphorylation but not that of Akt or p38 MAP kinase. A high concentration of HNE stimulated dephosphorylation of IκB but promoted activation of p38 MAP kinase. Taken together, these results suggest that NF-κB and p38 MAP kinase lie in separate signal pathways for serum deprivation-stimulated iNOS expression and NO production. HNE selectively suppresses the former pathway, targeting a site downstream of Akt. J. Cell. Biochem. 83: 271–280, 2001. © 2001 Wiley-Liss, Inc.

Key words: macrophage; NF-κB; p38 MAP kinase; nitric oxide; 4-hydroxynonenal

Nitric oxide (NO), a potent messenger molecule produced by nitric oxide synthase (NOS), mediates a wide range of biological effects involved in neurotransmission, antimicrobial defense, and vascular homeostasis. Among the three major types of NOS, inducible NOS (iNOS), expressed mainly in activated macrophages, is transcriptionally controlled following general or local inflammatory response. While they contribute to a principle mechanism of macrophage cytotoxicity [Nathan, 1992; MacMicking et al., 1997], iNOS and concomitant NO are also thought to be involved in many pathological conditions, including infections [Koprowski et al., 1993], ischemia [Iadecola et al., 1997], and multiple sclerosis [Merrill et al., 1993]. The signal pathway for transcriptional regulation of iNOS is not yet clear, although it has been extensively studied in iNOS expression in murine macrophages that is induced by proinflammatory cytokines, such as tumor necrosis factor (TNF), interferon-γ (IFN-γ), or interleukin-1 (IL-1).

A known main regulator of iNOS gene expression is the transcription factor NF-κB, a DNA-binding protein important for regulation of various genes involved in immune and in-

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flammatory responses. Activation of NF-κB requires ubiquitin-dependent degradation of the corresponding inhibitory proteins IκB that retain inactive NF-κB in the cytosol [Thanos and Maniatis, 1995; Mercurio et al., 1997]. Cytokines lead to the phosphorylation of IκB by IκB kinase (IKK) at two serine residues at its N-terminus, and phosphorylated IκB is then removed from the NF-κB complex, allowing the translocation of NF-κB into the nucleus [Traenckner et al., 1995; Mercurio et al., 1997; Alpert et al., 1999]. It has become clear that the phosphorylation of IκB at the two serines is in most cases the key step in NF-κB activation. In addition to proinflammatory stimuli, many other factors induce IκB phosphorylation, such as insulin in Chinese hamster ovary cells [Bertrand et al., 1998], PDGF in fibroblasts [Romashkova and Makarov, 1999], and αvβ3 integrin in endothelial cells [Scatena et al., 1998], through a PI3-kinase/Akt-dependent or independent signal pathway. In those cases, the activation of NF-κB is related to anti-apoptosis and cell survival maintenance. Recently, it has been reported that TNF activation of NF-κB also requires Akt activation [Ozes et al., 1999], implying a role of this serine-threonine kinase in the regulation of iNOS at an upstream step of NF-κB.

On the other hand, recent studies have indicated that the mitogen-activated protein (MAP) kinase cascades play an important role in cytokine-enhanced iNOS expression and that the relative contributions of the three MAP kinase subfamilies to iNOS induction vary, depending on the cell type and the agonists. For IL-1β-induced expression of iNOS, activation of JNK and p38 are necessary in rat glomerular mesangial cells [Guan et al., 1999], and ERKs and p38 are required in cardiac myocytes [LaPointe and Isenovic, 1999]. In RAW 264.7 cells, both ERKs and p38 are required for the up-regulation of iNOS by lipopolysaccharide (LPS) plus IFN-γ but not for that by LPS alone [Ajizian et al., 1999]. It has also been reported that JNK and its upstream kinases, but not ERKs or p38, is involved in the regulation of iNOS by costimulation of mouse macrophages with IFN-γ and TNF-α [Chan et al., 1999].

Oxidized low-density lipoprotein (oxLDL) is known to alter the expression of inflammatory gene products in mononuclear phagocytes. The effects of oxLDL on atherogenesis may be partly mediated by altering NO production in vascular cells. The results of many studies have suggested that oxLDL decreases the production of NO from macrophages stimulated by LPS and IFN-γ through inhibition of iNOS expression [Dulak et al., 1999; Huang et al., 1999], though it has also been reported that oxLDL decreases iNOS enzyme activity without altering iNOS protein and mRNA levels [Yang et al., 1994]. However, the mechanism and the active constituents in oxLDL involved in the inhibition of iNOS expression have not been elucidated.

In this study, we report that withdrawal of serum from mouse macrophage culture stimulates iNOS expression and NO production, which are co-operatively controlled by Akt-mediated IκB phosphorylation and p38 activation. Activation of p38 MAP kinase contributes to the iNOS expression in an IκB phosphorylation-independent pathway. 4-hydroxy-2-nonenal (HNE), an ingredient of oxLDL, suppresses iNOS synthesis through inhibition of IκB phosphorylation at a step downstream of Akt.

MATERIALS AND METHODS

Antibodies and Reagents

Rabbit polyclonal anti-iNOS antibody was purchased from Transduction Laboratories. Rabbit polyclonal anti-phospho-IκBα and anti-IκBα, anti-phospho-Akt and anti-Akt, and anti-phospho-p38 MAP kinase and anti-p38 MAP kinase antibodies were obtained from New England Biolabs. The anti-phospho-IκBα antibody is specific for phosphorylated serine 32 of IκBα, the anti-phospho-Akt antibody is specific for phosphorylated serine 473 of Akt, and the anti-phospho-p38 antibody is specific for phosphorylated threonine 180 and tyrosine 182 of p38 MAP kinase. SB203580, wortmannin, and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma and were dissolved in dimethyl sulfoxide. HNE, purity ≥98%, was obtained from Cayman Chemical as a solution in ethanol. For use in experiments, the inhibitors and HNE were added in the culture medium and were 1,000-times diluted to the final concentrations. Control experiments demonstrated that treatment of cells with the same concentration of dimethyl sulfoxide or ethanol alone had no effect on the ability of all agents tested to inhibit serum deprivation-induced iNOS induction.

Cell Culture

RAW 264.7 mouse macrophage cells (American Type Culture Collection, Manassas, VA) were cultured in plastic plates with RPMI 1604 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% CO_2, 95% air incubator. Before use in experiments, the cells were washed twice with PBS, and the culture media were replaced by RPMI 1640 with no serum.

Immunoblotting

Western blot analysis was performed essentially as described previously [Liu et al., 1999]. Briefly, cells were lysed with sample buffer (×2: 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol and 10% glycerol). Equal amounts of denatured proteins were loaded and separated on 10% SDS polyacrylamide gels, and were then transferred to a polyvinylidene difluoride membrane. After blocking with 2% BSA or 5% milk, the membrane was stained with a polyclonal specific first antibody, followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Tago, Burlingame, CA), which was visualized using the Western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA).

Determination of NO Synthesis

The amount of NO was estimated from the accumulation of stable NO metabolite nitrite in the medium by Griess assay [Green et al., 1982]. Briefly, equal volumes of culture supernatants and Griess reagents (1% sulfanilamide/0.1% *N*-[naphthyl] ethyl-enediamine dihydrochloride in 2.5% H_3PO_4) were mixed, and the absorbance was determined at 532 nm using a microplate reader. The amount of nitrite was calculated from a sodium nitrite standard curve.

RESULTS

Serum Withdrawal Stimulates iNOS Expression and NO Production That can be Inhibited by HNE

The results of many studies have suggested that oxLDL decreases NO production from macrophages [Jorens et al., 1992; Bolton et al., 1994; Yang et al., 1994]. To investigate the role of HNE in this process, we stimulated cultured RAW cells with HNE in the absence of serum. Unexpectedly, we found that mere serum-deprivation stimulated iNOS expression. As shown in Figure 1A, withdrawal of serum from the culture medium for 24 h induced the expression of iNOS protein, which is usually undetectable in non-stimulated cells. Treatment of the cells with 20 μM of HNE prevented the increase in iNOS expression, and this effect of HNE lasted up to 48 h and disappeared at 72 h after treatment (Fig. 1A, top and middle). An experiment using different concentrations of HNE showed that the inhibitory effect of HNE on iNOS expression was dose-dependent (Fig. 1A, bottom). To determine whether serum withdrawal-induced expression of iNOS is followed by an increase in NO production, we measured the amount of nitrite accumulated in the culture medium. As shown in Figure 1B, serum-cultured macrophages produced very low amounts of NO, whereas detectable levels of NO were present in the serum-depleted macrophage cultures. HNE treatment decreased NO production by ~75% in macrophages activated by serum deprivation, correlating with the results of iNOS expression.

Serum Withdrawal Induces Phosphorylation of IκB, Akt, and p38 MAP Kinase

Cytokine-mediated induction of iNOS involves an increase in IκB phosphorylation for NF-κB activation. To determine whether IκB phosphorylation was involved in the signaling of serum deprivation-triggered iNOS expression, we examined the effect of the removal of serum from the culture on IκB phosphorylation. As shown in Figure 2A, Western blot using a specific anti-phospho-IκBα antibody that recognizes IκBα only when activated by phosphorylation at Ser-32 showed a time-dependent increase in activated IκBα in response to serum deprivation, while the IκBα protein level was not affected. The phosphorylation of Akt, which was recently reported to be required for NF-κB activation by TNF through phosphorylation of IKKα [Ozes et al., 1999], was also stimulated rapidly. We next investigated whether serum deprivation activates the p38 MAP kinase, which is in many cases necessary for iNOS expression mediated by cytokines. As shown in Figure 2B, Western blot using a specific anti-phospho-p38 antibody that detects the p38 MAP kinase only when activated by phosphorylation at Thr180/Tyr182 showed an increased activation of p38 MAP kinase when the serum was removed from the culture for 4 h.

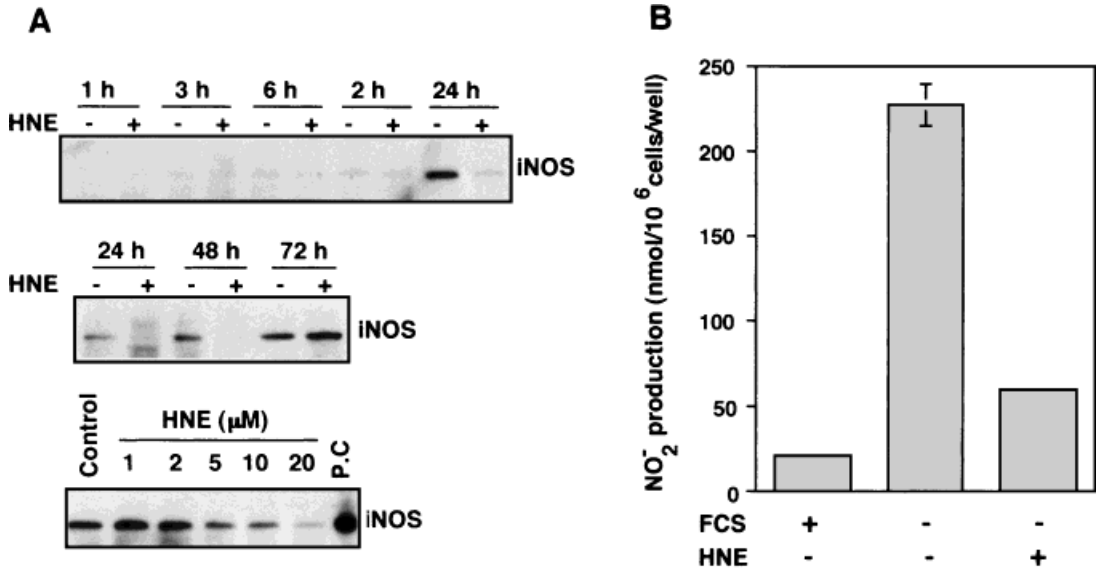

Fig. 1. Induction of iNOS expression and NO production by serum deprivation in RAW 264.7 cells and its inhibition by HNE. **A**: RAW 264.7 macrophages were incubated in a serum-free medium with or without 20 μM of HNE for indicated time periods (top and middle) or with different amounts of HNE for 24 h (bottom), for measurement of the iNOS expression level by immunoblotting. PC, positive control. These data are representative of two independent experiments. **B**: RAW 264.7 macrophages were incubated in the indicated medium for 24 h, and nitrite levels in the culture medium were assayed as described in Materials and Methods. The results are presented as means±SE of three independent triplicate measurements.

IκB Phosphorylation and p38 MAP Kinase Activation are Required for Serum Deprivation-Mediated iNOS Expression and NO Production

To determine whether IκB phosphorylation and p38 MAP kinase activation are needed for serum deprivation-mediated induction of iNOS, we examined the effects of inhibition of Akt, NF-κB, or p38 MAP kinase with wortmannin, a specific inhibitor of PI-3 kinase, pyrrolidine dithiocarbamate, an inhibitor of NF-κB, or SB203580, a specific inhibitor of p38 MAP kinase, on iNOS expression. Using different amounts of the inhibitors, we showed that each

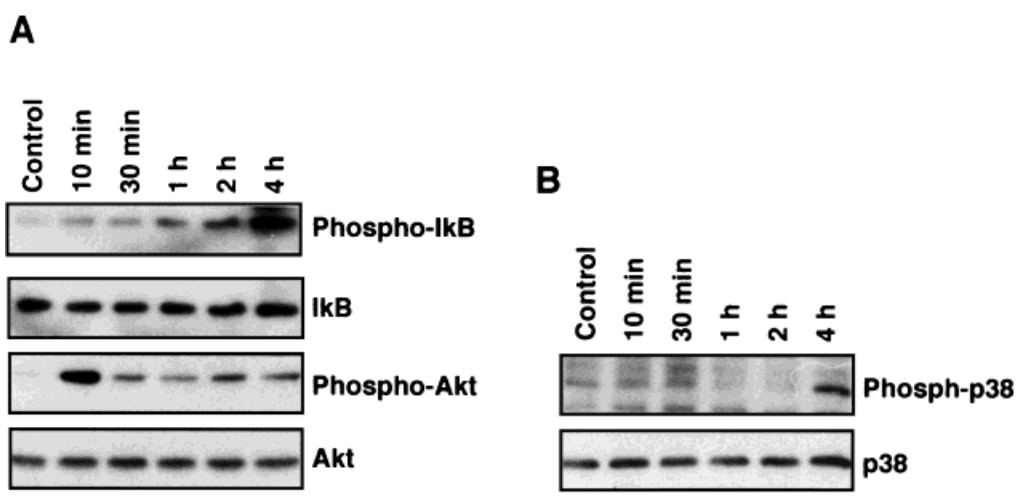

Fig. 2. Serum withdrawal stimulates phosphorylation of IκB, Akt, and p38 MAP kinase. **A**: RAW 264.7 macrophages were incubated in a serum-free medium for the indicated time periods, for measurement of phosphorylated and total IκB and Akt levels by immunoblotting. **B**: RAW cells were serum-deprived for the indicated time periods for measurement of phosphorylated and total p38 MAP kinase levels. The experiments were repeated three times and the results were reproducible.

of the inhibitors partially attenuated serum deprivation-triggered iNOS expression in a clear dose-dependent manner (Fig. 3A). We then tested the action of a combination of any two of the inhibitors on iNOS formation. The combination of SB203580 with wortmannin or PDTC showed a more extensive effect than did any of the three inhibitors alone, whereas the combination of wortmannin and PDTC did not result in a greater degree of inhibition (Fig. 3B). In a parallel experiment, we examined the effect of these inhibitors on NO production. In agreement with the results in iNOS expression, each inhibitor reduced NO formation, and a marked synergistic effect was observed in the case of SB203580 plus wortmannin treatment (Fig. 3C).

SB203580 Does not Block Phosphorylation of Akt and IκB

Previous studies have shown that p38 MAP kinase regulates iNOS expression mediated by cytokines at a dominant transcriptional level [Da Silva et al., 1997; Chan et al., 1999]. In our present system, we therefore tried to determine the possible regulatory pathway through p38 MAP kinase on NF-κB. As shown in Figure 4, treatment with any of a wide range of concentrations of SB230580, which obviously attenuated iNOS expression, failed to show any

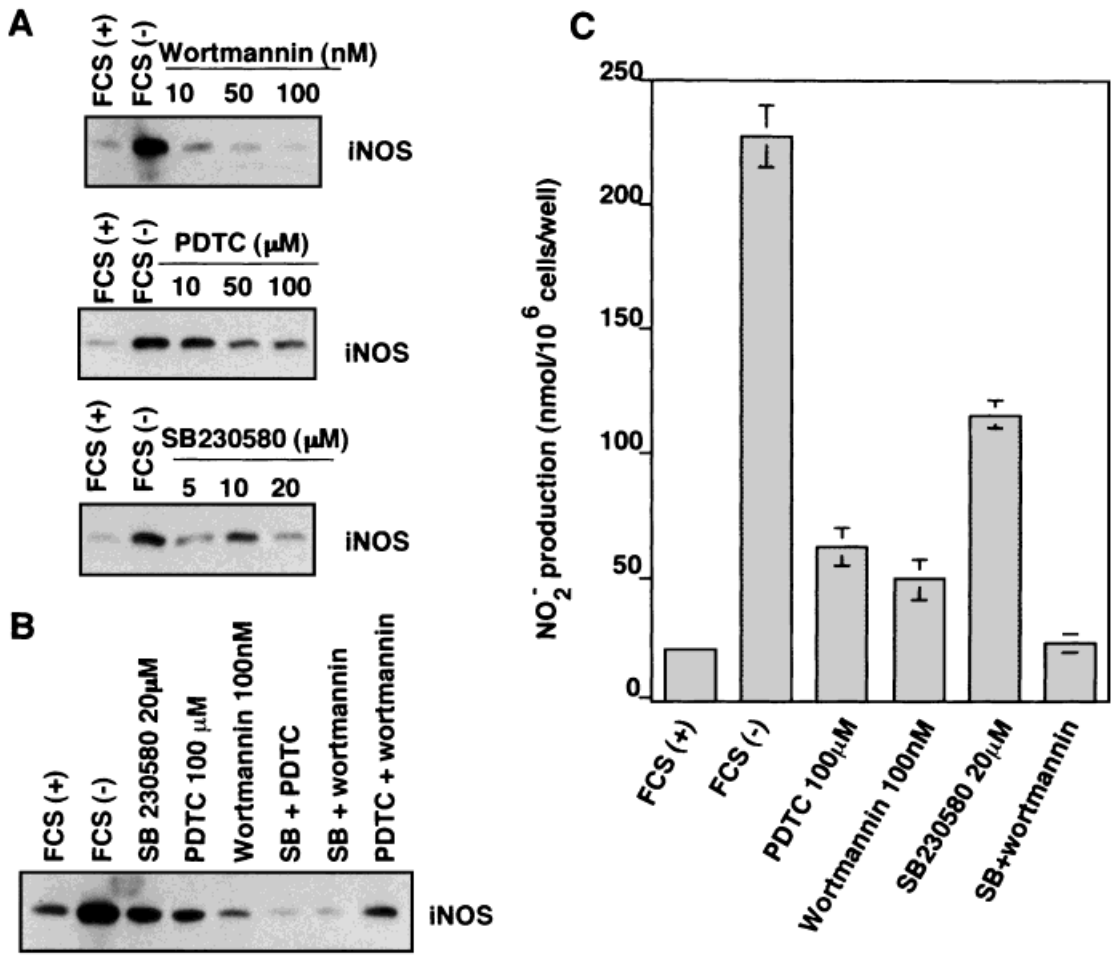

Fig. 3. Phosphorylation of IκB and p38 MAP kinase are both needed for full iNOS expression and NO production. **A** and **B**: RAW 264.7 macrophages were incubated in a serum-free medium with or without the indicated reagents for 24 h for measurement of the iNOS level by immunoblotting. The data are representative of three independent experiments (**C**). RAW 264.7 macrophages were incubated in the indicated medium for 24 h, and nitrite levels in the culture medium were assayed as described in Materials and Methods. The results are presented as means±SE of three independent triplicate measurements.

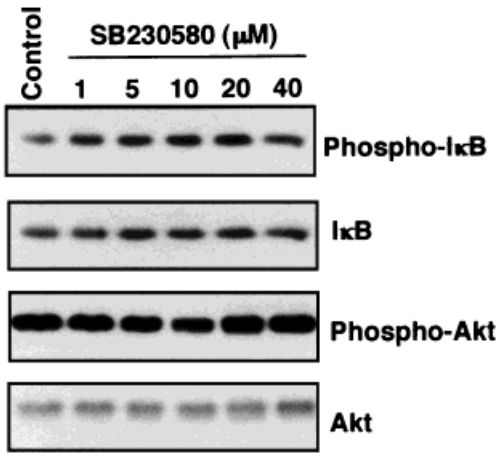

Fig. 4. SB230580 does not block Akt and IκB phosphorylation. RAW 264.7 macrophages were incubated in serum-free medium with or without the indicated concentrations of SB230580 for 4 h (upper 2 lanes) or for 10 min (lower 2 lanes) for measurement of phosphorylated and total IκB and Akt levels by immunoblotting. These data are representative of three independent experiments.

inhibitory effect on IκB phosphorylation. The same concentrations of SB230580 did not inhibit the serum withdrawal-stimulated phosphorylation of Akt either. Taking together with the data shown in Figure 3, these results indicate that p38 MAP kinase is unlikely to regulate NF-κB in this iNOS inductive process.

HNE Inhibits Phosphorylation of IκB but not Akt or P38 MAP Kinase

The observation that HNE inhibits serum withdrawal-stimulated iNOS expression, which involves the phosphorylation of IκB and activation of p38 MAP kinase prompted us to investigate whether HNE displays its inhibitory effect through influencing either of the two signaling events. As shown in Figure 5A, addition of 20 μM of HNE to the culture of RAW 264.7 cells at the start of serum-depleted culture for 4 h clearly blocked IκB phosphorylation, whereas the same concentration of HNE failed to show any inhibitory effect on serum withdrawal-stimulated phosphorylation of Akt and p38 MAP kinase. To further characterize the effect of HNE, we next treated the cells that had been previously serum-starved for 4 h with HNE for 15 min and examined the phosphorylation levels of IκB and Akt. As shown in Figure 5B, serum starvation-induced phosphorylation of IκB was partially impaired with treatment of 20 μM of HNE and completely disappeared with treatment of higher concentrations (50–100 μM) of HNE for 15 min, whereas the serum starvation-induced phosphorylation of Akt was not affected. This result raises the possibility that HNE not only inhibits phosphorylation promotion but also promotes dephosphorylation of IκB. In contrast, the addition of high concentrations (50–100 μM) of HNE into the culture promoted phosphorylation of p38 kinase time-dependently (Fig. 5C). In order to test the possibility of involvement of p38 phosphorylation in the mechanism of IκB phosphorylation prevention by HNE, we treated the cells with 20 μM of HNE in the absence or presence of different amounts of SB230580. We found that SB230580 did not change the level of the HNE-mediated IκB phosphorylation level reduction, whereas it evidently promoted the inhibition by HNE of iNOS expression (Fig. 5D). Collectively, these data demonstrated that HNE inhibits iNOS expression through selective reduction of the IκB phosphorylation level, independent of Akt or p38 kinase. The results shown in Figure 5D further confirm that p38 MAP kinase is not an upstream regulator of IκB but plays an independent role in serum deprivation-triggered signal transduction.

DISCUSSION

In this study, we demonstrated that removal of serum from the culture of a mouse macrophage cell line stimulates iNOS expression and NO production, which were mediated by both IκB phosphorylation and p38 MAP kinase activation. We further showed that the p38 MAP kinase-mediated signal is not a regulator of IκB phosphorylation in this process. We also obtained evidence showing that the lipid peroxidation product HNE inhibits iNOS synthesis through selective suppression of IκB phosphorylation, independent of p38 kinase.

Compared to the high-output production of NO induced by cytokines, serum withdrawal triggered a low-level induction of NO correlated to the weak expression of iNOS. This observation is significant because it may reflect the different functions of NO in the normal activity of macrophages. Whereas the cytokine-induced release of NO contributes the cytotoxicity of macrophages in host defense, inflammation, and immunity, serum deprivation-induced production of iNOS and NO may reflect the response of macrophages to extracellular stress

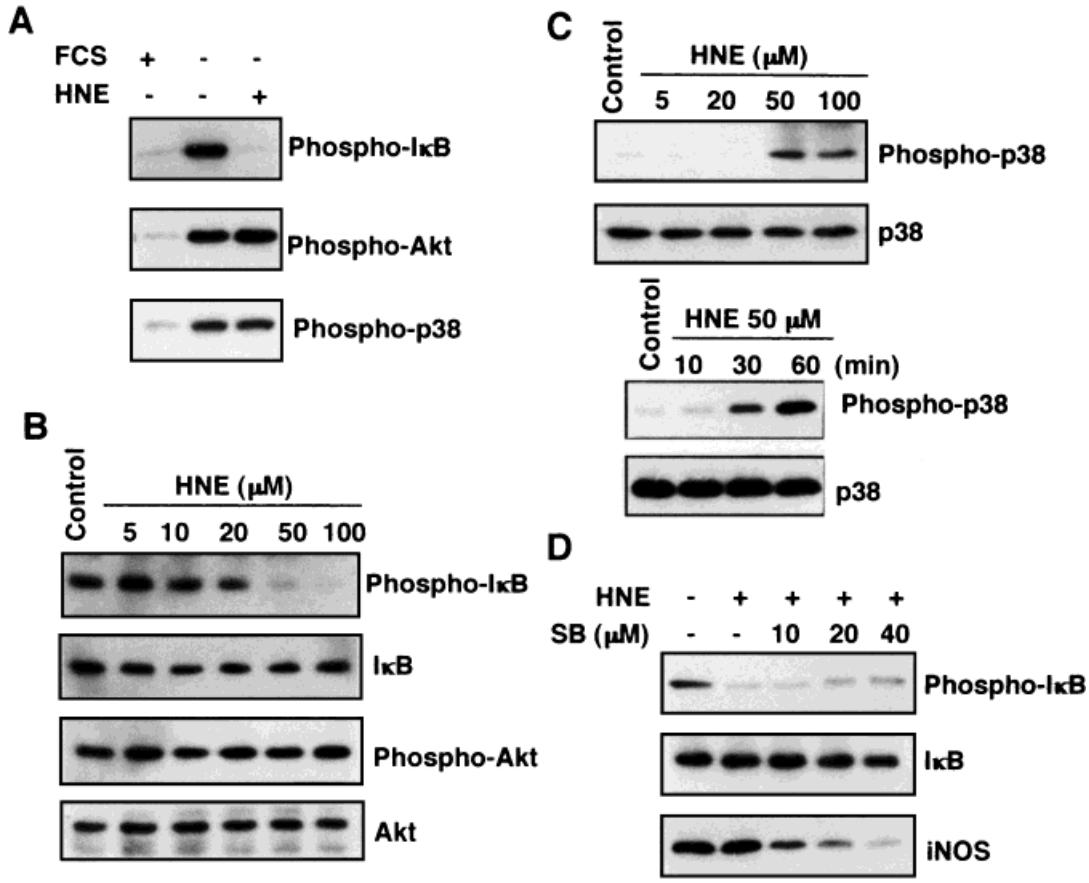

Fig. 5. Effects of HNE on IκB phosphorylation and p38 MAP kinase activation. **A**: RAW 264.7 macrophages were incubated in RPMI with 10% FCS or in a serum-free medium with or without 20 μM of HNE for 4 h (top and bottom) or for 10 min (middle) for measurement of phosphorylated and total IκB, Akt, and p38 kinase levels by immunoblotting. **B**: RAW 264.7 cells were starved in a serum-free medium for 4 h and were then treated with the indicated amounts of HNE for 15 min for measurement of phosphorylated and total IκB and Akt levels by immunoblotting. **C**: RAW 264.7 macrophages were incubated in a serum-free medium with or without the indicated concentrations of HNE for 1 h (upper 2 lanes), or with 50 μM of HNE for the indicated time periods (lower 2 lanes) for measurement of phosphorylated and total p38 MAP kinase levels. **D**: RAW 264.7 cells were treated with 20 μM of HNE in the presence or absence of the indicated amounts of SB230580 in a serum-free medium for 4 h (top and middle) or for 24 h (bottom) for measurement of phosphorylated and total IκB and iNOS levels. These data are representative of three independent experiments.

in maintaining cell homeostasis. In fact, accumulating evidence has demonstrated that iNOS is expressed not only in inflammatory cells but also in many other types of cells in which iNOS and NO are involved in the cell physiology [Kuo et al., 1997; Hattori et al., 1999] and that the effect of NO varies to a great extent depending on its concentration [Shen et al., 1998].

We analyzed the signaling pathway for the serum deprivation-induced iNOS expression and NO production promotion, and we found that the serum withdrawal-triggered iNOS expression and NO production require both p38 MAP kinase and NF-κB. Although the potential roles of $p42/44^{ERK}$ and $p54^{JNK}$ were not ruled out, we focused our study on the role of p38 MAP kinase in the mechanism of iNOS expression because serum withdrawal led to no or only slight activation of $p42/44^{ERK}$ and $p54^{JNK}$ (data not shown). Serum withdrawal stimulated p38 activation, and inhibition of p38 with its specific inhibitor SB230580 markedly suppressed the iNOS expression. However, the effect of SB230580 was incomplete, and full inhibition required the combination of SB230580 with wortmannin or PDTC, demonstrating that activation of p38 is essential but not sufficient for full serum deprivation-induced iNOS expression. Although earlier studies have shown that both p38 MAP kinase and NF-κB are involved in some cytokine-stimulated iNOS expression, the relation between these two signals

is not clear. In the present study, we tested the potential linkage between p38 MAP kinase and NF-κB linked signals for serum deprivation-induced iNOS expression and NO production promotion. The lack of an inhibitory effect by SB230580 on IκB phosphorylation, however, rendered the involvement of p38 MAP kinase in the regulation of NF-κB unlikely, demonstrating that p38 MAP kinase and NF-κB lie in two distinct pathways. p38 MAP kinase has been found to be capable of activating several transcription factors, including CREB [Iordanov et al., 1997], ATF-1 [Tan et al., 1996], and MEF2C [Han et al., 1997], and is therefore involved in the control of many processes for gene expression. P38 MAP kinase can also regulate gene expression at a post-transcriptional level [Pietersma et al., 1997; Ridley et al., 1997]. Therefore, in serum deprivation-induced iNOS expression, activation of p38 MAP kinase results in a cooperative effect with NF-κB, possibly through affecting its downstream substrate serving as a *trans*-activating factor or at the level of post-transcription.

We demonstrated that serum deprivation-stimulated iNOS expression involves activation of serine/threonine kinase Akt. It has recently been suggested that in RAW cells, LPS mediates NO production via a PI-3 kinase-independent post-translational pathway [Salh et al., 1998], because PI-3 kinase inhibitors failed to reduce PI-3 kinase and Akt were activated by LPS. However, our present study showed that serum deprivation rapidly activated Akt and that wortmannin dramatically inhibited iNOS induction, demonstrating a clear involvement of the PI-3 kinase/Akt signal in serum deprivation-mediated iNOS expression. This finding is in line with recent observations that Akt works as an upstream regulator of NF-κB: TNF-induced activation of NF-κB requires Akt [Ozes et al., 1999] and NF-κB is a target of Akt in anti-apoptotic PDGF signaling [Romashkova and Makarov, 1999]. For serum deprivation-stimulated iNOS expression, Akt serves in the NF-κB signal pathway in which IκB phosphorylation is promoted. Interestingly, HNE, a pro-oxidant that was previously demonstrated to participate in intracellular signaling either by direct binding and activation/inactivation of intracellular enzymes [Liu et al., 1999] or by changing the cellular redox status [Uchida et al., 1999; Liu et al., 2000], was found to specifically inhibit the phosphorylation of IκB without suppression of Akt. The data shown in Figure 5B also imply a possible activation of a protein phosphatase by HNE that specifically dephosphorylates phosphorylated IκB but not phosphorylated Akt. It is thought that HNE directly attacks Akt-downstream IκB regulators, possibly including IκB kinase and specific phosphatase for either inactivation or activation.

Inducible NO production in macrophages is considered to be important during atherosclerosis [Buttery et al., 1996]. However, NO was also reported to exert anti-atherogenic effects [Bath et al., 1991; Radomski et al., 1993; Dhaunsi et al., 1997]. Because macrophages incubated with oxLDL assume many characteristics typical of foam cells found in early atherosclerotic lesions [Aviram, 1996], the interactions of oxLDL with macrophages are thought to be early events leading to the development of atherosclerotic plaques. In the present study, HNE showed marked inhibitory effects on serum deprivation-stimulated iNOS expression and NO production, indicating that it is an appropriate model for elucidating the interactions of oxLDL with macrophages in a near-physiological condition. Our data suggest that HNE is an active constituent of oxLDL for inhibition of iNOS expression and NO production. Suppression of NO and other possible NF-κB-activated genes by HNE may contribute to the oxLDL-mediated induction of atherosclerosis, in which the homeostasis impairment may be crucial for advanced lesion progression [Ball et al., 1995].

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