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Okadaic Acid Induces Phosphorylation of p65NF-κB on Serine 536 and Activates NF-κB Transcriptional Activity in Human Osteoblastic MG63 Cells

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Abstract Nuclear factor-kappa B (NF-κB) is an essential transcription factor in the control of expression of genes involved in cell growth, differentiation, inflammation, and neoplastic transformation. Previously, we reported that okadaic acid (OA), which is a specific inhibitor of serine/threonine protein phosphatases, induced apoptosis in cells of human osteosarcoma cell line MG63. However, to date, it is not clear whether the phosphorylation status of NF-κB could be affected by the treatment with OA. In this report, we demonstrate that treatment of MG63 cells with OA enhanced the phosphorylation level of NF-κB, as judged from the results of Western blot analysis and a λ protein phosphatase dephosphorylation assay. The phosphorylation level of NF-κB was enhanced in both time- and dose-dependent manners. In the cells treated with 100 nM OA for 3 h, consequential translocation of NF-κB from the cytosol to the nucleus occurred. Western blotting experiments with an anti-phospho-p65NF-κB antibody disclosed that the NF-κB was phosphorylated on serine 536. Furthermore, OA stimulated the transcriptional activity of NF-κB in MG63 cells, as judged from the results of a luciferase assay. Our findings indicate that OA elicit phosphorylation of NF-κB on serine 536 in MG63 cells, resulting in the translocation of phospho-NF-κB to the nucleus, thereby promoting transcriptional activity of genes. J. Cell. Biochem. 99: 1275–1284, 2006.

Key words: apoptosis; kinases; phosphatases; transcriptional factor; transactivation

The nuclear factor-kappa B (NF-κB) pathway is a key component of cellular responses to a variety of extracellular stimuli. NF-κB is induced in response to signals that lead to cell growth, differentiation, inflammation, and neoplastic transformation [Pahl, 1999; Gilmore et al., 2002]. NF-κB has also been shown to have a role in regulating the apoptotic program, either as essential for the induction of apoptosis or as a blocker of apoptosis [Barkett and Gilmore, 1999; Karin and Lin, 2002]. NF-κB is present in the cytoplasm as two major precursor forms, either as the p50-p65 complex with the inhibiting IκBα or as a heterodimeric p65-p105 complex [Baeuerle and Henkel, 1994]. Several stimulators, including infection or LPS, induce the phosphorylation and degradation of IκBα in the target cells to activate NF-κB [Baeuerle and Henkel, 1994; Didonato et al., 1996; Barkett and Gilmore, 1999; Karin and Lin, 2002]. For activation of NF-κB, IκBα is phosphorylated, ubiquitinated, and then degraded by the protease, thereby allowing translocation of the liberated NF-κB from the cytoplasm to the nucleus where it regulates the expression of the target genes that modulate biological responses. Optimal induction

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of NF-κB target genes also requires the phosphorylation of NF-κB proteins, such as p65. However, whether and, if so, how phosphorylation of NF-κB modulates its function remains unclear. Several kinases and phosphatases are considered as candidate enzymes for involvement in the phosphorylation of NF-κB [Vermeulen et al., 2002; Viatour et al., 2005].

Okadaic acid (OA) is a toxic polyether fatty acid produced by several dinoflagellates and is a potent inhibitor of serine/threonine protein phosphatases type 1 (PP1) and type 2A (PP2A). The use of this agent has led to the understanding that the phosphorylation and dephosphorylation status is related to cellular regulation, including the biological end point, apoptosis [Fernandez et al., 2002; Haneji, 2005]. We previously reported that OA induced apoptosis in human osteoblastic osteosarcoma cells [Morimoto et al., 1997, 1999; Kito et al., 2003], human oral squamous carcinoma cells [Fujita et al., 1999, 2004; Goto et al., 2002; Okamura et al., 2004, 2005]. Protein kinases and phosphatases were reported to be involved in transcriptional stimulation through activation of the NF-κB pathway [Yang et al., 2001; Tan et al., 2002]. We recently reported that the PKR/eIF-2α pathway was activated and that NF-κB translocation occurred during the OA-induced apoptosis [Morimoto et al., 2004]. However, details of the mechanisms of OA-mediated NF-κB phosphorylation are still obscure. In the present study, we examined the phosphorylation status of NF-κB in MG63 cells treated with OA. Our results indicate that the p65 component of NF-κB is phosphorylated in the cytosol and translocated to the nucleus. Our results also indicate that the OA treatment enhanced the phosphorylation status of serine residue positioned at 536 in p65NF-κB in MG63 cells.

MATERIALS AND METHODS

Materials

OA was purchased from Wako (Osaka, Japan), and stock solutions (100 μM), prepared in dimethyl sulfoxide and protected from the light, were diluted to the appropriate concentrations with medium. Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS); and α-modified Eagle's minimal essential medium (α-MEM), from Gibco BRL (Gaithersburg, MD). Plastic dishes were from Iwaki (Chiba, Japan). Anti-p65NF-κB rabbit polyclonal antibody (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p65NF-κB (Ser276) and anti-phospho-p65NF-κB (Ser529) rabbit polyclonal antibodies were obtained from Rockland Immunochemicals (Gilbertsville, PA). Anti-phospho-p65NF-κB (Ser536) rabbit polyclonal antibody was obtained from Cell Signaling Technology (Beverly, MA). Other materials used were of the highest grade commercially available.

Cell Culture

Human osteosarcoma cell line MG63 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in α-MEM containing 10% (v/v) FBS and were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was replenished every 3 days. Confluent cells were treated with various concentrations of OA for variable periods of time. Cell modification was monitored with the use of an Olympus IMT-2 phase-contrast microscope. For immunocytochemistry MG63, cells were grown on sterile 18-mm round glass coverslips placed in 60-mm plastic dishes and cultured for the desired periods.

Immunocytochemistry

The confluent cells were sequentially treated with OA for 3 h, washed three times with PBS, fixed with 3.7% formaldehyde in PBS for 30 min at ambient temperature, and permeabilized with methanol for an additional 20 min at −20°C. Nonspecific binding sites were blocked with 4% BSA in PBS for 30 min at 20–22°C in a humidified atmosphere. The cells were rinsed with cold PBS and incubated with specific anti-p65NF-κB and anti-phospho-Ser536 p65NF-κB antibodies diluted 1:100 to 1:400 in 4% BSA for 60 min at 20–22°C. After three washes with PBS containing 0.05% Tween-20 (PBS-Tween) over a 15-min period at 20–22°C, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel-Organon Teknika, Turnhout, Belgium) diluted 1:200 in 4% BSA in PBS for another 60 min at 20–22°C. The coverslips were washed as described above and mounted while wet with PermaFluor aqueous mounting medium (Lipshow, Pittsburgh, PA). The samples were examined under an Olympus BX50 microscope equipped with epifluorescence illumination with a U-MNIBA filter for FITC. Photomicrographs

were recorded on a computer (Olympus, DP70-WPCXP).

SDS–PAGE and Western Blot

Cultured cells were washed twice with phosphate buffered-saline (PBS), scraped into lysate buffer containing 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM EGTA, and protein phosphatase inhibitor cocktail (Sigma, St. Louis, MO) in PBS. Nuclear and cytosol fractions were obtained from the cells by using a CelLytic NuCLEAR extraction kit (Sigma). The protein concentration was evaluated by using Protein Assay Reagent (Bio-Rad, Hercules, CA), and each sample was diluted to a protein concentration of 1 mg/ml with lysate buffer followed by the addition of Laemmli's 5 × SDS-sample buffer. Each sample of proteins and prestained molecular weight markers (Gibco BRL) were separated by SDS–PAGE and then transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were incubated for 2 h at 20–22°C in a blocking solution for phosphorylated proteins (Blocking One-P, Nacalai Tesque, Kyoto, Japan). Then they were washed briefly in PBS-Tween and incubated overnight at 4°C in a blocking solution containing specific antibodies. Next the membranes were washed four times for 30 min with PBS-Tween and subsequently incubated for 1 h at ambient temperature in 5% skim milk in PBS-Tween containing horseradish peroxidase-conjugated second antibodies. The membranes were washed again and the proteins identified using the appropriate antibodies and visualized using an ECL detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden). To re-use the membrane, the antibody was stripped off by treatment with 2% SDS and 0.35% 2-mercaptoehanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50°C. The antibody stripped-membranes were then blocked again and re-incubated with another appropriate antibody.

In Vitro Dephosphorylation

Whole cell lysates were prepared from untreated MG63 cells or treated with 100 nM OA. Cell lysates (10 μg) were incubated for 45 min at 30°C with 1,000 U of λ protein phosphatase (New England BioLabs, Berverly, MA) in the reaction buffer (50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij, and 35.2 mM $MnCl_2$). After incubation, 5 × SDS sample buffer was added to the reaction mixture, and the proteins were denatured for 5 min in boiling water. The samples were subjected to SDS–PAGE and analyzed by Western blot using the polyclonal anti-p65NF-κB antibody.

DNA Transfection and Luciferase Assay

The luciferase plasmid pNF-κB-Luc was obtained from Stratagene (La Jolla, CA). MG63 cells transfected with the plasmid by using Lipofectamine reagent (Gibco BRL) were treated with 100 nM OA. The luciferase assay was performed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). As an internal control, cells were co-transfected with the Renilla luciferase gene expression vector (pRL-TK, Promega). Luciferase assays were performed as recommended by the vendor and according to the manufacturer's instructions. The luciferase levels were normalized to the level of Renilla luciferase activity in each transfectant.

RESULTS

Phosphorylation of NF-κB on Ser536 Residue by OA Treatment

Figure 1A shows staining reaction of antibodies against phospho-Ser276 p65NF-κB (upper panel), phospho-Ser529 p65NF-κB (middle panel), and phospho-Ser536 p65NF-κB (lower panel) with the proteins prepared from the 100 nM OA-treated cells. The anti-phospho-Ser536 p65NF-κB antibody time-dependently interacted with a major band having an estimated molecular weight of 65 kDa. It appeared after 2 h of OA-treatment, and the level of staining intensity remained unchanged up to 8 h of treatment (Fig. 1A). The anti-phospho-Ser536 p65NF-κB antibody also recognized a more slowly migrating band (Fig. 1A). However, the anti-phospho-Ser276 p65NF-κB and anti-phospho-Ser529 p65NF-κB antibodies did not react with any protein. The bound antibodies were stripped off the membranes by SDS and 2-mercaptoethanol, and each membrane was then re-incubated with anti-p65NF-κB antibody (Fig. 1B). This antibody interacted with a major band having an estimated molecular weight of 65 kDa (Fig. 1B). The anti-p65NF-κB antibody also recognized a more slowly migrating band. This band was not detected in the extracts prepared from control cells. It appeared after 2 h of treatment with OA, and the level of staining intensity remained

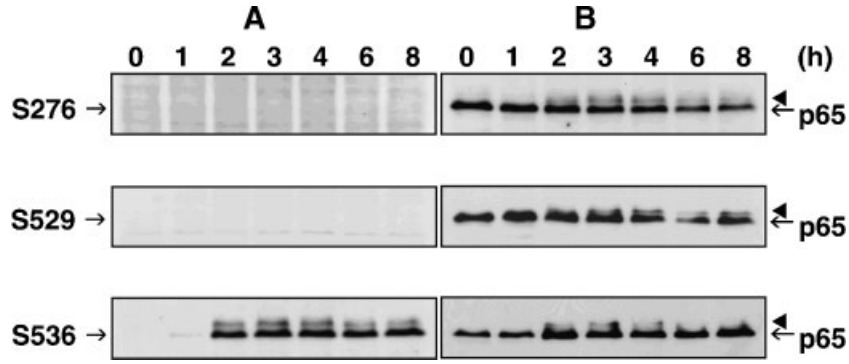

Fig. 1. Western blot analysis of NF-κB in OA-treated MG63 cells. After having reached confluence, MG63 cells were treated with 100 nM OA for the variable time periods indicated and cell lysates were prepared from each type of culture. **A**: Ten microgram samples were separated on a 10% of SDS–PAGE gel, and transferred to a PVDF membrane. Each membrane was then incubated with the anti-phospho-Ser276 NF-κB (**upper panel**, Ser276), anti-phospho-Ser529 NF-κB (**middle panel**, S529), or anti-phospho-Ser536 NF-κB (**lower panel**, S536) antibodies. **B**: Each membrane had the antibody stripped off and re-incubated with anti-p65NF-κB antibody (p65). ◀ means slower migrated form of p65NF-κB.

unchanged up to 8 h of treatment (Fig. 1B). The corresponding bands were not detected when the membranes were incubated with normal rabbit serum (data not shown).

The cells were treated for 6 h with various concentrations of OA (0–200 nM). Figure 2 shows that the anti-phospho-Ser536 p65NF-κB antibody interacted with a 65-kDa band in a dose-dependent manner (Fig. 2, upper panel). When the bound antibody was stripped off the membrane and the membrane re-incubated with anti-p65NF-κB antibody, staining of the 65 kDa protein occurred (Fig. 2, lower panel). The slower migrating form was also found in cells treated with 10 nM and higher doses of OA. The staining intensity of this band increased in a dose-dependent manner (Fig. 2).

In Vitro Dephosphorylation

To characterize the slower migrating form of p65NF-κB, we prepared lysates of OA-treated cells and incubated them with λ protein phosphatase, a residue-nonspecific protein phosphatase. Figure 3 shows that treatment of the cell lysates with λ protein phosphatase resulted in the disappearance of the slower migrating band of p65NF-κB (Fig. 2, lane 4); whereas this band was retained in the untreated lysates (Fig. 3, lane 2). The slower migrating form of p65NF-κB was detected in cells treated with OA and absent in untreated cells (Fig. 3, lanes 1 and 3). The present result shows that the slower migrating band is a phosphorylated form of p65NF-κB since it is dephosphorylated by λ protein phosphatase.

Translocation and Activation of NF-κB by OA

Cytosolic and nuclear fractions were prepared from MG63 cells incubated for 6 h with or without OA, and the expression of p65NF-κB analyzed by Western blot. The cytosolic fraction of control or 20 nM OA-treated cells showed

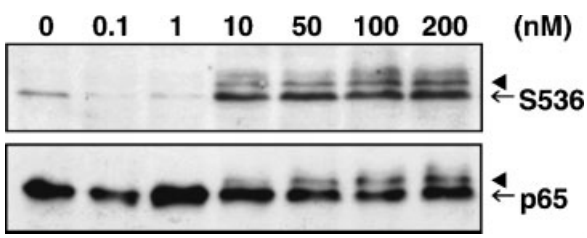

Fig. 2. Western blot analysis of NF-κB in OA-treated MG63 cells. The cells were also treated for 6 h with various concentrations of OA as indicated. The samples were analyzed by Western blot using anti-phospho-Ser536 NF-κB antibody (**upper panel**, S536). The antibody was stripped off the membrane and replaced with anti-p65NF-κB antibody (**lower panel**, p65). ◀ means slower migrated form of p65NF-κB.

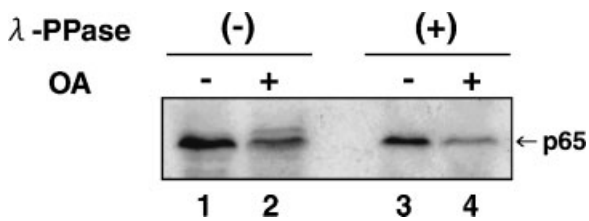

Fig. 3. Dephosphorylation of NF-κB with λ protein phosphatase. MG63 cells were untreated (**lanes 1** and **3**) or treated for 6 h with 100 nM OA (**lanes 2** and **4**). Cell lysates prepared from each type of culture were incubated for 45 min at 30°C without (lanes 1 and 2) or with 1,000 units of λ protein phosphatase (lanes 3 and 4). The samples were then analyzed by Western blotting using the anti-p65NF-κB antibody.

intense staining of p65NF-κB while the staining intensity of p65NF-κB was less with 100 nM OA-treated cells (Fig. 4, upper panel, lanes 1–3). A low level of p65NF-κB was detected in the nuclear fraction of control and 20 nM OA-treated cells (Fig. 4, upper panel, lanes 4 and 5), whereas intense staining of p65NF-κB occurred with the nuclear fraction prepared from the cells treated with 100 nM OA (Fig. 4, upper panel, lane 6). The slower migrating band of p65NF-κB was also detected in the nuclear fraction of 100 nM OA-treated cells (Fig. 4, middle panel, lane 6). The same amounts of the cell fractions were analyzed for phospho-Ser536 p65NF-κB by Western blot. The anti-phospho-Ser536 p65NF-κB antibody did not react with proteins in the cytosolic and nuclear fractions of control unstimulated cells (Fig. 4, middle panel, lanes 1 and 4, respectively). This antibody reacted weakly with fractions prepared from cells treated with 20 nM OA (Fig. 4, middle panel, lanes 2 and 5). However, intense staining of phospho-Ser536 p65NF-κB occurred with both the cytosolic and nuclear fractions of cells treated with 100 nM OA (Fig. 4, middle panel, lanes 3 and 6). The bound antibody on the membranes was stripped off and replaced with anti-B23 (nucleophosmin) antibody to determine the purity of the cell fractions. This antibody did not interact with any protein in the cytosolic fractions (Fig. 4, lower panel, lanes 1 to 3). However, the anti-B23 antibody interacted with the 38-kDa protein in the nuclear fractions of untreated and OA-treated cells (Fig. 4, lower panel, lanes 4–6), validating the high purity of the nuclear preparation.

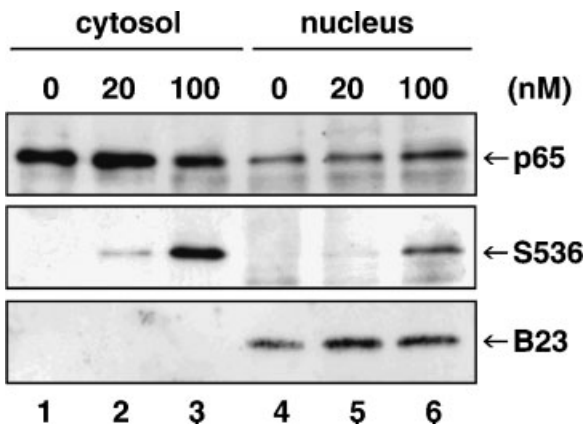

Fig. 4. Nuclear translocation NF-κB in MG63 cells treated with OA. Untreated MG63 cells (**lanes 1** and **4**), treated for 6 h with 20 nM (**lanes 2** and **5**) or 100 nM (**lanes 3** and **6**) OA were fractionated to prepare the cytosolic (lanes 1–3) and nuclear (lanes 4–6) fractions. Proteins prepared from each fraction were subjected to Western blot analysis using anti-p65NF-κB antibody (**upper panel**). Equivalent amounts of proteins were also subjected to Western blot analysis using anti-Ser536 p65NF-κB antibody (**middle panel**). In addition, the antibody was stripped off the membrane, and replaced with anti-B23 antibody (**lower panel**).

To examine whether treatment with OA could alter the cellular localization of p65NF-κB, MG63 cells were incubated with 100 nM OA for 3 h and then subjected to staining with anti-phospho-Ser536 p65NF-κB and anti-p65NF-κB antibodies. The staining of the cytoplasm and nuclei of unstimulated control cells using anti-phospho-Ser536 p65NF-κB antibody was weak (Fig. 5A). In contrast, the staining of unstimulated cells with anti-p65NF-κB antibody was intense (Fig. 5C). Staining was restricted mostly to the cytoplasm as only a weak reaction was elicited within the nuclei (Fig. 5C). Intense fluorescence for phospho-Ser536 p65NF-κB (Fig. 5B) or for p65NF-κB (Fig. 5D) was observed both in the nucleus and cytoplasm in cells treated with OA. These findings taken together with the results of Western blot analysis mentioned above indicate that phosphorylation of p65NF-κB at serine 536 occurred in cytoplasm was translocated to nucleus.

Stimulation of NF-κB Promoter Activity by OA

The activation of NF-κB by OA was determined by measuring the luciferase activity in MG63 cells that had been transiently transfected with pNF-κB-Luc. Forty-eight hours after transfection, the cells were treated with 100 nM OA for variable periods of time. Cell lysates were prepared and luciferase activity determined. Figure 6 shows that the luciferase activity in MG63 cells transfected with pNF-κB-Luc and subsequently treated with OA increased in a time-dependent manner up to 6 h. The luciferase activity in the cells treated with OA for 6 h was 20-fold greater than that in untreated cells.

DISCUSSION

We explored the effects of OA on the phosphorylation status and activation of transcription factor NF-κB in human osteoblastic MG63 cells. We demonstrated that p65NF-κB was phosphorylated on serine residue at position 536 by the OA treatment and that the translocation of p65NF-κB from the cytosol to the nucleus was induced by OA. OA is one of many stimuli

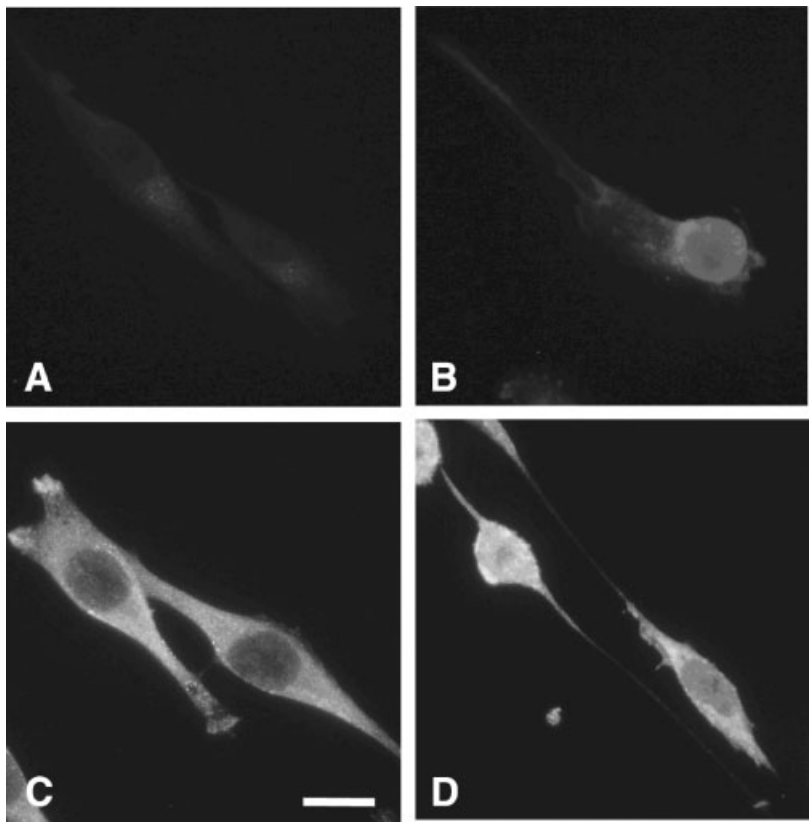

Fig. 5. Immunocytochemical identification of NF-κB in MG63 cells treated with OA. Untreated cells (**panels A** and **C**) and cells incubated for 3 h with 100 nM OA (**panels B** and **D**) were stained with anti-phospho-Ser536 NF-κB antibody (panels A and B) or anti-p65NF-κB antibody (panels C and D). Bar represents 10 μm.

that activate NF-κB in cultured cells. OA penetrate the cell membrane and inhibits the intracellular protein phosphatases, thus promoting phosphorylation of target proteins. Besides OA, NF-κB is activated by a wide variety of other agents, including LPS and TNF. Phosphorylation and activation of p65NF-κB by TNF or LPS becomes evident 5–10 min with some cell lines [Ashikawa et al., 2002; Yang et al., 2003; Doyle et al., 2005]. In the present study, however, phosphorylation of p65NF-κB was evident at 2 h after OA-treatment. This finding indicates that OA-mediated phosphorylation of p65NF-κB occurs slower than that induced by receptors associated with TNF or LPS. The induced signal transduction pathways differ among the agents used [Viatour et al., 2005]. The nature of molecules in the pathways of p65NF-κB phosphorylation on which OA might affect is not clear at present.

In the present study, anti-phospho-Ser536 p65NF-κB antibody interacted with the 65-kDa band, which was also recognized by anti-p65NF-κB antibody. This finding indicates that phosphorylation of this site does not influence the migration of p65NF-κB when subjected to SDS–PAGE. In addition to the 65-kDa band, anti-phospho-Ser536 p65NF-κB and anti-p65NF-κB antibodies also stained the slower migrating band. The finding that this band was altered on treatment with λ protein phosphatase shows that it is a phosphorylated form of p65NF-κB. Since several distinct residues have been identified on p65NF-κB [Viatour et al., 2005], the antibodies we used should recognize super-phosphorylated form of p65NF-κB. The precise mapping should be performed to clarify this point.

NF-κB is sequestered in the cytoplasm by inhibitory proteins, such as IκBα, which mask the nuclear localization signal of NF-κB [Beg et al., 1992]. Degradation of IκBα liberates the NF-κB complex, thereby allowing translocation of NF-κB from the cytoplasm to the nucleus

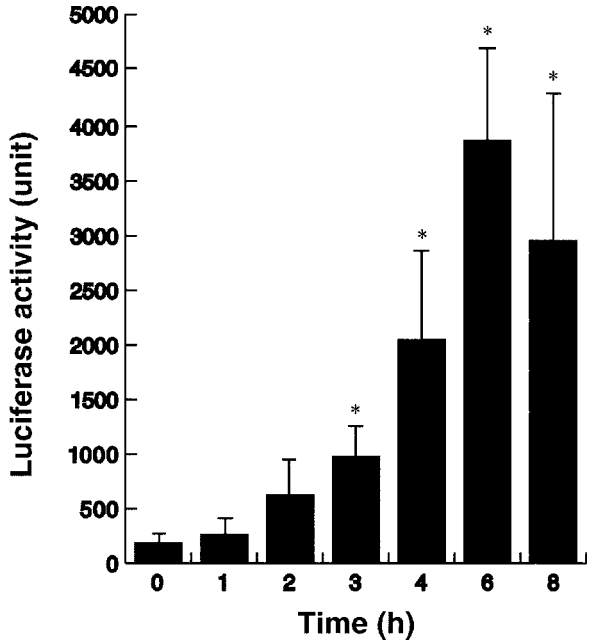

Fig. 6. Determination of NF-κB activity in OA-treated MG63 cells using luciferase assay. MG63 cells were transfected with pNF-κB-Luc plasmid as described in the Materials and Methods section and cultured for 48 h. The level of luciferase activity was measured in MG63 cells treated with 100 nM OA for variable periods of time. The luciferase levels were normalized to that of Renilla luciferase, yellow fluorescent protein, in each transfectant. The error bars represent standard deviations. $*P < 0.05$, OA-treated versus untreated MG63 cells by Student's *t*-test.

where it is phosphorylated and regulates the expression of the target genes that modulate biological responses [Karin and Ben-Neriah, 2000]. In human neutrophils and HL-60 cells, OA and orthovanadate, the latter being an inhibitor of phosphotyrosine phosphatase, stimulated the activation of NF-κB and rapid degradation of IκBα. The activation of NF-κB was caused by the OA-induced inhibition of PKCδ and IKK phosphatases or by the OA-induced activation of ERK1, a member of the MAP kinase family [Sonoda et al., 1997; Miskolci et al., 2003]. These reports indicate that the phosphorylation and degradation of IκBα was influenced by an OA-sensitive phosphatase. However, it was reported that OA-induced activation of NF-κB did not depend on the inhibitory properties of OA but rather on the production of reactive oxygen intermediates [Schmidt et al., 1995]. Thus, NF-κB might be regulated by an IκBα-independent pathway [Beyaert et al., 1996]. Previously, we demonstrated that the degradation of IκBα did not occur in the OA-treated MG63 cells [Morimoto et al., 2005]. Recently, it was also reported that phosphorylation of Ser536 on p65NF-κB is not involved in the process leading to IκBα degradation [Doyle et al., 2005]. There is also evidence that the phosphatidylinositol-3 kinase pathway is involved in IκB-independent regulation of NF-κB [Sizemore et al., 1999]. These findings indicate that phosphorylation of NF-κB does not depend on IκBα degradation.

Changes in NF-κB transcriptional activity have been attributed to the phosphorylation of p65NF-κB by a large variety of kinases in response to different stimuli [Anrather et al., 1999; Leitges et al., 2001; Mascat et al., 2003; Tsai et al., 2004; Doyle et al., 2005]. The N-terminal domain of p65NF-κB was shown to be phosphorylated by PKC-ζ [Anrather et al., 1999; Leitges et al., 2001]. In fact, PKC-ζ can directly and efficiently phosphorylate p65NF-κB; and more importantly, TNF-α-induced phosphorylation of p65NF-κB was seriously inhibited in PKC-$\zeta^{-/-}$ embryo fibroblasts [Leitges et al., 2001]. It should be remembered that the phosphorylation status of p65NF-κB is determined not only by kinase activities but also by the interplay of both kinases and phosphatases. Indeed, PP2A is physically associated with p65NF-κB in unstimulated melanocytes and is able to dephosphorylate p65NF-κB after IL-1 stimulation [Yang et al., 2001].

The majority of p65NF-κB phosphorylation occurs at serine residues; but, as yet, only several phosphorylation sites have been mapped precisely [Viatour et al., 2005]. As OA enhanced the phosphorylation level of p65NF-κB, mapping the phosphorylation sites targeted by OA is quite important to understand how this phosphatase inhibitor functions in the cells. Phosphorylation of Ser276, situated in the Rel-homology-domain (RHD) in NF-κB, was reported to be essential for p65NF-κB-dependent cellular responses [Zhong et al., 1997; Okazaki et al., 2003]. However, in our present study, phosphorylation of Ser276 was not detected in MG63 cells by OA treatment, indicating that phosphorylation of Ser276 is not essential for the OA-activated NF-κB transcription. In response to TNF-α, p65NF-κB was phosphorylated in HeLa cells at Ser529 by a casein kinase II [CKII; Wang et al., 2000]. The catalytic subunit of CKIIα bound to PP2A in vitro and in mitogen-starved cells. Thus, CKIIα may regulate the deactivation of the mitogen-activated protein kinase pathway. PP2A associates with free CKIIα, but not with CKII holoenzyme.

These results suggest that binding of kinase-active CKIIα to PP2A may enhance PP2A activity toward MEK1 in vitro [Hériché et al., 1997]. Ser536 was phosphorylated by IKK complex [Sakurai et al., 1999; Madrid et al., 2001; Yang et al., 2003]. Overexpression studies with the activated Akt revealed that IKK is necessary for enhanced p65NF-κB transactivation, whereas mutation of either Ser529 or Ser536 abolishes this effect [Madrid et al., 2001]. The involvement of both IKKα and IKKβ in phosphorylation of Ser529 and Ser536 in p65 NF-κB was recently confirmed by using IKKα$^{-/-}$ or IKKβ $^{-/-}$ mouse embryonic fibroblasts [Sizemore et al., 2002]. In our present study, we demonstrated that OA enhanced the phosphorylation level of Ser536 but not Ser276 and Ser529 on p65NF-κB in MG63 cells. These findings indicate that phosphorylation of Ser536 on p65NF-κB functions in NF-κB activation in the OA-treated cells. In earlier experiments, the phosphorylation of Ser529 on p65NF-κB was shown not to contribute to NF-κB activity in response to TNFα or LPS, suggesting only a minor role for Ser529 phosphorylation on p65NF-κB in NF-κB function [Sakurai et al., 2003; Yang et al., 2003]. Phosphorylation of Ser536 on p65NF-κB can be detected in the cytoplasm and in the nucleus [Sakurai et al., 2003; Yang et al., 2003]. However, it is unclear if this was due to the shuttling of phosphorylated p65NF-κB or to the presence of Ser536 protein kinase in both the cytoplasm and nucleus. In the present study, phosphorylated p65NF-κB was localized in both cytoplasm and nucleus in the OA-treated cells, whereas in the unstimulated cells, it was detected only in the cytoplasm. These results strongly suggest that in OA-treated cells, phosphorylation of Ser536 of p65NF-κB occurs in the cytoplasm and translocated into the nucleus. More detailed experiments are needed to clarify these points.

To determine whether the OA could affect the activity of NF-κB reporter gene expression, we performed a luciferase reporter gene assay using the pNF-κB-Luc construct. We demonstrated that OA increased the NF-κB reporter activity in MG63 cells. The luciferase activity in MG63 cells transfected with constructs increases following OA treatment in a time-dependent manner. Expression of a p65 S536A mutant in a p65$^{-/-}$ background revealed that Ser536 is required for TNF- or LPS-induced activation of a NF-κB reporter gene [Yang et al., 2003]. Our present results suggest that phosphorylated Ser536 is required for OA-induced NF-κB reporter gene expression.

Recent data suggest that gene expression varies in response to different signals [Francastel et al., 2000; Stenoien et al., 2000]. Different kinases and phosphatase inhibitors may be acting at various stages of the transcription process, including nuclear transport, DNA-binding capacity, and transcriptional initiation or elongation. The phosphorylation of p65NF-κB is a process highly regulated by both cell- and stimulus-dependent activating kinases and phosphatases.

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