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Okadaic Acid Induces Apoptosis through Double-Stranded RNA–Dependent Protein Kinase/Eukaryotic Initiation Factor-2α Pathway in Human Osteoblastic MG63 Cells

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Double-stranded RNA-dependent protein kinase (PKR) is a participant in the cellular antiviral response and phosphorylates the α-subunit of eukaryotic translation initiation factor 2α (eIF-2α) to block protein synthesis. Treatment of human osteosarcoma cell line MG63 cells with a serine and threonine protein phosphatase inhibitor, okadaic acid, at the concentration of 100 nM, but not at 20 nM, induced apoptosis. To investigate the functional relationship between phosphatases and apoptosis, we examined the phosphorylation levels of PKR and eIF-2α by Western blot analysis. During treatment of cells with it at the higher concentration (100 nM), okadaic acid increased the level of phosphorylated PKR in MG63 cells, this kinase phosphorylating eIF-2α. However, at the lower concentration (20 nM), okadaic acid did not affect the level of phosphorylated PKR. In the cells treated with 100 nM okadaic acid, activation of NF-κB also occurred. Even though inhibition of translation occurred simultaneously in MG63 cells, the expression of pro-apoptotic proteins Fas and Bax was not affected by 100 nM okadaic acid in these cells. We concluded that the inhibition of translation decreased anti-apoptotic protein expression, thus resulting in apoptosis. Our results also suggest that the inhibition of the protein phosphatase activity by okadaic acid induced apoptosis in MG63 cells through PKR and eIF-2α.

Key words: apoptosis, eIF-2α, okadaic acid, PKR, protein phosphatase.

Abbreviations: eIF-2α, eukaryotic initiation factor-2α; FBS, fetal bovine serum, OA, okadaic acid; PKR, double-stranded RNA–dependent protein kinase; PP1, protein phosphatase 1; PP2, protein phosphatase 2.

Apoptosis is an important and well-controlled form of self-regulated cell death. The main morphological features of apoptosis are cell rounding and shrinking, condensation and fragmentation of the nucleus, cytoplasmic blebbing, and DNA fragmentation producing DNA laddering (*1–3*). The apoptotic process has been recognized to be of major importance for embryonic development, tissue homeostasis, autoimmune diseases, carcinogenesis, and virus infections (*1–3*). Apoptosis can be regulated by extrinsic factors, including hormones, growth factors, cell-surface receptors, and cellular stresses. The action of apoptosis-related factors is often affected by a change in the phosphorylation status of key elements participating in the apoptotic processes (*4*).

Okadaic acid (OA) is a potent inhibitor of serine/threonine protein phosphatase types 1 (PP1) and 2A (PP2A). The IC_{50} for PP1 is 100 times higher than that for PP2A. By blocking dephosphorylation, OA increases the levels of phosphorylated cellular proteins (*5*). The use of this agent has led to the understanding that protein phosphorylation and dephosphorylation are important for the regulation of the cellular signals involved in diverse cellular processes including the biological end point, apoptosis. We previously reported that OA induced apoptosis in human osteoblastic cells (*6*, *7*), human oral squamous carcinoma cells (*8*), and human submandibular gland cells (*9*). However, OA has also been reported to inhibit apoptosis in some instances (*10–13*), but reasons for these opposite effects have not yet been given.

Double-stranded RNA-dependent protein kinase (PKR) is an abundantly expressed serine/threonine protein kinase that is induced by interferon, double-stranded RNAs (dsRNAs), cytokines, and stress signals (*14*, *15*). Once activated, the enzyme phosphorylates the α-subunit of protein synthesis initiation factor-2α (eIF-2α), thereby inhibiting translation (*16*). The PKR-eIF-2α cascade has been implicated as a general transducer of apoptosis in response to a variety of stimuli (*17–21*). PKR is activated through autophosphorylation and reversibly inactivated through dephosphorylation via the action of serine/threonine protein phosphatases. It was reported that PKR was dephosphorylated and negatively regulated by PP1 (*22*, *23*). PP1 binds directly to PKR and reduces dsRNA-mediated auto-activation of PKR (*24*). eIF-2α is inactivated through phosphorylation by the activated PKR and activated through dephosphorylation. In cells infected with herpes simplex virus 1, PP1 interacted with virus-derived $\gamma_1$34.5 protein and dephosphorylated eIF-2α (*25*). The dephosphorylation of eIF-2α by PP1 is a viral tactic against cellular defense mechanisms

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that inhibit *de novo* protein synthesis including that of viral proteins. PP1 may regulate the activities of both PKR and eIF-2α by dephosphorylating them, and thus might block protein synthesis and apoptosis. In the present study, we examined possible mechanisms of PKR and eIF-2α activation controlled by protein phosphatases by treating MG63 cells with OA.

MATERIALS AND METHODS

Materials—OA was purchased from Wako (Osaka, Japan), and α-modified Eagle's minimal essential medium (α-MEM), from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). Plastic dishes were from Falcon Plastics (Los Angeles, CA, USA). Anti-PKR antibodies were obtained from Upstate (Lake Placid, NY, USA). Anti-phospho-PKR and anti-phospho-eIF-2α polyclonal antibodies were obtained from BIOSOURCE International (Camarillo, CA, USA). Anti-eIF-2α (C-20), anti-NF-κB p65 (F-6), anti-IκBα (FL), anti-Bax (N-20), and anti-Fas (B-10) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other materials used were of the highest grade commercially available.

Cell Culture and Identification of Apoptotic Cell Death—Human osteosarcoma cell line MG63 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in α-MEM containing 10% (v/v) FBS, and were maintained at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. Cell modification was monitored under an Olympus IMT-2 phase-contrast microscope. Morphological changes characteristic of apoptosis were monitored by staining cell nuclei with Hoechst 33342. After appropriate incubation, coverslips were removed from the dishes and placed directly into 3.7% formalin in PBS for 10 min at ambient temperature, followed by permeabilization with methanol for 10 min at –20°C. After washing three times with cold PBS, the coverslips were incubated with Hoechst 33342 (10 μg/ml) for 10 min at ambient temperature. After rinsing with PBS, the coverslips were mounted while wet in PermaFluor aqueous mounting solution (Lipshaw, Pittsburgh, PA, USA). The cells were examined under an Olympus BX-50 microscope equipped for epifluorescence illumination (Olympus BX-FLA) and for photomicroscopy (Olympus PM-30). Fluorescent photomicrographs were taken using Fuji Presto 400 film.

DNA Isolation and Agarose Gel Electrophoresis—Purification of DNA from cultured MG63 cells was started by lysis of the cells in cold 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.5% Triton X-100. After lysis, debris was removed by centrifugation at 15,000 × *g* for 20 min. DNAse-free RNAse (Sigma) was added to the lysates to a final concentration of 40 μg/ml, and then the lysates were incubated with gentle shaking for 1 h at 37°C. Proteinase K (Sigma) was added to the RNAse-treated lysates to a final concentration of 40 μg/ml. The lysates were further incubated for 1 h at 37°C with gentle shaking. DNA was precipitated with 2-propanol and sodium chloride overnight at –20°C. After centrifugation and drying, the DNA was dissolved in TE-buffer (10 mM Tris, pH 8.0, containing 1 mM EDTA). Agarose gel electrophoresis of DNA was performed using 2.0% agarose gels containing 0.5 μg/ml ethidium bromide. 100 bp DNA markers (New England BioLabs, MA, USA) were run in the same gels. To visualize apoptotic alterations of DNA integrity, we observed the DNA bands with an UV transilluminator. Photographs were taken with a Polaroid DS-300 camera.

SDS-PAGE and Western blotting—MG63 cells were washed twice with PBS, and then scraped into lysate buffer comprising 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM EGTA, 50 mM NaF, and 1 mM Na_3VO_4 in phosphate-buffered saline (PBS). Cell fractions were obtained from the cells using a CelLytic NuCLEAR extraction kit (Sigma). Proteins concentration were determined by using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA), which was diluted to a protein concentration of 1 mg/ml with lysate buffer before the addition of Laemmli's 5× sample buffer. Twelve micrograms of samples and prestained molecular weight markers (GIBCO, BRL, Gaithersburg, MD, USA) were separated by SDS-PAGE and then transferred to PVDF membranes (Immobilon; Millipore, Bedford, MA, USA). Next the membranes were incubated for 2 h at 20–22°C in a blocking solution comprising 5% non-fat skim milk in PBS containing 0.05% Tween-20 (PBS-Tween), washed briefly in PBS-Tween, and then incubated overnight at 4°C in PBS-Tween containing specific antibodies. After the membranes had been washed 4 times within 30 min in PBS-Tween, they were incubated for 1 h at 20–22°C in PBS-Tween containing horseradish peroxidase–conjugated anti-rabbit IgG or anti-goat IgG. The membranes were washed again as described above, and then the proteins recognized by the antibodies were visualized with an ECL detection kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions. To strip off the antibodies the membranes were treated for 30 min at 50°C with 2% SDS and 0.35% 2-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8). The antibody stripped-membranes were then blocked again and re-incubated with another antibody.

Measurement of Protein Synthesis—Subconfluent cells were treated with a low (20 nM) or high (100 nM) concentration of OA for 6 h. During the final 1 h, the cells were labeled with 10 μCi/ml (0.37 MBq/ml) of L- [^{35}S] methionine (Amersham Pharmacia Biotech). Proteins extracted from the cells were subjected to SDS-PAGE and then stained with Coomassie Brilliant Blue. The same gel was used for autoradiography. The relative radioactivity of each band was estimated with a BAS 2000 imaging analyzer (Fuji, Tokyo, Japan).

RESULTS

The Higher Concentration of Okadaic Acid Induced Apoptosis in MG63 Cells—Figure 1 shows the phase-contrast and Hoechst 33342 staining appearance of MG63 cells treated with OA for 6 h. In the cultures treated with the inhibitor at the concentration of 100 nM, cell rounding occurred and most cells became detached from the culture dishes (Fig. 1C). The cells treated with 20 nM OA, however, showed no morphological changes within 6 h (Fig. 1B), as their morphology was similar to that of the

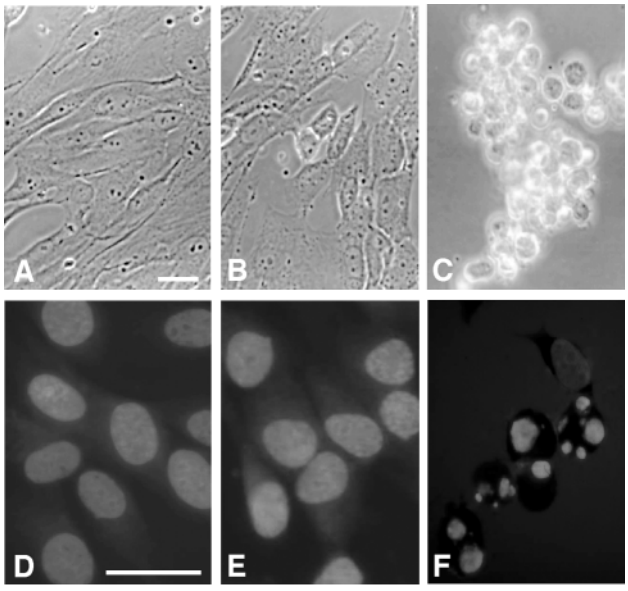

Fig. 1. **Phase-contrast and Hoechst-staining appearance of MG63 cells treated with OA.** After they had reached subconfluence, MG63 cells were left untreated (A and D) or treated for 6 h with OA at the concentration of 20 nM (B and E) or 100 nM (C and F). Nuclear fragmentation was caused by OA-treatment in MG63 cells. The cells were detached and stained with Hoechst 33342. Bars represent 20 μm.

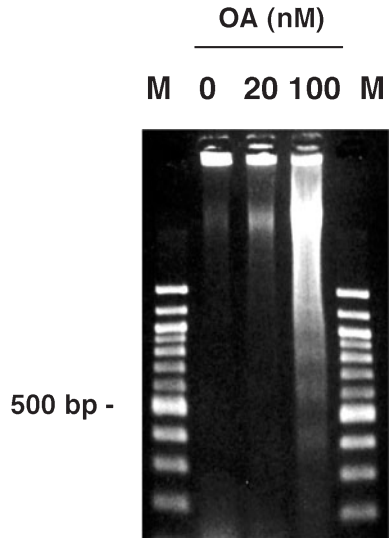

Fig. 2. **DNA ladder formation in MG63 cells treated with OA.** MG63 cells were exposed for 8 h to OA at the concentrations indicated. DNA was extracted and analyzed on an agarose gel. The gel was stained with ethidium bromide and observed on an UV transilluminator. Lane M, standard DNA markers (bp).

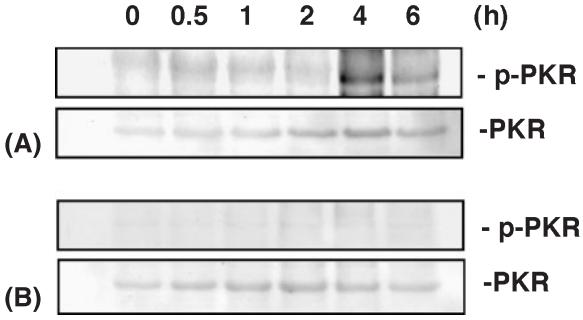

Fig. 3. **PKR expression and phosphorylation on OA treatment.** The expression of PKR and its phosphorylation form was analyzed in OA-treated MG63 cells by Western blotting. The cells were treated with 100 nM (A) or 20 nM (B) OA for various times, as indicated. Twelve micrograms of protein of each sample was separated on a 10% of SDS-PAGE, transferred to a PVDF membrane, and then incubated with an anti-phospho-PKR antibody (upper panel). The antibody was then stripped off the membrane, which was subsequently re-incubated with the whole PKR-recognizing antibody (lower panel).

control cultures (Fig. 1A). To determine if the OA-induced morphological change of MG63 cells was due to apoptosis, we examind for the presence of nuclear fragmentation and condensation in MG63 cells treated with OA. Cells treated with or without OA for 6 h were removed with trypsin and EDTA, fixed, permeabilized, and stained with Hoechst 33342. A control culture of MG63 cells (Fig. 1D) or cells treated with 20 nM OA (Fig. 1E) did not show any apoptotic features. However, in the cells treated with OA at 100 nM, nucleic acid staining with Hoechst 33342 revealed typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin and fragmentation of chromatin, indicating cells experiencing apoptosis (Fig. 1F). To further confirm that the 100 nM OA-induced cytotoxicity and nuclear fragmentation in MG63 cells was due to apoptosis, we looked for the presence of nuclear fragmentation in the cells treated with 20 nM or 100 nM OA for 8 h. The extracted DNA was analyzed by agarose gel electrophoresis and stained with ethidium bromide. For the 100 nM OA-treated cells, a DNA fragmentation pattern comprising a ladder of multiples of 185–200 bp was observed (Fig. 2). However, the cells treated with 20 nM OA showed no DNA ladder pattern.

Okadaic Acid Stimulated the Phosphorylation of PKR and eIF-2α—The PKR-eIF-2α cascade is a pathway regulated by the phosphorylation states of its components and is associated with apoptosis. To determine whether or not the level of active PKR could be increased by OA, we examined the activated form of PKR, *i.e.*, its phosphorylated form, by Western blotting using an anti-phospho-PKR specific antibody. Figure 3 shows that OA at 100 nM increased both the amount of PKR and the phosphorylation status of PKR. The phosphorylated form of PKR was detected in the lysates prepared from the cells treated with OA for 4 to 6 h (Fig. 3A, upper panel). This antibody was then stripped off from the PVDF membrane, followed by re-probing with an anti-panPKR antibody, which recognizes all forms of PKR. OA at 100 nM stimulated the expression of PKR in a time-dependent manner (Fig. 3A, lower panel). When the cells were treated with 20 nM OA, the phosphorylated form was not detected (Fig. 3B, upper panel) and the amount of PKR did not change (Fig. 3B, lower panel). Thus the level of activated PKR was increased by 100 nM OA treatment, but not by 20 nM OA, indicating a high dose threshold for the effect. The phosphorylation level of eIF-2α was also determined by Western blotting using an anti-phospho-eIF-2α antibody. Figure 4 shows that OA at 100 nM increased the amount

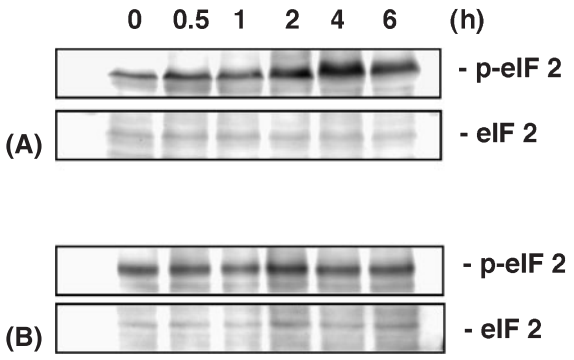

Fig. 4. **eIF-2α expression and phosphorylation on OA treatment.** The expression of eIF-2α and its phosphorylation form was analyzed in OA-treated MG63 cells by Western blotting. The cells were treated with OA at 100 nM (A) or 20 nM (B) for various times, as indicated. Twelve micrograms of protein of each sample was separated on a 10% of SDS-PAGE, transferred to a PVDF membrane, and then incubated with an anti-phospho-eIF-2α specific antibody (upper panel). The antibody was then stripped off the membrane, which was subsequently re-incubated with the whole eIF-2α recognizing antibody (lower panel).

of the phosphorylated form of eIF-2α in a time-dependent manner (Fig. 4A, upper panel). When the cells were treated with 20 nM OA, phosphorylation of eIF-2α did not occur (Fig. 4B, upper panel). The amount of eIF-2α did not change with either 100 nM or 20 nM OA-treatment (Fig. 4A, lower panel and Fig. 4B, lower panel).

Okadaic Acid Inhibited Protein Synthesis in MG63 Cells—OA-treated MG63 cells were pulse-labeled with L-[^{35}S] methionine. Figure 5A shows the Coomassie Blue staining of the proteins prepared from the cells treated with the two concentrations of OA, which indicated that equal amounts of proteins had been loaded in the lanes (Fig. 5A). Figure 5B shows that *de novo* protein synthesis in MG63 cells was inhibited by the 100 nM OA-treatment, as determined from the incorporation of L-[^{35}S] methionine. However, there was no difference in protein synthesis between the control cells and the cells treated with 20 nM OA. Autoradiography analysis showed that in cells treated with 100 nM OA, protein synthesis had decreased to 50% of the control cell level but that the level was not changed by the treatment with OA at 20 nM (Fig. 5C).

Okadaic Acid Induced NF-κB Activation in MG63 Cells—To determine the effect of OA on the activity of NF-κB in MG63 cells, we treated the cells with the low or high concentration of OA for 6 h and then examined the phosphorylation status of IκBα. The IκBα isolated from the cells treated with 100 nM OA migrated more slowly on SDS-PAGE compared with the basal form of IκBα (Fig. 6A). The slower migration of IκBα might be due to its phosphorylation. However, phosphorylation of IκBα did not occur when the cells were treated with 20 nM OA or in the control cultures (Fig. 6A). Figure 6B shows the nuclear localization of NF-κB in MG63 cells treated with OA. NF-κB was not detected in the nuclear fraction of the control cells (lane 2) or that of the cells treated with 20 nM OA (lane 4). However, NF-κB was detected in the nuclear fraction of the cells treated with 100 nM OA (lane 6). In the cells treated with 20 nM or 100 nM OA, the

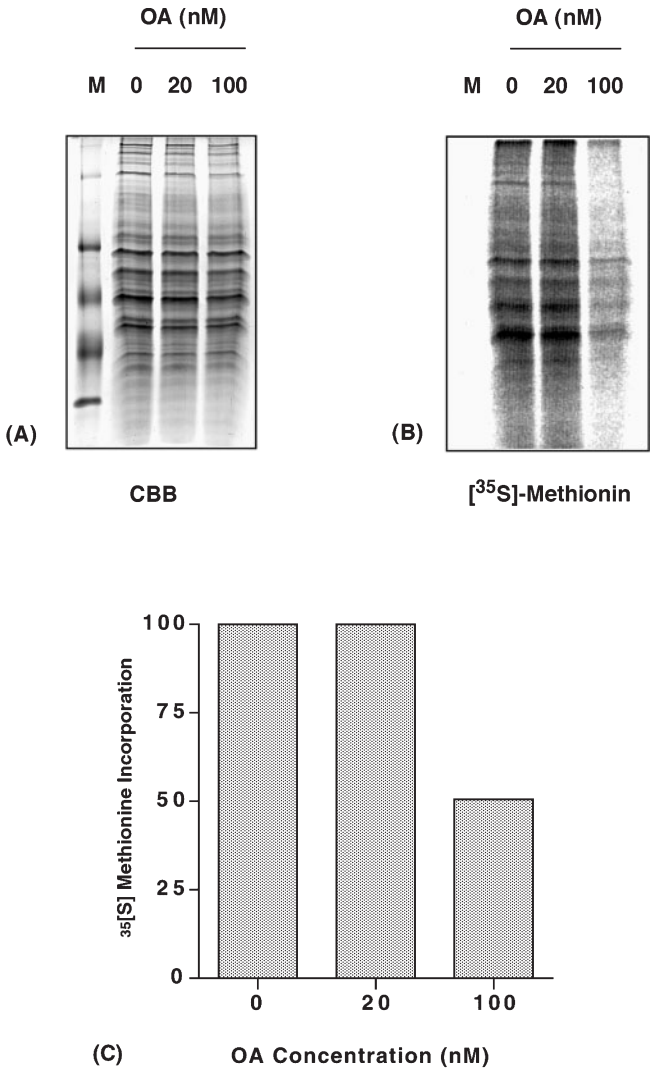

Fig. 5. **Analysis of *de novo* protein synthesis.** Cells were untreated or treated for 6 h with OA at 20 nM or 100 nM. Cells were labeled for the last 1 h with [^{35}S]-methionine, and then cell extracts were prepared. Equal amounts of total proteins were separated by 12.5% SDS-PAGE. The gel was stained with Coomassie Blue (A), and then autoradiographed (B). The density of each lane was determined and compared (C).

expression levels of Fas (Fig. 7, upper panel) and Bax (Fig. 7, lower panel) were not affected.

DISCUSSION

Okadaic acid is a toxic polyether fatty acid produced by several dinoflagellates and was isolated from marine sponges that feed on them (*26*). In the present study we demonstrated that short-term treatment with OA at the higher concentration used induced obvious morphologic changes, DNA fragmentation and DNA ladder formation, a hallmark of apoptosis, in MG63 cells. There are conflicting reports on the effect of OA on apoptosis. In some cases, OA induced apoptosis (*6–9*), whereas in other situations OA could prevent apoptosis induced by various stimuli (*10–13*). Since OA inhibits PP1 and PP2A at different concentrations (*26*), the opposite effects of OA on

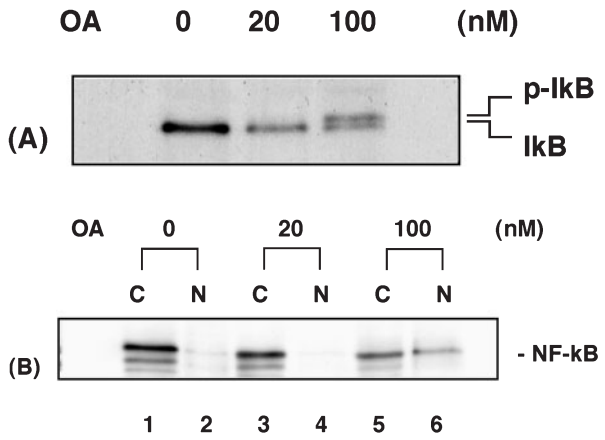

Fig. 6. **Activation of NF-κB by OA.** (A) MG63 cells were untreated or treated for 6 h with 20 nM or 100 nM OA. Twelve micrograms of protein of each sample was separated on a 12.5% of SDS-PAGE, transferred to a PVDF membrane, and then incubated with an anti-IκBα-specific antibody. (B) MG63 cells were untreated (lanes 1 and 2) or treated with 20 nM (lanes 3 and 4) or 100 nM (lanes 5 and 6) OA, and then fractionated to obtain cytosolic (lanes 1, 3, and 5) and nuclear (lanes 2, 4, and 6) fractions. Proteins prepared from each fraction were subjected to Western blot analysis for NF-κB.

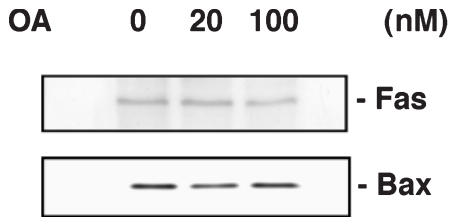

Fig. 7. **Apoptotic protein expression. MG63 cells were untreated or treated for 6 h with 20 nM and 100 nM OA.** Twelve micrograms of protein of each sample was separated on a 12.5% of SDS-PAGE, transferred to a PVDF membrane, and then incubated with an anti-Fas (upper panel) or anti-Bax (lower panel) specific antibody.

apoptosis may due to the combination of inhibited enzymes. OA at the concentration that induced apoptosis increased the level of activated form of PKR and, consequentially, that of phosphorylated eIF-2α in MG63 cells. The same concentration of OA inhibited protein synthesis. These findings suggest that apoptosis induced by OA occurs in part via the PKR-eIF-2α pathway and that protein phosphatases play a key role in apoptosis.

PKR was initially identified and characterized as a translational inhibitor in an antiviral pathway regulated by interferons (*27*). PKR is auto-phosphorylated and thus activated in response to many stimuli. The activated PKR then phosphorylates eIF-2α, resulting in shutdown of general protein synthesis (*28*). PKR contains the (R/K)(V/I/L) X (F/W/Y) motif, which binds PP1 (*24*). eIF-2α also binds PP1 through a virus-derived $\gamma_1$34.5 protein that contains the PP1 binding motif (*25*). Therefore both PKR and eIF-2α may be candidate substrates of PP1. PP1 may thus dephosphorylate both PKR and eIF-2α, and negatively control the function of the PKR-eIF-2α pathway. In the present study, 100 nM OA treatment resulted in elevated levels of the phosphorylated forms of both PKR and eIF-2α, and inhibited protein synthesis. However, 20 nM OA treatment affected neither these levels nor protein synthesis. These findings suggest that the higher concentration of OA could modulate this PKR and eIF-2α pathway through PP1 inhibition.

PKR can function as a signal transducer mediating transcriptional activation in response to dsRNA *via* its ability to phosphorylate IκBα, thus disabling it, which results in the activation of NF-κB. In the present study, OA at 100 nM led to an increased level of phosphorylated IκBα and its degradation. Degradation of IκBα liberates the NF-κB complex, which is able to migrate to the nucleus, where it triggers gene expression (*29*). NF-κB initiates the gene expression of apoptotic factors including Fas, which results in apoptosis (*30*, *31*). 100 nM OA treatment for 6 h caused the translocation of NF-κB from the cytosol to the nucleus, where it would activate NF-κB. In this study, the higher concentration of and short-term treatment with OA activated NF-κB. Although under the conditions used in the present study, the expression of the Fas receptor or Bax protein was not altered by the OA treatment, protein synthesis was inhibited at the translational level. We previously reported that a low concentration of OA induced Fas receptor and Fas ligand expression in HSG and SCC-25 cells (*32*, *33*). We also demonstrated that the induction of new cell death genes and ongoing protein synthesis might be needed for OA-induced apoptosis in MG63 cells (*7*). However, the duration of the treatment was much longer in the previous studies than in the present study. In the present study, the activation of NF-κB did not occur with 20 nM OA treatment for 6 h, and the same concentration of OA did not induce the expression of the Fas and Bax proteins in MG63 cells.

In eukaryotic cells, protein synthesis is tightly controlled at the translational level by the phosphorylation status of eIF-2α. Inactivated (*i.e.*, phosphorylated) eIF-2α inhibits the recruitment of the methionyl-initiator tRNA to the start codon of mRNAs (*19*, *34*). However, inactivated eIF-2α has also been reported to preferentially allow translation of the Fas receptor and Bax proteins (*35*). It has been reported that OA-induced apoptosis was associated with the up-regulation of the Fas receptor and Fas ligand (*32*, *33*), and Bax (*36*) at both the mRNA and protein levels. In the present study, the higher concentration of OA inhibited protein synthesis, whereas the lower one did not. The protein levels of pro-apoptotic molecules such as Fas and Bax did not increase with either concentration. These results indicate that the suppression of translation-dependent anti-apoptotic proteins rather than the synthesis of apoptotic proteins was stimulated in the cells treated with 100 nM OA. There may be two pathways for apoptosis induction, one pathway already exists and is suppressed by anti-apoptotic factors, and the other pathway requires the synthesis of apoptotic proteins. The contrary effects of OA would be due to these two pathways. If the expression of apoptotic proteins is suppressed by OA treatment, apoptosis would be inhibited; whereas if that of anti-apoptotic proteins is suppressed, apoptosis would be induced.

We conclude that OA-induced elevation of the levels of activated PKR and eIF-2α plays important roles in apoptosis and that protein synthesis is suppressed strictly

during 100 nM OA-induced apoptosis. PP1 may play an important role in the PKR-eIF-2α pathway, which responds to interferon or virus infection as a host defense mechanism.

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