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Activation of p38 Mitogen-Activated Protein Kinase and Mitochondrial Ca^{2+}-Mediated Oxidative Stress Are Essential for the Enhanced Expression of *grp78* Induced by the Protein Phosphatase Inhibitors Okadaic Acid and Calyculin A

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Abstract We have reported that treatment with okadaic acid, a potent protein phosphatase inhibitor, has the ability to enhance the synthesis of the 78-kDa glucose-regulated protein (GRP78). This article reports our investigation of another protein phosphatase inhibitor, calyculin A, demonstrating the signaling pathways elicited by the protein phosphatase inhibitors that lead to the induction of *grp78*. Our data showed that the induction process is abolished by SB203580, a specific inhibitor of p38 mitogen-activated protein kinase ($p38^{MAPK}$). Phosphorylation-activation of $p38^{MAPK}$ in the treated cells was indicated by its own phosphorylation, as shown by double Western blotting analyses and directly confirmed by the in vitro kinase assay using MAPK-activated protein kinase-2, a well-known downstream effector of $p38^{MAPK}$, as a substrate. The involvement of $p38^{MAPK}$ in this process is further substantiated by using transient transfection assays with a plasmid, pGRP78-Luc, which contains a 0.72-kbp stretch of the *grp78* promoter. By exploiting the same transfection assay, we demonstrated that the up-regulation of the *grp78* promoter by the protein phosphatase inhibitors is suppressed in the presence of the cytoplasmic calcium chelator bis(aminophenoxy)ethane N,N′-tetraacetic acid, the mitochondria calcium uniporter inhibitor ruthenium red as well as the antioxidants N-acetyl cysteine and pyrrolidinedithiocarbamate. Taken together, our results lead us to conclude that treatment with the protein phosphatase inhibitors would activate the signaling pathways involving $p38^{MAPK}$ and mitochondrial calcium-mediated oxidative stress and that these pathways must act in concert in order to confer the induction of *grp78* by okadaic acid and calyculin A. J. Cell. Biochem. 76:585–595, 2000. © 2000 Wiley-Liss, Inc.

Key words: p38 mitogen-activated protein kinase; mitochondrial calcium; oxidative stress; glucose-regulated protein; protein phosphatase

The 78-kDa glucose-regulated protein (GRP78) is a molecular chaperone [Lee, 1992; Little et al., 1994] found in the endoplasmic reticulum (ER) [Haas, 1994] and cytoplasm [Liao et al., 1997], as well as on the cytoplasmic membrane [Delpino et al., 1998]. Induction of GRP78 is critical for maintaining the viability of cells subjected to a variety of stressful conditions [Liu et al., 1997; Morris et al., 1997; Watowich and Morimoto, 1998]. The induction process can be elicited by glucose starvation [Shiu et al., 1977] treatment with Ca^{2+} ionophore A23187 [Resendez et al., 1985; Drummond et al., 1987; Li et al., 1993], ER Ca^{2+}-ATPase inhibitor thapsigargin [Li et al., 1993; Delpino et al., 1998], reducing agent 2-mercaptoethanol [Kim and Lee, 1987; Kim et al., 1987], protein glycosylation blocker tunicamycin [Watowich and Morimoto, 1998], protein transport blocker brefeldin A [Liu et al., 1992], and protein phosphatase (PPase) inhibitor okadaic acid (OA) [Hou et al., 1993]. Transactivation of the *grp78* gene has been extensively analyzed in terms of the promoter activities and it is regulated by a complex interplay of several *cis*-acting elements and transcription factors that bind to these sites. It has been shown that transcription elements/motifs such as a CCAAT box [Resendez et al., 1988; Roy and Lee, 1995;

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Roy et al., 1996], a CRE-like element [Alexandre et al., 1991; Chen et al., 1997, 1998], and a *grp* core element [Resendez et al., 1988; Li et al., 1997a,b], as well as the corresponding transcription factors including CBF/NF-Y [Roy and Lee, 1995; Roy et al., 1996], CREB, ATF-2 [Chen et al., 1997, 1998], YY1, and YB1 [Li et al., 1997a,b] are essentially involved in the basal and/or induced expression of the *grp78* gene. However, the signaling mechanism(s) that lead(s) to the activation of the transcription factors responsible for GRP78 induction remain(s) largely unknown.

PPases consist of a large number of members that belong to a superfamily, as a whole, the enzymes catalyze the dephosphorylation of cellular proteins and play pivotal roles in the regulation of a wide range of cellular activities [Cohen, 1989]. The ser/thr specific family of PPases can be divided into four groups—PPase 1 (PP1), PP2A, PP2B, and PP2C—based on their preference of substrate, dependence of divalent ions, as well as sensitivity to inhibitor proteins or naturally occurring toxins [Cohen, 1989, 1991]. More recently, based on the phylogenetic and structural data, it is suggested that the ser/thr PPases are more appropriately classified into family I (PP1, PP2A, and PP2B) and family II (PP2C) [Cohen, 1993; Barford, 1995; Wera and Hemmings, 1995]. Nevertheless, among its numerous members, PP1 and PP2A are considered the two principal PPases because of their ubiquitous abundance and broad substrate specificity [Wera and Hemmings, 1995]. Owing to the central importance of PP1 and PP2A in the regulation of reversible phosphorylation of proteins in cells, their specific inhibitors, including OA and calyculin A (CL-A), have widely been used as research tools in studying the roles of PPases in many cellular processes, including transactivation of specific genes [Cohen et al., 1990].

OA, a toxin that causes diarrheic shellfish poisoning in humans, is a potent inhibitor of PP1 and PP2A [Bialojan and Takai, 1988]. It binds to and inhibits PP1 and PP2A with dissociation constants (K_i) of 147 and 0.032 nM, respectively [Takai et al., 1995]. By contrast, CL-A has a higher affinity for PP2A than PP1 with, respectively, $K_i \simeq 0.12$ and 1 nM [Takai et al., 1995]. These two functionally related toxins share two biological effects. First, they are very specific and potent inhibitors of PP1 and PP2A, as mentioned. Second, both toxins have non-phorbol tumor promoting activity, meaning that their actions may be not mediated by protein kinase C (PKC) [Suganuma et al., 1988; Gopalakrishna et al., 1992]. We have previously reported that, in 9L rat brain tumor (RBT) cells, OA specifically induces the synthesis of GRP78 and the maximal induction of the protein can be obtained by treating the cells with 200 nM OA for 15 h [Hou et al., 1993]. However, the induction process can be markedly accelerated when the OA-treated cells (200 nM, 1 h) were subjected to a subsequent heat shock treatment at 45°C for 15 min [Chen et al., 1996]. Under this rapid induction of *grp78*, we showed that a CRE-like element (TGACGTCA) is involved in this process [Chen et al., 1997]. Moreover, the transcription factor that is activated by the tandem treatment has been determined to be a heterodimer consisting of CREB and ATF-2, which are shown to be, respectively, phosphorylated-activated by the signaling pathways involving protein kinase A and $p38^{MAPK}$ [Chen et al., 1997, 1998].

The goal of this study is to unravel the signaling events specifically elicited by OA that lead to the induced synthesis of GRP78. CL-A, another well-studied PP1 and PP2A inhibitor, was included for comparative studies. We specifically address the role of $p38^{MAPK}$, mitochondrial Ca^{2+} homeostasis, and oxidative stress in the transactivation of the *grp78* gene induced by OA and CL-A in 9L RBT cells. Within this context, we exploit the effects of $p38^{MAPK}$ inhibitor SB203580, intracellular Ca^{2+} chelator bis(aminophenoxy)ethane N,N′-tetraacetic acid (BAPTA), mitochondrial Ca^{2+} uniporter inhibitor ruthenium red (RR), as well as antioxidants N-acetyl cysteine (NAC) and pyrrolidinedithiocarbamate (PDTC) on the induction process (Table I). Our results demonstrated that the signaling pathways involving $p38^{MAPK}$ and mitochondrial calcium-mediated oxidative stress are essential for the expression of *grp78* induced by the PPase inhibitors.

MATERIALS AND METHODS

Materials

OA, CL-A, and culture medium components were purchased from Gibco Laboratories (Grand Island, NY). [γ-^{32}P]ATP (5,000 Ci/mmol), [γ-^{32}P]CTP (3,000 Ci/mmol), horseradish peroxidase (HPO)-conjugated antibodies, and the ECL Western blotting detection kits were purchased from Amersham (Buckinghamshire, UK). Anti-

TABLE I. Cellular Targets of the Employed Inhibitors

Inhibitors	Target	References
SB203580	p38^{MAPK}	Lee et al. [1994]; Cuenda et al. [1995]
BAPTA	Cytosolic free Ca^{2+}	Collatz et al. [1997]; Tymianske et al. [1997]
RR	Mitochondria Ca^{2+} uniporter	Rigoni et al. [1980]; Bernardi et al. [1984]
NAC	Oxidants	De Vries and De Flora [1993]; Satoh and Sakagami [1995]
PDTC	Oxidants	Verhaegen et al. [1995]; Chinery et al. [1998]

bodies against p38^{MAPK} and phospho-p38^{MAPK} were purchased from New England Biolabs (Beverly, MA). Synthetic oligonucleotides were ordered from Gibco-BRL (Gaithersburg, MD) and the rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) oligonucleotide probe was purchased from Clontech (Palo Alto, CA). Chemical reagents for electrophoresis and were from Bio-Rad (Richmond, CA). BAPTA-acetoxymethyl ester (BAPTA-AM), RR, NAC, PDTC were from Sigma Chemical Co. (St. Louis, MO). SB203580 was obtained from Calbiochem (La Jolla, CA). The fusion protein, MAPK-activated protein kinase-glutathione-S-transferase (MAPKAPK-2-GST), used in the in vitro p38^{MAPK} assays, was from Upstate Biotechnology (Lake Placid, NY). Other chemicals were obtained from Sigma or Merck (Darmstadt, Germany).

Cell Culture

The 9L RBT cells [Weizsaecker et al., 1981] were maintained in Eagle's minimum essential medium plus 10% fetal calf serum (FCS) supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin in a 37°C incubator under 5% CO_2 and 95% air. Prior to each experiment, stock cells were plated in 25-cm^2 flasks or six-well plates at a density of 4–6 × 10^4 cells per cm^2. Exponentially growing cells at 80–90% confluency were used.

Drug Treatment

The drug stocks were prepared as follows: OA (10 µM), CL-A (10 µM) and BAPTA-AM (5 mM), were dissolved in 10% dimethylsulfoxide (DMSO), while RR (10 mM), NAC (500 mM), and PDTC (20 mM) were in distilled water. The stocks were stored in aliquots at −70°C. For treatment, the stocks were added directly to the cultured cells to the final concentrations as follows: OA, 200 nM; CL-A, 10 nM; BAPTA-AM, 25 µM; RR, 50 µM; NAC, 10 mM; PDTC, 100 µM. For treatment with the PPase inhibitors, cells were incubated with 200 nM OA or 10 nM CL-A for ≤8 h at 37°C. To study the effects of other drugs, cells were preincubated with the drugs at the specified concentrations for 1 h before exposing to the PPase inhibitors.

SDS-PAGE and Immunoblotting Analysis

After treatment, the cells were washed with phosphate-buffered saline (PBS) and then lysed in sample buffer [Laemmli, 1970]. Equal amounts of cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970] with 10% (w/v) acrylamide for resolving gels and 4.75% acrylamide for stacking gels using a mini-gel apparatus (Hoefer, San Francisco, CA). The PAGE-resolved proteins were electrotransferred onto nitrocellulose membranes (Hybond-C Super, Amersham) and respectively probed with specific antibodies as described [Chen et al., 1998]. Antibodies against GRP78 (diluted 1:1,000), p38^{MAPK} (diluted 1:1,000), phospho-p38^{MAPK} (diluted 1:500) were employed, respectively, as the primary antibodies, whereas goat anti-mouse/rabbit IgG conjugated with HPO (diluted 1:2,000) was used as the secondary antibody. The immune complexes were visualized by using the ECL Western detection kit according to the manufacturer's instructions.

RNA Isolation and Northern Blotting

Total RNA was isolated from 9L RBT cells according to methods of Chomczynski and Sacchi [1987], with minor modifications as previously described [Chen et al., 1998]. Equal amounts of total RNA isolated from different treatment conditions were blotted onto a nylon membrane and fixed using an ultraviolet (UV) crosslinker (Stratagene, La Jolla, CA). The template for the *grp78* hybridization probe was a polymerase chain reaction (PCR) product from isolated 9L genomic DNA. PCR primers for producing the *grp78* probe were 5′-TCGTGGCTCCTCCTG-3′ (forward) and 5′-CAACCACCATGCCTA-3′ (backward) according to the exon 1 of rat *grp78* gene. *grp78* and G3PDH oligo-

nucleotide probes were labeled with [α-^{32}P]dCTP by Rediprime DNA labeling system (Amersham). After prehybridization, hybridization, and autoradiography, membranes were stripped off the probes by boiling in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) containing 0.01% SDS for 20 min and then rehybridized with other probes. Autoradiograms were quantified by densitometric scanning in 2-D mode (Molecular Dynamics, Sunnyvale, CA).

Construction of Plasmids and Transient Transfection Assays

The construction of pGRP78-Luc plasmid was derived from ligation of a rat *grp78* promoter fragment with the pGL2-Basic vector containing firefly luciferase as the reporter (Promega, Madison, WI). A 0.72-kb *bgl*II/*kpn*I fragment spanning −725 to −4 of the rat *grp78* promoter was inserted into the *bgl*II/*kpn*I cloning site to generate pGRP78-Luc. To achieve normalization of the transfection efficiency in each experiment, we co-transfected the cells with the pβgal-Control vector, which contains the *LacZ* gene as an internal standard.

The cells were transfected by LipofectAMINE™ transfection system (Life Technologies). Briefly, cells were seeded at a density of 1.2–3.0 × 10^6 cells per 100-mm dish and allowed to attach for 16 h. The cells were then incubated in 5 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) containing LipofectAMINE reagent under standard growth conditions for 6 h before transfection. After transfection, DMEM containing 10% serum was added to the tissue culture dish and further incubated for 24 h. The cells were then subjected to PPase inhibitor treatments with or without SB203580, BAPTA-AM, RR, NAC, or PDTC. Subsequently, the cells were lysed in extraction buffer (100 mM NaH_2PO_4, pH 7.9, 5% Trition X-100, 1 mM dithiothreitol), and the cell lysates were assayed for either luciferase with luciferin (Boehringer-Mannheim, prepared in 1 mM Tris-HCl, pH 7.0) or β-galactosidase with o-nitrophenyl β-D-galactopyranoside (Sigma, 4 mg/ml in 100 mM NaH_2PO_4, pH 7.5) as substrates. The luciferase activity was measured by using a luminometer, while that of β-galactosidase was determined spectrophotometrically at 420 nm. For each sample, the luciferase activity was normalized against the β-galactosidase activity and the *grp78* promoter activity was determined by the ratio of relative luciferase activity as measured from treated and untreated cell lysates.

Assays for p38^{MAPK} Activity

p38^{MAPK} activity in the treated cells was determined by immunoprecipitation of the enzyme and by using MAPKAPK-2 as the substrate. The immunoprecipitation step was performed according a previously protocol published [Chen et al., 1998]. After treatment, cell lysate was prepared in lysis buffer and then clarified by centrifugation. Protein concentration of the cell lysate was determined by the Bradford assay (Pierce). Equal amounts of cellular proteins were incubated with anti-p38^{MAPK} for 2 h at 4°C. Immunocomplexes were precipitated with protein G-Sepharose and washed twice with lysis buffer and once with assay buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM dithiothreitol). For the kinase assay, 0.5 mg MAPKAPK-2-GST, 0.1 mM ATP, 15 mM $MgCl_2$, and 10 mCi/ml [γ-^{32}P]ATP were incubated in a total volume of 30 µl and the reaction was allowed to proceed at 30°C for 10 min. The labeled proteins were analyzed by autoradiography and densitometric analysis.

RESULTS

SB203580 Inhibits OA- and CL-A-Induced GRP78 Expression and *grp78* Promoter Activity

We previously demonstrated that treatment with OA for a prolonged duration will augment GRP78 production in 9L RBT cells [Hou et al., 1993]. To investigate the possible involvement of p38^{MAPK} in this process, the cells were exposed for up to 8 h in OA- or CL-A-containing medium, in the absence and presence of SB203580. Then, the amount of GRP78 protein and the correspondent mRNA were determined, respectively, by Western blotting after 8 h or Northern blotting after 4 h of treatment (Fig. 1A). We found that, in the presence of SB203580, the accumulation of GRP78 protein and mRNA were completely abolished in the PPase inhibitor-treated cells (Fig. 1). We subsequently determined whether the transcriptional activity of rat *grp78* promoter was activated by OA or CL-A treatment and whether the activation was affected by adding increasing amounts of SB203580. The 0.72-kbp *grp78* promoter-luciferase reporter construct (designated pGRP78-

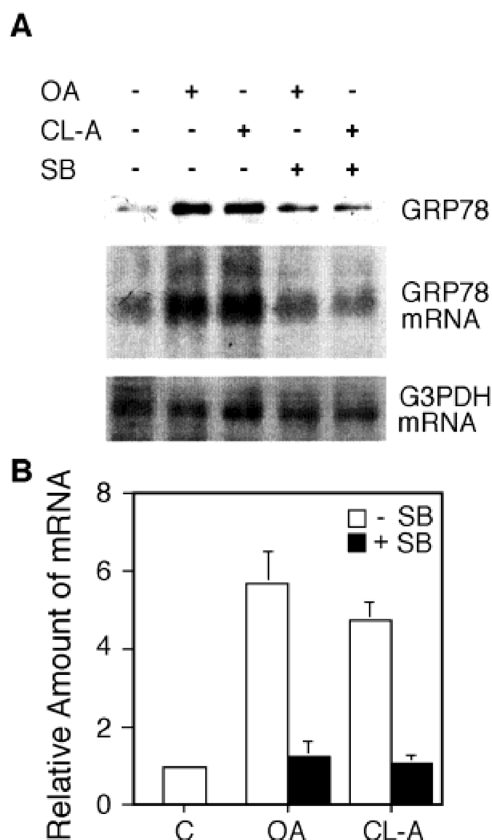

Fig. 1. Inhibitory effect of SB203580 on the induction of GRP78 protein and mRNA in 9L rat brain tumor (RBT) cells treated with okadaic acid (OA) and calyculin A (CL-A). Cells were preincubated with 20 μM SB203580 for 1 h and then treated with 200 nM OA or 10 nM CL-A for 4 (for Northern blotting) or 8 h (for Western blotting) in the presence of SB203580. Treated cells were then subjected, respectively, to Northern and Western blotting analysis, using ^{32}P-labeled *grp78* segments and anti-GRP78 antibodies as probes. **A:** Repeated hybridization of the filters with a G3PDH-specific probe confirms similar RNA loading in all lanes. **B:** Bands of interest in the resulting blots were quantified by densitometric scanning. Data are the means ± SD of three independent experiments.

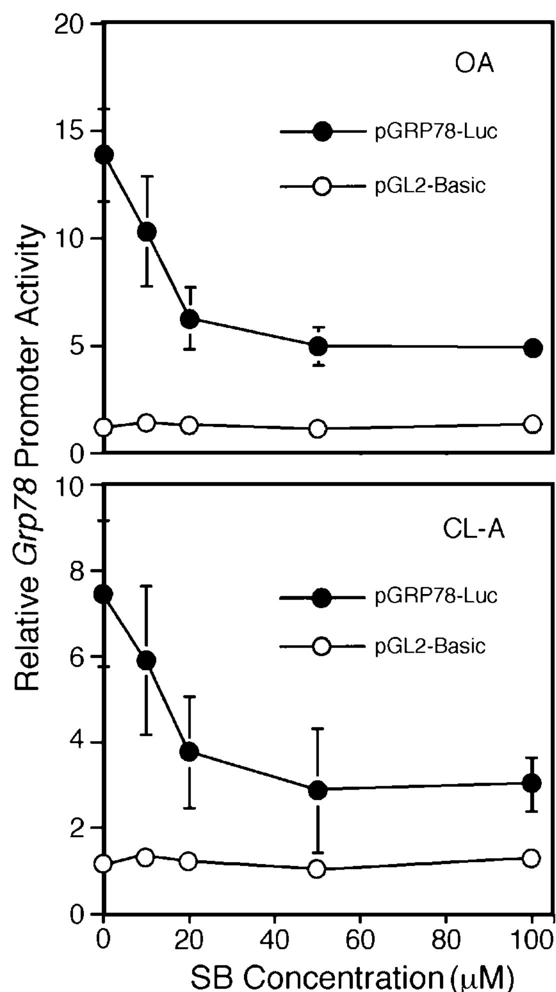

Fig. 2. Dose-dependent effect of SB203580 on the up-regulation of the promoter activity of rat *grp78* gene activated by okadaic acid (OA) and calyculin A (CL-A). 9L rat brain tumor (RBT) cells were transfected with a recombinant plasmid containing a rat *grp78* promoter fragment and firefly luciferase as the reporter (pGRP78-Luc) or with a control plasmid (pGL2-Basic). The cells were also co-transfected with a pβgal-Control vector. The transfectants were then preincubated with different concentrations of SB203580 for 1 h and then treated with OA or CL-A for 4 h as specified in the legend of Fig. 1. Luciferase activity in cell lysates was then quantified and presented as relative to that of the untreated control after normalization. Data are the means ± SD of three independent experiments.

Luc) was therefore tested in the transient transfection assay. pGRP78-Luc showed much higher activity in cells after 4-h treatment of both OA and CL-A (13.81- and 7.43-fold relative to the untreated control) and was markedly suppressed in the presence of SB203580 (Fig. 2). These data clearly demonstrate that SB203580 inhibits the induction of *grp78* by PPase inhibitors, indicating the involvement of $p38^{MAPK}$ in this process.

OA and CL-A Activate the $p38^{MAPK}$ Signal Transduction Pathway in 9L RBT Cells

To determine whether OA and CL-A indeed may activate the $p38^{MAPK}$ pathway in the treated cells, we first studied the effects of OA and CL-A on phosphorylation of $p38^{MAPK}$. Tyrosine phosphorylation of $p38^{MAPK}$ was determined by double immunoblotting, using an anti-phospho-$p38^{MAPK}$ (Tyr-182) antibody and an anti-$p38^{MAPK}$ antibody to assess the changes in phosphorylation and total expression of $p38^{MAPK}$. The OA and CL-A treatment resulted in a 7.6- and 4.5-fold increases in $p38^{MAPK}$ phosphorylation on Tyr-182, respectively, while the phosphoryla-

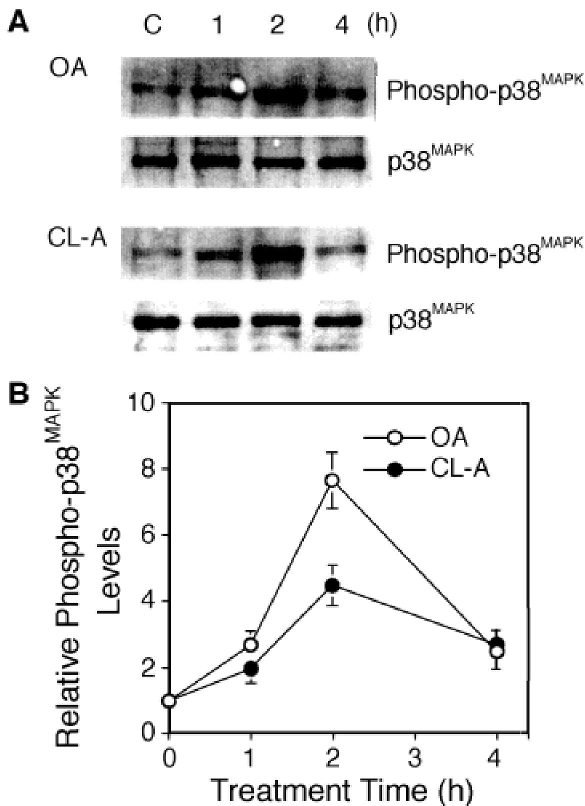

Fig. 3. Time-dependent activation of the mitogen-activated protein kinase ($p38^{MAPK}$) pathway in 9L rat brain tumor (RBT) cells treated with okadaic acid (OA) and calyculin A (CL-A). Cells were preincubated with 20 μM SB203580 for 1 h and then treated with 200 nM OA or 10 nM CL-A in the presence of SB20358 for up to 4 h. At the time intervals indicated, the cells were lysed and the cell lysates were analyzed by immunoblotting with anti-phospho-$p38^{MAPK}$ (specific for Tyr-182 in $p38^{MAPK}$) and anti- $p38^{MAPK}$. **A:** Protein-antibody complexes were visualized by enhanced chemiluminscence. **B:** Levels of phosphorylation of $p38^{MAPK}$ were quantified by densitometric scanning. Data represent the means ± SD of three independent experiments.

tion level reached a maximum at 2 h of drug treatment. The immunoblot showed that the $p38^{MAPK}$ protein remains constant during the treatments (Fig. 3). It has been shown that $p38^{MAPK}$ is a major activator of MAPKAPK-2 [Rouse et al., 1994]. To determine the effect of SB203580 on the activity of endogenous $p38^{MAPK}$ induced by PPase inhibitors, the cells were placed for 2 h in OA- and CL-A-containing medium in the presence of increasing concentrations of SB203580. $p38^{MAPK}$ was immunoprecipitated from the cell lysates and reacted with MAPKAPK-2-GST for in vitro kinase assay. As shown in Figure 4, treatment with OA and CL-A for 2 h, respectively, caused 5- and 3.7-fold increases in MAPKAPK-2-GST phosphorylation, which was significantly suppressed by increasing concentrations of SB203580, with an IC_{50} of around 20–25 μM.

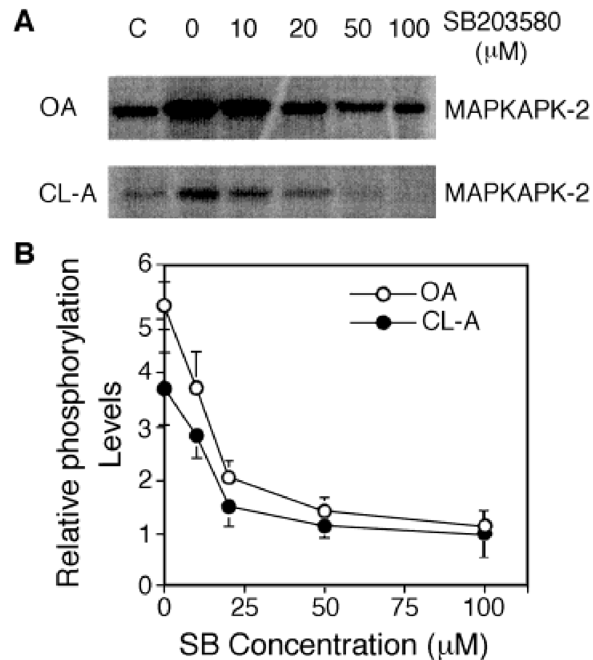

Fig. 4. Dose-dependent effect of SB203580 on the activation of mitogen-activated protein kinase ($p38^{MAPK}$) by okadaic acid (OA) and calyculin A (CL-A). 9L rat brain tumor (RBT) cells were treated with 200 nM or 10 nM CL-A in the presence of 0, 10, 20, 50, or 100 μM SB203580 for 2 h. After treatment, the cells were lysed and the $p38^{MAPK}$ in the cell lysates were immunoprecipitated by anti-$p38^{MAPK}$ and protein G-Sepharose. While still on the beads, the $p38^{MAPK}$ enzymatic activity was assayed using recombinant MAPKAPK-2-GST as a substrate in the presence of [γ-^{32}P]ATP at 30°C for 10 min. Phosphorylation of MAPKAPK-2-GST was assessed by autoradiography (**A**), followed by densitometric scanning (**B**). Data represent the means ± SD of three independent experiments.

Pretreatment of BAPTA, RR, NAC, and PDTC Abolishes the Induction of *grp78* Promoter Activity by OA and CL-A

We used cytoplasmic Ca^{2+} chelator BAPTA, mitochondria Ca^{2+} uniporter inhibitor RR, as well as antioxidants NAC and PDTC to investigate the possible roles of $[Ca^{2+}]_{mit}$ homeostasis and ROIs in OA and CL-A-induced *grp78* expression. The cells containing pGRP78-Luc were pretreated with the drugs for 1 h and then co-treated with OA or CL-A for another 2 h. The normalized luciferase activity was used as an indicator for the promoter activity of *grp78*. It was found that chelation of free cytoplasmic calcium with BAPTA and inhibition of mitochondrial Ca^{2+} uniporter by RR can significantly

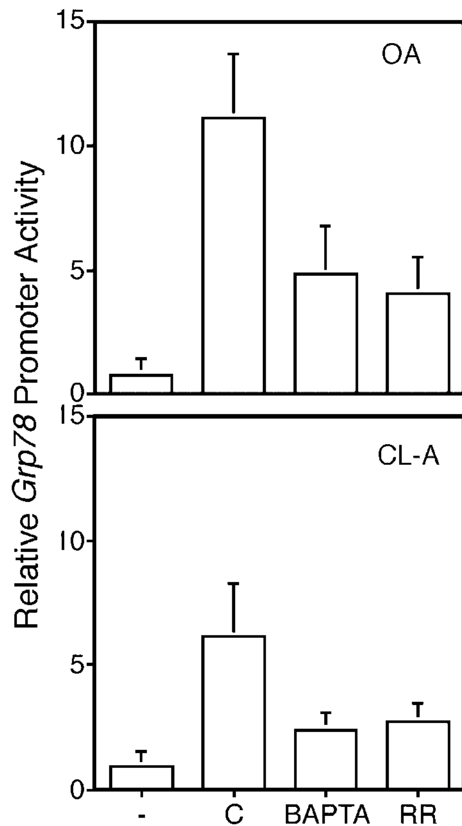

Fig. 5. Inhibitory effect of bis(aminophenoxy)ethane N,N′-tetraacetic acid (BAPTA) and ruthenium red (RR) on the up-regulation of the promoter activity of rat *grp78* gene activated by okadaic acid (OA) and calyculin A (CL-A). 9L rat brain tumor (RBT) cells were transfected with a recombinant plasmid containing a rat *grp78* promoter fragment and firefly luciferase as the reporter (pGRP78-Luc). Cells were also co-transfected with a pβgal-Control vector. The transfectants were then preincubated with 25 μM BAPTA-AM or 50 μM RR for 1 h and then treated with 200 nM OA or 10 nM CL-A for 4 h. Luciferase activity in cell lysates was then quantified and presented relative to that of the untreated control after normalization. Data are the means ± SD of three independent experiments. -, untreated control.

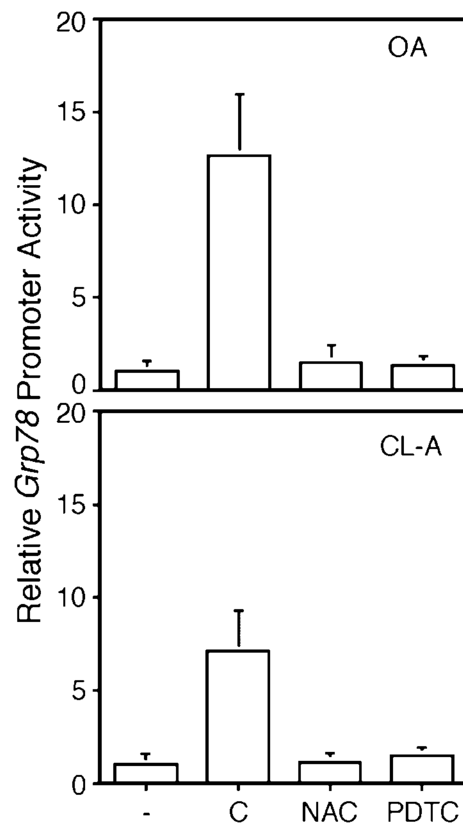

Fig. 6. Inhibitory effect of N-acetyl cysteine (NAC) and μM pyrrolidinedithiocarbamate (PDTC) on up-regulation of the promoter activity of rat *grp78* gene activated by okadaic acid (OA) and calyculin A (CL-A). 9L rat brain tumor (RBT) cells were transfected with a recombinant plasmid containing a rat *grp78* promoter fragment and firefly luciferase as the reporter (pGRP78-Luc). Cells were also co-transfected with a pβgal-Control vector. The transfectants were then preincubated with 10 mM NAC or 100 PDTC for 1 h and then treated with 200 nM OA or 10 nM CL-A for 4 h. Luciferase activity in cell lysates was then quantified and presented relative to that of the untreated control after normalization. Data are the means ± SD of three independent experiments. -, untreated control.

reduce the up-regulation of the *grp78* promoter activity by OA and CL-A (Fig. 5), indicating that the increase in $[Ca^{2+}]_{mit}$ may be important for the induction of *grp78*. Moreover, when the cells were pretreated with either NAC or PDTC followed by treatment with OA or CL-A, the promoter activity of *grp78* gene induced by the PPase inhibitors was completely abolished (Fig. 6). This finding suggested that OA and CL-A rely on the induction of oxidative stress for the activation of the *grp78* gene.

DISCUSSION

Our data show that activation of the p38 ^{MAPK} and of the oxidative stress pathways is involved in the up-regulation of *grp78* by OA and CL-A in 9L RBT cells. p38^{MAPK} belongs to a family of kinase known as MAPKs; these proline-directed serine/threonine kinases are themselves activated through phosphorylation on specific tyrosine and threonine residues in response to signaling pathways induced by mitogens or stress conditions. Interestingly, the distinct MAPK cascades can be activated either independently or simultaneously [Cano and Mahadevan, 1995]. The prototypes of this family are p42/p44 MAPKs, also termed ERK1/2, which are highly related and strongly activated by a variety of mitogens through a well-characterized signaling pathway [Pelech and Sanghera,

1992; Marshall, 1994]. $p54^{MAPK}$, also known as SAPKs/JNKs [Kyriakis et al., 1994; Verheij et al., 1996], and $p38^{MAPK}$ [Rouse et al., 1994; Han et al., 1994; Lee et al., 1994], are subsequently identified. These latter types of MAPK are found to be only activated weakly by mitogens, but very strongly by stress stimuli, endotoxin, inflammatory cytokines, and tumor necrosis factor-α [Rouse et al., 1994; Kyriakis et al., 1994; Lee et al., 1994; Raingeaud et al., 1995; Wang and Ron, 1996; Crawley et al., 1997]. In mammalian cells, $p38^{MAPK}$ phosphorylates/activates MAPKAPK-2 [Rouse et al., 1994] as well as transcription factors including ATF-2, Max, CREB-homologue/growth arrest DNA damage 153 (CHDP/GADD153) [Molnar et al., 1997]. Recently, $p38^{MAPK}$ has been shown to be activated by OA in several cell types, and its activation appears to be mediated by signaling pathway(s) that may or may not be related to the oxidative stress [Moriguchi et al., 1996; Wesselborg, 1997].

Conversely, both OA and CL-A may act as strong inducers of oxidative stress; their effects can be suppressed by pretreatment of cells with antioxidants [Schreck and Baeuerle, 1994; Schmidt et al., 1995]. It has been shown that many types of oxidative stress may also induce GRP78. For instance, the ROI inducer CCl_4 causes oxidative stress and increases GRP78 mRNA accumulation in rat liver [Schiaffonati and Tiberio, 1997]. It was also found that xanthine oxidase plus hypoxanthine-induced oxidative stress could enhance the expression of GRP78 protein in human umbilical vein endothelial cells [Dreher et al., 1995]. Our findings support the notion that OA and CL-A are strong inducers of oxidative stress and also indicate that this pathway is involved in the induction of *grp78* by these inhibitors. However, the relationship between the generation of ROIs induced by oxidative stress and the transactivation of *grp78* gene remains to be elucidated. Moreover, several lines of evidence suggest an intimate relationship between transmembrane mitochondrial Ca^{2+} exchange and oxidative stress. The accumulation of mitochondrial Ca^{2+} results in a permeability increase of the inner mitochondrial membrane that is mediated by the opening of a complex channel, the Ca^{2+}-dependent mitochondrial permeability transition pore. Recent work indicates that the mitochondrial transition pore is highly regulated [Bernardi and Petronilli, 1996] and that the opening may be involved in the release of apoptogenic factors [Kroemer et al., 1997]. An increase in $[Ca^{2+}]_{mit}$ may also lead to oxidative damage of mitochondria, such as increased H_2O_2 production [Cadenas and Boveris, 1980; Castilho et al., 1995] and increased HO· production from H_2O_2 by mobilizing intramitochodrial Fe^{2+} [Merryfield and Lardy, 1982; Castilho et al., 1995]. We have shown that pretreatment with BAPTA, RR, NAC, and PDTC (Table I), which are expected to prevent an increase of $[Ca^{2+}]_{mit}$, as well as production of ROIs, also blocks the up-regulation of *grp78* in cells treated with OA and CL-A; This finding indicates that the PPase inhibitors may also activate an oxidative stress pathway that results from an increase in $[Ca^{2+}]_{mit}$ and is required for the transactivation of the *grp78* gene.

Regulation of the *grp78* gene has undergone rigorous investigation for more than a decade. At least two mechanisms for induced expression of GRP78 have been proposed, one involving a phosphorylation step that requires de novo protein synthesis and another mechansim that is independent of both of these steps [Price et al., 1992]. It is noteworthy that most, if not all, GRP78 inducers, including OA and CL-A, require a lag time of several hours for observation of the enhanced synthesis of GRP78. However, rapid induction of GRP78 can be obtained by treating cells with OA, followed by heat shock, under which enhanced synthesis of GRP78 can be detected almost immediately after the combined treatment [Chen et al., 1996, 1998]. We have shown that a number of signaling pathways such as those mediated by protein kinase A and $p38^{MAPK}$ are involved in the regulation of the *grp78* gene. In this article, we demonstrate for first time that treatment with OA or CL-A alone indeed elicits the activation of the $p38^{MAPK}$ as well as the oxidative stress pathways and that both pathways are essentially participated in the PPase inhibitor-induced expression of GRP78. These findings shed new light on the elucidation of the regulatory mechanisms of the *grp78* gene, which encodes a molecular chaperone with increasing importance.

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