Thapsigargin Induces Expression of Activating Transcription Factor 3 in Human Keratinocytes Involving Ca\(^{2+}\) Ions and c-Jun N-Terminal Protein Kinase

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Received July 19, 2010; accepted August 16, 2010

ABSTRACT

Thapsigargin is a specific inhibitor of the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase of the endoplasmic reticulum. Here, we show that stimulation of human HaCaT keratinocytes with nanomolar concentrations of thapsigargin triggers expression of activating transcription factor (ATF) 3, a basic-region leucin zipper transcription factor. ATF3 expression was also up-regulated in thapsigargin-stimulated glioma cells, hepatoma cells, and airway epithelial cells. Thapsigargin-induced up-regulation of ATF3 expression in keratinocytes was attenuated by BAPTA-acetoxymethyl ester or by sigargin-induced up-regulation of ATF3 expression in keratinocytes involving activation of JNK, biosynthesis of ATF3, and attenuation of thapsigargin-induced expression of ATF3. The up-regulation of caspase-3/7 activity in thapsigargin-stimulated HaCaT cells was attenuated by inhibition of JNK. Together, these data show that stimulation of HaCaT cells with thapsigargin induces a specific signaling pathway in keratinocytes involving activation of JNK, biosynthesis of ATF3, and up-regulation of caspase-3/7 activity.

Introduction

The sesquiterpene lactone thapsigargin selectively inhibits the Ca\(^{2+}\) ATPases of the sarco/endoplasmic reticulum without showing effects upon the Ca\(^{2+}\) ATPases of the plasma membrane (Thastrup et al., 1990). As a result, the Ca\(^{2+}\) store of the endoplasmic reticulum is emptied in thapsigargin-treated cells, leading to an increase in the concentration of cytosolic free Ca\(^{2+}\). The treatment of cells with thapsigargin was used in numerous studies as a tool to determine the role of intracellular Ca\(^{2+}\) stores on cellular signaling. However, thapsigargin exhibits additional effects at the concentration frequently used (\(\approx 1 \mu M\)) [for example, the modulation of Ca\(^{2+}\) entry pathways including the activation of CRAC channels (Zweifach and Lewis, 1993; Taylor and Broad, 1998)].
ATF3 belongs to the family of basic region leucine zipper transcription factors that binds to DNA via its basic domain and dimerizes with other basic leucine zipper proteins via its leucine zipper region. Expression of ATF3 is low in quiescent cells. However, exposure of cells to physiological and pathological stimuli, including genotoxic agents, proteasome inhibitors, ischemia, and hypoxia, induces a strong up-regulation of ATF3 expression (Hai et al., 1999). The ATF3 encoding gene has been described as an “adaptive response gene” (Lu et al., 2007), that allows the cells to cope with changes in their environment. ATF3 functions as a transcriptional repressor when bound as a homodimer to DNA. ATF3 has been shown to regulate cell cycle progression, cell growth, apoptosis, and stress response, dependent on the cell type (Hai et al., 1999). In keratinocytes, enhanced expression of ATF3 has been observed as a result of skin injury (Harper et al., 2005).

It has been shown that ATF3 is synthesized in thapsigargin-stimulated HaCaT keratinocytes (Kang et al., 2003). In the present study, we have analyzed the signal transduction by which stimulation of HaCaT keratinocytes with thapsigargin leads to the activation of ATF3 expression. We show that the intracellular signaling cascade connecting thapsigargin stimulation with ATF3 expression requires an elevated intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) and activation of c-Jun N-terminal protein kinase (JNK). MAP kinase phosphatases 1 and 5 (MKP-1 and MKP-5) are probably part of a negative feedback loop that inactivates signal-induced ATF3 gene transcription. Functional experiments revealed that nanomolar concentrations of thapsigargin induce apoptosis in keratinocytes.

Materials and Methods

Materials. Cells were incubated for 24 h in medium without serum before stimulation. Stimulation with thapsigargin (Calbiochem, Darmstadt, Germany), dissolved in DMSO, was performed as indicated under Results. The MAP kinase kinase inhibitor PD98059 (Oncogene Science, Manhasset, NY) was dissolved in DMSO and used at a final concentration of 20 nM. The p38 protein kinase inhibitor SB203580 (Sigma, St. Louis, MO) was dissolved in DMSO and used at a concentration of 10 to 20 µM. The Leucine zipper region. Expression of ATF3 is low in quiescent cells. However, exposure of cells to physiological and pathological stimuli, including genotoxic agents, proteasome inhibitors, ischemia, and hypoxia, induces a strong up-regulation of ATF3 expression (Hai et al., 1999). The ATF3 encoding gene has been described as an “adaptive response gene” (Lu et al., 2007), that allows the cells to cope with changes in their environment. ATF3 functions as a transcriptional repressor when bound as a homodimer to DNA. ATF3 has been shown to regulate cell cycle progression, cell growth, apoptosis, and stress response, dependent on the cell type (Hai et al., 1999). In keratinocytes, enhanced expression of ATF3 has been observed as a result of skin injury (Harper et al., 2005).

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Lentiviral Gene Transfer. All lentiviral transfer vectors used in this study are based on plasmids pFUW or pFUWG (Lois et al., 2002). The transgenes were expressed under the control of the human ubiquitin-C promoter. The lentiviral transfer vectors pFUW-MKP-1, pFUW-MKP-5, and pFUWmATF3 have been described previously (Bauer et al., 2007; Mayer et al., 2008a, 2009; Rössler and Thiel, 2009). An expression plasmid encoding MEKK1Δ, a truncated form of mitogen-activated/extracellular signal responsive kinase kinase (MEK) kinase-1 (MEKK1) (Minden et al., 1994), was a kind gift of Michael Karin (University of California, San Diego, CA). The expression vector pCMV-FLAG-MEKK1Δ encoding FLAG-tagged MEKK1Δ has been described elsewhere (Bauer et al., 2007). The coding region was excised from plasmid pCMV-FLAG-MEKK1Δ and inserted into the HpaI site of plasmid pFUW, generating the lentiviral transfer vector pFUW-FLAG-MEKK1Δ. Plasmid pFUW-FLAG-MK6E, encoding a FLAG-tagged, constitutively active MKK6, was generated by cutting plasmid pcDNA3-FLAG-MKK6E (Raingeaud et al., 1996), a kind gift of Roger Davis (University of Massachusetts Medical School, Worcester, MA), with HindIII and Bsp120I and filling in with the Klenow fragment of DNA polymerase I. The fragment was cloned into HpaI-cut pFUW. The lentiviral transfer vector pFWATF3luc was generated by replacing the EGF coding region of plasmid pFUWG with that of mCherry. Plasmid pCMV-PV-NES-GFP was a kind gift of Anton Bennett (Yale University, New Haven, CT) (Pulci et al., 2002). We replaced the GFP coding region with that of mCherry and cloned the fragment encoding a NES-tagged parvalbumin-mCherry fusion protein into plasmid pFUWG. Cloning details can be obtained upon request. The lentiviral transfer vector HIV-1/2-gal was a kind gift of Jing-Kuan Yee (Department of Virology, Beckman Research Institute, City of Hope, California, CA) (Kowolik and Yee, 2002). The viral particles were produced as described previously (Stefano et al., 2006) by triple transfection of 293T/17 cells with the gag-pol-rev packaging plasmid, the env plasmid encoding VSV glycoprotein and the transfer vector.

Reporter Assays. The lentiviral transfer vector pFWATF3luc has been described elsewhere (Mayer et al., 2008a). Cell extracts of stimulated cells were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and analyzed for luciferase activities as described previously (Thiel et al., 2000). Luciferase activity was normalized to the protein concentration.

[Ca\(^{2+}\)]\(_{i}\), Imaging. HaCaT cells (8 × 10\(^4\) cells/well) were seeded in each well of a six-well plate on poly-l-ornithine–coated (0.1 mg/ml) Sigma glass cover slips in culture medium containing 10 mM HEPES (C. Roth GmbH, Karlsruhe, Germany) and grown under standard culture conditions for 2 days. Cells were loaded at room temperature for 30 min with 2 mM fura-2/AM (Invitrogen GmbH, Karlsruhe, Germany), washed, and incubated at room temperature for 10 min before use. The cover slip was assembled into a self-made sandwich chamber, which allowed a complete solution exchange in <1 s. Cells were measured on the stage of an Olympus IX 70 microscope (Olympus, Tokyo, Japan) equipped with a 20× (universal apochromatic/340; numerical aperture, 0.75) objective and were alternatively illuminated at 340 and 380 nm with the Polychrome IV Monochromator (TILL Photonics, Gräfelfing, Germany). Fluorescence emissions \(\pm\) 440 nm were captured with a charge-coupled device camera (TILL Imago), digitized, and analyzed using TILL Vision software. Ratio images were recorded at intervals of 5 s.

Apoptose Assays. Caspase 3/7 activities were measured using the Caspase-Glo assay kit (Promega) as described previously (Volkmann et al., 2007). Cell pellets were lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM MgCl\(_2\), 150 mM NaCl, 0.5% nonylphenolpolyethylene glycol-40, 1× Complete medium (Roche, Mannheim, Germany), and 10 mM dithiothreitol. The protein concentration was determined by using the BCA protein assay reagent (Pierce, Rockford, IL) and 5% trichloroacetic acid. Caspase-Glo substrate (Promega) was prepared according to the manufacturer’s instructions.
manufacturer’s instructions) for 1 h at room temperature. The luminescence was measured in a luminometer. To detect the fragmentation of chromatin in apoptotic cells, we used the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique, after which the cells were labeled with tetramethylrhodamine dUTP (Roche, Mannheim, Germany). Hoechst 33258 (Sigma-Aldrich) was used to stain the chromatin in both TUNEL-positive and -negative cells. Cells were seeded in triplicate at a density of 2 × 10⁴ cells/well in 96-well plates in serum-containing medium. Twenty-four hours later, the medium was removed and replaced by medium without serum. The cells were incubated for 24 h and the serum-lacking medium was renewed before application of the cytotoxic compounds. Twenty-four hours later, cells were fixed with 4% paraformaldehyde and the TUNEL stain was performed according to the manufacturer’s protocol. The cells were additionally labeled with Hoechst 33258 (1 µg/ml, 10 min), dissolved in phosphate-buffered saline, to visualize the DNA of the cells. The analysis was performed with a fluorescent microscope (Axiovert 200 M; Zeiss, Wetzlar, Germany) with Cy3 and 4,6-diamidino-2-phenylindole filter sets to detect both TUNEL and Hoechst fluorescence. Images were captured using 10× and 20× objectives with the Axiovision software. Quantitation of TUNEL-positive cells was performed in three nonoverlapping randomly chosen microscopic fields per sample. Presented data are averages of at least two independent experiments after counting of approximately 200 cells per microscopic field. To avoid personal bias, apoptosis assays and quantitation of apoptosis (blind coded) were performed by different persons. The average apoptosis percentages were determined and values are compared by S.D. of the means. Data are presented as means ± S.D. in three replicates. To discriminate between early apoptotic and dead cells, we used the Guava Nexin reagent (Millipore, Billerica, MA), which contains phycoerythrin-labeled annexin-V to stain phosphatidylserine on the cell surface and the cell-impermeant dye 7-amino-actinomycin D (7-AAD) to detect dead cells by intercalating into double-stranded nucleic acids. Cells were centrifuged once at 300g and dispersed using ViaCount cell dispersal reagent (Millipore) before they were analyzed on a Guava easyCyte SHT device.

Western Blots. Whole-cell extracts and nuclear extracts were prepared as described previously (Kaufmann and Thiel, 2002). Proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted, and incubated with antibodies directed against ATF3 (Santa Cruz Biotechnology, Heidelberg, Germany), Egr-1 (Santa Cruz Biotechnology), c-Jun (Santa Cruz Biotechnology), the phosphorylated form of c-Jun and HDAC1 (Santa Cruz Biotechnology). The antibody directed against HDAC1 was used as a loading control. To detect FLAG-tagged proteins, we used the M2 monoclonal antibody directed against the FLAG epitope (Sigma-Aldrich, Steinheim, Germany) at a 1:3000 dilution. Immunoreactive bands were detected via enhanced chemiluminescence using a 1:1 combination of solution 1 and solution 2 (2.5 mM Luminol, 400 µM p-coumaric acid, 100 mM Tris-HCl, pH 8.5). Densitometric analysis of signal intensities was performed by using QuantityOne quantification analysis software (Bio-Rad Laboratories, München, Germany).

Statistics. Statistical analysis (caspase-3/7 activity, luciferase activity, number of TUNEL-positive cells, densitometric analysis of Western blot signal intensities) were done by using the two-tailed Student’s t test. Data shown are mean ± S.D. from three independent experiments. Values were considered significant when P < 0.05.

Results

Biosynthesis of ATF3 in Thapsigargin-Stimulated HaCaT Keratinocytes. HaCaT keratinocytes were serum-starved for 24 h and then stimulated with different concentrations of thapsigargin. The cells were harvested 2 h after stimulation, nuclear extracts were prepared, and ATF3 expression was analyzed via immunoblotting. ATF3 immunoreactivity was undetectable in the absence of stimulation. In contrast, stimulation with nanomolar concentrations of thapsigargin strikingly increased ATF3 expression (Fig. 1A). The

![Fig. 1. Thapsigargin triggers the biosynthesis of ATF3 in HaCaT keratinocytes. A, induction of ATF3 expression in thapsigargin-stimulated HaCaT cells. Cells were serum-starved for 24 h and then stimulated with thapsigargin for 2 h as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against ATF3. The antibody directed against HDAC1 was used as a loading control. B, the biosynthesis of ATF3 after thapsigargin stimulation is sustained. HaCaT cells were stimulated with thapsigargin (10 nM) for the indicated periods. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against ATF3. C, schematic representation of the integrated provirus encoding luciferase under the control of the human ATF3 promoter (sequence from −1850 to +34). D, HaCaT cells were infected with a recombinant lentivirus encoding an ATF3 promoter/luciferase reporter gene. Cells were serum-starved for 24 h and then stimulated with thapsigargin (10 nM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration (***, P < 0.001). Each experiment illustrated here and in all subsequent figures was repeated a minimum of three times with consistent results.](molpharm.aspetjournals.org)
ATF3 encoding gene has frequently been labeled as an “immediate-early gene,” suggesting that the stimulus-dependent ATF3 expression is robust but transient. We therefore stimulated HaCaT cells with thapsigargin (10 nM) and harvested the cells 1, 3, 5, and 8 h after stimulation. Figure 1B shows that ATF3 expression in thapsigargin-stimulated HaCaT cells lasted for at least 8 h. The up-regulation of ATF3 expression in thapsigargin-stimulated HaCaT cells was corroborated by enhanced ATF3 promoter activity. We implanted an ATF3 promoter/luciferase reporter gene into the chromatin of HaCaT cells to ensure that the reporter gene is packed into an ordered nucleosomal structure. Figure 1C shows a schematic depiction of the integrated provirus. Cells were serum-starved for 24 h and stimulated with thapsigargin (10 nM). Figure 1D shows that thapsigargin stimulation significantly induced ATF3 promoter/luciferase reporter gene transcription ($P < 0.001$).

**Thapsigargin Stimulation Up-Regulates ATF3 Expression in Different Cell Types.** Next, we tested whether thapsigargin-induced up-regulation of ATF3 is specific for keratinocytes. We stimulated human U87-MG glioma cells (Fig. 2A), human ARPE-19 retinal pigment epithelial cells (Fig. 2B), human HepG2 hepatoma cells (Fig. 2C), and human A549 airway epithelial cells (Fig. 2D) with different concentrations of thapsigargin as indicated. The results show that ATF3 was synthesized in these cells after treatment with nanomolar concentrations of thapsigargin, indicating that the effect of thapsigargin on ATF3 gene transcription is not restricted to keratinocytes.

**Thapsigargin Stimulation Results in Elevated Ca$^{2+}$/H11545 Levels in HaCaT Cells.** Thapsigargin, normally used at micromolar concentrations, blocks the SERCA of the endoplasmic reticulum, thus attenuating the transport of Ca$^{2+}$/H11001 ions from the cytosol to the endoplasmic reticulum. This results in an elevated cytosolic Ca$^{2+}$/H11001 concentration (Thastrup et al., 1990; Taylor and Broad, 1998). To test whether stimulation with nanomolar concentrations of thapsigargin leads to elevated intracellular Ca$^{2+}$/H11001 concentrations [Ca$^{2+}$/H11001], in HaCaT cells, imaging experiments were performed. Application of 10 nM thapsigargin induced [Ca$^{2+}$/H11001] in almost all cells ranging from 10 nM to more than 150 nM as indicated by the red color (Fig. 3A) and the average trace (Fig. 3B).

**Expression of ATF3 in Thapsigargin-Treated Keratinocytes Requires an Influx of Ca$^{2+}$/H11545 from Internal Stores.** We assessed the importance of elevated cytosolic Ca$^{2+}$/H11001 levels for the signaling cascade connecting thapsigargin stimulation with enhanced ATF3 expression. HaCaT cells were preincubated in the presence or absence of the acetoxymethyl ester of the cytosolic Ca$^{2+}$/H11001 chelator BAPTA (25 $\mu$/M, 30 min). When the thapsigargin-induced elevation of the intracellular Ca$^{2+}$/H11001-concentration was precluded by the preincubation with BAPTA-AM, the stimulus-induced biosynthesis of ATF3 was completely blocked (Fig. 3C). Hence, a rise of cytosolic Ca$^{2+}$/H11001 levels was essential for the induction of the biosynthesis of ATF3 after treatment of HaCaT cells with thapsigargin. In contrast, expression of HDAC1 was not altered in the presence or absence of thapsigargin or BAPTA-AM. The importance of elevated cytosolic Ca$^{2+}$/H11001 levels for the signaling cascade in thapsigargin-stimulated HaCaT cells was further demonstrated using expression of the Ca$^{2+}$/H11001-binding protein parvalbumin. Figure 3D shows the modular structure of the PVmCherry fusion protein, which contains a nuclear export signal (NES) derived from MKK1 on its N terminus to direct expression to the cytosol (Pusl et al., 2002).

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**Fig. 2.** Thapsigargin triggers the biosynthesis of ATF3 in glioma, hepatoma, and epithelial cells. Top, human U87-MG glioma cells (A), human ARPE-19 retinal pigment epithelial cells (B), human HepG2 hepatoma cells (C), and human A549 airway epithelial cells (D) were serum-starved for 24 h and then stimulated with thapsigargin for 2 h as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against ATF3. The antibody directed against HDAC1 was used as a loading control. Bottom, U87-MG cells (A), ARPE-19 cells (B), HepG2 cells (C), and A549 cells (D) were stimulated with thapsigargin (10 nM) for the indicated periods. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against ATF3.
Fig. 3. Essential role of cytoplasmic Ca\(^{2+}\) for the thapsigargin-induced expression of ATF3 in HaCaT keratinocytes. A and B, Ca\(^{2+}\)-imaging of HaCaT cells stimulated with 10 nM thapsigargin. Application of thapsigargin induces [Ca\(^{2+}\)]\(_i\) increases in HaCaT cells. A, an infrared picture and two time points of fura-2 ratio pictures of a typical imaging experiment are shown, one under resting conditions, one after stimulation with 10 nM thapsigargin in the culture medium. Warmer colors indicate higher [Ca\(^{2+}\)]\(_i\). B, average of 313 cells of nine experiments. C, HaCaT cells were serum-starved for 24 h and preincubated for 30 min with the acetoxymethyl ester of the Ca\(^{2+}\) chelator BAPTA (25 \(\mu\)M). Incubation of the cells with thapsigargin (10 nM) was for 2 h. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against ATF3. The antibody directed against HDAC1 was used as a loading control (***, \(P < 0.001\)). D, schematic representation of the NES-PVmCherry fusion protein. E, phase contrast and fluorescence images of HaCaT cells that had been infected with a lentivirus encoding either mCherry (top) or NES-PVmCherry (bottom). F, HaCaT keratinocytes cells were infected with a lentivirus encoding either mCherry or NES-PVmCherry. The cells were serum-starved for 24 h. Stimulation with thapsigargin (10 nM) was performed for 2 h. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against ATF3. The antibody directed against HDAC1 was used as a loading control (***, \(P < 0.001\)).
Expression of Either MKP-1 or MKP-5 blocks ATF3 Expression in Thapsigargin-Stimulated HaCaT Keratinocytes. Stimulus-induced signaling requires a negative feedback loop to inactivate the signaling cascade. The MAP kinases extracellular-signal regulated protein kinase (ERK), p38, and JNK are translocated to the nucleus as phosphorylated activated enzymes, whereas dephosphorylation leads to inactivation. We tested whether overexpression of the dual-specific phosphatase MKP-1 or MKP-5 counteracts the stimulus-induced biosynthesis of ATF3 in HaCaT cells. MKP-1 dephosphorylates ERK, p38, and JNK, whereas MKP-5 specifically dephosphorylates p38 protein kinase and JNK (Ta
oue et al., 1999). Figure 4 shows that thapsigargin-induced expression of ATF3 was impaired in MKP-1- and MKP-5-expressing HaCaT keratinocytes. In contrast, the expression levels of HDAC1 did not change in the presence or absence of MKP-1 or MKP-5. The fact that both MKP-1 and MKP-5 dephosphorylate p38 and JNK indicates that either one or both of these kinases mediate the signal transduction connecting elevated Ca^{2+} concentrations with enhanced ATF3 gene transcription.

Activation of ERK Is Not Involved in the Signaling Cascade Connecting Thapsigargin Stimulation with ATF3 Expression. Expression of ATF3 has been shown to be controlled, at least in part, by ERK in vascular endothelial cells (Inoue et al., 2004). The role of ERK activation for the thapsigargin-triggered up-regulation of ATF3 expression was assessed using PD98059, a compound that inhibits the phosphorylation of the MAP kinase kinase. Cells were preincubated for 8 h and then stimulated with thapsigargin. Figure 5A shows that preincubation with PD98059 did not block the biosynthesis of ATF3 in thapsigargin-stimulated HaCaT cells, indicating that activation of ERK is not required for the induction of ATF3 gene transcription. These data were corroborated by experiments involving HaCaT keratinocytes expressing the catalytic domain of A-Raf as a fusion protein with the ligand binding domain of the murine estrogen receptor (HaCaT-ARaf:ER cells). Addition of 4OHT leads to an enhancement of A-Raf protein kinase activity and to a selective activation of the ERK signaling pathway (Rössler and Thiel, 2004). Figure 5B shows that conditional activation of the ERK signaling pathway in HaCaT keratinocytes expressing a ΔARaf-estrogen receptor fusion protein induced expression of Egr-1, a zinc finger transcription factor, but not of ATF3 as a result of 4OHT treatment. The up-regulation of Egr-1 was blocked in cells that had been preincubated with PD98059 before stimulation with thapsigargin (Fig. 5C), showing that Egr-1 was synthesized as a result of an activation of the ERK signaling pathway.

p38 Protein Kinase Regulates the Up-Regulation of ATF3 Expression in Anisomycin-Treated but Not Thapsigargin-Treated HaCaT Cells. The experiments involving overexpression of either MKP-1 or MKP-5 (Fig. 4) indicated that either p38 protein kinase or JNK connects thapsigargin stimulation of HaCaT cells with enhanced ATF3 expression. First, we tested whether forced activation of p38 protein kinase is sufficient to induce ATF3 expression in HaCaT keratinocytes in the absence of thapsigargin stimulation. We expressed a constitutively active form of MKK6, a potent upstream activator of p38 protein kinase, in HaCaT cells using lentiviral gene transfer. MKK6 is activated by phosphorylation of serine residue 207 and threonine residue 211. The MKK6E mutant contains two point mutations of these phosphoacceptor sites, S207E and T211E, thus generating a constitutively active form of MKK6 with a negative charge on these sites (Raingeaud et al., 1996). MKK6E additionally contains a FLAG epitope on its N terminus. Expression of MKK6E was verified by Western blot analysis using antibodies targeting the FLAG epitope on its N terminus. Expression of MKK6E was verified by Western blot analysis using antibodies targeting the FLAG epitope (Fig. 6A). The functional consequence of MKK6E expression is depicted in Fig. 6B. ATF3 expression was up-regulated in MKK6E-expressing cells. Preincubation of the cells with the p38-specific inhibitor SB203580 attenuated ATF3 expression induced by forced expression of MKK6E. As a control, cells were infected with a lentivirus encoding β-galactosidase. In addition, we used a pharmacological approach to assess the role of p38 protein kinase activation on the thapsigargin-induced signal-
ing cascade leading to ATF3 expression. Figure 6C shows that preincubation of the cells with SB203580 did not block the thapsigargin-induced up-regulation of ATF3 in HaCaT cells. In contrast, anisomycin-induced expression of ATF3 was impaired by preincubation of the cells with SB203580. Together, these results showed that although activation of the p38 pathway led to ATF3 expression, p38 protein kinase was not involved in the signaling cascade that connects thapsigargin stimulation and ATF3 expression in HaCaT cells.

**JNK Is Required for ATF3 Expression in Thapsigargin-Stimulated HaCaT Keratinocytes.** The experiments involving forced expression of MKP-5 (Fig. 4) revealed that p38 protein kinase, JNK, or both are required to connect the thapsigargin-induced Ca^{2+} signals with enhanced expression of ATF3. Using the compound SB203580, we were able to exclude an involvement of p38, thus leaving JNK as the likely candidate that connects elevated [Ca^{2+}], with ATF3 gene transcription. Thus, we analyzed the impact of JNK in the thapsigargin-triggered signaling cascade in HaCaT keratinocytes. The activity of JNK is controlled by the MAP3 kinase MEKK1. To stimulate JNK activity, we infected HaCaT keratinocytes with a lentivirus expressing MEKK1Δ. Figure 7A shows that FLAG-tagged MEKK1Δ was expressed in HaCaT cells as expected. The analysis of the ATF3 expression levels revealed that ATF3 was synthesized in MEKK1Δ-expressing cells but not in HaCaT cells infected with a β-galactosidase-expressing lentivirus (Fig. 7B). MEKK1Δ-induced up-regulation of ATF3 expression was impaired in HaCaT cells that had been preincubated with the JNK inhibitor SP600125. However, ATF3 expression resulting from MKK6E overexpression was not attenuated by this compound (Fig. 7C). Next, we stimulated HaCaT keratinocytes that had been pretreated with SP600125 or vehicle with either thapsigargin or anisomycin. Figure 7D shows that thapsigargin-stimulated up-regulation of ATF3 was significantly impaired in HaCaT cells that had been pretreated with SP600125, whereas this compound had no effect upon anisomycin-induced ATF3 expression. SP600125 also did not influence the expression of Egr-1 in 4OHT-treated HaCaT-ΔRaf:ER cells (Fig. 7E), indicating that the ERK signaling pathway was not altered in SP600125-treated keratinocytes. The importance of the JNK signaling pathway in thapsigargin-treated keratinocytes was further demonstrated using a dominant-negative mutant of SEK1/MKK4, a MAP kinase kinase that is activated by MEKK1 via phosphorylation of Ser204 and Ser207 and directly phosphorylates and activates JNK. Mutation of Ser204 and Ser207 to alanine or leucine, respectively, generates a dominant-negative mutant, SEK-AL. Expression of SEK-AL in murine fibroblasts revealed that JNK but not p38 activation was specifically impaired (Zanke et al., 1996). We generated a lentiviral transfer vector encoding FLAG-tagged SEK-AL. Western Blot analysis showed that the SEK-AL mutant was correctly expressed in HaCaT cells infected with a lentivirus encoding FLAG–SEK-AL. Expression of SEK-AL blocked the up-regulation of ATF3 in thapsigargin-stimulated HaCaT cells, whereas anisomycin-induced ATF3 expression was not influenced under these conditions (Fig. 7F). Hence, JNK activation is essential within the signaling cascade that leads to an up-regulation of ATF3 in thapsigargin-stimulated HaCaT keratinocytes.

**Up-Regulation of Caspase-3/7 Activity in ATF3-Expressing HaCaT Keratinocytes.** ATF3 expression has been correlated with enhanced cell death in various cell types (Hai et al., 1999). Therefore, we expressed ATF3 in HaCaT cells using lentiviral gene transfer. As a control, cells were infected with a lentivirus expressing β-galactosidase- or ATF3-galactosidase. Figure 8A shows that ATF3 was expressed as expected. Next, we measured caspase-3/7 activity in either β-galactosidase- or ATF3-expressing HaCaT cells. Figure 8B shows that caspase-3/7 activity was up-regulated on the order of 2-fold in ATF3-expressing HaCaT cells.

**Fig. 5.** Activation of ERK is not required for ATF3 induction in thapsigargin-stimulated HaCaT cells. A, HaCaT cells were serum-starved for 24 h and then maintained in the presence or absence of PD98059 (50 μM). Cells were stimulated with thapsigargin (10 nM) for 2 h. Nuclear extracts were prepared and analyzed for ATF3 expression. B, HaCaT cells expressing a conditionally active form of the protein kinase A-Raf (HaCaT-ΔRaf:ER cells) were serum-starved for 24 h and then stimulated with 4OHT (200 nM) as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against either Egr-1 or HDAC1. C, HaCaT-ΔRaf:ER cells maintained in the presence or absence of PD98059 (50 μM) were stimulated with 4OHT (200 nM) for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against either Egr-1 or HDAC1.
Induction of Programmed Cell Death in Thapsigargin-Stimulated HaCaT Keratinocytes. Many studies showed that stimulation with micromolar concentrations of thapsigargin induces programmed cell death in a variety of cell types, including HaCaT cells (Pani et al., 2006). We measured caspase-3/7 activity in HaCaT cells stimulated with different concentrations of thapsigargin. Caspases-3/7 play a critical role in the execution phase of apoptosis. The enzymes are normally expressed as an inactive pro-enzyme, but can be activated via proteolysis in apoptotic signaling cascades. Figure 9A shows increased caspase-3/7 activity in HaCaT cells treated with increased concentrations of thapsigargin. Stimulation with 10 nM thapsigargin led to a 2-fold up-regulation of caspase-3/7 activity. A similar increase has been observed in HaCaT cells forced to ATF3 (Fig. 8B). Incubation with the compound SP600125 blocked the activation of caspase-3/7 in thapsigargin-stimulated HaCaT cells (Fig. 9B), because it blocked the thapsigargin-triggered expression of ATF3 (Fig. 7D), suggesting that thapsigargin stimulation activated ATF3 expression and activation of caspase-3/7 via JNK. The induction of programmed cell death was confirmed by analyzing the fragmentation of the chromatin (TUNEL assay). As a control, etoposide-induced chromatin fragmentation was assessed. It is noteworthy that stimulation of the cells with low concentration of thapsigargin already significantly increased the number of cells with fragmented chromatin (Fig. 9, C and D). To discriminate between early apoptotic and dead cells we used annexin-V to stain phosphatidylserine on the cell surface and the cell impermeant dye 7-AAD to detect dead cells by intercalating into double-stranded nucleic acids. Although the number of annexin-V (+)/7-AAD (−) cells increased 2.5- to 3-fold in cells treated with either 10 nM or 1 μM thapsigargin, we did not detect a significant increase of the number of 7-AAD-positive cells. These data indicate that cell death induced by thapsigargin in HaCaT keratinocytes is apoptotic and not necrotic. As a control, we treated the cells with hydrogen peroxide, which is known to induce necrosis at higher concentrations (Csordas et al., 2006). Although the number of annexin-V (+)/7-AAD (−) cells increased 2- to 2.5-fold in cells treated with H₂O₂ (1 and 5 mM), we also observed an increase of the number of 7-AAD-positive cells, indicating that H₂O₂ induces both apoptosis and necrosis in HaCaT cells.

Discussion

Thapsigargin is a very frequently used compound; almost 7000 publications in the PubMed database involve thapsigargin. Treatment of cells with thapsigargin is used to empty intracellular Ca²⁺ stores and to increase the cytoplasmic Ca²⁺ concentration. The up-regulation of [Ca²⁺]i induces a variety of signaling events in the cells. It has been observed

Fig. 6. Activation of p38 protein kinase is not required for ATF3 induction in thapsigargin-treated HaCaT keratinocytes. A, HaCaT keratinocytes were infected with a recombinant lentivirus encoding a FLAG-tagged form of MKK6E. As a control, HaCaT cells were infected with a lentivirus encoding β-galactosidase (β-Gal). The Western blot was probed with an antibody against the FLAG tag. Molecular mass markers (in kiloDaltons) are shown on the left. B, HaCaT cells were infected with lentiviruses encoding either β-galactosidase or MKK6E. The cells were serum-starved for 24 h and cultured in the presence or absence of SB203580 (10 μM). Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against ATF3. The antibody directed against HDAC1 was used as a loading control. C and D, HaCaT cells were serum-starved for 24 h and then cultured in the presence or absence of SB203580 (10 μM) for 1 h. Cells were stimulated with either thapsigargin (10 nM) (C) or anisomycin (10 ng/ml) (D) for 2 h. Nuclear extracts were prepared and analyzed for ATF3 and HDAC1 expression.
that thapsigargin treatment triggers the biosynthesis of the transcription factor ATF3 in HaCaT cells (Kang et al., 2003), indicating that there is a communication between the rise of 
\[ \text{Ca}^{2+} \] and the gene expression pattern in the nucleus. The objective of this study was to analyze the signaling cascade that leads to the expression of ATF3 in HaCaT keratinocytes after stimulation with thapsigargin.

Given the central role of an increased concentration of cytosolic free Ca\(^{2+}\) in thapsigargin-treated cells, we studied the Ca\(^{2+}\) influx properties with selected pharmacological compounds. The thapsigargin-induced up-regulation of ATF3 expression was not blocked in HaCaT cells treated with either EGTA or nifedipine, indicating that Ca\(^{2+}\) influx from outside (i.e., involving L-type voltage gated Ca\(^{2+}\) channels) was not involved in the signaling cascade. Likewise, a role for the ryanodine receptors in thapsigargin signaling could be excluded, based on experiments involving dantrolene. In contrast, experiments using the acetoxymethyl ester of the cytosolic Ca\(^{2+}\) chelator BAPTA revealed that the rise of cytosolic Ca\(^{2+}\) concentration is essential for induction of ATF3 biosynthesis after thapsigargin treatment. In addition, we used an alternative approach to assess the role of cytoplasmic Ca\(^{2+}\) ions for the stimulus-induced up-regulation of ATF3 expression. We expressed a parvalbumin-mCherry fusion

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**Fig. 7.** Activation of JNK is necessary for ATF3 induction in thapsigargin-treated HaCaT keratinocytes. A. HaCaT keratinocytes were infected with recombinant lentiviruses encoding either a FLAG-tagged form of MEKK1 or—as a control—\( \beta \)-galactosidase (\( \beta \)-Gal). The Western blot was probed with an antibody against the FLAG tag. Molecular mass markers (in kiloDaltons) are shown on the left. B. HaCaT cells were infected with lentiviruses encoding either \( \beta \)-galactosidase or MEKK1 and serum-starved for 24 h. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against ATF3. Molecular mass markers (in kiloDaltons) are shown on the left. C. HaCaT cells infected with lentiviruses encoding either \( \beta \)-galactosidase, MEKK1, or MKK6E were serum-starved for 24 h and then cultured in the presence or absence of SP600125 (5 \( \mu \)M). Cells were stimulated with thapsigargin (10 nM) for 2 h as indicated. Nuclear extracts were prepared and analyzed for ATF3 and HDAC1 expression. D, noninfected HaCaT cells were serum-starved for 24 h and then cultured in the presence or absence of SP600125 (5 \( \mu \)M). Cells were stimulated with either thapsigargin (10 nM) or anisomycin (10 ng/ml) for 2 h. Nuclear extracts were prepared and analyzed for ATF3 and HDAC1 expression (***, \( P < 0.001 \)). E, HaCaT-\( \Delta \)ARaf:ER cells were serum-starved for 24 h and then maintained in the presence or absence of SP600125 (5 \( \mu \)M). Cells were stimulated with thapsigargin (10 nM) for 2 h as indicated. Nuclear extracts were prepared and analyzed for ATF3 and HDAC1 expression. F, Western blot analysis of HaCaT cells infected with a recombinant lentivirus encoding \( \beta \)-galactosidase and thapsigargin. G, HaCaT cells were infected with lentiviruses encoding either \( \beta \)-galactosidase or SEK-AL. The cells were serum-starved for 24 h and then cultured in the presence or absence of SP600125 (5 \( \mu \)M). Cells were stimulated with thapsigargin (10 nM) or anisomycin (10 ng/ml) for 2 h as indicated. Nuclear extracts were prepared and analyzed for ATF3 and HDAC1 expression (***, \( P < 0.001 \)).
protein in the cytosol of HaCaT cells to buffer Ca$^{2+}$ in this compartment. Accordingly, expression of ATF3 was blocked when the thapsigargin-induced up-regulation of Ca$^{2+}$ was buffered. Together, these data show that the rise of [Ca$^{2+}$]$_i$ from internal stores is essential for up-regulation of ATF3 expression in thapsigargin-stimulated HaCaT keratinocytes.

A rise of [Ca$^{2+}$]$_i$ is in many intracellular signaling cascades the trigger to activate the ERK1/2 (i.e., after activation of G$_{q}$-coupled receptors) (Mayer et al., 2008b; Rössler et al., 2008; Rössler and Thiel, 2009; Thiel et al., 2010). However, activation of ATF3 expression in thapsigargin-stimulated HaCaT cells was independent of ERK. As a further proof, we showed that the selective activation of the ERK signaling pathway in HaCaT cells expressing a conditionally active form of A-Raf protein kinase induced expression of the zinc finger protein Egr-1 but not expression of ATF3. Egr-1 has been shown to transactivate the ATF3 gene in sulindac sulfide- or troglitazone-treated human colorectal cancer cells or GnRH-stimulated gonadotrophs (Bottone et al., 2005, Mayer et al., 2008a). The results presented here showed that Egr-1 plays no role in the thapsigargin-stimulated up-regulation of ATF3 in HaCaT keratinocytes. Even forced expression of Egr-1 in HaCaT cells expressing an active A-Raf protein kinase failed to induce ATF3 expression. Thus, the regulation of ATF3 gene transcription by Egr-1 is cell type- and stimulus-specific.

Expression experiments involving an up-regulation of MKP-1 and MKP-5 in HaCaT cells showed that MAP kinase phosphatases are key enzymes for blocking the thapsigargin-induced signaling cascade leading to enhanced ATF3 expression. Although MKP-1 is known to dephosphorylate and inactivate ERK1/2, p38 protein kinase, and JNK, MKP-5 selectively dephosphorylates p38 and JNK. These experiments, together with the previous exclusion of ERK1/2 as an integral part of the thapsigargin-induced signaling cascade, directed our attention to the role of p38 protein kinase and JNK as mediators between elevated [Ca$^{2+}$]$_i$ and enhanced ATF3 gene transcription. p38 protein kinase was a likely candidate to mediate the connection between a rise of [Ca$^{2+}$]$_i$, and expression of ATF3, because it has been shown that p38 is essential for the biosynthesis of ATF3 in anisomycin-stimulated HeLa cells (Lu et al., 2007). Moreover, expression of a constitutively active MKK6 in HaCaT cells induced expression of ATF3. However, incubation of the cells with the p38-specific inhibitor compound SB203580 blocked anisomycin-triggered up-regulation of ATF3 but had no effect upon thapsigargin-stimulated ATF3 biosynthesis, indicating that p38 protein kinase is essential to induce the biosynthesis of ATF3 in anisomycin-stimulated HaCaT cells but not in HaCaT cells that had been stimulated with thapsigargin.

The essential role of JNK in the up-regulation of ATF3 expression in HaCaT cells was assessed using three different approaches: ectopic expression of a truncated form of MEKK1, expression of SEK-AL, a dominant-negative mutant of SEK1/MKK4, and treatment of the cells with the compound SP600125. MEKK1 is a MAP3 kinase that controls the activity of JNK. ATF3 was up-regulated in HaCaT cells expressing an N-terminal truncated constitutively active form of MEKK1. These experiments showed that activation of the JNK pathway is sufficient to induce ATF3 biosynthesis. In contrast, expression of a dominant-negative mutant of SEK1/MKK4 that inhibits phosphorylation and activation of JNK blocked the thapsigargin-induced up-regulation of ATF3 in HaCaT cells. The dominant-negative mutant SEK-AL has also been shown to impair JNK but not p38 activation in mouse fibroblasts. Likewise, the activation of JNK, but not the activation of p38, was impaired in SEK1/MKK4-deficient cells (Zanke et al., 1996; Yang et al., 1997). The importance of JNK within the signaling cascade that connects thapsigargin stimulation and ATF3 gene transcription was further corroborated in experiments using the compound SP600125, often described as “JNK inhibitor.” However, SP600125 inhibits the activity of several other protein kinases as well. In the framework of this study, it was legitimate to use this compound, because experiments involving ectopic expression of MKP-5 had already indicated that either p38 protein kinase or JNK mediates the connection between the thapsigargin-induced rise of [Ca$^{2+}$]$_i$, and the biosynthesis of ATF3. Together, these data show that activation of JNK is an essential part of the signaling cascade that leads to enhanced ATF3 expression in thapsigargin-stimulated HaCaT cells.

**Fig. 8.** Up-regulation of caspase-3/7 activity in ATF3-expressing HaCaT keratinocytes. A, HaCaT cells were infected with lentiviruses encoding either ATF3 or β-galactosidase. Two days later, nuclear extracts were prepared and analyzed for ATF3 expression using an antibody against ATF3. Molecular mass markers (in kilodaltons) are shown on the left. B, HaCaT cells were infected with lentiviruses encoding either ATF3 or β-galactosidase. Cells were serum-starved for 24 h. Caspase-3/7 activity was measured 2 days later.
Thapsigargin stimulation induces programmed cell death in many different cell types, including HaCaT cells (Pani et al., 2006). Likewise, ATF3 expression has been correlated with enhanced cell death (Hai et al., 1999). ATF3 expression in thapsigargin-stimulated HaCaT cells lasts for at least 8 h, indicating that, in this case, ATF3 biosynthesis is not transient but sustained. The fact that the caspase-3/7 activity is up-regulated either in thapsigargin-stimulated HaCaT cells or in cells that are forced to overexpress ATF3 indicates that the synthesized ATF3 protein was biologically active. In addition, we could show that inhibition of JNK attenuated stimulus-induced ATF3 expression and up-regulation of caspase-3/7 activity as well. The connection between JNK activation, ATF3 biosynthesis, and programmed cell death has also been reported to occur in K⁺-deprived cerebellar granule cells (Mei et al., 2008).

In summary, we have shown that stimulation of HaCaT keratinocytes with nanomolar concentrations of thapsigargin...
induces a signaling cascade involving rise of [Ca$^{2+}$], activation of JNK, and up-regulation of ATF3 expression. A negative feedback loop is established by the protein phosphatases MKP-1 and MKP-5, which dephosphorylate and inactivate JNK. Thapsigargin stimulation as well as overexpression of ATF3 led to an up-regulation of caspase-3/7 activity in HaCaT keratinocytes, underlining the proapoptotic activity of ATF3.

Acknowledgments

We thank Verena Dexheimer for initial work on this project, Heike Bantel for help in setting up the caspase-3/7 assay, and Frank Schmitz and Karin Schwarz for help in microscopy. We thank N. E. Fusseneg for HaCaT cells; Roger Davis, Michael Karin, and James R. Woodgett for plasmids; Karl Bach for excellent technical help; and Libby Guetlein for critical reading of the manuscript.

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Woodgett for plasmids; Karl Bach for excellent technical help; and

Fusenig for HaCaT cells; Roger Davis, Michael Karin, and James R.

Bantel for help in setting up the caspase-3/7 assay, and Frank

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