

# Mitochondrial Cytochrome *c* Release Mediates Ceramide-induced Activator Protein 2 Activation and Gene Expression in Keratinocytes\*

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**The intracellular signaling pathway(s) through which second messenger ceramides induce gene expression in human cells has not yet been characterized. In the present study, ceramide-induced expression of intercellular adhesion molecule-1 (ICAM-1), which requires activation of transcription factor activator protein 2 (AP-2), was found to be mediated through a mitochondrial pathway. Inhibitors of mitochondrial electron transport chain (e.g. rotenone, thenoyltrifluoroacetone, and antimycin A) reduced ceramide-induced ICAM-1 expression. Stimulation of human keratinocytes with cell-permeant ceramides at concentrations that did not induce apoptosis (no activation of caspases 3, 8, and 9 and no nucleosomal fragmentation) but caused AP-2 activation and ICAM-1 induction released cytochrome *c* (cyt *c*) from mitochondria into the cytoplasm of cells. This cyt *c* release was an indispensable prerequisite for effective ceramide signaling, because its inhibition by modulating the mitochondrial megachannel with bongkrekic acid or carboxyatractyloside prevented ceramide-induced AP-2 activation and ICAM-1 expression. Analysis of the interaction between cyt *c* and AP-2 revealed that cyt *c* oxidized AP-2 and that this redox regulation greatly enhanced the DNA binding capacity of AP-2. Mitochondria thus have a previously unrecognized function in signaling ceramide-induced transcription factor activation and gene regulation.**

Ceramides play a well recognized role as second messenger in the stress response of human cells to a variety of different stimuli including the inflammatory cytokines tumor necrosis factor- $\alpha$  and interleukin-1,  $\gamma$ -radiation, and solar UV B (290–320 nm) and A (320–400 nm) radiation (1–3). Previous research has focused on the mechanisms by which ceramides are being generated under these conditions. It has been shown that ceramides can be produced enzymatically, either through *de novo* synthesis involving the enzyme ceramide synthase (4) or through hydrolysis of cell membrane sphingomyelin by acid or

neutral sphingomyelinases (1, 2). In addition, evidence has been provided for a third pathway, in which singlet oxygen mediates the nonenzymatic hydrolysis of ceramide from cell membrane sphingomyelin (3).

Relatively little is currently known about the intracellular signaling pathways that allow ceramides to up-regulate transcriptional expression of genes. By stimulating NHEK with cell-permeant ceramides (e.g. D-erythro-sphingosine, N-acetyl-(C2-ceramides)<sup>1</sup> or D-erythrosphingosine, N-hexanoyl-(C6-ceramides)) it has recently been observed that ceramide-induced expression of the human ICAM-1 gene is mediated through activation of AP-2, indicating that ceramides are capable of activating transcription factors in human cells through a yet unknown mechanism (3).

In addition to regulating gene expression, second messenger ceramides have also been shown to induce apoptosis in human cells (5, 6). Ceramide-induced apoptosis was found to be due to the release of cyt *c* from mitochondria into the cytoplasm (7) and the subsequent activation of caspases (8). The precise mechanism by which ceramides cause cyt *c* release from mitochondria is not known, but it has been suggested that ceramides are able to exert direct effects on mitochondria. Studies employing isolated mitochondria have demonstrated that ceramides affect the mitochondrial electron transport chain (9), which plays a critical role in controlling the release of cyt *c* through the mitochondrial permeability transition pore/mitochondrial megachannel into the cytoplasm (10, 11). It is currently not known whether mitochondria are involved in an analogous fashion in ceramide-induced transcription factor activation and gene expression. In this regard, it is of interest that induction of apoptosis requires ceramide concentrations that are severalfold higher than those necessary to induce gene regulatory effects (12, 13). This suggested to us the possibility that stimulation of human cells with nonapoptogenic ceramide concentrations could have more subtle effects on mitochondrial functions, which would lead to gene induction rather than apoptosis. In the present study, we have tested this hypothesis by studying the role of mitochondria in ceramide-induced AP-2 activation and ICAM-1 expression in NHEK. As UVA radiation may exert other effects on mitochondria (14) we did not expose cells to UVA irradiation to induce an endogenous ceramide

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This paper is dedicated to Professor Lothar Jaenicke, Institute of Biochemistry at the University of Cologne, Germany, on the occasion of his 80th birthday in September 2003, in recognition of his important contributions to biochemistry.

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<sup>1</sup> The abbreviations used are: C2-ceramide, D-erythro-sphingosine, N-acetyl-; C6-ceramide, D-erythro-sphingosine, N-hexanoyl-; ICAM-1, intercellular adhesion molecule-1; AP-2, activator protein-2; NHEK, normal human epidermal keratinocyte(s); cyt *c*, cytochrome *c*; Bka, bongkrekic acid; Cat, carboxyatractyloside; IFN- $\gamma$ , interferon- $\gamma$ ; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; fpu, foot-printing unit; RT, reverse transcription; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; GSSG, glutathione disulfide.

release. Instead, synthetic short chain ceramides were employed, which permeate through the cytoplasmic membrane and have been successfully used in previous studies to address mechanistic questions (15–18).

#### EXPERIMENTAL PROCEDURES

**Materials**—C2-ceramide, C6-ceramide, *N*-oleylethanolamine, rotenone, thenoyltrifluoroacetone, antimycin A, interferon- $\gamma$ , bongkrekic acid, carboxyatractyloside, camptothecin, staurosporine, DNase I, *N*-acetyl-L-cysteine, and human recombinant AP-2.

**Cell Culture**—Long term cultured, primary normal human keratinocytes (NHEK) prepared from neonatal foreskin were cultured as described (19). Epidermoid KB cells obtained from ATCC (American Type Culture Collection, Manassas, VA) served as a keratinocyte line with a similar reaction pattern concerning cytokine and adhesion molecule expression as NHEK (20). Osteosarcoma cells depleted for mitochondrial DNA (Rho 0 cells) served as controls (21). Human dermal fibroblasts prepared from neonatal foreskin were cultured as described (22). HeLa cells were obtained from ATCC and cultured as described (23).

**Antibodies, Cytokines, and Chemicals**—Rotenone, thenoyltrifluoroacetone, antimycin A, DNase I, glutathione disulfide, and glutathione reductase were purchased from Sigma. Interferon- $\gamma$  (IFN- $\gamma$ ) was obtained from R&D Systems (Wiesbaden, Germany). C2-ceramide (D-erythro-sphingosine, *N*-acetyl-), C6-ceramide (D-erythro-sphingosine, *N*-hexanoyl-), bongkrekic acid, carboxyatractyloside, camptothecin, *N*-oleylethanolamine, and *N*-acetyl-L-cysteine were delivered by Calbiochem-Novabiochem (Bad Soden, Germany). Ceramides were dissolved in ethanol as stocks (10 mM) and added to the culture medium in dilutions of 1:1000 to obtain a final concentration of 10  $\mu$ M. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Roth (Karlsruhe, Germany). Human recombinant AP-2 was obtained from Promega (Mannheim, Germany). Staurosporine was purchased from Alexis (Gruenberg, Germany).

**Differential RT-PCR**—Total RNA was isolated using RNeasy Total RNA Kits (Qiagen, Hilden, Germany). Expression of ICAM-1 was measured by differential reverse transcriptase-PCR using the RT-PCR core kit (Applied Biosystems, Darmstadt, Germany) and a specific primer pair for ICAM-1 (5'-TGACCAGCCCAAGTTGTTGG-3', 5'-ATCTCTCC-TACCAGCACCG-3'). Semiquantitative analysis of the RT-PCR products was done using ion exchange chromatography connected to an on-line UV spectrophotometer (absorption at 260 nm) (22, 24). Gene expression was assessed in cells treated with 10  $\mu$ M C6-ceramide or 1000 units/ml IFN- $\gamma$ . The effect of inhibitors of mitochondrial respiratory chain such as rotenone (2.5  $\mu$ M), thenoyltrifluoroacetone (20  $\mu$ M), or antimycin A (1  $\mu$ M) on gene expression was analyzed in both types of stimulations. *N*-Acetylcysteine (20  $\mu$ M) was added to cells in order to increase the endogenous glutathione level.

**Gel Electrophoresis Mobility Shift Assay**—Nuclear extracts were prepared according to the Dignam protocol as described (25). The AP-2 consensus oligonucleotide (top strand, 5'-GACCCTCTCGGCCCGGC-ACCCT-3') was deduced from the ICAM-1 promoter (26). Specificity controls were performed as done before (19). For assays using the recombinant AP-2 protein, the gel electrophoresis standard buffer was diluted four times.

**Immunoprecipitation and Western Blot**—Release of cyt *c* from mitochondria was analyzed in cytosolic extracts prepared as described in detail. In order to separate intact cells into cytosolic and mitochondrial fractions (27), NHEK were harvested using ice-cold phosphate-buffered saline and washed. Cell pellets were resuspended in buffer A (10 mM HEPES/KOH, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol) and disrupted with 20 strokes of a Dounce homogenizer and a tight pestle (B-type). After centrifugation, a one-eleventh volume part of buffer B (0.3 M HEPES/KOH, pH 7.9, 0.03 M MgCl<sub>2</sub>, 1.4 M KCl) was added, and the extract was centrifuged for 1 h at 4 °C at 355,000  $\times g$ . Supernatant was dialyzed overnight at 4 °C against buffer D (20 mM HEPES/KOH, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% (v/v) glycerol). Proteinase inhibitor Complete™ containing EDTA (Roche Applied Science) was added to all of the buffers used. The purified cytosolic extract was analyzed for protein content and stored at -80 °C. The purity of all fractions regarding mitochondrial contamination was controlled by Western blot analysis (Fig. 2B) for the mitochondrial marker protein cyt *c* oxidase subunit II (28), yielding a specific band at 18 kDa (29). The anti-cyt *c* oxidase subunit II monoclonal antibody (clone 12C4) was obtained from Molecular Probes Europe (Leiden, The Netherlands).

For detection of cyt *c* in the cytosolic fraction immunoprecipitation technique was used according to Ref. 30. One hundred  $\mu$ g of cytosolic extract were adjusted to 1 ml using buffer A, 5  $\mu$ g of mouse anti-human

cyt *c* antibody MAB898 (R&D Systems, Wiesbaden, Germany) was added, and the extract was incubated on a rotating platform using end-over-end inversion at 4 °C overnight. After the addition of 7  $\mu$ g of rabbit anti-mouse IgG (heavy + light chain, Zymed Laboratories Inc., Zytomed GmbH, Berlin, Germany), the incubation was continued for another 30 min. Fifty  $\mu$ l of protein A-agarose (Roche Applied Science) were added, and the extract was further incubated for 30 min. The extract was centrifuged for 30 s at 12,000  $\times g$  at 4 °C. After discarding the supernatant, the pellet was washed three times using ice-cold phosphate-buffered saline and transferred to a new reaction tube. The pellet was resuspended in 50  $\mu$ l of 2 $\times$  SDS gel sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, pH 6.8, 10% glycerol, and bromophenol blue) by vortexing and then incubated for 3 min at 95 °C. The immunoprecipitate was fractionated through SDS-PAGE (16% polyacrylamide) (31) and immunoblotted on nitrocellulose membrane (Trans-blot; Bio-Rad). Human recombinant cyt *c* (R&D Systems, Wiesbaden, Germany) served as control on gels. Detection of cyt *c* was done using anti-holo-cyt *c* antibody (clone 2CYTC-199, R&D Systems, Wiesbaden, Germany). The cyt *c* was visualized by chemiluminescence (ECL; Amersham Biosciences).

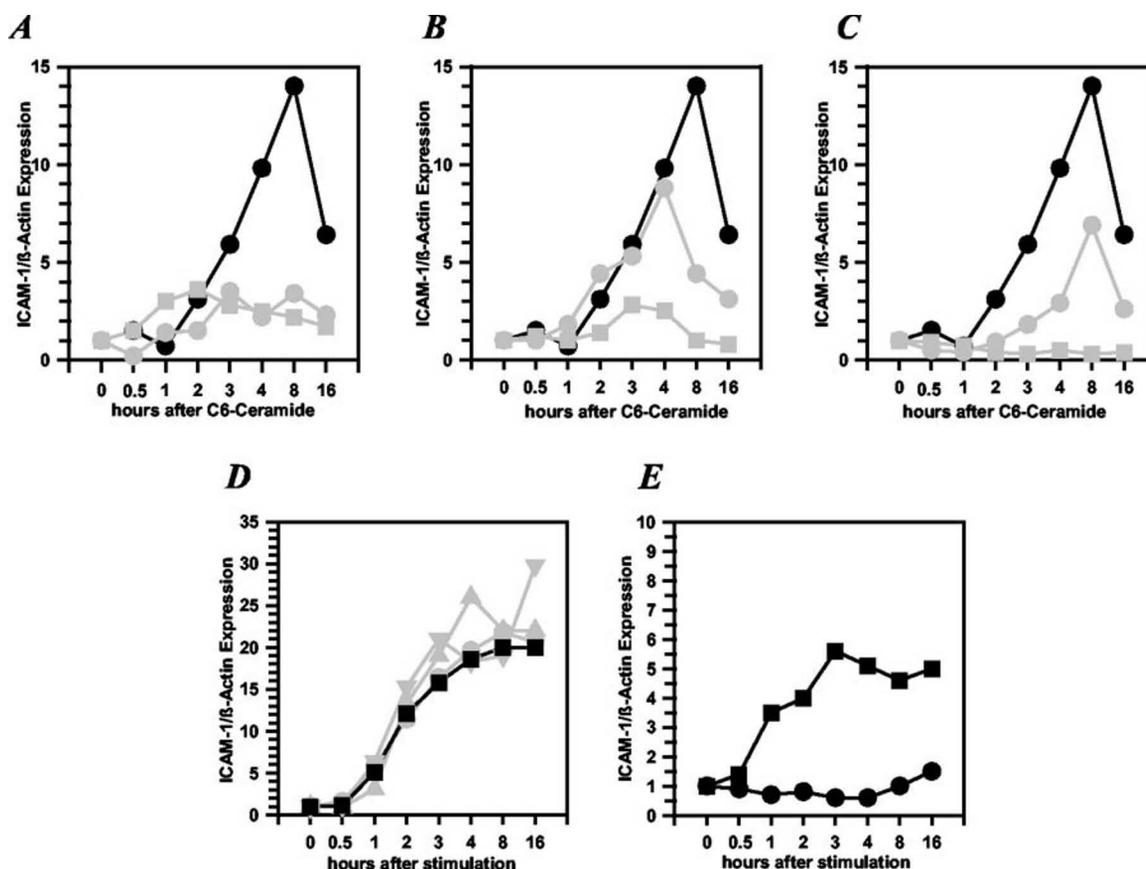
**Apoptosis Assays**—Detection of nucleosomal fragmentation was assessed using Cell Death Detection ELISA<sup>plus</sup> (Roche Applied Science). The enrichment of mono- and oligonucleosomes released into the cytoplasm of cell lysates at 0.5, 16, and 24 h after stimulation with 10–100  $\mu$ M C6-ceramide or 5  $\mu$ M camptothecin was detected by biotinylated anti-histone and peroxidase-coupled anti-DNA-antibodies and was calculated by using the formula: absorbance of sample cells/absorbance of control. For detection of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), cells were stained according to the manufacturer's description by the *in situ* cell death detection kit (Roche Applied Science). Briefly, 2  $\times 10^6$  cells were fixed with 100  $\mu$ l of 2% paraformaldehyde for 60 min at room temperature, washed once, resuspended in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, and washed again twice. Cells were incubated with the TUNEL reaction mixture for 60 min at 37 °C in the dark in a humidified atmosphere. After washing twice, the cells were analyzed by fluorescence-activated cell sorting analysis using a FACScan (BD Pharmingen, Heidelberg, Germany). Cells treated with DNase I (9 units/sample) served as positive controls.

Effector caspase 3 was analyzed by fluorometric immunoabsorbent enzyme assay (FIENA; Roche Applied Science) in NHEK stimulated with either 10  $\mu$ M C6-ceramide or 2–4  $\mu$ M staurosporine. Caspase 8 and caspase 9 were detected by colorimetric activity assays (R&D Systems, Wiesbaden, Germany) in NHEK stimulated with 10  $\mu$ M C6-ceramide or 1–4  $\mu$ M staurosporine. Camptothecin and staurosporine served as positive controls to induce apoptosis.

**Viability Using MTT Assay**—Cytotoxicity of ceramide and/or of substances that modulate the mitochondrial megachannel such as bongkrekic acid (5  $\mu$ M) or carboxyatractyloside (20  $\mu$ M) was evaluated using the MTT colorimetric assay according to Ref. 32. Briefly, NHEK were seeded in 96-well plates at 15,000 cells/200  $\mu$ l in each well. The next day, the cells were treated with the substances of interest for 16 h. After 16 h, the medium was exchanged, 25  $\mu$ l of MTT (2 mg/ml phosphate-buffered saline) were added, and the mixture was incubated for another 3 h. Finally, solutions were removed, formazan crystals were dissolved in 200  $\mu$ l of Me<sub>2</sub>SO, and absorption was measured using a microplate reader (Labsystems, Global Medical Instruments Inc., Albertsville, MI) at 540 nm. Viability was calculated as a percentage of control from three individual experiments (33).

**Assay of Cytochrome *c* Reduction**—AP-2 oxidation by cyt *c* (from horse heart, purity 95%; Sigma) was determined *in vitro* by monitoring the changes in absorption A<sub>550</sub> on a DU-70 spectrophotometer (Beckman Coulter GmbH, Unterschleißheim, Germany) with the bandwidth 0.5 nm (34). Ten  $\mu$ M cyt *c* solution in 0.1 M HEPES, pH 7.0 (35), was analyzed in the presence or absence of different amounts of transcription factor human recombinant AP-2 (Promega, Heidelberg, Germany) quantified in footprinting units (fpu) or bovine serum albumin (molecular biology grade). The footprinting units are defined as the amount of protein required to give a full DNase I protection pattern on the SV40 early promoter (*i.e.* depending on the lot 0.5 fpu/ $\mu$ l, meaning ~20–150 ng of protein). Calculation of cyt *c* in the reduced form according to the Lambert-Beer law was done by using the extinction coefficient (36) of cyt *c*, 19 mM<sup>-1</sup> cm<sup>-1</sup> at 550–540 nm.

**Glutathione Assay**—Determination of total glutathione content was done using the 5,5'-dithiobis-(2-nitrobenzoic acid)-glutathione disulfide (GSSG) reductase recycling assay for GSSG (37). All samples including the standards were assayed in duplicate. A graph of the rate of change of absorbance *versus* the amount of GSSG was drawn, and the values for



**FIG. 1. Abrogation of ceramide-induced expression of ICAM-1 mRNA in NHEK by inhibitors for mitochondrial electron transport chain complexes I (rotenone), II (thenoyltrifluoroacetone), and III (antimycin A).** *A*, ICAM-1/ $\beta$ -actin expression in NHEK after stimulation of cells with  $10 \mu\text{M}$  C6-ceramide (black circles), after preincubation with  $2.5 \mu\text{M}$  rotenone (gray squares) for 60 min, or after preincubation with  $2.5 \mu\text{M}$  rotenone (gray circles) for 60 min and stimulation with  $10 \mu\text{M}$  C6-ceramide. Results represent one of three essentially identical experiments. *B*, ICAM-1/ $\beta$ -actin expression in NHEK after stimulation of cells with  $10 \mu\text{M}$  C6-ceramide (black circles), after preincubation with  $20 \mu\text{M}$  thenoyltrifluoroacetone (gray squares) for 60 min, or after preincubation with  $20 \mu\text{M}$  thenoyltrifluoroacetone (gray circles) for 60 min and stimulation with  $10 \mu\text{M}$  C6-ceramide. Results represent one of three essentially identical experiments. *C*, ICAM-1/ $\beta$ -actin expression in NHEK after stimulation of cells with  $10 \mu\text{M}$  C6-ceramide (black circles), after preincubation with  $1 \mu\text{M}$  antimycin A (gray squares) for 60 min, or after preincubation with  $1 \mu\text{M}$  antimycin A (gray circles) for 60 min and stimulation with  $10 \mu\text{M}$  C6-ceramide. Results represent one of three essentially identical experiments. *D*, ICAM-1/ $\beta$ -actin expression in NHEK after stimulation with 1000 units/ml IFN- $\gamma$  without inhibitors of electron transport chain (black squares) or after preincubation for 60 min with  $2.5 \mu\text{M}$  rotenone (gray upright triangles),  $20 \mu\text{M}$  thenoyltrifluoroacetone (gray circles), or  $1 \mu\text{M}$  antimycin A (gray inverse triangles). Incubation with inhibitors alone is shown in A–C. Results represent one of three essentially identical experiments. *E*, ICAM-1/ $\beta$ -actin expression in cells depleted for mitochondrial DNA (Rho 0 cells) stimulated either with 1000 units/ml IFN- $\gamma$  (black squares) or  $10 \mu\text{M}$  C6-ceramide (black circles). Results represent one of three essentially identical experiments.

the samples were calculated using a linear regression program. Concentrations are obtained by multiplying with the appropriate sample dilution factor.

**Determination of Ceramides**—Lipid extracts based on 500  $\mu\text{g}$  of protein were prepared as described in Ref. 3. Briefly, lipids extracted in chloroform/methanol (2:1, v/v) were hydrolyzed in mild alkaline, and the lower lipid phase was evaporated. The lipids were dissolved in chloroform/methanol and resolved by analytical high performance thin layer chromatography using a CAMAG Automated Multiple Development 2 device (CAMAG, Berlin, Germany) (3). Quantification was performed after postchromatographic derivatization with manganese chloride dipping (3). Colored lipids were detected by a CAMAG TLC Scanner III using CATS software 4.06 (CAMAG, Berlin, Germany) in absorption mode at 550 nm with a tungsten lamp, monochromator bandwidth of 20 nm, and slit width of  $0.45 \times 6$  mm. Quantification was performed using a second-order polynomial calibration curve with four standard mixes in the range 50–1000 ng.

## RESULTS

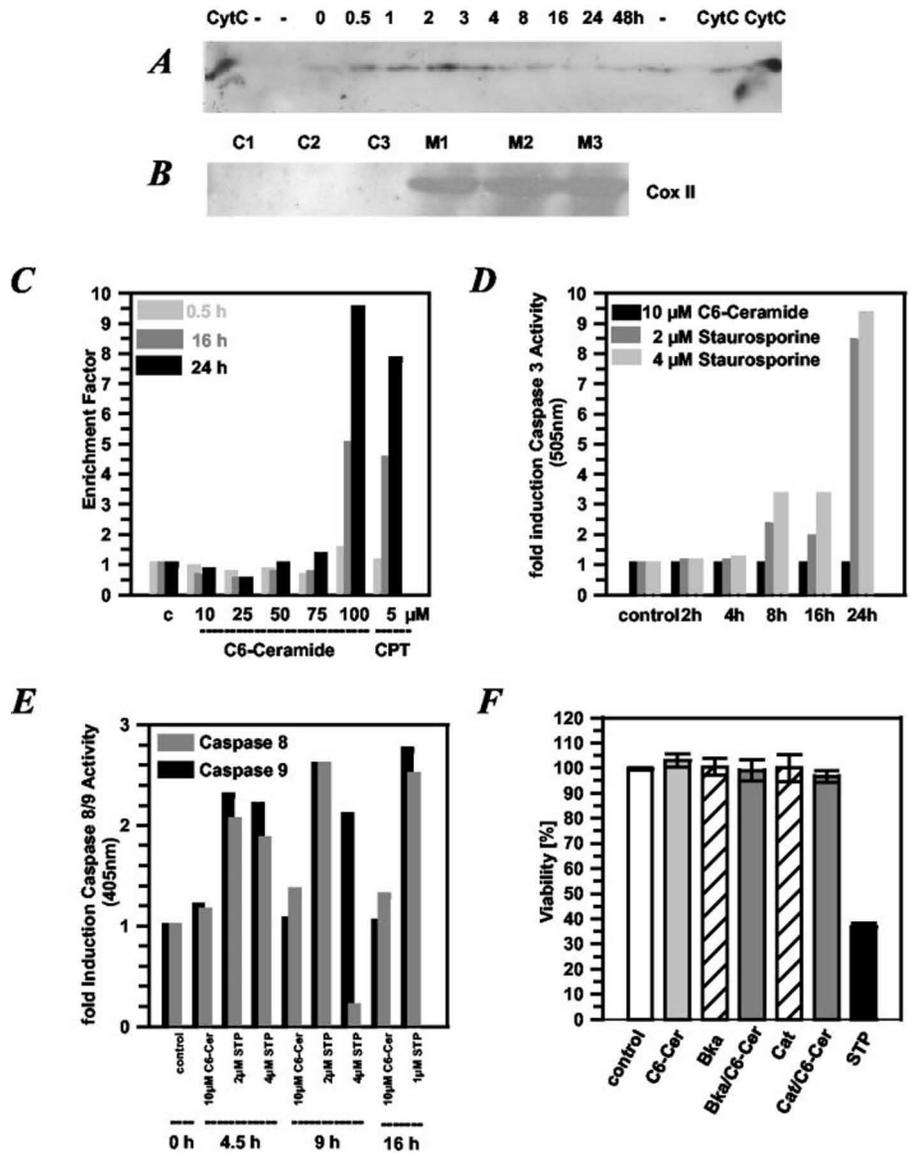
**Inhibition of Ceramide-induced ICAM-1 Expression by Mitochondrial Inhibitors and Depletion of the Mitochondrial Respiratory Chain**—To determine whether mitochondria are involved in ceramide-induced ICAM-1 expression in NHEK, cells were ceramide-stimulated in the presence or absence of specific inhibitors that prevent electron flow between certain complexes of the respiratory chain. The optimal effective nontoxic

concentrations for preincubation with these inhibitors were determined by dose effect studies with NHEK (data not shown). Significant inhibition of ceramide-induced ICAM-1 mRNA expression was achieved upon treatment of cells with the complex I inhibitor rotenone (38), the complex II inhibitor thenoyltrifluoroacetone (39), and the complex III inhibitor antimycin A (40) (Fig. 1, A–C). These inhibitory effects were specific, because identical concentrations of these inhibitors did not affect interferon- $\gamma$ -induced ICAM-1 mRNA expression in the same cells (Fig. 1D). The involvement of the mitochondrial electron transport chain in ceramide-induced ICAM-1 expression was corroborated in experiments in which epithelial cells (Rho 0 cells) were used from which mitochondrial DNA had been depleted through repetitive treatment with ethidium bromide (21). Rho 0 cells constitutively expressed ICAM-1 mRNA, and this expression could be up-regulated upon stimulation of cells with IFN- $\gamma$ , whereas ceramide stimulation failed to do so (Fig. 1E). Taken together, these results indicate that ceramide-induced ICAM-1 expression requires a functionally active mitochondrial electron transport chain.

**Ceramide Stimulation Causes the Release of Mitochondrial *cyt c* into the Cytoplasm without the Induction of Apoptosis**—The mitochondrial electron transport chain inhibitors em-

### FIG. 2. Ceramide-induced *cyt c* release without induction of apoptosis.

**A**, immunoprecipitation of 100  $\mu\text{g}$  of cytosolic extracts that were prepared from NHEK at the indicated time points after stimulation with 10  $\mu\text{M}$  C6-ceramide. Human recombinant *cyt c* (100 and 300 ng) served as controls as indicated. Results represent one of three essentially identical experiments. **B**, mitochondrial (*M*) and cytosolic (*C*) fractions (30  $\mu\text{g}$  of protein) were routinely analyzed for mitochondrial contamination using *cyt c* oxidase subunit II by Western blot. Here the contamination controls of three independent isolations are indicated as *C1–C3* and *M1–M3*. **C**, nucleosomal fragmentation (enzyme-linked immunosorbent assay) of NHEK 0.5, 16, and 24 h after stimulation with increasing amounts of C6-ceramide or 5  $\mu\text{M}$  camptothecin (*CPT*). Results represent one of three essentially identical experiments. **D**, effector caspase 3 activity was measured in NHEK stimulated either with 10  $\mu\text{M}$  C6-ceramide or 2 or 4  $\mu\text{M}$  staurosporine using a fluorometric immunoabsorbent enzyme assay. Caspase 3 activity in unstimulated cells at the indicated time points was arbitrarily set as 1. Activity in the stimulated cells was expressed as -fold increase. Results represent one of three essentially identical experiments. **E**, caspase 8 and 9 activity was assessed in NHEK using a colorimetric activity assay. Unstimulated NHEK served as controls and were set as 1. Activity of cells stimulated either with 10  $\mu\text{M}$  C6-ceramide or increasing amounts of staurosporine (*STP*) as indicated is shown as -fold induction. Results represent one of three essentially identical experiments. **F**, viability of the NHEK 16 h after stimulation was assessed using the MTT assay. Cells were stimulated with C6-ceramide (10  $\mu\text{M}$ ), preincubated for 90 min with inhibitors of mitochondrial megachannel (5  $\mu\text{M}$  Bka, 20  $\mu\text{M}$  Cat), or treated with C6-ceramide after preincubation with inhibitor. Staurosporine (4  $\mu\text{M}$ ) served as control for cytotoxicity. These data represent the results of three individual experiments.



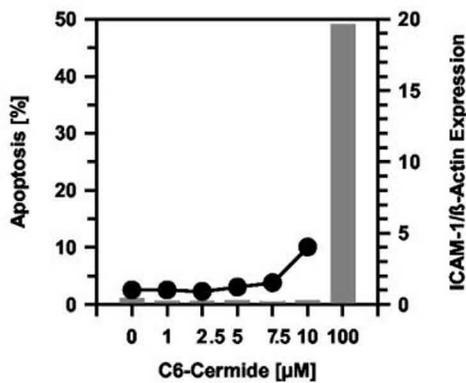
ployed in the present study differed in their capacity to prevent ceramide-induced ICAM-1 mRNA expression, with rotenone being superior to antimycin A and thenoyltrifluoroacetone. These results indicated that the electron flow proximal to the ubiquinone complex was of particular relevance to ceramide-induced gene regulation. Electron entry from complex I to ubiquinone is important for the transition of *cyt c* from mitochondria into the cytoplasm (41). We therefore asked whether ceramide-induced ICAM-1 expression might be associated with a release of mitochondrial *cyt c* into the cytoplasm. The immunoblots in Fig. 2A show that stimulation of NHEK with cell-permeant ceramides at concentrations that induced AP-2 activation and ICAM-1 expression caused the time-dependent release of mitochondrial *cyt c* into the cytoplasm of the cells. The *cyt c* signal detected in the cytosolic fraction of ceramide-stimulated cells was not due to a mitochondrial contamination, because the mitochondrial marker enzyme *cyt c* oxidase subunit II could not be detected in the same fractions (Fig. 2B). An increase in mitochondria-derived holo-*cyt c* was detected in cytosol preparations from ceramide-stimulated NHEK as early as 30 min after stimulation with a maximum at 2 h. It is important to note that under these stimulation conditions, ceramides did not induce apoptosis in NHEK as was determined by employing four independent apoptosis assays includ-

ing nucleosomal fragmentation; activation of caspase 3, 8, and 9; and the TUNEL method (Figs. 2, C–E, and 3A). This was not due to an inability of NHEK to undergo apoptosis, because in the same experiments, NHEK apoptosis was induced upon stimulation of cells with camptothecin, staurosporine, and ceramides, if they were added in 10-fold higher concentrations (Figs. 2, C–E, and 3A). Moreover, there was no evidence for apoptosis-independent cell death in ceramide-stimulated cells, whereas staurosporine caused significant cytotoxicity (Fig. 2F).

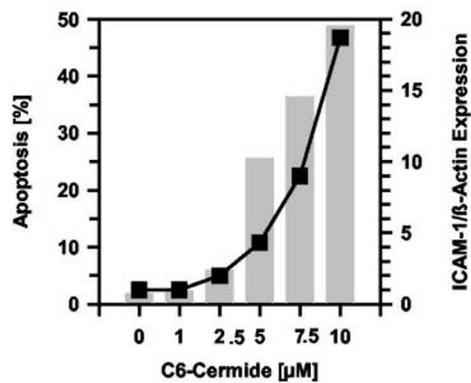
We next addressed the question whether C6-ceramide stimulation can induce ICAM-1 expression in the absence of apoptosis in other cells or cell types as well. Similar to keratinocytes, stimulation of primary human dermal fibroblasts with C6-ceramide in the range of 0–10  $\mu\text{M}$  dose-dependently induced ICAM-1 expression up to 15-fold without the induction of apoptosis, which required 3-fold higher ceramide concentrations (Fig. 3C). This was in contrast to cells from the human epithelial cell line HeLa, in which dose responses for C6-ceramide-induced apoptosis and gene expression did not significantly differ (Fig. 3B).

It should be noted that up-regulation of ICAM-1 expression could also be achieved in human keratinocytes if endogenous ceramide levels were increased by treatment of cells with *N*-oleylethanolamine, an inhibitor for acid ceramidases (42, 43).

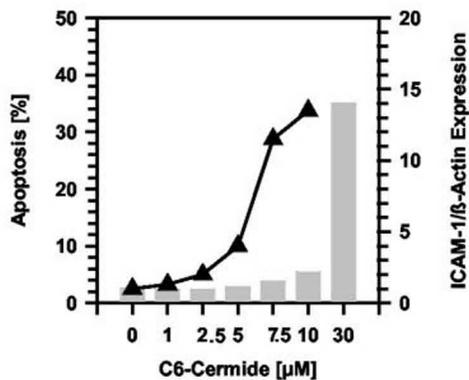
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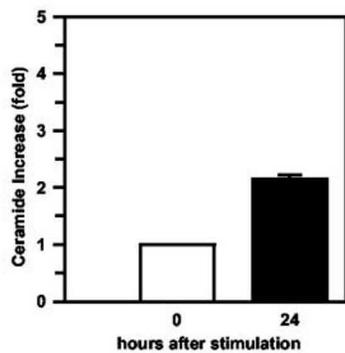
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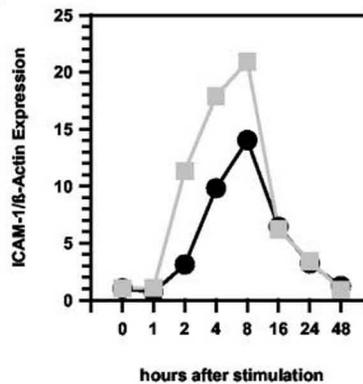
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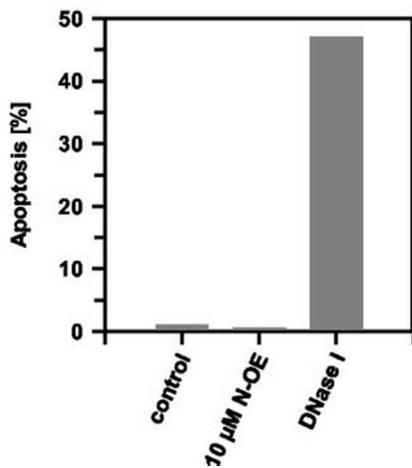
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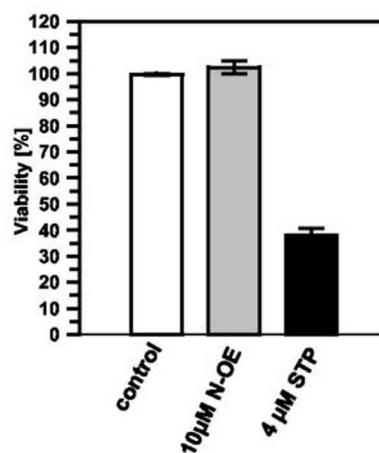
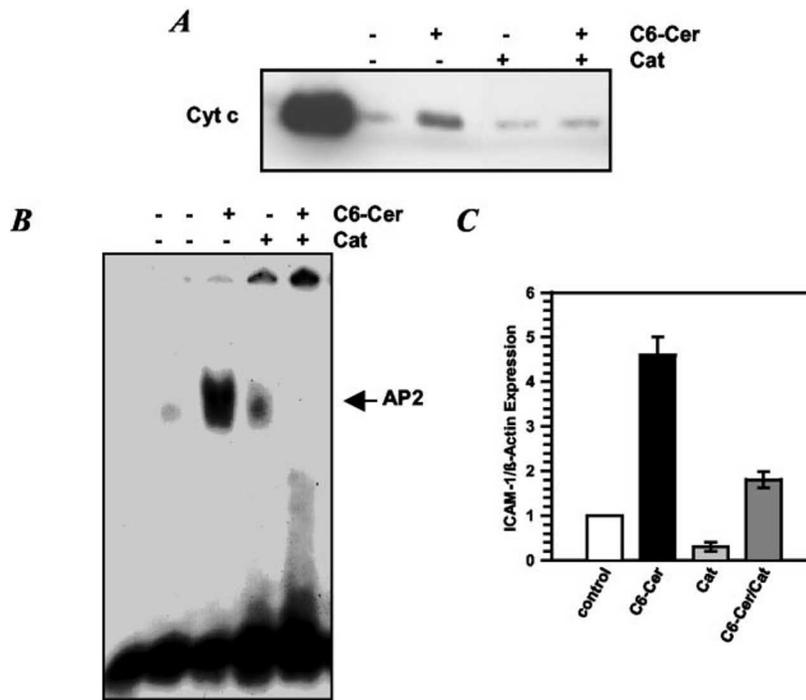


FIG. 3. Induction of ICAM-1/β-actin expression versus apoptosis in NHEK, HeLa cells, and primary dermal fibroblasts upon ceramide stimulation. NHEK (A), HeLa cells (B), and primary dermal fibroblasts (C) were stimulated with C6-ceramide at the indicated concentrations. After 24 h, cells were analyzed for ICAM-1 expression (black circles) and apoptosis (TUNEL assay; gray bars). Results represent



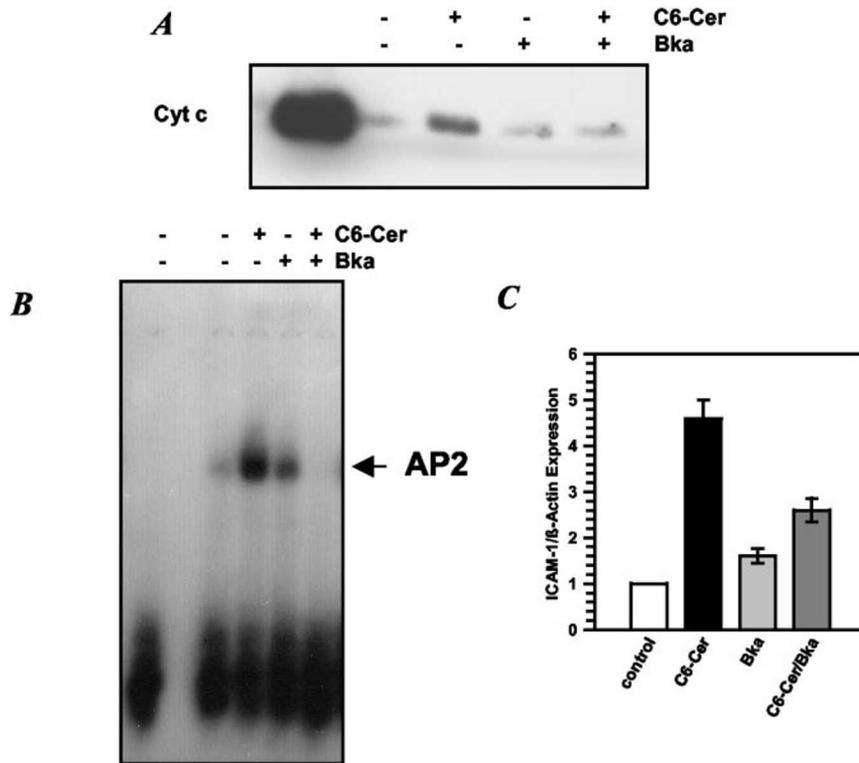
**FIG. 4. The inhibitor of the mitochondrial megachannel carboxyatractyloside inhibits ceramide-induced cytochrome *c* release, AP-2 activation, and ICAM-1 expression in NHEK.** *A*, immunoprecipitation of cytochrome *c* from cytosolic extracts of NHEK treated with 10  $\mu$ M C6-ceramide, after preincubation with 20  $\mu$ M Cat for 90 min, or after preincubation with Cat for 90 min followed by a treatment with 10  $\mu$ M C6-ceramide. Cells were harvested immediately before and 0.5 h after stimulation with C6-ceramide. 100 ng of human recombinant cytochrome *c* served as control. These data represent one representative of four individual experiments. *B*, gel electrophoresis mobility shift assay with nuclear extracts of NHEK treated with 10  $\mu$ M C6-ceramide, after preincubation with 20  $\mu$ M Cat for 90 min, or after preincubation with 20  $\mu$ M Cat for 90 min followed by a treatment with 10  $\mu$ M C6-ceramide for 0.5 h. *Lane 1*, control with radiolabeled AP-2 oligonucleotide alone; *lane 2*, nuclear extract of untreated control cells; *lane 3*, nuclear extract of cells treated with 10  $\mu$ M C6-ceramide; *lane 4*, nuclear extract of cells treated with 20  $\mu$ M Cat; *lane 5*, nuclear extract of cells treated with both substances. These data represent one representative of four individual experiments. *C*, ICAM-1/ $\beta$ -actin expression in control NHEK compared with NHEK treated with 10  $\mu$ M C6-ceramide alone, preincubated with 20  $\mu$ M Cat alone, or after both treatments 2 h after stimulation with C6-ceramide. These data represent the results of three individual experiments.

As is shown in Fig. 3*D*, *N*-oleylethanolamine treatment up-regulated endogenous ceramide levels by a factor of 2, and this was associated with an increased ICAM-1 expression (Fig. 3*E*). The time kinetics of C6-ceramide-induced and *N*-oleylethanolamine-induced ICAM-1 expression were essentially identical, whereas the magnitude of the latter response exceeded that observed after the addition of exogenous ceramides. Note that treatment of NHEK with *N*-oleylethanolamine did not induce apoptosis (Fig. 3*F*) or decrease cell viability (Fig. 3*G*).

**Cyt *c* Release Is Required for Ceramide-induced AP-2 Activation and ICAM-1 Expression**—Transition of cytochrome *c* from mitochondria into the cytoplasm occurs through the mitochondrial megachannel (permeability transition pore), which is formed by proteins from both the inner mitochondrial membrane including the adenine nucleotide translocator and the outer membrane, which is the voltage-dependent anion channel (10, 11). In order to study the functional relevance of ceramide-induced cytochrome *c* release for ceramide-induced AP-2 activation and ICAM-1 expression, NHEK were next treated with Cat, a specific inhibitor of the adenine nucleotide translocator (44), or Bka, which inhibits the voltage-dependent anion channel (44). Both inhibitors, Cat and Bka, have been shown to block the transport of nucleotides via the mitochondrial ADP/ATP car-

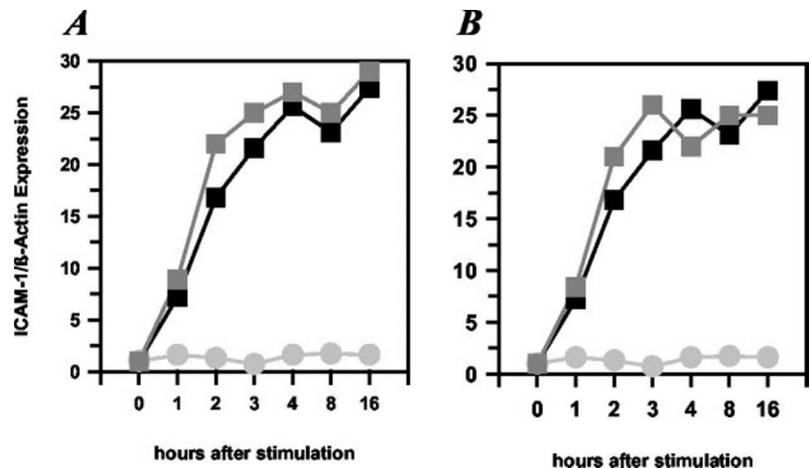
rier (45, 46). As expected, cytosolic extracts, which had been prepared from ceramide-stimulated cells and which were negative for cytochrome *c* oxidase subunit II (data not shown), contained increased amounts of holo-cytochrome *c*, as compared with extracts from unstimulated control cells, and this increase was inhibited upon treatment of ceramide-stimulated NHEK with Cat or Bka (Figs. 4*A* and 5*A*). The observed higher molecular weight bands have been reported before and are due to the formation of multimers and to the short chain of IgG (47). We are aware that ceramides can induce pore formation in phospholipid membranes and can increase the permeability of the outer mitochondrial membrane in isolated mitochondria (48, 49), but Figs. 4*A* and 5*A* indicate an involvement of the mitochondrial megachannel in ceramide-induced cytochrome *c* release in our system. The inhibition of cytochrome *c* release by Cat or Bka acid was associated in both instances with inhibition of ceramide-induced AP-2 activation (Figs. 4*B* and 5*B*) and ceramide-induced up-regulation of ICAM-1 mRNA expression (Figs. 4*C* and 5*C*). These inhibitory effects were specific, because identical concentrations of the inhibitors did not affect IFN- $\gamma$ -induced ICAM-1 mRNA expression (Fig. 6, *A* and *B*). Also, treatment of ceramide-stimulated NHEK with Bka or Cat did not reduce cell viability (Fig. 2*F*). In aggregate, these results indicate that

one of three essentially identical experiments each. *D*, NHEK were stimulated with 10  $\mu$ M *N*-oleylethanolamine (black bar) and analyzed for ceramide increase 24 h after stimulation in comparison with untreated controls (white bar). These data represent the results of three individual experiments. *E*, NHEK were stimulated with 10  $\mu$ M *N*-oleylethanolamine (gray squares) or 10  $\mu$ M C6-ceramide (black circles) and analyzed for ICAM-1/ $\beta$ -actin expression at the indicated time points. These data represent the results of three individual experiments. *F*, NHEK were stimulated with 10  $\mu$ M *N*-oleylethanolamine (*N*-OE) for 24 h and analyzed for apoptosis (TUNEL assay). DNase I-treated cells served as positive control for induction of apoptosis. Results represent one of three essentially identical experiments each. *G*, viability of NHEK 24 h after stimulation with 10  $\mu$ M *N*-oleylethanolamine (*N*-OE) was assessed using the MTT assay. Staurosporine (4  $\mu$ M) served as control for cytotoxicity. These data represent the results of three individual experiments.



**FIG. 5. The inhibitor of the mitochondrial megachannel Bka prevents ceramide-induced cyt *c* release, AP-2 activation and ICAM-1 expression in NHEK.** *A*, immunoprecipitation of cyt *c* from cytosolic extracts of NHEK treated with 10  $\mu\text{M}$  C6-ceramide, after preincubation with 5  $\mu\text{M}$  Bka for 90 min, or after preincubation with Bka followed by a treatment with 10  $\mu\text{M}$  C6-ceramide. Cells were harvested immediately before and 0.5 h after stimulation with C6-ceramide. 100 ng of human recombinant cyt *c* served as control. These data represent one representative of four individual experiments. *B*, gel electrophoresis mobility shift assay with nuclear extracts of NHEK treated with 10  $\mu\text{M}$  C6-ceramide, after preincubation with 5  $\mu\text{M}$  Bka for 90 min, or after preincubation with Bka followed by a treatment with C6-ceramide for 0.5 h. *Lane 1*, control with radiolabeled AP-2 oligonucleotide alone; *lane 2*, nuclear extract of untreated control cells; *lane 3*, nuclear extract of cells treated with 10  $\mu\text{M}$  C6-ceramide; *lane 4*, nuclear extract of cells treated with 5  $\mu\text{M}$  Bka; *lane 5*, nuclear extract of cells treated with both substances. These data represent one representative of four individual experiments. *C*, ICAM-1/ $\beta$ -actin expression in control NHEK compared with NHEK treated with 10  $\mu\text{M}$  C6-ceramide alone, 5  $\mu\text{M}$  Bka alone, or both 2 h after stimulation with C6-ceramide. These data represent the results of three individual experiments.

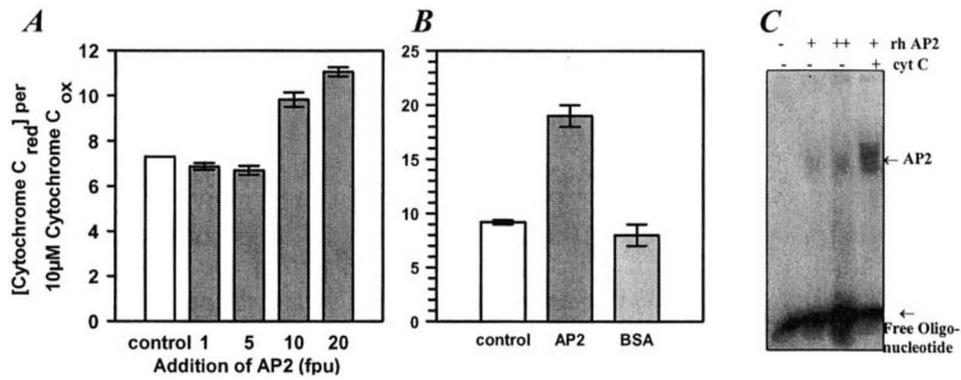
**FIG. 6. Inhibition of mitochondrial megachannel does not inhibit IFN- $\gamma$  induced ICAM-1 expression.** *A*, ICAM-1/ $\beta$ -actin expression was assessed in NHEK incubated with 1000 units/ml IFN- $\gamma$  (black squares), after preincubation with 20  $\mu\text{M}$  Cat (gray circles) for 90 min, or after a combination of both (dark gray squares) for the indicated time points. These data represent the results of three individual experiments. *B*, ICAM-1/ $\beta$ -actin expression was assessed in NHEK incubated with 1000 units/ml IFN- $\gamma$  (black squares), after preincubation with 5  $\mu\text{M}$  Bka (gray circles) for 90 min, or after a combination of both (dark gray squares) at the indicated time points. These data represent the results of three individual experiments.



transition of cyt *c* from the mitochondria into the cytoplasm is required for ceramide-induced AP-2 activation and ICAM-1 expression.

**Cytochrome *c*-induced Oxidation of AP-2 Results in Enhanced DNA Binding**—In order to study the mechanism by which cyt *c*, after its release into the cytoplasm, might be capable of activating transcription factor AP-2, we next performed *in vitro* coinubation experiments with cyt *c* and recombinant human AP-2. Photometric analysis of the redox state of cyt *c* revealed that the addition of increasing concentrations of human recombinant AP-2 dose-dependently reduced cyt *c* (Fig.

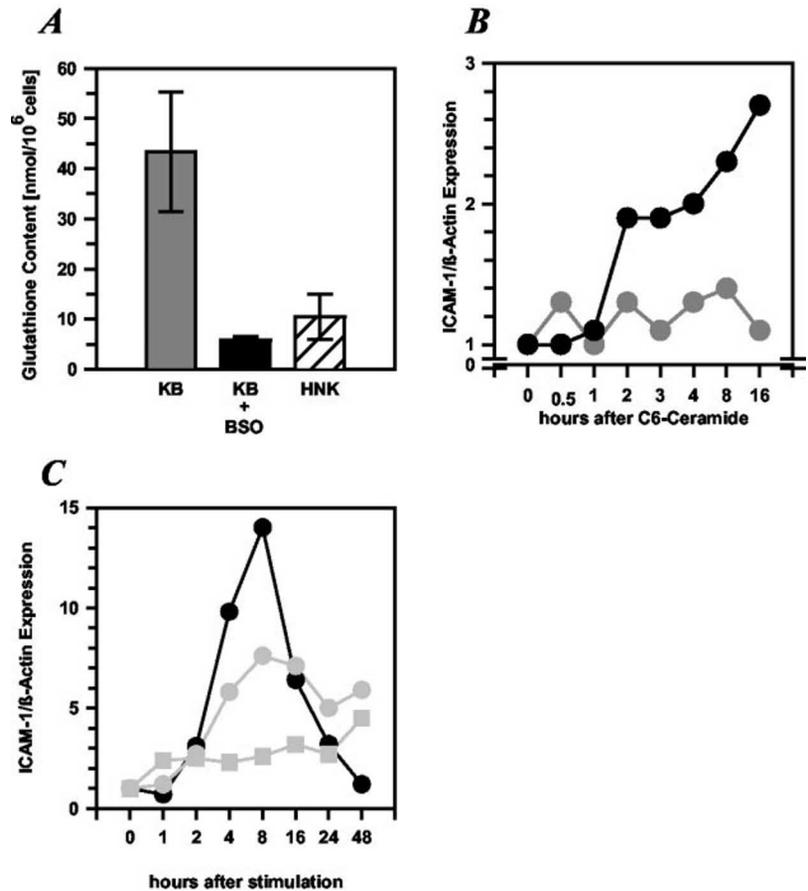
7A). This redox regulation was not induced when equivalent concentrations of bovine serum albumin were added to cyt *c* (Fig. 7B). Redox regulation is thought to be one mechanism by which transcription factors can be activated (50). We therefore next assessed the impact of cyt *c* coinubation on the DNA binding capacity of AP-2. As is shown in Fig. 7C, binding of human recombinant AP-2 to AP-2 oligonucleotides, which were deduced from the AP-2 binding site that is present within the human ICAM-1 promoter, was significantly enhanced if human recombinant AP-2 was first coinubated with cyt *c* to allow for AP-2 oxidation. In fact, DNA binding of oxidized AP-2 was even



**FIG. 7. Oxidation of AP-2 by cyt *c* in vitro is dose dependent, specific and increases DNA binding capacity.** *A*, in a solution containing 10  $\mu\text{M}$  oxidized cyt *c* (*Cytochrome C<sub>ox</sub>*), the content of reduced cyt *c* (*Cytochrome C<sub>red</sub>*) was assessed spectrophotometrically at 550 nm. Cyt *c* containing solutions are either left untreated (control) or incubated with increasing amounts of human recombinant AP-2 quantified in footprinting units (fpu; 0.5 fpu/ $\mu\text{l}$   $\sim$  20–150 ng/protein, varying from lot to lot). Original and double amounts were taken from the identical lot. These data represent the results of three individual experiments. *B*, in a solution containing 10  $\mu\text{M}$  oxidized cyt *c* (*Cytochrome C<sub>ox</sub>*), the content of reduced cytochrome *c* (*Cytochrome C<sub>red</sub>*) was assessed spectrophotometrically at 550 nm. Cyt *c*-containing solutions are either left untreated (control) or incubated with 10 fpu human recombinant AP-2 or an equimolar amount of bovine serum albumin (molecular biology grade). These data represent the results of three individual experiments. *C*, gel electrophoresis mobility shift assay with radiolabeled AP-2 consensus oligonucleotide coincubated with buffer only (lane 1), human recombinant AP-2 (lane 2), double amount of human recombinant AP-2 (lane 3), and human recombinant AP-2 plus cyt *c* (lane 4). These data represent one representative of three individual experiments.

**FIG. 8. Endogenous glutathione content determines reactivity toward ceramide-induced ICAM-1 expression.**

*A*, glutathione content (GSH + GSSG) in nmol/ $10^6$  cells in NHEK and KB cells with and without a 24-h preincubation with buthionine sulfoximine (BSO; 1 mM). These data represent the results of three individual experiments. *B*, ICAM-1/ $\beta$ -actin expression in epidermoid carcinoma KB cells stimulated with 10  $\mu\text{M}$  C6-ceramide either without (gray circles) or with 1 mM buthionine sulfoximine pretreatment for 24 h (black circles). These data represent the results of three individual experiments. *C*, ICAM-1/ $\beta$ -actin expression in NHEK, which were pretreated for 16 h with 20  $\mu\text{M}$  *N*-acetyl-L-cysteine (gray circles) or not (black circles) and subsequently stimulated for the indicated time with 10  $\mu\text{M}$  C6-ceramide. Control cells were pretreated for 16 h with 20  $\mu\text{M}$  *N*-acetyl-L-cysteine and incubated without further stimulation for the indicated time (gray squares). These data represent the results of three individual experiments.



superior to that observed if the concentration of unoxidized human recombinant AP-2 was doubled.

In order to study the *in vivo* relevance of the redox state of AP-2 for ceramide-induced gene expression in NHEK, we next compared long term cultured NHEK with cells from the human epidermoid carcinoma cell line KB. This cell line, which has previously been employed as a model to study cytokine-induced ICAM-1 expression (51), differs in its antioxidant status from NHEK, as is demonstrated by 4-fold higher endogenous glutathione levels (Fig. 8A). We speculated that increased endogenous glutathione levels would inhibit the redox-dependent

AP-2 activation and thereby prevent ceramide-induced ICAM-1 expression in these cells. As is shown in Fig. 8B, ceramide stimulation failed to up-regulate ICAM-1 mRNA expression in KB cells, but these cells could be rendered ceramide-responsive (Fig. 8B) if their endogenous glutathione levels were lowered through pretreatment with buthionine sulfoximine (Fig. 8A), a potent inhibitor of glutathione synthesis (52). Vice versa, NHEK could be rendered insensitive toward C6-ceramide-induced ICAM-1 expression when their endogenous glutathione levels were increased. Accordingly, the addition of *N*-acetyl-L-cysteine (53) to NHEK caused an increase in endogenous glu-

tathione levels by 30% (data not shown), which was associated with a decrease in their capacity to mount an ICAM-1 response upon C6-ceramide stimulation (Fig. 8C). These *in vivo* results indicate that the antioxidant status of keratinocytes determines their capacity to increase AP-2-dependent gene transcription upon ceramide stimulation.

#### DISCUSSION

Previous work has demonstrated that ceramide-induced ICAM-1 expression requires activation of transcription factor AP-2 (3). We have shown here that ceramide-induced AP-2 activation occurs through a mitochondrial pathway that requires a functional electron transport chain and depends on the transition of cyt *c* from the mitochondria into the cytoplasm. We have also provided *in vitro* and *in vivo* evidence that the resulting interaction between cyt *c* and AP-2 leads to activation of the transcription factor through a redox regulation during which AP-2 is oxidized. These studies describe a previously unknown function that mitochondria serve in transcription factor activation and regulation of gene transcription in mammalian cells.

The ceramide-induced gene regulatory pathway described here shares several features, in particular the release of cyt *c* from mitochondria, with the signaling cascade that has been described for ceramide-induced apoptosis (5, 6, 8). In the present study, stimulation of NHEK with ceramides at concentrations that caused gene transcription did not induce caspase activation (Fig. 2, *D* and *E*). It should be noted that apoptosis can also occur in a caspase-independent manner, which, depending on the experimental system, may or may not involve ceramide signaling (54–56).

In this regard, it is important that in the present study, ceramide-induced AP-2 activation and gene expression was not associated with DNA fragmentation, which was assessed by means of two independent assays (Figs. 2C and 3A) and is a common final endpoint of caspase-dependent as well as -independent cell death. Increased gene expression in the absence of apoptosis was also observed in primary human dermal fibroblasts (Fig. 3C) and thus is not specific for keratinocytes. It is not, however, a general phenomenon, because no such concentration-dependent discrepancy between these two ceramide-induced biological effects could be observed in HeLa cells (Fig. 3B). Accordingly, sensitivity toward ceramide-induced apoptosis was recently reported to differ between different cell types. In these studies, similar to our observation, tumor cells glare to be more sensitive toward ceramide-induced apoptosis as compared with primary cells (57, 58).

The amount of mitochondrial cyt *c* in the cytoplasm that was sufficient for AP-2 activation and ICAM-1 induction was relatively small and could only be detected by immunoprecipitation (Figs. 2A, 4A, and 5A), whereas in cells undergoing ceramide-induced apoptosis, mitochondrial cyt *c* was found to be released at larger quantities, which could be detected by Western blot analysis (8). It thus appears that mitochondria by virtue of their capacity to release smaller or larger amounts of cyt *c* can serve a role either in transcription factor activation and gene regulation or caspase activation and apoptosis. Our model provides a mechanistic explanation for previous work that suggests the existence of a signal communication between the mitochondrial and nuclear compartment in several prokaryotic and eukaryotic systems in which changed mitochondrial functions affected expression of nuclear genes (59–62).

Our results do not allow the identification of the molecular mechanism by which cyt *c*-induced oxidation activates AP-2. The DNA binding domain of AP-2 contains five cysteine residues at positions 243, 308, 345, 375, and 392 (63), and oxidation of one of these (*e.g.* in position 243) could lead to a conforma-

tional change that would enhance the DNA binding capacity of AP-2. There are also nine lysine and arginine residues present in the DNA binding domain of AP-2, and they could serve as reactants during a redox regulation as well. The analysis of redox changes of all these possible reactants is beyond the scope of the present study. Redox regulation as a mechanism for transcription factor activation, including AP-2 (64) and NF- $\kappa$ B (50), has been reported before.

Our results provide further insights into the signaling cascade that mediates ceramide-induced gene regulation in human cells. They also indicate new ways by which stress responses of human cells that are induced (*e.g.* by cytokines (1, 2) or solar ultraviolet radiation (3, 19)) and that depend on ceramide-induced gene transcription may be altered by manipulation of mitochondrial functions. Ceramides have been shown *in vitro* to activate signal-transducing kinases such as protein kinase  $\zeta$  and ceramide-activated protein kinase (67) and ceramide-activated protein phosphatase. Direct ceramide targeting of one or several of these molecules could be part of the mechanism that transmits the ceramide signal into the mitochondria and thereby induces cyt *c* transition. In conclusion, our results provide fundamental new insights into intracellular ceramide signaling and the way mitochondria regulate transcription factor activation and gene transcription in human cells.

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