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Ambient Temperature Regulation of Apoptosis in Brown Adipose Tissue

Erk1/2 PROMOTES NOREPINEPHRINE-DEPENDENT CELL SURVIVAL*

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Brown adipose tissue hyperplasia is a fundamental response to low ambient temperature. We show here that cold exposure of an animal markedly increased the phosphorylation of mitogen-activated protein kinase (p42/p44) Erk1 and Erk2 in brown adipose tissue, and protected cells in the tissue from apoptosis. We also show that cessation of the sympathetic stimulus, by transferring cold-adapted animals to 28 °C, caused an increased rate of apoptosis in the tissue. In primary cultures of brown adipose tissue, norepinephrine (NE) stimulated both the phosphorylation and the activity of Erk1/2 via the Erk kinase MEK, and protected the cells form apoptosis. Similarly, agonist stimulation of α_1- and β-adrenergic receptors and increases in the intracellular level of Ca^{2+} and cAMP stimulated the phosphorylation of Erk1/2. Agonist stimulation of α_1- and β-adrenergic receptors, and increased intracellular cAMP level also promoted the cell survival. Furthermore, NE stimulated the expression and secretion of basic fibroblast growth factor (bFGF), which further promoted the cell survival via MEK-dependent activation of Erk1/2. In essence, we show that Erk1/2 has a critical role in promoting NE- and bFGF-dependent survival of brown adipocytes, and propose that NE- and bFGF-dependent regulation of the cell survival is involved in the cold-induced hyperplasia of brown adipose tissue.

The complex balance between cell proliferation and the normal cell death in a tissue is crucial for the maintenance of tissue homeostasis. It is fundamental for the normal physiology of the organism, conferring functionality and viability. Alterations in this balance, leading to excessive cell death, cell survival, cell proliferation, or a combination of some of them, could play a role in a great number of disease states (1).

The regulation of mitotic cell growth has been extensively studied, and the dependence of growth factors and their signaling pathways in different cell types established (2). There is convincing evidence that cells also depend on hormone and/or growth factor stimulation for their survival. In the absence of signals from other cells, the cell may activate an intrinsic suicide program and kill itself with the typical features of programmed cell death (3).

Several peptide growth factors (*e.g.* NGF,[1] insulin-like growth factor-1, bFGF, and platelet-derived growth factor) have been shown to promote cell survival of specific cell types, and the characterization of their corresponding signaling pathways has identified proteins that are critical mediators of cell survival. For example, insulin-like growth factor-1 was recently shown to promote the survival of cerebellar neurones, which was mediated via phosphatidylinositol 3-kinase-dependent activation of the protein kinase Akt, also known as PKB or RAC (4). Basic fibroblast growth factor (bFGF) has been shown to suppress tumor necrosis factor-α mediated apoptosis in L929 cells via a Erk1/2 dependent pathway (5). Similarly, it was recently also shown that insulin-like growth factor-1 attenuated tumor necrosis factor-α induced apoptosis in cultured fetal brown adipocytes (6).

Norepinephrine (NE) is a neurotransmitter with pleiotropic effects on brown adipocytes. It has been implicated both in the regulation of brown adipose tissue (BAT) hyperplasia and in the differentiation of brown adipocytes. Furthermore, the acute thermogenic response to low ambient temperature is mediated by NE released from sympathetic neurones innervating the tissue. Two phases can be distinguished during physiological activation (*i.e.* cold exposure of an animal) of BAT: an initial phase responsible for the acute heat production; a recruitment phase involving mitochondriogenesis, increased protein, and DNA synthesis, ultimately resulting in an increase in the number of cells in the tissue (7). Likewise, when a cold adapted animal is transferred to higher ambient temperature there is an abrupt cessation of the thermogenic activity in the tissue and a new adaptation process starts. This adaptation involves rapid degradation of RNA and proteins, decreasing mitochondrial content, the amount of nuclear DNA in the tissue decreases, and eventually the BAT mass is decreased (8).

Although the regulation of the cell number in BAT has long been considered in terms of mitotic cell growth (9–14), physiological regulation of the cell survival may also play an important part in the tissue hyperplasia. The concerted action of these two processes, decreased apoptosis accompanied by increased mitotic cell growth, would be a way to rapidly increase the tissue size to meet the metabolic demand for increased thermogenesis.

In the present study we demonstrate that low ambient temperature (4 °C) caused a rapid increase in the phosphorylation of Erk1/2 in BAT, and promoted the survival of cells in the tissue. Termination of the stimulus, by transferring cold

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[1] The abbreviations used are: NGF, nerve growth factor; BAT, brown adipose tissue; MAPK, mitogen-activated protein kinase; NE, norepinephrine; bFGF, basic fibroblast growth factor; Erk, extracellular regulated protein kinase; MEK, MAP/Erk kinase; PKA, protein kinase A; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; JNK, c-Jun N-terminal; CREB, cAMP response element-binding protein; CRE, cAMP response element.

adapted animals to 28 °C, caused a marked increase in the rate of apoptosis in the tissue. We further show that in primary cultures of BAT Erk1/2 has a critical role in promoting NE- and bFGF-dependent cell survival. NE stimulated the Erk cascade through both α_1- and β-adrenergic receptors mediated by Ca^{2+} and cAMP via the Erk kinase MEK. NE also stimulated the expression and secretion of bFGF from the cells, which further promoted the cell survival via MEK-dependent activation of Erk1/2. This is, to our knowledge, the first demonstration of adrenergic regulation of cell survival mediated via the Erk1/2 cascade.

MATERIALS AND METHODS

Animals and Treatments—3-week-old male mice (NMRI out-bred strain; Eklunds, Stockholm, Sweden) were kept at 28 °C for at least 7 days with free access to food and water. At the start of the experiments the animals were either maintained at 28 °C or transferred to 4 °C for the indicated times. The animals were sacrificed (CO_2 euthanasia) and the interscapular BAT dissected, frozen in liquid nitrogen, and stored at −70 °C until analyzed. For primary cultures of BAT, mice were kept at 22 °C with free access to food and water for 2–3 days before sacrifice.

Cell Culture—Brown adipocyte precursor cells were isolated from the interscapular, the axillary, and the cervical BAT depots of 3-week-old male mice, and grown in culture as earlier described (15, 16). Briefly, pooled tissues was minced in a Hepes-buffered solution containing 0.2% (w/v) collagenase type II (Sigma) and digestion was allowed for 30 min at 37 °C. The digest was filtered through 250- and 25-μm nylon filters to remove undigested parts and mature cells. The precursor cells were pelleted by centrifugation (700 × *g*), washed in Dulbecco's modified Eagle's medium, pelleted, and resuspended in 0.5 ml of culture medium/mouse. The precursor cells were inoculated into multiwell culture dishes (Corning, Falcon) at a density of 1–2 × 10^4 cells/cm^2. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 4 nM insulin, 25 μg/ml sodium ascorbate, 10 mM Hepes, 4 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin at 37 °C in an atmosphere of 8% CO_2 in air. The cells were washed with Dulbecco's modified Eagle's medium on day 1 and the medium was changed on day 1, 3, 5, and 7 of culture.

Hoescht DNA Content Determination—The DNA amount in cell cultures and tissue homogenate was measured by a method described by Downs and Wilfinger (17), and modified by West *et al.* (18). Calf thymus DNA (Sigma), the concentration determined spectrophotometically at 260 nm, was used as standard. Tissue homogenates and cultured cells were sonicated and 50 μl of the cell suspension was treated with 1.45 ml of ice-cold 10 mM EDTA (pH 12.3) in order to dissolve the DNA. Samples were incubated at 37 °C for 30 min and subsequently cooled on ice, neutralized with 75 μl of 1 M KH_2PO_4, and left on ice for 10 min. One volume of Hoescht 33258 (Serva) dissolved in Tris buffer, pH 7.0 (0.2 μg/ml), was added to the samples and the fluorescence measured in a Perkin-Elmer fluorescence spectrophotometer (MPF-2A; excitation λ 350 nm, emission λ 455 nm).

Agarose Gel Assay for DNA Fragmentation—Interscapular BAT from warm adapted (28 °C, 5 weeks) and cold adapted (4 °C, 5 weeks) mice were homogenized in a lysis buffer containing 5 mM Tris, 20 mM EDTA, and 0.5% Triton X-100 (pH 8.0) and left on ice for 30 min. The tissue homogenates were centrifuged (14,000 rpm, 30 min) to separate the cytosolic and the nuclear fraction. The cytosolic fraction from the same amount of cells (determined by Hoescht 33258 DNA measurements) were treated with 200 μg/ml proteinase K (Sigma) overnight at 50 °C. Protein was extracted once with phenol/chloroform followed by one chloroform extraction. DNA was precipitated with 0.1 volume of 2.5 M NH_4Ac and 2 volumes of 100% ethanol. Precipitated DNA was dissolved in 100 μl of TE (10 mM Tris, 1 mM EDTA, pH 7.4) and treated with 100 μg/ml RNase (Ambion) for 90 min at 37 °C. DNA was precipitated as above, and the DNA fragments were separated on a 1.5% SEAPLAQUE GTG (FMC BioProducts) agarose gel and visualized with ethidium bromide under UV light.

Enzyme-linked Immunosorbent Assay for Internucleosomal DNA Fragmentation—DNA fragmentation in the tissues and cell cultures was quantified by measuring cytosolic histone-associated DNA fragments using an enzyme-linked immunosorbent assay kit (Boehringer Mannheim). Interscapular BAT was homogenized in the lysis buffer and left on ice for 30 min. The homogenates were centrifuged at 14,000 rpm in an Eppendorf centrifuge for 30 min at 4 °C to separate the cytosolic and nuclear fractions.

For the cell cultures, medium was discarded and the cells were lysed directly in the well with lysis buffer for 30 min on ice. The cells were harvested by scraping in the lysis buffer and centrifuged 14,000 rpm for 30 min at 4 °C. Histone-associated DNA fragments was measured in the supernatant (cytosolic fraction) according to the manufacturer's recommendation. Briefly, microtiter plate wells (Costar) were coated with anti-histone antibody for 60 min at room temperature or 4 °C overnight. The samples were added and incubated in the wells for 90 min at room temperature allowing antibody histone binding. To ensure equal loading of cells in the assay a volume corresponding to the same amount of DNA, determined in the cell homogenates/lysates, from each sample was analyzed. The wells were washed, and an anti-DNA-antibody conjugated with peroxidase added to the wells, incubated for 90 min at room temperature and washed. The immunocomplex was detected by a substrate solution containing ABST® (2,2′-azino-di-3-ethylbenzthiazoline sulfonate) and the absorbance measured at 405 nm in a Titrtek Multiscan. The absorbance values were used to calculate the percentage of DNA fragmentation compared with control, with the control values set to 100%.

TUNEL Assay—Primary cultures of BAT were grown in culture on glass coverslips until day 8. Medium was discarded and the cells were washed and fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 min at room temperature. Cells were incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature to block endogenous peroxidase. Staining was performed with the *In Situ* Cell Death Detection Kit POD from Boehringer Mannheim according to the manufacturers instructions. The amount of positive (apoptotic) cells were analyzed by light microscopy.

Erk1/2 Kinase Activity—Erk1/2 kinase activity was measured using a Erk1/2-dependent Elk-1 phosphorylation assay (New England BioLabs), according to the manufacturer's instructions. After the indicated times, cells were washed once with ice-cold phosphate-buffered saline and lysed directly in the well with the provided lysis buffer supplemented with 2 mM Pefabloc®SC (Boehringer Mannheim) protease inhibitor. Activated Erk1/2 was immunoprecipitated from 200 μl of cell lysates using 4 μl of a phospho-specific (human Tyr(P)-204) Erk1/2 antibody (rabbit polyclonal IgG) and 20 μl of anti-rabbit IgG-agarose (50% beads in phosphate-buffered saline; Sigma). Immunocomplexes were incubated for 30 min at 30 °C in 50 μl of kinase buffer containing 1 μg of GST-Elk1 fusion protein (expressed in *Escherichia coli*) (New England BioLabs). The phosphorylation of Elk-1 was analyzed by Western blotting with a phospho-Elk-1 (Ser-383) specific antibody (rabbit polyclonal IgG) and chemiluminescence.

Western Blot—Proteins were separated on 12.5% polyacrylamide gels and electrotransferred to Hybond-C Extra nitrocellulose membranes (pore size, 0.45 μm; Amersham) with a semidry electroblotter. After transfer, the membranes were allowed to soak in Tris-buffered saline (TBS) for 5 min followed by quenching (5% non-fat dry milk, 0.1% Tween 20 in TBS) of nonspecific binding for 3 h at room temperature or overnight at 4 °C. The membranes were incubated with primary antibodies, Erk1/2 phosphospecific antibody (human Tyr(P)-204, New England BioLabs), Erk2 antibody (New England BioLabs), B-Raf antibody (Santa Cruz Biotechnology), or Rap-1 antibody (Transduction Laboratories), overnight at 4 °C. The primary antibody was detected with the Phototope®-horseradish peroxidase Western blot Detection kit (New England BioLabs) according to the manufacturer's recommendation, or by using a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma) and enhanced chemiluminescence (ECL, Amersham). The blots were exposed to Kodak X-Omat RP films and quantified on a Molecular Dynamics densitometer.

Chemicals—Basic fibroblast growth factor (heparin stabilized, human), L-norepinephrine bitartate (arterenol), isoprenaline (L-isoproterenol D-bitrate), phorbol 12-myristitate 13-acetate (TPA), A23187, forskolin, and collagenase (type II) were obtained from Sigma. Cirazoline was from RBI. CGP-12177 was a gift from Ciba-Geigy. Anti-bFGF neutralizing antibody was from R&D Systems. Bovine serum albumin (albumin, fraction V) was from Boehringer Mannheim. PD98059 was from New England BioLabs. All agents were freshly dissolved in water except TPA, forskolin, and PD98059 which were dissolved in dimethyl sulfoxide.

Statistical Analysis—Results are presented as the mean values ± S.E. The Student's *t* test (unpaired) was used to test for differences between the different treatments and/or controls (*, $p \leq 0.05$; **, $p \leq 0.01$; (***, $p \leq 0.001$).

RESULTS

Decreased Rate of Apoptosis in Brown Adipose Tissue of Cold Exposed Animals—In an attempt to investigate the role of

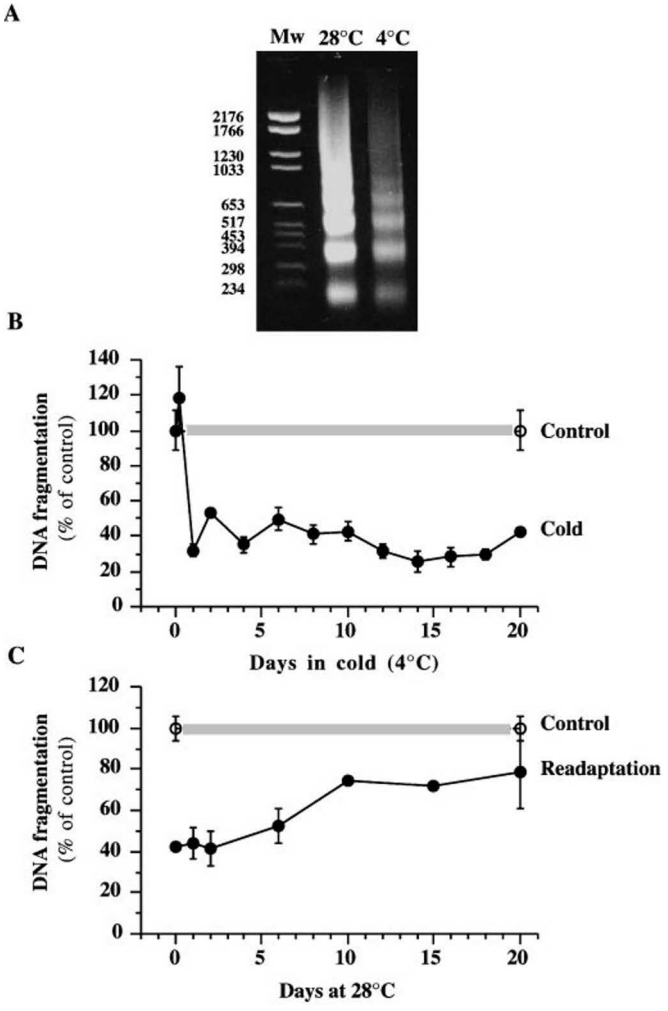

FIG. 1. **Ambient temperature regulation of apoptosis in mouse brown adipose tissue.** *A,* mice were cold exposed (4 °C), or kept at near thermoneutral temperature (28 °C) for 5 weeks, and DNA fragmentation in the interscapular BAT was analyzed by an agarose gel assay, as described under "Materials and Methods." *Mw*, DNA molecular weight markers. *B,* time course of DNA fragmentation in the interscapular BAT of mice exposed to cold for different lengths of time. Mice were pre-acclimated to 28 °C for at least 7 days, and then cold exposed (*Clod*), or remained at 28 °C (*Control*), for the indicated times. All animals were sacrificed on the same day, except for some of the control animals that were also killed at the start of the experiment. DNA fragmentation was measured using an enzyme-linked immunosorbent assay, as described under "Materials and Methods." *C,* time course of DNA fragmentation in the interscapular BAT of cold-adapted animals transferred to near thermoneutral temperature. Mice were acclimated to 4 °C for at least 3 weeks and then transferred to 28 °C for the indicated times (*Readaptation*). Control animals were kept at 28 °C (*Control*). The animals were sacrificed and DNA fragmentation was analyzed as described in *B*. Results are normalized to the mean value of the controls. Values are mean ± S.E. from two animals per point. Data are representative of two to four independent experiments each done in duplicate.

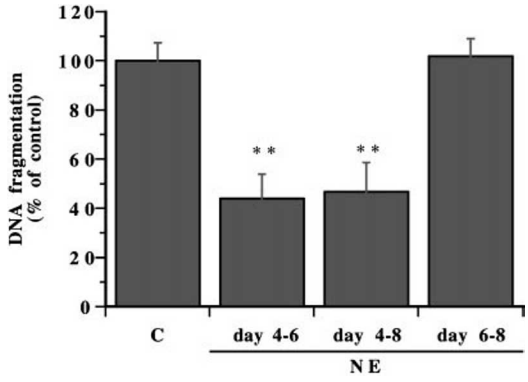

FIG. 2. **The effect of chronic NE stimulation on internucleosomal DNA fragmentation in brown adipocytes.** BAT precursor cells were grown in culture for 4 days. On day 4 the cells were stimulated, except the controls (*C*), with 1 μM NE for 48 h. On day 6 the cultures were deprived of NE, and grown for an additional 48 h; or 4-day cultures were stimulated with 1 μM NE for 96 h; or 6-day cultures were stimulated with 1 μM NE for 48 h. Medium was changed on day 1, 3, 5, and 7. Fresh NE was added every 24 h. All cells were harvested on day 8, and assayed for DNA fragmentation as described under "Materials and Methods." Results are normalized to the mean value of the controls. Values are means ± S.E. of two to four experiments each done in duplicate, **, $p < 0.01$ indicate differences compared with control.

programmed cell death or apoptosis in the physiological regulation of brown adipose tissue hyperplasia cytosolic fragmented DNA, a hallmark of apoptosis, was analyzed in mice cold exposed (4 °C) for 5 weeks. It was found that the amount of cytosolic fragmented DNA was markedly decreased in BAT of the cold adapted animals (Fig. 1*A*). We next analyzed the rate of DNA fragmentation in the tissue of mice exposed to cold for up to 20 days. It was found that after 1 day of cold exposure DNA fragmentation in the tissue was significantly decreased by more than 50% ($p \leq 0.05$), which was sustained for the whole 20-day experimental period (Fig. 1*B*). The effect of cold was specific to the brown fat cell lineage, since epididymal white fat did not show any alteration in the DNA fragmentation under these physiological conditions (not shown).

Increased Rate of Apoptosis in Brown Adipose Tissue of Cold Adapted Animals Transferred to Thermoneutral Temperature—We further investigated if the reversal process (*i.e.* increased apoptosis) could be seen in BAT of cold adapted animals (*i.e.* 4 °C for at least 3 weeks) transferred to near thermoneutral temperature (28 °C). It was found that DNA fragmentation increased significantly in the tissue within 10 days at 28 °C ($p \leq 0.01$), reaching control levels after 20 days (Fig. 1*C*). The data show that cessation of the sympathetic stimulus leads to an increased rate of apoptosis in the tissue.

Taken together, these data indicate that physiological stimulation (*i.e.* sympathetic nerve activation) of BAT promotes the survival of cells within the tissue, and thus that the regulation of cell survival could be a process involved in the cold-induced hyperplasia of BAT.

NE Promoted Cell Survival in Primary Cultures of Brown Adipose Tissue—In order to study the signaling pathways and the molecular mechanisms behind the cold-dependent cell survival we used the primary culture system of mouse BAT. These cells are able to fully differentiate *in vitro*, as shown both by electron microscopy and their ability to express the UCP-1 gene (16, 19, 20). The cells show two distinct stages associated with time after plating, preconfluent proliferating cells (0–5 days in culture) and confluent differentiating cells (6 days and older). Besides the difference in cell density, there is also a marked difference in fat accumulation between these two stages, which further indicate the differentiation state of the cells (15, 19). Since NE has been implicated in the regulation of both proliferation and differentiation of brown adipocytes (16, 20, 21), we hypothesized that NE also could be involved in promoting the survival of the cells. In order to test this, primary cultures of BAT were stimulated on day 4 or day 6 with NE (1 μM) for different lengths of time (*i.e.* 2 to 4 days of stimulation). In order to ensure sustained hormonal activity, NE was added to the cultures every 24 h. It was found that NE significantly decreased (≈50%) the DNA fragmentation in cultures treated with the hormone, compared with control cells (Fig. 2). Furthermore, the effect of NE stimulation on cell survival showed a clear temporal dependence, since only preconfluent prolifer-

TABLE I
TUNEL staining of cultures of brown adipose tissue after chronic NE treatment

BAT precursor cells were grown in culture for 4 days. The cells were treated for 4 days, except controls, with 1 μM NE. Cells were fixed and stained for DNA breaks with the *in situ* Cell Death Detection Kit according to the manufacturers instructions. The number of positive cells was determined by light microscopy.

Treatment	TUNEL cell number		% Apoptotic cells
	Positive	Negative	
Control	90	917	8.9%
NE	57	1119	4.8%

ating cells were found to respond to the NE treatment with decreased DNA fragmentation (Fig. 2). Once the cells started the differentiation process NE was without effect. Moreover, the effect of NE treatment on the cell survival remained for at least 2 days despite removal of the hormone (Fig. 2), indicating some kind of cellular memory. Alternatively, the expression of an autocrine/paracrine growth factor may be induced by the NE stimulation, and secreted from the cells ensuring cell viability.

We also analyzed the percentage of apoptotic cells in control and NE-treated cultures, by counting positive cells in a TUNEL assay. It was found that 8.9% of the cell in the control cultures were apoptotic, and that NE stimulation markedly decreased the number of apoptotic cells to 4.8% of the total cell population (Table I). Thus, in the growing cultures ≈10% of the cell population are dying by apoptosis. NE treatment of the cultures markedly decreases the number of apoptotic cells (TUNEL positive) by 50%, which correlates well to the NE-induced decrease in DNA fragmentation (*cf.* Fig. 2).

Characterization of the Adrenergic Receptors and Intracellular Signaling Pathways Involved in Promoting Cell Survival—Since NE binds to both α_1- and β-adrenergic receptors, we treated preconfluent cells (day 4) for 48 h with specific agonists for either α_1- or β-receptors (*i.e.* cirazoline and isoprenaline, respectively). It was found that cirazoline (1 μM) was able to fully mimic the effect of NE on cell survival, whereas isoprenaline (1 μM) partially mimicked the effect of NE (Fig. 3). Similarly, when the cells were treated with the β_3-receptor agonist CGP12177 (1 μM) (22) DNA fragmentation was decreased to the same level as in isoprenaline-treated cells (not shown).

Activation of α_1-receptors has been shown to cause increases in the intracellular Ca^{2+} level and stimulation of protein kinase C (PKC) activity (23, 24). Agonist stimulation of β-receptors leads to activation of adenylyl cyclase, resulting in increased intracellular cAMP level and activation of protein kinase A (PKA). In order to further characterize the adrenergic signals involved in the regulation of cell survival we analyzed the effect of these post-receptor signals by treating the cells with the PKC activator TPA (50 nM), and the adenylyl cyclase activator forskolin (5 μM) for 48 h. As shown in Fig. 3, TPA treatment of the cells did not significantly alter the cell survival. However, there was a tendency for increased apoptosis in the TPA-treated cell. Forskolin, on the other hand, was found to mimic the effect of NE on cell survival, indicating that PKA-dependent phosphorylation could be involved in promoting cell survival.

NE-induced bFGF Expression and an Autocrine/Paracrine Effect of bFGF—It was recently shown that NE could induce bFGF expression in primary cultures of rat BAT (25). Similarly, in primary cultures of mouse BAT we found that NE increased the expression of bFGF mRNA and that the cells expressed FGF-receptor-1 mRNA.[2] In order to investigate the

[2] J. M. Svensson and S. Rehnmark, unpublished data.

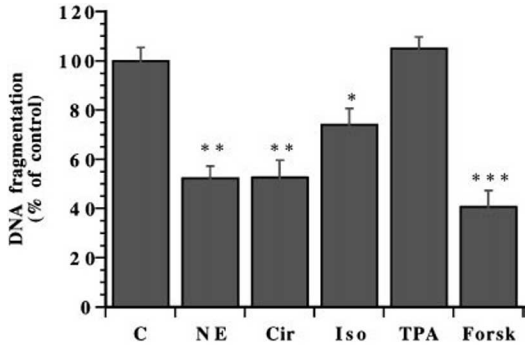

FIG. 3. **The effect of adrenergic agonists, TPA, and forskolin on internucleosomal DNA fragmentation in brown adipocytes.** BAT precursor cells were grown in culture for 4 days. On day 4 the cells were stimulated for 48 h, except the controls (*C*), as indicated: 1 μM NE, 1 μM cirazoline (*Cir*), 1 μM isoprenaline (*Iso*), 50 nM TPA, 5 μM forskolin (*Forsk*). Medium was changed after 24 h and fresh agents added. Cells were harvested on day 6, and assayed for DNA fragmentation as described under "Materials and Methods." Results are normalized to the mean value of the controls. Values are means ± S.E. of four to eight experiments each done in duplicate; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ indicate differences compared with control.

role of bFGF and a possible autocrine effect of bFGF, we stimulated the cells with bFGF, or NE together with a bFGF neutralizing antibody, and analyzed the effects on cell survival. It was found that bFGF stimulation of the cells promoted cell survival to the same extent as NE stimulation did, and that the bFGF antibodies attenuated the NE-dependent survival (Fig. 4). In a control experiment we also tested whether the bFGF antibody could inhibit the effect of bFGF on cell survival. It was found that the effect of bFGF was abrogated by the bFGF antibody (not shown), which show that the antibody indeed inactivated bFGF. These data indicated that part of the NE effect was mediated by bFGF, and suggested a possible interaction of the NE and bFGF signal in promoting the survival. In order to test for such an interaction, NE and bFGF were added together to the cells. As shown in Fig. 4, simultaneous NE and bFGF stimulation of the cells significantly decreased the DNA fragmentation by 74 and ≈50% compared with control cells and NE- or bFGF-stimulated cells, respectively. These results suggest an interaction of the NE and bFGF signal in promoting the survival of brown adipocytes.

NE- and bFGF-induced Activation of Erk1/2 in Primary Cultures of Brown Adipose Tissue—Since bFGF, via its receptor, is known to activate the Erk1/2 signaling pathway, and NE recently also was shown to be a potent activator of Erk1/2 (26, 27), the data suggested that Erk1/2 may have a role in promoting NE- and bFGF-dependent survival of brown adipocytes. In order to investigate the effect of NE and bFGF on Erk1/2 activation in primary cultures of BAT, we first analyzed the effects of NE and bFGF on Erk1/2 phosphorylation with a phospho-specific Erk1/2 antibody. As shown in Fig. 5*A*, stimulation of preconfluent cells with either NE (1 μM) or bFGF (10 ng/ml) for 10 min caused a ≈5-fold increase in Erk1/2 phosphorylation. We next analyzed whether NE and bFGF stimulated Erk1/2 phosphorylation corresponded to increased Erk1/2 kinase activity, by measuring Erk1/2-dependent Elk-1 phosphorylation in an *in vitro* kinase assay. As shown in Fig. 5*B*, stimulation of the cells with either NE or bFGF for 10 min resulted in a ≈5-fold increase in Erk1/2 activity. In order to exclude the possibility that the effect of NE on Erk1/2 activation was mediated by NE induced secretion of bFGF and an autocrine effect of bFGF, we measured the effect of NE stimulation in the presence of the bFGF antibody. It was found that the NE stimulated Erk1/2 kinase activity was unaffected by the presence of the bFGF antibody (data not shown). These results

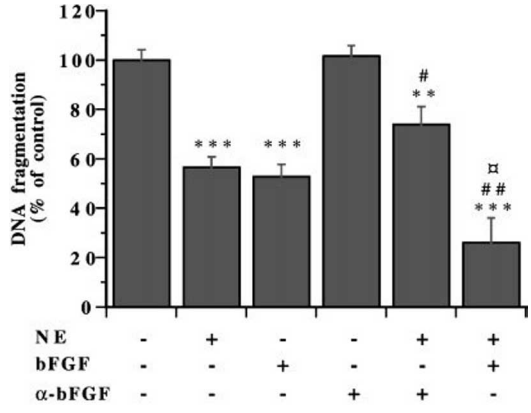

FIG. 4. **The effect of bFGF, NE, and bFGF neutralizing antibody on internucleosomal DNA fragmentation in brown adipocytes.** BAT precursor cells were grown in culture for 4 days. On day 4 the cells were treated for 48 h, except the controls (*C*), as indicated: 1 μM NE, 10 ng/ml bFGF, and 10 μg/ml bFGF neutralizing antibody, cells were pretreated with α-bFGF for 1 h before NE addition (*α-bFGF*). Medium was changed after 24 h and fresh agents added. Cells were harvested on day 6, and DNA fragmentation was assayed as described under "Materials and Methods." Results are normalized to the mean value of the controls. Values are mean ± S.E. of three to eight independent experiments each done in duplicate: **, $p < 0.01$, and ***, $p < 0.001$ indicate differences compared with control. #, $p < 0.05$; ##, $p < 0.01$; and ✧, $p < 0.05$ indicate differences compared with NE and bFGF, respectively.

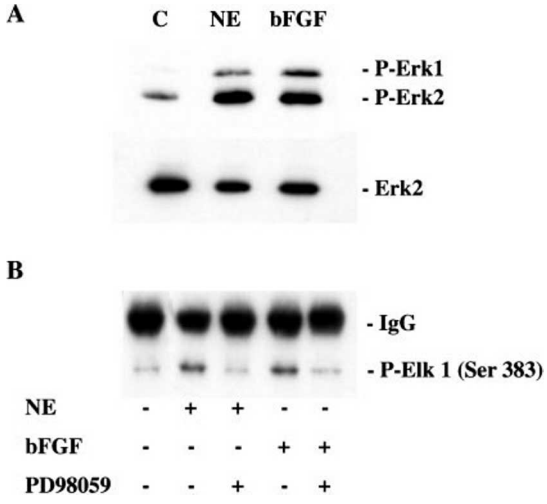

FIG. 5. **NE- and bFGF-induced phosphorylation and activation of Erk1/2 in brown adipocytes, and inhibition by PD98059.** *A,* BAT precursor cells were grown in culture for 3 days. On day 3 the medium was changed and the cells preincubated in culture medium containing 0.5% serum for 2 h. The cells were stimulated for 10 min, as indicated: 1 μM NE, 10 ng/ml bFGF. Erk1/2 phosphorylation (*P-Erk1/2*) was measured using Western blotting with a phospho-specific Erk1/2 antibody, an Erk2 antibody was used as a loading control, as described under "Materials and Methods." *B,* BAT precursor cells were grown in culture for 5 days. On day 5 the cells were treated for 10 min, as indicated: 1 μM NE, 10 ng/ml bFGF, 50 μM PD98059, the cells were pretreated for 1 h before NE or bFGF addition (PD98059). The Erk1/2 kinase activity was analyzed in an *in vitro* kinase assay, measuring Elk-1 phosphorylation at Ser-383 with a phospho-specific Elk-1 antibody, as described under "Materials and Methods." The IgG band represents the antibody used for immunoprecipitation. Data are representative of two independent experiments each done in duplicate.

show that acute NE stimulation of brown adipocytes cause the phosphorylation and activation of Erk1/2. It further shows that the acute effect of NE is mediated through the adrenergic receptors and not via an autocrine/paracrine effect of bFGF.

We also tested whether it was possible to inhibit the activation of Erk1/2 by pretreating the cells with the MAP/Erk kinase (MEK) inhibitor, PD98059 (50 μM), for 1 h before NE or bFGF stimulation of the cells. As shown in Fig. 5*B*, PD98059 abrogated the NE and bFGF induced activation of Erk1/2. These data show that in brown adipocytes NE and bFGF activates the Erk cascade through a MEK-dependent signaling pathway.

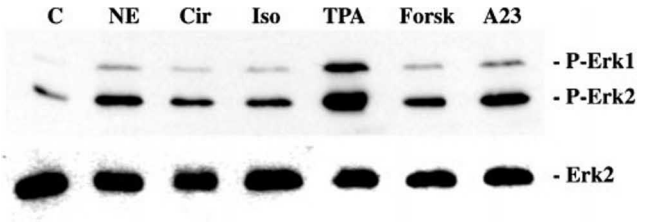

FIG. 6. **α_1- and β-adrenergic receptor mediated Erk1/2 phosphorylation in brown adipocytes.** BAT precursor cells were grown in culture for 3 days. On day 3 the medium was changed and the cells preincubated in culture medium containing 0.5% serum for 2 h. The cells were treated for 10 min, as indicated: 1 μM NE, 1 μM cirazoline (*Cir*), 1 μM isoprenaline (*Iso*), 50 nM TPA, 5 μM forskolin (*Forsk*), or 1 μM A23187 (*A23*). Erk1/2 phosphorylation (*P-Erk1/2*) was measured using Western blotting with a phospho-specific Erk1/2 antibody, an Erk2 antibody was used as a loading control, as described under "Materials and Methods." Data are representative of two independent experiments each done in duplicate.

α_1- and β-Adrenergic Receptor-mediated Phosphorylation of Erk1/2—In order to characterize the adrenergic receptors involved in the activation of Erk1/2, the cells were stimulated for 10 min with specific α_1- and β-receptor agonists (*i.e.* cirazoline and isoprenaline, respectively). As shown in Fig. 6, both cirazoline (1 μM) and isoprenaline (1 μM) markedly increased the phosphorylation of Erk1/2 (2–3-fold) compared with control cells. Since α_1-receptor stimulation cause increases in the intracellular Ca^{2+} level and activation of PKC, the cells were treated with the Ca^{2+} ionophore A23187 (1 μM) and the PKC activator TPA (50 nM) for 10 min, and the phosphorylation of Erk1/2 assayed. As shown in Fig. 6, both A23187 and TPA markedly increased the phosphorylation of Erk1/2 (5- and 10-fold, respectively) compared with control cells. Notably, TPA was the most potent inducer of Erk1/2 phosphorylation tested. In order to mimic β-receptor stimulation, cells were treated with forskolin (5 μM) (*i.e.* increasing the intracellular cAMP level) for 10 min and the phosphorylation Erk1/2 analyzed. As shown in Fig. 6, forskolin caused a ≈3-fold increase in the phosphorylation of Erk1/2, which show that in brown adipocytes cAMP is a potent activator of Erk1/2.

Expression of Rap1 and B-Raf in Brown Adipocytes—It has recently been shown that cAMP activates the Erk cascade through a PKA-, Rap1-, and B-Raf-dependent signaling pathway (28). Although Rap1 and B-Raf are most abundantly expressed in cells of neuronal origin, expression has been shown in other cell types (29, 30). In order to investigate whether brown adipocytes expressed B-Raf and Rap1, 4-day-old primary cultures of BAT were analyzed by Western blot with specific antibodies against B-Raf and Rap1. As shown in Fig. 7*A*, the 67-kDa short form of B-Raf was the predominantly expressed isoform in the brown adipocytes. Similarly, we also found that Rap1 was expressed in the brown adipocytes (Fig. 7*B*), indicating that a cAMP > PKA > Rap1 > B-Raf signal cascade is present in brown adipocytes.

Inhibition of MEK Abrogates the Effect of NE and bFGF on the Cell Survival—Our data showed that both NE and bFGF activated Erk1/2 in primary cultures of BAT, and suggested that NE- and bFGF-dependent cell survival could be mediated by the Erk1/2 signaling pathway. In order to investigate the role of Erk1/2 on cell survival we exposed the cells to either NE or bFGF in combination with the MEK inhibitor PD98059 (50 μM). As shown in Fig. 8, inhibition of MEK abolished the effect of NE and bFGF on cell survival, indicating a critical role for

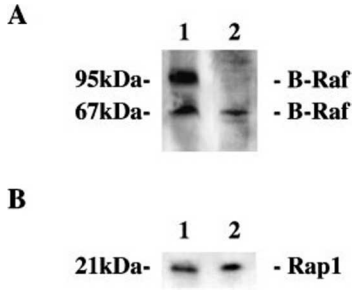

FIG. 7. **Expression of B-Raf and Rap1 in brown adipocytes.** Western blot analysis of of B-Raf expression (*A*): mouse cerebellum (positive control) (*lane 1*) and 4-day old primary cultures of BAT (*lane 2*). Western blot analysis of of Rap1 expression (*B*): A431 cells (positive control) (*lane 1*) and 4-day old primary cultures of BAT (*lane 2*).

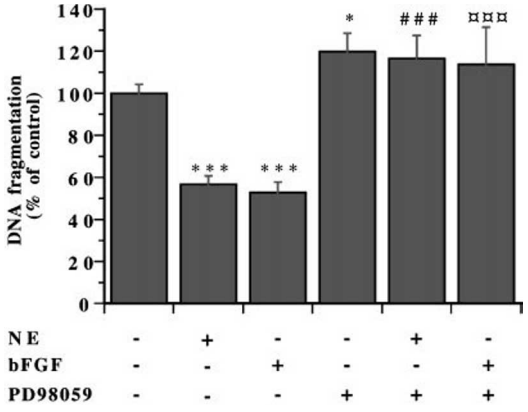

FIG. 8. **The effect of PD98059 on NE- and bFGF-dependent survival of brown adipocytes.** BAT precursor cells were grown in culture for 4 days. On day 4 the cells were treated for 48 h, as indicated: 1 μM NE, 10 ng/ml bFGF, 50 μM PD98059, the cells were pretreated for 1 h before NE or bFGF addition (PD98059). Medium was changed after 24 h and fresh agents added. Cells were harvested on day 6 and the DNA fragmentation was assayed, as described under "Materials and Methods." Values are mean ± S.E. of five to eight experiments done in duplicate: *, $p < 0.05$; ***, $p < 0.001$ indicate differences compared with control. ###, $p < 0.001$ and ◇◇◇, $p < 0.001$ indicate differences compared with NE and bFGF, respectively.

Erk1/2 in promoting NE- and bFGF-dependent cell survival. Furthermore, PD98059 in itself significantly increased cell death compared with untreated control cells, suggesting that an intact Erk1/2 signaling pathway also is important for the "normal" cell survival.

Cold-induced Phosphorylation of Erk1/2 in Brown Adipose Tissue—The results form the cell cultures showed a critical role for Erk1/2 in promoting NE- and bFGF-dependent cell survival. In order to investigate whether the cold-dependent cell survival in BAT could be correlated to Erk1/2 activation, we analyzed the effects of cold exposure on Erk1/2 phosphorylation in BAT. Mice were cold exposed for different lengths of time (*i.e.* 30 min to 20 days) and the phosphorylation of Erk1/2 was assayed in BAT. It was found that within 30 min of cold exposure the phosphorylation of Erk1/2 was markedly increased, and that the phosphorylation level of Erk1/2 was sustained during the whole 20-day experimental period (Fig. 9). These data show a clear correlation between Erk1/2 activation and cold-dependent cell survival, and indicate that the Erk1/2 signaling pathway may have a critical role in promoting cold-dependent cell survival in BAT.

Although the *in vivo* data could be interpreted as if sustained activation of the Erk cascade is obligatory to decrease the rate of apoptosis in the tissue, the *in vitro* data showed that the cells only had to be stimulated for 48 h with NE to decrease cell death (*cf.* Fig. 2). The *in vitro* data further showed that the

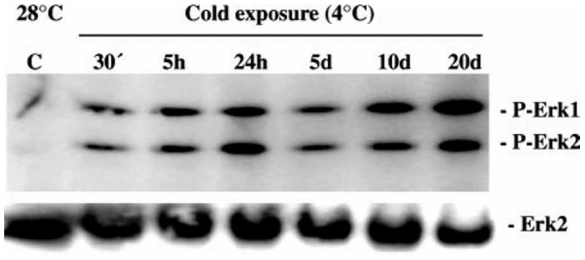

FIG. 9. **Cold-induced Erk1/2 phosphorylation in mouse brown adipose tissue.** Three-week-old mice were pre-acclimated to 28 °C for 7 days and then placed in the cold (4 °C), or remained at near thermoneutral temperature (28 °C), for the times indicated. All animals were sacrificed on the same day. Total protein from interscapular brown adipose tissue was analyzed for Erk1/2 phosphorylation (*P-Erk1/2*) by Western blotting with a phospho-specific Erk1/2 antibody, an Erk2 antibody was used as a loading control, as described under "Materials and Methods." The gel show one representative series from one experiment done in duplicate.

effect of NE treatment on cell survival remained for at least 2 days despite removal of the hormone (*cf.* Fig. 2), indicating that sustained activation of the Erk cascade may not be necessary to induce a decrease in the rate of apoptosis.

Different Kinetics of the NE- and bFGF-induced Activation of Erk1/2—In order to investigate the kinetics of the NE- and bFGF-induced phosphorylation of Erk1/2, we stimulated 4-day-old BAT primary cultures for different lengths of time with NE (1 μM) or bFGF (10 ng/ml). As shown in Fig. 10, *A* and *B*, NE caused a rapid increase in the phosphorylation of Erk1/2 (≈8-fold) within 10 min of stimulation. The level of phosphorylated Erk1/2 decreased within 30 min of NE stimulation to ≈3-fold over basal, and was back to control levels within 4 h of stimulation. In contrast, bFGF caused sustained phophorylation of Erk1/2; the activation of Erk was sustained for more than 4 h following bFGF stimulation. However, the initial kinetics of bFGF- and NE-induced Erk1/2 activation was similar.

Different Kinetics of the NE- and bFGF-promoted Cell Survival—Since NE and bFGF showed different kinetics in the activation of the Erk cascade, it was possible that the effect of NE and bFGF stimulation also could show different kinetics in promoting the cell survival. In order to test this, the cells were stimulated on day 5 for 24 h with either NE (1 μM) or bFGF (10 ng/ml). As shown in Fig. 11, 24 h bFGF stimulation of the cells decreased the rate of apoptosis. In contrast, 24 h of NE stimulation was not enough to alter the cell survival, irrespective of when the stimulation was initiated (*i.e.* day 4–5 (not shown) or day 5–6).

DISCUSSION

In the present study we demonstrate that cold acclimation (4 °C) of an animal is accompanied by a decreased rate of apoptosis in BAT. Although acute cold exposure (less than 5 h) did not alter the rate of normal cell death (apoptosis) significantly, the rate of apoptosis in BAT was markedly reduced already within 24 h of cold exposure. The reduced rate of apoptosis was sustained as long as the animals were exposed to the cold, that is, at least for up to 5 weeks of cold exposure. In analogy, when cold-adapted animals were transferred to near thermoneutral temperature (28 °C), leading to decreased sympathetic stimulation of the tissue, the rate of apoptosis increased within 10 days, reaching control level after 20 days. Thus, the data show that the regulation of cell survival is a process involved in the cold-induced hyperplasia of BAT, as well as in eliminating redundant cells during the transition from the thermogenic to the non-thermogenic state. The data further indicated that some factors, released during the cold-dependent recruitment of the tissue, could promote the sur-

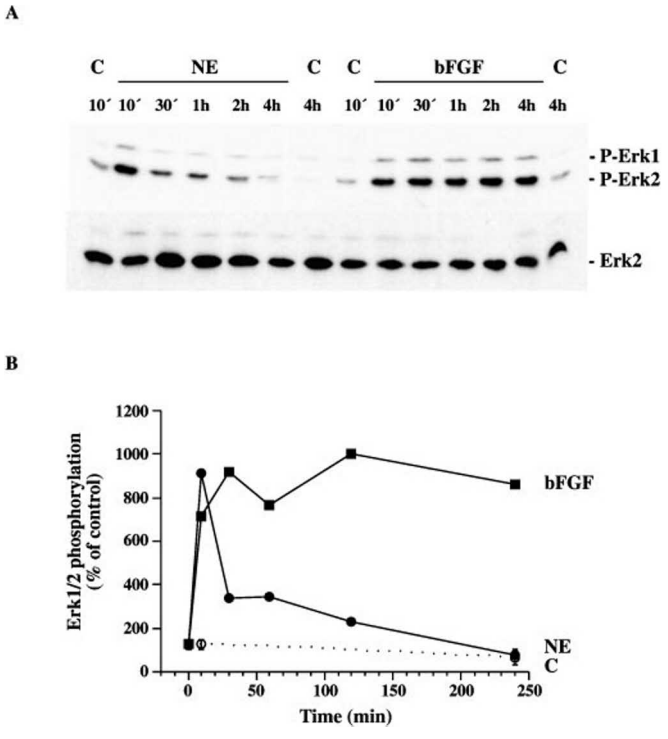

FIG. 10. **Kinetics of the NE- and bFGF-induced Erk1/2 phosphorylation in primary cultures of brown adipose tissue.** *A,* BAT precursor cells were grown in culture for 4 days. On day 4 the medium was changed and the cells preincubated in culture medium containing 0.5% serum for 2 h and then treated with 1 μM NE or 10 ng/ml bFGF for the times indicated. Erk1/2 phosphorylation (P-Erk1/2) was measured using Western blotting with a phospho-specific Erk1/2 antibody, an Erk2 antibody was used as a loading control, as described under "Materials and Methods." *B,* computation of the results obtained in *A*, the control level was set to 100%. The sum of P-Erk1 and P-Erk2 are shown.

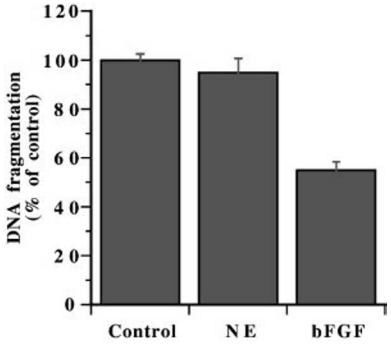

FIG. 11. **Kinetics of the NE- and bFGF-induced cell survival.** BAT precursor cells were grown in culture for 5 days. On day 5 the cells were treated for 24 h, except the controls (*C*), as indicated: 1 μM NE, 10 ng/ml bFGF. All cells were harvested on day 6, and assayed for DNA fragmentation as described under "Materials and Methods." Results are normalized to the mean value of the controls.

vival of the cells, and that certain cells in the tissue apparently are destined to undergo apoptosis unless rescued by these survival factors.

There are several examples of growth factor-dependent cell survival in the developing vertebrate. For example, the survival of developing neurones depend on neurotrophic factors (*e.g.* NGF for sympathetic neurones) that are secreted by the target cells they innervate; those that fail to get enough neurotrophic factors (*e.g.* NGF) die by typical programmed cell death (31, 32). However, there are only a few examples of hormonal or growth factor-dependent regulation of cell survival (or apoptosis) in tissues of adult animals. In adult hamsters, for example, the reproductive activity is determined by the photoperiod; long days stimulate testis development, while short days induce testis regression. An increased rate of testis cell apoptosis has been shown in Djungarian hamsters exposed to short photoperiods, which correlated with decreases in serum testosterone levels. Similarly, when the hamsters were transferred to long photoperiods testis cell apoptosis was decreased, correlating with increased testosterone levels (33).

The immediate response of an animal to a cold environment is the activation of the sympathetic nervous system; NE is released at the nerve terminals triggering several physiological responses in BAT (34). We show here that NE stimulation, agonist stimulation of α_1- and β-receptors (cirazoline and isoprenaline, respectively), and bFGF stimulation of primary cultures of BAT promoted the survival of the cells. NE and bFGF stimulation (48 h) of the cells caused a similar decrease in the rate of apoptosis ($\approx$50% decrease in DNA fragmentation). Furthermore, the effect of NE treatment on the cell survival remained for at least 2 days despite removal of the hormone. Similarly, cessation of the sympathetic stimulus, in the intact animal, caused a delayed increase in the DNA fragmentation in BAT. These data suggest that both the NE and the cold stimulus caused alterations in the cellular composition of relatively stable character. The anti-apoptotic effect of NE also showed a clear temporal dependence; once the cells reached confluence and started differentiating NE was without effect. It could be speculated that this may also be the case in physiologically activated BAT, and thus that sympathetic stimulation of BAT may only promote the survival of the proliferating precursor cells in the tissue.

Intracellular Signals Promoting Cell Survival—As the NE-dependent cell survival apparently was mediated via both α_1- and β-receptors, it was possible that Ca^{2+}- and cAMP-dependent signaling pathways could be involved in promoting the survival. We found that increasing the intracellular cAMP level by treating the cells with forskolin mimicked the effect of NE. However, forskolin was found to be more potent than selective β-receptor stimulation (*i.e.* isoprenaline), which may be due to different levels of intracellular cAMP generated by these agents (forskolin being the more potent), and the duration of the signal. Thus, it is possible that the magnitude and duration of the cAMP signal may be critical for the physiological response of the cell.

Increases in the intracellular cAMP level has been shown to either induce apoptosis or protect cells from apoptosis, depending on the cell type and the circumstances. For example, in neutrophils and PC12 cells, cAMP raising agents have been shown to protect the cells from apoptosis (35–37) whereas it induces apoptosis in a variety of other cell types (38–41). Clearly, our data show that in brown adipocytes increases in the intracellular cAMP level promotes the cell survival.

In contrast to the effect of the α_1-receptor agonist cirazoline, TPA activation of PKC did not affect cell survival. Similarly, in PC12 cells, growth factor withdrawal-induced cell death cannot be rescued by TPA treatment (37). It has been suggested that Erk and the stress-activated kinase, c-Jun N-terminal kinase (JNK) and p38 MAPK, cascade may have opposing effects on nerve cells, and that the dynamic balance between Erk and the JNK/p38-MAPK signaling pathways is important in determining whether a cell survives or undergoes apoptosis (42). The induction of apoptosis in PC12 cells is likely dependent on the balance between anti-apoptotic and apoptotic signals (*i.e.* Erk and JNK/p38-MAPK, respectively) (42, 43). It has recently been shown that TPA activates the p38 MAPK, Erk, and JNK cascades in A3.01 T cells, and that both p38 MAPK and JNK were synergistically activated by TPA and ionomycin (Ca^{2+} ionophore) (44). It is possible that TPA treatment of brown

adipocytes also could lead to activation of stress-activated JNK/p38 MAPK cascades. Thus, the inability of TPA to rescue brown adipocytes from apoptosis, despite that it was found to cause the most pronounced phosphorylation of Erk1/2, could be due to simultaneous stimulation of the Erk and the stress-activated JNK/p38 MAPK cascade by TPA, and this may alter the balance between anti-apoptotic and apoptotic signals. Moreover, the observation that TPA stimulation of brown adipocytes showed a tendency to increase the cell death, rather that the opposite, gives further support to this notion.

Activation of Erk1/2 in Brown Adipocytes—In agreement with previous findings in rat brown adipocytes and rat cardiomyocytes (26, 27, 45), we found that NE, α_1-, and β-receptor agonists activated the Erk cascade in mouse brown adipocytes. In cardiomyocytes, α_1- and β-receptor agonists synergistically activate Erk through PKA- and PKC-dependent pathways; the activation of Erk was also shown to be dependent on the influx of extracellular Ca^{2+} (27, 45). Although we have not directly analyzed for a possible synergism between α_1- and β-receptor stimulation on Erk activation, our results show that, with the concentrations used, NE stimulation of the cells resulted in a ≈5-fold increase in Erk1/2 phosphorylation, whereas cirazoline or isoprenaline stimulation of the cells caused a 2–3-fold increase in Erk1/2 phosphorylation. These data indicate that NE-induced Erk activation is mediated via both α_1- and β-adrenergic receptors through increases in intracellular Ca^{2+} and cAMP levels. The data further suggest that NE-induced Erk activation could be mediated via Ca^{2+}- and cAMP-dependent kinases. Apparently, in brown adipocytes the signals from α_1- and β-receptors converge at some point in the activation of Erk.

In PC12 and HEK-293 cells, extracellular signals that increase the intracellular Ca^{2+} level activates the Ras > Raf > MEK > Erk pathway through Pyk2 (*i.e.* the proline-rich tyrosine kinase, belonging to the family of non-receptor protein tyrosine kinases) (46, 47). It has been suggested that Ca^{2+}/calmodulin could be involved in the activation of Pyk2 (47). Since we here show that both α_1-receptor stimulation and Ca^{2+} ionophore treatment of the cells induces Erk activation, it could be suggested that α_1-receptor activation of the Erk cascade in brown adipocytes could be mediated by a Ca^{2+}/calmodulin-dependent Pyk2 pathway.

cAMP elevating agents has been shown to have both stimulatory and inhibitory effects on Erk activation depending on the cell type (48), and a number of possible sites for the interaction of cAMP/PKA in the Erk signaling pathway has been described (28, 48–51). In brown adipocytes, forskolin (*i.e.* increased intracellular cAMP level) caused a ≈3-fold increase in Erk1/2 phosphorylation, which was similar to the effect of β-receptor stimulation. These results indicate that β-receptor-stimulated Erk activation is mediated by cAMP/PKA. It has recently been shown that cAMP/PKA activates the Erk cascade through a B-Raf and Rap1-dependent pathway (28). Since we here show that brown adipocytes express both Rap1 and B-Raf, it is possible that cAMP may activate the Erk cascade through the PKA > Rap1 > B-Raf > MEK signaling pathway.

bFGF stimulation of the cells also results in Erk activation, similar to the effect of NE. However, the kinetics of NE- and bFGF-induced Erk1/2 phosphorylation was markedly different. NE caused transient Erk activation (*i.e.* the phosphorylation of Erk1/2 peaked within 10 min, was markedly reduced within 30 min, and was back to control level within 4 h of NE stimulation), while bFGF caused sustained Erk activation (*i.e.* the phosphorylation of Erk1/2 was sustained for more than 4 h following bFGF stimulation). Similarly, in PC12 cells, the activation of Erk is sustained for several hours following NGF stimulation, while epidermal growth factor stimulation cause

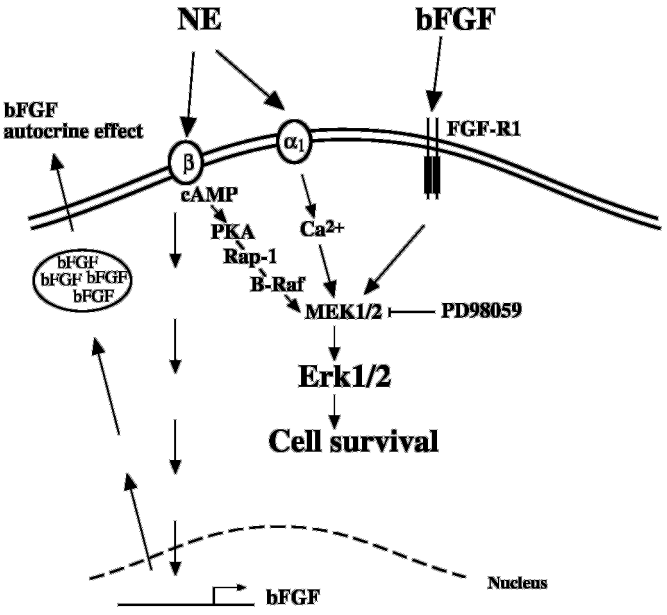

FIG. 12. **Tentative model for the NE-dependent survival of brown adipocytes.** NE stimulates the Erk cascade via both α_1- and β-adrenergic receptors through increases in the intracellular Ca^{2+} and cAMP level. NE also increases the expression and secretion of bFGF, which leads to further activation of Erk. Erk has a critical role in promoting NE- and bFGF-dependent survival of brown adipocytes.

transient Erk activation. These differences may in part be due to the differences in receptor down-regulation of the epidermal growth factor receptor and the NGF receptor (TrkA) (52). However, York *et al.* (53) recently reported that the activation of Erk by NGF, in PC12 cells, involves two distinct pathways: an initial rapid phase of Erk activation mediated by Ras, and a sustained phase of Erk activation mediated by Rap1. It was further shown that the cAMP analogue 8-(4-chlorophenylthio)-cAMP was able to activate Rap1 for prolonged periods. cAMP raising agents has also been shown to induce sustained Erk activation in PC12 cell (54).

As NE stimulation cause increases in the intracellular Ca^{2+} and cAMP level, it could be suggested that NE activates both the Ras pathway and the Rap1 pathway in brown adipocytes. In analogy with NGF (Ras and Rap1 activator), NE could be expected to cause sustained Erk activation in brown adipocytes. However, as mentioned above, NE induces transient Erk activation in brown adipocytes. This could not be explained by a similar transient increase in the intracellular cAMP level; the cAMP level in brown adipocytes remains markedly elevated after 30 min of NE stimulation.[3] These apparently contradictory results could perhaps be explained by some form of interaction between different signals activated by NE. One possibility is that NE also activates inhibitory signals, such as a protein phosphatase(s), that could limit the activation of Erk. Pretreating the cells with the MEK inhibitor PD98059 abrogated the NE- and bFGF-induced activation of the Erk cascade, which show that, in brown adipocytes, the signaling pathways used by G-protein-coupled receptors (adrenergic) and tyrosine kinase receptors (FGFR) intersect at MEK or upstream of MEK.

We further show that cold exposure of the animals caused a rapid increase in the phosphorylation of Erk1/2 in BAT. Already within 30 min of cold exposure the phosphorylation of Erk1/2 was markedly increased, and the phosphorylation level was sustained as long as the animals were exposed to the cold. Since the cold-induced activation of Erk was both rapid and

[3] G. Bronnikov personal communication.

sustained in the tissue, it could be suggested that the initial activation of Erk is due to the acute effect of sympathetic stimulation mediated via adrenergic receptors, and that bFGF could be responsible for the sustained activation. Interestingly, the cold-induced phosphorylation pattern of Erk1 and Erk2 was similar between the two isoforms, whereas in the cultured cells Erk2 was the more heavily phosphorylated isoform (*cf.* Figs. 6 and 9).

Erk1/2-dependent Survival of Brown Adipocytes—Since both NE and bFGF were found to activate the Erk signaling pathway, it was possible that the signal for cell survival could be mediated via the Erk pathway. We show here that both the NE- and bFGF-dependent cell survival are dependent on Erk activation; inhibition of MEK with PD98059 abrogated the anti-apoptotic effect of both bFGF and NE. We further show that the anti-apoptotic effect of NE was slower than the effect of bFGF; it was necessary to stimulate the cells for 48 h with NE to reduce the rate of apoptosis, while the effect of bFGF appeared within 24 h of stimulation. This together with the finding that NE, in contrast to bFGF, causes transient Erk activation may suggest that additional NE-dependent signals (*i.e.* separate from the Erk pathway) could be involved in promoting cell survival. However, it may also be explained by down-regulation of the adrenergic receptors involved in transmitting the signal. It has previously been shown that NE stimulation of brown adipocytes cause a rapid down-regulation of β_3-adrenergic receptor mRNA; within 2 h of NE stimulation the cells were almost devoid of β_3-receptor mRNA. However, the down-regulation was transient to its nature and within 18 h of NE stimulation the level of β_3-receptor mRNA was back to control level (55). Similarly, NE has also been reported to cause a transient down-regulation of α_1-adrenergic receptors (56, 57). Thus, the transient down-regulation of the adrenergic receptors may in part explain the differences between NE and bFGF in promoting cell survival (*i.e.* 48 h NE exposure is required, while 24 h bFGF exposure is sufficient). It could be suggested that the initial exposure of the cells to NE, causing transient Erk activation, induces the expression of genes that are anti-apoptotic. However, as the initial exposure to NE also causes a transient receptor down-regulation, NE needs to be readded to the cells at a time when the receptors have reappeared. Since we followed this scheme, the readdition of NE to the cells may induce a second peak of Erk activation, which could further enhance the expression of genes that are anti-apoptotic.

Alternatively, since our data indicate that NE promotes the cell survival partly by increasing the expression of bFGF and partly by a direct effect of NE (Fig. 12), the differences between NE and bFGF in promoting cell survival may in part be explained by an autocrine/paracrine effect of bFGF. It is possible that 24 h of NE stimulation is not sufficient for the accumulation of bFGF to a biologically relevant level, but within 48 h of NE stimulation such a level could be reached. However, since the neutralizing bFGF antibody only partially inhibited the NE-dependent cell survival, additional NE-dependent signals may be involved. The observation that cell survival was markedly enhanced by simultaneous NE and bFGF stimulation, compared with either NE or bFGF alone, may support the notion that additional NE-dependent signals (*i.e.* separate from the Erk pathway) could be involved. These data also indicate that NE- and bFGF-dependent signals in some way cooperates in promoting the cell survival. Although Erk activation is required for the anti-apoptotic effect of both NE and bFGF, PKA- and Ca^{2+}-dependent kinases may augment the Erk-dependent cell survival. It is possible that co-stimulation of these pathways (*e.g.* Erk and PKA) could enhance the expression of genes that are anti-apoptotic. Interestingly, it was recently shown that NGF-induced sustained Erk activation, nuclear translocation of Erk, and maximal activation of Elk-1, a member of the the Ets family of transcription factors, was attenuated by PKA inhibition (54). Thus, the cooperation between the Erk pathway and PKA may dictate the physiological response to growth factors and neurotransmitters, such as NE.

PKA and Erk can activate common transcription factors, such as Elk-1 and the cAMP response element-binding protein, CREB (28, 58–61). Activated CREB binds to specific sites, cAMP responsive elements (CRE), present in the promoter of cAMP responsive genes. Elk-1 binds together with a serum response factor to serum response elements present in the promoter of many genes. As CREB and Elk-1 can be activated both by the PKA pathway and the Erk pathway, the transcription of genes that are anti-apoptotic could be regulated by either pathway. Furthermore, simultaneous stimulation of the Erk pathway and the PKA pathway may enhance the transcription of genes containing both serum response element and CRE sites in their promoters. Although the Bcl-2 promoter containes a functional CRE and Bcl-2 expression in B cells is dependent on CREB phosphorylation (62), we could not detect increased Bcl-2 expression in cold exposed animals.[2] However, the transcription of other anti-apoptotic genes in brown adipocytes could perhaps be dependent on CREB and/or Elk-1 activation. Although the anti-apoptotic effect of NE and bFGF could be dependent on transcriptional activation of genes that are anti-apoptotic, post-translational modifications may be important as well. The phosphorylation of pro-apoptotic proteins (*i.e.* the Bcl-2 family member, Bad) can inactivate the cell-intrinsic death machinery (63, 64), while the phosphorylation of anti-apoptotic proteins (*i.e.* Bcl-2) can promote cell death (65). Since the serine/threonine kinase Erk, due to its rather broad nature of substrate recognition (Pro-Leu-(Ser/Thr)-Pro or (Ser/Thr)-Pro), can phosphorylate a large number of proteins (66), the anti-apoptotic effect of NE and bFGF could be due to Erk-dependent phosphorylation of already existing proteins. This does not necessarily mean that activated Erk is directly involved in the phosphorylation and regulation of anti-apoptotic or pro-apoptotic proteins, but the activity of other kinases or phosphatases could be activated via the Erk pathway. Taken together, Erk may promote the survival of brown adipocytes by regulating the expression of genes that are anti-apoptotic and pro-apoptotic, and/or via post-translational modifications inactivate the cell-intrinsic death machinery; the specific mechanism(s) remains to be elucidated.

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