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N-Terminal Proopiomelanocortin Acts as a Mitogen in Adrenocortical Tumor Cells and Decreases Adrenal Steroidogenesis

MARTIN FASSNACHT, STEFANIE HAHNER, IMMO A. HANSEN, TANJA KREUTZBERGER, MARTINA ZINK, KNUT ADERMANN, FRANZ JAKOB, JAKOB TROPPMAIR, AND BRUNO ALLOLIO

Department of Medicine, Endocrine and Diabetes Unit (M.F., S.H., I.A.H., T.K., M.Z., B.A.), Department of Orthopedics (F.J.), and Institut fuer Medizinische Strahlenkunde und Zellforschung (J.T.), University of Wurzburg, Wurzburg 97080, Germany; and IPF PharmaCeuticals GmbH (K.A.), 30625 Hannover, Germany

There is evidence that proopiomelanocortin (POMC)-derived peptides other than ACTH are involved in pituitary-dependent adrenal growth. We have synthesized the human N-terminal POMC fragment 1-28-POMC with the disulfide bridges in the correct position between cysteine residues 2–24 and 8–20 and studied the activity of these peptides in adrenocortical tumor cells *in vitro*. 1-28-POMC stimulated cell proliferation in human NCI-h295 and mouse Y-1 adrenal cancer cell lines and also in primary cultures of bovine adrenocortical cells in a concentration-dependent manner. 1-28-POMC led to rapid activation of the MAPKs extracellular signal-regulated kinases-1 and -2, but not c-Jun N-terminal kinase and p38, pathways. Steroid hormone production (cortisol, 17-hydroxyprogesterone, and dehydroepiandrosterone sulfate) in NCI-h295 cells was decreased by 1-28-POMC in a concentration-dependent fashion. However, protein levels of important regulators of steroidogenesis [steroidogenic factor-1, DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome 1), steroidogenic acute regulatory protein, and cytochrome P450 side-chain cleavage enzyme] remained unaffected by 1-28-POMC treatment. Our results provide evidence that synthetic 1-28-POMC induces adrenal tumor cell proliferation, inhibits adrenal steroidogenesis, and mediates its action by signaling via the extracellular signal-regulated kinase pathway. The distinct roles of 1-28-POMC and ACTH in the regulation of adrenal growth and steroidogenesis suggest that the adrenal cortex is under the dual opposing control of fragments from the same mother peptide POMC. (*J Clin Endocrinol Metab* 88: 2171–2179, 2003)

THE PATHOGENESIS OF adrenocortical carcinoma is poorly understood. There is little known about the proliferative pathways that are involved in adrenal tumor growth. Only a few adrenal mitogens have been identified, of which basic fibroblast growth factor (bFGF) and IGF-I and -II are the most widely studied (1–10). Pituitary-derived factors play a crucial role in adrenal growth, as hypophysectomy leads to adrenal atrophy, whereas chronic adrenal overstimulation, *e.g.* in Cushing's disease, causes adrenal hyperplasia (11–14). Accordingly, long-standing hypersecretion of proopiomelanocortin (POMC) in patients with inadequately treated congenital adrenal hyperplasia can cause adrenal tumor formation (15, 16).

Based on experiments using purified ACTH to prevent adrenal atrophy in dexamethasone-treated hypophysectomized animals, ACTH has been proposed to be the major trophic and mitogenic stimulus for the adrenal cortex (12, 17, 18). This view is supported by the observation that inactivating mutations of the ACTH receptor are associated with adrenocortical hypoplasia (19). However, there is clear evidence that ACTH has antimitogenic activity *in vitro* (20, 21). Moreover, *in vivo* experiments with immunoneutralized ACTH demonstrated that ACTH has no role in compensatory adrenal growth after unilateral adrenalectomy (22). These findings led to the concept that other peptides of pituitary origin are probably involved in the control of adrenal proliferation. In the 1980s, Lowry and co-workers (22–24) demonstrated adrenal mitogenic activity of peptides derived from the N terminus of POMC (N-POMC). Although human 1-76-POMC (pro-γMSH), the major N-terminal POMC peptide secreted by pituitary corticotrophs, had no trophic effect on the adrenals (25), shorter N-POMC peptides, not containing the γMSH sequence, showed mitogenic activity. This was demonstrated *in vivo* by administration of 1-28-POMC to intact rats (23) and to hypophysectomized rats after enucleation of the adrenals (24, 26). In addition, antisera against 1-48-POMC were shown to inhibit compensatory adrenal growth after unilateral adrenalectomy (22), and incubation with 1-28-POMC led to an increase in [^{3}H]thymidine incorporation in cultured rat adrenocortical cells (23). It was suggested that 1-76-POMC undergoes postsecretional cleavage resulting in the generation of smaller N-POMC peptides with mitogenic activity (22, 23), and recently an adrenal serine protease specifically cleaving rat POMC has been identified (27).

Although these observations offer an attractive explanation for seemingly conflicting experimental data, the evi-

Abbreviations: AsP, Adrenal secretory protease; bFGF, basic fibroblast growth factor; DAX-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome 1; DHEA-S, dehydroepiandrosterone sulfate; ESIMS, electrospray ionization mass spectrometry; FCS, fetal calf serum; IgG, immunoglobulin G; JNK, c-Jun N-terminal kinase; MEK, MAPK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; N-POMC, N-terminal proopiomelanocortin; 17-OH-P, 17-hydroxyprogesterone; P40scc, cytochrome P450 side-chain cleavage enzyme; POMC, proopiomelanocortin; SF-1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein.

dence was based on the use of pituitary extracts possibly contaminated by ill-defined peptides and has not yet been confirmed using synthetic peptides. Moreover, as pituitary extracts are scarce, further investigations in this field have virtually ceased until recently (28, 29). A major problem with results from earlier studies investigating the mitogenic activity of N-POMC is the use of synthetic peptides with undefined disulfide bonds (30). The disulfide bridge arrangement of native N-POMC has been unambiguously determined (30, 31) and is crucial for its biological activity (32, 33).

We have synthesized 1-28-POMC with Cys^2-Cys^{24} and Cys^8-Cys^{20} disulfide bridges corresponding to the structure of endogenous POMC and used this peptide to study its effects on proliferation and steroidogenesis in adrenocortical tumor cells. Our findings support the concept that potent adrenal mitogenic activity resides in the extreme N terminus of POMC.

Materials and Methods

Materials

RPMI 1640, MEM, fetal calf serum (FCS), horse serum, BSA, antibiotics, and trypan blue were supplied by Invitrogen (Eggenstein, Germany). IGF-I, bFGF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), antibiotics, DMEM/Ham's F-12, collagenase II, deoxyribonuclease I, insulin, transferrin, selenium, glutamine, HEPES, and Percoll were obtained from Sigma-Aldrich Corp. (Deisenhofen, Germany). Synthetic $ACTH_{1–24}$ was purchased from Novartis Pharma (Nuernberg, Germany); UO 126 was obtained from Promega Corp. (Mannheim, Germany). Rabbit polyclonal antibodies against phosphorylated extracellular signal-regulated kinase-1 (ERK-1), ERK-2, total ERK, DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome 1), and steroidogenic factor 1 (SF-1) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse anti-β-actin was obtained from Sigma-Aldrich Corp., and antirabbit immunoglobulin G (IgG) and antimouse IgG was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). Antibodies against phosphorylated JNK, phosphorylated p38, and p38 were obtained from Cell Signaling Technology (Frankfurt, Germany).

Rabbit antibodies against cytochrome P450 side-chain cleavage enzyme (P450scc) (34) and steroidogenic acute regulatory protein (StAR) (35) were gifts from W. Miller (University of California, San Francisco, CA). Recombinant rat 1-74-POMC (36) was provided by C. Denef (University of Leuven, Leuven, Belgium).

Synthesis of human 1-28-POMC and 1-48-POMC

1-28-POMC and 1-48-POMC were synthesized by standard solid phase chemistry on a TentaGel trityl-Leu-F-moc resin (Rapp Polymere; substitution level, 0.23 mmol/g) at a scale of 0.1 mmol on a 433A peptide synthesizer (PE Applied Biosystems, Weiterstadt, Germany) (37). Activation of F-moc-protected amino acids was carried out with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. To selectively introduce the two disulfide bonds, cysteine residues in positions 2 and 24 were trityl-protected, while cysteines in positions 8 and 20 were acetamidomethyl-protected. Residues 16–18 (Asn, Leu, Leu) were double-coupled. For cleavage and deprotection, the dry peptidyl resin was treated with trifluoroacetic acid/ethanedithiol/water (94:3:3, vol/vol/vol) for 3 h. The crude product was precipitated by adding cold tert-butylmethylether. The material obtained was dried, dissolved in water, filtered, and prepurified by preparative reverse phase HPLC (Vydac C_{18}, The Separations Group, Hesperia, CA). The molecular mass determined by electrospray ionization mass spectrometry (ESIMS) was 3341.8 Da, exactly corresponding to the calculated value. Fractions containing the reduced product were lyophilized (yield, 360 mg) and subsequently subjected to oxidation at room temperature for 70 h. The peptide concentration was 0.5 mg/ml, and pH was adjusted to 7.8 with diluted ammonia. The resulting solution was directly loaded onto a preparative reverse phase C_{18} HPLC column (Vydac, 300 Å, 15–20 μm), and the monocyclic intermediate containing a Cys^2-Cys^{24} disulfide was isolated. The molecular mass determined by ESIMS was 3339.3 Da (calculated, 3339.8 Da). To cleave acetamidomethyl groups and to introduce the second disulfide bond, 25 mg monocyclic peptide were dissolved in a mixture of acetic acid and 0.1 M HCl (4:1, vol/vol), and treated with 20 equivalents of iodine for 2 h at room temperature. Excess iodine was reduced by the addition of fresh sodium ascorbate solution. The resulting mixture was diluted with water (1:1) and subjected to a final reverse phase HPLC purification on a Vydac C_{18} column. The product-containing fractions were combined and lyophilized (yield: 19.5 mg). The product was shown to be homogeneous by analytical C_{18} HPLC [Vydac, 250 × 4.6 mm, 300 Å, 5 μm; eluent A, 0.1% trifluoroacetic acid in water; eluent B, 0.1% trifluoroacetic acid in acetonitrile/water (4:1); linear gradient, 10–70% B in 30 min; flow rate, 0.8 ml/min; UV detection at 215 nm]. The molecular mass determined by ESIMS was 3195.4 Da (calculated, 3195.6 Da).

Cell culture

The human adrenocortical cancer cell line NCI-h295 (38) was obtained from American Type Culture Collection (Manassas, VA). Adherent cells were maintained in DMEM/Ham's F-12 medium supplemented with transferrin, insulin, selenium, and 5% FCS as described previously (39). The cells were grown at 37 C under an atmosphere of 5% CO_2-95% air. For experiments the FCS concentration was reduced to a maximum of 2%.

The mouse adrenocortical cancer cell line Y-1 (40) was obtained from Cell Line Service (Heidelberg, Germany). The adherent cells were maintained in Ham's F-10 medium supplemented with 2.5% FCS, 7.5% horse serum, and 200 nM L-glutamine. The cells were grown at 37 C under an atmosphere of 5% CO_2-95% air. For experiments, the serum concentration was reduced to a maximum of 2%.

The primary culture of bovine adrenocortical cells was performed as described in detail by Weber and Michl (41). Briefly, adrenal glands from 2- to 3-yr-old steers were obtained from the local slaughterhouse. The tissue was dissected and cells of the zona fasciculata and zona reticularis were obtained by digestion with collagenase II and deoxyribonuclease I. To purify dissociated cells and to remove contaminating red blood cells and cell debris, a Percoll centrifugation was performed. After washing, the final cell pellet was resuspended in DMEM/Ham's F-12 medium supplemented with 10% FCS, 5% horse serum, and antibiotics. Cells were plated in 75-cm^2 flasks in a concentration of 10^6 cells/ml. Medium was changed after 24 h to remove cell debris and unattached cells. During the following days, medium was replenished every 24–48 h. Seventy-two hours after seeding, cells were incubated with different concentrations of 1-28-POMC or 10 nM ACTH, respectively.

In all proliferation experiments IGF-I (10 nM) or bFGF (10 nM) served as the positive control.

Dye exclusion assay

Cells were seeded at an initial concentration of 10^6 cells/ml in six-well cell culture plates. Peptides were added at the specified concentrations. At various time points viability was determined by trypan blue staining (42). The total number of viable cells excluding the dye was calculated, and the percentage of viable cells recovered in the treatment groups was determined by comparison with untreated control cells.

MTT assay

In this assay the tetrazolium salt MTT is converted to a colored formazan product by enzymes active only in living cells (43). Cells were seeded into 96-well microtiter plates at a density of 50,000 cells/well. Twenty-four hours after inoculation, 1-28-POMC (final concentration, 1 nM to 10 μM) was added to the wells (n = 8 replicates). The cells were then incubated for an additional 72–96 h, followed by addition of MTT reagent (5 mg/ml stock solution) to a final concentration of 0.5 mg/ml. After incubation at 37 C for 3 h, the product was dissolved by addition of 100 μl cell lysis buffer (isopropanol with HCl), and absorbance of the lysate was measured at 570 nm using an ELISA plate reader (400 SF, SLT Lab Instruments, Crailsheim, Germany).

Western blotting

Analysis of signal transduction pathways. Both NCI-h295 and Y-1 cells, were starved (36 h without serum). Thereafter, cells were incubated for 5, 10, 15, 20, 30, and 60 min with 1-28-POMC, and proteins were extracted according to the Laemmli method (44) in a lysis buffer containing bromophenol blue (0.04%), glycerol (10%), sodium dodecyl sulfate (2%), 2-mercaptoethanol (5%), and Tris base (0.0625 M), pH 6.8. Equal amounts of lysates were electrophoresed on 10% polyacrylamide-sodium dodecyl sulfate gels at 150 V for 1 h. SDS-PAGE-resolved proteins were transferred onto a nitrocellulose membrane by a tank-blotting procedure. The membrane was treated with blocking buffer (5% nonfat dry milk dissolved in PBS containing 0.02% Tween 20) for 60 min, followed by overnight probing with the different specific antibodies against phosphorylated ERK-1 and -2 (pERK-1 and-2; 1:1000), total ERK-1 and -2 (1:1000), phosphorylated c-Jun N-terminal kinase (1:1000), phosphorylated p38 (1:1000), and total p38 (1:1000) at 4 C. All antibodies were diluted in blocking buffer. After three washes with 5% nonfat dry milk dissolved in PBS containing 0.02% Tween 20, the membrane was incubated for 1 h with horseradish peroxidase-labeled goat antirabbit (1: 3000) or antimouse (1:1500) IgG as secondary antibody. The nitrocellulose sheet was then washed three times for 10 min each time, and the antigen-antibody complex was visualized by enhanced chemiluminescence using a Western blotting detection kit (Amersham Pharmacia Biotech). NCI-h295 cells were also stimulated for 15 min with 1-28-POMC after preincubation (60 min) with the MEK kinase inhibitor UO 126 (0.5–25 μM), and pERK-1 and -2 and total ERK-1 and -2 were analyzed. Normalization of the levels of phosphorylated protein was performed by reprobing the blot with an antibody recognizing total ERK-1 and -2.

Steroidogenic enzymes and nuclear factors. NCI-h295 cells were incubated with 1000 nM 1-28-POMC for 1, 6, 12, 18, and 72 h, and total protein was extracted as described above. Protein levels of StAR, P450scc, DAX-1, and SF-1 were detected by Western blotting using specific antibodies in dilutions of 1:15000, 1:1000; 1:200, and 1:2400, respectively. For normalization, the blots were stripped and reprobed with an antibody against β-actin (1:5000), followed by incubation with horseradish peroxidase-labeled antimouse IgG.

The relative intensity of the signals was quantified by scanning densitometry using the Image Gauge V3.4 (Fujifilm, Düsseldorf, Germany) and normalized against β-actin.

Steroid determination

NCI-h295 cells and bovine adrenocortical cells were incubated for 48 h with 1-28-POMC (10–1000 nM). Each experiment was performed in triplicate. In bovine adrenocortical cells, incubation with 10 nM ACTH with and without 100 nM 1-28-POMC was performed. At the end of the incubation period, steroid hormones [cortisol, 17-hydroxyprogesterone (17-OH-P), and dehydroepiandrosterone sulfate (DHEA-S), respectively] were determined in the cell supernatants by commercially available RIAs (DPC Biermann, Bad Nauheim, Germany). The intra- and interassay coefficients of variation were less than 8% and less than 12%, respectively, for all assays.

Statistical analysis

Significance of differences was evaluated by ANOVA using the statistical software program StatView 4.51. $P < 0.05$ was considered statistically significant, with *post hoc* analysis carried out by Fisher's protected least significant difference test. Each experiment was performed with three replicates (MTT assays with eight). All results are expressed as the mean ± SD. Data given are from one representative experiment. Similar results were obtained in at least three experiments.

Results

N-POMC enhances cell proliferation in adrenocortical tumor cells

In the human adrenocortical cancer cell line NCI-h295, treatment with 1-28-POMC led to a significant concentration-dependent increase in cell proliferation to 131% of unstimulated cells as measured by MTT assay (Fig. 1A). This effect was paralleled by an increase in cell number to 148% of unstimulated cells, as determined by trypan blue staining (10 nM 1-28-POMC, $1.43 \pm 0.12 \times 10^6$; 1000 nM 1-28-POMC, $1.63 \pm 0.06 \times 10^6$; untreated control cells, $1.10 \pm 0.10 \times 10^6$ vital cells/well; $P < 0.001$; Fig. 1B). On a molar basis, the proliferative activity of 1-28-POMC was comparable or superior to that of the established adrenal mitogens IGF-I or bFGF (Fig. 1).

In mouse Y-1 adrenocortical cancer cells, 1-28-POMC caused a significant concentration- and time-dependent increase in cell proliferation. The absorbance in the MTT assay after incubation with 1-28-POMC of Y-1 cells for 4 d increased concentration-dependently (1 nM, 127 ± 14; 100 nM, 137 ± 18; 10 μM, 162 ± 10; untreated control cells, 100 ± 22; $P < 0.001$ for all *vs.* control cells; Fig. 2A), with a time-dependent significant increase in cell number after 3 and 6 d for 10 and 1000 nM 1-28-POMC (Fig. 2B). After 6 d, vital cell numbers in cells treated with 100 nM and 10 μM 1-28-POMC reached 123 ± 9% and 136 ± 8%, respectively, in comparison with untreated control cells.

The proliferation index in the MTT assay also increased significantly after 72 h of treatment with 1-28-POMC in non-neoplastic bovine adrenocortical cells in primary culture (10 nM 1-28-POMC, 168 ± 15%; 1000 nM 1-28-POMC, 179 ± 11%; untreated control cells, 100 ± 14%; $P < 0.001$ for all *vs.* control

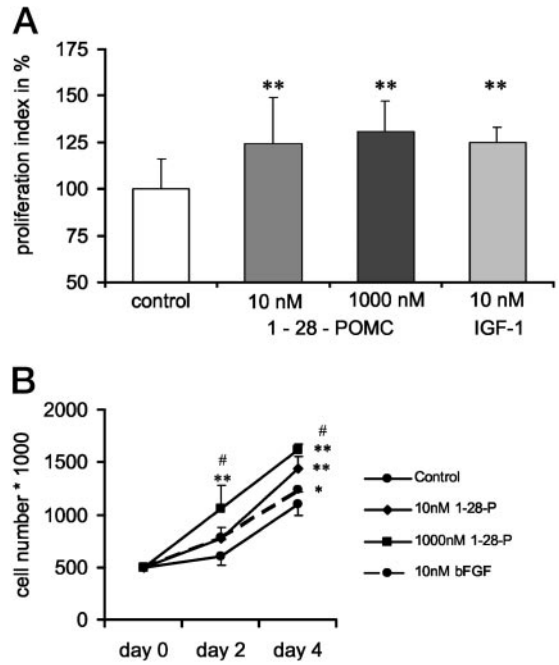

FIG. 1. Concentration- and time-dependent effect of 1-28-POMC on cell viability and proliferation in the human NCI-H295 cell line as measured by the MTT assay (A) and cell counting after trypan blue staining (B). Cells were treated with 10–1000 nM 1-28-POMC or 10 nM IGF-I and 10 nM bFGF as indicated (for details, see *Materials and Methods*). Results in A are the percentage of untreated control cells (100%) and are the mean ± SD of eight wells; in B, cell number per well (mean ± SD of three dishes) is given. *, $P < 0.05$; **, $P < 0.01$ (treatment groups *vs.* untreated controls at the same time point); #, $P < 0.05$ (10 nM 1-28-POMC *vs.* 1000 nM 1-28-POMC). Similar results were obtained in three experiments.

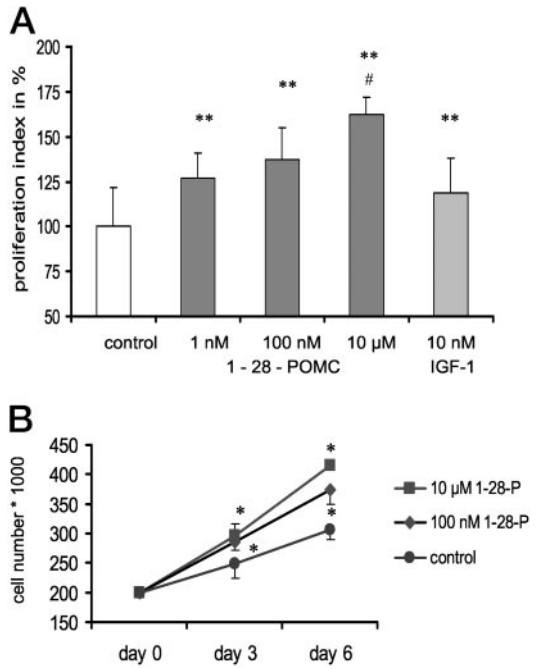

FIG. 2. Concentration- and time-dependent effect of 1-28-POMC on cell viability and proliferation in the mouse Y-1 cell line as measured by the MTT assay (A) and cell counting after trypan blue staining (B). Cells were treated with 1 nM to 10 μM 1-28-POMC as indicated or 10 nM IGF-I (for details, see *Materials and Methods*). Results in A are the percentage of untreated control cells (100%) and are the mean ± SD of eight wells. **, $P < 0.01$ (treatment groups *vs.* untreated controls); #, $P < 0.01$ (10 μM *vs.* 1 and 100 nM 1-28-POMC). In B, cell number per well (mean ± SD of three dishes) is given. *, $P < 0.05$ (treatment groups *vs.* untreated controls at the same time point). Similar results were obtained in three experiments.

cells). In contrast, incubation with ACTH (10 nM) led to a significant decrease in the proliferation index to 80 ± 4% of that observed in control cells ($P < 0.05$).

In addition, we investigated the activity of larger N-POMC peptides, 1-48-POMC and recombinant rat 1-74-POMC, on adrenal tumor cell proliferation. Incubation of NCI-h295 cells with all three N-POMC fragments stimulated the proliferation index in the MTT assay in a significant manner (Fig. 3). There were no significant differences between the N-POMC peptides. Similar results were seen in Y-1 cells, thereby demonstrating that the proliferative activity of N-POMC depends on the extreme N terminus, 1-28-POMC, which we, therefore, termed adrenoproliferin.

1-28-POMC stimulates MAPK in adrenocortical tumor cells

To further characterize the proliferative effect of 1-28-POMC, we analyzed its impact on intracellular signaling pathways, which elicit transcriptional and nontranscriptional events required for cell cycle entry. Previous work has demonstrated the critical role of the Ras-Raf-MAPK kinase (MEK)-ERK cascade in this process (45, 46). Activation of the MAPK ERK-1 and -2 depends on the phosphorylation of critical amino acids by upstream kinases, which can be visualized using phosphorylation-specific antibodies. Similar to treatment with the mitogen IGF-I, incubation with 1-28-POMC resulted in a time-dependent activation of ERK-1 and

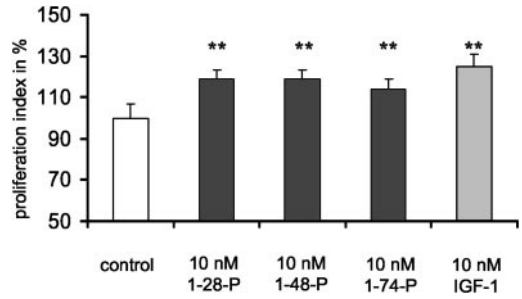

FIG. 3. Effects of synthetic human 1-28-POMC and 1-48-POMC and recombinant rat 1-74-POMC on cell viability and proliferation in the human NCI-h295 cell line as measured by the MTT assay. Cells were treated with 10 nM of the different N-POMC peptides or with 10 nM IGF-I (for details, see *Materials and Methods*). Results are the percentage of untreated control cells (100%) and are the mean ± SD of eight wells. **, $P < 0.01$ (treatment groups *vs.* untreated controls).

-2 in both adrenocortical tumor cell lines, NCI-h295 and Y-1 (Fig. 4). Maximum phosphorylation was detected between 10–20 min after stimulation with 1-28 POMC. In contrast, phosphorylation of JNK and p38, which normally do not or only poorly respond to mitogenic stimuli (47), was not found to be altered. Activation of ERK-1 and -2 requires a dual specificity upstream kinase (MEK), which, in turn, represents the only *in vivo* substrate known to date for the protein serine/threonine kinases of the Raf family (48). Recently, U0126 has been identified as a specific inhibitor of the signal transfer from Raf to MEK (49). In our experiments the involvement of this signaling cascade was demonstrated by the inhibition of 1-28-POMC-induced phosphorylation of ERK by UO 126 (Fig. 5) in the NCI-h295 cell line. Thus, adrenoproliferin specifically activates the MEK pathway with downstream signaling via ERK-1 and ERK-2, but not JNK and p38, pathways.

N-POMC diminishes steroidogenesis

Forty-eight hours of incubation with 1-28-POMC in NCI-h295 cells led to a concentration-dependent reduction of cortisol, 17-OH-P, and DHEA-S in NCI-h295 cell supernatants (Fig. 6). In contrast, IGF-I and bFGF did not decrease hormone secretion (data not shown). Similar results were obtained in primary culture of bovine adrenocortical cells (cortisol: 1000 nM 1-28-POMC, 47 ± 3%; control cells, 100 ± 22% ($P < 0.01$); 17-OH-P: 1000 nM 1-28-POMC, 58 ± 8%; control cells, 100 ± 16% ($P < 0.01$)]. In addition, 1-28-POMC significantly attenuated ($P < 0.05$) the ACTH-induced cortisol increase in bovine adrenocortical cells (100 nM 1-28-POMC plus 10 nM ACTH, 295 ± 9%; 10 nM ACTH, 337 ± 29%; untreated control cells, 100 ± 12%).

The observed decrease in steroid secretion suggested a general suppressive effect of 1-28-POMC on steroidogenesis. Therefore, we investigated the protein levels of the two first and rate-limiting steps in steroidogenesis, StAR and side-chain cleavage enzyme (P450scc), and of two important transcription factors that are involved in the regulation of steroidogenesis, SF-1 and DAX-1. Incubation of the NCI-h295 cells with 1-28-POMC for 1–72 h did not lead to a significant change in protein expression of StAR and P450scc (Fig. 7). The protein levels of the nuclear receptors SF-1 and DAX-1

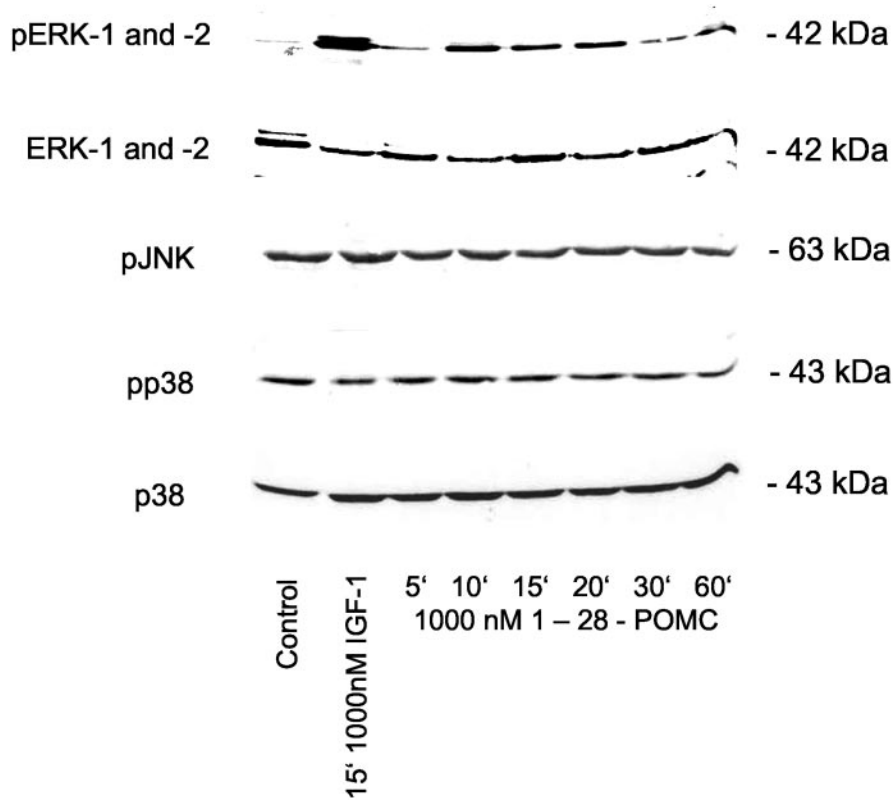

FIG. 4. Effect of 1-28-POMC on MAPK in the human NCI-h295 cell line. Cells were starved (36 h without serum) and thereafter stimulated with 1000 nM 1-28-POMC (5–60 min) or 1000 nM IGF-I (15 min). Western blot analysis was performed as described in *Materials and Methods* with specific antibodies against phosphorylated ERK-1 and -2, phosphorylated JNK, phosphorylated p38, total ERK-1/2, and total p38.

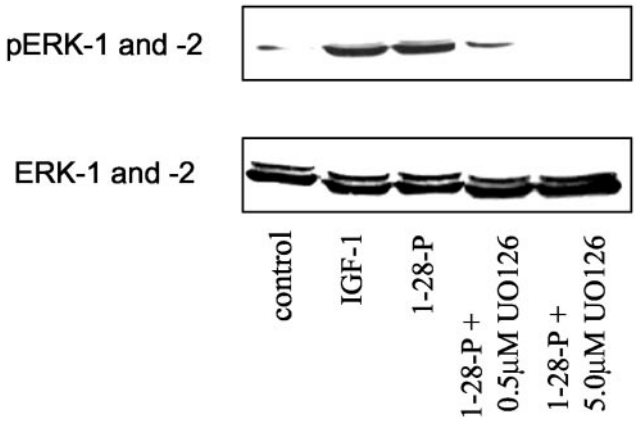

FIG. 5. Effect of the MEK inhibitor UO 126 on 1-28-POMC-induced phosphorylated ERK-1 and -2 in the human NCI-h295 cell line. Cells were stimulated with 1000 nM 1-28-POMC for 15 min with or without preincubation for 60 min with 0.5 or 5 μM UO 126. Western blotting was performed as described in *Materials and Methods* with specific antibodies against phosphorylated ERK-1 and -2 and total ERK.

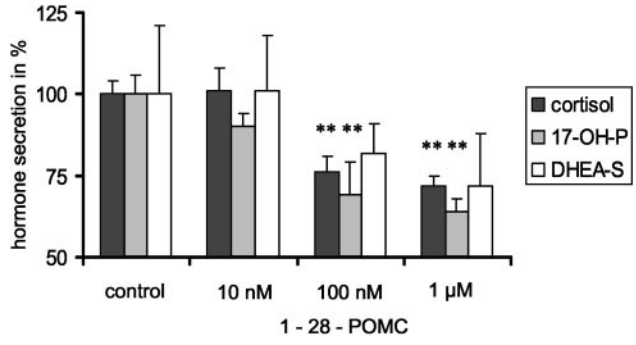

FIG. 6. Effects of 1-28-POMC on steroidogenesis in the human NCI-h-295 cell line. Cells were treated for 48 h with 1-28-POMC (10–1000 nM). Cortisol, 17-OH-P, and DHEA-S were measured in cell supernatant by RIA. Hormone secretion in untreated control cells was 17.5 ± 0.4, 8.7 ± 0.5, and 37.9 ± 4.8 ng/10^6 cells, respectively. Results are the percentage of untreated control cells (100%) and are the mean $\pm$ SD of three dishes. **, $P < 0.01$ (treatment groups *vs.* untreated controls). Similar results were obtained in three experiments.

also remained unaffected by 1-28-POMC after incubation of NCI-h295 cells for 1–72 h (Fig. 7).

Discussion

The successful synthesis of 1-28-POMC and 1-48-POMC with the physiological disulfide bridges Cys^2-Cys^{24} and Cys^8-Cys^{20} provided us with a tool to analyze the effects of N-POMC peptides in adrenocortical tumor cells. We found a concentration-dependent increase in cell number after treatment with synthetic 1-28-POMC in two adrenocortical tumor cell lines from different species (human and mouse) and also in primary cultures of normal bovine adrenocortical cells. Thus, our study supports the concept proposed by Lowry and co-workers in the 1980s (23) that the pituitary-derived trophic and mitogenic stimulus for the adrenal cortex resides, at least in part, in the N terminus of POMC. Using pituitary extracts, this group demonstrated stimulation of adrenal growth in rats *in vivo* by 1-28-POMC (22, 24, 26). They found an increase in [^{3}H]thymidine incorporation in rat adrenocortical cells and quenching of compensatory adrenal growth by antibodies raised against 1-48-POMC. More recently, Ross *et al.* (29) reported that intrafetal infusion of

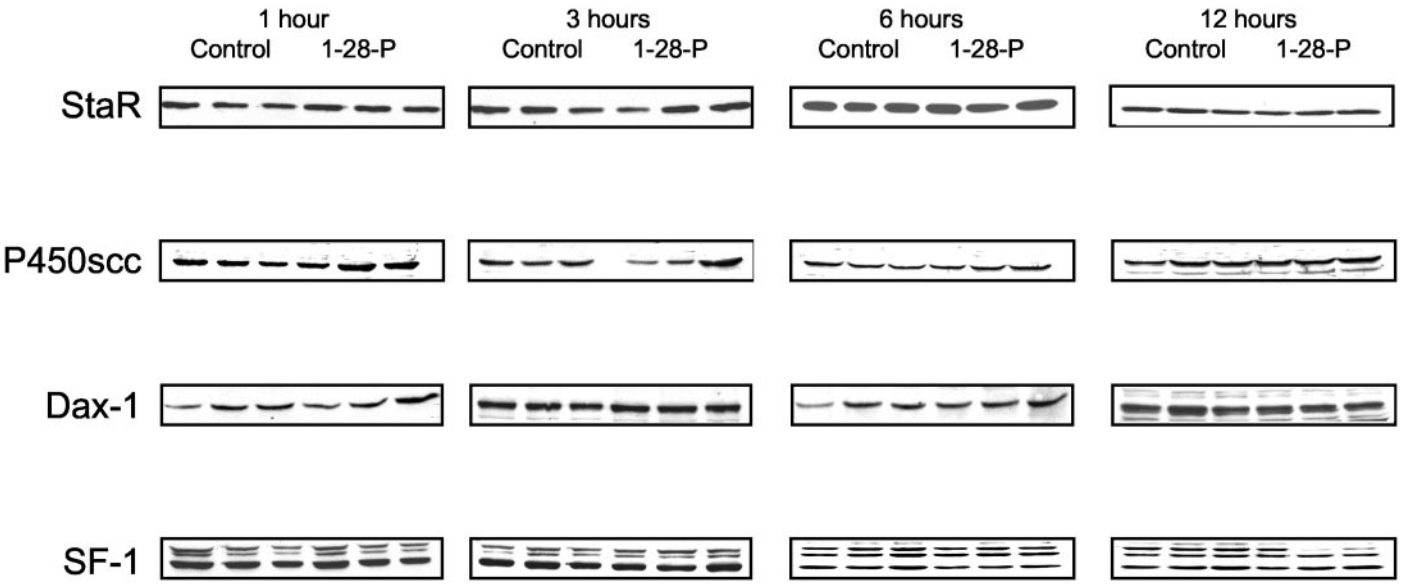

FIG. 7. Effects of 1-28-POMC on steroidogenic enzymes and nuclear factors in the human NCI-h295 cell line. Cells were incubated with 1000 nM 1-28-POMC for 1–12 h. Western blotting was performed as described in *Materials and Methods* with specific antibodies against StAR, P450scc, DAX-1, and SF-1. Specific bands were seen at 32, 53, 53, and 50 kDa, respectively. Experiments were performed in triplicate, and blots were stripped and reprobed with an antibody against β-actin (data not shown). No significant changes in protein level were detectable.

bovine 1-77-POMC in sheep resulted in an increased adrenal weight compared with saline-infused sheep, suggesting an important role of 1-77-POMC for fetal adrenal growth. This view is further supported by the study by Saphier *et al.* (50), who observed 1-77-POMC concentrations 10–50 times higher than those of ACTH in the circulation of fetal sheep. In addition, ACTH exerts inhibitory activity on the proliferation of normal adrenal cells *in vitro* (51–53), which is in keeping with our results.

The most widely studied adrenal growth factors are bFGF and IGF-I/II. At present, bFGF is regarded as the most potent adrenal mitogen and has been studied in a variety of *in vitro* systems, including human NCI-h295 cells (10), mouse Y-1 cells (20), cultured bovine adrenocortical cells (54, 55), and human fetal adrenocortical cells (7, 56–58). However, the responses of normal adrenal cells and adrenocortical tumor cells to growth stimulators have been invariably moderate. As rather differentiated cells, adrenal tumor cells grow slowly and respond only moderately to mitogenic stimuli. The maximum effect reported in NCI-h295 cells was an increase in cell number to 138% of controls after 7 d of treatment with bFGF (10). The activity of IGF-I is comparable and has been observed not only in bovine adrenocortical cells (3, 6) and fetal adrenocortical cells (4, 7), but also in NCI-295 cells (59). The concentrations of IGF-I and bFGF used in our studies and the resulting increases in cell number are in agreement with these reports. As the effect of N-POMC peptides observed in our experiments is on the same order of magnitude, it is likely that stimulation of adrenal growth by N-POMC is physiologically relevant. The N-POMC peptide concentrations used in this study are higher then expected in the circulation, although in some situations plasma concentrations of N-POMC may be 1–2 orders of magnitude higher than plasma ACTH concentrations. However, based on the experience with ACTH effects on adrenocortical cells *in vitro* it seemed prudent to use concentrations at least in the range commonly used for the study of ACTH action (60–63). Clearly, more work is required to define the physiological role of N-POMC in the multitude of factors involved in adrenal proliferation *in vivo*.

At present, it is uncertain which POMC-derived peptide is the physiological adrenoproliferin. In our hands, human 1-28-POMC, 1-48-POMC, and recombinant rat 1-74-POMC were equally potent in adrenocortical tumor cells, indicating that the mitogenic activity resides in the extreme N terminus. This part of POMC is highly conserved between species, indicating that the hairpin structure of the molecule serves an important physiological role. Intriguingly, the administration of either 1-28-POMC or 2-59-POMC stimulated adrenal growth *in vivo* and *vitro* in rats (22, 23), whereas human 1-76-POMC was inactive (25). These observations suggest that cleavage of human 1-76-POMC is required to generate mitogenically active peptides. This hypothesis is supported by the recent discovery and cloning of a serine protease [adrenal secretory protease (AsP)], which is up-regulated in the adrenal during compensatory adrenal growth and is capable of cleaving human 1-76-POMC into shorter fragments (27).

On the other hand, in fetal sheep only full-length bovine 1-77-POMC increased adrenal weight, whereas the smaller N-terminal peptide 1-49-POMC was inactive. Possibly glycosylation protects 1-77-POMC from rapid proteolytic degradation in the circulation, whereas smaller nonglycosylated peptides such as 1-49-POMC may be relatively labile *in vivo*. The importance of the glycosylation status of N-POMC has recently been shown by Bert *et al.* (36). In lactotroph cells, natural glycosylated human 1-76-POMC did not stimulate the MC-3 receptor, whereas recombinant nonglycosylated rat 1-74-POMC did. Thus, the stimulation of adrenal cell proliferation by recombinant rat 1-74-POMC in our study

may reflect the lack of glycosylation. Alternatively, tumor cells may express sufficient amounts of AsP to cleave full-length N-POMC to generate mitogenic activity *in vitro*, as it has been shown that Y-1 cells express AsP (27).

Our report sheds the first light on some components of signal transduction involved in the trophic activity mediated by 1-28-POMC. These are major mitogenic/antiapoptotic pathways, which are also activated by the administration of IGF-I. There is evidence that bFGF, endothelin, and adrenomedullin mediate at least some of their actions via the ERK cascade (64–66). There are conflicting data on the effect of ACTH on activation of ERK kinases. In Y1 cells, a short pulse of ACTH induced rapid activation of ERK (63) and accumulation of c-Fos protein (67), suggesting a growth-promoting effect of ACTH, whereas continuous treatment with ACTH (14 h) inhibited cell cycle progression via a cAMP-dependent pathway (67). However, it has also been shown that ACTH antagonizes the mitogenic action of bFGF in Y1 cells and is rather a poor activator of ERK kinases (68). Recently, Lotfi *et al.* (69) reported clear activation of ERK kinases by a commercial preparation of porcine pituitary corticotropin A, whereas synthetic 1-39-ACTH and 1-24-ACTH were largely inactive. Fractionation of the pituitary extract revealed multiple peaks including γMSH and βMSH, with variable activity concerning ERK activation. They concluded that this activation of ERK kinases resulted from unknown contaminating peptides (69), which, in the light of our results, may well have been N-terminal fragments of POMC. Watanabe *et al.* (70) demonstrated that synthetic ACTH decreases ERK activity in rats *in vivo* while inducing the JNK pathway. Activation of JNK has been linked to negative growth regulation and induction of apoptosis (71) and could, therefore, be related to the inhibitory activity of ACTH on adrenal growth. From these observations partially opposing activities of ACTH and 1-28-POMC in adrenocortical cells can be derived: 1-28 POMC activates ERK-1/ERK-2, leaving JNK activity unaltered, whereas ACTH increases JNK and has minor or even negative effects on ERK activation. Further elucidation of 1-28-POMC signal transduction is of major interest, as constitutive activation of these pathways may be involved in adrenal tumorigenesis.

There have been conflicting reports on the effects of N-POMC-derived peptides on steroid production. Pedersen and Brownie (72) observed that 16-kDa N-POMC, a glycosylated fragment prepared from the mouse pituitary tumor cell line AtT-20, stimulated steroidogenesis in isolated rat adrenocortical cells. This effect was enhanced by trypsinization, suggesting that this activity is restricted to a fragment of 16 kDa POMC (73). Further studies by these researchers suggested that this stimulative effect is related to Lys-γ_3MSH (rat 50-74-POMC) (73). In contrast, Jornot *et al.* (74) showed no effect of the 16-kDa fragment in isolated rat adrenal cells, and Cathiard *et al.* (75) demonstrated no change in the presence of γ_3MSH in bovine adrenal cells. Al-Dujaili *et al.* (76, 77) observed ACTH-potentiating effects of the naturally occurring human N-POMC glycopeptide on steroidogenesis in perfused rat and human adrenal cells. This effect was delayed and required a priming dose of N-POMC. A small enhancing effect of the small 16-kDa fragment of POMC on cortisol production was also observed more recently in guinea pig adrenal cells (78). There are several possible explanations for these conflicting reports. It is to be assumed that the steroidogenesis-enhancing activity resides in the C-terminus of N-POMC. Thus, depending on the experimental system used, variable activities of N-terminal 16-kDa POMC fragment preparations may lead to variable effects on steroidogenesis. Moreover, contamination by undetermined peptides and species differences may also have contributed to the conflicting findings. The use of synthetic 1-28-POMC fully excludes interfering steroidogenic activities of other N-POMC fragments and thereby unmasks the inhibitory potential of the extreme N terminus of POMC on steroidogenesis.

The concordant inhibitory effect of 1-28-POMC on all steroids measured suggests a general suppressive effect rather than inhibition of a specific steroidogenic enzyme. Protein levels of StAR, P450scc, DAX-1, and SF-1 remained unaffected by 1-28-POMC. However, we cannot exclude that posttranslational modifications such as changes in phosphorylation may contribute to the inhibitory action of 1-28-POMC on steroidogenesis. In contrast to our findings, Coulter *et al.* (28) found suppression of StAR mRNA by 1-77-POMC in fetal sheep *in vivo*. However, they did not investigate protein levels. Although their findings in fetal sheep differ from our observation *in vitro*, they also point to an inhibitory potential of N-POMC on steroidogenesis.

In differentiated thyroid carcinoma, suppression of TSH secretion is an established therapeutic principle to inhibit the growth of tumor cells and maintain clinical remission (79). It is uncertain whether a similar concept holds true also in adrenocortical cancer. Our data suggest that suppression of POMC secretion may potentially inhibit the growth of some adrenocortical carcinomas. Such inhibition of POMC secretion would be easily achievable by administration of exogenous glucocorticoids. However, any possible benefit must be balanced against the side-effects of glucocorticoid-induced POMC suppression.

In conclusion, we demonstrate for the first time that synthetic physiological 1-28-POMC and 1-48-POMC are adrenal mitogens that act via activation of MAPKs while simultaneously attenuating steroidogenesis. The distinct activities of 1-28-POMC and ACTH indicate that the adrenal cortex may be under the dual opposing control of fragments from the same mother peptide.

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Address all correspondence and requests for reprints to: Martin Fassnacht, M.D., Department of Medicine, Endocrine and Diabetes Unit, University of Wurzburg, 97080 Wurzburg, Germany. E-mail: Fassnacht-M@medizin.uni-wuerzburg.de.

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Present address for J.T.: Daniel-Swarovski Research Lab, Department of Surgery, University of Innsbruck, 6020 Innsbruck, Austria.

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