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Cerebellin and des-cerebellin exert ACTH-like effects on corticosterone secretion and the intracellular signaling pathway gene expression in cultured rat adrenocortical cells - DNA microarray and QPCR studies

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Abstract. Precerebellins (Cbln) belong to the C1q/TNF superfamily of secreted proteins which have diverse functions. They are abundantly expressed in the cerebellum, however, three of them are also expressed in the rat adrenal gland. All members of the Cbln family form homomeric and heteromeric complexes with each other *in vitro* and it was suggested that such complexes play a crucial role in normal development of the cerebellum. The aim of our study was to investigate whether Cbln1-derived peptides, cerebellin (CER) and des-Ser[1]-cerebellin (desCER) are involved in regulating biological functions of rat adrenocortical cells. In the primary culture of rat adrenocortical cells, 24 h exposure to CER or desCER notably stimulated corticosterone output and inhibited proliferative activity and similar effects were evoked by ACTH. To study gene transcript regulation by CER, desCER and ACTH, we applied Oligo GEArray® DNA Microarray: Rat Signal Transduction Pathway Finder™ . In relation to the control culture, 13 of the 113 transcripts present on the array were differentially expressed. These transcripts were either up- or down-regulated by ACTH and/or CER or desCER treatment. Validation of DNA Microarray data by QPCR revealed that only 5 of 13 genes studied were differentially expressed. Of those genes, Fos and Icam1 were up-regulated and Egr1 was down-regulated by ACTH, CER and desCER. The remaining two genes, Fasn (insulin signaling pathway) and Hspb1 (HSP27) (stress signaling pathway), were regulated only by CER and desCER, but not by ACTH. Thus, both CER and desCER have effects similar to and different from corticotrophin on the intracellular signaling pathway gene expression in cultured rat adrenocortical cells.

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Abbreviations: Cbln, precerebellins; Cbln1-4, precerebellin 1-4; CER, cerebellin; desCER, des-Ser1-cerebellin

Key words: cerebellin, ACTH, adrenal proliferation, corticosterone secretion, DNA microarray, signaling pathways, Fos, Icam1, Egr-1, Fasn, hot shock protein 27, QPCR

Introduction

Precerebellins (Cbln) belong to the C1q/TNF superfamily of secreted proteins which have diverse functions. Proteins of this superfamily, among others are involved in the classical pathway of complement activation, host defense, inflammation, apoptosis, autoimmunity, cell differentiation, organogenesis, hibernation, and insulin-resistant obesity (1). The subfamily of Cbln, on the other hand, is highly expressed in various regions of the brain and recent data suggest they belong to a new class of transneuronal regulators of synapse development and synaptic plasticity (2).

At present four precerebellins (Cbln1-4) are known. Cbln1 is homologous to originally described human precerebellin (3) while Cbln2 is homologous to rat and murine cerebellin-like proteins identified by Wada and Ohtani (4) and Kavety *et al* (5). Cbln3 binds specifically to Cbln1 (6) and recently Cbln4 was identified (7) (NCBI accession number NM_175631). All members of the Cbln family form homomeric and also heteromeric complexes with each other *in vitro* and it was suggested that such complexes play a crucial role in normal development of the cerebellum (2,8). However, not only entire Cbln molecules are able to act as a transneuronal cytokines. In 1984 Slemmon *et al* (9) isolated two Cbln1 derived polypeptides from the rat cerebellum. One identified hexadecapeptide was named cerebellin (CER) while its N-terminal truncated version, des-Ser[1]-cerebellin, was called des-cerebellin (desCER). It is generally assumed that desCER is a metabolite of CER, its formation is catalyzed by serine aminopeptidase (9-11).

Cbln are also expressed in extra-cerebellar tissues, including the neuroendocrine system (12) and there is growing evidence that both CER and desCER exert a modulatory action on the human and rat adrenal gland (13-20). We present DNA microarray and QPCR based data suggesting that CER and desCER

Table I. Functional gene groupings on Oligo GEArray® DNA Microarray: Rat Signal Transduction Pathway Finder™ .

Pathway	Studied genes
Mitogenic	Egr1 (egr-1), Fos, Jun (c-jun), Nab2
Wnt	Cdh1, Ccnd1 (cyclin D1), Fgf4, Jun, Lef1, Myc (c-myc), Pparg, Tcf7, Vegf, Vegfc, Wisp1, Wisp2
Hedgehog	Bmp2, Bmp4, Mo-En-1 (engrailed), Foxa2, Hhip, Ptch, Ptch1, Wnt1, Wnt2, Wsb1
TGFß	Cdkn1a (p21Waf1, p21Cip1), Cdkn1b (p27), Cdkn1c (p57Kip2), Cdkn2a (p16Ink4), Cdkn2b (p15Ink2b), Cdkn2c (p18, cdk4 inhibitor), Cdkn2d (p19)
Survival: PI3 Kinase / AKT	Bcl2, Ccnd1, Fn1 (fibronectin), Jun, Mmp7 (matrilysin), Myc, Pten
Survival: Jak / Src	Bcl2, Bcl2l1
Survival: NFκB	Bcl2a1, Birc1b, Birc3, Birc7, Tert
p53	Bax, Cdkn1a, Ei24 (Pig8), Gadd45a, Igfbp3, Mdm2, Tnfrsf10b (TrailR/DR5), Tnfrsf6
Stress	Atf2, Fos, Hsf1 (tcf5), Hspb1 (hsp25), Hspca, Hspcal3, Myc, Tp53 (p53)
NFκB	Ccl20, Cxcl1, Icam1, Ikbkb, Il1a, Il2, Lta (TNFß), Nfkb1 (NFκB), Nfkbia (IκBα), Nos2 (iNOS), Pecam, Tank, Tnf (TNFα), Vcam1
NFAT	Cd5, Il2, Tnfsf6 (FasL)
CREB	Cyp19a1 (aromatase p450), Egr1, Fos
Jak-Stat	Csn2 (ß-casein), Cxcl9 (Mig), Il4, Il4r, Irf1, Mmp10 (stromelysin-2), Nos2 (iNOS), Pzp
Estrogen	Bcl2 (Bcl-2), Brca1, Ctsd (cathepsin D), Egfr, Igfbp4, Pgr (PR), Trim25
Androgen	Cdk2, Cdkn1a (p21Waf1/p21Cip1), Egfr, Klk3 (Klkb1), Ngfg, Tmepai (N4wbp4), TMPRSS9
Calcium and Protein Kinase C	Csf2 (GM-CSF), Fos, Il2, Il2ra, Jun, Myc, Odc1, Prkca, Prkcb1, Prkce, Tfrc
Phospholipase C	Bcl2, Egr1, Fos, Icam1, Jun, Junb, Nos2,Ptgs2 (cox-2), Vcam1
Insulin	Cebpb, Fasn, Gys1, Gys2, Hk2, Lep (Ob)
LDL	Ccl2 (Scya2/mcp-1), Csf2, Sele (ELAM-1), Selp (P-selectin), Vcam1
Retinoic acid	Ctsd, Mo-En-1, Hoxa1, Hoxb1, Rbp1 (CRBPI), Rbp2 (CRABPII), Stra6

exert ACTH-like effects on corticosterone secretion and expression of intracellular signaling pathway genes of cultured rat adrenocortical cells.

Materials and methods

Chemicals. Cerebellin and (des-Ser[1])-cerebellin (desCER) were purchased from Bachem Feinchemikalien AG (Bubbendorf, Switzerland), ACTH (Synacthen) from Ciba (Basle, Switzerland). Unless stated otherwise, all other chemicals and reagents were provided by Sigma-Aldrich (St. Louis, MO, USA) or POCh (Gliwice, Poland).

Animals. Studies were performed on intact 20-22-day-old male rats of the Wistar strain. Animals were maintained under standardized conditions of light (14:10 h light-dark cycle, illumination onset 06.00 a.m.) at 23˚C with free access to standard pellets and tap water. The study protocol was approved by the local Ethics Committee for Animal Studies.

Adrenocortical cell culture. Details of primary adrenocortical cell culture were published previously (20-28). Briefly, for the preparation of one primary culture of adrenocortical cells, 12 rats were sacrificed by decapitation. The adrenal glands were immediately removed, placed in Dulbecco's MEM/Nutrient mix from Gibco (15.57 g/l) and the fat and connective tissue were cleaned away. Each adrenal gland was cut into small pieces. Tissue fragments were dissociated to cell suspensions using enzymatic digestion in Dulbecco's MEM/Nutrient mix (15.57g/l) supplemented with 1 g/l collagenase, 0.1 g/l trypsin inhibitor, 0.3 g/l BSA and 4.75 g/l HEPES (all from Sigma) for 30 min at 37˚C in shaking water bath. The cells were harvested by centrifugation and suspended in Dulbecco's medium with 1.125 g/l sodium bicarbonate (POCh), 10% fetal bovine serum (Gibco) and a designated concentration of antibiotics (penicillin-streptomycin-fungizone mixture; Sigma). Cells were counted with the Cell Counter and Analyser Systems (CASY), Model TT (Schaerfe System GmbH, Reutlingen, Germany). Prepared suspensions were placed either in 96-well cluster dishes (NUNC Brand Products), 10,000 cells per well, and cultured for 96 h at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every 24 h. At day 5 of culture, CER or desCER were added at the following concentrations, $1x10^{-10}$ M, $1x10^{-8}$ M and $1x10^{-6}$ M. As a positive control, ACTH at concentration $1x10^{-7}$ M was used. The collected culture medium was stored at -36˚C until the corticosterone assay.

Adrenocortical cell proliferation assay. Proliferation of cultured rat adrenocortical cells was estimated with EZ4U Nonradioactive Cell Proliferation and Cytotoxicity Assay (Biomedica, Vienna, Austria). This assay depends on the reduction of non-toxic tetrazolium salts to intensely colored formazan derivatives. The reduction process requires functional mitochondria, which are inactivated within a few minutes after cell death (29). EZ4U assay system is highly compatible with the standard ^{3}H-thymidine incorporation assay and formed formazan derivatives are quantified by a microplate

Table II. Validation of DNA Microarray data by QPCR.

cDNA	GenBank Accession number	Primer	Primer sequence (5'-3')	Position	PCR product size (bp)
Egr 1	NM_012551	S	GAGCCGAGCGAACAACCCTA	187-296	82
		A	CCACCAGCGCCTTCTCGTTA	249-268	
Icam1	NM_012967	S	GCCACCATCACTGTGTATTCGT	343-364	92
		A	CAGCGCAGGATGAGGTTCTT	415-434	
Cxcl1	NM_030845	S	AACCACAACTTGCGGACCTCT	269-287	184
		A	AGGCTTGCCTTGACCCTGA	434-453	
Rbp1	NM_012733	S	GGCATAGATGACCGCAAGTG	323-342	187
		A	GGGCCGCTCAGTGTACTTT	491-509	
Pgr	NM_022847	S	GCCGGAAGAAATGATTGCAT	1780-1799	143
		A	AGGGCTCTCATAACTCGGACTT	1901-1922	
Rbp2	NM_012640	S	CGTTCCGCAACTATGACCTA	225-244	181
		A	TACAGCTTGTCTCCCTCGAC	386-405	
Fasn	NM_017332	S	CCGTGATGGGGTTGTGAA	5517-5534	183
		A	GTCTTGGAGATGGCGGAAAT	5680-5699	
Jun	NM_021835	S	GCCACCGAGACCGTAAAGA	318-336	61
		A	CCTGTGCGAGCTGGTATGAGTA	357-378	
Fos	NM_022197	S	TTTCAACGCGGACTACGAG	167-185	164
		A	AGTTGGCACTAGAGACGGACA	310-330	
Cdkn2a	NM_031550	S	TCTCCGAGAGGAAGGCGAACT	3-23	204
		A	GAGCTGCCACTTTGACGTTG	87-206	
Il4ra	NM_133380	S	CATCTCCTGCATCTGCATCCTA	967-988	174
		A	GACTCCTGGCTTCGGGTCT	1122-1140	
Hspcal3	XM_216334	S	CTCAGTTTATTGGCTACCC	632-650	133
		A	TCTATTTCAGGCTTGTCATC	745-764	
Hspb1	NM_031970	S	TCACTGGCAAGCACGAAGA	432-450	182
		A	GGTGATCTCCGCTGATTGTG	594-613	
HPRT	NM_012583	S	CAGTCAACGGGGGACATAAAAG	391-412	146
		A	ATTTTGGGGCTGTACTGCTTGA	515-536	

In relation to the control, listed genes were differentially expressed in adrenocortical cells exposed to ACTH, cerebellin, and des-cerebellin. Reference gene, HPRT, hypoxanthine-guanine phosphoribosyltransferase. Oligonucleotide sequences for sense (S) and antisense (A) primers are shown.

reader. In this assay adrenocortical cells cultured in the presence of either neuropeptide (CER or desCER) or ACTH were additionally incubated with EZ4U while an extinction was recorded.

Corticosterone estimations. Corticosterone was extracted from incubation medium and its concentration was measured by RIA, using [1,2,6,7-^{3}H]-corticosterone (Amersham, UK; S.A., 1.96 Tbq/mmol) and antisera developed in rabbit (Sigma, St. Louis, MO, USA). Corticosterone RIA sensitivity was 50 pg/ml, cross-reactivity, corticosterone and cortisol, 100%; 11-deoxycorticosterone and progesterone, 2%; other steroids, <0.001%. Intra- and inter-assay variations, 7 and 9%, respectively (21-23).

Total RNA isolation, purification and cRNA labeling. From cultured cells [control ones and those exposed to ACTH (10^{-7} M), CER (10^{-10} M) and desCER (10^{-10} M)] total RNA was isolated using Tri-Reagent (Sigma) method and purified on columns (RNeasy Mini Kit, Qiagen) (20,30-32). The contaminating DNA was digested by DNase-I (RNase-Free DNase Set, Promega, Madison, WI). Total RNA was determined by measuring optical density at 260 nm and purity was estimated by 260/280 nm absorption ratio, which was consistently >1.8. RNA integrity was assessed by electrophoresis in 1.5% agarose gel with ethidium bromide. For labeling of cRNA, TrueLabeling-AMP™ 2.0 kit (SuperArray Bioscience Corp., Frederick, MD) was applied. This system is designed to rapidly amplify and label antisense RNA for hybridization to the OligoGEArray® from SuperArray Bioscience. Starting from total RNA, this kit utilizes a proprietary and patent-pending linear RNA amplification and labeling procedure to synthesize labeled antisense RNA (aRNA), also known as labeled cRNA target. As a first step 1 μg of RNA was reverse transcribed using the classical method (42˚C, 50 min). cDNA was used as a template in the labeling reaction with Biotin-16-UTP (Roche Applied Science), RNA polymerase enzyme (SP6) and RNA polymerase buffer. The reaction mixture was incubated for 5 h at 37˚C. The obtained cRNA was purified using ArrayGrade cRNA Cleanup Kit. The amount of cRNA was determined by measuring optical density at 260 nm.

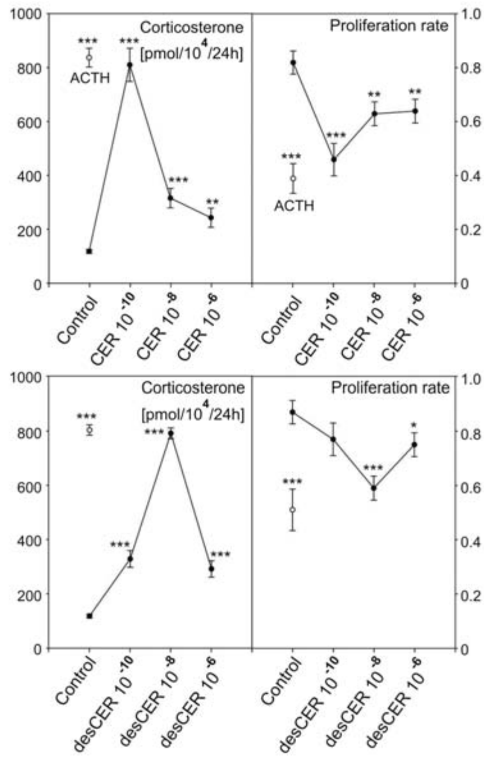

Figure 1. Effects of cerebellin (CER) and des-cerebellin (desCER) on corticosterone secretion (pmol/10^4 cells/24 h) in primary culture of rat adrenocortical cells and their proliferative activity (absorbance). Cells were exposed for 24 h to CER or desCER ($1x10^{-6}$ - $1x10^{-10}$ M). As a positive control, ACTH ($1x10^{-7}$ M) was applied. Results are expressed as means ±SE. In each group n=6. Statistical comparison of differences by the Student's t-test, as compared to those obtained in the control group, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

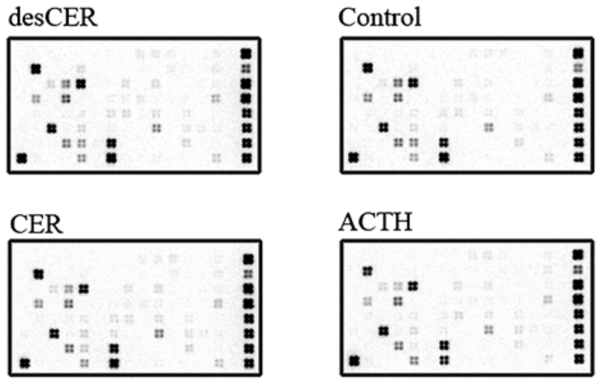

Figure 2. Raw images of the gene arrays after hybridization with biotin-labeled cDNA probes, detected by chemiluminescence. Groups, control culture of rat adrenocortical cells, and cells exposed for 24 h to CER ($1x10^{-10}$ M), desCER ($1x10^{-10}$ M), and ACTH ($1x10^{-7}$ M).

Oligo Array Hybridization. Oligo GEArray® DNA Microarray, Rat Signal Transduction Pathway Finder™ was applied. This system profiles the expression of 113 genes, representative of the 18 signal transduction pathways (Table I). For each Oligo Array about 4 μg of cRNA was used. Four of the arrays were placed into Multi-Chamber HybPlate in order to perform the pre-hybridization step. Each array was made wet with 2 ml H_2O per 3 min. Prehybridization step was performed by adding 2 ml pre-warmed to 60˚C GEAhyb Hybridization Solution (without cRNA) and by incubation for 1 to 2 h at 60˚C. Hybridization solution was prepared by adding 4 μg cRNA target to a 2.0 ml aliquot of warm GEAhyb Hybridization Solution. The mixture was incubated overnight at 60˚C. After washing steps, signal of hybridization was developed by Chemiluminescent Detection Kit. Signal was captured by using an image station from UVP factory. The pictures were saved in grayscale 16-bit tiff files and were analyzed by GEArray Expression Analysis Suite in relation to the following reference genes, ribosomal protein L32, lactate dehydrogenase and aldolase A and glyceraldehyde-3-phosphate dehydrogenase. Genes were considered to be expressed differentially if their expression level was altered by ≥+2 or ≤-2 in relation to the control group.

Validation of results by QPCR. In order to validate the gene expression data obtained from the microarray, we analyzed the mRNA by QPCR of differentially expressed genes. QPCR methods applied were described previously (20,31-36). The obtained cDNA was used as a template in QPCR. Real-time PCR was carried out in a Roche LightCycler 2.0 with software version 4.05, using the following program, denaturation step (95˚C for 10 min), and 45 cycles of three step amplification (denaturation, 95˚C for 10 sec; annealing, 58˚C for 5 sec; and extension, 72˚C for 10 sec). Subsequently, melting curve (60-90˚C with a heating rate of 0.1˚C/sec) was performed to check the specificity of amplification products and the presence of by-products. All samples were amplified in duplicate, and the HPRT gene was used as a reference to normalize the data. The primer sequences are shown in Table II.

Statistical treatment of results. Data on corticosterone output and proliferation are expressed as means ±SE, and statistical comparison was done using the unpaired Student's t-test.

Results

Corticosterone secretion and adrenocortical cell proliferation. In the primary culture of rat adrenocortical cells, 24 h exposition to CER or desCER notably stimulated corticosterone output, and the lower peptide concentrations ($1x10^{-10}$ M and $1x10^{-8}$ M) were more effective than the higher one ($1x10^{-6}$ M) (Fig. 1). Stimulating effects of both CER and desCER are comparable to those evoked by ACTH ($1x10^{-7}$ M). Both CER and desCER notably inhibited proliferative activity of studied cells and a similar effect was evoked by ACTH.

Gene transcript regulation by CER, desCER and ACTH. Raw images of the gene arrays after hybridization with biotin-labeled cDNA probes, detected by chemiluminescence, are shown in Fig. 2. Using the normalization process described in the Oligo Array Hybridization section, 13 of the 113 transcripts present on the array were differentially expressed in relation to the control culture. These transcripts were either up- or down-regulated by ACTH, and/or CER or desCER treatment. Table II lists transcripts affected by the applied experimental conditions and they were further examined by QPCR. As revealed by QPCR, only 5 of 13 genes studied were differ-

Table III. Validation of DNA Microarray data by QPCR.

Position on array	GeneBank	Symbol	Description	ACTH[a]	CER[a]	desCER[a]
34	NM_012551	Egr1	Early growth response 1	-9.54	-2.66	-4.54
36	NM_017332	Fasn	Fatty acid synthase	-	-3.12	-6.00
39	NM_022197	Fos	FBJ murine osteosarcoma viral oncogene homolog	6.23	7.4	15.4
49	NM_031970	Hspb1	Heat shock 27 kDa protein 1	-	3.33	4.35
52	NM_012967	Icam1	Intercellular adhesion molecule 1	12	10	5

Gene array analysis was done using Oligo GEArray® DNA Microarray, Rat Signal Transduction Pathway Finder™ . [a]Fold-change values in relation to cultured rat adrenocortical cells of the control group, as revealed by QPCR. Cells exposed to ACTH, cerebellin (CER) and des-cerebellin (desCER).

entially expressed in relation to the control group of cultured rat adrenocortical cells (Table III). Of those genes, Fos and Icam1 were up-regulated and Egr1 was down-regulated by ACTH, CER and desCER. The remaining two genes, on the other hand, Fasn and Hspb1 (HSP27), were regulated by CER and desCER, but not by ACTH.

Discussion

Expression of Cbln-related genes and Cbln1 peptide in the human and rat adrenal gland is well documented, however, physiologic relevance of Cbln1 peptide and Cbln1-derived polypeptides (CER and desCER) in control of adrenocortical activity still remains an open question (for review see 12,37). In this regard, the performed studies are the first to demonstrate that both CER and desCER exert ACTH-like effects on intracellular signaling pathways of cultured rat adrenocortical cells.

Previous reports of CER effects on the adrenal gland revealed a stimulating effect of CER on aldosterone, cortisol and catecholamine secretion by human adrenal slices (13). Numerous experiments presented in these studies suggested that the CER-stimulating effect on corticosteroidogenesis is mediated paracrinally by medullary catecholamines. Similar results were found in an *in situ* perfused rat adrenal gland (14). Furthermore, acute CER administration resulted in elevation of blood aldosterone and corticosterone levels, an effect independent from changes in ACTH levels (16,17). On the other hand, neither CER nor desCER changed serum corticosteroid levels in rats with enucleation-induced adrenocortical regeneration (15). A study revealed that 24 h exposure of cultured rat adrenocortical cells to CER and desCER resulted in notable stimulation of corticosterone output, an effect comparable to that evoked by ACTH. Of interest is, that lower CER and desCER concentrations were more effective than the higher one ($1x10^{-6}$ M), the finding reported previously by our group (20).

Previous studies revealed that prolonged administration of CER and desCER resulted in a decrease of the total number of rat adrenocortical cells (16). Furthermore, both compounds modulated proliferative activity of regenerating rat adrenocortical cells (15). We have also confirmed that apart from the effect of CER and desCER on adrenocortical steroidogenesis, both polypeptides directly inhibit proliferative activity of the studied cells (20). Thus, as far as proliferative activity is concerned, both CER and desCER have ACTH-like effects, which are known to directly inhibit mitotic activity of adrenocortical cells (38,39).

Since both CER and desCER exerted ACTH-like effects on cultured rat adrenocortical cells, we subsequently assessed how these polypeptides alter expression of the signal transduction pathway genes in studied cells. For this purpose DNA Microarray system, profiling the expression of 113 genes representative of the 18 signal transduction pathways, was applied. Thirteen of 113 transcripts present on the array were differentially expressed in relation to the control culture, however, validation of microarray data by QPCR revealed that only 5 of them were differentially expressed. Of those genes Fos and Icam1 were up-regulated and Egr1 down-regulated by ACTH, CER and desCER. The remaining two genes, Fasn and Hspb1, were regulated by CER and desCER, but not by ACTH. Thus, both CER and desCER have effects similar to and different from corticotrophin on the expression of certain genes in rat adrenocortical cells.

In past reports on global profiles of gene expression induced by ACTH in the Y1 mouse adrenal cells, 1,386 of 5,655 transcripts were affected by corticotrophin, and ~45% of them were up-regulated (40,41). The up-regulated transcripts are involved mainly in steroid biosynthesis and metabolism while the down-regulated transcripts are connected with cell proliferation, nuclear transport and RNA processing. The protein kinase A-dependent signaling pathway accounted for 56% of ACTH effect, while protein kinase C-dependent signaling accounted only for 6%.

Our study revealed that Fos and Icam1 genes were up-regulated and Egr1 down-regulated by ACTH, CER and desCER. The Fos gene belongs to mitogenic, CREB, calcium and protein kinase C, phospholipase C, and stress intracellular signaling pathways. The ubiquitously-expressed fos gene is a proto-oncogene that encodes leucine zipper proteins that dimerize with proteins of the Jun family, thereby forming the transcription factor complex AP-1 (42). As such, the Fos proteins were implicated as regulators of cell proliferation, differentiation, and transformation. In the adrenal gland ACTH, but not cAMP, stimulates expression of Fos and Jun mRNA and proteins (43-46). ACTH- or angiotensin II (AII)-induced activation of both Fos and Jun genes also stimulates activation of genes responsible for adrenocortical steroidogenesis, among other Star gene (47,48). Previous studies demonstrated that induction of c-Fos and c-Jun genes regulates proliferative activity of adrenocortical cells. Products encoded by these genes regulate transition of mouse Y1 and H295R cells from G_0/G_1 to S phase of the cell cycle (43,49). Similar mechanisms control proliferative activity of rat adrenocortical cells *in vivo* (50). Our study revealed that ACTH, CER and desCER have a potent stimulatory effect on expression of the Fos gene in cultured rat adrenocortical cells, however, expression of Jun gene was unaffected. Lack of changes in Jun gene expression, as found in the present study, may depend on time of exposure of cultured cells to ACTH, CER and desCER. Activation of that gene by ACTH or AII is rapid (42) while our cells were exposed to tested compounds for 24 h.

Only scanty data are available on the expression and role of ICAM (intercellular adhesion molecule) gene and protein in the adrenal cortex. This gene belongs to NFκB and phospholipase C signaling pathways and two forms of Icam proteins are known: Icam-1 which is membrane bound and its soluble form (sIcam-1). Experiments on mice demonstrated a significantly longer time of survival of adrenal transplants obtained from ICAM-1-deficient BALB/c (H-2d) mice than transplants obtained from wild mice (51). Expression of ICAM1 gene was also described in human adrenal gland (52). Levels of circulating sICAM, on the other hand, were higher in patients with adrenal carcinomas than in adrenal adenomas and control (53). In our study, in the primary culture of rat adrenocortical cells, ACTH and CER or desCER notably stimulated expression of the Icam-1 gene. Thus, the obtained data indicate that this gene is expressed in parenchymal cells of the rat adrenal cortex, and regulated by corticotrophin. However, its specific role in the adrenal cortex remains to be elucidated.

Egr-1 is an immediate-early response gene induced transiently and ubiquitously by numerous mitogenic stimuli and also involved in initiation of differentiation (54,55). Protein encoded by that gene belongs to a zinc-finger family of transcription factors. In the applied array, the Egr-1 gene is described as a gene involved in mitogenic, CREB and phospholipase C signaling pathways. Some studies, however, suggest that the Egr-1 gene also contains anti-apoptotic effects (56-59). Of interest is the interrelationship between glucocorticoid receptor (GR), MAPK signaling pathway and Egr-1. Activation of GR increases the expression and enzymatic activity of proteins in the MAPK signaling pathway and leads to an increase in the levels of both Egr-1 mRNA and protein (60). In the adrenal gland, Egr-1 is involved in the neural regulation of phenylethanolamine N-methyl-transferase (PNMT) and tyrosine hydroxylase genes expression of chromaffin cells (61-62). Expression of Egr-1 gene was also present in the adrenal cortex. Its expression in adrenal carcinomas is 8-fold lower, and in adrenal adenomas 3-fold lower than in a normal adrenal gland (63). The current experiments revealed that in the primary culture rat adrenocortical cells, expression of Egr-1 gene is down-regulated by prolonged exposure to ACTH, CER or desCER. At the same time all these compounds inhibit proliferative activity and stimulate corticosterone output by cultured cells. Thus, the obtained data suggest that down-regulation of the Egr-1 gene inhibits mitotic activity and stimulates differentiated (specialized) functions of adrenocortical cells.

The two other studied genes are differentially expressed in relation to the control culture; they were controled by CER and desCER, but not by ACTH. Those genes are Fasn (fatty acid synthase), involved in insulin signaling pathway, and Hspb1, gene of stress signaling pathway. Fasn gene plays a pivotal role in lipid metabolism and is highly expressed in organs such as the brain, liver and adrenals (64-66). This gene is also known to regulate proliferative activity of numerous embryonic tissues and its expression is controled by various hormones, among others by insulin, glucagon, glucocorticoids, and thyroid hormones. In endometrium, Fasn expression highly correlates with the degree of expression of progesterone and estrogen receptors and with proliferative activity of endometrial cells. As revealed by our experiments, Fasn gene expression is down-regulated by both CER and desCER.

Heat shock proteins (HSP) are conserved proteins with variable molecular mass and they are divided into few classes, among others HSP90, 70, 60, while those with molecular mass 15-42 kDa are known as small HSPs (67). Their transcriptionally regulated expression is increased in cells exposed to stress. One role of HSPs, among others, is to protect cytosolic proteins, they also regulate the MAPK/p38 signaling pathway (68). HSP27 is the only small HSP which is stress induced (69,70). Furthermore, HSP27 has an important suppressive effect on apoptosis (71). HSP70 was present in the rat adrenal cortex and its expression was regulated by ACTH (72). Also, heat stress stimulated HSP27 expression in the rat adrenal gland (69). As demonstrated by immunocytochemistry, HSP27 is highly expressed in normal human adrenal glands and reduced in adrenals obtained from Cushing's syndrome patients (73). In those studies, expression of HSP70 was lower than HSP27, and its level was also lowered in the adenomas. However, expression of HSP60 was significantly increased in adrenal Cushing's tumors. The cited authors suggest that changes observed in HSP protein expression is connected to lowered plasma ACTH levels in patients with Cushing's syndrome. Our experiments revealed that in cultured rat adrenocortical cells expression of Hspb1, meaning HSP27, is not regulated by ACTH. On the contrary, prolonged exposure of cultured cells to both CER and desCER resulted in nearly a 10-fold increase in expression of the gene.

Since in cultured rat adrenocortical cells ACTH had no effect on Fasn and HSP27 gene expression, CER and desCER induced potent up-regulation of HSP27 and down-regulation of Fasn cannot be connected with either regulation of adrenocortical steroidogenesis or with proliferative activity of studied cells. The physiological significance of these findings remains unclear and requires further investigation.

Thus, our study is the first to demonstrate that Cbln1-derived peptides, CER and desCER, have ACTH-like direct effects on corticosterone output and proliferative activity of cultured rat adrenocortical cells. Like ACTH, CER and desCER have similar effects on Fos, Icam1 and Egr1 gene expression. However, in studied cells, CER and desCER also have effects different from those evoked by corticotrophin, and such effects were observed in regulation of Fasn and HSP27 gene expression.

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