Extracellular Nampt (eNampt/visfatin/PBEF) directly and indirectly stimulates ACTH and CCL2 protein secretion from isolated rat corticotropes

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Conflict of interest
None declared

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Abstract

Background. Nicotinamide phosphoribosyltransferase (Nampt/visfatin/PBEF) acts both as an enzyme in the nicotinamide adenine dinucleotide (NAD) synthesis pathway as well as an extracellular hormone (eNampt). Among its effects, eNampt exerts potent pro-inflammatory effects. We have recently shown that, in rats, eNampt stimulates corticosterone secretion by acting through the pituitary rather than the hypothalamus.

Objectives. To investigate the mechanism of action of eNampt on the secretion of adrenocorticotropic hormone (ACTH) and chemokine (C-C motif) ligand 2 (CCL2), which are cytokines secreted by pituitary neuroendocrine tumors.

Materials and methods. The research was carried out on the AtT-20 murine cell line, primary rat pituitary cell culture, isolated pituitary corticotropes, and in vivo. The effects of the performed experiments were examined using the following methods: gene expression profiling using microarrays, quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA).

Results. The results suggest that eNampt stimulates ACTH secretion from rat corticotropes both directly and indirectly. Indirect action most likely occurs through interleukin (IL)-6 secreted by folliculostellate cells of the pituitary gland. In isolated ACTH cells of the rat pituitary gland, eNampt stimulates the expression of genes involved in the immune response. Among them, the protein encoded by the *CCL2* gene seems to also be involved in the regulation of corticotropin-releasing hormone (CRH)-dependent metabolism. Unlike rat corticotropes, murine AtT-20 corticotropic cells do not react to either eNampt or Fk866 (the inhibitor of Nampt enzymatic action).

Conclusions. The eNampt stimulates the secretion of ACTH from rat corticotropes indirectly and directly, likely by stimulating IL-6 secretion from folliculostellate cells of the pituitary gland. This effect was not observed in the AtT-20 corticotropic cell cancer cell line.

Key words: ACTH, Nampt, pituitary gland, CCL2, IL-6

Background

Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme for nicotinamide adenine dinucleotide (NAD) salvage synthesis in mammals, thereby influencing NAD-dependent enzymes and constituting a strong endogenous defense system against various stresses. Nampt, apart from its intracellular function (iNampt), is secreted outside the cells where it circulates in the bloodstream as a hormone (eNampt), also called visfatin or pre-B cell colony-enhancing factor (PBEF).[1–3] eNampt is secreted mainly by adipose tissue, but has also been proven to be secreted by many other types of cells.[4] eNampt can be detected in the human bloodstream and other extracellular fluids, where it exerts pro-inflammatory, prochemotactic (promoting migration of the cells), proangiogenic, and insulin-like effects. The exact mechanism of action of eNampt is still unclear.[4] In the literature, there are 3 not necessarily mutually exclusive theories: 1) eNampt binds to toll-like receptor 4 (TLR4), C-C motif chemokin receptor 5 (CCR5) or a yet unidentified receptor; 2) eNampt is enzymatically active in the extracellular matrix; and/or 3) eNampt is carried in the systemic circulation in extracellular vesicles (EV) and liberated upon internalization, enhancing NAD+ biosynthesis.[4–7]

Our earlier studies demonstrated that intraperitoneal (i.p.) administration of eNampt within 1 h significantly increased levels of corticosterone, but not aldosterone and adrenocorticotropic hormone (ACTH), in rat serum.[8] Under experimental conditions, proopiomelanocortin (POMC) mRNA levels in the pituitary glands of the examined rats increased. Moreover, eNampt protein did not affect the secretion of corticotropin-releasing hormone (CRH) from rat hypothalamic explants and inhibited the release of CRH, induced by potassium ions. In anterior pituitary fragments, eNampt did not stimulate ACTH but did increase POMC mRNA expression. The obtained results suggest that the stimulating effect of eNampt protein on corticosterone secretion in rats is dependent on the pituitary gland. However, the mechanism of action of eNampt on changes in the pituitary gland of rats described above remains unexplained.

The anterior lobe of the pituitary gland (adenohypophysis) is a complex structure composed of many different hormone-secreting cells, such as corticotropes, thyrotropes, gonadotropes, somatotropes, lactotropes, a small population of mammosomatotropes, and hormonally nonactive folliculostellate cells, as well, as blood vessels and fibroblasts.[9,10] Under the influence of hypothalamic CRH, the corticotropes of the adenohypophysis secrete ACTH, the main hormone regulating the growth, differentiation and secretory activity of adrenocortical cells.[11] Stimulation of pituitary secretion of ACTH also occurs in response to inflammatory factors, such as interleukin (IL)-1 or tumor necrosis factor alpha (TNF-α).[12] However, these factors do not act directly on corticotropes, but rather exert their biological effect through folliculostellate cells.[13,14] Under the influence of these inflammatory factors, folliculostellate cells secrete a variety of pro- and anti-inflammatory factors, including interleukin(IL)-6.[15,16] There are experiments showing that secreted IL-6 exerts a stimulating effect on ACTH secretion by corticotropes. It is suggested that, due to this mechanism, *CRH* gene silencing in mice does not prevent pituitary secretion of ACTH.[17] It appears that only neutralization of IL-6 with specific antibodies completely inhibits activation of the hypothalamo–pituitary–adrenal (HPA) axis in mice with *CRH* gene knockout.[18,19]

As mentioned above, the stimulating effect of eNampt protein on corticosterone secretion in rats most likely occurs at the pituitary level.[8] In this respect, it should be stressed that pituitary folliculostellate cells, in response to stimulation of CD14 and TLR4 receptors, secrete IL-6.[13,14] It has also been shown that eNampt has the ability to bind to TLR4 receptors.[6] Moreover, there are reports that eNampt protein stimulates IL-6 secretion by human leukocytes.[20] In view of these studies, it seems that the stimulating effect of eNampt on corticotropes may be mediated by pituitary folliculostellate cells. To investigate this hypothesis, we performed several experiments with the AtT-20 murine corticotropic cell line as well as with isolated rat corticotropes. These cells were exposed to eNampt as well as CRH, IL-6 and Fk866 – an inhibitor of Nampt enzymatic action – and their effects were determined using enzyme-linked immunosorbent assay (ELISA), microarray analysis of gene expression and quantitative polymerase chain reaction (qPCR). Considering the fact that pituitary neuroendocrine tumors secrete numerous cytokines, including CCL2,[21] in isolated ACTH cells of the rat pituitary gland exposed to eNampt or ACTH, the expression of various cytokines with particular attention to CCL2 was characterized.

Objectives

To verify the main research hypothesis presented above, we formulated several specific objectives. The 1st objective was to investigate the effect of eNampt on ACTH secretion in the AtT-20 cell line, rat primary pituitary cell culture and isolated corticotropes. The 2nd objective was to determine the effect of eNampt CRH and IL-6 on the transcriptome profiling of isolated rat corticotropes. The last objective was to investigate the effect of eNampt on CCL2 biosynthesis in rat primary pituitary cell culture, isolated corticotropes and a rat in vivo study.

Materials and methods

Reagents

If not stated otherwise, all reagents were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) or Avantor Performance Materials Poland S.A. (Gliwice, Poland).

AtT-20 murine cell line

The mouse pituitary corticotrope AtT-20/D16v-F2 tumor cell line was bought from Sigma-Aldrich (94050406). The cells were cultured within Dulbecco's Modified Eagle Medium (DMEM)/F12 (without phenol red) medium supplemented with 10% fetal bovine serum (FBS) and 1× AA solution (Sigma-Aldrich; A5955). The cells were cultured in 96-well plates at ~10,000 cells/well.

Primary rat pituitary cell culture

The 21-day old Wistar rats were obtained from the Animal House of Wielkopolska Centre for Advanced Technologies (Poznań, Poland). The brains of the rats were removed directly after decapitation and the adenohypophyses were isolated using sterile surgical tools and transferred to DMEM/F12 (no phenol red) medium supplemented with 10% FBS and 1× AA solution. Subsequently, the glands were fragmented with scissors and treated with 0.9 mg/mL collagenase I (collagenase type I; Sigma-Aldrich) in phosphate-buffered saline (PBS) solution for 20 min at 37°C. Subsequently, the cells were centrifuged (1000 × g for 7 min), suspended in 15 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) in phosphate-buffered saline (PBS) solution, and incubated for 10 min at 37°C. After such treatment, the cells were filtered with a Corning® 70-µm cell strainer nylon membrane (Sigma-Aldrich ref. No. 431751). The cells were then centrifuged (1000 × g for 7 min) and resuspended in DMEM/F12 with 1× AA solution, 10% FBS and 0.05 µg/L fibroblast growth factor (FGF) solution (Thermo Fisher Scientific, Waltham, USA; cat. No. 1263344C). The described procedure delivered the isolated rat pituitary cells, of which ~60% were alive. The living cells percentage was estimated using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific; cat. No. A27974) in the presence of trypan blue. The cells were cultured in 96-well plates at ~10,000 cells/well.

Isolation of corticotropes

After 2 days of isolated rat pituitary cell culture, the cells were treated with 1× trypsin solution (Sigma-Aldrich; 59427C) and collected from culture plates. The corticotropic cells were separated from culture using CELLection™ Biotin Binder Kit (Thermo Fisher Scientific – Invitrogen; lot: 11533D). For cell separation, the anti CRHR1 antibody was used (Alomone Labs, Jerusalem, Israel; ACR050AN0150) with secondary biotin-labeled antibody (Abcam, Cambridge, UK; anti-IgG ab6720). After separation, the cells were cultured with DMEM/F12 with 1× AA solution, 10% FBS and 0.05 µg/L FGF. The cells were cultured in 96-well plates at ~10,000 cells/well.

Animals

As an additional observation, we measured CCL2 levels in animal blood plasma obtained from experiments described in our previous publication.[8] Briefly, experiments were performed on 15 adult (3–4 months old, 250–300 g body weight) male rats. The eNampt protein was administered by ip. injection at a dose of 4 µg/100 g, while ACTH (Cortrosyn®; Organon Pharmaceuticals, Merck KGaA, Darmstadt, Germany) was given at a dose of 2.5 µg/100 g. Rats in the control group were administered 0.2 mL physiological saline. Each group (control, eNampt and ACTH) consisted of 5 animals. Rats were decapitated 1 h after injection. Trunk blood was collected on EDTA (150 mM, pH 8, 300 µL/5 mL) and centrifuged at 1000 × g for 10 min at 4°C. The serum was collected in fresh tubes and stored at –20°C until analysis. The study protocol was approved by the independent Local Ethics Committee for Animal Studies in Poznań (protocol No. 75/2016).

Hormone administration

If not stated otherwise, the studied substances were administered in medium at final concentrations of 10^{-8} M for Nampt (BioVendor R&D Products, Brno, Czech Republic), 10^{-6} µM for CRH (CRH Ferring®; Ferring Pharmaceuticals, Saint-Prex, Switzerland) and 50 pg/mL of IL-6 (Sino Biological, Beijing, China). The culture media were collected 24 h after administration of the tested substances and frozen at –20°C. The cells were subsequently subjected to RNA isolation.

Hormone level detection

The culture media were analyzed using ELISA to determine the concentration of ACTH (Phoenix Europe GmbH, Karlsruhe, Germany; cat. No. EK-001-21), IL-6 (Invitrogen; BM5625) and CCL2 – MCP1 (CCL2) Rat ELISA Kit (Abcam; ab100778). All determinations were performed according to the manufacturers' protocols.

RNA isolation

After incubation, the cells were washed with PBS. Total RNA was extracted from 96-well plates using 100 µL of TRIzol Reagent (Thermo Fisher Scientific; cat. No. 15596026). Further isolation was carried out according to the protocol and reagent proportions stated in the manufacturer's protocol. The amount of total mRNA was determined by optical density at 260 nm and its purity was estimated by the 260/280 nm absorption ratio (>1.8; NanoDrop ND-1000 spectrophotometer; Thermo Fisher Scientific).

Microarray assay

The microarray study was carried out as described elsewhere.[22–24] A 5 ng/sample of total RNA from isolated corticotropic cells was subjected to microarray analysis. The microarray procedure was performed using GeneChip™ WT Pico Kit (Thermo Fisher Scientific; cat. No. 902622) and GeneChip™ Hybridization, Wash and Stain Kit (Thermo

Fisher Scientific; cat. No. 900720). Biotin-labeled fragments of cDNA were hybridized to the GeneChip™ Rat Gene 2.1 ST Array Strip (Thermo Fisher Scientific; cat. No. 902126, 48°C/20 h). Next, the microarrays were washed and stained according to the technical protocol using the Affymetrix GeneAtlas™ Fluidics Station. Subsequently, the array strips were scanned using the Imaging Station of GeneAtlas System. Preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ Operating Software. The quality of gene expression data was checked according to quality control criteria provided by the software. The obtained CEL files were imported into downstream data analysis software. All of the presented analyses and graphs were performed using Bioconductor and the R programming language (R Foundation for Statistical Computing, Vienna, Austria). For analysis we used following bioconductor packages pd.ragene.2.1.st (3.14.1) (a), limma (3.48.1) (b) and arrayQualityMetrics (3.48.0) (c). Each CEL file was merged with a description file. In order to correct the background, and normalize and summarize results, we used the robust multiarray averaging (RMA) algorithm.

Statistical significance of the analyzed genes was assessed with moderated t-statistics using the empirical Bayes method. The obtained p-values were corrected for multiple comparisons using the Benjamini–Hochberg false discovery rate (1995). The selection of significantly changed gene expression was based on p-values <0.05, a false discovery ratio <20% and an expression fold change higher than 2.

Finally, interactions between differentially expressed genes and their protein products were investigated using STRING10 software (Search Tool for the Retrieval of Interacting Genes; https://string-db.org/).[25] The list of gene names was used as a query for an interaction prediction. The search criteria were based on the co-occurrences of genes/proteins in scientific texts (text mining), co-expression and experimentally observed interactions. The results of such analyses generated a gene/protein interaction network, where the intensity of the edges reflects the strength of the interaction score.

RT-qPCR

The reverse transcription (RT) was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland; cat. No. 04379012001). It was performed according to the manufacturer's protocol. The primers used for qPCR (Table 1) were designed by Primer 3 software v. 0.4.0 (Whitehead Institute for Biomedical Research, Cambridge, USA) and purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warszawa, Poland). The qPCR was performed using a CFX96 Deep Well Real-Time System (Bio-Rad, Hercules, USA).

Using the aforementioned primers, a SYBR Green detection system was applied, as described previously.[8,23,26–28] Every 20 µL of reaction mixture contained 2 µL template cDNA (standard or control), 0.5 µM specific primers and a previously determined optimum concentration of $MgCl_2$ (3.5 µM per reaction). LightCycler FastStart DNA Master SYBR-Green I mix (Roche Applied Science, Penzberg, Germany) was used. The qPCR program included a 10-min denaturation step at 95°C to activate the Taq DNA polymerase, followed by 45 cycles of a three-step amplification program: denaturation at 95°C for 10 s; annealing at 56°C for 5 s; and extension at 72°C for 10 s. The specificity of the reaction products was checked by determination of the melting points (0.1°C/s transition rate). The gene expression was normalized to the *HPRT* and *B2M* genes using the Pfaffl ratio method.[29]

Statistical analyses

Statistical analyses of the microarray experiments are described above. For the ELISA assay and qPCR analysis, we used the Kruskal–Wallis test with Dunnett's post hoc test for comparison of multiple experimental groups and the Wilcoxon test for comparison of 2 groups.

Table 1. The quantitative polymerase chain reaction (qPCR) starters sequences

Gene	Forward	Reverse	Accession	Product size
CCL2	ATGCAGTTAATGCCCCACTC	TTCCTTATTGGGGTCAGCAC	NM_031530.1	167
C3	TGCTTCATGCATCAGTCACA	TTTAGGGCGTTTCTGCACTT	NM_016994.2	233
Cp	CAGTTGCTCCAACGTTACCA	TTCCGACAAACAATCAATGG	NM_001270961.1	172
Sod2	AAGGAGCAAGGTCGCTTACA	GGGCTTCACTTCTTGCAAAC	NM_017051.2	215
Lcn2	TCACCCTGTACGGAAGAACC	CAGGTGATTCTCTGGCAACA	NM_130741.1	237
Tlr4	CCCTGGTGTTGGATTTTACG	TCGTTTCTCACCCAGTCCTC	NM_019178.1	223
Cd14	GGCTGGAGCACGTACCTAAA	GAGCAAAGCCAAAGTTCCTG	NM_021744.1	236
Pomc	CATGACGTACTTCCGGGGAT	TCACCACGGAAAGCAACCTG	XM_017594033	192
IL-6	TGATGGATGCTTCCAAACTG	GAGCATTGGAAGTTGGGGTA	NM_012589.2	230
Nampt	TGATCCCAACAAAAGGTCGAA	CCCACTCACACAAAAGCCTA	NM_177928	238
B2m	CTTGCAGAGTTAAACACGTCA	CTTGATTACATGTCTCGGTC	NM_012512.2	70
Hprt	ATAGAAATAGTGATAGGTCCA	TCTGCATTGTTTTACCAGT	XM_008773659	177

Results

AtT-20 cells

When planning our research, we intended to perform experiments on the AtT-20 cell line. However, for these cells, 24-h eNampt exposure at concentrations of 10^{-10} M to 10^{-7} M did not change the basal secretion of ACTH (Fig. 1). Similarly, 24-h exposure of AtT-20 cells to the iNampt inhibitor Fk866 did not affect the basal output of corticotrophin. It is interesting that the combined addition of Fk866 and CRH to the incubation medium reduced ACTH secretion by the examined cells (Fig. 2).

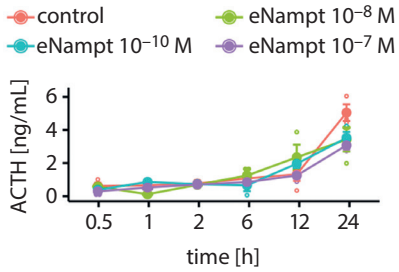

Fig. 1. The concentration of adrenocorticotropic hormone (ACTH) [pg/mL] in the incubation medium of cultured murine AtT-20 cells exposed to different concentrations of eNampt (10,000 cells/well). Each circle indicates an individual measurement. Data are presented as mean ± standard error of the mean (SEM)

Isolated rat corticotropes

Since the experiments with AtT-20 mouse cancer cells did not show any effect of eNampt on ACTH secretion, we decided to change the experimental model to use rat pituitary cells. In this case, we used 2 models: primary rat pituitary cell culture and isolated rat corticotropes. It appeared that eNampt, CRH and IL-6 stimulated ACTH output by cultured isolated rat corticotropes. Although eNampt stimulated IL-6 production in primary rat pituitary cell culture, no such effect was observed in cultured isolated rat corticotropes (Fig. 3).

Gene expression profiling using microarrays

In the 2nd series of experiments, we performed microarray analysis on isolated rat corticotropes cultured for 24 h in the presence of CRH, eNampt or IL-6. As shown in Fig. 4, under these conditions, the expression level of only a small number of genes was upregulated: CRH upregulated

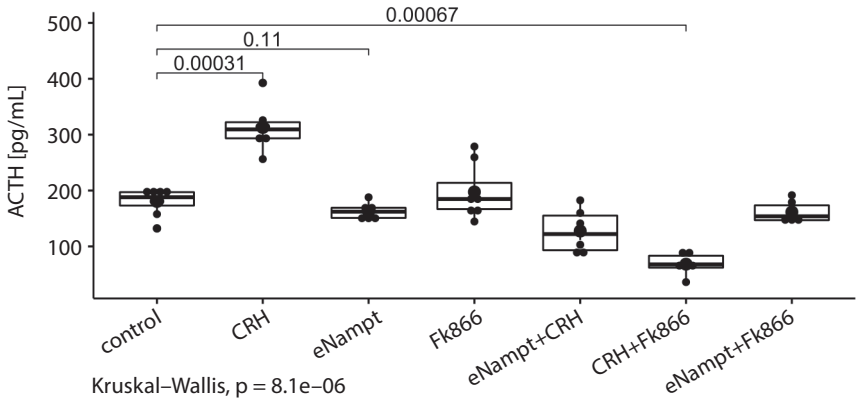

Fig. 2. The concentration of adrenocorticotropic hormone (ACTH) [pg/mL] in the incubation medium of cultured murine AtT-20 cells exposed for 24 h to corticotropin-releasing hormone (CRH), eNampt, Fk866, and their combinations. The figure shows the median and quartiles. Each symbol indicates an individual measurement (10,000 cells/well). Significant differences were observed between the control and CRH groups (p = 0.00031) as well as between the control and CRH+Fk866 groups (p = 0.00067). No significant differences between other experimental groups were observed

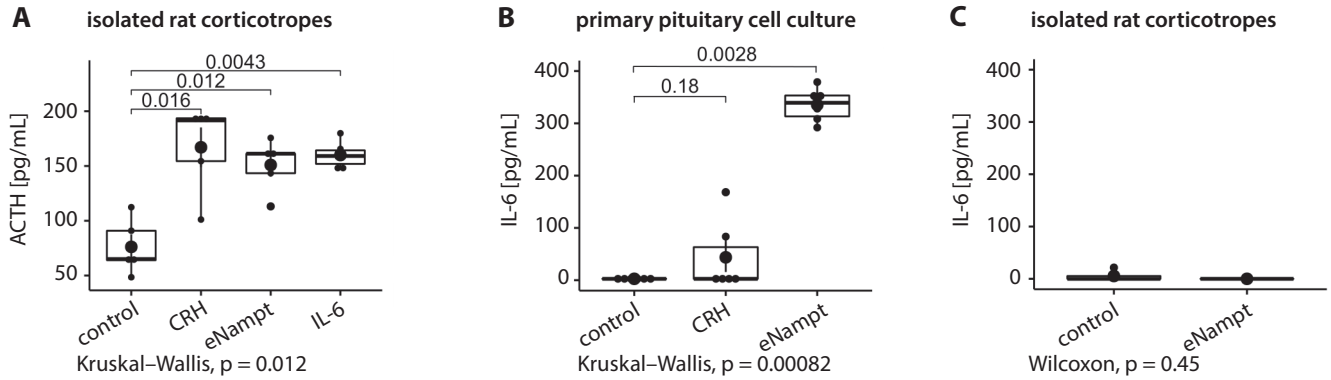

Fig. 3. The response of primary pituitary cell cultures to eNampt, CRH and IL-6. The data are presented as median and quartiles. A. All 3 studied compounds stimulated adrenocorticotropic hormone (ACTH) production of isolated rat corticotropes. Significant differences were observed between the control and corticotropin-releasing hormone (CRH) groups (p = 0.016); control and eNampt groups (p = 0.012); and control and IL-6 groups (p = 0.0043); B. eNampt stimulated IL-6 production in primary rat pituitary cell culture. A significant difference was observed between the control and eNampt groups (p = 0.0028). There was no significant difference between the control and CRH groups (p = 0.18); C. eNampt did not stimulate IL-6 secretion in isolated rat corticotropes culture (p = 0.45)

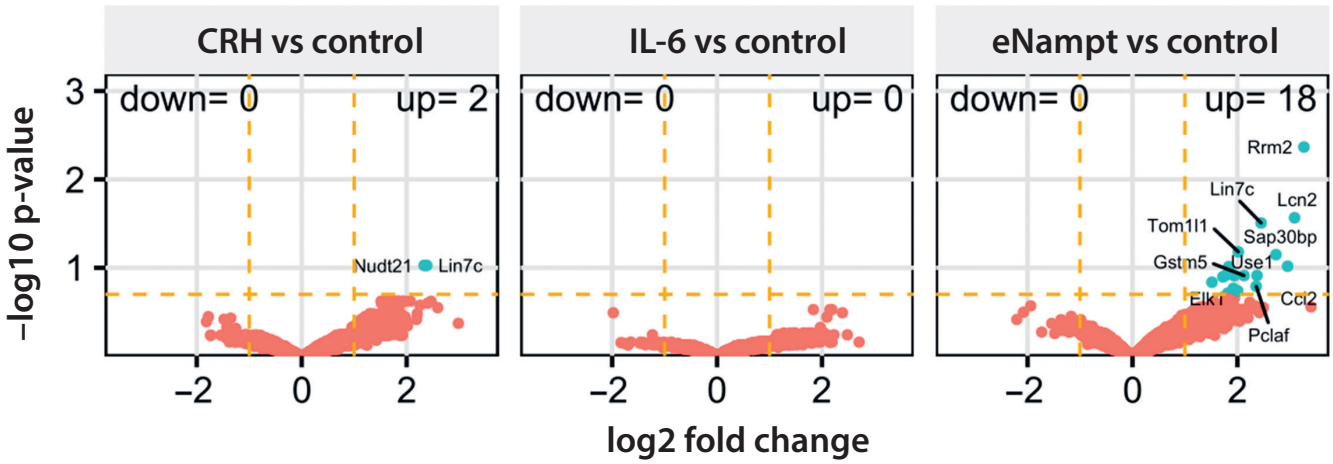

Fig. 4. Volcano plot. Each dot represents 1 gene. Genes with a fold ratio >2 and false discovery rate below 20% are marked in cyan. The names of the 10 most upregulated and downregulated genes are show in the figure

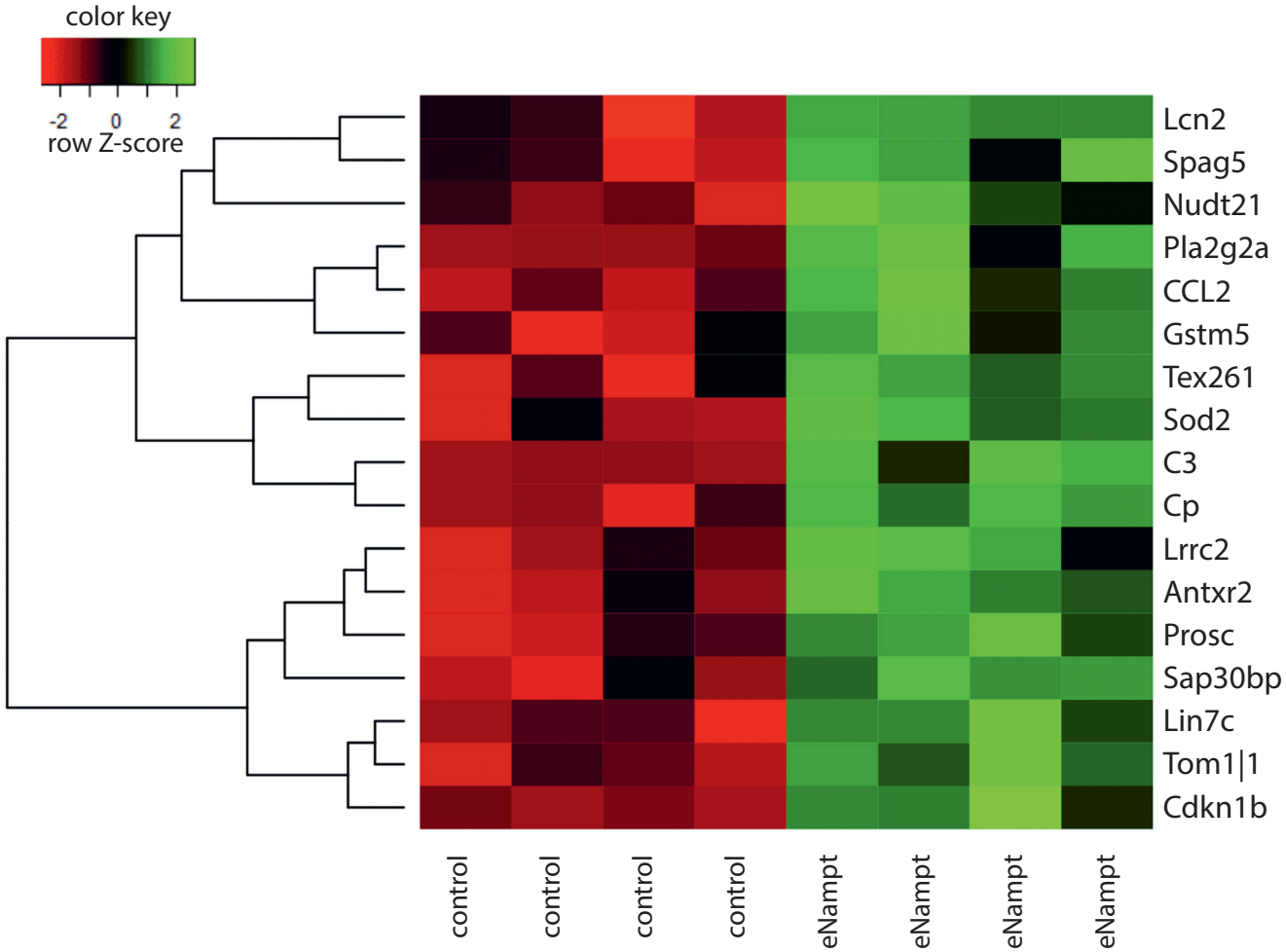

Fig. 5. Heatmap presenting the differences in gene expression between control and eNampt-treated isolated corticotropic cells. Gene expression is presented as a color gradient where red symbolizes the lowest expression level and green symbolizes the highest expression level. This gradient is presented separately for each gene. The histogram on the left shows the clusterization pattern. The genes with the most similar expression patterns are grouped together

2 genes, eNampt upregulated 18 genes and IL-6 did not affect the expression of any of the genes studied.

The results obtained for the microarray analysis were validated with the qPCR method. Only corticotropes treated with eNampt were subjected to these studies. The results of this analysis are presented as heatmap graphs. As Fig. 5 shows, the results of gene expression level determination with qPCR of isolated corticotropes

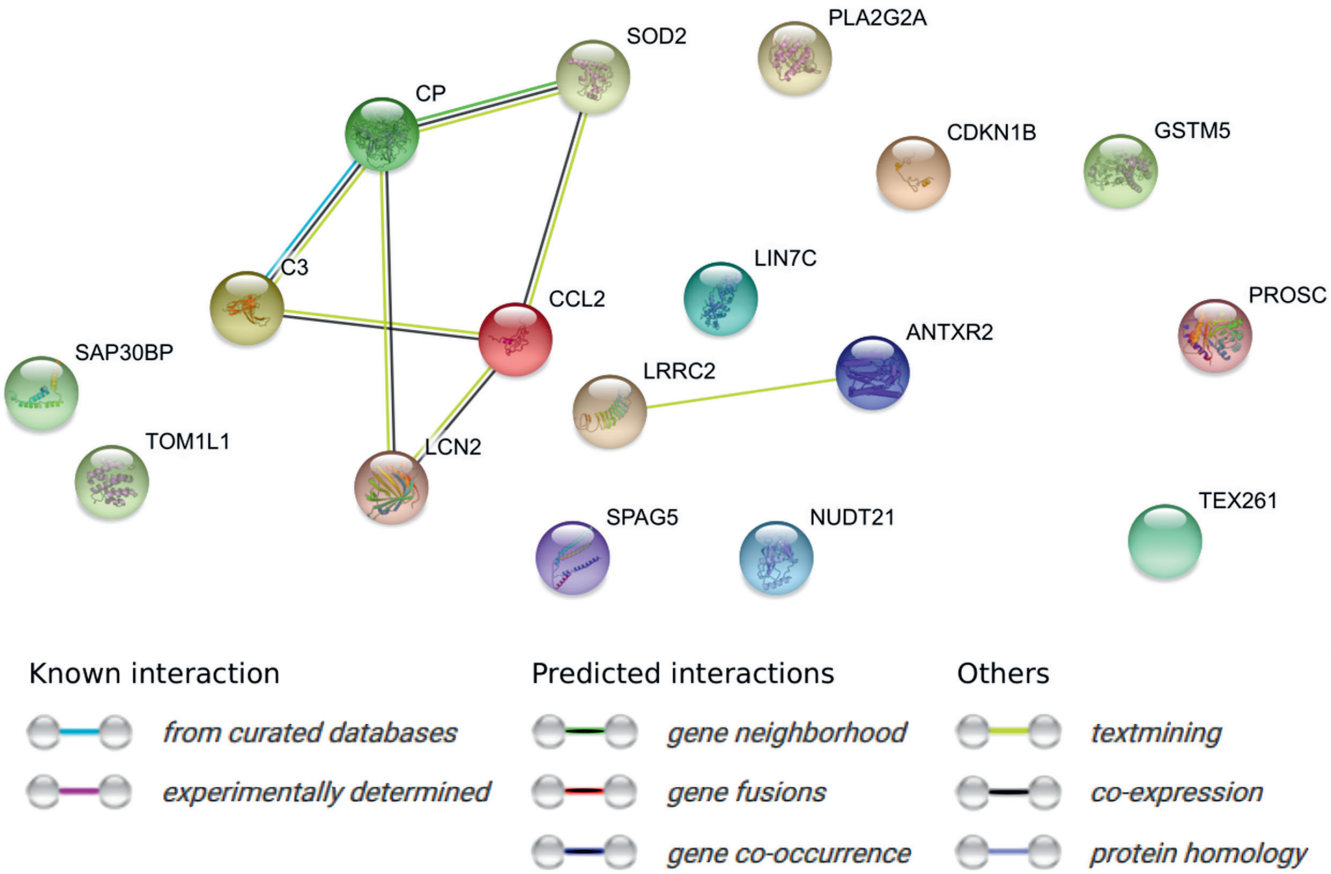

Fig. 6. STRING-generated interaction network with differently expressed genes of the eNampt-treated isolated corticotropes. The color of the edges reflects the type of interaction

cultured in the presence of eNampt are consistent with the results obtained using microarray analysis.

These genes were then subjected to analysis with STRING-db software via its browser API. The STRING-generated interaction network provides information about molecular interactions formed between the protein products of the studied genes (Fig. 6). The STRING analysis showed that genes such as *CCL2* (chemokine (C-C motif) ligand 2), *Sod2* (superoxide dismutase 2), *LCN2* (lipocalin-2), *C3* (complement component 3), and *CP* (ceruloplasmin) are functionally connected. They were mostly shown to be co-expressed. Most of these genes seem to be involved in inflammatory processes.

In the next stage of the study, we compared the expression levels of selected genes in primary rat pituitary cell culture, with those observed in cultured isolated rat corticotropes. Cultured cells were exposed for 24 h to CRH, eNampt or IL-6, and the expression levels of the studied genes were evaluated using qPCR. For these studies, we chose genes for which the level of expression changed significantly after exposure to eNampt. As presented in Fig. 7, in the primary rat pituitary cell culture, the influence of the investigated substances on the expression level of the studied genes was negligible. In both experimental models, eNampt did not change the expression level of the *Nampt* and *IL-6* genes. Similarly, no effect was observed for the genes *Cd14* (cluster of differentiation 14) and *Tlr4* (toll-like receptor 4) (results not shown). In contrast, in isolated corticotropes, eNampt increased the expression levels of the *Sod2*, *LCN2*, *CCL2,* and *C3* genes. In the employed experimental models, CRH increased the expression level of the *POMC* (proopiomelanocortin) gene only in isolated corticotropes, whereas exposure of the tested cells to IL-6 did not change the expression level of any of the studied genes (in both experimental models).

CCL2 protein secretion in vivo and in vitro

In our study, both the microarray and qPCR analysis data indicated that, under the applied experimental conditions and after cell exposure to eNampt, the *CCL2* gene expression level increased significantly. In this regard, the literature shows that CCL2 protein is secreted from human mammary epithelial cells after eNampt stimulation.[30,31] Moreover, CCL2 is secreted by pituitary neuroendocrine tumors.[21] Based on the literature data, we decided to investigate CCL2 protein secretion both in vivo and in vitro. As shown in Fig. 8, 60 min after the injection of eNampt, the level of CCL2 protein in rat blood serum increased significantly, while administration of CRH did not change the CCL2 protein level.

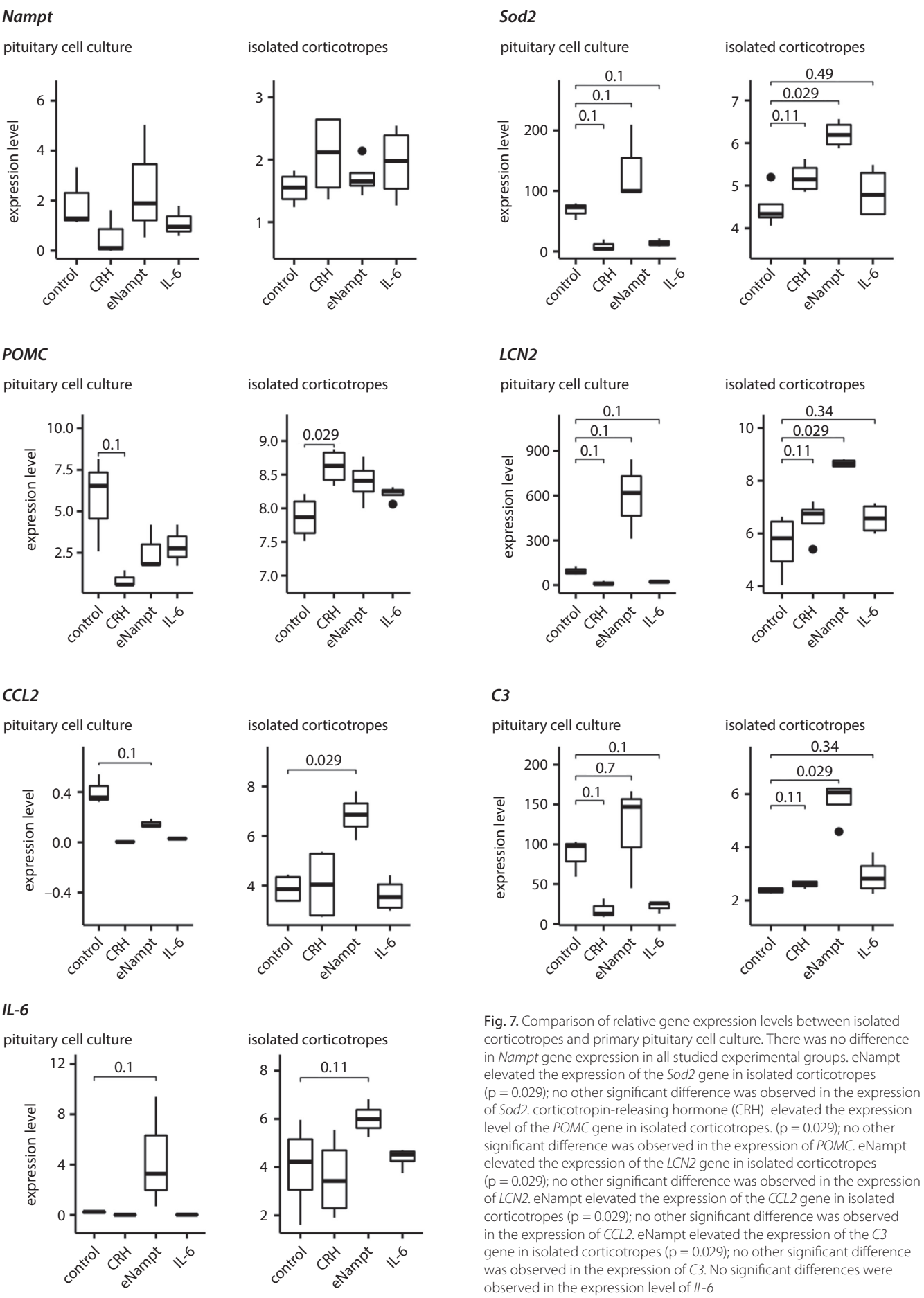

Fig. 7. Comparison of relative gene expression levels between isolated corticotropes and primary pituitary cell culture. There was no difference in *Nampt* gene expression in all studied experimental groups. eNampt elevated the expression of the *Sod2* gene in isolated corticotropes ($p = 0.029$); no other significant difference was observed in the expression of *Sod2*. corticotropin-releasing hormone (CRH) elevated the expression level of the *POMC* gene in isolated corticotropes. ($p = 0.029$); no other significant difference was observed in the expression of *POMC*. eNampt elevated the expression of the *LCN2* gene in isolated corticotropes ($p = 0.029$); no other significant difference was observed in the expression of *LCN2*. eNampt elevated the expression of the *CCL2* gene in isolated corticotropes ($p = 0.029$); no other significant difference was observed in the expression of *CCL2*. eNampt elevated the expression of the *C3* gene in isolated corticotropes ($p = 0.029$); no other significant difference was observed in the expression of *C3*. No significant differences were observed in the expression level of *IL-6*

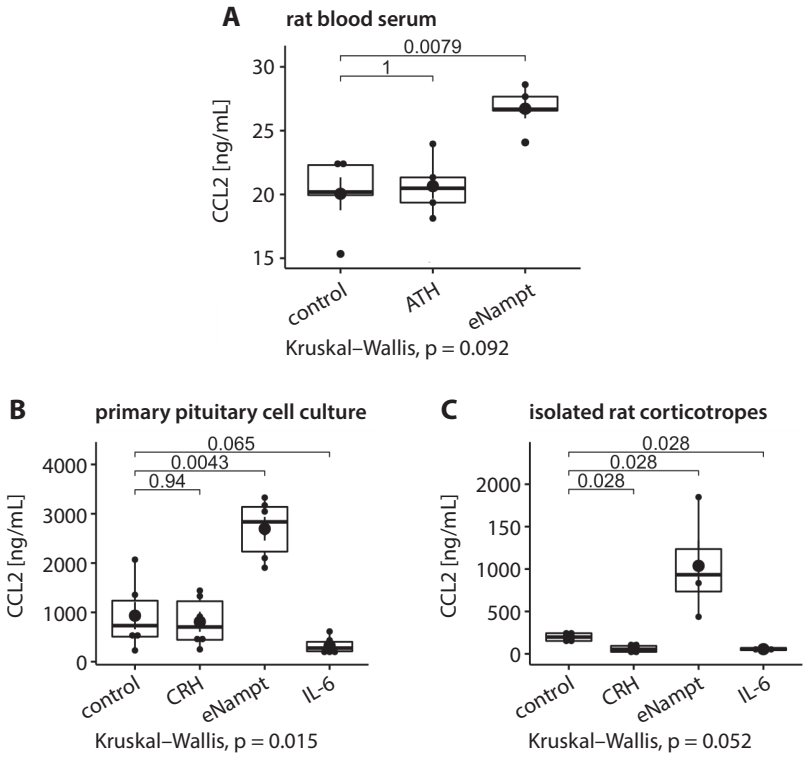

Fig. 8. CCL2 levels measured in rat blood serum, culture medium of primary pituitary cell culture and rat isolated corticotropes. A. Intraperitoneal administration of eNampt elevated CCL2 levels in rat serum (p = 0.0092); B. eNampt administration increased secretion of CCL2 in primary pituitary cell culture (p = 0.0043); C. CRH and IL-6 decreased secretion of CCL2 in isolated rat corticotropes (p = 0.028 and p = 0.028, respectively), while eNampt elevated secretion level by these cells (p = 0.028)

eNampt also increased the CCL2 protein concentration in the incubation medium of the primary pituitary cell culture and cultured isolated corticotropes. It is interesting that, in case of the isolated corticotrope culture, both CRH and IL-6 decreased the secretion of the analyzed protein.

Discussion

It is well known that ACTH secreted by corticotropic cells of the anterior pituitary lobe plays an essential role in the regulation of differentiation, growth, and function of the adrenal cortex. In turn, secretion of ACTH depends on the hypothalamic hormone CRH.[32–35]

As we showed in an earlier publication, one of the factors regulating pituitary secretion of ACTH is eNampt.[8] Our in vitro experiments showed that in the anterior pituitary lobe fragments, eNampt increases *POMC* gene expression and ACTH secretion into the incubation medium. However, the mechanism of eNampt action on pituitary ACTH cells is not known. Therefore, the aim of the present study was to explain the mechanism of action of eNampt on the secretory function of rat corticotropic cells.

Initially, we planned to perform the projected studies in the AtT-20 mouse pituitary tumor cell line, which secretes a huge amount of ACTH. However, under a wide range of eNampt concentrations, these cells did not change the level of secretion of ACTH in response to eNampt, nor did they react to CRH and Fk866 added to the medium. The lack of reaction of AtT-20 cells to the applied compounds forced us to use another experimental models. These experimental models included primary rat pituitary cell culture and cultured isolated rat corticotropes. In the primary rat pituitary cell culture, all of the cells of the anterior pituitary lobe are present, which likely retains the ability for possible functional interactions of all gland cells. Such interactions take place in the pituitary, and the pituitary folliculostellate cells play an essential role in this process.[9]

The classical pathway of HPA axis activation, i.e., via CRH secreted by the hypothalamus, is not the only pathway leading to ACTH secretion. ACTH might also be secreted after IL-6 stimulation, which is secreted by folliculostellate cells.[9,12,15–19,36] In this respect, it should be noted that eNampt also stimulates the secretion of IL-6 in isolated human leukocytes.[20] Moreover, *Nampt* gene expression positively correlates with serum levels of IL-6 and CRP.[37]

Based on these observations, we decided to check whether eNampt affects the secretion of ACTH by the paracrine route through IL-6 secreted by pituitary folliculostellate cells. To test this hypothesis, we isolated corticotropic cells from the primary pituitary cell culture and compared the effects of eNampt and IL-6 on ACTH secretion in both experimental systems. It appeared that both eNampt and IL-6 increased ACTH secretion by isolated rat corticotropes. It also appeared that eNampt increased the secretion of IL-6 in primary pituitary cell culture, but did not show such an effect in the case of isolated rat pituitary ACTH cells. These results suggest that eNampt may have an indirect effect (via IL-6) on ACTH secretion by isolated rat corticotropes. However, we have also shown that eNampt can directly stimulate corticotropin secretion from the examined cells. These observations suggest that

the stimulating effect of IL-6 on the secretion of ACTH by rat corticotropes may occur both directly and indirectly, mediated by the studied interleukin.

The lack of influence of eNampt on ACTH secretion in the case of the AtT-20 mouse pituitary tumor cell line is noteworthy, yet there was a clear stimulating effect on corticotropin secretion by isolated rat corticotropes. Differences in the effect of eNampt on normal and tumor cells were also observed in our previous studies.[38] In these studies, in primary culture, eNampt did not affect the rate of proliferation of rat adrenocortical cells, but it did stimulate proliferation of the H295R adrenocortical cancer cell line. It is difficult to explain the causes of the different effects of eNampt on normal and neoplastic cells.

It is well known that Nampt is a protein essential for the life of cells and organisms. For example, whole body deletion of the *Nampt* gene results in embryonic lethality, and muscle-specific Nampt deficient mice exhibit progressive muscle degeneration.[39,40] Moreover, retina-specific Nampt deficient mice exhibit severe vision loss.[41,42] In humans and mice, the level of eNampt in circulation decreases significantly with age. On the other hand, increasing the eNampt level in the blood of aged mice by adipose tissue-specific overexpression of Nampt increases NAD+ levels in multiple tissues, thereby enhancing their functions and extending the lifespan in female mice.[5] However, beyond its physiological function, Nampt has been indicated as one of the most important factors in cancer malignances.[43–45] Its expression was found to be higher in tumor cells than in normal cells.[43,46–55] It should be noted that eNampt serum concentrations in various types of cancer are usually elevated,[4,56–63] and at least part of the circulating protein is derived from the tumor itself.[64]

In this regard, it can be suggested that in the case of AtT-20 cells where the level of Nampt is most likely to be elevated (maximal stimulation), eNampt added to the culture may no longer increase ACTH secretion. The AtT-20 cell line is characterized by autonomous ACTH secretion; therefore, it might be difficult to further increase its ACTH secretion. Moreover, various factors, such as an inhibitor of the Jak2 signaling pathway (Lapatinib),[65] somatostatin analog (SOM230)[66] and DNA replication inhibitor (Aphidiloclin),[67] have been proven to decrease ACTH secretion of this cell line. It is also surprising and incomprehensible to note that Fk866 added to the AtT-20 cell culture also did not change ACTH secretion by these cells.

It is also worth mentioning that eNampt has been proven to promote stemness and dedifferentiation of cancer cells, which is critical for tumor initiation, progression, therapy resistance, and metastasis.[45,68–72] Moreover, previous studies strongly suggest that the effects of eNampt, as a cytokine, are independent of iNampt enzymatic activity.[45,70–73] Since eNampt has a strong and dedifferentiating effect on cancer cells, it is possible that the molecular mechanism of eNampt-induced secretion of ACTH was lost or altered in AtT-20. However, the reasons behind the observed differences between AtT-20 and primary corticotropes remain unknown and require further study.

In the next stage of the study, we attempted to explain the changes in gene expression levels that accompany the actions of CRH, eNampt and IL-6 on isolated rat corticotropes. We used microarray analysis for this purpose, which revealed changes in the expression level of only a few genes of the studied corticotropes, among which there were no Pomc and Nampt genes. It should be noted that, under the applied conditions, all examined substances increased ACTH secretion of isolated corticotropes. In our previous work,[8] we reported that 2 h of exposure to eNampt stimulated *POMC* and *Nampt* gene expression levels in isolated rat pituitary explants. It seems that after 24 h of culture, the expression levels of these genes were already normalized.

However, under these experimental conditions, 17 genes still showed elevated expression levels after administration of eNampt (24-h exposure). In further research, we focused on these genes. This group includes genes such as *CCL2* (chemokine (C-C motif) ligand 2), *Sod2* (superoxide dismutase 2), *LCN2* (lipocalin-2), *C3* (complement component 3), and *CP* (ceruloplasmin). This group of genes is functionally connected and the proteins they encode take part in the immune response. For example, CCL2 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation evoked by either tissue injury or infection.[74,75] Sod2 protein plays an antiapoptotic role against oxidative stress, ionizing radiation and inflammatory cytokines.[76] LCN2 is involved in innate immunity by sequestrating iron, thus limiting bacterial growth,[77] and C3 plays a central role in the complement system and contributes to innate immunity.

It should be noted that eNampt affects inflammatory processes and it is commonly recognized as a pro-inflammatory cytokine.[4,78–81] Therefore, among the genes that are controlled by eNampt in isolated corticotropes, the stimulation of *CCL2* gene expression seems interesting.

Previous data indicate that pituitary neuroendocrine tumors secrete numerous cytokines, including CCL2.[21] Since we observed an elevated *CCL2* gene expression level in isolated rat corticotropes after eNampt exposure, we decided to measured level of secreted CCL2 protein in the in vitro model as well as in rat serum from an in vivo experiment described earlier.[8] It appears that, in all cases (in vivo as well as in vitro), eNampt leads to an increase in CCL2 protein secretion. This observation indicates that part of the CCL2 protein found in rat serum originates from pituitary ACTH cells.

CCL2 is implicated in the pathogenesis of several diseases characterized by monocytic infiltrates, such as psoriasis, rheumatoid arthritis and atherosclerosis,[82] as well as various diseases of the central nervous system (CNS) characterized by neuronal degeneration.[83–89] Moreover, gene and protein expression of CCL2 is significantly

increased in the blood and tumors of renal cell carcinoma patients.[90] Some studies have indicated that CCL2 protein is also involved in regulating metabolism. CCL2 impairs insulin signaling in skeletal muscle cells and significantly reduces insulin-stimulated glucose uptake in myocytes.[91] In parallel, the CCL2 protein regulates liver and muscle metabolism and mitochondrial biogenesis, and participates directly or indirectly in the progression of obesity-related metabolic complications or aging.[92,93]

In light of these observations, it seems reasonable to suggest that stimulated eNampt secretion of CCL2 protein by isolated rat corticotropes is not only related to the regulation of immune response, but may also be related to the regulation of metabolism.[94] This is further suggested by the inhibition of CCL2 protein secretion by CRH, which we observed in isolated ACTH cells. It is well documented that the synthesis and secretion of CRH are regulated by various neuropeptides that regulate, among others, feeding and appetite, thus regulating metabolism.[95–100] Therefore, the results obtained suggest that crosstalk between CRH and CCL2 may be involved in regulating metabolism.

Limitations

A main limitation of this study is the lack of experiments involving isolated folliculostellate cells. The rat pituitary primary cell culture model used in the current study contained many different cell types derived from the pituitary gland. Therefore, it is possible that the observed eNampt-dependent stimulation of IL-6 secretion may occur via other cell types than folliculostellate cells. Unfortunately, to the best of our knowledge, folliculostellate cells do not possess any specific surface marker that would allow their isolation and thus the establishment of a specific cell culture model.

The results of our findings suggest that eNampt is involved in the stimulation of ACTH, IL-6 and CCL2 secretion, but do not clarify the molecular mechanism of eNampt action. Regarding IL-6 secretion, it is known that eNampt binds to the TLR4 receptor of human cell lines. It is also known that eNampt administration results in IL-6 secretion from human lymphocytes. In the present study, we did not examine whether a similar mechanism occurs in folliculostellate cells or primary corticotropes. This aspect requires further studies.

Conclusions

The results of this study suggest that eNampt stimulates ACTH secretion from rat corticotropes both directly and indirectly. Indirect action most likely occurs through IL-6 secreted by folliculostellate cells of the pituitary gland. In isolated ACTH cells of the rat pituitary gland, eNampt stimulates the expression of genes involved in the immune response. Among them, the protein encoded by the *CCL2* gene seems to also be involved in the regulation of CRH-dependent metabolism. Unlike rat corticotropes, murine AtT-20 corticotropic cells do not react to either eNampt or Fk866 (the inhibitor of Nampt enzymatic action).

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