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Nicotinamide phosphoribosyltransferase and the hypothalamic-pituitary-adrenal axis of the rat

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Abstract. Nicotinamide phosphoribosyltransferase (Nampt), also termed visfatin, catalyses the rate-limiting step in the nicotinamide adenine dinucleotide (NAD) salvage pathway. In addition to its intracellular function (iNampt), extracellular Nampt (eNampt) also affects numerous intracellular signalling pathways. The current study investigated the role of Nampt in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis in rats. At 1 h after intraperitoneal administration of eNampt (4 μg/100 g) in adult male rats, serum adrenocorticotropic hormone(ACTH) and aldosterone levels remained unchanged, while corticosterone levels were notably elevated compared with the control group, as determined by ELISA. The results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) demonstrated that, in the hypothalami of eNampt-treated rats, the mRNA expression levels of Fos proto-oncogene, which is also termed c-Fos, were not significantly different compared with the control group; however, the mRNA expression levels of proopiomelanocortin (POMC) were markedly increased in the pituitary gland of eNampt-treated rats compared with the control group. Furthermore, in hypothalamic explants, ELISA results demonstrated that the addition of the eNampt protein exhibited no effect on corticotropin-releasing hormone (CRH) release into the incubation medium and prevented potassium ion-induced CRH release. Additionally, the eNampt-induced increase in ACTH output by pituitary gland explants was not statistically significant, compared with the control group. However, RT-qPCR indicated that exposure of pituitary gland explants to eNampt and CRH increased the levels of POMC mRNA expression; the effect of eNampt, but not CRH, was inhibited by FK866, which is a specific Nampt inhibitor. In primary rat adrenocortical cell cultures, eNampt exhibited no effect on basal aldosterone or corticosterone secretion, while increases in aldosterone and corticosterone levels in response to ACTH were retained. To assess the potential role of iNampt in the regulation of adrenal steroidogenesis, experiments involving a specific Nampt inhibitor, FK866, were performed. Exposure of cultured cells to FK866 notably lowered basal aldosterone and corticosterone output compared with the control group, and completely eliminated the response of cultured cells to ACTH. The results of the present study indicated that the injected eNampt may have increased the corticosterone serum levels by acting at the pituitary level. In addition, iNampt may exert a tonic stimulating effect on the secretion of aldosterone and corticosterone from rat adrenocortical cells, as normal iNampt levels were required to retain the response of cultured rat adrenocortical cells to ACTH. Thus, these data suggest an important physiological role of both iNampt and eNampt in the regulation of the HPA axis activity in the rat.

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Introduction

Nicotinamide phosphoribosyltransferase (Nampt), which is also termed pre-B-cell colony-enhancing factor 1 or visfatin, is a protein with a molecular weight of 52-kDa that exhibits various functions (1-3). Two isoforms of Nampt exist, which include intracellular Nampt (iNampt) and extracellular, or secreted Nampt (eNampt). iNampt catalyses the rate-limiting step in the nicotinamide adenine dinucleotide (NAD) salvage pathway. At present, four pathways for NAD biosynthesis have been identified. NAD may be synthesized from tryptophan, which is a primary mechanism of biosynthesis, or via NAD salvage pathways from various niacins, including nicotinamide (NAM), nicotinic acid and nicotinamide riboside. In mammals, 90% of the substrates for Nampt activity originate from NAM (4,5).

Although it is a central metabolic enzyme, eNampt is considered to be an important secreted cytokine. eNampt is primarily secreted by adipose tissues; however, it may also be secreted by other cell types, including macrophages, hepatocytes, chondrocytes, leucocytes, inflamed endothelial cells, human peripheral blood lymphocytes, chronic lymphocytic leukaemia lymphocytes, gastric cancer cells and antigen-presenting cells (6). Despite this, the mechanism of eNampt secretion has not yet been fully elucidated. Notably,

the 3T3-L1 murine adipose cell line secretes high levels of eNampt (7). iNampt is a cytosolic protein that localises outside of intracellular vesicles. The secretion of eNampt is not inhibited by brefeldin A or monensin, which indicates that eNampt is not secreted by a classical endoplasmic reticulum-Golgi apparatus-mediated pathway and instead occurs via an alternative pathway (8). Furthermore, it has been reported that sirtuin (SIRT)1 is required for eNampt secretion; in the white and brown adipose tissue of wild-type FVB mice, deacetylation of iNampt by the mammalian NAD^+-dependent deacetylase SIRT1 mediates the secretion of the protein by adipocytes (7).

It has been previously reported that iNampt functions in numerous oncogenic cellular activities, including DNA repair, autophagy, apoptosis, tumour metastasis, inflammation and angiogenesis (2,5-8). In addition, iNampt has a central role in maintaining the equilibrium of NAD degradation and NAD synthesis at stable levels. Through this function, iNampt stimulates numerous NAD-dependent enzymes and transcriptional factors, including SIRT1, CD38, BRCA1 and poly(ADP ribose) polymerase 1, and influences the downstream pathways of those proteins (6). Furthermore, eNampt has been proven to circulate in the blood and exhibits robust NAD biosynthetic activity (9). Importantly, the Nampt receptor has not yet been identified.

Despite numerous reports concerning the action of eNampt on various aspects of organs and the function of regulatory systems, limited data is currently available concerning its involvement in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. eNampt is present in human serum and cerebrospinal fluid (CSF); however, the levels of eNampt in the CSF are much lower (~10%) compared with levels in the serum. The origin of the CSF eNampt is not known (10). Regarding this, 24 h after administration of Nampt protein into the arcuate nucleus of the rat hypothalamus, increased food intake was observed alongside reduced corticotropin-releasing hormone (CRH) and cocaine and amphetamine-regulated transcript (CART) gene expression, while proopiomelanocortin (POMC) gene expression remained unaltered (11).

A limited amount of experimental data has indicated the presence of an association between adrenocorticotropic hormone (ACTH) and Nampt. ACTH stimulation reduces the Nampt gene expression level in white adipose tissue cell culture by 60% after 4 h of treatment (12). In addition, in adrenocortical cells, ACTH-stimulated accumulation of reduced NAD (NADH) induced the transcription of cytochrome P450 family 17 subfamily A member 1 by promoting the dissociation of corepressor carboxyl-terminal-binding proteins from the promoter (13). Recently, Reverchon *et al* (14) demonstrated that eNampt induces and improves *in vitro* insulin-like growth factor (IGF)1-induced steroidogenesis in bovine ovary primary cell culture. They reported that human recombinant eNampt (10 ng/ml) enhanced cellular progesterone and oestradiol output, an effect that was associated with an increase in the protein expression of steroidogenic acute regulatory protein (STAR), increased hydroxy-Δ-5-steroid dehydrogenase, 3 β- and steroid Δ-isomerase 1 (HSD3B) activity and enhanced phosphorylation of the IGF1 receptor (IGF1R) and the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK)1/2 in the presence or absence of IGF1 (10 nM) (14). These reports indicate that eNampt may directly affect steroidogenic cells (bovine ovary primary cell culture). As iNampt is a rate-limiting enzyme for NAD^+ production, which is used by the cytochrome P450 family in steroidogenesis, a similar effect may be observed in the adrenal cortex. Concerning this, to the best of our knowledge, no data are currently available, and the present study therefore aimed to investigate the role of Nampt in the regulation of rat HPA axis activity.

Materials and methods

Animals and reagents. Wistar rats were obtained from the Laboratory Animal Breeding Centre of the Department of Toxicology, Poznan University of Medical Sciences (Poznan, Poland). The animals were maintained under standard light conditions (14:10 h light/dark cycle, illumination onset at 6.00 a.m.) at 23˚C, 50-60% air humidity, 8-10 air changes per hour (mechanical, via HEPA filters) with free access to standard pellets and tap water. The studies were performed on total of 90 sexually mature wistar rats. 15 rats were used for *in vivo* experiments while 75 were used for all *in vitro* experiments. The number of rats, their sex, age and weight used in the current study are given in the descriptions of the individual experiments or descriptions of the figures. The study protocol was approved by the independent Local Ethics Committee for Animal Studies in Poznań (protocol no. 75/2016). If not otherwise stated, all reagents were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) or Avantor Performance Materials Poland S.A. (Gliwice, Poland). Recombinant human Nampt protein was purchased from BioVendor R&D Products (Brno, Czech Republic) and the specific Nampt inhibitor FK866 was purchased from ApexBio Technology (Houston, TX, USA).

eNampt and ACTH administration in vivo. Experiments were performed on 15 adult (3-4 months old, 250-300 g body weight) male rats. The eNampt protein was administered by intraperitoneal (i.p.) 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 with 0.2 ml physiological saline (in each group-control, eNampt and ACTH- n=5). Rats were decapitated 1 h after injection. Trunk blood was collected on EDTA (150 mM, pH 8, 300 µl/5 ml) and centrifuged at 1,000 x g, for 10 min at 4˚C. The serum was collected in fresh tubes and stored at -20˚C until analysis. Simultaneously, the adrenal glands, pituitary glands, hypothalami and periadrenal adipose tissue were collected, preserved in RNAlater™ (ThermoFisher Scientific, Inc., Waltham, MA, USA) and stored at -20˚C until RNA isolation. Doses of administered proteins were selected according to previous studies (9,15).

Adrenal compartment isolation and freshly isolated rat adrenocortical cells for in vitro experiments. The methods described by Trejter *et al* (16) were followed. Immediately after decapitation, adrenal glands of intact 15 adult males, ~3-4 months old, ~250-300 g body weight, rats were removed and freed of adherent fat. Subsequently, under a stereomicroscope, glands were decapsulated to separate the

zona glomerulosa (ZG) from the zona fasciculata/reticularis (ZF/R). Pieces of connective tissue capsule with adjacent ZG cells and pieces of the ZF/R compartment were mechanically chopped using surgical scissors. The mechanically isolated compartments were then digested with collagenase (collagenase type I; Sigma-Aldrich; Merck KGaA) in Krebs-Ringer solution (1 mg/ml). Digestion was performed at 37˚C for 60 min in a water bath with continuous shaking (300 oscillations per min). The obtained suspensions were subjected to mechanical grinding by pipetting (10-15 times) and filtered through a nylon membrane. Cells were centrifuged (200 x g for 10 min at room temperature) and washed three times in Krebs-Ringer solution supplemented with 0.3% glucose and 0.2% bovine serum albumin (Sigma-Aldrich; Merck KGaA). Finally, cells were suspended in Dulbecco's modified Eagle's medium (DMEM)/F12 medium without phenol red (Sigma-Aldrich; Merck KGaA) with 10% fetal bovine serum (FBS, F7524, Sigma-Aldrich; Merck KGaA) and Antibiotic Antimycotic Solution (A5955, Sigma-Aldrich; Merck KGaA). Incubation of cells (5,000 cells/ml) with eNampt (0.1 and 10 nM) and/or ACTH (1 μM) was performed in a water bath at 37˚C for 2 h. This is a modified version of the technique described in greater detail by Hinson *et al* (17), Malendowicz *et al* (18,19) and Rucinski *et al* (20). The concentrations of eNampt were selected based on a previous study by Benito-Martin *et al* (21). The incubation medium was centrifuged (200 x g for 10 min at room temperature) and the collected cells were frozen at -36˚C. Cells were treated with TRI Reagent (Sigma-Aldrich; Merck KGaA) for RNA isolation. The obtained material was used for further experiments.

Primary culture of rat adrenocortical cells. The method used for culturing rat adrenocortical cells has been described previously (16,22). Briefly, adrenal glands were obtained from 35 ~60-80 g, 21-day-old, male rats. Glands were immediately transferred into a vessel with culture medium (DMEM/F12 without phenol red), mechanically chopped and digested with collagenase type I (1 mg/ml in DMEM/F12 medium; Sigma Aldrich; Merck KGaA) in a water bath at 37˚C for 30 min. The suspension was further mechanically disintegrated using a glass pipette and poured through a nylon filter into a test tube, followed by centrifugation (200 x g for 10 min at room temperature). The collected cells were subsequently suspended in DMEM/F12 with 10% FBS (FBS, F7524, Sigma-Aldrich; Merck KGaA) and Antibiotic Antimycotic Solution (A5955, Sigma-Aldrich; Merck KGaA) and plated on culture plates (Nalge Nunc International, Penfield, NY, USA) at $1x10^4$/well. The culture was incubated in 37˚C and 5% CO_2. The culture medium was changed every 24 h. At day 4 of culture, the test substances, including eNampt (1 and 100 nM), ACTH (1 μM) and FK866 (10 nM), were added and cells were harvested after 24 h incubation. For the group treated with a combination of eNampt + FK866, 1 μM eNampt and 10 nM FK866 were employed. The incubation medium was centrifuged (200 x g for 10 min at room temperature) and frozen at -36˚C. Obtained cells were treated with TRI Reagent for RNA isolation.

Pituitary gland explants. Rat pituitary glands from 20 ~3-4 months old, 250-300 g, male rats were collected immediately following decapitation. Following neural lobe removal, the glands were halved, preincubated for 30 min (without test substances) and incubated at 37˚C, with continuous shaking (300 oscillations per min), in DMEM/F12 supplemented with 10% FBS and the addition of test substances, including eNampt (10 nM), CRH (1 μM; CRH Ferring®; Ferring Pharmaceuticals, Saint-Prex, Switzerland) and FK866 (10 nM, Selleck Chemicals, Houston, TX, USA). Pituitary glands were harvested after 2 h of incubation. Pituitary gland halves were treated with TRI Reagent (Sigma-Aldrich; Merck KGaA) for RNA isolation. Incubation medium was frozen at -36˚C.

Hypothalamic explants. Details of this method have been described previously by Rucinski *et al* (23). Briefly, rat hypothalami were collected from 20 ~3-4 months old, 250-300 g, male rats immediately following decapitation, preincubated for 30 min (without test substances) and incubated at 37˚C, with continuous shaking (300 oscillations per min), in DMEM/F12 supplemented with 10% FBS and the addition of eNampt (10 nM), KCl (60 mM) or both. Hypothalami were harvested after 2 h of incubation. Explants were treated with TRI Reagent for RNA isolation. Incubation medium was frozen at -36˚C.

Hormone level detection. The sera and incubation media were analysed by ELISA to determine the concentration of aldosterone (cat. no. DE5298 Demeditec Diagnostics GmbH, Kiel, Germany), corticosterone (cat. DEV9922 Demeditec Diagnostics GmbH), ACTH (cat. no. EK-001-21 Phoenix Europe GmbH, Karlsruhe, Germany) and CRH (cat. no. OKEH00625 Aviva Systems Biology, Corp., San Diego, CA, USA). All determinations were performed according to the manufacturers' protocols.

RNA isolation. The methods used have been described previously (24-28). Total RNA was extracted from collected cells, samples of adrenal zones, entire adrenal glands, periadrenal adipose tissue, pituitaries and hypothalami using TRI Reagent and subsequently purified on columns (NucleoSpin RNA XS; Macherey-Nagel, Düren, Germany). 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, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT was performed using the Transcriptor First Strand cDNA Synthesis Kit (cat. no 04379012001 Roche Diagnostics, Basel, Switzerland). RT was performed according to the manufacturer's protocol. The primers used for qPCR (Table I) were designed by Primer 3 software (version 0.4.0, Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). qPCR was performed using a LightCycler 2.0 instrument 4.05 software version (Roche Diagnostics). Using the aforementioned primers, a SYBR Green detection system was applied, as described previously (24-29). Every 20 μl reaction mixture contained 2 μl template cDNA (standard or control), 0.5 μM specific primers and a previously determined optimum $MgCl_2$ concentration (3.5 μM for one reaction). The LightCycler

Table I. Primer sequences used for quantitative polymerase chain reaction.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank accession number	Product length, bp
Nampt	TGATCCCAACAAAAGGTCGAA	CCCACTCACACAAAAGCCTA	NM_177928	238
POMC	CATGACGTACTTCCGGGGAT	TCACCACGGAAAGCAACCTG	XM_017594033	192
Fos	TTTCAACGCGGACTACGAG	AGTTGGCACTAGAGACGGAC	NM_022197	164
HPRT	ATAGAAATAGTGATAGGTCCA	TCTGCATTGTTTTACCAGT	XM_008773659	177

The HPRT gene was used as a reference gene for expression levels. Nampt, nicotinamide phosphoribosyltransferase; POMC, proopiomelanocortin; Fos, Fos proto-oncogene; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

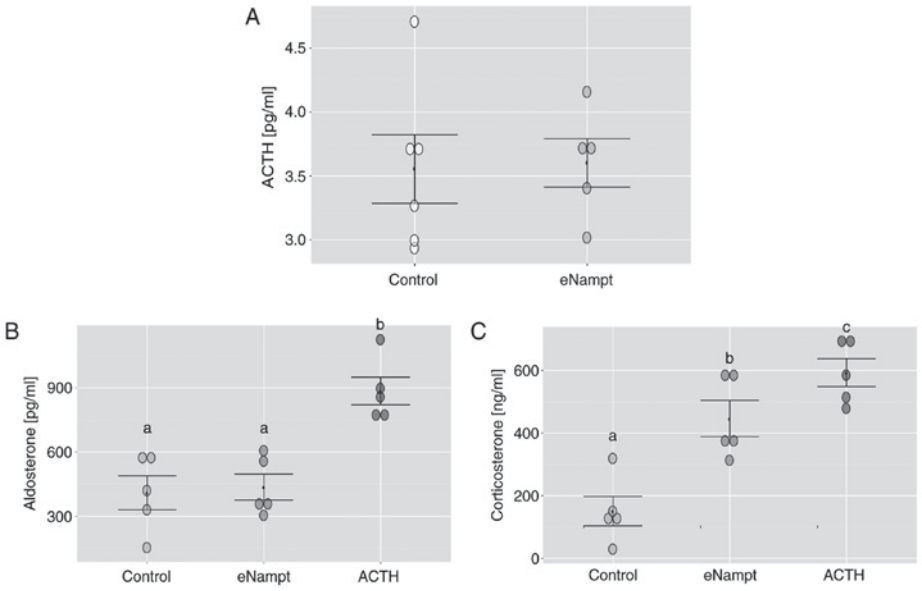

Figure 1. Serum levels of ACTH, aldosterone and corticosterone following i.p. administration of eNampt in rats. At 1 h after i.p. administration of eNampt (4 μg/100 g), ACTH (2.5 μg/100 g) or 0.2 ml saline as a control, the serum levels of (A) ACTH, (B) aldosterone and (C) corticosterone were measured by ELISA. n=5 per group. Data are presented as the mean ± standard error of the mean. Each dot represents an individual result. Groups sharing the same letter are not significantly different, while different letters indicate groups that are significantly different to each other, with P<0.05. ACTH, adrenocorticotropic hormone; i.p., intraperitoneal; eNampt, extracellular nicotinamide phosphoribosyltransferase.

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 a 45 cycles of three-step amplification program: Denaturation at 95˚C for 10 sec, annealing at 56˚C for 5 sec and extension at 72˚C for 10 sec. The specificity of the reaction products was checked by determination of the melting points (0.1˚C/sec transition rate). The gene expression was normalized to HPRT with Pfaffl Ratio method (30) by 4.05 LightCycler 2.0 software.

Statistical analysis. Data are presented as the mean ± standard error of the mean. For multiple comparisons, statistical analysis of the data was performed by using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Calculations were performed using R x64 3.4.1 software with the multcomp library. Following one-way ANOVA, if P<0.05 was obtained, Tukey's post-hoc test was performed and differences were considered to be statistically significant when P<0.05. On the figures, the results of the Tukey's post-hoc test are marked by letters. Groups sharing the same letter are not significantly different to each other, according to the Tukey's post-hoc test. When only two groups were compared, a statistical evaluation of the differences was performed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

In vivo experiments. At 1 h after i.p. eNampt administration, and in the saline-treated control group, CRH was not detectable in the serum of rats (data not shown). At this time-point, serum ACTH levels remained unchanged in the eNampt group compared with the control group (Fig. 1A). Notably, the i.p. eNampt injection did not affect serum aldosterone concentration compared with the control group; however, corticosterone levels were notably elevated in the eNampt group compared with the control group, reaching levels comparable to those induced by ACTH administration (Fig. 1B and C).

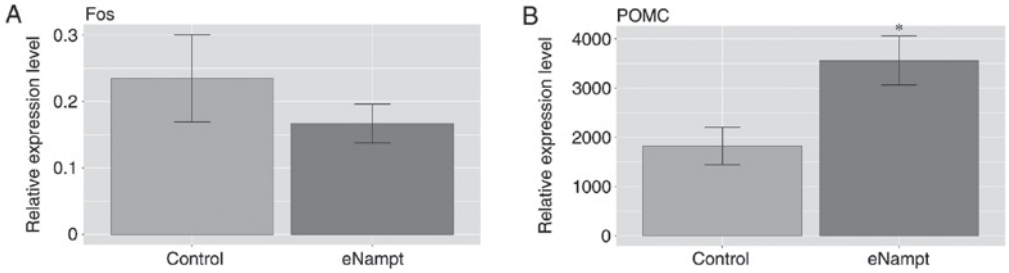

Figure 2. Expression of Fos in the hypothalamus and POMC in the pituitary gland following i.p. eNampt administration in rats. (A) Hypothalamic mRNA expression of Fos was determined at 1 h after administration of eNampt (4 μg/100 g) or 2 ml physiological saline as a control by RT-qPCR. (B) Expression of POMC mRNA in the pituitary gland of rats at 1 h after i.p. administration of eNampt (4 μg/100 g) or 0.2 ml physiologic saline as a control was determined by RT-qPCR. n=5 per group. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. control group. Fos, Fos proto-oncogene; POMC, proopiomelanocortin; i.p., intraperitoneal; eNampt, extracellular nicotinamide phosphoribosyltransferase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

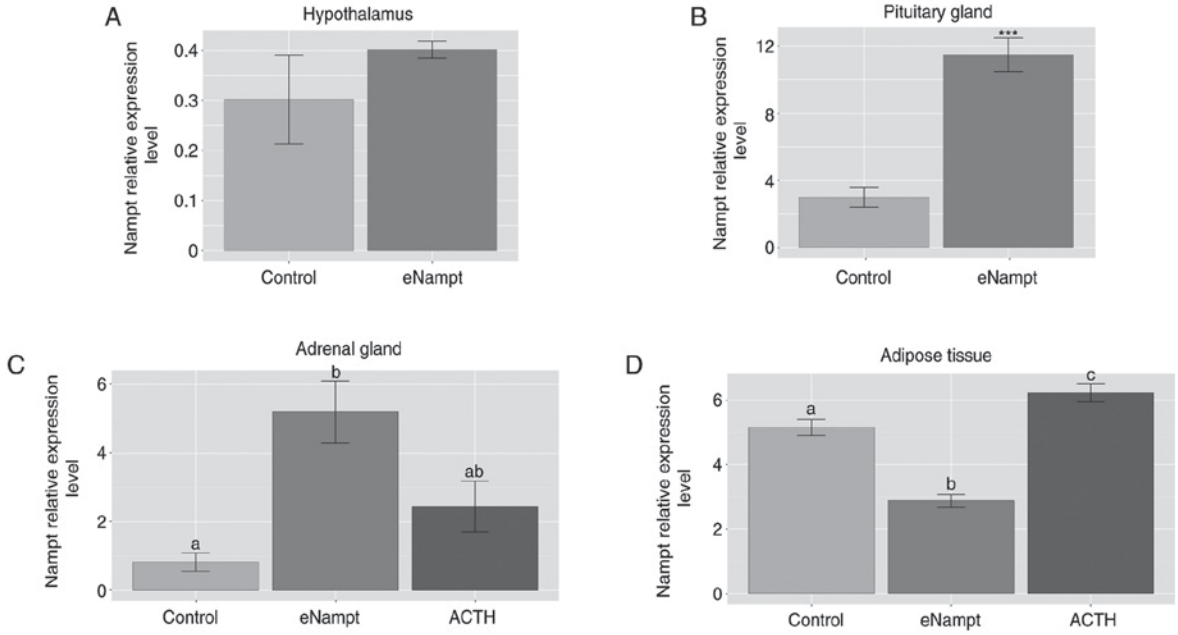

Figure 3. Relative mRNA expression of the Nampt gene in the hypothalamus, pituitary gland, adrenal glands and periadrenal adipose tissue following i.p. eNampt administration in rats. At 1 h after i.p. eNampt (4 μg/100 g), ACTH (2.5 μg/100 g) or 0.2 ml physiological saline administration in rats, reverse transcription-quantitative polymerase chain reaction was performed to determine the mRNA expression levels of Nampt in the (A) hypothalamus, (B) pituitary gland, (C) adrenal gland and (D) periadrenal adipose tissue. n=5 per group. Data are presented as the mean ± standard error of the mean. In part B, ***P<0.001 vs. control group; in parts C and D, groups sharing the same letter are not significantly different, while different letters indicate groups that are significantly different to each other, with P<0.05. Nampt, nicotinamide phosphoribosyltransferase; i.p. intraperitoneal; eNampt, extracellular Nampt; ACTH, adrenocorticotropic hormone.

In the hypothalami of eNampt-treated rats, the mRNA expression levels of Fos proto-oncogene (Fos), also termed c-Fos, were not significantly different compared with levels in the saline-treated group (Fig. 2A). By contrast, in the pituitary glands of eNampt-treated rats, the mRNA expression level of POMC was significantly higher compared with levels in the saline-treated control group (Fig. 2B).

In addition, the present study also investigated whether i.p. administration of eNampt affected Nampt gene expression in the hypothalamus, pituitary glands, adrenal glands and adipose tissue. In eNampt-treated rats, Nampt gene expression was unchanged in the hypothalamus (Fig. 3A), markedly elevated in the pituitary and adrenal glands (Fig. 3B and C), and lowered in the periadrenal adipose tissue (Fig. 3D), compared with the control group. In addition, following administration of ACTH, the expression of Nampt mRNA was increased in the adipose tissue compared with the control group.

In vitro hypothalamic explants. Treatment of hypothalamic explants with eNampt protein exhibited no effect on CRH release into the incubation medium, compared with the control group (Fig. 4A). As expected, treatment with KCl notably increased CRH release compared with the control group, an effect that was prevented by the addition of eNampt. Furthermore, in hypothalamic explants exposed to eNampt, there was a significant increase in Fos and Nampt gene expression compared with the control group (Fig. 4B and C).

In vitro pituitary gland explants. The secretion of ACTH by the pituitary explants was increased in the presence of eNampt,

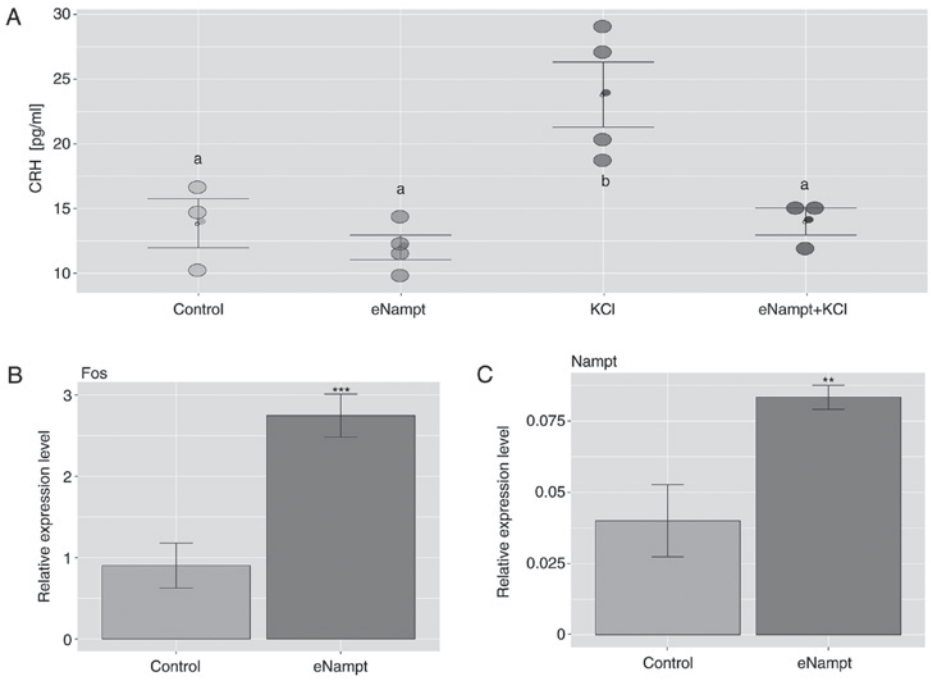

Figure 4. Effects of eNampt on CRH release by hypothalamic explants and the expression of Fos and Nampt genes. (A) ELISA was performed on incubation media following 2 h exposure of hypothalamic explants to eNampt (10 nM), KCl (60 mM) or a combination of both to determine the level of CRH release. Each dot represents an individual result. After 2 h exposure of hypothalamic explants to eNampt (10 nM), KCl (60 mM) or a combination of both, reverse transcription-quantitative polymerase chain reaction was performed to determine the mRNA expression of (B) Fos and (C) Nampt in the hypothalamic explants. Data are presented as the mean ± standard error of the mean. n=4 per group. In part A, groups sharing the same letter are not significantly different, while different letters indicate groups that are significantly different to each other, with $P<0.05$; in parts B and C, $^{**}P<0.01$, $^{***}P<0.001$ vs. control group. Nampt, nicotinamide phosphoribosyltransferase; eNampt, extracellular Nampt; CRH, corticotropin-releasing hormone; Fos, Fos proto-oncogene.

compared with the control group; however, this increase was not statistically significant. By contrast, CRH significantly increased the release of ACTH into the incubation medium, compared with the control group, and this effect was not modified by the presence of eNampt (Fig. 5A). The stimulatory effect of eNampt on the expression of POMC in the pituitary glands was inhibited by administration of FK866, a specific Nampt inhibitor (Fig. 5B). Furthermore, in the pituitary glands, eNampt or CRH administration alone did not alter the level of Nampt gene expression, while joint administration of the two substances significantly increased the level of Nampt gene expression compared with the control group (Fig. 5C).

Freshly isolated adrenocortical cells and primary rat adrenocortical cell cultures. In freshly isolated ZF/R cells, eNampt at a concentration of 0.1 nM led to an increase in the corticosterone output compared with the control group; an effect that was not observed at the higher eNampt concentration 10 nM (Fig. 6A). Notably, in freshly isolated adrenocortical cells, eNampt did not affect ACTH-stimulated corticosterone secretion (Fig. 6B).

Subsequent experiments were performed on primary rat adrenocortical cell cultures, and the cells were exposed to test substances at day 4 for 24 h. The results demonstrated that eNampt at concentrations of 1 and 100 nM did not alter basal aldosterone and corticosterone secretion from primary adrenocortical cell cultures, while the cell response to ACTH was retained (Fig. 7A and B). In cells exposed to eNampt, an increase in Nampt gene expression levels was observed compared with the control group; however, ACTH administration did not affect the expression level of this gene (Fig. 7C).

To assess the potential role of iNampt in the regulation of adrenal steroidogenesis, the present study also performed experiments using FK866, a specific iNampt inhibitor. Exposure of cultured rat adrenocortical cells to FK866 notably lowered basal aldosterone output compared with the control group, and these effects were not altered by the addition of eNampt (Fig. 8A). Furthermore, the stimulatory effect of ACTH on aldosterone output in cultured rat adrenocortical cells was eliminated by FK866 administration (Fig. 8B). Similar effects were observed on corticosterone output following FK866 treatment (Fig. 8C and D).

Discussion

iNampt has an important role in the regulation of intracellular NAD levels and, due to this function, is essential for various metabolic processes. Secreted Nampt (eNampt) is a newly discovered hormone whose function is not yet fully understood. As a result, the role of eNampt in the regulation of the HPA axis remains to be established. Therefore, the current study aimed to investigate the role of Nampt (both iNampt and eNampt) in the regulation of the HPA axis in the rat. The results demonstrated that i.p. injection of eNampt increased the serum levels of corticosterone, potentially by acting at the pituitary level. Furthermore, the results of *in vitro* experiments in the present study indicated that iNampt may exert a tonic stimulatory effect on the basal secretion of aldosterone and corticosterone from cultured rat adrenocortical cells.

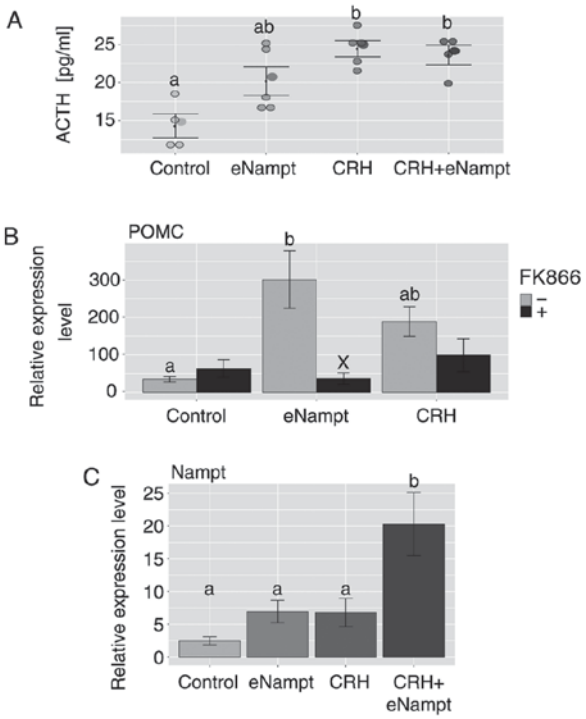

Figure 5. Effects of eNampt, CRH and FK866 on ACTH release by rat pituitary gland explants and the expression of POMC and Nampt genes. (A) ELISA was performed on incubation media following 2 h exposure of pituitary gland explants to eNampt (10 nM), CRH (1 μM) or a combination of both to determine the level of ACTH release. Each dot represents an individual result. (B) After 2 h exposure of pituitary gland explants to eNampt (10 nM) or CRH (1 μM), with or without FK866 (10 nM), RT-qPCR was performed to determine the mRNA expression of POMC in the pituitary gland explants. (C) After 2 h exposure of pituitary gland explants to eNampt (10 nM), CRH (1 μM) or a combination of both, RT-qPCR was performed to determine the mRNA expression of Nampt in the pituitary gland explants. Data are presented as the mean ± standard error of the mean. n=4 per group. Groups sharing the same letter are not significantly different, while different letters indicate groups that are significantly different to each other, with $P<0.05$. In part B, X is used to indicate a statistically significant difference compared with the respective FK866 negative group, with $P<0.001$. Nampt, nicotinamide phosphoribosyltransferase; eNampt, extracellular Nampt; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; POMC, proopiomelanocortin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Furthermore, normal iNampt levels were required to retain the normal response of cultured rat adrenocortical cells to ACTH.

As previously mentioned, administration of eNampt by i.p. injection in rats resulted in a large increase in the serum corticosterone concentration, while the aldosterone levels remained unchanged. This observation indicates that eNampt may stimulate hypothalamic CRH and/or pituitary ACTH secretion. Although eNampt is present in the CSF, its origin has not been explained (10). In the brain, Nampt is primarily expressed in neurons with a cytoplasmic localization (31,32) and it is unlikely that this large protein is able to pass through the blood-brain barrier, which may explain the lack of effect of eNampt on CRH and ACTH secretion at 1 h after injection of this protein. In the present study, at 1 h after i.p. eNampt administration and in control rats, serum CRH was undetectable, and serum ACTH levels remained unchanged in the eNampt group compared with the control group. As the half-life of CRH and ACTH is short (33,34), the current study also investigated the expression of Fos in the hypothalamus and POMC in the pituitary gland of experimental rats. Fos, an immediate-early response gene, is expressed in neurons, and the expression of this gene is employed as a marker of neuroendocrine cell activation (35,36). In the current study, following i.p. eNampt administration, the expression level of Fos in the hypothalamus was unaltered, while POMC gene expression in the pituitary gland was significantly increased, compared with the control group. These results indicate that injected eNampt may not be able to activate hypothalamic CRH neurons, at least acutely. This lack of activation may be due to the fact that large eNampt molecules do not cross the blood-brain barrier. This hypothesis is further supported by the observation that, in hypothalamic explants, eNampt significantly stimulated the expression of the Fos gene following 2 h treatment.

The literature concerning the effect of eNampt on the hypothalamus is scarce. One group reported that administration of eNampt directly into the arcuate nucleus of the rat hypothalamus increased food intake within 24 h, and this effect was accompanied by decreased CRH and CART gene expression, while POMC gene expression remained unaltered (11). Importantly, in their study, the mRNA was isolated from the entire hypothalamus. Another group recently reported that in mouse hypothalamic explants, eNampt enhanced NAD^+/SIRT1 and the neural activity of cells, as estimated by Fos gene expression (7). Thus, regarding the interaction between eNampt and the Fos gene in hypothalamic explants, the observations in rats in the present study confirm previous observations in mice.

Based on the results of the current study and the available literature, it may be hypothesised that the primary site of action of exogenous eNampt within the HPA axis is the pituitary gland. To test this hypothesis, the present study performed experiments *in vitro* with rat pituitary glands. These experiments demonstrated that the exposure of pituitary explants to eNampt did not significantly stimulate ACTH secretion, but the expression of the POMC gene in the pituitary gland was significantly increased, compared with the control group. The effect of eNampt on POMC gene expression was inhibited by the specific inhibitor of Nampt enzymatic activity, FK866 (37). Notably, based on these results, it is unclear whether eNampt enzymatic activity is responsible for the biological effect exerted by eNampt. In addition, whether the enzymatic activity of eNampt is inhibited by FK866 is also debatable, as it is not clear yet whether eNAMPT actually carries out its enzymatic activity in the extracellular environment. The plasma concentrations of the Nampt substrates (Nam, ATP and PRPP) are insufficient to support the Nampt reaction (38) as well as the NMN presence in plasma remains controversial (9,38-40). Therefore, the mechanism of eNampt action remains unclear. Despite this, the results of *in vivo* and *in vitro* experiments in the current study indicate that eNampt may exhibit a direct stimulatory effect on the secretion of ACTH by the pituitary and on POMC gene expression in this gland.

In the existing literature, several publications have reported the presence of iNampt in the pituitary gland; however, they do not provide any information regarding the association of iNampt and eNampt with ACTH. For example, in the pars tuberalis of the sheep pituitary gland, Nampt gene

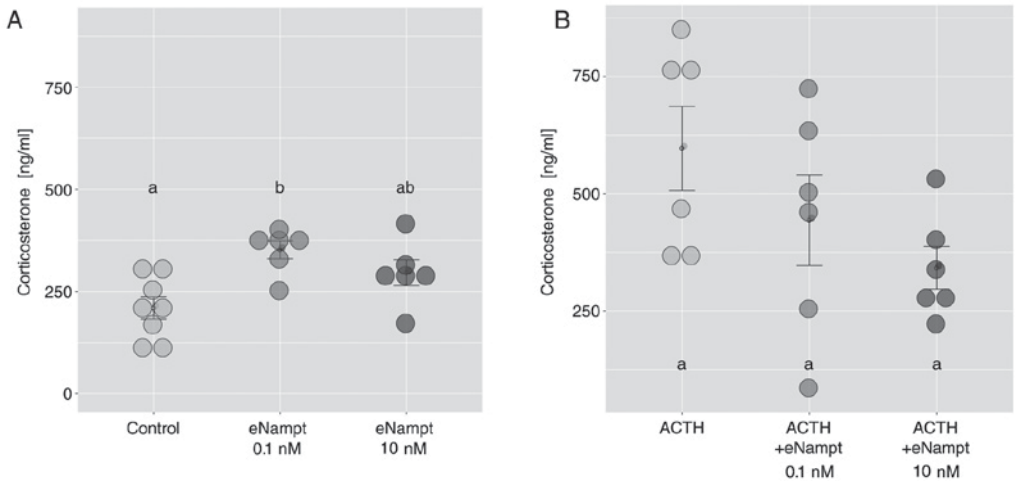

Figure 6. Effects of eNampt on basal and ACTH-stimulated corticosterone output by freshly isolated zona fasciculata/reticularis adrenocortical cells. (A) Cells were exposed to eNampt (0.1 and 10 nM) for 2 h and the levels of corticosterone in the incubation media were determined by ELISA. (B) Cells were exposed to ACTH (1 μM) with or without eNampt (0.1 and 10 nM) for 2 h, and the levels of corticosterone in the incubation media were determined by ELISA. Data are presented as the mean ± standard error, n=8. Each dot represents an individual result. Groups sharing the same letter are not significantly different, while different letters indicate groups that are significantly different to each other, with P<0.05. eNampt, extracellular nicotinamide phosphoribosyltransferase; ACTH, adrenocorticotropic hormone.

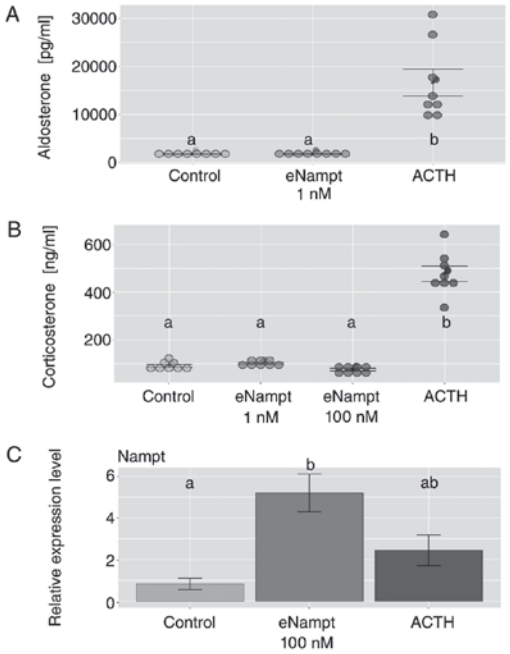

Figure 7. Effects of eNampt on aldosterone and corticosterone output in primary cultures of rat adrenocortical cells. At day 4 of culture, cells were exposed to eNampt (1 and 100 nM) or ACTH (1 μM), and medium was collected after 24 h to perform ELISA to measure the levels of (A) aldosterone and (B) corticosterone in the media. Each dot represents an individual result. (C) At day 4 of culture, cells were exposed to eNampt (100 nM) or ACTH (1 μM), and cells were collected after 24 h for reverse transcription-quantitative polymerase chain reaction analysis of Nampt mRNA expression. Data are presented as the mean ± standard error, n=8. Groups sharing the same letter are not significantly different, while different letters indicate groups that are significantly different to each other, with P<0.05. Nampt, nicotinamide phosphoribosyltransferase; eNampt, extracellular Nampt; ACTH, adrenocorticotropic hormone.

expression was reported to be regulated by melatonin (41,42). Importantly, the pars tuberalis is a structurally distinct part of the adenohypophysis with few or no ACTH cells (43).

The role of eNampt in the regulation of steroidogenesis has been previously investigated in relation to the ovaries and testes and, to a lesser extent, the adrenal cortex. Data have indicated that eNampt is involved in the regulation of reproductive function. Nampt gene expression was identified in primary human granulosa cells and in a human ovarian granulosa-like tumour cell line (44). In these cells, eNampt increased IGF-1-induced steroidogenesis (secretion of progesterone and oestradiol) and cell proliferation. The Nampt gene was also reported to be expressed in bovine granulosa, theca and luteal cells (42). In cultured bovine granulosa cells, eNampt also exhibited a stimulatory effect on the secretion of progesterone and oestradiol, an effect that was associated with increased protein levels of STAR and HSD3B activity (42). The same group also conducted research on the role of eNampt in the gonads of chickens and turkeys (45,46). The expression of the Nampt gene in chicken and turkey steroidogenic cells was similar to that described in humans and cattle. However, in cultured chicken granulosa cells, eNampt notably inhibited progesterone secretion, an effect that was associated with decreased STAR and HSD3B protein levels. In another type of steroidogenic cell, Leydig cells of the testis, the expression of the Nampt gene has also been reported in prepubertal and adult chicken testes (47). However, in the existing literature, no reliable data concerning the effect of eNampt on the secretion of steroid hormones by the Leydig cells was available.

To the best of our knowledge, there is only one report concerning the association between Nampt and steroidogenesis in the adrenal glands (48). The authors investigated the role of eNampt in the H295R human adrenal cell line. In these cells, eNampt markedly induced steroid release into the cell culture media within 4 h, an effect that was associated with the upregulation of STAR mRNA and protein expression. The authors also provided data indicating that the effects of eNampt on steroidogenesis may be mediated through the MAPK and phosphatidylinositol 3-kinase/Akt signalling pathways.

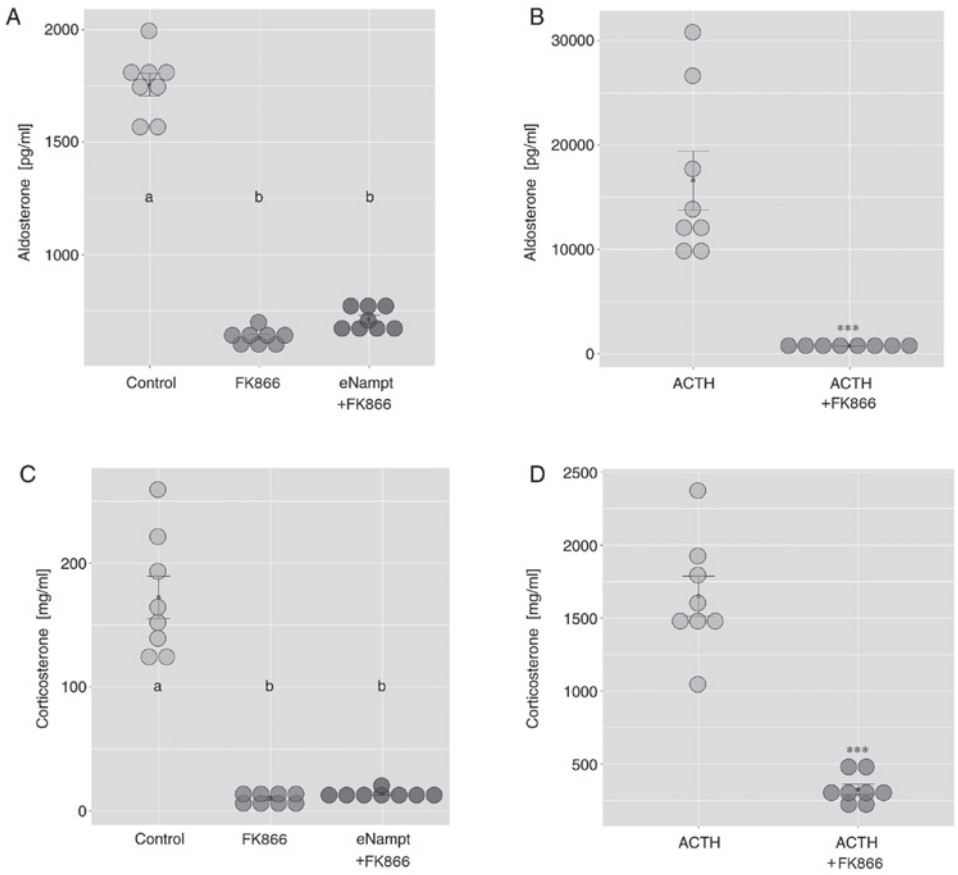

Figure 8. Effects of FK866 on basal and ACTH-stimulated aldosterone and corticosterone output in primary cultures of rat adrenocortical cells. At day 4 of culture, cells were exposed to FK866 (10 nM) with or without eNampt (1 μM), or ACTH (1 μM) with or without FK866 (10 nM), and medium was collected after 24 h. The effect of FK866 on (A) basal and (B) ACTH-stimulated aldosterone release was determined by ELISA. The effect of FK866 on (C) basal and (D) ACTH-stimulated corticosterone release was determined by ELISA. Each dot represents an individual result. Data are presented as the mean ± standard error, n=8. In parts A and C, groups sharing the same letter are not significantly different, while different letters indicate groups that are significantly different to each other, with $P<0.05$; in parts B and D, $^{***}P<0.001$ vs. ACTH alone group. ACTH, adrenocorticotropic hormone; eNampt, extracellular Nampt.

As mentioned earlier, in the present study, i.p. administration of eNampt significantly increased the serum corticosterone levels within 1 h, but not aldosterone levels. In these rats, the serum corticosterone levels were comparable to those induced by ACTH administration. In addition, in the adrenal gland, Nampt gene expression levels were notably elevated following eNampt administration, compared with the control group. Based on these results, it was further investigated, using *in vitro* experiments, whether eNampt exerts a direct effect on adrenal steroidogenesis. In freshly isolated ZF/R cells of the rat adrenal cortex, eNampt at a concentration of 0.1 nM, but not 10 nM, led to an increase in corticosterone output. In addition, none of the eNampt concentrations influenced the ACTH-stimulated corticosterone output of freshly isolated adrenocortical cells. Based on these results, in freshly isolated adrenocortical cells, the effects of eNampt on adrenal steroidogenesis may depend on the concentration of this adipokine; lower doses of eNampt appear to exert a stimulatory effect on steroidogenesis. However, the role of iNampt in the regulation of steroidogenesis in the adrenal glands of rats remains an open question. To investigate this, the present study performed experiments involving FK866, a specific Nampt enzymatic action inhibitor. Exposure of cultured rat adrenocortical cells to FK866 markedly lowered basal aldosterone and corticosterone output within 24 h, and these effects were also observed in the presence of 1 μM eNampt. Furthermore, in the presence of FK866, the stimulatory effects of ACTH on aldosterone and corticosterone secretion were eliminated. To exclude a potential toxic effect of FK866 on cultured rat adrenocortical cells, the proliferation rate of cells was also investigated (data not shown); the results demonstrated that FK866 did not interfere with the proliferation or survival rates of cells.

Therefore, regarding the effect of eNampt on adrenal steroidogenesis in the rat, the results of the current study indicate that low concentrations of this adipokine may stimulate the secretion of corticosteroids by cultured adrenocortical cells. Furthermore, for the first time, the present study obtained indicating a physiological role of iNampt in the regulation of steroidogenesis. iNampt appeared to be necessary for the normal responses of adrenocortical cells to ACTH. Finally, the results of the current study also suggest that iNampt may exert a tonic stimulatory effect on the secretion of aldosterone and corticosterone by cultured rat adrenocortical cells, evidence of which is provided by the reduction in the basal secretion of these hormones in the presence of FK866, a specific Nampt enzymatic activity inhibitor (40).

In the steroidogenic pathway, cytochrome P450 enzymes are responsible for the hydroxylation and cleavage of the steroid intermediates and hormones, and their function is dependent

on reduced NAD phosphate (49). The mitochondria of cells that secrete steroid hormones are able to generate NADP from NAD in a reaction that is catalysed by nicotinamide nucleotide transhydrogenase (NNT) (50). Thus, the availability of NAD in adrenocortical cells may determine normal steroidogenesis. Importantly, in this regard, FK866 is a highly specific non-competitive inhibitor of iNampt enzymatic action (40). Therefore, in the present study, the decline in the basal secretion of aldosterone and corticosterone by rat adrenocortical cells cultured in the presence of FK866 indicates that iNampt may exert a tonic stimulatory effect on the secretion of corticosteroids. This hypothesis is consistent with the latest clinical reports that indicate that NNT mutations maybe responsible for primary adrenal insufficiency, which refers to combined mineralocorticoid and glucocorticoid deficiency (14,42-53). Patients with these genetic mutations also suffer from oxidative stress.

Another important observation described in the current study is the stimulation of Nampt gene expression by eNampt. This effect was observed in hypothalamic and pituitary gland explants, and in cultured rat adrenocortical cells. A similar effect was also observed in primary bovine ovary cells exposed to eNampt, as reported in a previous study (14). It is possible that this effect may be associated with the regulation of redox imbalance and oxidative stress in these cells (54-56). However, in the present study, Nampt gene expression following i.p. administration of eNampt differed notably compared with *in vitro* results. In the experimental model, Nampt gene expression was unchanged in the hypothalamus, notably elevated in pituitary and adrenal glands, and lowered in adipose tissue. These results support our suggestion that eNampt administered by i.p. injection does not cross the blood-brain barrier. However, the results of *in vivo* experiments may not be comparable to the results obtained in *in vitro* experiments. It is established that alterations *in vivo* reflect the eNampt-induced alterations in the whole body and may not necessarily represent a direct impact of eNampt on a specific organ.

In conclusion, the present study has demonstrated that, in the rat, i.p. injection of eNampt increased the serum levels of corticosterone, which may occur via action at the pituitary gland level. The results of *in vitro* experiments also demonstrated that eNampt treatment increased the level of POMC gene expression in isolated pituitary glands and induced ACTH secretion. It was also indicated that iNampt may exert a tonic stimulatory effect on the basal secretion of aldosterone and corticosterone from cultured rat adrenocortical cells. In addition, the results indicate that physiological iNampt enzymatic activity is required to maintain the normal response of adrenocortical cells to ACTH. Finally, proper understanding of endocrine crosstalk between hypothalamo-pituitary-adrenal axis and other tissues could help better understand mechanisms of metabolic and mineral disorders.

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