ORIGINAL PAPER

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# Mitochondrial sirtuins in the rat adrenal gland: location within the glands of males and females, hormonal and developmental regulation of gene expressions

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**Abstract**

**Introduction.** Sirtuins are NAD dependent class III histone deacetylases. In adrenal cortex mitochondria are able to transform — *via* nicotinamide nucleotide transhydrogenase (NNT) — NAD into NADPH, which is required for steroidogenesis. These findings suggest that sirtuins expressed in mitochondria, Sirt3, Sirt4 and Sirt5, may be associated with adrenal steroidogenesis. Therefore, the purpose of this study was to characterize the expression of mitochondrial sirtuins (Sirt3–5) in individual compartments of rat adrenal cortex, their developmental regulation and to demonstrate whether their expression is dependent on adrenocorticotrophic hormone (ACTH) and Nampt (nicotinamide phosphoribosyltransferase also known as visfatin/PBEF), the rate-limiting enzyme in the regulation of mammalian NAD synthesis.

**Material and methods.** Studies were performed on rat adrenal glands or on primary culture of rat adrenocortical cells. Expression of mitochondrial sirtuins (Sirt3–5) was evaluated by Affymetrix microarray system or QPCR. The bulk of data were extracted from our earlier experiments which have been reanalyzed in regard to Sirt3–5 mRNAs expression levels and — if necessary — validated by QPCR.

**Results.** Sirt3–5 were expressed throughout the rat adrenal, with the highest expression level of Sirt5. The level of expression of all sirtuins is higher in the zona glomerulosa (ZG) and zona fasciculata/reticularis (ZF/R) than in the adrenal medulla. Sirt3 and Sirt5 expression levels were similar in adult male and female rats, while Sirt4 expression level was higher in females. As revealed by analysis of the available open database, no significant changes in Sirt3–5 expression levels in whole adrenal glands were observed up to week 104 of life of both male and female rats. Moreover, 60 min after intraperitoneal ACTH injection the expression level of Sirt3 in the entire gland was elevated while Sirt5 expression level lowered. On the other hand, chronic ACTH infusion (48 h) did not change expression of studied sirtuins. In cultured cells, ACTH greatly increased the expression levels of the Sirt4 and Sirt5. In cultured cells, Fk866 — a highly specific competitive inhibitor of Nampt — reduced expression level of Sirt5 only. In enucleation-induced regenerating rat adrenal, the expression levels of all studied sirtuins were significantly reduced in relation to the control group. Finally, in primary rat adrenal culture the FCS depletion elevates the Sirt3 and Sirt4 expression levels and downregulates Sirt5 expression.

**Conclusions.** Sirt3–5 are expressed throughout the rat adrenal, with the highest expression levels in adrenal cortex. Performed experiments (ACTH stimulation, FCS depletion, regeneration) suggest that in the adrenal cortex, the mitochondrial Sirt5 is the primary mitochondrial sirtuin involved in regulating the biological activity of adrenocortical cells. Our results also suggest that normal levels of intracellular Nampt (iNampt) enzymatic activity are required to maintain normal (control) levels of Sirt5 mRNA in cultured cells. *(Folia Histochemica et Cytobiologica 2017, Vol. 55, No. 4, 190–202)*

**Key words:** rat; adrenal gland; mitochondrial sirtuins; ACTH; ontogenesis; adrenal regeneration; Nampt; microarrays; QPCR

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## Introduction

Sirtuins (Sirt), important energy status sensors, are class III histone deacetylases, which need NAD for their catalytic activity [1]. Sirtuins have been implicated in the regulation of metabolism, stress responses, neuroprotection and aging. Because of their dependency on NAD+, they are highly sensitive to the metabolic state of the cell, among other to caloric restrictions. Of importance is that sirtuins can also control mitochondrial biogenesis. The mammalian genome encodes seven sirtuin proteins (Sirt1 through Sirt7). Three mammalian sirtuins, Sirt3, 4, and 5, are localized to mitochondria [2–8].

It is well known that mitochondria are crucial intracellular organelles involved in energy production, metabolism, and intracellular signaling. Moreover they also play the pivotal role in steroidogenesis. In this aspect, the role of Sirt3, which is located in the mitochondrial matrix, is not fully understood. It has been demonstrated that Sirt3 may regulate the activity of acetyl-CoA synthetase (AceCS), an enzyme involved in acetyl-CoA formation, an intermediate which is also required for cholesterol and fatty acid synthesis [6, 9]. Furthermore, convincing data indicate that in humans, Sirt4, *via* ADP-ribosylation, controls the activity of glutamate dehydrogenase regulating the amount of $\alpha$-ketoglutarate produced. Subsequently, $\alpha$-ketoglutarate can be used in the Krebs cycle, leading to the production of ATP [5, 6]. The least known is the Sirt5 function. It has been suggested that this Sirt isoform modulates numerous acyl modifications and glutarylation in both mitochondrial and extra-mitochondrial compartments [10, 11].

It has been suggested that all sirtuins function as sensors of the cellular energy status represented by NAD or NAD/NADH ratio [7, 12]. Nampt (nicotinamide phosphoribosyltransferase), on the other hand, is the rate-limiting enzyme of the most important pathway for the regulation of mammalian NAD synthesis [13–15]. Two isoforms of Nampt exist, an intracellular form (iNampt) and an extracellular form (eNampt). iNampt plays a critical role in maintaining the activity of NAD-dependent enzymes while the biological role of eNampt, which is secreted by various types of cells, is not fully recognized [13, 14]. Therefore we suggest that eNampt levels may influence sirtuin expression levels.

Recent findings provide evidence of the important physiological role of Nampt in the regulation of adrenocortical secretory function. Adrenocortical cells show a high level of specialization associated with the biosynthesis and secretion of steroid hormones. In these cells mitochondria play an important role in

the transport of electrons, a process which is linked to the biosynthesis of steroids [16–19].

In steroidogenic pathway cytochrome P450 enzymes are responsible for the hydroxylation and cleavage of the steroid intermediates and hormones, and their function is dependent on NADPH [20]. It is well documented that mitochondria of steroidogenic cells are able to generate NADPH *via* three major pathways; one of them is catalyzed by nicotinamide nucleotide transhydrogenase (NNT) [21]. Thus, the availability of NAD in adrenocortical cells may determine normal steroidogenesis [22]. This suggestion is supported by recent findings demonstrating that NNT mutations can be the cause of primary adrenal insufficiency (combined mineralocorticoid and glucocorticoid deficiency) [22–24]. Patients with these genetic mutations also suffer from oxidative stress.

Our recent studies show that in rats, acute intraperitoneal (*i.p.*) administration of extracellular Nampt (eNampt) elevates plasma corticosterone levels. However, eNampt seems to not have the direct effect on adrenal cortex cells, but rather through stimulating the pituitary gland [25]. Both, sirtuin dependence on NAD produced by iNampt enzymatic activity and partial dependence of adrenal steroidogenesis from NNT, transforming NAD into NADP, suggest that mitochondrial sirtuins may be associated with adrenal steroidogenesis. Therefore, the purpose of this study was to characterize the expression of mitochondrial sirtuins in individual compartments of rat adrenal gland. Moreover we investigated Sirt3–5 developmental regulation and their expression levels in relation to ACTH action. In this work we have used our own data from numerous microarray experiments (reanalyses) and the data available from open database (meta-analysis). Furthermore, we performed experiments on the effect of ACTH, Nampt and the Fk866 (a highly specific competitive inhibitor of Nampt) on Sirt3, Sirt4 and Sirt5 expression levels in rat adrenal gland.

## Material and methods

**Animals and reagents.** Male and female Wistar rats from the Laboratory Animal Breeding Center, Department of Toxicology, Poznan University of Medical Sciences were used. The animals were maintained under 14:10 h light-dark cycle (illumination onset at 06.00 a.m.), at 23°C, with free access to standard diet and tap water. The number of rats, their sex, age and body mass used in the study are given in the descriptions of the individual experiments or descriptions of the figures. The study protocols were approved by the Local Ethics Committee for Animal Studies (protocols no. 11/2015 and 75/2016). If not otherwise stated, all reagents

were obtained from Sigma-Aldrich (St. Louis, MO, USA) or from Avantor Performance Materials Poland S.A. (Gliwice, Poland).

**Sampling of the rat adrenal gland.** After decapitation (between 09:00 and 10:00 h), adrenal glands were promptly removed, freed of adherent fat and processed for study. Studies were performed either on the entire adrenals or on fragments of adrenal compartments. In the latter case, adrenal glands were decapsulated to separate zona glomerulosa (ZG). From the remaining part of the gland pieces of zona fasciculata/reticularis (ZF/R) and adrenal medulla (M) were taken. Identification of adrenal compartments was anatomy-based and conducted under a stereomicroscope. Samples were used for microarray experiments, as described earlier [26, 27].

***In vivo* experiments. *Effects of ACTH on sirtuin genes expression in adrenal glands.*** In these experiments 32 rats (4 rats per group) were used. Adult male rats were administered with ACTH (Organon Pharmaceuticals, West Orange, USA) and/or antagonist of ACTH receptor. ACTH receptor antagonist with amino acid sequence GKVLKKRR [28, 29] was synthesized by Nowazym (Poznan, Poland).

In acute experiments ACTH was administered intraperitoneally (*i.p.*) at a dose 3 nmol/100 g body weight and/or ACTH receptor antagonist (6 nmol/100 g) and animals were sacrificed after 60 min. Control rats were i.p. administered with 0.9% of saline solution.

In experiments with chronic ACTH and/or ACTH receptor antagonist administration Alzet osmotic minipumps (Model103D) were implanted subcutaneously. Rats were infused with ACTH at the dose 2 nmol/24 h/100 g and/or ACTH receptor inhibitor at a dose 4 nmol/24 h/100 g. Minipumps filled with 0.9% saline were implanted into the control group. Animals were sacrificed 48 h after minipumps implantation. The adrenal glands were freed from adherent fat, weighted and subjected to the RNA isolation.

***Enucleation-induced rat adrenal gland regeneration.*** In this experiment, we used the results of our earlier research using Affymetrix microarray, as described in earlier publications [30, 31]. These microarrays were re-analyzed in relation to the expression of tested mitochondrial sirtuins. In the 18 rats (female Wistar rats, final body weight 100–150 g), under standard ketamine and xylazine anesthesia, *via* dorsal approach, both adrenal glands were enucleated according to the classic method, as described earlier [30–33]. The operated rats were given *ad libitum* 0.9% NaCl to drink for 3 days. One, 2, 3, 5, 8 and 15 days after surgery the rats were sacrificed, and their regenerating adrenals immediately removed, freed of adherent tissue and frozen at –20°C for gene expression studies by means of microarray. Adrenals from 3 sham operated rats (day 1 after sham surgery) served as a control adrenal glands.

**Rat primary adrenocortical cell culture.** Method of culturing rat adrenocortical cells was described earlier [34–36]. Briefly, adrenals were obtained from 40, 20–22-day-old male rats. After decapitation adrenal glands were immediately transferred into vessel with culture medium (DMEM//F12, without Phenol Red, Sigma-Aldrich), mechanically chopped and digested with collagenase (Collagenase type I, Sigma-Aldrich, SCR103, mg/mL), in water bath at 37°C for 30 minutes. The suspension was further mechanically disintegrated using glass pipette and then poured through a nylon filter into a test tube and centrifuged for 10 minutes at $1000 \times$ g. The collected cells were then suspended in DMEM/F12 with 10% fetal calf serum (FCS) and Antibiotic Antimycotic Solution (penicillin, streptomycin and amphotericin, all purchased from Sigma-Aldrich) and plated into 96-well culture plates (Nunc International, Rochester, NY, USA) ($1 \times 10^4$ living cells/well). Cells were cultured for 96 h, culture medium was changed every 24 h.

***Expression of adrenal mitochondrial sirtuins in FCS-depleted culture medium.*** At day 4 of culture, incubation was carried out in the presence or absence of FCS (10%) (3 repetitions per each condition). Cells were harvested and processed for microarray analysis. This data comes from the reanalysis of data presented by Trejter *et al.* [26].

***Effects of ACTH and eNampt on expression of mitochondrial sirtuins in cultured cells.*** At day 3 of culture (4 repetitions per each group) (medium with FCS) ACTH ($1 \times 10^{-6}$ M), eNampt ($1 \times 10^{-8}$ M) and/or Fk866 ($1 \times 10^{-7}$ M) were added and cell harvested after 24 h. Incubation medium was centrifuged and frozen in $-36°C$. Obtained cells were proceeded to RNA isolation. Recombinant human eNampt protein was purchased from Biovendor R&D (Brno, Czech Republic) and specific Nampt inhibitor Fk866 from ApexBio (Houston, USA).

**Analysis of microarray dataset obtained from adrenals of male and female rats during postnatal ontogenesis.** Data on the expression of mitochondrial sirtuins in adrenal glands of male and female rats during postnatal ontogenesis were obtained from open database. These studies were performed on Fischer 344 strain rats aged 2, 6, 21 and 104 weeks [37]. Experimental data were obtained from Gene Expression Omnibus database, accession number: GSE53960 (available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53960) (January 2017).

**RNA isolation.** From collected cells (0.2–1.2 $\times 10^6$ cells), a sample of adrenal zones and of entire adrenal glands (for ZG and adrenal medulla 3–5 mg, for ZF/R 10–20 mg) total

**Table 1.** QPCR analyzes of mitochondrial sirtuins (Sirt3–5). Oligonucleotide sequences for sense (S) and antisense (A) primers are shown. HPRT (hypoxanthine guanine phosphoribosyltransferase) was the reference gene

| Gene | Forward | Reverse | Forward primer position | Reverse primer position | Product length (bp) | Accession number |
|------|---------|---------|-------------------------|-------------------------|---------------------|------------------|
| Sirt3 | CTTTGCTCTGAGTCCTCGAA | ATAGTCTGTCTTATCCGTCCT | 795-814 | 955-935 | 140 | NM_001106313.2 |
| Sirt4 | ACACTGGGCTCTGAGCAACT | AGTCTGCTCCCCACAGCTC | 610-629 | 766-748 | 157 | NM_001107147.1 |
| Sirt5 | CAGCCACCAACAGATTCAGG | GTCACTTAGCCGACTGTCT | 886-905 | 1122-1104 | 218 | NM_001004256.1 |
| HPRT | ATAGAAATAGTGATAGGTCCA | TCTGCATTGTTTTACCAGT | 402-423 | 578-560 | 177 | XM_008773659 |

RNA was extracted using TRI Reagent (Sigma-Aldrich) and then, purified on columns (Nucleospin RNA XS, Macherey-Nagel, Düren, Germany). The amount of total RNA was determined by optical density at 260 nm. The purity was estimated by 260/280 nm absorption ratio (NanoDrop spectrophotometer — ND-1000, Thermo Scientific).

**Reverse transcription.** Reverse transcription with oligodT primers was performed using Transcriptor High Fidelity Reverse Transcriptase enzyme blend for high fidelity two-step RT-PCR of RNA (Roche, Basel, Switzerland). Reverse transcription was performed according to manufacturer's protocol. Final volume of transcription reaction = 20 $\mu$L.

**QPCR.** QPCR was performed by means of the Lightcycler 2.0 instrument (Roche) with the 4.05 software version. SYBR green detection system was applied as described earlier [30, 31, 38–40]. Every of 20 $\mu$L reaction mixtures contained 2 $\mu$L template cDNA (standard or control), 0.5 $\mu$M of specific primer and a 3.5 $\mu$M MgCl$_2$. LightCyclerFast-Start DNA Master SYBR Green I mix (Roche) was used. The real-time PCR program included 10 min denaturation step to activate the Taq DNA Polymerase, followed by a 45 cycles of 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. Specificity of reaction products was checked by determination of melting points (0.1°C/s transition rate).

The primers used (Table 1) were designed by Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). HPRT was used as the reference gene. The primers were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

**Affymetrix microarray.** Presented in this publication, all data obtained with the microarray methodology was obtained from the reanalysis of our previous experiments, which have been partially published [26, 31]. Affymetrix Rat Gene 1.1 ST Array method was used as described above. Shortly, isolated total mRNA (100 ng) was subjected to two rounds sense cDNA amplification (Ambion WT Expression Kit, Thermo Fischer Scientific, Waltham, Mass., USA). The obtained cDNA was used for biotin labeling and fragmentation by AffymetrixGeneChip WT Terminal Labeling and Hybridization (Affymetrix). Microarray results were analyzed using Bioconductor package of R language. The obtained CEL files were imported into downstream data analysis software. All of presented analysis and graphs were performed by Bioconductor and R programming language [41]. Each CEL file was merged with a description file. In order to conduct background correction, normalization and summarization of results, we used Robust Multiarray Averaging (RMA) algorithm. Statistical significance of analyzed genes was examined by moderated *t*-statistics from the empirical Bayes method. The obtained *p* values were corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate (statistical method incorporated into Bioconductor calculations) [42]. From such prepared data expression of mitochondrial sirtuins were extracted.

**Statistical methods for QPCR analysis.** QPCR data were expressed as means ± SE. In the case of multiple comparisons statistical analysis of the data was performed by using one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Calculation was made by means of R environment with multcomp library. Results were considered as statistically significant when *p* values from ANOVA were lower than 0.05. In such cases *post-hoc* Tukey's HSD test was performed. In this case, the threshold of statistical significance was set again to 0.05. On the figures results of Tukey's HSD test were marked by letters. Groups sharing the same letter are not significantly different according to Tukey's HSD test. In the case of comparisons of two groups, a statistical evaluation of the differences was carried out using the Student's t test.

# Results

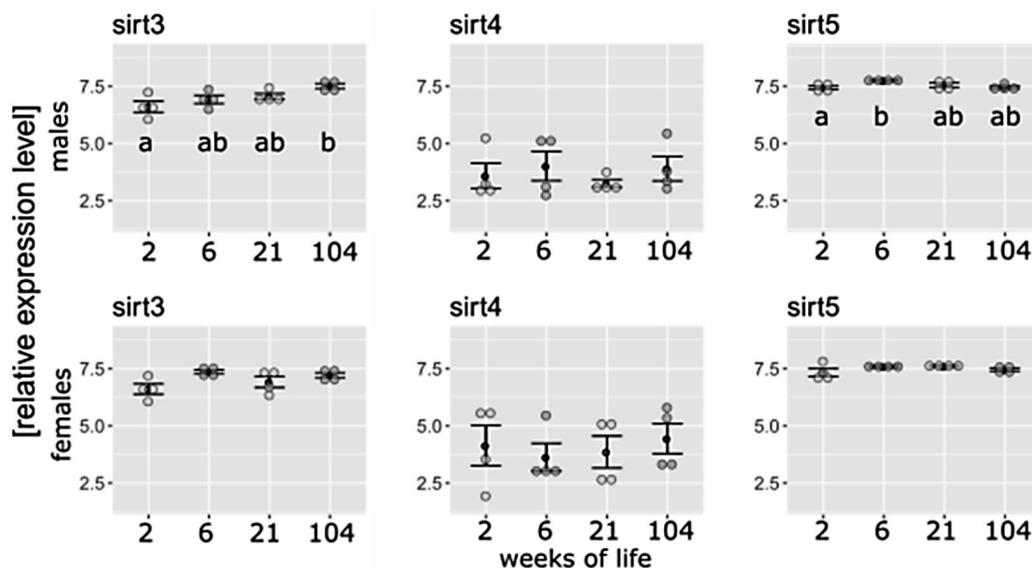## *Mitochondrial sirtuins expression in rat adrenal gland*

As demonstrated in Figure 1A, Sirt3–5 are expressed throughout the rat adrenal, with the highest expres-
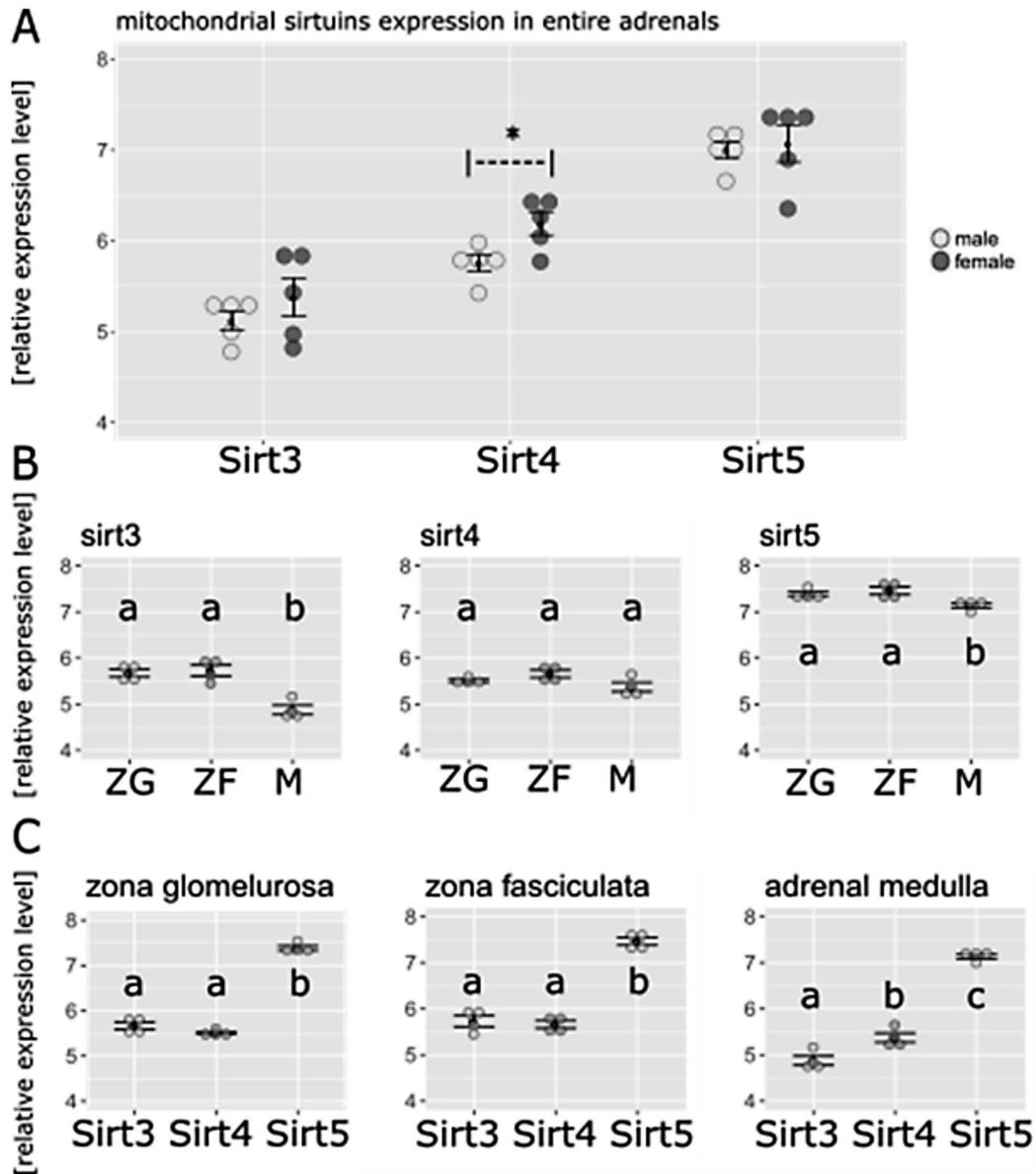
**Figure 1.** Relative expression levels of mitochondrial sirtuins in rat adrenal glands. **A.** Expression levels in samples from entire adrenal glands of adult male and female Wistar rats; **B.** Expression in individual compartments of adrenal gland of adult males; **C.** Expression levels of mitochondrial sirtuins in glomerulosa (ZG) and fasciculata zones (ZF) and in medulla (M) of adult male rats. Reanalyzed microarray data obtained from [26, 27]. Data expressed as means ± SE. Each circle represents a single rat. A. Comparisons of data in relation to male rats — Student's t test: *$p < 0.05$; B and C. One-way analysis of variance (ANOVA) followed by Tukey's HSD test. Groups sharing the same letter are not significantly different according to Tukey's HSD test.

sion level of Sirt5. Sirt3 and Sirt5 expression levels are similar in mature male and female rats, while Sirt4 expression is higher in females. The levels of expression of Sirt3 and 5 are higher in the ZG and ZF/R zones than in the adrenal medulla while no difference is seen in case of Sirt4 (Fig. 1B). In all adrenal compartments, the Sirt5 expression level is highest. In adrenal medulla expression levels of Sirt4 and Sirt5 are higher than Sirt3 (Fig. 1C).

To gain an insight into the developmental regulation of mitochondrial sirtuins in the rat we performed the analysis of data deposited by Yu *et al*. [37]. Studies were performed on Fischer 344 strain rats aged 2, 6, 21 and 104 weeks, and the entire glands were sampled (Fig. 2). Only in male rats Sirt3 gene expression level was statistically significantly higher at 104 weeks of age than at 2 weeks of age. We also revealed an increase in Sirt5 gene expression level in the adrenal glands

**Figure 2.** Relative expression levels of mitochondrial sirtuins in entire adrenal glands of Fischer 344 male and female rats in course of ontogenesis. The analysis of data published by Yu *et al.* [37]. Experimental data were obtained from Gene Expression Omnibus database, accession number: GSE53960 (available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53960) (January 2017). Data expressed as means ± SE. Each circle represents a single rat. Statistical analysis of the data was performed by using one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Groups sharing the same letter are not significantly different according to Tukey's HSD test. In the remaining groups *p* values from ANOVA were higher than 0.05.

of 6-week male rats. In contrast to males, females did not show any changes in the Sirt3–5 expression level in the examined periods of ontogenesis. There were also no differences in the gene expression levels of the tested sirtuins between males and females.

### ACTH and adrenal mitochondrial sirtuins expression
The administration of ACTH within 60 minutes increases the Sirt3 gene expression level in the entire adrenal gland of the rat (Fig. 3). The Sirt5 gene's expression level decreases after administration of the ACTH antagonist, an effect reversed by corticotropin administration. In acute experiment, neither ACTH nor its inhibitor change in the adrenal glands expression of the Sirt4 gene. In the experiment with chronic administration of ACTH and/or its antagonist, the Sirt3–5 genes' expression level does not change.

Next, we investigated the effect of ACTH on mitochondrial sirtuins expression in primary adrenocortical cell culture. As shown in Figure 4, 24-hour exposure of cells to ACTH greatly increases the expression of Sirt4 and Sirt5 genes in cultured cells.

### FCS depletion experiment
In another experiment, we traced the expression of mitochondrial sirtuins in adrenocortical cells cultured in FCS-depleted medium. Under such conditions, in cultured rat adrenocortical cells the expression levels of Sirt3 and Sirt4 increase, while Sirt5 expression decreases (Fig. 5).

### The dependence of Sirt3–5 genes expression in adrenocortical cells on Nampt
In these experiments, rat adrenocortical cells were cultured in the presence of eNampt and/or Fk866, a specific iNampt inhibitor. Compared to the control group, the 24-hour exposure of cultured cells to eNampt does not change the Sirt3–5 expression in the examined cells. However, in the presence of Fk866 expression level of the Sirt5 gene decreases significantly and is not modified by eNampt administration (Fig. 6). In contrast, the expression of the Sirt4 gene increases in the presence of eNampt and Fk866, while neither eNampt nor Fk866 influence the expression of the gene.

### Mitochondrial sirtuins expression during enucleation-induced adrenal regeneration
In the regenerating rat adrenal, the expression of all sirtuins studied was significantly reduced in relation to the control group (Fig. 7). In the postoperative period expression of mitochondrial sirtuins gradually increased. The levels of expression of the tested sirtuin genes are not statistically different from the level of expression of these genes in the adrenal glands from
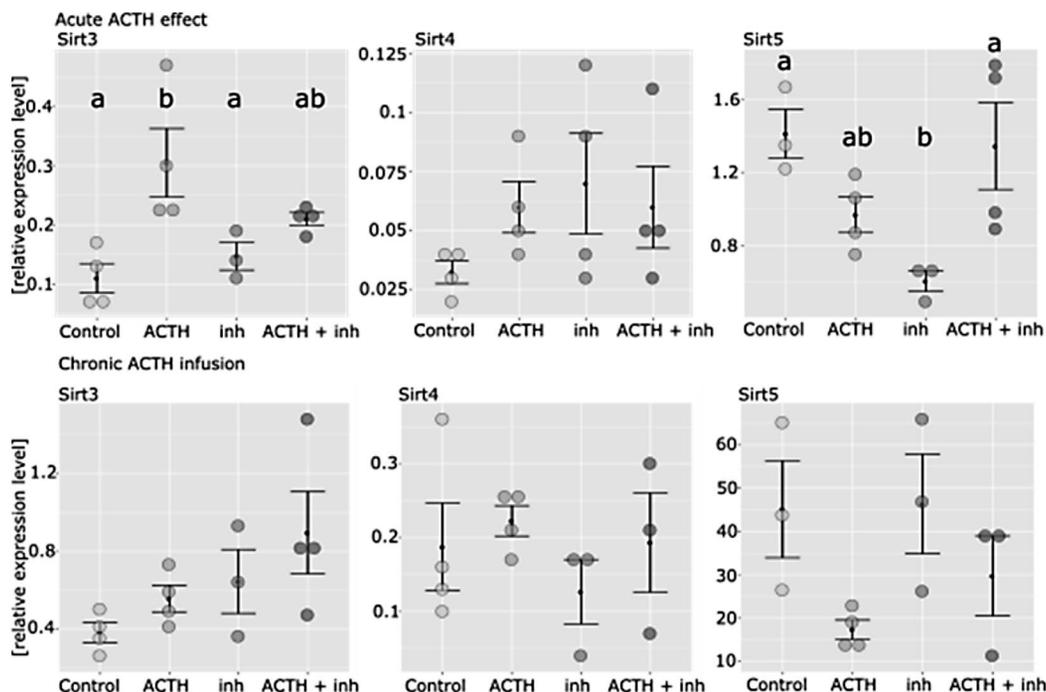
**Figure 3.** Effects of ACTH on mitochondrial sirtuin genes expression levels in adrenal glands of adult male rats. Acute experiment: ACTH was administered *i.p.* (3 nmol/100 g body weight) and/or antagonist of ACTH receptor (inh; 6 nmol/100 g body weight) and sacrificed after 60 min. In chronic experiment ACTH and/or ACTH receptor antagonist were infused for 48 h. Infusion rates: ACTH — 2 nmol/24 h/100 g; ACTH receptor antagonist — 4 nmol/24 h/100 g. QPCR was performed on samples from entire adrenals. Data expressed as means ± SE. Each circle represents a single rat. Statistical analysis of the data was performed by using one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Groups sharing the same letter are not significantly different according to Tukey's HSD test. In the remaining groups *p* values from ANOVA were higher than 0.05.
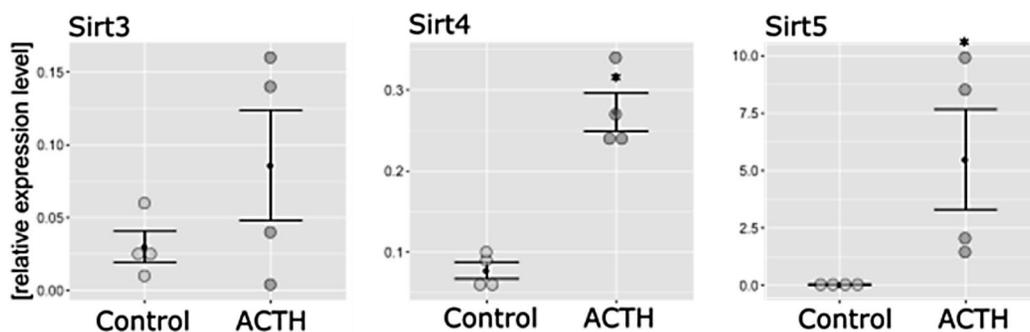


**Figure 4.** Effects of ACTH on relative expression levels of mitochondrial sirtuins in primary culture of rat adrenocortical cells. At day 3 of culture ACTH ($1 \times 10^{-6}$ M) was added and cell harvested after 24 h. Data obtained by QPCR. Data expressed as means ± SE. Each circle represents an independent culture. Comparisons of data in relation to control group — Student's t test: *$p < 0.001$.

the following days after enucleation: Sirt3 — day 5, Sirt4 and Sirt5 — day 8.

## Discussion

Numerous reports point to the important, multidirectional effects of sirtuins in cells, organs and the entire body. However, only few data are available on the expression and role of mitochondrial sirtuins (Sirt3–5) in the adrenals or in other steroidogenic cells. Our study characterized the expression of mitochondrial sirtuins in individual components of the rat adrenal gland, their developmental regulation, and their response to ACTH, the major regulator of adrenal growth and function.
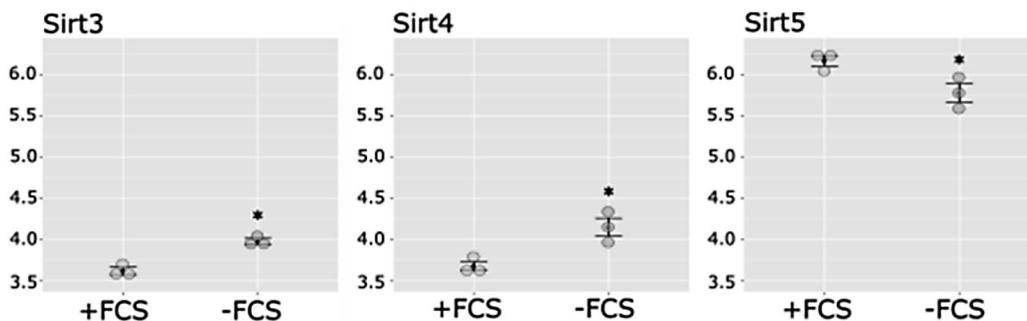
**Figure 5.** Relative expression levels of mitochondrial sirtuins in rat adrenocortical cells cultured in fetal calf serum (FCS)--depleted medium. At day 4 of culture, incubation was carried out in the presence or absence of FCS (10%). Cells were harvested and processed for microarray analysis. Reanalyzed data from Trejter *et al.* [26]. Data expressed as means ± SE. Each circle represents an independent culture. Comparisons of data in relation to control group — Student's t test: *$p < 0.05$.
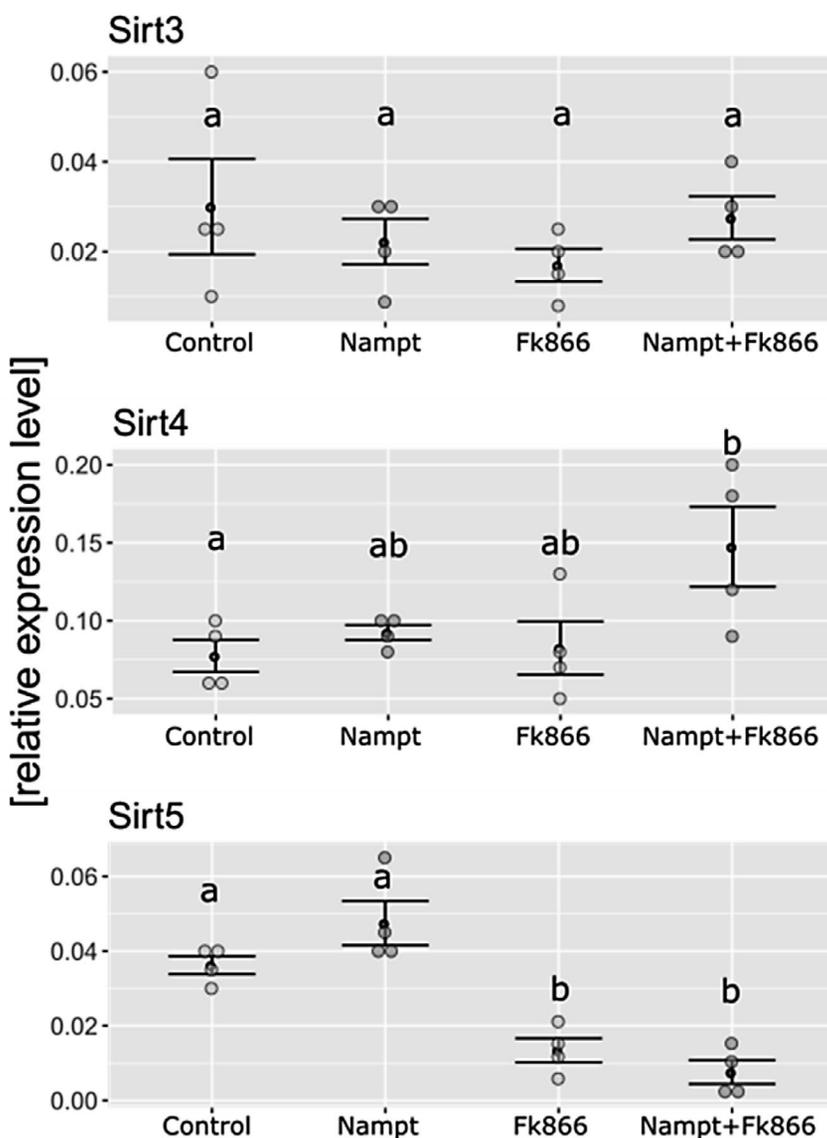


**Figure 6.** Effects of eNampt on relative expression levels of mitochondrial sirtuins in primary culture of rat adrenocortical cells. At day 3 of culture eNampt ($1 \times 10^{-8}$ M) or/and Fk866 (a highly specific competitive inhibitor of Nampt, $1 \times 10^{-7}$ M) were added and cell harvested after 24 h. Data obtained by QPCR. Data expressed as means ± SE. Each circle represents an independent culture. Statistical analysis of the data was performed by using one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Groups sharing the same letter are not significantly different according to Tukey's HSD test.
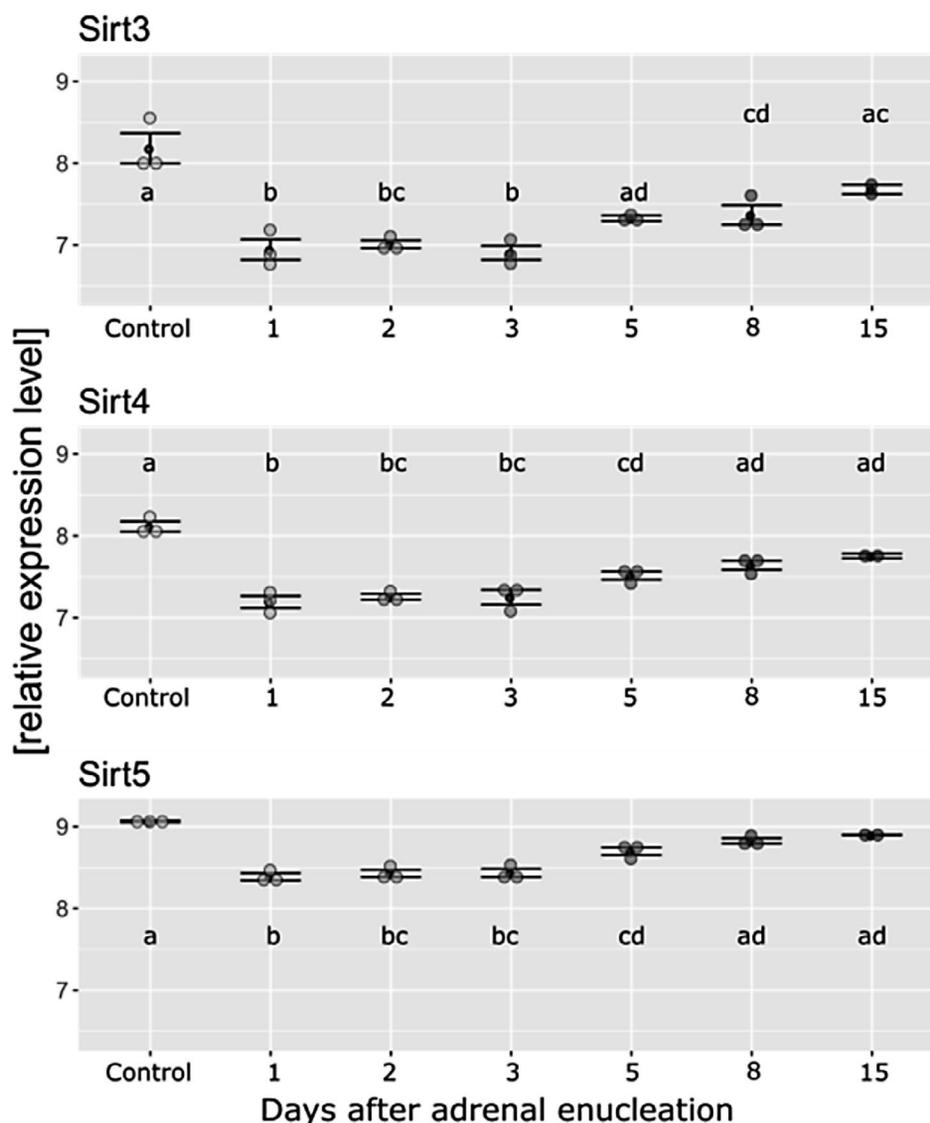
**Figure 7.** Relative expression levels of adrenal mitochondrial sirtuins in the course of enucleation-induced adrenal regeneration. Rats were sacrificed 1, 2, 3, 5, 8 and 15 days after surgery. Reanalyzed microarray data were obtained from earlier study [31]. Data expressed as means ± SE. Each circle represents a single rat. Statistical analysis of the data was performed by using one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Groups sharing the same letter are not significantly different according to Tukey's HSD test.

Without taking immunohistochemical studies into account, the literature does not contain data on the expression of mitochondrial sirtuin genes in individual adrenal gland compartments. Our results indicate that the highest expression of the investigated sirtuins is present in the ZG and ZF/R zones of the rat adrenal cortex, while significantly lower in adrenal medulla. Of sirtuins studied, in all adrenal compartments the level of Sirt5 mRNA is highest compared to Sirt3 and Sirt4. Further interesting data on mitochondrial rat adrenal sirtuins expression were obtained from analysis of data deposited in the Gene Expression Omnibus database

by Yu *et al*. [37]. Authors performed RNA-Seq on samples from 11 organs, including entire adrenal glands of male and female Fischer 344 rats aged 2 ("juvenile"), 6 ("adolescent"), 21 ("adult") and 104 ("aged") weeks. Obtained data revealed neither sex nor clear developmental differences in expression levels of mitochondrial sirtuins in this strain of rats. In the present study, in samples of entire adrenal gland of Wistar rats expression levels of Sirt4 are higher in adult females. These differences may be due to different strains of rats and the technique used, our results are derived from DNA microarray, while Yu *et al*. [37] data from RNA-Seq.

As far as immunohistochemical localization of mitochondrial sirtuins is concerned, Human Protein Atlas presents related data on Sirt3, Sirt4 and Sirt5 of human adrenal glands. In all cases sirtuin-like immunoreactivity is demonstrated by the presence of reaction product in groups of parenchymal cells, while it is absent in interstitial compartment of the gland (Release date: 2017.01.31) http://www.proteinatlas.org. Figure no: HPA022002 [34]. This location pattern of mitochondrial sirtuins is consistent with our research, which indicates the expression of the Sirt3–5 genes in the primary culture of rat adrenocortical cells, *i.e.* in parenchymal cells of the adrenal cortex.

Subsequent experiments aimed to answer whether the expression of mitochondrial sirtuin genes in the rat adrenal gland is regulated by ACTH. In chronic experiment, ACTH infusion for 48 h did not alter expression of mitochondrial Sirt3–5 in samples taken from the entire adrenal gland, while an increase in adrenal gland weight was observed (31.6 ± 0.5 mg *vs.* 36.6 ± 0.9 mg; control *vs.* ACTH; n = 8; means ± SE; p < 0.001). In contrast, within 60 min bolus *i.p.* ACTH administration notably elevated levels of Sirt3 mRNA and lowered that of Sirt5 mRNA. Both of these effects did not occur after joint ACTH and ACTH receptor antagonist administration. These data suggest that *in vivo*, acute ACTH-induced changes in mitochondrial sirtuin genes expression are mediated by corticotrophin interaction with its receptor. We also investigated the effect of ACTH on the expression of mitochondrial sirtuins in primary culture of adrenocortical cells. It appeared that 24 h exposure of cultured cells to ACTH resulted in small increase in the expression of Sirt4 and great increase in Sirt5 mRNA level. Despite the opposite results of experiments on ACTH-induced changes in Sirt5 expression *in vivo* and *in vitro*, the data obtained indicate that in the rat adrenal gland Sirt5 gene is the most potently regulated mitochondrial sirtuin by ACTH. The present study is the first to our knowledge that directly tested the role of the ACTH in regulation of adrenal mitochondrial sirtuins expression. It is difficult to explain why Sirt5 expression *in vivo* is inhibited and *in vitro* stimulated by corticotrophin. Be as it might, these findings are still to be explored. It is all the more interesting that the latest data associate Sirt5 gene expression disorders with such diseases as cancer, Alzheimer's disease, or Parkinson's disease [11].

Numerous literature data indicate that different kinds of starvation, like short-term calorie restriction can influence sirtuins expression levels in different cells and tissues [10, 44, 45]. It is also known that low serum doses or serum depletion differentially affect cell growth and protein expression in many cells

[46–49]. In experimental techniques serum depletion is mostly used for reduction of analytical interference. However serum depletion is also used for reduction of basal cellular activity and making the population of proliferating cells more homogenous [49]. Therefore, we have investigated the effect of FCS depletion on the expression of mitochondrial sirtuins in primary adrenocortical cell culture. We analyzed the data from the short-term FCS restriction by omitting FCS in incubation medium. It appeared that under the serum depletion conditions the expression levels of Sirt3 and Sirt4 increased, while Sirt5 expression levels decreased. To our knowledge, to date there are no publications examining the expression of Sirt3, 4 and 5 in cells cultured in FCS depleted medium. However, under the conditions of caloric restriction similar changes are observed in other cell types. For example, culture of H9c2 myoblasts (a permanent cell line derived from rat cardiac tissue) in low concentration of glucose had elevated mRNA expression levels of Sirt1–4 and 7, while Sirt5 mRNA expression did not differ from control [45].

In cells Nampt (nicotinamide phosphoribosyltransferase) catalyzes the rate-limiting step in the NAD salvage pathway. Our previous studies [25] demonstrated that intraperitoneal administration of eNampt elevates rat plasma corticosterone levels. eNampt, however, did not influence the corticosterone production in primary culture of rat adrenocortical cells. All sirtuins are NAD+-dependent enzymes, therefore we have also checked whether eNampt affects expression levels of mitochondrial sirtuins in such a culture. It appeared that 24-hour exposure of cultured cells to eNampt did not affect the expression levels of mitochondrial sirtuins studied. We also studied the possible role of intracellular Nampt (iNampt) in regulation of mitochondrial sirtuins expression in adrenocortical cells. To achieve this goal cultured cells were exposed to Fk866, a highly specific noncompetitive inhibitor of Nampt [50]. Under the experimental conditions applied Fk866 reduced expression of Sirt5 only, and this effect was also observed in the presence of eNampt. These findings suggest that normal levels of iNampt enzymatic activity are required to maintain normal (control) levels of Sirt5 mRNA in cultured cells. It is an original observation that suggests the physiological role of iNampt in the regulation of mitochondrial expression of Sirt5 gene in rat adrenocortical cells.

The role of mitochondrial sirtuins in the regulation of steroidogenesis is not known. To our knowledge, only few reports are directly related to this issue. As reported by Li *et al.* [51], in H295R adrenocortical cells stable overexpression of Sirt3 gene increased

the basal levels of P450scc (cholesterol side-chain cleavage enzyme) and resulted in a higher amounts of cortisol secretion. Furthermore, overexpression of Sirt3 or Sirt5 decreased the acetylation of P450scc, while suppression of these mitochondrial sirtuins (siRNA method) exerted opposite effects on the cellular amount of acetylated P450scc. These results suggest that both, Sirt3- and Sirt5-dependent deacetylation of P450scc is involved in regulation of steroidogenesis at the step of cholesterol side-chain cleavage. As this is the first step in the biosynthesis of all steroid hormones, these data suggested that the mitochondrial Sirt3 and Sirt5 may exert similar effects in other steroid hormone secreting cells. Recently, however, Marti *et al*. [52] reported that in human adrenocortical H295R cells Sirt1, 3 and 5 overexpression did not affect steroidogenesis.

In human luteinized granulosa cells Sirt3 depletion (RNAi system) resulted in decreased mRNA expression of aromatase, $17\beta$-hydroxysteroid dehydrogenase 1, StAR, P450scc, and $3\beta$-hydroxysteroid dehydrogenase and these effects were accompanied by decreased progesterone secretion [53]. As it follows from the above literature, in adrenocortical H295R cells overexpression of Sirt3 rather increases expression of P450scc and cortisol secretion, while Sirt3 depletion in human granulosa cells decreases expression of P450scc and progesterone secretion. Thus, in steroidogenic cells Sirt3 expression seems to be directly related to the control of steroidogenesis. However, our observations indicate that in the rat adrenal cortex the most sensitive to acute ACTH action is Sirt5. It is difficult to explain this phenomenon. But these data suggest that in the adrenal cortex, the Sirt5 is the primary mitochondrial sirtuin involved in regulating the biological activity of adrenocortical cells. Further research is needed to confirm our hypothesis.

In a model of enucleation-induced adrenal regeneration expression levels of all mitochondrial sirtuins were notably downregulated. The largest decrease in expression of these sirtuins was observed within 3 days after surgery, later on their expression increased. Such a pattern of Sirt3–5 expression may result from severe metabolic disorders caused by a decrease in secretion of corticosteroids as well as by very high proliferation rate of cells in regenerating adrenal glands. As it is known, sirtuins, in particular Sirt3 and Sirt4, are suppressors of the proliferation of different types of cells [54–56]. Therefore, the low expression of these sirtuins in the regenerating rat adrenal seems to be justified.

Thus, in our research we have obtained a number of original results concerning the mitochondrial sirtuin expression in the adrenal glands of rat. Our studies

have revealed that expression of mitochondrial sirtuins in rat adrenal is the highest in ZG and ZF/R zones. Expression of these sirtuins is not significantly altered during postnatal ontogenesis. Chronic infusion of ACTH did not alter the expression of mitochondrial sirtuins in adrenocortical cells while acutely administered ACTH increases Sirt3 expression and lowers Sirt5. In the primary culture of adrenocortical cells ACTH leads to a very significant increase in Sirt5 expression. The obtained data shows that among the examined mitochondrial sirtuins of adrenocortical cells, the most strongly controlled is expression of the Sirt5 gene. This is not a phenomenon specific for adrenal cortex parenchymal cells. A similar high Sirt5 gene reactivity to various factors is also observed in other cell types [11]. However, it should be stressed that the role of this sirtuin in the regulation of steroidogenesis is still to be clarified.

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## Conflict of interest

The authors declare no conflict of interest.

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