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The Effect of Fetal Hypoxia on Adrenocortical Function in the 7-Day-Old Rat

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Fetal hypoxia in late gestation is a common cause of postnatal morbidity. The purpose of the present study was to evaluate adrenal function in vivo and in vitro in 7-d-old rat pups previously exposed to normoxia or hypoxia (12% O_2) during the last 2–3 d of gestation. Seven-day-old rats exposed to fetal hypoxia had a small, but significant decrease in plasma aldosterone despite no decreases in plasma ACTH or renin activity. There was a small (approx 20%) but significant decrease in the aldosterone and corticosterone response to cAMP in vitro in dispersed cells from 7-d-old pups exposed to fetal hypoxia. The aldosterone, corticosterone, and cAMP response to ACTH, however, was not altered by prior fetal hypoxia. There was also no effect of fetal hypoxia on steroidogenic enzyme expression or zonal dimension in 7-d-old rats. We conclude that fetal hypoxia in late gestation results in a subtle decrease in cAMP-stimulated steroidogenesis. Fetal hypoxia appears to have minimal effects on subsequent adrenal function in the neonatal rat.

Key Words: Hypoxia; fetus; adrenal cortex; corticosterone; aldosterone.

Introduction

Hypoxia in the fetus can result from umbilical/placental compromise *(1)*, as well as cardiopulmonary disease in the mother *(2)*. Fetal hypoxia is a significant cause of subsequent dysfunction in the neonate and adult. These morbidities include neurological *(3–5)*, behavioral *(5–7)*, neuroendocrine *(5)*, metabolic *(8)*, sympathoadrenal *(9)*, and cardiopulmonary *(10,11)* systems. Detailed examination of the controllers of adrenocortical function and adrenal steroidogenesis have not been carefully examined in the normoxic newborn exposed to hypoxia in late gestation.

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We have recently performed a systematic analysis of the control of steroidogenesis in the 7-d-old rat exposed to hypoxia from birth *(12)* and in the adult rat exposed to hypoxia *(13)*. The adult rat showed a decrease in aldosterone during hypoxia due to a decrease expression of the late pathway enzyme P450c11AS, while the neonate did not and was therefore able to maintain steroidogenesis. We had no prediction as to what the effect of exposure of pregnant dams to hypoxia in late gestation would have on the their offspring at 7 d of age (reared from birth under normoxic conditions).

The purpose of the present study was to evaluate the effect of prenatal exposure to hypoxia on subsequent plasma hormonal controllers and adrenal steroids, on steroidogenesis in vitro, and on expression of steroidogenic enzyme mRNAs and zonal morphology of the adrenal gland in the 7-d-old rat pup.

Results

There were no differences in plasma ACTH and PRA in 7-d-old rats exposed to late-gestation fetal hypoxia (**Fig. 1**). Corticosterone tended to be lower in rat pups who had been exposed to fetal hypoxia, although levels were sufficiently low at baseline (compared to those reported for adult rats *[13]*) such that no significant difference could have been detected. However, there was a small (approx 20%) but significant decrease in plasma aldosterone in 7-d-old rats exposed to hypoxia in late gestation as compared to their normoxic controls.

There was no difference in plasma glucose in 7-d-old rats exposed to fetal hypoxia (150 ± 9 mmol/L) as compared to normoxic controls (148 ± 4 mmol/L). Body weight was significantly lower at 7 d of age in rats exposed to hypoxia (11.6 ± 0.3 g; n = 43) as compared to normoxic controls (13.0 ± 0.2 g; n = 54; $p < 0.001$).

Figure 2 shows aldosterone production from dispersed adrenal cells. There was no effect of prenatal hypoxia on basal steroidogenesis in cells from 7-d-old rat pups. However, cells from 7-d-old rats exposed to prenatal hypoxia demonstrated significant decreases in cAMP-stimulated aldosterone production (entire pathway) and in the conver-

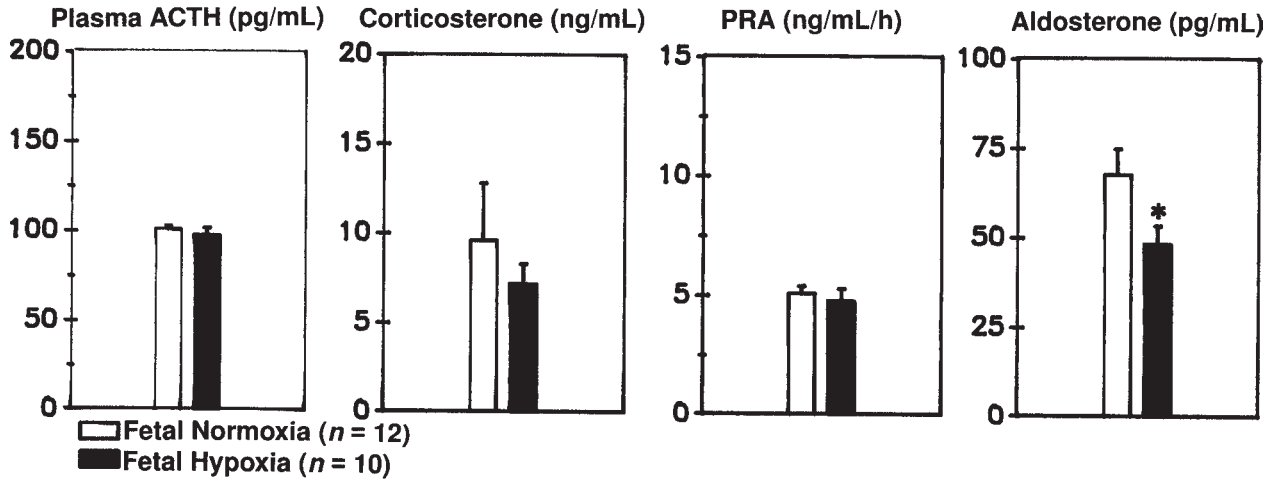

Fig. 1. Plasma hormone levels in 7-d-old rat pups exposed to fetal normoxia or hypoxia. * indicates hypoxia different from normoxia.

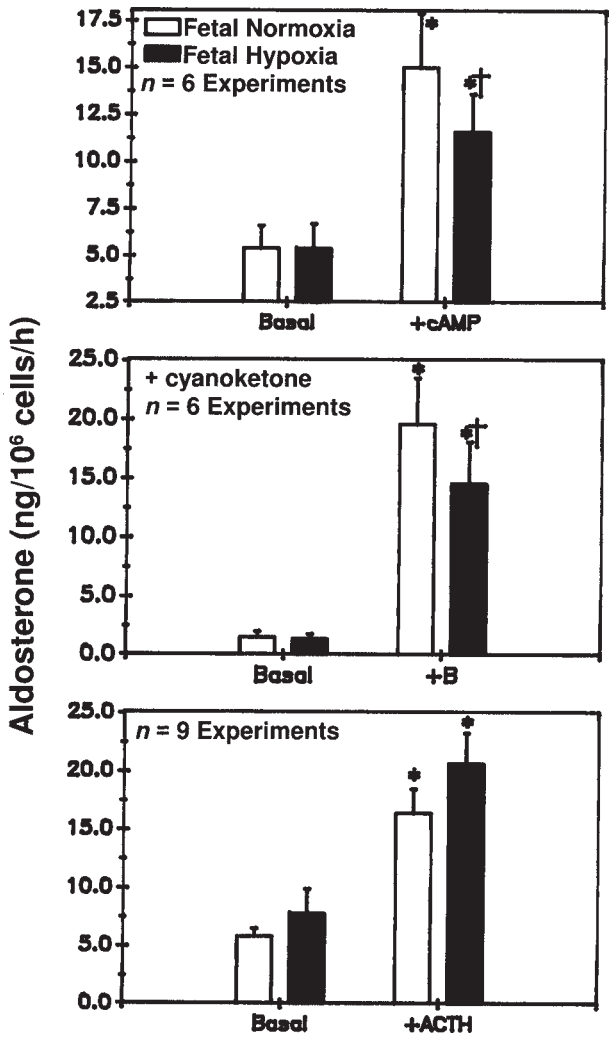

Fig. 2. Aldosterone production in vitro in response to dibutyryl cAMP (3 m*M*), in response to addition of corticosterone (B; 7.2 μ*M*) in the presence of cyanoketone (P450c11AS activity), and in response to ACTH (20 ng/mL) in dispersed adrenal cells from 7-d-old rat pups exposed to fetal normoxia or hypoxia. * indicates greater than Basal within same treatment; † indicates hypoxia different from normoxia.

sion of corticosterone to aldosterone in the presence of cyanoketone (P450c11AS activity). However, ACTH-stimulated aldosterone production was not different between the cells from the two groups of rats.

Figure 3 shows basal, cAMP, and ACTH-stimulated corticosterone production (presumably from ZF/ZR cells) as well as cAMP-stimulated pregnenolone production in the presence of cyanoketone (P450scc activity) and ACTH-stimulated cAMP production. cAMP-stimulated corticosterone and pregnenolone production was significantly lower in cells from 7-d-old rat pups exposed to fetal hypoxia, although the effect was subtle. In contrast, there was no effect on ACTH-stimulated corticosterone or cAMP production.

There were no significant differences in mitochondrial steroidogenic enzyme mRNA expression measured by RT-PCR between adrenals (6/group) from 7-d-old rats exposed to fetal normoxia vs hypoxia (**Table 1**). If anything, P450scc and P450c11AS mRNAs tended to be higher in adrenal glands from 7-d-old rats exposed to fetal hypoxia as compared to normoxic control.

Figure 4 shows representative images of immunofluorecent staining for P450c11AS protein used to define the width of the zona glomerulosa (ZG) in adrenals from 7-d-old rat pups. There was no effect of fetal hypoxia on the width of the ZG (Table 1). Although zona fasciculata/reticularis width cannot be quantitated owing to the elipsoid shape of the adrenals, qualitative analysis did not suggest major differences between the two groups of rats.

Discussion

This study examined postnatal hormone levels and adrenocortical function in vitro in 7-d-old rats exposed to late-gestational fetal hypoxia vs. normoxic control. We focused on the 7-d-old rat because this has become a standardized model for evaluation of metabolic and adrenocortical function in the neonatal rat *(12,14,15)*. We found that

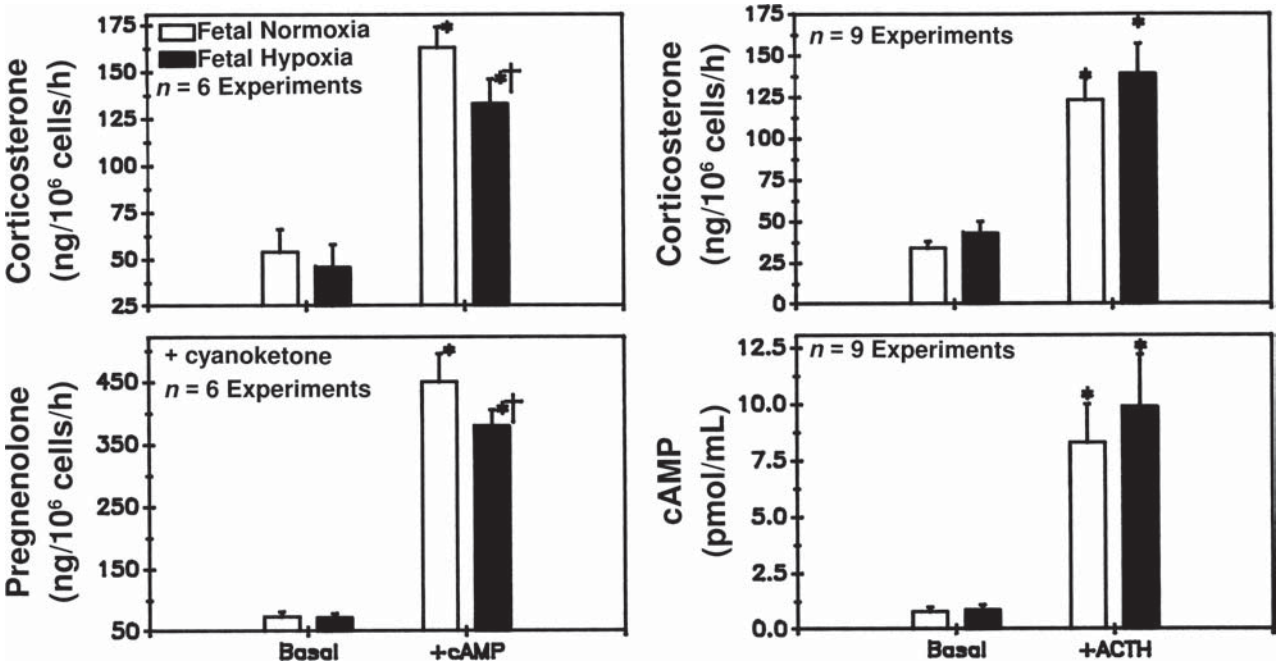

Fig. 3. Corticosterone response to cAMP and ACTH, pregnenolone response to cAMP in the presence of cyanoketone (P450scc activity), and the endogenous cAMP response to ACTH in dispersed adrenal cells. Symbols and concentrations the same as Fig. 2.

Table 1
Mitochondrial Steroidogenic Enzyme mRNAs and Zona Glomerulosa (ZG) Width in Adrenals from 7-D-Old Rats Exposed to Fetal Hypoxia[a]

	P450scc	P450c11B	P450c11AS	ZG Width
Fetal Normoxia	0.66 ± 0.04	0.35 ± 0.02	0.07 ± 0.02	39.4 ± 1.6 μ*M*
Fetal Hypoxia	0.75 ± 0.03	0.38 ± 0.09	0.11 ± 0.01	38.7 ± 3.8 μ*M*

[a]Data are mean ± SEM. P450 mRNA data expressed in arbitrary units (target mRNA/β-actin mRNA).

plasma aldosterone levels were lower in newborn rats exposed to fetal hypoxia despite the fact that plasma ACTH and renin activity levels were not different. A decrease in plasma corticosterone could not be detected because the levels were already extremely low, consistent with previous studies in 7-d-old rats *(12,14,15)*. Whereas plasma glucose was not different between groups, the rats exposed to fetal hypoxia still had lower body weight at 7 d of age that indicated some degree of growth retardation. cAMP-stimulated corticosteronogenesis and aldosteronogenesis in vitro were significantly inhibited in cells from newborn rats exposed to fetal hypoxia. Although the effect was rather subtle, it was highly consistent. The conversion of corticosterone to aldosterone (late pathway) was also inhibited in the absence of secretagogue stimulation. Interestingly, ACTH stimulation of adrenal cells in vitro did not reveal any differences. These data suggest that exposure to hypoxia in the fetus results in subtle alterations in adrenocortical function in the newborn rat.

This effect was not explained by changes in steroidogenic enzyme expression as reflected in mRNA levels measured by PCR. In fact, if anything, P450scc and P450c11AS mRNA tended to be higher in newborn rats exposed to fetal hypoxia. Furthermore, changes in aldosterone secretion cannot be attributed to any obvious change in zona glomerulosa size or morphology as analyzed by P450c11AS protein staining and immunohistofluorescence. We can only surmise that the effect detected in this study is a functional one.

What might be the explanation for a decrease in cAMP-stimulated but not ACTH-stimulated steroidogenesis, since ACTH is thought to operate via cAMP generation? It has recently become clear that a component of the action of ACTH is exerted through cAMP-independent pathways *(16)*. This suggests that this cAMP-independent effect may restore steroidogenesis to normal in the 7-d-old rats exposed to fetal hypoxia. This may also account for decreased basal plasma steroid levels even though ACTH concentration was not different. There are also other oxygen-sensitive phenomena within the adrenal gland that could account for differences between cAMP-stimulated and ACTH-stimulated steroidogenesis *(17,18)*. Although more light could

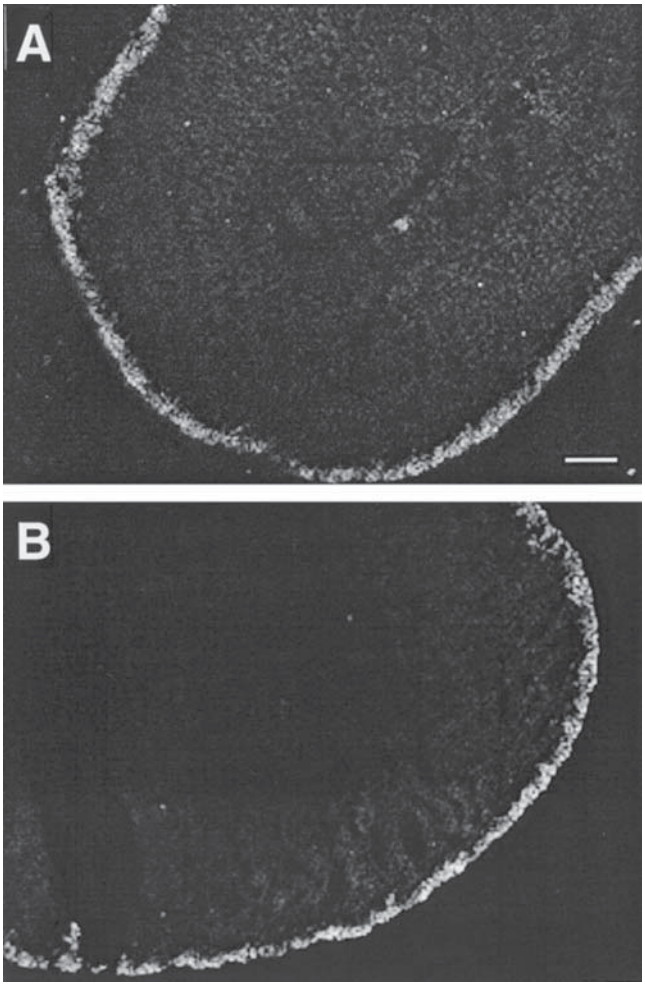

Fig. 4. Representative images of immunofluorecent staining for P450c11AS protein used to define the width of the zona glomerulosa (ZG) in adrenals from 7-d-old rat pups exposed to fetal normoxia **(A)** or hypoxia **(B)**. There was no effect of fetal hypoxia on the width of the ZG. Bar is 100 μm.

have been shed on this phenomenon by either measuring steroidogenesis at different time points or by doing extensive dose response curves in vitro, we were limited by the number of cells we could generate from the number of rat pups that could be exposed to hypoxia at one time.

There was a significant difference between the findings of the current experiment with fetal hypoxia and our previous findings of the effects of hypoxia from birth to 7 d of age *(12)*. In that previous study, hypoxia from birth resulted in an augmentation of aldosteronogenesis also not explained by changes in steroidogenic enzyme mRNA levels or histomorphometry. Furthermore, that previous study with postnatal hypoxia found no effect on corticosteronogenesis. Although the physiological significance of these differences from the current findings is not obvious at this point, it does merit further investigation.

The effect of fetal hypoxia on subsequent adrenal function was quite consistent, but admittedly subtle. This suggests that the long-term effects of fetal hypoxia on subsequent adrenal function is not particularly serious as compared to the effects on other biological systems studied *(3–11)*. That the response in vitro to an endogenous secretagogue (i.e., ACTH) was normal further suggests that fetal hypoxia in late gestation does not lead to major alterations in adrenocortical function in the neonate.

Materials and Methods

Animal Treatment

All animal experimentation had IACUC approval. Timed pregnant Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, n=32) were obtained at 14 d gestation and maintained on a standard sodium diet (Richmond Standard Diet, Brentwood, MO) and water ad libitum in a controlled environment (0600–1800 lights on). At 19 d gestation, dams in their home cages were moved to environmental chambers and exposed to normoxia (21% O_2) or hypoxia (12% O_2) as described in detail previously *(19–21)*. We have previously shown that this exposure leads to arterial PO_2 levels in adults of about 50–55 torr with sustained hypocapnia and alkalosis *(20,21)*. Parturition occurred on the afternoon of gestational d 21 during which rats were kept under observation. As soon as a litter was completely delivered, the dam and her pups (8–10/litter) were immediately moved to normoxia. Therefore, the duration of exposure to hypoxia was approx 50–56 h.

At 0800 h of d 7, dams were quickly removed from the chambers. Then, rat pups were quickly decapitated and trunk blood from 3–4 pups was pooled for the measurement of plasma ACTH, renin activity, aldosterone, and corticosterone. Adrenal glands were quickly removed and randomly assigned for processing for each technique described below.

Dispersed Cells

Whole adrenal glands were minced and dispersed with collagenase. Although Feuillan and Aguilera *(15)* were able to separate the capsule (ZG) from the subcapsule (ZF/ZR) in 7-d-old rat adrenals, we were unable to get a clean enough separation of adrenals from 7-d-old rats to generate sufficient capsular (ZG) cells to perform the experiments described. Therefore, P450scc activity represents the early pathway from all adrenal zones. The dispersed cells were washed and placed in cold Krebs–Hepes–calcium buffer at a concentration of 50,000 cells/mL. Cells were always studied the day they were dispersed. Cells were placed in test tubes, incubated for 2 h at 37°C in a shaking water bath, and activities of the different mitochondrial enzymatic steps of the steroidogenic pathways were assessed as described previously *(13)*. Each treatment within an experimental day (basal, cAMP, cyanoketone) was performed in triplicate. Briefly, the entire aldosterone (ZG) and corticosterone (ZF/ZR) pathways were assessed

by stimulation with dibutyryl cAMP (3 m*M*) or ACTH (20 ng/mL), P450scc activity (mostly ZF/ZR cells by mass) was assessed by stimulation of the conversion of endogenous cholesterol to pregnenolone with cAMP in the presence of cyanoketone (3βHSD inhibitor; 10 μ*M*) generously donated by Sterling-Winthrop (Collegeville, PA), and P450c11AS activity (ZG) was measured by the conversion of corticosterone (7.2 μ*M*; Sigma Chemical, St. Louis, MO) to aldosterone in the presence of cyanoketone. Furthermore, cAMP generation was measured without and with addition of ACTH (20 ng/mL). Triplicate replications for each treatment on an experimental day was averaged and treated as one value. ns in figure legends represent the numbers of different cell preparations (experimental days).

Reverse Transcription-Competitive Polymerase Chain Reaction (RT-cPCR) of P450scc, P450c11B, and P450c11AS mRNA

Whole adrenal glands were quickly frozen and stored in liquid nitrogen. The RNA extraction, reverse transcription, and PCR primers have all been described in detail previously *(13,22)*. Briefly, total cellular RNA was extracted with guanidine thiocyanate and single-strand cDNA generated using Superscipt preamplification reagents (Life-Technologies, Bethesda, MD). PCR was performed using specific primers for P450c11AS (CYP11B2), P450c11B (CYP11B1), and P450scc (CYP11A1). PCR products were separated by electrophoresis in 2% agarose gel and analyzed by ethidium bromide staining and a CCD camera/video gel documentation/image analysis system and software (Bio-Rad, Hercules, CA). The expression of each enzyme mRNA was normalized to β-actin mRNA expression.

Zona Glomerulosa Width

Immunohistofluorescent staining for P450c11AS protein was performed on adrenal sections as described previously *(23)*. Briefly, adrenal sections were fixed with Zamboni's fixative, blocked with normal donkey serum, and incubated overnight with rabbit anti-P450c11AS antibody (provided by C. Gomez-Sanchez, U. Mississippi Medical Center). Sections were incubated with Cy3-labeled donkey anti-rabbit secondary antibody and coverslipped. Control slides incubated with the anti-P450c11AS antibody preabsorbed with a truncated version of the immunizing peptide were negative for staining. Optical images were collected using a CCD camera. Using the presence of P450c11AS protein staining to define the zona glomerulosa, the size of the zone was estimated for each adrenal by measuring the average width of a minimum of 10 sections taken from the central region of each adrenal (i.e., presence of adrenal medulla).

Measurements and Assays

Plasma ACTH, renin activity, corticosterone, and aldosterone were measured by radioimmunoassay *(12,20,21, 24)*. The concentrations of pregnenolone, corticosterone, and aldosterone in cell dispersion were measured by radioimmunoassay *(13)*. cAMP in the supernatant was measured as described previously *(25)* using an RIA with reagents purchased from Amersham (Piscataway, NJ). Plasma glucose was measured spectrophotometrically using reagents purchased from Sigma (St. Louis, MO) as described previously *(26)*. Body weight was measured using an Ohaus LS200 electronic balance (Florham Park, NJ).

Statistical Analysis

Data were analyzed by *t*-test, two-way analysis of variance, and Duncan's multiple range test. Replicates on each experimental day were averaged and treated as 1 datum; n values in each figure legend are number of different cell or mitochondrial preparations. $p < 0.05$ was considered significant. Data are presented as mean ± SEM.

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