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Adrenocortical changes and arterial hypertension in lipoatrophic A-ZIP/F-1 mice

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Summary

The A-ZIP/F-1 transgenic mouse is a model of lipotrophic diabetes with severe insulin resistance, hyperglycemia and hyperlipidemia. Recently, a regulatory role of adipose tissue on adrenal gland function and blood pressure has been suggested. To further explore the importance of adipose tissue in the regulation of adrenal function and blood pressure, we studied this mouse model of lipodystrophy. A-ZIP/F-1 mice exhibit significantly elevated systolic and diastolic blood pressure values despite lack of white adipose tissue and its hormones. Furthermore, A-ZIP/F-1 lipotrophic mice have a significant reduction of adrenal zona glomerulosa, while plasma aldosterone levels and aldosterone synthase mRNA expression remain unchanged. On the other hand, lipotrophic mice present elevated corticosterone levels but no adrenocortical hyperplasia. Ultrastructural analysis of adrenal gland show significant alterations in adrenocortical cells, with conformational changes of mitochondrial internal membranes and high amounts of liposomes. In conclusion, lipodystrophy in A-ZIP/F-1 mice is associated with hypertension, possibly due to hypercorticosteronemia and/or others metabolic-vascular changes.

Keywords

Adrenal gland, adipose tissue, A-ZIP/F-1 lipotrophic mice, hypertension.

Introduction

Obesity is a major health problem, increasing the risk of diabetes, hypertension, congestive heart failure, coronary artery disease, and certain types of cancer (Kopelman 2000). Numerous studies in the last decade have revealed that adipose tissue, besides its role in energy storage, has to be considered an endocrine organ, secreting a wide variety of cytokines and proteins, commonly referred to as adipokines (Kim and Moustaid-Moussa 2000; Trayhurn and Beattie 2001).

Obesity is strongly associated with hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis. The abnormalities in HPA axis regulation in obesity, either located centrally or peripherally at the level of the adrenal itself, have important consequences on the stress system and blood pressure regulation (Chrousos 2000; Björntorp 2001). Besides ACTH and angiotensin II (Ang II), adrenal gland function is regulated by several additional systems, including cytokines and neurotransmitters (Nussdorfer and Mazzocchi 1998). Recently, a stimulatory effect of adipose tissue-derived factors on adrenal steroidogenesis has been observed (Ehrhart-Bornstein et al. 2003), thus suggesting a direct involvement of adipose tissue in the regulation of adrenal secretory function, and possibly mediating hypertension in obesity (Lamounier-Zepter et al. 2005).

To further understand the physiological role of adipose tissue in the regulation of adrenal function, we studied the A-ZIP/F-1 transgenic mice. These transgenic mice express a dominant-negative protein named A-ZIP/F-1, which inhibits the DNA binding and function of transcription factors critical for fat tissue development. The resulting transgenic mice have virtually no white adipose tissue and a significantly reduced amount of brown adipose tissue. Thus, the phenotype of these mice resembles the human clinical condition of lipodystrophy, an inherited or acquired

disorder characterized by the absence of white adipose tissue (Moitra et al. 1998). Similarly to humans with lipodystrophic disorders, these mice suffer from lipoatrophic diabetes with severe insulin resistance, hyperglycemia, hyperlipidemia, hepatic steatosis and organomegaly (Moitra et al. 1998). Interestingly, A-ZIP/F-1 mice have increased corticosterone levels, which contribute to the metabolic phenotype of these mice (Haluzik et al. 2002; Gavrilova et al. 1999). Whether the increase of corticosterone synthesis results from central dysregulation of HPA axis or directly from altered adrenal function is still unclear. Based on our previous data suggesting a direct link between adipose tissue and adrenal gland function, we measured blood pressure and examined the adrenal gland structure and function in this lipoatrophic animal model.

Methods

Animals

Male A-ZIP/F-1 hemizygous mice (n=8) for the transgene on the FVB/N background and wild-type (n=8) littermates at 16 weeks of age were studied. Animals were housed under controlled temperature (20 ± 2 °C) and lighting (12-h light/dark cycle - 6 am/6 pm) with free access to water and standard pellet chow diet (Velaz, Czech Republic). At the end of experiments mice were killed by anesthesia overdose. Adrenals were collected and frozen in liquid nitrogen for RNA extraction or fixed in glutaraldehyde solution for electron microscopy or in a glycol solution (Tissue-Tek O.C.T. Compound, Sakura Finetek, Zoeterwoude, The Netherlands) for light microscopy. Animal experiments were approved by the Animal Care and Use Committee of the First Faculty of Medicine, Charles University in Prague.

Electron Microscopy

Adrenal glands were fixed in 0.1 M cacodylate buffer at pH 7.2 with 2.5% (vol/vol) glutaraldehyde. Tissue slices were dehydrated in ethanol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined at 80 kV in a CM 10 electron microscope (Philips, Eindhoven, The Netherlands).

Morphometric Analysis

To determine the size of adrenal sections, morphometric analysis was performed by using a computer-supported imaging system connected to a light microscope (Axio Imager M1, Carl Zeiss, Jena). The area of several sections was measured in triplicate. The four largest sections were evaluated for an approximation of the longest diameter in each gland.

Blood pressure measurements

The animals were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Blood pressure was measured by direct puncture of the carotid artery using Stattham P23Db transducer and Hewlett-Packard recorder as previously described (Kunes and Jelinek 1984).

Hormonal assays

Mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) and blood was withdrawn through the retro-orbital plexus. Blood samples were centrifuged at 30 x g for 10 min at 20°C; serum was collected and quickly frozen at -80°C for hormone measurements. Glucose was measured using a Glucometer Elite (Bayer, Elkhart, IN). Triglycerides and free fatty acids were measured using colorimetric kits (Sigma-Aldrich, St. Louis, MO, USA and Roche Molecular Biochemicals, Mannheim, Germany; respectively).

Aldosterone levels were measured using a radioimmunoassay kit (DSL, Sinsheim, Germany). Corticosterone and ACTH levels were measured using an ELISA kit for corticosterone (IBL, Hamburg, Germany) and for ACTH (Biomerica, Hannover, Germany), respectively. Serum insulin levels were measured by RIA (Linco

Research, St. Charles, MI, USA). Serum leptin, adiponectin and resistin levels were measured using an ELISA kit (BioVendor, Brno, Czech Republic).

Isolation of total RNA and reverse transcription

Total RNA was extracted from adrenal gland by standard procedures using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the provider's instructions. Mice adrenal gland was mechanically disrupted in lysis buffer with a Qiashreder (Qiagen) before column-based RNA isolation. For all RNA extractions a DNase digestion step (RNase-free DNase, Qiagen) was included according to the provider's instructions. Quality and integrity of RNA was verified by agarose gel electrophoresis. 1 µg of RNA was reversely transcribed using oligo-dT primer in a standard 33 µl reaction by the Ready-to-go T-primed first-strand kit (Amersham Biosciences, Freiburg, Germany).

Quantitative PCR

The mRNA expression levels of the mouse aldosterone synthase (CYP11B2) gene were determined by a real-time PCR assay using the Mx4000 Multiplex Quantitative PCR System (Stratagene, Amsterdam, The Netherlands). mRNA quantity was measured applying PCR protocols based on SYBR Green I detection (QuantiTect SYBR Green PCR Kit; Qiagen) and the exon-spanning primers CYP11B2_for (5'-CAGTGGCATTGTGGCGGAACTAATA-3') / CYP11B2_rev (5'-GGTCTGACATGGCCTTCTGAGGATT-3'). The PCR protocol consisted of a pre-denaturation step (2 min at 95°C) and 40 amplification cycles (30 s at 95°C, 1 min at 62°C, 30 s at 72°C). The CYP11B2 transcript levels were normalized to the quantity of β-actin transcripts. The SYBR Green I-based quantification of β-actin was performed with the exon-spanning primers β-actin_for (5'-

GAGCACAGCTTCTTTGCAGCTCCTT-3') / β -actin_rev (5'-TGCCATGTTCAATGGGGTACTTCAG-3') and the same thermal cycling profile as used for quantification of the CYP11B2 mRNA.

For the PCR reactions 2 μ l of 1:10 diluted cDNA products were used as template. All primers had a final concentration of 0.5 μ M in the assays. Serial dilutions of plasmid DNA containing the appropriate PCR fragments over 7 log scales (10^2 - 10^8 copies per reaction) were used as template standards. All determinations were carried out in triplicate for each cDNA sample. The molecule ratios of CYP11B2 to β -actin transcripts were calculated from the mean values.

Statistical analysis

The data are expressed as means \pm SE. Statistical significance between the groups was determined using Student's *t* test.

Results

Blood pressure

Both systolic (SBP) and diastolic blood pressure (DBP) of A-ZIP/F-1 mice was significantly higher in comparison with wild-type mice. Basal SBP was 98 ± 6 mmHg in wild-type and 135 ± 6 mmHg in A-ZIP/F-1 mice ($p < 0.01$). Basal DBP was 64 ± 5 mmHg in wild-type and 84 ± 5 mmHg in A-ZIP/F-1 mice ($p < 0.05$).

Biochemical analysis

Body weight of lipotrophic adult A-ZIP/F-1 mice was significantly higher relative to wild-type littermates (32 ± 1.7 g vs. 25 ± 1.4 g, $p < 0.01$). A-ZIP/F-1 mice exhibited severe insulin resistance and diabetes (blood glucose 17.2 ± 3.2 mmol/l, serum insulin 116 ± 14 ng/ml) relative to wild-type littermates (blood glucose 7.1 ± 0.6 mmol/l, serum insulin 0.7 ± 0.1 ng/ml, $p < 0.01$ for both). They have also markedly increased circulating triglyceride and free fatty acid levels (triglyceride 80.9 ± 2.3 mg/dl, free fatty acid 1385 ± 98 μ mol/l) relative to wild-type mice (triglyceride 24.8 ± 1.8 mg/dl, free fatty acid 721 ± 51 μ mol/l, $p < 0.01$ for both).

A-ZIP/F-1 mice exhibited reduced serum levels of leptin and resistin (leptin 184.2 ± 51.7 pg/ml, resistin 496.4 ± 118.5 pg/ml) relative to wild-type mice (leptin 1019 ± 469 pg/ml, $p = 0.11$; resistin 6158 ± 457 pg/ml, $p < 0.001$). Furthermore, A-ZIP/F-1 mice have almost undetectable serum levels of adiponectin (0.28 ± 0.03 μ g/ml vs. wild-type 11.9 ± 2.1 μ g/ml, $p < 0.001$).

Morphometric analysis

No differences were observed in both adrenal gland cortex and medulla size between A-ZIP/F-1 and wild-type mice (data not shown). However, morphometric analysis of

the three layers of adrenal cortex separately showed a significant reduction of zona glomerulosa width of lipotrophic A-ZIP/F-1 mice (Figure 1A and B).

Adrenal steroid synthesis

Interestingly, lipotrophic A-ZIP/F-1 mice have comparable aldosterone levels to their wild-type littermates (904 ± 116 pmol/l vs. 823 ± 115 pmol/l, respectively, $p = 0.062$) despite significantly reduced width of zona glomerulosa (Figure 1C) where aldosterone synthesis takes place. Furthermore, lipotrophic A-ZIP/F-1 mice have 2.8-fold elevated corticosterone plasma levels (288 ± 118 nmol/L vs. wild-type 103 ± 19 nmol/L, $p = 0.15$), but similar ACTH levels in comparison to wild-type controls (261 ± 66 pg/ml vs. wild-type 192 ± 111 pg/ml, $p = 0.61$).

Interestingly, no differences were observed in mRNA expression of aldosterone synthase CYP11B2 (Figure 1D) and of the steroidogenic enzymes StAR and CYP21A1 (data not shown). Thus, a compensatory increase of aldosterone synthesis combined with decreased zona glomerulosa area and the elevated corticosterone synthesis can not be explained by an increased transcription of steroidogenic enzymes, but possibly by an enhancement of cholesterol supply for steroidogenesis.

Structure of adrenal gland in A-ZIP/F-1 mice

Light microscopy

Lipotrophic A-ZIP/F-1 mice are devoid of subcutaneous and visceral white fat tissue as previously described (Moitra et al. 1998). Normal adrenals in wild-type animals are surrounded by periadrenal adipose tissue (Figure 2A). Interestingly in A-ZIP/F-1 mice remnant periadrenal adipose tissue is frequently found in a subcapsular location even infiltrating the adrenal cortex (Figure 2B). Thus fat cells are found in direct contact with adrenocortical cells primarily of the zona glomerulosa (Figure 2C).

Furthermore, adrenocortical cells in direct juxtaposition with fat cells exhibit a high content of lipid droplets (Figure 2D).

Adipose tissue penetrating under the adrenal gland capsule was observed in all A-ZIP/F-1 mice, but only in 25% of the wild-type mice. Penetration of fat cells into or under the organ capsule occurred at various locations around the adrenal gland and was not limited to any pole of the organ. In 50 % of the A-ZIP/F-1 mice fat cells come into direct contact with zona glomerulosa cells whereas this was not observed in any of the wild-type animals.

Electron microscopy

The most prominent organelles of normal steroid producing cells of the adrenals are mitochondria and vast amounts of smooth endoplasmic reticulum (SER). Furthermore, normal adrenocortical cells in wild-type animals exhibit liposomes, Golgi apparatus and filopodia (Figure 3A). In contrast, adrenocortical cells of lipoatrophic mice present ample lipid droplets filling large areas of the cytoplasm (Figure 3B). These liposomes are frequently observed in direct contact with mitochondria (Figure 3B).

Zona glomerulosa cells in normal wild-type mice are arranged in small clusters and exhibit a smaller cytoplasm and round or oval nuclei. The most characteristic organelles are the mitochondria. Mitochondria in the zona glomerulosa cell are frequently oval-shaped (Figure 3C). The internal mitochondrial membranes form vesicular or short tubular structures. The mitochondrial matrix is clear and prominent (Figure 3C).

In lipoatrophic mice there are significant structural alterations in adrenocortical cells. Mitochondria are more variable in size and frequently present with circular internal membranes (Figure 3D). Internal membranes are completely bridging the matrix,

while some mitochondria are almost devoid of internal membranes. Some mitochondria form whirl-like structures with enlarged or blurry internal membranes in direct contact with elongated smooth endoplasmic reticulum (Figure 3F). Furthermore, adrenocortical cells in A-ZIP/F-1 mice have large amounts of glycogen particles and prominent plasma membrane changes. Adrenocortical cells are frequently interconnected by cellular junctions (Figure 3E) and a budding of the plasma membrane is observed (Figure 3F).

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Discussion

In the present study we observed that lipoatrophic A-ZIP/F-1 mice have remarkable structural changes on adrenal gland, which were accompanied by development of arterial hypertension.

Although there is a highly significant reduction in the size of the adrenal zona glomerulosa in lipoatrophic mice, there is no significant reduction in the expression of aldosterone synthase or the secretion of aldosterone. There may be several explanations for this phenomenon. Steroid synthesis takes place in the smooth endoplasmic reticulum and the mitochondria. Efficiency of steroid synthesis depends on the number, size and conformational arrangement of the internal mitochondrial membranes and their interplay with membranes of the SER (Bornstein et al. 1992). Therefore, it is well conceivable that the increased number of internal mitochondrial membranes, the circular arrangement and the juxtaposition with SER facilitate steroid production in the A-ZIP/F-1 mice, further compensating for the reduction of zona glomerulosa cell mass.

Another interesting characteristic of A-ZIP/F-1 mice concerns the residual brown adipose tissue. As described previously, the lipoatrophic A-ZIP/F-1 mice have virtually no white adipose tissue and reduced amount of brown adipose tissue (Moitra et al. 1998). In accordance with previously published data, the periadrenal BAT in A-ZIP/F-1 mice showed a single lipid droplet per cell, and peripheral nuclei, resembling WAT, or inactive BAT as suggested by Moitra et al. (Moitra et al. 1998). Contrary to the wild-type mice, periadrenal BAT was not restricted to pericapsular, but was observed mainly subcapsular, in direct contact with adrenocortical steroid-producing cells. We have previously observed that adipocyte-derived factors directly stimulate adrenal aldosterone secretion (Ehrhart-Bornstein et al. 2003), partially through the

Wnt-signalling pathway (Schinner et al. 2007). Thus, the residual BAT in A-ZIP/F-1 mice, infiltrating the zona glomerulosa, may exert a paracrine effect on aldosterone synthesis and/or release, compensating for the reduced number of aldosterone-producing cells within the adrenal gland. Furthermore, the A-ZIP/F-1 mice showed reduced but detectable serum levels of leptin and resistin, even in the absence of WAT. Thus, these adipokines may be produced by the residual BAT in A-ZIP/F-1 mice.

In accordance with our previous studies (Haluzik et al. 2002), we observed increased corticosterone levels in A-ZIP/F-1 mice. Interestingly, the increase of plasma corticosterone levels verified in the present study was not accompanied by an increase of basal ACTH secretion. Leptin has been identified as an important metabolic signal to the adrenal gland, inhibiting adrenal glucocorticoid but not aldosterone release (Bornstein et al. 1997; Glasow et al. 1998). Previous studies have demonstrated that chronic leptin treatment of A-ZIP/F-1 mice partially reduced corticosterone plasma levels, thus suggesting that leptin deficiency is one of the causes of increased adrenal glucocorticoid production (Haluzik et al. 2002). However, leptin treatment did not normalize the increased fasting levels of corticosterone, so that other causes may be implicated. The present finding of elevated corticosterone levels without a parallel increase of ACTH levels suggests a peripheral alteration of adrenal gland itself.

A-ZIP/F-1 mice, despite lack of WAT, have significantly increased body weight relative to wild-type mice. The main reason for this finding is, as previously described (Moitra et al. 1998), the presence of organomegaly, especially hepatomegaly but also increased other organs such as spleen, kidney and heart. The organomegaly observed in A-ZIP/F-1 mice is most likely due to the IGF-1-like effects of insulin.

Insulin levels are extremely high in the A-ZIP/F-1 mice, therefore probably leading to enlargement of viscera through activation of IGF-1 receptor.

A further characteristic of A-ZIP/F-1 mice observed in the present study is an increased arterial blood pressure. Thus, the absence of WAT resembles the metabolic syndrome in humans, with hypertension, hyperlycemia, hyperinsulinemia, and dyslipidemia. Previous studies have suggested an important role for leptin in the regulation of blood pressure. Izawa-Abe et al. (Izawa-Abe et al. 2000) have demonstrated increased blood pressure in skinny transgenic mice with leptin overexpression relative to wild-type littermates while leptin-deficient *ob/ob* mice had completely normal blood pressure. These data would suggest that leptin deficiency might protect against arterial hypertension induced by severe insulin resistance as seen in *ob/ob* mice. Here we demonstrated that this is not the case in lipoatrophic A-ZIP/F-1 mice despite having markedly reduced serum leptin levels.

The presence of arterial hypertension thus resembles the situation in some patients with human lipodystrophies. These otherwise clinically heterogeneous disorders, characterized by increased insulin resistance, hyperinsulinemia, dyslipidemia and hepatic steatosis, are not always accompanied by hypertension (Garg 2004). Acquired lipodystrophies are more common than the inherited forms, and among these, lipodystrophies in HIV-patients treated with protease inhibitors are the most prevalent type observed to date. Recent studies have observed increased arterial blood pressure in these subjects (Sattler et al. 2001). The mechanisms underlying insulin resistance, metabolic complications and hypertension in lipodystrophic subjects are still unclear. It is supposed that the increased circulating fatty acids and ectopic triglyceride storage play an important pathogenetic role. Interestingly, the most severe arterial hypertension and insulin resistance is found in inherited lipodystrophy associated with PPARG mutations, even with moderate fat loss

(Hegele and Leff 2004). PPARG^{p465L/+} mice have minimal insulin resistance accompanied by a mild adipose tissue redistribution, but have severe hypertension (Tsai et al. 2004). Thus, hypertension in lipodystrophy can be explained not only by hyperinsulinemia and increased circulating fatty acids, but also by other mechanisms such as PPARG mutations. Recently it has been suggested that altered vascular function in lipoatrophic mice may partially explain the development of hypertension in these mice (Takemori et al. 2007). A further possible mechanism underlying the development of hypertension may be related to altered adrenal function, as observed in the present study.

In conclusion, A-ZIP/F-1 lipodystrophic mice exhibit marked morphological and functional adrenal changes accompanied by hypertension. These findings further support an important crosstalk between adipose tissue and adrenal function.

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Figure 1.

A, Micrograph of adrenal gland of wild-type (**A**) and A-ZIP/F-1 (**B**) mice showing width of adrenal zona glomerulosa (ZG) in μm . **B**, Morphometric analysis of adrenal ZG in μm of wild-type (open bars) and A-ZIP/F-1 (solid bars) mice. Data are mean \pm SEM, $n = 4$ animals per group. **C**, plasma aldosterone levels of wild-type (open bars) and A-ZIP/F-1 (solid bars) mice. Data are mean \pm SEM, $n = 12$ animals per group. **D**, Quantification of aldosterone synthase (CYP11B2) mRNA expression of adrenal gland from wild-type (open bars) and A-ZIP/F-1 (solid bars) mice. CYP11B2 mRNA copies were normalized to the copies of β -actin. Data are mean \pm SEM, $n = 5-6$ animals per group. *** $p < 0.001$.

Figure 2.

Histology of adrenal gland. **A**, wild-type mice show periadrenal adipose tissue surrounding adrenal gland. **B**, **C**, A-ZIP/F-1 mice show residual periadrenal adipose tissue in a subcapsular location, invading the adrenal cortex. **D**, adrenocortical cells of A-ZIP/F-1 mice in direct contact with fat cells show a high content of lipid droplets. Toluidine blue staining, magnification 20x (**A**, **B**); 40x (**C**, **D**).

Figure 3.

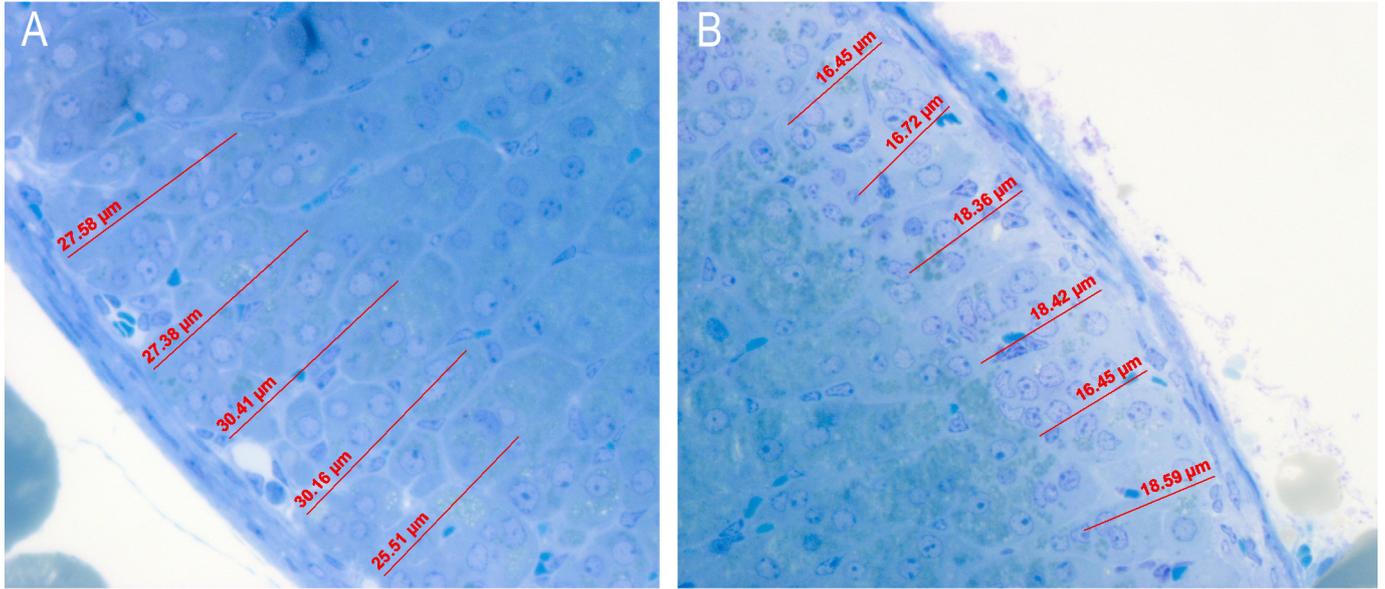
Electron micrographs of adrenocortical cells of wild-type (**A**, **C**) and A-ZIP/F-1 (**B**, **D**, **E**, **F**) mice. Adrenocortical cells of A-ZIP/F-1 mice show high amount of lipid droplets in the cytoplasm, frequently in direct contact with mitochondria (**B**, arrows). Mitochondria in the zona glomerulosa are normally oval-shaped, with vesicular and short tubular structures (**C**). Mitochondria in the zona glomerulosa of A-ZIP/F-1 mice are variable in size, with circular internal membranes (**D**). Some mitochondria form whirl-like structures in direct contact with elongated smooth endoplasmic reticulum

(F). Adrenocortical cells are interconnected by cellular junctions (E, arrows) and show a budding of the plasma membrane (F, arrows). Glomerulosa cells in A-ZIP/F-1 mice have large amounts of glycogen particles (D, E). Mit., mitochondria; Nuc., nucleus; SER, smooth endoplasmic reticulum; Lip., liposomes; Glyc., glycogen particles.

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Figure 1

A



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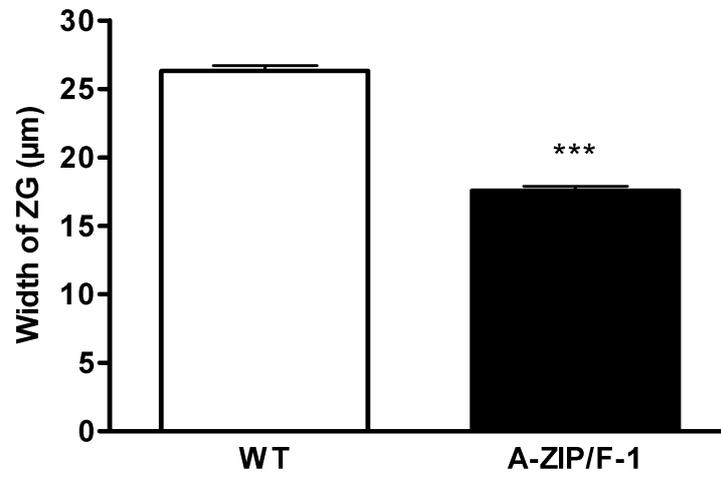
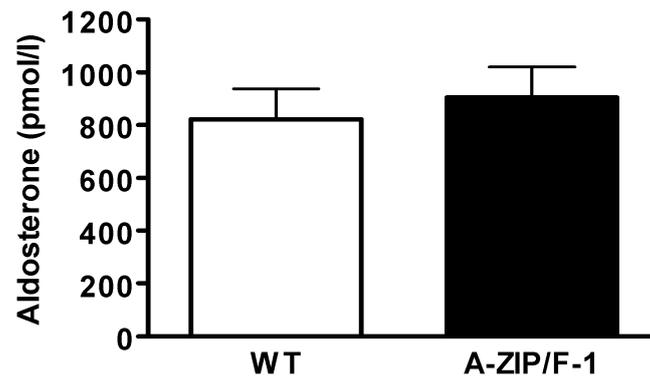
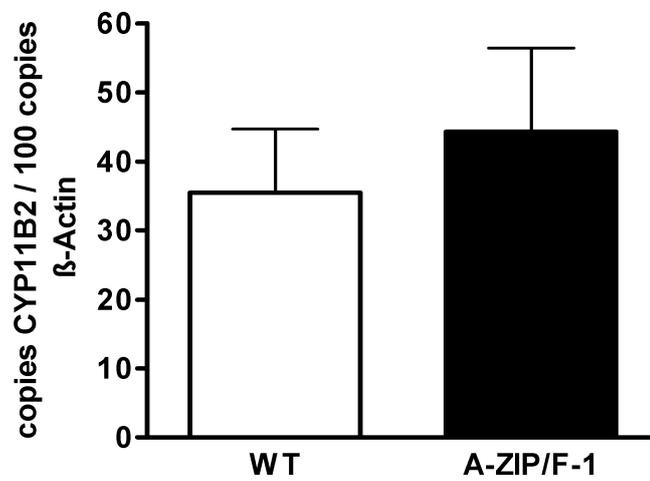
B**C****D**

Figure 2

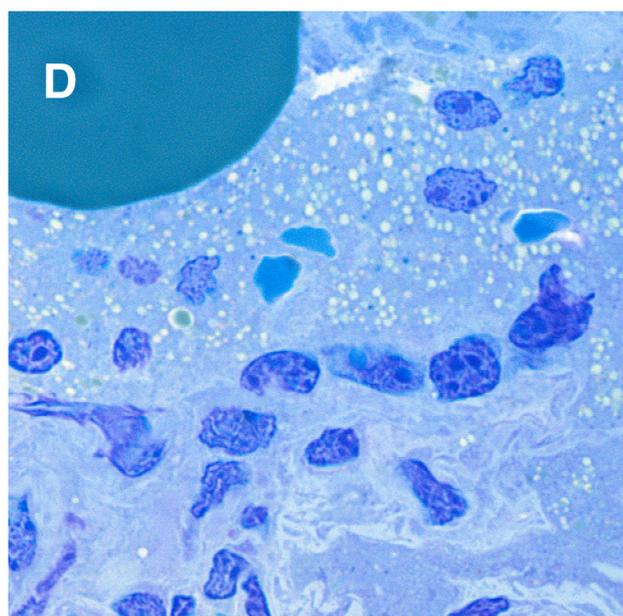
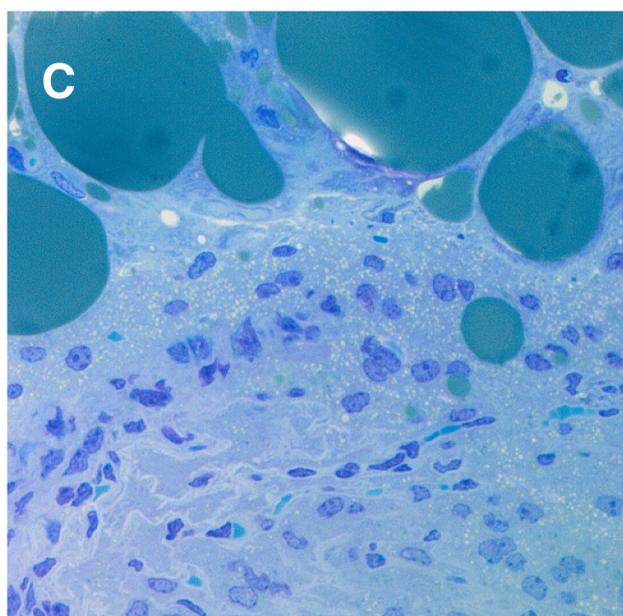
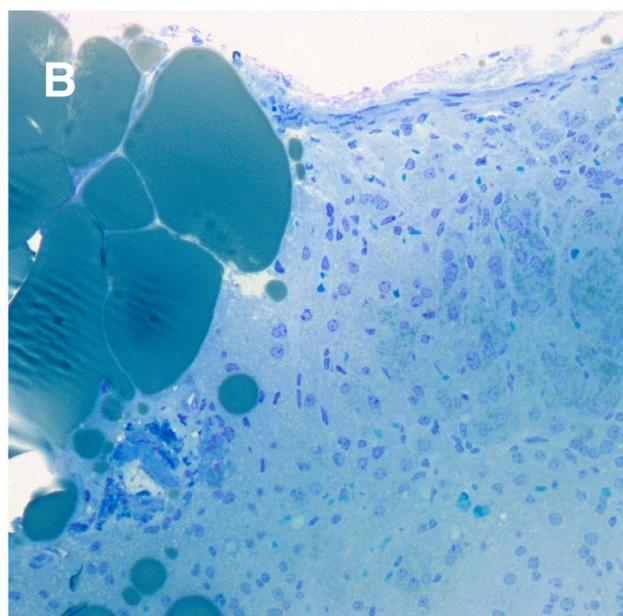
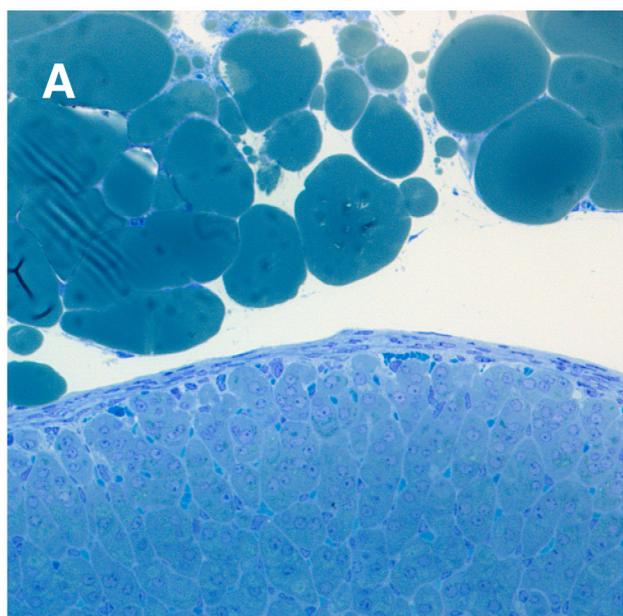


Figure 3

