SOCS3 expression in SF1 cells regulates adrenal differentiation and exercise performance

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Abstract

Many hormones/cytokines are secreted in response to exercise and cytokine signaling may play a pivotal role in the training adaptations. To investigate the importance of cytokine signaling during vertical ladder climbing, a resistance exercise model, we produced mice lacking SOCS3 protein exclusively in steroidogenic factor-1 (SF1) cells (SF1 Socs3 KO mice). SF1 expression is found in steroidogenic cells of the adrenal cortex and gonads, as well as in neurons of the ventromedial nucleus of the hypothalamus. Histological markers of the fetal adrenal zone (or X-zone in rodents) were still present in adult males and postpartum SF1 Socs3 KO females, suggesting a previously unrecognized effect of SOCS3 on the terminal differentiation of the adrenal gland. This change led to a distinct distribution of lipid droplets along the adrenal cortex. Under basal conditions, adult SF1 Socs3 KO mice exhibited similar adrenal weight, and plasma ACTH and corticosterone concentrations. Nonetheless, SF1 Socs3 KO mice exhibited a blunted ACTH-induced corticosterone secretion. The overall metabolic responses induced by resistance training remained unaffected in SF1 Socs3 KO mice, including changes in body adiposity, glucose tolerance and energy expenditure. However, training performance and glucose control during intense resistance exercise were impaired in SF1 Socs3 KO mice. Furthermore, a reduced counter-regulatory response to 2-deoxy-D-glucose was observed in mutant mice. These findings revealed a novel participation of SOCS3 regulating several endocrine and metabolic aspects. Therefore, cytokine signaling in SF1 cells exerts an important role to sustain training performance possibly by promoting the necessary metabolic adjustments during exercise.

Key Words

- ▶ adrenal cortex
- ▶ counter-regulatory response
- ▶ cytokine signaling
- ▶ steroidogenic factor-1
- ▶ ventromedial nucleus.

Journal of Endocrinology
(2017) **235**, 207–222

Introduction

Cytokines normally regulate cellular behavior via the activation of plasma membrane receptors (Krebs & Hilton 2001). Many cytokine receptors recruit the Janus kinase/signal transducer and activator of transcription

(JAK/STAT) pathway, which controls the transcription of numerous genes and thereby regulates cell proliferation, differentiation and apoptosis (Krebs & Hilton 2001). Cytokine signaling is modulated by proteins from the

family of suppressors of cytokine signaling (SOCS). SOCS proteins comprise 8 proteins that can bind tyrosine-phosphorylated residues of cytokine receptors or JAK, blocking their capacity to activate intracellular signaling pathways (e.g., activation of STAT proteins) or leading them to proteosomal degradation (Krebs & Hilton 2001, Howard & Flier 2006). Interestingly, cytokine signaling can robustly increase the SOCS expression, indicating that SOCS proteins regulate cytokine signaling via a negative feedback loop (Krebs & Hilton 2001, Howard & Flier 2006).

Among the different SOCS proteins, SOCS3 has received considerable attention because several studies described its effects controlling important metabolic aspects. Obese animals exhibit increased SOCS3 expression in different tissues (Bjorbaek *et al.* 1998, Jorgensen *et al.* 2013). Notably, brain-specific or muscle-specific SOCS3 ablation improves insulin sensitivity and may partially prevent diet-induced obesity (Mori *et al.* 2004, Jorgensen *et al.* 2013, Pedroso *et al.* 2014). These effects occur because SOCS3 inhibits several cytokines/hormones that regulate the metabolism, such as leptin, insulin, growth hormone, prolactin, interleukin-6 and insulin-like growth factor-1 signaling (Krebs & Hilton 2001, Howard & Flier 2006).

Physical exercise produces robust metabolic consequences (Laing *et al.* 2016). Importantly, the levels of many hormones/cytokines are markedly altered by acute or chronic exercise, including those inhibited by SOCS3 (Strasser *et al.* 2012, Golbidi & Laher 2014). Exercise increases SOCS3 expression in different tissues (Spangenburg *et al.* 2006, Trenerry *et al.* 2007, Pereira *et al.* 2015), suggesting that SOCS3 may have a profound impact on cytokine signaling during exercise, which highlights a possible participation of this protein in the regulation of training adaptations. In the present study, we hypothesize that exercise-induced cytokine signaling plays a pivotal role in the training adaptations, which may depend on the activation of signaling pathways either in specific brain areas or peripheral tissues. For example, earlier studies observed that physical exercise activates cytokine signaling pathways in the hypothalamus (Pereira *et al.* 2015) or skeletal muscle (Spangenburg *et al.* 2006, Trenerry *et al.* 2007, Begue *et al.* 2013).

Since whole-body SOCS3-knockout mice exhibit embryonic lethality (Roberts *et al.* 2001), we employed the Cre-loxP system to produce tissue-specific SOCS3 ablation, as previously described (Mori *et al.* 2004, Jorgensen *et al.* 2013, Pedroso *et al.* 2014, Zampieri *et al.* 2015, Bohlen *et al.* 2016). To investigate the potential role of SOCS3

in exercise-induced metabolic adaptations, we generated mice lacking SOCS3 only in cells that express the nuclear receptor steroidogenic factor-1 (SF1). In the central nervous system, SF1-positive cells are specifically found in the ventromedial nucleus of the hypothalamus (VMH) (Ikeda *et al.* 1995, Segal *et al.* 2005, Kim *et al.* 2011), which comprises an important neuronal population involved in the regulation of the energy balance, glucose homeostasis and autonomic nervous system (Brobeck 1946, Powley 1977, Ikeda *et al.* 1995, Majdic *et al.* 2002, Seoane-Collazo *et al.* 2015). Additionally, VMH neurons express receptors for several hormones whose levels change in response to physical exercise, including insulin (Klockener *et al.* 2011), leptin (Nagaishi *et al.* 2014), growth hormone (Furigo *et al.* 2017), prolactin (Furigo *et al.* 2014, 2017) and interleukin-6 (Schobitz *et al.* 1993). Therefore, cytokine signaling in the SF1/VMH cells may promote metabolic adjustments during or after exercise. In fact, a recent study showed that a hypothalamic SF1 expression is required for the beneficial metabolic effects of exercise (Fujikawa *et al.* 2016). Pituitary gonadotropic cells as well as steroidogenic cells of the adrenal cortex and gonads also express SF1, which controls the transcription of key enzymes that synthesize steroid hormones (Ikeda *et al.* 1995, Zhao *et al.* 2001, Parker *et al.* 2002). Since exercise performance is also affected by glucocorticoids and sex hormone levels, the SOCS3 expression in these endocrine cells possibly modulates training adaptations as well. Thus, the objective of the present study was to characterize the endocrine and metabolic consequences of SOCS3 ablation in SF1 cells. Subsequently, these conditional knockout mice were subjected to a resistance exercise protocol in order to investigate whether the SF1-specific SOCS3 expression regulates training performance and the metabolic effects of exercise.

Material and methods

Animals and genotyping

Mice expressing the Cre recombinase under SF1 promoters (Tg(Nr5a1-cre)7Lowl/J, The Jackson Laboratory, Bar Harbor, ME, USA) were bred with animals carrying a loxP-flanked *Socs3* allele (B6.129S4-Socs3^{tm1Ayo}/J, The Jackson Laboratory). Mice carrying an SF1-specific SOCS3 inactivation (SF1 *Socs3* KO group) were homozygous for the loxP-flanked *Socs3* allele and carried the Cre transgene, whereas the control group was composed of littermate mice carrying only the loxP-flanked *Socs3* allele

in homozygosity. SF1-Cre animals were also bred with the Cre-inducible tdTomato-reporter mouse (B6;129S6-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J, The Jackson Laboratory). The purpose of this breeding was to allow the visualization of SF1-positive cells through the red fluorescent tdTomato protein. Mice were weaned at 3–4 weeks of age and genotyped through PCR using DNA extracted from the tail tip (REDExtract-N-Amp Tissue PCR Kit, Sigma-Aldrich). Mice were bred and maintained under standard conditions of light (12-h light/darkness cycle; lights on at 8:00 h) and temperature ($22 \pm 2^\circ\text{C}$). In all experiments, animals received a regular rodent chow diet (2.99 kcal/g; 9.4% calories from fat; Quimtia, Colombo, Brazil). The animal procedures were approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo (protocol number 71/2016).

Characterization of SF1 Socs3 KO mice

To identify Cre-mediated DNA recombination, the hypothalamus, cerebellum, adrenal gland and liver of SF1 Socs3 KO mice were collected. After the same DNA extraction protocol employed for genotyping, we performed a PCR reaction to identify a 250-bp DNA fragment that would indicate Cre-mediated SOCS3 ablation (Mori *et al.* 2004). Control primers were also used to confirm that DNA extraction and PCR reaction did not fail in negative controls (cerebellum and liver). To analyze the effects of SF1-specific SOCS3 ablation in the hypothalamus, SF1-Cre/tdTomato-reporter or SF1 Socs3 KO mice ($n=3$) were transcardiacally perfused with formalin and their brains were prepared for histological analysis, as previously described (Furigo *et al.* 2014, Nagaishi *et al.* 2014). The distribution of tdTomato-expressing cells in the brain was assessed. Additionally, the VMH cytoarchitecture was analyzed through DAPI staining using Dapi-Fluoromount-G mounting medium (EMS, Hatfield, PA, USA), standard thionin staining and after a peroxidase reaction to label estrogen receptor- α immunoreactivity (ER α -ir) in the VMH of control and SF1 SOCS3 KO mice, as previously described (da Silva *et al.* 2014). To analyze the consequences of SF1-specific SOCS3 ablation in the gonadal function, the testicles were weighed and plasma samples were collected to assess testosterone levels by enzyme-linked immunosorbent assay (Calbiotech; TE187S-100; Spring Valley, CA, USA). Additionally, fertility was evaluated in males and females by measuring the time required for control and SF1 Socs3

KO mice to produce litters. The potential effects in the adrenal gland were evaluated by measuring adrenal weight, and basal plasma corticosterone (DetectX Enzyme Immunoassay Kit, Arbor Assays, Ann Arbor, MI, USA) and adrenocorticotrophic hormone levels (ACTH; Milliplex MAP Mouse Bone Magnetic Bead Panel, Millipore Corporation). An ACTH stimulation test was also performed in 3-month-old male mice as previously described (Scheys *et al.* 2011). Briefly, mice received i.p. injections of 5 mg/kg dexamethasone at the lights off 1 day before and at 10:00 h on the test day to suppress the hypothalamic–pituitary–adrenal (HPA) axis. Two hours later, a blood sample was collected (time 0), followed by an i.p. injection of 1 mg/kg ACTH (Bachem, Torrance, CA, USA). Mice were killed after 60 min to assess serum corticosterone levels. The histological analysis of the adrenal gland was evaluated in another group of adult males (5 months) and in virgin or postpartum adult females. In the latter case, females were allowed to nurse the pups for 5 days. Then, the pups were removed and the adrenals were collected after approximately 5 days. The counter-regulatory response to 2-deoxy-D-glucose (2DG) was evaluated by assessing changes in glycemia after an i.p. infusion of 0.5 mg/kg 2DG (Sigma) for 3 h (Flak *et al.* 2014). Serum ACTH and corticosterone levels were also determined 60 min after an i.p. injection of 0.5 mg/kg 2DG.

Experimental design

Experimental design is summarized in Fig. 1. Four-month-old male mice were distributed in 4 groups according to genotype and training condition: sedentary control ($n=20$), exercise control ($n=20$), sedentary SF1 Socs3 KO ($n=16$) and exercise SF1 Socs3 KO ($n=16$). The exercise groups were subjected to an 8-week resistance exercise protocol, as described in detail later, whereas sedentary groups were exposed to the same experimental conditions, except for the training protocol. Exercise training was performed in the light period from 14:00 h to 16:00 h (lights off at 20:00 h) and researchers were blinded to the experimental groups. Along the experiment, mice were weighted weekly. Performance was measured through the increments of the weight lifted during the 8-week training period and by one repetition maximum (1RM) tests performed at the beginning of training protocol and after 4 and 8 weeks of training. Glycemia was assessed just before the beginning of the last 1RM test and immediately after exhaustion using a portable glucometer (OneTouch, Johnson & Johnson).

Figure 1

Scheme that summarizes the experimental design. 1RM, one repetition maximum; CLAMS, Comprehensive Lab Animal Monitoring System; GTT, glucose tolerance test; ITT, insulin tolerance test.

After 4 and 5 weeks of training, mice were subjected to a glucose tolerance test (GTT; 2 g glucose/kg; i.p.) and an insulin tolerance test (0.8 IU insulin/kg; i.p.), respectively. During the last 2 weeks of training, mice were placed in the OxyMax/Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA) to analyze O_2 consumption (VO_2), CO_2 production (VCO_2), respiratory exchange ratio (RER; VCO_2/VO_2), food intake, water intake and locomotor activity (through infrared beam sensors). After 3 days of adaptation inside the CLAMS, the metabolic parameters of each mouse were evaluated for 4 consecutive days. Therefore, the results presented here were the average of this period. Both sedentary and exercise groups were evaluated in the CLAMS, and the exercise groups were maintained in their regular training protocol. The data produced by the CLAMS were presented as pre-training (the light cycle before the training session from 8:00 h to 14:00 h), post-training (the light cycle immediately after the training session from 16:00 h to 20:00 h) and dark cycle (from 20:00 h to 8:00 h). In the following day after the last training session, food was removed from cages for 4 h and mice were killed by decapitation (approximately from 14:00 h to 16:00 h). Body adiposity was calculated by summing the weight of the perigonadal, subcutaneous and retroperitoneal fat pads. The soleus muscle was removed in order to assess cross-sectional area (CSA) of individual fibers after a standard hematoxylin and eosin (HE) staining. Quantification and evaluation of fiber CSA (150 fibers per muscle) were performed as previously described (Fortes *et al.* 2015). After removing the soleus muscle, sections from the midportion region were obtained. A cryostat (Leica CM3050 S; Leica Microsystems, Nussloch GmbH, Germany) was used to acquire a 10 μ m thick section from each muscle.

Sections were imaged using an optical microscope with camera (Nikon DXM 1200; Nikon Instruments). Digitized images were analyzed using the AxioVision software (version 4.8.1.0, Carl Zeiss).

Resistance exercise protocol

A 110 cm high and 18 cm wide ladder angled at 80° and with a 2 cm spacing between the steps (Hornberger & Farrar 2004) was used for the training sessions. A 20 × 20 × 20 cm chamber is located at the top of the ladder and serves as a shelter during the rest periods between the series of climbs. Initially, the animals from the exercise groups were familiarized to the apparatus for 3 days, which consisted of three climbing attempts. Before the first attempt, mice were kept in the shelter for 90 s to feel safe. On the first attempt, the mouse was placed at the ladder top portion at an approximate distance of 35 cm from the door of the shelter. On the second attempt, the animal was positioned at the middle of the ladder at a distance of approximately 55 cm from the door of the shelter. On the third attempt, the animal was placed at the bottom of the ladder at a distance of approximately 110 cm from the door of the shelter. Afterward, animals were adapted to the exercise protocol. The resistance training started after the adaptation period, which consisted of 4 series of 8 climb repetitions during 1 week without any load. The protocol was adapted from Kim and coworkers and consisted of climbing a vertical ladder with progressive loads attached to the mouse tail base (Kim *et al.* 2015). To determine the initial training workload, all mice were subjected to a 1RM test, which consisted of climbing repetitions carrying a progressively heavier load. In the initial climbing, mice had to carry 75% of the animal's own body mass load. When mice reached the top of the ladder, a rest of 2 min was given before the next attempt. During subsequent attempts, an additional 5 g load was added until the animal was unable to climb the ladder completely. Thus, the load carried in the last successful climbing was considered as the 1RM load. The training protocol consisted of 4 series of climbing repetitions with progressive overload. Each series contained an average of 8 repetitions. In the first series, animals had to carry 75% of 1RM load. An additional 5–10% 1RM load was added in the following series. The load was kept until the animal could climb the whole ladder. If the animal could perform all series, the load of the last series would be the initial load on the following day. Resistance ladder climbing exercise was performed 5 days per week for 8 weeks.

Adrenal and pituitary analysis

For the HE staining, left adrenal glands from 5-month-old males and 3-month-old virgin or postpartum females of control and SF1 Socs3 KO groups (5 per group) were removed, fixed in formalin and embedded in paraffin. Serial histological sections (10 µm thickness) were used for a standard HE staining procedure. The cell morphology and the cortical areas images were captured under a light microscope (Nikon Eclipse 80i) and analyzed by Neurolucida and Neurolucida Explorer software (MBF Bioscience). For the quantification of the adrenal X-zone, images were captured with 10× magnification. The X-zone area was manually delimited based on the strong eosin staining. The area occupied by eosinophilic cells in the corticomedullary junction was determined using ImageJ software (ImageJ 1.51n, Wayne Rasband, National Institute of Health, Bethesda, MA, USA). For the oil red O (ORO) staining and immunofluorescence (IF) against anti-cytochrome P450 family 17 subfamily A member1 (Cyp17a1), right adrenal glands were collected and the excess fat was removed. The tissue was rapidly frozen in dry ice and embedded in optimal cutting media (OCT, Sakura) for cryosection. For the ORO staining and quantification, we performed a standard protocol, as previously described (Mehlem *et al.* 2013). The relative quantification of ORO staining was performed in 15 different images of the adrenal cortex per animal and analyzed using the ImageJ software, following the protocol previously described (Mehlem *et al.* 2013). For the IF, sections (3 animals from each experimental group) were fixed in ice-cold acetone (10 min) and washed in PBS. Blocking solution (10% donkey serum) was applied in each section and incubated for 1 h. After washing, primary anti-Cyp17a1 (ab134910; Abcam) at 1:250 dilution (PBS+0.3% Triton X+1% donkey serum) was applied and sections were incubated overnight at 4°C. Sections were washed and incubated with 1:1000 secondary donkey anti-rabbit IgG Alexa Fluor 594 (Jackson ImmunoResearch Laboratories). Sections were mounted on antifade reagent (ProLong Gold, Life Technologies) and analyzed under a fluorescent microscope (Nikon Eclipse 80i). Fresh pituitary glands from adult male mice were collected to analyze the mRNA levels of pituitary hormones. The protocol to extract the total RNA, produce the complementary DNA and perform the RT-PCR has been described in previous studies from our group (Pedroso *et al.* 2014, 2016, Zampieri *et al.* 2015, Bohlen *et al.* 2016). The expression of the constitutive gene cyclophilin A was used to normalize the data. The following primers were used: cyclophilin A (forward: tatctgcactgccaagactgagt;

reverse: cttcttgctggtcttgccattcc), GH (forward: gcttctcagagaccatcccg; reverse: agcgagaagcgaagcaattc), POMC (forward: tagatgtgtggagctggtgc; reverse: ccagcgagaggtcgagtttg), LHβ (forward: agttctgcccagctctgcatc; reverse: actcggaccatgctaggaca) and FSHβ (forward: aataccacttggtgtgcggg; reverse: gccaggcaatcttacggtct).

Statistical analysis

When data from control and SF1 Socs3 KO groups were compared, independently of training protocol, the unpaired two-tailed Student's *t*-test was used. To analyze the effects of exercise training and/or SF1-specific SOCS3 ablation, a two-way ANOVA test followed by Bonferroni posttests was employed. GraphPad Prism software was used for the statistical analyses and the results were expressed as mean ± S.E.M. Only *P* values <0.05 were considered to be statistically significant.

Results

Characterization of SF1 Socs3 KO mice

It is well established that the hypothalamus and adrenal cortex contain SF1-expressing cells, whereas no SF1 expression is normally found in cerebellum and liver (Ikeda *et al.* 1995, Kim *et al.* 2011). Using primers that amplify a 250-bp DNA fragment, which is only expressed after Cre-mediated recombination in Socs3 gene (Mori *et al.* 2004), we confirmed that SF1 Socs3 KO mice had SOCS3 ablation in the hypothalamus and adrenal gland, but not in the cerebellum or liver (Fig. 2A). To identify the distribution of SF1-expressing cells in the brain, we used a Cre-inducible tdTomato-reporter mouse. So, SF1-positive cells could be visualized by the red fluorescent protein tdTomato. In accordance with previous studies that mapped the distribution of SF1 expression in the brain (Ikeda *et al.* 1995, Segal *et al.* 2005, Kim *et al.* 2011), the tdTomato expression was abundantly found in the VMH and only few scattered cells were present in other brain nuclei (Fig. 2B). Therefore, central SOCS3 ablation essentially affected VMH neurons. SF1 is critically involved in the VMH formation (Ikeda *et al.* 1995, Majdic *et al.* 2002) and ablation of SF1 in the central nervous system drastically reduces the expression of steroid receptors in the VMH (Kim *et al.* 2010). We assessed VMH cytoarchitecture and integrality through thionin staining (Fig. 2C and D) and by the presence of ERα in the ventrolateral part of the VMH (VMHvl; Fig. 2E and F), but no changes were observed in SF1 Socs3 KO mice.

Figure 2

Characterization of SF1 Socs3 KO mice.

(A) Agarose gel showing the products of a PCR reaction from different tissues of SF1 Socs3 KO mice. A 250-bp DNA fragment indicates Cre-mediated recombination in the Socs3 gene, whereas a ~800-bp fragment is a positive control band. (B) Epifluorescence photomicrograph illustrating the distribution of SF1 neurons in the ventromedial nucleus of the hypothalamus (VMH), using a reporter mouse that expresses the red fluorescent tdTomato protein under Cre recombination. DAPI staining was used as an anatomical reference. (C and D) Thionin staining illustrating a normal VMH cytoarchitecture in SF1 Socs3 KO mice. (E and F) Peroxidase reaction to label estrogen receptor- α immunoreactivity (ER α -ir) in the VMH of control and SF1 Socs3 KO mice. 3V, third ventricle; ARH, arcuate nucleus of the hypothalamus; VMHc, central part of the VMH; VMHdm, dorsomedial part of the VMH; VMHvl, ventrolateral part of the VMH. Scale bar = 200 μ m.

SOCS3 expression in SF1 cells is required for the terminal differentiation of the adrenal gland and maximal adrenal steroidogenic capacity

Previous studies have indicated that prolactin signaling induces the SOCS3 expression in the adrenal cortex of rats (Tam *et al.* 2001), and maternal dietary restriction can induce adrenocortical hypertrophy by upregulating cytokine signaling (Zhang *et al.* 2013). Whether SOCS3 plays a role in the adrenal cortex physiology remains unclear. Thus, here we investigated morphological and functional aspects of the adrenal cortex of SF1 Socs3 KO mice. HE staining revealed a different pattern of the cortex morphology in the medullary boundary (Fig. 3), with some clusters of eosinophilic cells matching the description and localization of the fetal adrenal zone or X-zone in rodents (Jones 1952). Indeed, these cells were positive for the steroidogenic enzyme Cyp17A1 (Fig. 4A and B), which is a marker for X-zone in rodents (Keeney *et al.* 1995).

ORO staining showed that the presence of this zone disturbed the morphology of the adjacent zones, especially the distribution of lipid droplets along the cortex (Fig. 4C, D, E and F). Due to this change, the total amount of lipids in adrenal cortex was significantly decreased ($P=0.0011$) in the SF1 Socs3 KO animals compared to control mice (Fig. 5A). We then evaluated possible functional consequences in the adrenal of SF1 Socs3 KO mice. Under basal conditions, control and SF1 Socs3 KO mice exhibited similar adrenal weight (Fig. 5B) as well as plasma ACTH (Fig. 5C) and corticosterone (Fig. 5D) levels. However, when the adrenal capacity to secrete corticosterone was evaluated during an ACTH stimulation test, SF1 Socs3 KO mice exhibited a blunted response in comparison with control animals (Fig. 5E). These results indicate a lower

Figure 3

SF1-specific SOCS3 ablation induces histological alterations in the adrenal cortex. (A, B, C, D, E, F, G and H) Photomicrographs of the adrenal gland of control (A, C, E and G) and SF1 Socs3 KO (B, D, F and H) mice after hematoxylin/eosin staining. The arrows indicate the localization of the fetal adrenal zone (or X-zone in rodents) in the medullary boundary, which is visible only in SF1 Socs3 KO mice. m, adrenal medulla; x, X-zone; zf, zona fasciculata; zg, zona glomerulosa. Scale bars=0.05 mm.

maximal adrenal steroidogenic capacity in SF1 Socs3 KO mice, even though their adrenal function seems to be normal during basal conditions.

SF1-specific SOCS3 ablation leads to a lower testis weight without affecting testosterone levels, fertility or pituitary function

SF1 expression is also found in the gonads (Ikeda *et al.* 1995, Kim *et al.* 2011), indicating that SOCS3 ablation may have occurred in the testis of SF1 Socs3 KO mice.

In fact, SOCS3 can modulate leptin and interleukin-6 signaling and affect testicular functions (Yuan *et al.* 2014, Huang *et al.* 2016). To assess the consequences of SF1-specific SOCS3 ablation in testicular functions, we initially measured testis weight. SF1 Socs3 KO mice exhibited significantly lighter testes compared to control animals (Fig. 6A). However, no changes in plasma testosterone levels were observed between groups (Fig. 6B). In addition, fertility was not impaired in male SF1 Socs3 KO mice (Fig. 6C), indicating that despite the lower testis weight, SF1 Socs3 KO mice

Figure 4

Presence of X-zone in adrenal glands of SF1 Socs3 KO mice. (A and B) Photomicrographs of the adrenal gland of control (A) and SF1 Socs3 KO (B) mice after immunofluorescence against Cyp17a1 ($n=3/\text{group}$). Insets show a high-powered field of the adrenal cortex in the medullary boundary. (C, D, E and F) Photomicrographs of the adrenal gland of control (C and D) and SF1 Socs3 KO (E and F) mice after oil red O staining. m, adrenal medulla; x, X-zone; zf, zona fasciculata; zg, zona glomerulosa. Scale bars: (A, B and C) E=0.1 mm; D and F=0.05 mm; insets=0.01 mm.

showed no apparent reproductive dysfunction. Pituitary-specific SF1 inactivation causes infertility due to a robust reduction in LH and FSH expressions, but does not affect other pituitary cell types, such as somatotropes, corticotropes and thyrotropes

(Zhao *et al.* 2001). To investigate whether SF1-specific SOCS3 ablation affects the pituitary gland, we analyzed the mRNA levels of several pituitary hormones. SF1 Socs3 KO male mice showed similar mRNA levels of GH, POMC, LH β and FSH β in the pituitary gland, compared

Figure 5

Effects of SF1-specific SOCS3 ablation in the adrenal gland function. (A) Quantification of oil red O (ORO) staining, which represents the total amount of lipids in adrenal cortex ($n=18/\text{group}$). (B, C and D) Basal adrenal weight (B) and plasma ACTH (C) and corticosterone (D) levels in control ($n=3-5$) and SF1 Socs3 KO ($n=3-6$) mice. (E) Serum corticosterone levels during an ACTH stimulation test ($n=7/\text{group}$). *significant ($P<0.05$) different than control group (unpaired two-tailed Student's t -test).

Figure 6

Effect of SF1-specific SOCS3 ablation in gonadal and pituitary functions. (A) Testis weight in control ($n=8$) and SF1 Socs3 KO ($n=9$) mice. (B) Plasma testosterone levels in control ($n=5$) and SF1 Socs3 KO ($n=4$) mice. (C) Fertility test evaluated by measuring the number of days required for control ($n=20$) and SF1 Socs3 KO ($n=30$) males to produce litters when breeding with female mice. (D) Gene expression analysis in the pituitary gland of control ($n=4$) and SF1 Socs3 KO ($n=4$) mice. (E) Quantification of the area occupied by eosinophilic cells in the corticomedullary junction, representing the X-zone. Unpaired two-tailed Student's *t*-test was used in the statistical analyses. *, significant ($P < 0.05$) different than virgin control and postpartum SF1 Socs3 KO groups.

to control animals (Fig. 6D). Therefore, SF1-specific SOCS3 inactivation does not induce apparent defects in the pituitary function.

SOCS3 expression in SF1 cells is required for X-zone regression in postpartum adult females

In female mice, the X-zone atrophy disappears after the first pregnancy (Tanaka & Matsuzawa 1995). As expected,

a histological analysis in the adrenal gland of virgin control females confirmed the presence of the X-zone in the medullary boundary (Figs 6E and 7A, D and G), which disappeared in postpartum control females (Figs 6E and 7B, E and H). Remarkably, SF1 Socs3 KO postpartum females retained the X-zone in the adrenal cortex (Figs 6E and 7C, F and I), indicating that the SOCS3 expression in SF1 cells is required for X-zone regression in postpartum adult females. The fertility of female mice was also

Figure 7

Presence of X-zone in adrenal gland of SF1 Socs3 KO postpartum female mice. (A, D and G) Photomicrographs of the adrenal gland of a virgin control female. (B, E and H) Photomicrographs of the adrenal gland of a postpartum control female. (C, F and I) Photomicrographs of the adrenal gland of postpartum SF1 Socs3 KO female. Black arrows indicate the X-zone. m, adrenal medulla. Scale bar = 0.02 mm.

analyzed. However, the number of days breeding until giving birth (control: 27.8 ± 2.5 days; SF1 Socs3 KO: 27.3 ± 1.7 days) and the number of pups per litter (control: 5.9 ± 0.4 ; SF1 Socs3 KO: 6.7 ± 0.6) were similar between the groups. Thus, SF1-specific SOCS3 ablation did not affect the fertility.

Resistance exercise reduces body adiposity and improves glucose tolerance

We assessed the metabolic consequences of 8-week resistance training. Compared to sedentary animals, exercise training reduced the body weight (Fig. 8A), weight gain (Fig. 8B) and body adiposity (Fig. 8C). Furthermore, glucose tolerance was improved by exercise training (Fig. 8D). These effects were equally observed in control and SF1 Socs3 KO mice (Fig. 8A, B, C and D). SOCS3 deletion or resistance exercise had no significant effect on insulin sensitivity (Fig. 8E). Therefore, SF1-specific SOCS3 deletion did not change the beneficial effects of exercise on energy balance and glucose homeostasis.

Metabolic changes induced by exercise training or SF1-specific SOCS3 ablation

Additional metabolic parameters were obtained in the last 2 weeks of training when mice were evaluated by the Oxymax/CLAMS (Fig. 9). During the post-training period (4h after the exercise session), exercise groups exhibited increased VO_2 (Fig. 9B), as well as reduced RER (Fig. 9E) and locomotor activity (Fig. 9H) compared to sedentary animals, independently of the genotype. Exercise also increased water intake during the dark cycle (Fig. 9O). We observed a significant effect of SF1-specific SOCS3 ablation by reducing the RER in the dark cycle (Fig. 9F),

the food and water intake during the pre-training period (Fig. 9J and M) and the water intake in the post-training period (Fig. 9N).

Impaired training performance, glucose control during intense exercise and counter-regulatory response to 2DG in SF1 Socs3 KO mice

Training performance was initially assessed through the weight lifted by the animals during the training sessions. During the first weeks of training, SF1 Socs3 KO and control mice carried similar loads during the climbing series (Fig. 10A). However, while control animals progressively increased the weight lifted, SF1 Socs3 KO mice were not able to lift similar loads from the fifth week of training, compared to control animals (Fig. 10A). The 1RM tests also confirmed the impaired exercise performance of SF1 Socs3 KO mice along the training protocol (Fig. 10B). Regarding the 1RM tests performed in the first and fourth weeks of training, SF1 Socs3 KO mice exhibited a similar performance compared to control animals (Fig. 10B). However, at the eighth week of training, SF1 Socs3 KO mice did not present improvements in their performance to the same extent as control animals (Fig. 10B). The analysis of the CSA of individual fibers of the soleus muscle revealed an interaction effect between the training status and the genotype ($P=0.0494$). While exercise training increased in 22% soleus CSA of individual fibers in control animals, the exercise SF1 Socs3 KO group exhibited no increment compared to sedentary mutants (data not shown).

An adequate blood glucose level is necessary to maintain performance during exercise (Coyle 1999). Since both the adrenal cortex and the VMH are related to the control of blood glucose (Verberne *et al.* 2014, Meek *et al.* 2016), we investigated whether SOCS3 ablation in SF1 cells affects the glycemia regulation during exercise. Thus,

Figure 8

Resistance exercise reduces body adiposity and improves glucose tolerance. (A, B, and C) Body weight (A), weight change (B) and body adiposity (C) at the end of experiment in sedentary control ($n=20$), exercise control ($n=20$), sedentary SF1 Socs3 KO ($n=16$) and exercise SF1 Socs3 KO ($n=16$). (D and E) Glucose tolerance test (D) and insulin tolerance test (E) after 4 and 5 weeks of training, respectively ($n=13/\text{group}$). *, significant ($P < 0.05$) effect of exercise (two-way ANOVA test).

Figure 9

Metabolic changes induced by exercise training or SF1-specific SOCS3 ablation. (A, B, C, D, E, F, G, H, I, J, K, L, M, N and O) VO_2 (A, B and C) respiratory exchange ratio (RER, D, E, and F), locomotor activity (G, H, and I), food intake (J, K and L) and water intake (M, N and O) in sedentary control ($n=14$), exercise control ($n=15$), sedentary SF1 Socs3 KO ($n=13$) and exercise SF1 Socs3 KO ($n=14$). These data represent the average analysis of 4 consecutive days and were presented as pre-training (the light cycle before the training session from 8:00 h to 14:00 h), post-training (the light cycle immediately after the training session from 16:00 h to 20:00 h) and dark cycle (from 20:00 h to 8:00 h). *significant ($P < 0.05$) effect of exercise (two-way ANOVA test). †significant ($P < 0.05$) effect of SF1-specific SOCS3 ablation.

we assessed glycemia just before the beginning of the last 1RM test and immediately after exhaustion. Despite the high intensity of the 1RM test, in which mice carried approximately 4 times their body weight during the ladder climbing, control animals were able to sustain the same pre-exercise glycemia values at the fatigue moment (Fig. 10C). On the other hand, SF1 Socs3 KO mice exhibited a significantly lower glycemia at the exhaustion compared to pre-exercise glycemia (Fig. 10C). Therefore, the SOCS3 expression in SF1 cells is required to sustain training performance and glycemia during exercise. SF1-positive neurons in the VMH are critically involved in the glucoregulatory responses required to reestablish glycemia under metabolic challenges like hypoglycemia or 2DG administration (Flak *et al.* 2014, Verberne *et al.* 2014, Meek *et al.* 2016). Thus, we assessed whether SF1

Socs3 KO mice exhibit changes to the counter-regulatory response evoked by 2DG administration. Notably, the conditional knockout mice showed an attenuated glucoregulatory response compared to control animals (Fig. 10D and E). This defect occurred despite a similar activation of the HPA axis, since serum ACTH (Fig. 10F) and corticosterone (Fig. 10G) levels showed no differences between the groups after 2DG infusion.

Discussion

In the present study, we investigated whether the SOCS3 expression in SF1 cells regulates adrenal, gonadal and pituitary functions, resistance training performance as well as the metabolic effects of exercise. Our hypothesis was that exercise-induced cytokine signaling exerts an

Figure 10

Impaired training performance, glucose control during intense exercise and counter-regulatory response to 2-deoxy-D-glucose (2DG) in SF1 Socs3 KO mice. (A) Changes in the weight lifted by control ($n=9$) and SF1 Socs3 KO ($n=8$) mice along the 8-week training period. (B) Maximal weight lifted during 1RM tests performed in the first, fourth and eighth weeks of training ($n=9$ /group). (C) Glycemia measured just before the last 1RM test and immediately after exhaustion ($n=9$ /group). (D) Evaluation of the counter-regulatory response induced by an acute injection of 0.5 mg/kg 2DG. (E) Area under the curve in the glycemia after 2DG administration ($n=10$ –12/group). (F and G) Serum ACTH (F) and corticosterone (G) levels in control ($n=12$) and SF1 Socs3 KO ($n=8$) mice 60 min after an i.p. injection of 2DG. Unpaired two-tailed Student's *t*-test was used in the statistical analyses.

important modulation in the training adaptations, and SF1-specific SOCS3 ablation might change the metabolic responses to exercise. Our results revealed that SF1 Socs3 KO mice exhibit histological and functional changes in the adrenal gland. The overall metabolic responses induced by resistance training remained unchanged in SF1 Socs3 KO mice. However, resistance training performance and glucose control during intense exercise were impaired in mice carrying SF1-specific SOCS3 ablation. Furthermore, a reduced counter-regulatory response to hypoglycemia was also observed in SF1 Socs3 KO mice. Therefore, our findings suggest a previously unrecognized participation of SOCS3 modulating several endocrine and metabolic aspects.

The main role of the adrenal gland is to maintain body homeostasis by secreting corticosteroids, such as corticosterone and aldosterone in rodents. Multiple signaling pathways have already been described to promote steroid hormone biosynthesis in steroidogenic cells, including cytokine-induced cAMP-independent pathways (Stocco *et al.* 2005). Our results show that SOCS3 ablation in SF1-positive cells apparently did not alter the adrenal gland function under basal conditions. For example, adrenal weight and basal corticosterone and ACTH level were not affected in SF1 Socs3 KO mice.

However, a reduction in the total amount of lipids along the adrenal cortex was observed in SF1 Socs3 KO mice, which suggests a lower capacity to produce steroid hormones, since steroidogenic cells do not store significant amounts of steroid hormones, and there is a need for a constant supply of cholesterol (including from lipid droplets) to synthesize new steroid hormones when the cell is stimulated (Shen *et al.* 2016). In accordance with these results, SF1 Socs3 KO mice showed a blunted adrenal steroidogenic capacity when corticosterone secretion was evaluated during an ACTH stimulation test. Therefore, these findings indicate that although SF1 Socs3 KO mice are able to maintain normal adrenal function during basal conditions, their maximal adrenal steroidogenic capacity seems to be limited.

One of the most intriguing phenotypes observed in SF1 Socs3 KO mice was the retention of the fetal adrenal X-zone in adult animals. In rodents, a transient cell layer, named the X-zone, is located in the corticomedullary junction and regresses at puberty in males and at the first pregnancy in females (Holmes & Dickson 1971). Interestingly, even with the presence of this zone in SF1 Socs3 KO mice, the atrophy of the adult cortex resulted in an adrenal weight equivalent to control animals. Knockout mice for the *Stat5a* and *Stat5b* genes

have no profound effect on X-zone morphology or on the expression of 20 α -hydroxysteroid dehydrogenase, which is a steroidogenic enzyme expressed in X-zone cells (Hershkovitz *et al.* 2007). Although the molecular mechanisms that drive fetal zone regression and terminal differentiation of the adrenal cortex are not yet clearly defined, our findings suggest that SOCS3 is a previously unrecognized factor required for the terminal differentiation of the adrenal gland.

The beneficial metabolic effects of exercise are well established (Strasser *et al.* 2012, Golbidi & Laher 2014, Sarvas *et al.* 2015). Chronic exercise improves energy and glucose homeostasis (Laing *et al.* 2016). Interestingly, several studies reported increased SOCS3 expression in response to exercise in the skeletal muscle (Spangenburg *et al.* 2006, Trenerry *et al.* 2007, Guerra *et al.* 2011, Begue *et al.* 2013) or hypothalamus (Pereira *et al.* 2015). Although these results seem to be conflicting since increased SOCS3 levels are associated with metabolic imbalances, such as obesity and insulin resistance (Bjorbaek *et al.* 1998, Mori *et al.* 2004, Howard & Flier 2006, Jorgensen *et al.* 2013), an increased SOCS3 expression may simply indicate an acute effect of exercise caused by cytokine/hormone secretion. In fact, chronic exercise causes no changes or even a reduction in the SOCS3 expression in different tissues, which may help to explain the higher insulin and leptin sensitivity observed in trained animals (Steinberg *et al.* 2004, Yaspelkis *et al.* 2010, Guerra *et al.* 2011, Olmedillas *et al.* 2011, Kang *et al.* 2013, Sarvas *et al.* 2015).

Previous studies have shown improved body adiposity and glucose homeostasis after SOCS3 ablation (Mori *et al.* 2004, Pedroso *et al.* 2014, 2016, Zampieri *et al.* 2015, Bohlen *et al.* 2016). Zhang *et al.* (2008) also studied mice carrying SF1-specific SOCS3 deletion. These authors observed no changes in body weight and adiposity in mutant mice exposed to normal chow or high-fat diet. However, different from our results, they found improved glucose homeostasis and lower energy expenditure in SF1 Socs3 KO mice (Zhang *et al.* 2008). Comparing control and SF1 Socs3 KO mice, independently of the training status, we only found very small metabolic differences. For example, we observed a lower food and water intake in SF1 Socs3 KO mice during the pre-training period. A previous study also showed that SOCS3 deletion in SF1 cells reduces food intake in mice, an effect attributed to increased leptin sensitivity in the VMH (Zhang *et al.* 2008). The reduced water intake was probably secondary to a lower food intake, since water intake is important for swallowing the dry pelleted feed. Furthermore, our data

confirmed some expected metabolic effects induced by exercise. Trained animals showed a lower body weight and adiposity, and an improved glucose tolerance compared to sedentary groups. Regarding the acute effects induced by the training session, we observed a significant effect of exercise during the post-training period by increasing VO₂, probably representing the excess post-exercise oxygen consumption (Maehlum *et al.* 1986) and by reducing RER and locomotor activity, which may indicate, respectively, a higher fat oxidation and a tiredness state. Water intake also increased in the dark period possibly indicating a post-exercise rehydration. However, none of these parameters were affected by SF1-specific SOCS3 ablation.

SF1 Socs3 KO mice showed a remarkably lower performance during the last weeks of training. The lack of changes in the first weeks of training may indicate that at lower intensities, SF1-specific SOCS3 ablation causes no significant biological effects for the exercise adaptations. However, when the intensity progressively increased, mutant mice were not able to lift similar loads during training sessions or 1RM test, compared to control animals. Interestingly, SF1 Socs3 KO mice were also unable to maintain circulating glucose levels during the last 1RM test. An incapacity to control glycemia at situations that consume high amounts of glucose is certainly a factor that can impair exercise performance and thereby training adaptations (Coyle 1999). A reduced adrenal capacity to respond to stress-secreting glucocorticoids could impair high-intensity exercise training by disrupting glycemia control.

Defects in VMH neurons could also affect glucose homeostasis and other metabolic changes. It is well established that VMH neurons control the energy balance, glucose homeostasis and autonomic nervous system (Brobeck 1946, Powley 1977, Ikeda *et al.* 1995, Majdic *et al.* 2002, Seoane-Collazo *et al.* 2015). Pharmacological manipulations in VMH neurons can decrease plasma glucose levels, hepatic glycogenolysis, lipolysis and fatty acid oxidation during exercise, leading to a reduced performance (Vissing *et al.* 1989, Balkan *et al.* 1991, Wanner *et al.* 2010, Miyaki *et al.* 2011). Exercise also increases noradrenergic and dopaminergic tonus to VMH neurons (Kitaoka *et al.* 2010), which in turn modulate the autonomic nervous system in order to produce the required adjustments in the cardiovascular system, nutrient mobilization and glucose homeostasis (Ball 2015, Seoane-Collazo *et al.* 2015). Accordingly, VMH SF1-positive neurons send projections to autonomic sympathetic and parasympathetic centers of the

hypothalamus and hindbrain, which innervate white and brown adipose tissue, heart, liver, pancreas and skeletal muscles (Lindberg *et al.* 2013, Seoane-Collazo *et al.* 2015). VMH SF1-positive neurons are also fundamental components of the neurocircuitry that regulates counter-regulatory responses to hypoglycemia, ensuring a rapid and efficient recovery of glucose levels (Flak *et al.* 2014, Verberne *et al.* 2014, Meek *et al.* 2016). In the present study, a lower glucoregulatory response to 2DG was observed in SF1 Socs3 KO mice. Since VMH neurons trigger the counter-regulatory response evoked by 2DG and the reduced counter-regulatory response occurred despite a similar activation of the HPA axis by 2DG, these results suggest a possible involvement of VMH SF1 neurons in the metabolic defects observed in SF1 Socs3 KO mice.

In conclusion, our study showed for the first time that SOCS3 expression in SF1 cells is required to maintain training performance and glucose control during intense resistance exercise. Additionally, SF1-specific SOCS3 ablation decreased testis weight, although it did not affect the fertility, and caused histological and functional changes in the adrenal cortex. Furthermore, an attenuated counter-regulatory response to hypoglycemia was also observed in SF1 Socs3 KO mice. Therefore, our results revealed a novel participation of SOCS3 regulating several endocrine and metabolic aspects. These findings provide new insights into how cytokine signaling can modulate multiple biological functions, especially regarding physical exercise and the terminal differentiation of the adrenal gland.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP; grant numbers 10/18086-0, 12/23535-4, 13/21722-4, 13/24409-5, 14/11752-6, 14/50140-6 and 15/10992-6).

Author contribution statement

J A B P and J D designed the study; J A B P, I T, V L P, L B L, I C F and D N B performed the exercise training and *in vivo* analyses; M A S F did the muscle analysis; P O R M, T B A, I C C and C F P L did the adrenal analysis; A M R L performed the brain histology; J A B P, M A S F, P O R M, T B A, I C C, C F P L and J D analyzed the data; J A B P, P O R M and J D wrote the paper. All authors read and approved the final version of the manuscript.

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Received in final form 15 August 2017

Accepted 12 September 2017

Accepted Preprint published online 12 September 2017