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BROWN ADIPOSE TISSUE FUNCTION IN SHORT-CHAIN ACYL-COA DEHYDROGENASE DEFICIENT MICE

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Abstract

Brown adipose tissue is a highly specialized organ that uses mitochondrial fatty acid oxidation to fuel nonshivering thermogenesis. In mice, mutations in the acyl-CoA dehydrogenase family of fatty acid oxidation genes are associated with sensitivity to cold. Brown adipose tissue function has not previously been characterized in these knockout strains. Short-chain acyl-CoA dehydrogenase (SCAD) deficient mice were found to have increased brown adipose tissue mass as well as modest cardiac hypertrophy. Uncoupling protein-1 was reduced by 70% in brown adipose tissue and this was not due to a change in mitochondrial number, nor was it due to decreased signal transduction through protein kinase A which is known to be a major regulator of uncoupling protein-1 expression. PKA activity and *in vitro* lipolysis were normal in brown adipose tissue, although in white adipose tissue a modest increase in basal lipolysis was seen in SCAD−/ − mice. Finally, an *in vivo* norepinephrine challenge of brown adipose tissue thermogenesis revealed normal heat production in SCAD−/− mice. These results suggest that reduced brown adipose tissue function is not the major factor causing cold sensitivity in acyl-CoA dehydrogenase knockout strains. We speculate that other mechanisms such as shivering capacity, cardiac function, and reduced hepatic glycogen stores are involved.

Keywords

brown adipose tissue; fatty acid oxidation; nonshivering thermogenesis; acyl-CoA dehydrogenase; uncoupling protein-1; protein kinase A

Introduction

Brown adipose tissue (BAT) generates tremendous amounts of heat through uncoupling protein -1 (UCP1)-mediated dissipation of the mitochondrial membrane potential. BAT is essential for defending core body temperature in small mammals and newborn humans [1].

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The heat comes largely from the oxidation of fatty acids and the whole process is regulated by norepinephrine signaling through β3 adrenergic receptors, cAMP formation, and protein kinase A (PKA)[1]. PKA activation induces numerous downstream events including increased lipolysis and UCP1 gene expression.

Knockout and transgenic mouse models have greatly facilitated our understanding of BAT and the factors that contribute to non-shivering thermogenesis. Dopamine β-hydroxylase knockout mice, which completely lack norepinephrine, cannot survive an acute cold challenge and neither can UCP1 knockout mice [2,3]. Surprisingly, mice deficient in any of the acyl-CoA dehydrogenase (ACAD) family of mitochondrial fatty acid oxidation enzymes are as cold sensitive, if not more so, than the UCP1 knockout strain [4,5,6]. ACADs display overlapping substrate specificities and thus knocking out any one ACAD enzyme results in only a partial block of mitochondrial fatty acid oxidation. Short-chain acyl-CoA dehydrogenase (SCAD) optimally utilizes C_4-CoA, medium-chain acyl-CoA dehydrogenase (MCAD) prefers C_8-CoA, long-chain acyl-CoA dehydrogenase (LCAD) prefers substrates 12–16 carbons in length, and very long-chain acyl-CoA dehydrogenase (VLCAD) is most active against acyl-CoAs between 16 and 20 carbons in length.

Cold sensitivity has become an important marker of dysfunctional mitochondrial fatty acid oxidation. But it remains to be determined why partial blocks in fatty acid oxidation are sufficient to cause lethal cold sensitivity and what mechanisms are involved. We hypothesized that adrenergic signaling may be impaired in ACAD-deficient mice. SCAD, LCAD, and VLCAD mice all fail to increase UCP1 mRNA during cold exposure [7,8]. Rather, all three strains show several-fold increases in UCP2 mRNA. SCAD null mice also have increased mRNA levels for the β1-adrenoreceptor subtype [7] which does not normally mediate adrenergic signaling in mature BAT. In the present studies we characterized PKA activity, UCP1 abundance, lipolysis, and adrenergic responsiveness in SCAD−/ − mice.

Materials and Methods

Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Eight-week old male BALB/cByJ mice (hereafter referred to as SCAD−/ −), which harbor a naturally occurring deletion in the SCAD gene [9,10], and BALB/cJ wildtype controls were acquired from Jackson Laboratories (Bar Harbor, ME). Snap-frozen LCAD−/ − and VLCAD−/ − BAT samples were a kind gift of Philip Wood (Burnham Institute, Lake Nona, FL).

Preparation of BAT tissue extracts

Frozen BAT was homogenized for 30 seconds in 250 μl of lysis buffer (250 mM sucrose, 25 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1% Triton-X 100) supplemented with Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL). Following homogenization the samples were placed in a sonicating ice bath for 5 min, incubated on ice for 30 min, and cleared by centrifugation. Protein concentrations were determined using the Bio-Rad D_c Protein Assay Reagent (Bio-Rad, Hercules, CA).

ACAD enzyme activity

Enzyme activity was measured with the anaerobic electron transferring flavoprotein (ETF) fluorescence reduction assay as previously described [11]. The reaction was started by the addition of either C_4-CoA (SCAD) or C_{16}-CoA (LCAD, VLCAD) at a final concentration of 10 μM. Activities were calculated as mU of activity per mg of total protein.

BAT DNA content

Entire BAT depots were carefully excised, weighed, and minced. The tissue was digested overnight with proteinase K (0.5 mg/mL) in NTES buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS) at 55°C. Total DNA was isolated by standard phenol-chloroform extraction, precipitated with isopropanol, and washed twice with ethanol. DNA was solubilized at 37°C in 100 ul of 10 mM Tris-HCl and the concentration measured by A_{260}.

Western blotting

Western blotting was conducted as previously described [11]. Rabbit anti-mouse UCP1 (Alpha Diagnostic International, San Antonio, TX) and anti-human HSL (Cell Signaling Technology, Beverly, MA) were used at a 1:1000 dilution. Anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control at 1:1000. HRP-conjugated secondary antibodies were used at 1:3000.

Mitochondrial DNA copy number

DNA was isolated from BAT (~20mg) using silica membrane spin columns (QIAamp DNA Mini; Qiagen, Valencia, CA). Quantitative real-time PCR was used to determine mitochondrial DNA copy number relative to nuclear DNA using the protocol published by Trinei et al [12]. Samples were run in triplicate on an ABI 7900HT Sequence Detection System. Specific primers were used to amplify a fragment of the mitochondrial D-loop region and a fragment of the telomerase gene (TERT) as a nuclear DNA control [12]. The threshold cycle values (CT) for each sample were calculated using Applied Biosystem's SDS software, version 2.2. Relative copy number was calculated using the $2^{-\text{deltaCT}}$ method where delta $CT = CT^{\text{mtDNA}} - CT^{\text{TERT}}$.

In vitro lipolysis

Lipolysis was measured as the release of glycerol from BAT and white adipose tissue (WAT) explants. Non-fasted mice were sacrificed early in the light period. Epididymal WAT and interscapular BAT were excised and placed in cold Krebs-Ringer buffer pH 7.4 (Sigma, St. Louis, MO) with 2% fatty acid-free BSA. Each tissue was minced into 8–10 pieces which were weighed on a digital balance. Tissue pieces were incubated in 500 μl of the Krebs-Ringer/BSA buffer at 37°C on an orbital shaker set to 40 rpm. After 10 minutes of pre-incubation, norepinephrine was added to half of the tissue pieces at a final concentration of 5 μM. After 20 minutes of incubation for BAT or one hour for WAT, the media was removed and frozen at −80°C. Glycerol was measured in the media using a colorimetric glycerol reagent (Sigma, St. Louis, MO). Data were expressed as ng of glycerol/mg tissue/hour.

PKA activity

Frozen BAT tissue was homogenized for 20 s in 500 ul of cold extraction buffer (25 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 0.5 mm EGTA, 10 mM β-mercaptoethanol) supplemented with protease inhibitors. Homogenates were cleared by centrifuging at 14,000 X g for five minutes at 4°C. Supernatants were immediately analyzed for PKA activity using the SignaTECT kit (Promega, Madison, WI). Each sample was assayed in duplicate without added cAMP (basal PKA activity) and also in duplicate with exogenous cAMP (5 μM). Background activity was determined by assaying samples without added PKA substrate.

In vivo norepinephrine challenge

Mice were anesthetized with an intraperitoneal injection of pentobarbital (75 mg/kg) and placed onto a heated surgical pad calibrated to 37°C. A rodent rectal temperature probe and

digital thermometer (Physitemps Instruments, Clifton, NJ) was used to monitor core body temperature for 15 min or until a stable baseline was achieved, defined as no drift in core temperature for at least 3 min. Then norepinephrine was injected (0.2 mg/kg in sterile saline) and body temperature monitored for 25 minutes. After 25 minutes the animals were sacrificed.

Statistical analysis

All statistical comparisons were done by Student's *t*-test and the corresponding *P* values are provided in the figure legends.

Results

SCAD−/ − mice have larger BAT depots and enlarged hearts

Fatty acid oxidation supplies 85% or more of the energy requirements of BAT and heart. Both tissues were found to be significantly enlarged in SCAD−/ − mice (Table 1). Additionally, many of the SCAD−/ − hearts had visible fatty streaks suggesting the possibility of a heart phenotype in these animals that has not previously been reported. While BAT mass was increased approximately 25% compared to wildtype mice, there was a non-significant trend (P=0.073) for decreased BAT cellularity demonstrated by less total DNA content per BAT depot (Table 2). BAT mitochondrial DNA content was the same between genotypes indicating equal numbers of mitochondria (Table 2). The increased weight of BAT was most likely due to increased fat storage as was previously suggested from histological examination of BAT tissue [4].

UCP1 protein is reduced in SCAD−/ − mice but not LCAD−/ − or VLCAD−/ − mice

The basal UCP1 protein content in SCAD−/ − mice housed at standard temperatures was about 3-fold less than in controls (Figure 1). The experiment was repeated on another group of animals with similar results. In contrast, LCAD−/ − and VLCAD−/ − BAT did not show any change in UCP1 content.

Lipolysis is normal in SCAD−/ − BAT but slightly increased in WAT

Based on the increased BAT mass, trend for decreased cellularity, and decreased UCP1 protein content of SCAD−/ − BAT we hypothesized that adrenergically-regulated processes such as lipolysis may be suppressed. No significant difference was seen for *in vitro* BAT lipolysis between SCAD−/ − and control mice (Figure 2). However, there was a modest but statistically significant increase in basal lipolysis for SCAD−/ − white adipose tissue (WAT). Interestingly, glycerol release from BAT explants was unresponsive to stimulation with norepinephrine while WAT showed an approximate doubling in glycerol release. The lack of an increase in glycerol release from BAT during norepinephrine stimulation may be due to recycling of glycerol as BAT possesses glycerol kinase while WAT does not [13].

PKA responds normally to cAMP in SCAD−/ − BAT

Flash-frozen BAT samples were analyzed for PKA activity. There was no detectable basal PKA activity in BAT from either genotype (not shown), probably due to the lability of endogenous cAMP. When the BAT extracts were stimulated with exogenous cAMP equal activity was detected between genotypes (Table 2). These data indicate that PKA is present in normal amounts in SCAD−/ − BAT and activates normally when exposed to cAMP.

SCAD−/ − mice respond normally to norepinephrine in vivo

UCP1 knockout mice do not increase core body temperature in response to injected norepinephrine [14]. We applied this paradigm to SCAD−/ − mice as an indicator of BAT

function *in vivo*. The animals were anesthetized and given intraperitoneal injections of norepinephrine. A rectal probe was used to monitor changes in core body temperature. In both genotypes core body temperature began to increase within 3–5 minutes of injection and the peak response ranged from 0.5 to 1.4 °C. The peak response to norepinephrine did not statistically differ between SCAD−/ − and control mice (Table 2).

SCAD−/ − mice have 30% residual enzyme activity in BAT

Residual ACAD enzyme activity has not previously been measured in BAT from ACAD-deficient mice. The highly sensitive and specific ETF fluorescence reduction assay was used to measure ACAD activity in SCAD−/− BAT extracts with a physiological concentration of C_4-CoA (10 μM) as substrate.SCAD−/− BAT exhibited approximately 30% residual enzyme activity due to the combined activities of other ACADs (Figure 3). LCAD−/− and VLCAD−/− BAT extracts were then tested for enzymatic activity using C_{16}-CoA as substrate. LCAD−/− BAT displayed approximately 50% activity toward C_{16}-CoA while VLCAD−/− BAT had 80% residual activity.

Discussion

It was hypothesized that adrenergic signaling may be disrupted in SCAD−/− mice based on previously reported defects in cold-induced UCP1 mRNA expression. In isolated BAT there was substantially less UCP1 protein but no difference between SCAD−/− and wildtype BAT with regards to lipolysis and PKA activity. Finally, SCAD−/− BAT appeared to produce heat normally when mice were injected with norepinephrine. While other tissues besides BAT are sensitive to norepinephrine, the increase seen in core body temperature is a BAT effect as evidenced by the lack of a thermal response in UCP1 knockout mice under the same conditions [14]. However, the acute norepinephrine challenge experiment has several limitations. Most importantly, it does not allow an accurate determination of the rate of heat production or how long it might be sustained. SCAD−/− mice might mount a normal response to a single large bolus of norepinephrine but still be incapable of the constitutive heat production that would be required to survive in the cold for long periods of time. Also, it is possible that SCAD−/− mice have reduced innervation of BAT or an impaired central nervous system (CNS)-mediated release of norepinephrine in BAT when shifted to a cold environment.

Our enzyme activity assays revealed that SCAD−/ −, LCAD−/ −, and VLCAD−/ − BAT retain 30%, 50%, and 80% of residual enzyme activity, respectively, due to overlapping substrate specificities among the ACADs. These numbers correlate well with previous measures in liver and muscle [15,16]. Interestingly, enzyme activity in the control mice with C_4-CoA (optimal SCAD substrate) was 40–50 times lower than activity with C_{16}-CoA (substrate for LCAD, VLCAD)(Figure 3). Additionally, we have previously noted that C_{16}-CoA ACAD activity in BAT is about 10 times higher than liver activity, which is in keeping with the known high metabolic rate of BAT [11]. In contrast, the C_4-CoA BAT activity measured in this study is several times lower than C_4-CoA activity that we previously recorded in liver [17]. SCAD may constitute a metabolic bottleneck in BAT that is not present in other tissues. VLCAD, on the other hand, appears to play a minimal role in BAT (Figure 3). Yet even mice heterozygous for VLCAD deficiency have been reported to display cold sensitivity after a period of fasting [6]. The minimal reduction of BAT C_{16}-CoA ACAD activity in VLCAD−/− mice and the ability of SCAD−/− mice to generate heat (at least acutely) after norepinephrine injection both point to non-BAT factors as playing a major role in cold sensitivity in these strains.

The stearoyl-CoA desaturase-1 (SCD1) knockout model illustrates how a cold sensitive phenotype can have surprising causes. SCD1 is the rate-limiting enzyme for synthesizing

long-chain monounsaturated fatty acids. SCD1−/− mice have increased basal BAT adrenergic signaling resulting in increased lipolysis, fatty acid oxidation and UCP1 content [18]. Yet they quickly succumb when transferred to a 4°C environment. Later, a skin-specific SCD1 knockout was developed which displays the cold-sensitive phenotype [19]. Changes in the composition of skin lipids rendered the animals less able to retain body heat. It was further shown that cold sensitivity could be ameliorated by maintaining the animals on a high-fat diet which significantly increased hepatic glycogen content and prevented hypoglycemia during cold exposure. Like SCD1−/− mice, the ACAD-deficient strains rapidly deplete their glycogen stores and become hypoglycemic when fasted or cold exposed [6,10]. This was shown to occur in VLCAD+/− heterozygous animals as well [6]. We thus speculate that rapid consumption of glycogen in liver, and also probably in muscle, contributes to the cold sensitivity seen in ACAD knockout strains. VLCAD−/− mice show reduced capacity for exercise manifesting as slower running speed and distance on a treadmill test [20]. Additionally, VLCAD−/−, LCAD−/−, and MCAD−/− mice all display cardiac abnormalities [5,21], and the current study detected a modest cardiac hypertrophy and fatty streaks in SCAD−/ − hearts (Table 1). Impaired ability to shiver and impaired cardiac function could also be factors that contribute to the severe cold sensitivity in these mouse strains.

In conclusion, SCAD−/− mice have low UCP1 protein consistent with a previous report of low mRNA [7]. However, 30% of normal UCP1 is enough to generate heat when injected with norepinephrine which is consistent with studies showing it takes a severe ablation of BAT (90%) to induce cold sensitivity [22]. We speculate that SCAD−/− mice and the other ACAD deficient strains manifest cold sensitivity as a combination of defects in several tissues including liver, muscle, and heart. Further studies are needed to determine whether these strains can be slowly acclimated to the cold and survive on non-BAT sources of thermogenesis as UCP1 knockout mice can [22]. Furthermore, studies are needed to examine innervation and CNS regulation of BAT. The inability of ACAD-deficient strains to induce UCP1 mRNA when placed in the cold remains unexplained.

RESEARCH HIGHLIGHTS

- Cold-sensitive SCAD−/− mice have enlarged brown adipose tissue and hearts.
- SCAD−/− mice have 70% less uncoupling protein-1 in brown adipose tissue.
- Protein kinase A activity and *in vitro* lipolysis are normal in SCAD−/− mice.
- SCAD−/− brown adipose tissue produces heat normally in response to norepinephrine.

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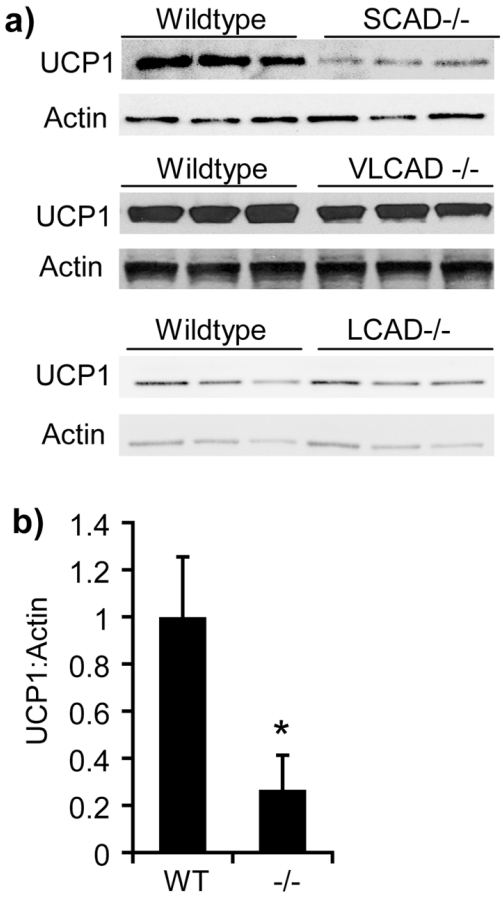

Figure 1. Reduced UCP1 protein in SCAD−/− BAT

a) Total SCAD−/−, LCAD−/−, and VLCAD−/− BAT protein (10μg) was subjected to SDS-PAGE followed by western blotting with anti-UCP1 antibody. Anti-β-actin was used as a loading control. b) Densitometry was used to quantitate the UCP1 and actin signals from SCAD−/− BAT. The bar graph shows the means and standard deviations of the UCP1: actin ratio for wildtype (WT) and SCAD deficient (−/−) mice, **P*<0.001.

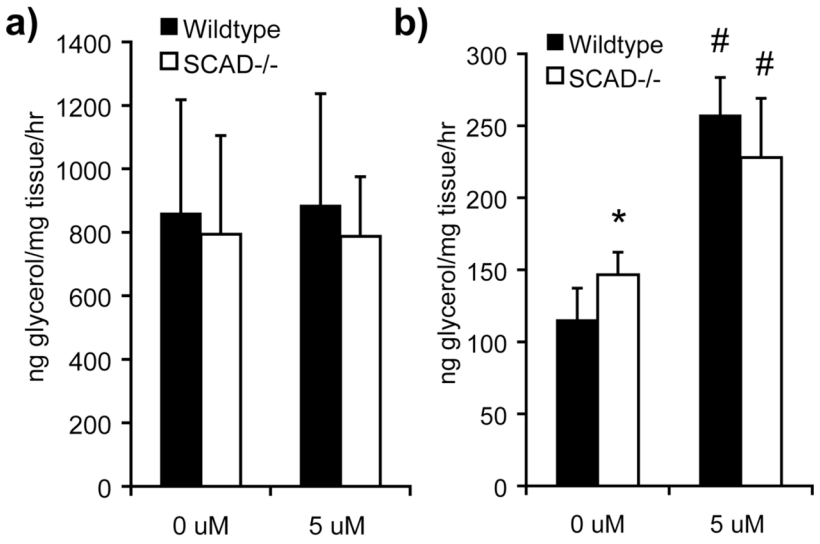

Figure 2. In vitro *lipolysis in SCAD−/− mice*

Lipolysis was measured in BAT (a) and epididymal WAT (b) explants *in vitro* by measuring the appearance of glycerol in the incubation media. Adipose tissues were incubated without (0 μM) and with norepinephrine in the media (5 μM final concentration). Each tissue was analyzed in duplicate. Bar graphs represent the means and standard deviations from N=4 animals per genotype. *P< 0.05 SCAD−/− versus wildtype mice; #, 5 μM norepinephrine significantly stimulated lipolysis over the basal rates for each genotype, P< 0.001.

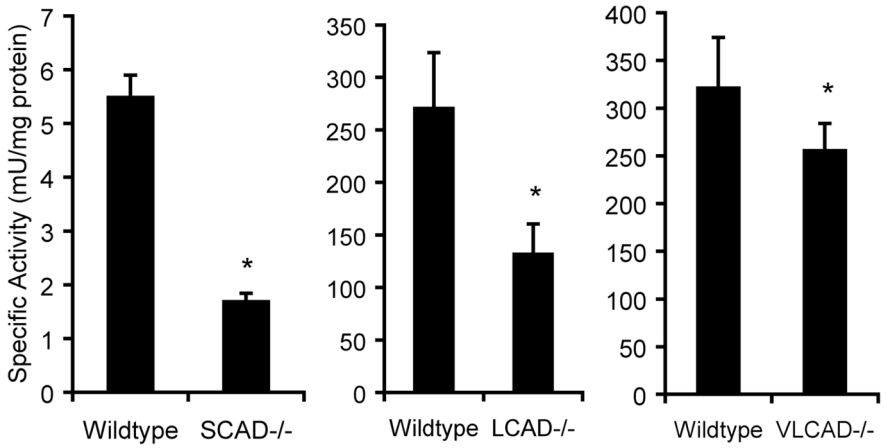

Figure 3. Residual enzyme activity in BAT from ACAD deficient mouse strains

SCAD−/− (*N*=4), LCAD−/− (*N*=3), and VLCAD −/ − (*N*=4) BAT extracts were used for the anaerobic ETF fluorescence reduction assay as described in *Materials and Methods.* BAT extracts prepared from age, sex, and strain-matched mice were used as wildtype controls. The substrates used for the assay were C_4-CoA (10 μM) for SCAD−/− BAT and C_{16}-CoA (10 μM) for LCAD−/− and VLCAD−/− BAT. Bars represent means and standard deviations. **P*<0.05.

TABLE 1

Tissue weights in SCAD−/− mice.

	Wildtype *(N)*	**SCAD−/ − *(N)***	**P Value**
Body weight (g)	27.6 ± 1.2 *(12)*	26.8 ± 1.5 *(12)*	0.163
BAT weight (g)	98.6 ± 17.8 *(12)*	121.1 ± 21.2 *(12)*	*0.010
eWAT weight (g)	0.201 ± 0.035 *(10)*	0.202 ± 0.037 *(10)*	0.926
Heart Weight (g)	0.139 ± 0.009 *(12)*	0.148 ± 0.020 *(12)*	*0.015
Heart : Body Weight Ratio	0.48 ± 0.03 *(12)*	0.55 ± 0.07 *(12)*	*0.003

Abbreviations: BAT, brown adipose tissue; eWAT, epididymal white adipose tissue. Presented are means and standard deviations. The number of animals per group is in parentheses.

*Statistically significant at $P<0.05$.

TABLE 2

Brown adipose tissue characteristics and function in SCAD−/− mice.

	Wildtype *(N)*	**SCAD−/ − *(N)***	**P Value**
Total DNA (μg /BAT depot)	164.4 ± 21.1 *(4)*	136.6 ± 14.7 *(4)*	0.073
mtDNA (arbitrary units)	6489 ± 2127 *(5)*	6768 ± 2234 *(5)*	0.845
Total Protein (μg/mg BAT)	16.6 ± 1.7 *(3)*	16.8 ± 3.9 *(3)*	0.912
PKA Activity (pmol ATP/min/μg)	4.0 ± 0.4 *(5)*	4.6 ± 0.5 *(5)*	0.083
In Vivo Response to NE (°C)	0.9 ± 0.3 *(5)*	1.1 ± 0.3 *(5)*	0.348

Abbreviations: BAT, brown adipose tissue; mtDNA, mitochondrial DNA; PKA, protein kinase A; NE, norepinephrine. Presented are means and standard deviations. The number of animals per group is in parentheses.

*Statistically significant at $P<0.05$