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Intracerebroventricular tempol administration in older rats reduces oxidative stress in the hypothalamus but does not change STAT3 signalling or SIRT1/AMPK pathway

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Abstract

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Hypothalamic inflammation and increased oxidative stress are believed to be mechanisms that contribute to obesity. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (tempol), a free radical scavenger, has been shown to reduce inflammation and oxidative stress. We hypothesized that brain infusion of tempol would reduce oxidative stress, and thus would reduce food intake and body weight and improve body composition in rats with age-related obesity and known elevated oxidative stress. Furthermore, we predicted an associated increase in markers of leptin signalling, including the silent mating type information regulator 2 homolog 1 (SIRT1)/5' AMP-activated protein kinase (AMPK) pathway and the signal transducer and activator of transcription 3 (STAT3) pathway. For this purpose, osmotic minipumps were placed in the intracerebroventricular region of young (3 months) and aged (23 months) male Fischer 344 x Brown Norway rats for the continuous infusion of tempol or vehicle for 2 weeks. Tempol significantly decreased ($p < 0.01$) nicotinamide adenine dinucleotide phosphate oxidase activity in the hypothalamus but failed to reduce food intake or weight gain and did not alter body composition. SIRT1 activity and Acetyl p53 were decreased and phosphorylation of AMPK was increased with age, but they were unchanged with tempol. Basal phosphorylation of STAT3 was unchanged with age or tempol. These results indicate that tempol decreases oxidative stress but fails to alter feeding behaviour, body weight, or body composition. Moreover, tempol does not modulate the SIRT1/AMPK/p53 pathway and does not change leptin signalling. Thus, a reduction in hypothalamic oxidative stress is not sufficient to reverse age-related obesity.

Keywords

tempol; intracerebroventricular (icv); brain; hypothalamus; aging; obesity; SIRT1; AMPK; leptin; p53; FOXO; STAT3; oxidative stress

Introduction

The hypothalamus is a critical brain region that regulates food intake, body weight, and glucose homeostasis. The hypothalamus directly senses and controls the metabolic signals from the periphery and projects neuronal inputs to the corresponding tissues and organs accordingly. In recent years, abundant research has demonstrated that normal reactive oxygen species (ROS) levels produced in mitochondria are vital physiological sensors for hypothalamic glucose and fatty acids (Benani et al. 2007). ROS are chemically reactive molecules containing oxygen (O_2). They are formed as a natural by-product of the normal metabolism of O_2 and play important roles in cell signalling and homeostasis. However, the excessive production of O_2 radicals, superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite cause excitotoxicity and impair the energy metabolism of cells. Indeed, elevated ROS production causes cellular dysfunction and death (Gyengesi et al. 2012). The endogenous antioxidant system (i.e., glutathione peroxidase, superoxide dismutase (SOD), catalase, and uric acid) serves to neutralize and/or convert these ROS into less toxic derivatives, thus preventing the reaction of ROS with DNA, RNA, lipids, or proteins. The production of excessive amounts of ROS depletes the endogenous antioxidants, resulting in increased peroxidation of membrane lipids or oxidation of proteins, leading to DNA fragmentation and inhibition of the mitochondrial electron transport system (Devasagayam et al. 2004). Because of the delicate balance required for efficient cellular function,

increased ROS production is thought to be a causative factor in multiple disease states, including obesity, and is also regarded as a possible cause of aging (Bonomini et al. 2015).

Within the hypothalamus, there are several important nuclei, including the lateral hypothalamic area and the paraventricular, dorsomedial, ventromedial, and arcuate hypothalamic nuclei, that regulate appetite and body weight. Some neurotransmitters have been shown to stimulate food intake and increase body weight, whereas others have an anorectic effect (Ahima and Antwi 2008). Several studies indicate that 5' AMP-activated protein kinase (AMPK) in the hypothalamus regulates energy metabolism by integrating inputs from hormones, peptides, neurotransmitters, and nutrients. Leptin, synthesized in adipose tissue, is one of the most important peptides involved in energy homeostasis, and this hormone communicates the nutritional-satiety state to the hypothalamus and other important reward centers. The anorectic effect of leptin occurs via the increased phosphorylation of signal transducer and activator of transcription 3 (STAT3) (pSTAT3) as well as via its interaction with the AMPK/silent mating type information regulator 2 homolog 1 (SIRT1) pathway (Fig. 1). In the hypothalamus, leptin inhibits AMPK, thus leading to decreased food intake and increased energy expenditure (Lim et al. 2010). SIRT1 and AMPK activity have been shown to control the intracellular energy balance, and SIRT1, in particular, affects longevity (Lim et al. 2010). SIRT1 expression decreases FOXO1 acetylation, suggesting that SIRT1 regulates the central melanocortin system in a FOXO1-dependent manner. In addition, hypothalamic Sirt1 regulates S6K (mammalian target of rapamycin (mTOR) pathway) signalling in such a way that inhibition of the fasting-induced Sirt1 activity results in upregulation of the S6K pathway (Cakir et al. 2009). Impaired hypothalamic leptin signalling (leptin resistance) is associated with increased adiposity and obesity. Furthermore, leptin resistance increases with age, as does the incidence of obesity and inflammation (Koenig et al. 2014).

Hypothalamic inflammation and increased oxidative stress are thought to be one of the underlying mechanisms of obesity (Cai and Liu 2012; Williams 2012). Moreover, aged rats demonstrate an even greater increase in oxidative stress (Erdos et al. 2011). Therefore, we hypothesized that the central infusion of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (tempol), a free radical scavenger, would reduce oxidative stress in the hypothalamus of aged obese rats and would potentially decrease food intake and body weight via restoration of the AMPK/SIRT1-dependent pathway or the leptin-coupled pSTAT3 pathway.

Materials and methods

Animals

Male 3- and 23-month-old Fischer 344 x Brown Norway rats ($N = 5$ per group) were obtained from the National Institute on Aging Colony at Harlan Laboratories (Indianapolis, Ind., USA). These rats constitute a good model for aging studies (Wolden-Hanson 2006, Tümer et al. 2014). The animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals*, and the protocol was approved by the University of Florida Institutional Animal Care and Use Committee (No. 201203230). The rats were maintained on a 12-h light/12-h dark cycle and were provided a standard rodent

chow (17% kcal from fat, 25% kcal from protein, no sucrose, 3.1 kcal/g) (diet 7912, Harlan Teklad, Madison, Wis., USA) and water ad libitum throughout the experimental protocol.

Surgical procedure and treatment

The rats were anesthetized with isoflurane (2%–3%), and their heads were prepared for surgery. They were placed into a stereotaxic frame, and a small incision (1.5 cm) was made over the mid-line of the skull to expose the landmarks of the cranium (the bregma and the lambda). The following coordinates were used for injection into the third ventricle: 1.1 mm posterior to the bregma and 1.6 mm ventral from the skull surface on the midline (medial fissure), with the nose bar set at 4 mm below the ear bars (below zero). A small hole was drilled through the skull, and a 23-gauge stainless steel guide cannula was placed into the third ventricle. Two thousand four hundred milligrams of tempol was dissolved in 4 mL of saline. Either artificial cerebrospinal fluid (ACSF) or tempol was infused (5 µg/min) by an Alzet osmotic minipump (Alza, Palo Alto, Calif., USA) into the lateral ventricle via an implanted cannula, as described previously (Scarpace et al. 2007). This maintained a tempol dose of 300 µg/h. The tempol dose was based on our previous study (Erdos et al. 2006) and on other studies (Kang et al. 2009; Xue et al. 2011). The cannula was attached to the minipumps by a polyethylene 50 tubing long enough to implant on the back, between or slightly posterior to the scapulae. A small incision was made at the base of the neck, and a subcutaneous pocket to receive the pump was created by blunt dissection. All the pumps were filled with ACSF. After a recovery and equilibrium period of a week, the dummy pumps were replaced with saline- or tempol-containing pumps, and brain infusion was continued for 2 more weeks (Fig. 2).

Food intake, energy intake, and body weight

The rats were housed individually, and food consumption and body weight were recorded in grams daily throughout the experiment.

Body composition of fat and lean mass

Body composition was assessed using time-domain nuclear magnetic resonance in restrained but fully conscious rats (TD-NMR Minispec; Bruker Optics, The Woodlands, Tex., USA) 2 days before and 2 weeks after the pump change.

Serum leptin

Enzyme immunoassays were used to determine the levels of leptin (rat leptin ELISA kit, EZRL-83K, Millipore, Mass., USA). Leptin was assayed in fed-state blood that was collected at death.

Tissue harvesting and preparation

The rats were euthanized with isoflurane. The circulatory system was perfused with 20 mL of ice-cold saline, and perirenal and retroperitoneal white adipose tissues, brown adipose tissue (BAT), and the hypothalamus were excised. The hypothalamus was removed by making an incision medial to the piriform lobes, caudal to the optic chiasm, and anterior to the cerebral crus, to a depth of 2 to 3 mm. The hypothalamus was sonicated in 50 mol/L

Tris-HCl, pH 6.8, plus protease inhibitors. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, Calif., USA).

Nicotinamide adenine dinucleotide phosphate oxidase activity

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was measured with a lucigenin-enhanced chemiluminescence assay using hypothalamus homogenates. The microplate containing approximately 15 µg of hypothalamus homogenates was maintained at 37 °C, while luminescence was recorded using a microplate reader. Relative light units were obtained for 30 min in the presence of nicotinamide adenine dinucleotide hydride (NADH) (474 µm) and lucigenin (218 µmol/L), and background-corrected values were normalized to protein content.

Western blot analyses

Briefly, an equal amount of protein for each sample was separated by 10%–12.5% SDS-PAGE for 1 h at 100 mA. After electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20. All membranes were incubated with primary antibody overnight at 4 °C. Immunoreactivity was visualized by the ECL Plus detection system (GE Healthcare, Piscataway, N.J., USA) and quantified by ImageQuant TL (GE Healthcare).

NADPH subunits

NADPH oxidase subunits (phox p47, p67, and gp91) were assessed by Western blot assays. Ten micrograms (for phox p47), 5 µg (for p67), and 15 µg (for gp91) of protein were loaded, and antibodies (all from Millipore, Billerica, Mass., USA) were used in 1:2000 concentration.

Total antioxidant capacity

Total antioxidant capacity in whole-brain homogenates was measured using the total antioxidant capacity kit (cat. no. ab65329, Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, plasma was allowed to reduce Cu^{2+} for 1.5 h at room temperature. Reduced Cu^{+} was chelated with a colorimetric probe, and absorbance was measured at 570 nm. Results were expressed as trolox equivalent according to a trolox standard curve.

SOD

SOD activity in whole-brain homogenates was determined using a commercial kit (ab65354, Abcam). SOD activity was determined by using the xanthine oxidase (XO) method based on $\text{O}_2^{\bullet-}$ -generation. The rate of the reduction with a superoxide anion is linearly related to the XO activity and is inhibited with SOD. The inhibition activity of SOD was determined using a colorimetric method.

Catalase

Catalase was assessed to determine antioxidant capacity in the hypothalamus. Six micrograms of protein was loaded, and antibody (Anti-Catalase, EMD Chemicals) was used in a 1:2000 concentration.

Reduced total glutathione (GSH) assay

Glutathione measurements were performed using a modification of the Ellman procedure (Beutler et al. 1963). Briefly, after centrifugation at 2000 *g* for 10 min, 0.5 mL of supernatant was added to 2 mL of 0.3 mol/L Na₂HPO₄·2H₂O solution. A 0.2-mL solution of 5,5'-dithiobis-2-nitrobenzoic acid (0.4 mg/mL 1% sodium citrate) was added, and the absorbance at 412 nm was measured immediately after mixing. The results are expressed as micromoles of GSH per gram of tissue.

AMPK pathway and SIRT1 activity

Total AMPK, phosphorylation of AMPK (pAMPK), AcP53, FOXO1, and SIRT1 were assayed by Western blot analysis. Twenty micrograms (for AMPK, pAMPK, and FOXO1), 10 µg (for AcP53), and 40 µg (for SIRT1) of protein were loaded, and antibodies (AMPK, pAMPK, FOXO1, SIRT1 (Cell Signaling, Danvers, Mass. USA) and AcP53 (Abcam, Cambridge, Mass. USA)) were used in 1:1000 (AMPK, pAMPK, and SIRT1) and 1:2000 (AcP53, FOXO1) concentrations.

Leptin signalling

Leptin signalling was assessed by total STAT and pSTAT protein, which were assayed by Western blot analysis. Sixty micrograms (for pSTAT) and 30 µg (for STAT) of protein was loaded, and antibodies (Cell Signaling) were used in 1:5000 (STAT) and 1:800 (pSTAT) concentrations.

Statistical analyses

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, Calif., USA). All data are expressed as means ± SE. Groups were compared with the Kruskal–Wallis test, followed by the Dunn's test for multiple comparisons or by Student's *t* test. Values of *p* < 0.05 were considered significant.

Results

Before tempol treatment, body weight was different across age (*p* < .001) but not within age groups (Fig. 3). Treatment with tempol did not affect food intake or body weight when compared with respective control animals. In addition, the cumulative food consumption was not significantly different among groups.

As expected, adipose tissue weight at death was greater in the aged compared with the young animals, but there was no influence as a result of tempol treatment (Table 1). Similarly, BAT was more abundant in aged vs young rats with no change with tempol treatment. When percent whole-body lean mass and fat mass were examined by TDNMR,

both measures were significantly higher in the older compared with the younger rats, but again, there was no difference as a result of tempol treatment (Table 1).

NADPH oxidase catalyzes the 1-electron reduction of O₂ into superoxide using either NADPH or NADH as the electron donor. Aged control rats had a significantly higher level of NADPH oxidase enzyme, and this was reversed partially by intracerebroventricular tempol treatment. However, subunits of NADPH oxidase, p-47, p67, and gp-91 were not significantly different among groups (Fig. 4).

Total antioxidant capacity and SOD activity were determined in whole brain, and antioxidant enzyme levels, including catalase enzyme and total GSH, were determined in the hypothalamus. With age, there was a decline in total antioxidant capacity and SOD activity in the brain. Antioxidant capacity was elevated significantly by tempol treatment in the older rats, whereas the increase in SOD activity was not significant (Figs. 5a and 5b). Hypothalamic catalase levels were significantly lower in the older rats (Fig. 5c); however, tempol failed to reverse this decline. Total GSH levels in the hypothalamus were not different among groups (Fig. 5d).

At death, several signalling molecules that are normally affected by feeding were examined. The rats were euthanized in the early morning, during a time when feeding was normally minimal; thus, these baseline values reflect this non-food-consumption state.

SIRT1 was significantly lower ($p < 0.001$) in the hypothalamus of the older rats, and tempol treatment failed to reverse this decline (Fig. 6a).

No significant change was detected in FOXO1 protein, whereas acP53 was significantly lower ($p < 0.05$) in the older control rats. Again, tempol treatment did not have any effect on either of these signalling molecules (Figs. 6b and 6c).

The baseline pAMPK levels were increased with age in the hypothalamus ($p < 0.05$), whereas total AMPK levels remained unchanged. Thus, the pAMPK/AMPK ratio was also significantly higher in the older control rats. Tempol treatment prevented the increased pAMPK and pAMPK/AMPK ratio that occurred with age (Figs. 6d, 6e, and 6f).

Basal levels of pSTAT and total STAT protein were not significantly different among age groups and were not affected by tempol treatment (Fig. 7).

Discussion

Hypothalamic inflammation and increased oxidative stress are thought to be 2 mechanisms underlying obesity and aging (Cai and Liu 2012; Williams 2012; Erdos et al. 2011). Tempol, a free radical scavenger, has been shown to decrease sympathetic activity in the brain (Lu et al. 2004) and to possess neuroprotective effects in various models of brain injuries, including stroke (Wilcox 2010; Hall et al. 2010; Dohare et al. 2014). It has been proposed that tempol's neuroprotective efficacy is largely caused by its ability to catalytically scavenge peroxynitrite radicals (Xiong et al. 2009). In this study, we hypothesized that central infusion of tempol, a free radical scavenger, would reduce oxidative stress in the

hypothalamus and decrease food intake and body weight by affecting leptin signalling through the AMPK/SIRT1-dependent pathway or pSTAT3 pathway in rats with age-related obesity. For this purpose, we administered tempol at a dose of 5 µg/min as a continuous infusion for 2 weeks. Other studies (Kang et al. 2009; Xue et al. 2011) have demonstrated that the effective dose ranged between 1.3 and 8.6 µg/min. Therefore, our dose may be considered a medium dose.

However, our results indicate that centrally administered tempol affected neither food intake nor body composition (lean and fat mass), despite the significantly decreased oxidative stress in the hypothalamus of the older rats as a result of tempol treatment. There are 3 categories of antioxidant species: enzyme systems (GSH reductase, SOD, catalase, peroxidase, etc.), small molecules (ascorbate, uric acid, GSH, vitamin E, etc.), and proteins (albumin, transferrin, etc.). Total antioxidant capacity measurement can detect combinations of both small-molecule antioxidants and proteins or small molecules alone. Measurement of the combined nonenzymatic antioxidant capacity provides an indication of the overall capability of the tissue to counteract ROS, resist oxidative damage, and combat oxidative stress-related diseases.

In this study, catalase activity, which is responsible for the conversion of hydrogen peroxide into water and O₂, was slightly increased with tempol in the younger rats, but there was no effect in the older rats. On the other hand, in aged rats, tempol reduced NADPH oxidase, a marker of oxidative stress, in the hypothalamus. Both total SOD enzyme activity and the antioxidant capacity of the whole brain also decreased significantly with age. Tempol treatment significantly increased brain antioxidant capacity in the older rats, whereas a slight, nonsignificant increase was observed in SOD. Other researchers have shown that tempol treatment was effective in increasing SOD activity and GSH activity in young brains (Ali et al. 2016). However, our findings did not confirm any increase in antioxidant enzymes in either the brain or the hypothalamus. On the other hand, we detected an increase in total antioxidant capacity, which was conferred mainly by non-enzymatic antioxidants. Our findings can be interpreted as tempol's failure to increase enzymatic antioxidants in the aged brain. A reason for this failure may be the irreversible decrease in anti-oxidant enzyme activity that cannot be restored. Hence, early and/or long-term treatment can be the key. Overall, tempol treatment reduced oxidative stress by enhancing the antioxidant system but failed to alter feeding behaviour, body weight, or body composition, suggesting that oxidative stress plays a minimal role in age-related obesity in rats.

Our hypothesis was based on the idea that decreasing oxidative stress would improve aging-related obesity and impaired hypothalamic signalling in the brain. Aging, however, is a multifactorial process. Besides the increased oxidative stress, a decrease in SIRT1 and AMPK activity has been shown to control the intracellular energy balance and to affect longevity (Lim et al. 2010). Cell cycle regulation by AMPK is mediated by inhibition of the mTOR pathway as well as by the upregulation of the p53, FOXO1/FOXO3a axis. The mTOR pathway is a major controller of protein biosynthetic processes (Wang et al. 2011). It was shown previously that pAMPK levels in peripheral tissues decrease with age (Reznick et al. 2007); however, it seems this is not the case in the brain. Previously, Liu and colleagues (2012) reported that baseline levels of phosphorylated AMPK were higher in the brains of

aged mice brains compared with those of young mice (Liu et al. 2012). In keeping with the research by Liu and colleagues (2012), our study demonstrates significantly higher pAMPK in older control vs younger control rats, although all groups had similar total AMPK levels.

Recently, studies have focused on the relation among SIRT1, AMPK, and p53 in terms of caloric restriction and longevity. p53 is a tumor suppressor protein that regulates autophagy; it interacts with the SIRT1 and mTOR pathways (Paraíso et al. 2013; Lee and Gu 2013; Salminen et al. 2013; Duan 2013; Carling 2004). A stress signal such as glucose starvation or DNA damage rapidly activates p53 and AMPK. Increases in AMPK can also induce p53. Increased p53 levels result in mTOR inhibition, decreased levels of pS6K, and activation of autophagy (Tucci 2012). Another downstream marker in this pathway is FOXO1; its expression in the brain was shown previously to decrease with aging in the frontal, parietal, and occipital cortex and in the hippocampus (Zemva et al. 2012). In the current study, FOXO1 levels in the hypothalamus displayed a variation among groups, although without significance. Moreover, acetyl p53 levels were significantly higher in the older rats, and tempol had a tendency to increase the levels in these rats.

We also evaluated the pSTAT3/STAT3 levels, a marker for leptin signalling, because we demonstrated previously that leptin receptor blockade disrupts body weight regulation (Matheny et al. 2014). Although there was a tendency toward a decline of leptin signalling in older versus younger rats, neither baseline levels nor levels after tempol treatment were significantly different.

In conclusion, these results indicate that although tempol decreases oxidative stress, it fails to modulate the leptin-signalling pathways. Moreover, tempol fails to alter feeding behaviour, body weight, or body composition. Thus, a reduction in hypothalamic oxidative stress is not sufficient to reverse age-related obesity.

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Fig. 1.

Aging is a complicated process, often characterized by increased ROS and inflammation. Whether this increase is a cause or an effect is still unclear. There is evidence that aging and increased ROS production lead to impaired FOXO1, AMPK, and SIRT1 signaling, possibly leading to a decrease in the catabolic pathways promoting obesity. In addition, leptin resistance is associated with aging and may further dampen catabolic signaling, which may lead to more inflammation and ROS production. AMPK, 5' AMP-activated protein kinase; GSH, reduced glutathione; IL, interleukin; NADPH, nicotinamide adenine dinucleotide phosphate; NF- κ B, nuclear factor kappa B; ROS, reactive oxygen species; SIRT1, silent mating type information regulator 2 homolog 1; TNF- α , tumour necrosis factor- α . [Colour online.]

Fig. 2.

Schematic representation of the experimental protocol. ACSF, artificial cerebrospinal fluid; Td-NMR, time-domain nuclear magnetic resonance. sac, sacrifice. [Colour online.]

Fig. 3.

(a) Body weight (BW), (b) delta BW, (c) food intake, and (d) cumulative food intake after pump change in vehicle- or tempol-treated young and old rats. BW, body weight. [Colour online.]

Fig. 4.

NADPH oxidase activity and subunits in the hypothalamus of vehicle- or tempol-treated young and old rats. NADPH, nicotinamide adenine dinucleotide phosphate. RLU, relative light unit. *, $p < 0.05$ vs young control; †, $p < 0.01$ old control vs old tempol.

Fig. 5.

(a) Total antioxidant capacity, (b) SOD activity in brain, (c) catalase, and (d) GSH levels in the hypothalamus of vehicle- or tempol-treated young and old rats. GSH, reduced glutathione; SOD, superoxide dismutase. *, $p < 0.05$ old control vs young control; [†], $p < 0.05$ old control vs old tempol.

Fig. 6.

Cellular signalling markers of the SIRT1/AMPK pathway in the hypothalamus of vehicle- or tempol-treated young and old rats. (a) SIRT1. (b) Acetyl p53. (c) FOXO1. (d) pAMPK. (e) Total AMPK. (f) pAMPK/AMPK. AMPK, 5' AMP-activated protein kinase; OC, old control; OT, old tempol; pAMPK, phosphorylation of AMPK; SIRT1, silent mating type information regulator 2 homolog 1; YC, young control; YT, young tempol. *, $p < 0.05$ vs young control; †, $p < 0.05$ old control vs old tempol.

Fig. 7.

Leptin signalling markers in the hypothalamus of vehicle- or tempol-treated young and old rats. OC, old control; OT, old tempol; pSTAT, phosphorylation of STAT; STAT, signal transducer and activator of transcription; YC, young control; YT, young tempol.

Table 1

Serum leptin, distribution of fats, and lean and fat mass % of young and old rats treated with vehicle or tempol.

	Young control	Young tempol	Old control	Old tempol
Serum leptin	9.06±1.49	7.75±0.76	17.03±1.00 *	19.53±2.13 *
Type of fats				
RTWAT (g)	2.85±0.16	3.07±0.70	8.53±1.00 *	9.21±0.47 *
PWAT (g)	0.87±0.07	0.83±0.16	1.67±0.12 *	1.72±0.13 *
EWAT (g)	3.78±0.17	4.36±0.73	9.75±0.90 *	10.09±0.27 *
Sum of WATs (g)	7.50±0.32	8.26±1.57	19.96±1.88 *	21.03±0.47 *
BAT (g)	0.35±0.01	0.35±0.02	0.64±0.06 *	0.65±0.04 *
Lean mass %				
Pre	59.66±1.1	59.23±0.3	56.43±0.9 *	56.45±0.7 *
Post	58.98±1.3	58.61±1.1	54.38±0.8 *	54.82±1.0 *
Fat mass %				
Pre	24.61±0.7	23.49±1.4	27.59±0.6 *	28.22±0.4 *
Post	24.83±0.6	25.41±1.3	27.92±0.7 *	28.41±0.7 *

Note: Results are presented as means±SE. RTWAT, retroperitoneal white adipose tissue; PWAT, perirenal white adipose tissue; EWAT, epididymal white adipose tissue; WATs, white adipose tissues; BAT, brown adipose tissue; Pre, before treatment; Post, after treatment.

* $p < 0.05$ old vs young.