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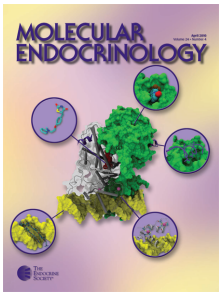

A Functional Leptin System Is Essential for Sodium Tungstate Antiobesity Action

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Sodium tungstate is a novel agent in the treatment of obesity. In diet-induced obese rats, it is able to reduce body weight gain by increasing energy expenditure. This study evaluated the role of leptin, a key regulator of energy homeostasis, in the tungstate antiobesity effect. Leptin receptor-deficient Zucker *fa*/*fa* rats and leptin-deficient *ob*/*ob* mice were treated with tungstate. In lean animals, tungstate administration reduced body weight gain and food intake and increased energy expenditure. However, in animals with deficiencies in the leptin system, treatment did not modify these parameters. In *ob*/*ob* mice in which leptin deficiency was restored through adipose tissue transplantation, treatment restored the tungstate-induced body weight gain and food intake reduction as well as energy expenditure increase. Furthermore, in animals in which tungstate administration increased energy expenditure, changes in the expression of key genes involved in brown adipose tissue thermogenesis were detected. Finally, the gene expression of the hypothalamic neuropeptides, *Npy*, *Agrp*, and *Cart*, involved in the leptin regulation of energy homeostasis, was also modified by tungstate in a leptin-dependent manner. In summary, the results indicate that the effectiveness of tungstate in reducing body weight gain is completely dependent on a functional leptin system. (***Endocrinology*** **150: 642–650, 2009)**

Obesity is caused by a prolonged energy homeostasis imbalance as a consequence of a greater energy intake than energy expenditure (1). This imbalance causes a complex metabolic disorder characterized by an excessive accumulation of body fat and associated comorbidities, such as diabetes, hypertension, cardiovascular disease, and cancer. The prevalence of obesity has increased during the last decades, reaching pandemic proportions. Currently available antiobesity drugs have limited effects on body weight loss and display several adverse effects (2). Consequently, the development of more effective and safer drugs is becoming imperative.

As we previously reported, oral administration of sodium tungstate decreases body weight gain and adiposity in diet-induced obese rats, without modifying food intake, intestinal fat absorption, or growth rate (3). Tungstate also ameliorates dyslipemia and insulin resistance in these animals and reduces body weight gain in lean control rats (4). These effects are mediated by an increase in whole-body energy dissipation and, in adipose tissue, by changes in the expression of genes and proteins involved in fatty acid oxidation and mitochondrial uncoupling (4, 5). Compared with other inorganic compounds with similar insulin-like properties, tungstate has a rather low toxicity in rats (6). Short- or long-term administration of tungstate in rodents has neither hepatotoxic nor nephrotoxic effects, as several studies have shown (4, 6, 7). Taken together, these data support the use of tungstate as a safe and effective antiobesity drug.

Leptin is a key adipokine in the regulation of energy homeostasis (8, 9). In both rodents and humans, defects in the genes encoding either leptin (9, 10) or its receptor (11–13) lead to severe obesity. Leptin is produced mainly by adipose tissue and is released into the circulatory system in correlation with fat content. Leptin binds to its receptors located in the brain and

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Abbreviations: AGRP, Agouti-related peptide; AU, arbitrary unit; BAT, brown adipose tissue; CART, cocaine- and amphetamine-related transcript; CO-I, cytochrome oxidase subunit 1; CO-IV, cytochrome oxidase subunit 4; eWAT, epididymal adipose tissue; FAS; fatty acid synthase; NEFA, nonesterified fatty acids; NPY, neuropeptide Y; PGC, peroxisomal proliferator-activated receptor-γ coactivator; POMC, proopiomelanocortin; TG, triglycerides; UCP, uncoupling protein.

produces a decrease in food intake and an increase in energy expenditure (14). Specifically, leptin acts on the arcuate nucleus of the hypothalamus, a key target area of the brain, in which it simultaneously inhibits orexigenic neuropeptide Y (NPY)/agouti-related peptide (AGRP) expressing neurons (15, 16) and activates anorexigenic proopiomelanocortin (POMC)/cocaine- and amphetamine-related transcript (CART) expressing neurons (17, 18). The strong involvement of leptin in body weight and energy expenditure regulation led us to examine whether the effects of tungstate on the regulation of body weight are leptin dependent. The purpose of the study was to improve our understanding of the mechanisms of tungstate antiobesity effects and unravel the role of the leptin system.

Materials and Methods

Animals and treatment

Twelve-week-old male lean (+/?) and obese leptin receptor-deficient (*fa/fa*) Zucker rats or male lean (+/?) and obese leptin-deficient (*ob/ob*) mice (Charles River Laboratories, Sta. Perpetua de Mogoda, Spain) were caged individually in a 12-h light, 12-h dark cycle in a temperature- and humidity-controlled environment. Animals were fed *ad libitum* with standard chow diet (type A04; Panlab, Barcelona, Spain).

Treatment was orally administered through a solution of 2 mg/ml sodium tungstate ($Na_2WO_4 \times 2H_2O$; Carlo Erba, Milano, Italy) in distilled drinking water for 30 d *ad libitum*. Using this concentration, the tungstate dose ingested daily was 225 mg/kg body weight for rats and 180 mg/kg body weight for mice. Throughout the experimental period, water, food and body weight of all animals were recorded periodically. At the end of the treatment period, animals were killed with inhaled isofluorane overdose, with the exception of mice used for hypothalamus isolation, which were decapitated. In decapitated mice, brain was rapidly removed from the skull and hypothalamus was immediately dissected using consistent landmarks, being bordered by the optic chiasma, mammillary bodies, and hypothalamic sulcus. Then was placed in an Eppendorf tube, weighed, and frozen. Interscapular brown adipose tissue (BAT) was excised and rapidly frozen in liquid nitrogen. All procedures were conducted in accordance with the guidelines of laboratory animal care (European Union regulations; O. J. of the European Communities L358/1, and local government guidelines) and approved by the Animal Research Committee of the University of Barcelona.

Adipose tissue transplantation

Adipose tissue transplantation was performed in 12-wk-old *ob/ob* male mice as previously described (19) with several modifications. Four explants of 100–150 mg of epididymal adipose tissue (eWAT) from lean mice were transplanted sc in four incisions divided between the scapular and lumbar zones. Transplanted animals were divided into two groups: untreated (*ob/ob txUT*) and tungstate-treated (*ob/ob txT*) mice. An additional group of *ob/ob* mice transplanted with *ob/ob* eWAT was used as a leptin deficiency transplant control (*ob/ob txCN*). The experiment was performed over 4 wk. After this period, the transplanted fat depots were surgically excised in some of the *ob/ob txT* mice; in these animals tungstate treatment was either maintained for an additional 14 d or withdrawn.

Recombinant-leptin treatment

Recombinant murine leptin (Sigma-Aldrich, St. Louis, MO) was dissolved following the manufacturer's instructions. Either vehicle (NaCl, 154 mmol/liter) or leptin (0.5 μg/g body weight) was administered twice daily to 12-wk-old male *ob/ob* mice by ip injection at 0800 h and 1900 h for 5 d. Half of the leptin-treated mice were simultaneously cotreated orally with tungstate, as described above.

Metabolic measurements

All animals were fasted for 6 h before metabolic measurements. Blood samples were collected from the tail vein using a capillary blood collection system with EDTA (Sarstedt, Nümbrecht, Germany). Plasma triglycerides (TGs) and nonesterified fatty acids (NEFA) levels, were measured using colorimetric kits TG PAP-150 (BioMérieux, Marcy l'Etoile, France) and NEFA C (Wako, Neuss, Germany), respectively. Plasma leptin levels were determined using a specific mouse ELISA kit (Linco Research, Inc., St. Charles, MO).

Oxygen consumption measurements

Volume of O_2 consumption in Zucker rats and *ob/ob* mice was measured using an open-circuit indirect calorimetry system (MM-100 metabolic monitor system; CWE Inc., Ardmore, PA) as previously described (3). Basal oxygen consumption was measured on 16-wk-old untreated animals, within the 3 h after the start of the light cycle; no differences were observed between animals within the same group. Then either a single dose of tungstate, equivalent to 8 h consumed dose during chronic *ad libitum* treatment (75 mg/kg body weight for rats and 60 mg/kg body weight for mice), or vehicle (NaCl, 154 mmol/liter), was administered by gavage, and oxygen consumption was measured during the after 8 h. In eWAT transplanted mice, measurements were performed for 23 h (from 1000 to 0900 h the next day) on 4-wk-treated or untreated transplanted mice. All animals were previously housed overnight in the measurement chamber to ensure acclimatization.

RNA isolation and quantitative RT-PCR analysis

BAT total RNA was purified by guanidine isothiocyanate-phenol chloroform extraction (Invitrogen, Carlsbad, CA). For hypothalamus total RNA purification RNA extraction minikit (Invitrogen) was used. cDNA was synthesized using first-strand cDNA synthesis (Invitrogen) from 1 μg RNA following the manufacturer's instructions. Synthesized cDNA was subjected to quantitative RT-PCR (7900HT fast real time

TABLE 1. Quantitative RT-PCR primer pairs

Gene	Forward (5′–3′)	Reverse (5′–3′)
Ucp1	cgatgtccatgtaccacaaggaa	tcgcagaaaagaagccacaa
CO-I	cgccatcatattcgtaggagtaaa	tctgagtagcgtcgtggtattcc
CO-IV	ccgactggagcagcctttc	tcggcgaagctctcgttaaa
Pgc1α	tgatgacagtgaagatgaaagtgataaac	ggcgacacatcgaacaatga
Acacα	gacttgcagaagaaatacgccata	cttgtatcccttgtagggatcttca
Fasn	tgctcccagctgcaggc	gcccggtagctctgggtgta
Npy	cgctctgcgacactacatcaa	gggctggatctcttgccat
Agrp	ggtgctagatccacagaaccg	ccaagcaggactcgtgcag
Pomc	agaggccactgaacatctttgtc	gcagaggcaaacaagattgga
Cart	ttctttcctcttgaagtgcttgtg	gggaatatgggaaccgaaggt

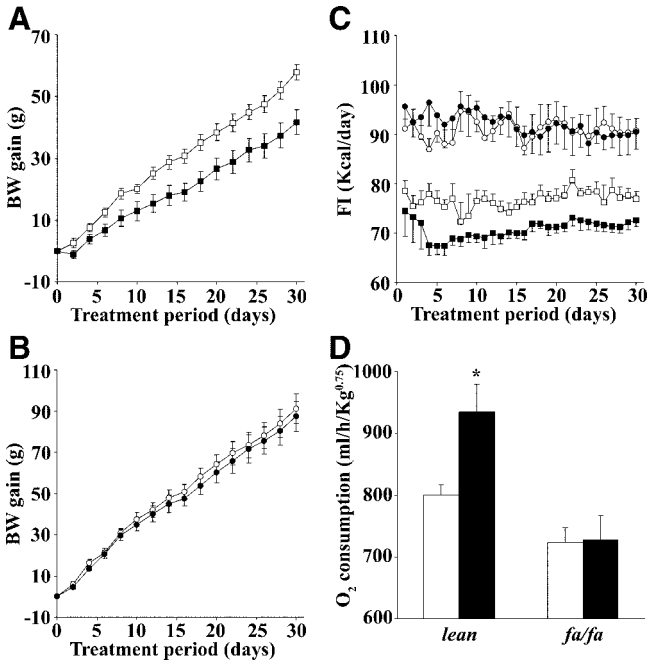

FIG. 1. Oral tungstate treatment neither induces a negative energy balance nor reduces body weight (BW) gain in leptin receptor-deficient Zucker rats. Evaluation of 30 d tungstate treatment in 12-wk-old male obese Zucker *fa/fa* and Zucker lean rats (n = 9/group). Measurement of body weight gain evolution during the treatment in lean (A; *white squares*, untreated; *black squares*, treated) and *fa/fa* (B; *white circles*, untreated; *black circles*, treated) rats. C, Measurement of cumulative food intake (FI; *white squares*, lean untreated; *black squares*, lean treated; *white circles*, *fa/fa* untreated; *black circles*, *fa/fa* treated; n = 9/group). D, Oxygen consumption measurements in untreated obese Zucker lean and Zucker *fa/fa* rats in response to a gavage administration of tungstate or vehicle (*white bars*, vehicle; *black bars*, tungstate; n = 6/group). Data are expressed as mean ± SEM. *, $P < 0.05$ for untreated *vs.* treated animals.

PCR system; Applied Biosystems, Foster City, CA), using in all cases Power SYBR Green PCR master mix (Applied Biosystems), with the exception of 18S rRNA, in which predesign Taqman probe and primers (Applied Biosystems) were used. Cycle threshold values were calculated using AbI Prism SDS version 2.1 software (Applied Biosystems), and data were normalized to values obtained for 18S rRNA. Results were expressed as arbitrary units (AU) normalized to values obtained for untreated mice from each experimental group. The primers used are described in Table 1.

Statistical analysis

The data were statistically evaluated by a two-way mixed model ANOVA, treatment fixed and time random. Subsequently we compared all possible pairs of subgroups using the multiple comparisons Tukey's method with a 5% level of confidence. Differences between two means were determined by Student's test.

Results

Tungstate treatment decreased body weight gain and adiposity in Zucker lean rats but not in *fa/fa* rats

Zucker rats were treated with tungstate to determine whether tungstate was able to decrease body weight gain in this leptin signaling-deficient model. As shown in Fig. 1A, oral administration of tungstate for 30 d reduced the body weight gain in Zucker lean rats by 27% compared with untreated rats, the difference being significant ($P < 0.001$). Moreover, a significant reduction in leptin levels was observed in treated Zucker lean rats (4.23 ± 0.36 ng/ml for untreated *vs.* 2.98 ± 0.18 ng/ml for treated rats, $P < 0.05$). However, tungstate did not induce any significant change in body weight gain and leptin levels in obese Zucker *fa/fa* rats (Fig. 1B). As in diet-induced obese rats, tungstate treatment improved the lipid profile of lean rats, decreasing circulating levels of TG and NEFA compared with untreated rats (Table 2). As expected, no improvement was observed in treated obese Zucker *fa/fa* rats when compared with untreated rats.

To determine the mechanism of tungstate action involved in the control of body weight, food intake measurements and indirect calorimetry were performed. Tungstate treatment induced a significant reduction in cumulative food intake ($P < 0.01$) (Fig. 1C), bringing the average daily difference in food intake 5.90 ± 0.32 kcal. Tungstate also increased oxygen consumption by 16% ($P < 0.05$) (Fig. 1D) in Zucker lean rats. However, as shown in Fig. 1, C and D, no changes in either cumulative food intake or oxygen consumption were detected in tungstate-treated obese Zucker *fa/fa* rats compared with untreated rats. Thus, tungstate treatment did not change the energy balance in obese Zucker *fa/fa* rats.

Interestingly, when Zucker animals were fed with high palatable diet (cafeteria diet), body weight changes were similar to the ones observed with standard diet. No changes in leptin receptor-deficient rats were observed, whereas a significant reduction in lean rats was detected when treated with tungstate (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). In contrast, no differences in food intake were observed among the groups as a consequence of tungstate treatment (supplemental Fig. 1). This effect, as is discussed in *Discussion*, could be related to the hyperphagic stimulus induced by high palatable diet.

TABLE 2. Oral tungstate treatment does not improve lipid profile in leptin-deficient animal models

	Triglycerides (mg/ml)		NEFA (nm)	
	UT	T	UT	T
Zucker lean rats	83.52 ± 7.60	65.35 ± 3.77[a]	0.78 ± 0.05	0.61 ± 0.04[a]
Zucker *fa/fa* rats	375.04 ± 27.39	375.35 ± 47.58	1.90 ± 0.11	1.94 ± 0.16
Lean mice	132.20 ± 5.34	105.41 ± 8.91[a]	0.85 ± 0.08	0.61 ± 0.08[a]
ob/ob mice	156.45 ± 7.93	148.25 ± 7.57	1.38 ± 0.13	1.30 ± 0.12

Measurements of biochemical parameters at the end of 30 d tungstate treatment in Zucker lean and *fa/fa* rats and in lean and *ob/ob* mice (n = 6/group). UT, Untreated; T, treated. Data are expressed as mean ± SEM.

[a] $P < 0.05$ T *vs.* UT.

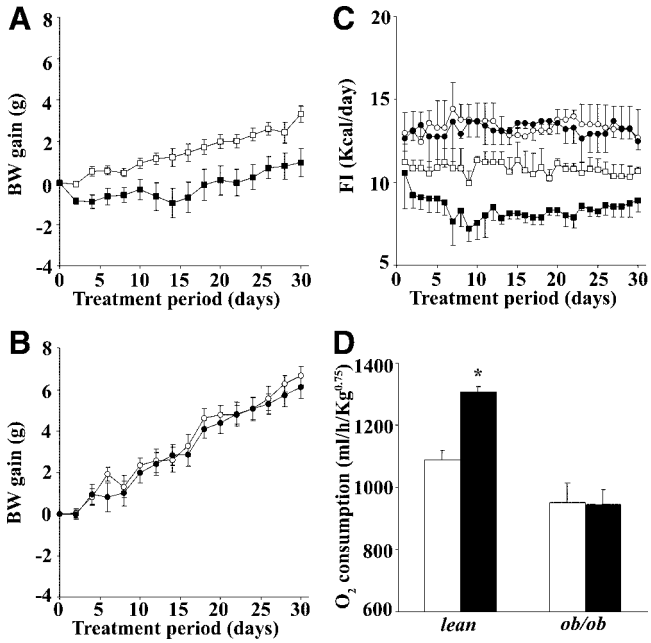

FIG. 2. Oral tungstate treatment neither induces a negative energy balance nor reduces body weight gain in leptin-deficient *ob/ob* mice. Evaluation of 30 d tungstate treatment in 12-wk-old male *ob/ob* and lean mice (n = 7/group). Measurement of body weight gain evolution during the treatment in lean (A; *white squares*, untreated; *black squares*, treated) and *ob/ob* (B; *white circles*, untreated; *black circles*, treated) mice. C, Measurement of cumulative food intake (FI; *white squares*, lean untreated; *black squares*, lean treated; *white circles*, *ob/ob* untreated; *black circles*, *ob/ob* treated; n = 9/group, n = 7/group). D, Oxygen consumption measurements in untreated *ob/ob* and lean mice in response to a gavage administration of tungstate or vehicle (*white bars*, vehicle; *black bars*, tungstate; n = 6/group). Data are expressed as mean ± SEM. *, $P < 0.05$ for untreated *vs.* treated mice.

Tungstate decreased body weight gain and adiposity in lean but not *ob/ob* mice

To further analyze the effectiveness of tungstate treatment on body weight regulation, we used an *ob/ob* mouse model, which lacks the expression of leptin. As expected, tungstate administration promoted a significant reduction of body weight gain in lean mice compared with age- and sex-matched untreated ones ($P < 0.001$) (Fig. 2A). This reduction in body weight cannot be associated with a reduction in food intake, as is shown in pair-fed experiments in which mice food intake was restricted to the daily amount of chow eaten by *ad libitum*-fed-treated animals (supplemental Fig, 2). In these animals, tungstate treatment induce a significant reduction of body weight gain ($P < 0.05$) independently of food intake because no differences were observed in food intake during the experiment (10.60 ± 0.06 kcal/d for untreated restricted food intake lean mice *vs.* 10.58 ± 0.09 kcal/d for tungstate-treated restricted food intake lean mice). As in Zucker rats, tungstate did not change body weight gain in *ob/ob* mice (Fig 2B). The lipid profile of lean mice was improved by tungstate through a decrease in TG and NEFA blood levels compared with untreated lean mice (Table 2). Nevertheless, tungstate treatment did not change these parameters in *ob/ob* mice.

The study of energy balance in these animals also showed that tungstate was effective only in lean mice, not in the *ob/ob* mice. On the one hand, tungstate treatment significantly reduced food intake ($P < 0.05$) (Fig. 2C), with an average daily food intake difference of 2.40 ± 0.12 kcal and simultaneously increased oxygen consumption by a 20% ($P < 0.05$) (Fig. 2D) in lean mice. On the other hand, *ob/ob* mice showed no significant changes due to tungstate treatment in either cumulative food intake or oxygen consumption (Fig. 2, C and D). Overall, these results indicate that leptin is necessary for tungstate to exert its effect on body weight.

Leptin restoration in ob/ob mice allows tungstate to exert its effect on body weight

Given the inability of tungstate to decrease body weight gain in animals with leptin deficiency, we studied whether its effect might be restored in *ob/ob* mice with recovered leptin function. To do so, we established an adipose tissue transplantation protocol to partially restore circulating leptin levels in *ob/ob* mice. In this transplantation protocol, eWAT from lean mice was transplanted sc into *ob/ob* mice. From the second week after the transplant until the end of the experiment, *ob/ob txUT* mice showed a significant decrease in body weight gain compared with *ob/ob txCN* mice, which were used as a control (−4.99 ± 0.92 for *txUT vs.* 5.38 ± 0.31 g for *txCN* mice, $P < 0.001$) (Fig. 3A).

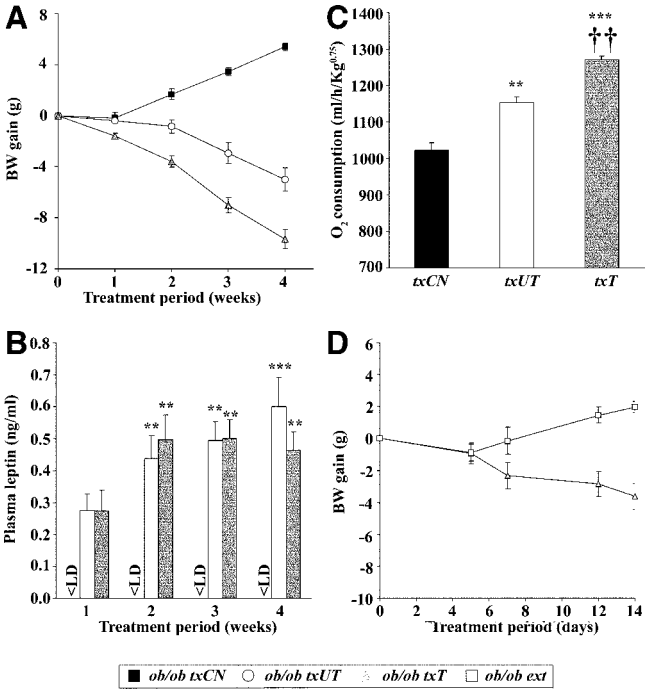

FIG. 3. Restoration of leptin deficiency in *ob/ob* mice enables tungstate to exert its antiobesity effects. Evaluation of 4 wk tungstate treatment in 12-wk-old *ob/ob* mice transplanted with epididymal adipose tissue from *ob/ob* (*ob/ob txCN*, n = 6) or lean mice (*ob/ob txUT* or *ob/ob txT*, n = 12/group). Measurement of body weight gain evolution (A; *black squares*, untreated *ob/ob txCN* mice; *white circles*, *ob/ob txUT* mice; *gray triangles*, *ob/ob txT* mice) and oxygen consumption (B) was measured after 4 wk of transplantation, in overnight cage-acclimatized mice and during 23 h (*ob/ob txCN* and *ob/ob txUT*, n = 3/group; *ob/ob txT*, n = 5). C, Measurement of plasma leptin levels during the treatment. D, Evaluation of tungstate treatment effects on transplanted adipose tissue removal from treated transplanted *ob/ob* mice [surgically excised mice (*ob/ob ext*) and *ob/ob txT*, n = 3/group]. Measurement of body weight gain evolution at the end of the 2-wk treatment period (*black triangles*, *ob/ob txT* mice; *white squares*, *ob/ob ext* mice). Data are expressed as mean ± SEM. **, $P < 0.01$ and ***, $P < 0.001$ for *ob/ob txUT* or *ob/ob txT vs. ob/ob txCN*; ††, $P < 0.01$ for *ob/ob txUT vs. ob/ob txT*. <LD values, Measurements under the limit detection of the method.

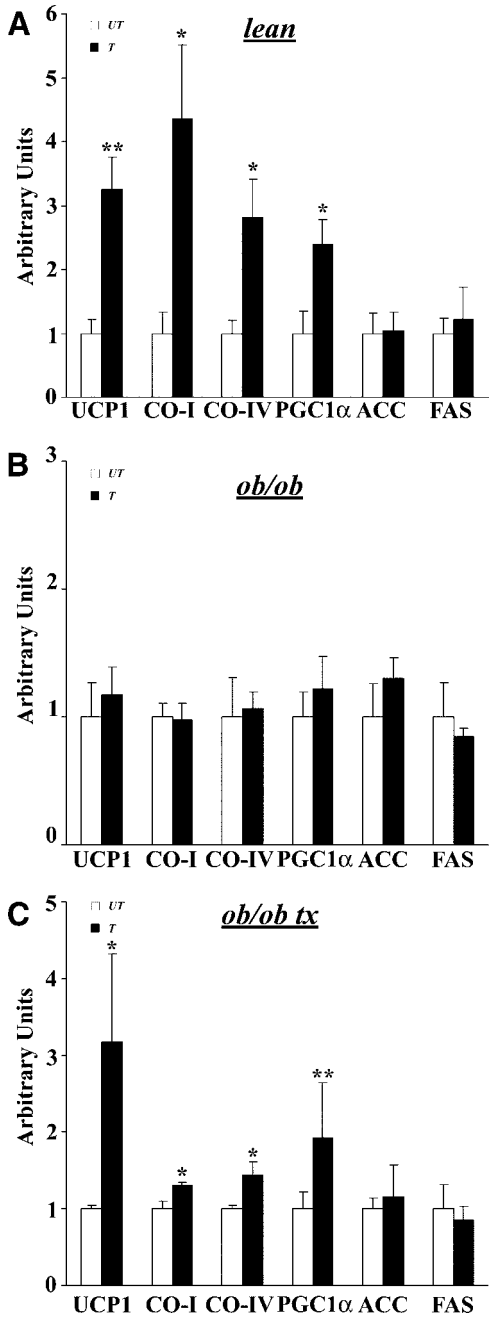

FIG. 4. Tungstate treatment induces BAT gene expression profile changes in lean and *ob/ob* transplanted mice but not *ob/ob* mice. Measurement of *Ucp1*, CO-I (mitochondrial DNA encoded), CO-IV (nuclear DNA encoded), Pgc1α, ACC (acetyl-Co-A carboxylase), and *Fasn* mRNA expression in BAT from lean (A), *ob/ob* (B), and transplanted *ob/ob* (C) mice [*ob/ob tx*; *white bars*, untreated (UT) mice; *black bars*, tungstate treated (T) mice; n = 4–5/group]. In the interests of clarity, gene expression levels were expressed as arbitrary units normalized to values obtained for untreated mice of each experimental group. Data are expressed as mean ± SEM. *, $P < 0.05$ and **, $P < 0.01$ for untreated *vs.* treated animals in each experimental group.

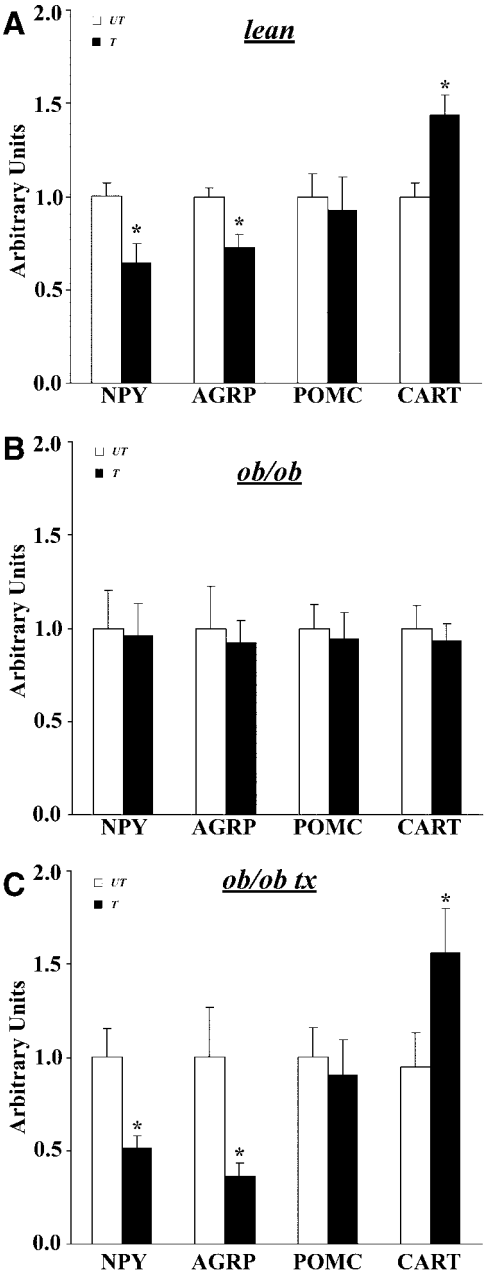

FIG. 5. Tungstate treatment induces changes in the hypothalamic neuropeptide gene expression profile in lean mice but not *ob/ob* mice. Measurement of hypothalamic neuropeptide mRNA expression in lean (A), *ob/ob* (B), and transplanted *ob/ob* (C) mice [*white bars*, untreated (UT) mice; *black bars*, tungstate treated (T) mice; n = 4–6/group]. Data are expressed as mean ± SEM. *, $P < 0.05$, for untreated *vs.* treated animals.

Whereas circulating leptin levels were undetectable in *ob/ob txCN* mice throughout the experiment, in *ob/ob txUT* mice, a slight increase in leptin levels was observed a week after transplant, which become significant after 2 wk and reached stable levels afterward (Fig. 3B). These results indicate that this transplant procedure partially increases leptin blood levels and also restores the leptin functional system, leading to a decrease in *ob/ob* mice body weight. Moreover, a significant reduction in cumulative food intake (586 ± 21 kcal per 4 wk for *txCN* mice *vs.* 373 ± 15 kcal per 4 wk for *txUT*; $P < 0.001$) and an increase in oxygen consumption (Fig. 3C) were observed in *ob/ob txUT* mice compared with *ob/ob txCN* mice, leading to a change in energy homeostasis which is in agreement with the reduction in body weight gain detected.

When these transplanted animals were treated with tungstate, the reduction of body weight accelerated. The difference was already significant after 2 wk of treatment, and the end of the experiment (4 wk), *ob/ob txT* mice had lost nearly twice the body

weight lost by untreated animals (*ob/ob txUT)* ($P < 0.001$) (Fig. 3A). Tungstate administration did not induce any change in circulating leptin levels in the transplanted mice, suggesting that the body weight decrease induced by tungstate was not due to an increase in leptin (Fig. 3C). In this model of leptin deficiency restoration, tungstate also reduced accumulated food intake (373 ± 15 kcal per 4 wk for *txUT vs.* 319 ± 10 kcal per 4 wk for *txT* mice; $P < 0.05$), with a daily average difference of 1.93 ± 0.06 kcal. Furthermore, as shown in Fig. 3B, tungstate increased oxygen consumption by 10% compared with untreated transplanted mice (*ob/ob txUT*) ($P < 0.01$).

After 4 wk of treatment, transplanted fat depots were surgically excised in some of the *ob/ob txT* mice; tungstate administration was then prolonged for 14 d more. The excision of transplanted eWAT led to a rapid disappearance of circulating leptin (data not shown) and an increase in the body weight gain in these animals compared with the treated animals in which the transplant was not removed (1.97 ± 0.35 g for fat depots in excised mice *vs.* -3.63 ± 0.80 for *txT* mice; $P < 0.001$), showing that with the excision of fat depots the effectiveness of tungstate disappears (Fig. 3D). Similar results were found in an additional group of mice in which fat depots were excised and treatment was then withdrawn. In these mice, leptin disappeared completely, and body weight gain reached similar values (2.57 ± 0.62 g) as in the excised mice treated with tungstate. Interestingly, when treatment was withdrawn in transplanted mice, a slow-down in body weight gain reduction was induced compared with transplanted mice in which treatment continued throughout the experiment (1.13 ± 0.52 g; $P < 0.01$ *vs. ob/ob txT* mice). This reinforces the results that indicate that tungstate acts on body weight in intact leptin system models.

To finally confirm the key role of leptin in the effects of tungstate on body weight, a short-term treatment of recombinant leptin was performed. After 5 d of leptin administration, a significant reduction in body weight gain was observed in comparison with untreated *ob/ob* mice (-5.34 ± 0.47 g for leptin treated *ob/ob* mice *vs.* 1.26 ± 0.19 g for leptin untreated *ob/ob* mice, $P < 0.001$). A higher decrease in body weight gain was detected in those animals receiving oral tungstate at the same time as ip leptin treatment (-6.52 ± 0.18 g for leptin- and tungstate-treated *ob/ob* mice, $P < 0.05$). Despite the differences in body weight gain in these tungstate-treated animals, no differences were detected in blood leptin levels between the two groups (2.13 ± 0.05 ng/ml for leptin-treated *ob/ob* mice *vs.* 2.10 ± 0.13 ng/ml for leptin and tungstate treated *ob/ob* mice).

Tungstate treatment induced changes in the expression levels of genes involved in energy expenditure in BAT of lean and *ob/ob* transplanted mice but not in *ob/ob* mice

Previous studies have determined an increase in BAT thermogenic-related genes and proteins (4, 5) in diet-induced obese rats. To assess whether in our models BAT was also responsible for the increase in whole-body energy expenditure in tungstate-treated animals, we studied the expression pattern of several genes involved in the thermogenic process. As expected, uncoupling protein (UCP)-1 expression was decreased in *ob/ob* mice (a 2.7-fold reduction). Furthermore, leptin restoration in *ob/ob* mice due to adipose tissue transplantation restored UCP1 expression (1.00 ± 0.29 AU for lean mice; 0.37 ± 0.09AU for *ob/ob* mice and 1.27 ± 0.15 AU for *ob/ob txUT* mice). As shown in Fig. 4, tungstate administration increased UCP1 expression 3-fold in both lean and transplanted *ob/ob* mice ($P < 0.01$ and $P < 0.05$, respectively) compared with untreated mice, whereas in *ob/ob* mice no significant treatment-related differences were observed. Furthermore, the analysis of peroxisomal proliferator-activated receptor-γ coactivator (PGC)-1α expression, a gene involved in mitochondrial biogenesis, and cytochrome oxidase subunits 1 (CO-I) and 4 (CO-IV), belonging to the oxidative phosphorylation system, showed a similar expression pattern to UCP1, *i.e.* a significant increase in lean and *ob/ob txT* mice due to tungstate treatment ($P < 0.05$ or $P < 0.01$), and no changes in *ob/ob* mice. The expression levels of genes involved in lipid synthesis, such as acetyl-CoA carboxylase and fatty acid synthase (FAS), was unchanged by tungstate administration in all experimental models. This finding suggests an adaptation of BAT to generate a mitochondrial proton gradient that can be used by UCP1 to increase thermogenesis and the lack of any compensatory effects on the opposite process, lipogenesis.

Tungstate administration modifies hypothalamic neuropeptide gene expression profile in lean mice but not *ob/ob* mice

To assess whether tungstate affects the gene expression of hypothalamic neuropeptides involved in energy homeostasis, which are classically regulated by leptin, we analyzed the expression of NPY, AGRP, POMC, and CART in the isolated hypothalamus in treated and untreated lean, *ob/ob*, and transplanted *ob/ob* mice. In lean mice, tungstate induced a significant decrease of NPY ($P < 0.05$) and AGRP ($P < 0.05$), and an increase in CART ($P < 0.05$) gene expression (Fig. 5A), but no changes were observed in POMC gene expression. Moreover, no differences in these genes were observed between *ob/ob* treated and untreated animals (Fig. 5B). In contrast, when leptin deficiency in *ob/ob* mice was restored through adipose tissue transplantation, neuropeptide expression changes due to tungstate administration were similar to the ones observed in treated lean mice (Fig. 5C). These results suggest that the effects of tungstate on body weight may be mediated centrally, via action on some of the neuropeptides involved in the regulation of energy homeostasis.

Discussion

The results presented here improve our understanding of the molecular mechanisms by which tungstate exerts its antiobesity effects. In our previous studies (3), we reported that sodium tungstate was able to reduce body weight gain and adiposity in diet-induced obese rats, without modifying food intake or intestinal lipid absorption. The main mechanism by which the treatment reduces body weight gain is through an increase in energy expenditure. Taking into account the key role that leptin plays in the regulation of energy expenditure, we decided to investigate

whether this adipokine was important for tungstate's antiobesity activity. We demonstrate clearly that the leptin system is essential for tungstate action because when either leptin or its receptor is absent, tungstate is ineffective. Moreover, when leptin is restored in the *ob/ob* model either by injection or transplant, tungstate effectiveness returns. Therefore, the antiobesity activity of tungstate depends entirely on a functional leptin system.

The data also show that adipose tissue transplantation is a valid approach for restoring leptin deficiency in *ob/ob* mice. After the transplant, a significant decrease in body weight is observed, simultaneous to the appearance of detectable circulating leptin levels. However, our adipose tissue transplantation protocol could not completely restore leptin levels in comparison with lean mice, reaching only a third of the lean mouse levels. This is probably due to the fact that we transplanted only 500 mg of eWAT, which is approximately 10% of the total fat in lean mice. Although eWAT is a depot with a high rate of leptin production, it is unable to attain the leptin levels present in lean mice. Nevertheless, the presence of circulating leptin levels, the reduction of body weight and food intake as well as the increase in energy expenditure reported here show the viability of transplantation. Furthermore, the increase observed in the vascular regeneration of the transplanted tissue (data not shown) probably ensures a physiological leptin secretion in response to other circulating factors such as NEFA or insulin (20, 21). Finally, we cannot rule out a central contribution to the regulation of leptin secretion in the transplanted depots, although Wang *et al.* (22) showed that no regeneration of sympathetic enervation was observed after 4 wk of fat transplantation.

In Zucker *fa/fa* rats, which are leptin receptor deficient, tungstate was unable to decrease body weight gain or food intake or increase energy expenditure. We conclude that tungstate needs an intact leptin signaling system to exert its effects on energy homeostasis. Furthermore, the fact that similar results were obtained in treated *ob/ob* mice supports the hypothesis that tungstate needs the endogenous hormone to act on body weight, and suggests that tungstate does not act as an agonist of the leptin receptor, but rather as a leptin enhancer. In both models, tungstate does not modify TG and NEFA levels. Therefore, tungstate is only able to improve the lipid profile when the treated animals lose body weight, suggesting that this improvement is secondary to the body weight reduction.

The importance of a functional leptin system is finally confirmed by the restitution of the tungstate effect on body weight, food intake, and energy expenditure observed in the eWAT transplanted *ob/ob* mice with a partially restored leptin function. Interestingly, in the treated transplanted mice, no increase in leptin levels was observed. This result suggests that functional leptin system is essential for the antiobesity action of sodium tungstate because with the same levels of the hormone, the treated transplanted mice had a higher decrease in body weight. To rule out the possible contribution to tungstate's antiobesity activity of other adipokines secreted by the transplanted depots, we performed a short-term treatment with recombinant leptin. As expected, leptin administration reduced the body weight of *ob/ob* mice, and, as in the case of the transplanted mice, tungstate treatment further enhanced this decrease. We therefore conclude that leptin is the main adipokine involved in tungstate's mechanism of action.

The oxygen consumption experiments performed in all our animal models suggest that the energy expenditure increase associated with tungstate administration is mediated by leptin. BAT is the most important thermogenic tissue in rodents (23). UCP1, widely expressed in BAT, plays a key role in adaptive thermogenesis by uncoupling ATP production from the oxidative phosphorylation system, a process that has long been recognized as a protective mechanism against the development of obesity (24). To evaluate the possible adaptation of BAT in the tungstate increase in whole-body energy expenditure, we studied the expression pattern of several genes involved in the thermogenic process. Our results show clearly that tungstate increases UCP1, PGC1α, CO-I, and CO-IV gene expression only in animals with a functional leptin system. These results are in agreement with previous results observed in diet-induced obese rats (4, 5). This increase correlates perfectly with the rise in oxygen consumption observed in the same experimental groups. In contrast, the expression levels of genes involved in lipid synthesis (acetyl-CoA carboxylase and FAS) remain unchanged in all tungstate-treated animals. Taken together, these data indicate that tungstate promotes an adaptation of BAT to generate a mitochondrial proton gradient that can be used by UCP1 to increase thermogenesis and energy expenditure and exert a positive effect on weight loss and avoid the use of this mitochondrial proton gradient in the lipid synthesis process.

In earlier experiments performed with diet-induced obese rats, tungstate administration had no effect on food intake, and we postulated that the decrease in body weight gain was the sole cause of the increase in energy expenditure (3). However, the results presented in this paper show that tungstate treatment reduced food intake in animals with a functional leptin system and, in combination with the lack of tungstate aversion in drinking water (supplemental Fig. 2) determined by two bottle preference test performed in Wistar and C57BL/6 mice, suggests an anorectic effect of tungstate when no hyperphagic stimulus, due to hypercaloric diet, is present. Previous studies reported a decrease in food intake in other animal models (5). These differences may be due to the hyperphagia induced in diets with high palatability (25), which is the case of the cafeteria diet used in diet-induced obesity experiments. Diets of this type could induce powerful orexigenic signals (26) that may mask the anorectic effects of tungstate. Although the hyperphagic stimulus due to palatability usually subsides within a few days and food intake stabilizes afterward, this is not the case of the cafeteria diet model, which, as we and others have reported, does not normalize within 30 d (27). However, because no data for longer studies are available, we cannot conclude that the disappearance of anorectic effects of tungstate is due exclusively to diet palatability. Food intake regulation involves the activation of several neuronal centers, such as reward, satiety, or hunger perception (28, 29), and further analysis is needed to determine whether tungstate modulates any of them. Another possible explanation for the differences observed in food intake is the rapid leptin resistance induced by high-fat diets (30). In this situation, it may be that tungstate cannot enhance leptin's suppressive effect on food

intake. In any case, further studies are underway to determine the causes of the differences in anorectic behavior recorded in the two dietetic groups.

Leptin regulates food intake and energy expenditure by acting on several brain centers, the arcuate nuclei being the main one (14–18). It is well established that leptin only regulates UCP1 mRNA expression by acting centrally through the sympathetic innervation and not directly on BAT (19, 31, 32). Interestingly, when tungstate is administered intracerebroventricularly, it induces a decrease in food intake (Claret, M., personal communication). Moreover, previous studies on the toxicology and pharmacology of sodium tungstate showed that when administered orally, tungstate could be detected in the brain by using inducted coupled plasma techniques (Barbera, A., personal communication). Therefore, it can be reasonably assumed that tungstate can cross the blood-brain barrier and exert its action on the regulation of energy homeostasis neuropeptides.

Finally, we wanted to assess whether tungstate effects on energy homeostasis involved a central mechanism, through the enhancement of leptin action. To do so, we studied the expression levels of the key hypothalamic neuropeptides involved in the energy balance, which are regulated by leptin (15–18, 33). The results presented here show a leptin-mediated regulation of NPY, AGRP, and CART, but not POMC gene expression, in response to tungstate treatment. These results suggest a hypothalamus-mediated effect of tungstate on leptin-regulated genes, resulting in the modulation of food intake and energy expenditure as mechanisms of body weight regulation. However, no differences were observed in POMC gene expression due to tungstate treatment. This lack of effect of tungstate on POMC has also been observed in other models with altered leptin signaling pathway: suppressor of cytokine signaling haploinsufficient mice (34), small heterodimer partner-2 (35), and Src homology-2B (36)-deficient mice. All these proteins are involved in leptin receptor downstream signaling regulation, suggesting that leptin-mediated energy homeostasis regulation could be exerted by POMC alternative neural signals. Obviously, due to the wide distribution of leptin receptor expression in a variety of tissues (37), we cannot rule out the possibility that tungstate may act either peripherally or in other neuronal centers to exert its effects on energy expenditure. We are currently performing a range of experiments to assess these possibilities. The preliminary results indicate that tungstate does not affect the dopaminergic stimulus on the accumbens nuclei, suggesting that it does not affect this reward center. However, further studies in this and other areas of the brain are necessary to determine their contribution on tungstate actions on food intake and energy expenditure.

In summary, the body weight reduction effect of tungstate is dependent on a functional leptin system. Tungstate acts by reducing food intake and increasing BAT-mediated thermogenesis (mechanisms that promote the reduction of body weight gain) in a leptin-dependent manner. Furthermore tungstate action is mediated, at least in part, by an increase in the expression of neuropeptides related to energy homeostasis regulation.

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