Integration of DIGE and Bioinformatics Analyses Reveals a Role of the Antiobesity Agent Tungstate in Redox and Energy Homeostasis Pathways in Brown Adipose Tissue*

Silvia Barceló-Batlíori‡§, Susana G. Kalko¶, Yaiza Esteban‡, Silvia Moreno‡, María C. Carmona‡, and Ramon Gomis‡

Our previous results demonstrated that tungstate decreased weight gain and adiposity in obese rats through increased thermogenesis and lipid oxidation, suggesting that brown adipose tissue was one of the targets of its antiobesity effect. To identify potential targets of tungstate, we used DIGE to compare brown adipose tissue protein extracts from the following experimental groups: untreated lean, tungstate-treated lean, untreated obese, and tungstate-treated obese rats. To distinguish direct targets of tungstate action from those that are secondary to body weight loss, we also included in the analysis an additional group consisting of obese rats that lose weight by caloric restriction. Hierarchical clustering of analysis of variance test contrasts clearly separated the different experimental groups. DIGE analysis identified 20 proteins as tungstate obesity direct targets involved in Krebs cycle, glycolysis, lipolysis and fatty acid oxidation, electron transport, and redox. Protein oxidation was decreased by tungstate treatment, confirming a role in redox processes; however, palmitate oxidation, as a measure of fatty acid β-oxidation, was not altered by tungstate, thus questioning its putative function in fatty acid oxidation. Protein network analyses using Ingenuity Pathways Analysis highlighted peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) as a potential target. We confirmed by real time PCR that indeed tungstate up-regulates PGC-1α, and its major target, uncoupling protein 1, was also increased as shown by Western blot. These results illustrate the utility of proteomics and bioinformatics approaches to identify targets of obesity therapies and suggest that in brown adipose tissue tungstate modulates redox processes and increases energy dissipation through uncoupling and PGC-1α up-regulation, thus contributing to its overall antiobesity effect. Molecular & Cellular Proteomics 7:378–393, 2008.

Obesity has emerged as one of the major global epidemics of the 21st century and is reaching frightening proportions. According to the World Health Organization, more than one billion adults are overweight, and 300 million are clinically obese. Prevalence is growing exponentially worldwide both in developed and developing countries, and most noticeably, childhood obesity has also increased dramatically over the last decades (1, 2). An increase in food availability together with environmental, genetic, and lifestyle changes seems to account for the development of this pandemic. Obese subjects have a decreased life quality and expectancy as well as an increased risk of developing comorbidities such as insulin resistance and type 2 diabetes, cardiovascular disease, hepatic steatosis, pulmonary and muscular pathologies, cancer, and psychological disorders among others. On the other hand, management of this epidemic and its comorbidities is a major economic burden for society. Consequently prevention, treatment, and understanding of obesity etiology ought to be urgent priorities for both scientific and government communities.

Obesity is a complex disease characterized mainly by an increase in body fat mass that results from an imbalance between energy intake and expenditure. Energy homeostasis regulation is complex and involves many molecules, genes, and different tissues. A simplified view of this topic is that the central nervous system, and specifically the hypothalamus, regulates and integrates food intake and energy expenditure, whereas the peripheral tissues such as liver, muscle, and adipose tissues are responsible for fat and carbohydrate metabolism, storage, and thermogenesis (3). Current therapeutic approaches are directed to the modulation of these pathways leading to a negative energy balance and consequently body weight and fat loss (4).

A promising new potential therapy for the treatment of obesity may be sodium tungstate. Recently we described that oral administration of tungstate reduced body weight gain and adiposity without affecting food intake and without any major side effects in cafeteria diet-induced obese rats (5). Additionally the treatment ameliorated dyslipemia and insulin resist-
targets of tungstate in BAT. This strategy consisted of defining several experimental groups of obese and lean rats that would allow us to specifically pick up proteins implicated in obesity and regulated directly by tungstate action. The combination of DIGE technology and bioinformatics analyses revealed that tungstate modulated oxidative stress and thermogenic pathways and identified peroxisome proliferator-activated receptor (PPAR) γ coactivator 1α (PGC-1α) as a key target of the tungstate antiobesity effect in BAT.

EXPERIMENTAL PROCEDURES

All animal procedures were conducted in accordance with principles of laboratory animal care (European Community and local government guidelines) and approved by the Animal Research Committee of the University of Barcelona. Diet-induced obesity and tungstate treatment in male Wistar rats were done essentially as described previously (5). Male Wistar rats (IFFA CREDO, L’Arbresle, France) weighing 200–240 g were individually caged, subjected to a 12-h light, 12-h dark cycle, and randomly divided into two dietary sets. Control lean animals were fed a standard chow diet (UL) during 30 days. Obesity was induced by feeding animals during 30 days with a cafeteria diet containing standard chow and a daily intake of cookies, liver pâte, bacon, and whole milk supplemented with 333 g/liter sucrose and 10 g/liter mineral and vitamin complex (Gevral; Cynamid Ibérica, Madrid, Spain) (5). The diet composition was 12.7% proteins, 38.7% lipids, and 36.1% carbohydrates. This diet resulted in 65% of the energy being derived from lipids, whereas for the standard diet the energy derived from lipids was only 8%. All food items were weighed daily and presented in excess. Food spillage was also collected and weighed. Daily caloric intake was calculated by multiplying the consumption of each item in the diet by its caloric density provided by the manufacturer. After the initial 30-day diet period, sodium tungstate was administered during 25 days in drinking fluids (2 mg/ml in distilled water and milk) to both lean (treated lean (TL)) and obese animals (treated obese (TO)) being fed chow and cafeteria diet, respectively. Some obese animals continued on the cafeteria diet until the end of the treatment period (untreated obese (UO)). An additional group of obese animals were calorie-restricted (after the initial 1-month obesity induction) by removing milk from their diet and feeding them half the amount of bacon and biscuits than obese rats (the other foods were kept at the same proportion; caloric restriction-treated obese (CRO)). Animal body weight and food and liquid intake were measured daily and analyzed by ANOVA. The day prior to sacrifice animals were fasted overnight and then anesthetized by inhalation of isoflurane, and interscapular BAT and other tissues were removed, snap frozen in liquid nitrogen, and kept at −80 °C until use. Animals were then killed by administration of an excess of isoflurane followed by decapitation. Experimental procedures for cafeteria diet and animal treatments require that each animal is housed in an individual cage to monitor food and drink intake. As a consequence of this, there is a limitation on the number of animals that can be used within the same set of experiments. Because the calorie-restricted obese group had not been characterized previously, because of the higher variability of the diet-induced obesity model compared with standard chow-fed lean animals, and because of the importance of tungstate treatment in obese animals for this study, we decided to use a greater number of animals for the obesity groups (three conditions, n = 6 each) than for the lean groups (two conditions, n = 3 each). DIGE experiments were designed to contain the minimum number of tissue replicates necessary to achieve statistical significance, and validation studies (Western blot, palmitate oxidation, and RT-PCR) included the same replicates used for DIGE analyses plus additional animals.
**Immunohistochemistry—** A small fraction of BAT was formalin-fixed for immunohistochemistry. Briefly tissue specimens were dehydrated, embedded in paraffin, and cut into 4-μm-thick sections. Adipose tissue sections were stained with hematoxylin and eosiin following standard protocols.

**Sample Preparation for DIGE Analyses—** BAT from UL (n = 3), TL (n = 3), OU (n = 4), TO (n = 4), and CRO (n = 4) was homogenized in a buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 65 mM DTT, 0.5% (v/v) IPG buffer, and 10 mM sodium orthovanadate. Proteins were extracted during 30 min at 4 °C, and lysates were clarified by centrifugation at 14,000 rpm at 4 °C for 30 min. The interface between the low density lipid layer and the insoluble pellet was carefully collected and centrifuged again. Protein extracts were then prepared following general guidelines recommended for subsequent DIGE labeling. Briefly proteins were precipitated using the 2D clean-up kit (GE Healthcare) and resuspended in a buffer containing 30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH adjusted to 8.5, and finally protein content was quantified using the DC RC protein assay kit (Bio-Rad). Sample extraction and homogeneity were checked by visualization of Coomassie Blue-stained proteins separated by 10% SDS-PAGE. The concentration of all samples was adjusted to 7 μg/μl.

**2D DIGE and Image Analyses—** Samples were minimally labeled with Cy3 or Cy5 fluorescent dyes (50 μg of protein/400 pmol of dye) during 30 min at 4 °C following the manufacturer’s instructions (GE Healthcare). To minimize system and inherent biological variation, half of the samples from each group were labeled with Cy3, and the other half of the samples were labeled with Cy5. An internal standard was prepared by mixing equal amounts of all samples analyzed and was labeled with Cy2 fluorescent dye. Sample multiplexing was also randomized (Table I) to produce unbiased results. IPG strips (pH 3–10, 17 cm, GE Healthcare) were cup loaded with 50 μg of each Cy2-, Cy3-, and Cy5-labeled sample in a buffer containing 7 M urea, 2% thiourea, 2% (w/v) CHAPS, 65 mM DTT, and 1% (v/v) IPG buffer. Isoelectric focusing was carried out in a Protean IEF cell (Bio-Rad) at 62 kV-h in different phases as follows: 10 min at 50 V, 1 h-ramp up to 500 V, 1 h at 500 V, 2-h ramp up to 1000 V, 10-h ramp up to 10,000 V, and 30 min at 10,000 V. Second dimension SDS-PAGE was run by overlaying the strips on 10% isocratic Laemmli gels (24 × 20 cm), which were cast in low fluorescence glass plates, on an Ettan DALT VI system. Gels were run at 20 °C at a constant power of 2.5 watts/gel during 30 min followed by 17 watts/gel until the bromphenol blue tracking front had run off the gel. Fluorescence images of the gels were acquired on a Typhoon 9400 scanner (GE Healthcare), Cy2, Cy3, and Cy5 images for each gel were scanned at 488/520-532/580-, and 633/670-nm excitation/emission wavelengths, respectively, at 100-μm resolution, thus obtaining a total of 27 images (9 × 3).

Image analysis was performed using DeCyder version 5.0 software (GE Healthcare) following published and the manufacturer’s recommendations. The differential in-gel analysis module was used for intragel co-detection of samples and internal standard protein spots. Artifactual spots (dust and others) were filtered (maximum slope, <2.5; maximum peak height, <150) and removed. Analyses were done in duplicate setting the initial spot detection number at 1600 to detect low abundance spots and at 600 to detect high abundance spots as a single spot. The biological variation analysis (BVA) module was used for intergel matching of internal standard and samples across all gels and performing comparative cross-gel statistical analyses of all spots based on spot volumes (10), permitting the detection of differentially expressed spots between experimental conditions (ANOVA and Student’s t test, p < 0.05). Only spots present in 21 of the 27 gel images were considered. BVAs were also done from both 1600- and 600-spot differential in-gel analyses, and results were pooled. Finally matches and data quality of proteins of interest were manually checked to avoid false positives.

**Protein Digestion, Mass Spectrometry, and Protein Identification—** The same gels used for DIGE analyses were used as preparative gels and were silver-stained using an MS-compatible protocol (11). Molecular weight and calibration using 2D standards (Bio-Rad) was done in small format minigels (12), and then data were transferred by image analyses to the preparative gels. Proteins were excised from the different gels (n = 9), silver destained, and in-gel digested with trypsin at 37 °C overnight (Promega). Peptide extraction was performed, and ZipTip concentrating and desalting was done as described previously (6, 13). Peptides were analyzed in a Voyager DE Perspective instrument (Applied Biosystems, Foster City, CA) in the reflector/delayed extraction mode as described previously (6). Data Explorer version 4.2 (Applied Biosystems) was used for spectra analyses and generating peak picking lists. Peaks were calibrated externally using a standard peptide mixture (Bruker) and internally using trypsin autolysis peptides. The peak list was exported to an Excel data sheet, and peak intensity was used to select from 100 up to a maximum of 250 peaks (increasing in 50) for peptide mass fingerprinting. Trypsin, keratin, and matrix-derived peaks were removed when they were the most intense peptides in the spectra using contaminant database list from PeakErazor (Lighthouse data, Odense, Denmark) and Aldente (www.expasy.org/tools/aldente/). Proteins were identified by peptide mass fingerprinting using Aldente software (versions 01/12/2005 and 24/05/2006, ExPASy) and protein databases Swiss-Prot and TrEMBL releases 49 and 50.2, restricted to mammalian taxonomy containing 54,384 and 172,240 sequence entries, respectively, for each software version and database release. Searches were performed using a mass tolerance of 50 ppm, a single trypsin missed cleavage, iodoacetamide as the modification for cysteine, and methionine oxidation as a variable modification. Proteins were considered as identified only when they had a positive score (p < 1e–06), they had a molecular weight/pl similar to the experimental values found from the 2D gels, the following non-homologous protein had a score of at least 2 orders lower than the first hit, and the most abundant peptides in the spectra were assigned as the identified protein. If peptides matched to multiple members of a protein family, the highest scoring rank was reported as the identified protein. Similarly if peptides matched different isoforms of the same protein, the highest scored isoform was reported. When the highest score matched the same protein from several species (or a difference of 1 order was present), the taxonomy Rattus norvegicus was selected because this was the origin of the sample; otherwise the first species identity was reported. Searches that did not fulfill the criteria described above were further analyzed by MS/MS using a MALDI-TOF/TOF 4700 Proteomics Analyzer (Applied Biosystems). Data Explorer version 4.2 (Applied Biosystems) was used for spectra analyses and generating peak picking lists. Peaks were calibrated externally using a standard peptide mixture (Bruker) and internally using trypsin autolysis peptides. The peak list was exported to an Excel data sheet, and peak intensity was used to select the most intense peaks (up to 350 fragment ions). Peptide masses from MS analyses and their fragments obtained from MS/MS spectra were combined and submitted to Sequence Query Mascot software from Matrix Science. Searches were performed using Swiss-Prot 52.1 as the database and mammals as taxonomy (50,864 sequences), 0.07-Da error for peptide mass tolerance (MS), 0.8 Da for fragment mass tolerance (MS/MS), a single trypsin missed cleavage, and iodoacetamide as the modification for cysteine. Proteins were considered identified when (a) the first hit was the same as that identified by Aldente and had a Mascot score above 25, (b) the peptide MS/MS had an ion score of at least 5, (c) a minimum of 10 ions were matched to the precursor ion from the candidate protein, and the highest peaks of the MS/MS spectra were assigned, and (d) the protein had a molecular weight/pl similar to the experimental values found from the 2D gels. This threshold was selected.
and used as a confirmation of the previous identification with Aldente when the score was $p < 1e^{-06}$ and the following non-homologous protein had a score of at least 1 order lower than the first hit. In addition, selected peptides from proteins already identified by PMF were also fragmented to confirm and validate previous results.

**Bioinformatic Analyses**—The relative expression values of the proteins identified as significant by statistical analysis (ANOVA or t test) for the 18 samples were considered for hierarchical clustering analysis (14). Heat map representation of hierarchical clustering analysis done in both components, proteins and samples, may reveal important correlations. Indeed clustering revealed that one of the samples from the TO group was an outlier (not shown). Furthermore examination of body weight evolution revealed that this animal had an anomalous behavior, and therefore it was removed from the subsequent data analyses.

UniProt codes of proteins that were identified as direct targets of tungstate action in obesity and their $p$ values (t test) were submitted as “Focus proteins” to the Ingenuity Pathways Analysis (Ingenuity Systems) server to discover and explore relevant biological networks. When entries were from species other than R. norvegicus the corresponding homologous rat entry was searched in the UniProt/Swiss-Prot database and submitted to Ingenuity.

**Western Blot**—For Western blot from 2D gels, samples were homogenized in 7 M urea, 2 M thiourea, 25% (v/v) CHAPS, 65 mM DTT, 0.5% (v/v) IPG buffer, and 10 mM sodium orthovanadate and quantified, and 100 µg were loaded on IPG strips (7 cm, pH 3–10) and run on a Protean IEF cell at 7300 V·h. The second dimension was run as described previously (12) in 10% acrylamide gels.

For conventional SDS-PAGE, samples were homogenized in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, phosphatase inhibitors (10 mM sodium phosphate, 10 mM sodium fluoride, and 1 mM sodium orthovanadate), and protease inhibitor mixture (Sigma). Proteins were quantified, separated by conventional SDS-PAGE, and transferred to PVDF membranes. For semi-quantitative Western blot, increasing amounts of BAT protein extract were loaded on IPG strips (7 cm, pH 3–10) and run on a Protean IEF cell at 7300 V·h. The second dimension was run as described previously (12) in 10% acrylamide gels.

**Functional Proteomics of Tungstate in Obesity**

**Fatty Acid Oxidation**—Palmitate oxidation rates were measured as an indicator of fatty acid oxidation as described previously (15). Briefly small fragments of BAT were homogenized, and protein was quantified and incubated for 30 min at 37 °C in oxidation buffer containing substrate [1-14C]palmitic acid (200 µM, 200 µCi/ml, Amersham Biosciences) and cofactors ATP (5 mM), NAD+/NADH (1 mM), cytochrome c (25 µM), coenzyme A (0.1 mM), L-carnitine (0.5 mM), and L-malate (0.5 mM). The reaction was stopped by addition of perchloric acid. Palmitate oxidation rates were calculated from the sum of [14C]CO₂ and [14C]perchloric acid-soluble products and expressed in picomoles of palmitate/minute/milligram of protein.

**Real Time PCR**—Total RNA was extracted from BAT using the RNeasy Lipid tissue kit (Qiagen Sciences), and DNase I was used to remove genomic contamination. One microgram of total RNA was reverse transcribed in a buffer solution containing 25 mM MgCl₂, 100 mM Tris (pH 8.3), 500 mM KCl, 39 units/ml RNaseguard (GE Healthcare), 200 units/ml Moloney murine leukemia virus reverse transcriptase (Invitrogen), 10 mM deoxyribonucleotide triphosphates, and random hexamer primers (d(N)₅-P₉O₇GE Healthcare). Pgc-1α was amplified by RT-PCR from synthesized cDNA (16 ng) using Power SYBR Green PCR Master Mix and ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Pgc-1α primers were 5’-GAGCGGAGATAAAGCCAACA-3’ and 3’-GCGAGGGCGGTCTAAGG-5’. A standard curve was generated from four serial dilutions of liver synthesized cDNA. Samples were analyzed in triplicate, negative controls were included, and PCR products were verified using dissociation curve analysis immediately after RT-PCR. Results were analyzed using SDS2.1 software (Applied Biosystems) and normalized to 18S as the housekeeping gene (18 S rRNA primed designed TaqMan probe and primers Applied Biosystems), and statistics were analyzed by Student’s t test ($p < 0.05$).

**RESULTS**

**Experimental Design for the Identification of Direct Targets of Tungstate Antibiody Action in BAT**—Induction of obesity by cafeteria diet during 1 month resulted in a 19% increase in body weight in relation to standard diet. Tungstate treatment of obese rats (2 g/liter, 25 days) reduced their body weight by 17%. These results were similar to our previous data where both cafeteria model and tungstate effect were characterized (5). To identify specific targets of tungstate related to its antiobesity effect, we originally designed a strategy consisting of finding proteins that were modified by tungstate treatment in obese rats and that were not altered when lean rats were treated, thus selecting tungstate obesity targets. However, and because obese treated animals dramatically lose weight, this comparison would reveal tungstate-induced direct changes and those secondary to body weight loss, both of which are undistinguishable using this approach. To overcome this limitation, a strategy was designed to include an additional control consisting of obese animals that lost weight by means other than tungstate treatment. Accordingly the study design consisted of five groups of animals: UL, TL, UO, TO, and CRO. Criteria for obesity induction and tungstate treatment effectiveness are described in our previous studies (5). Fig. 1A shows the body weight gain evolution of the different experimental groups over the treatment period. Calorie-restricted obese rats showed a decrease in their body weight gain by 51% compared with UO on cafeteria diet.
similar to tungstate treatment (59%) (UO versus TO/CRO, p < 0.001 by ANOVA; TO versus CRO, n.s.). Cumulative caloric intake was decreased in the caloric restriction group (supplemental Fig. 1) but remained unchanged in the tungstate-treated obese rats as shown previously (5).

BAT tissue weight was not changed by tungstate treatment of obese rats compared with untreated animals (TO, 0.70 ± 0.06 g compared with UO, 0.77 ± 0.07 g, statistically not significant; UL, 0.46 ± 0.03 g, p < 0.05 compared with UO); nevertheless morphological changes were evident (Fig. 1B). BAT from UO had bigger adipocytes and higher fat content and presented large single vacuoles when compared with lean animals. Remarkably tungstate treatment reduced all these features, and morphologically, BAT from TO was very similar to tissue from lean animals. These observations together with our published results indicated that BAT was a target of tungstate and led us to identify proteins regulated by tungstate in this tissue.

**DIGE Analyses**—BAT protein homogenates from the different animal groups were prepared; minimally labeled with Cy2, Cy3, and Cy5; and processed for DIGE analysis as described under “Experimental Procedures” and in Table I. To minimize system and inherent biological variation, samples were labeled and multiplexed following these guidelines: (a) half of the samples from each group were either Cy3- or Cy5-labeled, (b) two samples from the same experimental group were never included in the same gel, (c) samples from each group were contrasted with the maximal number of different groups, and (d) samples from the same group were run twice in different gels but with the opposite dye labeling pattern. An internal standard consisting of a pool of the different samples was Cy2-labeled and included in each gel.

Protein spots were detected automatically using the software DeCyder. To allow detection of low abundance proteins, we originally set up the initial number of spots as 1600, and from this initial point, the software detected 1625 ± 88 spots (mean ± S.D., n = 27 gel images). DeCyder software does not allow manual spot detection to reduce user-subjective manipulation. This is generally considered an improvement and advantage over other 2D analysis programs where this kind of operation is feasible; nevertheless in certain circumstances it can be a drawback. For instance, we found that highly abundant spots were often detected as multiple spots, thus complicating the quantification and analysis of these proteins. To overcome this limitation, we performed a parallel analysis setting the initial number of spots as 600, resulting in a total of 555 ± 39 spots detected, and indeed, abundant proteins were mostly perceived as a single spot. Intragel analysis

![Image](image_url)
yielded normalized values for each sample with respect to the Cy2 standard and direct comparisons between groups. Matching between the different gels was done by means of internal standards using the BVA module, and only spots present in 21 of the 27 gel images were considered suitable for analysis. Thus, 1027 and 330 spots were matched across the different gels and analyzed in the 1600- and 600-spot analyses, respectively.

DIGE analyses rendered 27 spots that exhibited statistically significant expression changes across all groups (ANOVA, \( p < 0.05 \)). Despite the small number of spots considered, hierarchical clustering analysis of their expression values clearly separated the different experimental groups with two major branches, lean and obese phenotypes (Fig. 1C). In addition, individual obese samples were correctly clustered within their corresponding group, and it was remarkable that the TO group showed major similarity to the lean group, coincident with what we observed in the histological analysis of BAT tissue (Fig. 1B). The UO animals were more distant from the lean animals as expected. Analysis of t test contrast between the different conditions also correctly segregated the different experimental groups (supplemental Fig. 2). The correct “protein signature” of the samples gave us confidence on the whole experiment, and it was a good starting point for the identification of direct targets of tungstate action.

**Identification of Direct Targets of Tungstate Antiobesity Action**—Targets of tungstate action in obesity were selected as those spots that were modified by the treatment in obese rats but not in lean (\( p < 0.05 \) TO versus UO and n.s. TL versus UL). Direct targets were distinguished from targets secondary to body weight reduction by including from the previous selection only those spots different between TO and CRO (\( p < 0.05 \) TO versus CRO). Fig. 2 shows a diagram of the selection criteria used for the identification of direct targets of tungstate in obesity. Spots fulfilling these conditions were identified by MALDI-TOF MS as described under “Experimental Procedures” and are shown in a representative image of a 2D gel from BAT homogenates (Fig. 3). The majority of spots contained only single proteins, but in some cases MS analyses indicated a protein mixture. Conversely multiple spots flagged the same protein identity, thus indicating the existence of posttranslational modifications or different isoforms. Proteins, their calculated and experimental molecular weight/pl in the gels, PMF identification parameters, and the ratio between TO and UO expression levels are listed in Table II. Additional details on protein identification by PMF and MS/MS and spectra data are shown for each hit as
supplemental data (supplemental Fig. 3). Proteins were classified by functionality and included tricarboxylic acid-Krebs cycle and lipolysis and fatty acid oxidation groups that were both up- and down-regulated. Glycolysis and redox groups were remarkably up-regulated, and in contrast the electron transport group was generally down-regulated.

Protein expression changes across the different experimental groups are shown in Fig. 4. The expression pattern illustrates the different behavior between TO and CRO, criteria that were established to characterize tungstate-specific targets. Approximately half of the spots were up-regulated by tungstate treatment in obese rats (Fig. 4A), and in general UO showed a decrease or no change compared with UL. Interestingly all proteins related to redox processes fell in this category, and moreover, some of them, heat shock 70 group, were remarkably up-regulated, and in contrast the electron transport group was generally down-regulated.

### Table II

| UniProt accession no. | Protein ID | Name | Ratio TO/UO | p value ID | Molecular weight | No. pep | Seq% | p value | % |
|-----------------------|------------|------|-------------|------------|------------------|--------|------|---------|---|
| P20004                | ACON       | Acnotin, mitochondrial | 2.34 | 0.002 | 82/7.1 | 67/6.86 | 11/109 | 18 | 3.2e-07 |
| P9ER34                | ACON       | Acnotin, mitochondrial | 1.83 | 0.027 | 82/7.1 | 71/7.19 | 11/75 | 16 | 5.4e-08 |
| Q99NA5                | IDH3       | Isocitrate dehydrogenase (NAD) subunit a | 1.32 | 0.030 | 37/5.7 | 40/5.36 | 6/94 | 24 | 9.5e-06 |
| P14408                | FUMH       | Fumarate hydratase, cytoplasmic-mito. | -1.31 | 0.017 | 50/8.3 | 48/8.54 | 9/82 | 34 | 1.5e-09 |
| Glycolysis            | P04797     | G3P  | Glyceraldehyde-3-phosphate dehydrogenase | 1.91 | 0.032 | 36/8.5 | 39/7.48 | 12/138 | 33 | 3.3e-08 |
| Lipolysis, fatty-acid β-oxidation | P15651     | ACADS | Acyl-CoA dehydrogenase, short chain, mito. | 1.52 | 0.060 | 42/6.4 | 41/7.04 | 11/89 | 34 | 5.9e-10 |
| P13437                | THIM       | 3-Ketoacyl-CoA thiase mitochondrial | -1.34 | 0.017 | 42/8.1 | 44/9.10 | 16/84 | 49 | 8.3e-14 |
| Q9WWK7                | HCDH       | Short-chain 3-hydroxyacyl-CoA dehydrogenase, mito. | -1.43 | 0.045 | 33/8.3 | 37/9.19 | 8/82 | 33 | 4.1e-08 |
| P13437                | THIM       | 3-Ketoacyl-CoA thiase mitochondrial | -1.38 | 0.002 | 42/8.3 | 44/9.21 | 15/185 | 46 | 3.7e-10 |
| P45953                | ACADV      | Acyl-CoA dehydrogenase, very long chain, mito. | -1.48 | 0.005 | 66/8.1 | 62/8.5 | 30/120 | 57 | 1.5e-20 |
| Electron transport    | Q8CAQ8-2   | IMMT | Mitochondrial inner membrane protein, mitoflin | 1.32 | 0.001 | 83/6.2 | 70/5.07 | 15/235 | 25 | 4.6e-08 |
| P32551                | UQCR2      | Ubiquinol-cytochrome c reductase complex | -1.38 | 0.002 | 47/8.8 | 44/9.21 | 11/189 | 51 | 1e-10 |
| P15999                | ATPA       | ATP synthase α chain | -1.45 | 0.004 | 55/8.3 | 53/8.67 | 13/116 | 34 | 4.1e-11 |
| Q6UPE1                | ETFD       | Electron transfer flavoprotein-ubiquinone oxidoreductase | -1.46 | 0.039 | 64/6.5 | 59/6.6 | 17/183 | 34 | 3.3e-08 |
| Redox, antioxidant, chaperones | P48721     | GRP75 | Stress-70 mitochondrial | 1.66 | 0.020 | 69/5.5 | 67/5.24 | 9/191 | 17 | 1.3e-06 |
| P50137                | TKT        | Transketolase | 1.65 | 0.035 | 68/7.2 | 63/7.22 | 11/139 | 27 | 8.9e-07 |
| P63093                | CH60       | 60-kDa heat shock protein mitochondrial | 1.72 | 0.044 | 58/5.3 | 58/5.08 | 12/156 | 29 | 7.7e-09 |
| P63093                | CH60       | 60-kDa heat shock protein mitochondrial | 1.28 | 0.068 | 58/5.3 | 58/5.18 | 15/85 | 34 | 3.5e-13 |
| P48721                | GRP75      | Stress-70 mitochondrial | 1.26 | 0.056 | 69/5.5 | 67/5.33 | 11/109 | 22 | 6.6e-08 |
| P40762                | CATA       | Catalase | 1.25 | 0.049 | 60/7.1 | 57/7.07 | 13/167 | 28 | 5.3e-09 |
| P81178                | ALDH2      | Aldehyde dehydrogenase, mitochondrial | 1.25 | 0.038 | 54/5.8 | 52/6.13 | 12/138 | 29 | 1.6e-09 |
| P48721                | GRP75      | Stress-70 mitochondrial | 1.13 | 0.065 | 69/5.5 | 67/5.43 | 18/118 | 38 | 5.7e-14 |
| P50137                | TKT        | Transketolase | -1.34 | 0.046 | 68/7.2 | 64/7.80 | 15/176 | 40 | 7.4e-10 |
| Others                |            |      |            |          |                  |        |      |         |   |
| P14480                | FIBB       | Fibrinogen β chain | 1.37 | 0.013 | 52/6.4 | 56/7.21 | 11/89 | 23 | 2e-10 |
| P09495                | TPM4       | Tropomyosin α -4 chain | 1.30 | 0.044 | 33/4.7 | 33/4.04 | 8/76 | 33 | 5.7e-08 |
| Q92322                | TPM1       | Tropomyosin 1α | -1.28 | 0.027 | 33/4.7 | 39/4.97 | 12/138 | 29 | 8.5e-09 |

* Ratio of protein expression levels were calculated using DeCyder software as the -fold change between normalized spot volume between TO and UO BAT homogenates (Student’s t test was based on the log of the ratio between TO and UO).

* Molecular weight and pI as determined by UniProt database.

* Molecular weight and pI as experimentally found in our 2D gels.

* Number of peptide masses matched/number of masses not matched.

* Protein sequence coverage by peptide mass fingerprinting.

* p value as calculated by Aldente from ExPASy server. Full details on protein identification by PMF are given in supplemental Fig. 3.

* Selected peptides were also analyzed by MS/MS to confirm protein identity; corresponding spectra, results, and fragments annotated are shown in supplemental Fig. 3.

* Protein entries were not statistically significant between TO and UO (p > 0.05); however, they were included in the table to illustrate variations in different isoforms from the same protein.
tein (CH60), and aldehyde dehydrogenase 2 (ALDH2) were also identified as tungstate targets in our previous study in WAT (6). Two proteins that participate in the tricarboxylic acid cycle, aconitase (ACON, two spots) and isocitrate dehydrogenase (IDH3), as well as mitochondrial inner membrane protein, also named mitofilin (IMMIT), involved in electron transport and glyceraldehyde-3-phosphate dehydrogenase (G3P) important for glycolysis, were also up-regulated by tungstate. Acyl-CoA dehydrogenase short-chain mitochondrial (ACADS) expression also had a tendency to increase upon tungstate treatment. Down-regulated proteins (Fig. 4B) included three proteins engaged in electron transport and mitochondrial respiration (ATP synthase α chain (ATPA), ubiquinol-cytochrome c reductase complex core protein 2 (UQCR2), and electron transfer flavoprotein-ubiquinone oxidoreductase (ETFD)), three proteins engaged in fatty acid oxidation and lipolysis...
(acyl-CoA dehydrogenase very-long-chain mitochondrial (ACADV), 3-ketoacyl-CoA thiolase mitochondrial (THIM), and short-chain 3-hydroxyacyl-CoA dehydrogenase mitochondrial (HCDH)), and one protein from the Krebs cycle (fumarate hydratase (FUMH)).

Another set of proteins consisted of those that were distributed over several spots and for which the expression pattern was not the same for the different forms. For instance, transketolase (TKT) was detected as two spots with the same mass but different isoelectric point (Fig. 4C), and tungstate increased the expression of the most acidic spot, whereas it reduced the basic form. Finally tropomyosin (TPM) was also present in two spots of slightly different mass and pl identified as isoforms 1 and 4 and were down- and up-regulated, respectively, by tungstate (Fig. 4D). Tropomyosin isoforms due to alternative splicing have been described (16).

Another classification of the identified proteins could be according to their cellular distribution. It was noticeable that 63% of the proteins are located in mitochondria; however, this was not implausible considering that brown adipose tissue is very rich in mitochondria (7). The question arises whether tungstate could modulate mitochondriogenesis rather than regulate protein expression. To clarify this, we analyzed expression levels of some mitochondrial subunits as mitochondrial markers. Western blot analyses (supplemental Fig. 4) showed no changes between UO and TO BAT homogenates, thus suggesting a direct effect of tungstate on protein expression rather than on the number of mitochondria.

**Tungstate Modulates Redox Processes but Does Not Alter Lipid Oxidation**—One of the major group of proteins affected by tungstate included chaperones and proteins involved in redox and antioxidant regulation processes. GRP75/hsp70 was one of these proteins and was identified as several spots, although only the most acidic spot was significantly in-
creased. We validated this result by 2D Western blotting and indeed observed that only the most acidic spot was significantly increased by tungstate, although the next spot had a tendency to increase (Fig. 5A).

Chaperones (GRP75/hsp70 and CH60), described to attenuate free radical production; the antioxidant enzyme catalase, and transketolase and ALDH2, both key enzymes of the polyol pathway but also implicated in reduction of reactive oxygen species (ROS) (17, 18), were identified here as direct targets of tungstate. These results together with our previous study on tungstate obesity targets in WAT (6) prompted us to investigate the effect of tungstate on protein oxidation, a consequence of ROS attack on protein side chains. To this aim, we analyzed carbonyl groups in homogenates from BAT by Western blotting and found that tungstate significantly decreased protein oxidation in obese rats (Fig. 5B). Protein oxidation in UO was increased 41% with respect to lean animals, in agreement with the well established link between obesity and oxidative stress (19), and tungstate reduced it by a 60%. These results indicate that tungstate protects proteins from oxidative damage and suggest a potential role in ROS reduction.

Another important group of proteins regulated by tungstate was that involved in lipolysis and fatty acid oxidation. Because some of the proteins were up-regulated whereas others were decreased, we wanted to assess the overall contribution of these pathways. Thus, palmitate oxidation was measured in BAT homogenates as an indicator of maximal fatty acid oxidation capacity. Palmitate oxidation was reduced in obese compared with lean animals as described previously (15); however, tungstate treatment did not alter lipid oxidation in obese rats (Fig. 6A). In addition, expression of MCAD, a mitochondrial enzyme that catalyzes the first step in the β-oxidation of fatty acids, was not altered by tungstate (Fig. 6B). These results suggest that although protein expression of some specific protein isoforms implicated in lipolysis and fatty acid oxidation (i.e. ACDV and THIM) was reduced by the treatment, on the whole, tungstate did not modify fatty acid oxidation capacity.

**Fig. 6.** Tungstate does not alter medium chain fatty acid oxidation. A, palmitate oxidation was measured as an indicator of fatty acid oxidation in homogenates of BAT in UO and TO rats (n = 4; mean ± S.E.; *, p < 0.05, t test). B, semiquantitative Western blot of MCAD. Homogenates were processed by Western blotting as described under “Experimental Procedures,” and the linear regression was used to quantify the differences between bands. C, optical density values obtained from the Image Gauge 4.0 software were extrapolated from the linear regression (D) and expressed as the mean ± S.E. (n = 4, Student’s t test). prot, protein. Error bars represent the S.E. of the mean.
Protein Network Analyses Implicate Tungstate in Regulating Energy Homeostasis Pathways—The 2D DIGE approach identified 20 different proteins involved mainly in carbohydrate and lipid metabolism, electron transport, and redox pathways as direct targets of tungstate in obesity. Functional analyses indicated that redox regulation may indeed be affected by tungstate, whereas a role in fatty acid oxidation was questionable. To gain further insight into the potential mechanisms of tungstate action, a new data mining strategy was exploited in our proteomics data. We performed “Ingenuity Pathways Analysis” to investigate whether our list of tungstate direct targets belongs to specific pathways and to explore relevant networks in which they are involved. Each “Focus protein” (a protein from our list) was mapped onto the Ingenuity Pathways Knowledge Base, which is a database containing important curated information of interactions between genes, proteins, and other biological molecules. Networks are displayed graphically as nodes (proteins) and edges (the biological relationship between the nodes). Highly interconnected networks are likely to represent significant biological functions, and a score is calculated as the likelihood that Focus proteins are in the network due to random chance. The overlapping of the first and second scored networks of our study is shown in Fig. 7.

Network analyses highlighted several nodes of “non-focused” proteins that were not present in our list. In the current
context of obesity and body weight reduction, leptin and PPARGC-1A, namely PPARγ coactivator 1α (PGC1α), were the most attractive. A role for leptin in tungstate action has recently been described by our group.2 PGC1α is a transcription co-activator and metabolic regulator in both brown and white fat, muscle, and liver (21). In BAT, PGC1α regulates components of the adaptive thermogenesis program and also seems to be necessary for brown adipocyte differentiation. This together with our network analysis results points to a potential role of this factor in tungstate action in BAT. To investigate this hypothesis, PGC-1α mRNA levels were quantified by real time PCR in BAT from TO and found to be significantly increased by 2.5-fold with respect to UO (Fig. 8A). Next we examined whether tungstate had an effect on UCP-1, one of the main targets of PGC-1α, that is specifically expressed in BAT and essential for non-shivering thermogenesis in small mammals (22). Likewise we measured UCP-1 protein levels by semiquantitative Western blot and found that tungstate also increased UCP-1 expression by 3-fold (Fig. 8B). In the DIGE analysis UCP-1 was probably masked by highly abundant proteins present in the region of the gel corresponding to its molecular mass of 33 kDa and pI of 9.2 (Fig. 3, just on the right of HCDH; molecular mass, 33 kDa; pI, 9.19). Alternatively we performed 2D Western blot of UCP-1 and detected the protein at a molecular weight and pI very similar to the theoretical values, and its expression was clearly increased in TO compared with UO (supplemental Fig. 5). These results were in agreement with our previous findings where tungstate increased UCP-1 mRNA levels in BAT and whole body metabolic rate, thus demonstrating a role of tungstate in energy dissipation in BAT.

DISCUSSION

The exponential and alarming growth of the obesity epidemic has led scientists to begin to take advantage of proteomics to identify obesity molecular targets and to study the mechanisms of action of potential obesity therapies. Proteomics analyses have been proven useful in the characterization of the adipocyte proteome (23), in the identification of

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2 I. Canals, M. C. Carmona, M. Amigó, A. Bortolozzi, F. Artigas, and R. Gomis, manuscript submitted.
obesity targets in different models of experimental obesity, and to characterize targets of several agents such as the insulin sensitizer rosiglitazone (24–26) or our recently discovered antiobesity agent tungstate (6). Although they are highly informative, these strategies often generate large amounts of data and long lists of proteins that are difficult to analyze and understand their biological importance. An additional intricacy to accomplish our goal of identifying tungstate targets was to distinguish direct targets from those secondary to body weight loss. Here we benefit from the recently described DIGE technology (8) to design an approach to reduce large amounts of data that are not relevant and to focus our search on finding direct specific targets of tungstate. Thus, multiplexing samples into the same gel together with an internal standard permitted us to analyze replicates from different experimental conditions with statistical confidence. The inclusion of untreated and tungstate-treated lean animals allowed us to select targets of tungstate specific to its antiobesity function, and the addition of obese rats that lost weight by caloric restriction let us distinguish between tungstate direct targets and those secondary to body weight loss. Using this approach, we identified 20 proteins as direct targets of tungstate that were implicated in redox, glycolysis, Krebs cycle, electron transport and mitochondrial respiration, and fatty acid oxidation and lipolysis. Functional analyses confirmed a role of redox processes in tungstate action. Furthermore bioinformatics analyses of our list of proteins using Ingenuity Pathways Analysis revealed for the first time that PGC-1α could be a direct target of the tungstate antiobesity effect in brown adipose tissue. PGC-1α protein expression could not be detected in our study because the half-life of the protein is very short (2.3 h) (27), and the experimental model required fasting of animals before sacrifice, which in BAT results in decreased PGC-1α protein levels and tissue activity. Although detection of PGC-1α protein remains an important issue requiring future experiments, we demonstrated increased PGC-1α mRNA levels and increased protein expression of its direct targets, IDH3 and UCP-1, thus suggesting that this factor is indeed a central regulator of many of the proteins modulated by tungstate.

In BAT, the transcriptional co-activator PGC-1α is increased during brown adipocyte differentiation, regulates several aspects of mitochondrial biogenesis and function, and also controls energy digestion and expression of UCP-1 (21). The thermogenic capacity specific to BAT results uniquely from the expression of UCP-1 in the mitochondrial membrane, which uncouples the electrochemical proton gradient from ATP synthesis during respiration (22). Thus UCP-1 facilitates the re-entry of protons, reduces ADP phosphorylation, and decreases ATP energy storage and as a consequence increases energy expenditure as heat. Our proteomics approach demonstrates that tungstate treatment to obese rats increased PGC-1α and UCP-1 expression and concomitantly reduced ATP synthase, consequently resulting in increased energy dissipation in BAT, which contributes to the overall reduction in energy balance and body weight loss (5). PGC-1α has also been associated with mitochondrial biogenesis and brown adipocyte differentiation. However, we measured mitochondrial subunit expression as a marker of mitochondria number and did not find an increase upon tungstate treatment. Remarkably a recent study demonstrates that although function and thermogenic activity are indeed regulated by this co-activator brown fat differentiation and mitochondrial biogenesis were unaffected (29). This suggests that indeed tungstate could alter PGC-1α expression and BAT function without affecting mitochondrial biogenesis.

An alternative function of UCP proteins, including UCP-1, is to reduce ROS generated during mitochondrial respiration (7, 22). Oxidative stress results from the imbalance between the production of ROS and its elimination by antioxidant defense mechanisms. In obesity, oxidative stress can result from increased fatty acids in tissues and plasma, hyperleptinemia, hyperglycemia, a deficient antioxidant system, or an increased production of free radicals (19). Additionally recent studies found an increase in protein oxidation and carbonylation in adipose tissue of obese C57Bl/6J mice (29) in agreement with our present results in cafeteria Wistar obese rats. A major finding of this study was that tungstate could reduce ROS in BAT from obese animals as demonstrated by a reduction of protein oxidation and carbonylation and an increased expression of proteins that regulate redox status. Thus tungstate may reduce ROS in obese rats by increasing expression of proteins that reduce oxidative stress such as UCP-1, heat shock proteins (30, 31), transketolase (18), catalase, and ALDH (17). Remarkably our previous proteomics study on WAT also pointed to a role of tungstate in reducing ROS in obesity, and actually we found that HSPs and ALDH were also increased by tungstate, thus reinforcing this hypothesis. Additional evidence in this direction is provided by the increase in ACON and G3P by tungstate because both proteins have been associated with decreased ROS levels (32). Recent studies have also suggested a potential role of ROS in regulating adipocyte differentiation (33). It is noteworthy that administration of different antioxidants decreased body weight and adipose tissue of experimental models of obesity (34, 35). What is more, the antioxidant resveratrol protects mice from diet-induced obesity and insulin resistance by interacting with PGC-1α and its regulator SIRT1 (36). Whether tungstate-induced fat and body weight reduction is a consequence of its ROS reducing potential as well as the potential role of antioxidants in preventing and ameliorating the obesity phenotype remains and ought to be further explored.

DIGE analyses suggested that tungstate regulated proteins involved in glycolysis and fatty acid β-oxidation and lipolysis. Glycolysis seemed to be up-regulated because increases in G3P and the majority of Krebs cycle components were observed upon tungstate treatment. In rodents, glucose can also
be an important fuel for BAT in vivo. Under physiological insulin stimulation, BAT can reach 10% of the total glucose turnover rate in the rat, and a number of metabolic genes are induced (7). Thus it is conceivable that tungstate, an insulin mimetic in models of diabetes mellitus (37, 38), may also stimulate glycolysis similarly to insulin. Besides increased glycolysis would provide an additional source of ATP to compensate for the reduced mitochondrial supply of ATP during thermogenesis. Concerning the effect of tungstate on fat oxidation and lipolysis, our results seemed ambiguous. Whereas proteomics analyses rendered up- and down-regulation of proteins involved in these pathways, with down-regulated proteins the majority, palmitate oxidation as a measure of β-oxidation was unaltered. Several explanations may account for this discrepancy. First, protein expression does not always correlate with activity. Second, posttranslational modifications usually alter function and also can modify the expression pattern of proteins in 2D gels; in fact the identified forms of ACADV and THIM had an experimental pi that was different from the calculated pi, suggesting posttranslational modifications. Third and most important, the rate-limiting step of fatty acid oxidation is carnitine palmitoyltransferase I/II system, which facilitates the transport of fatty acyl-CoA into the mitochondria (39); consequently alterations in other enzymes in this pathway (18), was also identified as two different spots in our study, and different phosphorylation sites have been described in vitro. This was in agreement with the different forms identified here with identical mass but different pi. Then tungstate may actually modify some proteins identified here by phosphorylation. On the other hand, a new prospect on protein oxidation emerges from our results. Protein carbonylation and oxidation can indeed regulate protein activity, and tungstate can modify these posttranslational modifications; nevertheless the contribution of these modifications to the tungstate effect found here needs further investigation. Regardless it seems clear that tungstate can regulate phosphorylation, oxidation, and perhaps additional posttranslational modifications. Phosphorylation has been long known to regulate transcription factor activity. For instance, cAMP-response element-binding protein transcription factor phosphorylation is essential for PGC-1α gene expression (21), and PGC-1α phosphorylation by p38 is also required for UCP-1 transcription and expression (52). The possibility of tungstate-induced p38 phosphorylation contributing to UCP-1 expression is a tempting hypothesis.

Sodium tungstate (Na₂WO₄) is a salt of tungsten and is present as a trace element in the form of oxoanions in metals in nature. Although no data exist on its availability in the human diet, it seems unlikely to have a natural contribution to human metabolism. In rats and dogs, administered sodium tungstate has a rather low toxicity as demonstrated by its oral...
LD<sub>50</sub> value (13–20 times higher than therapeutic dose), high bioavailability, and total plasma clearance and half-life elimination rate (53–55). In addition, antiobesity effects of tungstate occur without any adverse effects such as gastrointestinal discomfort, the main side effect described for vanadate, which is also an insulin mimic (5, 53). Toxicity data in humans have only been reported upon occupational and environmental exposure (56). Nevertheless tungstate as an obesity therapy has satisfactorily passed phase I clinical trials, and the second phase trials will start shortly. 4 It is noteworthy that chromium derivatives, transition metals with insulin mimetic actions, attenuate body weight gain and increase insulin sensitivity in clinical trials in subjects with type 2 diabetes (57). Despite the fact that the mechanism of action of tungstate on the insulin cascade (48, 49) may diverge from that of chromium (20), collectively these data create expectation on the therapeutic use of tungstate in obesity.

In summary, the findings of this study suggest that the antiobesity effect of tungstate results, at least in part, from the reduction of redox status and increased energy dissipation through up-regulation of PGC-1<sub>a</sub> and possibly modulation of posttranslational modifications. This opens new directions in the search for mechanisms of action of this attractive therapy for the treatment of obesity. Furthermore these results highlight the value of DIGE technology and bioinformatics tools to discover new targets for fighting against obesity and encourage its application to other pathologies important for human health.

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To whom correspondence may be addressed: Laboratory of Experimental of Diabetes and Obesity, IDIBAPS, C/ Villarroel 170, E-08036 Barcelona, Spain. Tel.: 34-93-2275400, ext. 2910; Fax: 34-93-4516638; E-mail: sbarcelo@clinic.ub.es.

To whom correspondence may be addressed: Endocrinology and Diabetes Unit, Hospital Clinic, C/ Villarroel 170, E-08036 Barcelona, Spain. Tel.: 34-93-2279846; Fax: 34-93-4516638; E-mail: rgomis@clinic.ub.es.

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