Inorganic Nitrate Promotes Glucose Uptake and Oxidative Catabolism in White Adipose Tissue through the XOR Catalyzed Nitric Oxide Pathway

Running Title: Nitrate Enhances Adipose Tissue Glucose Metabolism

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Abstract

An ageing global population combined with sedentary lifestyles and unhealthy diets has contributed to an increasing incidence of obesity and type 2 diabetes. These metabolic disorders are associated with perturbations to nitric oxide (NO) signaling and impaired glucose metabolism. Dietary inorganic nitrate, found in high concentration in green leafy vegetables, can be converted to NO in vivo and demonstrates anti-diabetic and anti-obesity properties in rodents. Alongside tissues including skeletal muscle and liver, white adipose tissue is also an important physiological site of glucose disposal. However, the distinct molecular mechanisms governing the effect of nitrate on adipose tissue glucose metabolism, and the contribution of this tissue to the glucose tolerant phenotype, remain to be determined. Using a metabolomic and stable-isotope labeling approach, combined with transcriptional analysis, we found that nitrate increases glucose uptake and oxidative catabolism in primary adipocytes and white adipose tissue of nitrate-treated rats. Mechanistically, we determine that nitrate induces these phenotypic changes in primary adipocytes through the xanthine oxidoreductase catalysed reduction of nitrate to nitric oxide and independently of Peroxisome Proliferator-Activated Receptor α. The nitrate-mediated enhancement of glucose uptake and catabolism in white adipose tissue may be a key contributor to the anti-diabetic effects of this anion.

According to the World Health Organization, there will be more than 650 million people worldwide with type 2 diabetes mellitus (T2D) by 2040. A lack of bioavailable nitric oxide (NO) observed in patients with T2D is considered a significant contributing risk factor for cardiovascular ill health (1). Polymorphisms in the endothelial-NOS (eNOS) gene, responsible for in vivo NO production, are associated with cardiovascular disease, T2D and insulin resistance in humans (2), supporting the view that perturbation of NO homeostasis contributes to the metabolic syndrome. Furthermore, eNOS-deficient mice are hypertensive, dyslipidemic, insulin resistant and glucose intolerant (3). Inorganic nitrate, found in high concentrations in green leafy vegetables, can be reduced in vivo to form NO. Complementary studies of dietary nitrate administration to the eNOS-deficient mouse and Sprague-Dawley rats report reduced adiposity and improved glucose and insulin homeostasis following nitrate treatment (4; 5). Inversely, long-term dietary nitrate deficiency induced the metabolic syndrome in mice (6). Therefore, nitrate may provide a therapeutic avenue for the treatment of aspects of the metabolic syndrome.

Recently, the mechanisms behind the beneficial metabolic effects of nitrate have been explored. In skeletal muscle nitrate induces effects associated with endurance exercise training including fatty acid β-oxidation and fibre-type switching (7; 8) which may contribute to observations that nitrate improves exercise tolerance (9). In the liver, nitrate may prevent steatohepatitis through effects on β-oxidation and lipogenesis (10).

In white adipose tissue (WAT), nitrate induces the browning response and β-oxidation (11). Whether nitrate directly regulates glucose metabolism in WAT remains unknown. We use metabolic profiling to identify a distinct glucose metabolic phenotype associated with WAT of nitrate-treated rats. We characterise the effect of nitrate on glucose uptake and metabolism in primary adipocytes using stable-isotope substrate labeling and define the mechanisms through which nitrate regulates glucose metabolism in WAT. This study provides evidence

for a novel mechanism in WAT through which nitrate may mediate glucose metabolism.

Methods

Animal experimentation. Male Wistar rats (6 weeks old) (269 ± 2 g; n = 12) (Charles River Laboratories) were weight matched and received either distilled water containing 0.7 mM NaCl or water containing 0.7 mM sodium nitrate ($NaNO_3$) (Ultra-pure, Sigma-Aldrich) ad libitum for 18 days with food and water intake monitored (n = 6 / group). Animals were housed in conventional cages at room temperature with a 12-hour/12-hour light/dark photoperiod.

All procedures involving live animals were carried out by a licence holder in accordance with UK Home Office regulations, and underwent review by the University of Cambridge Animal Welfare and Ethical Review Committee. The rats used in this work were also used in separate, but parallel studies (8; 11), with a view to reducing the total numbers of animals used in accordance with UK Home Office best practice. Where relevant data (e.g. nitrate intakes has been reported previously, we refer to the previous paper (Supplementary Table 1).

Tissue collection. Rats were fasted overnight and then euthanized with sodium pentobarbital (200 mg/ml, Vétoquinol UK Ltd.). Blood was taken by cardiac puncture and processed for plasma and WAT was removed and frozen in liquid nitrogen.

Culture and differentiation of primary adipocytes. Primary WAT stromal vascular cells were fractionated from 6 – 10 week old C57BL6 mice or PPARα null mice (Ppara null mice were a kind gift of Frank Gonzalez [National Institutes of Health (NIH), Bethesda, MD, USA]) as previously described (11; 12). Cells were counted using a Scepter™ Cell Counter (Millipore) according to the manufacturer's instructions. Cells were seeded at 10,000/cm^2. Cells were then cultured and differentiated into adipocytes according to published methods (11; 12). During the 6 day differentiation, cells were cultured with either saline (control) or

500 μM $NaNO_3$ (Ultra-pure, Sigma-Aldrich) or 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) (50 μM) or KT5823 (1 μM) (Santa Cruz Biotechnology) or 100 nM insulin. Cells were treated with PTIO, KT5823 and insulin with and without 500 μM $NaNO_3$. $NaNO_3$ was added at day 1 and PTIO or KT5823 at day 5 of differentiation. For both Glut4 membrane biotinylation and immunoprecipitation assays and insulin-stimulated glucose uptake assays, after differentiation, primary mouse adipocytes were serum starved for 4 hours before being treated with 100 nM insulin, 500 μM nitrate or both 100 nM insulin and 500 μM nitrate together for 10 mins.

Cell viability. Cell viability was determined using a resazurin assay. Briefly, cells were washed with phosphate buffered saline and incubated with 44 μM resazurin in culture media for 2 hrs. Resazurin fluorescence was then measured (excitation 530 nm, emission 590 nm, cut-off 550 nm) with a fluorescence microplate reader.

siRNA xanthine oxidoreductase knockdown. FlexiTube siRNA against XOR, AllStars negative control siRNA and HiPerFect Transfection Reagent were purchased from Qiagen. Adipocyte transfection was carried out as per the manufacturer's instructions (75 ng siRNA, 3 μl transfection reagent per well, 10 nM final siRNA concentration) on day 2 and 4 of differentiation.

Primary adipocyte ^{13}C-glucose substrate labeling. Following the six-day 500 μM $NaNO_3$ treatment during differentiation, cells were cultured in low-glucose serum free media supplemented with U-^{13}C-labelled glucose (3100 mg/L) for 24 hours. Cellular metabolites were then extracted and analysed by Gas Chromatography-Mass Spectrometry (GC-MS) as described below.

Metabolite extraction. Metabolites were extracted from WAT, blood plasma and primary adipocytes using a modified Bligh and Dyer method (14). Frozen WAT (20 mg) was pulverized using a Tissue Lyser II (Qiagen). Methanol-chloroform (2:1, 600 µl) was added to the WAT, plasma (50 µl) or primary adipocytes and the samples were sonicated for 15 minutes. Chloroform-water (1:1) was then added (400 µl). Samples were centrifuged (16,100 g, 20 minutes) the aqueous phase was separated, dried under nitrogen and stored at -80°C until analysis.

GC-MS analysis. Dried aqueous phase samples were derivatized using methoxyamine hydrochloride solution (20 mg/ml in pyridine; Sigma-Aldrich) and 30 µl of N-methyl-N-trimethylsilyltrifluoroacetamide (Macherey-Nagel, Duran, Germany) using the method described previously (14). GC-MS and data analysis were performed according to published methods (14). All GC-MS analyses were made using a Trace GC Ultra coupled to a Trace DSQ II single-quadrupole mass spectrometer (Thermo Scientific, Cheshire, UK). Derivatized aqueous samples were injected with a split ratio of 10 onto a 30 m × 0.25 mm 5% phenylpolysilphenylene-siloxane column with a 0.25 µm ZB-5 ms stationary phase (Phenomenex). The injector temperature was 230°C, and the helium carrier gas was used at a flow rate of 1.2 ml/min. The initial column temperature of 70°C was increased by 10°C/min to 130°C and then increased at a rate of 5°C/min to 230°C followed by an increase of 20°C/min to 310°C and held for 5 min [transfer line temperature = 250°C; ion source = 250°C; electron ionization (EI) = 70 eV]. The detector was turned on after 240 s, and full-scan spectra were collected using 3 scans/s over a range of 50–650 m/z.

GC-MS chromatograms were processed using Xcaliber (version 2.0; Thermo Scientific). Each individual peak was integrated and then normalized. Overlapping peaks were separated using traces of single ions. Peak assignment was based on mass fragmentation patterns matched to the National Institute of Standards and Technology (USA) library and to previously reported literature.

Gene expression analysis. Total RNA extraction from WAT and adipocytes, cDNA conversion and quantitative RT-PCR was performed according to published protocols (12). All data were normalized to 18SrRNA (mouse primary adipocytes) or RLPL1 (rat WAT) and quantitative measures obtained using the Δ-Δ-CT method.

Biotinylation, immunoprecipitation and Western blot of cell surface Glut4. Surface Glut4 biotinylation, immunoprecipitation and Glut4 Western blot were carried out according to published protocols (15). Briefly, cells were incubated for 1 hour at 4°C with 0.5 mg/ml Biotin sulfo-NHS (Sigma-Aldrich). Cell lysates were pre-cleared by incubation for 30 minutes with 0.5% (w/v) protein A-Sepharose. The protein A-Sepharose was pelleted by centrifugation for 1 minute at 13,000 g and the supernatant removed and incubated overnight with 0.5% (v/v) anti-Glut4 antibody (1F8, Cell Signaling). Protein A-Sepharose was added to 0.5% (w/v) to the samples and incubation continued at 37°C for 1 hour. Immunocomplexes were pelleted at 13,000 g for 1 minute and the pellet washed three times with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 1% (v/v) Nonidet P-40.

Immunoprecipitated biotinylated complexes were mixed with dissociation buffer (125 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 100 mM dithiothreitol, Bromophenol

Blue) and boiled for 5 minutes. Glut4 was resolved by electrophoresis through 10% polyacrylamide gels, and then transferred to Hybond-P polyvinylidene difluoride membrane. The membrane was blocked for 1 hour in phosphate-buffered saline (PBS; 1.5 mM KH2PO4, 2.7 mM Na2HPO4, 150 mM NaCl, pH 7.4) containing 5% (w/v) dried milk powder and 0.1% (v/v) Tween-20, followed by incubation with peroxidase-conjugated streptavidin [1:1000 dilution in PBS containing 0.1% (v/v) Tween-20] for 1 hour. Bound peroxidase conjugates were visualised using an enhanced chemiluminescence detection system (Amersham Biosciences). Quantitation of immunoblots was performed using ImageJ.

Glucose uptake assay. Cells were grown and differentiated in 96-well plates. Cells were washed with Dulbecco's phosphate buffered saline (DPBS) and placed in low glucose (1g/L) serum-free Dulbecco Modified Eagle Medium (DMEM) for 24 hours. Media was replaced with low glucose serum-free DMEM for 1 hour. Following media aspiration DPBS containing 6-deoxy-6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (6-NBDG) (200 μM) was added for 1 hour and cells were kept at 37ºC, 5% CO_2. Cells were washed three times with DPBS and fluorescence measured using a microplate reader (excitation 485 nm, emission 528 nm).

Multivariate data analysis. Metabolomics data analysis was performed using Metaboanalyst version 4.0 (16). Data sets were auto scaled and analyzed using partial least squares-discriminant analysis (PLS-DA). Metabolite changes responsible for clustering or regression trends within the pattern recognition models were identified by interrogating the corresponding loadings plot. Metabolites identified in the variable importance in projections/coefficients plots were deemed to have changed globally if they contributed to separation in the models with a confidence limit of 95%. Plasma metabolomics data was

analysed using univariate volcano plots with a fold change cut off of 1.2 and P-value cut off of 0.05 to identify significantly different metabolites.

Data resource availability. All data including metabolomics datasets for GC-MS analysis of nitrate-treated rat adipose tissue and nitrate-treated primary adipocytes are available from the corresponding author on reasonable request.

Results

Metabolomic profiling identifies nitrate-mediated effects on glucose metabolism in white adipose tissue

Male Wistar rats were treated with either 0.7 mmol/L NaCl or 0.7 mmol/L $NaNO_3$ via the drinking water for 18 days. Nitrate intake for the control group was 1 mg/kg/day compared with 8 mg/kg/day in the nitrate treated group, whilst water and food intake was not significantly different between the groups (Supplementary Table 1) (11). Metabolomic profiling and analysis using PLS-DA multivariate statistics identified a distinct metabolic signature differentiating subcutaneous inguinal WAT of nitrate-treated animals from controls (Fig. 1A). Interrogation of the corresponding loadings plots identified a distinct reprogramming of glucose and fatty acid metabolism in the WAT of nitrate treated rats (Fig 1B). The decrease in the WAT concentration of the fatty acids propanoic, heptanoic, nonanoic, 3-hydroxyoctanoic, oleic, linoleic, and arachidonic acid in nitrate treated animals was consistent with our previous findings that nitrate drives WAT browning and β-oxidation (11). In a novel observation we identified a distinct glucose metabolism phenotype in the WAT of nitrate treated rats. The key glycolytic intermediates glucose 6-phosphate (Fig. 1C), and 3-phosphoglycerate (Fig 1D) alongside the pentose phosphate pathway – glycolysis metabolite glycerate (Fig 1 E) and the pentose phosphate pathway metabolite D-altro-heptulose (Fig 1F) were decreased in nitrate-treated rat WAT. Metabolic profiling of the plasma from nitrate treated animals indicated a corresponding decrease in glucose concentration (Fig 1 G & H).

To investigate whether nitrate functions to increase glucose uptake and metabolism in adipose tissue, and possibly contribute to systemic glucose clearance, the expression of genes encoding the insulin-regulated glucose transporter type 4 (Glut4), the insulin-independent glucose transporter (Glut1) and a key rate regulating enzyme in glycolysis, hexokinase 2

(Hk2), which converts glucose to glucose 6-phosphate, was interrogated using RT-qPCR (Fig 1i). Nitrate significantly increased the expression of these glucose import and metabolism genes. These data suggest that nitrate increases glucose uptake and disposal in adipose tissue.

Nitrate increases glucose uptake and catabolism in white adipocytes

To determine whether nitrate functions directly on WAT to increase glucose uptake and metabolism, stromal vascular fraction-derived primary adipocytes isolated from inguinal WAT of mice were treated with nitrate. A $NaNO_3$ concentration of 500 μM was chosen (11). Consistent with the results in vivo, metabolomic profiling identified that nitrate decreased the intracellular concentration of the glycolytic intermediates glucose 6-phosphate (Fig 2A) and 3-phosphoglycerate (Fig 2B) in adipocytes. Nitrate treatment also significantly decreased the glucose concentration of the media (Fig 2C). The expression of genes key to glucose import and metabolism Glut4, Glut1 and Hk2 was increased in the nitrate-treated adipocytes (Fig 2D).

To functionally assess the effect on glucose metabolism observed in the WAT of nitrate treated rats, the stable isotope substrate U-^{13}C-glucose was employed to evaluate enrichment of glucose-derived carbon through glucose import, the glycolytic pathway and into the TCA cycle. Primary adipocytes were incubated in serum-free media containing U-^{13}C-glucose and treated with 500 μM nitrate. GC-MS analysis was used to define the relative enrichment of metabolites. The labeled glucose enters the glycolytic pathway and is catabolized to pyruvate which is either converted to lactate, or labeled acetyl-CoA which enters the TCA cycle (Fig 2E). An unlabeled metabolite is detected as the molecular ion (M) in the mass spectrum. Additional ^{13}C-carbon atoms introduced to the specific molecule give rise to an increase in mass of 1 (M1, M2, M3, and so forth). Nitrate treatment increased the labeling of lactate (Fig

2F) and the M2 isotopologues of TCA cycle intermediates, citrate (Fig 2G), malate (Fig 2H) and the amino acid glutamate (Fig 2I), which is in fast exchange with 2-oxoglutarate from the TCA cycle. We noted a higher fractional enrichment of the M1 isotopologues of citrate (Fig. 4H) in nitrate-treated adipocytes, which may be indicative of a moderate increase in malic enzyme (ME) activity. Relative enrichment of the M3 isotopologues of TCA cycle intermediates was elevated in nitrate-treated cells (Fig. 2 H-I). Increased labelling of M3 occurs through the action of pyruvate carboxylase (PC).

In summary, nitrate confers a functional effect on adipocytes, not only increasing glucose uptake and flux through glycolysis but also oxidative catabolism.

Nitrate increases plasma membrane Glut4 and insulin-stimulated glucose uptake in adipocytes

We then investigated whether nitrate increased Glut4 presentation at the plasma membrane of primary adipocytes at baseline and following insulin-stimulation. Using a cell surface protein biotinylation and immunoprecipitation approach we identified that nitrate increased adipocyte plasma membrane Glut4 concentration (Fig 3A & B) (Supplementary Figure 1). We also observed that nitrate had an additive effect on plasma membrane Glut4 expression when combined with insulin (Fig 3A & 3B). Next, the functional uptake of glucose into adipocytes treated with nitrate was measured with the fluorescent glucose analog 6-NDBG in combination with insulin (100 nM) (Fig. 3C). Nitrate and insulin treatments did not affect cell viability (Supplementary Figure 2). Nitrate increased adipocyte glucose uptake and had an additive effect on insulin-stimulated glucose uptake into primary adipocytes.

Nitrate-induced expression of glucose metabolism genes is independent of PPARα in adipocytes

We next probed the mechanisms through which nitrate mediates its effects on glucose metabolism in adipocytes. Several of the metabolic effects of nitrate, including increased β-oxidation and lactate dehydrogenase activity in muscle, occur through downstream PPARα signalling (8; 17). We examined the effect of nitrate on glucose import and catabolic gene expression in primary adipocytes differentiated from the stromal vascular fraction of inguinal WAT from PPARα null mice. The lack of PPARα had no effect on nitrate-induced expression of glucose metabolism genes within the adipocytes (Fig 4A). Nitrate treatment significantly increased the expression of Glut4, Hk2 and Glut2. Thus, nitrate regulates glucose metabolic gene expression in adipocytes independently of PPARα.

Nitrate promotes glucose uptake into adipocytes via nitrate-NO signaling

NO can be generated in vivo from nitrate via XOR catalysed serial reduction of nitrate to nitrite and then to NO via the nitrate-nitrite-NO pathway (18; 19). We found nitrate induced the browning process in WAT through this pathway (11) . Therefore, we speculated that nitrate might be functioning via NO to increase glucose import and metabolism in WAT. Primary adipocytes were differentiated in the presence of nitrate and the NO scavenger PTIO. Nitrate and PTIO treatments did not affect cell viability (Supplementary Figure 3). PTIO abrogated the nitrate-induced expression of glucose metabolism genes, Glut4, Glut1 and Hk2 (Fig 4B). Next, the functional uptake of glucose into primary adipocytes treated with nitrate was measured with 6-NDBG in combination with PTIO (Fig. 4C). PTIO inhibited nitrate-mediated glucose uptake into adipocytes. NO increases glucose uptake through the activity of cyclic GMP-dependent protein kinase G (PKG) (20). We have previously shown that nitrate

increases intracellular cGMP concentrations in adipocytes (11). We speculated that nitrate may signal via PKG to regulate adipocyte glucose metabolism. The pharmacological PKG inhibitor KT5823 blocked the nitrate-induced expression of Glut4, Glut1 and Hk2 in the adipocytes (Fig. 4D). These data demonstrate that nitrate signals via NO to enhance glucose uptake.

As mentioned above the reduction of nitrate to NO in mammals can proceed via an enzymatic mechanism catalyzed by XOR (18; 19). XOR is expressed in WAT, and has a role in adipocyte homeostasis and the nitrate-induced browning mechanism (11; 21). To determine the role of this enzyme in the nitrate-mediated enhancement of glucose catabolism, XOR in primary adipocytes was knocked down using siRNA (Supplementary Fig 4) (11). Nitrate and XOR knockdown did not affect cell viability (Supplementary Figure 5). Knockdown of XOR abrogated the increased expression of glucose import and catabolism genes, Glut4, Glut1 and Hk2 in adipocytes treated with nitrate (Fig 4E). Functionally, the effect of XOR knockdown on glucose uptake into nitrate-treated primary adipocytes was measured using 6-NDBG (Fig 4F). XOR knockdown inhibited nitrate-induced glucose uptake into primary adipocytes.

These data indicate nitrate induces glucose uptake and oxidative catabolism in WAT via the XOR catalysed reduction of nitrate and downstream NO signaling.

Discussion

Inorganic nitrate, found in high concentration in green leafy vegetables, may restore impaired NO signaling to treat aspects of cardiometabolic diseases including T2D (22). Nitrate regulates glucose homeostasis in rodent models of T2D (4-6; 23), however the mechanisms for nitrate-mediated improvements in metabolism remain to be fully elucidated. In WAT, nitrate promotes the browning response and enhanced fatty acid oxidation (11). The effects of nitrate on WAT glucose metabolism remained undefined. We are the first to determine that inorganic nitrate directly enhances both glucose uptake and oxidative catabolism through the activity of XOR, NO and PKG signaling. These findings are consistent with the effects of NO on glucose uptake, and Glut4 expression, in WAT (24-26). Interestingly, NO exhibits both insulin-dependent and insulin-independent effects on glucose uptake (24). We observe that nitrate enhances insulin-stimulated glucose uptake and plasma membrane expression of the insulin sensitive glucose transporter Glut4 in adipocytes. Nitrate also stimulates the expression of the insulin-independent transporter Glut1. This is consistent with our previous observation that nitrate induces WAT browning, since the browning phenomenon enhances Glut1 expression and may partly uncouple WAT glucose uptake from insulin action (27).

Several of the metabolic effects of nitrate, including enhanced lactate dehydrogenase activity, in skeletal muscle and heart are mediated through the nuclear receptor PPARα (8; 17). Our data show that nitrate-mediated expression of glucose uptake and metabolism genes was independent of PPARα signaling. Nitrate's effects on Glut4, Glut1 and Hk2 were preserved in PPARα-null adipocytes. This may, in part, reflect the low levels of expression of this nuclear receptor in WAT.

Although both WAT browning and enhanced mitochondrial biogenesis and β-oxidation in muscle are likely to substantially contribute to the anti-obesity and anti-diabetic effects of

nitrate, increased glucose uptake and oxidative catabolism will be a distinct factor mediating the effect of the bioactive anion on glucose homeostasis.

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Author Contributions

Dr. Lee Roberts is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors declare they have no conflicts of interest. B.D.M., A.M, N.T.W, T.A. and LDR carried out the majority of experiments. S.M. assisted with experiments throughout. S.A.M, B.D.M, L.D.R and J.L.G assisted with metabolomic screens and ^{13}C isotope studies. R.C.M and M.T.K provided intellectual input. A.J.M and TA designed and led the animal studies. L.D.R designed and led the studies, interpreted the results and wrote the paper with input from all co-authors.

Figure Legends

Figure 1. Metabolic profiling identifies inorganic nitrate reprograms glucose metabolism in white adipose tissue. A) Partial least squares-discriminant analysis (PLS-DA) plot showing the clustering of metabolites measured using GC-MS from adipose tissue of nitrate-treated rats (n = 6, green circles) compared with the control rats (n = 6, red circles (R2(X) = 99%, Q2 = 67%). 95% confidence regions are displayed. B) PLS-DA loadings plot showing separation of nitrate-treated rat adipose tissue is driven by a decrease in glucose metabolites and fatty acids. The concentration of C) glucose 6-phosphate, D) 3-phosphoglycerate, E) glycerate and F) D-altro-heptulose in nitrate-treated rat adipose tissue normalised to control. G) Volcano plot of metabolic profiling data of nitrate-treated rat plasma (metabolite concentrations are higher in the control group). H) The concentration of glucose in nitrate-treated rat plasma normalised to control. I) The expression of glucose transporter (Glut 4 and Glut 1) and metabolism (Hk2) genes in nitrate-treated rat subcutaneous adipose tissue (n = 6). Bar graph data is displayed as Mean ± SEM. *, $P \leq 0.05$. **, $P \leq 0.01$.

Figure 2. Nitrate enhances glucose catabolism in primary white adipocytes. The intracellular concentrations of, and representative chromatographic peaks for, A) glucose 6-phosphate and B) 3-phosphoglycerate in nitrate-treated primary adipocytes normalised to control. The concentration of, and representative chromatographic peak for, C) glucose in the media of nitrate-treated primary adipocytes normalised to control. D) The expression of glucose transporter (Glut 4 and Glut 1) and metabolism (Hk2) genes in nitrate-treated (500 μM) primary adipocytes. E) Schematic representation of glucose carbon tracing through glycolysis, the TCA cycle, malic enzyme (ME1), pyruvate carboxylase (PC) and glutamate

dehydrogenase (GDH) reactions in control and nitrate-treated (500 μM) primary adipocytes in presence of U-^{13}C glucose. Ball diagrams indicate isotopologues. Open circles represent ^{12}C, black circles represent ^{13}C through glycolysis and pyruvate dehydrogenase-initiated TCA cycle reactions. Green arrow indicates ^{13}C from PC-initiated TCA reactions (subsequently identified by the green circles). Purple arrow indicates the ME1 pathway and purple circles represent ^{13}C derived from the ME1 reaction. Not all possible labeled metabolites are shown. Gas Chromatography-Mass Spectrometry stable isotope analysis of methoximation and silylation-derivatized F) lactate, G) citrate, H) malate and I) glutamate extracted from control and 500 μM nitrate-treated primary adipocytes. Graphs show the ratio enrichments in the metabolite isotopologues. The X-axis indicates the mass isotopomers (which are M1, M2…Mn, where n is the number of labelled atoms in the molecule) in the specified metabolites (corrected for^{13}C natural abundance) (n = 4). Data is displayed as Mean ± SEM. *, $P \leq 0.05$. **, $P \leq 0.01$, ***, $P \leq 0.001$, ****,$P \leq 0.0001$.

Figure 3. Nitrate enhances Glut4 expression at the cell membrane and insulin-stimulated glucose uptake. A) Immunoprecipitation blots of cell surface biotinylated Glut4 from primary mouse adipocytes treated with 500 μM nitrate with and without 100 nM insulin (n = 3). **B)** Quantitation of cell surface biotinylated and immunoprecipitated Glut4 from primary mouse adipocytes treated with 500 μM nitrate with and without 100 nM insulin (n = 3). **C)** Glucose uptake in primary adipocytes treated with 500 μM nitrate with and without 100 nM insulin ($n \geq 29$). Data is displayed as Mean ± SEM. *, $P \leq 0.05$. **, $P \leq 0.01$.

Figure 4. Nitrate promotes glucose import and metabolism through a xanthine oxidoreductase catalysed reduction of nitrate to nitric oxide. A) The expression of

glucose import (Glut4, Glut1) and catabolic (Hk2) genes in primary adipocytes differentiated from the stromal vascular fraction of inguinal WAT from PPARα null mice and treated with 500μM nitrate (n = 3). B) Glucose import and metabolism gene expression in primary adipocytes treated with the NO scavenger PTIO (50 μM) with and without 500 μM nitrate (n = 3). C) Glucose uptake in primary adipocytes treated with the NO scavenger PTIO (50 μM) with and without 500 μM nitrate (n = 5). D) Glucose import and metabolism gene expression in primary adipocytes treated with the Protein Kinase G inhibitor KT5823 (1 μM) with and without 500 μM nitrate (n = 4). E) Glucose uptake and metabolism gene expression in primary adipocytes treated with negative control siRNA or siRNA against XOR with and without 500 μM $NaNO_3$ (n = 3). F) Glucose uptake in primary adipocytes treated with negative control siRNA or siRNA against XOR with and without 500 μM $NaNO_3$ ($n \geq 8$). Data is displayed as Mean ± SEM. *, $P \leq 0.05$. **, $P \leq 0.01$, ***, $P \leq 0.001$, ****, $P \leq 0.0001$.

References

1. Low Wang CC, Hess CN, Hiatt WR, Goldfine AB: Clinical Update: Cardiovascular Disease in Diabetes Mellitus: Atherosclerotic Cardiovascular Disease and Heart Failure in Type 2 Diabetes Mellitus - Mechanisms, Management, and Clinical Considerations. Circulation 2016;133:2459-2502
2. Monti LD, Barlassina C, Citterio L, Galluccio E, Berzuini C, Setola E, Valsecchi G, Lucotti P, Pozza G, Bernardinelli L, Casari G, Piatti P: Endothelial nitric oxide synthase polymorphisms are associated with type 2 diabetes and the insulin resistance syndrome. Diabetes 2003;52:1270-1275
3. Cook S, Hugli O, Egli M, Vollenweider P, Burcelin R, Nicod P, Thorens B, Scherrer U: Clustering of cardiovascular risk factors mimicking the human metabolic syndrome X in eNOS null mice. Swiss Med Wkly 2003;133:360-363
4. Carlstrom M, Larsen FJ, Nystrom T, Hezel M, Borniquel S, Weitzberg E, Lundberg JO: Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice. Proc Natl Acad Sci U S A 2010;107:17716-17720
5. Essawy S, Khaled AS, Amani E: Comparing the Effects of Inorganic Nitrate and Allopurinol in Renovascular Complications of Metabolic Syndrome in Rats: Role of Nitric Oxide and Uric Acid. Acta Endocrinol-Buch 2012;8:387-401
6. Kina-Tanada M, Sakanashi M, Tanimoto A, Kaname T, Matsuzaki T, Noguchi K, Uchida T, Nakasone J, Kozuka C, Ishida M, Kubota H, Taira Y, Totsuka Y, Kina SI, Sunakawa H, Omura J, Satoh K, Shimokawa H, Yanagihara N, Maeda S, Ohya Y, Matsushita M, Masuzaki H, Arasaki A, Tsutsui M: Long-term dietary nitrite and nitrate deficiency causes the metabolic syndrome, endothelial dysfunction and cardiovascular death in mice. Diabetologia 2017;60:1138-1151

7. Roberts LD, Ashmore T, McNally BD, Murfitt SA, Fernandez BO, Feelisch M, Lindsay R, Siervo M, Williams EA, Murray AJ, Griffin JL: Inorganic Nitrate Mimics Exercise-Stimulated Muscular Fiber-Type Switching and Myokine and gamma-Aminobutyric Acid Release. Diabetes 2017;66:674-688
8. Ashmore T, Roberts LD, Morash AJ, Kotwica AO, Finnerty J, West JA, Murfitt SA, Fernandez BO, Branco C, Cowburn AS, Clarke K, Johnson RS, Feelisch M, Griffin JL, Murray AJ: Nitrate enhances skeletal muscle fatty acid oxidation via a nitric oxide-cGMP-PPAR-mediated mechanism. BMC biology 2015;13:110
9. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B: Effects of dietary nitrate on oxygen cost during exercise. Acta physiologica 2007;191:59-66
10. Cordero-Herrera I, Kozyra M, Zhuge Z, McCann Haworth S, Moretti C, Peleli M, Caldeira-Dias M, Jahandideh A, Huirong H, Cruz JC, Kleschyov AL, Montenegro MF, Ingelman-Sundberg M, Weitzberg E, Lundberg JO, Carlstrom M: AMP-activated protein kinase activation and NADPH oxidase inhibition by inorganic nitrate and nitrite prevent liver steatosis. Proc Natl Acad Sci U S A 2019;116:217-226
11. Roberts LD, Ashmore T, Kotwica AO, Murfitt SA, Fernandez BO, Feelisch M, Murray AJ, Griffin JL: Inorganic nitrate promotes the browning of white adipose tissue through the nitrate-nitrite-nitric oxide pathway. Diabetes 2015;64:471-484
12. Roberts LD, Bostrom P, O'Sullivan JF, Schinzel RT, Lewis GD, Dejam A, Lee YK, Palma MJ, Calhoun S, Georgiadi A, Chen MH, Ramachandran VS, Larson MG, Bouchard C, Rankinen T, Souza AL, Clish CB, Wang TJ, Estall JL, Soukas AA, Cowan CA, Spiegelman BM, Gerszten RE: beta-Aminoisobutyric Acid Induces Browning of White Fat and Hepatic beta-Oxidation and Is Inversely Correlated with Cardiometabolic Risk Factors. Cell Metab 2014;19:96-108
13. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-917
14. Roberts LD, Murray AJ, Menassa D, Ashmore T, Nicholls AW, Griffin JL: The contrasting roles of PPARdelta and PPARgamma in regulating the metabolic switch between oxidation and storage of fats in white adipose tissue. Genome Biol 2011;12:R75
15. Taylor DR, Watt NT, Perera WS, Hooper NM: Assigning functions to distinct regions of the N-terminus of the prion protein that are involved in its copper-stimulated, clathrin-dependent endocytosis. J Cell Sci 2005;118:5141-5153
16. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, Wishart DS, Xia J: MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic acids research 2018;
17. Horscroft JA, O'Brien KA, Clark AD, Lindsay RT, Steel AS, Procter NEK, Devaux J, Frenneaux M, Harridge SDR, Murray AJ: Inorganic nitrate, hypoxia, and the regulation of cardiac mitochondrial respiration-probing the role of PPARalpha. FASEB J 2019;33:7563-7577
18. Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, Freeman BA, Frenneaux M, Friedman J, Kelm M, Kevil CG, Kim-Shapiro DB, Kozlov AV, Lancaster JR, Jr., Lefer DJ, McColl K, McCurry K, Patel RP, Petersson J, Rassaf T, Reutov VP, Richter-Addo GB, Schechter A, Shiva S, Tsuchiya K, van Faassen EE, Webb AJ, Zuckerbraun BS, Zweier JL, Weitzberg E: Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol 2009;5:865-869
19. Jansson EA, Huang L, Malkey R, Govoni M, Nihlen C, Olsson A, Stensdotter M, Petersson J, Holm L, Weitzberg E, Lundberg JO: A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. Nat Chem Biol 2008;4:411-417
20. Bergandi L, Silvagno F, Russo I, Riganti C, Anfossi G, Aldieri E, Ghigo D, Trovati M, Bosia A: Insulin stimulates glucose transport via nitric oxide/cyclic GMP pathway in human

vascular smooth muscle cells. Arteriosclerosis, thrombosis, and vascular biology 2003;23:2215-2221
21. Cheung KJ, Tzameli I, Pissios P, Rovira I, Gavrilova O, Ohtsubo T, Chen Z, Finkel T, Flier JS, Friedman JM: Xanthine oxidoreductase is a regulator of adipogenesis and PPAR gamma activity. Cell Metabolism 2007;5:115-128
22. Lundberg JO, Carlstrom M, Larsen FJ, Weitzberg E: Roles of dietary inorganic nitrate in cardiovascular health and disease. Cardiovasc Res 2011;89:525-532
23. Hezel MP, Liu M, Schiffer TA, Larsen FJ, Checa A, Wheelock CE, Carlstrom M, Lundberg JO, Weitzberg E: Effects of long-term dietary nitrate supplementation in mice. Redox biology 2015;5:234-242
24. Tanaka T, Nakatani K, Morioka K, Urakawa H, Maruyama N, Kitagawa N, Katsuki A, Araki-Sasaki R, Hori Y, Gabazza EC, Yano Y, Wada H, Nobori T, Sumida Y, Adachi Y: Nitric oxide stimulates glucose transport through insulin-independent GLUT4 translocation in 3T3-L1 adipocytes. European journal of endocrinology 2003;149:61-67
25. McGrowder D, Ragoobirsingh D, Brown P: Modulation of glucose uptake in adipose tissue by nitric oxide-generating compounds. Journal of biosciences 2006;31:347-354
26. Zhang X, Ji J, Yan G, Wu J, Sun X, Shen J, Jiang H, Wang H: Sildenafil promotes adipogenesis through a PKG pathway. Biochem Biophys Res Commun 2010;396:1054-1059
27. Mossenbock K, Vegiopoulos A, Rose AJ, Sijmonsma TP, Herzig S, Schafmeier T: Browning of white adipose tissue uncouples glucose uptake from insulin signaling. PLoS One 2014;9:e110428