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Nitrite augments glucose uptake in adipocytes through the Protein Kinase A-dependent stimulation of mitochondrial fusion

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Abstract

Though it is well accepted that adipose tissue is central in the regulation of glycemic homeostasis, the molecular mechanisms governing adipocyte glucose uptake remain unclear. Recent studies demonstrate that mitochondrial dynamics (fission and fusion) regulate lipid accumulation and differentiation in adipocytes. However, the role of mitochondrial dynamics in glucose homeostasis has not been explored. The nitric oxide oxidation products nitrite and nitrate are endogenous signaling molecules and dietary constituents that have recently been shown to modulate glucose metabolism, prevent weight gain and reverse the development of metabolic syndrome in mice. While the mechanism of this protection is unclear, the mitochondrion is a known subcellular target for nitrite signaling. Thus, we hypothesize that nitrite modulates mitochondrial dynamics and function to regulate glucose uptake in adipocytes. Herein, we demonstrate that nitrite significantly increases glucose uptake in differentiated murine adipocytes through a mechanism dependent on mitochondrial fusion. Specifically, nitrite promotes mitochondrial fusion by increasing pro-fusion protein mitofusin 1 while concomitantly activating protein kinase A (PKA), which phosphorylates and inhibits the pro-fission protein, dynamin-related protein 1 (Drp1). Functionally, this signaling augments cellular respiration, fatty acid oxidation, mitochondrial oxidant production and glucose uptake. Importantly, inhibition of PKA or Drp1 significantly attenuates nitrite-induced mitochondrial respiration and glucose uptake. These findings demonstrate that mitochondria play an essential metabolic role in adipocytes, a novel role for both nitrite and mitochondrial fusion in regulating adipocyte glucose homeostasis and have implications for the potential therapeutic use of nitrite and mitochondrial modulators in glycemic regulation.

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Disclosures

none

Author Contributions

NKHK and SS planned study, analyzed and interpreted data and wrote the manuscript. LM, SZ, CKP, FG-B, LL and YW collected and analyzed data.

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Keywords

adipocyte; nitrite; mitochondria; glucose uptake; fusion

Introduction

Chronic elevation of circulating glucose levels is a central component of the metabolic syndrome and type 2 diabetes and is due primarily to an inability of peripheral organs to perform glucose clearance. Though white adipose tissue has been shown to contribute significantly less to overall glucose metabolism than skeletal muscle (1), specific inhibition of adipocyte insulin signaling or glucose transport leads to glucose intolerance and disruption of insulin action in muscle and liver (2). Despite the recognition that adipose tissue plays a vital role in glucose clearance, the signaling factors and molecular events regulating glucose uptake in the adipocyte remain unclear.

Nitrate (NO_3^-), constitutively present in blood and tissues, is derived from the diet as well as the oxidation of endogenously generated nitric oxide (NO) (3,4). While once considered to be physiologically inert, it is now recognized that nitrate, through a reductive pathway, can be bioactivated *in vivo* to nitrite (NO_2^-), a more active metabolite that mediates physiological signaling either directly (5,6) or through its further reduction to NO (3,7). Notably, nitrate and nitrite have recently been associated with the reversal of symptoms of the metabolic syndrome in a murine model of NO deficiency. In endothelial NO synthase (eNOS) knockout mice, dietary nitrate supplementation improved glucose tolerance, decreased fasting blood glucose levels and significantly attenuated levels of glycosylated hemoglobin. Further, nitrate treated mice had decreased visceral fat compared to untreated controls suggesting that adipocytes may be a target for the actions of nitrate (8). While the beneficial effects in this model were associated with an increase in plasma nitrite concentration, the sub-cellular targets and mechanisms by which nitrite regulates glucose homeostasis remain unclear. Additionally, the potential role of nitrite in regulating adipocyte function is unexplored.

The mitochondrion is a well-established target of nitrite signaling as well as a regulator of adipocyte function. Nitrite modulates mitochondrial oxidative phosphorylation rates in heart (9,10) and liver (11), increases efficiency in skeletal muscle (12), stimulates mitochondrial biogenesis in hypoxic smooth muscle cells (6), and has recently been shown to induce mitochondrial fusion in cardiomyocytes (13). In the adipocyte, the efficiency of oxidative phosphorylation and the rate of fatty acid oxidation have been shown to modulate lipid accumulation (14,15) and differentiation (16,17) as well as alter reactive oxygen species (ROS) generation to affect downstream signaling (18,19). Consistent with this central role of mitochondrial function in adipocyte physiology, the induction of mitochondrial biogenesis is protective in a number of models of obesity and insulin resistance (20–22). Most recently, changes in mitochondrial dynamics (fission and fusion), resulting in altered mitochondrial tubular networks within the cell, have been described to occur in differentiating adipocytes (17). Inhibition of the fission regulatory protein, dynamin related protein-1 (Drp1), or overexpression of the fusion promoting mitofusin 2, resulting in a net increase in

mitochondrial networks, decreased triglycerol accumulation in 3T3-L1 adipocytes (23). While emerging data suggests that mitochondrial dynamics impact adipocyte function, it is unclear whether alterations in mitochondrial fission and fusion affect adipocyte glucose uptake. Further, the effect of nitrite on mitochondrial dynamics, number or function in the adipocyte has previously not been explored.

Herein, we hypothesize that nitrite modulates mitochondrial dynamics and function to positively regulate glucose homeostasis in adipocytes. We demonstrate that nitrite augments adipocyte glucose uptake through the stimulation of mitochondrial fusion and subsequent increase in mitochondrial respiration. These data suggest that nitrite-induced glucose uptake may at least partially contribute to the mechanism of nitrate-induced reversal of metabolic syndrome symptoms and their physiological, dietary and therapeutic implications will be discussed.

Materials and methods

Materials

All reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise specified.

Cell culture

3T3-L1 fibroblasts were purchased from ATCC (Rockville, MD) and maintained in DMEM (Invitrogen; Carlsbad, CA) containing 10% calf serum (Invitrogen) and 1% penicillin/streptomycin prior to initiation of the differentiation protocol. For differentiation, at 2 days post confluence, the 3T3-L1 fibroblasts were treated with differentiation cocktail (1 µg/ml insulin, 1 mM dexamethasone and 0.5 mM isobutylmethylxanthine) for 2 days in adipocyte medium (DMEM; 10% fetal bovine serum (Hyclone; Logan, UT); 1% penicillin/streptomycin). Cells were then maintained in adipocyte medium supplemented with 1 µg/ml insulin for 2 days. On day 4, the cells were cultured in adipocyte medium for a minimum of 2 additional days before treatment. At least 90% of the cell population showed the adipocyte phenotype with the accumulation of lipid droplets. Cells were grown at 37°C in 95% air and 5% CO₂. 3T3-L1 fibroblasts were used until passage 10.

Animals

eNOS^{-/-} mice were treated in accordance with regulation of the NIH and University of Pittsburgh IACUC. Mice were fed ad libitum and placed on drinking water with containing no nitrite (control) or 500 mg/L sodium nitrite for 5 days. Mice were euthanized and visceral adipose tissue was harvested.

Glucose uptake

Adipocytes were treated with sodium nitrite for 24 h. For pharmacological inhibition, adipocytes were pretreated with inhibitors for 30 min followed by 24 h nitrite treatment. During the last 4 h of the treatment period, adipocytes were serum-starved in DMEM in the presence of pharmacological inhibitors and nitrite. The adipocytes were rinsed and incubated in Krebs-Ringer HEPES buffer (121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 0.33 mM CaCl₂, and 12 mM HEPES, pH 7.4). Separate wells were treated with insulin (100

nM) which was used as a positive control. Glucose uptake was initiated by the addition of 200 μ M 2-[3 H]deoxy-d-glucose (the final concentration; 0.5 μ Ci/500 μ l). After a 10-min incubation with 2-[3 H]deoxy-d-glucose, glucose uptake was stopped by washing three times with ice-cold Krebs-Ringer HEPES buffer. The cells were lysed in 0.1 M NaOH and assayed for 2-[3 H]deoxyglucose uptake in disintegrations/min/mg of protein by liquid scintillation counting. Protein concentrations were determined using BCA kit (Pierce Chemical Co; Rockford, IL) and used to normalize the counts as specific activity. All measurements were made in duplicate and corrected for non-carrier-mediated uptake as determined in the presence of 10 μ M cytochalasin B, which was below 10% of the total uptake.

Bioenergetic measurements

Oxygen consumption rate (OCR) was measured in differentiated adipocytes using the Seahorse XF24 Extracellular Flux analyzer (Seahorse Bioscience, Billerica MA) as described previously (6). 3T3-L1 cells were plated at 12,000/well in Seahorse 24-well V7-PET and then differentiated into adipocytes. Following a 24 hour treatment with nitrite, adipocytes were rinsed with PBS, and kept in growth media for 1h. Growth media was then replaced with unbuffered media before initiating OCR readings. After 28 min of equilibration, oligomycin (2 μ M) was injected into each well, followed by FCCP (1.5 μ M) at 45 min, and rotenone (20 μ M) at 62 min. Fatty acid oxidation rates were obtained by measuring basal respiration and OCR after the addition of palmitate (100 μ M). Etomoxir (20 μ M) was added to ensure the measurement of fatty acid oxidation.

PKA activity

PKA activity was measured by ELISA using the PKA activity kit (Enzo Life Sciences; ADI-EKS-390A) as per manufacturer's instructions.

Western blot analysis

Following treatment, adipocytes were rinsed in ice cold phosphate buffer saline (PBS) and lysed in RIPA buffer containing protease cocktail inhibitor (11873580001, Roche; Indianapolis, IN), 10 mM NaF and 1 mM sodium orthovanadate. Protein determination and Western blot analysis were performed as previously described (24).

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and was reverse-transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA) as previously described (24). Gene expression was determined by quantitative RT-PCR (qPCR) using TaqMan gene expression assays-on-demand (Applied Biosystems; Foster City, CA) and normalized to 18S ribosomal RNA or actin using the comparative Ct method.

Measurement of mitochondrial superoxide

Intra-mitochondrial superoxide levels were quantified using MitoSOXTM Red (Invitrogen, Carlsbad, CA) as previously described (13). Adipocytes were incubated at 37°C with MitoSOX Red (5 μ M in PBS) and fluorescence (510/580 nm) was monitored kinetically.

using a Synergy plate reader (BioTek Instrument Inc., Winooski, VT). After the maximal rate was determined following the addition of nitrite, MitoTEMPO (Sigma-Aldrich, St. Louis, MO) was added to the adipocytes and fluorescence monitored kinetically.

Adenovirus gene transfer

Adenoviruses (Ad.Empty and Ad.MnSOD) were replication defective (25) and purchased from the University of Iowa Gene Transfer Vector Core Facility. Briefly, differentiated adipocytes were infected with Ad.MnSOD at the indicated multiplicity of infection (MOI) for 24 h after which the infection medium was removed and replaced with fresh medium for an additional 24 h. Ad.Empty (25 MOI) was used as a control for adenovirus infection.

Transmission electron microscopy

Coverslips were fixed in 2.5% glutaraldehyde in PBS for 1 hour at room temperature and post fixed in 1% OsO₄ with 1% Fe₆CN₃ for 1 h at 4°C. They were subsequently dehydrated in a graded series of alcohol (30%, 50%, 70% and 90%, 10 min each) with three changes in 100% ethanol (15 min each) and then followed in epon (3x, 1 hr each). After the last change of epon, inverted beam capsules full of resin was added and allowed to polymerize at 37° C overnight and then 48 h at 60° C. Cross sections (60 nm) were obtained on a Riechart Ultracut E microtome, poststained in 4% uranylacetate, and viewed on a Jeol JEM1011 transmission electron microscope (Jeol, Peabody, MA, USA) at 80kV. Images were taken using an AMT2K digital camera (Danvers, MA, USA). Mitochondrial length was quantified using ImageJ software as described in (26) by a technician blinded to the experimental conditions. The mean length of mitochondria in each treatment group is shown.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical analysis was performed with GraphPad Prism and the data were analyzed by 1-way or 2-way ANOVA with Tukey's multiple comparison post hoc comparisons and Student's t-test. All results are considered significant at $p < 0.05$.

Results

Nitrite induces adipocyte glucose uptake

To determine the effect of nitrite on adipocyte function, in the first series of experiments differentiated 3T3-L1 adipocytes were treated with nitrite (10–100 μ M) and glucose uptake measured 24 h later. While untreated cells showed measurable glucose uptake, nitrite significantly increased glucose uptake in a concentration dependent manner (Fig. 1A). Notably, nitrate (200 μ M) had no effect on glucose uptake. This was likely due to the inability of the adipocytes to reduce nitrate to its bioactive mediators, nitrite and NO.

Given the evidence of nitrite-stimulated increased glucose uptake in vivo and in vitro, we next sought to determine the mechanism of nitrite-mediated glucose uptake. A number of the physiological responses mediated by nitrite are due to its hypoxic reduction to NO and subsequent cGMP production (3,7). Though hypoxia was not required for nitrite-induced glucose uptake, we tested whether the effects of nitrite were due to its conversion to NO.

Treatment of 3T3-L1 differentiated adipocytes with nitrite (100 μ M) did not increase the generation of cGMP in the cells (26.3 ± 7.1 vs 24.2 ± 5.1 fmol/mg). Further, inhibition of soluble guanylate cyclase by ODQ (5 μ M) did not affect the ability of nitrite to stimulate glucose uptake (Fig. 1B). Collectively, these data demonstrate that nitrite stimulates adipocyte glucose uptake through a mechanism independent of NO formation.

Nitrite stimulates mitochondrial fusion but not mitochondrial biogenesis

Given the established role of mitochondria in adipocyte physiology, we next tested whether nitrite modulated mitochondrial number or morphology in these cells. Electron microscopy of untreated differentiated adipocytes and those treated with nitrite (100 μ M) demonstrated a clear change in mitochondrial morphology (Fig. 2A). Assessment of the mitochondrial biogenesis pathway suggested this change was not due to stimulation of mitochondrial synthesis. Measurement of the mRNA expression of key genes in the biogenesis pathway demonstrated that nitrite did not significantly increase the mRNA expression of PPAR γ co-activator 1 α (PGC1 α), the master regulator of mitochondrial biogenesis. Moreover, no change was observed in the downstream genes regulating biogenesis including mitochondrial transcription factor A (Tfam) and nuclear respiratory factor 1 (Fig. 2C). Further, the enzymatic activity of complexes I and IV in the electron transport chain as well as the matrix enzyme citrate synthase were unchanged by nitrite treatment (Fig. 2D).

Though no change in absolute mitochondrial number was observed in the cells, the cells displayed a change in morphology characterized by elongated mitochondrial networks (Fig. 2A&B) consistent with mitochondrial fusion. Semi-quantitative analysis revealed a significant increase in mitochondrial length in the nitrite treated adipocytes compared to the untreated controls (762.7 ± 60.3 vs 487.8 ± 36.3 , Fig. 2B). Mitochondrial elongation is determined by a balance of fusion and fission (27). Thus, to determine the mechanism underlying this nitrite-induced mitochondrial fusion phenotype, we next investigated the effect of nitrite on proteins that catalyze both fusion and fission. Compared to untreated cells, nitrite (10–100 μ M; 24 h) stimulated a concentration dependent increase in the expression of the pro-fusion mitofusin proteins. Specifically, mitofusin 1 showed a robust increase, while nitrite induced a slight upregulation of mitofusin 2 that did not reach statistical significance (Fig. 2E). Examination of the principal catalyst of fission, dynamin related protein-1 (Drp1), showed no change in total protein expression after nitrite treatment. However, the activity of Drp1 is predominantly regulated by post-translational modification, most significantly through the phosphorylation of serine⁶⁵⁶ which inhibits Drp1 activity, resulting in the inhibition of fission (28). Notably, nitrite stimulated the phosphorylation of Drp1 (ser⁶⁵⁶) in a concentration dependent manner (Fig. 2F). Collectively, these data suggest nitrite simultaneously inhibits Drp1-catalyzed fission and stimulates mitofusin-dependent fusion to enhance elongated mitochondrial networks in adipocytes.

Nitrite induced mitochondrial fusion is dependent on PKA activation

Phosphorylation of Drp1 is mediated predominantly by protein kinase A (PKA) (28). To determine whether nitrite treatment activates PKA resulting in Drp1 phosphorylation, adipocytes were treated with nitrite. The treatment of nitrite, but not nitrate, significantly increased PKA activity dose-dependently (50 and 100 μ M) for 24 h (Fig. 3A) and as early as

1 and 3 h (Fig. 3B). Further, in the presence of the PKA inhibitor (PKI), nitrite was unable to increase PKA activity (Fig. 3C) or mediate phosphorylation of Drp1 (Fig. 3D). Pharmacologic inhibition of PKA also attenuated nitrite-induced upregulation of mitofusin 1 (Fig. 3D). To determine whether this PKA-induced increase in fusion was relevant in an *in vivo* model, eNOS knockout mice were administered high nitrite in the drinking water (500mg/L) for 5 days and PKA and fusion signaling were measured. Mice drinking nitrite showed a significant increase in plasma nitrite levels (2.4 ± 0.3 vs. $0.21 \pm 0.08 \mu\text{M}$ in controls) along with significant increases in Mfn-1 expression, phosphorylation of Drp-1 and PKA activity (Supplemental Fig 1). In aggregate, these data are consistent with nitrite mediated activation of PKA, which ultimately inhibits fission and increases fusion in adipocytes.

Nitrite augments mitochondrial function

Mitochondrial fusion is known to regulate bioenergetic function (29–31) and changes in mitochondrial function can modulate adipocyte function. Thus, we next tested whether nitrite altered adipocyte mitochondrial function. To assess bioenergetic function, intact differentiated 3T3-L1 adipocytes treated with or without nitrite (0–100 μM) were subjected to extracellular flux analysis. A bioenergetic profile was generated in which basal respiration rate was measured followed by the rate of non-ATP linked respiration (induced by the addition of oligomycin, and inhibitor of complex V; 2 μM) and then the maximal uncoupled respiratory rate (induced by FCCP, an uncoupler of oxidative phosphorylation; 1.5 μM). The rate of non-mitochondrial oxygen consumption was also assessed by inhibiting the electron transport chain with rotenone (a complex I inhibitor; Fig. 4A). While no significant change was observed in proton leak, nitrite induced a concentration dependent increase in both basal and maximal respiratory rates. Notably, nitrate (200 μM) had no effect on any respiratory parameter measured (Fig. 4B). Measurement of cellular oxygen consumption in the presence of palmitate demonstrated that nitrite-treated adipocytes showed a small, though not statistically significant, increase in the rate of fatty acid β -oxidation which was inhibited by the CPT-1 inhibitor etomoxir (Fig. 4C).

Alteration in mitochondrial electron transport chain function can induce mitochondrial reactive oxygen species (ROS) formation, which have been demonstrated to mediate downstream cellular signaling (32,33). Measurement of mitochondrial superoxide formation in differentiated adipocytes showed that nitrite treatment (100 μM) significantly increased mitochondrial superoxide generation (Fig. 4D). Adenoviral transduction of MnSOD concentration-dependently increased MnSOD protein expression (Fig. 4E) and attenuated the nitrite-dependent increase in mitoSox signal, confirming the generation of superoxide (Fig. 4F). Additionally, the mitochondrially targeted antioxidant mitoTEMPO (20 μM) blunted the nitrite-induced mitoSox signal (Fig. 4F).

Mitochondrial fusion is required for nitrite-mediated glucose uptake

We next determined whether PKA activation and Drp1 phosphorylation was required for nitrite induced changes in mitochondrial function and improved glucose uptake. Treatment of adipocytes with PKI (10 μM) or mdivi-1 (10 μM), a Drp1 inhibitor, prevented nitrite-induced augmentation of mitochondrial respiration (Fig. 5A, B). The effect of PKI and

mdivi-1 on nitrite stimulated glucose uptake was next assessed. Consistent with the requirement for PKA activity and the ability to inhibit fusion, nitrite was unable to stimulate glucose uptake in the presence of PKI or mdivi-1 (Fig. 5C). In aggregate, these data demonstrate that nitrite-induced PKA activation results in mitochondrial fusion and stimulation of respiration which is required for augmented glucose uptake in adipocytes.

Discussion

Though it is recognized that white adipose tissue plays an essential role in glucose clearance, the physiological mediators and mechanisms that regulate adipocyte glucose uptake remain unclear. The current study demonstrates that the intrinsic signaling molecule nitrite stimulates adipocyte glucose uptake. Mechanistically, this augmented uptake is dependent on the activation of PKA leading to the inhibition of mitochondrial fission and the promotion of mitochondrial fusion (**graphical abstract**). These data demonstrate a novel role for nitrite as a regulator of adipocyte function and represent a potential mechanism underlying the beneficial effects of nitrate in metabolic syndrome previously reported (8). Additionally, these data expand the role of the mitochondrion in regulating adipocyte function as they suggest that mitochondrial dynamics regulate glucose uptake.

Accumulating evidence suggests that nitrite signaling mediates cardiovascular and metabolic protection in obese pre-diabetic and diabetic models (8,34). Most recently, Carlström and colleagues showed that dietary supplementation of nitrate, through its reduction to nitrite (and likely further reduction to NO), decreased visceral fat accumulation and improved glucose tolerance in a murine model of metabolic syndrome (8). Despite these studies, the effect of nitrite on adipocyte biology is unknown. The data presented herein reveal that nitrite regulates adipocyte function, and to our knowledge, are the first to directly demonstrate that nitrite regulates glucose uptake in peripheral organ cells. Prior studies have established that white adipose tissue is a central regulator of glucose homeostasis. For example, specific ablation of glucose transporter 4 (GLUT4) in adipocytes significantly impairs skeletal muscle insulin sensitivity in mice (2,35). Likewise, chronic increases in adipocyte glucose uptake improve whole body insulin sensitivity (36–38). Thus, it is possible that the ~50% increase in glucose uptake observed in nitrite-treated adipocytes in this study represents a mechanism underlying the nitrite-dependent beneficial regulation of glucose homeostasis in metabolic syndrome previously described.

While once considered to be physiologically inert, it is now recognized that nitrite can react with a number of enzymes in vivo, typically in hypoxia, to be bioactivated to NO (3,7). Once generated, NO can activate soluble guanylate cyclase to generate cGMP and mediate downstream responses (39). Prior studies suggest the effects of nitrite in obese models are at least partially dependent on this canonical nitrite-NO-cGMP signaling (8,34,40). However, the current study provides evidence that the effect of nitrite in adipocytes is independent of cGMP production and instead reliant on the activation of the cAMP-dependent kinase PKA. The molecular mechanism by which nitrite activates PKA is not entirely clear, but ongoing studies are focusing on nitrite-dependent post-translational modification of adenylate cyclase and phosphodiesterase activities, which regulate cAMP levels and hence PKA activation. Notably, PKA is a known regulator of adipocyte metabolism, thermogenesis and lipolysis

(41,42). Adipose tissue PKA expression is decreased in obese individuals and activation of PKA in adipose tissue has been shown to diminish weight gain in ob/ob mice and high fat diet models (43–45). Thus, while the current study focused on the short term (24 h) effects of nitrite, it is interesting to speculate that nitrite may also regulate lipid accumulation and body weight through the activation of PKA.

Independent of the effects of nitrite, the data present here establish a role for mitochondrial dynamics in regulating adipocyte glucose homeostasis. A number of studies provide evidence that decreased adipose tissue mitochondrial number is apparent with the onset of obesity (21). Further, declining oxidative phosphorylation and fatty acid oxidation are key pathogenic features in obesity-induced glucose intolerance and insulin resistance (30,46). It is now recognized that mitochondrial dynamics, fission and fusion events leading to the modulation of mitochondrial networks, represents an essential rapid regulatory mechanism of mitochondrial function (47). While fusion, known to preserve and enhance mitochondrial function, has been associated with augmented glucose uptake in hepatocytes (48) and skeletal muscle (49), its role in adipocyte biology has thus far been limited to regulating differentiation and preventing lipid accumulation (23). The finding that mitochondrial fusion enhances adipocyte glucose uptake, oxidative phosphorylation and fatty acid oxidation suggests that a pro-fusion phenotype promotes adipocyte health on many levels and may represent a therapeutic target.

Elucidation of the exact mechanism by which mitochondrial fusion increases glucose uptake in the adipocyte is ongoing. It is likely that mitochondrial ROS act on several downstream targets. AMP Kinase, a central metabolic sensor that is widely accepted to regulate glucose homeostasis, is one such target. Traditionally, AMPK is activated through alterations in the balance of cyclic nucleotides, with increases in AMP leading to activation of the protein. Alternatively, low levels of hydrogen peroxide, including those derived from the mitochondrion, are known to oxidize AMPK leading to its subsequent auto-phosphorylation and activation (32,50). Recent work by our group demonstrates in cardiomyocytes that nitrite-mediated fusion increases mitochondrial ROS generation leading to AMPK oxidation and subsequent activation (13). Indeed, nitrite-dependent mitochondrial fusion induced AMPK oxidation is potentially operational in the adipocyte. However, we cannot exclude the involvement of downstream protein targets beyond AMPK, such as the activation of MAP signaling or direct effects on glucose transporters.

It is interesting to consider the role of nitrite as both a physiological regulator of adipocyte function as well as a therapeutic. Nitrite concentration in peripheral tissue is reported to be 1–10 μM (5). However, this concentration may increase significantly in inflammatory conditions or after exercise (51). For example, a greater than five-fold increase in adipocyte nitrite concentration has been reported after cytokine stimulation of iNOS (52). In these chronic inflammatory conditions, tissue nitrite concentration may approach the levels used in this study (50–100 μM). On a dietary and therapeutic level, prior studies have speculated that nitrite is a key component in the Mediterranean diet (53) which lowers the risk of cardiovascular incidence (54). In one study, the switch of overweight subjects at high risk for heart to a Mediterranean diet decreased the incidence of heart attacks, strokes and deaths by 30% without inducing weight loss (54). This type of data suggests that improved

adipocyte function may account for some of the beneficial effects of this diet. While mitochondrial dynamics have not been measured in subjects on a Mediterranean or high nitrate/nitrite diet, the current study suggests that pursuing this avenue may provide insight into dietary management of obesity and metabolic syndrome.

In conclusion, nitrite treatment stimulated the phosphorylation of PKA and Drp-1, indicative of a pro-mitochondrial fusion phenotype. This nitrite-induced signaling pathway increased mitochondrial respiration and glucose uptake in cultured adipocytes. These findings suggest that nitrite-mediated mitochondrial fusion may play a role in the regulation of glucose homeostasis and improved mitochondrial function in adipocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Mitochondrial dynamics play an essential metabolic role in adipocytes

Nitrite promotes mitochondrial fusion by increasing pro-fusion protein mitofusin 1

Nitrite stimulates phosphorylation (and inhibition) of the pro-fission protein, DRP1

Nitrite increases PKA activity and generates superoxide

Inhibition of PKA or Drp1 blunts nitrite-induced respiration and glucose uptake

Fig. 1. Nitrite induces glucose uptake in cultured adipocytes

A: 3T3-L1 adipocytes were treated with NO_2^- (0–100 μM) or NO_3^- (200 μM) for 24 h. **B:** 3T3-L1 adipocytes were pretreated with ODQ (5 μM), an inhibitor of soluble guanylate cyclase, for 30 min followed by 24 h incubation with NO_2^- (100 μM) and then 2-Deoxy- $[\text{}^3\text{H}]$ glucose uptake was measured. Values represent means $\pm$ SEM; $n = 5\text{--}8$. * $p < 0.05$ compared with cells not treated with NO_2^- .

Fig. 2. Nitrite stimulates mitochondrial fusion but not mitochondrial biogenesis

A: Representative electron micrographs of 3T3-L1 adipocytes treated with and without NO_2^- (100 μM , 24 h). Mitochondria are denoted by black arrows. Magnification is 25000x. **B:** Semi-quantitative analysis of mitochondrial length from the electron micrographs were determined as described in *Materials and methods*. **C:** mRNA expression of respective mitochondrial biogenesis markers were analyzed in 3T3-L1 adipocytes incubated with NO_2^- (100 μM) for 6, 12 and 24 h. **D:** Enzymatic activity of complex I, IV and citrate synthase in 3T3-L1 adipocytes were determined following treatment with NO_2^- (100 μM , 24 h). **E:** Representative Western blot of Mfn 1 (top) and densitometric analysis (bottom) to β -actin. **F:** Representative Western blot (top) of Drp-1 phosphorylation and densitometric quantification (bottom) of total Drp-1. Values represent means $\pm$ SEM; n = 5–10. * p < 0.05 compared with cells not treated with NO_2^- .

Fig. 3. Nitrite stimulates Drp-1 phosphorylation and increases Mfn1 in a PKA-dependent manner

A–C: PKA activity was determined in 3T3-L1 adipocytes following treatment with: NO₂⁻ (50 and 100 μM) or NO₃⁻ (200 μM) for 24 h (**A**), 50 μM NO₂⁻ for 1 and 3 h (**B**) and PKI for 30 min (pre-treatment) and then incubation with NO₂⁻ (100 μM) for 24 h. **D:** Representative Western blot (top) and densitometric analysis (bottom) for P-Drp1 and Mfn-1 of adipocyte lysate from experimental design in Panel 3B. Values represent means ± SEM; n = 3–8. *p < 0.05 compared with cells not treated with NO₂⁻.

Fig. 4. Nitrite increases adipocyte mitochondrial respiration

A: 3T3-L1 adipocytes were treated with NO_2^- (0–100 μM) or NO_3^- (200 μM) for 24 h. Representative bioenergetic profile of 3T3-L1 adipocytes were generated using the Seahorse XF24 Analyzer. **B:** Composite of all bioenergetic experiments showing baseline and maximal respiration. **C:** 3T3-L1 adipocytes were treated with NO_2^- (100 μM , 24 h). Fatty acid oxidation was measured in the presence of 100 μM palmitate with and without 20 μM Etomoxir, to ensure the measurement of fatty acid oxidation. **D:** The MitoSOX fluorescence rate was determined in 3T3-L1 adipocytes loaded with 5 μM mitoSOX for 30 min and then treated with NO_2^- (50 or 100 μM). **E:** 3T3-L1 adipocytes were infected with 5 and 25 MOI Ad.MnSOD as described in *Materials and methods* and lysate collected at 48 h. Representative Western blot of MnSOD and GAPDH (top) and densitometric quantification (bottom). **F:** MitoSOX fluorescence rate determined in Ad.MnSOD (and Ad.Empty)-infected 3T3-L1 adipocytes loaded with 5 μM mitoSOX for 30 min and then treated with NO_2^- (100 μM). After initial rate was determined

following addition of nitrite, mitoTempo (20 μ M) was added. Values represent means $\pm$ SEM; n = 4–12. *p< 0.05 compared with cells not treated with NO₂⁻.

Fig. 5. Nitrite-induced mitochondrial respiration and glucose uptake is dependent on mitochondrial fusion signaling

A: 3T3-L1 adipocytes were pretreated with PKI (10 μ M) for 30 min followed by NO₂⁻ (100 μ M) for 24 h. Representative bioenergetic profile of 3T3-L1 adipocytes (left) and quantification of baseline and maximal respiration of all bioenergetic experiments (right). **B:** 3T3-L1 adipocytes were pretreated with mdivi-1 (10 μ M), a Drp1 inhibitor, for 30 min followed by NO₂⁻ (100 μ M) for 24 h. Representative bioenergetic profile of 3T3-L1 adipocytes (left) and quantification of baseline and maximal respiration of all bioenergetic experiments (right). **C:** 2-Deoxy-[³H]glucose uptake was measured in 3T3-L1 adipocytes pretreated with PKI (10 μ M) or mdivi-1 (10 μ M) for 30 min followed by NO₂⁻ (100 μ M; 24 h). Values represent means $\pm$ SEM; n = 6–11. *p < 0.05 compared with cells not treated with NO₂⁻.