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Review

Modulation of mitochondria and NADPH oxidase function by the nitrate-nitrite-NO pathway in metabolic disease with focus on type 2 diabetes

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ABSTRACT

Mitochondria play fundamental role in maintaining cellular metabolic homeostasis, and metabolic disorders including type 2 diabetes (T2D) have been associated with mitochondrial dysfunction. Pathophysiological mechanisms are coupled to increased production of reactive oxygen species and oxidative stress, together with reduced bioactivity/signaling of nitric oxide (NO). Novel strategies restoring these abnormalities may have therapeutic potential in order to prevent or even treat T2D and associated cardiovascular and renal co-morbidities. A diet rich in green leafy vegetables, which contains high concentrations of inorganic nitrate, has been shown to reduce the risk of T2D. To this regard research has shown that in addition to the classical NO synthase (NOS) dependent pathway, nitrate from our diet can work as an alternative precursor for NO and other bioactive nitrogen oxide species via serial reductions of nitrate (i.e. nitrate-nitrite-NO pathway). This non-conventional pathway may act as an efficient back-up system during various pathological conditions when the endogenous NOS system is compromised (e.g. acidemia, hypoxia, ischemia, aging, oxidative stress). A number of experimental studies have demonstrated protective effects of nitrate supplementation in models of obesity, metabolic syndrome and T2D. Recently, attention has been directed towards the effects of nitrate/nitrite on mitochondrial functions including beiging/browning of white adipose tissue, PGC-1α and SIRT3 dependent AMPK activation, GLUT4 translocation and mitochondrial fusion-dependent improvements in glucose homeostasis, as well as dampening of NADPH oxidase activity. In this review, we examine recent research related to the effects of bioactive nitrogen oxide species on mitochondrial function with emphasis on T2D.

1. Introduction

Metabolic disorders including type 2 diabetes (T2D) are progressively increasing worldwide and have reached epidemic proportions in many countries. The risk of developing T2D is strongly associated with overweight and obesity, which are closely coupled to sedentary lifestyle and unhealthy dietary habits [1]. In 2016, WHO estimated T2D to be the seventh leading cause of death worldwide. Extensive research efforts have been made to understand the underlying pathophysiological mechanisms contributing to the initiation of metabolic abnormalities (including abdominal obesity, insulin resistance, hypertension, and hyperlipidemia) and the progression to T2D, as well as adverse vascular complications associated with the disease. Mitochondria play a fundamental role in maintaining cellular metabolic homeostasis, and T2D has been coupled with mitochondrial dysfunction, increased production of reactive oxygen species and oxidative stress, together with reduced bioactivity of the gaseous signaling molecule nitric oxide (NO) [2,3]. Novel nutritional or pharmacological approaches that restore these abnormalities may have therapeutic potential in prevention or treatment of T2D [4]. In addition to the NO generated from L-arginine by NO synthase (NOS) activity, an alternative nitrate-nitrite-NO pathway exists which can be boosted by our everyday diet (Fig. 1). Here we review the latest research progress of nitrate-nitrite-NO dependent signaling with regard to mitochondrial function in pathologies associated with metabolic disease and specifically focus on T2D.

2. Link between dietary approaches and the nitrate-nitrite-NO pathway

Considering the important link between changed dietary habits and development of obesity and T2D, extensive research has been carried out focusing on what type of diet that should be promoted in addition to the obvious strategy of lowering total caloric intake [1]. Based on meta-analysis of prospective cohort studies on T2D, protective effects have

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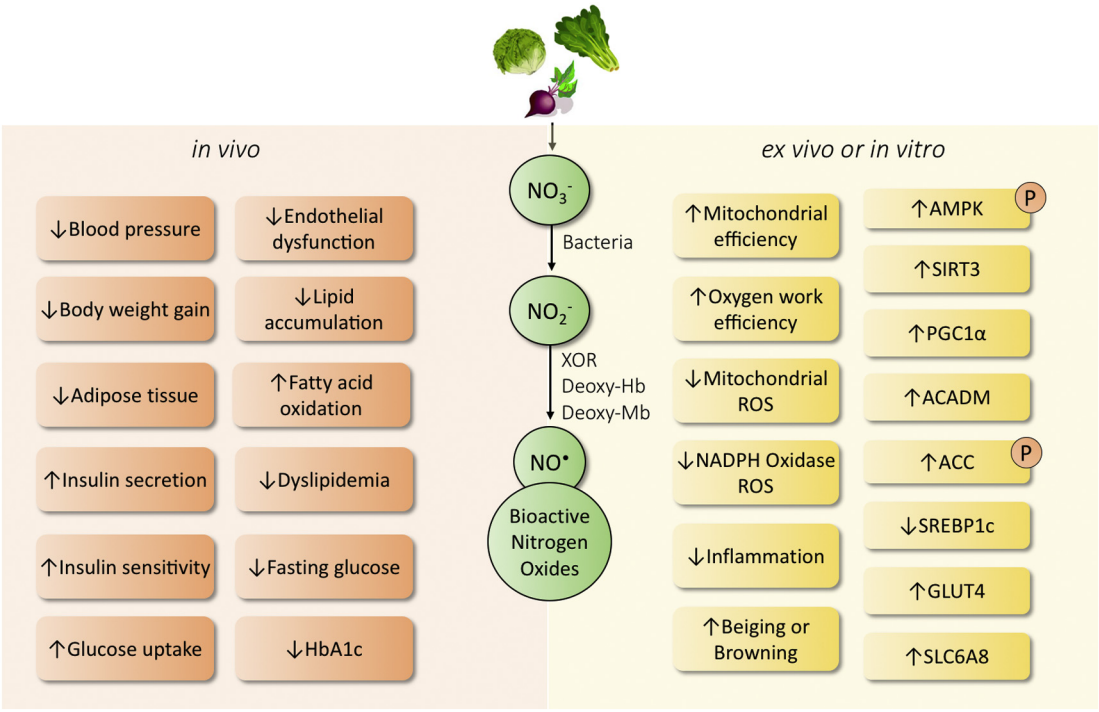

Fig. 1. Summary of various effects on cardiovascular and metabolic function/signaling of dietary nitrate facilitated by the nitrate-nitrite-NO pathway in vivo, ex vivo or in vitro. Nitrate reductase containing commensal bacteria in the oral cavity, and potentially also in the gut, reduce nitrate into nitrite which is further reduced to nitric oxide and other bioactive nitrogen species via several non-enzymatic and enzymatic mechanisms.

been associated with a diet rich in green leafy vegetables [5]. However, trials with single nutrients such as fibers, vitamins and minerals have mostly been negative. Green leafy vegetables contain high concentrations of nitrate and after it became apparent that nitrate can be endogenously reduced to bioactive NO in the 1990s [6–8], the interest for this anion has increased tremendously.

Beneficial effects of dietary nitrate have since then been identified in several pathological conditions related to blood pressure regulation, ischemia reperfusion injury and platelet aggregation [9–12]. As recently reviewed [4], accumulating studies during the last decade have also demonstrated favorable metabolic effects of inorganic nitrate in different models of T2D, [13–22], which together emphasizes potential benefits in patients with metabolic syndrome and T2D patients. However, lack of beneficial effects of dietary nitrate on metabolic disease-associated alterations has also been demonstrated [23]. Although more efforts are needed to explain some of the controversies between experimental studies, it will be even more important to investigate the potential therapeutic metabolic effects of dietary nitrate in human trials.

In vivo, nitrate undergoes reduction to nitrite via commensal bacteria [24–27] that possess effective nitrate reductase activity, which mammalian cells are not thought to do [28]. Nitrite can then be further reduced to NO and other bioactive nitrogen oxides via both non-enzymatic mechanisms (e.g. acidic and hypoxic conditions) and by several different proteins such as hemoglobin, myoglobin, neuroglobin, xanthine oxidoreductase and complexes in the mitochondrial respiratory chain [28,29] (Fig. 1). In addition to classical NO-sGC-cGMP signaling, effects following stimulation of the nitrate-nitrite-NO pathway have also been associated with PKA activation [30] and H_2O_2 dependent PKG oxidation [31].

It is well known that NO synthase (NOS)-derived NO interacts with mitochondrial function, biogenesis and redox state of the cells [32–34] (Fig. 2). NO has been implicated in mitochondrial biogenesis (i.e. increased mitochondrial mass/content in the cells) via guanylate cyclase activation, generation of cyclic GMP and PGC-1α activation [35,36] (Fig. 2). Downregulation of genes related to mitochondrial biogenesis and oxidative phosphorylation have been observed in T2D [37] (discussed below in Sections 3.1 and 3.2).

The most well-known effect of NO on mitochondria is its direct interaction with cytochrome *c* oxidase to inhibit respiration, a binding that occurs at nM levels of NO in competition with oxygen [32]. Today we know that NO and other reactive nitrogen species generated from the nitrate-nitrite-NO pathway also target mitochondria via other mechanisms. Larsen and colleagues demonstrated, for the first time, that nitrate administration in humans improves mitochondrial efficiency in skeletal mitochondria [38]. In addition, as mentioned above NO inhibits cytochrome *c* oxidase, the terminal enzyme in the electron transport system which may have effects on mitochondrial efficiency [39] (Fig. 2), oxygen homeostasis and ROS production in vivo [40]. Nitrite dependent reversible S-nitrosation of complex-I that dynamically attenuates mitochondrial ROS generation has been shown to confer protection in ischemia/reperfusion (I/R) injury in several organs (Fig. 2) [41,42] and offers protection against Parkinson's disease in experimental animal models [43]. Moreover, nitrite and NO can react with non-heme iron to form dinitrosyl iron complexes (DNIC) that may be involved in cytoprotective effects after I/R injury [44]. Furthermore, DNICs may have implications in the cytotoxic effects observed at high NO concentrations [44]. In a recent study, it was also demonstrated that dietary nitrate increased nitrosyl-Hb in blood and tissue and levels of DNIC in a mouse model of obesity and T2D, which was associated with reduction of oxidative stress and favorable metabolic effects [45]. Recently, a new hypothesis has been proposed with regard to NO signaling where instead of freely diffusing NO, mobile/exchangeable NO-ferroheme species may activate sGC in a more efficient and coordinated way [46]. However, caution is needed as increased oxidative stress in combination with increased NO levels may increase the formation of peroxynitrite [47], which in vivo has been implicated in the mechanisms underlying pathogenesis of conditions such as diabetes, heart disease, chronic inflammatory diseases, and cancer [48]. In a randomized, double-blinded, crossover study in well-trained male subjects,

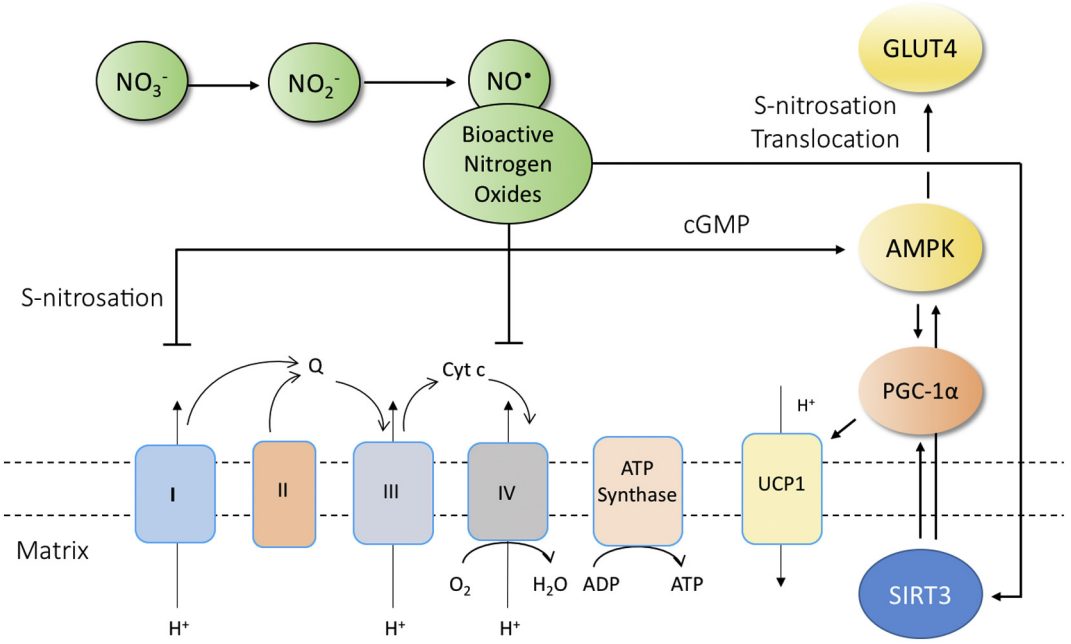

Fig. 2. Effects of Nitrate-nitrite-NO pathway on mitochondria. Nitric oxide is a potent complex IV inhibitor which can affect oxygen homeostasis, ROS production and mitochondrial efficiency. Reversible nitrite dependent S-nitrosation of complex I contributes to dynamically regulated ROS production, suggested to be protective in ischemia/reperfusion. Dietary nitrate contributes to browning/beiging of white adipose tissue, which at least in part involves cGMP-dependent activation of AMPK and upregulation of PGC-1α and UCP1. Moreover, nitrate activates SIRT3 which promotes AMPK activation and S-nitrosation followed by membrane translocation of GLUT4 and mitochondrial biogenesis via PGC-1α.

Gholami and colleagues reported increased circulatory peroxynitrite only after administration of high dose of nitrate (24 mmol) in combination with high intensity exercise [47]. However, since nitrate-nitrite-NO pathway may attenuate iNOS activity and NADPH oxidase-derived ROS (discussed below, Sections 4 and 5), the risk of increased peroxynitrite formation may be limited. In LPS-activated macrophages, nitrite treatment actually reduced peroxynitrite formation [49].

In conclusion, the underlying mechanisms contributing to the salutary metabolic effects of dietary nitrate supplementation are still not fully understood but involve interaction with mitochondria and AMPK signaling as well as modulation of oxidative stress.

3. Mitochondrial targets by the nitrate-nitrite-NO pathway

The sections below focus on metabolic effects of the nitrate-nitrite-NO pathway and specifically highlight connections to browning, activation of SIRT3, AMPK and GLUT4, modulation of mitochondrial dynamics.

3.1. Induction of browning

Adipocytes are present in three phenotypically different types, namely white, brown and beige/brite [50]. White adipose tissue (WAT) stores energy as lipid and contain droplets of triglycerides and few mitochondria due to the low energy demand [51]. Brown adipose tissue (BAT) contains lipid droplets that are tightly associated with a dense mitochondrial network [52,53], capable of burning a large amount of fatty acids [53]. Apart from brown fat recruitment during prolonged cold exposure, white adipocytes containing UCP1-expressing mitochondria and multilocular fat droplets appear, a process referred to as browning of the WAT depots. Brown-like adipocytes in WAT can arise via trans-differentiation of differentiated white adipocytes or through the development of distinct subpopulations [54]. Brown-like adipocytes have been named brite (brown-in-white) or beige adipocytes. It has been proposed that brite fat cells may originate from *de novo* differentiation of precursor cells [55,56] or develop through the bi-directional interconversion between brite and white adipocyte phenotypes [57–59]. BAT and brite cells share many molecular similarities, although there is a differential expression of certain genes including metabolic proteins (e.g., Slc27a1), inflammatory proteins (e.g., CD40 and CD137) and transcription factors (Tbx15 and Zic1) [56,60]. BAT located in the neck of adult humans contains a mixture of brite and classical brown cells [61,62].

The high levels of uncoupling protein-1 (UCP-1) present in BAT contribute to proton leak across the inner mitochondrial membrane thereby generating heat [63]. Although present in small amounts in adult humans, approximately 50 g of BAT is capable of burning up to 20% of basal caloric requirements [64]. BAT is reduced in obese individuals [65]. Browning of WAT is demonstrated to have anti-diabetic effects [66,67]. Basal UCP-1 expression level in brite cells is low, but they have an intrinsic capacity to substantially increase the expression of BAT associated genes. Numerous activators have been identified that can contribute to the browning response such as, cardiac natriuretic peptides [68], irisin [69], β-aminoisobutyric acid [70] and fibroblast growth factor 21 [71]. Moreover, NO is involved in the regulation of mitochondrial biogenesis [35], and hence supplementation with inorganic nitrate to boost NO production has emerged as a potential approach to induce browning (Fig. 3). Following 18-days of nitrate administration via the drinking water in rats, the expression of UCP-1 and PGC-1α were increased in a dose dependent manner with a concomitant increase in basal cell respiration [72]. Nitric oxide synthase (NOS) inhibition with L-NAME did not alter the effect whereas knock down of xanthine oxidoreductase (XOR) abrogated the effect. Thus, XOR was required for the induction of BAT-specific proteins [72]. The effect correlated with increased cGMP levels whereas the effect was abolished upon guanylate cyclase inhibition with ODQ. In addition, downstream signaling was investigated via protein kinase G (PKG) inhibition by KT5823 which abrogated the nitrate-induced expression of BAT-specific genes [72]. In accordance with this study, Peleli and colleagues demonstrated increased expression of UCP1 in nitrite-treated primary mouse adipocytes exposed to high palmitate concentrations [73]. In addition, chronic supplementation with nitrate attenuated

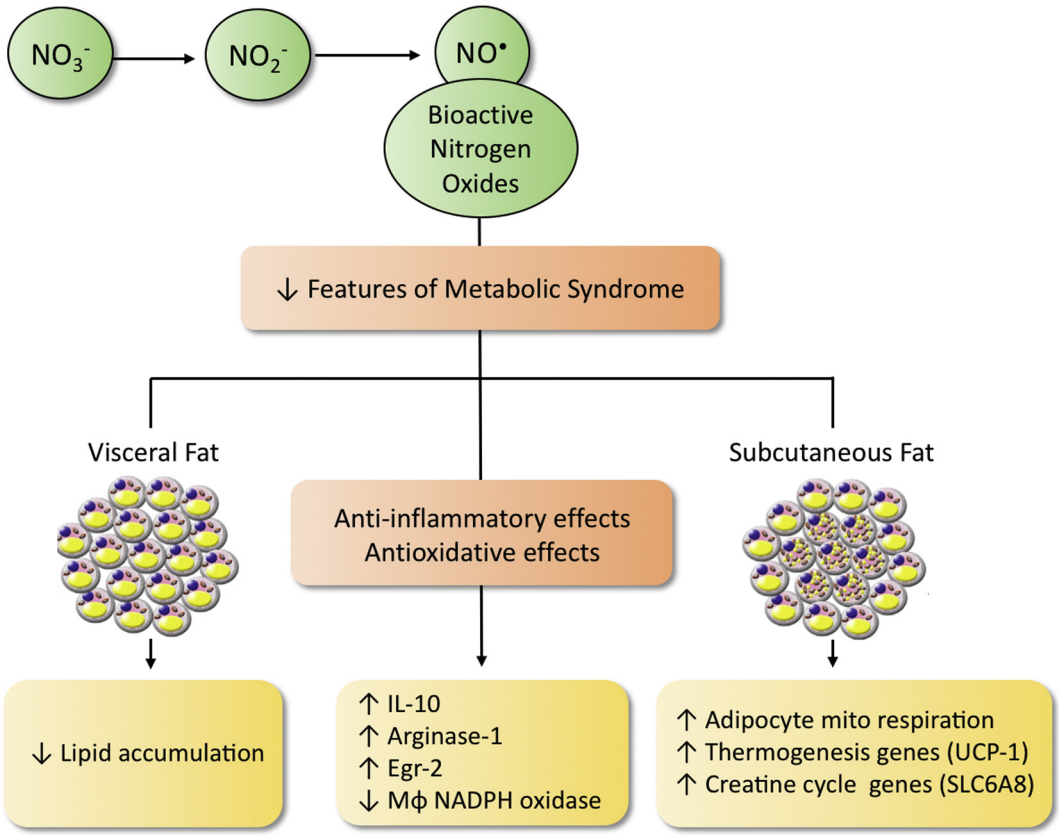

Fig. 3. Effects of Nitrate-nitrite-NO pathway on fat phenotype in metabolic syndrome. Dietary nitrate has been shown to reverse many features of metabolic syndrome (e.g. lowering blood pressure, restore glucose/insulin homeostasis and reduce fat accumulation). Recent studies show that nitrate-mediated reduction of lipid accumulation in models of high-fat diet induced metabolic dysfunction is coupled with anti-inflammatory and antioxidative effects. Moreover, boosting of the nitrate-nitrite-NO pathway was associated with increased mitochondrial respiration in primary mouse subcutaneous adipocytes and upregulation of thermogenic genes and creatine cycle genes.

body weight gain, accumulation of fat, reduced fasting glucose and improved glucose and insulin tolerance in mice chronically fed with high fat diet. Interestingly, nitrite also increased expression of the sodium- and chloride-dependent creatine transporter 1 (Slc6a8) in adipocytes exposed to palmitate [73]. The creatine phosphate cycle has recently been connected to mitochondrial uncoupling independent thermogenesis [74] where ablation of Slc6a8 is shown to impair thermogenesis and induces obesity [75].

In conclusion, XOR dependent nitrate-nitrite reduction to NO leads to a cGMP-dependent induction of BAT-specific genes, possibly contributing to antidiabetic effects (Fig. 3).

3.2. Activation of SIRT3, AMPK and GLUT4

SIRT3 is a mitochondria-localized member of the sirtuin family of lysine deacetylases [76] known to be involved in suppression of ROS and stimulation of mitochondrial biogenesis [77,78]. More than 20% of mitochondrial proteins are regulated by lysine acetylation [79]. SIRT3 deficiency has been linked to the development of metabolic syndrome [80] and pulmonary arterial hypertension [81]. Moreover, SIRT3 expression is shown to be markedly decreased in islets from patients with T2D [82].

AMPK is an attractive target for T2D as its activation improves glucose homoeostasis and insulin sensitivity. Numerous animal models demonstrate decreased AMPK activity in muscle [83] also confirmed in human skeletal muscle [84] and in adipose tissue [85].

GLUT4 is responsible for facilitating glucose transport into the cells in response to insulin and considered a vital regulator of entire body glucose homeostasis [86]. Hyperglycemia and oxidative stress can disturb the homeostatic functioning of endoplasmic reticulum that are involved in synthesis, folding, packaging and transport of proteins which results in ER stress [87]. A number of studies failed to detect a difference in GLUT4 expression in skeletal muscle homogenates with a mixture of insulin-sensitive slow-twitch fibers (type 1) contraction-sensitive fast-twitch fibers (type 2) among T2D patients [88–91]. However, when separating type 1 and type 2 muscle fibers, Gaster and colleagues observed a type 1 muscle fiber specific reduction in GLUT4 expression in T2D patients [92]. In addition, hyperglycemia was inversely related to GLUT4 contribution from type 1 fibers and directly related to GLUT4 contribution from type 2 fibers [92]. Numerous studies have also reported impaired insulin and contraction dependent GLUT4 translocation in T2D patients [93–95].

Exercise training and caloric restriction increases SIRT3 expression whereas high-fat diet is shown to reduce it [96,97]. SIRT3 is activated by NAD^+ and thereby closely connected to cellular energy status [76]. SIRT3 is also connected to mitochondrial dynamics through activation of optic atrophy 1 (OPA1) proteins [98]. Recently, SIRT3 has also been shown to activate AMPK [97,99], that is involved in membrane translocation of GLUT4 [100] (Fig. 2). Moreover, SIRT3 promotes mitochondrial biogenesis through PGC-1α activation, facilitate DNA repair via deacetylation of 8-oxoguanine DNA glycosylase 1 and attenuate mitochondrial fragmentation (i.e. result of increased fission activity) via ablation of translocation of DRP1 via deacetylation of Ku70 [101]. In addition, over expression of SIRT3 enhance anti-oxidative capacity via Nrf-2 activation and deacetylation of SOD2 [101,102]. SIRT3 activation has also been implicated in the protection against advanced glycation end products accumulation in nucleus pulposus leading to cell apoptosis, this via AMPK-PGC-1α signaling [103].

Knock down of SIRT3 resulted in lowered insulin secretion which correlated with increased ROS production and IL1β [82]. On the contrary, overexpression of SIRT3 improves whole body glucose homeostasis in mice [104]. Lai and colleagues demonstrated that nitrate and nitrite administration counteracted hyperglycemia in obese ZSF1 rats independent of changes in body weight [15]. The effect was lost in SIRT3-deficient human skeletal muscle cells and high fat diet fed SIRT3 KO mice. The nitrate/nitrite effect was not dependent on SIRT3 levels but rather the level of SIRT3 activation recognized by the short 28 kDa active form of SIRT3. In support, SIRT3 mRNA levels and upstream PGC-1α levels were unchanged [15]. This was paralleled with a reduction in mitochondrial protein acetylation. The effect was a result of nitrite dependent AMPK activation and GLUT4 membrane translocation but was insulin and NO-independent. This conclusion is in somewhat contrast with earlier findings by Bryan and colleagues who demonstrated that nitrite-mediated translocation of GLUT4, in two different cell types, was linked to NO-mediated nitrosation [17]. However, the phosphorylation level of the AMPK upstream LKB1 and CaMKII was unchanged further supporting SIRT3 dependent AMPK activation. Nitrite dependent activation of SIRT3 was proposed to be ROS dependent as the ROS scavengers peg-SOD and peg-CAT abolished the SIRT3 activation. However, the interpretation is complex as antioxidant treatment alone restored the SIRT3 levels upon palmitic acid, glucose, and insulin treatment, whereas the nitrite-induced SIRT3 activation was reduced by antioxidant treatment [16,105,106]. Previous studies suggest a nitrite mediated AMPK-activation through attenuation of oxidative stress [16,105]. Cordero-Herrera and colleagues observed a nitrate dependent prevention of reduced p-AMPK and liver steatosis in mice fed a high fat diet which was correlated to reduced oxidative stress via nitrite-dependent inhibition of NADPH-oxidase [45]. The protective effect of dietary nitrate was absent in germ-free mice demonstrating the obligatory role of host microbiota in the bioactivation of nitrate [26,45]. Further studies are clearly required to clarify how nitrite signals under normoxic conditions, and downstream targets AMPK activation as well as dampening of oxidative stress.

3.3. Modulation of mitochondrial dynamics

Mitochondrial fission and fusion are ongoing processes reflecting the highly dynamic properties of mitochondria [107–109]. Increased fusion activity results in mitochondrial elongation with long filamentous mitochondria whereas increased fission promotes mitochondrial fragmentation resulting in short rods and spherical mitochondria (Fig. 4). A certain defined set of proteins are associated with the regulation of fission and fusion. Mitofusins, Mfn1 and Mfn2 are known to promote fusion [110] in cooperation with optic atrophy protein 1 (OPA1) [111]. Dynamin-related protein 1 (Drp1) and mitochondrial fission 1 protein (Fis1) are required for mitochondrial fission [112] (Fig. 4). Recently, the actin-depolymerizing protein cofilin1 has emerged as a negative regulator of mitochondrial DRP1 activity [113]. Hyperglycemia is shown to promote mitochondrial fission [114,115] reflected by increased expression of Fis1 and Drp1 [116]. T2D is related to a reduced expression of Mfn2, which may be linked to impaired mitochondrial function in skeletal muscle [117]. Montaigne and colleagues observed a T2D related mitochondrial fragmentation in myocardium and a substantial decrease in Mfn1 expression [118]. A recent paper demonstrated a nitrite dependent increase in mitochondrial length in adipocytes that correlated with a robust increase in Mfn1 expression [30]. Drp1 levels were unchanged, however nitrite dose-dependently increased phosphorylation of Ser656 [30] which inhibits Drp1 activity [119]. Interestingly, this was suggested to be obtained in an NO-independent manner since cGMP did not increase and the effect of nitrite was not abolished by pharmacological inhibition of sGC [30]. This study rather emphasized nitrite-induced PKA activity [30] that predominantly mediates Drp1 phosphorylation. The mechanism of nitrite mediated induction of PKA activity is still unclear. Importantly, nitrite augmented glucose uptake in adipocytes that was dependent on mitochondrial fusion. Drp1 inhibition abolished this effect [30]. The authors concluded that nitrite simultaneously stimulates Mfn-1 dependent fusion and inhibits Drp1-catalyzed fission in adipocytes (Fig. 4).

In conclusion, further investigation is needed to reveal the mechanistic explanation of the nitrite dependent induced PKA activity leading to augmented mitochondrial fusion.

4. Interaction between mitochondrial and NADPH oxidase-derived reactive oxygen species

Hyperglycemia-induced ROS generation is considered to contribute to the development and progression of diabetes and vascular disease [120]. Both mitochondria [121] and NADPH oxidases (NOXs) [122] have been recognized as major sources of ROS generation, contributing to oxidative stress, during conditions of hyperglycemia. In recent years, a cross-talk between mitochondria and NOXs leading to a feed-forward regulation has been reported that may contribute to a vicious cycle of ROS production [123] (Fig. 5).

Mechanistically, mitochondrial ROS (mtROS) facilitate the opening of the mitochondrial permeability transition pore (mPTP) resulting in the escape of mtROS to the cytosol, which in turn activates NOX2 and NOX1 via PKC or tyrosine kinase resulting in a more potent cytosolic ROS formation [124,125], with subsequent uncoupling of eNOS [125]. Mitochondrial ROS dependent activation of NOX2 is prevented by mPTP inhibition or mitochondria targeted antioxidants [124–128].

Reverse cross-talk exists mediated by angiotensin II via diacylglycerol formation which activates PKC and subsequently NOX2 or NOX1 [129–134]. The NOX dependent ROS in turn contribute to activation of redox-sensitive PKCε or MAPK leading to impaired calcium hemostasis, mitochondrial alkalinization, altered mitochondrial membrane potential and finally increased mitochondrial superoxide/hydrogen peroxide formation/release [135–137]. Potential involvement of redox-sensitive mitochondrial ATP-sensitive potassium channels (mtK_{ATP}) has been reported with subsequent $p66^{Shc}$, monoamine oxidase, respiratory complex activation or impairment of mitochondrial antioxidant defense leading to induction of mtROS formation [138] (summarized in Fig. 5).

NOX2 is upregulated in heart and kidney in diabetes [139] and NOX2 inhibition has been reported to be protective in diabetic cardiomyopathy [140]. Increased expression of NOX2 has also been coupled to ultrastructural alterations in the central auditory system in zucker diabetic fatty rats (ZDF) [141]. NOX2 deficiency preserves islet function via reductions in ROS and β cell apoptosis in streptozotocin-induced diabetic mice [142]. NOX2 is also activated in the retinal vasculature [143] together with increased arginase activity in diabetes [144] leading to reduced bioavailability of nitric oxide eventually causing diabetic retinopathy. Schuhmacher and colleagues demonstrated an inverse correlation between loss of endothelial function and NOX activity in type 1 diabetes [145].

Evidence of a redox-based NOX4 activation is more limited and has been thought to be regulated mainly by transcriptional control [146]. However, a recent study suggested that NOX4 activity is negatively regulated by ATP to work as an energetic sensor [147]. NOX4 has been suggested to be localized in mitochondria [148,149], and contribute to significant amounts of ROS in the heart [150], and kidney cortex [148]. In addition to mitochondria, NOX4 is also localized in other subcellular localizations such as the endoplasmic reticulum, nucleus, and plasma membrane [148,151–153]. NOX4 is shown to be upregulated in kidney cortex of diabetic rats [148]. The therapeutic value of modulating NOX4 activity in diabetes is debated and inhibition of this enzyme has been associated with both benefits and harm in the renal and cardiovascular system [154].

In conclusion, diabetes induced upregulation of NADPH oxidases and their cross-talk with mitochondria may contribute to a vicious cycle of ROS production contributing to progression of disease.

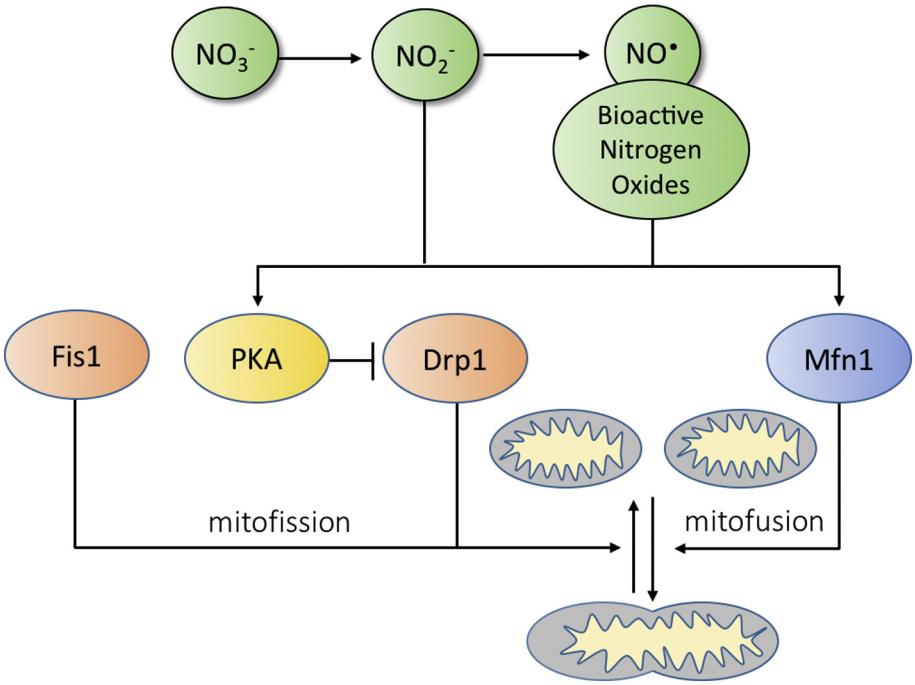

Fig. 4. Nitrate-nitrite-NO pathway and mitochondrial dynamics. Nitrite-dependent increase in mitochondrial fusion in adipocytes via increased mitofusin1 expression and PKA dependent phosphorylation of dynamin-related protein 1 have been shown to shown to augment glucose uptake in adipocytes.

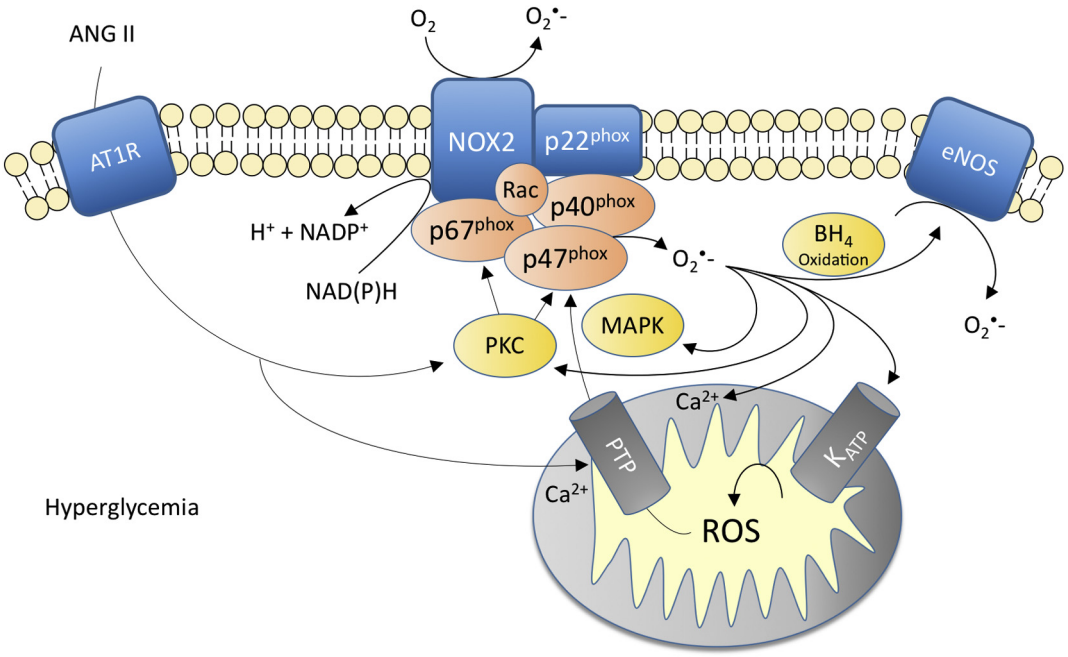

Fig. 5. Crosstalk between Mitochondria and NOX-derived ROS generation. Conditions with hyperglycemia have been associated with abnormal NADPH oxidase and mitochondrial functions leading excessive ROS formation and oxidative stress. In addition, this can be triggered by elevated levels of angiotensin II (ANG II). Both hyperglycemia and stimulation of ANG II type 1 receptor (ATR1) activate e.g. PKC, which leads to translocation of NOX2 subunits (p47phox, p67phox, Rac) to merge/assemble with the plasma membrane NOX2 and increase its activity. By somewhat different mechanisms, ANG II may also activate NOX1, NOX4 and NOX5 (not shown). The cytosolic ROS in turn contribute to activation of MAPK, PKCε and Ca^{2+} signaling leading to altered mitochondrial membrane potential and increased mitochondrial ROS production. Mitochondrial ROS facilitate opening of the mitochondrial permeability transition pore resulting in the escape of ROS from mitochondria to the cytosol, which in turn further activates NOX via PKC or tyrosine kinase leading oxidation of BH_4 and subsequent eNOS uncoupling (i.e. switch to superoxide production instead of generating NO). NADPH oxidase-dependent ROS may also activate ATP-sensitive potassium channels resulting in respiratory complex activation or impaired mitochondrial antioxidant defense leading to induction of mitochondrial ROS formation.

5. Modulation of NADPH oxidase activity by the nitrate-nitrite-NO pathway

In three independent studies from 2011, Carlstrom, Montenegro and Sindler et al. demonstrated for the first time that boosting the nitrate-nitrite-NO pathway dampened NOX expression or activity, reduced vascular oxidative stress, and attenuated hypertension in experimental models of cardiovascular and renal disease as well as during aging

[155–157]. Since then, numerous studies using similar disease conditions have confirmed these favorable effects on oxidative stress following nitrate and nitrite treatment [158]. More recently several studies have investigated potential modulation of NOX-derived ROS during metabolic disease including obesity and diabetes. Cordero-Herrera and colleagues showed that chronic supplementation with nitrate attenuated high-fat diet induced liver steatosis via mechanisms that involve 1) increased formation of NO and other bioactive nitrogen oxide species, 2) inhibition of NOX activity and activation of AMPK signaling [45]. Although further studies are warranted, the authors suggested that NOX inhibition was mainly related to reduction of NOX2 activity rather than NOX4 [45]. Moreover, nitrate treatment improved insulin responses together with reduced blood pressure in old rats and attenuated contractility to angiotensin II in resistance vessels [159], an effect observed also in superoxide dismutase-1 knockout mice [105]. In addition, acute treatment with nitrate (0.1 mmol/kg, given i.p) improved glucose clearance and preserved HOMA-IR along with reduced NOX activity in livers of adenosine A_{2B} receptor knockout mice, which display features of metabolic syndrome [16]. Interestingly, Peleli and colleagues showed that this dietary dose of nitrate induced similar therapeutic effects as metformin, administered by the same route and dose (0.1 mmol/kg) [16]. In agreement, in a model diet-induced cardiometabolic disease head-to head comparison with metformin showed that nitrate therapy was equally effective in restoring the metabolic profile, whereas nitrate was superior regarding reducing the cardiovascular risk [160]. In another study from the same group, increased mitochondrial respiration and expression of mitochondrial complexes in adipose tissue was associated with reduced NOX-derived superoxide production in bone-marrow derived macrophages from mice treated with dietary nitrate [73]. The underlying mechanisms contributing to dampen NOX activity following nitrate/nitrite are still not fully clear. At least in activated mouse macrophages, nitrite-mediated inhibition of NOX activity cannot be explained by S-nitrosation of the NOX enzyme, but rather changes in NOX2 expression and XOR function may contribute [161].

In conclusion, boosting the nitrate-nitrite-NO pathway contributes to attenuation of oxidative stress via reduced NOX activity and improved glucose clearance, hence dietary nitrate supplementation may be a suitable complement to metformin treatment in conditions with T2D.

6. Summary and future perspectives

Based on accumulating experimental evidence, prevention of T2D and associated complications by targeting mitochondria and oxidative stress via nitrate supplementation to boost the nitrate-nitrite-NO pathway looks promising. The research field is still at an early-stage to revealing the mechanistic explanations for the various effects of nitrate and nitrite on mitochondrial function, but has been associated with AMPK-activation and modulation of oxidative stress. Moreover, the proposed NO-independent activation of PKA and PKG requires further investigation, and may involve other bioactive nitrogen oxide species. A general concern regarding the extrapolation from rodents to humans in terms of nitrate-induced browning of white adipose tissue is the well accepted nitrate-induced reduction of oxygen consumption at the whole body level in humans both during work [162] and at rest [163]. This is intriguing as browning expects to increase whole body oxygen consumption, especially at rest. Future studies may elucidate the exact signaling pathways involved in the nitrate and nitrite dependent positive effects on mitochondrial function and interaction with NOX-derived ROS with respect to T2D.

Based on epidemiological studies, high intake of nitrate-rich vegetables has been associated with a reduced risk of metabolic disease. However, if these favorable effects can be attributed to nitrate is not clear. In one small trial, using beet root juice (250 ml once daily), nitrate supplementation for 2 weeks had no significant effect on glucose homeostasis in patients with T2D [164]. We speculate that this lack of effect could be related to the dose regime or the fact that majority of the patients were treated with metformin which shares similar mechanisms of action as nitrate. Indeed, in a recent study in mice we saw independent antidiabetic effects of metformin and nitrate, but little further improvement when the two compounds were combined [160]. Future prospective and placebo-controlled clinical studies are warranted to further investigate the potential favorable metabolic effects of inorganic nitrate and nitrite supplementation in humans with pre-diabetes or T2D. If such effect could be demonstrated, this would open for new and cost-efficient nutritional and pharmacological approaches to prevent development and progression of the disease.

Declaration of competing interest

J.O.L and E.W. are co-inventors on patent applications related to the therapeutic use of inorganic nitrate. The other authors have no conflicts of interest.

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