frontiers in Physiology

ORIGINAL RESEARCH
published: 07 August 2015
doi: 10.3389/fphys.2015.00222

In adenosine A_{2B} knockouts acute treatment with inorganic nitrate improves glucose disposal, oxidative stress, and AMPK signaling in the liver

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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *17 June 2015*
Accepted: *22 July 2015*
Published: *07 August 2015*

Citation:
Peleli M, Hezel M, Zollbrecht C, Persson AEG, Lundberg JO, Weitzberg E, Fredholm BB and Carlström M (2015) In adenosine A_{2B} knockouts acute treatment with inorganic nitrate improves glucose disposal, oxidative stress, and AMPK signaling in the liver Front. Physiol. 6:222. doi: 10.3389/fphys.2015.00222

Rationale: Accumulating studies suggest that nitric oxide (NO) deficiency and oxidative stress are central pathological mechanisms in type 2 diabetes (T2D). Recent findings demonstrate therapeutic effects by boosting the nitrate-nitrite-NO pathway, which is an alternative pathway for NO formation. This study aimed at investigating the acute effects of inorganic nitrate on glucose and insulin signaling in adenosine A_{2B} receptor knockout mice ($A_{2B}^{-/-}$), a genetic mouse model of impaired metabolic regulation.

Methods: Acute effects of nitrate treatment were investigated in aged wild-type (WT) and $A_{2B}^{-/-}$ mice. One hour after injection with nitrate (0.1 mmol/kg, i.p.) or placebo, metabolic regulation was evaluated by intraperitoneal glucose and insulin tolerance tests. NADPH oxidase-mediated superoxide production and AMPK phosphorylation were measured in livers obtained from non-treated or glucose-treated mice, with or without prior nitrate injection. Plasma was used to determine insulin resistance (HOMA-IR) and NO signaling.

Results: $A_{2B}^{-/-}$ displayed increased body weight, reduced glucose clearance, and attenuated overall insulin responses compared with age-matched WT mice. Nitrate treatment increased circulating levels of nitrate, nitrite and cGMP in the $A_{2B}^{-/-}$, and improved glucose clearance. In WT mice, however, nitrate treatment did not influence glucose clearance. HOMA-IR increased following glucose injection in the $A_{2B}^{-/-}$, but remained at basal levels in mice pretreated with nitrate. NADPH oxidase activity in livers from $A_{2B}^{-/-}$, but not WT mice, was reduced by nitrate treatment. Livers from $A_{2B}^{-/-}$ displayed reduced AMPK phosphorylation compared with WT mice, and this was increased by nitrate treatment. Finally, injection with the anti-diabetic agent metformin induced similar therapeutic effects in the $A_{2B}^{-/-}$ as observed with nitrate.

Conclusion: The $A_{2B}^{-/-}$ mouse is a genetic mouse model of metabolic syndrome. Acute treatment with nitrate improved the metabolic profile in it, at least partly via reduction in oxidative stress and improved AMPK signaling in the liver.

Keywords: insulin resistance, metabolic syndrome, NADPH oxidase, nitric oxide, nitrite, superoxide, obesity, type 2 diabetes

Introduction

Metabolic syndrome, which worsens during aging and obesity, is a cluster of biochemical and physiological abnormalities that increase the risk of developing cardiovascular disease and type 2 diabetes (T2D) (Eckel et al., 2005; Carlström, 2011). Reduced nitric oxide (NO) production from endothelial nitric oxide synthase (eNOS) and augmented oxidative stress are proposed to be central events in metabolic syndrome (Litvinova et al., 2015). In the past decade, an alternative pathway for NO formation has been described where inorganic nitrate is serially reduced to nitrite and then NO and other bioactive nitrogen oxides (Lundberg et al., 2008, 2009, 2011). We have shown that several features of metabolic syndrome present in aged eNOS-deficient mice can be reversed by dietary supplementation with inorganic nitrate (Carlström et al., 2010). A recent study showed that chronic nitrite supplementation through increased phosphorylation of the skeletal muscle AMP activated kinase (AMPK) improved some metabolic syndrome components in a model of obesity (Singamsetty et al., 2015). Moreover, chronic treatment with nitrate attenuates oxidative stress and high blood pressure in models of renal and cardiovascular disease (Carlström et al., 2011a; Gao et al., 2015).

Adenosine is another important regulator of metabolism, and signaling via its different receptor subtypes, A_1, A_{2A}, A_{2B}, and A_3, has also gained a lot of interest (Chen et al., 2013). In a recent publication we demonstrated that abrogation of adenosine A_1 signaling improves metabolic regulation in aged mice by modulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and immune responses (Yang et al., 2015a). Besides the A_1 receptor, signaling via both A_{2A} and A_{2B} receptors play important roles in modulating glucose homeostasis and fat mass (Johnston-Cox et al., 2012; Gnad et al., 2014). Another recent study suggested gene deletion of adenosine A_{2B} receptor as a suitable model for metabolic syndrome (Csóka et al., 2014). The authors showed that A_{2B} knockout mice ($A_{2B}^{-/-}$), fed a regular chow, displayed increased body weight and fat mass, impaired glucose and insulin homeostasis, together with dysregulated insulin, adipokine, triglyceride, and cholesterol metabolism compared with wild-type (WT) control mice. Food consumption was similar between genotypes, but daily walking time was reduced in the $A_{2B}^{-/-}$ mice. Moreover, Johnston-Cox et al. reported that a high fat diet (HFD) aggravated the abnormal metabolic phenotype in $A_{2B}^{-/-}$, whereas Csoka and colleagues did not observe this.

The current study aimed at investigating the acute effects of inorganic nitrate treatment on metabolic functions in aged A_{2B} receptor knockout mice ($A_{2B}^{-/-}$). Considering previous findings about nitrate- or nitrite-mediated modulation of both NADPH oxidase (Montenegro et al., 2011; Carlström et al., 2011a; Gao et al., 2015; Yang et al., 2015b) and AMPK (Kamga Pride et al., 2014; Singamsetty et al., 2015), we hypothesized that nitrate could improve abnormal metabolic functions during aging and increased fat mass by increasing AMPK activation and moderating oxidative stress. We observed improved metabolic regulation in the $A_{2B}^{-/-}$ mice after nitrate treatment and this was indeed associated with decreased NADPH oxidase-derived superoxide production in the liver, possibly mediated via restored AMPK activation.

Materials and Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC) in Stockholm, and performed according to the National Institutes of Health guidelines for the conduct of experiments in animals. Experiments were conducted on aged (12–16 months) adenosine A_{2B} receptor gene-deleted and WT mice from heterozygous breeding pairs. $A_{2B}^{-/-}$ mice (a gift from professor M. Sitkovsky at Northwestern University, Boston, Mass) were backcrossed 11 times to a C57BL/6J background at Northwestern University. Both sexes were used, with equal distribution for all experimental series. Mice were housed in temperature-controlled rooms with 12 h light/dark cycles and received a standard rodent chow (4% fat, R34, Lactamin AB, Kimstad, Sweden) and tap water *ad libitum*. An overview of the experimental protocol is shown in **Figure 1**.

Intraperitoneal Glucose, Insulin, and Pyruvate Tolerance Tests

Glucose tolerance tests (IPGTT) were performed following 6 h of fasting, as described previously (Yang et al., 2015a). Inorganic nitrate ($NaNO_3$; 0.1 mmol/kg body weight) or placebo (NaCl, 0.1 mmol/kg body weight) was administered intraperitoneally 60 min prior to the tolerance tests. In a human (70 kg) this dose of nitrate corresponds to around 450 mg; an amount found in a single serving of a nitrate rich vegetable such as spinach, beetroot, or lettuce (Weitzberg and Lundberg, 2013). A bolus of D-glucose or pyruvate was injected (2 g/kg body weight; 30% in saline) and tail blood was sampled at 0, 15, 30, 60, and 120 min. Plasma glucose was determined using a portable glucose meter (FreeStyle Lite, Abbot Diabetes Care Inc, CA). In a cohort of $A_{2B}^{-/-}$ mice we also investigated the effects on glucose disposal with the anti-diabetic drug metformin. Metformin (0.1 mmol/kg body weight) or placebo (NaCl, 0.1 mmol/kg body weight) was administered intraperitoneally 60 min prior to the IPGTT. Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated at baseline, at 60 min after injection with nitrate or placebo, and again 30 min after injection with glucose.

In order to investigate the acute effects of nitrate in a model with more pronounced obesity, IPGTT were performed as described above in WT mice given a HFD (34.9% fat, D12492, Research Diets Inc., New Brunswick, NJ) for 14 months (Supplementary Material).

Intraperitoneal insulin tolerance tests (IPITT) were performed similarly to IPGTT without fasting. A bolus of insulin (0.75 IE/kg body weight; Novorapid 100 IE/ml, Novo Nordisk A/S, Denmark) was injected (0.2 IE/ml in saline) and blood samples were obtained for plasma glucose measurements.

Plasma Analysis

Insulin content was measured using ELISAs purchased from Mercodia (Uppsala, Sweden). Plasma samples containing IBMX

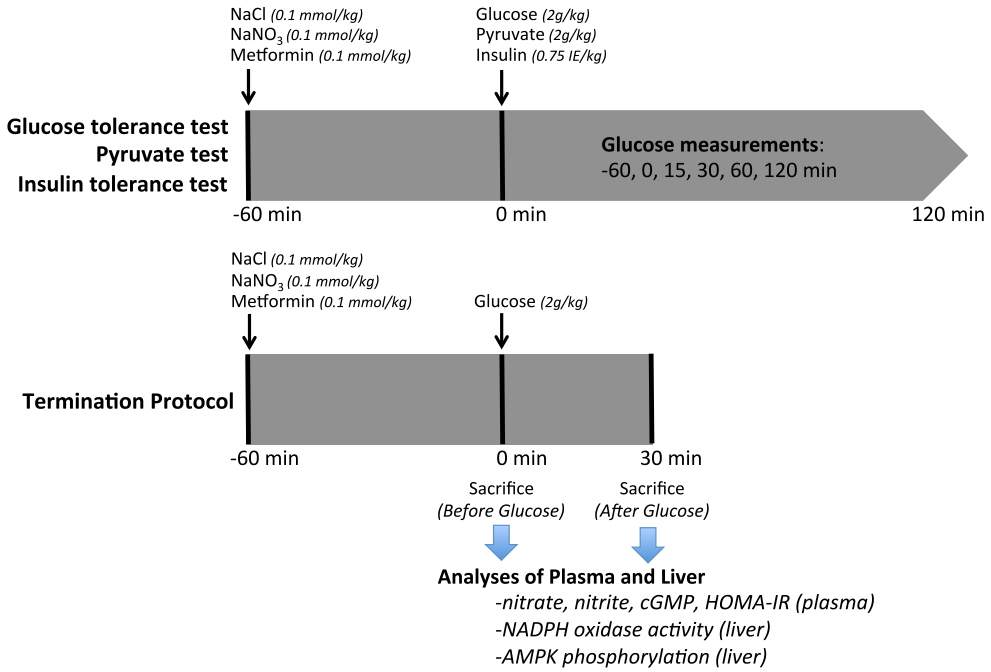

FIGURE 1 | Experimental Protocol. Diagram of the experimental procedures on aged WT or A_{2B}KO mice undergoing intraperitoneal glucose (IPGTT), pyruvate or insulin (IPITT) tolerance tests. In the termination series, plasma and liver were collected at 0 or 30 min. NaCl, sodium chloride; $NaNO_3$, sodium nitrate; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test.

(10 μM) were analyzed for cGMP using ELISA method (EIA system; GE Healthcare). All kits were run according to manufacturers' instructions. Nitrite and nitrate were analyzed by HPLC (ENO-20) and autosampler (840, EiCom, Kyoto, Japan), as described previously (Carlström et al., 2010; Hezel et al., 2015). The plasma samples were extracted using methanol (1:2) and then centrifuged for 10 min (4°C; 10.000 g), separated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. The nitrite was then derivatized using Griess reagent to form diazo compounds and analyzed by detection at 540 nm.

NADPH Oxidase Activity

NADPH oxidase-mediated superoxide formation was detected by lucigenin-dependent chemiluminescence assay (Carlstrom et al., 2013; Yang et al., 2015a). Livers were separately homogenized and used for subsequent activity measurement.

Western Blotting of AMPK

Livers obtained from mice under (1) basal condition, (2) after pretreatment with placebo, nitrate or metformin, and (3) after stimulation with glucose were weighed and homogenized using 0.5 mm zirconium oxide beads (Bullet Blender™, Next Advance, Inc., Stockholm, Sweden) in 2.5 volumes of lysis buffer containing 10 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 60 mM N-octyl glucoside, 1% Triton X-100, protease, and phosphatase inhibitor cocktails (Sigma-Aldrich, Stockholm, Sweden). After centrifugation and protein quantification of the soluble fraction (Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Solna, Sweden), equal amounts of protein were separated by SDS-PAGE followed by transfer to a PVDF membrane (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in Tween-containing TBS, incubated with specific primary antibody for phosphorylated AMPK (Thr172; Cell signaling/BioNordika, Stockholm, Sweden) and anti-rabbit secondary antibody (horseradish peroxidase-conjugated goat antibody to rabbit IgG, Santa Cruz, Heidelberg, Germany). To detect total AMPK, Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific™, Göteborg, Sweden) was applied followed by blocking and re-probing the membranes with primary antibody for AMPK (Cell Signaling/BioNordika) and anti-rabbit secondary antibody. Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad), intensities were quantified using densitometry (Image Lab 5.2.1 software, Bio-Rad) and results are reported as relative optical density of the specific proteins.

Statistical Analysis

Values are presented as means ± SEM. Single comparisons between normally distributed parameters were tested for significance using the Student's paired or unpaired *t*-test as appropriate. For multiple group comparisons, One-Way ANOVA followed by Bonferroni's *post-hoc* test was used to allow for more than one comparison with the same variable. Statistical significance was defined as $p < 0.05$.

Results

Animal Characteristics

Body weight was significantly higher in aged (12–16 months) $A_{2B}^{-/-}$ (36.5 ± 0.8 g; $n = 42$) compared with age-matched WT mice (32.5 ± 0.9 g; $n = 40$), and plasma glucose levels in non-fasting mice were also higher in the $A_{2B}^{-/-}$ mice (9.5 ± 0.4 vs. 7.6 ± 0.2 mmol/L). WT mice fed with HFD for 14 months were more obese (body weight; 60.6 ± 3.3 g; $n = 10$) compared with the aged-matched mice on a regular chow ($P < 0.05$).

Glucose Tolerance Tests

To investigate the ability of acute inorganic nitrate treatment to modulate the metabolic phenotype in aged $A_{2B}^{-/-}$ mice we performed glucose tolerance tests in aged $A_{2B}^{-/-}$ and WT mice 1 h after nitrate injection ($NaNO_3$; 0.1 mmol/kg body weight). Fasting blood glucose levels were similar in $A_{2B}^{-/-}$ and WT mice and nitrate treatment had no influence on glucose clearance in WT mice (**Figure 2A**). Interestingly, the impaired glucose tolerance in $A_{2B}^{-/-}$ mice compared to WT was significantly improved after acute nitrate treatment (**Figure 2B**). Administration of the anti-diabetic drug metformin to the $A_{2B}^{-/-}$ mice resulted in an even more pronounced increase in glucose tolerance (**Figure 2C**). Similar to that observed in $A_{2B}^{-/-}$ mice, acute treatment with nitrate also improved glucose disposal (AUC 1493 ± 91 vs. 1779 ± 118; $n = 10$) in HFD-treated mice (Supplementary Material).

Pyruvate Tolerance Test

We probed whether nitrate influences gluconeogenesis in $A_{2B}^{-/-}$ mice, which could contribute to the production and clearance of glucose. To this end, the administration of the gluconeogenic substrate precursor pyruvate showed that there was no difference in glucose production in mice with nitrate pretreatment. Hence, nitrate had no significant impact on the gluconeogenesis pathway. However, upon glucose production (after 60 min), the clearance rates of glucose were again significantly faster in nitrate treated $A_{2B}^{-/-}$ mice compared to placebo group (**Figure 2D**; 120 min), confirming a promotion in glucose clearance upon acute treatment with inorganic nitrate.

Insulin Tolerance Tests and HOMA-IR

Insulin sensitivity after insulin injection (IPITT) was lower in $A_{2B}^{-/-}$ mice (**Figure 3B**) compared to WT (**Figure 3A**) resulting

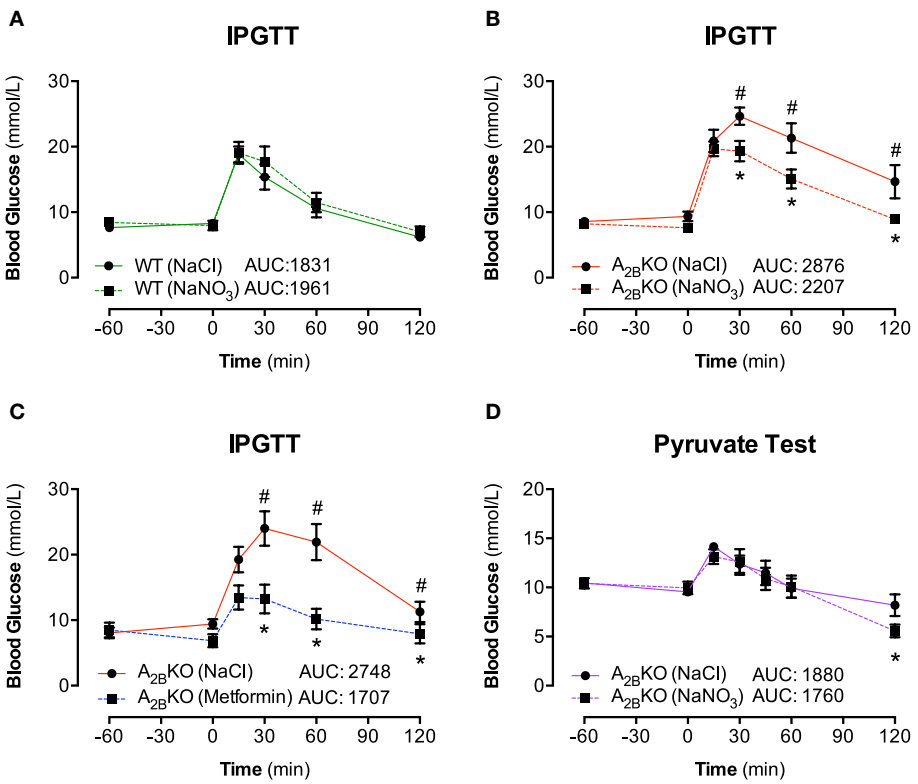

FIGURE 2 | IPGTT and Pyruvate test. The effect of inorganic nitrate on glucose tolerance was determined by measuring plasma glucose levels in WT mice **(A)** and A_{2B}KO mice **(B)** after placebo (NaCl) or nitrate injection ($NaNO_3$). Glucose levels after injection of metformin were determined in A_{2B}KO mice to investigate the effect of the anti-diabetic drug in mice with metabolic syndrome **(C)**. The impaired glucose tolerance in A_{2B}KO mice compared to WT could be significantly improved both with $NaNO_3$ and with metformin. Finally, to assess any potential effect of nitrate on gluconeogenesis, a pyruvate tolerance test was performed **(D)**. The total AUC (mmol/L/min) for the 0–120 min period was calculated. Values are mean ± SEM, $n = 10$–16/group. $^*p < 0.05$ vs. A_{2B}KO (NaCl); $^\#p < 0.05$ vs. WT.

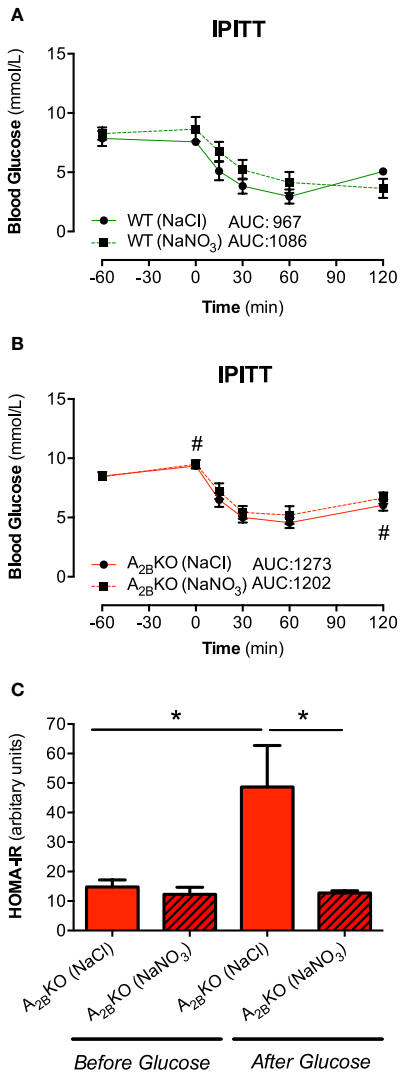

FIGURE 3 | IPITT and HOMA-IR. Plasma glucose levels in response to insulin injection were not influenced by nitrate treatment in WT **(A)** and A_{2B}KO mice **(B)**. HOMA-IR, a measure of insulin resistance, increased following glucose injection in A_{2B}KO mice, which was prevented by prior nitrate treatment **(C)**. Values are mean ± SEM, $n = 10$–16/group. $^{\#}p < 0.05$ vs. WT; $^{*}p < 0.05$ among the indicated groups.

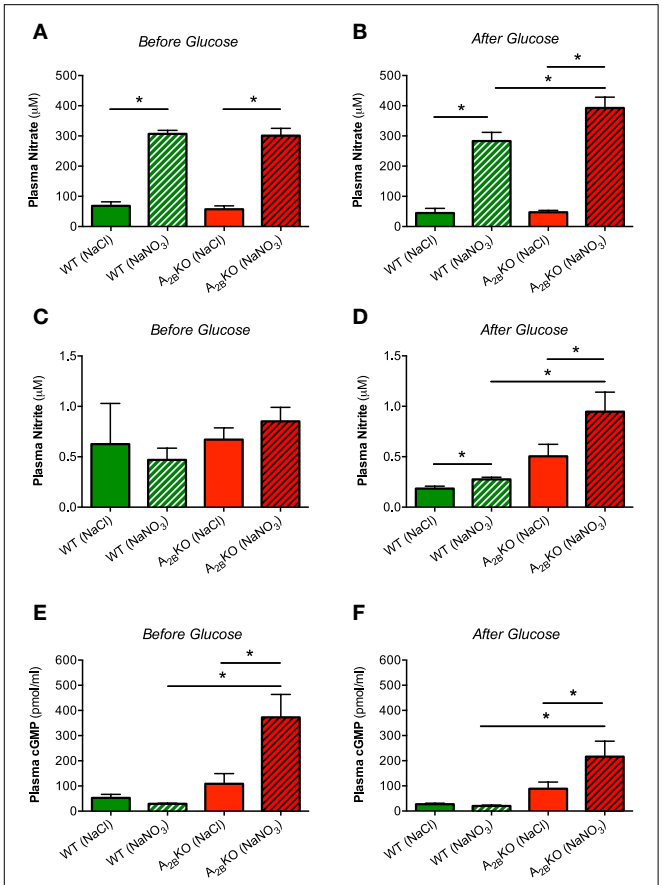

FIGURE 4 | Nitrate, nitrite and cGMP in plasma. Treatment with nitrate significantly increased plasma levels of nitrate in both genotypes before **(A)** and after glucose injection **(B)**. Plasma nitrite levels were significantly increased only in nitrate treated A_{2B}KO mice after glucose **(C, D)**. In A_{2B}KO mice, nitrate supplementation caused a significant increase of plasma cGMP levels both before **(E)** and after glucose injection **(F)**. Values are mean ± SEM, $n = 6$–14/group. $^{*}p < 0.05$ among the indicated groups.

in higher AUC. Glucose clearance did not differ between placebo or nitrate treated animals of both genotypes. In addition, insulin resistance (expressed as HOMA-IR) during fasting condition was similar in both genotypes but increased following glucose injection in the $A_{2B}^{-/-}$ mice (**Figure 3C**). This could be prevented by prior injection with nitrate.

Nitrate, Nitrite, and cGMP in Plasma

Nitrate treatment increased plasma nitrate levels in both WT and $A_{2B}^{-/-}$ mice as expected (**Figure 4A**) and this increase was even higher in $A_{2B}^{-/-}$ mice compared to WT after glucose injection (**Figure 4B**). Plasma nitrite levels were not different between groups during fasting (**Figure 4C**) but after glucose injection, $A_{2B}^{-/-}$ mice treated with nitrate showed significantly higher nitrite levels compared to WT and also $A_{2B}^{-/-}$ placebo group (**Figure 4D**). The second messenger cGMP, a central downstream NO signaling target, was not influenced by nitrate or glucose in WT mice (**Figures 4E,F**). However, in $A_{2B}^{-/-}$ mice treatment with nitrate resulted in a significant increase in plasma cGMP levels compared to the placebo group and WT mice.

NADPH Oxidase Activity in the Liver

NADPH oxidase-derived superoxide production in liver homogenates from $A_{2B}^{-/-}$ mice was significantly higher compared to WT mice (**Figure 5A**). Interestingly, nitrate treatment as well as injection of metformin significantly reduced superoxide production whereas nitrate had no effect on NADPH oxidase activity in WT mice. The same beneficial effects of

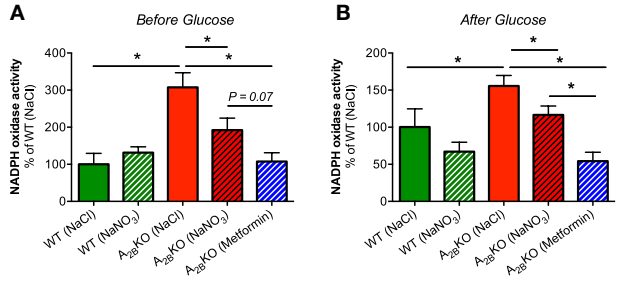

FIGURE 5 | NADPH oxidase activity in the liver. NADPH oxidase-derived superoxide formation was measured with lucigenin-dependent chemiluminescence signal in liver homogenates derived from A_{2B}KO and WT mice before **(A)** and after glucose injection **(B)**. A significantly increased NADPH oxidase activity was observed in A_{2B}KO compared to WT, which could be diminished by prior injection of nitrate or metformin. Values are mean ± SEM, $n = 6$–14/group. $^*p < 0.05$ among the indicated groups.

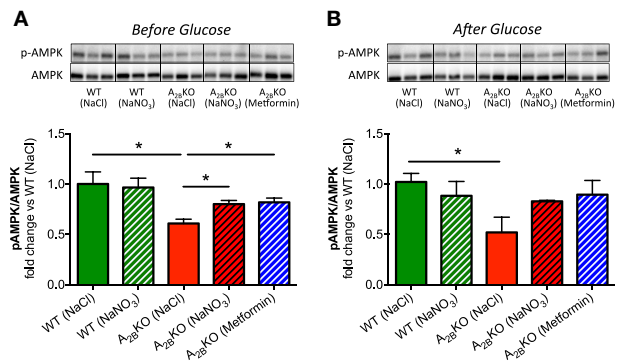

FIGURE 6 | AMPK regulation in the liver. Western blot was used to determine AMPK expression and phosphorylation in liver. Before **(A)** and after glucose injection **(B)**, A_{2B}KO mice showed significantly lower AMPK activation compared to WT. This could be improved by prior injection of nitrate or metformin. Three representative samples per group obtained from different gels are shown together with densitometric quantification, presented as ratio p-AMPK/AMPK. Values are mean ± SEM, $n = 4$/group. $^*p < 0.05$ among the indicated groups.

nitrate and metformin were observed after glucose injection (**Figure 5B**).

AMPK Regulation in the Liver

Expression and phosphorylation levels of AMPK were assessed in liver tissue derived from all animal groups. The ratio of phosphorylated AMPK to total AMPK, as a measure of AMPK activation, was significantly lower in $A_{2B}^{-/-}$ mice compared to WT (**Figures 6A,B**). This could be partially rescued by treatment with nitrate or metformin. As was the case with the NADPH oxidase activity, AMPK activation could also be improved with nitrate or metformin after glucose injection.

Discussion

Aged $A_{2B}^{-/-}$ Mice Present Characteristics of the Metabolic Syndrome

We confirmed previous studies (Johnston-Cox et al., 2012; Csóka et al., 2014) showing that aged $A_{2B}^{-/-}$ mice present several features of the metabolic syndrome; they are more obese than WT mice, display hyperglycemia and poor glucose clearance.

Impaired Liver AMPK Activation Could Contribute to the Metabolic Dysregulation in Aged $A_{2B}^{-/-}$ Mice

The mechanisms leading to the development of this impaired metabolic regulation in the $A_{2B}^{-/-}$ mice are still being investigated and there are many unanswered questions. Major contributing factors are elevated hepatic inflammation and IRS-2 expression (Johnston-Cox et al., 2012) and augmented classical macrophage activation in the adipose tissue (Csóka et al., 2014). In this study we focused mainly on the liver since it is an organ of high importance in glucose metabolism and T2D development (Bechmann et al., 2012) and has not been extensively investigated in $A_{2B}^{-/-}$ mice. AMPK is an important intracellular energy sensor and one of the key players in maintaining liver glucose homeostasis (Viana et al., 2006; Wang et al., 2012), which is often downregulated under hyperglycemic conditions (Kraegen et al., 2006). The ability to activate liver AMPK is a major reason why the anti-diabetic drugs metformin and 5-amino-4-imidazolecarboxamide riboside (AICAR) were developed and used as treatments (Towler and Hardie, 2007). In our study we show for the first time that aged $A_{2B}^{-/-}$ mice present lower phosphorylation levels of liver AMPK compared to normoglycemic WT mice of similar age. In agreement with that, the AMPK activator metformin elevated liver AMPK phosphorylation levels together with a remarkable improvement of glucose clearance. Thus, one may speculate that impaired AMPK activation is one of the factors contributing to metabolic dysfunction in this animal model.

Elevated Liver NADPH Oxidase Activity Could Contribute to the Metabolic Dysregulated Phenotype of the Aged $A_{2B}^{-/-}$ Mice

Another key regulator of liver glucose uptake and metabolism is the enzyme family of NADPH oxidases. It is well known that elevated levels of NADPH oxidase-derived superoxide in the liver diminish glucose uptake and contribute to the development of hyperglycemia (Guichard et al., 2008). To our knowledge, this is the first study showing that $A_{2B}^{-/-}$ mice present higher levels of liver NADPH oxidase activity compared to WT, both before and after glucose treatment. Therefore, the investigation of pharmacological interventions to target liver NADPH oxidases may be of great interest for the improvement of the metabolic phenotype when the A_{2B} receptors are ablated.

AMPK and NADPH Oxidases are Closely Linked

Several studies have indicated that pharmacological activation of AMPK can reduce NADPH oxidase activity and expression

in various target organs and cells like liver (Adachi and Brenner, 2008), cardiomyocytes (Balteau et al., 2014), podocytes (Piwkowska et al., 2010), and human umbilical vein endothelial cells (Ceolotto et al., 2007). One mechanism leading to reduced production reactive oxygen species (ROS) might be via upregulated mRNA expression of the antioxidant enzymes SOD2 and catalase, as it was seen in activated hepatic stellate cells treated with AMPK activators AICAR or metformin (Adachi and Brenner, 2008). Another mechanism of how activated AMPK can inhibit NADPH oxidase activity was shown in activated human neutrophils where AMPK activation with AICAR prevented phosphorylation and membrane translocation of the cytosolic NADPH oxidase subunit p47phox, which are both crucial to the enzyme activation (Alba et al., 2004). However, it is still unknown if this inhibition of NADPH oxidases and ROS production by p-AMPK can lead to improved glucose clearance. Speaking in favor of this concept, activation of AMPK by inorganic nitrate or metformin in our study was clearly associated with both reduced NADPH oxidase activity and improved glucose tolerance in $A_{2B}^{-/-}$ mice. Functional AMPK may therefore be important as an early warning system for oxidative stress to trigger compensatory antioxidant effects, which in turn improve glucose uptake. Future studies are needed to investigate if inorganic nitrate exerts its beneficial effect via upregulation of antioxidant enzymes, prevention of p47phox phosphorylation and translocation or another mechanism.

The Nitrate-Nitrite-NO Pathway is Upregulated in the Aged $A_{2B}^{-/-}$ Mice

We and other groups have previously shown that long-term treatment with inorganic nitrate or nitrite improves glucose clearance, insulin sensitivity, and reduces visceral fat levels during aging and obesity (Carlström et al., 2010; Hezel et al., 2015; Singamsetty et al., 2015). However, the underlying mechanisms of how inorganic nitrate and nitrite mediate their beneficial effects remain to be elucidated. In the current study we investigated if acute treatment with inorganic nitrate can exert similar effects as observed with long-term supplementation. Interestingly, nitrate treatment significantly increased the plasma levels of nitrite and cGMP only in $A_{2B}^{-/-}$ mice, but not in the WT, and the increase in plasma nitrate levels was higher in $A_{2B}^{-/-}$ mice compared with WT after glucose load. Since the baseline levels of nitrate, nitrite and cGMP were similar between WT and $A_{2B}^{-/-}$ it seems that the nitrate-nitrite-NO pathway is sensitized in the absence of A_{2B} receptors. Activation of the A_2 receptors has been linked with increased NOS activation, and therefore it is likely that the NOS function is compromised in the A_{2B} knockouts. There are publications showing that A_2 receptor signaling is associated with higher NO production in the renal microvasculature (Carlström et al., 2011b) and in the liver during ischemia/reperfusion injury (Peralta et al., 1999). Moreover, A_2 receptor activation, especially of the type 2B, leads to higher NO production in coronary artery endothelial cells (Olanrewaju and Mustafa, 2000) and enhances vasorelaxation in mouse aorta (Ansari et al., 2007). Another mechanism potentiating the action of nitrate in the $A_{2B}^{-/-}$ mice but not in WT might be via the higher superoxide levels. Several studies in redox biology suggest that stimulating NO production or antioxidant systems are more potent when there are higher levels of ROS and in particular superoxide (Wink et al., 2001; Silva et al., 2012; Araujo and Wilcox, 2014). Since activation of A_{2B} receptors can facilitate NO production and mice lacking these receptors already present higher levels of liver superoxide one could speculate that this oxidative stress leads to a more prominent and faster activation of the alternative nitrate-nitrite-NO pathway.

The Nitrate-Nitrite-NO Pathway in T2D: Clinical and Experimental Data

In recent bibliography there are several experimental *in vivo* and *in vitro* studies showing favorable effects of inorganic nitrate and nitrite in T2D (Bahadoran et al., 2015). The proposed mechanisms involve compensation for disturbed eNOS-derived NO generation (Carlström et al., 2010), improved antioxidant capacity (Khalifi et al., 2015), and increased pancreatic islet blood flow and insulin secretion (Nyström et al., 2012). Moreover, nitrate/nitrite-mediated NO production may also improve insulin resistance and glucose uptake by activation of glucose transporter 4 (GLUT4) (Jiang et al., 2014; Ohtake et al., 2015). Apart from the experimental reports some clinical studies have been conducted. So far there is evidence that inorganic nitrate or nitrite could have beneficial effects on overweight or slight obese patients or in T2D despite no clear correlation between T2D and plasma or urinary levels of nitrate and nitrite (Bahadoran et al., 2015). Joris et al. showed that supplementation with beetroot juice, which is high in inorganic nitrate, improved postpranial endothelial function in slight overweight or obese men (Joris and Mensink, 2013). In patients with T2D, a single dose of inorganic nitrate was suggested to lower basal plasma glucose and improve oral glucose insulin sensitivity index, however no improvement in glucose tolerance was observed (Cermak et al., 2015). In another small study in patients with T2D, Gilchrist and colleagues did not observe improvement in endothelial function or insulin sensitivity (Gilchrist et al., 2013), but their findings suggested that dietary nitrate could improve cognitive function in diabetic patients (Gilchrist et al., 2014). Taken together, despite several studies reporting beneficial properties with inorganic nitrate and nitrite, the data from small size clinical studies are still contradictory and clearly show the need for a carefully conducted large-scale, long-term follow up trial in patients.

In summary, the present study demonstrates an important influence of acute inorganic nitrate treatment in modulating metabolic functions. In aged A_{2B} receptor knockout mice, characterized by metabolic syndrome, inorganic nitrate improved their glucose clearance and this was associated with increased AMPK activation and reduced NADPH oxidase activity in the liver. Similar favorable effects of acute nitrate on glucose disposal was also observed in HFD-treated obese WT mice. Intriguingly, the dose of nitrate was similar to what is found in a single serving of a green leafy vegetable; the predominant dietary source of nitrate. These findings suggest that the beneficial effects of inorganic nitrate act not only long-term but also acutely and future studies should be

aimed at determining the therapeutic value of dietary nitrate supplementation in patients with metabolic disease.

Acknowledgments

We thank Eva Lindgren, Annika Olsson, Carina Nihlen, and Margareta Stensdotter (Department of Physiology and Pharmacology, Karolinska Institutet) for excellent technical contribution. This work was supported by grants from the Swedish Research Council (521-2011-2639), the Swedish Heart and Lung Foundation (20140448), David and Astrid Hagelén Foundation, Jeanssons Foundation (JS2013-00064), Stockholm City Council (ALF), the Bodossaki Foundation (Athens, Greece), and by KID-funding from the Karolinska Institutet.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2015.00222

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Conflict of Interest Statement: Jon O. Lundberg and Eddie Weitzberg are co-inventors on patent applications related to the therapeutic use of inorganic nitrate. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.