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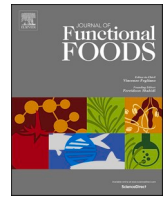
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Chronic nitrite treatment activates adenosine monophosphate-activated protein kinase-endothelial nitric oxide synthase pathway in human aortic endothelial cells

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ABSTRACT

Endothelial dysfunction, with impaired bioavailability and/or bioactivity of the vasoprotective molecule, nitric oxide, appears to be a vital step in the initiation of atherosclerosis. Several studies have shown that dietary nitrate/nitrite can have significant benefits on human cardiovascular homeostasis. Although serum nitrite concentrations can reach micromolar levels, the physiological significance of nitrate/nitrite in normal tissues has not been fully elucidated. We investigated *in vitro* the chronic effects of nitrate/nitrite on endothelial nitric oxide synthase (eNOS) to determine the potential vasoprotective effects of nitrate/nitrite and the underlying molecular mechanisms. Our results demonstrate the expression of phosphorylated eNOS at Ser1177 and phosphorylated adenosine monophosphate activated protein kinase (AMPK) at Thr172 in human aortic endothelial cells were increased after nitrite treatment. We suggest that nitrite stimulation may enhance eNOS activation, which is due, in part, to AMPK activation. The AMPK-eNOS activation by nitrite may be a possible molecular mechanism underlying the vascular protective effects of dietary nitrate.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide (Roth et al., 2017). The aetiology and pathogenesis of CVD is multifactorial, including genetic, environmental, dietary and lifestyle factors (Balakumar, Maung, & Jagadeesh, 2016). Major adverse events of CVD, such as myocardial infarction and stroke, are predominately due to reduced and ultimately blocked blood flow in the corresponding tissues and organs. This arises secondary to a thickening of the inner layer of arteries and the formation of obstructive atherosclerotic plaques, which can impede flow or dislodge and travel elsewhere to block blood flow (Gimbrone & Garcia-Cardena, 2016). Endothelial dysfunction is characterised by disturbed vasodilator and anticoagulant function, increased inflammation and breakdown of barrier function, a vital step in the initiation of atherosclerosis (Gimbrone & Garcia-Cardena, 2016; Vanhoutte, Shimokawa, Tang, & Feletou, 2009).

Endothelium-derived nitric oxide (NO) is an important signalling molecule and is involved in the regulation of cardiovascular homeostasis (Daiber et al., 2019). NO is a potent vasodilator, it inhibits platelet

activation and aggregation, prevents leucocyte adhesion and migration, decreases smooth muscle cell proliferation and migration, and suppresses oxidation of LDL (Cahill & Redmond, 2016). Reduced NO availability is considered the major cause of endothelial dysfunction. In the artery wall, NO deficiency activates a series of atherogenic processes involving: vasoconstriction, adhesion, migration and differentiation of monocytes, proliferation of smooth muscle cells, thrombosis and impaired coagulation, and eventually atherosclerosis (Versari, Daghini, Viridis, Ghiadoni, & Taddei, 2009).

Endothelial nitric oxide synthase (eNOS) is constitutively expressed in endothelial cells. It converts the amino acid L-arginine into L-citrulline and NO (Bondonno, Croft, Ward, Considine, & Hodgson, 2015). eNOS activity is intimately regulated by protein phosphorylation, which is modulated by kinases, phosphatases and protein-protein interactions (Kolluru, Siamwala, & Chatterjee, 2010). Phosphorylation of eNOS occurs on threonine, tyrosine residues, as well as serine 1177 (Ser1177) in the reductase domain (Mount, Kemp, & Power, 2007). eNOS is directly regulated by protein kinase B (Akt) in endothelial cells in response to a variety of cellular stimuli. Activation of Akt at Ser473 leads to eNOS

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phosphorylation, thereby enhancing eNOS activity, and consequently increasing NO generation (Sessa, 2004). Metabolic stress can also trigger the breakdown of ATP, which can stimulate adenosine monophosphate-activated protein kinase (AMPK) to phosphorylate eNOS (Michell et al., 2002; Sessa, 2004).

Inorganic nitrate and nitrite from endogenous and dietary sources are emerging as an alternative pathway for NO generation, especially when the nitric oxide synthase (NOS) pathway is compromised (Lundberg, Weitzberg, & Gladwin, 2008; Machha & Schechter, 2011). In the human body, absorbed dietary nitrate is efficiently converted into nitrite by commensal facultative anaerobic bacteria, and subsequently nitrogen oxides including NO is formed through reduction of nitrite (Lundberg et al., 2008). It has been suggested that the nitrate–nitrite–NO pathway may be critical in maintaining bioactive NO levels and might play a key role in maintaining cardiovascular homeostasis (Bondonno, Croft, & Hodgson, 2016).

eNOS is a major contributor to bioactive NO generation and maintains cardiovascular homeostasis. However, the interactions between nitrate/nitrite and eNOS under physiological conditions have not been clarified to date. We hypothesized that eNOS exerts a significant action in the nitrate–nitrite–NO pathway under normoxic conditions. We investigated the effects *in vitro* of chronic treatment with nitrate and nitrite on eNOS and associated molecules in human aortic endothelial cells (HAECs) to determine potential molecular mechanisms underlying the beneficial effects of nitrite and nitrate.

2. Materials and methods

2.1. Cell cultures and stimulations

HAECs (Lonza Pty Australia) were cultured in commercially available EBM-2 media (Lonza Pty Australia) containing 10% Foetal Bovine Serum (Corning) in T-75 tissue culture flasks (Thermo Fischer Scientific, USA). Cells were grown at 37°C with 5% CO₂ and 18.6% O₂ in a humidified incubator and maintained in a logarithmic growth phase by routine passages every 2–3 days at a 1:3 split ratio. All experiments were performed with cells from passage three to nine in 6-well culture plates (Thermo Fischer Scientific, USA).

The cells were maintained in regular media prior to all experiments. Following a 4 hr serum starvation, the cells were treated with sodium nitrite (NaNO₂) (final concentration of 0.1 μM and 1 μM) or sodium nitrate (NaNO₃) (final concentration of 1 μM and 10 μM) for 30 min or 24 hr. To assess the contribution of eNOS on the response to NaNO₂, cells were pre-incubated with 100 μM N (gamma)-nitro-L-arginine methyl ester (L-NAME, NOS inhibitor) for 30 min. The role of AMPK in modulating eNOS activation was determined by incubation with 20 μM Compound C (AMPK inhibitor) or 1.25 mM 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR, AMPK activator) for 1 hr. At the end of the treatment periods, cell lysates were collected and stored at –80 °C until further analysis.

2.2. Immunoblotting

The cells were washed twice with ice-cold PBS then lysed and harvested in 200 ml Laemmli's SDS-sample buffer (Bio-Rad, Australia), containing 250 mM Tris-HCl, 8% SDS, 40% glycerol, 8% β-mercaptoethanol, 0.02% bromophenol blue, 5% 2-mercaptoethanol and a phosphatase inhibitor cocktail (Roche Diagnostics, Australia). Thirty micrograms of protein per sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 4%–10% polyacrylamide gels (Bio-Rad, Australia), and transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, USA) overnight at 4°C. The membranes were blocked with 5% skim milk for 1 hr at room temperature and probed with primary antibody overnight at 4°C (1:500 or 1:1000). Membranes were washed in Tris-buffered saline containing 0.1% Tween followed by incubation with HRP-conjugated secondary

antibodies (1:5000) at room temperature for 1 hr. Proteins were visualized using the enhanced chemiluminescence kit (Thermo Fischer Scientific, USA) under a ChemiDoc XRS system (Bio-Rad). Protein bands were analysed using the image lab software (Bio-Rad) and normalized to the parent protein or Pan-actin.

2.3. Antibodies and chemicals

Antibodies for AKT (#4691), p-AKT^{Ser473} (#4060), AMPKα (#2603), p-AMPK^{Thr172} (#2535), ACC (#3676), p-ACC^{Ser79} (#3661), Pan-Actin (#4968) and p-eNOS^{Ser1177} (#9571) were purchased from Cell Signaling Technologies (Denver, MA, USA). Antibodies for eNOS (#610298) was purchased from BD Biosciences (San Jose, CA, USA). NaNO₂, NaNO₃, potassium iodide, iodine, Compound C, L-NAME and AICAR were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were obtained from RPH Medical Research Foundation. Stock solutions of NaNO₂ (20 mM) and NaNO₃ (20 mM) were prepared with fresh Milli-Q water.

2.4. Statistics

All immunoblots shown are the representative results from 4 independent experiments. Values are presented as means ± SEM. Comparisons between groups were performed by one-way ANOVA followed by Tukey–Kramer or Dunnett's post-hoc test. A value of *p* < 0.05 was considered statistically significant. All analyses were carried out using the GraphPad Prism 7.

3. Results

3.1. Effects of nitrite and nitrate on eNOS phosphorylation in HAECs

To confirm eNOS is involved in the actions of nitrite and nitrate, we determined the level of phosphorylated eNOS at Ser1177. Following nitrite treatment for 24 hr, the expression of p-eNOS was significantly increased (Fig. 1). In contrast, treatment of HAECs with nitrate did not alter the ratio of phosphorylated eNOS/total eNOS (data not shown). And the level of phosphor-eNOS (Ser 1177) in HAEC stimulated by sodium nitrate or nitrite for 30 min was not changed (data not shown). To further confirm the role of eNOS activation, we treated cells with nitrite in the presence of L-NAME and saw significant reductions in p-eNOS (Fig. 2).

3.2. Nitrite stimulation induces AMPK activation without Akt activation

As AMPK activation is known to contribute to eNOS phosphorylation and activation in endothelial cells, we compared the phosphorylation of AMPK in response to nitrite treatment and observed a significant increase in the level of AMPK phosphorylation at Thr172 (Fig. 3). This also resulted in an increase in eNOS phosphorylation (Fig. 3) and acetyl-CoA carboxylase (ACC) Ser79 phosphorylation (Fig. 3). In contrast, the level of phosphorylated Akt was not significantly increased following nitrite treatment (Fig. 3).

3.3. AMPK lies upstream to the eNOS activation

Compound C, as an inhibitor of AMPK, was used to verify whether AMPK lies upstream of the eNOS nitrite interaction. The effects of nitrite on the phosphorylation of AMPK and eNOS were both almost completely abolished in the presence of Compound C (Fig. 4), suggesting AMPK activation takes place first.

4. Discussion

NO is one of the most important biological mediators involved in maintaining vascular homeostasis. In addition to its ability to influence

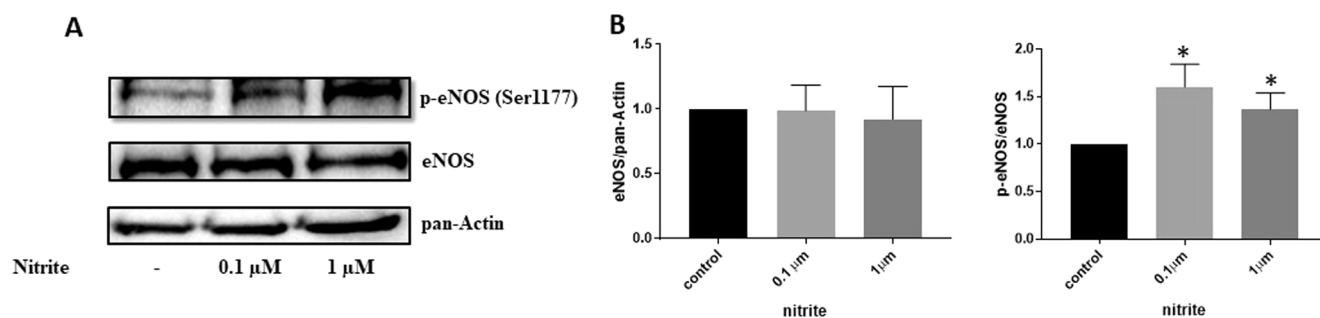


Fig. 1. The expression and activity of eNOS in HAECs treated with sodium nitrite (0.1 μ M and 1 μ M, 24hr). (A) Western blot protein expression of total eNOS, eNOS phosphorylation (Ser1177) and Pan-actin in HAECs. (B) The expression level of p-eNOS and eNOS was measured by the densitometry of the corresponding bands and normalised to Pan-actin using Image Lab. Values are means \pm SEM, n = 4/group, *p < 0.05 vs control. p-eNOS: phosphorylated endothelial nitric oxide synthase; eNOS: endothelial nitric oxide synthase; HAECs: human aortic endothelial cells.

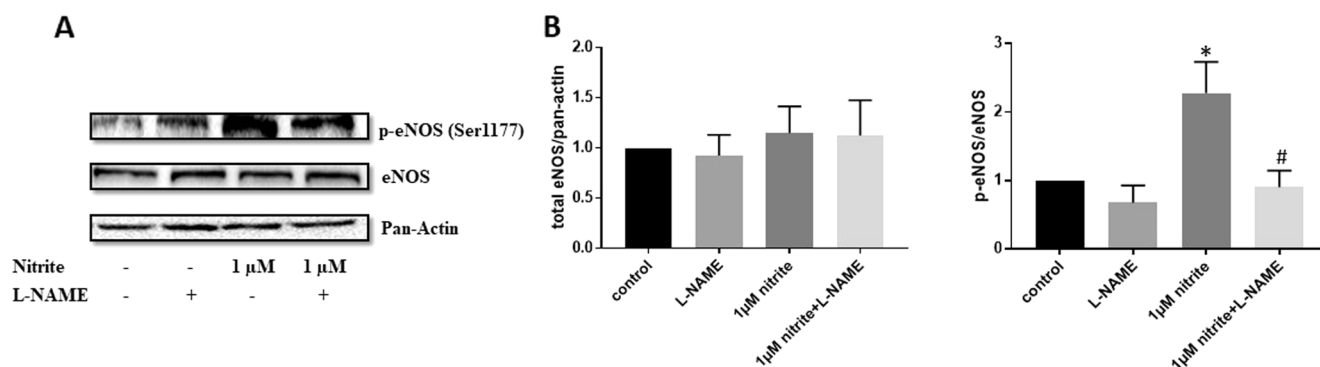


Fig. 2. The expression and activity of eNOS in HAECs treated with sodium nitrite (1 μ M, 24hr) in the presence and absence of L-NAME (100 μ M). (A) Western blot protein expression of total eNOS, phosphorylated eNOS (Ser1177) and Pan-actin in HAECs. (B) The density of the western blotting band analyzed by Image Lab. Values are means \pm SEM, n = 4/group, *p < 0.05 vs control, #p < 0.05 presence of inhibitor vs respective treatment. p-eNOS: phosphorylated endothelial nitric oxide synthase; eNOS: endothelial nitric oxide synthase; HAECs: human aortic endothelial cells; L-NAME: N-Nitroarginine methyl ester.

basal vascular tone, NO is also recognized as a key determinant of vascular health by modulating platelet-endothelial interactions, exerting antithrombotic, and anti-inflammatory properties within the vasculature. Impaired NO bioavailability represents the central feature of endothelial dysfunction, a common abnormality found in many vascular diseases. Atherosclerosis, hypercholesterolemia, hypertension, diabetes mellitus, thrombosis and stroke have all been linked to abnormalities in NO signaling (V. W. T. Liu & Huang, 2008). There are two major pathways for the NO formation in the body—the endogenous NOS-dependent system and the exogenous nitrate-nitrite-NO pathway from dietary uptake of green leafy vegetables (Bleckenhorst et al., 2018; Bondonno et al., 2016). The dietary pathway is considered a NO reservoir, which contributes to overall NO availability (Milsom, Fernandez, Garcia-Saura, Rodriguez, & Feelisch, 2012). Dietary nitrate is converted to nitrite by anaerobic bacteria in the mouth. The nitrite anion is a cell-signaling molecule, which is considered as a storage pool of NO (Y. Liu, Croft, Hodgson, Mori, & Ward, 2020). Once formed, nitrite can be utilized via various nonenzymatic and enzymatic pathways and converted to bioactive NO in the circulatory system and tissues (Kim-Shapiro & Gladwin, 2014; Weitzberg, Hezel, & Lundberg, 2010). Under hypoxic conditions, nitrite may serve as a substrate for endothelium-derived generation of NO (Gautier, van Faassen, Mikula, Martasek, & Slama-Schwok, 2006). Here, we suggest a possible new way that diet derived nitrate/nitrite can activate eNOS in normoxic conditions, with nitrite increasing eNOS phosphorylation in cultured HAECs. This finding is consistent with a previous report showing eNOS phosphorylation in response to treatment with sodium nitrite (Ling, Lau, Murugan, Vanhoutte, & Mustafa, 2015).

We used HAECs as a cell model and observed that 0.1 μ M to 1 μ M nitrite treatment for 30 min did not significantly change AMPK

phosphorylation and eNOS activation (data not shown). However, with a prolonged treatment (24 hr), we demonstrated that nitrite activates AMPK and eNOS. In contrast, a previous study showed that 30 min nitrite treatment stimulates eNOS phosphorylation in an AMPK dependent manner in human glomerular endothelial cells (HGEC) (Miyamoto et al., 2017). However, that study used a higher nitrite concentration (10 μ M) and a different cell line (Miyamoto et al., 2017). The physiological concentration of nitrite in blood is likely <1 μ M (Gladwin et al., 2000).

AMPK is an important monitor of cellular metabolism and energy homeostasis and regulates almost all aspects of cellular function, including autophagy, mitochondrial homeostasis, cell polarity, cell growth and cell proliferation (Hardie, 2011; Mihaylova & Shaw, 2011). In addition to being activated in response to stimuli that deplete cellular ATP supplies, including oxidative stress, nutrient deprivation or hypoxia, AMPK can also be stimulated following exposure to shear stress, growth factors, and cytokines (Fisslthaler & Fleming, 2009). In mammals, AMPK is a heterotrimeric complex composed of a catalytic subunit (α), and two regulatory subunits (β and γ) (Hardie, 2007). Phosphorylation of Thr172, which lies in the activation loop of the α -subunit is required for AMPK activation (Stein, Woods, Jones, Davison, & Carling, 2000). During the last decade, a number of upstream kinase activities have been identified (Mihaylova & Shaw, 2011). Among them, calcium/calmodulin-dependent protein kinase kinase (CAMKK) and liver kinase B1 (LKB1) are now widely accepted to be the predominant AMPK upstream kinases (Carling, Sanders, & Woods, 2008). Previous studies have shown that both LKB 1 and CAMKK exist in endothelial cells and mediate AMPK activation under different conditions (Stahmann, Woods, Carling, & Heller, 2006). *In vitro* studies have demonstrated that activated AMPK can prevent oxidative stress injury in HAECs via increased phosphorylation of eNOS at Ser1177 (Li, Reif, Craige, Kant, & Keaney, 2016). A

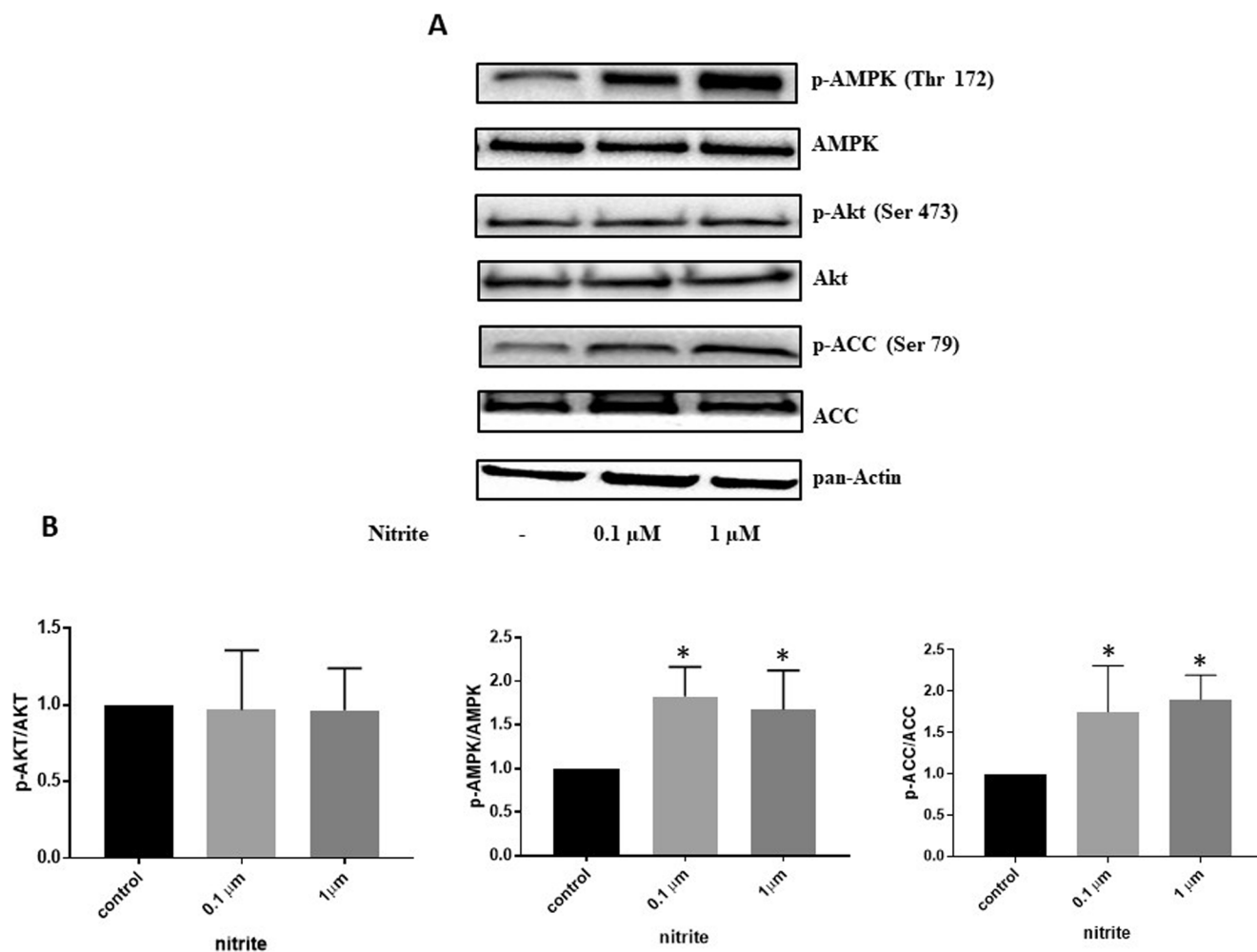


Fig. 3. The expression and activity of Akt, AMPK and in HAECs treated with sodium nitrite (0.1 μM and 1 μM, 24hr). (A) Western blot protein expression of total AMPK, phosphorylated AMPK (Thr172), total Akt, phosphorylated Akt (Ser473), total ACC and phosphorylated ACC (Ser79) and in HAECs. (B) The density of the western blotting band analyzed by Image Lab. Values are expressed as means ± SEM, n = 4/group, *p < 0.05 vs control. p-Akt: phosphorylated Protein kinase B; Akt: Protein kinase B; p-AMPK: phosphorylated 5' adenosine monophosphate-activated protein kinase; AMPK: 5' adenosine monophosphate-activated protein kinase; p-ACC: phosphorylated acetyl-CoA carboxylase; ACC: acetyl-CoA carboxylase; HAECs: human aortic endothelial cells.

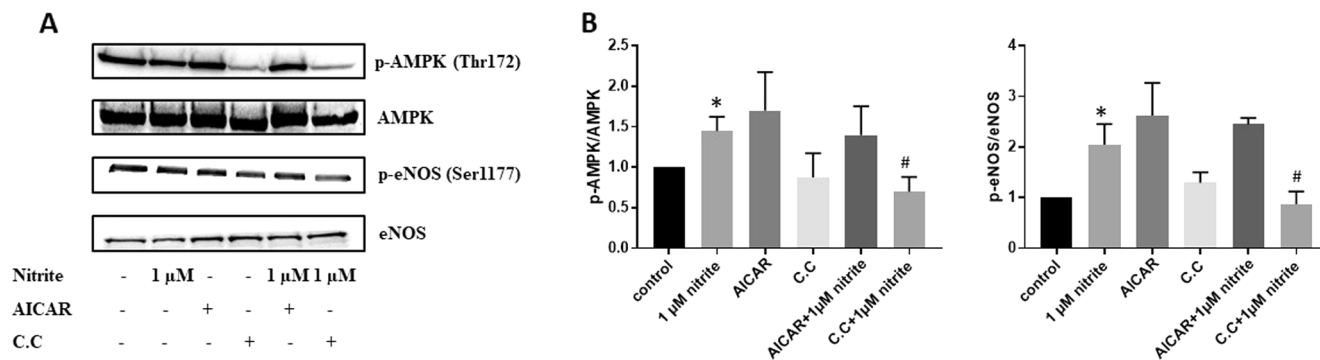


Fig. 4. The expression and activity of phosphorylated AMPK and phosphorylated eNOS in HAECs treated with sodium nitrite (1 μM, 24hr) in the presence and absence of AICAR (AMPK activator, 1.25 mM) or Compound C (20 μM). (A) Western blot protein expression of total AMPK, phosphorylated AMPK (Thr172), total eNOS and phosphorylated eNOS (Ser117) in HAECs. (B) The density of the western blotting band analyzed by Image Lab. Values are expressed as means ± SEM, n = 4/group, *p < 0.05 vs control, #p < 0.05 presence of inhibitor vs respective treatment. p-eNOS: phosphorylated endothelial nitric oxide synthase; eNOS: endothelial nitric oxide synthase; p-AMPK: phosphorylated 5' adenosine monophosphate-activated protein kinase; AMPK: 5' adenosine monophosphate-activated protein kinase; HAECs: human aortic endothelial cells; AICAR: 5-Aminoimidazole-4-carboxamide ribonucleotide; Compound C: dorsomorphin.

recent study has shown that nitrite restored phosphorylated AMPK levels in steatosis HepG2 cells (Cordero-Herrera et al., 2019). This supports our present findings, where we saw increased AMPK

phosphorylation at significantly lower doses of nitrite, in a human cell line that is more directly related to cardiovascular disease. Prolonged supplementation with 10 μM nitrite can also increase activation of

AMPK in insulin-resistant human skeletal muscle cells (Lai et al., 2016). It has also been reported that AMPK can be activated to stimulate mitochondrial biogenesis in response to nitrite exposure (50 μ M, 3hr) in rat aortic smooth muscle cells under hypoxic conditions (Mo et al., 2012). The protection mediated by nitrite and thought to be dependent on AMPK activation is optimized under the conditions of anoxia and low pH. In addition, nitrite not only confers cardioprotection when administered during ischaemia, but also when transiently present hours prior to the onset of an ischaemic episode (Kamga Pride et al., 2014). Consistent with our results, nitrite activates AMPK in normoxia, but the underlying mechanisms are unclear. When intracellular ATP is low, AMPK might be activated by both LKB1 and CAMKK. As a result, activated AMPK mediates eNOS activation in a LKB1 dependent manner (Thors, Halldorsson, & Thorgeirsson, 2011). Conversely, under conditions where intracellular ATP is unchanged, there is less NO generation and activation of AMPK is only dependent on CAMKK. Thus eNOS is phosphorylated and activated independently of AMPK (Thors et al., 2011). Although we did not measure ATP concentration in culture media, it may be speculated that ATP levels will be decreased by nitrite stimulation over time. Thus, it is likely that eNOS phosphorylation is AMPK dependent and this is supported by the fact that the activation of AMPK and eNOS were almost completely abolished by treatment with Compound C. Unfortunately due to the gas phase chemiluminescence methodology used to measure NOx we were unable to confirm increased NO production in our study as the concentration of NOx measured in the culture media also included the nitrite which was added to the cells (data not shown).

It is well-established that the phosphatidylinositol 3-kinase (PI3K)–Akt cascade is a commonly activated signalling pathway that regulates eNOS activation in endothelial cells (Fleming & Busse, 2003). eNOS Ser 1177 has been shown to be phosphorylated by Akt as well as AMPK (Zhang et al., 2009). However, Akt is unlikely to promote eNOS activation mediated by nitrite, as no significant change in Akt phosphorylation levels was detected following nitrite treatment. Therefore, nitrite is proposed to mediate eNOS activation in an AMPK-dependent, but not Akt-dependent manner. AMPK's role as an energy sensor has been well established in many tissues and organs such as the skeletal muscle and heart. However, its exact role in endothelial cell metabolism remains incompletely understood. It is believed that endothelial cells exist in a more or less permanent state of "metabolic hypoxia" (Fisslthaler & Fleming, 2009) and AMPK may have a more important role in the maintenance of endothelial function. AMPK is the only kinase identified to date that can potentially phosphorylate eNOS on Ser633 (Chen et al., 2009) which might be more efficacious than Ser1177 in augmenting the eNOS-derived NO bioavailability (Bauer et al., 2003). Furthermore, phosphorylation of Ser633 seems to occur after eNOS Ser1177 phosphorylation, where this post-translational modification of Ser633 is proposed to maintain persistent eNOS activity after its initial activation (Boo et al., 2002). Future investigation is needed to clarify whether phosphorylation of Ser633 is stimulatory for eNOS activity by nitrite stimulation.

In conclusion, we have shown that nitrite at physiological concentrations is an activator of the AMPK–eNOS pathway in human aortic endothelial cells. Nitrite, may increase NO production via activating AMPK, followed by eNOS activation in addition to non-enzymatic pathway as previously reported. The AMPK–eNOS activation by nitrite could be a potential molecular mechanism underlying the cardioprotective effects of dietary nitrate.

Author contribution

The work described in this manuscript was developed by NCW and KDC in conjunction with the other authors. Laboratory work was carried out by YL and all authors contributed to the analysis, interpretation and presentation of the findings.

Ethical statement

This work did not include any human or animal subjects or samples.

Declaration of Competing Interest

The authors declare no conflict of interest.

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