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Nitrite activates protein kinase A in normoxia to mediate mitochondrial fusion and tolerance to ischaemia/reperfusion

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Aims Nitrite (NO_2^-), a dietary constituent and nitric oxide (NO) oxidation product, mediates cardioprotection after ischaemia/reperfusion (I/R) in a number of animal models when administered during ischaemia or as a pre-conditioning agent hours to days prior to the ischaemic episode. When present during ischaemia, the reduction of nitrite to bioactive NO by deoxygenated haem proteins accounts for its protective effects. However, the mechanism of nitrite-induced pre-conditioning, a normoxic response which does not appear to require reduction of nitrite to NO, remains unexplored.

Methods and results Using a model of hypoxia/reoxygenation (H/R) in cultured rat H9c2 cardiomyocytes, we demonstrate that a transient (30 min) normoxic nitrite treatment significantly attenuates cell death after a hypoxic episode initiated 1 h later. Mechanistically, this protection depends on the activation of protein kinase A, which phosphorylates and inhibits dynamin-related protein 1, the predominant regulator of mitochondrial fission. This results morphologically, in the promotion of mitochondrial fusion and functionally in the augmentation of mitochondrial membrane potential and superoxide production. We identify AMP kinase (AMPK) as a downstream target of the mitochondrial reactive oxygen species (ROS) generated and show that its oxidation and subsequent phosphorylation are essential for cytoprotection, as scavenging of ROS prevents AMPK activation and inhibits nitrite-mediated protection after H/R. The protein kinase A-dependent protection mediated by nitrite is reproduced in an intact isolated rat heart model of I/R.

Conclusions These data are the first to demonstrate nitrite-dependent normoxic modulation of both mitochondrial morphology and function and reveal a novel signalling pathway responsible for nitrite-mediated cardioprotection.

Keywords Ischaemia • Protein kinase A • Pre-conditioning • Nitrite/nitrate • Mitochondria

1. Introduction

Nitrite, a dietary constituent and nitric oxide (NO) oxidation product, has emerged as an intrinsic signalling molecule that mediates cytoprotection during ischaemia/reperfusion (I/R) in a number of organ systems.[1–7] In the heart, nanomolar increases in the circulating nitrite concentration significantly decrease infarct size in murine and canine models of myocardial infarction.[8–10] This protection, thought to be dependent on the reduction of nitrite to bioactive NO by myoglobin and xanthine oxidoreductase in the heart, is optimized in the ischaemic conditions of anoxia and low pH.[5,10–13] Notably, we and others have observed that, in a manner mimicking ischaemic pre-conditioning (IPC), nitrite not only confers cardioprotection when administered during ischaemia, but also when transiently present hours prior to the onset of the ischaemic episode.[1,9,14,15] While this phenomenon suggests that nitrite is able to mediate signalling even in non-ischaemic conditions, the mechanisms underlying this normoxic nitrite-dependent pre-conditioning are unknown.

Modulation of mitochondrial function contributes to the cardioprotection conferred by a number of pre-conditioning agents including

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sub-lethal ischaemia,[16,17] volatile anaesthetics,[18] and adenosine.[19] For example, the inhibition of ATP production and the incremental augmentation of mitochondrial reactive oxygen species (ROS) production have been linked to cardioprotection induced by IPC.[20] These alterations in mitochondrial function induce protective downstream adaptive responses including the activation of the metabolic sensor AMPK.[21,22] More recently, accumulating evidence suggests that changes in mitochondrial dynamics (fission and fusion), resulting in altered mitochondrial tubular networks within the cell, can modulate the cellular response to I/R.[16] Pharmacological inhibition of the fission regulatory protein, dynamin-related protein-1 (Drp1), or overexpression of the fusion promoting mitofusins (Mfn1 and 2), resulting in the formation of elongated cellular mitochondrial networks, has been shown to decrease infarct size in rodent models of myocardial infarction.[16,23] While the activity of Drp1 is modulated by post-translational modifications such as protein kinase A (PKA)-dependent phosphorylation,[24] the signalling mechanisms that underlie PKA-mediated phosphorylation of Drp1 in the cardiomyocyte remain unknown. While we have previously shown that nitrite modulates mitochondrial function during I/R by reversibly inhibiting complex I activity, concomitantly decreasing reperfusion ROS generation and preventing permeability pore opening,[14] the effect of nitrite on mitochondrial dynamics, particularly during non-ischaemic conditions, has not been explored.

Herein, we investigate the mechanism by which normoxic nitrite confers pre-conditioning. We demonstrate for the first time in H9c2 cardiomyocytes that nitrite modulates mitochondrial dynamics by activating protein kinase A to phosphorylate and inhibit Drp1, leading to the enhancement of mitochondrial fusion in normoxia. Functionally, this augments mitochondrial superoxide production, which oxidizes and activates AMPK, an essential step in nitrite-mediated pre-conditioning. These data are the first to show that nitrite regulates mitochondrial morphology and function in normoxia and that this modulation is important for cardioprotection. The implications of these results will be discussed in the contexts of mitochondrial physiology as well as the role of nitrite not only as a potential therapeutic agent, but also as a natural component of cardioprotective diets.

2. Experimental procedures

2.1 Materials

All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) except where indicated. H9c2 cardiomyocytes were purchased from ATCC (Rockville, MD, USA). All antibodies were commercially available and detailed information as well as western blot conditions are supplied in Supplementary material online, Methods.

2.2 Animals

Twelve-week-old-male Sprague–Dawley rats (Harlan Sprague Dawley, Inc. Indianapolis, IN, USA) were anaesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium, and anaesthesia was monitored by pinching of the toe.

2.3 Hypoxia/reoxygenation (H/R)

Control cells were maintained in normoxia (21% O_2, 5% CO_2) throughout. Ischaemia was simulated by subjecting cells to hypoxia, (1% O_2, 5% CO_2, 94% N_2; 5 h), in modified Esumi buffer [137 mmol/L NaCl, 12 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 0.9 mmol/L $CaCl_2$, 20 mmol/L HEPES; 20 mmol/L 2-deoxy-D-glucose (2-DG), pH 6.2][25] as described by previous publications.[26–28] Cells were then reoxygenated in the same buffer in normoxia (21% O_2, 5% CO_2) for 1 h. IPC was achieved by incubating cells in Esumi buffer without 2-deoxy-D-glucose (2-DG) in hypoxia (1% O_2, 5% CO_2, 94% N_2; 30 min), followed by a delay time (1 h) in normoxia (in DMEM–FBS cell growth medium) before H/R (as described above) was initiated. Nitrite treatment (0–100 μM) was for 30 min in normoxia in growth medium, followed by washing of the cells with PBS once and a delay time (0–6 h in growth medium) prior to the onset of H/R (*Figure 1A*).

2.4 Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity in the media was measured spectrophotometrically by measuring the decrease in NADH at 340 nm and was expressed as a per cent of total LDH in the media and lysed cells.

2.5 Oxygen consumption

Oxygen consumption rate was measured using the Seahorse XF24 Extracellular Flux analyser (Seahorse Bioscience, Billerica MA) as described in Mo *et al.*[29] and Supplementary material online, Methods.

2.6 Membrane potential

Membrane potential was measured using TMRM (Molecular Probes, Eugene, CA, USA), according to manufacturer's instructions. Cells (in PBS buffer) were incubated with 1 μmol/L TMRM and fluorescence monitored at 544/574 nm prior to and after the addition of nitrite, oligomycin, and FCCP. Membrane potential was quantified as a per cent of the total range (determined by the difference between Oligomycin and FCCP signals).

2.7 Superoxide measurement

Intra-mitochondrial superoxide levels were quantified using MitoSOX^{TM} Red (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. Briefly, cells were pre-loaded with MitoSOX Red (5 μmol/L; 37°C; 15 min). After three washes, fluorescence (510/580 nm) was read using a Synergy plate reader (BioTek Instrument, Inc., Winooski, VT, USA) prior to and after the injection of nitrite into the well. Alternatively, cells were pre-loaded with MitoSOX Red, washed three times and confocal microscopy images were taken over time.

2.8 Aconitase activity

Aconitase activity was measured by spectrophotometrically monitoring the conversion of citrate to isocitrate as previously described.[30]

2.9 Generation of Rho 0 cells

Mitochondrial DNA-depleted (Rho 0; ρ^0) cells were created by growing H9c2 cells in media supplemented with ethidium bromide (0.634 μmol/L), uridine (0.205 mmol/L), and sodium pyruvate (1 mmol/L) for 7–21 days.

2.10 Adenoviral transfection

The constructs for the Adenoviruses AdCMVCatalase (AdCat) and Adempty (Vector) were manufactured by inserting catalase and LacZ genes into the E1 region of an Ad5 E1/partial E3-deleted replication-deficient adenoviral vector.[31]

2.11 Silencing of AMPKα

Cells were transfected with either rat AMPKα1 siRNA (sc-270142; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or control siRNA

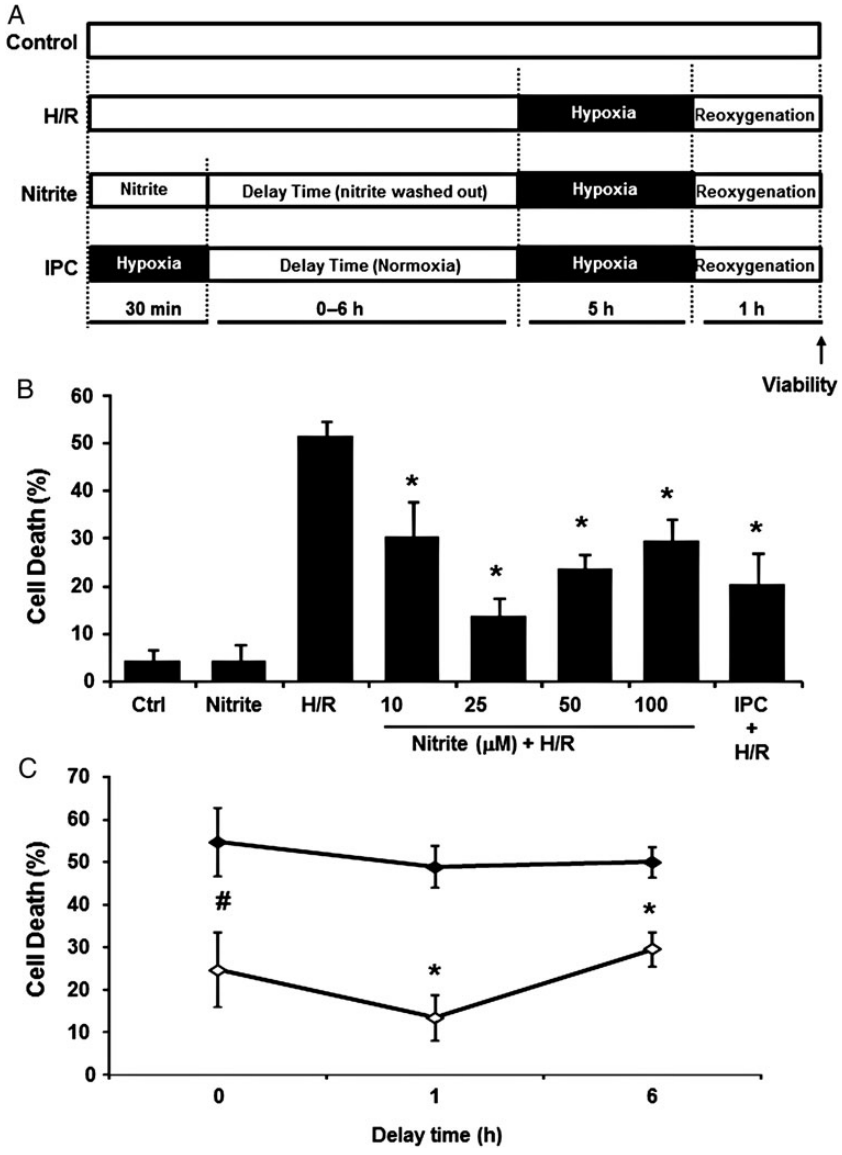

Figure 1 Nitrite mediates cytoprotection after *in vitro* H/R. (*A*) Experimental model to simulate I/R. H9c2 cells were subjected to hypoxia (1% O_2, 5% CO_2; 20 mmol/L 2-DG; pH 6.2; 5 h), followed by reoxygenation (21% O_2, 1 h). Nitrite was administered at 21% O_2 for 30 min, after which nitrite was washed out and cells kept in normoxia for a delay time of 0–6 h prior to H/R. IPC treatment was 1% O_2 (30 min). (*B*) Percentage of dead cells (measured by cellular LDH release) after H/R initiated 1 h after nitrite (0–100 μmol/L) treatment. $n = 5$; $n = 3$ for normoxic nitrite, 100 μmol/L and IPC. (*C*) Percentage of H/R-induced death in cells treated with (open symbols) or without (filled symbols) nitrite (25 μmol/L) 0–6 h prior to H/R. Asterisks indicate $P < 0.01$ and hash indicates $P < 0.05$ vs. H/R; $n = 4$ per group.

(sc-37007), according to manufacturer's instructions. Knockdown of AMPKα1 expression was verified by western blot using anti-AMPKα1 (C-20) (sc-19128) antibody.

2.12 GFP-Drp1 plasmid transfection

Drp1 mutant plasmids containing either wild-type Drp1 or the S656A mutant Drp1 (with shRNA to silence wild-type Drp1) were a kind gift from Dr Stefan Strack and were used as characterized and described in Cribbs and Strack.[32]

2.13 AMPK oxidation

Oxidation was assessed by a modified biotin switch method adopted from Wang *et al.*,[33] and described in Supplementary material online, Methods.

2.14 PKA activity

PKA activity was measured by determining the level of PKA-substrate phosphorylation by ELISA using the PKA activity kit (Enzo Life Sciences; ADI-EKS-390A).

2.15 Immunofluorescence microscopy

Cell imaging was performed as described in Supplementary material online, Methods. Briefly, cells were treated with nitrite (25 μM) and, following nitrite removal, were fixed using 1% paraformaldehyde, and then stained with rabbit anti-TOM20 (SantaCruz, clone sc-11415, 1:1000) and imaged for mitochondrial morphology using a Provis AX70 research microscope (Olympus, Center Valley, PA, USA) using a ×60 objective lens, and processed with MagnaFire (Optronics, Goleta, CA, USA). In parallel experiments, cells were transfected with the CellLight® Mitochondria-GFP reagent (Molecular Probes, Invitrogen) according to manufacturer's instructions, and the next day, treated with or without nitrite, and then imaged by microscopy.

2.16 Mitochondrial isolation

For measurement of Drp1 translocation, cells were lysed and subjected to differential centrifugation as described previously. LDH activity was measured in the mitochondrial fraction to determine purity of the preparation and found to be undetectable.

2.17 Isolated perfused heart

Hearts were isolated from male Sprague–Dawley rats (250 g) and subjected to retrograde perfusion with Krebs–Henseleit (KH) buffer as previously described in Curtis *et al.*[34] and in Supplementary material online, Methods. Infarct size was determined at 2 h by TTC staining and left ventricular developed pressure monitored throughout as previously described.[14,34]

2.18 Mitochondrial permeability transition pore assay

Mitochondrial permeability transition pore (MPTP) opening was assessed by measuring quenching of calcein-AM fluorescence as described in Hausenloy *et al.*[35] H9c2 cells were treated with or without nitrite, in the presence or absence of compound C, and then after 1 h of washout, were subjected to H/R. Cells were co-loaded with calcein-AM (1 μmol/L; Molecular Probe) and cobalt-chloride ($CoCl_2$ 1 mmol/L) at 37°C for 20 min, resulting in mitochondrial localization of calcein fluorescence. MPTP opening was measured as the decrease in mitochondrial calcein signal (expressed as the percentage of the initial value) using a microplate reader (emitting at 488 nm and detecting at 505 nm).

2.19 Image analysis of mitochondrial morphology and density measurements

To quantify mitochondrial morphology, custom-written macros were developed for the NIH Image J software (1.44) (http://www.imagejdocu.tudor.lu), as previously described in Dagda *et al.*[36] and Supplementary material online, Methods.

2.20 Statistics

All values are means ± SEM of at least three independent experiments. *N* values for each experiment are listed in the figure legend. Single comparisons were tested for significance by two-tailed Student's *t*-test. Multiple comparisons were made using ANOVA followed by Bonferroni correction. Significance is noted when $P < 0.05$.

3. Results

3.1 Nitrite mediates cytoprotection in an *in vitro* model of hypoxia/reoxygenation

To determine whether normoxic nitrite-mediated protection occurs at the level of the myocyte, an *in vitro* model of hypoxia/reoxygenation (H/R) using H9c2 cells was established to simulate I/R (*Figure 1A*). After subjecting cells to hypoxia (1% O_2; 5 h), then reoxygenation (21% O_2; 1 h), significant cell death (51.3 ± 3.4%), measured by LDH release, was observed compared with normoxic controls (4.3 ± 2.4%) (*Figure 1B*). To determine whether nitrite could prevent death in this model, cells were treated with nitrite (10–100 μmol/L) in normoxia for 30 min, after which they were washed to remove nitrite. Measurement of nitrite levels (372 ± 8.4 nmol/L in nitrite-treated cells vs. 382 ± 9.3 nmol/L in control cells) after washout demonstrated that all nitrite was removed for the washout period. The cells were then incubated in normoxia for a delay time of 1 h before being subjected to H/R. Concentrations of nitrite 10 μmol/L and above significantly attenuated cell death when administered 1 h before the onset of hypoxia (*Figure 1B*), demonstrating that nitrite mediates pre-conditioning in this model. Nitrite-mediated protection showed a biphasic concentration response, in which the maximal effect was observed at 25 μmol/L and was similar in magnitude to the protection mediated by IPC, a well-characterized classical cytoprotective program[37,38] (*Figure 1B*). The inclusion of sodium bicarbonate (25 mM) to maintain a stable pH during H/R did not affect the ability of nitrite to mediate protection, suggesting that nitrite-dependent pre-conditioning was not pH dependent (Supplementary material online, *Figure S1*). A time course, in which the delay time between the end of nitrite treatment and the initiation of hypoxia, was varied (0–6 h), revealed that nitrite could effectively prevent cell death when transiently present up to 6 h prior to the hypoxic episode (*Figure 1C*). Collectively, these data demonstrate that nitrite mediates tolerance to H/R in myocytes over a wide range of concentrations and times.

3.2 Nitrite-dependent PKA activation and mitochondrial fusion are required for cytoprotection

We next sought to determine the mechanism of nitrite-mediated pre-conditioning. Modulation of mitochondrial function has been implicated in ischaemic and pharmacological pre-conditioning and changes in mitochondrial dynamics, particularly increased mitochondrial fusion, confer cardioprotection.[16,39] Thus, we first examined the effect of nitrite on mitochondrial morphology. Cells were treated with or without nitrite and then stained with antibodies specific for the translocase of the outer mitochondrial membrane of 20 kDa (TOM20) (*Figure 2A*) or mitochondrially targeted GFP (Supplementary material online, *Figure S2A*) to visualize mitochondrial networks. Nitrite significantly increased mitochondrial interconnectivity (0.37 ± 0.03 vs. 0.26 ± 0.01 in untreated cells), consistent with increased mitochondrial fusion (*Figure 2A*).

Mitochondrial networks are altered in response to changes in the balance between mitochondrial fusion and fission. To determine whether nitrite-mediated fusion was a result of increased fusion or the inhibited fission, the effect of nitrite on Drp1 and Mfn1, the major catalytic enzymes that mediate fission and fusion, respectively, was assessed. There was no significant change in Mfn1 expression 1 h after nitrite treatment (data not shown). Drp1 is regulated predominantly by post-translational modification, with phosphorylation of the protein at serine 656 (S656) leading to the inhibition of its activity and an attenuation of fission.[24,32].

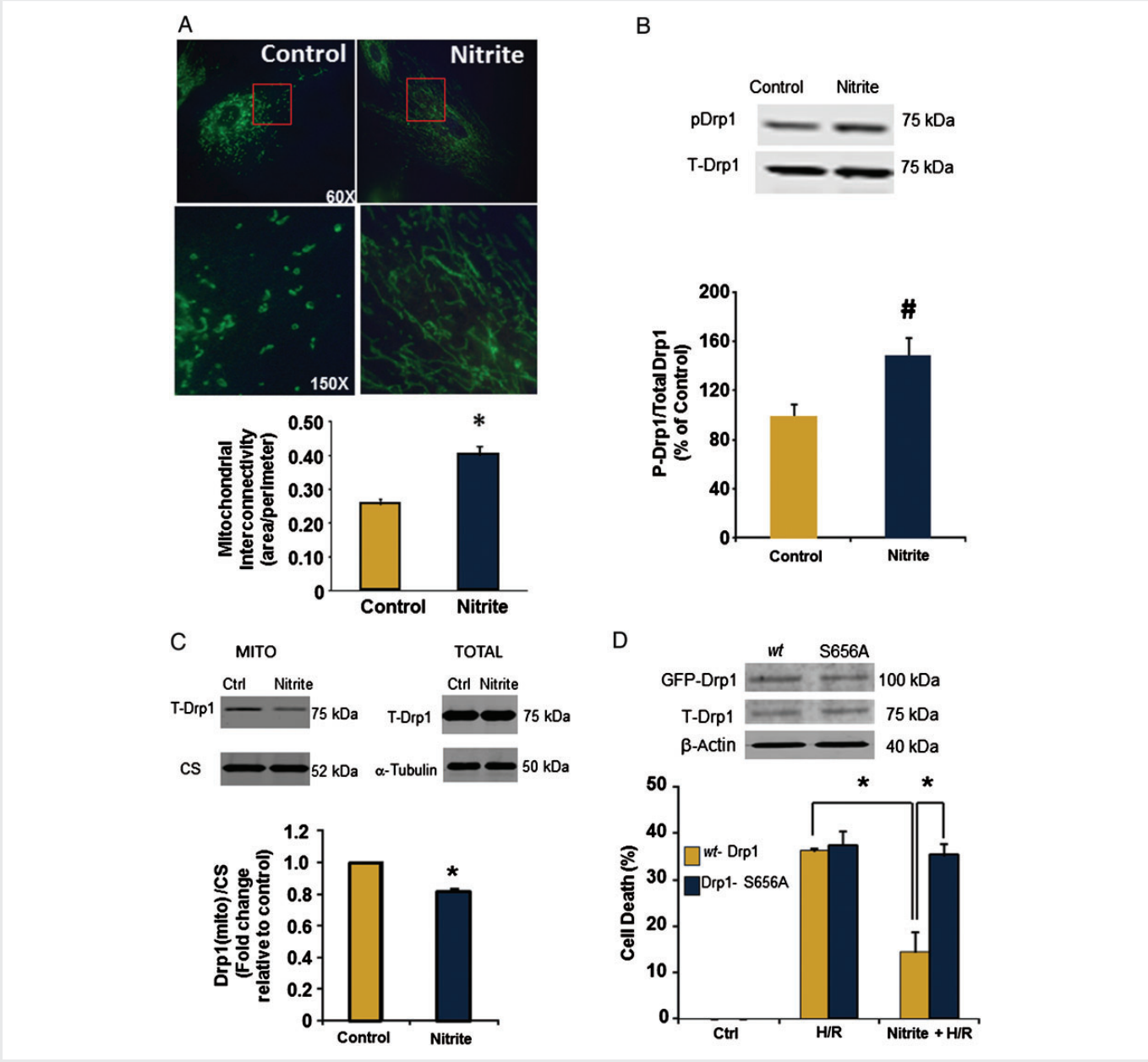

Figure 2 Nitrite promotes mitochondrial fusion by increasing Drp1 phosphorylation. Cells were treated with or without nitrite (25 μmol/L; 21% O_2, 30 min) and collected 1 h after nitrite removal. (*A*) Cells stained with antibodies to TOM20. Top panel is ×60 magnification and bottom panel is ×150 magnification of image section in red box. Quantification of interconnectivity using $n = 20–23$ fields/group. (*B*) Representative immunoblot for phosphorylated and total Drp1 and quantification of Phospho-Drp1 levels (normalized to total Drp1). $n = 4$. (*C*) Representative immunoblot and quantification of Drp1 expression (and citrate synthase; CS) in whole cells and the mitochondrial fraction of cells treated with or without nitrite (25 μmol/L). (*D*) Protein expression of exogenous (GFP-Drp1) and endogenous (T-Drp1) in transfected cells. Cell death after H/R in wild-type (yellow) or cells expressing the S656A mutant (blue) treated with or without nitrite. Asterisks indicate $P < 0.01$ and hash indicates $P < 0.05$ vs. control; $n = 5$ per group.

While there was no change in total Drp1 protein expression, phosphorylation of S656 was significantly increased 1 h after normoxic nitrite treatment (*Figure 2B* and Supplementary material online, *Figure S2B*). Nitrite treatment also decreased the expression of Drp1 in the mitochondrial fraction of the cells, consistent with nitrite-mediated inhibition of Drp1 translocation from the cytosol to the mitochondrion (*Figure 2C*).

To test whether the phosphorylation of Drp1 at S656 was required to mediate nitrite-dependent cytoprotection after H/R, cells stably transfected with GFP-tagged plasmids encoding *wildtype* Drp1 or a non-phosphorylatable mutant of Drp1 (S656A) (*Figure 2D*) were treated with nitrite and subjected to H/R. Importantly, the plasmid containing the S656A plasmid also included shRNA to silence wild-type Drp1 protein such that only S656A was expressed.[32] Consistent with the necessity for Drp1 phosphorylation at S656 to mediate cytoprotection, nitrite-attenuated cell death after H/R in cells expressing *wt*Drp1 ($14.5 \pm 4.5\%$ vs. 36.2 ± 0.5 H/R alone), but did not mediate protection in cells transfected with the non-phosphorylatable mutant S656A ($35.2 \pm 2.3\%$ vs. 37.3 ± 3.2 in H/R alone) (*Figure 2D*).

Drp1 phosphorylation at S656 is mediated by PKA.[24] PKA activity levels were significantly greater in cells collected 1 h after a normoxic

nitrite treatment (*Figure 3A*). Furthermore, pre-treatment of cells with PKI (5 μmol/L), a pharmacological inhibitor of PKA, decreased PKA activity by ~80% and inhibited the nitrite-mediated phosphorylation of Drp1 (*Figure 3B*). Additionally, the inhibition of PKA with PKI abolished nitrite-dependent cytoprotection, when H/R was initiated 1 h after nitrite treatment (*Figure 3C*). Collectively, these findings demonstrate that nitrite-induced activation of PKA and subsequent phosphorylation of Drp1 at S656 are essential for nitrite-mediated normoxic preconditioning of cells against H/R injury.

3.3 Nitrite-mediated Drp1 phosphorylation augments mitochondrial superoxide production

To determine whether nitrite-mediated Drp1 phosphorylation alters mitochondrial function, cells were treated with nitrite (normoxia, 30 min), and then mitochondrial respiration, membrane potential (MMP), and superoxide production were assessed. Respiration was measured basally and in the presence of the ATPase inhibitor oligomycin (2 μmol/L), the uncoupler FCCP (7.5 μmol/L), and the complex I inhibitor rotenone (2 μmol/L) to determine the rates of basal, non-ATP linked, maximal, and non-mitochondrial oxygen consumption, respectively, in the intact cells. Nitrite treatment significantly increased the maximal respiratory rate (12.1 ± 0.4 vs. 8.1 ± 0.1 pmol O_2/min/103 cells), but had no significant effect on basal respiration or proton leak (*Figure 4A*). Consistent with prior studies demonstrating that mitochondrial fusion increases membrane potential,[40–42] nitrite treatment also resulted in increased MMP (73.5 ± 7.7% vs. 38.6 ± 8.8% in untreated) as measured by the potentiometric dye TMRM (*Figure 4B*). Not surprisingly, this increase in MMP was associated with augmented mitochondrial superoxide production in nitrite-treated cells (893.4 ± 95.5 vs. 447 ± 84.7 RFU/min/mg in control) as measured by MitoSOX Red (*Figure 4C* and *D*; Supplementary material online, *Figure S3A*). This nitrite-induced increase was confirmed by electron paramagnetic spin resonance (EPR) using the spin trap CMH (1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine) (Supplementary material online, *Figure S3B*). Further, the activity of the redox-sensitive protein aconitase was significantly decreased after nitrite treatment, also indicative of increased oxidant production (Supplementary material online, *Figure S3C*). This oxidation of aconitase was abrogated by inhibition of PKA or scavenging of ROS by the mitochondrially targeted scavenger mitoTEMPO (Supplementary material online, *Figure S3C*). Furthermore, nitrite-induced increased superoxide generation was dependent on the phosphorylation of Drp1, as it was not observed in cells expressing the non-phosphorylatable mutant Drp1 protein (*Figure 4D*).

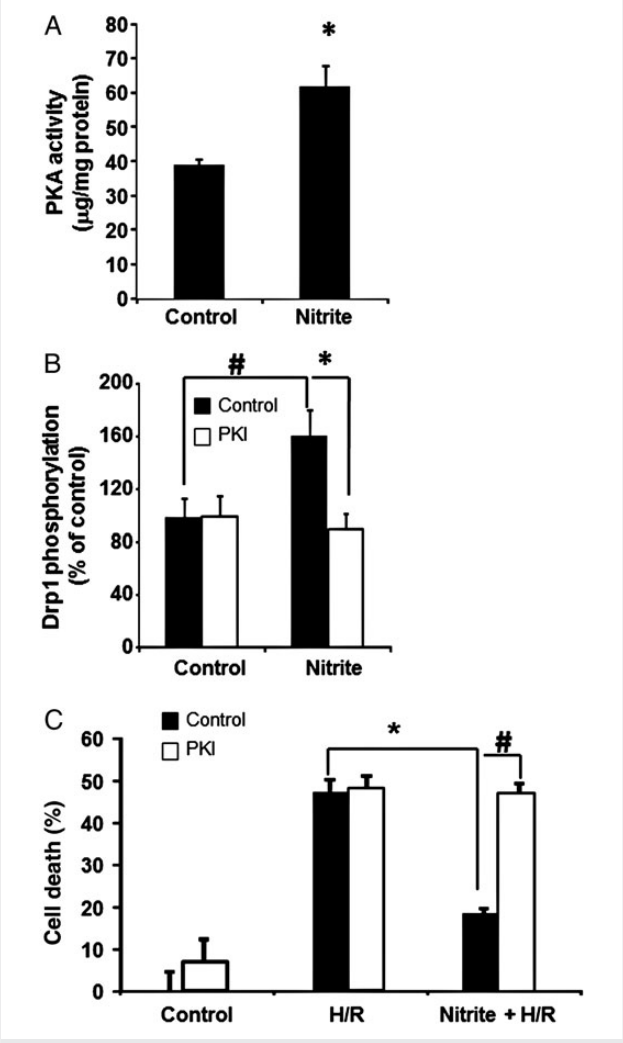

Figure 3 Nitrite-induced Drp1 phosphorylation is dependent on increased PKA activity. (*A*) PKA activity in control and nitrite-treated cells 1 h after nitrite removal. (*B*) Phospho-Drp1 levels (normalized to total Drp1) measured 1 h after cells were treated with saline or nitrite (25 μmol/L), with and without PKI (5 μmol/L) $n = 4$. (*C*) Cell death 1 h after nitrite treatment in untreated cells or those treated with PKI. Asterisks indicate $P < 0.01$ and hashes indicate $P < 0.05$ vs. control; $n = 3$ per group.

3.4 Nitrite-mediated cytoprotection is dependent on mitochondrial superoxide generation

Given that nitrite-mediated cytoprotection was dependent on Drp1 phosphorylation and Drp1 phosphorylation led to mitochondrial superoxide production, we next tested whether mitochondrial reactive oxygen species generation was required for nitrite-dependent cytoprotection after H/R. The requirement for global mitochondrial signalling in nitrite-mediated cytoprotection was first tested by generating ρ^0 H9c2 cells lacking a functional electron transport chain. The lack of a respiratory chain was confirmed by a significant decrease in the activity of complex IV in these cells (4.9 ± 3.0 μmol cytochrome c/min/mg vs. 21.8 ± 2.0 wild-type). Nitrite was unable to protect ρ^0 cells from H/R initiated 1 h after nitrite treatment (72.0 ± 6.7% cell death). Although ρ^0 cells have altered metabolism compared with wild-type cells, these data suggested that a functional mitochondrial respiratory chain was involved in the mechanism of protection (*Figure 4E*).

To more specifically test the role of mitochondrial ROS generation in nitrite-mediated protection, cells were co-treated with the mitochondrially targeted ROS scavenger mitoTEMPO (2 μmol/L), which had no significant effect on cell death mediated by H/R at this concentration, but abrogated the protective effect of nitrite after H/R (*Figure 4F*). Mitochondrial superoxide is rapidly converted to the more stable oxidant hydrogen peroxide (H_2O_2), which can diffuse out of the mitochondrion. To determine whether H_2O_2 was responsible for the nitrite-dependent cytoprotection, adenovirus-mediated overexpression of catalase was used to scavenge H_2O_2 (*Figure 4F*,

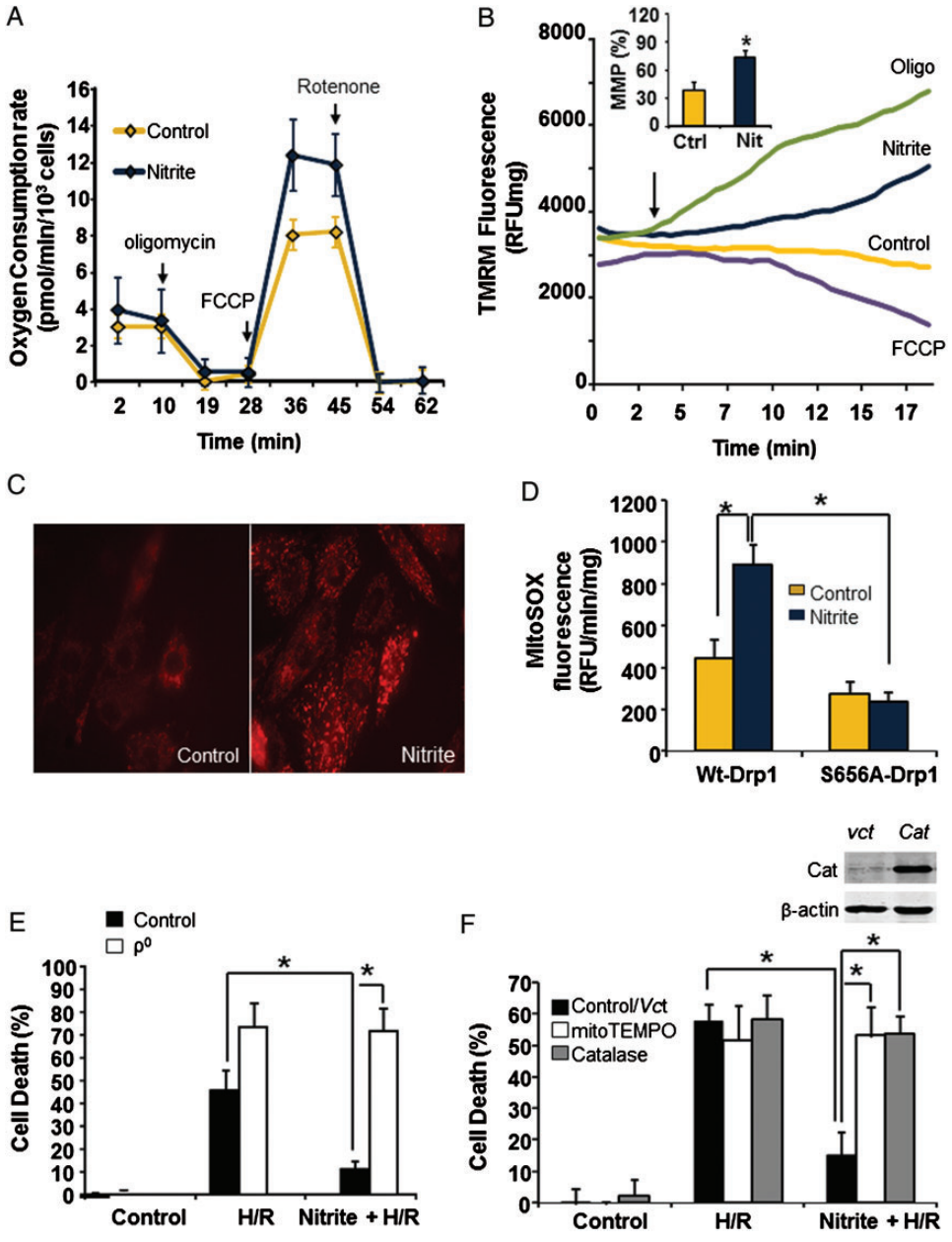

Figure 4 Nitrite-mediated Drp1 phosphorylation augments mitochondrial superoxide that is essential for cytoprotection. Cells were treated with or without nitrite (25 μmol/L) at 21% O_2 (30 min). (*A*) Representative traces of oxygen consumption rates in control (yellow) and nitrite-treated (blue) cells 1 h after nitrite removal. (*B*) Representative TMRM traces for cells treated with nothing (yellow), nitrite (blue), Oligomycin (5 μmol/L; green), and FCCP (10 μmol/L; purple). Arrow notes the addition of each treatment. Inset: quantification of the membrane potential as a per cent of the total TMRM range (determined by FCCP and oligomycin), $n = 4$. Asterisks indicate $P < 0.01$. (*C*) Representative confocal microscopy images of control (left panel) and nitrite-treated (right panel) cells stained with MitoSox Red. (*D*) Quantitation of the rate of mitochondrial superoxide production in *wt*-Drp1 or S656A-transfected cells treated with or without nitrite. $n = 4$. Asterisks indicate $P < 0.01$. (*E*) Cell death after H/R initiated 1 h after nitrite treatment in control or ρ^0 cells. $n = 4$. Asterisks indicate $P < 0.0001$. (*F*) Cell death after H/R initiated 1 h after saline or nitrite treatment in untreated cells, those treated with MitoTEMPO (2 μmol/L) or those overexpressing catalase (*Cat*). Representative western blot of catalase expression in cells transfected with adenovirus containing vector (*Vct*) or catalase (inset). Asterisks indicate $P < 0.01$ vs. control. $n = 4$

inset). While cells transfected with the empty vector were protected by nitrite after H/R (15.1 ± 7.6% cell death vs. 57.9 ± 5.3 in H/R alone), nitrite-induced cytoprotection was abolished in catalase-overexpressing cells (53.8 ± 5.6% cell death vs. 58.4 ± 7.8 in H/R alone) (*Figure 4F*). Taken together, these data reveal that the production of mitochondrial ROS is required for nitrite-mediated cytoprotection after H/R.

3.5 Nitrite-mediated protection is dependent on the oxidation and phosphorylation of AMPK

We next sought to determine the downstream cytoprotective target of nitrite-induced mitochondrial ROS. AMPK is an integral metabolic sensor whose activation is implicated in cytoprotection after I/R.[43]

Furthermore, recent studies have shown that oxidation of AMPK by H_2O_2 can lead to its auto-phosphorylation and subsequent activation.[21] Thus, we next tested whether nitrite could activate AMPK and whether this activation was required for nitrite-mediated cytoprotection. Treatment of cells with nitrite for 30 min in normoxia showed a significant increase in the phosphorylation of AMPK at Thr172 compared with untreated cells (*Figure 5A*). Additionally, this AMPK activation was required for nitrite-mediated cytoprotection as treatment with compound C (5 μmol/L), a pharmacological inhibitor of AMPK, abolished nitrite-mediated cytoprotection after H/R (53.8 ± 5.6 vs. 53.3 ± 2.0% cell death in H/R alone) (*Figure 5B*). The requirement of AMPK activation for nitrite-induced cytoprotection was further confirmed in cells transfected with siRNA against the α1 catalytic subunit of AMPK, in which nitrite did not prevent cell death after subsequent H/R (*Figure 5B*).

To determine whether nitrite-induced mitochondrial ROS could oxidize AMPK leading to its auto-phosphorylation, we first determined whether AMPK was oxidized by nitrite. Cells were treated with nitrite (25–50 μmol/L; 21% O_2) and subjected to a modified biotin switch assay in which oxidized proteins were labelled with maleimide conjugated biotin. As shown in *Figure 5C*, significant AMPK oxidation was observed in cells treated with nitrite compared with untreated cells (*Figure 5C*). To determine whether nitrite-induced mitochondrial ROS were responsible for the AMPK oxidation observed, cells were co-treated with nitrite and mitoTEMPO. Scavenging of mitochondrial ROS significantly attenuated both nitrite-dependent AMPK oxidation (*Figure 5C*) and phosphorylation (*Figure 5A*). Taken together, these results indicate that nitrite-mediated cytoprotection after H/R is dependent on the oxidation and subsequent activation of AMPK.

Opening of the mitochondrial permeability pore is a major event in the propagation of apoptotic cell death after I/R. Since the permeability pore is a known downstream target of AMPK,[44,45] we next determined whether nitrite-mediated activation of AMPK inhibited pore opening. Measurement of the quenching of the fluorescent dye calcein by cobalt chloride loaded into the cells at the time of reoxygenation demonstrated that H/R stimulated permeability pore opening and that nitrite significantly inhibited this. Furthermore, inhibition of AMPK by compound C attenuated the ability of nitrite to prevent permeability transition (*Figure 5D* and Supplementary material online, *Figure S4*), demonstrating that inhibition of the permeability transition pore is a downstream mechanism by which AMPK mediates nitrite-induced protection.

3.6 Nitrite mediates cytoprotection through the PKA-Drp1-AMPK pathway in the perfused heart

We next sought to determine whether nitrite elicited the PKA-dependent cytoprotective pathway in the intact heart. Adult rat hearts were isolated and perfused with nitrite (10 μmol/L) for 10 min, followed by a washout period (10 min) during which the heart was perfused with nitrite-free buffer. After this washout period, nitrite-treated hearts showed a significant increase in PKA activity (*Figure 6A*) as well as phosphorylation of Drp1 and AMPK (*Figure 6B* and *C*), consistent with the nitrite-dependent activation of the PKA-Drp1-AMPK pathway. The hearts were then subjected to global no-flow ischaemia (20 min) and reperfusion (2 hours) during which left ventricular developed pressure (LVDP) was measured. In these hearts, I/R induced a 55.4 ± 10.1% decrease in LVDP consistent with I/R damage and nitrite significantly protected against this decrease (*Figure 6C* and Supplementary material online, *Table S1*). Notably, perfusion with a higher concentration of nitrite (25 μmol/L) also significantly increased PKA activity, phosphorylation of Drp1, and AMPK as well as protected against I/R-induced decrease in LVDP (data not shown). To determine whether this nitrite-mediated protection was dependent on the activation of PKA, heart were perfused with nitrite (10 μmol/L) in the presence or absence of the PKA inhibitor PKI (0.1 mmol/L) for 10 min and then perfused with nitrite-free buffer for a washout period of 10 min before being subjected to I/R. Inhibition of PKA abolished the nitrite-induced increase in PKA activity (*Figure 6A*) and phosphorylation of AMPK (*Figure 6B*) and Drp1 (*Figure 6C*) as well as attenuated nitrite-mediated protection (*Figure 6D*). Inhibition of PKA also significantly abrogated the ability of nitrite to decrease infarct size (measured at the end of reperfusion) in the heart (*Figure 6E* and *F*). These data demonstrate that nitrite activates the PKA-Drp1-AMPK pathway in the isolated heart and that nitrite-mediated protection is dependent on the activation of PKA.

4. Discussion

Nitrite was initially tested as a potential I/R therapeutic based on its ability to preferentially mediate NO-based signalling in ischaemic hypoxic conditions.[1,5] Almost a decade later, it is clear that nitrite is equally protective when administered prior to ischaemia, as a pharmacological pre-conditioning agent.[9,14,15] However, the normoxic signalling resulting in this delayed protection remains virtually unexplored. The current study demonstrates that nitrite-mediated cardiomyocyte protection is dependent on the modulation of mitochondrial morphology and function, leading to the downstream activation of AMPK. Specifically, nitrite enhances the PKA-dependent phosphorylation of Drp1, resulting structurally in increased mitochondrial fusion and functionally in augmented mitochondrial membrane potential and mitochondrial-derived ROS generation. This production of mitochondrial ROS is essential for the oxidation and subsequent activation of the protective metabolic sensor AMPK (Supplementary material online, *Figure S5*). We show that this pathway is activated by nitrite in the intact adult heart. Furthermore, this study is the first demonstration of protective nitrite-dependent normoxic signalling in the mitochondrion of cardiomyocytes.

Prior work from our group suggests that the S-nitrosation of complex I resulting in the inhibition of mitochondrial ROS production at the time of reperfusion is essential in nitrite-mediated cardioprotection after I/R.[14,46] Notably, this significant inhibition of complex I was present only after mitochondria were subjected to I/R. This previously described mechanism likely complements the results presented in the current study as nitrite appears to mediate differing modes of cardioprotection in two temporally distinct windows. Moreover, the initiation of these two mechanisms is likely governed in tissue by oxygen and pH levels. When administered prior to ischaemia/hypoxia at physiological oxygen tensions, nitrite activates PKA to modulate mitochondrial dynamics resulting in augmented ROS generation with no inhibition of complex I (demonstrated by the lack of inhibited respiration; *Figure 4A*). However, during ischaemia, as tissue becomes anoxic and acidotic, nitrite mediates S-nitrosation to significantly inhibit complex I and decrease reperfusion ROS generation. Hence, future studies are required to determine whether cross-talk exists between these two mechanisms.

As mentioned above, we and others have previously described ischaemic hypoxic nitrite signalling, which is dependent on the reduction of nitrite to bioactive NO by proteins such as myoglobin and xanthine oxidoreductase in the heart.[5,10] However, the current study demonstrates

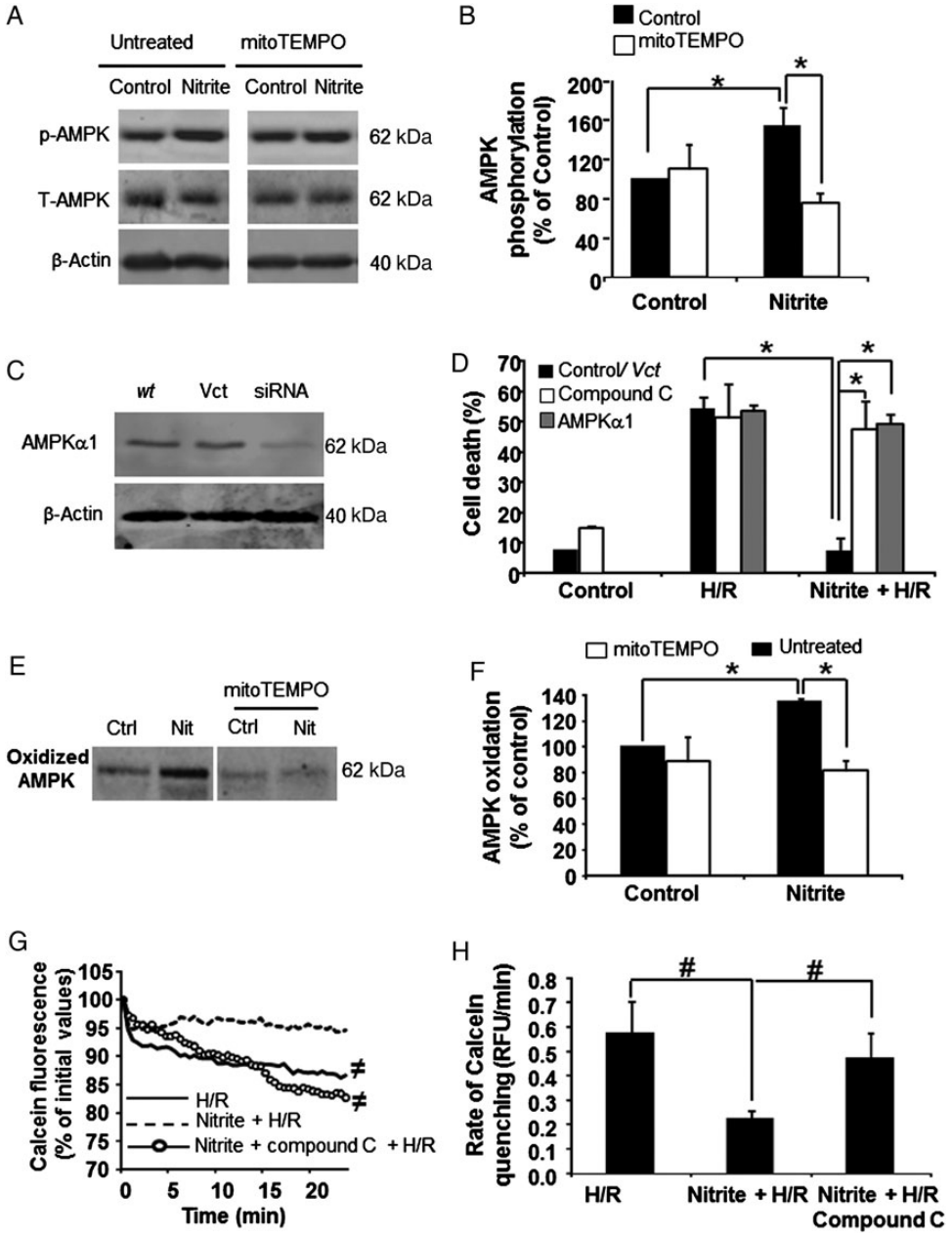

Figure 5 Nitrite-mediated cytoprotection is dependent on AMPK activation. Cells were treated with or without nitrite (25 μmol/L; 21% O_2) in the presence or absence of mitoTEMPO (2 μmol/L; 30 min). (*A*) Western blot of phospho-AMPK (p-AMPK), total AMPK (t-AMPK) and β-actin, and quantitation of p-AMPK/t-AMPK/β-actin 1 h after nitrite removal. $n = 4$ for untreated and $n = 3$ for mitoTEMPO. (*B*) Cells were transfected with an empty vector (*Vct*) or siRNA against AMPKα1. Representative immunoblot of AMPKα1 expression 48 h after transfection. Cell death after H/R initiated 1 h (grey) after treatment with nitrite in cells transfected with siRNA to AMPKα1. Cell death measured after H/R initiated 1 h following nitrite treatment is also shown for control cells and those pre-treated with compound C (5 μmol/L; open bars) $n = 4$. (*C*) Western blot representing oxidized AMPK and blot quantitation after nitrite treatment in untreated cells and those co-treated with mitoTEMPO. Asterisks indicate $P < 0.01$, $n = 4$. (*D*) Representative calcein fluorescence traces and quantification of permeability transition pore opening in cells after H/R in the absence and presence of nitrite (50 μmol/L) as well as in the presence of compound C and nitrite. $n = 4$. Hashes indicate $P < 0.05$.

that nitrite increases PKA activity in normoxia, a condition in which it is unlikely that nitrite generates NO. While the chemistry behind normoxic signalling in this system remains unclear, nitrite can be oxidized to nitrogen dioxide ($NO_2^{\cdot}$) through a haem catalysed peroxidase reaction in the presence of H_2O_2.[47] Indeed, Wang *et al.* have recently shown that $NO_2^{\cdot}$ generation potentially underlies the mechanism of nitrite-mediated wound healing in normoxic airway epithelial cells.[48] It is possible that identical chemistry leads to the nitration of adenylate cyclase or phosphodiesterases to alter their activity and increase cellular cAMP levels, leading to activation of PKA in myocytes. Alternatively, nitrite may react *in vivo* to form electrophilic fatty acids, some of which have been observed to increase adenylate cyclase activity.[49] Notably, prior proteomic studies have associated the cardioprotective effects of nitrite with increased PKA protein expression.[50] Additionally, PKA

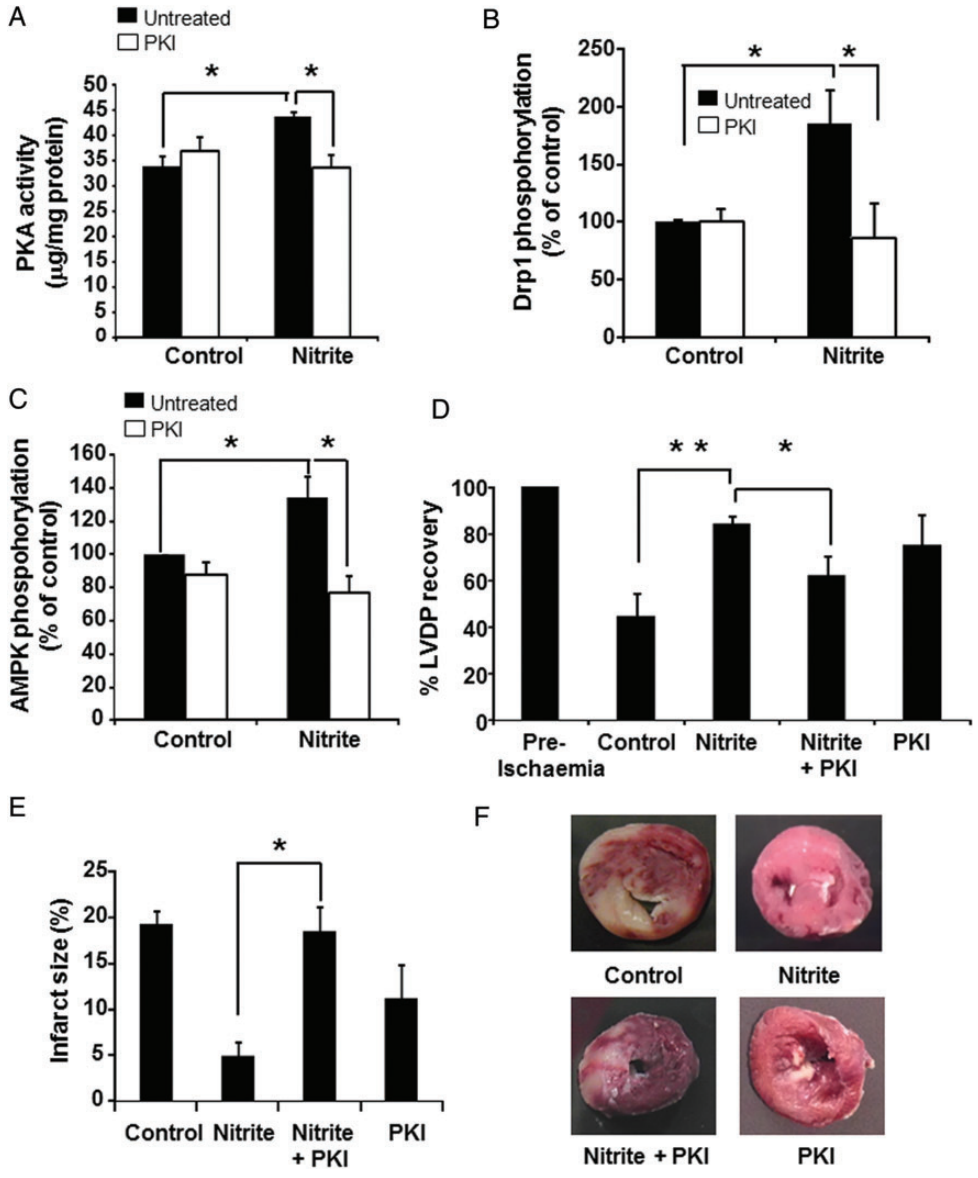

Figure 6 Nitrite activates the PKA-Drp1-AMPK pathway and mediates delayed cardioprotection in perfused hearts. (*A*) PKA activity, (*B*) phosphorylation of Drp1, and (*C*) phosphorylation of AMPK in isolated rat hearts treated with or without nitrite (10 μmol/L) in the presence (white bars) or absence (black bars) of PKI (5 μmol/L). (*D* and *F*) Recovery of LVDP (*D*) and infarct size (*E* and *F*) of perfused hearts subjected to global I/R with and without nitrite pre-treatment (10 min of nitrite perfusion followed by a washout period), in the presence or absence of the PKA inhibitor PKI. $n = 4$ per group. Asterisks indicate $P < 0.01$ and double asterisk indicates $P < 0.001$. (*F*) Representative images of TTC stained heart sections used to determine infarct size.

has been shown to increase nitrite levels through the induction of eNOS activity.[51] However, this study is the first to report direct evidence of PKA activation by nitrite, particularly in normoxia.

Our data confirm prior studies demonstrating the cardioprotective effect of mitochondrial fusion. Mitochondrial fission, particularly mediated by shear stress-dependent NO, has been associated with reoxygenation injury in endothelial cells.[52] Moreover, the elongation of mitochondrial networks as a result of either the pharmacological inhibition of Drp1 or overexpression of Mfn2 has previously been shown to decrease infarct size in murine models of myocardial infarction.[16] Notably, the cytoprotective effects mediated by mitochondrial fusion have been attributed predominantly to the inhibition of the translocation of the pro-apoptotic proteins Bax and Bak to the mitochondrion which ultimately prevents cytochrome c release.[53] While nitrite prevents cell death and pore opening in our model, we show that this is dependent on the production of mitochondrial ROS and activation of AMPK. This is consistent with prior studies, demonstrating that the permeability transition pore is a downstream target for AMPK.[44,45] Though the current study shows an effect of AMPK on pore opening, this may not be the only mechanism by which AMPK mediates cytoprotection in this system. Activation of this metabolic sensor is also known to mediate cardioprotection through the opening of ATP-sensitive potassium channels,[54] the modulation of autophagy,[55,56] and the stabilization of hypoxia-inducible factor-1α.[57,58] Interestingly, a number of preconditioning agents, including IPC, are known to augment mitochondrial ROS production[18,20] in a manner similar to that observed with nitrite in

this study. Additionally, ROS-induced activation of AMPK has been previously observed with sevoflurane-induced pre-conditioning in isolated Langendorff-perfused rat hearts.[59] However, this report is the first to link the modulation of mitochondrial dynamics to the activation of AMPK in the context of pre-conditioning and begs the question of whether the induction of mitochondrial fusion is a central mechanism common to all preconditioning agents.

We and others have previously established that administration of oral nitrite from 24 h to 1 week prior to ischaemia mediates tissue protection after I/R.[9,15] It has been speculated that given the abundance of nitrate (reduced to nitrite by oral bacteria) in the Mediterranean Diet, nitrite may be the active cardioprotective agent in this diet.[15] The presence of mitochondrial fusion has not been compared in subjects on this diet vs. other diets. We show here that nitrite mediates increased PKA activity and phosphorylation of Drp1 in the rat heart. However, the effect of a nitrite-rich diet is potentially an interesting avenue for further future investigations. In addition to enhancing myocardial tissue viability after I/R injury, activation of AMPK has been shown to improve cardiac function in pathologies such as type II diabetes[60] and drug-related cardiomyopathy.[61] Notably, oral nitrite also mediates protection in these conditions,[62,63] suggesting that nitrite-mediated mitochondrial fusion and subsequent AMPK activation may play a role in these protective effects.

In conclusion, the results presented here describe a novel mechanism by which nitrite mediates delayed cytoprotection after I/R. These data for the first time demonstrate nitrite-dependent regulation of mitochondrial dynamics as well as normoxic modulation of mitochondrial function. Together, these data greatly expand the role of nitrite in the regulation of cellular bioenergetics as well as in the adaptation to the response.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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