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RESEARCH ARTICLE

Bioenergetic and volume regulatory effects of mitoK$_{ATP}$ channel modulators protect against hypoxia–reoxygenation-induced mitochondrial dysfunction

John O. Onukwufor, Don Stevens and Collins Kamunde*

ABSTRACT

The mitochondrial ATP-sensitive K^+ (mitoK$_{ATP}$) channel plays a significant role in mitochondrial physiology and protects against ischemic reperfusion injury in mammals. Although fish frequently face oxygen fluctuations in their environment, the role of the mitoK$_{ATP}$ channel in regulating the responses to oxygen stress is rarely investigated in this class of animals. To elucidate whether and how the mitoK$_{ATP}$ channel protects against hypoxia–reoxygenation (H-R)-induced mitochondrial dysfunction in fish, we first determined the mitochondrial bioenergetic effects of two key modulators of the channel, diazoxide and 5-hydroxydecanoate (5-HD), using a wide range of doses. Subsequently, the effects of low and high doses of the modulators on mitochondrial bioenergetics and volume under normoxia and after H-R using buffers with and without magnesium and ATP (Mg-ATP) were tested. In the absence of Mg-ATP (mitoK$_{ATP}$ channel open), both low and high doses of diazoxide improved mitochondrial coupling, but only the high dose of 5-HD reversed the post-H-R coupling-enhancing effect of diazoxide. In the presence of Mg-ATP (mitoK$_{ATP}$ channel closed), diazoxide at the low dose improved coupling post-H-R, and this effect was abolished by 5-HD at the low dose. Interestingly, both low and high doses of diazoxide reversed H-R-induced swelling under mitoK$_{ATP}$ channel open conditions, but this effect was not sensitive to 5-HD. Under mitoK$_{ATP}$ channel closed conditions, diazoxide at the low dose protected the mitochondria from H-R-induced swelling and 5-HD at the low dose reversed this effect. In contrast, diazoxide at the high dose failed to reduce the swelling caused by H-R, and the addition of the high dose of 5-HD enhanced mitochondrial swelling. Overall, our study showed that in the presence of Mg-ATP, both opening of mitoK$_{ATP}$ channels and bioenergetic effects of diazoxide were protective against H-R in fish mitochondria, while in the absence of Mg-ATP only the bioenergetic effect of diazoxide was protective.

KEY WORDS: Mitochondrial bioenergetics, Volume regulation, Swelling, Diazoxide, 5-Hydroxydecanoate

INTRODUCTION

The mitochondrial ATP-sensitive K^+ (mitoK$_{ATP}$) channel was first identified in rat liver (Inoue et al., 1991) as a highly selective, small conductance K^+ channel located in the inner mitochondrial membrane. Since then it has also been identified in heart (Paucek et al., 1992; Wojtovich and Brookes, 2009), brain (Bajgar et al., 2001; Debska et al., 2001), skeletal muscle (Gurke et al., 2000; Debska et al., 2002) and kidney (Cancherini et al., 2003). Pharmacological modulation of the mitoK$_{ATP}$ channel has been instrumental in elucidating the pathophysiology of ischemia–reperfusion (IR) injury in mammals (Garlid et al., 1997; Jaburek et al., 1998; Grover et al., 2001). In this regard, the mitoK$_{ATP}$ channel opener (diazoxide) was found to protect against IR injury (Garlid et al., 1997), and this protection was inhibited by 5-hydroxydecanoate (5-HD), a blocker of the channel (Jaburek et al., 1998). Although the exact mechanisms of protection are still debated, several potential explanations have been proposed (Garlid and Paucek, 2003; Ardehali and O'Rourke, 2005; Costa et al., 2006). First, it is hypothesized that opening of the mitoK$_{ATP}$ channel increases mitochondrial matrix volume through uptake of K^+ from the cytosol into the matrix, thus preventing intermembrane space contraction (Garlid, 2000; Garlid et al., 2003). Second, opening of the mitoK$_{ATP}$ channel is believed to cause mild mitochondrial uncoupling that triggers reactive oxygen species (ROS) production with activation of kinases that protect against IR injury (Krenz et al., 2002; Andrukhiv et al., 2006). Support for this hypothesis is provided by the finding that ROS scavengers blocked the protection conferred by mitoK$_{ATP}$ (Vanden Hoek et al., 1998; Pain et al., 2000; Baines et al., 2001; Cohen et al., 2001). Third, it has been argued that it is the reduction/inhibition of ROS production that causes protection (Ferranti et al., 2003; Facundo et al., 2007; Kulawiak et al., 2008), while others have suggested that the opening of the mitoK$_{ATP}$ channel would result in matrix alkalinisation triggering increased ROS production (Costa et al., 2006). Lastly, it has been suggested that functions attributed to the mitoK$_{ATP}$ channel may essentially be the effects of pharmacological modulators used to study the effect of the channel on mitochondrial function (Holmuhamedov et al., 1999, 2004; Dröse et al., 2006; Kopustinskiene et al., 2010).

Overall, empirical evidence from the use of pharmacological modulators of the mitoK$_{ATP}$ channel suggest two general bases of the mechanisms of protection against IR/H-R-induced deleterious effects: modulation of mitochondrial bioenergetics (Holmuhamedov et al., 1999, 2004; Dröse et al., 2006; Kopustinskiene et al., 2010) and/or volume (Garlid et al., 1997; Jaburek et al., 1998; Costa et al., 2006). However, Garlid (2000) argued that bioenergetic effects of mitoK$_{ATP}$ channel modulators (diazoxide and 5-HD) were observed at high (toxic) concentrations with the channel already open because of the absence of Mg-ATP in the assay buffer. Given the controversies cast above, the present study sought to elucidate the relative contribution of bioenergetic and volume regulation modes of mitoK$_{ATP}$ channel protection against H-R-induced stress with a focus on mechanisms not mediated by ROS. First, we predicted that the effects of the mitoK$_{ATP}$ opener (diazoxide) and blocker (5-HD) on mitochondrial respiration and volume would be diametrically opposite and dose-dependent. Second, we predicted that in the

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List of abbreviations

5-HD	5-hydroxydecanoate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
H-R	hypoxia–reoxygenation
IR	ischemia reperfusion
mitoK$_{ATP}$	mitochondrial ATP-sensitive K$^+$ channel
MRB	mitochondrial respiratory buffer
NADH	nicotinamide adenine dinucleotide (reduced)
OXPHOS	oxidative phosphorylation
RCR	respiratory control ratio
ROS	reactive oxygen species
TEA$^+$	tetraethylammonium ion

absence of Mg-ATP, diazoxide and 5-HD would modulate mitochondrial bioenergetics but not volume. Third, we predicted that low doses of diazoxide in the presence of Mg-ATP would protect against H-R-induced effects only by altering mitochondrial volume. Fourth, we predicted that high doses of both diazoxide and 5-HD in the presence and absence of Mg-ATP would alleviate H-R-induced damage by altering mitochondrial bioenergetics. The only previous studies on the mitoK$_{ATP}$ channel in non-mammals have been concerned with the sarcolemmal mitoK$_{ATP}$ channel; to the best of our knowledge, ours is the first study to probe the role of the mitoK$_{ATP}$ channel in H-R-induced stress in isolated fish mitochondria.

MATERIALS AND METHODS

Ethics

The procedures for experimental animals in the present study were approved (protocol no. 11-034) by the University of Prince Edward Island Animal Care Committee in accordance with the Canadian Council on Animal Care.

Fish

Juvenile rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] weighing 150±4.8 g (mean±s.e.m.) at sampling were obtained from Ocean Farms (Brookvale, PE, Canada) and kept in a 400-litre tank supplied with flow-through aerated well water at the Atlantic Veterinary College Aquatic Facility. Water temperature and pH were 10±1°C and 7.7, respectively. The fish were fed at 1% body weight daily with commercial trout chow (Corey Feed Mills, Fredericton, NB, Canada).

Mitochondrial isolation

To isolate mitochondria, fish were randomly sampled from the tank, stunned with a blow to the head, decapitated and immediately dissected to remove the liver. Mitochondria were isolated according to the method of Onukwufor et al. (2014) and re-suspended in a 1:3 (weight to volume) ratio of mitochondrial respiration buffer [MRB: 10 mmol l^{-1} Tris, 25 mmol l^{-1} KH_2PO_4, 100 mmol l^{-1} KCl, 1 mg ml^{-1} bovine serum albumin (BSA, fatty acid free), 2 µg ml^{-1} aprotinin, pH 7.3]. Mitochondrial protein concentration was measured by spectrophotometry (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA, USA) according to Bradford (1976) with BSA as the standard.

Measurement of mitochondrial respiration

Mitochondrial respiration rates were measured using Clark-type oxygen electrodes (Qubit Systems, Kingston, ON, Canada) in 2 ml cuvettes after a two-point calibration at 0 and 100% air saturation at the ambient atmospheric pressure. Temperature during the assays was maintained at 13°C using a recirculating water bath (Haake, Karlsruhe, Germany). After the calibration, 1.45 ml of MRB and 100 µl of mitochondrial suspension containing 2.2–3.1 mg of protein were loaded into the cuvettes and continuously stirred. To spark the Krebs cycle, 5 mmol l^{-1} malate was added and respiration was supported with a saturating concentration (5 mmol l^{-1}) of glutamate, a complex I substrate. The addition of 250 µmol l^{-1} ADP initiated the state 3 respiration, which transitioned to state 4 upon depletion of ADP. All rates of oxygen consumption were monitored using Logger Pro 3 with the Vernier Labpro interface (Vernier Software and Technology, Beaverton, OR, USA). The respiratory control ratios (RCR; ratio of state 3 to state 4) were calculated according to Chance and Williams (1955).

Exposure of mitochondria to hypoxia–reoxygenation

The protocol used for H-R was based on the study of Onukwufor et al. (2014). Briefly, mitochondrial complex-1-driven oxygen consumption was measured under normoxic conditions as described above. To make the MRB hypoxic, nitrogen gas was bubbled into the cuvettes depleting the partial pressure of oxygen (P_{O_2}) to <2 Torr (1 Torr=133 Pa; actual concentrations were 0.002–0.003 mg O_2 l^{-1}) under prevailing environmental conditions. This concentration is below the 2.25–3.75 Torr intracellular level of oxygen typically encountered by rat mitochondria *in vivo* (Gnaiger and Kuznetsov, 2002). Once the P_{O_2} reached the desired level, the cuvettes were sealed and the hypoxic conditions were maintained for 15 min. At the end of the hypoxia exposure period, the cuvettes were opened and fully re-oxygenated (100% air saturation) and ADP (250 µmol l^{-1}) was added to impose the second phosphorylation with measurements of state 3 and 4 respiration rates. The difference between the first and second set of respiration parameters represented the effect of H-R on mitochondrial bioenergetics.

A control experiment was done to test the effect of bubbling on mitochondrial respiration. In this experiment we bubbled with air rather than nitrogen but for the same length of time (2 min) as the H-R trials. The results showed that there was no effect of bubbling with air on respiration rate during state 3 (Student's *t*-test, t=−0.56, P=0.59) and state 4 respiration (t=0.00, P=0.99) or RCR (t=−0.73, P=0.50).

Bioenergetic effects of the mitoK$_{ATP}$ channel

The prediction that diazoxide (mitoK$_{ATP}$ channel opener) would protect against H-R damage by modulating mitochondrial bioenergetics and 5-HD (mitoK$_{ATP}$ channel blocker) would reverse this effect was tested by measuring mitochondrial respiration before and after 15 min H-R at 13°C. We first carried out dose–response experiments for diazoxide (10, 25, 100, 200 and 500 µmol l^{-1}) and 5-HD (50, 100, 200, 500 and 1000 µmol l^{-1}) and thereafter selected low (25 µmol l^{-1} diazoxide and 50 µmol l^{-1} 5-HD) and high (500 µmol l^{-1} diazoxide and 1 mmol l^{-1} 5-HD) dose combinations and assessed their effects on post H-R respiration rates using respiration buffers with and without 1 mmol l^{-1} $MgCl_2$ and 200 µmol l^{-1} ATP (Mg-ATP). The goal here was to delineate the direct effects of diazoxide and 5-HD on mitoK$_{ATP}$ channels from their potential effects on oxidative phosphorylation (OXPHOS) by carrying out the assay with the mitoK$_{ATP}$ channel closed (with Mg-ATP) and open (without Mg-ATP). In these experiments, the mitochondria were pre-incubated with diazoxide alone and in combination with 5-HD for 5 min in the respiratory cuvettes after

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the measurement of normoxic respiration, and thereafter exposed to H-R followed by a second measurement of respiration rates.

Volume regulatory effects of the mitoK$_{ATP}$ channel

Mitochondrial volume was measured using a spectrophotometric method (Onukwufor et al., 2015) under normoxic conditions and after H-R, with and without diazoxide alone, and in combination with 5-HD using buffers with and without Mg-ATP. In this assay, 100 µl of mitochondrial suspension was first energized with 5 mmol l^{-1} glutamate and 5 mmol l^{-1} malate in the cuvette and exposed to 15 min of hypoxia followed by reoxygenation. At the end of the reoxygenation the mitochondrial suspension was recovered and diluted with air-saturated buffer (10 mmol l^{-1} Tris, 25 mmol l^{-1} KH_2PO_4, 100 mmol l^{-1} KCl) to give a mitochondrial protein concentration of 1 mg ml^{-1}. Volume changes were then measured at 25°C, with 200 µmol l^{-1} Ca as a positive control for swelling, by spectrophotometric monitoring of changes in absorbance at 540 nm every 10 s for 30 min. In this assay, a decrease in absorbance indicates swelling. To assess the role of the mitoK$_{ATP}$ channel on normoxic and H-R-induced mitochondrial volume changes, energized mitochondria were pre-incubated with 25 or 500 µmol l^{-1} diazoxide alone and in combination with 5-HD at either a low (50 µmol l^{-1}) or high (1 mmol l^{-1} 5-HD) dose, respectively, for 5 min, and changes in absorbance at 540 nm under normoxia were measured every 10 s for 30 min. For post H-R swelling, mitochondrial suspensions were incubated with diazoxide and 5-HD for 5 min and exposed to hypoxia for 15 min followed by full reoxygenation and measurement of changes in absorbance as described above. Lastly, to confirm that the swelling response was mediated by the mitoK$_{ATP}$ channel, K^+ was replaced with tetraethylammonium (TEA^+) and changes in absorbance at 540 nm in the presence of 25 µmol l^{-1} diazoxide alone and in combination with 50 µmol l^{-1} 5-HD were measured.

Data analysis

Data were tested for normality of distribution (chi-square test) and homogeneity of variances (Cochran's *C* test) before submission to one- or two-way ANOVA or analysis using a mixed-model repeated-measures general linear model (Statistica version 13.0, Dell Statistica, Tulsa, OK, USA). Significantly different means were separated using Tukey's *post hoc* test at $P<0.05$. The data are reported as means±s.e.m. except the kinetics of volume changes, which are means ($n=5$) without s.e.m.

RESULTS

Effects of mitoK$_{ATP}$ channel modulators on OXPHOS

We first measured the effects of diazoxide and 5-HD on OXPHOS with the mitoK$_{ATP}$ channels open (i.e. in the absence of Mg-ATP) to characterize their bioenergetic effects. Diazoxide (0-500 µmol l^{-1}) did not alter state 3 respiration ($F_{5,24}=0.92$, $P=0.49$; Fig. 1A) but did stimulate state 4 respiration ($F_{5,24}=4.69$, $P=0.004$; Fig. 1B), leading to a significant dose-related reduction in RCR ($F_{5,24}=6.64$, $P=0.0005$; Fig. 1C). Similarly, 5-HD did not significantly alter state 3 respiration ($F_{5,24}=2.47$, $P=0.06$; Fig. 1D) but did reduce state

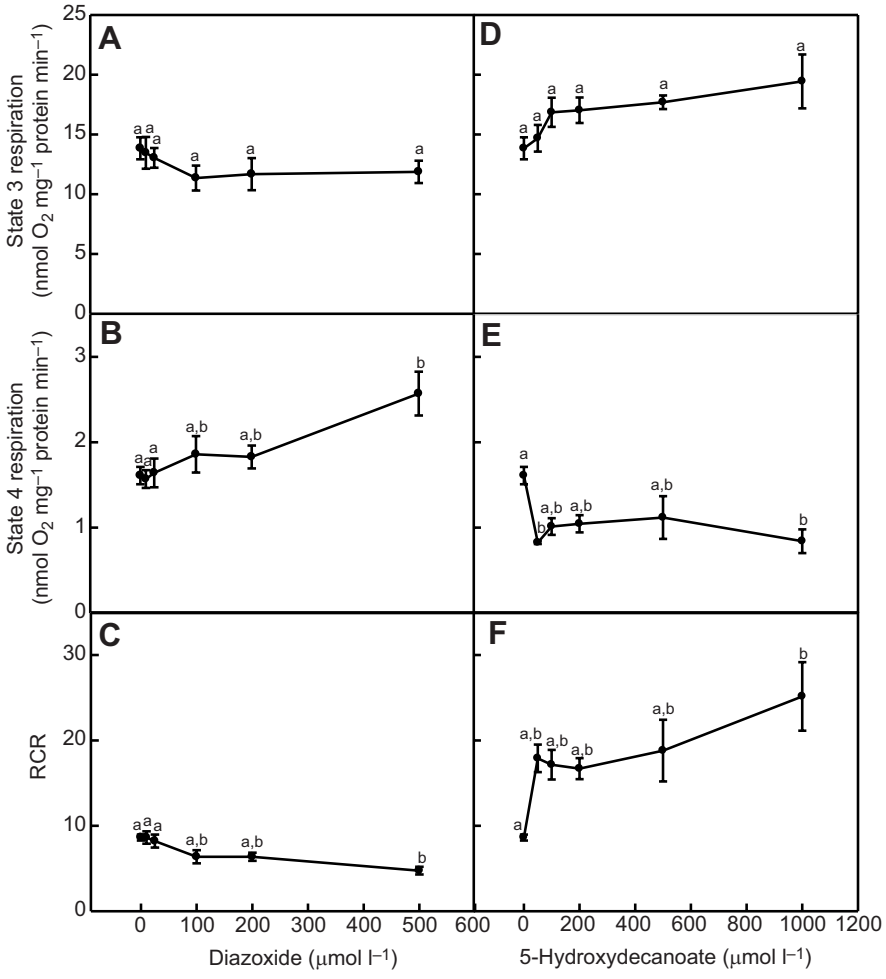

Fig. 1. The effect of different doses of diazoxide and 5-hydroxydecanoate (5-HD) on normoxic mitochondrial respiration. Effect of (A) diazoxide on state 3 respiration, (B) diazoxide on state 4 respiration, (C) diazoxide on respiratory control ratio (RCR), (D) 5-HD on state 3 respiration, (E) 5-HD on state 4 respiration and (F) 5-HD on RCR. Isolated mitochondria were exposed to diazoxide (10, 25, 100, 200 and 500 µmol l^{-1}) or 5-HD (50, 100, 200, 500 and 1000 µmol l^{-1}). Data are means±s.e.m. ($n=5$). Points with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P<0.05$).

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4 ($F_{5,24}$=4.37, P=0.006; Fig. 1E). These effects of 5-HD on state 3 and 4 respiration resulted in dose-related enhancement of RCR ($F_{5,24}$=4.61, P=0.004; Fig. 1F).

We then used combinations of low doses without bioenergetic effects (25 µmol l^{-1} diazoxide and 50 µmol l^{-1} 5-HD) and high doses with bioenergetic effects (500 µmol l^{-1} diazoxide and 1 mmol l^{-1} 5-HD) and tested their effects on post-H-R respiration using buffers without and with Mg-ATP. Mg-ATP blocks mitoK$_{ATP}$ channels on the cytosolic (*cis*) side (Bednarczyk et al., 2005) and in its absence the channels are open (Garlid, 2000) and therefore not amenable to modulation by diazoxide or 5-HD. We found that state 3 and state 4 respiration were slightly higher while RCR was slightly lower during normoxia when tested in the absence of Mg-ATP; however, these results were from different experiments and were not compared statistically.

In the absence of Mg-ATP (Fig. 2A), H-R ($F_{1,20}$=53.9, P<0.0001), treatment with mitoK$_{ATP}$ modulators ($F_{4,20}$=8.21, P=0.0004) and their interaction ($F_{4,20}$=6.18, P=0.002) significantly altered state 3 respiration. This response was primarily driven by the inhibitory effect of H-R in controls and that of H-R combined with the low and high doses of diazoxide. Interestingly, diazoxide at both low and high doses in the presence of 5-HD resulted in restoration of state 3 respiration to the normoxic level. Similarly, state 4 respiration (Fig. 2B) was altered by H-R ($F_{1,20}$=121, P<0.0001) and mitoK$_{ATP}$ modulators ($F_{4,20}$=37.8, P<0.0001), as well as their interaction ($F_{4,20}$=23.0, P<0.0001). Specifically, H-R stimulated state 4 respiration, with the high but not the low dose of diazoxide inhibiting this stimulation. Moreover, diazoxide at the high dose in the presence of 5-HD (high dose) exacerbated the stimulatory effect of H-R on state 4 respiration. Consistent with their effects on state 3 and 4 respiration, H-R ($F_{1,20}$=728, P<0.0001) and mitoK$_{ATP}$ modulators ($F_{4,20}$=25.1, P<0.0001) reduced the RCR and showed a significant interaction effect ($F_{4,20}$=11.45, P=<0.0001; Fig. 2C). Here, except for the combined high doses of diazoxide and 5-HD, all of the treatments minimized the RCR-reducing effect of H-R.

With Mg-ATP in the buffer (Fig. 2D), the interaction between treatment with mitoK$_{ATP}$ modulators and H-R was significant for state 3 ($F_{4,20}$=3.50, P=0.03) but their individual effects were not. Interestingly, the low dose of diazoxide alone and with a low dose of 5-HD did not affect state 3 respiration, but their combination at high doses reduced state 3 respiration relative to the controls. In contrast,

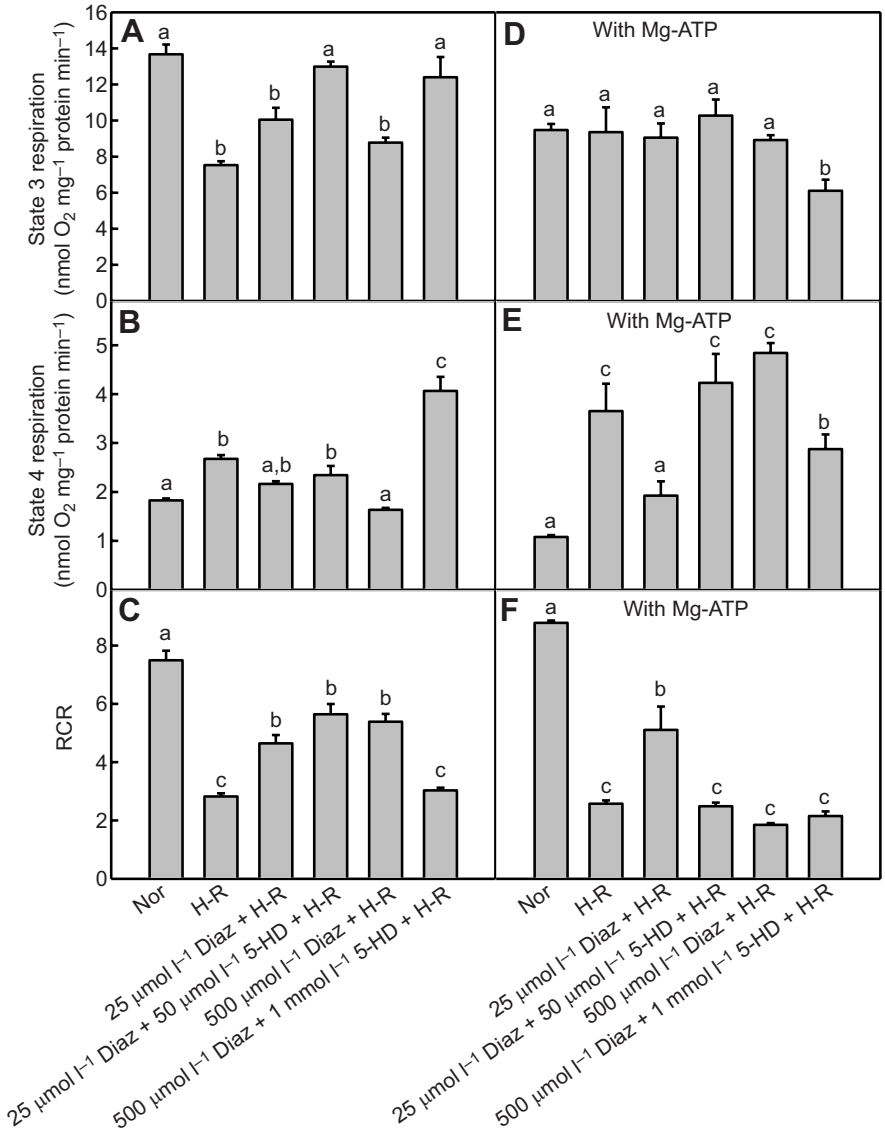

Fig. 2. The effects of diazoxide and 5-HD on mitochondrial oxidative phosphorylation capacity during hypoxia–reoxygenation (H-R) in respiration buffer with and without Mg-ATP. (A) State 3 respiration, (B) state 4 respiration and (C) RCR in Mg-ATP-free respiration buffer, and (D) state 3 respiration, (E) state 4 respiration and (F) RCR in buffer containing Mg-ATP. Low doses [25 µmol l^{-1} diazoxide (Diaz) and 50 µmol l^{-1} 5-HD] and high doses (500 µmol l^{-1} and 1 mmol l^{-1} 5-HD) were tested under normoxia (Nor) and after 15 min H-R at 13°C. Data are means±s.e.m. (n=5). Bars with different letters are statistically different from each other (mixed model repeated measures ANOVA with Tukey's HSD, P<0.05).

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H-R ($F_{1,20}$=179, P<0.0001), treatment with mitoK$_{ATP}$ modulators ($F_{4,20}$=6.55, P=0.0015) and their interaction ($F_{4,20}$=8.27, P=0.0004) significantly altered state 4 respiration (Fig. 2E). Here, as expected, H-R stimulated state 4 respiration, and the low but not the high dose of diazoxide reversed the H-R effect. Importantly, the low dose of 5-HD reversed the inhibitory effect of diazoxide on state 4 respiration, essentially restoring it to the H-R level. However, the high dose of 5-HD reduced the stimulatory effect of the high dose of diazoxide on state 4 respiration after H-R. Lastly, the RCR was significantly reduced by H-R ($F_{1,20}$=897, P<0.0001), treatment with mitoK$_{ATP}$ modulators ($F_{4,20}$=8.29, P=0.0004) and their interaction ($F_{4,20}$=13.89, P<0.0001; Fig. 2F). The low dose of diazoxide partially protected against the RCR-reducing effect of H-R, and this protection was blocked by low dose of 5-HD. In contrast, the high dose of diazoxide alone and in combination with 5-HD (high dose) did not alter the RCR-reducing effect of H-R.

Role of mitoK$_{ATP}$ channel in mitochondrial function and volume homeostasis

Under normoxia without Mg-ATP (Fig. 3A,B), the high dose of diazoxide and Ca (positive control) caused mitochondrial swelling of similar form and amplitude. The low dose (25 µmol l^{-1}) of diazoxide without and with the low dose of 5-HD did not significantly alter mitochondrial volume, whereas its high dose induced significant swelling that was reversed by the high dose of 5-HD. Mitochondrial swelling following exposure to H-R alone was similar to that resulting from exposure to combined H-R and Ca or Ca alone (Fig. 3C,D). Incubating mitochondria with low and high doses of diazoxide before exposure to H-R resulted in less swelling relative to H-R alone, and 5-HD at both low and high doses did not reverse these effects of diazoxide (Fig. 3C,D).

In the presence of Mg-ATP, mitochondria displayed high-amplitude swelling, as shown by the Ca-positive control

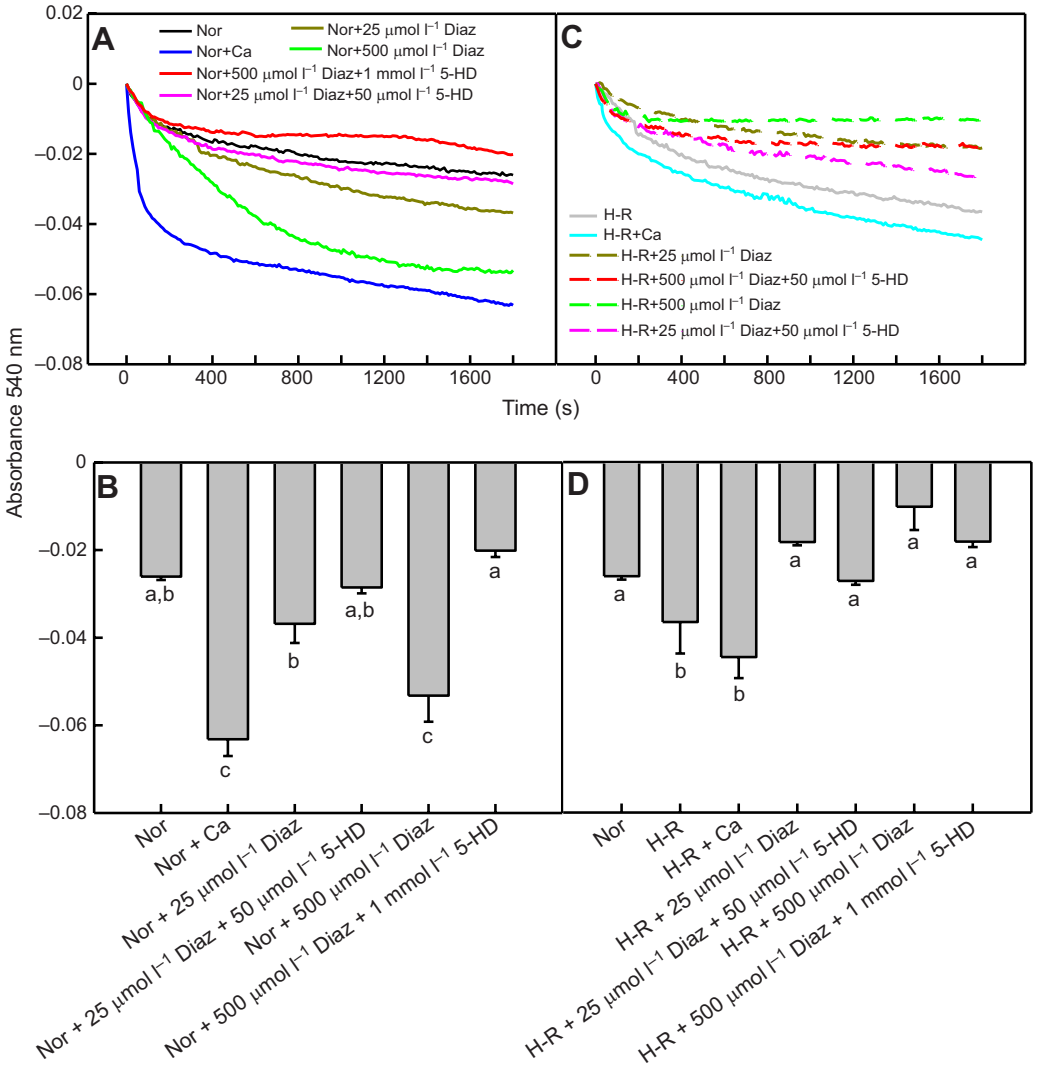

Fig. 3. The effects of diazoxide and 5-HD on mitochondrial volume under normoxia (Nor) and after 15 min H-R in Mg-ATP-free swelling buffer at 24°C. (A) Normoxic swelling kinetics, (B) normoxic swelling amplitude at 30 min, (C) H-R swelling kinetics and (D) H-R swelling amplitude at 30 min. Low doses [25 µmol l^{-1} diazoxide (Diaz) and 50 µmol l^{-1} 5-HD] and high doses (500 µmol l^{-1} and 1 mmol l^{-1} 5-HD) were tested with 200 µmol l^{-1} Ca as positive control. Swelling was monitored every 10 s for 30 min as absorbance changes at 540 nm, and the kinetics and terminal amplitude of volume changes after 30 min are shown. The trend lines in A and C represent means of swelling data from five independent mitochondrial preparations (i.e. n=5). Bars with different letters in B and D are statistically different from each other (one-way ANOVA with Tukey's HSD, P<0.05, n=5).

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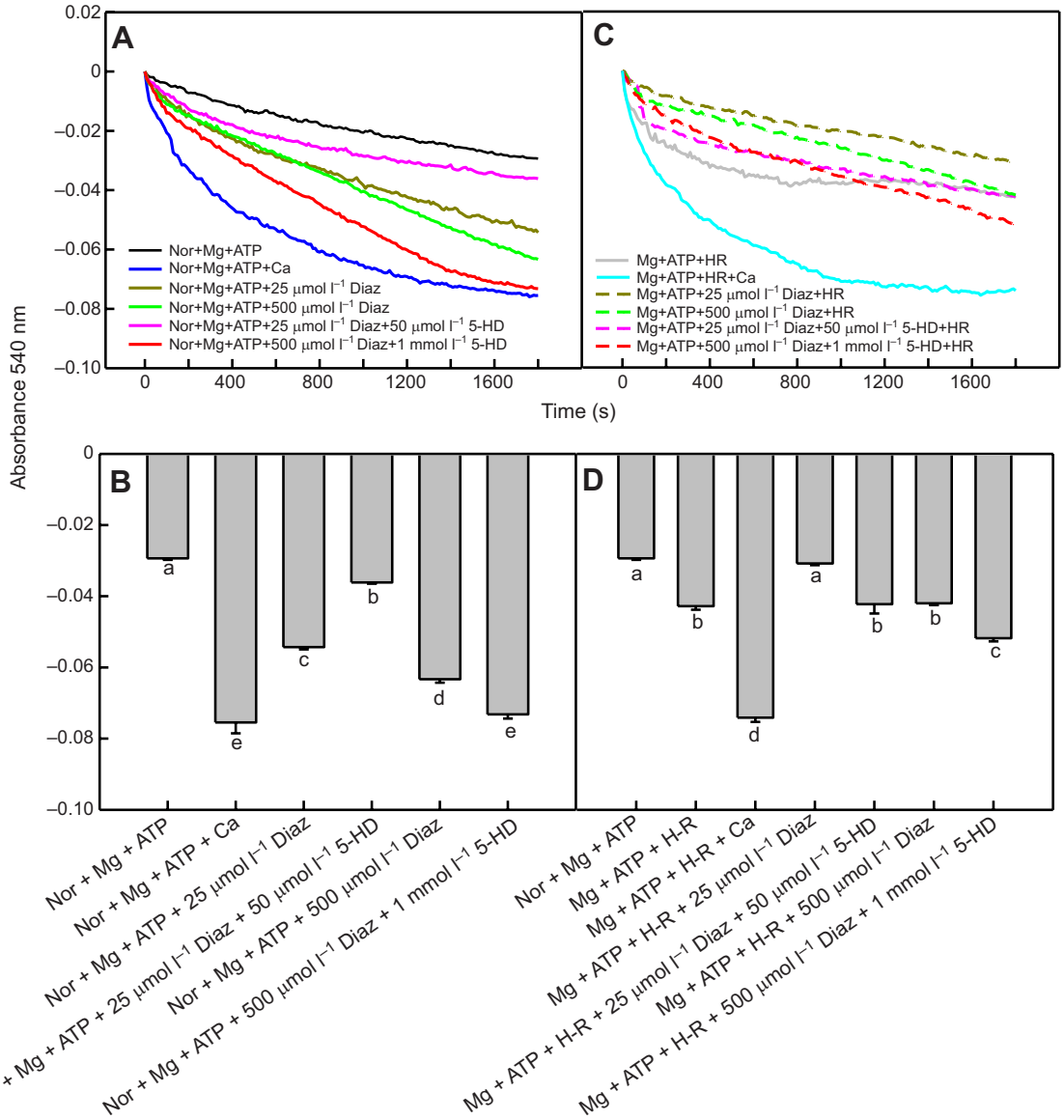

Fig. 4. The effects of diazoxide and 5-HD on mitochondrial volume under normoxia (Nor) and after 15 min H-R in the presence of Mg-ATP in swelling buffer at 24°C. (A) Normoxic swelling kinetics, (B) normoxic swelling amplitude at 30 min, (C) H-R swelling kinetics and (D) H-R swelling amplitude at 30 min. Low doses [25 μmol l^{-1} diazoxide (Diaz) and 50 μmol l^{-1} 5-HD] and high doses (500 μmol l^{-1} and 1 mmol l^{-1} 5-HD) were tested with 200 μmol l^{-1} Ca as positive control. Swelling was monitored every 10 s for 30 min as absorbance changes at 540 nm, and the kinetics and terminal amplitude of volume changes after 30 min are shown. The trend lines in A and C represent means of swelling data from five independent liver mitochondrial preparations (i.e. n=5). Bars with different letters in B and D are statistically different from each other (one-way ANOVA with Tukey's HSD, P<0.05, n=5).

(Fig. 4A,B). Here, the low dose of diazoxide caused significant swelling that was reversed by the low dose of 5-HD. Moreover, the high dose of diazoxide resulted in greater swelling than that which resulted from its low dose, but this swelling was exacerbated by 5-HD at the high dose. Exposure of mitochondria to H-R resulted in swelling relative to normoxia; however, Ca-induced swelling after H-R was of similar magnitude as that observed under normoxia (Fig. 4). Importantly, the H-R-induced swelling was reversed by the low dose of diazoxide, and 5-HD at the low dose blocked this effect. In contrast, the high dose of diazoxide did not alter H-R-induced swelling, and the addition of a high dose of 5-HD exacerbated the swelling. The role of the mitoK$_{ATP}$ channel was corroborated by the observation that 5-HD reversed swelling in the presence of K^+ but not TEA^+ when Mg-ATP was present (Fig. 5).

DISCUSSION

Opening mitoK$_{ATP}$ channels is believed to protect against the deleterious effects of IR; however, the effects of the two widely used mitoK$_{ATP}$ channel modulators, diazoxide (channel opener) and 5-HD (channel blocker), are controversial. Specifically, Garlid (2000) argued that opening the mitoK$_{ATP}$ channel has minimal direct effects on mitochondrial bioenergetics and that bioenergetic effects observed by others (Holmuhamedov et al., 1999, 2004; Dröse et al., 2006; Kopustinskiene et al., 2010) reflect toxic effects secondary to the use of excessively high doses of the modulators. Thus, we sought to shed light on the controversies surrounding the effects of diazoxide and 5-HD on OXPHOS and mitochondrial volume. We found that under mitoK$_{ATP}$ channel open conditions (no Mg-ATP), both low and high doses of diazoxide improved mitochondrial coupling but only the high dose of 5-HD reversed the

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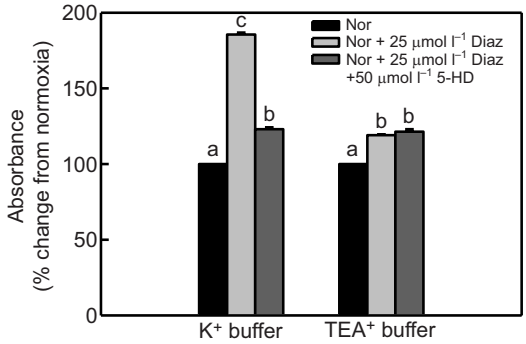

Fig. 5. Percent change in mitochondrial volume from control in K^+ and TEA^+ buffers containing Mg-ATP under normoxia (Nor). Volume changes were measured following exposure of mitochondrial suspension to 25 µmol l^{-1} diazoxide (Diaz) and 25 µmol l^{-1} diazoxide+50 µmol l^{-1} 5-HD. Swelling was monitored every 10 s for 30 min as absorbance changes at 540 nm. Data are means±s.e.m. (n=3) after 30 min. Bars with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, P<0.05).

post-H-R coupling-enhancing effect of diazoxide (Fig. 2C). Although 5-HD can be converted to 5-HD-CoA, which is metabolized via β-oxidation to provide NADH, which in turn supplies electrons to the electron transport system, thus stimulating mitochondrial respiration (Lim et al., 2002; Hanley et al., 2005), its high doses inhibit both β-oxidation and succinate dehydrogenase/complex II (Hanley et al., 2002, 2005; Lim et al., 2002). The 5-HD dose range tested in our study did not significantly alter state 3 respiration of mitochondria respiring on a complex I substrate, but inhibited state 4 respiration, thereby improving coupling. In tests carried out in the presence of Mg-ATP, the low dose of diazoxide alone had a bioenergetic effect evidenced by improved coupling post-H-R, and this effect was abolished by 5-HD at the low dose (Fig. 2F). Note that this low dose of diazoxide had no effect on complex-I-supported OXPHOS under normoxia (Fig. 1). That is, our findings are consistent with earlier reports on the beneficial effects of a low dose of diazoxide on mammalian mitochondrial coupling (Iwai et al., 2000; Dos Santos et al., 2002).

The primary role of the mitoK$_{ATP}$ channel is believed to be mitochondrial volume regulation (Jaburek et al., 1998; Garlid, 2000; Costa et al., 2006). In our study, diazoxide at the low dose had no effect in the absence of Mg-ATP under normoxic conditions, but its high dose caused mitochondria to swell, and 5-HD at the high dose reversed this effect. Because mitoK$_{ATP}$ channels were open throughout during this assay, these volume changes were likely mediated by mechanisms other than the mitoK$_{ATP}$ channel, e.g. opening of mitochondrial permeability transition pores (Hausenloy et al., 2004) and/or inhibition of the K^+/H^+ exchanger. Under these conditions, the reversal of the effect of diazoxide by 5-HD can be explained by their diametrically opposite effects on OXPHOS (Fig. 1). Additional evidence of diazoxide altering mitochondrial volume via mechanisms independent of the mitoK$_{ATP}$ channel was that its reversal of H-R-induced swelling was not sensitive to 5-HD. In contrast, in the presence of Mg-ATP under normoxia, swelling induced by diazoxide (low dose) was partially reversed by 5-HD and could therefore in part be attributed to the opening of the mitoK$_{ATP}$ channel. The fact that diazoxide at the high dose caused greater swelling relative to the low dose and the addition of 5-HD worsened this effect implies that this was a toxic response. Notably, the low dose of diazoxide protected the mitochondria from H-R-induced swelling, an effect that was reversed by the low dose of 5-HD, thus suggesting a role of the mitoK$_{ATP}$ channel in this phenomenon and in preserving state 3 respiration. In contrast, diazoxide at the high dose failed to reduce the swelling caused by H-R and the addition of 5-HD (high dose) enhanced mitochondrial swelling (Fig. 4). Because the mitoK$_{ATP}$ channel is closed by Mg-ATP, it is logical that H-R-induced swelling would be less pronounced in the presence of Mg-ATP than in its absence. Overall, our study showed that although both the opening of mitoK$_{ATP}$ channels and bioenergetic effects of diazoxide were protective against H-R-induced swelling in the presence of Mg-ATP, only the bioenergetic effect was protective in the absence of Mg-ATP. Furthermore, mitochondrial swelling was responsive to low doses of diazoxide and 5-HD in K^+ but not TEA^+ buffer, confirming the involvement of the mitoK$_{ATP}$ channel in mitochondrial volume regulation (Beavis et al., 1993; Garlid et al., 1996; Jaburek et al., 1998).

Several previous studies have reported that mitochondria shrink/contract at high phosphorylation state/state 3 respiration (Packer, 1960; Garlid, 2000; Hackenbrock, 1968; Kowaltowski et al., 2001) and swell in state 4 respiration (Packer, 1960; Hackenbrock, 1968; Bosetti et al., 2004). In our study, the relationship between H-R-induced swelling and state 3 respiration was positive in the presence of Mg-ATP and negative in its absence. In contrast, state 4 respiration (proton leak) was positively correlated with mitochondrial volume both in the presence and absence of Mg-ATP, but the leak was five times higher in the presence of Mg-ATP. A possible explanation of these findings is that during phosphorylation (state 3), the proton gradient that drives electrogenic K^+ entry into the mitochondria matrix is consumed for ATP synthesis, thus reducing the influx of K^+, and subsequently that of osmotically obliged water (Garlid, 2000). Conversely, the swelling in state 4 respiration was possibly due to the high membrane potential (proton gradient) that supports high rates of K^+ and water influx. Overall, it appears that assay conditions and the procedure of inducing swelling define the mitochondrial volume–functional state relationship.

Conclusions

The prediction that modulators (diazoxide and 5-HD) of the mitoK$_{ATP}$ channel would exhibit a dose-dependent opposite effect on mitochondrial bioenergetics was confirmed in our study, and explains their antagonistic effects on mitochondrial responses not mediated by mitoK$_{ATP}$ channel opening. The prediction that in the absence of Mg-ATP diazoxide and 5-HD would modulate only mitochondrial bioenergetics was corroborated by our findings and highlighted the physiological role of Mg-ATP in blocking the mitoK$_{ATP}$ channel. Notably, the low dose of diazoxide in the presence of Mg-ATP had both volume regulatory and bioenergetic effects. Finally, high doses of diazoxide and 5-HD alleviated H-R-induced damage by altering mitochondrial bioenergetics in the absence but not in the presence of Mg-ATP. Thus, both the opening of the mitoK$_{ATP}$ channel and bioenergetic effects of diazoxide protected against H-R-induced mitochondrial swelling and preserved their functional states.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.K., J.O.O.; Methodology: C.K., J.O.O., D.S.; Formal analysis and investigation: C.K., J.O.O., D.S.; Writing - original draft preparation: J.O.O.;

Writing - review and editing: C.K., D.S., J.O.O.; Funding acquisition: C.K., D.S.; Resources: C.K., D.S.; Supervision: C.K., D.S.

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Data availability
Data are available from the University of Prince Edward Island data repository (https://data.upei.ca/) at: https://data.upei.ca/islandora/object/researchdata%3A410

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