Ischaemic Preconditioning Protects Skeletal Myotubes Against Ischaemia-reperfusion Injury

Pauline Walsh¹, Kevin Mulhall².
¹University College Dublin, Dublin, Ireland, ²Mater Misericordiae University Hospital, Dublin, Ireland.

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Introduction:

Ischaemic preconditioning (IPC) is a well recognised and powerful phenomenon whereby a tissue becomes more tolerant to a period of prolonged ischaemia when it is first subjected to short bursts of ischaemia/reperfusion. While much is known about the ability of IPC to protect myocardial tissue against ischaemia-reperfusion injury, its potential to confer benefit in an orthopaedic setting by protecting skeletal muscle remains relatively unexplored to date. One mechanism by which IPC may induce protection is through a reduction in oxidative stress. Reactive oxygen species (ROS) are generated both during prolonged ischaemia and also upon reperfusion, thereby leading to an increase in oxidative stress. The transcription factor, NF-E2-related factor 2 (Nrf2), is a key regulator of the cells response to oxidative stress as it regulates the expression of a network of anti-oxidant/detoxifying enzymes. Nrf2 signalling has recently been shown to protect against ischaemia-reperfusion injury in both a kidney cell line and in liver biopsies, indicating that this transcription factor may play a key role in the protection provided by ischaemic preconditioning.

To date, the involvement of Nrf2 in the response of skeletal muscle to ischaemia-reperfusion has not been investigated. Thus, the aims of this study were to investigate the ability of ischaemic preconditioning to protect skeletal myotubes against ischaemia-reperfusion and to determine the role of Nrf2 signalling in this protection.

Methods:

C2C12 mouse myoblasts were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in DMEM containing 20% FBS. When cultures were approximately 90% confluent, myoblasts were differentiated to myotubes by changing to DMEM supplemented with 2% horse serum and culturing for 7-10 days. Differentiated myotubes were then exposed to varying periods of simulated ischaemia (1% O₂) followed by 2h re-oxygenation (21% O₂). To precondition myotubes, cells were subjected to 30 min of simulated ischaemia followed by 1 hour re-oxygenation prior to the prolonged ischaemic event.

Cell survival was assessed by lactate dehydrogenase release. Changes in Nrf2 expression were assessed using real-time PCR and Western blotting. Western blots were performed on nuclear extracts after protein separation by SDS-PAGE and probed with primary antibodies against Nrf2 (Abcam) and the nuclear marker, Lamin B1 (Santa Cruz). Changes in sequestosome-1/P62 (SQSTM1/P62), catalase (CAT), glutathione S-transferase theta-1 (GSTT1), heme oxygenase-1 (HO-1) expression were assessed using real-time PCR.

Statistical analyses were performed using the one-way
analysis of variance (ANOVA) and the paired t-test.

Results:

Preconditioned myotubes showed greater viability both after 4h of ischaemia, and after 4h ischaemia followed by 2h of re-oxygenation (p ≤ 0.05). This increase in cell viability was associated with increased nuclear expression of Nrf2. In addition, gene expression analysis revealed an up-regulation in the expression of a number of important oxidative stress defense genes, which are under the control of the Nrf2 transcription factor, in preconditioned myotubes including SQSTM1, and the antioxidant enzymes, CAT, GSTT1 and HO-1 (p ≤ 0.05).

Discussion: Our findings indicate that ischaemic preconditioning can protect skeletal myotubes against the effects of ischaemia-reperfusion in vitro. This protection is associated with increased Nrf2 signalling indicating that this transcription factor may play a role in mediating the protection induced by ischaemic preconditioning. These findings indicate that the induction of an oxidative stress response may be an important factor in the protection provided by ischaemic preconditioning.

Significance: Our findings show that ischaemic preconditioning increased the survival of skeletal myotubes exposed to ischaemia. This protection was associated with increased Nrf2 signalling indicating that this transcription factor may play an important role in mediating the protection induced by ischaemic preconditioning.

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References:
Figure 1 Effect of ischaemic preconditioning on skeletal myotube viability. A, exposure to increasing periods of simulated ischaemia (0 – 6 h) resulted in increased cellular injury as examined by LDH release. B, morphological changes of cells exposed to simulated ischaemia included cell shrinking and rounding (magnification x 10). C + D, IPC attenuated cellular injury following 4h of simulated ischaemia as examined by LDH release. Cntl – control, SI – simulated ischaemia, IPC = ischaemic preconditioning, * = p<0.05.
Figure 2 Effect of ischaemic preconditioning on oxidative stress defence gene expression and Nrf2 nuclear accumulation. Skeletal myotubes were preconditioned by exposing cultures to 30 min of simulated ischaemia (1% O2). Preconditioned cultures showed (A) increased oxidative stress gene expression and (B) increased nuclear accumulation of Nrf2. Cat = catalase, Gstt1 = glutathione S-transferase theta-1, Hmox1 = heme oxygenase-1, Sqstm1 = sequestosome-1, * = p<0.05.