

# High glucose sensitizes adult cardiomyocytes to ischaemia/reperfusion injury through nitrative thioredoxin inactivation

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## KEYWORDS

Diabetes;  
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**Aims** Ischaemic cardiac injury is significantly increased in diabetic patients, but its underlying mechanisms remain incompletely understood. The current study attempted to identify new molecular mechanisms potentially contributive to hyperglycaemic-exaggeration of myocardial ischaemic injury.

**Methods and results** Adult mouse cardiomyocytes were cultured in normal-glucose (NG, 5.5 mM) or high-glucose (HG, 25 mM) medium. Twelve hours after NG or HG pre-culture, cardiomyocytes were subjected to 3 h of simulated ischaemia (SI), followed by 3 h of reperfusion (R) in NG medium. Prior to and after SI/R, the following were determined: cardiomyocyte death and apoptosis, sustained oxidative/nitrative stress and thioredoxin (Trx) activity, expression, and nitration. Compared with NG-cultured cardiomyocytes, 12 h HG culture significantly increased superoxide and peroxynitrite production, increased Trx-1 nitration, and reduced Trx activity ( $P < 0.01$ ). Despite being subject to identical SI/R procedures and conditions, cells pre-cultured in HG sustained greater injury, evidenced by elevated lactate dehydrogenase release and caspase-3 activation ( $P < 0.01$ ). Moreover, SI/R induced greater superoxide/peroxynitrite overproduction and greater Trx-1 nitration and inactivation in HG pre-cultured cardiomyocytes than in NG pre-cultured cardiomyocytes. Finally, the supplementation of human Trx-1, superoxide scavenger, or peroxynitrite decomposition catalyst in HG pre-cultured cells reduced Trx-1 nitration, preserved Trx-1 activity, and normalized SI/R injury to levels observed in NG pre-cultured cardiomyocytes.

**Conclusion** High glucose sensitized cardiomyocytes to ischaemia/reperfusion injury through nitrative Trx-1 inactivation. Interventions restoring Trx-1 activity in the diabetic heart may represent novel therapies attenuating cardiac injury in diabetic patients.

## 1. Introduction

Cardiovascular disease is the most prevalent aetiology of morbidity and mortality among patients with diabetic mellitus.<sup>1</sup> Recent experiments have demonstrated that hyperglycaemia not only causes vascular injury that leads to ischaemic heart disease, but also adversely impacts ischaemic cardiomyocytes directly, resulting in larger infarct size and more severe heart failure after ischaemia/reperfusion. Enormous efforts have been made to identify the diabetic vascular injury mechanisms responsible for increased morbidity in diabetic patients suffering ischaemic heart disease. Many signalling pathways leading to diabetic vascular dysfunction/cellular injury have been reported.<sup>2</sup> However, the

molecular basis linking diabetes with increased vulnerability to ischaemia/reperfusion injury and resultant higher mortality has not been established.

Thioredoxin (Trx, including 12 kDa cytosolic Trx-1 and 18 kDa mitochondrial Trx-2) is a small protein ubiquitously expressed in living cells, with many protective biological functions.<sup>3</sup> Trx not only exerts cytoprotective functions against oxidative stress, but also regulates cell survival signalling pathways.<sup>4</sup> Clinical and experimental results have demonstrated that inhibition of Trx-1 promotes apoptosis.<sup>5</sup> Recent *in vitro* studies demonstrate that Trx-1 is inhibited via direct interaction with apoptosis-regulating kinase-1 (ASK1), a mitogen-activated protein (MAP) kinase that activates two pro-apoptotic kinases, p38 MAP kinase (MAPK) and c-Jun N-terminal kinase (JNK).<sup>6</sup> These results suggest Trx-1 may play a critical role in regulating the balance between cell proliferation and cell death. However, whether Trx-1 activity is reduced in the hyperglycaemic heart, thus

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contributing to increased diabetic cardiomyocyte death, has never been previously investigated.

Reactive oxygen species (ROS) have long been recognized to cause oxidative protein modification, and act as major mediators of tissue injury associated with hyperglycaemia.<sup>7</sup> However, the observed increased post-ischaemic cardiomyocyte apoptosis in diabetic animals (and the increased susceptibility of the diabetic heart to ischaemia/reperfusion injury) cannot be exclusively explained by ROS production, and many fundamental questions remain unresolved.<sup>8</sup> Recent data have identified nitric oxide-derived reactive nitrogen species (RNS), such as peroxynitrite (ONOO<sup>-</sup>), as critical contributors of protein modification and cell injury, providing potential targets for therapeutic interventions.<sup>9</sup> However, whether increased RNS production may increase cardiomyocyte apoptosis via post-translational modification of any specific protein remains unknown.

Therefore, the aim of the present study was (i) to determine whether Trx activity is reduced in cardiomyocytes exposed to hyperglycaemic conditions; (ii) if so, to identify the mechanism responsible for hyperglycaemia-induced Trx activity alteration; and (iii) to investigate whether treatments preserving Trx activity after hyperglycaemic exposure might attenuate subsequent ischaemia/reperfusion injury.

## 2. Material and methods

### 2.1 Adult mouse cardiomyocyte culture

All experiments were performed on adult (8–10 weeks) male C57BL6 mice. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the University Committee on Animal Care. Cardiomyocytes were isolated as reported in our previous studies.<sup>10,11</sup> In brief, mice were anesthetized with 2% isoflurane, and hearts were removed and perfused at 37°C for ~3 min with a Ca<sup>2+</sup>-free bicarbonate-based buffer. Enzymatic digestion was initiated by adding collagenase type B/D to the perfusion solution (Sigma Aldrich, St Louis, MO, USA). After appropriate digestion, 50 μM Ca<sup>2+</sup> was added to the enzyme solution. Approximately 7 min later, the left ventricle was removed, cut into several sections, and further digested for 10 min at 37°C. The supernatant containing the dispersed myocytes was filtered into a sterilized tube and centrifuged at 800 g for 1 min. The cell pellet was then resuspended in bicarbonate-based buffer containing 125 μM Ca<sup>2+</sup>. After the myocytes were pelleted by gravity for ~10 min, the supernatant was aspirated, and the myocytes were resuspended in bicarbonate-based buffer containing 250 μM Ca<sup>2+</sup>. Criteria for viable mouse cardiomyocytes were (i) rod shape, (ii) clearly defined sarcomeric striations, and (iii) quiescent state (no spontaneous contractile waves) for at least 5 min during observation.<sup>12</sup> With this method, we routinely obtained a high yield (>5.5 × 10<sup>6</sup> cells/left ventricle) and high percentage (>86%) of rod-shaped myocytes that were suitable for our studies. Myocytes were plated at 0.5–1 × 10<sup>4</sup> cells cm<sup>-2</sup> in culture dishes pre-coated in mouse laminin.

One hour after plating, cells were washed with PBS and non-adhering cells were removed from the culturing system. Cells were randomized to receive one of the

following treatments: (i) normal glycaemic (NG) control (culture medium containing 5.5 mM glucose and 19.5 mM mannitol); (ii) hyperglycaemia (HG) without treatment (culture medium containing 25 mM glucose<sup>13,14</sup>); (iii) hyperglycaemia with human Trx-1 treatment (HG+hTrx-1, culture medium containing 25 mM glucose plus 1 μg/mL Trx-1)<sup>15</sup>; (iv) hyperglycaemia with INO-4885 treatment (NG+INO-4885, culture medium containing 25 mM glucose plus 10 μg/mL INO-4885,<sup>16</sup> a peroxynitrite decomposition catalyst); (v) hyperglycaemia with Tiron treatment (HG+Tiron, culture medium containing 25 mM glucose plus 100 mM Tiron,<sup>17</sup> a cell membrane permeable superoxide scavenger); (vi) hyperglycaemia with 1400W treatment (HG+1400W, culture medium containing 25 mM glucose plus 20 μM 1400W<sup>11</sup>); and (vii) hyperglycaemia with diphenyleneiodonium (DPI) treatment ((HG+DPI, culture medium containing 25 mM glucose plus 10 μM DPI<sup>18</sup>).

### 2.2 Simulated ischaemia/reperfusion

After culture in the mediums specified above for 12 h (24 h for extended medium exposure), 50% of cardiomyocytes were used to determine cell viability, superoxide and peroxynitrite production, and Trx-1 assays (described in detail below). Another 50% of cardiomyocytes were subjected to sham-simulated ischaemia/reperfusion (SI/R) or SI/R as originally described by Isner and colleagues<sup>19</sup> and modified in our previous study.<sup>20</sup> In brief, glucose-free culture medium was first exposed to a hypoxic gas mixture (95%N<sub>2</sub>/5%CO<sub>2</sub>) for 5 min. Normal or high-glucose culture medium was quickly replaced with the hypoxia-hypoglycaemic medium, and cardiomyocytes were placed in a Napco 8000WJ hypoxia (1%O<sub>2</sub>/5%CO<sub>2</sub>/94%N<sub>2</sub>) incubator (Thermo Scientific, Waltham, MA, USA). After 3 h of hypoxia/hypoglycaemic exposure, the medium was replaced with medium of normal-glucose concentration. Cells were incubated for an additional 3 h under normoxic conditions in a CO<sub>2</sub> incubator before the below assays were performed. All cells were subjected to identical SI/R procedure. No additional treatments were instituted during the SI/R period.

### 2.3 Assessment of simulated ischaemia/reperfusion-induced cardiomyocyte injury

To determine cardiomyocyte death, lactate dehydrogenase (LDH) release into medium was determined by an enzyme activity assay kit (Sigma-Aldrich, St Louis, MO, USA). All values were expressed as percent LDH released relative to the value obtained following cell permeabilization with 50 μM digitonin. To determine cardiomyocyte apoptosis, caspase-3 activity was determined as reported in our previous study.<sup>11</sup> Caspase-3 activity was expressed as nmol pNA/h/mg protein.

### 2.4 Quantification of superoxide production and cellular nitrotyrosine content

Superoxide production, an index for oxidative stress, in viable cardiomyocytes was measured by lucigenin-enhanced chemiluminescence as described previously,<sup>11</sup> and expressed as relative light units (RLU) per second per milligram protein (RLU/s/mg protein). Cardiomyocyte nitrotyrosine content, an index of protein nitration and nitrate stress, was determined as described in our previous study.<sup>11</sup>

## 2.5 Trx activity assay

Trx activity was determined via the insulin disulfide reduction assay.<sup>21</sup> In brief, 40  $\mu$ g of cellular protein extracts were pre-incubated at 37°C for 15 min with 2  $\mu$ L activation buffer (100 mM HEPES, 2 mM EDTA, 1 mg/mL BSA, and 2 mM DTT) to reduce thioredoxin. After addition of 20  $\mu$ L reaction buffer (100 mM HEPES, 2.0 mM EDTA, 0.2 mM NADPH, and 140  $\mu$ M insulin), the reaction was initiated by addition of mammalian Trx reductase (1  $\mu$ L, 15 mU, Sigma) or water to controls. After incubation for 30 min at 37°C, the reaction was terminated by adding 125  $\mu$ L of stopping solution (0.2 M Tris-CL, 10 M guanidine-HCl, and 1.7 mM 3-carboxy-4-nitrophenyl disulfide, DTNB) followed by absorption measurement at 412 nm.

## 2.6 Western blot analysis

Cardiomyocytes were collected in lysis buffer. Equal amounts of protein (80  $\mu$ g protein/lane) were electrophoresed on a 14% SDS-polyacrylamide gel, and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% skim milk in TBS containing 0.05% Tween 20 at room temperature for 1 h, the membrane was incubated first with a monoclonal anti-murine Trx-1 antibody (Redox Bioscience, Japan), and subsequently with HRP-linked anti-mouse IgG (Cell Signaling, Danvers, MA, USA). The blot was developed with a Supersignal chemiluminescent detection kit (Pierce Chemical, Rockford, IL, USA), and visualized with a Kodak Image Station 400. The blot densities were analysed with Kodak 1D software.<sup>22</sup>

## 2.7 Detection of Trx-1 nitration

Cardiomyocytes were homogenized with lysis buffer. Endogenous Trx-1 was immunoprecipitated with a

monoclonal anti-murine Trx-1 antibody (Redox Bioscience, Japan). After sample separation, Trx-1 nitration was detected with a monoclonal antibody (Upstate, Charlottesville, VA, USA) against nitrotyrosine. The blot was developed with Supersignal-Western reagent (Pierce) and visualized with a Kodak Image Station 400. The blot densities were analysed with Kodak 1D software (version 3.6).

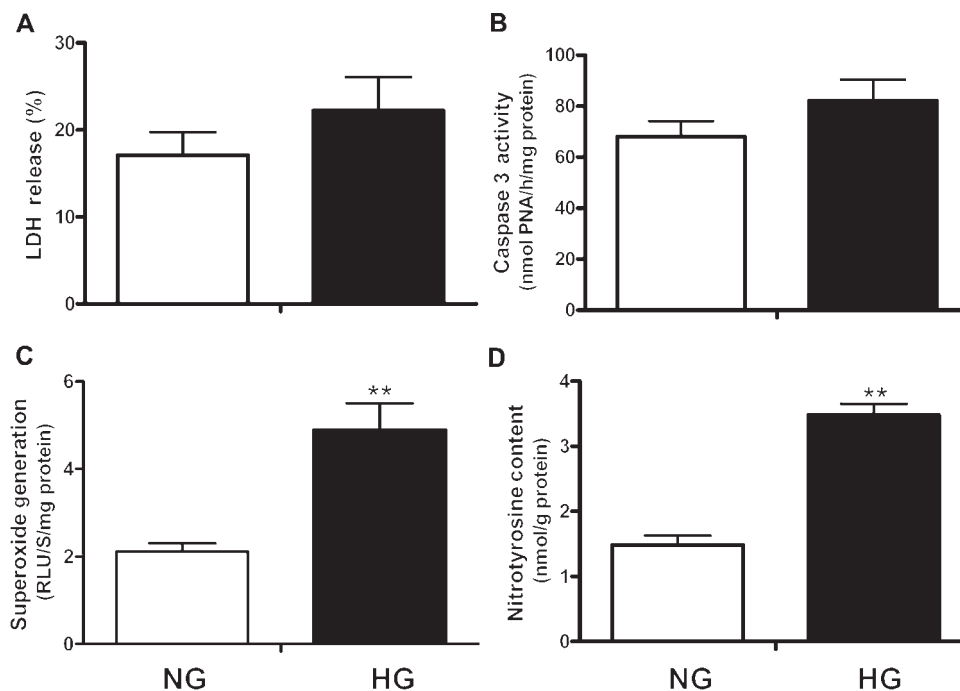
## 2.8 Statistical analysis

All values in the text and figures are presented as means  $\pm$  SEM of *n* independent experiments. All data (except Western blot density) were subjected to ANOVA, followed by Bonferroni correction for *post-hoc t*-test. Western blot densities were analysed with the Kruskal-Wallis test, followed by Dunn's *post hoc* test. Probabilities of 0.05 or less were considered to be statistically significant.

## 3. Results

### 3.1 Effect of high-glucose culture on cell viability and oxidative/nitrative stress

Our pilot experimental results demonstrated no difference in cell viability, oxidative/nitrative stress, and Trx-1 activity between cardiomyocytes cultured in 5.5 mM glucose vs. 5.5 mM glucose plus 19.5 mM mannitol. Cell cultured in 5.5 mM glucose and 19.5 mM mannitol (normal glucose with osmotic control) was thus used as a control group in all subsequent experiments. Twelve hours of high-glucose culture slightly increased cardiomyocyte LDH release and caspase-3 activity (Figure 1A and B). However, 12 h of high-glucose culture significantly increased superoxide production in cardiomyocytes (Figure 1C). Similarly, protein nitration (as assessed by nitrotyrosine content) was significantly increased in high-glucose-cultured cardiomyocytes (Figure 1D). Although cellular proteins can be nitrated



**Figure 1** Effect of 12 h high-glucose exposure on cardiomyocyte LDH release (A), caspase-3 activity (B), superoxide production (C), and peroxynitrite formation (D). *n* = 14–16 wells/group with cardiomyocytes isolated from 6 to 8 mice. \*\**P* < 0.01 vs. NG group.

*in vitro* by multiple chemical molecules, only two pathways (the ONOO<sup>-</sup> and myeloperoxidase pathways) are pathologically important regarding protein nitration *in vivo*. Since cardiomyocytes were cultured in high-glucose medium without the presence of leukocytes, it is conceivable that observed increased nitrotyrosine formation under our experimental conditions reflects an increased ONOO<sup>-</sup> production. Furthermore, significantly more cell death and apoptosis was observed in 24 h high-glucose cultured cells than in normal-glucose-cultured cells (LDH:  $35.9 \pm 2.08$  vs.  $26.5 \pm 2.14\%$ ; caspase-3 activity:  $119 \pm 6.1$  vs.  $84 \pm 5.9$  nmol pNA/h/mg protein,  $P < 0.05$ ).

### 3.2 Effect of high-glucose culture on Trx activity, Trx-1 expression, and Trx-1 nitration

The results described earlier indicate that oxidative/nitrative stress preceded cell death in high-glucose cultured cardiomyocytes, suggesting that oxidative/nitrative stress might play a causative role in high-glucose-cultured cell sensitivity to SI/R injury. Trx-1 is a potent anti-oxidant/cell survival molecule. Changes in thioredoxin's activity alter cell proliferation and cell death. In a recent study, we demonstrated that nitrative Trx-1 inactivation plays a causative role in myocardial ischaemia/reperfusion injury. To determine the effect of high glucose on the Trx-1 system, we determined Trx activity, Trx-1 expression, and Trx-1 nitration. Twelve hours of high-glucose culture significantly reduced Trx activity (Figure 2A) without altering its expression (Figure 2B). Moreover, Trx-1 nitration was not detected in normal-glucose-cultured cardiomyocytes. In contrast, clear Trx-1 nitration (2.3-fold over normal-glucose group) was detected in cardiomyocytes cultured in high-glucose medium (Figure 2C).

### 3.3 Effect of high-glucose pre-culture on simulated ischaemia/reperfusion-induced cardiomyocyte death

To determine whether high-glucose-induced Trx inactivation may increase cardiomyocyte sensitivity to ischaemia/reperfusion, cells pre-cultured in normal glucose or high glucose were subjected to either SI/R (6 h of normoxia/normal-glucose environment) or SI/R (3 h hypoxia-hypoglycaemic environment plus 3 h normoxia/normal glucose environment). As summarized in Figure 3, SI/R caused significant cardiomyocyte death and increased caspase-3 activity in cells cultured in normal-glucose. Most importantly, although all cardiomyocyte groups were subjected to identical SI/R procedures and conditions, those cells pre-cultured in high glucose for 12 h had significantly higher cell death as evidenced by elevated LDH release and increased caspase-3 activity (Figure 3). Since high glucose was introduced into experimental conditions only during the 12 h pre-culturing period, these results suggest that hyperglycaemia significantly sensitized adult cardiomyocytes to ischaemia/reperfusion injury.

### 3.4 Effect of high-glucose pre-culture on simulated ischaemia/reperfusion-induced oxidative/nitrative stress

Having demonstrated that pre-culture in high glucose increased SI/R injury, we further determined the effect of

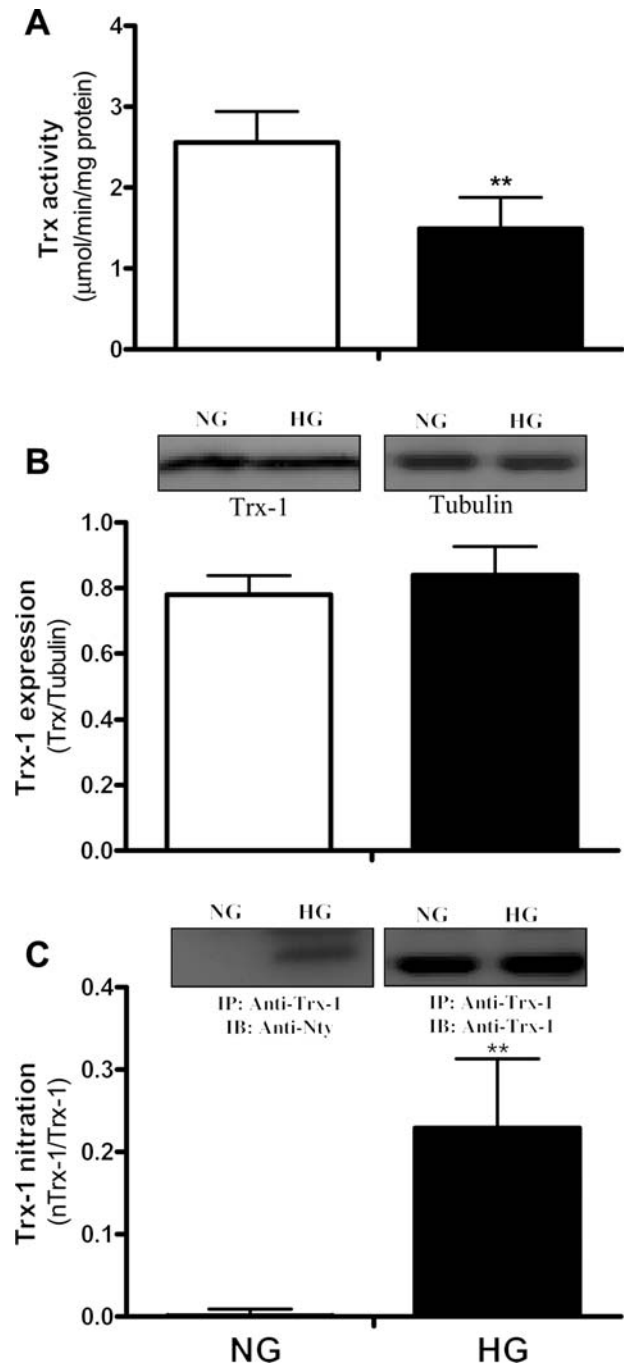
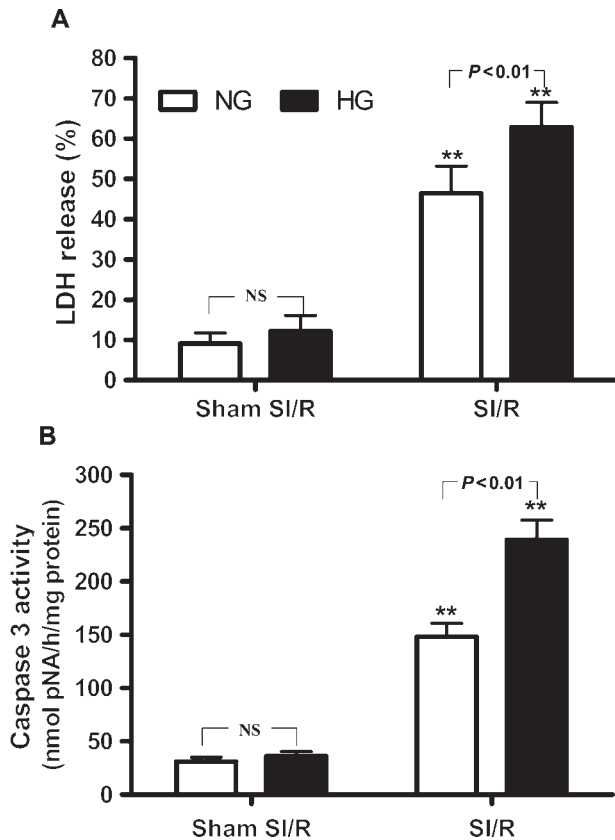


Figure 2 Effect of 12 h high-glucose exposure on cardiomyocyte Trx activity (A), Trx-1 expression (B), and Trx-1 nitration.  $n = 14-16$  wells/group with cardiomyocytes isolated from 6 to 8 mice. \*\* $P < 0.01$  vs. NG group.

high-glucose pre-exposure on SI/R-induced oxidative/nitrative stress, and possible involvement of the Trx-1 system. Although superoxide production was significantly increased in cells cultured in high glucose for 12 h (Figure 1C), no significant difference in superoxide production was observed between normal-glucose and high-glucose pre-cultured cardiomyocytes after 6 h of sham SI/R (Figure 4A, two left bars). This result indicated that culturing cardiomyocytes in normal-glucose medium with normal oxygen for 6 h (sham SI/R) normalized superoxide production. As expected, superoxide production was significantly increased after SI/R in cardiomyocytes pre-cultured in normal-glucose. Most importantly, although no



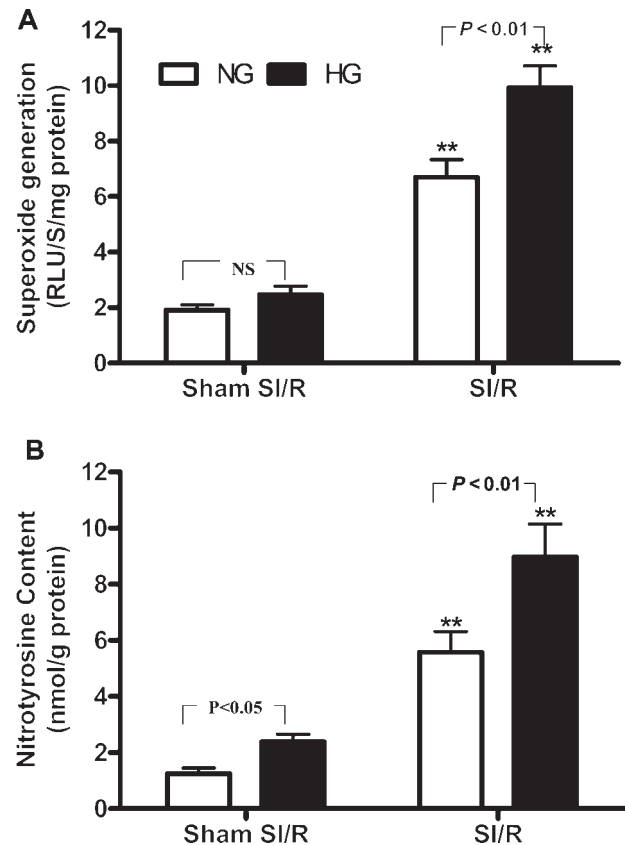
**Figure 3** Effect of high-glucose pre-culture on subsequent simulated ischaemia/reperfusion-induced cardiomyocyte LDH release (A) and caspase-3 activity (B).  $n = 14-16$  wells/group with cardiomyocytes isolated from 6 to 8 mice. \*\* $P < 0.01$  vs. sham SI/R group.

difference was observed in superoxide production in cells pre-cultured in normal glucose or high glucose after sham SI/R, SI/R-induced superoxide production was significantly higher in cells pre-cultured in high glucose (Figure 4A, right two bars).

In contrast to superoxide production, which normalized completely when glucose concentration was reduced to normal levels, nitrotyrosine content only partially normalized (Figure 4B) after 6 h culture with normal oxygen and normal glucose (sham MI/R), indicating that nitrative stress had a slower recovery time course after glucose normalization. Moreover, SI/R-induced peroxynitrite ( $\text{ONOO}^-$ ) overproduction significantly amplified in cells pre-cultured in high glucose (Figure 4B).

### 3.5 Effect of high-glucose pre-culture on simulated ischaemia/reperfusion-induced Trx-1 inactivation and nitration

To further identify the mechanisms responsible for high-glucose sensitization of cardiomyocytes to SI/R, both Trx activity and Trx-1 nitration were determined in cells subjected to sham SI/R or SI/R. As summarized in Figure 5A, Trx activity remained significantly lower in cells pre-cultured in high glucose and subjected to sham SI/R (left two bars). Moreover, nitrated Trx-1 can still be detected in high-glucose pre-cultured cells after sham SI/R. These results indicated that Trx-1 nitration and Trx activity did not return to normal 6 h after glucose normalization. More importantly, SI/R-induced Trx inactivation (Figure 5A) and

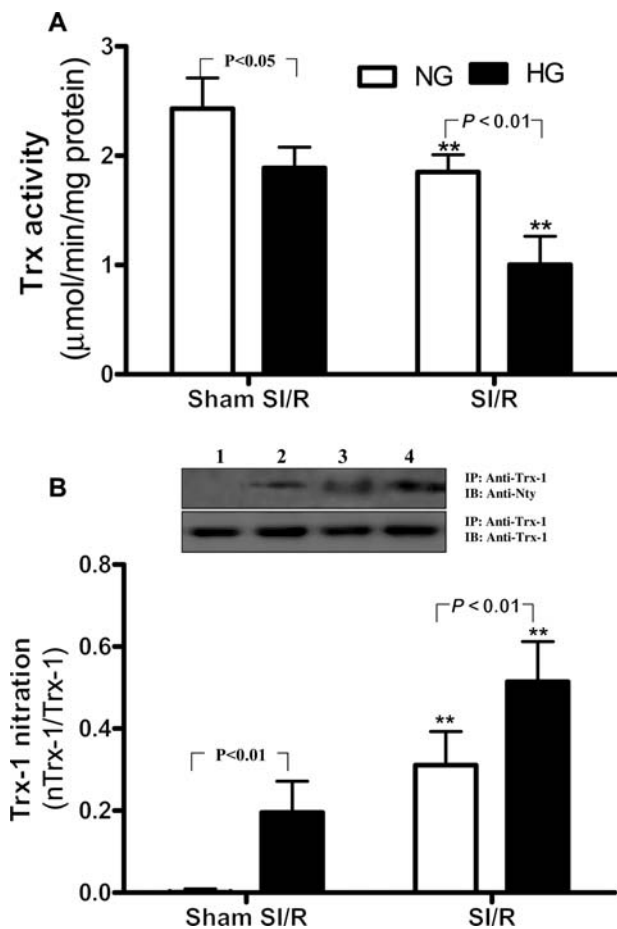


**Figure 4** Effect of high-glucose pre-culture on subsequent simulated ischaemia/reperfusion-induced superoxide (A) and peroxynitrite overproduction (B).  $n = 14-16$  wells/group with cardiomyocytes isolated from 6 to 8 mice. \*\* $P < 0.01$  vs. sham SI/R group.

Trx-1 nitration (Figure 5B) were significantly enhanced in cells pre-cultured in high glucose.

### 3.6 Prevention of high-glucose-induced Trx-1 nitrative inactivation normalized cardiomyocyte response to simulated ischaemia/reperfusion

These results clearly demonstrated that high-glucose culture increased oxidative/nitrative stress, caused Trx-1 nitration and inactivation, and sensitized cardiomyocytes to SI/R. However, whether high-glucose-induced nitrative Trx-1 inactivation is causatively related to accelerated cell death in these cells after SI/R remains uncertain. To directly address this issue, two additional series experiments were performed. In the first series of experiments, cardiomyocytes were treated with recombinant human Trx-1 (hTrx-1), INO-4885, or Tiron during the 12 h high-glucose culture period. Endogenous Trx-1 nitration (using anti-mouse Trx-1 for immunoprecipitation) and total Trx activity (including endogenously expressed and exogenously supplemented) were determined. Compared with normal-glucose control, a 2.7-fold increase in endogenous Trx-1 nitration and a 2-fold decrease in Trx activity were observed in the high-glucose culture group (vehicle in Figure 6). Supplementation with hTrx-1, treatment with a peroxynitrite decomposition catalyst (INO-4885), or with a cell membrane permeable superoxide scavenger (Tiron), significantly reduced Trx-1 nitration, increased Trx activity (hTrx-1 supplementation being the most, and Tiron administration being the least efficacious intervention) (Figure 6). These results provide



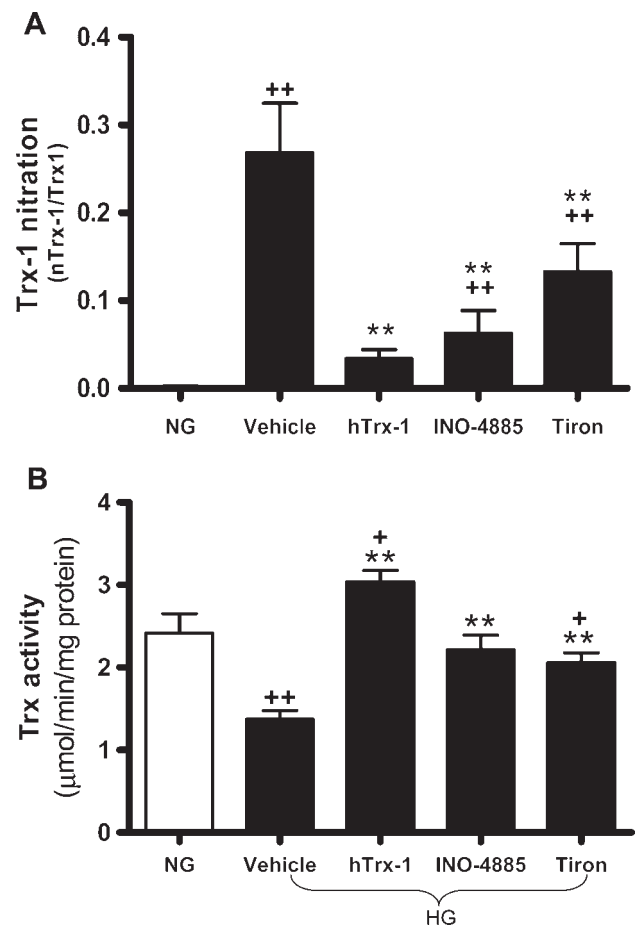
**Figure 5** Effect of high-glucose pre-culture on subsequent simulated ischaemia/reperfusion-induced Trx inactivation (A) and Trx-1 nitration (B).  $n = 14-16$  wells/group with cardiomyocytes isolated from 6 to 8 mice.  $**P < 0.01$  vs. sham SI/R group.

clear evidence that high-glucose-induced Trx-1 nitration is causatively related to high-glucose-induced cardiomyocyte Trx-1 inactivation.

In the second series of experiments, cells pre-cultured in high glucose with or without the afore-mentioned treatments were subjected to sham SI/R or SI/R (no drug treatments given during the SI/R period). Cell death and apoptosis were determined by LDH release and caspase-3 activity. As summarized in *Figure 7*, pre-treatment of cells with hTrx-1, INO-4885, or Tiron during the high-glucose culturing period significantly reduced SI/R-induced cell death and apoptosis to comparable levels seen in cells pre-cultured in normal glucose and subjected to SI/R (horizontal lines in *Figure 7*). These results emphasize that preventing high-glucose-induced Trx-1 nitrative inactivation normalized cardiomyocyte response to SI/R, suggesting that enhanced nitrative Trx-1 inactivation from high-glucose exposure plays a causative role in high-glucose-induced amplification of myocardial ischaemia/reperfusion injury.

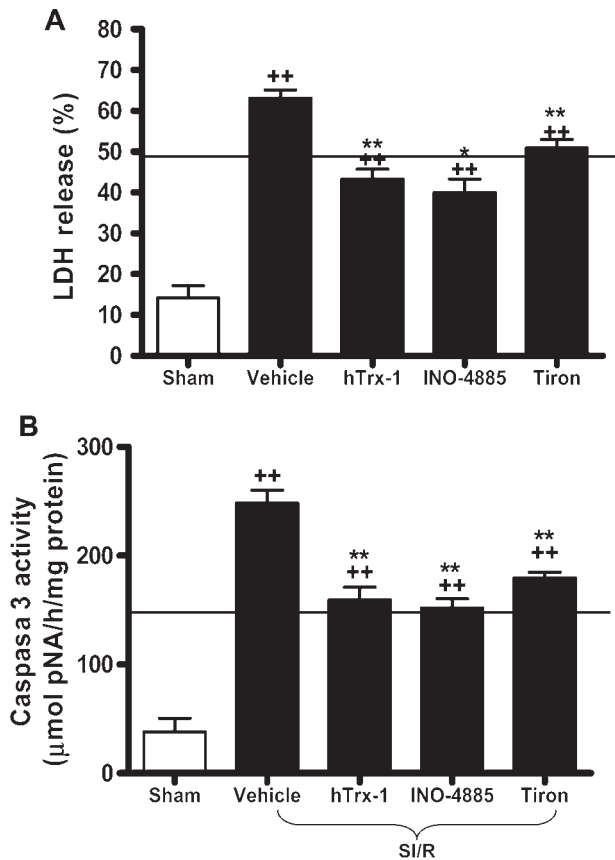
### 3.7 Effect of high-glucose pre-culture on simulated ischaemia/reperfusion-induced NADPH oxidase/iNOS expression

Superoxide reacts with NO in 1:1 ratio and peroxynitrite is maximally produced when both superoxide and NO production are simultaneously elevated. Although eNOS and



**Figure 6** Effect of supplementation of human recombinant Trx-1 or reduction of peroxynitrite levels with either INO-4885, a peroxynitrite decomposition catalyst, or Tiron, a cell membrane permeable superoxide scavenger, on high-glucose exposure-induced Trx-1 nitration (A) and Trx inactivation (B).  $n = 14-16$  wells/group with cardiomyocytes isolated from 6 to 8 mice.  $^+P < 0.05$ ,  $^{++}P < 0.01$  vs. NG group,  $**P < 0.01$  vs. HG treated with vehicle group.

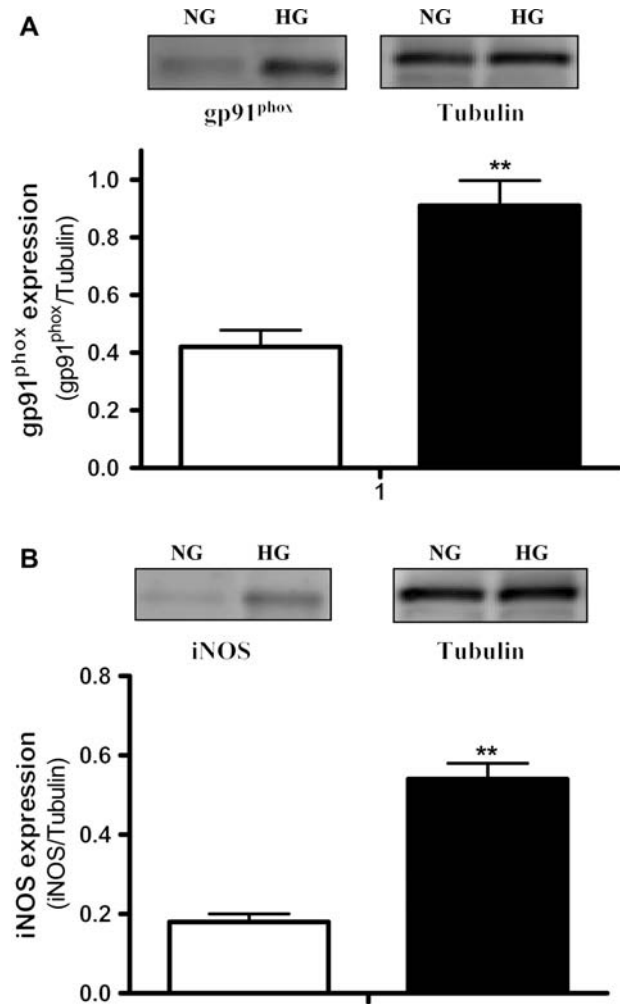
nNOS are expressed in cardiomyocytes, basal NO production from these forms of NOS is unlikely responsible for peroxynitrite formation due to their low-level NO production. To further determine the molecular sources of superoxide and NO overproduction caused by high glucose, NADPH oxidase (the most important cytosolic source for superoxide production in cardiomyocytes) and iNOS expression were determined. As illustrated in *Figure 8*, culturing cardiomyocytes with high glucose for 12 h significantly increased gp91<sup>phox</sup> (a major component of NADPH oxidase) and iNOS expression. More importantly, pre-treatment of cells with a selective NADPH oxidase inhibitor (DPI) or a selective iNOS inhibitor (1400W) during the high-glucose culturing period significantly reduced SI/R-induced cell death (LDH:  $46.5 \pm 3.1\%$  and  $49.9 \pm 4.2\%$  in DPI and 1400W treated group, respectively;  $P < 0.01$  vs. vehicle group) and apoptosis (Caspase 3 activity:  $168 \pm 4.1 \mu\text{mol pNA/h/mg protein}$  and  $172 \pm 6.8 \mu\text{mol pNA/h/mg protein}$  in DPI and 1400W treated group, respectively;  $P < 0.01$  vs. vehicle group) to comparable levels seen in cells pre-cultured in normal glucose and subjected to SI/R. These results demonstrated that NADPH oxidase and iNOS are important molecular sources responsible for superoxide and NO overproduction after high-glucose exposure.



**Figure 7** Effect of supplementation of hTrx-1 or reduction of peroxynitrite levels with INO-4885 or Tiron on high-glucose exposure-induced sensitization of cardiomyocytes to SI/R. (A) LDH release, (B) caspase-3 activity.  $n = 14-16$  wells/group with cardiomyocytes isolated from 6 to 8 mice.  $^{++}P < 0.01$  vs. sham SI/R group,  $^{**}P < 0.01$  vs. SI/R treated with vehicle group. Horizontal lines indicate the mean value of normal-glucose-cultured cardiomyocytes after SI/R.

#### 4. Discussion

Several important observations were made in this study. First, we have demonstrated that pre-exposure to a high-glucose environment accelerated simulated ischaemia/reperfusion-induced cardiomyocyte injury, despite normalization of ambient glucose levels during ischaemia and reperfusion. Many previously published studies have clearly demonstrated that hyperglycaemia not only causes vascular injury leading to ischaemic heart disease, but also has a direct adverse impact on ischaemic cardiomyocytes resulting in larger infarct size and more severe heart failure after ischaemia alone or ischaemia/reperfusion. However, these studies did not control blood glucose levels during ischaemia/reperfusion. Therefore, ambient high-glucose concentrations continually affected cardiomyocytes, enhancing reperfusion injury. It thus remains unclear from prior studies' data whether high-blood glucose levels in diabetic patients may sensitize cardiomyocytes to ischaemia/reperfusion injury, even if an individual patient's blood glucose levels were to become tightly controlled. To better mimic this clinical scenario, where blood glucose is aggressively controlled in an inpatient setting after diabetic patients have developed cardiovascular complications,<sup>23</sup> cardiomyocytes were only exposed to high-glucose concentrations during the 12 h pre-culture period, and normal-glucose concentration was used



**Figure 8** Effect of 12 h high-glucose exposure on cardiomyocyte gp91<sup>phox</sup> (A) and iNOS expression (B).  $n = 14-16$  wells/group with cardiomyocytes isolated from 6 to 8 mice.  $^{**}P < 0.01$  vs. NG group.

during simulated ischaemia/reperfusion. The results of the current study clearly demonstrate that adult cardiomyocytes exposed to high-glucose concentration for a relatively short-time period manifest no significant cytotoxicity *per se*, but are 'primed' for enhanced sensitivity to simulated ischaemia/reperfusion injury. Given advanced treatments currently available to diabetic patients suffering myocardial ischaemia, we believe that our experimental results are unique and important, because these results suggest that acute blood glucose normalization alone in diabetic patients after myocardial ischaemia/reperfusion may not be sufficient to normalize myocardial ischaemia/reperfusion injury. Additional therapeutic interventions are required to achieve a better outcome in diabetic patients suffering from myocardial ischaemia/reperfusion injury.

Secondly, we have provided the first direct evidence that nitrative Trx-1 inactivation plays a causative role in the high-glucose sensitization of cardiomyocytes to ischaemia/reperfusion injury. Previous studies have demonstrated that both nitric oxide (NO) and superoxide ( $O_2^-$ ) production are increased in diabetic or high-glucose-treated cardiomyocytes.<sup>24</sup> Peroxynitrite, the reaction product of superoxide and nitric oxide, is highly cytotoxic. However, the specific

proteins vulnerable to nitrative modification by peroxynitrite that are causatively related to high-glucose cytotoxicity sensitization remain completely unknown. In the current study, we demonstrated that nitrative Trx-1 inactivation is a major mechanism responsible for accelerated cardiomyocyte death after SI/R in cardiomyocytes pre-cultured in high glucose.

Trx-1 is a small protein expressed in all living cells. Clinical and experimental studies have demonstrated the markedly upregulated Trx-1 expression in cancer tissues, where it promotes cell survival.<sup>5</sup> Conversely, genetic ablation of Trx in mice causes massively increased apoptosis in E10.5-day embryos, leading to embryonic lethality.<sup>25</sup> These results demonstrate that the critical role alteration of Trx-1 expression plays in cell proliferation/cell death. Because we demonstrated cardiomyocyte Trx-1 expression is not reduced in high-glucose-cultured cardiomyocytes, reduced cardiomyocyte Trx activity in high-glucose-treated cells cannot be attributed to altered Trx-1 expression. It is well known that Trx-1 is sensitive to post-translational modification, and four forms of Trx-1 post-translational modification have been reported to date.<sup>26-30</sup> S-nitrosylation increases Trx-1 activity, and is thus irrelevant with the current experimental results. Oxidation inhibits Trx-1 function, and is the most common modification. However, the cardiomyocyte Trx-1 inhibition observed in the present study cannot be attributed to Trx-1 oxidation because all samples were pre-treated with dithiothreitol (DTT) before Trx activity assay. Glutathionylation occurs at Cys-73, and this post-translational modification significantly inhibits Trx activity *in vitro*.<sup>29</sup> However, whether Trx-1 glutathionylation may occur *in vivo* in diseased tissues remains unknown, and the role of this form of post-translational modification in regulating Trx function *in vivo* remains to be determined. Finally, we have recently demonstrated that (in addition to the three afore-mentioned post-translational Trx-1 modification avenues, all of which occur at a cysteine residue) Trx-1 can also be modified at the tyrosine residue in a peroxynitrite-dependent fashion known as protein nitration.<sup>30</sup> More interestingly, in contrast to the reversible oxidative Trx-inactivation (via Trx reductase), nitrative modification of Trx is an irreversible inactivation.<sup>30</sup> Our current study demonstrated that nitrative modification is responsible for cardiomyocyte Trx-1 inactivation from acute high-glucose exposure. More importantly, we have demonstrated that Trx-1, an increasingly recognized important intracellular protein, and its nitrative inactivation plays a causative role in high-glucose 'priming' of cardiomyocytes, enhancing sensitivity to ischaemia/reperfusion injury.

Thirdly, we have identified several interventions that effectively normalized cardiomyocyte ischaemic/reperfusion injury when utilized only during high-glucose exposure period. These results not only provided more evidence supporting our conclusion that high-glucose-induced nitrative Trx-1 inactivation is causatively related to accelerated cardiomyocyte death after ischaemia/reperfusion, but also have potential clinical application. As presented in *Figures 6 and 7*, supplementation of recombinant human Trx-1: (i) almost completely abolished high-glucose-induced endogenous Trx-1 nitration, (ii) elevated Trx-1 activity to a level even greater than normal-glucose control, and (iii) reduced SI/R induced LDH release and caspase-3 activation to similar levels observed in cardiomyocytes pre-cultured

in normal glucose. Previous studies have demonstrated that exogenously administered Trx-1 enters the cells through endocytosis.<sup>30</sup> Therefore, exogenously supplemented high concentration human Trx-1 may have reacted with intracellular peroxynitrite, thus effectively preventing endogenous Trx-1 nitration, preserving its activity.

INO-4885 is a second generation peroxynitrite decomposition catalyst, with a peroxynitrite degradation rate  $5 \times 10^7 \text{M/s}^{16}$ . This is at least 10 times faster than FP-15, the extensively investigated first generation peroxynitrite decomposition catalyst.<sup>31</sup> Our experimental results demonstrated that although INO-4885 was not as effective as human Trx-1 in blocking Trx-1 nitration and preserving Trx activity, it is the most effective agent among the three treatments in protecting cells from simulated ischaemia/reperfusion injury. Such overtly conflicting results might be explained by the fact that Trx activity measured in this study did not distinguish between endogenously expressed mouse Trx-1, and exogenously supplemented human Trx-1. A higher Trx activity observed in the exogenously supplemented hTrx-1 treated group may reflect the sum of endogenous *and* exogenous Trx-1. The more potent cytoprotective effect of INO-4885 over human Trx-1 indicates that, atop nitrative Trx-1 inactivation, the high-glucose-induced peroxynitrite overproduction sensitized cardiomyocytes to ischaemia/reperfusion injury through mechanisms not identified in the current study.

Treatment with Tiron, a cell-permeable superoxide scavenger, also significantly reduced Trx-1 nitration, preserved Trx activity, and partially blocked high-glucose sensitization of cardiomyocytes to ischaemia/reperfusion. However, among the three investigated interventions, Tiron is the least effective molecule in blocking high-glucose-induced adverse effects, despite very high utilized study concentrations. It has been shown previously that NO reacts with superoxide at near diffusion-limited rate to produce peroxynitrite, and the reaction between Tiron and superoxide is several orders of magnitude slower than the NO/superoxide reaction.<sup>32</sup> Therefore, addition of Tiron, even at very high concentration, may not be able to completely overwhelm the reaction between superoxide and nitric oxide. Thus, it is not surprising that high-glucose sensitization of cardiomyocytes to ischaemia/reperfusion was only partially blocked by Tiron treatment.

In summary, the results of the current study strongly suggest that increased nitrative Trx-1 modification is a novel pathologic pathway by which hyperglycaemia increases cardiomyocyte susceptibility to ischaemic/reperfusion injury. Blocking peroxynitrite formation, increasing peroxynitrite decomposition, or supplementation of exogenous Trx-1 significantly protected high-glucose pre-cultured cardiomyocytes from SI/R injury. These results suggest that therapeutic interventions preserving Trx-1 activity in the diabetic patient, in conjunction with glycaemic control (with insulin, for example), may further improve patient outcomes after myocardial ischaemia/reperfusion injury.

## 5. Limitations

Adult cardiomyocytes can only be cultured *in vitro* for a limited time period. Due to this inherent limitation of our *in vitro* model, cardiomyocytes were subjected to SI/R



immediately after glucose was 'normalized'. Although we have demonstrated that the high-glucose environment sensitized cardiomyocytes to ischaemia/reperfusion injury, the length of time this 'priming' effect lasts after glucose normalization remains unknown. This clinically important question is currently under investigation using an *in vivo* diabetic model.

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