

RESEARCH PAPER

Salvage of nicotinamide adenine dinucleotide plays a critical role in the bioenergetic recovery of post-hypoxic cardiomyocytes

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BACKGROUND AND PURPOSE

Ischaemic heart disease can lead to serious, life-threatening complications. Traditional therapies for ischaemia aim to increase oxygen delivery and reduce the myocardial ATP consumption by increasing the coronary perfusion and by suppressing cardiac contractility, heart rate or blood pressure. An adjunctive treatment option for ischaemia is to improve or optimize myocardial metabolism.

EXPERIMENTAL APPROACH

Metabolic suppression in the ischaemic heart is characterized by reduced levels of high-energy molecules: ATP and NAD⁺. Because NAD⁺ is required for most metabolic processes that generate ATP, we hypothesized that restoration of NAD⁺ would be a prerequisite for ATP regeneration and examined the role of the major NAD⁺ anabolic and catabolic pathways in the bioenergetic restoration process following oxygen–glucose deprivation injury in a cardiomyocyte cell line (H9c2 cells).

KEY RESULTS

Salvage of NAD⁺ via nicotinamide phosphoribosyl transferase was essential for bioenergetic recovery in cardiomyocytes. Blockade of nicotinamide phosphoribosyl transferase prevented the restoration of the cellular ATP pool following oxygen–glucose deprivation injury by inhibiting both the aerobic and anaerobic metabolism in the cardiomyocytes. NAD⁺ consumption by PARP-1 also undermined the recovery processes, and PARP inhibition significantly improved the metabolism and increased cellular ATP levels in cardiomyocytes.

CONCLUSIONS AND IMPLICATIONS

We conclude that the NAD⁺ salvage pathway is essential for bioenergetic recovery in post-hypoxic cardiomyocytes and PARP inhibition may represent a potential future therapeutic intervention in ischaemic heart disease.

Abbreviations

CVD, cardiovascular disease; FK866, (E)-N-[4-(1-benzoylpiperidin-4-yl)butyl]-3-(pyridin-3-yl)acrylamide; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide; MitoSOX Red, MitoSOX™ Red mitochondrial superoxide indicator; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NamPRT, nicotinamide phosphoribosyltransferase; NMNAT, nicotinamide mononucleotide adenylyl transferase; OGD, oxygen–glucose deprivation; PJ34, N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino) acetamide hydrochloride; RNF146, ring finger protein 146

Tables of Links

TARGETS

Enzymes

PARP-1

LIGANDS

FK866, daporinad

NAD⁺

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Ischaemic heart disease is a major cause of death and disability in developed countries. Cardiovascular disease (CVD) accounts for 31% of deaths, and coronary heart disease alone causes one of six deaths in the United States (Go *et al.*, 2014; Go *et al.*, 2014). Currently, CVD costs more than any other diagnostic group including cancer. The estimated cost of CVD was \$315.4 billion in the United States in 2010 while the costs of all cancer and benign neoplasms was \$201.5 billion (Go *et al.*, 2014). Ischaemic heart disease is caused by reduced coronary blood supply to the heart that stops to provide enough oxygen and energy source for the myocardium. This leads to decreased oxygen consumption and to diminished ATP and NAD⁺ levels in the heart (Nunez *et al.*, 1974; Cave *et al.*, 2000; Schriever *et al.*, 2013). The imbalance between oxygen delivery and myocardial ATP demand causes energetic failure that may result in myocardial dysfunction or serious complications including angina pectoris, heart failure and acute myocardial infarction.

Revascularization is the most effective therapy to limit the infarct size and preserve the systolic function, but it involves secondary damage to the myocardium (reperfusion injury). Traditional pharmacological therapies use haemodynamic approaches to restore the balance between ATP formation and utilization: increasing the blood flow (oxygen delivery) via coronary vasodilation or decreasing the heart rate, arterial blood pressure and cardiac contractility to reduce the oxygen consumption (Stanley, 2001). Alternative (or adjunctive) treatments address the reperfusion injury and aim to optimize the energy metabolism in the ischaemic heart. Various pharmacological agents were tested to address the oxidative stress (antioxidants, PARP inhibitors) (Liaudet *et al.*, 2001; Morrow *et al.*, 2009), the mitochondrial permeability transition pore opening (cyclosporine A) (Hausenloy *et al.*, 2002; Piot *et al.*, 2008) and the metabolism (glucose–insulin–potassium, atorvastatin and glucagon-like peptide exenatide) (Diaz *et al.*, 1998; Bell and Yellon, 2003; Sack and Yellon, 2003; Lonborg *et al.*, 2012) and even mechanical interventions (ischaemic post-conditioning, remote ischaemic conditioning and therapeutic hypothermia) were used with success in myocardial infarction (Staat *et al.*, 2005). Interestingly, these earlier approaches share a common mechanism of action: the activation of survival kinases, which were also designated as the reperfusion injury salvage kinase (RISK) pathway (Hausenloy and Yellon, 2004; Frohlich *et al.*, 2013; Hausenloy

and Yellon, 2013). Activation of survival kinases leads to inhibition of apoptotic effectors and also increases the translocation of glucose transporters to the cell membrane and improves glucose uptake (Sack and Yellon, 2003; Hausenloy and Yellon, 2004). However, the major limitation of ATP synthesis is not glucose deficiency in the ischaemic tissues but the reduced cellular NAD⁺ content (Nunez *et al.*, 1974). Because CVD is a disease of the elderly, the basal tissue NAD⁺ content is also lower as a result of age-related decrease in NAD⁺ biosynthesis enzymes (Braidly *et al.*, 2011; Stein and Imai, 2012). Furthermore, NAD⁺ deficiency may be aggravated by PARP-mediated NAD⁺ consumption in oxidative stress (Szabo *et al.*, 2004; Molnar *et al.*, 2006).

In mammalian cells, NAD⁺ is produced via two biosynthetic pathways: (1) the *de novo* (or kynurenine) pathway that uses tryptophan as substrate and (2) the salvage pathway that regenerates NAD⁺ from nicotinamide. The dominant route is the salvage pathway in which the rate-limiting step is catalysed by nicotinamide phosphoribosyltransferase (NamPRT) (Chiarugi *et al.*, 2012). Interestingly, there is also a secreted (extracellular) isoform of NamPRT (also called visfatin, pre-B cell colony-enhancing factor) that acts as a cytokine (Houtkooper *et al.*, 2010). NAD⁺ has a short half-life in the cells, and it is constantly recycled within a day; the estimated daily NAD⁺ synthesis is around 1 g kg⁻¹ tissue weight (Chiarugi *et al.*, 2012). Because NAD⁺ is compartmentalized in the cells, its distribution also needs to be considered in conditions of depletion. For instance, in cardiac myocytes, ~70% of the cellular NAD⁺ pool is in the mitochondria and cannot support glycolysis (Nikiforov *et al.*, 2011; Stein and Imai, 2012). Because NAD⁺ is a prerequisite for energy production, rapid resynthesis of the cellular NAD⁺ content is expected to promote the recovery following hypoxia. Counterintuitively, inhibition of NAD⁺ biosynthesis by blocking NamPRT was protective in myocardial infarction (Montecucco *et al.*, 2013). While this action was associated with blockage of the extracellular NamPRT and an anti-inflammatory mechanism was suggested, it should be noted that the currently available NamPRT inhibitors block both the extracellular and the intracellular isoforms of the enzyme.

To investigate whether NamPRT played an essential role in the recovery following hypoxia, we studied the bioenergetic restoration in an *in vitro* model of ischaemia-reperfusion injury, using a rat cardiomyocyte cell line (H9c2 cells). Because PARP is the most important NAD⁺ consumer in the cells, we also explored its role in the recovery process. We found that bioenergetic recovery was abolished by NamPRT inhibition but PARP inhibition significantly improved the cellular bioenergetics in the absence of NamPRT activity.

Methods

Cell culture

H9c2 rat cardiomyocytes were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM (Biochrom AG, Berlin, Germany) supplemented with 4 mM glutamine, 10% FBS (PAA Laboratories Inc, Westborough, MA), 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Invitrogen, Carlsbad, CA) at 37°C in 10% CO₂ atmosphere.

Oxygen–glucose deprivation injury

Oxygen–glucose deprivation (OGD) injury was conducted as previously described (Szabo *et al.*, 2011; Szoleczky *et al.*, 2012; Gero *et al.*, 2014) with slight modifications. H9c2 cells (10 000 per well) were plated on 96-well plates and cultured for 3 days prior to the induction of OGD. Cells transfected with siRNAs were subjected to OGD 48 h post-transfection. Prior to hypoxia induction, the culture medium was replaced with glucose-free DMEM. Then the plates were placed in gas-tight incubation chambers (Billups-Rothenberg Inc., Del Mar, CA), and the chamber atmosphere was replaced by flushing with 95% N₂/5% CO₂ mixture at 30 L min⁻¹ flow rate for 10 min. Hypoxia was maintained for 8 or 10 h (as indicated) by incubation at 37°C. All assay plates subjected to hypoxia included wells exposed to glucose-free medium (OGD) and medium containing 4.5 g L⁻¹ glucose (hypoxia). Cells were simultaneously exposed to glucose-free medium (glucose deprivation) or maintained in glucose-containing medium (CTL) under normoxia. Following the hypoxia, glucose and serum concentration was restored by supplementing the culture medium with glucose and FBS, and the cells were incubated for 16 h at 37°C (recovery). In select cases, cells were treated with an inhibitor of NamPRT, FK866 (10 µM; (E)-N-[4-(1-benzoylpiperidin-4-yl)butyl]-3-(pyridin-3-yl)acrylamide, NIMH F-901, Research Triangle Institute, Research Triangle Park, NC) and/or the PARP inhibitor PJ34 (3 µM; N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride, Sigma-Aldrich, St. Louis, MO) immediately following the hypoxic period. Measurement of cell viability, LDH release, ATP and NAD⁺ content, mitochondrial potential and oxidant production was carried out either immediately following the hypoxic period or at the end of the 16-h-long recovery period. Cellular viability was measured with MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide and by LDH release, as previously described (Gero *et al.*, 2007). A calibration curve was created by measuring the MTT converting capacity of serial dilutions of H9c2 cells to calculate the number of viable cells using Gen5 data reduction software (Biotek, Winooski, VT). Viability values are shown as percent of control mean cell count.

Oxidant-induced injury

Oxidant injury was carried out as previously described (Gero *et al.*, 2007; Gero *et al.*, 2014). H9c2 cells (10 000 per well) were plated on 96-well plates and grown for 3 days. The cells were treated with H₂O₂ in fresh culture medium and

incubated for 3 or 24 h. Cell viability was measured by the MTT and LDH assays (Gero *et al.*, 2007). Viability values are shown as percent of vehicle-treated control mean cell count. Cells transfected with siRNAs were subjected to H₂O₂ injury 48 h post-transfection. To test the oxidant sensitivity of the cells exposed to hypoxia and/or glucose deprivation, the cells were treated with H₂O₂ in fresh culture medium immediately following the hypoxia and incubated for 3 h prior to viability measurements. To assay the role of NamPRT, cells were pretreated with FK866 (10 µM) for 1 h prior to the addition of H₂O₂.

ATP and NAD⁺ assays

ATP concentration was determined by the commercially available CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) as previously described (Gero *et al.*, 2014). An ATP calibration curve was prepared by measuring the luminescent signal generated by serial dilutions of ATP. The ATP content of each well was calculated by Gen5 data reduction software (Biotek, Winooski, VT), and values are shown as percent of control mean ATP content.

The cellular NAD⁺ content was measured using an alcohol dehydrogenase-based assay (Queval and Noctor, 2007; Modis *et al.*, 2012). NAD⁺ extraction was performed by precipitating the cell samples in 0.2 M hydrochloric acid, then the lysates were centrifuged at 16 000×g for 15 min. The cleared supernatant (200 µL) was heated to 100°C for 3 min and neutralized by adding 20 µL 0.4 M NaH₂PO₄ and 160 µL 0.2 M NaOH. The NAD⁺ reaction mix was prepared by diluting MTT (250 µM), N-methylphenazonium methyl sulfate (1 mM) and alcohol dehydrogenase (7.5 U mL⁻¹) in reaction buffer (100 mM HEPES, 2 mM EDTA, 10 mM nicotinamide, pH 7.5). The sample or NAD⁺ calibration standard (20 µL) was added to 160 µL reaction mixture and the reaction started by addition of the substrate (ethanol, 7.5%). The reaction was monitored kinetically at 570 nm for 1 h. NAD⁺ concentration was calculated using a calibration curve generated from the maximal velocity values of simultaneously measured NAD⁺ dilution series. NAD⁺ content is shown as percent of control mean NAD⁺ values.

Mitochondrial potential and superoxide production

Mitochondrial potential was measured with JC-1 (Sigma-Aldrich, St. Louis, MO) fluorescent probe. The cells were loaded with the dye by exposing them to JC-1 stain solution (containing 10 µM JC-1 and 0.6 mM β-cyclodextrin (Sigma-Aldrich, St. Louis, MO)) for 30 min. Subsequently, the cells were washed in PBS, and the red (Ex/Em: 485/528 nm) and green (Ex/Em: 530/590 nm) fluorescence were measured on a microplate reader. The mitochondrial potential is expressed as the relative ratio of the mitochondrial J-aggregates (red fluorescence) and the cytoplasmic monomer form of the dye (green fluorescence).

Mitochondrial reactive oxygen species (ROS) production was measured using the mitochondrial superoxide sensor MitoSOX™ Red (Invitrogen, Carlsbad, CA) as previously described (Gero *et al.*, 2013).

siRNA-mediated gene silencing and gene expression assays

H9c2 cells (10000 per well) were plated on 96-well plates; the following day, the cells were transfected with Silencer Select *PARP-1*, *NMNAT1*, *NMNAT3* or negative control #1 siRNA (1 pmol per well, assay IDs: s62054, s151624, s221491 and ID: 4390844, Life Technologies, Carlsbad, CA) using Lipofectamine 2000 transfection reagent. The knock-down efficiency was evaluated by realtime PCR and by Western blotting 24 and 48 h post-transfection for *PARP-1* and by realtime PCR for *NMNAT1* and *NMNAT3* siRNAs 48 h following the transfection. RNA was isolated using a commercial RNA purification kit (SV total RNA isolation kit, Promega, Madison, WI) and reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) as previously described (Gero *et al.*, 2013; Gero *et al.*, 2013). *PARP-1*, *NMNAT1*, *NMNAT3* and *NamPRT* expressions were measured with Taqman assays (assay IDs: Rn00565018_m1, Rn01516826_m1, Rn01403958_m1 and Rn00822043_m1, Life Technologies, Carlsbad, CA) using TaqMan® Rodent *GAPDH* Control Reagents (Life Technologies, Carlsbad, CA) for normalization. Relative expression values are shown as percent of control mean expression.

Western blotting was performed as previously described using antibodies against *PARP-1* (Cell Signaling, Beverly, MA) and anti-ring finger protein 146 (*RNF146*) (Abnova, Walnut, CA) (Gero *et al.*, 2014). Blots were detected on a CCD camera-based detection system (GBox, Syngene USA, Frederick, MD) with enhanced chemiluminescent substrate, and signals were quantitated using Genetools analysis software (Syngene USA, Frederick, MD). Normalized expression values are shown as percent of control mean expression.

Bioenergetic profiling by extracellular flux analysis

An XF24 Analyzer (Seahorse Biosciences, Billerica, MA) was used to measure cellular bioenergetics following hypoxia in H9c2 cells (Gero *et al.*, 2013; Gero *et al.*, 2013). It measures oxygen and proton concentrations in real time via specific fluorescent dyes and calculates oxygen consumption rate (expressed in pmol min^{-1}) and extracellular acidification rate (expressed in pH change per minute) that represent measures of the aerobic and anaerobic metabolism.

H9c2 cells transfected with *PARP-1* or CTL siRNA were exposed to 8-h-long hypoxia or OGD. Subsequently, glucose concentration and oxygen tension were normalized, and

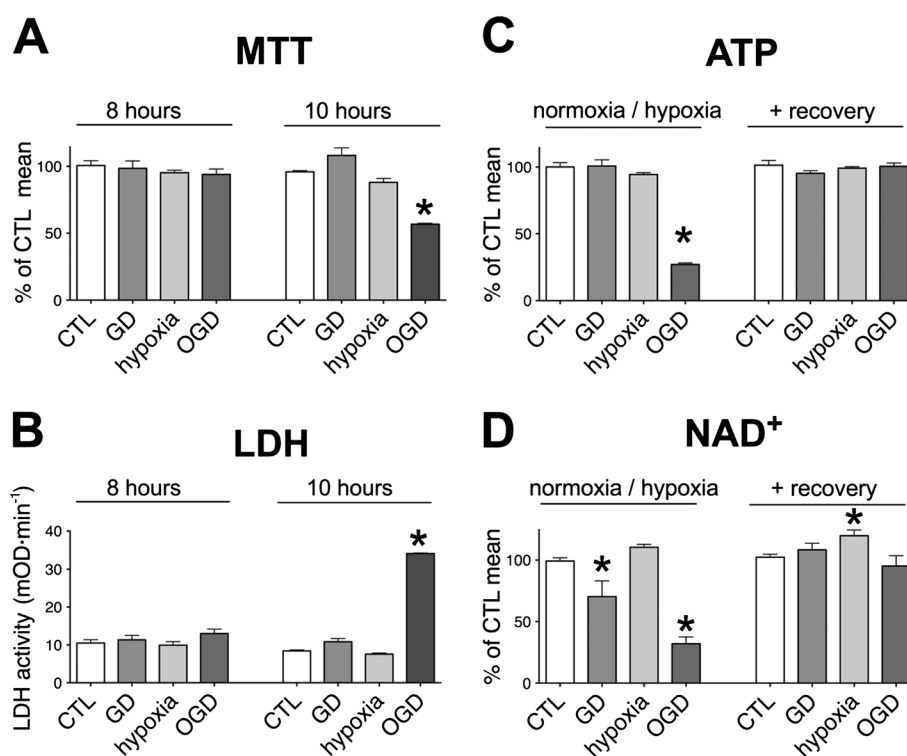


Figure 1

OGD induces reversible energy depletion. A, B: H9c2 cells were exposed to glucose deprivation (GD), hypoxia or OGD or maintained in glucose-containing culture medium at normoxia (CTL) for 8 or 10 h. The cellular viability was determined by the MTT assay (A), and LDH release (B) was measured in the supernatant following a 16-h-long recovery period. 8-h-long OGD did not affect the cellular viability, while 10-h-long exposure induced significant decrease in viability. C, D: H9c2 cells were exposed to GD, hypoxia or OGD for 8 h followed by a 16-h-long recovery period. The cellular ATP and NAD^+ contents were measured at the end of the 8-h-long hypoxic period and following the recovery. OGD induces significant decrease in cellular ATP and NAD^+ contents that are completely restored during the recovery period. $n = 4$, * $P < 0.05$ compared with CTL.

the cells were treated with FK866 (10 μ M) or vehicle and incubated for 16 h before analysing the cellular metabolism. For all bioenergetic measurements, the culture medium was changed to unbuffered DMEM (pH 7.4) containing 5 mM glucose, 2 mM L-glutamine and 1 mM sodium pyruvate. After determining the basal oxygen consumption rate and extracellular acidification rate values, oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone and antimycin A were injected (1 μ g mL⁻¹, 0.3 μ M and 2 μ g mL⁻¹, respectively) to determine the oxygen consumption linked to ATP production, the non-ATP-linked oxygen consumption (proton leak), the maximal respiration capacity and the non-mitochondrial oxygen consumption. Simultaneous

recording of the proton production was used to calculate the capacity of the cells to compensate via anaerobic metabolism.

Data analysis

Data are shown as means \pm SEM. One-way ANOVA was used to detect differences between groups or unpaired two-tailed Student's *t*-test to compare two groups. *Post hoc* comparisons were made using Tukey's test. A value of *p* < 0.05 was considered statistically significant. Non-linear regression curves were fitted to raw viability and LDH activity values with Prism 4 software to calculate 50% reduction or increase values respectively. All statistical

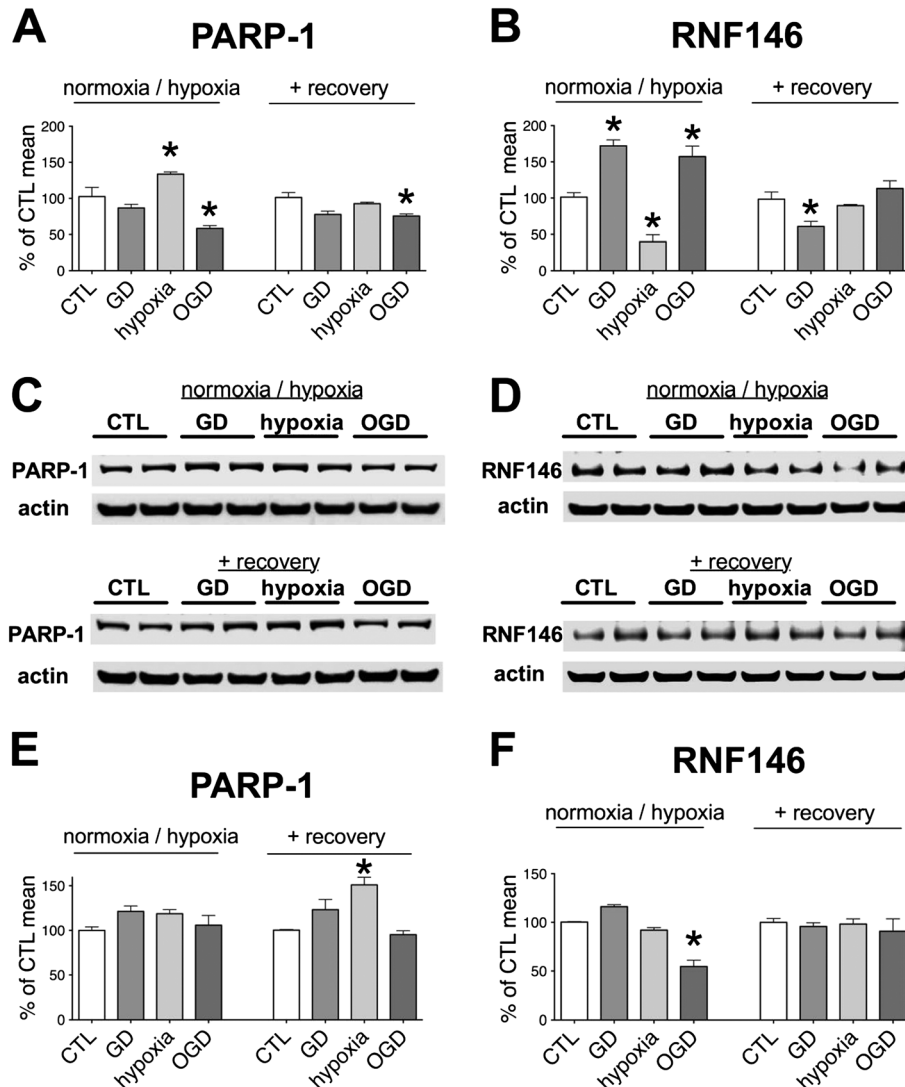


Figure 2

PARP-1 and RNF146 expression in hypoxia-reoxygenation injury. H9c2 cells were exposed to glucose deprivation (GD), hypoxia or OGD or maintained in glucose-containing culture medium at normoxia (CTL) for 8 h followed by a 16-h-long recovery period. A, B: *PARP-1* (A) and *RNF146* (B) mRNA level expression was measured by Taqman assays at the end of the hypoxic period and following the recovery. Relative expression levels were normalized to *GAPDH* expression. Hypoxia induces significant increase in PARP-1 mRNA level and a decrease in RNF146 expression, while GD and OGD increases the expression of RNF146. C–F: PARP-1 (C and E) and RNF146 (D and F) protein expression was measured by Western blotting at the end of the hypoxic period and following the recovery using actin normalization. Representative blots (C and D) and densitometric analysis results (E and F) are shown. Hypoxia increases the expression of PARP-1 at the end of the recovery period. OGD induces a reversible decrease in RNF146 expression. *n* = 4, **P* < 0.05 compared with CTL.

calculations were performed using Prism 4 analysis software (GraphPad Software, Inc., La Jolla, CA).

Results

OGD induces reversible injury that sensitizes the cells to oxidant injury

In ischaemia, the loss of blood flow decreases the oxygen and energy source (glucose) supply and results in cellular energy depletion (diminished ATP pool). To investigate the bioenergetic recovery processes from this state, we developed an *in vitro* model of reversible injury by exposing H9c2 cardiomyocytes to OGD. We found that 8-h-long OGD resulted in no change of cellular viability if the oxygen and energy source supplies were normalized subsequently (Figure 1A and B), while longer OGD periods clearly induced cell death (Figure 1A and B) (Szabo *et al.*, 2011). The 8-h-long OGD severely diminished the cellular ATP and NAD⁺ pools that were completely restored during the 16-h incubation with normalized oxygen and glucose levels (Figure 1C and D).

Glucose deprivation or hypoxia alone did not decrease the ATP pool (Figure 1C); thus, the energy source reserve in the cells was sufficient to produce ATP via aerobic metabolism and the hypoxic cells could switch to anaerobic metabolism and produce adequate amount of ATP using glucose for energy source. Interestingly, glucose deprivation alone also caused a slight decrease in NAD⁺ content, but it was not comparable to the effect of OGD (Figure 1D).

PARP-1 is the major NAD⁺ consumer in oxidative stress, and PARP inhibition (by genetic or pharmacological means) is protective in various heart disease models, including myocardial infarction (Pieper *et al.*, 2000; Liaudet *et al.*, 2001). We found that PARP-1 expression was decreased following severe ischaemic injury in the heart and the removal of PARP-1 was regulated by RNF146, an E3 ubiquitin ligase (Gero *et al.*, 2014). RNF146 directly interacts with PARP-1, and by capturing PARP-1, it can modulate the cell fate in oxidative stress. High levels of RNF146 protect against cellular injury, while decreased RNF146 levels augment the injury.

To test whether similar changes occur in this model, we measured the expression of PARP-1 and RNF146. We found

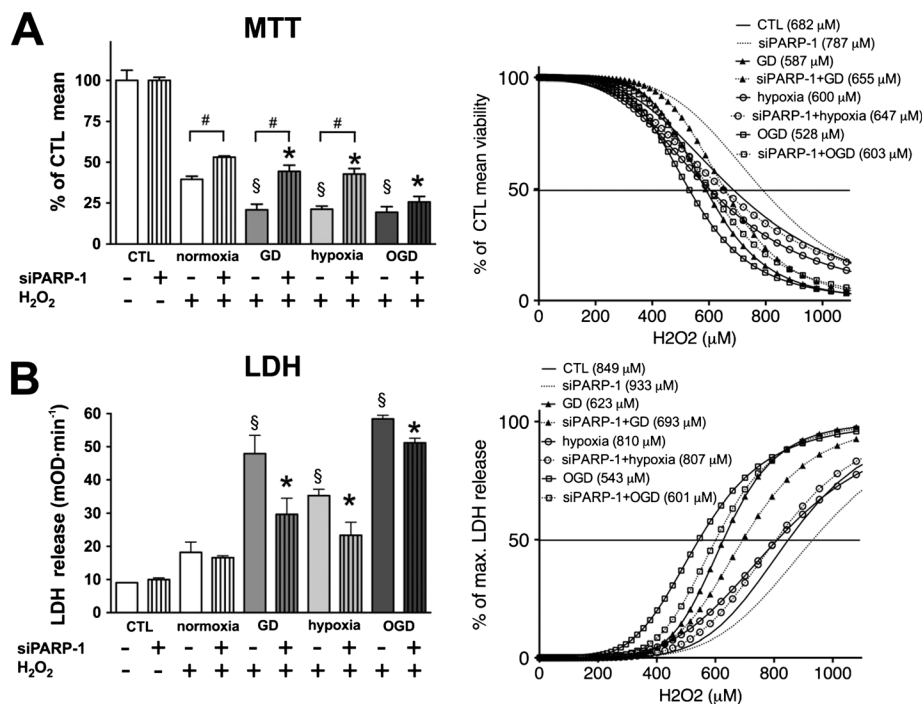


Figure 3

Oxygen and glucose deprivation increases the sensitivity to oxidants. H9c2 cells were transfected with PARP-1 (siPARP-1) or CTL siRNA, and 48 h later, the cells were exposed to glucose deprivation (GD), hypoxia or OGD for 8 h or maintained in glucose-containing culture medium at normoxia (CTL). Subsequently, the cells were exposed to H₂O₂ (0–1100 μM) for 3 h, and cellular viability was determined by the MTT assay (A) and LDH release (B) was measured in the supernatant. Non-linear curve-fitting was applied to the raw data, and the concentration of H₂O₂ that caused 50% reduction in viability (A) or 50% increase in LDH release (B) was determined. Raw data measured at 700 μM H₂O₂ are shown (left panels), and curve-fitting results with the concentrations that cause 50% reduction in MTT conversion or 50% increase in LDH release are shown (right panels). H₂O₂ induced significant reduction in cellular viability and induced significant increase in LDH release in all groups at 700 μM H₂O₂ concentration (not labelled on the graph). Hypoxia, GD and OGD augment the H₂O₂-induced injury, and PARP-1 silencing provides protection. GD and OGD result in narrower range of H₂O₂ tolerance (steeper curves). *n* = 4, #*P* < 0.05 PARP-1 silenced cells compared with respective CTL siRNA treated cells, \$*P* < 0.05 CTL siRNA-transfected, H₂O₂-treated cells exposed to GD, hypoxia or OGD compared with CTL siRNA-transfected cells maintained at normoxia, **P* < 0.05 PARP-1 siRNA-transfected, H₂O₂-treated cells exposed to GD, hypoxia or OGD compared with PARP-1 siRNA-transfected cells maintained at normoxia.

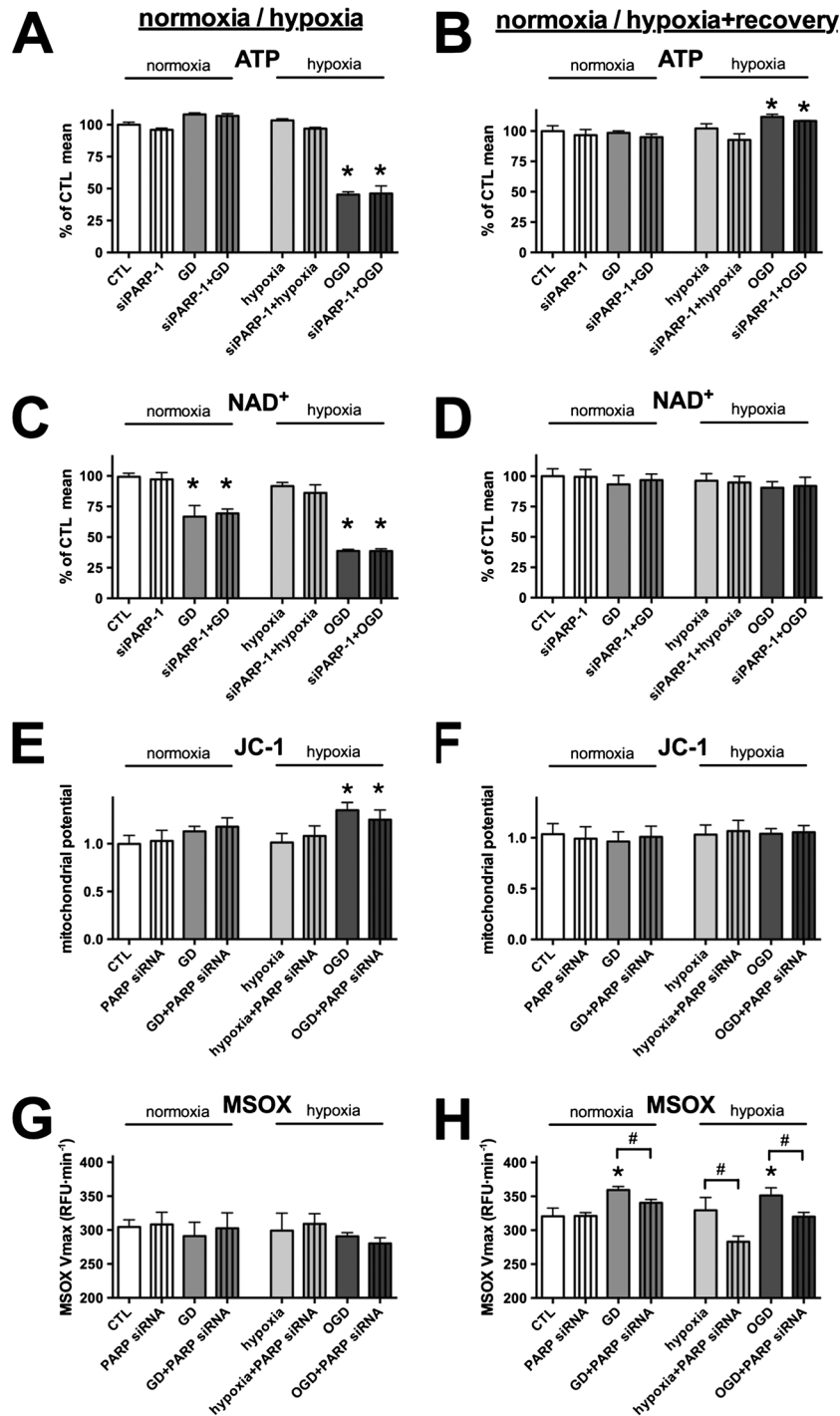


Figure 4

PARP-1 silencing decreases the mitochondrial oxidant production following hypoxia. A–H: H9c2 cells were transfected with PARP-1 (siPARP-1) or CTL siRNA, and 48 h later, the cells were exposed to glucose deprivation (GD), hypoxia or OGD for 8 h. Control cells (CTL) were maintained in glucose-containing culture medium at normoxia. Subsequently, the cells were subjected to a 16-h-long recovery. A–D: The cellular ATP (A and B) and NAD⁺ (C and D) concentrations were determined both at the end of the hypoxia (A and C) and following the recovery (B and D). E, F: The mitochondrial potential measured by JC-1 at the end of the hypoxia (E) and following the recovery (F). G, H: Mitochondrial superoxide production was measured using MitoSOX Red (MSOX) staining at the end of the hypoxia (G) and following the recovery (H). PARP-1 silencing does not affect the cellular ATP recovery or the mitochondrial potential following hypoxia but decreases the mitochondrial oxidant production. $n = 3$; * $P < 0.05$ compared with CTL, # $P < 0.05$ PARP-1 silenced cells compared with respective CTL siRNA treated cells.

that significant changes occur in the expression of PARP-1 and RNF146 at the mRNA level following hypoxia, but these mostly return to normal by the end of the recovery period (Figure 2A and B). At the protein level, RNF146 expression is significantly suppressed following OGD, but it is normalized by the end of the recovery period (Figure 2C–F). On the other hand, PARP-1 level becomes significantly elevated in the cells exposed to hypoxia alone, which can be the result of increased PARP-1 and decreased RNF146 expression during hypoxia, as detected at the mRNA level. It should be noted that changes at the mRNA level may not be directly translated to the protein levels in case of these proteins, because the degradation of PARP-1 and RNF146 can occur very rapidly (Gero *et al.*, 2014).

The increased amount of PARP-1 protein, and decreased amounts of RNF146 and NAD^+ (the substrate of PARP-1) may all predispose to oxidative stress-induced injury; thus, we tested whether hypoxia, glucose deprivation or OGD (that itself does not induce irreversible injury) increases the sensitivity of the cells to oxidants. To test the role of PARP-1 in the oxidant-induced damage, we utilized siRNA-mediated PARP-1 silencing (Figure S1). We found that cells subjected to glucose deprivation, hypoxia or OGD showed decreased tolerance to oxidants, but PARP-1 silencing had some protective effects in all cases (Figure 3). Cells exposed to OGD showed the weakest tolerance to oxidants, but cells subjected to glucose deprivation or hypoxia were also more sensitive than the cells maintained at normoxia. Glucose deprivation

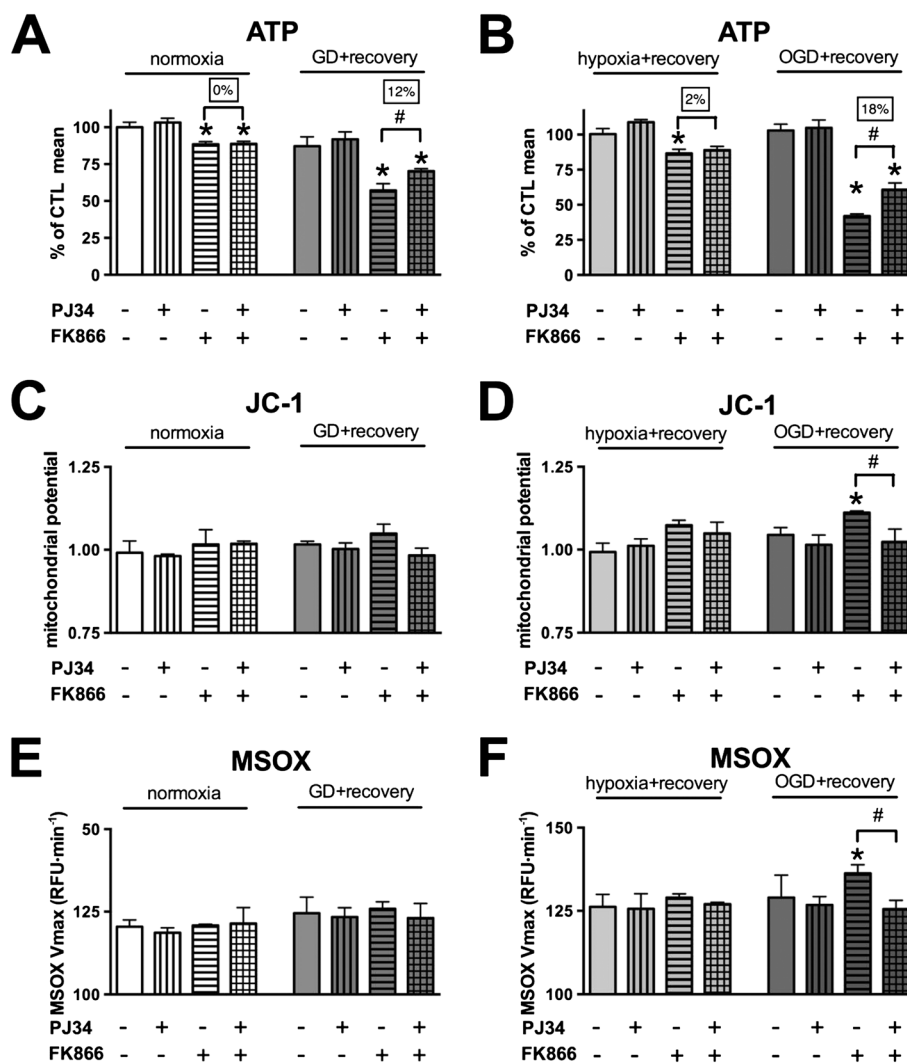


Figure 5

NamPRT is necessary for regeneration of the cellular ATP pool following OGD. A–F: H9c2 cells were exposed to glucose deprivation (GD), hypoxia or OGD or maintained in glucose-containing culture medium at normoxia (CTL) for 8 h. Subsequently, the cells were treated with NamPRT inhibitor FK866 (10 μ M), PARP inhibitor PJ34 (3 μ M) or vehicle and were subjected to 16-h recovery. A, B: The cellular ATP concentration was measured. C, D: The mitochondrial potential determined by JC-1. E, F: Mitochondrial ROS production was determined by measuring the oxidation of the MitoSOX (MSOX) superoxide sensor. NamPRT inhibition blocks the regeneration of the cellular ATP pool following OGD. PARP inhibition improves the ATP recovery following NamPRT inhibition, restores the mitochondrial potential and reduces the ROS production. $n = 3$; * $P < 0.05$ compared with CTL, # $P < 0.05$ compared with respective PARP inhibitor treated cells.

and OGD narrowed the concentration range of oxidants that was tolerated by the cells that might be the result of diminished NAD⁺ content of the cells. Hypoxia alone also sensitized the cells to oxidants, but it did not narrow the concentration range of oxidants that the cells survived.

We tested whether the resistance to oxidants mediated by PARP-1 silencing was associated with higher cellular ATP or NAD⁺ levels following hypoxia, but no difference was found between the PARP-1 silenced and control cells (Figure 4A–D). Similarly, no difference was detectable in the cellular ATP and NAD⁺ levels between the PARP-1 silenced and control cells following the recovery period. Glucose deprivation and OGD resulted in a recoverable increase in the mitochondrial potential following the 8-h-long exposure, and PARP-1 silencing resulted in no change (Figure 4E and F). A slight mitochondrial dysfunction was measurable following the recovery period in the glucose-deprived and oxygen–glucose-deprived cells, as an increase in the mitochondrial superoxide production that was beneficially affected by PARP-1 silencing (Figure 4G and H).

Overall, we conclude that both hypoxia and glucose deprivation sensitize the cells to oxidative stress and, when the two are combined, dysfunction and serious injury can be caused by levels of oxidative stress that scarcely cause damage in intact cells. Diminished cellular energy reserve (ATP and NAD⁺ pools) is responsible in large part for the increased oxidant sensitivity.

NamPRT is necessary for bioenergetic recovery following OGD

Because OGD not only decreased the cellular ATP content but also resulted in a severe decrease in the NAD⁺ level and both the ATP and NAD⁺ levels returned to normal during the recovery period, we hypothesized that NAD⁺ resynthesis was necessary for the ATP recovery. As mentioned in the introduction, NAD⁺ synthesis can occur via two major pathways: (1) the *de novo* synthesis and (2) the salvage pathway (Houtkooper *et al.*, 2010). We blocked the NAD⁺ salvage with the NamPRT inhibitor FK866 during the recovery period following hypoxia, glucose deprivation or OGD (Hassmann and Schemainda, 2003). NamPRT inhibition completely blocked the recovery of the cellular ATP content following OGD exposure and also resulted in significantly lower ATP content following glucose deprivation and hypoxia (Figure 5A and B), and it blocked the normalization of the mitochondrial potential and induced mitochondrial ROS production (Figure 5C–F). To test whether NAD⁺ consumption by PARP-1 affected the recovery, we blocked PARP activity with PJ34 during the recovery. Consistent with previous findings showing that silencing of PARP-1 did not affect ATP regeneration, PARP inhibition by itself had no effect on ATP recovery (Figure 5A and B). However, inhibition of PARP significantly increased the ATP level in glucose-deprived and OGD-exposed cells if NAD⁺ salvage was blocked, showing that PARP-1 was responsible for a significant basal

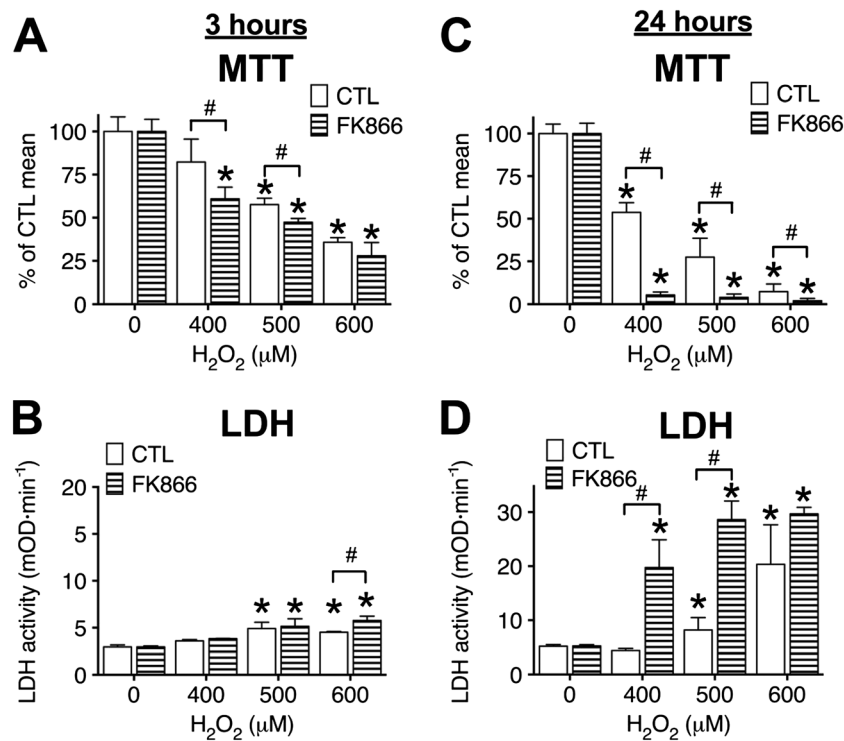


Figure 6

NamPRT is necessary for recovery following oxidant-induced injury. A–D: H9c2 cells were treated with NamPRT inhibitor FK866 (10 μM, 1 h) or vehicle (CTL) and exposed to H₂O₂ (400, 500 or 600 μM) for 3 (A and B) or 24 h (C and D). Cellular viability was determined by the MTT assay (A and C), and the LDH release (B and D) was measured in the supernatant. NamPRT inhibition augments cellular oxidant-induced cellular injury. *n* = 3; **P* < 0.05 compared with cells not exposed to H₂O₂, #*P* < 0.05 compared with respective CTL cells.

NAD⁺ consumption in the cells. PARP inhibition also restored the mitochondrial potential and reduced the mitochondrial ROS production (Figure 5C–F).

These data suggest that NAD⁺ synthesis from nicotinamide (the salvage pathway) plays a dominant role in NAD⁺ biosynthesis in cells following hypoxia. To test whether its dominant function is restricted to post-hypoxic recovery, we also exposed H9c2 cells to oxidant injury and measured the viability changes after 3 and 24 h. We found that NamPRT inhibition caused little difference 3 h following oxidant exposure, but it significantly augmented cell damage at 24 h

(Figure 6), suggesting that NamPRT was involved in the cellular recovery from oxidative stress.

To further investigate the role of NamPRT in cellular bioenergetics in post-hypoxic cells, we conducted extracellular flux analysis. Oxidative phosphorylation was slightly diminished in cells prior exposed to OGD, but the anaerobic metabolism was not affected (Figure 7A–D). NamPRT inhibition did not affect the basal respiration or the anaerobic metabolism in cells exposed to hypoxia only but strongly decreased both respiration and acid production in cells pre-exposed to OGD, confirming that NamPRT is necessary for bioenergetic recovery following OGD that caused

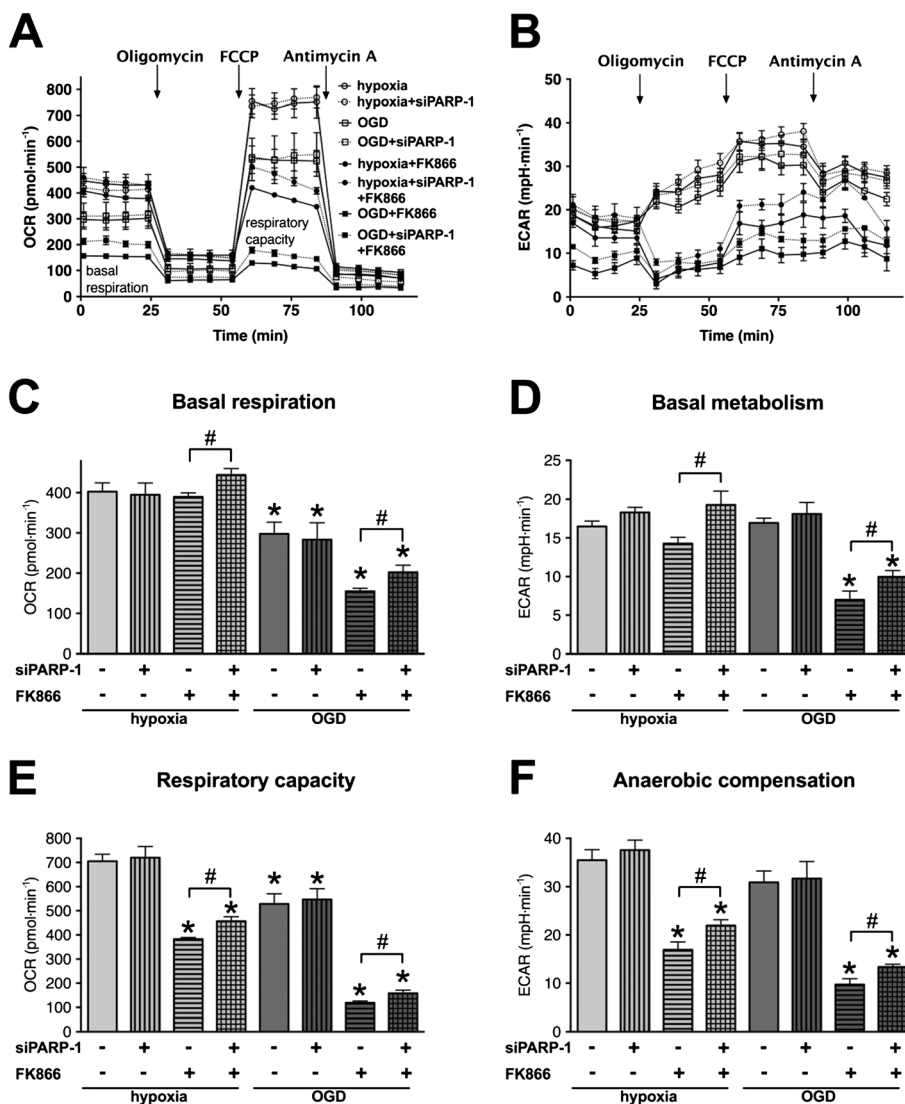


Figure 7

NamPRT is necessary for bioenergetic recovery following OGD. A–F: H9c2 cells were transfected with PARP-1 (siPARP-1) or CTL siRNA, and 48 h later, the cells were exposed to hypoxia or OGD for 8 h. Subsequently the cells were treated with NamPRT inhibitor FK866 (10 μM) or vehicle and were subjected to 16-h-long recovery. A, B: Metabolic profile of the cells was studied by sequential injections of oligomycin (1 μg · mL⁻¹), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (0.3 μM) and antimycin A (2 μg · mL⁻¹) and by measurement of (A) the cellular oxygen consumption rate (OCR) and (B) the extracellular acidification rate using extracellular flux analysis. C, D: Basal oxygen consumption (C) and acid production of the respective basal metabolism (D) are shown. E, F: Total respiratory capacity (E) was determined following the addition of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, and respective acid production via anaerobic compensation (F) is shown. NamPRT inhibition severely blocks the recovery of the respiratory capacity and prevents the anaerobic metabolic compensation. *n* = 3, **P* < 0.05 compared with hypoxia control, #*P* < 0.05 PARP-1 silenced cells compared with respective CTL siRNA treated cells.

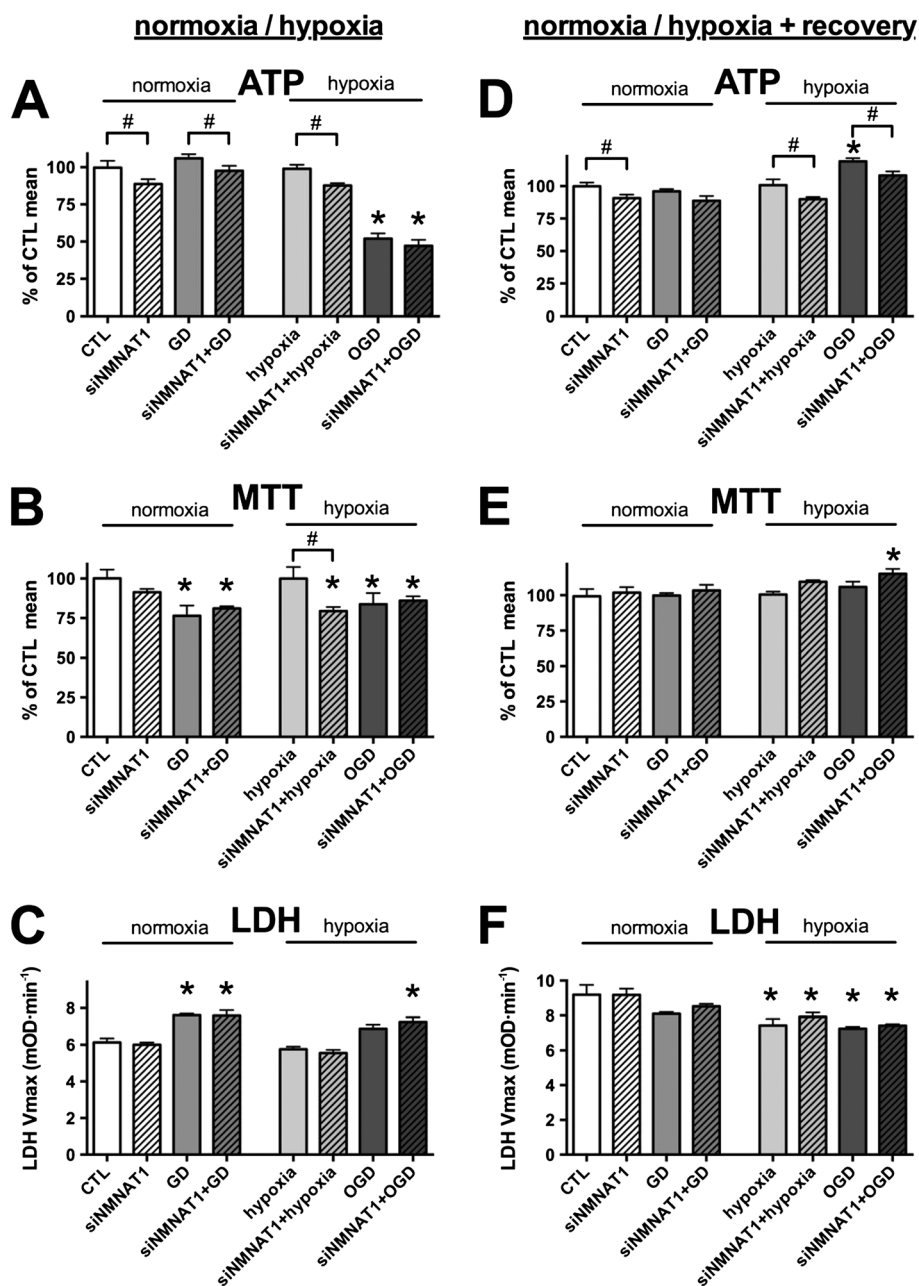


Figure 8

NMNAT1 silencing does not affect bioenergetic recovery following OGD. A–C: H9c2 cells were transfected with NMNAT1 siRNA (siNMNAT1) or CTL siRNA, and 48 h later, the cells were exposed to glucose deprivation (GD), hypoxia or OGD or maintained in glucose-containing culture medium at normoxia (CTL) for 8 h. D–F: Following the 8-h-long GD, hypoxic injury or OGD, glucose concentration and oxygen tension were normalized, and the cells were subjected to 16-h-long recovery. A, D: The cellular ATP content was measured at the end of the hypoxic period and following the recovery period. B, E: The cellular viability was measured by the MTT assay. C, F: LDH release was determined in the supernatant. NMNAT1 siRNA decreases the cellular ATP content but does not inhibit regeneration of the cellular ATP following OGD. $n = 3$, $*P < 0.05$ compared with normoxia CTL, $\#P < 0.05$ NMNAT1 silenced cells compared with respective CTL siRNA treated cells.

cellular NAD⁺ and ATP decrease. We used PARP-1 silencing to simultaneously test whether NAD⁺ consumption by PARP-1 affected the cellular metabolism. Consistent with our results of PARP inhibition (Figure 5), PARP-1 silencing significantly improved both the aerobic and anaerobic metabolism in the cells exposed to OGD, when resynthesis of NAD⁺ was blocked (Figure 7A–D).

Metabolic profiling also revealed diminished respiratory capacity in cells pre-exposed to OGD and the respiratory capacity was greatly reduced by NamPRT inhibition (Figure 7E). Importantly, anaerobic metabolism (glycolysis) showed an even higher degree of blockage in both post-hypoxic and post-OGD cells, when oxidative phosphorylation and NAD⁺ resynthesis were inhibited (by oligomycin and FK866, respectively)

(Figure 7F), showing that the lack of NAD⁺ seriously inhibited glycolysis. PARP-1 silencing significantly improved the respiratory capacity and anaerobic compensation, when NAD⁺ salvage was inhibited (Figure 7E and F). These results confirmed consumption of NAD⁺ by PARP-1.

The next step of NAD⁺ biosynthesis is catalysed by nicotinamide mononucleotide adenylyl transferases (NMNATs). There are three NMNAT isoforms in mammals: the (predominantly) nuclear NMNAT1 and the mitochondrial NMNAT3 are ubiquitous, while NMNAT2 is localized to the Golgi and the

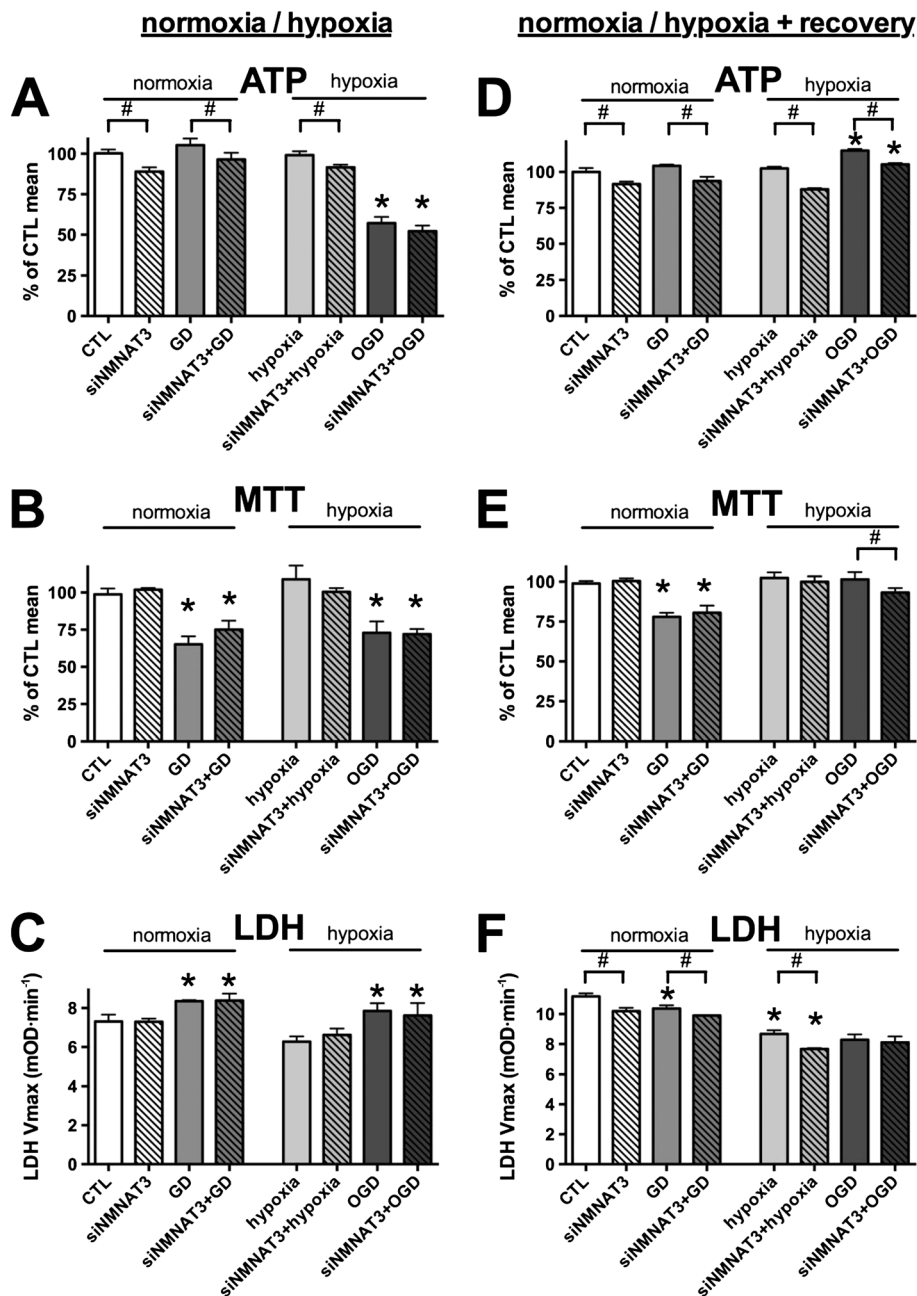


Figure 9

NMNAT3 silencing does not affect bioenergetic recovery following OGD. A–C: H9c2 cells were transfected with NMNAT3 siRNA (siNMNAT3) or CTL siRNA, and 48 h later, the cells were exposed to glucose deprivation (GD), hypoxia or OGD or maintained in glucose-containing culture medium at normoxia (CTL) for 8 h. D–F: Following the 8-h-long GD, hypoxic injury or OGD, glucose concentration and oxygen tension were normalized, and the cells were subjected to 16-h-long recovery. A, D: The cellular ATP content was measured at the end of the hypoxic period and following the recovery period. B, E: The cellular viability was measured by the MTT assay. C, F: LDH release was determined in the supernatant. NMNAT3 siRNA decreases the cellular ATP content but does not inhibit regeneration of the cellular ATP following OGD. $n = 3$, * $P < 0.05$ compared with normoxia CTL, # $P < 0.05$ NMNAT3 silenced cells compared with respective CTL siRNA treated cells.

cytoplasm but its expression is restricted to the brain (Berger *et al.*, 2005; Nikiforov *et al.*, 2011). To selectively test whether NMNAT1 or NMNAT3 is essential for the recovery from oxidant-induced or OGD-induced injury, we used siRNA-mediated gene silencing. NMNAT1 silencing induced a slight decrease in the basal ATP levels of the cells, but glucose deprivation and hypoxia did not result in any further decrease in the cellular ATP level, and neither the OGD-induced decrease nor the recovery was affected by the decreased NMNAT1 expression (Figure 8A–F). Similarly, reduced NMNAT3 level resulted in a slight decrease in the ATP content in the hypoxia injury, but it neither affected the OGD-induced decrease nor the recovery of the cellular ATP content (Figure 9A and D). NMNAT3 silencing did not affect the cellular viability in the OGD injury (Figure 9A–F), and unlike NMNAT1 silencing (Figure 8B), the reduced NMNAT3 expression did not result in decreased tricarboxylic acid cycle activity (as measured by the MTT assay) following hypoxia (Figure 9B). We also tested the effect of NMNAT silencing in oxidant-induced injury and found that neither NMNAT1 nor NMNAT3 silencing affected the cell survival or the recovery following the oxidant exposure (Figure S2 and S3).

Taken together, these data confirmed that NAD⁺ salvage by NamPRT played a significant role in bioenergetic recovery of post-hypoxic/post-OGD cardiomyocytes, and that the suppressed NAD⁺ pool following OGD inhibited both aerobic and anaerobic metabolism in these cells. On the other hand, NMNAT1 and NMNAT3 did not play essential roles in the bioenergetic recovery following OGD, or alternatively, they may compensate for the loss of each other in this process. PARP-1 consumed significant amounts of NAD⁺ in the post-hypoxic cells, thus affecting the cellular metabolism. The basal activity of this enzyme could delay the bioenergetic recovery from the suppressed state caused by restricted oxygen and glucose supply.

Discussion

The main findings and conclusions of the present study are the following: (1) post-hypoxic cells show increased sensitivity to oxidants due to diminished energy pools; (2) ATP regeneration is NAD⁺-dependent in hypoxia-reoxygenation injury; (3) NAD⁺ salvage by NamPRT is required for NAD⁺ and ATP regeneration; and (4) PARP-1 is a significant NAD⁺ consumer in post-hypoxic cells, and PARP inhibitors can be beneficial in ischaemia, even in the absence of acute complications.

ATP is the central coenzyme in the cells that functions as universal energy currency to transfer chemical energy. ATP is generated by catabolism of organic compounds via a series of redox reactions that involve the transfer of electrons from organic donor molecules to acceptor molecules (oxygen) to generate carbon dioxide and water. During these redox reactions, energy is 'collected' and stored mostly by reducing NAD⁺ to NADH in the cells. This energy is released from NADH to generate ATP using the proton gradient across the inner mitochondrial membrane as energy carrier in oxidative phosphorylation. Under basal conditions, fatty acids serve as major fuel substrates for energy production in the heart, but there is a metabolic switch in the ischaemic heart that leads to increased glucose utilization and glycolysis (Diaz *et al.*, 1998; Stanley, 2001), similar to the fuel substrate switch that

occurs in chronic hypoxia (induced by high altitude) (Essop, 2007). Glycolysis is also preferred to fatty acid utilization during the reperfusion because it is more oxygen-efficient (Lopaschuk and Stanley, 1997; Essop, 2007; Lopaschuk *et al.*, 2010). ATP is mostly generated via oxidative phosphorylation under normal conditions in the cells, with only a small portion of ATP provided by glycolysis. However, in hypoxia, the shortage of oxygen supply may completely stop mitochondrial respiration, and glycolysis will become the dominant ATP generation process.

Glycolysis is supplemented by lactic acid fermentation which helps regenerate the NAD⁺ molecules utilized through glycolysis. However, NAD⁺ consumed by the tricarboxylic acid cycle and by beta oxidation of fatty acids cannot be recycled in the absence of oxidative phosphorylation. Apart from the metabolic redox reactions catalysed by dehydrogenases, NAD⁺ is used in ADP-ribosylation reactions by PARPs and in deacetylation reactions by sirtuins in the cells (Hassa *et al.*, 2006; Hirsche, 2011). These reactions produce nicotinamide that can be re-used for NAD⁺ synthesis via the salvage pathway, but this pathway is an energy-requiring (endothermic) process that itself consumes ATP. Thus, in the ischaemic heart, NAD⁺ depletion is a result of several processes: (1) reduced NAD⁺ regeneration from NADH, (2) increased NAD⁺ consumption by PARP activated by oxidative stress and (3) decreased NAD⁺ re-synthesis from nicotinamide as a result of low metabolic output of the cells. The consequence of NAD⁺ insufficiency is the blockade of all processes in the cells that require NAD⁺ as coenzyme (Hassa *et al.*, 2006; Hirsche, 2011). In the ischaemic heart, the metabolic blockade and energy depletion are well documented, and they constitute a risk that the cells cannot fulfil the energy requirements of heart function (Nunez *et al.*, 1974; Stanley, 2001). Thus, the NAD⁺ metabolome is a rational therapeutic target in ischaemic heart disease.

There are two options to increase the cellular NAD⁺ content: (1) NAD⁺ generation may be increased and (2) NAD⁺ consumption can be decreased. Therapeutic approaches for intervention tried to improve NAD⁺ biosynthesis by providing more nicotinamide (substrate) to NamPRT or by avoiding this step of the synthesis and supplementing with nicotinamide mononucleotide. Both approaches have been effective in ischaemia-reperfusion injury of the heart (Sukhodub *et al.*, 2010; Yamamoto *et al.*, 2014).

Pharmacological interventions to decrease NAD⁺ catabolism have focused on PARP inhibition (Jagtap and Szabo, 2005). Unlike the energy-producing metabolic steps, PARP function can be lost for longer periods without severe adverse effects (Piskunova *et al.*, 2008). There are multiple PARP isoforms in the cells with various functions, but regarding NAD⁺ consumption and cellular bioenergetics, PARP-1 is the most important, because it shows the highest expression level and it can use the most NAD⁺ (Schreiber *et al.*, 2006). In preclinical studies, PARP inhibitors exerted a dramatic protective effect in myocardial reperfusion injury, and genetic ablation of PARP-1 confirmed a similar effect (Zingarelli *et al.*, 1997; Pieper *et al.*, 2000; Liaudet *et al.*, 2001). Beneficial effects of PARP inhibition has also been shown in various heart disease models including cardioplegic arrest, cardiopulmonary bypass and heart failure models and in age-induced cardiac dysfunction (Pacher *et al.*, 2004; Szabo *et al.*, 2004; Szabo *et al.*, 2004; Pacher *et al.*, 2006). However, faith was lost in PARP inhibitors when it turned out that the cardioprotection seen

with drug pretreatment was greatly reduced if PARP inhibitors were administered following the reperfusion (Morrow *et al.*, 2009; Roesner *et al.*, 2010). After the initial safety study of a single injection of the drug with 10 myocardial infarction patients per dose group, PARP inhibitor therapy was never tested in adequately powered cardiovascular clinical trials, but these inhibitors are now being tested in various cancer trials, which will provide data regarding their long-term safety (Morrow *et al.*, 2009; Garber, 2013). Because the compounds were shown to effectively block the enzyme in humans, we can expect that the beneficial effects of PARP inhibitors will be fully exploited in ischaemic heart disease in the coming years. We recently reported that PARP-1 leaves the nucleus in myocardial ischaemia-reperfusion injury and directly interacts with the ubiquitin E3 ligase protein RNF146 (Gero *et al.*, 2014). Overexpression of RNF146 can provide protection against oxidant-induced injury in cardiomyocytes, suggesting a novel option for reducing PARP-1 activity by capturing PARP-1 either with RNF146 or with a similar binding agent. Because this strategy is specific for PARP-1, it may represent a valuable alternative to non-specific inhibition of PARP.

In conclusion, metabolic therapy is a promising direction to supplement the current therapeutic options in ischaemic heart disease, and the NAD⁺ metabolome may be a prime target in this approach. Our results showed that NAD⁺ biosynthesis was crucial in bioenergetic recovery and blockade of NAD⁺ consumption by PARP-1 had beneficial effects in post-hypoxic cardiomyocytes. These are in line with previous data implying that both the NAD⁺ anabolic and catabolic processes can be exploited for therapeutic interventions in the ischaemic heart.

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Author contributions

DG and CS designed the experiments, DG performed the experiments, and DG and CS analysed the data and wrote the paper.

Conflict of interest

The authors declare that they have no competing interests as defined by *British Journal of Pharmacology* or other interests that might be perceived to influence the results and discussion reported in this paper.

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Supporting Information

Additional Supporting Information may be found in the on-line version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13252>

Figure S1 PARP-1 silencing in H9c2 cells

Figure S2 NMNAT1 silencing cause enhanced sensitivity to oxidant-induced injury. AG

Figure S3 NMNAT3 silencing does not affect the oxidant-induced injury. A-G