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Minocycline protects cardiac myocytes against simulated ischemia-reperfusion injury by inhibiting poly(ADP-ribose) polymerase-1

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Abstract

There is an increase in reactive oxygen and nitrogen species in cardiomyocytes during myocardial ischemia/reperfusion injury. This leads to oxidative DNA damage and activation of nuclear repair enzymes such as poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 activation promotes DNA repair under normal conditions. However, excessive activation of PARP-1 leads to cell death. Here we report that PARP-1 enzymatic activity is directly inhibited by minocycline, and we propose that one mechanism of minocycline cardioprotection is due to PARP-1 inhibition. Using cultured adult rat cardiac myocytes, we evaluated the mechanism of minocycline protection in which PARP-1 activation was induced by simulated ischemia/reperfusion (sI/R) injury using oxygen-glucose deprivation. We found an increase in reactive oxygen species production, PARP-1 activation, and PARP-1-mediated cell death after sI/R. Cell death was significantly reduced by the PARP inhibitors DPQ (10 μ M) and PJ-34 (500 nM), or by minocycline (500 nM). Cellular NAD⁺ depletion and poly(ADP-ribose) formation, which are biochemical markers of PARP-1 activation, were also blocked by minocycline. Finally, sI/R led to induction of the mitochondrial permeability transition (MPT), which was prevented by minocycline. Therefore, we propose that the protective effect of minocycline on cardiac myocyte survival is due to inhibition of PARP-1 activity.

Keywords

Mitochondria; PARP-1; ischemia; ischemia/reperfusion; oxidative injury

INTRODUCTION

Minocycline is a tetracycline derivative that is clinically approved for treatment of acne and other skin infections. It was demonstrated to have anti-inflammatory and cytoprotective effects unrelated to its antimicrobial properties, and its beneficial effects have been

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documented in various experimental models of neurological diseases since it was initially reported to be a neuroprotective agent(1). In these studies, the protective effects of minocycline have been attributed both to reduced inflammation and a direct effect on cell survival(1–6). Recently, minocycline has been reported to have protective effects in other injury models, such as cardiac myocytes.

Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant nuclear enzyme that plays a key role in cell death and survival under stress conditions(7), and is the most abundant of several PARP family members(8). Upon DNA damage, PARP-1 is activated and consumes NAD⁺ to form branched polymers of ADP-ribose (poly(ADP-ribose)) on target proteins, which include histones and PARP-1 itself. Poly(ADP-ribose)-ylation facilitates DNA repair by preventing chromatid exchange and by loosening histone wrapping. However, extensive PARP-1 activation can lead to cell death, presumably through mechanisms linked to NAD⁺ depletion, bioenergetic failure, and release of apoptosis inducing factor from the mitochondria(9–11). Cells from PARP-1 knockout mice or pharmacological inhibition of PARP-1 can promote cell and tissue survival in these settings(9,10,12–14).

Though the cytoprotective effects by minocycline have been extensively reported in many models of CNS injury, little is known about its potentially beneficial effects as a cardioprotective agent against ischemia/reperfusion (I/R) injury. One study reported cardioprotective effects through direct inhibition of various cell-death pathways(15). We have previously shown that low levels of minocycline protected neurons against the DNA alkylating agent, MNNG, by directly inhibiting PARP-1 activity(6). Therefore, we designed the present study to characterize and assess the mechanism and effectiveness of minocycline protection against I/R injury in primary cultures of rat cardiac myocytes. Here we show that minocycline blocks PARP-1 – mediated cardiac myocyte death in culture by directly inhibiting PARP-1 activity, and identify a novel mechanism by which minocycline can potentially influence survival after I/R injury.

MATERIALS AND METHODS

Cell culture reagents were purchased from CellGro, and all other reagents were purchased from Sigma-Aldrich unless otherwise noted.

Rat cardiac myocyte isolation and culture

Adult rat cardiac myocytes were isolated and cultured from 250–300 g male rats (Charles River) as described previously(16) using a modified method reported by Zhou et.al.(17). The animal use protocol was approved by the San Francisco Veterans Affairs Medical Center animal studies committee. The investigation conforms to 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). Rats were treated with heparin (50 U) and anesthetized by intraperitoneal injection with sodium pentobarbital (1.6 mg/10 g). The heart was quickly excised and the aorta cannulated for retrograde perfusion in a Langendorff apparatus at a constant flow rate (12 ml/min) at 37°C. The Langendorff system was cleaned prior to each use with 70% ethanol and rinsed thoroughly with sterile water to reduce contamination. All solutions used were sterilized using a 0.02 µm nitrocellulose filter. The heart was perfused for 9–10 min. with isolation buffer (120 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 5.6 mM glucose, 5 mM NaHCO₃, 10mM HEPES, 50 µM CaCl₂, 10 mM 2,3-butanedione monoxime (BDM) and 5 mM taurine, pH 6.8), followed by digestion for 20 minutes with collagenase II (1.5 mg/ml, Worthington) in isolation buffer. After digestion, the soft and flaccid heart was removed, and myocytes were suspended in isolation buffer. A series of four centrifugations (40 × g, 1min) and resuspensions were used for stepwise Ca²⁺ reintroduction from 50 µM to 1.0 mM, which was the final medium Ca²⁺ concentration.

The cells were suspended in minimum essential medium (MEM) with Hanks Buffered Salt Solution (HBSS), 10 µg/ml penicillin, 1.5 µM vitamin B12, and 10 mM BDM. For cell viability assays, myocytes were plated on 35 mm tissue culture dishes coated with laminin (10µg/ml) for a 1.5h attachment period in 2% CO₂/air at 37°C. For fluorescence imaging, myocytes were plated overnight on laminin-coated 15 mm glass cover slips for a 1.5h attachment period in 2% CO₂/air at 37°C. After this period of attachment, the medium was changed to MEM/HBSS containing 10 µg/ml penicillin, 1.5 µM vitamin B12, and 1 mM BDM, and incubated overnight. Experiments were performed the day following isolation and culture.

Experimental procedures

For cell viability, cardiomyocytes were exposed to conditions corresponding to each experimental group. Simulated ischemia/reperfusion (sI/R) was induced by placing cells in an anoxia chamber and replacing the media with glucose-free media previously equilibrated in the anoxia chamber (95% N₂, 5% CO₂ 37°C). This gives an experimental condition of oxygen and glucose deprivation (OGD). Reperfusion was initiated by removing the cells from the anoxia chamber and replacement with normal experimental media.

For fluorescence imaging, experiments were initiated by transferring the coverslips in the cell culture plates onto an open-bath imaging chamber (Warner Instruments, Hamden, CT), and replacing the culture medium with recording buffer containing (in mM): NaCl (120.4), CaCl₂ (1.0), KCl (4.7), MgSO₄ (1.2), Na₂HPO₄ (0.6), K₂HPO₄ (0.6), NaHCO₃ (4.6), glucose (5.6), HEPES (10.0), taurine (5.0), and 0.1% bovine serum albumin (BSA), and filtered through a 0.2 µm sterile filter before use. The imaging chamber was placed onto an inverted microscope for time-lapse fluorescence microscopy (see *Fluorescence microscopy* section). Pharmacological compounds were added immediately after reperfusion from concentrated stocks prepared in recording buffer.

Measurements of Cell Viability

Myocyte survival was determined by counting live cells in the experimental cultures compared to the number of live cells in the control cultures as determined by a trypan blue exclusion assay. Cells that were positive for trypan blue staining have a compromised plasma membrane integrity, indicating necrotic-type cell death. Myocytes were counted in media containing trypan blue (0.04%) after 10 min at room temperature. Live (non-blue) and dead (blue) myocytes in 10 random fields per condition per experiment were recorded at 100 × magnification in a Zeiss brightfield microscope. The number of cells that excluded trypan blue in the control cultures was defined as equal to 100%. To validate the trypan blue assay, percentages of live and dead myocytes were determined using calcein plus propidium iodide (C/PI) method (Molecular Probes, Eugene, OR, USA) as previously described in our laboratory(18,19). Similarly, cells that stained positive for PI and lose calcein retention were considered to have lost plasma membrane integrity, thus indicating necrotic-type cell death. Initial experiments were performed to compare the results obtained with the C/PI method versus those from trypan blue exclusion method (at 0.5 0 2 hr after SI/R). Subsequent cell death data are presented using C/PI method.

Quantitation of reactive oxygen species generation

Measurement of ROS generation was assayed using dihydroethidium (dHEt). dHEt is a reduced, nonfluorescent dye that increases in fluorescence upon oxidation to ethidium with increase in cellular ROS level(20,21). We also used the ROS scavengers Tempol and Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP). Both are cell-permeable superoxide dismutase (SOD) mimetics and peroxynitrite scavengers(22) (Calbiochem). Rat cardiac myocytes were loaded for at least 30 minutes with dHEt (1 nM). Experiments were

initiated by placing rat cardiac myocytes on glass cover slips mounted onto an open-bath imaging chamber. The dye was excited at 545 nm using a rhodamine excitation filter (Chroma Technology, Brattleboro, VT), and fluorescence emission was measured using a rhodamine dichroic mirror and emission filter set (590 nm).

Fluorescence microscopy

Fluorescence was collected on an inverted microscope (Zeiss Axiovert 200M) with a 40× oil objective, and equipped with a CCD digital camera (ORCA-ER; Hamamatsu Corporation, Bridgewater, NJ), operated with Openlab software (ImproVision, Boston, MA) on a Mac G4. Data collection and analysis were performed using Openlab software (Improvision, Lexington, MA). Fluorescence values were collected as a field average for each excitation / emission pair, with 1–2 cells assessed per coverslip. These values were averaged for each separate experiment and normalized to the baseline values obtained prior to SI/R or normal (control) exposure.

NAD⁺ measurements

NAD⁺ was assayed as previously described(19) using an enzymatic recycling assay(23) and normalized to total protein as measured by the bicinchonic acid method and bovine serum albumin standards(24).

Mitochondrial isolation for NAD⁺ assay

Mitochondrial isolation from cardiac myocytes were performed as described previously(19). Cells were grown on 6-well plates as described, and were harvested with a scraper in 0.5 mL of isolation medium (320 mM sucrose, 1 mM potassium EDTA, and 10 mM Tris–HCl pH 7.4). Cells were homogenized in a glass Teflon homogenizer, and were centrifuged at 500×g for 5 min at 4°C. The supernatant fractions (S1) were saved (at 4°C) for the subsequent centrifugation step. The pellet was re-homogenized and centrifuged at 500×g for 10 min at 4°C. The supernatant (S2) was combined with S1 and centrifuged at 500×g for 10 min at 4°C. The supernatant was collected, and centrifuged at 16,000×g for 30 min. The final pellet (mitochondrial fraction) was resuspended in 200 µl of isolation medium and kept on ice for biochemical studies (NAD⁺ assay and AIF western blot). Mitochondrial enrichment was quantified by Western blots using an antibody targeted to mitochondrial protein voltage-dependent anion channel (VDAC).

Western blots

Western blots were prepared as described(6). The membranes were probed with monoclonal anti-PARP-1 (Trevigen, Gaithersburg, MD), and antibody binding was visualized with peroxidase-conjugated anti mouse IgG (Vector Laboratories, Burlingame, CA) and the ECL^{TM} WB Detection kit (Amersham-Pharmacia Biotech). Total PAR immunoreactivity was measured in each lane in the high – molecular weight region spanning the 116 kD PARP-1 band to the top of the membrane and quantified with the NIH Image J program. Values for each lane were normalized to protein loading as measured by β-actin immunoreactivity and expressed as a percent of PAR staining in the control condition prepared in each experiment. Controls were performed in the absence of primary or secondary Abs showed no signal (not shown).

Statistics

For single comparisons, Students t-test was used. For multiple comparisons, either one-way analysis of variance or a repeated measures analysis of variance was carried out. Post-hoc analysis was performed using the Student-Newman-Keuls test. $P < 0.05$ was considered significant.

RESULTS

Cardiomyocyte death after sI/R is due to free radical generation

Primary rat cardiomyocytes were exposed to various durations of oxygen-glucose deprivation (OGD) followed by reperfusion with normal media (glucose-containing media equilibrated in air) as a model of sI/R injury. Longer exposure to OGD led to an increase in cell death (Fig. 1A), as assayed with either the trypan blue exclusion method or with the viability/death assay using calcein and propidium iodide (C+PI) method. Using a 1h exposure to OGD as our working model, we next wanted to see if cell death is mediated by an increase in ROS generation. Using either the small molecule cell-permeable superoxide dismutase (SOD) mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (Tempol), or another cell-permeable SOD mimetic Mn(III)tetrakis(4-Benzoic acid)porphyrin (MnTBAP), sI/R mediated injury was significantly reduced (Fig. 1B). Therefore, our model of cell death with sI/R injury is mediated by an increase in the generation of reactive oxygen species (ROS).

sI/R leads to an increase in ROS generation

To verify an increase in free radical production with sI/R-mediated cell death, we assayed ROS production after sI/R. Cells loaded with dHEt were exposed to sI/R, and dHEt fluorescence was assayed at various times immediately after reperfusion. There was a significant increase in dHEt fluorescence after sI/R compared to control (non-sI/R) conditions, indicating an increase in ROS production (Fig. 2A). The increase in ROS production was significant as early as 10 min after sI/R, and continued to increase 20 min after sI/R.

To verify that the increase in dHEt fluorescence after sI/R was due to an increase in ROS production, we treated the cells with the SOD mimetics Tempol and MnTBAP to scavenge free radicals (Fig. 2B). As predicted, treatment with either Tempol (250 μ M) or MnTBAP (100 μ M) prevented the increase in dHEt fluorescence, indicating a reduction in ROS production after sI/R. The increase in ROS production after sI/R was not significantly prevented by the PARP inhibitor PJ34 (500 nM) or by minocycline (500 nM). This is in support of the cardioprotective effects of Tempol and MnTBAP against sI/R-induced death shown in Figure 1.

Minocycline protects against sI/R-induced cell death

Prior studies have established that PARP-1 activation is a key factor mediating neuron death in stroke, and that either pharmacological inhibition or genetic deletion of PARP-1 is protective(11,25,26). Similarly, it was previously shown that PARP-1 mediates cardiac ischemia/reperfusion injury. This was confirmed in cardiac myocytes by near-complete inhibition of sI/R-induced cell death with the PARP inhibitors 3, 4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) or N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N, N-dimethylacetamide hydrochloride (PJ34) (Fig. 3A). Based on our previous findings in neurons, we hypothesized that minocycline can protect cardiomyocytes against I/R injury. Indeed, minocycline similarly protected against sI/R-induced cell death, with an IC₅₀ of 126.3 $\pm$ 24.2 nM (Fig. 3B). Thus, minocycline treatment is protective against sI/R-induced cardiomyocyte death, presumably through inhibition of PARP-1 activity. Therefore, we used 500 nM minocycline as our working concentration, which is 4-fold the IC₅₀ and therefore should provide maximal effect.

sI/R leads to increased ROS formation and PARP-1 activation

It is known that an increase in ROS production leads to DNA damage, which activates PARP-1. Activation of PARP-1 leads to an increase in poly(ADP)ribose (PAR) formation.

Also, PARP-1 is itself a substrate for poly(ADP)ribosylation (or PARylation). This was confirmed here by an increase in PARylation of PARP-1 (PAR-PARP-1) after sI/R (Fig. 4A,B). PAR-PARP-1 was reduced by treatment with the antioxidants Tempol or MnTBAP. Total PARP-1 protein levels did not change with sI/R or with treatment with either Tempol or MnTBAP. These data indicate that sI/R leads to an increase in ROS generation, which activates PARP-1. To further verify these findings, we assayed total PAR formation after sI/R. There was an increase in PAR formation after sI/R, which was prevented by treatment with either Tempol or MnTBAP (Fig. 4C,D). These data indicate that sI/R leads to an increase in oxidative DNA damage and PARP-1 activation.

sI/R leads to PARP-1-mediated NAD⁺ depletion due to ROS formation

PARP-1 activation consumes NAD⁺ as its substrate. NAD⁺ is the source of ADP-ribose for PAR formation on target proteins. Therefore, we assayed total cellular NAD⁺ levels after sI/R. As shown, there is a time-dependent decrease in total NAD⁺ after sI/R. Treatment with the free radical scavengers Tempol or MnTBAP prevented the decrease in NAD⁺ (Fig. 5A). Measurement of mitochondrial NAD⁺ revealed that NAD⁺ content in mitochondria did not significantly change 30 min after reperfusion, but was significantly depleted 90 min after reperfusion. This is in support of our previous findings that PARP-1 activation in cardiac myocytes initially depletes cytosolic NAD⁺, which comprises approximately 10–20% of total NAD⁺ in cardiac myocytes(19). Treatment with the free radical scavengers MnTBAP or Tempol prevented the decrease in mitochondrial NAD⁺ (Fig. 5B). These indicate that mitochondrial NAD⁺ is preserved by antioxidants, and is in support of our previous finding that PARP-1 activation primarily consumes cytosolic NAD⁺ levels(19).

Minocycline inhibits sI/R-mediated PARP-1 activation

Effects of minocycline on cardiac PARP-1 activity was determined by measuring poly(ADP-ribose) (or PAR) formation and NAD⁺ consumption(6,7,19,27). As mentioned earlier, PARP-1 is itself a target for PAR formation (PAR-PARP-1). An increase in PAR-PARP-1 was detected 30 minutes after sI/R (Fig. 6A,B). PAR formation was nearly completely suppressed by treatment with either the PARP inhibitor (PJ34), or with minocycline (500 nM). These results were corroborated by western blots of total PAR formation (Fig. 6C,D).

To verify that PARP-1 activation was inhibited by minocycline, we measured NAD⁺ consumption by PARP-1 in parallel studies by measuring NAD⁺ in cardiac myocytes after sI/R. The source of ADP-ribose and the substrate of PARP-1 activity is NAD⁺, and it has been shown that excessive PARP-1 activation leads to a decrease in NAD⁺ concentration(10). sI/R reduced total NAD⁺ levels as early as 30 min after reperfusion, and almost completely after 90 min of reperfusion (Fig. 5C). Minocycline (500 nM) attenuated the NAD⁺ depletion with the same efficacy as the PARP inhibitor PJ34. These data indicate that minocycline inhibits PARP-1 activity in cardiac myocytes, and strongly suggests that minocycline protection against sI/R injury is through direct inhibition of PARP-1 activity.

Mitochondrial NAD⁺ was also assayed 30 min and 90 min after sI/R (Fig. 5D). Mitochondrial NAD⁺ was not affected 30 min after sI/R, but was severely decreased 90 min after sI/R. The decrease in mitochondrial NAD⁺ was prevented by treatment with the PARP inhibitor PJ34, and by minocycline (500 nM). These data indicate that mitochondrial NAD⁺ levels are maintained in treatments shown to protect against sI/R injury, and suggest that maintenance of mitochondrial function is required for cell survival. The results further indicate that PARP inhibition, either by PJ34 or by minocycline, inhibited MPT induction and prevented mitochondrial NAD⁺ loss, as we and others have reported previously(19,28).

MPT inhibitors protect against sI/R injury but do not inhibit PARP-1 activity

Pharmacological inhibition of the mitochondria permeability transition (MPT) pore has been shown to protect against various forms of cell and tissue injury, including sI/R injury(10,19,28,29). To verify that the MPT inhibitors cyclosporine-A (CsA) and sanglifehrin-A (SfA) do not protect by inhibition of PARP-1, we assayed PAR formation after sI/R (Fig. 7A,B). The degree of PAR formation after sI/R was not affected by the MPT inhibitors CsA (500 nM) or SfA (500 nM), indicating that these compounds do not directly inhibit PARP-1 activity. Consistent with other studies(29–31), treatment with either CsA or SfA significantly protected against sI/R injury (Fig. 7C), suggesting that MPT pore opening plays a role in sI/R induced cell death in cultured cardiac myocytes.

Mitochondrial NAD⁺ depletion after sI/R is due to MPT pore opening

PARP-1 activation consumes NAD⁺ as its substrate and source of ADP-ribose for PAR formation on target proteins. We have previously shown that PARP-1 activation initially utilizes cytosolic, but not mitochondrial, NAD⁺(19). Therefore, we assayed total cellular NAD⁺ levels after sI/R. As shown, there is a time-dependent decrease in total NAD⁺ with sI/R. Treatment with the mitochondria permeability inhibitors cyclosporine-A (CsA) or sanglifehrin-A (SfA) did not prevent the initial decrease in total NAD⁺ 30 minutes after sI/R, but did prevent the final decrease 90 minutes after sI/R (Fig. 5E). This is in direct support of our previous findings that PARP-1 activation in cardiac myocytes initially depletes cytosolic NAD⁺, which comprises approximately 10–20% of total NAD⁺ in cardiac myocytes(19). Mitochondrial NAD⁺ is not depleted 30 min after sI/R, but is significantly decreased 90 min after sI/R alone(Fig. 5F). The decrease in mitochondrial NAD⁺ was prevented by the MPT inhibitors CsA and SfA. These data are in support of the finding that mitochondrial NAD⁺ is not available to PARP-1 until opening of the MPT pore, which prevents NAD⁺ release to the cytosol and where it becomes available as a PARP-1 substrate(28). Preservation of mitochondrial NAD⁺ by MPT inhibitors, as well as by the PARP inhibitor PJ34 and minocycline (Fig. 5D) indicated that PARP-1 activation promoted MPT induction and mitochondrial NAD⁺ loss.

DISCUSSION

These results identify a novel and potent mechanism of cardiac protection by minocycline against I/R injury. Using cultured rat cardiomyocytes, we first established that sI/R leads to increased ROS formation and ROS-mediated injury. We demonstrated for the first time that minocycline can protect cardiac myocytes against PARP-1-mediated toxicity at nanomolar concentrations. We verified the inhibitory effects of minocycline on biochemical markers of PARP-1 activation in the cardiomyocyte cultures, and found that minocycline was again a highly potent inhibitor.

In our previous study using purified PARP-1, data collected from kinetic studies of PARP-1 *in vitro* enzyme activity indicated that minocycline directly inhibited PARP-1 activity, and suggested a competitive interaction between minocycline and NAD⁺. We predicted that the carboxamide and aromatic ring structure shared between NAD⁺ and minocycline is the active site of interaction with PARP-1(6). Indeed, a common structural feature of competitive PARP inhibitors is this carboxamide group attached to an aromatic ring or the carbamoyl group built in a polyaromatic heterocyclic skeleton(6).

Minocycline has previously been reported to have several other effects that could contribute to improved cell survival, but at far higher concentrations (micromolar) than are shown here to inhibit PARP-1 activity. These effects include upregulation of mitochondrial bcl-2 expression(32); inhibition of caspase activation(15), calcium-induced cytochrome-C release

and mitochondrial permeability transition(4,5,33); direct scavenging of reactive oxygen species(34); and inhibition of mitogen activated protein kinases(35,36). By comparison, we found that minocycline blocked PARP-1 mediated cell death at nanomolar concentrations, which is consistent with our previous report(6), and is much lower than what is reported by others. Thus, under conditions causing PARP-1 - mediated cell death, our data suggest that the primary mechanism of minocycline protection is likely to be PARP-1 inhibition. However, it is important to note that these results do not exclude the possibility that other effects of minocycline may also contribute to cell survival, particularly under conditions where cell death is not mediated primarily by PARP-1 activation.

In the CNS, minocycline has also been shown to have effects other than its direct effects on neuron survival, such as inhibition of microglial proliferation and secretory activity induced by excitotoxicity or brain ischemia(1,2,37,38). These effects have been reported with minocycline concentrations of 20 nM in cell culture(37,38). These findings suggest that minocycline protection is not limited to cardiomyocytes, neurons, or microglia, and that its effects may include protection in other systems where oxidative stress plays a role in injury(39).

The proposed mechanism of protection is presented as a schematic diagram in Figure 8. Cardiac myocytes exposed to sI/R exhibit an increase in reactive oxygen species (ROS) generation, which promotes oxidative damage to cellular targets, such as proteins, lipid structures, and DNA. When ROS induces DNA damage, it activates various DNA repair enzymes, which include PARP-1. Upon activation, PARP-1 utilizes NAD⁺ as a substrate for PAR formation on target proteins, and excessive PARP-1 activation leads to NAD⁺ depletion. The consequence is a failure of NAD⁺-dependent processes, such as glycolysis, deacetylation reactions, and endogenous antioxidant recycling. Mitochondrial failure and cell injury can occur with significant cytosolic NAD⁺ depletion, and we and others have shown that opening of the MPT pore is a downstream effect of excessive PARP-1 activation(10,19,28,29). Irreversible MPT induction can lead to mitochondrial release of NAD⁺ as well as release of other mitochondrial proteins, which include various cell death factors. Minocycline protects from this cell death pathway by inhibiting PARP-1 activity.

In conclusion, our findings indicate that minocycline (and potentially other tetracycline derivatives) may be an invaluable source of future drug design for treatment of acute and chronic injury mediated by oxidative stress. In addition, because minocycline is already approved for clinical use, its potential use against heart failure, cardiomyopathies, and other cardiovascular conditions is highly possible.

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Figure 1. Cardiomyocyte death after sI/R is due to free radical generation

A) Effects of OGD duration to model simulated ischemia/reperfusion (sI/R) on cell survival. Viability was assayed using either trypan blue exclusion expressed as % cells positive for trypan blue, or calcein + propidium iodide (C/PI) expressed as % cells positive for P.I over calcein-positive + P.I.-positive cells. $n=3$ **

B) The superoxide dismutase mimetics Tempol (250 μ M) and MnTBAP (100 μ M) protects against simulated ischemia/reperfusion (sI/R) injury. OGD was induced for 1h, and subsequently switched to normal media. Cell death was assayed using C+PI method. $n=3$ ** $p < 0.01$ (vs. sI/R alone).

Figure 2. sI/R increases ROS formation in cultured cardiomyocytes

Cultured cardiac myocytes were exposed to 1h OGD, followed by reperfusion with normal media.

A) Dihydroethidium (dHEt) fluorescence was assayed at 0, 10, and 20 min after reperfusion. An increase in fluorescence indicates an increase in ROS production. Values were normalized to baseline (F_0) and presented as F/F_0 at indicated time along x-axis. $n=3$ * $p < 0.01$ (vs. sI/R alone) ** $p < 0.01$ (vs. sI/R alone).

B) Quantified dHEt fluorescence of various treatment groups 20 min after reperfusion, which include the PARP inhibitor (PJ34 - 500 nM), free radical scavengers (Tempol - 250 μ M; MnTBAP - 100 μ M), and minocycline (500 nM). $n=3$ ** $p < 0.01$ (vs. sI/R alone).

Figure 3. Minocycline protects against sI/R-induced cell death

A) PARP inhibitors PJ34 (500 nM) or DPQ (10 μ M) protected cultured cardiac myocytes against sI/R injury, indicating that sI/R led to PARP-1-mediated cell death. $n=3$ ** $p < 0.01$ (vs. sI/R alone).

B) Minocycline (filled triangles) dose-dependently protected against sI/R injury. The calculated IC_{50} for minocycline was 126.3 ± 16.3 nM. * $p < 0.05$ ** $p < 0.01$ compared to sI/R alone (filled diamond).

Figure 4. sI/R activates PARP-1 in cultured cardiomyocytes

A) Western blot of PAR formation on PARP-1 (PAR-PARP-1) in cardiac myocytes 30 min after sI/R. Control wells received vehicle (medium) only. The major PAR band (116 kD) corresponds to PAR labeling of PARP-1 itself. Staining for total PARP-1 (no PAR) indicates the same protein loading in each lane. Cells were treated with either Tempol (250 μ M) and MnTBAP (100 μ M) during onset of reperfusion.

B) Quantification of PAR-PARP-1 Western blots. Data are normalized to PARP-1 protein band density. Data are means $\pm$ s.e.m. of 3–4 independent experiments, each performed in triplicate. $n=3$; ** $p < 0.01$ (vs. sI/R alone).

C) Western blot of total PAR formation in cardiac myocytes 30 min after sI/R. Control wells received vehicle (medium) only. The β -actin bands indicate protein loading in each lane. Cells were treated with either Tempol (250 μ M) and MnTBAP (100 μ M) during onset of reperfusion.

D) Quantification of PAR Western blots. Data are normalized to β -actin protein band density. Data are means $\pm$ s.e.m. of 3–4 independent experiments, each performed in triplicate. $n=3$; ** $p < 0.01$ (vs. sI/R alone).

Figure 5. Minocycline prevents sI/R-induced decrease in cytosolic NAD⁺ by inhibiting PARP-1

A, C, E) Total NAD⁺ was assayed before and after sI/R, and presented as a time course of NAD⁺ depletion. sI/R was used to induce injury to cardiac myocytes with treatment of vehicle (sI/R alone), (A) with Tempol (250 μ M) or MnTBAP (100 μ M), (C) with PJ34 (500 nM), or minocycline (Mino; 500 nM), and (E) with CsA (500 nM) or sI/R with SfA (500 nM). n=3; * $p < 0.05$; ** $p < 0.01$ (vs. all other conditions).

B, D, F) Mitochondrial NAD⁺ was assayed 30 min (gray bars) and 90 min (black bars) after sI/R. Mitochondrial NAD⁺ did not decrease until 90 min after OGD in the sI/R treatment alone. Treatment with antioxidants (Tempol, MnTBAP), PJ34 (500 nM), minocycline (500 nM), and the MPT inhibitors CsA (500 nM) and SfA (500 nM) prevented the decrease in mitochondrial NAD⁺ at 90 min. n=3; ** $p < 0.01$ (vs. control).

Figure 6. Minocycline inhibits sI/R-mediated PAR formation

A) Western blot of PAR formation on PARP-1 (PAR-PARP-1) in cardiac myocytes 30 min after sI/R. Control wells received vehicle (medium) only. The major PAR band (116 kD) corresponds to PAR labeling of PARP-1 itself. Staining for total PARP-1 (no PAR) indicates the same protein loading in each lane. Cells were treated with either PJ34 (500 nM) or minocycline (Mino; 500 nM).

B) Quantification of PAR-PARP-1 Western blots. Data are normalized to PARP-1 protein band density. Data are means $\pm$ s.e.m. of 3 independent experiments, each performed in triplicate. $n=3$; ** $p < 0.01$ (vs. sI/R alone).

C) Western blot of PAR formation in cardiac myocytes 30 min after sI/R. Control wells received vehicle (medium) only. The β -actin bands indicate protein loading in each lane. Cells were treated with either PJ34 (500 nM) or minocycline (Mino; 500 nM) during onset of reperfusion.

D) Quantification of PAR Western blots. Data are normalized to β -actin protein band density. Data are means $\pm$ s.e.m. of 3 independent experiments, each performed in triplicate. $n=3$; ** $p < 0.01$ (vs. sI/R alone).

Figure 7. MPT inhibitors protect against sI/R injury but do not inhibit PARP-1 activity

A) Western blot of PAR formation on PARP-1 (PAR-PARP-1) in cardiac myocytes 30 min after sI/R. Control wells received vehicle (medium) only. The major PAR band (116 kD) corresponds to PAR labeling of PARP-1 itself. Staining for total PARP-1 (no PAR) indicate same protein loading in each lane. Cells were treated with the MPT inhibitors CsA (500 nM) or SfA (500 nM) during onset of reperfusion.

B) Quantification of PAR-PARP-1 Western blots. Data are normalized to PARP-1 protein band density. Data are means $\pm$ s.e.m. of 3 independent experiments, each performed in triplicate.

C) Cell death was evaluated after sI/R alone or with the MPT inhibitors CsA (500 nM) or SfA (250 nM) during onset of reperfusion. n=3; ** $p < 0.01$ (vs. sI/R alone).

Figure 8. Schematic Diagram of PARP-1-mediated cell death

Ischemia/Reperfusion (I/R) leads to an increase in reactive oxygen species (ROS) production, promoting oxidative damage to cellular targets, such as DNA. Upon DNA damage, the DNA repair enzymes are activated, which include PARP-1. PARP-1 activity utilizes NAD⁺ as a substrate, and excessive PARP-1 activation can lead to NAD⁺ depletion. Mitochondrial failure and cell injury can occur with significant cytosolic NAD⁺ depletion. Mitochondrial NAD⁺ is expelled from mitochondria upon MPT induction. Minocycline protects from this cell death pathway by inhibiting PARP-1 activity, and by so doing, inhibits MPT opening.