Cardioprotection by the Mitochondrial Unfolded Protein Response is Mediated by ATF5

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ABSTRACT

Rationale: The mitochondrial unfolded protein response (UPR^{mt}) is a conserved signaling pathway triggered by mitochondrial dysfunction. The transcription factor ATF5 is proposed to be central to mammalian UPR^{mt} signaling. We hypothesized that UPR^{mt} activation may be cardioprotective against ischemia-reperfusion (IR) injury, and that this protection would require ATF5. *Objective:* To determine whether pharmacologic UPR^{mt} activation protects mouse hearts from acute IR injury in an ATF5-dependent manner. *Methods and Results:* Loss of ATF5 did not affect baseline IR injury in an *ex-vivo* perfused heart model. However, *in-vivo* administration of the UPR^{mt} inducers oligomycin or doxycycline 6 h prior to *ex-vivo* IR injury was cardioprotective. Such protection was absent in hearts from *Atf5*$^{-/-}$ mice, as well as in hearts given doxycycline only during *ex-vivo* perfusion. Genes encoding known UPR^{mt}-linked chaperones exhibited modest but significant cardiac induction by oligomycin after 6 h. In addition, cardiac gene expression analysis by RNA-Seq revealed mild induction of numerous genes in an ATF5-dependent manner, which may be important for cardioprotection. *Conclusion:* ATF5 is required for cardioprotection induced by drugs that activate the UPR^{mt}.

Keywords: UPRmt, ATF5, Ischemia, Cardioprotection

INTRODUCTION

Cardiac ischemia-reperfusion (IR) injury is the underlying pathologic basis of myocardial infarction, which accounts for significant morbidity and mortality.[1] A large body of research has highlighted the importance of mitochondria as both mediators of cardiac IR injury and as targets for cardioprotective interventions.[2,3] An important concept in many cardioprotective paradigms is hormesis, wherein small sub-lethal insults can trigger signaling pathways that afford protection against subsequent larger injuries.[4] A classical example of mitohormesis in the heart is ischemic preconditioning (IPC), in which small ischemic periods induce the mitochondrial generation of reactive oxygen species (ROS) that can serve a signaling role to protect against large-scale pathologic ROS generation in subsequent IR injury.[3]

A relatively unstudied hormetic signaling pathway in mammals, and particularly in the heart, is the mitochondrial unfolded protein response (UPR^{mt}), a stress response pathway triggered by proteotoxic stress in the organelle.[5] Similar to other compartment-specific UPRs (e.g. the endoplasmic reticulum UPR), UPR^{mt} signaling upregulates chaperones to promote protein folding and proteases to digest misfolded proteins.[6] Furthermore, the UPR^{mt} also upregulates glycolysis[7] and down-regulates expression of mitochondrial respiratory chain subunits[8] to reduce the burden on mitochondrial translation and folding machinery.

While the UPR^{mt} was first discovered in mammals,[5] most insights into this pathway have emerged from work in *C. elegans*, including the discovery of its central mediating transcription factor ATFS-1.[9] The ATFS-1 protein contains both nuclear and mitochondrial targeting sequences,[7,9] and under normal conditions, ATFS-1 is transported into mitochondria, where it is degraded.[7] However, under conditions of mitochondrial proteotoxic stress, mitochondrial import of ATFS-1 is impaired, resulting in its translocation to the nucleus, where it acts as a transcriptional modifier.[7,8]

In mammals, the activating transcription factor ATF5 has been described as having roles in cancer,[10] cell cycle regulation,[11] apoptosis,[12] and neural differentiation.[13] It also plays a role in stress signaling[14] and was proposed to be the mammalian ortholog of ATFS-1 due to functional and structural similarities.[9] Notably, ATF5 expression rescues UPR^{mt} signaling in *C. elegans* lacking ATFS-1, demonstrating its orthologous role.[15]

Cardiac IR injury is known to induce bioenergetic dysfunction, protein misfolding, and oxidative stress.[16] Since the targets of UPR^{mt} signaling include glycolysis, chaperones, and antioxidants,[6] we hypothesized that UPR^{mt} induction may be cardioprotective against IR, as it protects against anoxia-reoxygenation in *C. elegans*.[17] Although there have been limited *in-vivo* mammalian studies of the UPR^{mt}, tetracycline antibiotics such as doxycycline and mitochondrial inhibitors such as oligomycin are known to

induce the response in mammalian cells,[15,18] likely via their disruptive effects on mitochondrial protein synthesis. Herein, we investigated the ability of these molecules to elicit cardioprotection in an ATF5-dependent manner using an *Atf5*$^{-/-}$ mouse.[19] Furthermore, we used RNA-Seq gene expression analysis to investigate both ATF5-dependent and -independent signaling.

METHODS

See Supplement for Detailed Methods.

Animals and reagents

Animal care and protocols were approved by the University of Rochester Committee on Animal Resources, in compliance with federal guidelines. *Atf5*$^{-/-}$ mice[19] of both sexes on a C57BL/6J background were used at 12-18 week-old (n=110). All reagents were from Sigma (St. Louis, MO) unless otherwise stated.

IR injury & drug treatments

An *ex-vivo* retrograde (Langendorff) perfused heart model was used, with glucose, fat, pyruvate and lactate provided as metabolic substrates.[20] Left ventricular developed pressure (LVDP) was recorded. For IR injury, 25 min global no-flow ischemia was followed by 60 min reperfusion. Infarcts were quantified by tetrazolium chloride (TTC) staining. For *in-vivo* testing, oligomycin (500 µg/kg) or doxycycline (70 mg/kg) were administered via intraperitoneal injection 6 h prior to experiments. For *ex-vivo* testing, doxycycline was dissolved at 20 µmol/L in perfusion buffer.

RT-qPCR and RNA-Seq

RNA was prepared from saline-flushed cardiac tissue and RT-qPCR was performed using standard protocols. Primers and their amplicon sizes are shown in Table S1. For RNA-Seq, library preparation and sequencing were performed using standard protocols for the Illumina HiSeq 2500 v4 platform (San Diego, CA). Data are available in SRA; accession number SUB4128289.

RESULTS

Baseline vulnerability to IR injury in Atf5$^{-/-}$ *mouse hearts*

Atf5$^{+/+}$ (WT), *Atf5*$^{+/-}$, and *Atf5*$^{-/-}$ mice were used (Fig. S1A/B). Consistent with previous reports,[19,21,22] a major confounder in these studies was the high neonatal mortality rate and reduced adult body weight of *Atf5*$^{-/-}$ mice relative to WT (Fig. S1C/D). Thus, both sexes were used and controls were

age- and sex-matched but not always littermates. Perfused *ex-vivo* hearts were subjected to global IR injury, with cardiac function tracked as rate × pressure product (RPP), and post-IR infarct assayed by TTC staining. *Atf5* genotype did not impact functional recovery or infarct size in response to IR (Fig. 1A/B, n=12 animals per genotype). A sub-analysis with all genotypes aggregated showed no differences between males and females (Fig. 1C/D, n=21 female, 15 male), and further stratification by both sex and genotype did not reveal any sex-specific genotype-dependent effects (not shown). Analysis of baseline cardiac function stratified by both sex and genotype showed modest sex effects but no genotype effects (Fig. S2A-E).

Activation of UPR^{mt} by oligomycin

Oligomycin, an inhibitor of mitochondrial ATP synthase, has been shown to induce the UPR^{mt} in mammalian cells.[15] *In-vivo* activation of the UPR^{mt} in WT mice was tested by intraperitoneal injection of 500 µg/kg oligomycin or vehicle. Cardiac mRNA was extracted 6 h later and RT-qPCR performed to obtain relative expression (normalized to reference genes) in oligomycin vs. vehicle treated hearts (ΔΔCq). Expression of *Hspa5*, which encodes the chaperone BiP in the endoplasmic reticulum UPR, was not affected by oligomycin (Fig. 2A). However, two UPR^{mt}-activated mitochondrial chaperones – *Hspd1* (HSP60) and *Hspa9* (mtHSP70) – were significantly induced by oligomycin, providing evidence of a UPR^{mt}-specific response.

ATF5-dependent cardioprotection by oligomycin

Having established cardiac UPR^{mt} induction by oligomycin, we next asked if this elicited cardioprotection. Hearts from WT mice treated with oligomycin exhibited significant improvement in post-IR functional recovery (oligomycin 52.4±4.4% vs. vehicle 23.7±4.5%, p=0.0018) and reduced infarct size (oligomycin 39.0±2.9% vs. vehicle 63.7±8.6%, p=0.042) (Fig. 2B/C). In contrast, the protective effect of oligomycin was lost in hearts from *Atf5*$^{-/-}$ mice (functional recovery: oligomycin 29.5±4.0% vs. vehicle 19.4±5.4%, p=0.18; infarct: oligomycin 57.8±6.3% vs. vehicle 57.5±7.6%, p=0.98) (Fig. 2D/E). Baseline cardiac function was similar across groups (Fig. S2F-J).

Cardioprotection by doxycycline requires ATF5

Doxycycline elicits cardioprotection against IR injury, via a mechanism thought to involve matrix metalloproteinases (MMPs).[23–26] However, since doxycycline also disrupts mitochondrial ribosomal function[27–29] and activates the UPR^{mt},[18,30] we hypothesized its cardioprotective effect may depend on UPR^{mt} and ATF5. Similar to oligomycin, doxycycline was administered *in-vivo* (70 mg/kg), and 6 h later,

IR injury was assessed in the *ex-vivo* perfused heart model. Hearts from WT mice treated with doxycycline exhibited significant improvement in post-IR functional recovery (doxycycline 66.6±5.0% vs. vehicle 25.8±5.8%, p=0.0064) and reduction in infarct size (doxycycline 36.4±1.9% vs. vehicle 65.9±6.7%, p=0.040) (Fig. 3A/B). This protective effect of doxycycline was lost in hearts from *Atf5*$^{-/-}$ mice (functional recovery: doxycycline 31.6±3.5% vs. vehicle 27.6±8.6%, p=0.70; infarct: doxycycline 48.83±0.9% vs. vehicle 58.9±7.4%, p=0.31) (Fig. 3C/D).

The ability of doxycycline to induce acute cardioprotection[30] was also tested, by delivery to perfused hearts immediately prior to ischemia and during reperfusion. No significant protective effect was found (functional recovery: doxycycline 40.2±19.0% vs. vehicle 33.8±9.0%, p=0.78; infarct size: doxycycline 65.6±6.3% vs. vehicle 49.3±4.9%, p=0.11) (Fig. 3E/F). However, doxycycline exhibited low solubility in Krebs-Henseleit perfusion buffer, and the filtration necessary for successful murine cardiac perfusion[31] may have lessened doxycycline bioavailability.

Targets of UPR^{mt} activation

To explore the cardioprotective signaling mechanism(s) of UPR^{mt} induction, we performed global expression analysis using RNA-Seq on cardiac mRNA from WT or *Atf5*$^{-/-}$ mice treated with vehicle, oligomycin, or doxycycline. Since both drugs are UPR^{mt} inducers, they were grouped for analysis, which focused on identifying genes induced by drug treatment in WT hearts (adjusted p<0.2), with reduced or no induction in *Atf5*$^{-/-}$ hearts (adjusted p<0.4). This yielded 69 genes, indicating an ATF5-dependent response in this model. However, several previously reported UPR^{mt}-induced genes such as *Hspd1* and *Hspa9* failed to emerge from RNA-Seq analysis, possibly due to the small magnitude of changes induced by drug treatment (as also seen in RT-qPCR, Fig. 2A). An unbiased pathway analysis did not yield useful insights, although it is possible that the timing of UPR^{mt} gene induction may be offset from that of cardioprotection (6 h).

DISCUSSION

The key findings of this study are that UPR^{mt}-induction is cardioprotective in IR injury and that ATF5 is necessary for UPR^{mt} activation and for cardioprotection by either oligomycin or doxycyline. Although a role for ATF5 in the UPR^{mt} has been proposed based on work in *C. elegans* and mammalian cell culture,[15] this is the first *in-vivo* demonstration that ATF5 is a necessary component of the mammalian UPR^{mt}. In addition, this is the first study to demonstrate a role for the UPR^{mt} in cardioprotection by tetracycline antibiotics.[23–26]

Herein, we activated the UPR^{mt} *in-vivo* with either oligomycin or doxycycline. Oligomycin inhibits ATP synthase, with UPR^{mt} induction likely occurring via impairment of mitochondrial protein synthesis[32] or import[33] due to restricted ATP availability. Consistent with a low rank of these processes in the hierarchy of ATP consumption in cells,[34] the dose of oligomycin used herein elicited no mortality and did not affect baseline cardiac function (Fig. S2F-J). Oligomycin has been previously used to activate the UPR^{mt} in mammalian cells,[15] and we confirmed the induction of UPR^{mt}-linked chaperones by oligomycin *in-vivo* (Fig. 2A). Doxycycline is a tetracycline antibiotic that disrupts both bacterial and mitochondrial ribosomal function.[27–29] It has been demonstrated to induce the UPR^{mt} in mammalian cells and mice *in-vivo,* by generating an imbalance between the nuclear and mitochondrial DNA-encoded subunits of the respiratory chain.[18]

A single bolus injection of either oligomycin or doxycycline 6 h prior to IR elicited significant cardioprotection in WT mice (Figs. 2B/C, 3A/B). As such, mitochondrial proteotoxic stress is a hormetic mechanism of cardioprotection, akin to other cardioprotective paradigms such as delayed IPC, wherein the primary stress induces a gene signature that protects against subsequent stresses of a similar nature.[35] In the case of UPR^{mt} , this gene signature includes chaperones, proteases, and a metabolic shift from mitochondria to glycolysis,[6–8] all of which would be predicted to confer benefits during IR injury. Cardioprotection conferred by the UPR^{mt} inducers oligomycin or doxycycline was absent in *Atf5*$^{-/-}$ mice (Figs. 2B-E, 3A-D), and importantly there was no change in baseline cardiac function or IR injury response in these mice (Fig. S2A-E). Thus, the loss of protection was not due to increased sensitivity to injury in *Atf5*$^{-/-}$.

A potential confounder on the use of doxycycline for UPR^{mt} activation[18] is that this drug is reported to confer cardioprotection by inhibiting MMPs.[23–26] Specifically, sub-microbicidal doses of doxycycline after ischemia were found to induce MMP-mediated cardioprotection,[25] and these findings have led to a human clinical trial (NCT00469261).[26] Proposed mechanisms for MMP inhibition by doxycycline include divalent metal chelation[36] and redox activity.[37] However, our data show that doxycycline-induced cardioprotection requires ATF5 (Fig. 3). To the best of our knowledge there is no connection between ATF5 and MMPs, and no MMP genes emerged from our RNA-Seq analysis (Fig. 4B). Furthermore, the structurally unrelated UPR^{mt}-inducer oligomycin also yielded ATF5-dependent cardioprotection (Fig. 2). Together these results suggest that doxycycline prophylaxis conveys cardioprotection independent of MMPs, via the UPR^{mt} and ATF5.

Herein, a 6 h delay between drug administration and IR injury experiments was chosen to permit induction of the UPR^{mt} gene program. However, a recent report claimed to observe acute cardioprotection by tetracycline antibiotics (delivery beginning 30 min prior to ischemia) via the UPR^{mt}.[30] We were unable

to reproduce this phenomenon at a similar dose (Fig. 3E/F), though this may have been due to solubility limitations for doxycycline, and such a time-frame is too short for induction of a stress response such as the UPR^{mt}, requiring translation and transcription.[38] Thus, acute tetracycline cardioprotection is likely attributable to MMP-inhibition, as previously shown.[23]

The current results are the first to cement a role for ATF5 as the central mediator of the mammalian UPR^{mt} at the whole organism level. While *C. elegans* UPR^{mt} studies have been extensive, this signaling pathway remains relatively unstudied in mammals, and there are important features of the UPR^{mt} at the whole organ or animal level that will require further mammalian studies. First, the nematode UPR^{mt} involves several proteins with potential but unproven orthologs in mammals, including DVE-1 (SATB2) and UBL-5 (UBL5),[39] which may play a role in the mammalian UPR^{mt} and in UPR^{mt}-mediated cardioprotection. Second, a key feature of the UPR^{mt} uncovered in *C. elegans* is its potential for cell non-autonomous activity, where UPR^{mt} induction in one cell or organ can trigger protection against stress in another.[40] In this regard, we observed only mild induction of the UPR^{mt} in hearts of oligomycin-treated animals (Fig. 2), but we utilized a whole body *Atf5*$^{-/-}$ mouse. As such, we cannot exclude the possibility that UPR^{mt} induction *in-vivo* may have occurred primarily in another tissue, with a blood-borne or other signal resulting in remote cardioprotection via ATF5-independent mechanisms. Further studies utilizing tissue-specific *Atf5*$^{-/-}$ animals may prove insightful.

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Figure 1: Role of ATF5 in Vulnerability to IR Injury. (A) Post-reperfusion functional recovery by genotype as measured by baseline-normalized rate × pressure product (RPP). Animals were of both sexes, n=12 for each genotype (WT: black, *Atf5*$^{+/-}$: green, *Atf5*$^{-/-}$: blue). *p<0.05 vs. *Atf5*$^{+/+}$. **(B)** Infarct sizes of the hearts in (A), as measured by TTC staining (bottom), with representative stained slices (top) and segmented images (middle: red healthy tissue, white infarct tissue). **(C)** Post-reperfusion functional recovery by sex. Hearts are the same as shown in (A) with mixed genotypes, n=21 for females (black) and n=15 for males (gray). **(D)** Infarct size of the hearts in (C). Data presented as means±SEM.

Figure 2: Effect of UPR^{mt} Induction by Oligomycin on IR Injury. (A) RT-qPCR was used to monitor relative expression (-ΔΔCq, vs. *Gapdh* and *Hprt1* reference genes) of *Hspa5* (BiP), *Hspd1* (HSP60), and *Hspa9* (mtHSP70) in hearts from oligomycin- vs. vehicle-treated hearts. Horizontal lines represent means. *p<0.05 compared to 0, n=6 for each group (female: dots, male: triangles). **(B)** Post-reperfusion functional recovery as measured by baseline-normalized rate × pressure product (RPP) in WT mice given oligomycin (orange) or vehicle (black). n=5 for each group. *p<0.05 compared to matching time point. **(C)** Infarct sizes of the hearts in (B), as measured by TTC staining (bottom), with representative stained slices (top) and segmented images (middle: red healthy tissue, white infarct tissue). *p<0.05 compared to vehicle control. **(D)** Post-reperfusion functional recovery of *Atf5*$^{-/-}$ hearts given oligomycin (pink) or vehicle (blue). n=5 for each group. *p<0.05 compared to matching timepoint. **(E)** Infarct sizes of the hearts in (D). Data presented as means±SEM.

Figure 3: Effect of UPR^{mt} Induction by Doxycycline on IR Injury. (A) Post-reperfusion functional recovery as measured by baseline-normalized rate × pressure product (RPP) in WT mice given doxycycline (red) or vehicle (black). n=3 for each group. *p<0.05 compared to matching time point. **(B)** Infarct sizes of the hearts in (A), as measured by TTC staining (bottom), with representative stained slices (top) and segmented images (middle: red healthy tissue, white infarct tissue). *p<0.05 compared to vehicle control. **(C)** Post-reperfusion functional recovery of *Atf5*$^{-/-}$ hearts given doxycycline (purple) or vehicle (blue). n=3 for each group. **(D)** Infarct sizes of the hearts in (C). **(E)** Post-reperfusion functional recovery of WT hearts given doxycycline during perfusion (red) or vehicle (blue). n=3 for each group. **(F)** Infarct sizes of the hearts in (E). Data presented as means±SEM.

Figure 4: Gene Analysis by RNAseq. (A) Schematic showing genotypes and treatment groups for RNA-Seq analysis on cardiac RNA extracts. **(B)** Heat map showing relative expression levels for the 69 genes

identified as being significantly up-regulated by drug treatment (oligomycin or doxycycline) in WT mice, as well as exhibiting a significantly blunted response to drug treatment in *Atf5*$^{-/-}$. Gene names listed in Table S2. Full data set available at SRA accession number SUB4128289.