Mitochondrial dysfunction induced by heat stress in cultured rat CNS neurons

Michael G. White†, Osama Saleh*, Doris Nonner*, Ellen F. Barrett†, Carlos T. Moraes#†
and John N. Barrett*†1

Departments of Physiology and Biophysics* and Neurology# and the Neuroscience Program†, Miller School of Medicine, University of Miami, Miami, FL 33136, USA

1Corresponding Author: John N. Barrett, Department of Physiology and Biophysics, University of Miami Miller School of Medicine, P.O. Box 016430, Miami FL 33101
Phone: (305) 243-6357; Fax: (305) 243-5931; Email: jbarrett@med.miami.edu

Abbreviations: BAPTA: 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; BAX:
Bcl-2 associated protein X; Bcl-2: B cell lymphoma/leukemia 2; Bcl-xL: long form of Bcl-
X, a homolog of Bcl-2; BH3: Bcl-2 homology 3; calpain inhibitor III: carbobenzoxy-valyl-
phenylalanal; CCCP: carbonyl cyanide m-chloro phenyl hydrazone; CsA: cyclosporin A;
DMSO: dimethylsulfoxide; ETC: electron transport chain; FCCP: carbonyl cyanide-p-
trifluoromethoxyphenylydrazone; G3P: glycerol 3-phosphate; GFP: green fluorescent
protein; γGC: γ-glutamyl cysteine; MnTBAP: Mn(III)tetrakis(4-benzoic acid)porphyrin
chloride; mPTP: mitochondrial permeability transition pore; NAD+: nicotinamide adenine
dinucleotide; PARP: poly ADP-ribose polymerase; PBN: N-tert-butyl-α-phenylnitrone;
PD150606: 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid; PI: propidium iodide;
qVD-OPH: quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone; ROS:

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reactive oxygen species; TMPD: N,N,N',N'-tetramethyl-1,4-phenylenediamine; TMRM: tetramethylrhodamine methylester; zVAD-fmk (Z-Val-Ala-Asp(OMe)-fluoromethylketone); $\Delta \Psi_m$: membrane potential across the mitochondrial inner membrane.

Running title: Heat stress-induced mitochondrial dysfunction
ABSTRACT

Previous work demonstrated that hyperthermia (43 °C for 2 hr) results in delayed, apoptotic-like death in striatal neuronal cultures. We investigated early changes in mitochondrial function induced by this heat stress. Partial depolarization of the mitochondrial membrane potential (ΔΨ_m) began about 1 h after the onset of hyperthermia, and increased as the stress continued. When the heat stress ended, there was a partial recovery of ΔΨ_m, followed hours later by a progressive, irreversible depolarization of ΔΨ_m. During the heat stress O2 consumption initially increased, but after 20-30 min began a progressive, irreversible decline to about half the initial rate by the end of the stress. The percentage of oligomycin-insensitive respiration increased during the heat stress, suggesting an increased mitochondrial leak conductance. Analysis using inhibitors and substrates for specific respiratory chain complexes indicated hyperthermia-induced dysfunction at or upstream of complex I. ATP levels remained near normal for ~4 h after the heat stress. Mitochondrial movement along neurites was markedly slowed during and just after the heat stress. The early, persisting mitochondrial dysfunction described here likely contributes to the later (>10 h) caspase activation and neuronal death produced by this heat stress. Consistent with this idea, proton carrier-induced ΔΨ_m depolarizations comparable in duration to those produced by the heat stress also reduced neuronal viability. Post-stress ΔΨ_m depolarization and/or delayed neuronal death were modestly reduced/postponed by nicotinamide adenine dinucleotide, a calpain inhibitor, and increased expression of Bcl-xL.

Keywords: heat stress, hyperthermia, mitochondria, respiration, ATP, apoptosis,
INTRODUCTION

Hyperthermia can cause brain damage and exacerbates the damage produced by stroke, traumatic brain injury, and certain drugs (Simon, 1993; Jones et al., 1994; Castillo et al., 1998; Brown and Kiyatkin, 2004; reviewed by White et al., 2007). Following a 2 h, 43 °C heat stress, cultured neurons exhibit a delayed (>10 h) death with apoptotic characteristics including shrunken and fragmented nuclei, caspase-3 activation and cytochrome c release (e.g., White et al., 2003, 2007). These findings may also have relevance in vivo, since some hyperthermia-induced neuronal death occurs after a delay of more than 10 h (e.g. Uney et al., 1993; Vogel et al., 1997), and cytochrome c release has been reported in the brain of heat-stressed murine embryos (Mirkes and Little, 2000).

The present study investigated earlier consequences of heat stress, focusing on changes in mitochondrial function, since studies in non-neuronal cells and isolated mitochondria demonstrate hyperthermia-induced disruption of mitochondrial energy production. For example, in isolated mitochondria hyperthermia increases the permeability of the mitochondrial inner membrane and impairs oxidative phosphorylation (Willis et al., 2000; Qian et al., 2004). Hyperthermia above 42°C induces mitochondrial uncoupling in rat cardiomyocytes (Qian et al., 2004) and in mitochondria isolated from heart cells (Zukiene et al., 2010). Other signs of damage/dysfunction in mitochondria isolated from rat hearts following in vivo hyperthermia (rectal temperature reaching 42 °C) include decreased ATP synthesis, a decreased respiratory control ratio, a greater tendency to open the mitochondrial permeability transition pore (mPTP) when exposed
to moderate Ca\textsuperscript{2+} loads (Qian et al., 2004), mitochondrial swelling, and a reduced number of cristae (Song et al., 2000). Mouse embryo fibroblasts stressed at 43 °C show BAX oligomerization and release of apoptotic factors from mitochondria that is promoted by BH3-only family proteins and reduced by Bcl-2 (Pagliari et al., 2005).

Consistent with these studies in non-neuronal tissues, we present evidence that hyperthermia produces a severe disruption of mitochondrial function in cultured rat central neurons, including a transiently reversible depolarization of $\Delta \Psi_m$ and an irreversible reduction in $O_2$ consumption. These changes in mitochondrial function occurred before, and thus may contribute to, the caspase-3 activation and neuronal death produced by this heat stress. To probe underlying mechanisms, we also tested whether inhibitors of various stress-activated pathways could reduce the heat-induced $\Delta \Psi_m$ depolarization. Caspase inhibitors had no significant effect, but a calpain inhibitor and addition of NAD$^+$ offered some protection.

MATERIALS and METHODS

Neuronal cultures

Striatal (and in some cases septal) tissue was dissected from embryonic day 15 (E15) rats, dissociated, and plated on poly-L-lysine-coated 72-well Terasaki plates (Nalge Nunc, Rochester, NY, USA) or glass coverslips at a density of ~1000–1800 cells/mm\textsuperscript{2}. Cultures were grown in a basic nutrient medium (N5, Kawamoto and Barrett, 1986) or in Neurobasal medium (Invitrogen, Carlsbad, CA, USA), supplemented with L-alanyl-L-glutamine (0.5 mM, GlutaMAX-1, Invitrogen) and a 55 kD serum fraction that supports prolonged neuronal survival with minimal proliferation of non-neuronal cells.
Cultures were maintained for 5-14 days in 5.5% CO₂/94.5% air at 36-37°C before use. Fresh medium was added every 5-7 days. Some respiration measurements used neurosphere cultures prepared from striatum or cerebral cortex, dissected and pooled from one E15 rat litter. Dissociated cells were suspended in 10 ml medium and plated into untreated 100 mm Optilux tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) for 5-14 days, during which time neurospheres formed (similar to those described in White et al., 1999).

**Heat stress protocol**

Cultures were stressed for 2 h at 43 °C on aluminum plates within a tissue culture incubator to ensure temperature uniformity. This is the minimum temperature and minimum duration that reliably produced delayed neuronal death (White et al., 2003). After the stress, cultures were transferred back to the standard 36-37 °C incubator. Cultures imaged during the heat stress were plated in glass bottom dishes (MatTek Corp., Ashland, MA) and heated in a small incubator on the microscope stage (5% CO₂ in air). The oil-immersion microscope objective was also heated to 43 °C using hot air from a hairdryer powered via a variable AC transformer (ISE, Inc., Cleveland, OH). The temperature close to the cells was monitored using a very small thermistor. The room was heated to approximately 30 °C to reduce temperature gradients and so allow better temperature control.

**Measurements of mitochondrial membrane potential (ΔΨₘ)**

Changes in ΔΨₘ produced by the heat stress were measured using tetramethyl rhodamine methyl ester (TMRM), a membrane-permeable, positively-charged dye that accumulates within the mitochondrial matrix in a Nernstian manner dependent on the
mitochondrial $\Delta \Psi_m$. Some of these experiments used high [K$^+$] bath solutions to completely depolarize the plasma membrane, thereby preventing changes in cytosolic TMRM concentrations caused by changes in the plasma membrane potential (in mM, 145 K gluconate, 10 NaCl, 1 MgCl$_2$, 5.5 glucose, and 1 pyruvate). In some experiments 145 mM KCl was used in place of K gluconate and 80 mM sucrose was added to prevent the cell swelling otherwise seen in this KCl solution.

For assays of $\Delta \Psi_m$ using a platereader (Wallac 1420 Victor, PerkinElmer, Boston, MA, USA) cells were exposed to 1 µM TMRM for 20 min and then stressed in the continued presence of TMRM (Figs. 1A,1B, 7A). At different post-stress intervals cultures were washed twice rapidly with the high [K$^+$] medium (lacking TMRM), and then solubilized with 50% DMSO in distilled water to release TMRM from the cells. This solubilization avoids errors due to self-quenching effects of high [TMRM] within mitochondria. Fluorescence was then assayed (535 nm excitation, >590 nm emission), with $\Delta \Psi_m$ depolarization detected as a decrease in TMRM fluorescence. Some culture wells were treated with a proton carrier, carbonyl cyanide m-chloro phenyl hydrazone (CCCP, 10 µM), to thoroughly depolarize $\Delta \Psi_m$, permitting measurement of background fluorescence.

Assays of $\Delta \Psi_m$ by microscopic imaging of single neurons used a lower concentration of TMRM (10 - 30 nM) to avoid self-quenching, so that the TMRM fluorescence would be monotonically related to $\Delta \Psi_m$. TMRM was added 20 min before beginning the imaging, and remained present throughout the experiment, allowing transiently depolarized mitochondria to re-accumulate the dye as they recovered their membrane potential. Images of TMRM fluorescence were collected using a Nikon
TE2000 inverted microscope with an Olympus 60X objective (1.45 NA) (Nikon, Inc. Melville, NY, USA; Olympus, Center Valley, PA, USA). Images were taken at a rate of one per minute with a 500-800 ms exposure time. A Cascade 512B CCD camera (Roper Scientific, Inc, Trenton, NJ, USA) and a shuttered monochromator (Photon Technology International, Inc., Birmingham, NJ, USA) with a slit attenuator enabled use of low excitation light intensities to minimize photodamage to dyes and cells.

Respiration measurements

Respiration was measured both in cultures of free-floating (unattached) neurospheres, and in standard cultures with cells attached to the substrate. Following heat stress applied to neurospheres, cultures were gently centrifuged (5 min at 250 x g) in 10 ml tapered conical tubes. The pellet was resuspended in 400 µl culture medium or (if to be permeabilized) in respiration medium (in mM: 220 D-mannitol, 60 sucrose, 3 KCl, 2 KH₂PO₄, 2 MgCl₂, and 2 HEPES) in a pre-warmed (37 °C) recording chamber with constant stirring. The oxygen pressure (pO₂) in the chamber was monitored continuously using a Clarke-type O₂ electrode, with respiration measured as the rate of O₂ consumption. Additional O₂ was introduced if chamber O₂ fell below 10-50 nmol/ml. Pyruvate (2 mM) and malate (2 mM) were added to ensure that respiration was not limited by substrates. To evaluate the function of individual electron transport chain (ETC) complexes, 2 mM ADP was added (to ensure state 3 respiration) and cells were permeabilized with 16 µM digitonin (e.g., Barrientos, 2002). The rate of O₂ consumption was then measured after each of the following sequential additions: 2 µM rotenone to inhibit complex I; 5 mM succinate and 5-10 mM glycerol-3-phosphate (G3P) to measure complex II- and complex III-driven respiration; 1 µM antimycin A to inhibit complex III;
500 µM TMPD (N,N,N',N'-tetramethyl-1,4-phenylenediamine) and 1 mM ascorbate to measure complex IV-driven respiration; and finally 12 mM KCN or NaCN to block complex IV, yielding a background rate (non-mitochondrial) of O₂ consumption. CCCP (2 µM) or the ATP synthase inhibitor oligomycin (5 ug/ml = 6 µM) was used in experiments examining proton leak. In some experiments, neurospheres were heat-stressed in the chamber itself, with temperature measured by a miniature thermistor.

To determine whether the heat stress induced similar changes in respiration in cells that remained attached to the polylysine substrate, O₂ consumption was measured using a modification of the flow-through technique of Jekabsons and Nicholls (2004). The heat stress was applied to cultures in a thin, O₂-impermeable chamber (10 x 40 x 0.1 mm) perfused with medium whose temperature was controlled by a Peltier device. Chamber temperature was monitored using two miniature thermistors. Fine quartz tubing (inner diameter 0.1 mm) connected the chamber inlet and outlet with the O₂ sensors. These O₂ sensors were located in a constant temperature 37°C water bath “decoupled” from the temperature of the chamber by a 15 cm loop of the quartz tubing within the water bath. The perfusing culture medium was first heated to 44 °C for 5 min to prevent bubble formation due to escape of dissolved gas from the heated culture chamber. This pre-heating helped ensure that the amount of dissolved O₂ and hence the concentration of O₂ passing over the cells in the chamber remained constant during the heat stress. The rate of O₂ consumption was measured as the flow rate (60 µL/min) times the difference in pO₂ values (converted to nM oxygen) measured at the inlet and outlet tubes with flow-through Clarke-type O₂ sensors (Microelectrodes Inc., Bedford, NH, USA). The output of the O₂ sensors was sampled at a rate of 0.5 Hz using a 16 bit...
analog-to-digital converter. Using this system, the rate of O$_2$ consumption at the normal 37 °C remained constant for many hours.

**Measurements of ATP, glutathione and intracellular [Ca$^{2+}$]**

ATP levels were measured using the CellTiter-Glo Luminescent assay (Promega, Madison, WI, USA), according to the manufacturer’s protocol.

Glutathione levels were measured in intact cells using monochlorobimane (50 µM), a cell-permeant, non-fluorescent compound that, via a reaction catalyzed by glutathione S-transferases, forms a fluorescent glutathione-monochlorobimane adduct that can be measured fluorometrically (excitation 355 nm/emission 460 nm, e.g. Chatterjee et al., 1999). Initial fluorescence was subtracted from fluorescence read 30 min later using a platereader.

Cytosolic free calcium ([Ca$^{2+}$]$_i$), was measured using fura-2 loaded as the acetoxymethyl ester (30 min incubation with 5 µM fura2-AM). Pairs of images using different excitation wavelengths (340 and 380 nM, provided using a TILL Photonics monochromator [Victor, NY, USA]; emissions monitored at 515 ± 20 nm) were used to calculate the 340/380 emission ratio, which increases with [Ca$^{2+}$]$_i$. [Ca$^{2+}$]$_i$ changes were calculated using the equation $[\text{Ca}^{2+}] = K_d(R - R_{\text{min}})/(R_{\text{max}} - R)$ (Gryniewicz et al., 1985) where $R_{\text{min}}$ is the 340/380 ratio when the cells are in zero Ca$^{2+}$ medium (no added Ca$^{2+}$ with 2 mM BAPTA) and $R_{\text{max}}$ is the maximal ratio measured in normal medium after addition of 100 µM ionomycin. The ionomycin solution was prepared fresh from a DMSO stock since it becomes inactive after just a few hours in water.

**Assays for cell survival and mitochondrial reducing ability**

Cell survival was measured with a live/dead imaging assay described in White et
al. (2003), using propidium iodide (PI, 15 µM) to label the nuclei of dead cells and Hoechst (bisbenzimide) 33342 (16 µM) to label the nuclei of all cells.

Cellular/mitochondrial reducing activity, an assay of cell viability, was measured using Alamar blue (Biosource International, Camarillo, CA, USA). The fluorescence of this water-soluble, membrane-permeable indicator increases when resazurin is reduced to resorufin by mitochondrial respiratory chain activity (as well as other cellular reductase systems). Alamar blue was added at a 1:20 dilution of the commercial stock solution, and its fluorescence was measured using a platereader (excitation 535 nm/emission 590 nm) before and after 2 h incubation at 37 °C.

**Measurement of mitochondrial movement**

Movement of Mitotracker green-labelled mitochondria (20 min incubation in 5 µM) along neurites was measured using a spinning disk confocal (Yokogawa CSU10, Solamere Technology Group, Salt Lake City, USA) equipped with a Cascade 512B CCD camera (Roper Scientific; excitation 488 nm, emission 535 nm). In most of these experiments the heat stress was applied in an incubator and the culture dish was then transferred to the microscope stage for imaging at 37 °C. Images were collected at 0.5 Hz using a 60x, 1.45 numerical aperture Olympus objective; movement was detected by subtracting successive images. The average velocity of each mitochondrion was calculated over a 20 s interval.

**Transfection**

Neuronal cultures (10-12 days in vitro) were transfected as described in Panickar et al. (2005), using plasmids containing cDNA for green fluorescent protein (GFP, pIRES-2EGFP, Clontech, Palo Alto, CA, USA) and Bcl-xL (gift from Dr. Larry Boise,
Boise et al., 1995). Stresses were applied 48 h following transfection.

Reagents

The pan-caspase inhibitor quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone (qVD-OPH) was from Enzyme Systems/ICN (Livermore, CA, USA). The calpain inhibitor PD150606 was from Calbiochem/EMD Biosciences (La Jolla, CA, USA). Fluorescent indicators, monochlorobimane, and the anti-oxidant N-tert-butyl-α-phenylnitrone (PBN) were from Molecular Probes/Invitrogen (Eugene, OR, USA). Other chemicals were from Sigma (St. Louis, MO, USA).

Statistics

Averages are expressed as mean ± SEM. To compare multiple groups to a common control, data were analyzed using a one-way analysis of variance followed by Dunnett’s test. The Student-Newman-Keuls test was used to compare all groups. Prism and InStat software (GraphPad Software, San Diego, CA, USA) were used.

RESULTS

Heat stress (2 hr, 43 °C) induces two phases of mitochondrial depolarization

Fig. 1A shows that in striatal cultures the heat stress partially depolarized $\Delta\Psi_m$, measured using TMRM fluorescence. Following the return to 37 °C, $\Delta\Psi_m$ partially repolarized, but subsequently began a second, slow phase of depolarization that was irreversible. Fig. 2A shows TMRM fluorescence changes tracked by imaging at higher frequency in a representative neuron, demonstrating that the initial $\Delta\Psi_m$ depolarization developed mainly during the second hour of the heat stress, and occurred in both soma and proximal processes. Because the measurements in Figs. 1A and 2A were made in
high [K+] medium (145 mM K+ with low Ca^{2+}, see Materials and Methods), one can be certain that the stress-induced loss of TMRM fluorescence was due to depolarization of ΔΨ_m rather than to a reduction in cytosolic [TMRM] resulting from depolarization of the plasma membrane potential.

The pattern of stress-induced changes in TMRM fluorescence was similar in experiments performed in normal culture medium, as shown by the platereader assays in Fig. 1B and the single neuron measurements in Fig. 2B. ΔΨ_m depolarization during the heat stress, and a transient ΔΨ_m repolarization following the stress, were consistent findings, though the magnitude of the depolarization and the time course and magnitude of the post-stress repolarization were variable. Note that one of the neurons in Fig. 2B exhibited a rapid decrease in TMRM fluorescence ~2 h after stress cessation, and also disappeared from the micrograph, suggesting total ΔΨ_m depolarization, possibly due to opening of the mitochondrial permeability transition pore (mPTP, see later section).

Thus the decline in the TMRM signal seen at late times following the heat stress in averaged records like those in Fig. 1A was probably due at least partially to sudden loss of TMRM from the mitochondria of some cells. Fig. 2C shows that TMRM fluorescence remained stable for many hours in non-stressed cultures.

**Heat stress transiently increases, then irreversibly decreases, mitochondrial respiration**

Fig. 3A plots changes in the rate of O_2 consumption of striatal neurosphere cultures during the heat stress. During the first hour the respiration rate was increased, reaching peak levels ~30% above pre-stress (control) levels by ~30 min. This initial hyperthermia-induced increase in O_2 consumption has also been noted in other cells.
(e.g. cardiac myocytes, Sammut et al., 2001; Sammut and Harrison, 2003). Following this initial increase, O₂ consumption fell, dropping to ~50% of control by the end of the 2 h stress. Fig. 3B shows that a similar pattern of heat stress-induced changes in O₂ consumption occurred in cells that remained attached to substrate, measured using the flow-through technique described in Materials and Methods. The decrease in respiration at the end of the heat stress persisted for many hours following return to 37 °C (Fig. 3C).

Hyperthermia reduces pyruvate/malate-dependent respiration

This persisting decrease in O₂ consumption suggests damage to the mitochondrial respiratory chain. The function of specific respiratory chain complexes was investigated by measuring O₂ consumption in neurosphere cultures following sequential addition of substrates and inhibitors specific to particular complexes (see Materials and Methods). Fig. 4 plots measurements made in non-stressed (left) and stressed (right) sister cultures (15-30 min post-stress). The only significant difference occurred in the pyruvate/malate solution, where stressed neurospheres consumed O₂ at about half the rate measured in non-stressed neurospheres (52.1 ± 4.9% of control). The nearly normal respiration measured in the presence of succinate/glycerol 3-phosphate and in TMPD/ascorbate indicates that the function of complexes II, III, and IV was not significantly altered at this early post-stress time. Thus the main hyperthermia-induced dysfunction of the respiratory chain probably occurred at (or upstream of) complex I.

Hyperthermia reduces mitochondrial movement and produces a delayed decrease in ATP
Fig. 5A shows that movement of Mitotracker green-labeled mitochondria along neurites was markedly slowed during the first hour following the heat stress (p<0.001). Partial recovery of movement occurred ~3 h after the end of the heat stress (not shown). ΔΨₘ depolarization produced by a proton carrier also causes cessation of mitochondrial movement (e.g. Bantseev and Sivak, 2005).

Fig. 5B shows that total cellular ATP levels remained at or above 75% of non-stressed control levels for at least 4 h following the stress. Consistent with this temporary maintenance of ATP levels, some cells exhibited continued ruffling movements of lamellipodia membranes (not shown) even when mitochondrial movement was greatly decreased. These results suggest that the rapid reduction in mitochondrial movement following the heat stress was not caused by generalized depletion of ATP, though ATP depletion in localized microdomains cannot be ruled out. Perhaps these young neurons have sufficient glycolytic potential to maintain ATP levels even when mitochondria are partially depolarized.

Proton carrier-induced ΔΨₘ depolarization reduces cell viability

To test whether the ΔΨₘ depolarization measured here (Figs. 1, 2) is relevant to the delayed cell death measured after the hyperthermia stress (White et al., 2003), we tested whether ΔΨₘ depolarization produced by a different technique would also reduce cell viability. Fig. 6 shows results of an experiment in which sister cultures were exposed to the proton carrier CCCP (20 µM) for 5-60 min, and then assayed for Alamar blue reducing activity 2-4 days later. When CCCP was added to normal culture medium (open bars), exposures longer than 20 min reduced viability assayed 4 days later. The 60 min CCCP exposure would be expected to produce a ΔΨₘ depolarization
similar in duration to that produced by the heat stress, since the heat stress-induced
\[ \Delta \Psi_m \] depolarization did not become marked until the 2\textsuperscript{nd} hour of the stress (Fig. 2A).
Since exposure to CCCP can also cause cytoplasmic acidification due to H\(^+\) influx
driven by the plasma membrane potential (Tretter et al., 1998), we also tested the
effects of CCCP exposure in medium containing high [K\(^+\)] to depolarize the plasma
membrane. Hatched bars in Fig. 6 show that a 60 min exposure to CCCP in high [K\(^+\)]
medium reduced cell viability assayed 2 days later. These results indicate that a
CCCP-imposed \[ \Delta \Psi_m \] depolarization of about the same duration as that produced
during the heat stress does indeed reduce cell viability assayed several days later,
consistent with the hypothesis that \[ \Delta \Psi_m \] depolarization (and/or associated downstream
effects) contribute to hyperthermia-induced neuronal death. Similar results were
obtained with FCCP (5 µM, not shown).

**Early hyperthermia-induced \[ \Delta \Psi_m \] depolarization is not mediated by increased ATP
consumption or caspase activation**

In an effort to determine the major mechanisms underlying heat stress-induced
cell damage, we tested whether inhibitors of various mitochondria-associated functions
might alter stress-induced changes in \[ \Delta \Psi_m \].

**Oligomycin blocks ATP synthesis by inhibiting the F\(_{1}\),F\(_{0}\)-ATP synthetase.** The
experiment of Fig. 7A tested oligomycin’s effect on hyperthermia-induced \[ \Delta \Psi_m \] changes.
Except for the point at 1.5 h post-stress, the pattern of \[ \Delta \Psi_m \] changes was similar in the
presence or absence of oligomycin. We also tested the effect of oligomycin on O\(_2\)
consumption, since one possible cause of the initial hyperthermia-induced increase in
O\(_2\) consumption (Fig. 3) is acceleration of ATP consumption. If this were true, then
most of the O$_2$ consumption during the heat stress should be ATP-dependent, and thus sensitive to oligomycin. However, Fig. 7B shows that oligomycin did not block the large, early hyperthermia-induced increase in O$_2$ consumption, and Fig. 7C shows that immediately following the heat stress O$_2$ consumption became more insensitive to oligomycin compared to non-stressed cultures. These findings, together with the finding that ATP levels were not immediately depleted following the heat stress (Fig. 5B), suggest that changes in $\Delta \Psi_m$ and O$_2$ consumption during the heat stress were not linked solely to changes in ATP consumption. Another possible cause of the transient increase in respiration during the heat stress is an increase in leak current across the inner mitochondrial membrane (i.e. uncoupling, see Discussion). By 70-80 min post-stress, the percentage of oligomycin-insensitive respiration was no longer significantly elevated, suggesting that any hyperthermia-induced increase in leak current was at least partially reversible.

A possible cause of increased mitochondrial leak as well as delayed cell death is opening of the mitochondrial permeability transition pore (mPTP, e.g. Qian et al., 2004; Yasuda et al., 2006). Data summarized in Table 1 show that an inhibitor of mPTP opening (cyclosporin A, CsA, 1 $\mu$M), did not reduce $\Delta \Psi_m$ depolarization during or following the heat stress. This finding does not completely rule out a role for mPTP opening, however, because for CNS mitochondria CsA may not be sufficient to block mPTP opening by severe stresses (Brustovetsky and Dubinsky, 2000 ).

Table 1 also shows that the pan-caspase inhibitor qVD-OPH (20 $\mu$M), which delays neuronal death following this heat stress (White et al., 2003), also did not significantly alter the post-stress time course of $\Delta \Psi_m$ depolarization. Thus the early
hyperthermia-induced changes in $\Delta \Psi_m$ studied here appear to be independent of and upstream of caspase activation, consistent with the finding that caspase-3 activation was first detected 10 h following this heat stress (White et al., 2003).

Oxidative damage and Ca$^{2+}$ may contribute to hyperthermia-induced neuronal death

Heat stress can increase the levels of reactive oxygen species (ROS, e.g., Venkataraman et al., 2004), and oxidative damage depletes NAD(H) in stressed mitochondria (Di Lisa and Ziegler, 2001; Du et al., 2003). An attempt to minimize this depletion by pretreatment with 1-10 mM NAD$^+$ beginning 24 h prior to the heat stress significantly increased TMRM fluorescence assayed 6.5 h post-stress, and pretreatment with 5 mM NAD$^+$ produced a modest but significant increase in cell survival (Table 2). NAD$^+$ had no significant effect on TMRM fluorescence or cell survival in non-stressed cultures. Stress-protective effects of applied NAD$^+$ or nicotinamide have also been reported in other neurons (e.g. transected dorsal root ganglion axons, Wang et al., 2005, Conforti et al., 2007; cultures stressed with glutamate and in vivo brain ischemia models, Liu et al., 2009).

Oxidative damage can also deplete glutathione. The heat stress decreased glutathione levels in neuronal cultures to 50 ± 2% of pre-stress control levels 6 h post-stress and 48 ± 2% 18 h post-stress, suggesting a persisting depletion of endogenous ROS defenses (measured by monochlorobimane fluorescence, see Materials and Methods, n=12 cultures each). $\gamma$-glutamylcysteine (500 μM), a precursor for reduced glutathione, increased glutathione concentrations for 48 h in both stressed and non-stressed cultures (not shown), but did not increase survival measured 2 days post-
stress (Table 2, live/dead assay in Materials and Methods). White et al. (2007) presents evidence that other antioxidants (500 μM PBN, 10 μM N-acetyl-cysteine, 100 μM MnTBAP) were likewise ineffective at improving post-stress survival. Thus there is evidence for oxidative damage following the heat stress, but only one of the tested antioxidative treatments (bath-applied NAD\(^+\)) significantly increased post-stress survival and it is likely that NAD\(^+\) acted via a mechanism independent of oxidative stress.

Imaging of cells loaded with the Ca\(^{2+}\) indicator fura-2 revealed that just after the end of the heat stress the average fura-2 340/380 nm ratio was 1.28 ± .06 times that measured in non-stressed sister cultures, corresponding to a 20 to 50 nM increase in intracellular [Ca\(^{2+}\)] (n=50 cells, p<0.001). However, in each of 4 experiments reduction of bath [Ca\(^{2+}\)] to 0-100 μM failed to prevent or reduce the ΔΨ\(_m\) depolarization during the heat stress (see Figs. 1A, 2A for records in low [Ca\(^{2+}\)] medium).

Elevated cytosolic [Ca\(^{2+}\)] can activate calpain proteases, some present in cytosol, others in mitochondria (e.g. Arrington et al., 2006). PD150606 inhibits a mitochondrial calpain (e.g., Mizukoshi et al., 2010) as well as cytosolic calpains. In 4 of 6 experiments PD150606 (100 μM) produced significant reductions in the ΔΨ\(_m\) depolarization recorded 4-6 h post-stress. Table 3 summarizes an experiment in which TMRM fluorescence measured 5 h post-stress was 2-fold higher in cultures treated with PD150606 than in stressed controls. PD150606 also transiently increased post-stress neuronal survival (Table 3). These results suggest that increased cytosolic [Ca\(^{2+}\)] and calpain activation may contribute to, but are not required for, hyperthermia-induced neuronal damage.

Transgenic expression of Bcl-x\(_L\) delays post-stress neuronal degeneration

If mitochondrial damage contributes importantly to the delayed neuronal death
following the heat stress, then enhanced expression of a mitochondria-protective, anti-apoptotic protein might be predicted to reduce post-stress neuronal death. Fig. 8 shows results of an experiment in which striatal cultures were co-transfected to (over)express Bcl-x<sub>L</sub> and Green Fluorescent Protein (GFP), using techniques detailed in Panickar et al. (2005). Fluorescence micrographs (Fig. 8A) taken before and 42 h following the heat stress show increased survival and more intact processes in stressed, GFP-labelled neurons transfected with Bcl-x<sub>L</sub> than in stressed neurons expressing GFP alone. Fig. 8B shows that expression of Bcl-x<sub>L</sub> increased the number of surviving neurons more than 2-fold when assayed 1 day post-stress. However, neurons continued to die; Bcl-x<sub>L</sub> transfection postponed, but did not prevent, hyperthermia-induced neuronal death.

**DISCUSSION**

Results presented here show that striatal neurons exposed to a 2 h, 43 °C heat stress exhibit mitochondrial dysfunction more than 10 h before the onset of caspase-3 activation and neuronal death described in White et al. (2003, 2007). This Discussion considers possible mechanisms underlying these early mitochondrial changes.

**Heat stress damages mitochondrial respiration and increases mitochondrial leak conductance**

During the first hour of the heat stress, O<sub>2</sub> consumption was increased and ΔΨ<sub>m</sub> depolarization was minimal. Heat-induced acceleration of metabolism probably contributes to this early increase in O<sub>2</sub> consumption but Fig. 7B suggests that mitochondrial leak conductance also increases within 10-20 min of hyperthermia onset,
which would also increase $O_2$ consumption. A 1 h heat stress at 43 °C produced no neuronal death (White et al., 2003), suggesting that any heat-induced damage during the first hour was reversible upon return to normal temperature.

During the second hour of the heat stress there was partial $\Delta\Psi_m$ depolarization accompanied by a ~50% decrease in $O_2$ consumption. In non-stressed cells $\Delta\Psi_m$ depolarization is usually accompanied by an increase in mitochondrial respiration to maintain the electrochemical proton gradient across the mitochondrial membrane (e.g., Brand and Nicholls, 2011). Our finding that the hyperthermia-induced partial depolarization of $\Delta\Psi_m$ was associated with decreased $O_2$ consumption suggests damage to mitochondrial respiratory complexes/enzymes, and indeed measurements made immediately following stress termination disclosed dysfunction at and/or upstream of complex I (see Fig. 4). Hyperthermia has been hypothesized to inactivate complex I in a manner that can be reversed by NADH (Grivennikova et al., 2001). Our finding that pretreatment with $NAD^+$ increased post-hyperthermia $\Delta\Psi_m$ and cell survival is consistent with this mechanism, assuming that some of the exogenously applied $NAD^+$ was converted to NADH within the cells. Another possibility is that protection was afforded by $NAD^+$ itself. $NAD^+$ applied nasally (Ying et al., 2007) or intraperitoneally (Zheng et al., 2012) has been reported to reduce brain damage in mice in transient brain ischemia models. One possible mechanism for $NAD^+$-mediated protection is that $NAD^+$ acts as a substrate for sirtuin 3, which can increase complex I activity by deacetylating certain components of complex I (Ahn et al., 2008).

Inhibition of poly-ADP-ribose (PARP) reduces organ damage after thermal injury (Avlan et al. 2005), and exogenous $NAD(H)$ can protect neurons from stresses involving
PARP activation (Alano et al., 2010). However, this mechanism seems unlikely, because a PARP inhibitor did not protect from this heat stress (White et al., 2007).

Other possible mechanisms underlying hyperthermia-induced dysfunction of mitochondrial respiration include depletion/oxidation of cardiolipin (a component of the mitochondrial inner membrane that enhances ETC function, Fry and Green, 1981; Paradies et al., 2004) or damage to enzymes that would affect complex I function (e.g. yeast aconitase is sensitive to hyperthermia, Bender et al., 2011).

The ΔΨ<sub>m</sub> depolarization that developed during the second hour of the heat stress may have been due in part to the decrease in respiration discussed above, but this is unlikely to be the sole mechanism because upon termination of the heat stress the ΔΨ<sub>m</sub> depolarization was partially (albeit transiently) reversible, whereas the decrease in respiration was not. The increase in oligomycin-insensitive respiration measured during and immediately after the heat stress suggests that the reversible component of the ΔΨ<sub>m</sub> depolarization was attributable to a hyperthermia-induced increase in leak current across the mitochondrial inner membrane (uncoupling). This hypothesized increased leak was probably not due solely to high-conductance openings of the mPTP, because stress-induced changes in ΔΨ<sub>m</sub> were not reduced by cyclosporin A. “Recycling” of Ca<sup>2+</sup> and Na<sup>+</sup> across the mitochondrial membrane can cause an apparent increase in mitochondrial leak (reviewed by Castaldo et al., 2009), but in this heat stress neither Ca<sup>2+</sup> nor Na<sup>+</sup> was essential for the ΔΨ<sub>m</sub> depolarization during the heat stress (Figs. 1A, 2A). The increased leak conductance is more likely related to the reversible, hyperthermia-induced increase in proton conductance reported in isolated mitochondria (e.g., Willis et al. 2000, Zukiene et al. 2010).
The hyperthermia-induced $\Delta \Psi_m$ depolarization likely contributes to the eventual neuronal death, because a proton carrier-induced $\Delta \Psi_m$ depolarization of comparable duration also reduced cell viability. CCCP-induced cell death in Jurkat and FL5.12 cells is also preceded by signs of mitochondrial damage and apoptosis (de Graaf et al., 2004). However, $\Delta \Psi_m$ depolarization-induced death is likely to involve additional susceptibility factors, since some cells are not readily killed by CCCP (e.g. osteosarcoma cells, Lim et al., 2001).

**Slow, post-stress $\Delta \Psi_m$ depolarization precedes apoptotic death**

Immediately following termination of the heat stress there was a partial recovery of $\Delta \Psi_m$. Since this post-stress repolarization occurred in the absence of an increase in respiration (suggesting persisting mitochondrial damage), this transient recovery of $\Delta \Psi_m$ was likely due to a decrease in the mitochondrial leak conductance.

A slowly-developing, irreversible depolarization of $\Delta \Psi_m$ followed this brief repolarization phase. The causes of this slow depolarization are not yet resolved, but our findings rule out some mechanisms. For example, the findings that this $\Delta \Psi_m$ depolarization was not significantly altered by cyclosporin A or by a pan-caspase inhibitor (qVD-OPH) that delays post-stress neuronal death suggest that the depolarization occurs independent of mPTP opening or caspase activation. Milleron and Bratton (2006) reported that a different pan-caspase inhibitor (zVAD-fmk = Z-Val-Ala-Asp(OMe)-fluoromethylketone) reduced the $\Delta \Psi_m$ depolarization in Jurkat T cells stressed by a 2 h exposure to 45 °C. Because the early post-stress depolarization was not accompanied by activation of any identified caspase, they suggested that heat stress activates an as-yet-identified zVAD-fmk-inhibited protease. A candidate for
this upstream protease is a calpain-like enzyme, since µ-calpain is inhibited by zVAD-fmk (Bizat et al., 2005). Consistent with this possibility, we found that a calpain inhibitor (PD150606) reduced the ΔΨₘ depolarization measured 5 h following the heat stress, and temporarily prolonged post-stress neuronal survival. The actual heat-sensitive target(s) of this inhibitor remain(s) to be resolved, but a mitochondrial calpain could contribute both to mitochondrial damage and indirectly to the later caspase activation. One possibility is that calpain-mediated cleavage of Bax could contribute to mitochondrial damage as found for damage to oligogendrocytes induced by excitotoxicity (Sánchez-Gómez et al. 2011).

The slow ΔΨₘ depolarization began prior to the onset of marked ATP depletion, suggesting that this late ΔΨₘ depolarization was not initiated by ATP depletion. However, the eventual ATP depletion, combined with reduced glutathione and reduced respiration, would make mitochondria more susceptible to further damage, perhaps tilting the balance between pro- and anti-apoptotic mechanisms. Pagliari et al. (2005) found that heat-stressed mitochondria showed Bax oligomerization that was enhanced by pro-apoptotic proteins and reduced by the anti-apoptotic protein Bcl-2. We found that transgenic expression of the anti-apoptotic protein Bcl-xL increased survival measured 1-3 days following the heat stress, but only delayed (rather than prevented) the eventual neuronal death, similar to the temporary protection afforded by caspase inhibition with qVD-OPH (White et al., 2003). Perhaps cellular damage from the heat stress triggers apoptotic pathways, but stress-induced damage to essential cellular components eventually results in death even when apoptotic mechanisms are inhibited.

In sum, measurements of mitochondrial function in cultured central neurons
demonstrate $\Delta \Psi_m$ depolarization and reduction in respiration both during and following a 2 h, 43 °C heat stress. An early, rapid phase of $\Delta \Psi_m$ depolarization, likely due to increased leak conductance, is partially reversible upon return to normal temperature, but a later slow phase of post-stress $\Delta \Psi_m$ depolarization is irreversible. Mitochondrial damage is likely to be an upstream event that helps bring about the delayed neuronal death that follows this heat stress.

Acknowledgements

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Qian L, Song X, Ren H, Gong J, Cheng S.


Qian L, Song X, Ren H, Gong J, Cheng S.


Fig. 1. Early and delayed phases of $\Delta \Psi_m$ depolarization associated with a 2 hr, 43 °C heat stress, recorded with a platereader in high $[K^+]$ (A) and normal $[K^+]$ (B) medium. A, TMRM fluorescence before (labeled -2 h) and following the heat stress, recorded in a high $K^+$ (145 mM), nominally zero $Ca^{2+}$ medium. $\Delta \Psi_m$ partially depolarized during the heat stress, recovered partially following the stress, and then exhibited a later, slow, irreversible depolarization. Mean ± s.e.m. for 30 culture wells. This biphasic pattern of heat stress-induced $\Delta \Psi_m$ depolarization was observed in >10 additional experiments. B, a similar pattern of $\Delta \Psi_m$ changes was recorded in normal culture medium (2 mM $Ca^{2+}$, 155 mM $Na^+$). In both A and B, TMRM fluorescence was normalized to values recorded prior to stress application (-2 h).

Fig. 2. Time course of changes in TMRM fluorescence in individual neurons heat-stressed in high $[K^+]$ (A), heat-stressed in normal $[K^+]$ (B), or non-stressed (C). A, TMRM fluorescence changes averaged over the soma of a representative neuron, demonstrating that $\Delta \Psi_m$ depolarization developed with a delay during the heat stress, and showed partial recovery after the stress. Micrographs show fluorescence images taken at the indicated times. The image at lower right in A shows the location of the region whose fluorescence was plotted. B, TMRM fluorescence in 4 neurons labeled a-d in micrographs taken at the indicated times. All cells exhibited transient partial post-stress recovery of TMRM fluorescence, but cell b lost its fluorescence relatively suddenly ~100 min following stress cessation. Magnification was lower than that in A allow $\Delta \Psi_m$ changes to be recorded simultaneously in several neurons in the same
microscope field. C, maintenance of TMRM fluorescence in 4 neurons in a non-stressed sister culture maintained at 37 °C throughout. Measurements in A,B were normalized to pre-stress controls. TMRM was present throughout (30 nM in A, 10 nM in B,C). Calibration bars: 20 µm in A, 100 µm in B. In the online version of this figure traces from individual neurons are colored.

Fig. 3. Heat stress first increases, then decreases O2 consumption. Measurements of O2 consumption during the heat stress applied to stirred suspensions of neurospheres (A and C), substrate-attached cultures (B). Data in A came from sister preparations analyzed after the indicated durations of heat exposure. Data in B reflect continuous measurements on the same culture, made using the flow-through O2 measurement system described in Materials and Methods. Data in C were obtained by exposing the cultures to the heat stress in a 43 ° incubator, then returning them to 37°C for the indicated times before assaying O2 consumption with a Clarke electrode. At all the indicated times post-stress O2 consumption was significantly below that measured in non-stressed cultures (p<0.001). All data were normalized to the O2 consumption measured before exposure to heat.

Fig. 4. Measurements of O2 consumption in digitonin-permeabilized neurospheres. Measurements were made on sister non-heated (left) and heated (right) neurospheres beginning 15-30 min following heat stress termination, using the sequence and drug concentrations described in Materials and Methods. The bar labelled pyruvate + malate measures respiration driven by complex I (with 2 mM ADP to ensure state 3
respiration). Rotenone (1 µM) was added to inhibit complex I (second bar from left).

Addition of succinate and glycerol 3-phosphate (G3P) then gives respiration mediated via complexes II and III. Antimycin A inhibits complex III. Addition of substrate TMPD plus ascorbate measures respiration mediated by complex IV. All O₂ consumption measurements were normalized to the O₂ consumption in the presence of pyruvate + malate in the non-heated condition. The only significant difference was in pyruvate + malate-driven respiration, indicating damage to complex I (and/or reactions upstream of complex I). **indicates significant difference from non-heated cultures, p < 0.01, two-tailed t-test.

Fig. 5. Hyperthermia slows mitochondrial movement (A) and produces a delayed decrease in cellular ATP (B). A, Cumulative plots of the percentage of Mitotracker green-labelled mitochondria in neurites with velocities equal to or greater than the x-axis value in heat-stressed (squares) and non-heated (triangles) sister cultures. Measurements were made at 37 °C, with >400 mitochondria assayed in each condition. Measurements in stressed cultures were made during the first hour post-stress. Differences between heated and non-heated cultures were significant at all movement velocities greater than 0.1 µm/s. Similar results were documented in >10 additional experiments; post-stress recovery of movement was slow and incomplete. B, Time course of hyperthermia-induced changes in ATP following the heat stress. Time zero on the x-axis indicates the end of the heat stress, when the temperature was returned to 37 °C. ATP was measured using the luminescence assay described in Materials and Methods. Each point represents the average of six substrate-attached sister cultures.
Three additional experiments yielded similar results.

Fig. 6. Exposure to a proton carrier (CCCP, 20 μM) reduces cell viability assayed 2-4 days later. Viability was measured as Alamar blue reducing activity (see Materials and Methods), normalized to values measured in sister cultures not exposed to CCCP or high [K⁺]. For results shown as open bars, sister cultures were exposed for 5-60 min to CCCP in normal medium, and assayed 4 days later. For results shown as hatched bars, sister cultures were all exposed to a high [K⁺] medium for 60 min (in mM, 145 KCl, 20 NaHCO₃, 5 glucose, 80 sucrose, 1 BAPTA), during which some cultures were also exposed to CCCP for the indicated times. Cultures were washed thoroughly with normal medium at the end of the CCCP and/or high [K⁺] exposure, and assayed 2 days later. * indicates significant difference from sister cultures not exposed to CCCP or high [K⁺] (Dunnett's test for multiple comparisons). The illustrated findings were confirmed in 2 additional experiments.

Fig. 7. Hyperthermia-induced changes in ΔΨₘ and O₂ consumption persist in oligomycin (5 μg/ml). A, Oligomycin did not block the ΔΨₘ depolarization during the heat stress or the transient ΔΨₘ repolarization upon return to 37 °C. Measurements were made on sister striatal cultures in high [K⁺] medium using TMRM, as in Fig. 1A. TMRM signals were normalized to those recorded prior to the heat stress. n=8 culture wells for each time point. Similar results were obtained when this experiment was repeated in normal [K⁺] medium. B, Effect of oligomycin on O₂ consumption before, during and immediately after a heat stress, normalized to pre-stress/pre-drug value.
Prior to the stress oligomycin reduced $O_2$ consumption to ~60% of control. Oligomycin did not block the hyperthermia-induced increase in $O_2$ consumption. $O_2$ consumption remained elevated above the pre-stress oligomycin value throughout the 75 min heat stress. C, Percentage of oligomycin-insensitive respiration in neurospheres before the heat stress and at two times following the 2 h heat stress, calculated by comparing $O_2$ consumption in cell aliquots in the absence and presence of oligomycin. The larger percentage of oligomycin-insensitive respiration prior to the heat stress in B may have occurred because the effect of oligomycin had not yet reached a steady-state when the heat stress was applied, or because of some damage to the cultures prior to the heat stress. *p<0.05 in A,C. $O_2$ consumption in B measured as in Fig. 3B; $O_2$ consumption in C measured as in Fig. 3A.

Fig. 8. Transgenic expression of Bcl-xL delays neuronal death following heat stress. A, Cultures were transfected to express GFP alone (top row) or GFP plus Bcl-xL (lower row). For each group a representative microscope field is shown before (left) and 42 h following the heat stress (right). B, Comparison of post-stress neuronal survival in GFP-positive cells ± Bcl-xL. Bcl-xL increased survival of GFP-positive neurons with processes on the first post-stress day (p<0.05, n>10 culture wells), but by the 3rd post-stress day most GFP-labeled process-bearing cells in both groups had disappeared.
Table 1. Inhibition of mPTP opening (cyclosporin A) or caspase activation (qVD-OPH) does not prevent ΔΨ<sub>m</sub> depolarization following heat stress.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclosporin A</th>
<th>No drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stress</td>
<td>83.8 ± 6.3</td>
<td>100 ± 7.5</td>
</tr>
<tr>
<td>Time post-stress (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>55.3 ± 2.6</td>
<td>56.8 ± 1.44</td>
</tr>
<tr>
<td>6.0</td>
<td>59.4 ± 3.2</td>
<td>70.6 ± 4.0</td>
</tr>
<tr>
<td>12.0</td>
<td>30.8 ± 2.9</td>
<td>32.6 ± 2.3</td>
</tr>
<tr>
<td>24.0</td>
<td>31.1 ± 2.3</td>
<td>39.2 ± 2.6</td>
</tr>
<tr>
<td>qVD-OPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No stress</td>
<td>88.0 ± 6.4</td>
<td>100 ± 3.9</td>
</tr>
<tr>
<td>Time post-stress (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>83.0 ± 2.3</td>
<td>84.2 ± 2.7</td>
</tr>
<tr>
<td>4.5</td>
<td>41.3 ± 2.3</td>
<td>34.8 ± 2.47</td>
</tr>
<tr>
<td>7.0</td>
<td>49.9 ± 4.8</td>
<td>41.6 ± 3.92</td>
</tr>
<tr>
<td>24.0</td>
<td>21.5 ± 4.6</td>
<td>29.7 ± 8.7</td>
</tr>
</tbody>
</table>

Platereader measurements of TMRM fluorescence in sister cultures in the presence or absence of the indicated drug. n = 12-18 culture wells. 1 μM cyclosporin A or 20 μM qVD-OPH was added 30 min before the stress; cyclosporin A was also supplemented 2 h before each post-stress measurement. Cyclosporin A findings were confirmed in 3 additional experiments, qVD-OPH findings were confirmed in 2 additional experiments.
Table 2. Effects of NAD\(^+\) and \(\gamma\)-glutamyl cysteine (\(\gamma\)GC) on post-stress survival and/or TMRM fluorescence

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No stress</th>
<th>No stress+drug</th>
<th>Stress alone</th>
<th>Stress+drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\gamma)GC 500 (\mu)M</td>
<td>93.7 ± 0.4 (12)</td>
<td>93 ± 0.6 (12)</td>
<td>32 ± 0.7 (24)</td>
<td>30.3 ± 0.8 (12)</td>
</tr>
<tr>
<td>NAD 5 mM</td>
<td>85.9 ± 0.8 (23)</td>
<td>81.3 ± 1 (12)</td>
<td>40.9 ± 0.8 (23)</td>
<td>50.5 ± 1 (12) #</td>
</tr>
</tbody>
</table>

TMRM fluorescence (% of no-stress control)

<table>
<thead>
<tr>
<th>[NAD(^+)]</th>
<th>No stress + NAD(^+)</th>
<th>Stress alone</th>
<th>Stress + NAD(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>96 ± 3 (12)</td>
<td>13 ± 1 (12)</td>
<td>45 ± 2 (12) *</td>
</tr>
<tr>
<td>10 mM</td>
<td>94 ± 3 (12)</td>
<td>13 ± 1 (12)</td>
<td>40 ± 4 (12) *</td>
</tr>
</tbody>
</table>

Cell survival was assayed 50 h post-stress for \(\gamma\)GC and 40 h post-stress for NAD\(^+\), using the live/dead assay described in Materials and Methods. TMRM fluorescence was assayed 6.5 h after stress termination. NAD\(^+\) was applied 24 h prior to the heat stress. Number of culture wells given in parentheses. *p < 0.001, #p<0.05 compared to stress alone (sister cultures, one-way ANOVA with Dunnett’s multiple comparison test). Similar results were obtained in two additional experiments.
Table 3

Effect of calpain inhibitor PD150606 (100 µM) on post-stress TMRM fluorescence and survival

TMRM fluorescence 5 h post stress (% no-stress control)

<table>
<thead>
<tr>
<th></th>
<th>Stress alone</th>
<th>Stress + PD150606 pretreat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress alone</td>
<td>12.5 ± 1.8 (12)</td>
<td>24.9 ± 2.6 (12)*</td>
</tr>
</tbody>
</table>

Cell survival (%)

<table>
<thead>
<tr>
<th></th>
<th>Stress alone</th>
<th>Stress + PD150606 pretreat</th>
<th>Stress + PD150606 stress onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress alone</td>
<td>26 ± 3</td>
<td>50 ± 3*</td>
<td>52.5 ± 4*</td>
</tr>
</tbody>
</table>

Pretreat indicates that PD150606 was present 2 h prior to, during and after the stress; stress onset indicates drug presence during and after the stress. TMRM fluorescence was measured 5 h post-stress (high [K+] medium throughout). Cell survival was measured 24 h post-stress (live/dead assay, see Materials and Methods). * indicates p < 0.001 compared to cultures stressed without drug. The protection afforded by PD150606 did not extend to 48 h post-stress, when survival measured in both PD150606-treated and non-treated cultures was similar (18-21%), a result repeated in 2 additional experiments. Long-term exposure to this drug may itself be toxic, because in non-heated cultures a 48 h exposure to PD150606 reduced survival from 90 to 39.5% measured by propidium iodide exclusion.
Fig. 1

A

High [K⁺]

Time after heat stress (h)

TMRM Fluorescence (normalized)

B

Normal [K⁺]

Time after heat stress (h)

TMRM Fluorescence (normalized)
Fig. 2

A

B

C

Heat
Fig. 3

A

O$_2$ Consumption (normalized)

Time (min)

0 25 50 75 100 125 150

0.25 0.50 0.75 1.00 1.25 1.50

B

O$_2$ Consumption (normalized)

Time (min)

0 25 50 75 100 125 150

0.25 0.50 0.75 1.00 1.25 1.50

C

O$_2$ Consumption (normalized)

Time after Stress (h)

no stress 0.5 1-2 6-8

0.0 0.5 1.0
Fig. 4

Comparison of % of non-heated oxygen consumption rate between non-heated and heated conditions with various treatments. The treatments included pyruvate + malate, rotenone, succinate + G3P, antimycin A, TMPD + ascorbate.
Fig. 5

(A) Graph showing the percentage of mitochondria over time after heat stress. The graph compares control and heat conditions. The Y-axis represents the percentage of mitochondria, and the X-axis represents time after heat stress (h).

(B) Graph showing the ATP levels over time after heat stress. The Y-axis represents ATP levels (nM/well), and the X-axis represents time after heat stress (h).
**Fig. 7**

A. TMRM Fluorescence (normalized) vs. Time (h)

B. Oligomycin and Heat effects on O₂ consumption (normalized)

C. % Oligomycin-insensitive respiration

- **non-heated**
- **0-15 min**
- **70-80 min**
A

Before heat
After heat

GFP only
GFP + Bcl-xL

B

% Surviving with processes

0.0 0.5 1.0

0 1 2 3

Time (days)

GFP
GFP + Bcl-xL

*