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Stephanie L.H. Zeiger, Jennifer R. Mckenzie, Jeannette N. Stankowski, Jacob A. Martin, David E. Cliffl, et al.. Neuron Specific Metabolic Adaptations Following Multi-Day Exposures to Oxygen Glucose Deprivation. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2010, 1802 (11), pp.1095. 10.1016/j.bbadis.2010.07.013 . hal-00623315

HAL Id: hal-00623315

<https://hal.science/hal-00623315>

Submitted on 14 Sep 2011

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Accepted Manuscript

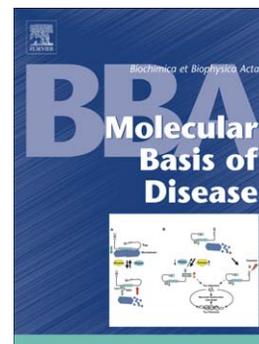
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PII: S0925-4439(10)00150-X
DOI: doi: [10.1016/j.bbadis.2010.07.013](https://doi.org/10.1016/j.bbadis.2010.07.013)
Reference: BBADIS 63138

To appear in: *BBA - Molecular Basis of Disease*

Received date: 2 June 2010
Revised date: 13 July 2010
Accepted date: 19 July 2010



Please cite this article as: Stephanie L.H. Zeiger, Jennifer R. McKenzie, Jeannette N. Stankowski, Jacob A. Martin, David E. Cliffl, BethAnn McLaughlin, Neuron Specific Metabolic Adaptations Following Multi-Day Exposures to Oxygen Glucose Deprivation, *BBA - Molecular Basis of Disease* (2010), doi: [10.1016/j.bbadis.2010.07.013](https://doi.org/10.1016/j.bbadis.2010.07.013)

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Neuron Specific Metabolic Adaptations Following Multi-Day Exposures to Oxygen Glucose Deprivation

Stephanie L. H. Zeiger^{a,e+}, Jennifer R. McKenzie^b, Jeannette N. Stankowski^{d,e}, Jacob A. Martin^a, David E. Cliffel^b, and BethAnn McLaughlin^{a,c,e*}

Departments of ^aNeurology, ^bChemistry and ^cPharmacology, ^dNeuroscience Graduate Program and ^eVanderbilt Kennedy Center, Vanderbilt University, Nashville, TN 37232

*Correspondence should be addressed to: Dr. BethAnn McLaughlin, Vanderbilt University, MRB III Room 8141, 465 21st Avenue South, Nashville, TN 37232-8548 USA Tel: (615) 936-3847; Fax: (615) 936-3747; email: bethann.mclaughlin@vanderbilt.edu

+Current address: Department of Medicine, Division of Nephrology, Vanderbilt University School of Medicine, Nashville, TN 32372

Abstract

Prior exposure to sub toxic insults can induce a powerful endogenous neuroprotective program known as ischemic preconditioning. Current models typically rely on a single stress episode to induce neuroprotection whereas the clinical reality is that patients may experience multiple transient ischemic attacks (TIAs) prior to suffering a stroke. We sought to develop a neuron enriched preconditioning model using multiple oxygen glucose deprivation (OGD) episodes to assess the endogenous protective mechanisms neurons implement at the metabolic and cellular level for stress adaptations. We found that neurons exposed to a five minute period of glucose deprivation recovered oxygen utilization and lactate production using novel microphysiometry techniques. Using the non-toxic and energetically favorable five minute exposure, we developed a preconditioning paradigm where neurons are exposed to this brief OGD for three consecutive days. These cells experienced 45% greater survival following an otherwise lethal event and exhibited a longer lasting window of protection in comparison to our previous *in vitro* preconditioning model using a single stress. As in other models, preconditioned cells exhibited mild caspase activation, an increase in oxidized proteins and a requirement for reactive oxygen species for neuroprotection. Heat shock protein 70 was upregulated during preconditioning, yet the majority of this protein was released extracellularly. We believe coupling this neuron enriched multiday model with microphysiometry will allow us to assess neuronal specific real-time metabolic adaptations necessary for preconditioning.

Key words: preconditioning, oxygen glucose deprivation, microphysiometry, reactive oxygen species, caspase activation, protein carbonyl formation, heat shock protein 70, ATP

Abbreviations: OGD, oxygen glucose deprivation; HSP70, heat shock protein 70; TIA, transient ischemic attack; HSC70, heat shock cognate 70; LDH, lactate dehydrogenase; ROS, reactive oxygen species; BSA, bovine serum albumin; LOx, lactate oxidase; MAP2, microtubule associated protein 2; PBN, N-tert-butyl- α -phenylnitrone; PBS, phosphate buffer solution; NMDA, N-methyl-D-aspartic acid.

1. Introduction

Stroke is the second leading cause of death and most common cause of long term adult disability worldwide [1]. Given that 87% of strokes are ischemic in nature [2], there is a pressing need to understand the pathophysiology of ischemic cell death. Developing neurotherapeutic agents based on our current understanding of the cytotoxic signaling pathways associated with loss of oxygen and glucose, however, has proven unsuccessful [3].

Like other organs, the CNS has a remarkable ability to exert endogenous protective pathways in the presence of non-toxic stress and these defenses are capable of providing significant protection against otherwise lethal injuries. This phenomena is referred to as ischemic preconditioning or tolerance and was first observed in the brain over 40 years ago [4]. Ischemic tolerance can be achieved by a host of stressful stimuli including low level exposure to oxidative stress, mitochondrial toxins, CNS-specific antigens and hypoxia [5-9]. Modifications of ion channel activity, kinase activation and release of adenosine and neurotransmitters occurs rapidly following the preconditioning stimuli and results in a very brief window of protection [10, 11]. More sustained changes in gene activation and protein synthesis which typically occurs over the course of hours to days is thought to lead to a longer window of cellular protection [9, 12, 13]. Indeed, hallmark features of preconditioning include a dependence on new protein synthesis, activation of ATP dependent potassium channels, and upregulation of heat shock proteins (HSPs) [14-16]. We have also shown that

activation of traditional cytotoxic agents such as reactive oxygen species (ROS) and caspases are also essential to elicit the subsequent neuroprotection [9].

The suggested clinical correlate to preconditioning is exposure to transient ischemic attacks (TIAs) prior to experiencing a stroke [17-19]. A TIA is defined by the TIA Working Group as 'a brief episode of neurologic dysfunction caused by focal brain or retinal ischemia, with clinical symptoms typically lasting less than 1 hour, and without evidence of acute infarction' [20]. Transient ischemic attacks have increasingly been recognized as one of the greatest immediate risk factors for ischemic stroke. Indeed, several studies have cited that up to 18% of patients presenting with ischemic stroke reported a history of TIA-like events [21-23]. Three major studies have demonstrated that patients with a history of TIA exhibit a better stroke outcome than those not experiencing a previous TIA [24-26]. There is evidence that these patients have smaller initial diffusion lesions along with less severe clinical deficits following a major stroke [25, 27] although this remains controversial [28].

Based on multiple models of preconditioning protection, there is clear linkage between the timing of the mild ischemic insults or other stressor and the protective window afforded. Patients who experience TIAs fewer than 4 weeks prior to stroke onset experienced less impairment one month following their stroke compared to those who either had no history of TIA or TIAs beyond the 4 week window [25, 26]. To date, TIA studies have been limited due to small patient sample size, variability between patient existing conditions, and the lack of a diagnostic test for TIA.

The protective effects of a prior angina before myocardial ischemia has, however, been more widely accepted and suggest that multiple or protracted periods of angina is more protective than a single episode alone (reviewed in [29]). Smaller studies in CNS have suggested that patients with a history of multiple TIAs had a higher proportion of favorable outcomes than patients with only one TIA [26]. Basic science models of preconditioning, however, more commonly rely on a single stressful insult to elicit protection suggesting a need to develop a multiple exposure model of preconditioning to capture the clinical realities of TIA and perhaps afford greater protection than a single episode of preconditioning stress.

In this work, we used a powerful new microphysiometry technique to measure in real time neuronal metabolic adaptations following brief glucose deprivation in order to develop a new model of preconditioning that more closely resembles a history of multiple TIA(s). Based on our findings of favorable ATP production following a five minute oxygen glucose deprivation (OGD), we exposed neurons to this mild stress repeatedly over several days to determine if these cells expressed hallmark features of preconditioning. These markers include including temporally and spatially controlled caspase-3 activation, production of reactive oxygen species and upregulation of the molecular chaperone heat shock protein 70 (HSP70)[9, 30-35].

2. Materials and methods

User-friendly versions of all of the protocols and procedures can be found on our website at <http://www.mc.vanderbilt.edu/root/vumc.php?site=mclaughlinlab&doc=17838> .

2.1. Materials and reagents

All media and media supplements were from Invitrogen (Carlsbad, CA) except for the microphysiometry experiments which used custom RPMI (1mM phosphate buffer, glucose and bicarbonate-free) from Mediatech, Inc. (Manassas, VA). Antibodies for western blot analysis and immunocytochemistry included rabbit cleaved caspase-3 polyclonal antibody (9661S) and anti-mouse IgG horseradish peroxidase conjugated secondary antibody from Cell Signaling Technology (Danvers, MA), rabbit HSP70 polyclonal antibody (SPA-811) and rabbit HSC70 polyclonal antibody (SPA-816) from Assay Designs (Ann Arbor, MI), mouse microtubule associated protein 2 (MAP2) monoclonal antibody and Hoescht 33342 (DAPI) from Sigma (St. Louis, MO), and anti-rabbit Cy-3 and anti-mouse Cy-2 from The Jackson Immunoresearch Laboratory (Bar Harbor, ME). The modular hypoxic chamber was purchased from Billups-Rothenberg Inc. (Del Mar, CA). Western Lightning[®] chemiluminescence reagent plus enhanced luminol reagents were from PerkinElmer Life Science (Waltham, MA). Commercial kits utilized include DC Protein Assay Kit II from Bio-Rad (Hercules, CA), OxyBlot[™] Protein Oxidation Detection Kit from Chemicon International (Billerica, MA), ViaLight[®] Plus Kit from Cambrex Bioscience (East Rutherford, NJ) and LDH Toxicology Assay Kit from Sigma.

Lyophilized alamethicin was obtained from A.G. Scientific, Inc. (San Diego, CA) and reconstituted with 1mL absolute ethanol. Nafion[®] (perfluorosulfonic acid-PTFE copolymer, 5% w/w solution) was from Alfa Aesar (Ward Hill, MA) while stabilized lactate oxidase (LOx) was purchased from Applied Enzyme Technology (Pontypool, UK). All Cytosensor[®] materials were purchased from Molecular Devices Corporation (Sunnyvale, CA). Sterile 20% glucose solution was purchased from Teknova (Hollister, CA). All other chemicals are from Sigma.

2.2. Cell culture

Cortical cultures were prepared from embryonic day 16 Sprague-Dawley rats as previously described with only minor modifications to include new media supplements supporting neurite outgrowth [36]. Briefly, cortices were dissociated and the resultant cell suspension was adjusted to 770,000 cells/well (6-well tissue culture plates containing poly-L-ornithine-treated coverslips) in growth media (80% Dulbecco's Modified Eagle Medium (DMEM), 10% Ham's with F12-nutrients, 10% bovine calf serum (heat-inactivated, iron-supplemented; Hyclone) with 24U/ml penicillin, 24µg/ml streptomycin, and 2mM L-glutamine). Following the inhibition of glial cell proliferation after two days in culture, neurons were maintained in Neurobasal media (Invitrogen) containing B27 and NS21 supplements [37], penicillin and streptomycin. All experiments were conducted three weeks following dissection (21-25 days *in vitro*).

2.3. Neuronal oxygen glucose deprivation (OGD)

In vitro OGD experiments were performed as previously described [38]. Mature neurons on glass coverslips were transferred to 35mm petri dishes containing glucose-free balanced salt solution that had been bubbled with an anaerobic mix (95% nitrogen and 5% CO₂) for 5 minutes immediately prior to the addition of cells to remove dissolved oxygen. Plates were then placed in a hypoxic chamber which was flushed with the anaerobic mix for 5 minutes, then sealed and placed at 37°C for 10 or 85 minutes for a total exposure time of 15 and 90 minutes. OGD treatment was terminated immediately following the 5 minute exposure or after the longer exposure periods by placing the glass coverslips into MEM media containing 10mM HEPES, 0.001% bovine serum albumin (BSA), and 2xN2 supplement (MEM/HEPES/BSA/2xN2) under normoxic conditions.

2.4. Toxicity assays

Twenty four hours following each period of OGD insult, 40µl of cell media was removed and used to assess cell viability using a lactate dehydrogenase (LDH)-based *in vitro* toxicity kit as previously described [9, 39]. In order to account for variation in total LDH content, raw LDH values were normalized to the toxicity caused by a 24 hour exposure to 100µM NMDA plus 10µM glycine. This stress has been shown to cause 100% cell death in this system [9, 38]. All experiments were performed using cells derived from at least three independent original dissections.

2.5. ATP assays

Measurements of ATP content were performed twenty four hours following 5, 15, or 90 minutes of OGD as described previously [38]. Briefly, each coverslip was removed from the toxicity plate and added to a new plate containing 300 μ l of Cell Lysis reagent from the ViaLight[®] Plus Kit. Following a 10 minute incubation time, 80 μ l of cell lysate and 100 μ l of ATP monitoring reagent were added to each well of a 96 well transparent plate. Bioluminescence due to the formation of light from the interaction of the enzyme luciferase with cellular ATP was measured on a Tecan Spectra Fluor Plus plate reader following two-minute incubation. Measurements were obtained in duplicate for each sample with an integration time of 1000ms and at a gain of 150 and normalized for protein levels. ATP levels are expressed as the mean from at least three independent experiments \pm standard error mean (S.E.M). Statistical significance was determined by two-tailed paired *t*-test with $p < 0.05$.

2.6. Microphysiometry analysis

Lactate-sensing electrode films were prepared similarly to that described previously [40, 41]. Briefly, 1.8mg of LOx was dissolved in 100 μ l of a BSA-buffer solution then quickly mixed with 0.8 μ l of 25% glutaraldehyde. Electrode films were then prepared by allowing a droplet of the enzyme solution to dry on the platinum electrode surface of a modified Cytosensor Microphysiometer plunger head described previously [40-42]. A droplet of the 5% Nafion solution was also applied to the oxygen electrode (127 μ m bare platinum wire) to reduce biofouling as shown in the literature [42, 43]. The solutions were prepared fresh for each experiment.

Lactate and oxygen measurements were performed with a multi-chamber bipotentiostat enabling us to monitor multiple analytes in four chambers simultaneously. The lactate sensing electrodes were held at a potential of +0.6V to oxidize the H_2O_2 produced within the enzyme film while the oxygen electrode was set at -0.45V to reduce dissolved oxygen. All potentials were set versus the Cytosensor Ag/AgCl reference electrode in the effluent stream.

Prepared cell inserts and modified sensor heads were placed in the four-channel microphysiometer as previously described [40]. Low-buffered 5mM glucose RPMI media was perfused through the chamber at 100 μ l per minute with the Cytosensor program maintaining a pump-on/pump-off cycle (80s pump-on, 40s pump-off). Lactate and oxygen signals were sampled by the potentiostat once per second for the entirety of the experiment.

The neurons were perfused for 90 minutes, at which point the media was replaced with an identical media containing no glucose and perfused for either 5 or 90 minutes. Next, neurons were perfused with 5mM glucose RPMI for an additional 120 minutes. Control experiments in which no glucose deprivation occurred were performed simultaneously. The neurons were perfused with 15 μ M alamethicin which leads to formation of pores in the cellular membrane and cellular death to allow for calibration of the lactate sensors.

Peak heights in nanoamps were calculated for each two minute pump-on/pump-off cycle. The average peak height measured after neuron death was subtracted from each point. Molar lactate production was calculated by comparing peak heights to the shifts in baseline during the calibration steps.

Molar oxygen was calculated by assuming the oxygen baseline to be the concentration of dissolved oxygen, ~ 0.24mM, and was calculated as described previously [44]. As each chamber of cells differs slightly in neuron density and metabolic activity, all signals were then normalized to 100% of the average signal of the thirty minutes before glucose deprivation. The data was boxcar smoothed and replicate chambers were compared and grouped into four time points encompassing between 20 to 30 minutes for visualization and include 0 minutes, 30 minutes, 1 hour, and 2 hours after glucose deprivation. The average activity for lactate and oxygen at each point was normalized to its control values and plotted as oxygen consumption or lactate production vs. time. Each group was compared to the control and statistical significance was determined by a two-tailed paired *t*-test with $p < 0.05$.

2.7. Preconditioning paradigm

Mature neuronal cultures were exposed to 5 minutes of OGD and then placed immediately into their original MEM/Hepes/0.01%BSA/2xN2 media. To determine if ROS were necessary for preconditioning, neurons were treated with 500 μ M N-tert-butyl- α -phenylnitrone (PBN) during the daily OGD exposure period. Twenty four hours later, neurons underwent an additional OGD exposure that was again repeated the following day for a total of three OGD exposures over three days. Every 24 hours following the exposures, toxicity was determined using LDH assays to evaluate cell death over time. Twenty four hours following the last OGD period, control, preconditioned only, or preconditioned with PBN neurons were exposed to 90 minute OGD and toxicity

was determined 24 hours later using the LDH assay. For longer survival studies, the 90 minute OGD period occurred 3 days following the last 5 minute OGD exposure. Values were normalized to 100 μ M NMDA toxicity. In order to assess if a multi-day treatment was more effective than a single stress, experiments were performed in which neurons were exposed to a single 5 minute OGD stress followed by the 90 minute OGD 24 hours later. Cellular death was then determined the following day using the LDH toxicity assay.

2.8. Immunocytochemistry and quantification

Neurons were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were then washed in phosphate buffer solution (PBS) for a total of 15 minutes and blocked with 8% BSA in PBS for 25 minutes. This was followed immediately by incubation in 1% BSA PBS solution containing cleaved caspase-3 primary antibody (1:500) and MAP2 primary antibody (1:1000) at 4°C overnight. Cells were then washed in PBS for a total of 30 minutes followed by incubation for 60 minutes in secondary antibodies diluted in 1% BSA. Cells were washed again in PBS for a total of 25 minutes followed by a 10 minute incubation in Hoescht 33342 (DAPI) to visualize nuclei [45]. Following an additional 15 minute PBS wash, coverslips were mounted on slides with Prolong Gold anti-fade reagent. Fluorescence was visualized with a Zeiss Axioplan microscope equipped with an Apotome optical sectioning filter as previously described [39]. The percentage of cleaved caspase-3-positive cells was determined by counting the number of cells that were positive for activated caspase and normalizing to the total number of neurons as determined by DAPI

staining. Data represent the average \pm SEM from at least three independent experiments done in duplicate by two independent blinded observers. Statistical significance was determined by a two-tailed paired *t*-test with $p < 0.05$.

2.9. Oxyblot™ methodology

Twenty four hours following the third preconditioning OGD exposure, neurons were harvested into 200 μ l of TNEB (50mM Tris, 2mM EDTA 150mM NaCl, 8mM β -glycerophosphate, 100 μ M orthovanadate, 1% Triton X-100 (1%), 1:100 protease inhibitor) and total oxidized proteins were determined using the OxyBlot™ Protein Oxidation Detection Kit. Following the cell harvest, 100 μ l of the lysate was immediately treated with 50mM DTT to prevent protein oxidation. The DTT treated lysates was split into two separate 50 μ l aliquots for the derivatization reaction containing 2,4-dinitrophenylhydrazin and the negative control containing derivatization control solution. Samples were stored at 4°C and within 7 days of protein derivatization, equal protein concentrations were analyzed by western blot using antibodies specific for the detection of oxidized proteins provided by the manufacturer. Data represent results from at least 3 independent experiments. Statistical significance was determined by two-tailed paired *t*-test with $p < 0.05$.

2.10. HSP70 analysis

Media was collected from control or preconditioned cultures immediately prior to the 90 minute lethal injury. To concentrate the extracellular media, 500 μ l of sample for each condition underwent centrifugal filtration using microcon YM-30 centrifugal filter devices from Millipore (Billerica, MA). The resulting

concentrated media was then used for western blot analysis to determine if HSP70 was present extracellularly. The underlying neuronal cell bodies were washed twice with ice cold PBS and harvested in 300 μ l of TNEB lysate buffer to evaluate intracellular HSP70 expression following preconditioning. The constitutively expressed form of HSP70, HSC70, was used as a loading control for intracellular HSP70. Similarly, neurons were exposed to 15 minutes of heat shock at 42°C. The following day, media was collected and neurons were harvested for western blot analysis of HSP70 and HSC70.

Equal protein concentrations as determined by BCA protein assay were separated using Criterion Tris-HCl gels (Bio-Rad), transferred to polyvinylidene difluoride membranes (Amersham Biosciences) and incubated in the appropriate antibody (HSP70, 1:1000 or HSC70, 1:1000) as previously described [9]. Image J software (NIH) was used to quantify the HSP70 western band intensity and data represents the mean from three independent experiments \pm S.E.M. Statistical significance was determined by two-tailed paired *t*-test with $p < 0.05$.

3. Results

3.1. Neuronal cells exhibit tolerance to brief OGD exposures

In order to establish a model of ischemic stress which more fully captures the potentially repetitive nature of TIA stress and allows us to assess neuronal specific adaptations necessary for preconditioning, we first exposed mature neuronal cultures to varying durations of OGD. Twenty four hours later, neuronal death was assayed by measuring the release of LDH into the culture media from dead and dying cells. Neither 5 nor 15 minutes of OGD affected neuronal viability or disrupted cytoarchitecture whereas a 90 minute exposure resulted in a significant increase in LDH release compared to control cells (Fig. 1A). Representative photomicrographs taken 24 hours following OGD demonstrate phase bright neurons with intact processes in control, 5 or 15 minute exposed cultures (Fig. 1B, C, D). In contrast, a 90 minute exposure resulted in neuronal soma shrinkage, near complete loss of phase bright cell bodies and evidence of neurite beading and retraction (Fig. 1E).

3.2. Neuronal energetic status is enhanced following mild stress

Given the immediate and profound metabolic consequences of a loss of oxygen and glucose, we next evaluated the effects of mild, moderate, and severe OGD on neuronal energetic status. Twenty four hours following 5, 15, or 90 minute OGD, we measured total neuronal ATP content and observed a significant enhancement in ATP levels following the non-toxic 5 minute exposure. Total ATP amounts were not significantly different from controls following at 15 minute OGD which did not result in cellular death (Fig. 2A). Neurons exposed to

the 90 minute OGD experienced a profound loss of energetic reserves as reflected by the 70% decrease in ATP which was statistically indistinguishable from that elicited by a lethal exposure to 100 μ M NMDA (Fig. 2A).

Recent advances allow for the simultaneous detection of multiple extracellular analytes to assess the relative contribution of aerobic and anaerobic pathways in maintaining metabolic tone. Using electrodes sensitive to lactate and oxygen, we performed real time measurements of neuronal oxygen consumption (aerobic) and lactate production (anaerobic) immediately following 5 or 90 minute glucose deprivation. Measurements of oxygen consumption revealed a failure of cells which experience lethal glucose deprivation to recover aerobic respiration. The non-toxic five minute exposure, however, resulted in an enhancement of aerobic respiration (Fig. 2B). Evaluation of anaerobic respiration by lactate generation revealed that loss of glucose significantly impaired anaerobic lactate production following both the 5 and 90 minute exposure. Within 30 minutes following the initial 5 minute exposure, however, lactate production was similar to that observed in control cells. In contrast, the lethal 90 minute exposed neurons were unable to recover anaerobic respiration and lactate production to control levels even two hours following stress (Fig. 2C).

3.3. Multiple OGD episodes results in preconditioning neuroprotection

Taken together, the toxicity and microphysiometry data suggest neurons are resistant to brief OGD episodes and that a single episode places neurons in an aerobically poised state. To determine if this state was neuroprotective, cultures were exposed to a single 5 minute OGD or multiple 5 minute OGD

periods on three consecutive days. The day following the last exposure, control, single or multi-day exposed neurons underwent 90 minute OGD (Fig. 3A). Representative photomicrographs demonstrate that following three days of preconditioning neurons still exhibit healthy processes and phase bright somas similar to control cultures (Fig. 3B, C, Supplementary Fig. 1). Using LDH assays, we monitored cell death 24 hours after each OGD exposure and found no difference between control, single or multi-day exposed cells reinforcing the visual inspection of the cells which revealed no injury (Fig. 3D). Moreover, multiple mild stressors evoked a preconditioning effect which did not occur following the single stress. The effect of the normally lethal 90 minute OGD was diminished by 45% in cells experiencing the multiple preconditioning priming (Fig. 3E, G). We also found a demonstrably increase in the protective window of this multi-day preconditioning compared to our previous *in vitro* single stress mixed culture model [46]. The preconditioning protection was still evident three days following the last OGD treatment (Fig. 3G).

3.4. Caspase-3 activation occurs following multi-day preconditioning

We have previously shown that spatially and temporally limited activation of caspase-3 is essential for eliciting preconditioning protection in our mixed neuronal/glia culture model of preconditioning protection [9]. In our model [47], limited caspase-3 proteolysis is held in check by existing chaperones which are depleted in an effort to target caspases for proteasomal degradation. This, in turn, leads to upregulation of HSP70 and other neuroprotective proteins necessary to survive a normally lethal stress.

As caspase activation occurs early in preconditioning following a single stress [9], we evaluated cleaved caspase-3 expression six hours following each daily five minute OGD using immunofluorescence. Quantification of caspase-3 positive cells as a percentage of total number of cells revealed a significant enhancement in caspase activation without cell death following the repetitive five minute OGD periods in comparison to either control or a single five minute treatment (Fig. 4A, B).

3.5. Neuronal preconditioning requires reactive oxygen species (ROS) generation

In addition to caspase-3 activation being an essential mechanism to deplete existing chaperones, without production of ROS, both neuronal as well as other types of cells do not evoke preconditioning defenses [9, 48]. In order to determine if ROS generation contributes to this new repetitive stress enriched neuronal model, we first determined the amount of protein carbonyl formation using the OxyBlot methodology. Following oxidative modification of proteins by free radicals and other reactive species, carbonyl groups are formed on protein side chains which when incubated with dinitrophenylhydrazine are derivatized into 2,4-dinitrophenylhydrazone which can be detected by western blot analysis. In these experiments, we observed a substantial increase in the amount of total oxidized proteins 24 hours following the last five minute OGD treatment compared to control cells represented as a greater intensity of staining in the preconditioned (Fig. 5A).

We used our previous strategy to determine if ROS production was necessary for the neuroprotective potential of this preconditioning [9] by blocking free radical interactions using the spin trap, PBN (500 μ m), during all three five minute OGD periods. Survival was compared to preconditioned neurons in the absence of PBN following 90 minute OGD. PBN treatment significantly decreased the neuroprotective effect of the multi-day preconditioning as exposure to 90 minutes of OGD resulted in 90% cell death as determined by LDH (Fig. 5B).

3.6. HSP70 is upregulated and released by neurons following multi-day preconditioning

New protein synthesis and upregulation of HSP70 are requisite features of neuronal preconditioning [5, 49] Using western blot analysis, we initially observed very little change in intracellular HSP70 expression 24 hours following the last day of preconditioning (Fig. 6A). As HSP70 has been shown by our group and others to alter cell fate when applied extracellularly [9, 50-53]) and has been shown to be released by tumor cells [54-57], we next evaluated if neurons were releasing the chaperone. We collected and concentrated the extracellular media 24 hours following the last day of preconditioning OGD and found a 3.3 fold increase in HSP70 expression in preconditioned media compared to control media (Fig. 6B). This data suggests HSP70 was, indeed, synthesized and released into the extracellular media following preconditioning.

4. Discussion

In this work, we developed a neuron-enriched culture system to mimic multiple TIAs and observed that repeated exposure to brief OGD provided robust neuroprotection against an otherwise lethal stroke-like event. This model demonstrates many conserved features of preconditioning including mild caspase activation, the necessity of ROS generation for protection, and increased protein synthesis as indicated by the upregulation of HSP70.

The loss of aerobic metabolism and limitation of glucose following ischemic occlusion has rapid and profound effects on CNS survival. With limited capacity for anaerobic respiration, and few alternatives to glucose as fuel, the loss of glucose rapidly impairs neuronal function. Until recently, understanding the dynamic behaviors of energetic pathways has been limited to static measures or indirect assessment of metabolism. Using a novel microphysiometry system, we were able to perform simultaneous measurements in real time of neuronal metabolic recovery following glucose deprivation for the first time and observed that neurons quickly adapt energetic challenge. The brief loss of glucose is not only non-toxic as determined by viability assays, but also allows for cells to rapidly recover as both oxygen consumption and lactate production were similar to control levels within 30 minutes. The fact that oxygen consumption which elevated 1-2h following 5 minute glucose deprivation suggesting that this stress does not fatally impair or cause lasting dysfunction in either the aerobic consumption of oxygen by the electron transport chain nor does it cause

irreversible reliance on anaerobic respiration as would be reflected in a larger increase in lactate.

In contrast, neurons exposed to the lethal 90 minute glucose deprivation did not recover aerobic respiratory capacity. Given that the five minute OGD was not toxic, these cultures can rapidly and efficiently adapt to non-lethal metabolic stress. Taken in conjunction with our ATP studies demonstrating higher ATP levels 24 hours following a 5 minute OGD, this data suggests neurons have adapted their metabolic pathways to build an ATP surplus necessary for surviving an otherwise lethal stress. These data are in contrast to our recently published report in mixed cultures comprised of 80% glia and 20% neurons where total ATP levels were simply comparable to control levels 24h after a single preconditioning event. Taken together, this work suggests that the neuronal enriched cultures exposed to multiday stressors evoke unique adaptive metabolic features which are not observed following a single stress in a mixed culture enriched for glia.

In addition to online assessment of lactate production and oxygen consumption, our microphysiometry system can be equipped with electrochemical detectors to measure extracellular glucose and pH [40, 42, 43]. While alterations in the acidification rate may not necessarily represent changes in anaerobic metabolism, as pH can be modified by CO₂ production, the combination of lactate and acidification sensors allows a more comprehensive view of neuronal metabolism [58]. We believe that this powerful four-analyte system will ultimately allow us to develop a neuronal metabolic biosignature to

determine the temporal windows of reliance upon aerobic and anaerobic metabolism in stressed cells and determine the best substrates to increase neural survival when oxygen or glucose is limiting.

As for the later signaling systems which are employed by neurons to evoke neuroprotection, we had already come to appreciate that preconditioning protection is strictly temporally limited. Using a single sub lethal stress, this window of protection lasts between 24 and 48 hours in our mixed neuron/glia preconditioning model [9]. Using this new neuron enriched multi-day preconditioning paradigm, we observed an enhanced window of protection lasting a *minimum* of three days. While not previously done in cortical or hippocampal systems, Gidday and colleagues have shown that that repetitive mild OGD stress over 12 days resulted in increased retinal ischemic tolerance from 1-3 days to weeks [59]. The current working model is that multiple episodes of sub-lethal stress likely evoke long term genomic reprogramming similar to that which occurs in hibernation or following exposure to high altitude and/or low oxygen conditions [12].

From a clinical standpoint, we recognize that the incidence of both TIA and stroke increases with age and risk factors such as diabetes, obesity and abnormal lipid profiles are strong risk factors for both forms of ischemia. The ability of neurons to induce many of the protective pathways outlined in this work and in other models of preconditioning, may reasonably be expected to be less vigorous or even already in place in the aged, patients experiencing chronic hypertension or hyperglycemia [60], so we look with great interest to developing

clinical measures which account for cumulative oxidative stress provided by increased lipid peroxidation or protein dysfunction in those at high risk of TIA or stroke as possible predictors of the ability to mount rigorous defenses against sublethal stress.

It is worth noting that while our neuronal preconditioning model shares a reliance on new protein synthesis, caspase activation and ROS production to elicit protection, the mechanism by which HSP70 evokes protection is more complicated than we originally anticipated. Most of the work on the neuroprotective action of chaperones has focused on HSP70 as an intracellular regulator of protection by limiting caspase activation, aiding in protein refolding or targeting proteins for proteasomal degradation [9, 61]. In this new multi-day stress model of preconditioning, HSP70 was found to be released into the extracellular media.

HSP70 release has been demonstrated following stress in glial tumors, neuroblastomas and following systemic or immune stress [62-68]. The release of HSP70 is thought to occur via exosomes and lipid rafts along with the activation of extracellular-signal-regulated kinase and phosphatidylinositol-3-kinase pathways and many of these signaling molecules and pathways have been linked to preconditioning as well [64, 66, 69-73]. This is the first work, however, demonstrating release of HSP70 following preconditioning. As cellular death was similar between control and preconditioned neurons, this extracellular pool of HSP70 is not due to a release from dead and dying cells. While heat shocked neurons exhibited a high level of intracellular HSP70, extracellular HSP70 was

only modestly impacted suggesting that following extreme stress HSP70 may be sequestered and recruited to sites of intracellular injury as its chaperone function is maximally required. In contrast, we hypothesize that a mild stress requires less of a role for intracellular HSP70 and allows release of it into the extracellular space. Taken in conjunction with the neuroprotective effect of purified HSP70 application to cells [9, 50, 74, 75], we believe this release may be important for preconditioning neuroprotection.

As our understanding of the role of released chaperones increases, we seek to develop a fuller understanding of the implications of the elevations in plasma and CSF levels of HSP70 and other chaperones that have been observed clinically [76-79]. As HSP70 is necessary for preconditioning cytoprotection, analyzing its release into the cerebral spinal fluid and other peripheral specimens may serve as a useful biomarker for CNS ischemia. Indeed, gene expression in peripheral blood varies between rats treated with brief focal ischemia or stroke [80]. Currently, TIAs are diagnosed primarily by patient history as there are no diagnostic markers of TIA if symptoms have resolved upon emergency room admittance [81, 82]. Given that 10% of emergency room TIA patients return within 48 hours with a stroke [83], a TIA biomarker may be beneficial in defining ischemic events and will allow us to determine if HSP70 release, markers of chronic stress or altered metabolic profile correlates with a better outcome upon secondary injury. We believe that comprehensive metabolic profiling in conjunction with traditional biochemical and high throughput screening will allow us to identify essential proteins for energetic

compensation as well as novel targets for stroke therapy as well as to defining best practices for glucose, oxygen, and lactate management in stroke patients.

Acknowledgements

This work was supported by NIH grants NS050396 (BM), Vanderbilt Neurogenomics training grant MH065215 (SLHZ), Vanderbilt Neuroscience Predoctoral Training Fellowship T32 MH064913 (JNS), NIH (NIAID) U01 AI061223 (DEC). Statistical and graphical support was provided by P30HD15052 (Vanderbilt Kennedy Center).

The authors wish to express their gratitude to Drs. Gregg Stanwood, Rachel Snider, and Pat Levitt for helpful suggestions. We also thank Mrs. Jacquelynn Brown for the generation and maintenance of primary neuronal cultures.

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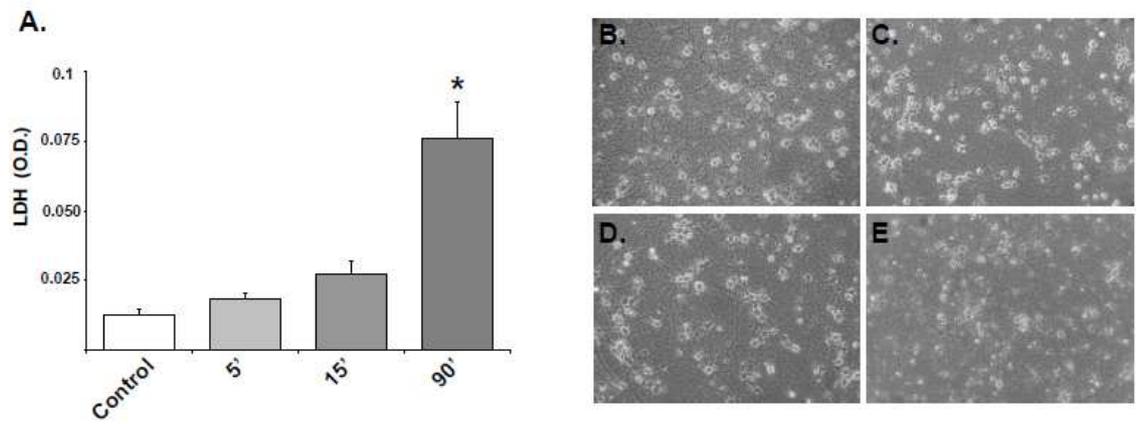


Figure 1

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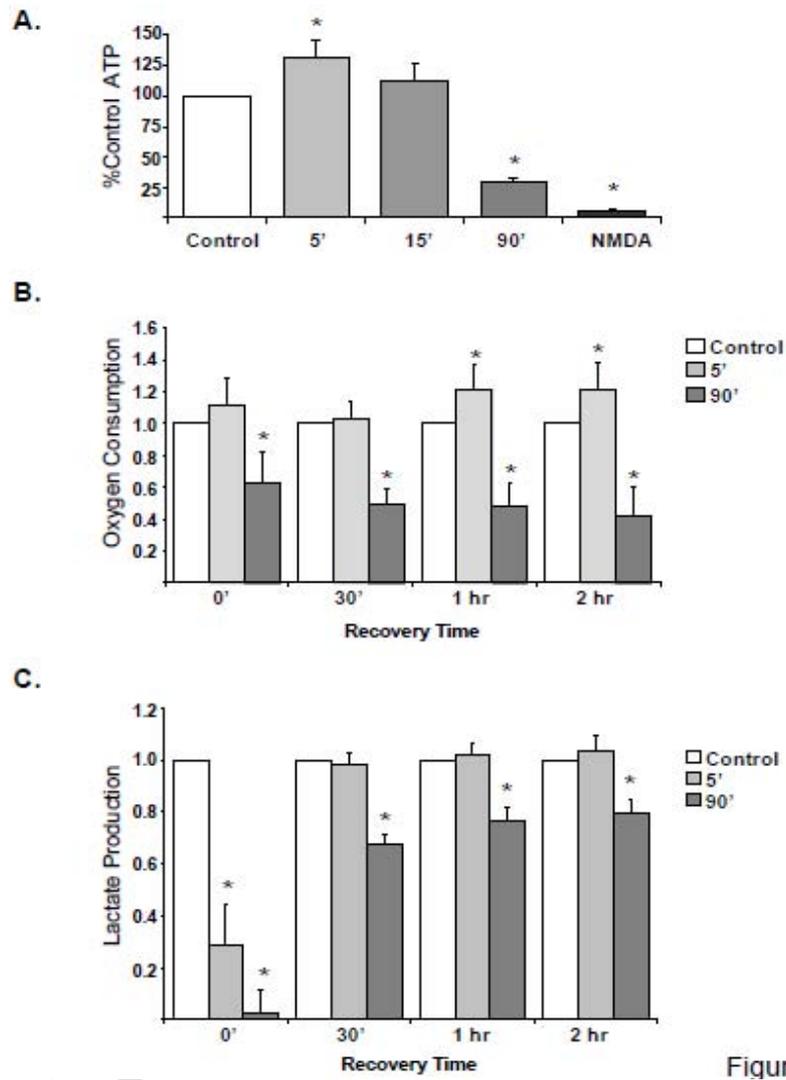


Figure 2

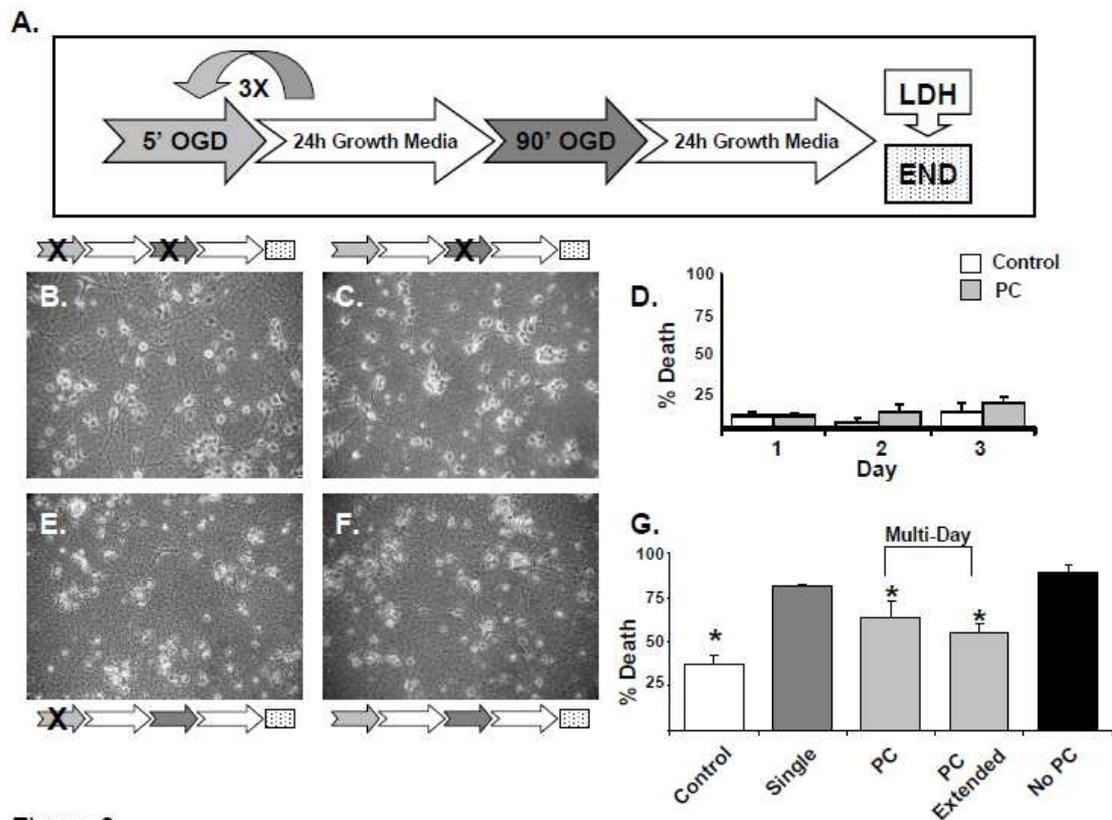


Figure 3

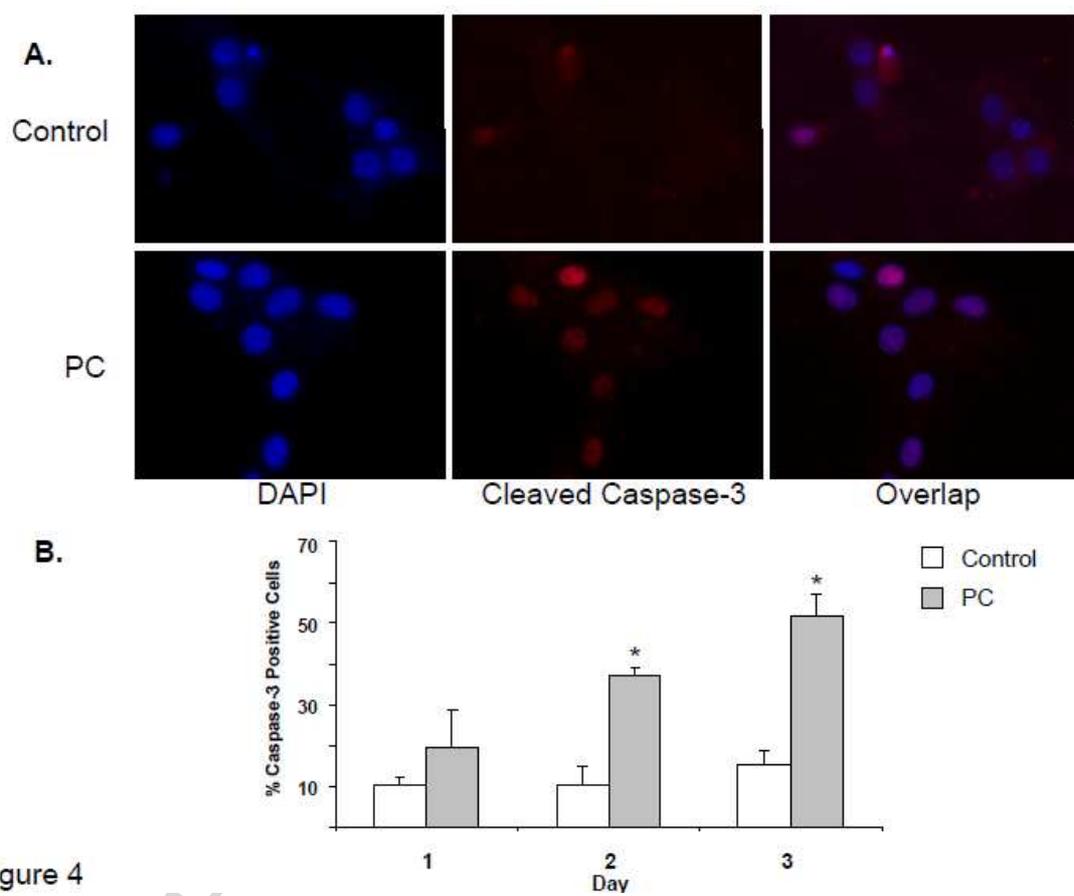


Figure 4

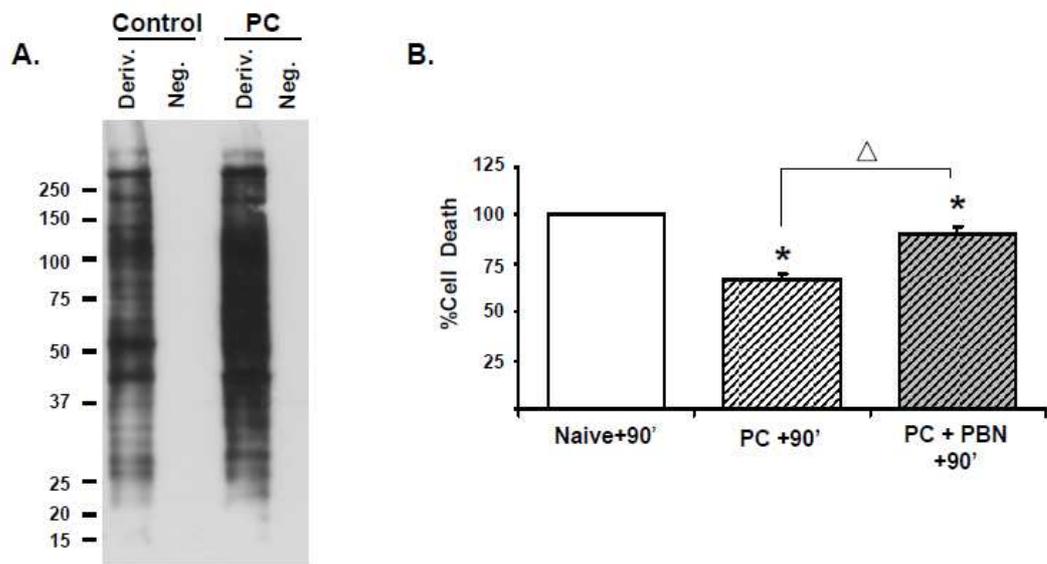


Figure 5

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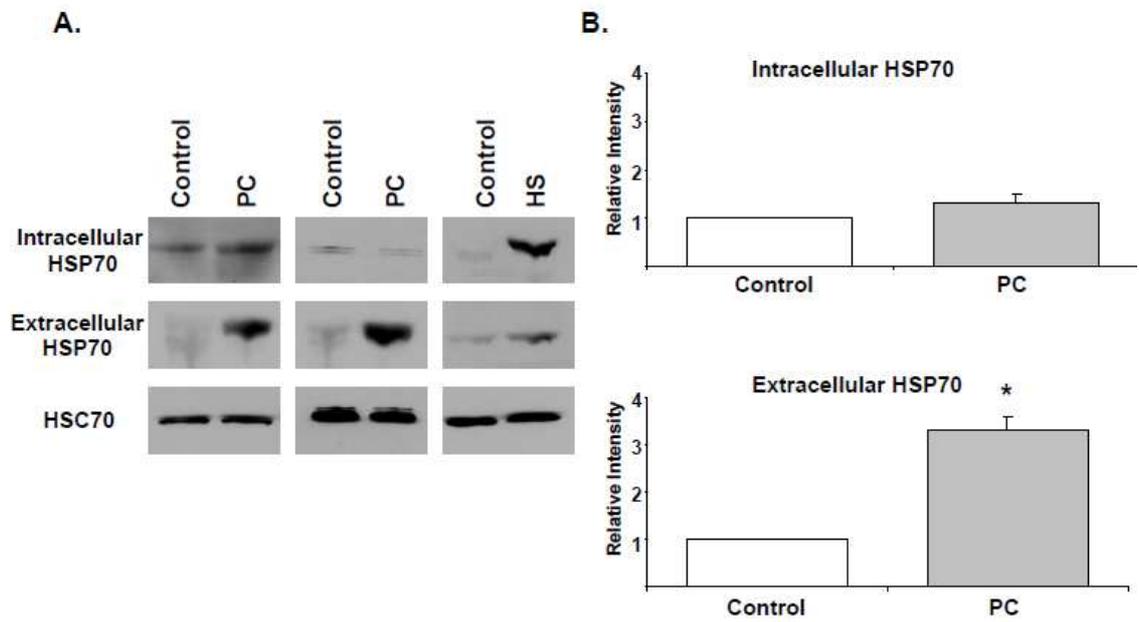


Figure 6