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Differential Nitric Oxide Resistance in Motor Neurons and Oigodendrocytes: Implications for Multiple Sclerosis

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Abstract

Oxidative stress plays a critical role in neurodegenerative diseases and spinal cord injuries. In multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS), 3-nitrotyrosine (3-NY) positive plaques have been observed, suggesting that peroxynitrite ($ONOO^-$) preferentially nitrates proteins after reaction of nitric oxide (NO) with superoxide. Our studies show a differential NO resistance between motor neurons and oligodendrocytes in that motor neurons are much more sensitive to NO damage than are oligodendrocytes. Western blot analysis shows that 3-NY formation correlates to cell death, indicating that peroxynitrite is the damaging agent, rather than NO per se. Resistance to NO can be induced in motor neurons after exposure to low levels of NO. When co-cultured, oligodendrocytes can bestow their resistance to motor neurons, and results suggest that the protective mechanism is due to a factor, rather than a morphological means. These findings elucidate the etiology of MS and the possible role of the heme oxygenase-1 pathway in the defense against this disease.

Introduction

Nitric oxide (NO) is a free radical gas and at low concentrations serves many physiological functions, including cellular differentiation, neurotransmission, and signal transduction (Packer *et al.* 2003). The concentrations at which these functions occur ranges from 50 to 500 nM. At high concentrations, NO is toxic and induces apoptosis in motor neurons. In pathological situations, microglia and astrocytes can produce NO at concentrations beyond 1 μM. Upon reaction with superoxide (O_2^-), peroxynitrite ($ONOO^-$), a reactive nitrogenous species, is produced (Pacher *et al.* 2007). Peroxynitrite preferentially nitrates proteins at tyrosine residues, forming 3-nitrotyrosine(3-NY) (Bishop *et al.* 2006). 3-NY plaques have been found in patients with multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and spinal cord injuries (Pacher *et al.* 2007).

In this study, motor neurons are primed for the cytotoxic dose of NO by induced adaptive resistance (IAR), a process under which the cells are exposed to a low dose of NO (~25 nM/s) prior to treatment (Bishop *et al.* 2009b). This mechanism of resistance is dependent on heme oxygenase-1 (HO-1) in a motor neuron cell line (Bishop *et al.* 2004). Furthermore, oligodendrocytes demonstrate a native resistance to NO at concentrations that are cytotoxic to motor neurons. When grown in co-culture, oligodendrocytes can bestow their resistance to motor neurons (Bishop *et al.* 2009a). This study investigates the implications of IAR and differential NO sensitivity for therapy in MS.

Materials and Methods

Cell lines

The NSC-34 cell line, a fusion of primary mouse spinal cord motor neurons with spinal neuroblastoma cells, was used as the motor neuron model. Upon serum reduction from 10% to 2%, the NSC-34 cells become NSC-34D, a further differentiated version that have characteristics

of differentiated motor neurons, such as expression of NMDA receptors, and a limited ability to divide (Eggett *et al.* 2000).

The MO3.13 cell line, an immortalized human-human hybrid cell line was used as the oligodendrocyte model. This line was created from the fusion of a 6-thioguanine resistant mutant of the human rhabdomyosarcoma with adult human oligodendrocytes. These cells also differentiate upon serum deprivation and have characteristics of mature primary oligodendrocytes, including the expression of myelin basic protein, CNPase, proteolipid protein, and O1 protein (McLaurin *et al.* 1995).

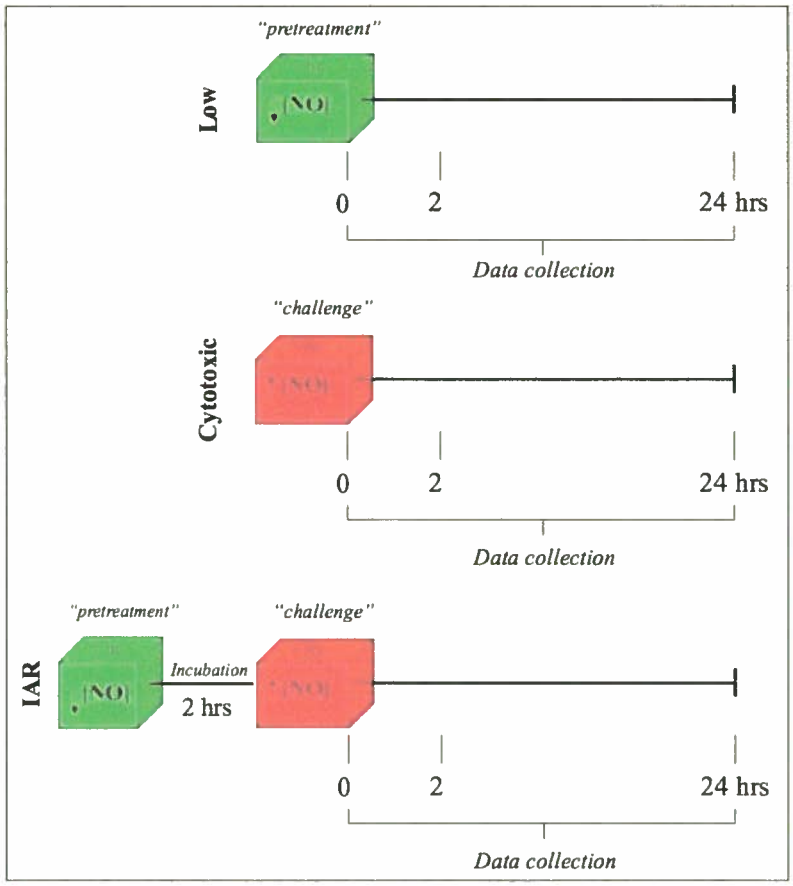
All cells were grown in a humidified 5% CO_2 environment in 1:1 Dulbecco's modified Eagle's medium (DMEM) without sodium pyruvate/Ham's F-12 mixture, 1% non-essential animo acids (NEAA), and 2% heat inactivated FBS.

Cell treatments

Motor neurons and oligodendrocytes were plated at the same density in six-well plates, fed with 1 mL of media at the same temperature and pH and treated with a range of NO doses. For treatment at "pretreatment" NO dose, the compound (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate, DETA NONOate with a half life of 20 hours at 37°C was used. For treatment at "challenge" NO dose, the compound (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate, spermine NONOate with a half life of 39 minutes at 37°C was used (Bishop *et al.* 2009a). The donors were prepared in aqueous solutions at pH 10 and 4°C to prevent NO release. After a 1 hour treatment with the prescribed NO donor, the media was changed to eliminate the NO donor. Cell counts were performed 24 hours after NO treatment because the onset of maximal damage occurs at this time (Bishop and Anderson 2005).

The induced adaptive resistance (IAR) protocol was performed by treating the cells with 10 μM DETA NONOate for 1 hour, refreshing the media with motor neuron-spent media, incubating for 2 hours, and challenging them with 1 mM spermine NONOate as in the cytotoxic protocol (Figure 1).

Figure 1. NO protocol



Other treatments included the addition of 20 μM zinc protoporhyrin-IX, a heme oxygenase-1 inhibitor (Akins *et al.* 2004) and 1 mM uric acid, a peroxynitrite scavanger (Pacher *et al.* 2007). These agents were tested alone and with NO donors to control for any toxicity with the agents, themselves. For the oligodendrocyte media overlay treatment, 48 hour-old media from the oligodendrocytes was transferred to six-wells containing motor neurons prior to NO treatment. The oligodendrocyte-spent media contained no oligodendrocyte cells because the cells are adherent to the plate. Trypsin was added to the spent media to determine whether a key protein was being secreted by the oligodendrocytes.

Cell survival assay

Cell viability was determined by Trypan blue exclusion, intact morphology, and neurite outgrowth. A minimum of 200 cells were counted from at least five randomly chosen fields.

Cell survival was calculated by dividing post-treatment totals by the post-treatment untreated cell counts and multiplying by 100%.

Western blot

Dead cells were collected by centrifugation and combined with live cells of the same treatment that were collected with lysis buffer. The cells were mechanically lysed with a 21 gauge syringe and preserved with protease inhibitor at -80°C. For western blot samples, the protein concentrations were determined using the Bio-Rad DC Protein Assay. Cell lysates were loaded with equal protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, run at 100 V for approximately 1.5 hours, and transferred onto nitrocellulose membranes for 2 hours at 80 V and 4°C. The blots were washed, blocked with 3% BSA, incubated with rabbit primary anti-3-NY (1:1000; Upstate Biotechnology 06-284) and developed by a colorimetric secondary antibody (Bio-Rad 170-5052). A biotintylated broad range molecular weight marker (Bio-Rad 161-0319)was used and nitrated albumin (Sigma-Aldrich N8159) was used as a positive control. Analysis of the membranes was conducted in UN-SCAN IT gel densitometry software (Silk Scientific).

Statistical analysis

Experiments were repeated a minimum of four times. The mean of the cell counts were taken, and the SEM was calculated. The data was analyzed by a two-tailed *t* test and significance (*p* value) was determined. A value of $p < 0.01$ was marked as significant.

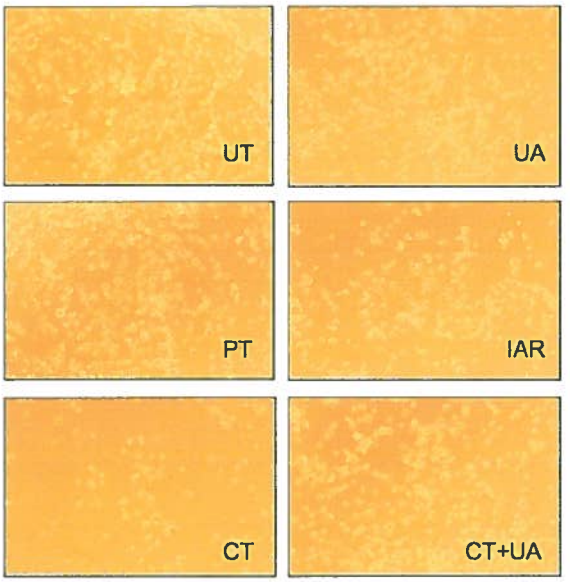
Results

Peroxynitrite-mediated cell death and IAR in motor neurons

Cell treatments were performed and pictures were taken 24 hours post treatment (Figure 2). Cell counts are quantified in Figure 3. Motor neurons treated with 1 mM spermine NONOate (CT) had most cell death with a survival rate of < 10%. The morphology of the cells in the

cytotoxic treatment changed such that there is little neurite outgrowth. The uric acid (UA) treatment and the addition of 10 μM DETA NONOate (PT) had little effect on the cells. The addition of uric acid in the cytotoxic treatment (CT+UA) restored cell viability. Also, pretreatment with a subtoxic dose, followed by exposure to the cytotoxic dose (IAR) saved the motor neurons from cell death.

Figure 2. Motor neuron morphology



The cell counts reflect the pictures of selected fields in the treatments. Survival of cells in IAR was 72% and 67% in the CT+UA treatment. The addition of 20 μM zinc protoporphyrin-IX in the IAR treatment (IAR+ZnPP) resulted in a loss of resistance.

Western blot analysis showed that there is a positive correlation between 3-nitrotyrosine and cell death (Figure 4). Whole lane analysis of the membranes, which were probed for 3-NY,

Figure 3. Motor neuron cell counts

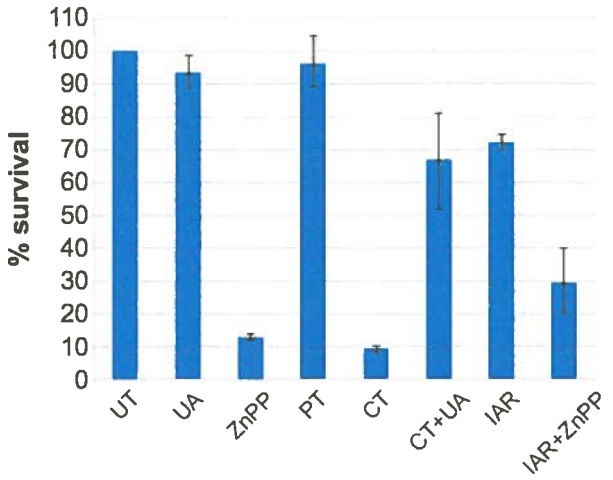
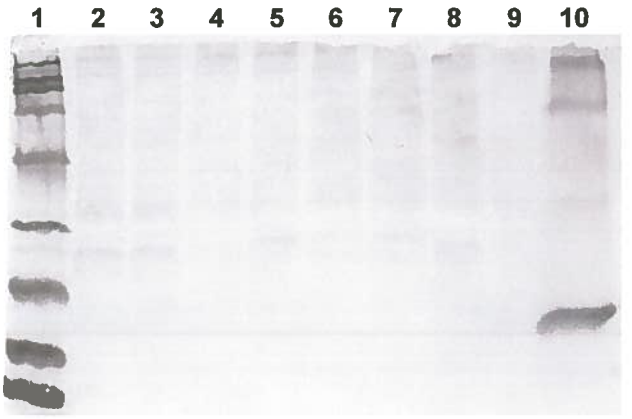


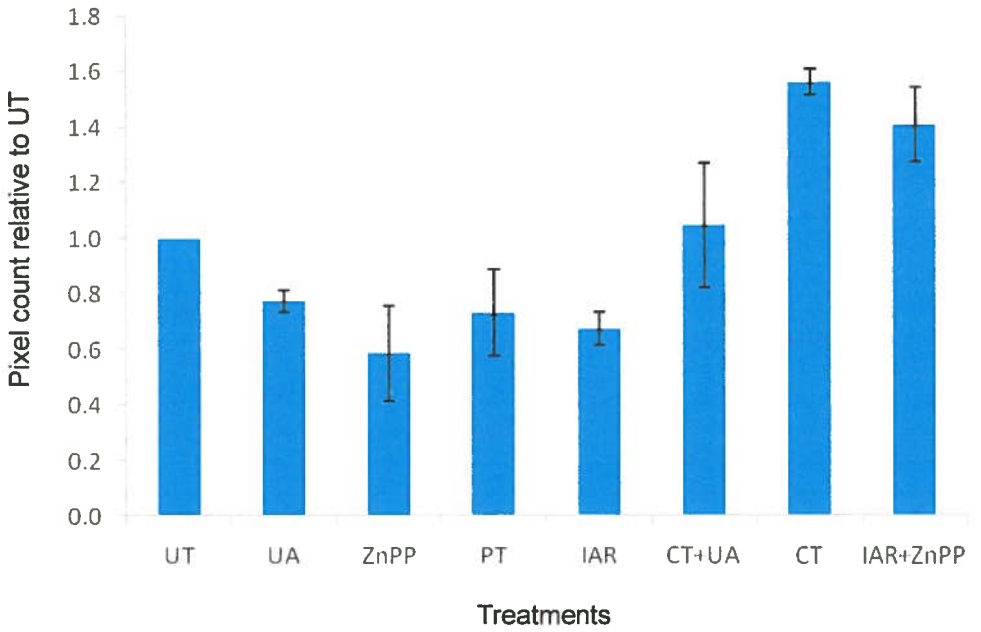
Figure 4. Western blot



1 molecular weight marker; 2 UT; 3 UA; 4 ZnPP; 5 PT; 6 CT; 7 CT+UA; 8 IAR; 9 IAR+ZnPP; 10 nitrated albumin (positive control)

demonstrated that CT had the most 3-NY formation when compared with the untreated (UT). IAR+ZnPP had 1.4 times more 3-NY residues than UT. All other treatments had equal or less 3-NY than the untreated (Figure 5).

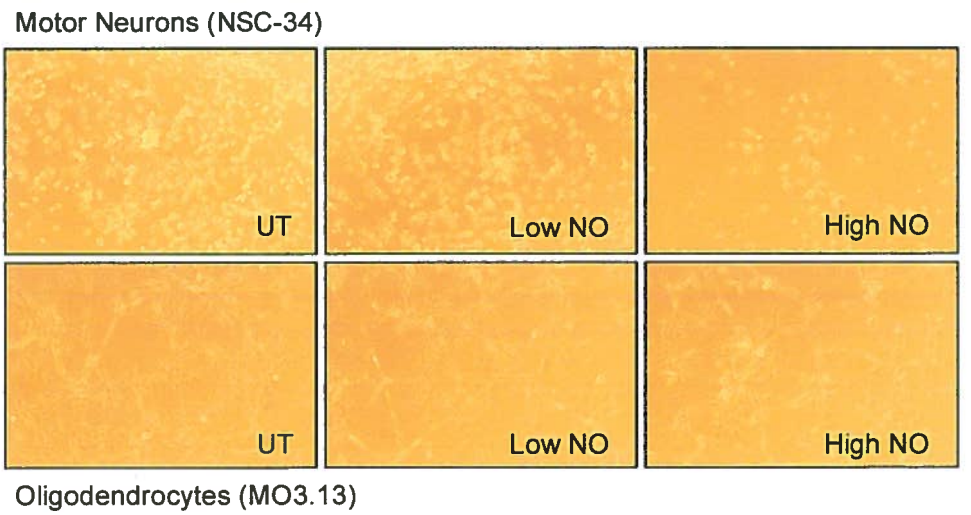
Figure 5. Densitometry analysis: treatments compared to untreated (UT)



Differential NO resistance in oligodendrocytes

Compared to motor neurons, oligodendrocytes are not as sensitive to NO damage (Figure 6). At the cytotoxic dose (high NO) nearly 80% of the oligodendrocytes survive, whereas there

Figure 6. Morphological differences between motor neurons and oligodendrocytes



is less than 10% survival in motor neurons (Figure 7). At low NO dose, there is relatively no change in either cell lines. To test whether the HO-1 pathway has a role in the native resistance of the oligodendrocytes, zinc protoporphyrin-IX (ZnPP)was added as a heme oxygenase-1 inhibitor. The control showed that ZnPP by itself is quite toxic, and when coupled with a high dose of NO (CT+ZnPP), resistance in oligodendrocytes is turned off. However, the addition of uric acid to the same treatment (CT+UA+ZnPP) restores viability, while using 4 mM of spermine NONOate (4XCT) results in only 37% cell survival (Figures 8 and 9).

Figure 7. Differential sensitivity to NO

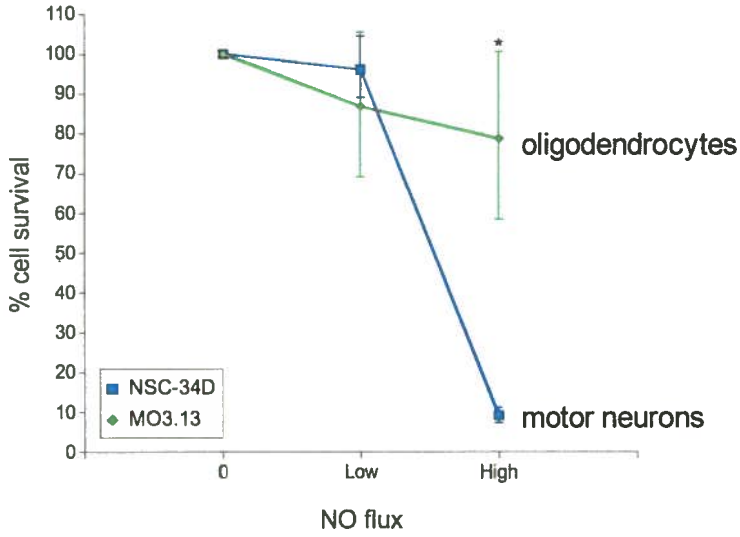


Figure 8. NO sensitivity in oligodendrocytes

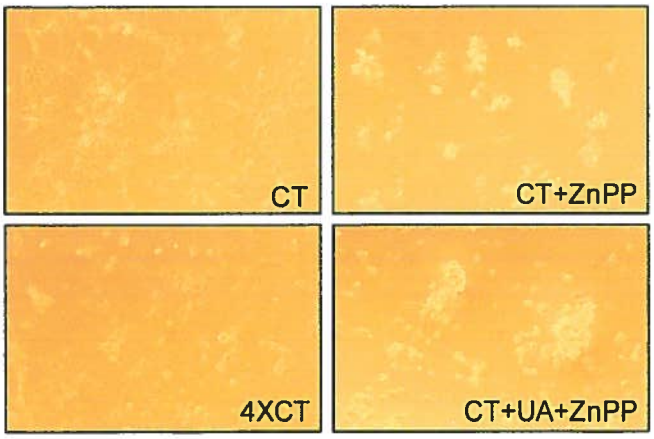
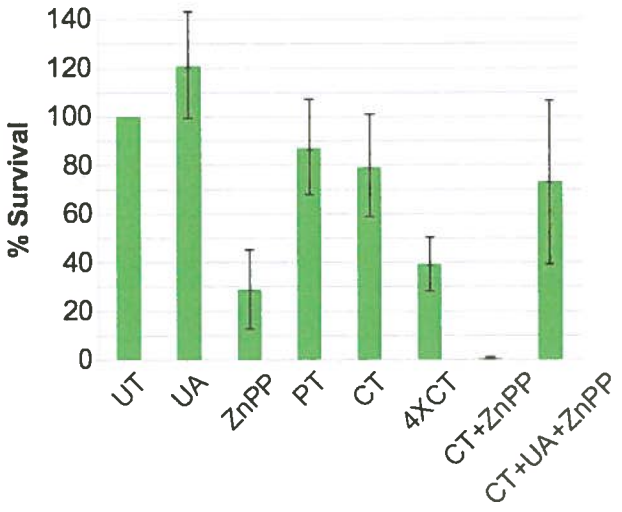


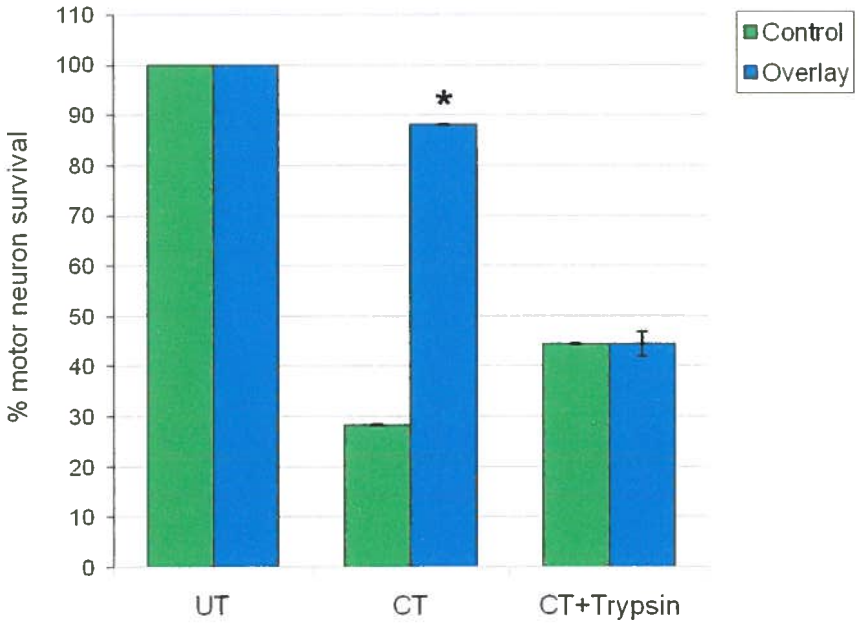
Figure 9. Oligodendrocyte cell counts



Increased motor neuron survival with oligodendrocyte aid

In previous studies by Bishop *et al.*, co-culturing motor neurons with oligodendrocytes showed that oligodendrocytes can bestow resistance on motor neurons (2009a). To determine whether the oligodendrocytes were saving the motor neurons morphologically or by secreting a

Figure 10. Motor neuron survival with oligodendrocyte media overlay



factor key to resistance, oligodendrocyte-spent media was placed on motor neurons prior to treatment. The media overlay showed in increase in motor neuron survival from 28% to 88%. Addition of trypsin, which cleaves proteins at lysine and arginine residues except when either is followed by a proline, resulted in a loss of resistance with survival of only 44% of the motor neurons (Figure 10).

Discussion

The results from this study shows that NO toxicity in motor neurons and oligodendrocytes is peroxynitrite-mediated. The addition of uric acid, a peroxynitrite scavenger, in the cytotoxic treatment (CT+UA) saved the sensitive motor neurons from the cytotoxic dose (Figure 3). Pictures of the cells demonstrate less neurite outgrowth, indicating that the motor neurons have been induced to apoptosis (Bishop *et al.* 2006). However, at the low NO dose (PT), cell survival remained high at 96%, suggesting NO is only harmful to motor neurons at high flux.

Induced adaptive resistance had a positive effect on motor neurons. The pretreatment of the cells with low dose before exposure to the high dose allowed cell survival to reach 72%, demonstrating that motor neurons can be induced to become resistant. The loss of resistance upon addition of zinc protoporphyrin-IX showed that the HO-1 pathway plays a critical role in resistance (Bishop *et al.* 2009b). Examination of 3-NY in the treatments revealed that cell death is positively correlated with 3-NY formation, and that the abrogation of 3-NY is important in therapy of diseases involving peroxynitrite-mediated cell death (Figure 5).

Furthermore, oligodendrocytes showed a native resistance to NO damage at the cytotoxic dose used in this study. Using 4 times the cytotoxic dose resulted in 37% oligodendrocyte survival, indicating that oligodendrocytes are sensitive to some degree, but are much more robust than the motor neurons. The inhibition of HO-1 by zinc protoporphyrin-IX showed a loss in resistance, supporting the data from the motor neuron experiments that the metabolites of HO-1

is necessary for the mitigation of 3-NY formation and subsequent cell death. In addition, treatment with uric acid for oligodendrocytes in this treatment (CT+UA+ZnPP) demonstrated again that cell death is peroxynitrite-mediated (Figure 9).

From co-culture studies by our lab, we have demonstrated that oligodendrocytes can lend their native resistance to motor neurons (Bishop *et al.* 2009a). The oligodendrocyte media overlay experiment showed that oligodendrocytes share this resistance by secreting a factor, rather than physically protecting the axons of the motor neurons. The difference in motor neuron viability from CT in the control to CT in the experimental treatment is significant, suggesting that the isolation of this factor has practical implications for drug development for MS. The trypsin treatment of the oligodendrocyte-spent media verifies that a protein secreted by oligodendrocytes is key to resistant activity (Figure 10).

Conclusion

Results from this study show that motor neurons are comparably more sensitive to NO than are oligodendrocytes, and that the toxicity to NO is from peroxynitrite nitrating proteins and affecting normal protein structure and function (Pacher *et al.* 2007). Moreover, inhibition of HO-1 results in a detrimental loss of this native resistance in oligodendrocytes and induced resistance in motor neurons by IAR. Since the metabolites of HO-1 are anti-oxidants, the oligodendrocytes may be secreting HO-1, itself, or a protein with HO-1 activity (Bishop *et al.* 2009a). The fact that media from oligodendrocytes contain a factor that is critical to motor neuron viability to the cytotoxic dose has tremendous therapeutic implications on MS. Identifying this unknown factor and understanding the mechanism by which protein nitration leads to apoptosis are important in elucidating the etiology of MS.

Acknowledgement

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