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AUTHOR SUMMARY

Oxidative stress plays a critical role in the immune defense by killing pathogens and tumor cells, but this form of stress also can promote disease processes (1). The vast number of oxidation products and their potential targets has made it difficult to identify the critical biological bases of such pathogenic mechanisms. Our previous work showed that a potent oxidant known as "peroxynitrite" ($ONOO^-$) activates distinct cell-death pathways in motor neurons and in PC12 cells, a cell line used to study neural cell growth and differentiation (1, 2). Oxidative damage to proteins by $ONOO^-$ can be detected by examining the addition of nitro groups to tyrosine residues. This process is not random and occurs at specific sites on surprisingly few proteins (3). Because cell death induced by $ONOO^-$ in motor neurons and PC12 cells can be prevented by small molecules that avert tyrosine nitration (4), we hypothesized that nitration of a particular protein might activate specific death-signaling pathways. The challenge has been to distinguish the nitrated proteins that act as causal drivers of pathology from the far more common sites of protein nitration that may constitute collateral damage with minimal functional consequences.

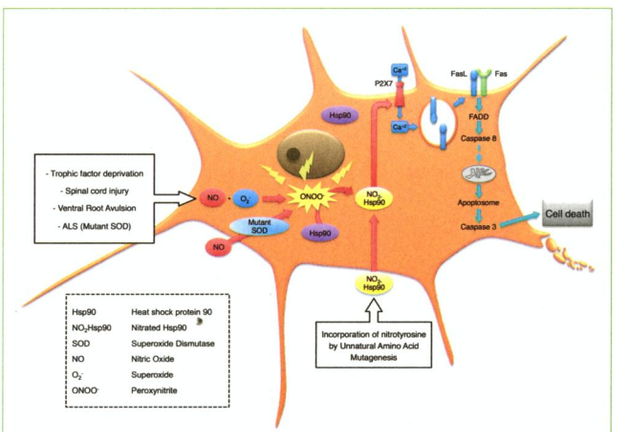

Fig. P1. Induction of motor neuron death by nitrated Hsp90. Many types of stress, including deprivation of growth factors and exposure to nitric oxide in cells that express mutant SOD, result in the endogenous production of $ONOO^-$. Nitration of either of two tyrosine residues on the prosurvival protein Hsp90 is sufficient to activate a gain of function that makes the protein toxic. The activation of the P2X7 receptor by nitrated Hsp90 results in an influx of calcium that mobilizes FasL to the plasma membrane and activates the FADD-mediated Fas pathway. These events culminate in cell death by apoptosis.

Mass spectrometry revealed that the prosurvival protein heat shock protein 90 (Hsp90) is a major target for $ONOO^-$ in vivo and that five of its 26 tyrosine residues are susceptible to nitration. Neuronal death was triggered by the intracellular delivery of purified Hsp90 treated with $ONOO^-$. Even without induction, constitutive Hsp90 expression accounts for ~3% of soluble protein in motor neurons. Delivery of only ~4% of nitrated Hsp90 relative to endogenous, unmodified Hsp90 was sufficient to induce motor neuron death, indicating that nitration of Hsp90 elicits a gain of function that turns the protein from prosurvival to toxic. The replacement of the five tyrosine residues susceptible to nitration by $ONOO^-$ to nitration-resistant phenylalanine residues prevented recombinant Hsp90 from becoming toxic after treatment with $ONOO^-$. Retention of either tyrosine residues 33 or 56 allowed this mutated form of Hsp90 to become toxic after treatment with $ONOO^-$, whereas tyrosine at the other three sites did not affect toxicity.

These results were supported further by using site-specific unnatural amino acid mutagenesis to incorporate a nitrotyrosine residue rather than a tyrosine residue directly at each of the five known sites of nitration in Hsp90; only nitrotyrosine incorporated at either position 33 or 56 on Hsp90 induced motor neuron death. Thus, incorporation of a single nitrotyrosine near the ATP-binding pocket of the protein was sufficient to induce motor neuron death at the same levels observed for the $ONOO^-$-treated recombinant Hsp90 protein.

Further study revealed that the toxic form of nitrated Hsp90 was present in motor neurons of spinal cords from sporadic amyotrophic lateral sclerosis (ALS) patients, in animal models of ALS, and in spinal cords following experimental spinal cord contusion. These results show that nitration of Hsp90 occurs in vivo in the cells and tissues most affected by chronic degenerative diseases like ALS as well as after acute trauma to the central nervous system.

Furthermore, we were able to show that nitration of Hsp90 induced motor neuron death by affecting P2X7-mediated activation of the Fas death pathway (Fig. P1). P2X7 is an extracellular ATP receptor/channel implicated in inflammation and cell death, and the binding of Hsp90 to its intracellular domain regulates P2X7 activity. Inhibitors of P2X7, the Fas pathway, and

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caspases—proteins that, when activated, are a hallmark of cell death by a process known as apoptosis—all prevented motor neuron death induced by nitrated Hsp90. In these cells, the activation of P2X7 resulted in a calcium influx that mobilized Fas ligand (FasL) from intracellular vesicles to the plasma membrane. Remarkably, the cell-death pathway that was activated by nitrated Hsp90 is the same pathway triggered by the expression of mutant superoxide dismutase (SOD), an antioxidant enzyme that, when mutated, is linked to familial ALS (5).

The approach used here provides conclusive evidence that selective nitration at a specific tyrosine residue converts Hsp90 from a prosurvival protein into a potent mediator of neuronal cell death. The high cellular abundance of Hsp90 makes it a potential sensor for $ONOO^-$. In pathological conditions such as ALS and spinal cord injury, nitration of Hsp90 may be the key identifier for highly damaged and subfunctional cells to activate death and removal pathways.

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