Science AAAS

Toxicity of ALS-Linked SOD1 Mutants
Toni L. Williamson, *et al.*
Science **288**, 399a (2000);
DOI: 10.1126/science.288.5465.399a

The following resources related to this article are available online at www.sciencemag.org (this information is current as of July 15, 2009):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:
http://www.sciencemag.org/cgi/content/full/288/5465/399a

This article **cites 20 articles**, 10 of which can be accessed for free:
http://www.sciencemag.org/cgi/content/full/288/5465/399a#otherarticles

This article has been **cited by** 3 articles hosted by HighWire Press; see:
http://www.sciencemag.org/cgi/content/full/288/5465/399a#otherarticles

This article appears in the following **subject collections**:
Neuroscience
http://www.sciencemag.org/cgi/collection/neuroscience
Technical Comments
http://www.sciencemag.org/cgi/collection/tech_comment

Information about obtaining **reprints** of this article or about obtaining **permission to reproduce this article** in whole or in part can be found at:
http://www.sciencemag.org/about/permissions.dtl

Downloaded from www.sciencemag.org on July 15, 2009

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. Copyright 2000 by the American Association for the Advancement of Science; all rights reserved. The title *Science* is a registered trademark of AAAS.

Toxicity of ALS-Linked SOD1 Mutants

Estévez *et al*. (*1*) focused on a potential mechanism through which dominantly inherited mutation in superoxide dismutase 1 (SOD1), an abundant, ubiquitously expressed antioxidant protein, triggers the selective death of motor neurons in amyotrophic lateral sclerosis (ALS). Each subunit of SOD1 binds one zinc and one copper atom. Dismutation of the superoxide radical to H_2O_2 or O_2 requires enzyme-bound copper, which alternates between reduced (Cu^{1+}) and oxidized (Cu^{2+}) forms during two asymmetric catalysis steps. Evidence from transgenic mice showed that the mutants confer disease independently of the level of SOD1 activity (*2*–*6*), findings widely interpreted to indicate that the mutations acquire one or more toxic properties. Several previous proposals have focused on potential sources of toxicity linked to aberrant copper-mediated catalysis (*7*–*9*), and all mutants examined do bind the copper in vivo (*10*).

Estévez *et al*. (*1*) proposed that, relative to wild-type SOD1, mutant subunits fail to bind or retain the zinc atom, thereby allowing rapid reduction of mutant SOD1 to the Cu^{1+} form by abundant intracellular reductants. The reduced SOD1 mutant would then run the normal catalytic step backwards, converting oxygen to superoxide; the superoxide so produced would combine with freely diffusing nitric oxide (NO), producing peroxynitrite, which would promote intracellular damage including protein nitration. The primary evidence supporting this view was that introduction by liposome fusion of purified, zinc-depleted SOD1 provoked rapid death of cultured motor neurons. Toxicity required both zinc depletion and bound copper.

This evidence, although persuasive in vitro, may have little relevance to the in vivo pathway of motor neuron death. Zinc-depleted wild-type SOD1 was just as toxic as the ALS-linked SOD1 mutants, even though the wild-type protein should be competent to acquire zinc in vivo and thereby moderate toxicity. Also, evidence for altered zinc binding—accelerated zinc release by four mutants relative to wild-type SOD1—required the presence of a protein denaturant and was not found at physiological ionic conditions (*9*). Although zinc-depleted mutant subunits have been found to compete less effectively with metal chelators for binding to free zinc (*9*), this in vitro measure should not be taken as evidence of the in vivo situation, because both protein folding and metal acquisition are facilitated in vivo. And none of the 67 identified disease-related mutations in the gene encoding the 153 amino acid SOD1 polypeptide (*11*) lies in any of the four residues that directly coordinate the zinc.

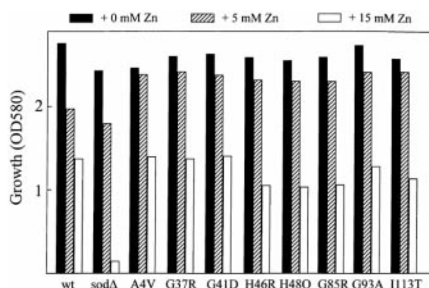

Fig. 1. FALS-SOD1 subunits buffer zinc as effectively as wild-type SOD1 in vivo. Growth of *sod1Δ cup1Δ* yeast (JS2004) harboring yeast SOD1-promoted FALS-SOD1 mutant expression plasmids (*10*) was monitored in media with varying levels of zinc.

In the cell culture model of Estévez *et al*. (*1*), inhibitors of neuronal NO synthase (nNOS) and immunocytochemical detection of nitrotyrosine, a footprint left by peroxynitrite, were used to demonstrate a dependence of toxicity on NO. Limiting NO production by loss of nNOS would therefore be predicted to ameliorate disease. Yet, in SOD1 mutant–mediated disease in mice, disruption of the gene for nNOS, accompanied by 14-fold reduction in detectable NOS activity, does not affect disease onset or progression (*12*).

Perhaps most compelling, neurofilaments, the subunits of which bind zinc in vitro, are an abundant component of motor neurons. As proposed by Estévez *et al*. (*1*), competition with neurofilaments would lower mutant SOD1-bound zinc; thus, raising the neurofilament content should exacerbate disease if reduction in SOD1-bound zinc produces toxicity. Just the opposite happens, however—in by far the most robust amelioration of ALS to date, raising the content of neurofilaments in motor neuron cell bodies by increasing the synthesis of neurofilament H (NF-H) slowed disease onset by six months (*13*).

We put the Estévez *et al*. (*1*) proposal to two additional in vivo tests, the first using yeast in which the yeast SOD1 gene had been replaced with human wild-type SOD1 or any of a series of ALS-linked mutants. When the mutant and wild-type proteins were allowed to accumulate to comparable levels (*10*), mutant strains protected yeast from toxicity of environmental zinc about as efficiently (and, thus, bound zinc about as effectively) as the wild type (Fig. 1). Second, a good target for SOD1-mediated nitration has been demonstrated in vitro to be neurofilament subunits (*14*). If peroxynitrite were generated by mutant SOD1, as in the Estévez *et al*. model, tyrosine residues on each of the three subunits should be robust targets for accumulated nitration, because the neuron-specific neurofilaments have biological half lives of at least several months and are especially abundant in motor neurons. We have tested this prediction using mass spectrometry to sequence neurofilament subunits isolated from end-stage animals that have developed motor neuron disease from expressing either the $SOD1^{G37R}$ or $SOD1^{G85R}$ mutants. With coverage of 100% of the 20 tyrosine-containing tryptic fragments of neurofilament L (NF-L), we have been unable to detect any nitrated peptide; nor were we able at any point in the course of disease in these two animal models to detect nitrotyrosine on any target protein using a variety of immunologic methods, including the one used in (*1*).

In sum, although in vitro evidence strongly suggests peroxynitrite-mediated nitration as a component of cell death arising from wild-type or mutant SOD1 introduced in a zinc depleted form (*1*), available in vivo tests offer no support for this mechanism as the one through which the mutants cause motor neuron disease.

Toni L. Williamson
Ludwig Institute for Cancer Research and Departments of Medicine and Neuroscience University of California at San Diego La Jolla, CA 92093, USA and Trophos, Inc. Parc de Luminy Batiment CCIMP - Case 922 13288 Marseille Cedex 9, France

Laura B. Corson
Ludwig Institute for Cancer Research and Departments of Medicine and Neuroscience University of California at San Diego and Samuel Lunenfeld Research Institute Mount Sinai Hospital 600 University Avenue Toronto, Ontario M5G 1X5, Canada

Lan Huang
Al Burlingame
Department of Pharmaceutical Chemistry University of California at San Francisco San Francisco, CA 92143, USA

Jian Liu
Ludwig Institute for Cancer Research and Departments of Medicine and Neuroscience University of California at San Diego

Lucie I. Bruijn
Bristol-Myers Squibb Company Pharmaceutical Research Institute Wallingford, CT 06492, USA

Don W. Cleveland
Ludwig Institute for Cancer Research and Departments of Medicine and Neuroscience University of California at San Diego

Downloaded from www.sciencemag.org on July 15, 2009

References

1. A. G. Estévez *et al., Science* **286**, 2498 (1999).
2. M. E. Gurney *et al., Science* **264**, 1772 (1994).
3. M. E. Ripps, G. W. Huntley, P. R. Hof, J. H. Morrison, J. W. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 689 (1995).
4. P. C. Wong *et al., Neuron* **14**, 1105 (1995).
5. L. I. Bruijn *et al., Neuron* **18**, 327 (1997).
6. L. I. Bruijn *et al., Science* **281**, 1851 (1998).
7. M. Wiedau-Pazos *et al., Science* **271**, 515 (1996).
8. J. S. Beckman, M. Carson, C. D. Smith, W. H. Koppenol, *Nature* **364**, 584 (1993).
9. J. P. Crow, J. B. Sampson, Y. Zhuang, J. A. Thompson, J. S. Beckman, *J. Neurochem.* **69**, 1936 (1997).
10. L. B. Corson, J. J. Strain, V. C. Culotta, D. W. Cleveland, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6361 (1998).
11. P. M. Andersen, M. Morita, R. H. Brown Jr., *Amyotrophic Lateral Sclerosis*, R. H. Brown Jr., V. Meininger, M. Swash, Eds. (Martin Dunitz, London, 2000), p. 223.
12. F. Facchinetti *et al., Neuroscience* **90**, 1483 (1999).
13. S. Couillard-Després *et al., Proc. Natl. Acad. Sci. U.S.A.* **95**, 9626 (1998).
14. J. P. Crow *et al., J. Neurochem.* **69**, 1945 (1997).

20 January 2000; accepted 14 March 2000

Response: Williamson *et al*. question the in vivo relevance of our hypothesis (*1*), relying mainly on results from transgenic mice. Although transgenic mice are powerful tools, their limitations necessitate a combination of different experimental approaches to unravel the pathogenesis of ALS. Indeed, our hypothesis actually helps rationalize many conflicting results obtained in transgenic mice, such as the failure of overexpressed wild-type SOD to protect mice against overexpressing ALS-SOD1 mutants—interpreted by Bruijn *et al*. (*2*) as ruling out a role for superoxide and oxidative stress in ALS. Using both in vitro biochemical studies and cultured motor neurons, we showed that zinc-deficient SOD1 could cause oxidative stress and motor neuron death even in the presence of large amounts of metal-replete Cu,Zn SOD (*1*).

Several points raised by Williamson *et al*. may actually support our proposed mechanism; others highlight the need for further investigation. First, Williamson *et al*. assert that, because zinc-deficient SOD can rebind zinc in vitro, the zinc-deficient forms of SOD1 should rebind zinc once inside the motor neurons and thus become non-toxic. Our results showed that this does not happen: If the SOD1s had become repopulated with zinc, they would have protected the motor neurons from trophic-factor deprivation. Even wild-type SOD did not protect, though both it and the ALS-SOD1 mutants are competent to bind zinc in vitro in seconds. Thus, motor neurons apparently limit the availability of free zinc—possibly an important clue to why toxicity of SOD1 mutants is manifested primarily in these cells in vivo.

Williamson *et al*. question the conditions used to measure relative zinc-binding affinities of mutant and wild-type SOD1. We reported previously that SOD1 mutants bound zinc less avidly than wild-type SOD1 under native conditions and, in a second set of experiments, used 2 M urea to hasten the equilibration between enzyme-bound and chelator-bound zinc and copper to quantify metal dissociation constants (*3*). SOD is an extremely stable protein retaining full enzyme activity in 6 M urea. Even in 2 M urea, there was a maximum 30-fold difference in the affinity of zinc between the most severe mutant (A4V) and wild-type SOD1. Indeed, both wild-type SOD1 and ALS-associated mutants can lose (or fail to acquire) zinc under a wide range of buffer conditions if a strong chelator is present—a phenomenon that could allow even wild-type SOD1 to become toxic under certain pathological conditions. Our motor neuron experiments offer compelling evidence that the absence of zinc in SOD1, rather than the mutation per se, is responsible for killing motor neurons by an oxidative mechanism.

Williamson *et al*. also ask why none of the 67 known SOD1 mutations associated with ALS involve the four amino acid ligands for zinc. Our model predicts that such SOD1 mutants would be constitutively toxic even in non-neuronal cells; consequently, such mutations would not allow carriers to survive long enough to develop ALS, or would result in widespread tissue damage producing disease symptoms unrelated to ALS.

Our results showed that nitric oxide greatly increased the toxicity of zinc-deficient SOD1 to motor neurons—consistent with a biochemical mechanism whereby zinc-deficient SOD1 catalyzes the formation of peroxynitrite from ascorbate, oxygen, and nitric oxide. Facchinetti *et al*. (*4*) reported that crossing G93A transgenic mice with an incomplete neuronal nitric oxide synthase (nNOS) knockout mouse failed to increase survival of G93A SOD1 mice, but also reported that a selective nNOS inhibitor produced a significant survival effect (*4*). Furthermore, nNOS is not the only source of NO in motor neurons, as they also express endothelial NOS (*5*, *6*). In the presence of zinc deficient SOD1, even nanomolar concentrations of nitric oxide become toxic. Thus, essentially all nitric oxide production in and near motor neurons may need to be genetically knocked out or pharmacologically inhibited over several months to observe a pronounced survival effect.

On the subject of neurofilaments, we have previously suggested that abnormal accumulations of NF-L may contribute to the accumulation of zinc-deficient SOD1 because this subunit is extremely abundant in motor neurons and has an exceptionally high capacity to bind zinc (*3*). In vitro, the affinity of disassembled NF-L is sufficient to compete with zinc-deficient SOD1 for binding zinc (*3*). When NF-L knockout mice were crossed with G85R mice, survival was increased (*7*), consistent with NF-L facilitating the accumulation of zinc-deficient SOD1. A second study showed even more pronounced survival when NF-H was overexpressed in G37R mice (*8*). Although the mechanism of protection by NF-H is unknown, one explanation consistent with our hypothesis is that overexpression of NF-H may minimize the binding of zinc by free NF-L subunits. Clearly, additional studies are needed to understand the interactions of the three different neurofilament subunits with zinc and with SOD1 itself.

The yeast experiments of Williamson *et al*. (their figure 1) support our finding that the ALS-SOD1 mutants can bind zinc to form fully functional and protective SOD1s. The ability of the mutants to do so is probably essential for humans to survive until mid to late life before developing ALS. Meanwhile, the failure of Williamson *et al*. to detect nitrotyrosine on neurofilament L peptides isolated from mouse spinal cord homogenates using mass spectrometry is at best weak negative evidence. Motor neurons account for considerably less than 1% of the cells in spinal cord, which results in an enormous dilution of motor neuron proteins upon homogenization. Immunohistochemical studies have revealed that nitrated proteins are most abundant in the soma of the motor neurons in both transgenic mice and human ALS patients (*5, 9–14*). Protein-bound nitrotyrosine has been observed by immunohistochemistry in motor neurons from human ALS and G93A mice, while free nitrotyrosine has been quantified by HPLC in CSF and spinal cord homogenates (*15–17*). Whether nitration is fundamentally related to the disease process remains to be determined. However, the presence of both free and protein-bound nitrotyrosine in human ALS and in transgenic mice indicates that peroxynitrite was formed in these tissues, and our studies have shown that motor neurons are particularly susceptible to endogenously produced peroxynitrite (*1, 18*).

Elucidating the mechanism by which SOD1 mutants cause ALS remains a challenging and complex problem. Transgenic mouse models have provided vital clues, but the existence of redundant compensatory systems limits their usefulness as a primary means of testing specific biochemical hypotheses. Results obtained in animals should be interpreted cautiously to avoid premature closure of promising research avenues. The issues raised by Williamson *et al*. underscore the need for a combined approach including human tissues, transgenic animals, neuronal culture models, and in vitro biochemistry. The ultimate test of our hypothesis regarding zinc-deficient SOD1 will lie in whether it can yield a useful treatment for stopping the progression of ALS.

Joseph S. Beckman
Departments of Anesthesiology,
Biochemistry and Molecular Genetics
and the Center for Free Radical Biology
University of Alabama at Birmingham

Downloaded from www.sciencemag.org on July 15, 2009

Birmingham, AL 35233, USA
E-mail: joe.beckman@ccc.uab.edu

John P. Crow
Departments of Anesthesiology, Pharmacology and Toxicology and the Center for Free Radical Biology University of Alabama at Birmingham

Alvaro G. Estévez
Department of Anesthesiology and the Center for Free Radical Biology University of Alabama at Birmingham

References

1. A. G. Estévez *et al.*, *Science* **286**, 2498 (1999).
2. L. I. Bruijn *et al.*, *Science* **281**, 1851 (1998).
3. J. P. Crow, J. B. Sampson, Y. Zhuang, J. A. Thompson, J. S. Beckman, *J. Neurochem.* **69**, 1936 (1997).
4. F. Facchinetti *et al.*, *Neuroscience* **90**, 1483 (1999).
5. K. Abe *et al.*, *Neurol. Res.* **19**, 124 (1997).
6. A. G. Estévez *et al.*, *J. Neurosci.* **18**, 3708 (1998).
7. T. L. Williamson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9631 (1998).
8. S. Couillard-Després *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9626 (1998).
9. K. Abe, L.-H. Pan, M. Watanabe, T. Kato, Y. Itoyama, *Neurosci. Lett.* **199**, 152 (1995).
10. S. M. Chou, H. S. Wang, K. Komai, *J. Chem. Neuroanat.* **10**, 249 (1996).
11. S. M. Chou, H. S. Wang, A. Taniguchi, *J. Neurol. Sci.* **139** (suppl.), 16 (1996).
12. R. J. Ferrante *et al.*, *Ann. Neurol.* **42**, 326 (1997).
13. R. J. Ferrante *et al.*, *J. Neurochem.* **69**, 2064 (1997).
14. C. I. Cha *et al.*, *Brain Res.* **853**, 156 (2000).
15. M. F. Beal *et al.*, *Ann. Neurol.* **42**, 646 (1997).
16. H. Tohgi *et al.*, *Ann. Neurol.* **46**, 129 (1999).
17. L. I. Bruijn *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7606 (1997).
18. A. G. Estévez *et al.*, *J. Neurosci.* **18**, 923 (1998).

25 February 2000; accepted 14 March 2000

Downloaded from www.sciencemag.org on July 15, 2009