Fas-Induced Caspase Denitrosylation

Joan B. Mannick,[1]* Alfred Hausladen,[2] Limin Liu,[3] Douglas T. Hess,[2] Ming Zeng,[2] Qian X. Miao,[1] Laurie S. Kane,[2] Andrew J. Gow,[2] Jonathan S. Stamler[2,3]*

Only a few intracellular S-nitrosylated proteins have been identified, and it is unknown if protein S-nitrosylation/denitrosylation is a component of signal transduction cascades. Caspase-3 zymogens were found to be S-nitrosylated on their catalytic-site cysteine in unstimulated human cell lines and denitrosylated upon activation of the Fas apoptotic pathway. Decreased caspase-3 S-nitrosylation was associated with an increase in intracellular caspase activity. Fas therefore activates caspase-3 not only by inducing the cleavage of the caspase zymogen to its active subunits, but also by stimulating the denitrosylation of its active-site thiol. Protein S-nitrosylation/denitrosylation can thus serve as a regulatory process in signal transduction pathways.

Programmed cell death, or apoptosis, must be tightly regulated in order to ensure appropriate cell survival. Nitric oxide (NO) and related molecules provide one such level of regulation by inhibiting apoptosis in many cell types (*1–3*). Recent studies suggest that this inhibition is achieved, at least in part, by S-nitrosylation of the active-site cysteine of caspases, a family of cysteine proteases that execute the death program (*2–4*). In particular, it has been shown that NO synthase (NOS) activity can lead to caspase inhibition by a mechanism independent of cyclic guanosine monophosphate, that caspases can be S-nitrosylated by NO donors in cellular and in vitro systems, that the S-nitrosylation takes place solely on the active-site cysteine, and that this modification inhibits caspase activity in a reversible manner (*2–4*). However, the biological significance of these observations is unclear in the absence of a demonstration that caspases are in fact S-nitrosylated endogenously. Furthermore, it is not known whether protein S-nitrosylation/denitrosylation serves as a component of apoptotic or other signaling pathways.

To address these issues, we immunoprecipitated caspase-3 from three different human B and T cell lines that express NOS (Fig. 1A) (*5*). Caspase-3 was efficiently immunoprecipitated with its specific antibody, but not with control antibody (Fig. 1B). Silver stains revealed that associated proteins did not significantly contaminate the caspase immunoprecipitates. In particular, the 32-kD unprocessed caspase-3 zymogen and a previously described 29-kD processing intermediate (*6*), which we identified with two antibodies to caspase-3 and which increased with other processed forms of caspase following Fas stimulation, were the only proteins specifically precipitated by caspase-3 antibody (Fig. 1C). Quantitative analyses indicated that 31 ± 7 nM (mean ± SEM, n = 19) of caspase-3 was immunoprecipitated (*5*). Similar results were obtained in two additional human T cell lines (H9 and CEM).

Nitrosylation of immunoprecipitated proteins was measured by photolysis-chemiluminescence (*7*). The concentration of NO detected in caspase-3 immunoprecipitates (21 ± 4 nM, mean ± SEM, n = 38) was higher than the NO content of paired immunoglobulin G2a (IgG2a) control immunoprecipitates (11 ± 4 nM, mean ± SEM, P = 0.05, n = 9) (Fig. 2A). Pretreatment of caspase-3 immunoprecipitates with $HgCl_2$, which selectively removes NO groups from S-nitrosothiols (SNOs) (*7*), reduced the NO content to control concentrations (Fig. 2A). Nitric oxide groups displaced in this manner from S-nitrosylated recombinant caspase-3 in vitro formed nitrite in solution (*8*). In an additional series of 22 immunoprecipitates, 16 produced nitrite following $HgCl_2$ treatment (*7*). Therefore, the NO detected in caspase-3 immunoprecipitates appears to be derived from SNO bonds. Taken together with studies in vitro that show that caspases are nitrosylated on a single cysteine (*2*), these results indicate that a significant proportion of caspase-3 is S-nitrosylated intracellularly.

Immunoprecipitates of caspase-8, which associates with Fas, also contained NO groups. However, the NO content was only slightly higher than that of control immunoprecipitates, and the differences did not reach statistical significance, perhaps because the concentration of

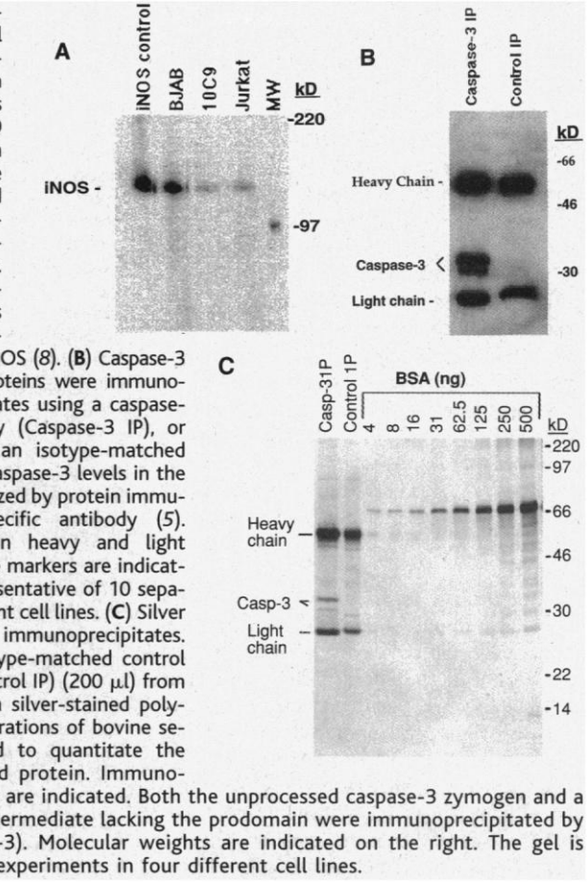

Fig. 1. NOS and caspase-3 expression in human lymphocyte cell lines. (**A**) NOS expression in human lymphocyte cell lines. Protein immunoblots of whole-cell lysates derived from 5×10^5 BJAB, 10C9 or Jurkat cells were done with an iNOS-specific antibody. A mouse macrophage cell lysate was used as an iNOS positive control. Molecular size markers (in kilodaltons) are indicated on the right. Lower amounts of iNOS are expressed in Jurkat and 10C9 lysates and higher amounts in BJAB lysates. Jurkat cells also contain nNOS (*8*). (**B**) Caspase-3 levels in immunoprecipitates. Proteins were immunoprecipitated from 10C9 cell lysates using a caspase-3–specific monoclonal antibody (Caspase-3 IP), or with equal concentrations of an isotype-matched control antibody (Control IP). Caspase-3 levels in the immunoprecipitates were visualized by protein immunoblot using a caspase-3-specific antibody (*5*). Caspase-3 and immunoglobulin heavy and light chains are shown. Molecular size markers are indicated on the right. The gel is representative of 10 separate experiments in three different cell lines. (**C**) Silver stain of caspase-3 and control immunoprecipitates. Caspase-3 (Casp-3 IP) and isotype-matched control IgG2a immunoprecipitates (Control IP) (200 μl) from Jurkat cells were analyzed on a silver-stained polyacrylamide gel. Various concentrations of bovine serum albumin (BSA) were used to quantitate the amount of immunoprecipitated protein. Immunoglobulin heavy and light chains are indicated. Both the unprocessed caspase-3 zymogen and a 29-kD caspase-3 processing intermediate lacking the prodomain were immunoprecipitated by the caspase-3 antibody (Casp-3). Molecular weights are indicated on the right. The gel is representative of 18 separate experiments in four different cell lines.

[1]Department of Adult Oncology, Dana Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA. [2]Department of Medicine, Divisions of Respiratory and Cardiovascular Medicine and Department of Cell Biology, and [3]Howard Hughes Medical Institute, Duke University Medical Center, Box 2612, 321 MSRB, Durham, NC 27710, USA.

*To whom correspondence should be addressed. E-mail: (J.S.S.) staml001@mc.duke.edu and (J.B.M.) Joan_mannick@dfci.harvard.edu

caspase-8 in the immunoprecipitates was very low (<5 nM). Therefore, we were unable to establish whether caspase-8 or an associated protein is S-nitrosylated intracellularly.

To determine if caspase-3 is S-nitrosylated endogenously on its active-site cysteine, MCF-7 cells [which do not express caspase-3 (*9*)] were transiently or stably transfected with plasmids expressing wild-type caspase-3 or a caspase-3 mutant in which the catalytic-site cysteine is replaced by an alanine (*10, 11*). Wild-type and mutant procaspase-3 were immunoprecipitated from the transfected cells, and the level of S-nitrosylation was measured by photolysis-chemiluminescence. The NO content of the wild-type caspase-3 immunoprecipitates was consistently higher than that of the mutant immunoprecipitates (Fig. 2B), although equal or lower amounts of wild-type caspase were immunoprecipitated from transfected cells (Fig. 2D) (*8*). Moreover, the molar SNO: caspase ratio in MCF-7 cells (Fig. 2, B and C) was similar to that found in human lymphocyte cell lines. We attribute the background signals in control immunoprecipitates (IgG2a or mutant caspase-3) to other basally nitrosylated proteins and to the small amounts of NO_x, which we detected in the cell lysates of samples used for immunoprecipitations (*5, 8*). Taken together with previous studies (*2, 3*), these data indicate that caspase-3 S-nitrosylation takes place on its active-site cysteine.

Because caspase S-nitrosylation is inhibitory (*2–4*), yet Fas promotes caspase activation, we reasoned that Fas induces caspase denitrosylation. To test this hypothesis, we immunoprecipitated caspase-3 from 10C9 (n = 7) and CEM (n = 6) cells that had been stimulated with Fas agonist antibody (*12*). Nine of 13 immunoprecipitates contained SNO at time zero; in these nine, SNO levels decreased an average of 77% ($P < 0.0005$) approximately 1.5 to 2 hours after Fas cross-linking (Fig. 3). Silver stains and protein immunoblots revealed that caspase levels were not changed significantly by Fas over this interval and that only a minority of caspase-3 zymogen had been cleaved to its active subunits (Fig. 3, A and B). Thus, in 10C9 and CEM cells, Fas activation decreased the S-nitrosylation of at least a subset of caspase zymogen before it was processed to its active form. Cleavage of zymogen before denitrosylation is not, however, precluded by these data.

The decline in S-nitrosylated caspase-3 could have resulted from either a decrease in the rate of S-nitrosylation or an increased rate of denitrosylation. To distinguish between these possibilities, we analyzed the extent of caspase-3 S-nitrosylation in cells grown in the presence or absence of the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMA) (*13*). Inhibition of intracellular NO production by L-NMA for 2 hours did not measurably decrease caspase-3 S-nitrosylation, although clear decreases were noted by 24 to 48 hours (*8*). Thus, the decline in caspase-3 S-nitrosylation 1.5 to 2 hours after Fas activation is evidently the result of an increase in denitrosylation activity. In support of this conclusion, we found no correlation between the NO content of cell lysates and the SNO content of immunoprecipitates from these lysates, suggesting that changes in whole-cell NOS activity do not account for the changes in caspase S-nitrosylation (n = 20, R^2 = 0.08).

S-Nitrosylation of recombinant caspase inhibits the enzyme in cell-free systems (*2–4*). To determine if caspase S-nitrosylation was functionally coupled to intracellular caspase activity, we measured caspase-3–like activity in lysates of cells grown in the presence or absence of L-NMA for 24 to 48 hours. Although L-NMA reduced caspase S-nitrosylation (*8*), this reduction was not associated with an increase in caspase-3–like activity (Fig. 4A) or poly(ADP-ribose) polymerase cleavage (*8*), suggesting that decreased S-nitrosylation alone is not sufficient to activate caspases. However, these lengthy treatments with L-NMA increased Fas-induced caspase activation within 2 hours of cross-linking (Fig. 4A) (*14*). Thus, caspase activation seems to require both denitrosylation of the active-site cysteine and cleavage of the zymogen. After longer periods of Fas activation (that is, more than 2 hours), L-NMA no longer increased Fas-induced caspase activity (*8*), probably because Fas alone had fully induced the denitrosylation of caspase at these time points (Fig. 3, A and B). In addition, a nitric oxide donor completely suppressed Fas-induced caspase-3 activation (*14*), consistent with our hypothesis that caspase-3 S-nitrosylation inhibits its intracellular activity (Fig. 4B).

Our results suggest that NO-related activity helps maintain caspase-3 zymogen in an inactive form and that this regulation is

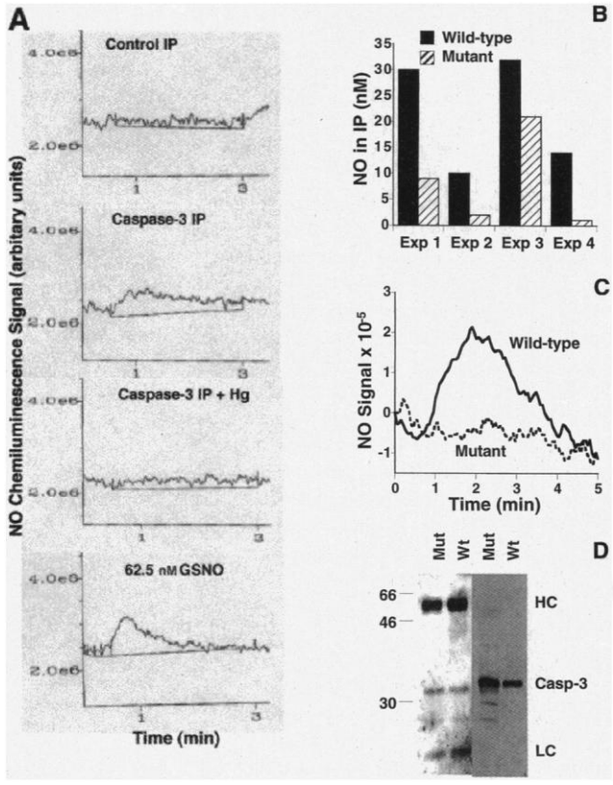

Fig. 2. S-Nitrosylation of caspase-3 zymogen on its catalytic-site cysteine. **(A)** S-nitrosylated caspase-3 in lymphocyte cell lines. Caspase-3 immunoprecipitates from 10C9 cells were divided into two samples, one of which was left untreated, and the other treated with $HgCl_2$, which selectively removes NO groups by breaking S-nitrosothiol bonds (*7*). NO content of the samples was then determined by photolysis-chemiluminescence. The NO-derived chemiluminescence signal (arbitrary units) is plotted on the y axis, and the time course over which the signal was measured is plotted on the x axis. The NO released from each sample is proportional to the area under the curve. Standard curves were generated using known concentrations of S-nitrosoglutathione (GSNO). Approximately 30 nM of NO was released from the untreated immunoprecipitate (Caspase-3 IP), whereas no detectable NO was released from the sample pretreated with $HgCl_2$ (Caspase-3 IP+Hg). NO released from the control IgG2a immunoprecipitate (Control IP) and from the 62.5 nM GSNO standard are shown for comparison. The data are representative of 9 (IgG2a), 38 (caspase-3), and 3 ($HgCl_2$) separate experiments in five different cell lines. **(B)** S-nitrosylation of the active-site cysteine. MCF-7 cells were transiently (Exp 1, 2) or stably (Exp 3, 4) transfected with plasmids expressing wild-type procaspase-3 (Wild-type) or procaspase-3 in which the catalytic-site cysteine was mutated to an alanine (Mutant) or vector alone. NO content of wild-type and paired mutant immunoprecipitates (IP) in four separate experiments (Exp) is shown. **(C)** NO signals from wild-type and mutant caspase-3. Raw data generated by photolysis-chemiluminesence from representative immunoprecipitates of stable clones expressing wild-type and mutant caspase-3, were fitted to a smoothed line by Kaleidagraph 3.0. The NO signals in wild type and mutant correspond to ~15 nM and ~ 0 nM, respectively. **(D)** Caspase-3 in immunoprecipitates from transfected MCF-7 cells. Protein immunoblot (right) and silver stain (left) of wild-type and mutant caspase-3 in the immunoprecipitates from transfected cells used in Exp 2 (right) and Exp 4 (left) in (B). The silver stain corresponds to ~20 nM caspase-3.

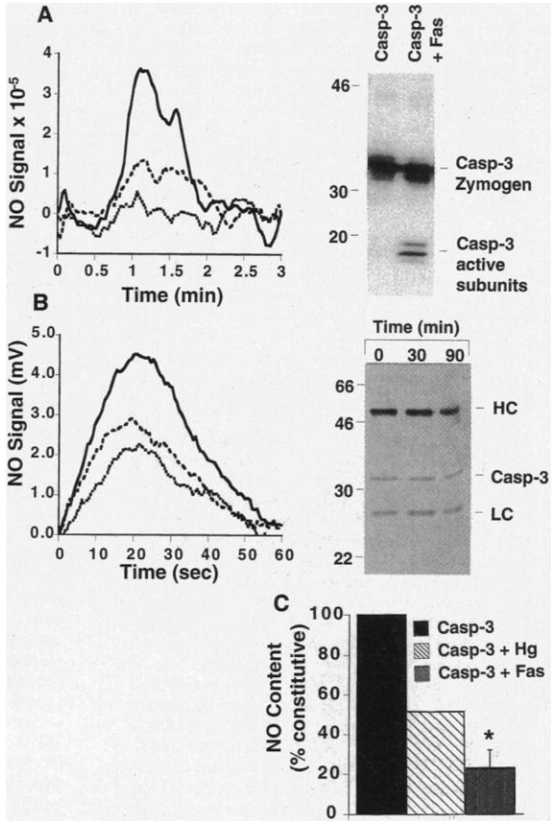

Fig. 3. Decreased caspase-3 S-nitrosylation after Fas activation. (**A**) Caspase-3 denitrosylation (photolysis). Caspase-3 was immunoprecipitated from 10C9 cells which had been grown for 2 hours in the presence of Fas agonist antibody (50 ng/ml; Casp-3 + Fas) or equal concentrations of isotype matched IgM control (Casp-3). NO content of the immunoprecipitates and the baseline (H_2O), as determined by photolysis-chemiluminescence, is shown on the left. NO chemiluminescence (signal) is expressed in arbitrary units over time (minutes of analysis). Raw data were fitted to a smoothed line by Kaleidagraph 3.0. Caspase-3 signal corresponds to 18 nM NO (solid line); Caspase-3 + Fas corresponds to 8 nM NO (dashed line); $H_2O \cong 0$ nM (dotted line). Caspase-3 protein immunoblots of the immunoprecipitates used in this experiment are shown on the right. The bands corresponding to caspase-3 zymogen and cleaved active subunits are identified. (**B**) Caspase-3 denitrosylation (chemical-reduction). The experiment described above was done with 10C9 cells that had been stimulated for 1.5 hours with Fas agonist antibody. NO content of caspase-3 was measured by chemical-reduction chemiluminescence (signal over time). Raw data were fitted to a smoothed line by Kaleidagraph 3.0. The signal difference between caspase-3 (solid line) and caspase-3 plus Fas (dashed line) corresponds to approximately 19 nM NO and the percent reduction by Fas is more than 80% relative to background buffer (dotted line). Silver stains of the immunoprecipitates used in this experiment at times indicated following Fas stimulation (Time), are shown on the right. Bands correspond to caspase-3 zymogen (caspase-3) and immunoglobulin heavy and light chains. Molecular size markers are shown on the left. (**C**) S-nitrosylation-denitrosylation of caspase-3. The NO content of untreated caspase-3 immunoprecipitates (Casp-3), caspase-3 immunoprecipitates derived from cells stimulated with Fas agonist antibody (Casp-3 + Fas), or caspase-3 immunoprecipitates pretreated with $HgCl_2$ (Casp-3 + Hg) was determined. The data are expressed as percent of constitutive caspase-3 nitrosylation in paired experiments and represent the mean of two (Casp + Hg), or nine (Casp-3 + Fas) separate experiments ± SEM. Asterisk indicates $P < 0.0005$ versus caspase-3, paired t test.

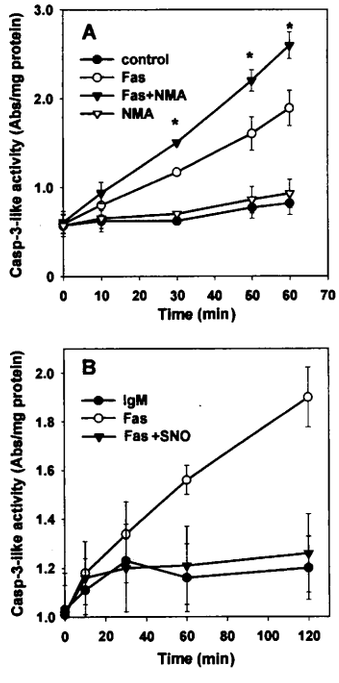

Fig. 4. NO inhibits caspase-3–like activity. (**A**) Intracellular NO production inhibits caspase-3–like activity. The 10C9 or Jurkat cells were left untreated (control), or were grown in the presence the NOS inhibitor L-NMA for 24 to 48 hours. L-NMA alone had no significant effect on caspase activity. The control and L-NMA–treated cells were then cultured for 1 hour in the presence or absence of Fas agonist antibody (100 ng/ml, clone CH-11, Upstate Biotech). Caspase-3–like activity in cytosolic extracts prepared from these cells was measured with Ac-DEVD-pNA (200 μM) as described (*4*). Absorbance of released pNA was read at 405 nm at the indicated times. The results are expressed as absorbance per milligram of protein, and represent the mean ± SEM of three separate experiments. Asterisk indicates $P < 0.05$ versus Fas, $n = 3$, paired t test. Similar results were obtained using PARP cleavage as the assay for caspase activity (*8*). (**B**) Nitrosothiol inhibits caspase-3–like activity. The experiments described above (A) were done with 10C9 and CEM cells grown in the presence of Fas agonist antibody (Fas), equal concentrations of isotype-matched IgM control (IgM), or Fas agonist antibody and 500 μM of the NO donor S-nitrosopenicillamine (Fas+SNO). The results are expressed as absorbance per milligram of protein and are the mean ± SEM of three separate experiments in two different cell lines.

achieved by S-nitrosylation of the catalytic-site cysteine. Upon activation of the Fas apoptotic pathway, caspase-3 zymogens were not only cleaved to their active subunits, but also denitrosylated, thereby freeing the active-site thiol. Thus, protein S-nitrosylation/denitrosylation appears to regulate the Fas apoptotic pathway. The function of ion channels, G proteins, respiratory proteins, transcription factors, and multiple enzymes can be altered by S-nitrosylation (*15, 16*). The finding that this protein modification may be dynamically regulated and coupled to cell-surface signals has potential implications for other signaling pathways and cellular control mechanisms.

References and Notes

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5. BJAB and 10C9 are human Epstein-Barr virus–negative Burkitt's lymphoma cell lines. Jurkat, H9, and CEM are human T leukemia cell lines. Cells were grown in RPMI medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) (Gemini), 2 mM glutamine (Cellgro), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL). For immunoprecipitations, 0.7×10^8 to 1.2×10^8 cells were lysed in

1 ml of NP-40 buffer [150 mM NaCl, 1.0% NP-40, 50 mM tris (pH 8.0)] containing protease inhibitors, and were precleared with protein A–Sepharose beads that had been incubated with normal rabbit serum. Cellular proteins were immunoprecipitated in the dark with 5 μg of an anti–caspase-3 IgG2a monoclonal antibody (Transduction Labs), 20 μl of an anti–caspase-3 rabbit polyclonal antiserum (Pharmingen), 5 μg of a control IgG2a antibody (Sigma), or control normal rabbit serum. The antibody/antigen complexes were isolated with protein A–Sepharose beads (Pharmacia) and then washed five times in high-salt buffer [500 mM NaCl, 1% NP-40, 50 mM tris (pH 8), 100 μM EDTA and protease inhibitors]. Antigen/antibody complexes were removed from the protein A–Sepharose beads by three 10-min incubations in 70 μl of 5 M $MgCl_2$ or 100 mM glycine (pH 3) at 4°C before NO measurements. To minimize the possibility of S-nitrosylation subsequent to immunoprecipitation, we raised the buffer pH to 5.5 in selected experiments and cleansed the solutions of contaminant nitrite by heating in sealed vessels for 2 hours at 95°C, at pH of 2.5 to 3.0. Any residual nitrite in buffers did not correlate with amounts of SNO in samples ($n = 55$, $R^2 = 0.03$). In addition, we adapted a methodology designed to exclude the possibility of artifactual S-nitrosylation by blocking free thiols in caspase-3 immunoprecipitates with 1 mM *N*-ethylmaleimide (NEM), included in wash buffers preceeding elution. Prior exposure to NEM completely blocked NO donor (pH 8)– or nitrite (0.5 N HCl)–mediated S-nitrosylation of recombinant caspase-3 or procaspase-3. Although NEM modestly reduced the NO signal derived from immunoprecipitated caspase-3 (~25%, $n = 7$), it also reduced by a similar amount the NO signal from highly pure SNO–caspase-3 [0.5 M NaCl, 1% NP-40, 50 mM tris (pH 8), 0.1 mM EDTA] that had been synthesized in vitro. Thus, this approach demonstrated that caspase-3 was S-nitrosylated intracellularly. For protein immunoblot analysis, whole-cell lysates or immunoprecipitated proteins were separated on 7% (NOS) or 12% (caspase-3) polyacrylamide gels, transferred to nitrocellulose, and incubated with 250 ng/ml of anti–caspase-3 monoclonal antibody, anti-nNOS monoclonal antibody, or anti-iNOS polyclonal antibody (Transduction Labs), or a 1:1,000 dilution of anti–caspase-3 rabbit polyclonal antibody (Pharmingen), followed by a 1:1000 dilution of secondary horseradish peroxidase antibody (Amersham), and then were developed by ECL (Amersham).

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. Photolysis-chemiluminesecence (assay for SNO) was performed as described [J. S. Stamler and M. Feelisch, in *Methods in Nitric Oxide Research*, J. S. Stamler and M. Feelisch, Eds. (Wiley, Chichester, UK, 1996), pp. 521–539]. Samples (200 μl) were injected directly into the photolysis unit. The chemical reduction–chemiluminescence assay for nitrite was performed according to the manufacturer (Sievers) and for SNO as described (*16*) with the following modifications: Immunoprecipitated proteins and cell extracts were assayed both with and without excess $HgCl_2$ in a paired radical-purged system, otherwise according to manufacturer's instructions (NO analyzer Sievers 280); standard curves were derived for both nitrite and S-nitrosoglutathione in the presence and absence of $HgCl_2$; data were normalized to baseline and integrated using Mathcad 7 Professional (MathSoft). SNO was calculated by subtracting the nitrite in samples without $HgCl_2$ from the nitrite in samples with $HgCl_2$ and by converting the nitrite difference to SNO from the standard curves of S-nitrosoglutathione (GSNO) ± $HgCl_2$. The photolysis and chemical reduction assays are linear to 1 pmol for SNO and nitrite, respectively, and can differentiate 1 pmol from zero (deionized water). S-Nitrosylated recombinant caspase-3 standards were detected by both methods; the standard curves for SNO–caspase-3 and GSNO were superimposeable in the photolysis assay. The chemical reduction method has not been extensively validated for SNO and thus was only used qualitatively to verify results from photolysis.

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11. We grew 9×10^7 MCF-7 cells to 75% confluence in T150 flasks containing Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gemini), 2 mM glutamine (Cellgro), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL). The cells were then either transiently transfected with 120 μg of plasmids—engineered to express either wild-type or mutant procaspase-3 in which cysteine-163 was mutated to alanine (*9*), using lipofectAMINE per the manufacturer's protocol (Life Technologies)—or further treated with G418 to select for stable clones. The coding sequence of each expression construct was sequenced in its entirety before use. The cells were lysed in NP-40 buffer (48 hours following transient transfection), and caspase-3 immunoprecipitations were done as above. The level of wild-type and mutant caspase-3 in the lysates and immunoprecipitates was determined by protein immunoblot or silver stain analysis.

12. Fas was cross-linked on the surface of cells with 50 ng/ml of anti-Fas IgM clone CH-11 (Upstate Biotech) for 5 min to 2 hours at 37°C. The cells were then washed at 4°C with phosphate-buffered saline (PBS), and immunoprecipitations were done at selected intervals (*6*).

13. Cells were grown for 2, 24, or 48 hours in the presence or absence of the NO synthase inhibitor L-NMA (5 mM versus 1 mM L-arginine in the medium) (Calbiochem). The cells were then washed, and immunoprecipitations were done (*6*).

14. We grew 1×10^7 10C9 or Jurkat cells for 24 to 48 hours in the presence or absence of L-NMA as described above. Fas agonist antibody (100 ng/ml, clone CH-11, Upstate Biotech) and S-nitrosopenicillamine (500 μM) were then added to the appropriate cultures for 50 to 75 min, after which the cells were washed with ice-cold PBS and resuspended in 140 μl of buffer A [100 mM Hepes (pH 7.4), 140 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 10 μg/ml leupeptin]. The cells were then lysed with three cycles of freezing and thawing, and the crude cytosol was obtained by centrifugation at 12,000*g* for 20 min at 4°C. We mixed 50 to 200 μg of cytosolic protein with 400 μM Ac-DEVD-pNA (Quality Biochemicals) in 150 μl of buffer B [100 mM Hepes (pH 7.4), 20% glycerol, and protease inhibitors] and incubated it at 37°C. The caspase-3–like activity was calculated by measuring the increased absorbence at 405 nm every 10 min. The reaction mixture without cell lysate or substrate was used as a control.

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Systemic Signaling and Acclimation in Response to Excess Excitation Energy in *Arabidopsis*

Stanislaw Karpinski,[1]* Helen Reynolds,[2] Barbara Karpinska,[1] Gunnar Wingsle,[1] Gary Creissen,[2] Philip Mullineaux[2]

Land plants are sessile and have developed sophisticated mechanisms that allow for both immediate and acclimatory responses to changing environments. Partial exposure of low light–adapted *Arabidopsis* plants to excess light results in a systemic acclimation to excess excitation energy and consequent photooxidative stress in unexposed leaves. Thus, plants possess a mechanism to communicate excess excitation energy systemically, allowing them to mount a defense against further episodes of such stress. Systemic redox changes in the proximity of photosystem II, hydrogen peroxide, and the induction of antioxidant defenses are key determinants of this mechanism of systemic acquired acclimation.

Large increases in light intensity for a short period can be beneficial for photosynthetic yields in low light (LL)–adapted plants (*1*). However, if these conditions persist, an imbalance can be created such that the energy absorbed through the light-harvesting complex is in excess of that which can be dissipated or transduced by photosystem II (PSII). This imbalance [excess excitation energy (EEE)] can be generated by excess light (EL) or chilling or both and can be strongly enhanced by a combination with other factors such as rapid and large increases in temperature and limitations in nutritional and H_2O status (*1–8*).