many small-scale studies (*1*, *5*, *10*, *12*). Instead, a more variable distribution with isolated pollination events was detected. The multiple pollinating agents (wind and insects) of canola and the large size of the source may contribute to the randomness of long-distance pollination events.

Varietal differences among canola sink fields were observed (Fig. 4), but no consistent effect of wind direction on pollen-mediated gene flow was detected (data not shown). The variety of canola may be a contributing factor in random pollination events at distance. Pollination has been shown to be affected by crop variety (*12*). Varieties have differences in flowering period, which will affect pollination events over such a large scale. Another explanation for these seemingly random events may also be related to insect behavior. Roaming insects may target single plants flowering early or late in a field, resulting in sporadic pollen movement. However, insects are more likely to remain in a single field if sufficient resources (e.g., flowers) are readily available (*13*).

Gene transfer is a complex process and is dependent on many factors (*14*–*16*), including environmental conditions, plant variety, insect behavior, and plant density. These observations, coupled with our data on long-distance pollen movement, indicate that laboratory and small-scale experiments may not necessarily predict pollination under commercial conditions. This study demonstrates that cross-pollination between commercial canola fields occurs at low frequencies but to considerable distance.

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# Regulation of Hypoxic Death in *C. elegans* by the Insulin/IGF Receptor Homolog DAF-2


**Barbara A. Scott,[1] Michael S. Avidan,[1] C. Michael Crowder[1,2]***



**To identify genetic determinants of hypoxic cell death, we screened for hypoxia-resistant (Hyp) mutants in *Caenorhabditis elegans* and found that specific reduction-of-function (rf) mutants of *daf-2*, an insulin/insulinlike growth factor (IGF) receptor (INR) homolog gene, were profoundly Hyp. The hypoxia resistance was acutely inducible just before hypoxic exposure and was mediated through an AKT-1/PDK-1/forkhead transcription factor pathway overlapping with but distinct from signaling pathways regulating life-span and stress resistance. Selective neuronal and muscle expression of *daf-2*(+) restored hypoxic death, and *daf-2*(rf) prevented hypoxia-induced muscle and neuronal cell death, which demonstrates a potential for INR modulation in prophylaxis against hypoxic injury of neurons and myocytes.**


Although genetically tractable model organisms have made longstanding contributions to our understanding of programmed cell death (*1*) and recently to identification of molecular mechanisms of hypoxic adaptation and sensing (*2*, *3*), direct genetic screens for hypoxia-resistant mutants have been relatively unexplored. To identify genes that regulate hypoxic cell death, we screened new and existing mutant strains for animals that survived exposure to either hypoxia or sodium azide (*4*), an electron-transport chain inhibitor used as a chemical surrogate for hypoxia. High-level resistance to hypoxia or azide was an uncommon phenotype. We identified only two new mutants and a few existing ones that had significantly improved survival. We found the strongest Hyp strains among existing mutants with reduced activity of the insulin/IGF receptor (INR) signaling pathway. *daf-2(e1370)*, which carries a rf mutation in the homolog of the human insulin/IGF receptor (*5*), was markedly azide resistant compared with wild-type strain N2 (13.2 ± 1.8% dead versus 80.8 ± 5.9%; $P < 0.0001$). Subsequent hypoxic incubation demonstrated that *daf-2(e1370)* was indeed Hyp (Fig. 1, Table 1). Genetic mapping confirmed the *e1370* mutation was responsible for the Hyp phenotype (*4*).

*daf-2(e1370)* not only survived but fully recovered normal locomotion behavior after as long as 20 hours of hypoxic incubation (Fig. 1A, movies S1 and S2). N2 displayed significant locomotion defects after recovery from a 6.5-hour incubation. Hypoxic sensitivity was not stage or age specific with the exception of N2 dauers (a long-lived alternative larval stage), which were Hyp (Fig. 1C). The Hyp phenotype of *daf-2(e1370)* was markedly sensitive to temperature; *e1370* animals were less


[1]Department of Anesthesiology, and [2]Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA.

*To whom correspondence should be addressed. E-mail: crowderm@morpheus.wustl.edu


**Table 1.** *daf-2* allelic variation for hypoxia resistance (Hyp). Animals were raised at 20°C except *sa187*, *e1369*, *e979*, which were raised at 15°C then shifted to 20°C 2 days before testing. Percent dead is reported as means ± SEM per trial. Adults 2 days post L4 were exposed to <0.3% oxygen at 28°C for 20 hours then scored after a 24-hour recovery period. Each trial was a completely independent experiment done on a different day.

| Genotype | Percent dead | Trials (*n*) | Animals (*n*) |
|---|---|---|---|
| +/+ | 95.5 ± 1.2 | 26 | 2568 |
| *daf-2(e1370)* | 3.6 ± 1.3* | 26 | 835 |
| *daf-2(sa219)* | 4.6 ± 2.4* | 4 | 699 |
| *daf-2(m579)* | 8.6 ± 3.8* | 5 | 110 |
| *daf-2(e1369)* | 46.4 ± 15.6* | 4 | 185 |
| *daf-2(m596)* | 47.0 ± 3.6* | 4 | 210 |
| *daf-2(sa187)* | 53.3 ± 6.3* | 5 | 318 |
| *daf-2(e979)* | 55.6 ± 12.4* | 4 | 330 |
| *daf-2(e1391)* | 59.0 ± 11.7* | 5 | 174 |
| *daf-2(e1368)* | 77.3 ± 7.7 | 4 | 344 |
| *daf-2(sa229)* | 80.1 ± 10.6 | 6 | 547 |
| *daf-2(e1365)* | 87.9 ± 6.7 | 4 | 330 |
| *daf-2(m577)* | 90.9 ± 6.7 | 3 | 199 |
| *daf-2(e1371)* | 95.8 ± 2.4 | 4 | 292 |

*$P < 0.01$ versus wild type by Mann-Whitney test.

Hyp when raised at 15°C than at 20°C (table S1). The temperature-sensitive period for *e1370* extended from early larval through adult stages, and switching to the restrictive temperature just before hypoxic incubation induced hypoxia resistance. Similar temperature elevation had the opposite effect in wild-type animals; warmer animals were more sensitive.

*daf-2*(rf) alleles including *e1370* have three well-characterized phenotypes. They were originally isolated based on their dauer constitutive (Daf-c) phenotype, forming dauer larvaewhen wild type normally does not. Adult *daf-2*(rf) mutants also have a prolonged life-span. Finally, *daf-2*(rf) adults are resistant to various environmental stresses, and this resistance correlates with prolonged life-span (*6*). To assess whether the hypoxia resistance of *daf-2(e1370)* is a consequence of the mechanisms producing its Daf-c, Age, and/or stress-resistance phenotypes, we tested 12 other *daf-2*(rf) alleles with various phenotypic severities (Table 1). Two additional alleles were strongly Hyp, five were weakly but significantly Hyp, and five others were non-Hyp. The Hyp phenotypes did not correlate well with life-span ($r$ = 0.32; $P$ = 0.36). Eight weak or non-Hyp alleles had significantly increased median life-spans as long as or longer than *e1370* (*7*). Similarly, four weak Hyp alleles (*e1369*, *sa187*, *e979*, *e1391*) have stronger Daf-c phenotypes than *e1370*, and non-Hyp *e1368* and *sa229* are as Daf-c as *e1370* (*5*, *7*). As for stress resistance, seven *daf-2* alleles (*e1369*, *m596*, *e979*, *e1391*, *e1368*, *e1365*, *e1371*) are significantly resistant to thermal stress (*7*) but are either weakly or non-Hyp. Thus, the hypoxia resistance of *daf-2*(rf) is highly allele specific and does not appear to be a consequence of mechanisms that regulate life-span, dauer formation, or stress resistance.

Seeking a molecular explanation for the allelic differences, we sequenced select Hyp alleles (*4*). *m579*, a strong Hyp, contained a missense mutation ($Arg^{437}$ to Cys) in a highly conserved residue in the cysteine-rich ligand-binding domain. The other strong alleles, *e1370* and *sa219*, carry missense mutations in conserved residues in the tyrosine kinase domain (*5*). Two weakly Hyp alleles had mutations in less well-conserved residues in the cysteine-rich region: *m596* ($Gly^{547}$ to Ser) and *e979* ($Gly^{383}$ to Glu), whereas *sa187* and *e1391* have mutations in highly conserved residues in the cysteine-rich and kinase domains, respectively (*5*). The non-Hyp alleles have mutations in poorly conserved residues in the ligand-binding domain (*5*). The location and nature of the mutations do not explain their phenotypic severities, but the strong Hyp mutations suggest that DAF-2 regulates hypoxic death through ligand-mediated activation of a downstream kinase cascade.

To define the *daf-2* hypoxic death pathway, we tested mutants in genes previously found to lie downstream of *daf-2*'s regulation of life-span and dauer formation (Table 2) (*4*). *age-1* codes for a phosphatidylinositol 3-kinase homolog that is a major output for the *daf-2* signaling cascade (*8*). Homozygous *age-1(mg44)*, a likely null, has a maternally rescued Daf-c phenotype and a zygotically prolonged life-span. $mg44^{m+z-}$ (maternal *mg44*/+, zygotic homozygous *mg44*) animals were weakly Hyp, which suggests either that *daf-2(e1370)*'s strong Hyp phenotype was mediated in part through an *age-1*-independent pathway or that there was maternal rescue of *mg44*. *mg44* can be propagated as a homozygote in the presence of gain-of-function mutations in *akt-1* and *pdk-1*, both of which suppress the Daf-c but not the long life-span phenotype of *mg44* (*9*, *10*). Neither *pdk-1*(gf) nor *akt-1*(gf) suppressed the Hyp phenotype of *age-1(mg44)* (Table 2). Indeed, the double mutants were more hypoxia resistant than *age-1(mg44)* alone, which indicates that the weak Hyp phenotype of *mg44*

**Table 2.** Hyp phenotype of mutants in *daf-2* life-span pathway. All animals were raised at 20°C. Adults 2 days post L4 were exposed to <0.3% oxygen at 28°C for 20 hours then scored after a 24-hour recovery period. The genotype *age-1(mg44)*$^{m+z-}$ is zygotic homozygous *mg44* from *mg44*/+ mothers.

| Genotype | Mutation | Percent dead | Animals (*n*) |
|---|---|---|---|
| +/+ | wild type | 95.5 ± 1.2 | 2568 |
| *age-1(hx546)* | rf | 78.8 ± 10.3† | 254 |
| *age-1(mg44)*$^{m+ z-}$ | null | 46.2 ± 3.6*† | 47 |
| *akt-1(mg144)* | gf | 79.3 ± 9.3† | 489 |
| *pdk-1(mg142)* | gf | 98.4 ± 1.0† | 518 |
| *pdk-1(sa680)* | rf | 42 ± 4.4*† | 56 |
| *daf-2(e1370)* | rf | 3.6 ± 1.3* | 835 |
| *age-1(mg44);akt-1(mg144)* | null;gf | 7.5 ± 3.5* | 343 |
| *daf-2(e1370);akt-1(mg144)* | rf;gf | 77.5 ± 3.8† | 461 |
| *age-1(mg44);pdk-1(mg142)* | null;gf | 12.5 ± 5.1* | 83 |
| *daf-2(e1370);pdk-1(mg142)* | rf;gf | 44.4 ± 5.6*† | 39 |
| *daf-18(nr2037)* | null | 100 ± 0† | 127 |
| *daf-2(e1370);daf-18(nr2037)* | rf;null | 97.2 ± 0.9† | 415 |
| *daf-2(e1370);daf-18(e1375)* | rf;rf | 27.6 ± 13.3*† | 57 |
| *daf-16(mgDf50)* | null | 100 ± 0† | 169 |
| *daf-16(mgDf47);daf-2(e1370)* | null;rf | 100 ± 0† | 100 |
| *daf-16(mg54);daf-2(e1370)* | null;rf | 96.0 ± 0.8† | 167 |
| *daf-16(m26);daf-2(e1370)* | null;rf | 86.8 ± 5.3† | 189 |
| *old-1(mk1)* | null | 79.8 ± 11.3† | 560 |
| *old-1(mk1);daf-2(e1370)* | null;rf | 3.0 ± 0.6* | 161 |

$P$ < 0.01 by Fisher's exact test: * Versus wild type; † Versus *e1370*.

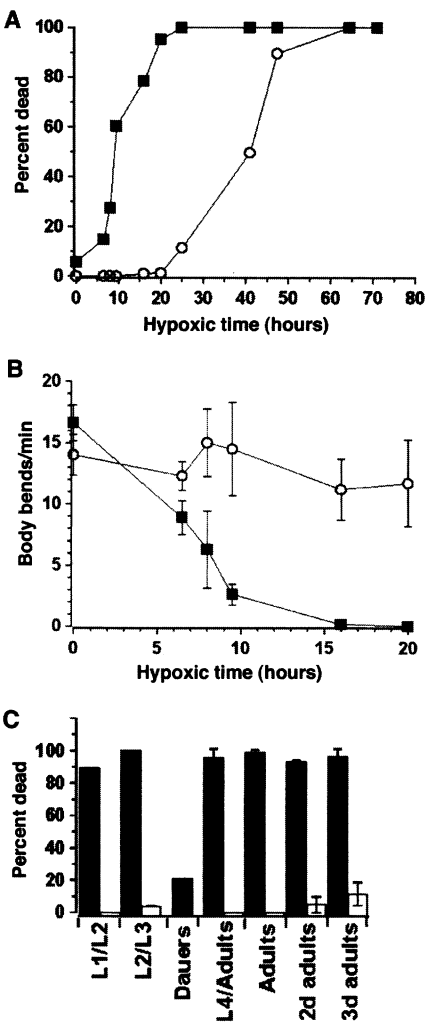


**Fig. 1.** Behavioral and lethal effects of hypoxia in wild-type (N2) (black squares) and *daf-2(e1370rf)* (open circles). All animals were scored 24 hours after recovery from incubation in a hypoxic chamber (<0.3% $O_2$ at 28°C). (**A**) Percent dead of N2 and *daf-2(e1370)* animals as a function of hypoxic incubation time. Animals without spontaneous or evoked body or pharyngeal movement were scored as dead. (**B**) Locomotion rate quantified as body bends per minute (mean ± SEM; 10 animals) after recovery from various durations of hypoxic incubation. Locomotion of N2 was significantly reduced at the 6.5-hour time point and thereafter ($P$ < 0.01; one-tailed $t$ test); *daf-2(e1370)* remained unchanged. (**C**) Hypoxic death of N2 and *e1370* as a function of developmental age. Hypoxic incubations were 20 hours for all stages. L1, L2, L3, and L4 are successive larval stages followed by adulthood. The dauer stage is an alternative third larval form tested only for N2. N2 dauers were Hyp compared with N2 adults but less hypoxia resistant than *daf-2(e1370)* adults ($P$ < 0.05; $\chi^2$ statistic).

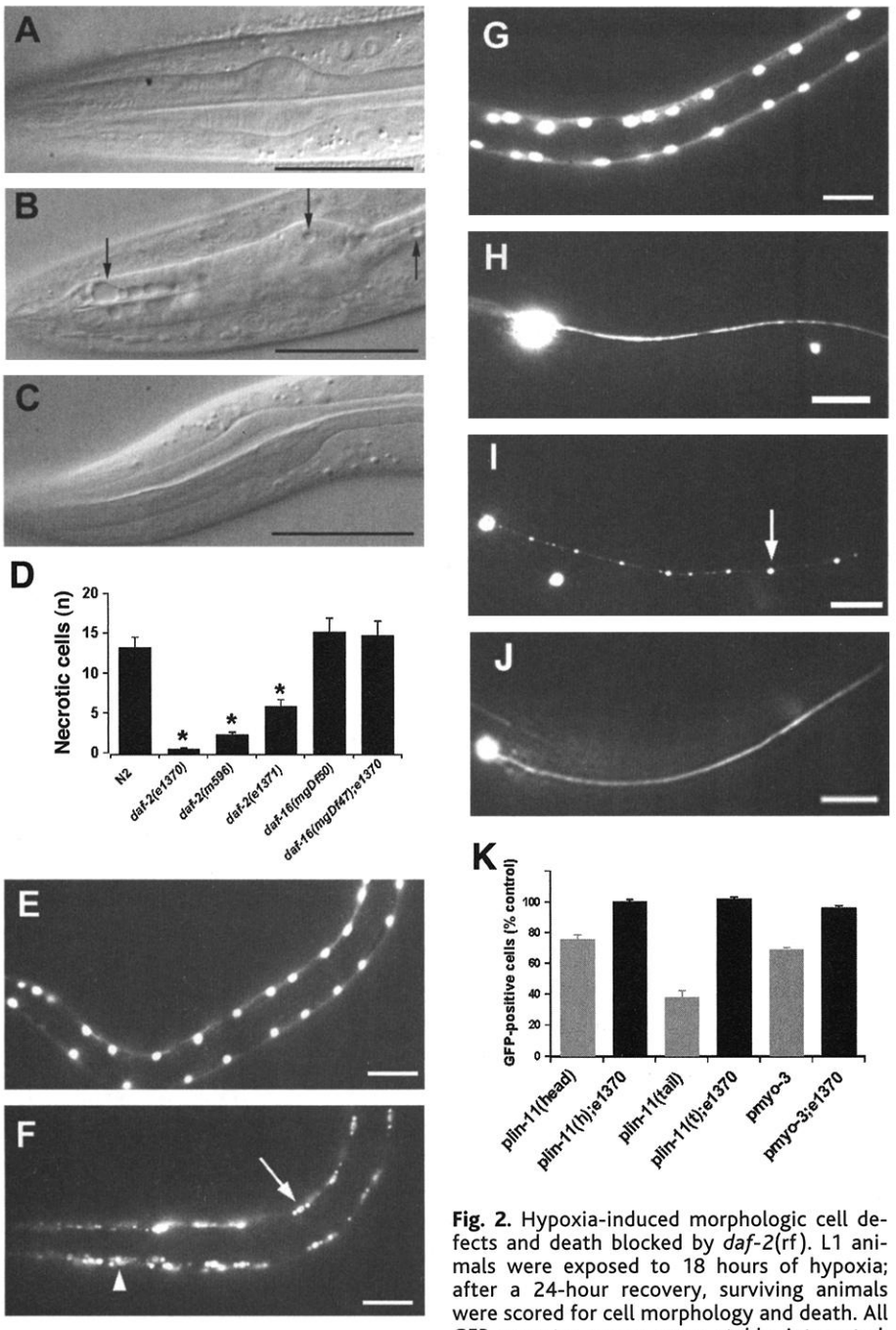


**Fig. 2.** Hypoxia-induced morphologic cell defects and death blocked by *daf-2*(rf). L1 animals were exposed to 18 hours of hypoxia; after a 24-hour recovery, surviving animals were scored for cell morphology and death. All GFP reporter genes were stably integrated. Scale bars = 20 μm. (**A**) Pharyngeal cells of untreated wild-type animal. (**B**) Hypoxia-treated wild-type animal with swollen necrotic pharyngeal cells (arrows). (**C**) Hypoxia-treated *daf-2(e1370)* with no evidence of necrotic cell death. (**D**) Necrotic cells per animal (mean ± SEM). Number of animals scored = 20 per strain except *daf-16(mgDf50)*, *daf-16(mgDf47)*; *daf-2(e1370)* ($n$ = 10). Asterisk indicates < wild type ($P < 0.01$; one-tailed $t$ test). *e1370* < *m596* < *e1371* ($P < 0.01$). (**E**) Nuclear-localized *pmyo-3::GFP* reporter gene expression (strain PD4251) (*25*) in body wall muscle nuclei of untreated animals. (**F**) Hypoxia-treated PD4251. Nuclear GFP expression was fragmented into two flanking (arrow) or multiple (arrowhead) fragments. (**G**) Hypoxia-treated *pmyo-3::GFP;daf-2(e1370)* animal with preservation of nuclear morphology. (**H**) GFP expression in untreated wild-type touch cell sensory neuron body and axon using *pmec-4::GFP* reporter gene. (**I**) *pmec-4::GFP* expression after hypoxia with axonal beading (arrow). (**J**) Normal *pmec-4::GFP* expression in *daf-2(e1370)* after hypoxia. (**K**) Remaining GFP-expressing neurons and muscle cells after hypoxia as a percent of untreated controls in *daf-2*(+) (shaded bars) and *daf-2(e1370)* (solid bars) backgrounds. A *plin-11::GFP* reporter gene (*26*) was used to score a subset of neurons in the head and tail ganglia. PD4251 was used to score muscle cells. GFP-positive neurons and muscle cells were reduced in *daf-2*(+) animals versus *daf-2(e1370)* and untreated animals ($P < 0.01$; one-tailed $t$ test).

homozygotes was due to maternal rescue. The lack of suppression of *age-1*(null) offers the possibility of an *akt-1/pdk-1* independent signaling pathway for hypoxic death. The weak Hyp phenotype of *pdk-1(sa680rf)*, a strong, perhaps null, allele is consistent with this alternative pathway (Table 2). The Hyp phenotype of *daf-2(e1370)* was strongly suppressed by both *akt-1*(gf) and *pdk-1*(gf), which suggests that DAF-2 signals exclusively through AKT-1 to PDK-1 to regulate hypoxic death or that the *e1370* mutation is not severe enough to reveal the alternative pathway suggested by *age-1*(null).

*daf-18* codes for a homolog of PTEN (phosphatase and tensin homolog deleted on chromosome 10) that functions to turn over phosphatidylinositol-1,4,5-trisphosphate and thereby inhibit the DAF-2/AGE-1 signaling cascade (*11*–*13*). *daf-18*(null) fully suppresses *e1370*'s Hyp phenotype (Table 2). However, *daf-18(e1375)*, a weaker allele that fully suppresses the long life-span of *daf-2(e1370)* (*14*), only weakly suppresses Hyp. Thus, long life-span is neither necessary as shown here nor sufficient as shown by the long-lived non-Hyp *daf-2* alleles to confer Hyp. *daf-16* codes for a forkhead/FKHRL1 transcription factor homolog, and *daf-16*(rf) mutants suppress the Daf-c and Age phenotypes of *daf-2*(rf) (*15*, *16*). *daf-16*(rf) also completely suppresses the Hyp phenotype of *daf-2(e1370)* (Table 2); therefore, *daf-2(e1370)* requires *daf-16* as well as *daf-18* function to produce hypoxia resistance. Finally, *old-1*, a receptor tyrosine kinase, has been proposed to function downstream of *daf-16* based on regulation of its expression by *daf-16* (*17*). *old-1*(gf) mutants are long-lived and stress resistant, whereas *old-1*(null) is short-lived and fully suppresses the long life-span and stress resistance of *daf-2(e1370)*. However, *old-1*(null) does not suppress the hypoxia resistance of *e1370* (Table 2). Thus, while *daf-2* signals through *daf-16* for regulation of aging, stress resistance, and hypoxic death, the mechanisms downstream of *daf-16* diverge (fig. S1).

To search for evidence of cell death protection by *daf-2*(rf), we examined the cell number and morphology of animals surviving hypoxia (Fig. 2). Compared with controls, hypoxia-exposed animals contained multiple strikingly swollen, necrotic-looking cells (Fig. 2, A and B). These necrotic cells were seen among multiple cell types and organs including pharynx, body wall muscle, gonad primordium, and other unidentified cells (Fig. 2B) (*18*). *daf-2(e1370)* significantly reduced the number of necrotic cells (Fig. 2, C and D); weaker *daf-2* alleles were also protective but less so than *e1370*. *daf-16*(rf) completely suppressed the cell-protective effect of *e1370*.

To focus on neuronal and muscle cell types, we used cell-type-specific promoters driving green fluorescent protein (GFP) expression in neurons and muscle (Fig. 2, E to K). Hypoxia

produced striking nuclear fragmentation in essentially all myocytes (Fig. 2, E and F). The fragmentation typically was not random; instead, the nuclear GFP was segregated into two satellite fragments flanking a shrunken or even absent nuclear remnant (Fig. 2F, arrow). In other myocytes, the GFP was diffusely fragmented, with no nucleus apparent by fluorescence or Normarski microscopy (Fig. 2F, arrowhead). The number of GFP-positive muscle nuclei was significantly reduced, which is consistent with cell death (Fig. 2K). *daf-2(e1370)* protected myocytes from both nuclear fragmentation and death (Fig. 2, H and K). In neurons with a cytoplasmic GFP marker, hypoxia induced a dramatic axonal beading morphology (Fig. 2I). Hypoxia also reduced the number of GFP(+) neurons. *e1370* mutants did not show neuronal loss and axonal pathology (Fig. 2, J and K).

Through which cells is *daf-2* regulating hypoxic death? Using cell type-specific promoters, Wolkow *et al.* showed that *daf-2*(+) expression in neurons, but not in muscle or intestine, could rescue the long life-span and dauer formation phenotypes of *daf-2(e1370)* (*19*). We used these strains to determine the cell types involved in *daf-2*-mediated organismal death (*4*). Pan-neuronal expression of *daf-2*(+) in a *daf-2(e1370)* background significantly increased hypoxia-induced death (65.0 ± 5.7 % dead; $P < 0.01$ versus *e1370*; Mann–Whitney nonparametric test) compared with *daf-2(e1370)* alone (4.0 ± 0.6% dead) . However, unlike its other phenotypes, *e1370*'s Hyp phenotype was also rescued by muscle expression of *daf-2*(+) (82.8 ± 7.9% dead; $P < 0.01$ versus *e1370*). Intestinal expression did not increase hypoxic death after the standard 20-hour incubation (10.0 ± 4.3 % dead) but it did after longer incubations (41-hour incubation: 100% dead versus 29.7% of *e1370*; $P < 0.01$). The potent rescue of Hyp by neuronal and muscle *daf-2*(+) expression confirms the assignment of the Hyp phenotype to *daf-2*. Consistent with the direct observation of *daf-2*-dependent neuronal and muscle cell death, these data also suggest that *daf-2*(+) expression induces hypoxic death of muscle and neuronal cell types, whose death, perhaps along with other cell types, then kills the organism. Alternatively, neuronal and muscle expression could induce death of other cell types responsible for organismal death. Indeed, cell nonautonomous effects of *daf-2* have been observed for both aging and dauer formation (*19*, *20*).

How might DAF-2 INR so potently regulate hypoxic death? We initially examined *daf-2* mutants because the INR signaling cascade had been found to regulate apoptosis of vertebrate cells. However, vertebrate INR cascades antagonize apoptosis (*21*); thus, reduction of DAF-2 signaling should, if anything, increase cell death. Hypoxic cell death is not, however, exclusively apoptotic, and after severe insults, it may be almost entirely necrotic (*22*). Given the role of the insulin receptor in regulating glucose utilization, alterations in metabolism by *daf-2*(rf) do provide an appealing mechanism for its hypoxia resistance. *daf-2(e1370)* has been found to have lower $O_2$ consumption than wild type, perhaps prolonging the time needed for depletion of energy stores and subsequent cell death (*23*). However, these results have been questioned on methodologic grounds and not all findings by van Voorhies are consistent with a metabolic mechanism for *daf-2*'s regulation of hypoxic sensitivity (*24*). Identification of additional Hyp mutants and genes downstream of *daf-16* should clarify the mechanisms underlying *daf-2*'s regulation of hypoxic cell death.

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**Supporting Online Material**

www.sciencemag.org/cgi/content/full/1072302/DC1
Materials and Methods
Figs. S1 and S2
Table S1
Movies S1 and S2


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# Myeloperoxidase, a Leukocyte-Derived Vascular NO Oxidase


**Jason P. Eiserich,[1,2,3*†] Stephan Baldus,[2,3*‡] Marie-Luise Brennan,[6] Wenxin Ma,[4] Chunxiang Zhang,[4] Albert Tousson,[5] Laura Castro,[2,3] Aldons J. Lusis,[6] William M. Nauseef,[7] C. Roger White,[3,4] Bruce A. Freeman[2,3†]**



**Myeloperoxidase (MPO) is an abundant mammalian phagocyte hemoprotein thought to primarily mediate host defense reactions. Although its microbicidal functions are well established in vitro, humans deficient in MPO are not at unusual risk of infection. MPO was observed herein to modulate the vascular signaling and vasodilatory functions of nitric oxide (NO) during acute inflammation. After leukocyte degranulation, MPO localized in and around vascular endothelial cells in a rodent model of acute endotoxemia and impaired endothelium-dependent relaxant responses, to which MPO-deficient mice were resistant. Altered vascular responsiveness was due to catalytic consumption of NO by substrate radicals generated by MPO. Thus MPO can directly modulate vascular inflammatory responses by regulating NO bioavailability.**


Vascular endothelial dysfunction is an established feature of acute inflammation (*1*, *2*) and is typified by compromised function of the endothelium-derived signaling molecule nitric oxide (NO), which serves to stimulate relaxation of vascular smooth muscle cells. Neutrophils contribute to endothelial dysfunction and altered NO signaling during in-