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Glucose Restriction Extends *Caenorhabditis elegans* Life Span by Inducing Mitochondrial Respiration and Increasing Oxidative Stress

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SUMMARY

Increasing cellular glucose uptake is a fundamental concept in treatment of type 2 diabetes, whereas nutritive calorie restriction increases life expectancy. We show here that increased glucose availability decreases *Caenorhabditis elegans* life span, while impaired glucose metabolism extends life expectancy by inducing mitochondrial respiration. The histone deacetylase Sir2.1 is found here to be dispensable for this phenotype, whereas disruption of *aak-2*, a homolog of AMP-dependent kinase (AMPK), abolishes extension of life span due to impaired glycolysis. Reduced glucose availability promotes formation of reactive oxygen species (ROS), induces catalase activity, and increases oxidative stress resistance and survival rates, altogether providing direct evidence for a hitherto hypothetical concept named mitochondrial hormesis or "mitohormesis." Accordingly, treatment of nematodes with different antioxidants and vitamins prevents extension of life span. In summary, these data indicate that glucose restriction promotes mitochondrial metabolism, causing increased ROS formation and cumulating in hormetic extension of life span, questioning current treatments of type 2 diabetes as well as the widespread use of antioxidant supplements.

INTRODUCTION

Reduced uptake of nutritive calories, termed calorie restriction (CR), is a long-standing intervention to extend life span in multicellular eukaryotes, including mammals (Weindruch and Walford, 1988). While it is currently unknown whether such interventions are capable of extending life span in humans, CR has recently been linked to desirable metabolic alteration in this species (Heilbronn et al., 2006). The idea that life span might be dependent on metabolic flux dates back to 1908, when Max Rubner suggested that maximum life span is inversely proportional to the amount of nutritive energy metabolized (Rubner, 1908). This hypothesis was extended in 1928 by Raymond Pearl, who proposed that an increased metabolic rate would decrease life span in eukaryotes (Pearl, 1928). Mechanistically, metabolism-induced production of reactive oxygen species (ROS) was introduced as the underlying cause, suggesting that increased mitochondrial metabolism would induce ROS formation and subsequently cause cellular and systemic damage cumulating in reduced life expectancy (Harman, 1956).

Recently, increasing evidence has emerged that the opposite might apply: It has been repeatedly hypothesized that induction of mitochondrial metabolism might cause a positive response to increased formation of ROS and other stressors, leading to a secondary increase in stress defense, cumulating in reduced net stress levels and possibly extended life span (Houthoofd et al., 2002b; Johnson et al., 2002; Kharade et al., 2005; Sinclair, 2005; Vanfleteren, 1993; Zarse et al., 2007). These interpretations are supported by the fact that exercise increases ROS formation (Chevion et al., 2003), while physical activity has been shown to extend life span in humans (Lindsted et al., 1991; Manini et al., 2006) independently of body mass (Hu et al., 2004). Accordingly, recent evidence suggests that antioxidant food supplements may decrease life span in humans (Bjelakovic et al., 2007).

In westernized countries, glucose represents a key dietary component since the most commonly ingested sugar, sucrose, contains equimolar amounts of glucose and fructose (Popkin and Nielsen, 2003). Specifically, in Americans, added dietary sugar accounts today for 15.8% of daily calorie intake (Malik et al., 2006). Nevertheless, it is a matter of debate whether glucose and other carbohydrates have a relevant effect on disease burden and mortality in humans, and experimental data in mammals warrant further investigation.

To directly address the putative role of glucose metabolism in a multicellular eukaryote in regards to life expectancy, we exposed the nematode *Caenorhabditis elegans* to a chemical inhibitor of glycolysis, producing a metabolic state resembling that of dietary glucose restriction (Ingram et al., 2006). We observed a significant AAK-2-dependent extension of life span in states of impaired glucose metabolism, induction of mitochondrial respiration, and

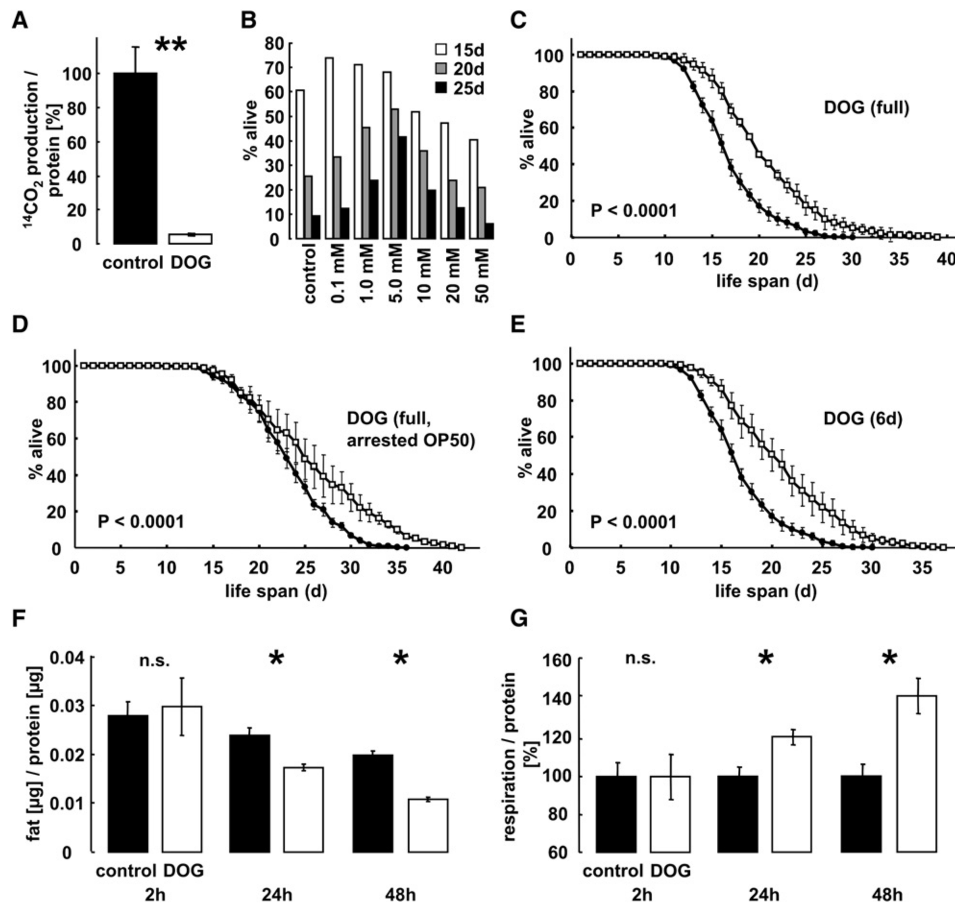

Figure 1. Increased Life Span Due to Increased Respiration in States of Impaired Glucose Metabolism

(A) Release of ^{14}C-labeled CO_2 from radioactively labeled D-glucose in control- and 2-deoxy-D-glucose (DOG)-treated nematodes. Black bars indicate control scores; white bars indicate scores of animals treated with DOG (also applies to Figures 1F and 1G, Figures 3E and 3F, and Figures S1A and S2). In this and all subsequent figures, error bars represent SEM; $^{*}p < 0.05$, $^{**}p < 0.01$.

(B) Preliminary screen of wild-type nematodes treated with different concentrations of DOG throughout their lifetime. Survival at three different time points is depicted; white bars indicate survival at day 15, gray bars indicate survival at day 20, and black bars indicate survival at day 25. Only dead animals were scored; no censoring events were incorporated in this analysis.

(C) Life-span assays for wild-type N2 *C. elegans* fed with live OP50-i bacteria and permanently treated with DOG. ● indicates untreated control; □ indicates animals with the respective treatment as indicated (also applies to Figures 1D and 1E, Figures 3G and 3H, and Figure S2D).

(D) Life-span assays for wild-type N2 *C. elegans* fed growth-arrested OP50 bacteria and permanently treated with DOG.

(E) Life-span assays for wild-type N2 *C. elegans* fed live OP50 bacteria and treated with DOG for 6 days only.

(F) Triglyceride content after 2, 24, and 48 hr of DOG administration for wild-type *C. elegans*.

(G) Oxygen consumption after 2, 24, and 48 hr following initiation of DOG treatment for wild-type *C. elegans.*

subsequently increased oxidative stress, which can be abolished by pretreatment with different antioxidants, supporting a process to be termed mitochondrial hormesis or "mitohormesis."

RESULTS AND DISCUSSION

Impaired Glycolysis in *C. elegans* Due to Treatment with 2-Deoxy-D-Glucose

To investigate the potential role of impaired glucose metabolism in life span in a multicellular eukaryote, we exposed adult *C. elegans* to 2-deoxy-D-glucose (DOG) at 5 mM concentration. DOG, which is efficiently phosphorylated by hexokinase but cannot be further metabolized, leads to a specific blockade of glucose metabolism and glycolysis, while breakdown of other nutrients remains unaffected (Sols and Crane, 1954). Accordingly, when maintained on DOG, nematodes almost completely lost the ability to oxidize exogenous, radioactively labeled glucose, as shown by impaired production of uniformly ^{14}C-labeled carbon dioxide (Figure 1A). When maintained on DOG, nematodes showed reduced fertility and a decrease in total progeny production (see Figure S1A in the Supplemental Data available with this article online), both of which are typical for states of extended longevity (Houthoofd et al., 2002b). We next aimed to establish whether chemically altered glucose, as in the case of DOG, is readily transported into nematodes. To test this possibility, we

exposed worms to 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (NBDG), which accumulated mainly in the gut but also appeared to be detectable, although to a much lesser extent, in other regions of the nematode (Figures S1B–S1E). To further investigate the transcriptional results of DOG treatment, nematodes were maintained on this compound for 48 hr, and RNA was extracted and analyzed by the Affymetrix *C. elegans* RNA chip. These analyses revealed an unambiguous induction of energy metabolism, and especially levels of transcripts encoding proteins involved in lipid storage and transport as well as fatty acid oxidation were found to be significantly upregulated. Interestingly, a number of genes involved in mitochondrial energy conversion, namely subunits of the F_1F_0-ATPase complex, showed increased expression (see grouped summary in Table S1). We identified further pathways of potential interest to be regulated, which have been summarized in the Table S1 and are additionally provided as a complete list of all regulated genes in Table S2. Of note, a large group of transcripts encoding collagen or collagen-like proteins displayed clear downregulation after DOG exposure (Table S1). Also, two additional groups, transthyretin-like proteins and carbohydrate-binding proteins (the latter of which are also known as lectins), were downregulated in a remarkably constant fashion (Table S1). Additionally, another group of genes was identified during our analysis that has been predicted to interact with each other in a previous study (Zhong and Sternberg, 2006) integrating a meta-analysis of genetic interactions in different species (Table S1).

Reduced Glucose Metabolism Extends Life Span

Nematodes were exposed to different concentrations of DOG, and preliminary survival rates at different time points showed a typical bell-shaped curve (Figure 1B). Nematodes maintained on their usual food source, live *E. coli*, showed a 25% extension of maximum life span and 17% extension of mean life span (Figure 1C; Table 1) when maintained on DOG. To exclude the possibility that DOG is metabolized by *E. coli*, resulting in nonspecific alterations of phenotypes, experiments were repeated with nematodes maintained on growth-arrested *E. coli*. Maximum life span was similarly increased by 23% and mean life span by 13% under these conditions (Figure 1D; Table 1).

To further evaluate whether life span could be extended by limiting the duration of DOG exposure, we placed nematodes on DOG-containing plates for the first 6 days of early adult life only; thereafter, they were maintained on DOG-free media. As shown in Figure 1E, this short-term treatment was sufficient to extend the worms' life span (see also Table 1).

Lastly, we wondered whether impaired availability of glucose could lead to increased metabolism of triglycerides, supported by a wide body of evidence in mammals (Malik et al., 2006). We observed a significant reduction of body fat in nematodes exposed to DOG (Figure 1F), suggesting increased β oxidation of fatty acids in states of glucose restriction. This is supported by our initial observation on the regulation of lipid metabolism-related transcripts (Table S1).

Taken together, these findings suggest that both short-term and continuous impairment of glucose metabolism extends *C. elegans* life span, a conclusion supported by previous findings suggesting that disruption of certain glycolytic genes may extend life span in nematodes (Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003b).

Reduced Glucose Metabolism Induces Respiration

In the unicellular eukaryote *Saccharomyces cerevisiae*, glucose restriction has been shown to extend life span by inducing respiration in a proposedly sirtuin-dependent fashion (Lin et al., 2002). Sirtuins are histone deacetylases that have been shown to extend *C. elegans* life span in states of overexpression where respiration has not been quantified (Tissenbaum and Guarente, 2001). Houthoofd and colleagues (2002a, 2002b) have found normal or even increased metabolic rates in genetically or environmentally food-deprived nematodes. The above-mentioned findings in *S. cerevisiae* have recently been challenged by demonstration of glucose-deprivation-dependent extension of life span in *S. cerevisiae* lacking mitochondrial DNA and hence presumably devoid of respiratory capacity (Kaeberlein et al., 2005).

Here, we quantified mitochondrial respiration in *C. elegans* in states of impaired glucose metabolism and observed a significant induction of oxygen consumption in nematodes exposed to DOG in comparison to control animals (Figure 1G). This suggests that *C. elegans* induces mitochondrial metabolism in response to impaired glycolysis, consistent with previously published findings for glucose-independent states of calorie restriction in *C. elegans* (Houthoofd et al., 2002a, 2002b) and glucose restriction in *S. cerevisiae* (Lin et al., 2002). Moreover, these findings are supported by recent findings suggesting a positive correlation of respiratory rate and life span in *C. elegans* independent of calorie restriction (Zarse et al., 2007).

Other Modifications of Glucose Metabolism Correspondingly Alter Life Span and Respiration

To exclude the remote possibility that alterations in life span following DOG treatment are caused by mechanisms other than impaired glycolysis, we tested whether treatment of *C. elegans* with RNA interference (RNAi) directed against a glycolytic enzyme, glucose phosphate isomerase (*gpi-1*), would exert a phenotype similar to that observed after DOG treatment. Of note, GPI-1 is an enzyme known to be competitively inhibited by DOG treatment. Consistent with previously published data (Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003b), we observed increased life span in states of impaired *gpi-1* expression that presumably mimic metabolic effects of DOG (Figure 2A; Table 1). As observed for states of DOG treatment (Figure 1G), impaired *gpi-1* expression accordingly increased respiration in *C. elegans* (Figure 2B).

We then questioned whether increased availability of glucose would conversely decrease life span in worms by impairing respiration. When nematodes were exposed

Table 1. Summary of Individual Life-Span Experiments

	Max Life Span in Days ± SEM	Mean Life Span in Days ± SEM	n	p Value versus Control
N2 control	27.9 ± 0.6	17.7 ± 0.4	7	
N2 5.0 mM DOG (full)	35.0 ± 1.7	20.7 ± 0.5	5	<0.0001
N2 5.0 mM DOG (6 days treatment)	34.3 ± 1.3	20.6 ± 0.7	4	<0.0001
N2 NAC (full)	29.0 ± 1.0	16.0 ± 0.1	2	n.s. (0.4792)
N2 NAC (9 days treatment)	29.5 ± 1.5	17.3 ± 0.2	2	n.s. (0.2613)
N2 NAC (full)/DOG (full)	28.5 ± 1.5	16.0 ± 1.2	2	n.s. (0.1745)
N2 NAC (full)/DOG (6d)	29.5 ± 0.5	15.8 ± 0.3	2	n.s. (0.1257)
N2 NAC (9 days)/DOG (full)	34.5 ± 0.5	18.8 ± 0.5	2	<0.0001
N2 NAC (9 days)/DOG (6 days)	27.5 ± 1.5	17.2 ± 0.6	2	n.s. (0.3510)
N2 DOG postreproductive (full)	35.0	20.4	1	<0.0001
N2 control (arrested bacteria)	33.3 ± 0.8	23.2 ± 0.3	6	
N2 5.0 mM DOG (full + arrested bacteria)	41.0 ± 1.0	26.2 ± 1.7	3	<0.0001
N2 5.0 mM D-glucose (full + arrested bacteria)	32.7 ± 0.6	21.9 ± 0.5	3	<0.0001
N2 50.0 mM D-glucose (full + arrested bacteria)	32.7 ± 0.6	21.4 ± 0.5	3	<0.0001
N2 control (+ethanol)	25.0	17.8	1	
N2 5 mM DOG (full + ethanol)	32.0	20.9	1	<0.0001
N2 vitamin C (full + ethanol)	26.0	19.0	1	n.s. (0.3050)
N2 vitamin C (full)/DOG (full + ethanol)	26.0	18.4	1	n.s. (0.7136)
N2 trolox (full + ethanol)	26.0	18.3	1	n.s. (0.6160)
N2 trolox (full)/DOG (full + ethanol)	28.0	18.4	1	n.s. (0.5363)
AAK-2 control	21.2 ± 1.1	14.9 ± 0.5	4	
AAK-2 DOG (full)	19.5 ± 1.5	13.8 ± 0.1	2	0.0190
AAK-2 DOG (6 days)	22.5 ± 0.5	14.0 ± 0.6	2	n.s. (0.0739)
RNAi control (vector L4440)	25.0	17.2	1	
RNAi *gpi-1*	28.0	20.5	1	<0.0001
sir2.1 control (live bacteria)	26.0	19.1	1	
sir2.1 DOG (6 days)	29.0	22.5	1	0.0014
sir2.1 DOG (full)	30.0	23.3	1	<0.0001
sir2.1/rrf-3 control (live bacteria)	24.0	16.8	1	
sir2.1/rrf-3 DOG (6 days)	26.0	18.9	1	0.0002
sir2.1/rrf-3 DOG (full)	27.0	20.0	1	<0.0001

Summary of maximum and mean life spans and statistical analysis (p values) for life-span experiments, including all strains and different treatments displayed in Figures 1C–1E, Figures 2A and 2C, Figures 3A, 3B, 3G, and 3H, Figures 5A–5C, and Figures S2A–S2D. Maximum and mean life spans summarize means of n independent experiments. n.s. = not significant.

to different concentrations of D-glucose (5 and 50 mM), they showed a significant decrease in mean life expectancy (Figure 2C; Table 1) that was paralleled by increased relative triglyceride content (Figure 2D) and reduced respiratory activity (Figure 2E). Hence, increased glucose availability displayed opposite effects as compared to the observations reported for nematodes treated with DOG, thereby not only providing further support for our hypothesis but also emphasizing the detrimental effect of glucose on the mitochondria in eukaryotic organisms.

The Histone Deacetylase Sir2.1 Is Dispensable for *C. elegans* Life-Span Extension

It has been previously shown for states of glucose restriction in *S. cerevisiae* that the histone deacetylase Sir2 is required for extension of replicative life span in yeast (Lin et al., 2000; Lin et al., 2002). We accordingly hypothesized that this protein might be responsible for extension of life span in *C. elegans*. Unexpectedly, we found that DOG treatment was capable of extending life span in two genetically different strains lacking Sir2.1, named *sir2.1*

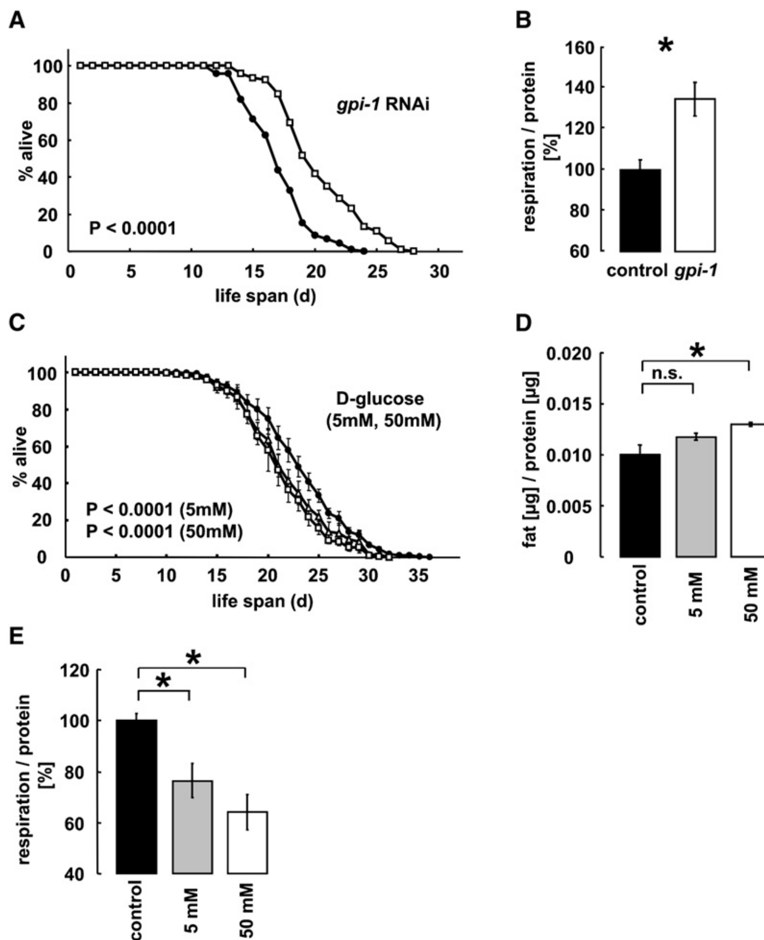

Figure 2. Modulation of Glycolytic Metabolism Is Inversely Correlated with Life Expectancy

(A and B) Life expectancy (A) and oxygen consumption (B) in *C. elegans* after RNAi-mediated reduction of glucose phosphate isomerase (*gpi-1*) expression. ● indicates nematodes fed control RNAi bacteria harboring the control RNAi vector L4440; □ indicates animals fed RNAi bacteria harboring the *gpi-1* RNAi-encoding plasmid. Black bar indicates control RNAi; white bar indicates *gpi-1* RNAi-treated animals.

(C–E) Life expectancy (C), triglyceride content (D), and oxygen consumption rate (E) following administration of various D-glucose concentrations. ● and black bars indicate controls; △ and gray bars indicate 5 mM glucose; □ and open bars indicate 50 mM glucose.

(Figure 3A; Table 1) and *sir2.1/rrf-3* (Figure 3B; Table 1), surprisingly suggesting that Sir2.1 signaling is not required for life-span extension in states of glucose restriction in *C. elegans*.

The *C. elegans* Homolog of AMPK, AAK-2, Is Required for Life-Span Extension

It has previously been shown that AAK-2, the *C. elegans* homolog of mammalian AMP-dependent kinase (AMPK) (Curtis et al., 2006), may play a role in life-span extension in response to various short-term stressors (Apfeld et al., 2004). Phosphorylation of AMPK at serine 172, and hence activity of this kinase, is induced by increasing intracellular levels of AMP and accordingly by decreasing levels of ATP (Kahn et al., 2005), both of which are to be anticipated in states of calorie restriction. Moreover, AMPK has been shown to specifically induce mitochondrial metabolism in mammalian cells (Kahn et al., 2005). Given the importance of both glycolysis and mitochondrial metabolism in providing sufficient amounts of ATP, we questioned whether phosphorylation of AAK-2 might be altered in states of DOG exposure and hence impaired glycolysis. We observed increased abundance of the phosphorylated form of AAK-2/AMPK following short-term exposure to DOG, while this induction became less pronounced after long-term exposure (Figure 3C; percent increase after normalization to α-tubulin: 2 hr, 110%; 24 hr, 24%; 48 hr, 21%).

We subsequently quantified the abundance of the phosphorylated form of AAK-2/AMPK in nematodes treated with high glucose concentrations (Figure 3D, lanes 1–3) and animals fed with RNAi bacteria directed against *gpi-1* (Figure 3D, lanes 4 and 5). While administration of high glucose concentrations reduced the abundance of phosphorylated AAK-2/AMPK (Figure 3D, columns 1–3; percent decrease after normalization to α-tubulin: 5 mM, 71%; 50 mM, 41%), RNAi directed against *gpi-1* mildly increased abundance of the phosphorylated form (Figure 3D, lanes 4 and 5; 27% increase after normalization to α-tubulin), which altogether provides a phosphorylation pattern resembling that of nematodes treated with DOG (Figure 3C). Importantly, phosphorylated AAK-2 protein was not detectable in the *aak-2* mutant strain in either the basal state (Figure 3C, lane 1) or the DOG-exposed state (data not shown). Taken together, these findings suggest that presence of AAK-2/AMPK is required for life-span extension in *C. elegans* under conditions of impaired glucose metabolism.

Since AMPK has been linked to altered β oxidation of fatty acids in mammalian systems (Kahn et al., 2005), we analyzed triglyceride content in nematodes lacking AAK-2 in the presence and absence of DOG (Figure 3E).

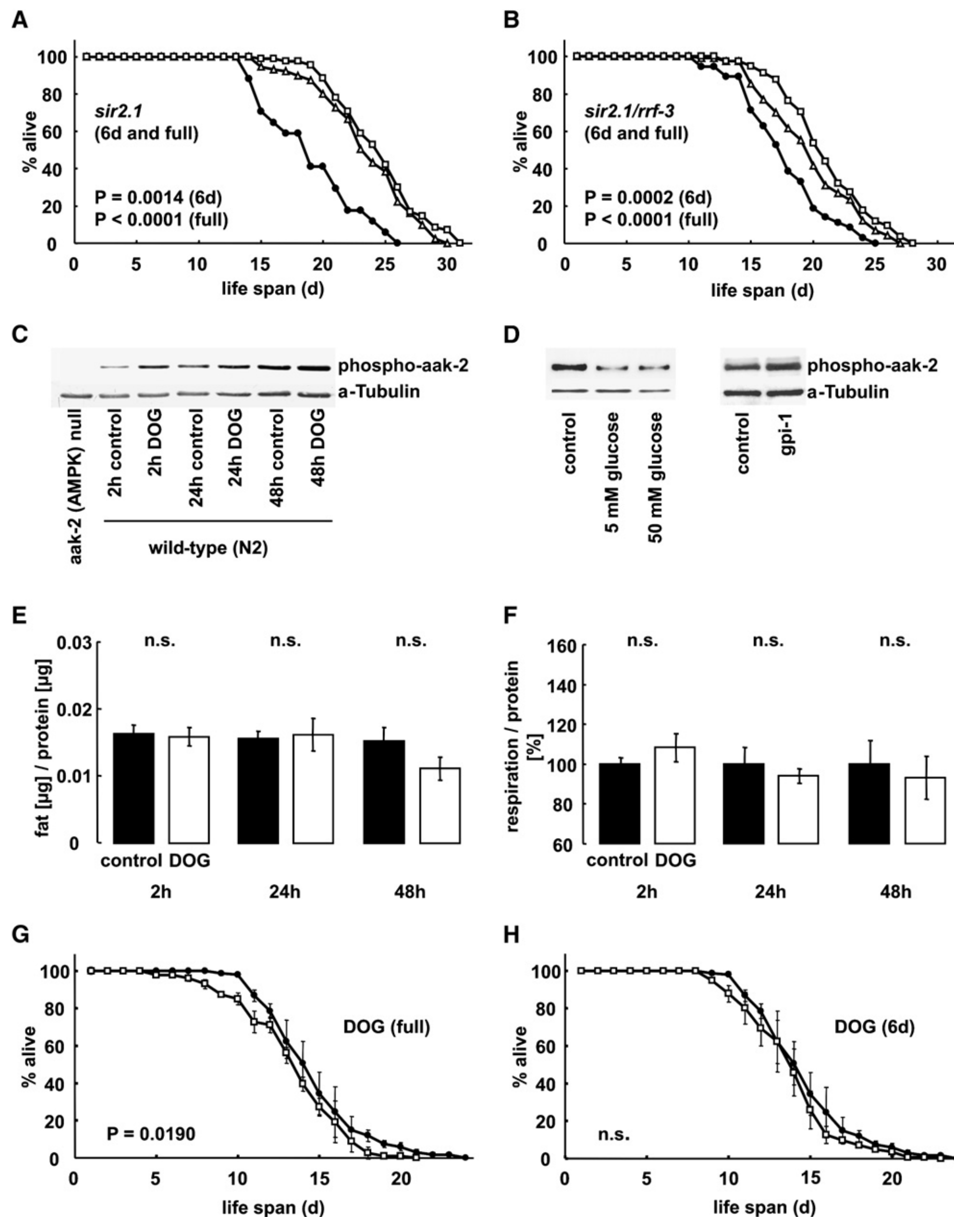

Figure 3. Increased Life Span in States of Glucose Restriction Requires AAK-2, the *C. elegans* AMPK Homolog

(A) Life-span analysis of the *sir2.1* mutant strain. ● indicates control animals; △ indicates animals exposed to DOG for 6 days; □ indicates animals exposed to DOG continuously (also applies to [B]).

(B) Life-span analysis of the *sir2.1/rrf-3* mutant strain.

(C) Abundance of the phosphorylated form of AAK-2/AMPK is increased after 2, 24, and 48 hr of DOG treatment (columns 2–7, upper row; percent increase versus control: 2 hr, 110%; 24 hr, 24%; 48 hr, 21%) in the wild-type N2 nematode. No phosphorylated protein could be detected for the *aak-2*-deficient strain (column 1, upper row). Thirty micrograms of protein lysate was loaded on each column; α-tubulin was used as a loading control and for signal normalization (lower row).

(D) Abundance of the phosphorylated form of AAK-2/AMPK upon exposure to high glucose concentrations after 48 hr of treatment (columns 1–3; percent decrease versus control: 5 mM, 71%; 50 mM, 41%) and after RNAi treatment directed against *gpi-1* (columns 4 and 5; percent increase versus control: 27%).

(E) Triglyceride content in *aak-2* mutant animals treated with DOG for 2, 24, and 48 hr.

(F) Oxygen uptake in the *aak-2*-deficient strain after 2, 24, and 48 hr of treatment with DOG.

(G) Life span of the *aak-2*-deficient strain fed live OP50-i bacteria and permanently exposed to DOG.

(H) Life span of the *aak-2*-deficient strain fed live OP50-i bacteria and exposed to DOG for 6 days.

Nematodes lacking AAK-2 showed reduced basal body fat content (Figure 3E versus Figure 1F), but no further reduction was achieved by applying DOG (Figure 3E). Accordingly, induction of oxygen consumption as observed in wild-type N2 worms following DOG exposure was completely abolished in *aak-2* mutant nematodes (Figure 3F). As the *aak-2* mutant strain displayed a shorter life expectancy than the wild-type strain, we questioned whether overall oxygen consumption rates might be lower in this strain. However, we observed only a slight and nonsignificant trend toward reduced respiration in *aak-2* mutant nematodes as compared to wild-type worms (data not shown).

If induction of respiration is required for extension of life span, life expectancy of nematodes lacking AAK-2 protein should not increase following glucose restriction. Indeed, DOG treatment of *aak-2* mutant nematodes failed to increase life span, in contrast to observations in wild-type N2 worms (Figures 1C–1E; Table 1) and *sir2*-deficient worms (Figures 3A and 3B), but rather showed a small but significant decrease in mean life span for states of continuous DOG treatment (Figure 3G; Table 1). Short-term DOG exposure for 6 days (performed as described above) similarly failed to produce any extension of life span as observed in wild-type worms (Figure 1E), but in contrast to continuous DOG treatment (Figure 3G) showed no significant difference in regards to life span in *aak-2*-deficient nematodes (Figure 3H; Table 1), suggesting that putative toxicity of DOG is unlikely to be involved in the phenotype observed. Taken together, these findings suggest that induction of respiration following glucose restriction is initiated by AAK-2 and that this kinase is required for extension of life span in glycolytically impaired *C. elegans*, while Sir2 appears to be dispensable.

Reduced Glucose Metabolism Induces ROS Formation and Subsequently Stress Resistance

Induction of respiration is commonly accepted to cause increased formation of ROS as an inevitable by-product of increased electron transfer within the respiratory chain (Dröge, 2002; Rea and Johnson, 2003). ROS are assumed to be detrimental to many biological processes (Harman, 1956). In contrast, it has been hypothesized at least in the case of glucose-restricted *S. cerevisiae* that ROS may induce enzymes capable of oxygen radical detoxification, ultimately leading to increased defense capacity against ROS (Kharade et al., 2005). Whether this process occurs in eukaryotes in a similar manner in regards to life-span extension has not been fully elucidated (Ingram et al., 2006; Johnson et al., 2002; Sinclair, 2005; Vanfleteren, 1993). Nevertheless, it has been suggested for both *C. elegans* (Houthoofd et al., 2002a, 2003; Johnson et al., 2002) as well as mice (Speakman, 2005; Speakman et al., 2004) that similar mechanisms, i.e., paradoxical induction of stress resistance in states of increased mitochondrial activity, might apply to multicellular eukaryotes for reasons widely unresolved. Concurrently, as long-term as well as short-term treatment with DOG is sufficient to increase life span, our current findings are in accord with the hormesis theory of aging, hormesis being defined as a short-lasting and nonlethal stressor that induces the stress response mechanisms of an organism and thereby increases not only stress resistance but also overall life expectancy (Masoro, 2006; Rattan, 2001; Southam and Ehrlich, 1943). Also, hormesis has been observed in *C. elegans* under different conditions of stress such as direct oxidative and thermal stress (Cypser and Johnson, 2002).

Therefore, we first tested whether impairing glycolysis indeed might induce ROS formation in *C. elegans*. In states of exposure to DOG for 48 hr, nematodes showed an approximately 3-fold increase in ROS formation (as quantified by DCF fluorescence; Kampkötter et al. [2007]) in comparison to untreated worms (Figure 4A, left pair of bars). Not surprisingly, pretreatment of nematodes with N-acetylcysteine (NAC), a membrane-permeable glutathione precursor known to ameliorate the effects of ROS by mimicking the radical-scavenging potential of reduced glutathione, significantly decreased ROS formation in DOG-treated nematodes (Figure 4A, right pair of bars). To see whether this induction of ROS formation due to DOG treatment might exert hormetic effects on *C. elegans*, we next quantified enzymes known to detoxify ROS in eukaryotic systems, namely superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). While no induction of SOD or GPX activity was observed at four time points evaluated (data not shown), CAT activity was found to be significantly increased in nematodes treated with DOG for 6 days (Figure 4B), though no significant alterations in CAT activity were observed after 2, 24, and 48 hr (data not shown). Hence, restriction of glucose metabolism might induce ROS defense by causing a secondary increase in CAT activity (not observed before 144 hr of treatment) in *C. elegans* following a primary increase in ROS formation (observed as early as after 48 hr of treatment). These findings suggest that increased antioxidant defense capacity (Figure 4B) is a consequence of increased ROS formation (Figure 4A) in states of impaired glucose metabolism.

Next, we exposed DOG-treated nematodes to sodium azide or paraquat, both of which are known to act as mitochondrial stressors and to be capable of inducing mitochondrial ROS formation. Treatment was initiated 6 days after DOG exposure began. DOG-treated worms showed significantly increased survival rates when exposed to paraquat (Figure 4C) or sodium azide (Figure 4D), demonstrating increased stress resistance despite elevated respiratory activity (Figure 1G) in states of impaired glycolysis.

Reactive Oxygen Species Are Required for Increase of Life Span

To investigate the possibility that induction of stress resistance in states of glucose restriction (Figures 4C and 4D) is dependent on DOG-induced ROS formation (Figure 4A), we again pretreated nematodes with NAC. As shown above, such pretreatment significantly reduced the induction of ROS following DOG exposure (Figure 4A) and, in parallel, completely abolished the previously observed (Figures 4C and 4D) increase in stress resistance, which

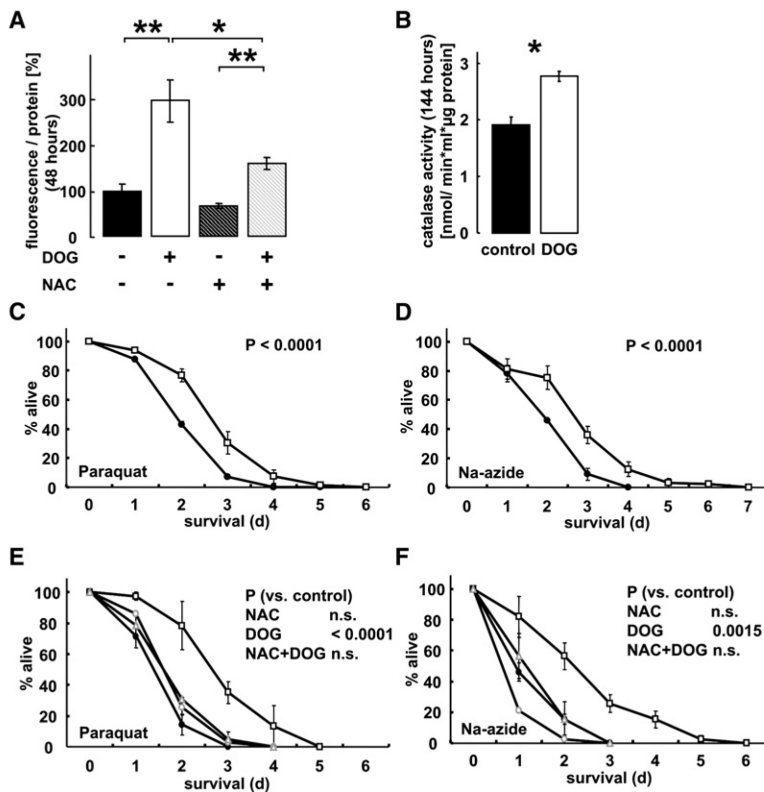

Figure 4. Increased Formation of Reactive Oxygen Species Promotes Stress Resistance in States of Glucose Restriction

(A) Relative formation of reactive oxygen species (ROS) after 48 hr of exposure to DOG with and without pretreatment with N-acetyl-cysteine (NAC). Black bar indicates control animals; white bar indicates DOG-exposed animals; dark gray bar indicates animals treated with NAC alone; light gray bar indicates exposure to NAC and DOG.

(B) Catalase activity in untreated control animals and animals after 6 days of DOG exposure. Black bar indicates control animals; white bar indicates DOG-treated animals.

(C and D) Survival of nematodes after 6 days of pretreatment with DOG on NGM plates additionally containing 50 mM paraquat (C) and 1 mM sodium azide (D). ● indicates untreated control; □ depicts nematodes treated permanently with DOG.

(E and F) Survival of nematodes maintained on NAC during development and 6 days of pretreatment with DOG on NGM plates additionally containing 50 mM paraquat (E) or 1 mM sodium azide (F). ● indicates untreated control nematodes; □ indicates animals treated with DOG only; ○ indicates animals treated with NAC only; △ indicates animals treated with NAC and DOG.

was now absent in states of both paraquat (Figure 4E) as well as sodium azide exposure (Figure 4F). These findings indicate that increased formation of ROS in glycolytically impaired worms is required for the hormetic induction of stress resistance.

Since pretreatment with the antioxidant NAC decreased both ROS formation and acute resistance to paraquat and sodium azide, we questioned whether the extension of life span induced by DOG (Figures 1C–1E) might be reduced by NAC treatment as well. We first observed that treatment of nematodes with NAC alone had no significant influence on life span (Figure 5A; Table 1), suggesting that this antioxidant is dispensable for and incapable of life-span extension in *C. elegans*.

More importantly, we next observed that pretreatment of nematodes with NAC completely abolished the effects of DOG on life-span extension under various combinations of short- and long-term exposure (Figure 5A; Table 1; Figures S2A–S2C), suggesting that induction of ROS must exceed a threshold (Figure 4A) to increase life expectancy in worms in states of impaired glycolysis. Of note, removal of NAC after 9 days of treatment in states of sustained DOG treatment failed to prevent extension of life span by glucose restriction (Table 1; Figure S2C). Similarly, initiation of DOG treatment in a later, postfertile period of the *C. elegans* life cycle was still capable of increasing life span in states of DOG exposure (Table 1; Figure S2D). To rule out the possibility that effects of NAC other than its ROS-scavenging properties might be responsible for the phenotype observed, we subsequently performed life-span experiments with two other well-established antioxidants, ascorbic acid (vitamin C) (Figure 5B) and the α-tocopherol (vitamin E) derivative trolox (Figure 5C). As observed with NAC treatment, administration of either antioxidant followed by DOG abrogated any extension of life span (Figures 5B and 5C), while the respective antioxidant alone had no significant effect on life expectancy (Figures 5B and 5C).

These latter findings tentatively suggest that the widespread use of antioxidants as human food supplements may exert undesirable effects, i.e., decreased cellular and possibly impaired systemic stress resistance that may decrease life expectancy in higher eukaryotes.

Mitohormetic Control of Eukaryotic Life Span

Glycolysis is a biochemical process generating ATP without requiring gaseous oxygen, in contrast to the process of mitochondrial respiration. Inhibition of this pathway by DOG causes a reduction of glycolytic ATP synthesis from glucose (Sols and Crane, 1954). Consistent with this, we observed a mild decrease in ATP levels after 48 hr of DOG treatment (data not shown). Our findings suggest that *C. elegans* compensates for decreased levels of glycolytic ATP by increased production of mitochondrial ATP, as reflected by increased oxygen consumption in states of glucose deprivation (Figure 1G). This metabolic shift from glycolytic to mitochondrial metabolism is initiated by AAK-2 (Figures 3C–3H). Disruption of *aak-2* has previously been shown to reduce life span in *C. elegans*, presumably due to impaired capacity of oxidative

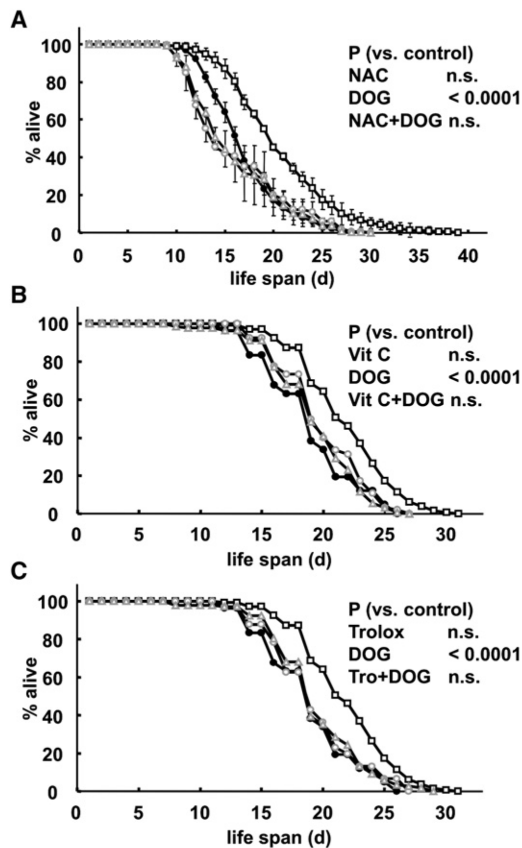

Figure 5. Glucose Availability Controls Life Span via Mitohormesis

(A) Life span of animals permanently exposed to DOG (as in Figure 1C), to NAC, or to DOG plus NAC. ● indicates untreated control nematodes; □ indicates results for animals treated with DOG only; ○ indicates animals treated with NAC only; △ indicates animals treated with NAC and subsequently with DOG.

(B and C) Life span of animals permanently exposed to DOG after pretreatment with the antioxidant ascorbic acid (vitamin C) (B) or the antioxidant trolox (a vitamin E derivative) (C) alone or in conjunction with DOG. ● indicates control nematodes treated with ethanol as solvent control; □ indicates results for animals treated with DOG only and ethanol as solvent control; ○ indicates animals treated with antioxidant only; △ indicates animals treated with the respective antioxidant compound and DOG.

phosphorylation and hence ATP production (Apfeld et al., 2004). Accordingly, we show here that AAK-2 is required to induce respiration, and hence oxidative ATP supply, in states of impaired glycolysis and glucose deprivation. These findings are supported by previous studies potentially linking carbohydrate metabolism to *C. elegans* life span (Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003b). However, at this time, we can not fully rule out other possible mechanisms acting in parallel or in conjunction with AMPK signaling to mediate the life-span-extending effects of DOG exposure.

Recently, two candidate genes have been identified that mediate the effects of food restriction in *C. elegans*, the neuron-localized transcription factor *skn-1* and the Foxa transcription factor family ortholog *pha-4* (Bishop and Guarente, 2007; Panowski et al., 2007). Clearly, more research is necessary to further dissect the mechanistic bases of different models of nutrient restriction, including DOG-mediated glucose restriction, mechanically impaired strains such as *eat-2* (Lakowski and Hekimi, 1998), dilution or complete removal of bacterial food source (Kaeberlein et al., 2006; Klass, 1977), axenic growth (Houthoofd et al., 2002a), and possibly others.

While calorie restriction is generally accepted to extend life span in a variety of model organisms (Ingram et al., 2006; Sinclair, 2005; Weindruch and Walford, 1988) and to exert health-promoting metabolic alterations in humans (Heilbronn et al., 2006), the underlying mechanisms are a matter of fierce debate (Kaeberlein et al., 2005; Lin et al., 2002; Sinclair et al., 2006). One line of evidence suggests that downregulation of mitochondrial metabolism causes decreased formation of ROS (Harman, 1956), a mandatory by-product of mitochondrial electron transfer (Dröge, 2002; Rea and Johnson, 2003). This hypothesis is essentially a modernized version of the "rate of living" theory (Pearl, 1928), which in later years focused on detrimental effects of ROS (Harman, 1956). The other and conflicting line of evidence suggests that induction of mitochondrial metabolism might induce a positive response to increased formation of ROS and other stressors, leading to a secondary, i.e., hormetic (Southam and Ehrlich, 1943), increase in stress defense and cumulating in reduced net stress levels (Houthoofd et al., 2002b; Johnson et al., 2002; Kharade et al., 2005; Sinclair, 2005; Vanfleteren, 1993; Zarse et al., 2007).

Of potential interest in further defining the role of ROS-mediated signaling in longevity are the well-established findings that inactivation of genes encoding proteins localized to the mitochondria can extend life span (Lee et al., 2003b). Although respiration is generally decreased in these strains, oxidative stress defense is often found to be activated, suggesting—on a hypothetical basis in regards to these strains, but in full congruence with our findings—that a primary deregulation of ROS formation might induce ROS defense and finally increase life expectancy. Notably, ROS production has recently been quantified in isolated mitochondria derived from the long-lived *daf-2* mutant strain (Brys et al., 2007). In full accordance with our findings, the authors observed elevated ROS formation in this strain and hypothesized ROS-induced hormetic effects as a possible mechanism of action leading to increased stress resistance and, ultimately, extended life expectancy. On a speculative basis, our current observations together with the latter findings in *daf-2* mutants suggest a generalized ROS-dependent signaling pattern that might apply to the vast majority of long-lived mutants. Future research is clearly warranted to resolve issues of a more generalized role of increased mitochondrial metabolism in extending longevity.

Lastly, it should be noted that increasing the cellular uptake of glucose is a fundamental pharmacological concept in treatment of human type 2 diabetes. While cellular glucose uptake is efficiently promoted by activation of the

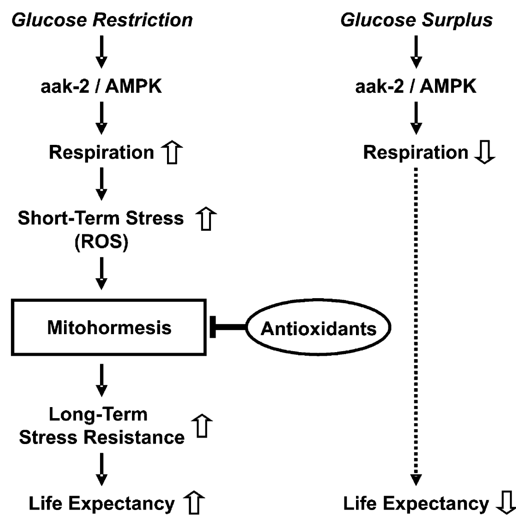

Figure 6. Nutritional Regulation of Life Span by Mitochondrial Hormesis in Response to Increased Oxidative Stress
Reduced availability of glucose induces mitochondrial respiration via AAK-2/AMPK. Increased respiration causes increased ROS formation, leading to mitohormetically increased catalase activity and stress resistance, cumulating in extension of life expectancy. Inversely, increased availability of glucose reduces mitochondrial activity, decreasing life span in nematodes.

insulin signaling cascade to translocate the glucose transporter GLUT4, disruption of this cascade has been shown to extend life span in many organisms, including *C. elegans* (Kenyon et al., 1993) and mice (Blüher et al., 2003; Taguchi et al., 2007). In light of our findings, the current body of evidence tentatively calls into question the efficacy of increasing cellular glucose uptake in diabetics and suggests that other methods of lowering blood glucose (Isaji, 2007; Wright et al., 2007) may be preferable to achieve normal life expectancy in human type 2 diabetes patients.

In summary, our findings suggest that nutritional interventions, and specifically reduction of glucose uptake and metabolism, may extend life span in an AAK-2-dependent fashion due to increased respiration causing a ROS-dependent induction of stress resistance (Figure 6). These findings, supported by recent epidemiological findings in humans (Bjelakovic et al., 2007), tentatively suggest that interventions aimed at decreasing ROS formation do not necessarily promote longevity and may rather reduce life span in multicellular eukaryotes.

EXPERIMENTAL PROCEDURES

Nematodes

The strains used in this study were wild-type Bristol N2, *aak-2*(ok524), *sir2.1*(ok434), and *rrf-3*(pk1426);*sir2.1*(ok434). All strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota). Nematodes were grown and maintained on NGM agar plates as described previously (Brenner, 1974; Zarse et al., 2007). All experiments were performed at 20°C. *C. elegans* stocks and prefertile animals were maintained on OP50 bacteria, while all experiments were performed with the streptomycin-resistant strain OP50-i as food source and with 200 μg/ml streptomycin added to the plates to prevent bacterial contamination except where explicitly stated otherwise. Plates containing experimental treatments were prepared from the same batch of NGM agar as the control plates except that the respective chemical was added to a final concentration of 5 mM from a sterile 0.5 M stock solution for DOG and to final concentrations of 5 and 50 mM from a 1 M aqueous stock for D-glucose. NAC was used at a final concentration of 5 mM from a 0.5 M aqueous stock. Ascorbic acid was used at a final concentration of 5 mM and the α-tocopherol derivative trolox was used at a final concentration of 100 μM (all from Sigma-Aldrich), using agar plates containing a final concentration of 17 mM ethanol (dilution 1:1000) for both ascorbic acid and α-tocopherol. It should be noted that ethanol appears to decrease *C. elegans* life span (compare NAC [no ethanol] with vitamin C/trolox [17 mM ethanol] in Figures 5A–5C). Animals were exposed to the antioxidants from the day of hatching in all experiments. For instance, short-term treatment with NAC lasted for 9 days in toto, while short-term treatment with DOG lasted for 6 days after initiation in the young adult stage. For all experiments involving high glucose concentrations, growth-arrested bacteria were employed as food source. To efficiently inhibit bacterial growth, 1000 μg/ml ampicillin was added to a fresh liquid culture of OP50, and bacteria were incubated for at least 5 hr. Bacteria were centrifuged and washed once with S buffer as described previously (Zarse et al., 2007). After recentrifugation, bacteria were kept frozen as pellets until use. Pellets were thawed and resuspended in one-tenth of the original culture volume with S buffer prior to use and were streaked onto agar plates containing 100 μg/ml ampicillin and 200 μg/ml streptomycin at least 24 hr before nematodes were placed onto the plates.

Fertility Assays

To determine fertility of nematodes, ten animals at the beginning of the egg-laying period were placed on NGM plates with or without DOG and were removed after an incubation period of 12 hr. Offspring were allowed to develop for 48 hr, and infertile eggs and hatched nematodes were quantified.

NBDG Staining

Staining with the fluorescent compound 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (NBDG) (Invitrogen) was performed in live animals. A stock of 0.1 mM NBDG in DMSO was diluted 1:500 in M9 buffer to obtain a final concentration of 200 μM. Young adult nematodes were washed off of a plate with M9 and washed three times before resuspending in the NBDG solution. After 60–90 min of staining, animals were briefly centrifuged and washed three times with 15 ml M9 without NBDG to remove all dye that was not incorporated into the nematodes. Animals were mounted on microscope slides covered with 1% agarose gel (w/v) in a small drop of 5 mM levamisol (Sigma-Aldrich) for anesthesia and covered with a coverslip.

Confocal photographs were taken with a Zeiss LSM 410 inverted laser scanning microscope connected to a Zeiss Axiovert 100 microscope. Magnification was 200-fold at an excitation wavelength of 488 nm and an emitted fluorescence wavelength ranging from 530 to 585 nm. Display items are representative for a series of photographs taken of stained and nonstained animals. All photographs were taken with identical settings to ensure comparability.

Life-Span Assays

Life-span analysis was carried out according to standard protocols (Kenyon et al., 1993; Zarse et al., 2007). Briefly, to obtain a synchronously growing population, eggs were prepared by treating a population of *C. elegans* with hypochlorite/NaOH solution and transferring the resulting eggs to NGM agar plates covered with *E. coli* strain OP50. At the prefertile young adult stage, 100 to 150 animals were transferred to NGM plates (with or without DOG) containing 200 μg/ml streptomycin that had been previously covered with the streptomycin-resistant bacterial strain OP50-i. This time point also represented the first day of

life-span analysis. To assay life spans on growth-arrested *E. coli* (as initially suggested by Garigan et al., 2002; Wilson et al., 2006), bacteria and NGM plates were additionally treated with ampicillin as described above. Nematodes were transferred to fresh plates daily during the progeny production period and after that were transferred every second to third day but monitored daily for dead animals. Nematodes that did not respond to gentle prodding and displayed no pharyngeal pumping were scored as dead. Animals that crawled off the plate or died due to internal hatching or protrusion of the gonads through the vulva were censored. Censoring describes an event where partial information on the life span of an individual animal is lost as a consequence of premature death. Thus, censored animals were included in statistical analysis only until the day of the censoring event. Replicates were carried out as indicated in Table 1, column 4. No censoring was applied in Figure 1B.

Fat Content Analyses

Eggs were prepared as above and allowed to hatch overnight in M9 buffer to obtain a highly synchronous population of L1-stage animals. Nematodes were then shifted to NGM plates covered with *E. coli* OP50, allowed to grow until the L4 stage, and transferred to plates containing 25 μM 5-fluoro-2′-deoxyuridine (Sigma-Aldrich) to prevent progeny formation. After an incubation of 16 hr, animals were transferred to the respective treatment groups and harvested at the indicated time points by three consecutive washes in ice-cold M9 buffer to separate nematodes from bacteria. Nematodes were flash frozen in liquid nitrogen and stored at −80°C until further processing. Approximately 25 mg was weighed and ground in a nitrogen-chilled mortar together with 250 μl of frozen phosphate buffer. The frozen material was gathered in a reaction tube and kept on ice. Extracts were sonicated three times and centrifuged for 7 min at 12,000 × g to clear the lysate off of insoluble matter. Fat content was determined with a commercially available triglyceride determination kit (Sigma-Aldrich) as previously described (Ristow et al., 2000) and normalized to protein content, which was determined according to the Bradford (1976) method. For each treatment condition and time point indicated, at least two independently generated biological samples were obtained, with double preparations of lysates from each sample and double measurements for every preparation.

Respiration Assays

Oxygen consumption rates were measured using a DW1/AD Clark-type oxygen electrode (Hansatech) as previously described (Lee et al., 2003a; Zarse et al., 2007). Age-synchronized animals were obtained as described above. Approximately 2000 nematodes were plated on NGM plates containing the corresponding treatment and 200 μg/ml streptomycin and covered with *E. coli* OP50-i. For every time point, at least two independent experiments were performed comprising at least three replicates per experiment and time point. At the time of measurement, animals were washed three times with M9 buffer and resuspended in 2 ml of M9 buffer. One milliliter aliquots were transferred into the chamber, and respiration was measured at 20°C for at least 10 min. Samples were carefully recovered from the chamber, centrifuged, and homogenized in a Dounce-type homogenizer (Homgen Plus, Schütt Labortechnik) for 30 s at 3000 rpm. After centrifugation at 8000 × g for 5 min, the supernatant was removed and used for protein quantification by the Bradford (1976) method.

RNA Interference

RNAi was induced as described (Oh et al., 2006; Zarse et al., 2007) according to standard protocols. For the life-span and respiration assays, slight modifications were applied: Animals were fed with RNAi bacteria from the day of hatching, and thus the age of the population tested in the respiration assay corresponds to that of the 48 hr DOG treatment groups. In addition, streptomycin in the plates was replaced by 100 μg/ml ampicillin, as the bacterial strain HT115 used for the RNAi feeding protocol contained an ampicillin-resistance plasmid. As a control, we employed HT115 bacteria harboring the L4440 plasmid without insertion as described before (Zarse et al., 2007). For induction of RNAi directed against glucose phosphate isomerase (*gpi-1*) gene transcripts, an RNAi plasmid described previously was used (Hansen et al., 2005; Kamath and Ahringer, 2003; Kamath et al., 2003). To ensure the presence of proper RNAi effects, the plasmid pLT61.1 from the Fire Lab Vector Kit, which causes a strong twitching phenotype in affected animals, was used as a parallel control in all RNAi experiments.

Stress Resistance Assays

Stress resistance was assayed by maintaining nematodes for 6 days, until progeny production ceased, on DOG or control plates. The 6 day treatment appeared necessary, as most animals died due to internal hatching if the stressor was applied earlier throughout the egg-laying period. After 6 days, nematodes were transferred to plates containing either 50 mM paraquat or 1 mM sodium azide (both from Sigma-Aldrich) in addition to DOG where applicable. Dead animals were scored as described above. NAC was applied similarly at a final concentration of 5 mM. Nematodes were maintained on NAC during development and during treatment with DOG (from L4 onward) before transferring to NGM plates containing paraquat or sodium azide in addition to the primary stressor where applicable.

Quantification of ROS Formation

ROS formation was quantified according to a modified protocol employing the membrane-permeable nonfluorescent dye 2,7-dichlorodihydrofluorescein-diacetate (H_2-DCF-DA) (Sigma-Aldrich) (Kampkötter et al., 2007). Upon entering the cell, H_2-DCF-DA is deacetylated and becomes membrane impermeable. H_2-DCF fluoresces upon oxidation to 2,7-dichlorofluorescein (DCF) by ROS. Briefly, animals were maintained and treated as described above. After 48 hr of exposure to the respective compounds, animals were washed off of the plates with cold M9 buffer. Bacteria were removed by three repeated washes and subsequent centrifugation at low speed. Animals were resuspended in M9 buffer, and a 50 μl volume of the suspension was pipetted in four replicates into the wells of a 96-well plate with opaque walls and transparent bottom and allowed to equilibrate to room temperature. In the meantime, a fresh 100 μM H_2-DCF-DA solution from a 100 mM stock solution in DMSO was prepared in M9 buffer, and a volume of 50 μl was pipetted to the suspensions, resulting in a final concentration of 50 μM. On each plate, control wells containing nematodes from each treatment without H_2-DCF-DA and wells containing H_2-DCF-DA without animals were prepared in parallel. Immediately after addition of H_2-DCF-DA, basal fluorescence was measured in a microplate reader at excitation/emission wavelengths of 485 and 520 nm. Plates were kept on a shaker at 20°C for 60 min and were measured under conditions equivalent to before. The initial fluorescence and the fluorescence signals of the control wells were subtracted from the corresponding signals of each well after the second measurement. One milliliter of the initial animal suspension from each sample was centrifuged, and the pellets were kept at – 80°C for later protein quantification to normalize the fluorescence signal. Assays were performed in independent duplicates.

Protein Analyses

Nematodes were washed three times with ice-cold M9 buffer as above, and pellets were shock frozen in liquid nitrogen. Protein extraction and western blot were performed as described previously (Schulz et al., 2006) except for minor adaptations due to the nature of the sample. Frozen pellets were ground in a nitrogen-chilled mortar and gathered in phosphate buffer containing protease and phosphatase inhibitors, Complete protease inhibitor cocktail (Roche), 2 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM PMSF, and 2 mM EDTA (all from Sigma-Aldrich). Extracts were sonicated three times and centrifuged for 7 min at 12,000 × g to clear the lysate off of insoluble matter. Supernatants were used for protein quantification, and a portion was boiled in sample buffer and applied for SDS-PAGE. Antibodies against basal and phosphorylated (Ser172) AMPK (both from

Cell Signaling Technology, Inc.) and α-tubulin (Sigma-Aldrich) were used. Antibody raised against basal AMPK gave no specific signal on protein extracts from *C. elegans*.

RNA Extraction and RNA Array Analyses

Animals were treated as described above for fat content analyses. Adult animals were placed on plates containing 5-fluoro-2′-deoxyuridine and the indicated treatment for 48 hr. Three independent replicates were prepared for both control- and DOG-treated animals (i.e., 6 samples in total). RNA was extracted from nematode samples according to the TRIzol method (Invitrogen) as described (Thierbach et al., 2005) and according to the manufacturer's instructions. Fifty milligrams of frozen nematode sample was used for each extraction. RNA was subsequently loaded onto an RNA binding column (QIAGEN) to remove contaminating genomic DNA and proteins before further processing. Total RNA content was assayed, and equal amounts of RNA were used for hybridization. For transcript-level analysis, the Affymetrix GeneChip *C. elegans* Genome Array was used according to the manufacturer's instructions. Analysis of the presented data was performed with Affymetrix GCOS 1.4 software by comparing means of signal intensities from RNA samples derived from control- and DOG-treated animals. Genes that displayed regulation in either direction were assembled and assigned a molecular function where possible by searching the Wormbase databank (http://www.wormbase.org/) for detailed information on the specific Affymetrix probe set identity numbers. Ratios for transcript-level regulation as control- over DOG-treated signal intensities were obtained to illustrate the extent of regulation (Affymetrix GCOS 1.4 software; threshold for downregulation was set to 1.50 and for upregulation to 0.66). As individual transcripts are represented by a set of different probes on the chip, transcripts were only considered to be regulated when at least two-thirds of probes for the same transcript displayed similar regulation. A subset of genes was grouped manually where applicable to facilitate interpretation of the data (Table S1); the complete set of genes is listed in Table S2.

Activity Assay for CAT, SOD, and GPX

Enzyme activities were assayed by employing commercially available kits (CAT and GPX, EMD Bioscience/Merck Biosciences; SOD, Sigma-Aldrich) and processed according to the respective manufacturers' instructions. For each time point and treatment, at least three independent samples were prepared at different days and extracted for further measurements. Extracts were measured in double determinations. Briefly, approximately 25 mg of frozen nematode sample was homogenized as described above in 50 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0), sonicated, and cleared by centrifugation. Supernatant samples were diluted 1:3 for CAT and SOD or used undiluted for GPX and assayed for enzyme activity.

[^{14}C]Glucose Oxidation

Uniformly labeled [^{14}C]D-glucose was purchased from GE Healthcare. Specific activity of the batch used was 281 mCi/mmol. Animals were maintained as described above on plates containing FUDR and the respective treatment for 48 hr. Equal numbers of animals were placed on each plate when treatment was initiated. After collection and three subsequent washes in S medium, worm pellets were resuspended in the incubation buffer. Two milliliters of the suspension was transferred to 4 cm Petri dishes. The latter were placed in 10 cm Petri dishes together with a second 4 cm Petri dish containing 1 ml of 0.1N KOH solution to trap CO_2 as described previously (Ristow et al., 2000). Hence, each 10 cm dish contained two 4 cm dishes, one carrying nematodes and the other containing KOH. As a substrate, labeled glucose was added to a final concentration of 35 μM U-[^{14}C]D-glucose (10 μCi/ml) in the nematode suspension. Nonradioactive glucose or DOG was added to a final concentration of 5 mM to the control samples or the samples pretreated with DOG, respectively. The 10 cm Petri dishes were covered, sealed with Parafilm in an air-tight manner, and incubated at 20°C for 60 min. Subsequently, an aliquot of 900 μl of KOH was immersed in 10 ml of scintillation fluid and placed in a liquid scintillation counter (Beckman LS 6000, Global Medical Instrumentation, Inc.) to quantify the amount of trapped $^{14}CO_2$ as described previously (Ristow et al., 2000).

Quantification of ATP

Animals were treated and samples were obtained as described above for fat content analyses. Quantification of ATP followed standard protocols for animal samples. Briefly, worm lysates were prepared as above by grinding approximately 20 mg of sample in a nitrogen-chilled mortar and collection in perchloric acid solution (0.6 M in 1 mM EGTA). Precipitated proteins were separated by centrifugation after 30 min incubation on ice and redissolved in 1 M sodium hydroxide solution for protein quantification after drying. The supernatant was adjusted to neutral pH with 3 M potassium hydroxide/potassium chloride solution and incubated for 10 min on ice. Precipitated salts were removed by centrifugation, and the supernatant was stored for later ATP quantification at −80°C. ATP content was determined as described previously (Ristow et al., 2000) by applying a commercially available assay kit (Sigma-Aldrich). Three independent samples were prepared for each treatment and time point, and double extractions were performed for each sample. Each extract was assayed for ATP content in duplicate.

Statistical Analyses

Statistical analyses for the fertility, fat content, oxygen consumption, and enzyme activity assays were performed by Student's t test after testing for equal distribution of the data and equal variances within the data set. For comparing significant distributions between different groups in the life-span assays and stress resistance assays, statistical calculations were carried out using the log-rank test. All calculations were performed using Microsoft Office Excel 2003 and SPSS version 13.0 as described previously (Schulz et al., 2006; Zarse et al., 2007). Experiments were performed in triplicate except where stated otherwise. Error measures were calculated from the variability of the independently prepared biological samples of the same treatment group where applicable. Differences were considered statistically significant at $p < 0.05$, thereby indicating a probability of error lower than 5%.

Supplemental Data

Supplemental Data include two figures and two tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/6/4/280/DC1/.

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