Caloric restriction, Ins/IGF-1 signalling and longevity in the nematode *Caenorhabditis elegans*

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ABSTRACT. Several mechanisms of life span extension in *C. elegans* have been described, including caloric restriction, reduced Ins/IGF-1 signalling, Clk mutation and germ line ablation. Here, we describe the effects of caloric restriction on metabolism and life span in *C. elegans* and examine whether Ins/IGF-1 signalling is involved in the life extension observed in calorically restricted worms. We show that life span extension in restricted worms is not caused by a reduced metabolic rate, but is accompanied by enhanced stress resistance. We further show that caloric restriction and Ins/IGF-1 have additive effects on life span extension and on the stress defense enzyme activities, and that caloric restriction acts independently of *daf-16*. Thus, caloric restriction extends life span by mechanisms distinct from those affected by the Ins/IGF-1-like pathway.

KEY WORDS : aging, longevity, metabolism, *C. elegans*, caloric restriction, Ins/IGF-1 signalling.

INTRODUCTION

During the last two decades, more than 40 genes have been described that, when mutated, increase the life span of *C. elegans* (JOHNSON et al., 2000). Mutations in *daf-2* and *age-1* induce inappropriate dauer formation, but larvae that bypass the dauer stage at lower temperature can develop into adults that are long-lived (FRIEDMAN & JOHNSON, 1988; KENYON et al., 1993). The longevity, or Age, phenotype is suppressed by mutations in the downstream gene *daf-16* (LIN et al., 1997). These and several other genes act in a common signalling pathway (GOTTLIEB & RUVKUN, 1994; DORMAN et al., 1995), which shows homology to the mammalian insulin and IGF-1 signalling pathways : *daf-2* was found to encode an insulin/IGF-1 receptor (KIMURA et al., 1997), and *age-1* a catalytic subunit of phosphoinositide-3-OH kinase (MORRIS et al., 1996). The protein encoded by *daf-16* is a forkhead transcription factor (OGG et al., 1997; LIN et al., 1997), which is inactive and resides in the cytoplasm when phosphorylated by the Ins/IGF-1 signal, and relocates to the nucleus and controls transcription when dephosphorylated (HENDERSON & JOHNSON, 2001; LIN et al., 2001). Longevity mutants with reduced activities of the Ins/IGF-1 pathway were recently discovered in *Drosophila* (CLANCY et al., 2001) and mice (COSCHIGANO et al., 2000), suggesting that this pathway for life span control has an ancient origin.

A nutritionally complete, but calorie restricted, diet can significantly extend life span in a wide variety of both vertebrate and invertebrate animals and presumably humans as well (FINCH, 1990; LANE, 2000). The mechanism by which caloric restriction prolongs life span is still unknown, however. It has been suggested that caloric restriction induces up-regulation of a variety of somatic maintenance functions (YU, 1994; MASORO, 1995; DHABHI et al., 2001) suggesting adaptive regulation and thus the existence of a specific pathway specifying the anti-ageing action of caloric restriction. This is sensible since a genetic program specifying enhanced maintenance in periods of food shortage would offer an appreciable selective advantage. Despite numerous suggestions that the Ins/IGF-1 pathway in worms may mediate the nematode response to caloric restriction (APFELD & KENYON, 1999; LANE, 2000; GUARENTE & KENYON, 2000), no critical tests have been done to test this hypothesis.

A contrasting and quite popular hypothesis assumes that caloric restriction acts by decreasing oxidative stress (SOHAL & WEINDRUCH, 1996). Considering that a decrease of metabolic activity would result in reduced fluxes of reactive oxygen species (ROS), produced as by-products of metabolism and believed to be proximal causes of the aging process, some have favored the idea that the beneficial effect of caloric restriction is associated with a hypometabolic state (LAKOWSKI & HEKIMI, 1998; LEE et al., 1999). Thus according to this view, life extension resulting from caloric restriction is under thermodynamic rather than direct genetic control.

We used axenic culture medium, diluted *E. coli* concentration in suspension cultures, and the *eat-2 (ad1113)* mutant to study caloric restriction and the interaction with the Ins/IGF-1 pathway. The *eat-2 (ad1113)* mutant has a defect in pharyngeal pumping, resulting in reduced food uptake. Axenic medium supports sustained growth of *C. elegans* in the absence of a bacterial food source. Worms raised in this medium show several symptoms of caloric restriction including slenderness, delayed maturation, substantially extended and reduced fecundity and extended life span, as well as several biochemical alterations shared with *eat-2* mutants and wild-type worms grown on a restrictive bacterial diet (HOUTHOOFD et al., 2002a, b).

MATERIAL AND METHODS

Culture conditions

Monoxenic (containing one additional species) age synchronous cultures were established as follows. Eggs were prepared by hypochlorite treatment of gravid adults and allowed to hatch overnight in S buffer (SULSTON & HODGKIN, 1988), and the resulting first stage larvae (L1) were inoculated onto freshly prepared cholesterol-supplemented nutrient agar (OXOID) plates containing a lawn of *E. coli* 9001 cells. Fourth stage larvae (L4) were transferred into Fernbach flasks containing 250 ml of aging medium, which was S buffer containing approx. 3×10^9 bacterial cells/ml and 50 µM FUdR (to prevent reproduction). The bacteria were added as frozen beads containing equal volumes of pelleted bacteria and S buffer. The flasks were shaken in a temperature controlled (24 °C) gyratory shaker at 120 oscillations/min.

Axenic cultures were established by two consecutive cycles of hypochlorite treatment to achieve sterility. L1s were inoculated into axenic culture medium. Axenic culture medium contained 3% (w/v) soy-peptone and 3% (w/v) dry yeast extract and was sterilized under standard conditions. After cooling, hemoglobin stock solution was diluted 100 fold into the basal medium. Hemoglobin stock solution was prepared by dissolving 5 g hemoglobin in 100 ml 0.1 N KOH, and autoclaving for no longer than 10 min at 121 °C to reduce excessive hydrolysis. To improve synchronous development, heat-killed *E. coli* cells were added at 3×10^9 cells/ml. As soon as the worms reached the fourth larval stage, they were washed with sterile S buffer and suspended into Fernbach flasks containing 250 ml axenic medium (without heat killed bacteria). FUdR was added at 50µM final concentration. Culture conditions were as described for the monoxenic cultures.

Metabolic analyses

An aliquot of a worm culture was harvested daily or every second day and cleaned using Percoll (FABIAN & JOHNSON, 1994) and sucrose density gradients, as previously described (BRAECKMAN et al., 2002). The respiration and heat output assays required live worms. Cleaned worm samples for other assays were frozen and stored at –75 °C. Consumption of dissolved oxygen by suspended worms was monitored using Clark electrodes, and heat output was measured by microcalorimetry (BRAECKMAN et al., 2002). Oxygen consumption was performed using worms that were suspended in liquid, as required for polarographic respirometry (Clark electrodes), but heat measurements were made under both growth conditions. Even so, all worms were suspended in liquid for the sucrose and Percoll washes, and we cannot guarantee that the metabolic activities in culture and under the assay conditions were identical. This is a caveat to comparing metabolic activity of worms raised on plates or in suspension.

ATP levels were measured by using the well-established luciferin-luciferase assay adapted for use in a microtiter plate format as described previously (BRAECKMAN et al., 2002). This assay is based on the reaction : luciferin + ATP + O_2 → oxyluciferin + AMP + pyrophosphate + CO_2 + light. The flash frozen nematode samples (100 µl) were taken from the –75 °C freezer and immediately submersed in a boiling water bath for 15 min to destroy ATPase activity and to allow diffusion of ATP out of the corpses. Dilutions were made using HPLC grade water (salts interfere with the assay) and the assay was performed according to the manufacturers' (Roche Diagnostics GmbH, Germany) instructions. This assay is several orders of magnitude more sensitive than colorimetric assays. We measured levels of approx. 1.4 mM ATP in young adult wild-type worms, approaching the range of values (2-10 mM) detected in other forms of life (LEHNINGER, 1981). Comparative assays using live worms that were boiled and sonicated to maximize ATP recovery indicated that our protocol underestimates true values by about 16%. We consider this mild underestimation fully acceptable in view of the convenience afforded by using stored worms.

We assessed total bioreduction capacity using XTT. Tetrazolium salts, especially MTT, are widely used for measuring the redox potential of cells as a parameter of viability (JOHNSON, 1995). This approach is based on the reduction of MTT, within active mitochondria of living cells, by succinate dehydrogenase to an insoluble blue formazan derivative. Tetrazolium salts can also be used to score dehydrogenase activity in cellular extracts. These methods commonly monitor phenazine methosulfate (PMS) -mediated reduction of tetrazolium by various NAD(P) dependent dehydrogenases (ALTMAN, 1972; RICHARDSON et al., 1986). Unlike most previous tetrazolium compounds, XTT [(2,3-bis-(2-methoxy-4 nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] is reduced to a water-soluble formazan, allowing direct monitoring of its appearance. We found no activity when these tetrazolium salts were added to worm homogenates (made 1% in CHAPS) in the absence of PMS. However, formazan formation proceeded upon addition of reduced nicotinamide cofactors. Conversely, reduced nicotinamide cofactors were unable to reduce XTT directly in the absence of appropriate electron acceptors such as phenazine methosulfate. Thus worm homogenate reduces XTT in the presence of NAD(P)H, and the amount of formazan produced increases linearly with the amount of tissue extract added (BRAECKMAN et al., 2002).

We found that exogenous SOD lowered XTT reduction by 50% suggesting that this amount is due to superoxide, and we infer that the activity that is not suppressible by SOD is contributed by unknown NAD(P)H-dependent reductase(s). This assay can be used to monitor changes in XTT bioreduction capacity that occur as a function of metabolic alterations, including those that accompany aging. Thus, although the precise biochemical targets of the assay are as yet obscure, we consider the XTT reduction capacity an excellent biomarker of aging.

SOD activity was measured by an assay based on the inhibition of superoxide-induced lucigenin chemiluminescence by SOD (CORBISIER et al., 1987). Aliquots of 6.7 µl were taken from a sample dilution series and added in duplicate to the wells of a microtiter plate. Next, 20 µl aliquots of xanthine oxidase reagent (xanthine oxidase diluted in double distilled water such that the blank reaction containing 6.7 µl water, 20 µl XO dilution and 174 µl

reaction mixture yielded approx. 1.2 x 10⁵ counts/s) and 174 μl of reaction mixture (5.2 ml 0.1 M glycine, 1 mM EDTA, adjusted to pH 9.0 with NaOH, 10 ml 0.108 mM xanthine, 2.1 ml 1 mM lucigenin, 1.2 ml water for a total of 18.5 ml) were added quickly by using a multichannel pipette. Luminescence was measured for 0.1 s during the time span required for 25 consecutive plate measurements at 25 ˚C using the Victor² Multilabel Counter. One unit of SOD activity is defined as the amount of SOD able to reduce the luminescence intensity by 50%. The homogenate fraction (dilution) reducing luminescence by 50% was derived mathematically from plots of the luminescence intensities measured as a function of the homogenate fraction. The sensitivity of this assay is superior to the standard cytochrome c assay (VANFLETEREN, 1993; VANFLETEREN & DE VREESE, 1995) and the numerical values of SOD activity are not comparable.

Catalase activity was assayed at 25 ˚C according to the method of AEBI (1984), adapted for use in microtiter plate format. Briefly 6.9 μl sample volumes were added to the wells of a 96-well flat bottom UV transparant microtiter plate (UV-star, Greiner). The reaction was started by adding 200 μl substrate (11.4 mM hydrogen peroxide in 50 mM Na_2HPO_4 : KH_2PO_4 (Sørensen) buffer, pH 7.0) using

a multichannel micropipette. The decrease in absorbance was monitored at 240 nm (Spectramax 190, Molecular Devices) for 25 reads (12 s interval, total measuring time : 4 min, 17s). The amount of peroxide decomposed was calculated using a molar coefficient of $\varepsilon_{240nm, 1cm}$ = 39.4. The enzyme activity decomposing 1μmole of hydrogen peroxide per min equals 1 unit catalase activity.

The experimental data were scaled to protein content to correct for differences in biomass. The metabolic measurements were repeated several times to reduce assay variation. The results were obtained from three or four independent source populations to control for environmental variation.

RESULTS AND DISCUSSION

Our primary interest was to determine if caloric restriction causes a reduction of the metabolic rate, as is often assumed. We therefore measured the oxygen consumption and the heat output of calorically restricted worms (Fig. 1a and 1b). We found that worms that undergo caloric restriction by three different means had significantly higher metabolic rates, suggesting that the life span extension of restricted worms is not caused by reduced
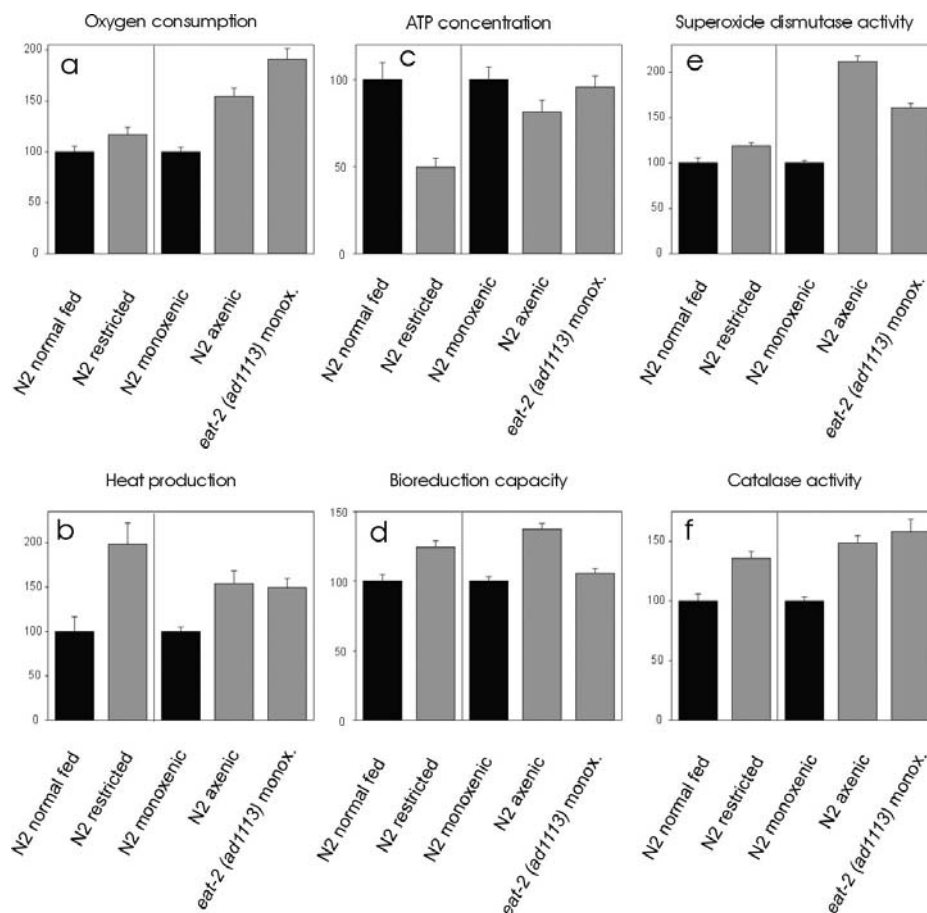

Fig. 1. – Metabolic alterations in response to caloric restriction. Experiment #1 (left panels) : N2 normal fed : two-day-old adults of N2, grown in Fernbach flasks. Average of cultures supplied with 15, 6 and 2.4 x 10⁹ *E. coli* cells/ml; N2 restricted : two-day-old adults of N2, grown in Fernbach flasks. Average of cultures supplied with 96, 38, 15 and 6 x 10⁷ *E. coli* cells/ml. Average of four replicates. Experiment #2 (right panels) : N2 and *eat-2 (ad1113)* monoxenic : worms grown in Fernbach flasks containing 3x10⁹ *E. coli* cells/ml S buffer. N2 axenic : N2 grown in axenic medium. Average over the first ten days of the adult life span of three-five replicates. All parameter values relative (%) to the normal fed wild type controls. All strains were grown at 24˚C.

metabolic activity. We reasoned that partial starvation may enhance ATP and NADPH consumption activity for *de novo* synthesis of biomolecules that are otherwise supplied with food. We therefore measured the ATP concentration in the worms and found less ATP in calorically restricted worms (Fig. 1c). We also measured the worms' ability to reduce XTT, since we have discovered that this capacity is strongly enhanced in non-feeding dauers (HOUTHOOFD et al., 2002c), which can survive several times the normal life span (KLASS & HIRSH, 1976). We found elevated XTT reduction capacity in worms grown in liquid medium, supplemented with less *E. coli* and in worms grown in axenic medium (Fig. 1d). However, the *eat-2 (ad1113)* mutant did not show elevated XTT reduction capacity, but the age dependent decline was slower (not shown), resulting in higher reduction capacity at older age. Perhaps the increase in bioreduction capacity partly serves the *de novo* synthesis of biomolecules. The observed metabolic effects were not caused by reduced progeny production, since a mutant lacking a germ line did show similar effects (not shown).

If a reduced metabolic rate is not the reason for the longevity phenotype of restricted worms, then one could assume that increased defense against oxidative stress could be the primary cause. We therefore measured the catalase and SOD activity of restricted worms. We found that the activity of both enzymes was increased in response to caloric restriction (Fig. 1e and 1f). We therefore conclude that caloric restriction increases the life span of *C. elegans* by increasing the ROS defense.

We further asked what pathway could regulate the increase in life span of calorically restricted worms. The Ins/IGF-1 pathway is an obvious candidate, since it is known that it controls life span in *C. elegans*. This phosphorylation cascade pathway tranduces a signal from the DAF-2 receptor to AKT proteins. AKT proteins regulate the localization of the transcription factor DAF-16 (HENDERSON & JOHNSON; 2001). Nuclear localization occurs in the absence of signalling due to lack of ligand binding or to mutation in one of the proteins in the pathway, and results in increased adult life span. To answer our question, we grew Ins/IGF-1 mutants under caloric restriction circumstances and determined the life span, stress defense and metabolic alterations.

We found that the mean life span of wild type was increased (153%; Fig. 2). Moreover, *daf-2(e1370)* results in life extension of 274% when the worms are grown in axenic medium, whereas this mutant extended life span by 69% relative to wild-type on plate cultures. Caloric restriction and reduced Ins/IGF-1 signalling thus lengthen the life span of *C. elegans* in a synergistic way, consistent with independence of caloric restriction from the Ins/IGF-1 pathway. This dramatic increase in longevity of *daf-2* under caloric restriction is the longest mean (90.9 days) and maximum (136 days) life span yet reported for *C. elegans* and argues dramatically for independence of the caloric restriction and *daf-2* pathways. We also investigated whether *daf-16* mutation, which has been reported to suppress the life extension of Ins/IGF-1 mutants to that of wild type (KENYON et al., 1993; GOTTLIEB & RUVKUN, 1994), can suppress caloric restriction-induced life extension. *daf-16* mutants showed increased life expectancy

averaging 152% in axenic culture, relative to *E. coli* plate cultures. These results indicate that life span extension caused by growth in axenic medium does not require *daf-16*. Since *daf-2* specified life span extension is *daf-16* dependent, caloric restriction and the Ins/IGF-1 pathway must be distinct.
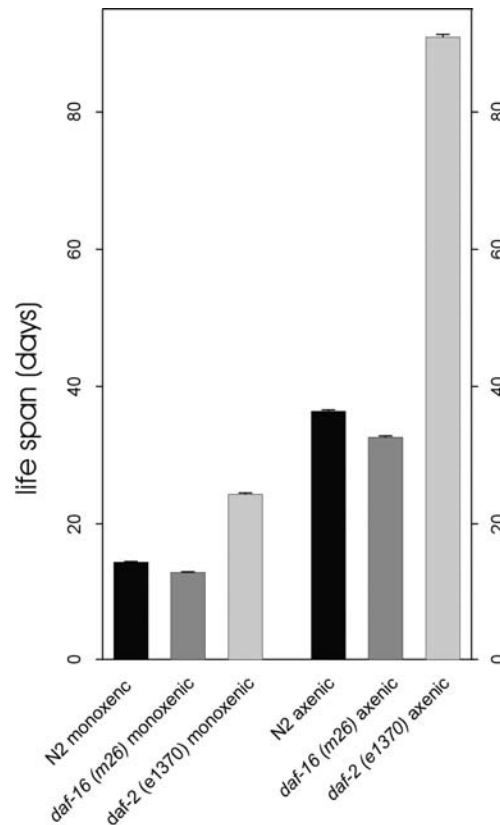

Fig. 2. – Life span in response to caloric restriction and Ins/IGF-1 signalling. Average life span of N2, *daf-16 (m26)* and *daf-2 (e1370)* at 24°C on monoxenic agar plates and in axenic medium.

Both SOD and catalase activities are elevated in *daf-2* and reduced in *daf-16* in bacterial culture and relative to wild-type (Fig. 3a and b). *daf-16* largely suppresses the *daf-2* SOD phenotype, consistent with other evidence that *daf-16* controls expression of SOD (HONDA & HONDA, 1999). However, growth in axenic medium causes elevation of SOD and catalase in all strains, suggesting that caloric restriction defines a separate pathway.

The DAF-16 protein is a transcription factor that becomes localized in the nucleus in response to upstream signals as well as to a series of stressors including heat, oxidative stress and starvation (HENDERSON & JOHNSON, 2001). If life span extension by caloric restriction is mediated by the Ins/IGF-1 pathway, then DAF-16 should respond to the stimulus by moving to the nucleus. A DAF-16::GFP construct, grown in axenic medium, shows no nuclear localization, however (not shown). This is consistent with the cytosolic localization of DAF-16::GFP in an *eat-2* mutant (HENDERSON & JOHNSON, 2001), and argues against a role for the Ins/IGF-1 pathway in mediating life extension by caloric restriction.
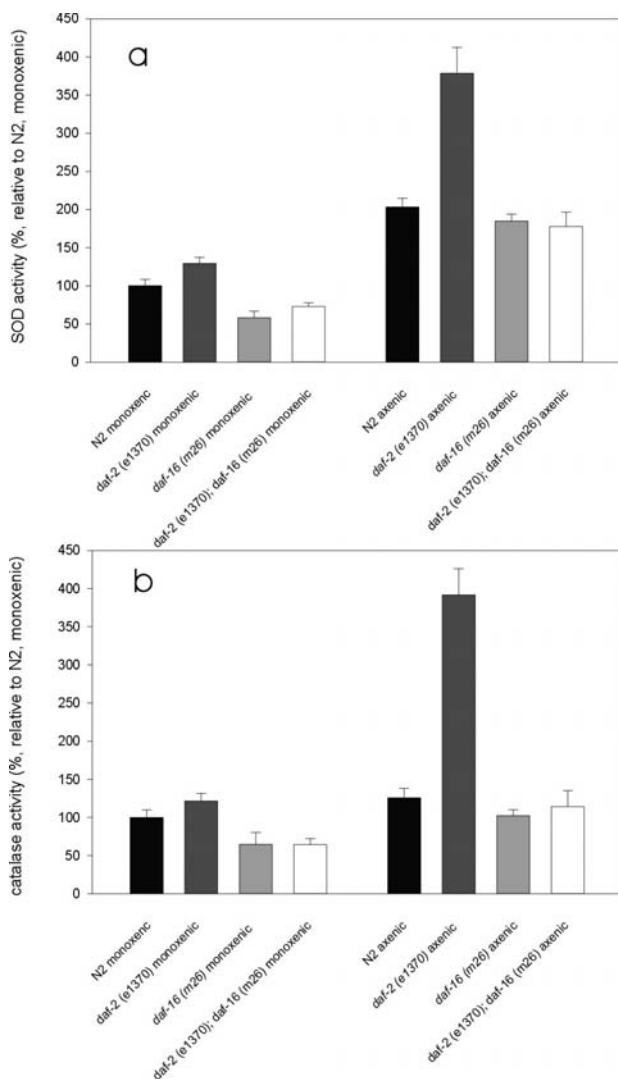
Fig. 3. – SOD and catalase activity in response to caloric restriction and Ins/IGF-1 signalling. Average activity over the total life span of N2, *daf-16 (m26)*, *daf-2 (e1370)* and *daf-2 (e1370); daf-16 (m26)* at 24°C. Monoxenic worms were grown in Fernbach flasks supplied with at least $3x10^9$ *E. coli* cells/ml. Axenic worms were grown in Fernbach flasks with axenic medium.

In conclusion, we found that caloric restriction and deficiencies in the Ins/IGF-1 pathway caused additive effects on life span and SOD and catalase activity. Furthermore, extension of life span and increased activity of SOD and catalase that are caused by caloric restriction, are also seen in *daf-16* mutants, suggesting that caloric restriction does not need intact *daf-16* activity. These conclusions are consistent with DAF-16 residing outside the nucleus in calorically restricted *eat* mutants and wild-type worms raised in axenic culture.

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APPENDIX : LIST OF ABBREVIATIONS

Ins :	Insulin
IGF-1 :	Insulin-like-growth-factor-1
ROS :	reactive oxygen species
PMS :	phenazine methosulfate
SOD :	superoxide dismutase
Note :	*daf-2* : gene, DAF-2 : gene product, Daf : phenotype (abnormal dauer formation)