

Dietary Restriction and Glycolytic Inhibition Reduce Proteotoxicity and Extend Lifespan via NHR-49

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Abstract

Mechanisms mediating protective effects of dietary restriction during aging are of great interest since activating such mechanisms protect against a wide range of age-related diseases. In mammals key metabolic responses to nutritional deprivation are mediated by the transcription factor PPAR-alpha, which is activated by free fatty acids and promotes lipid metabolism while inhibiting glucose metabolism. The *C. elegans* gene *nhr-49* appears to function similarly in *C. elegans*. Here we report that protective effects of dietary restriction and inhibition of glucose metabolism to increase lifespan wild-type *C. elegans* and reduce toxicity in a polyQ model of Huntington's disease in *C. elegans* are dependent on NHR-49 and its co-activator CREB-Binding Protein (CBP). We have previously demonstrated that inhibition of *cbp* blocks protective effects of dietary restriction and blocks the molecular switch from glucose metabolism to alternative substrates. Conversely, increased glucose concentration and inhibition of *cbp* reduce lifespan and increase proteotoxicity. Lactate and inhibition of ETC complex II mimicked toxic effects of glucose on proteotoxicity whereas pyruvate and inhibition of ETC complex I protected against glucose-enhanced proteotoxicity. These results support that PPAR-alpha-like activity mediates protective effects of dietary restriction by reducing glucose metabolism via reducing production of NADH, and corroborate and extend recent studies demonstrating that PPAR-alpha agonists increase lifespan in *C. elegans* dependent on NHR-49.

Keywords: Dietary restriction, glucose metabolism and PPAR

Introduction

Dietary restriction increases lifespan, slows the rate of aging [1], and protects against many age-related impairments [2], including in models of Huntington's disease [3,4]. The metabolic mechanisms by which dietary restriction is protective remain unclear, but studies from our laboratory have demonstrated that inhibition of CREB-binding protein (CBP) blocks all protective effects of dietary restriction, including in a model of Alzheimer's disease proteotoxicity [5]. Inhibition of CBP also prevents the effect of dietary restriction to shift metabolism away from glucose metabolism and towards lipid metabolism [5]. These data suggest that at least some protective effects of dietary restriction are mediated by a decrease in glucose and an increase in lipid metabolism, leading to an increased FADH₂:NADH ratio and thereby enhancing ETC complex II utilization relative to ETC complex I [6,7]. Since CBP is a transcriptional co-activator and does not directly bind DNA, these results raise the question of which transcription factors bind to the DNA of the relevant metabolic genes and thus mediate metabolic protection. This metabolic switch from glucose metabolism to lipid metabolism is thought to be

protective since glucose metabolism promotes utilization of electron transport chain complex I, which produces reactive oxygen species, whereas lipid metabolism promotes utilization of complex II, which does not produce reactive oxygen species, [6,7]. Experimentally reducing complex II activity reduces lifespan, whereas experimentally reducing complex I activity increases lifespan [8]. Furthermore, Huntington's Disease (HD) is characterized by relatively reduced complex II activity [9], and inhibition of complex II activity produces cellular phenotypes similar to those of HD [10].

In mammals the transcription factor PPAR-alpha is activated by free fatty acids during nutritional deprivation and mediates the switch from glucose metabolism to lipid metabolism [11]. This metabolic switch may mediate many of the protective effects of dietary restriction [6,7]. Furthermore a recent study has demonstrated that pharmacological activators of PPAR-alpha (e.g., fenofibrate) [12] increase lifespan in *C. elegans*, dependent on *nhr-49*, a functional homlog of *ppar-alpha* [13]. Here, we report that *nhr-49* is required for protective effects of dietary and glucose restriction on lifespan and proteotoxicity.

Research Design and Methods

C. elegans strains

The AM140 strain with the *rmIs132(P(unc-54)Q35::YFP)* transgene [14], the N2 strain and the RB1716 strain with the *ok2165* deletion in the *nhr-49* gene. All strains were obtained from the Caenorhabditis Genetic Center (CGC) in Minneapolis, MN. Worms were maintained at 20°C with the *E. coli* OP50 strain as their food source.

RNAi strains

cbp-1, *nhr-49*, *nuo-4* and *mev-1* dsRNA expressing bacterial strains were from the genomic RNAi library produced by J. Ahringer at the Wellcome/CRC Institute Sequences for RNAi strains are below. The bacterial strain containing the empty L4440 vector was used for control.

cbp-1: forward primer- CAACGATCGAAACAACATGG;
reverse primer- GGCTGGCTCATTCTGAAGAC

nhr-49: forward primer- ACCAACATCTGGAGCCAGTC;
reverse primer- TGTCGTCGAGAATGAACTCG

nuo-4: forward primer- TTTTGGCCAGTTTCAATTCC;
reverse primer- ACTTGCTGATCAACTCGGCT

mev-1: forward primer- AGAAAATCAGCATTGTTTCAGGA;
reverse primer- ACGGAAAAGAGAGAAAATCGAAG

Paralysis and lifespan analysis

Eggs were collected via a standard axenization procedure whereby gravid worms were treated with hypochlorite. Worms were then grown on nematode growth media (NGM) plates (100 mM) with control OP50 bacteria until they reached day 1 of adulthood in all studies except the mitochondrial complex study. For the mitochondrial RNAi study, worms were grown on *nuo-4* RNAi containing *E. coli* during development as well as during adulthood. AM140 worms were scored for paralysis and death every day starting on day 6-8 of adulthood. Paralysis was indicated by inability of the worm to travel forward after plate tapping and prodding with a platinum wire, but distinguished from death by waving of the head. N2 worms were scored starting on day 10 of adulthood and scored every 2-3 days.

RNA interference

Standard bacterial feeding methods were used for RNAi interference studies [15]. *E. coli*, which was transformed with respective RNAi construct, was grown overnight at 37°C. 3.5 mm NGM plates, supplemented with 5-fluorodeoxyuridine (FUdR) (0.1 g/L) (Sigma), IPTG (0.24 g/L) (Sigma) and ampicillin (0.1 g/L) (CellGro), were seeded with bacteria expressing the dsRNA (180-200 µL) and incubated for several hours-overnight at 37°C. On day 1 of adulthood, worms were transferred to these plates. Worms were exposed to RNAi during adulthood with one exception. When treating with *nuo-4* or *mev-1* RNAi, worms were treated during development as well as adulthood.

Dietary restriction

Dietary restriction was accomplished by complete removal of food [16] since this is the protocol previously used to study effects of dietary restriction on polyQ proteotoxicity [17]. Thus worms were put on 35 mM NGM plates containing no bacteria. For RNAi + dietary restriction studies, worms were kept on plates containing RNAi for the first 4 days of adulthood and then transferred to dietary restriction plates or to control plates containing bacteria harboring the L4440 vector.

Drug Studies

For the drug and metabolite studies, the drug/metabolite was added to the standard NGM agar with a final concentration of 100 mM glucose (Fisher), 100 mM sodium lactate (Sigma), 200 mM sodium pyruvate (Sigma) and 4 mM 2DG (Sigma), L4440 was used as the control bacteria except for the pyruvate and lactate studies which used OP50. The 2-DG + glucose study and pyruvate+glucose study were performed with 50 worms.

Statistical analysis

Paralysis/survival curves were compared using the Kaplan-Meier statistical test. Onset of paralysis (or death) was statistically compared using a Wilcoxon rank sum test, a nonparametric comparison of medians between two groups. All statistical analyses were carried out using the GraphPad Prism 5 Software. Criterion for significance was set at $p < 0.05$. Although the data presented here represents a single study with 60-80 worms each, each study was completely replicated 3 times unless otherwise specified [18].

Results

Protective effects of dietary restriction and glycolytic inhibition require *nhr-49*

Dietary restriction [19] and glycolytic inhibition [20] both increase lifespan in *C. elegans*, whereas elevated glucose reduces lifespan [21,22]. In mammals the transcription factor Ppar-alpha mediates the switch from glucose metabolism to lipid metabolism produced by nutritional deprivation [11,23] which may mediate protective effects of dietary restriction [6,7]. Furthermore, pharmacological activators of Ppar-alpha (e.g., fenofibrate) have also been shown to increase lifespan in *C. elegans* [12]. The transcription factor Creb-binding protein (CBP) serves as a transcriptional co-activator for PPARs [24]. Furthermore Cbp is required for protective effects of dietary restriction [5] and inhibition of miRNA 80 which produces responses similar to dietary restriction [25]. The transcription factor *nhr-49* appears to serve the same function in worms as PPAR-alpha in mammals [13].

We therefore assessed if genetic ablation of *nhr-49* would attenuate protective effects of dietary restriction and glycolytic inhibition. As shown in Figure 1a, ablation of *nhr-49* largely (by 67%), though not quite completely, prevented the protective effects of dietary restriction to increase lifespan.

Similarly, ablation of *nhr-49* completely prevented the effect of glycolytic inhibition via 2-DG to increase lifespan (Figure 1b). Furthermore, inhibition of CBP with RNAi also completely prevented the effects of 2-DG to increase lifespan (Figure 1c). As indicated above, we have already demonstrated the inhibition of CBP prevents protective effects of dietary restriction to increase lifespan and protect against proteotoxicity [5].

Inhibition of *nhr-49* and *cbp-1* blocks protective effect of dietary restriction on polyQ proteotoxicity

Several lines of investigation have suggested that proteotoxicity in polyQ models of Huntington's Disease is mediated by the inhibition of CBP activity [26-28]. To assess the role of *nhr-49* in polyQ proteotoxicity, we used the *C. elegans* AM140 strain which has a tract of 35 glutamine residues fused

to YFP expressed in the body wall muscles. The readout of proteotoxicity is paralysis [29]. We assessed if inhibition of *nhr-49* would block the protective effects of dietary restriction [17] in this model. In this study, *nhr-49* was inhibited with RNAi to avoid the complications of crossing the polyQ transgenic worm with the *nhr-49* knockout worm. As shown in Figure 2a, inhibition of *nhr-49* completely prevented the protective effects of DR on proteotoxicity, as indicated by age-related paralysis (Figure 2a). Similarly, as shown in Figure 2b, inhibition of *cbp-1* by RNAi also completely prevented the protective effects of DR on the polyQ proteotoxicity model.

Glucose accelerates polyQ toxicity while glycolytic inhibition delays proteotoxicity, dependent on *nhr-49* and *cbp-1*

Consistent with previous studies on lifespan [21], as shown in Figure 3a, elevated glucose enhanced polyQ proteotoxicity. Conversely, inhibition of glycolysis by 2DG substantially protected against polyQ proteotoxicity (Figure 3b). Furthermore, elevated glucose completely prevented the protective effects of 2-DG on paralysis (Figure 3b). Similarly, inhibition of *nhr-49* also completely prevented the protective effects of 2-DG on polyQ proteotoxicity (Figure 3c). Finally, inhibition of *cbp-1* also prevented the protection of 2DG in the polyQ model (Figure 3d).

NADH as enhancer of proteotoxicity

Several lines of evidence suggest that toxic effects of glucose metabolism are mediated in part by increasing NADH/NAD⁺ ratios [30] and protective effects of DR during aging are mediated by reducing this ratio [31]. NADH/NAD⁺ ratios are increased by lactate and reduced by pyruvate. As shown in Figure 4a, increased lactate enhanced polyQ-induced proteotoxicity (Figure 4a). In contrast, increased pyruvate produced a non-significant trend toward protection under standard conditions. However, increased pyruvate significantly protected against glucose-induced proteotoxicity (Figure 4b).

The role of electron transport chain complexes in proteotoxicity

A possible mechanism of NADH toxicity involves enhanced activity of electron transport chain (ETC) complex I, which is the site of NADH oxidation for production of ATP and which produces reactive oxygen species [30]. Indeed, inhibition of ETC complex I increases lifespan, whereas inhibition of ETC complex II reduces lifespan [8]. Similarly, as shown in Figure 4c, RNAi inhibition of complex I protein NADH ubiquinone reductase homolog, *nuo-4* significantly protected against proteotoxicity (Figure 4c). Conversely, inhibiting *mev-1*, a homolog of cytochrome b of complex II, enhanced proteotoxicity (Figure 4d).

Discussion

Mechanisms mediating the robust protective effects of dietary restriction on age-related diseases are the subject of great interest since they could lead to pharmacological treatments that mimic these effects. Previous studies suggested that the

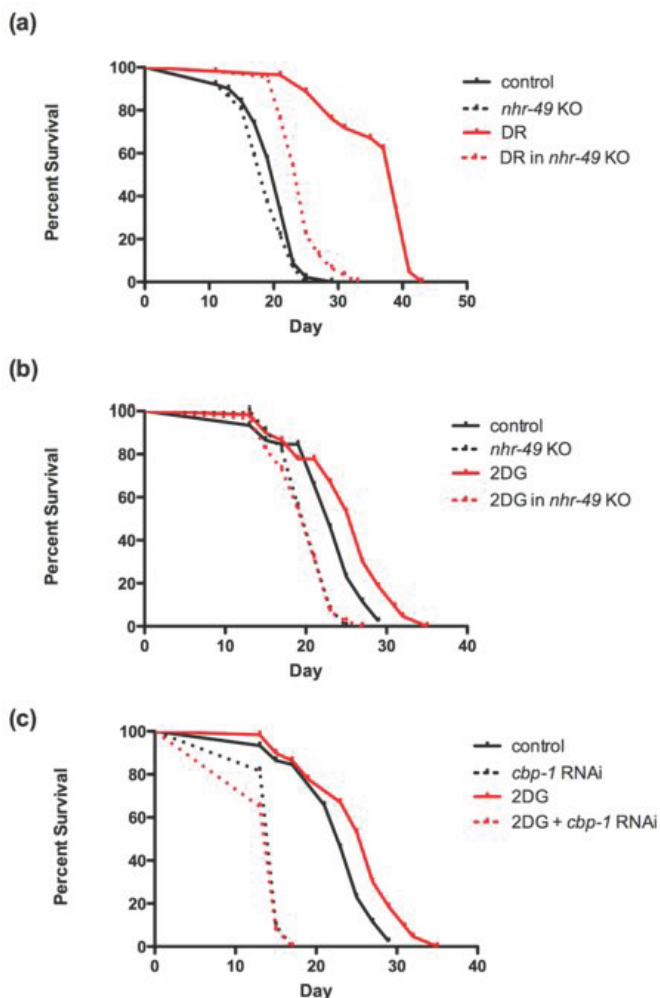


Figure 1: Metabolic protection on lifespan is dependent on PPAR and CBP. The *nhr-49* knockout strain shows (a) almost complete prevention of lifespan extension by dietary restriction and (b) complete prevention of lifespan extension by glycolytic inhibition. Extension of lifespan by glycolytic inhibition using 2-DG is completely reversed in the (c) when *cbp-1* is inhibited in the wild-type strain.

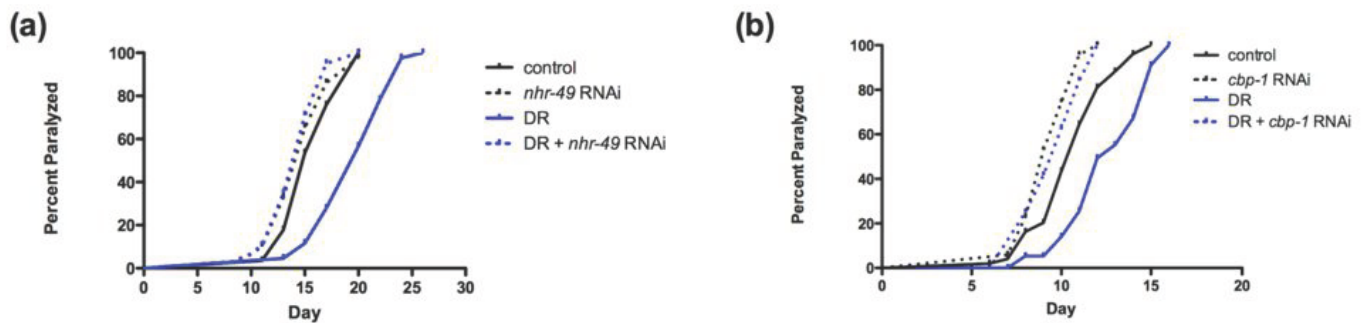


Figure 2: PPAR and CBP are necessary for protection of dietary restriction on proteotoxicity. Protection on proteotoxicity via dietary restriction is prevented with (a) *nhr-49* inhibition or (b) *cbp-1* inhibition.

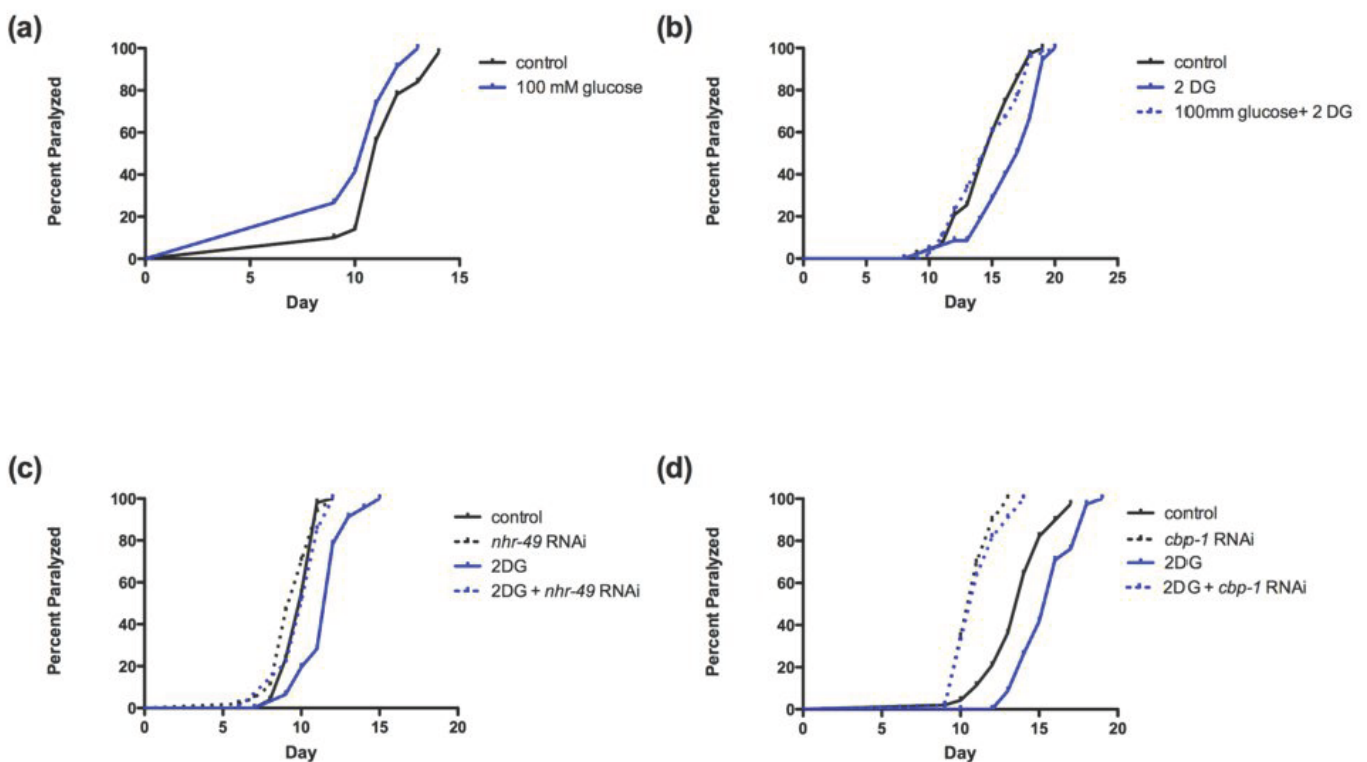


Figure 3: High glucose potentiates whereas glycolytic inhibition protects against polyQ proteotoxicity in a PPAR and CBP-dependent fashion. (a) High glucose (100 mM) potentiates polyQ toxicity as indicated by accelerating the median onset of paralysis ($p < 0.005$). (b) Glycolytic inhibition via 2-DG delays paralysis ($p < 0.0001$). These protective effects are reversed by administration of high glucose (100 mM). Protection via glycolytic inhibitor is prevented with (c) *nhr-49* or (d) *cbp-1* inhibition.

protective effects of dietary restriction on polyQ proteotoxicity require the activity of *hsf-1* [17]. We had similarly reported that inhibition of *hsf-1*, as well as *cbp-1*, blocked protective effects of dietary restriction and the *daf-2* mutation on lifespan [5]. Since HSF-1 interacts with and recruits CBP for transcription, we hypothesized that part of the protective effects of dietary restriction are mediated through this interaction [5]. However, since there is no evidence that HSF-1 regulates metabolic gene expression, especially the shift from glucose to lipid metabolism that appears to mediate at least some protective effects of dietary restriction [6,7,31], it was of interest to assess the role of

other transcription factors, more relevant to metabolism. Since inhibition of CBP prevents this molecular switch produced by dietary restriction [5], whereas PPAR homologs promote this molecular switch [11], we hypothesized that this family of transcription factors mediate these protective effects of dietary restriction. This hypothesis was greatly supported by the report that pharmacological activators of Ppar-alpha (e.g., fenofibrate) [12] increase lifespan in *C. elegans*, dependent on *nhr-49*, the functional homolog of Ppar-alpha in *C. elegans* [13]. Here we report that genetic ablation of the *ppar* homolog *nhr-49* largely blocks protective effects of dietary restriction, and completely

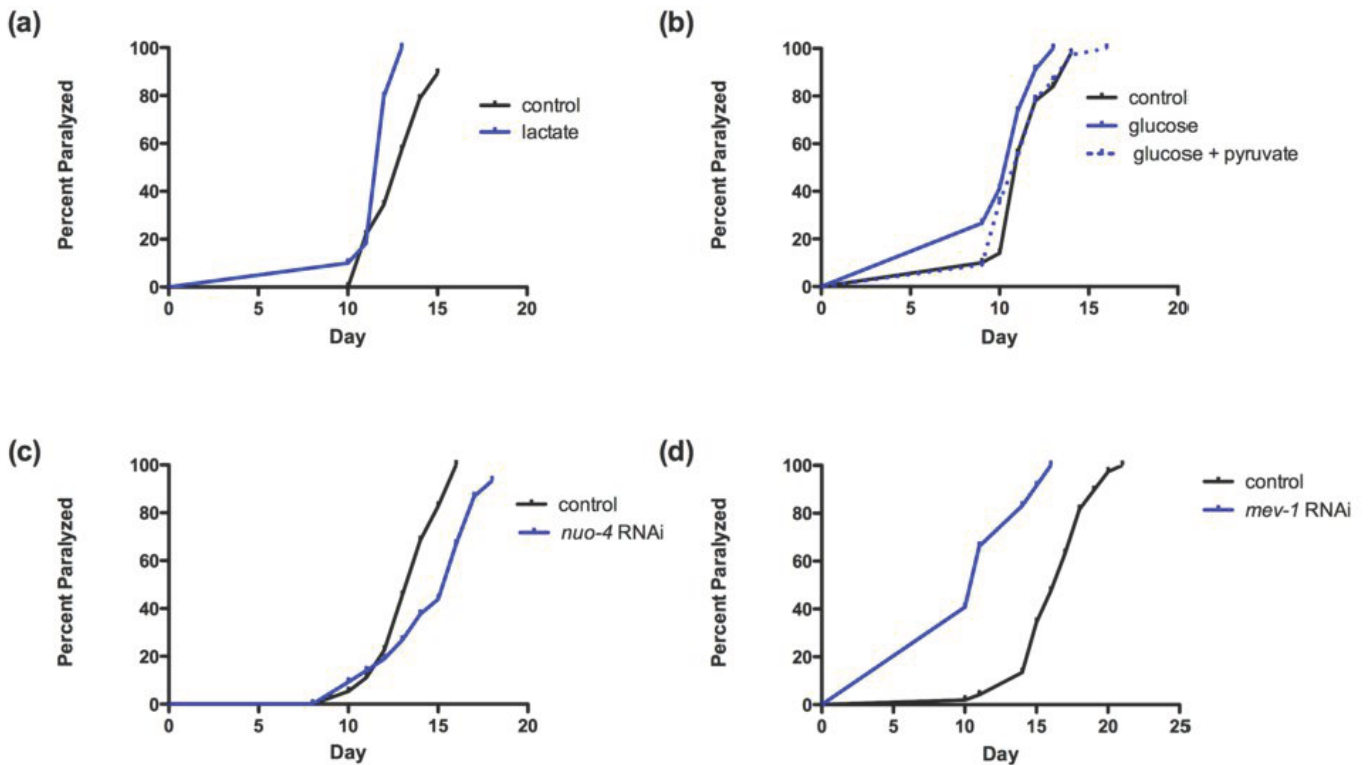


Figure 4: Role of NADH and ETC in proteotoxicity. NADH can be manipulated by the addition of pyruvate or lactate. **(a)** Addition of lactate (100mM) exacerbates proteotoxicity as reflected by an acceleration of paralysis ($p < 0.005$). **(b)** Addition of pyruvate (200 mM) protects against the toxic effect of glucose (100 mM) on the polyQ model. **(c)** RNAi inhibition of *nuo-4*, a homolog NADH ubiquinone reductase delays median day of paralysis by 2 days ($p < 0.001$). **(d)** RNAi inhibition of *mev-1*, a homolog of cytochrome b of complex II of the ETC chain, exacerbates proteotoxicity as evidenced by a 6-day acceleration of paralysis.

blocks effects of glycolytic inhibition to increase lifespan. As expected inhibition of *cbp-1* also completely blocked effects of glycolytic inhibition to increase lifespan.

Elevated glucose reduces lifespan [21,22] whereas inhibiting glucose metabolism by 2-DG increases lifespan [20] in *C. elegans*. The present studies confirm and extend these observations to polyQ proteotoxicity. Inhibition of the *C. elegans* homolog of AMPK prevented protective effects of 2-DG to increase lifespan [20]. Similarly in the present studies inhibiting *cbp-1* also completely prevented the protective effects of 2-DG on lifespan as well as on proteotoxicity. Of particular interest, inhibiting the *ppar* homolog *nhr-49* also completely blocked the protective effects of 2-DG on lifespan and proteotoxicity. Since increased glucose can reduce mammalian *ppar* gene expression by inhibiting AMPK [32], all of these studies are consistent with the hypothesis that 2-DG protects against proteotoxicity by activating AMPK activity, leading to increased PPAR activity and increased recruitment of CBP to genes that promote lipid metabolism and reduce glucose metabolism.

The mechanism by which glucose metabolism drives polyQ proteotoxicity remains to be established but our studies suggest that a key metabolic reaction is increased production of NADH (from NAD⁺). Our data support that one mechanism mediating

toxic effects of this reaction is increased utilization of ETC complex I, consistent with studies implicating this same complex in limiting lifespan [8]. This hypothesis is particularly attractive since Huntington's Disease is characterized by relatively reduced complex II activity vs. complex I activity [33]. On the other hand, the NAD⁺ mechanism also activates sirtuins, which are also implicated in mediating protective effects of dietary restriction [34], even in mammals [35]. A particularly attractive hypothesis is that some of the deleterious effects of NADH are mediated by inhibition of the NADH-dependent transcriptional co-repressor CtBP [36]. CtBP inhibits CBP function [37] and in turn inhibition of CtBP by RNAi increases lifespan [38]. Another mechanism by which glucose metabolism may lead to deleterious effects is inhibition of daf-16/FOXO [21], which also recruits CBP [39]. Conversely, we hypothesize that the profound effects of the insulin-like pathway on healthspan and lifespan [40] are mediated at least in part by reduced glucose metabolism. It is therefore of some interest that crossing the polyQ construct onto the *daf-2*-null background significantly reduces proteotoxicity (not shown).

Different protocols of DR entail somewhat different molecular mechanisms [19], although inhibition of *cbp-1* blocks protective effects of all protocols examined as well as of the *daf-*

2 mutation [5]. Therefore it will be of great interest to assess if inhibiting *nhr-49* blocks protective effects of other protocols of DR and the *daf-2* mutation. Furthermore, since reduction of lifespan by elevated glucose entails inhibition of *daf-16* [21], the role of *daf-16* in mediating effects of 2-DG on polyQ proteotoxicity is also of great interest. Similarly, the role of the metabolic switch away from glucose utilization in mediating protective effects of DR must also be assessed. Finally, it would be of great interest to determine if protective effects of DR on polyQ proteotoxicity specifically require induction of *nhr-49* in ASI neurons, as appears to be the case for *skn-1* [41].

In conclusion, we have demonstrated here that the PPAR family of transcription factors and CBP mediate at least some protective effects of dietary restriction and, especially, glycolytic inhibition on lifespan and proteotoxicity. These studies suggest that Ppar-alpha activators may be similarly protective to prevent age-related diseases in humans. Furthermore, these studies suggest that glucose toxicity on polyQ proteotoxicity is mediated, at least in part, by production of NADH and oxidation of NADH via ETC complex I. These studies also propose several targets for drug development: glycolytic inhibitors, pyruvate mimetics, and ETC complex I inhibitors.

Author Contributions

Bridget Marcellino completed most of the initial experiments many of which were replicated by Nydia Ekasumara. Bridget Marcellino analyzed data and wrote manuscript with help of Charles Mobbs.

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