Longevity of insulin receptor substrate1 null mice is not associated with increased basal antioxidant protection or reduced oxidative damage

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Abstract Insulin receptor substrate-1 null (*Irs1*^{−/−}) mice are long lived and importantly they also demonstrate increased resistance to several age-related pathologies compared to wild type (WT) controls. Currently, the molecular mechanisms that underlie lifespan extension in long-lived mice are unclear although protection against oxidative damage may be important. Here, we determined both the activities of several intracellular antioxidants and levels of oxidative damage in brain, skeletal muscle, and liver of *Irs1*^{−/−} and WT mice at 80, 450, and 700 days of age, predicting that long-lived *Irs1*^{−/−} mice would be protected against oxidative damage. We measured activities of both intracellular superoxide dismutases (SOD); cytosolic (CuZnSOD) and mitochondrial (MnSOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and reduced glutathione (GHS). Of these, only hepatic CAT was significantly altered (increased) in *Irs1*^{−/−} mice. In addition, the levels of protein oxidation (protein carbonyl content) and lipid peroxidation (4-hydroxynonenal) were

unaltered in *Irs1*^{−/−} mice, although the hepatic GSH/GSSG ratio, indicating an oxidized environment, was significantly lower in long-lived *Irs1*^{−/−} mice. Overall, our results do not support the premise that lifespan extension in *Irs1*^{−/−} mice is associated with greater tissue antioxidant protection or reduced oxidative damage.

Keywords Insulin receptor substrate-1 · *Irs1* · Lifespan · Antioxidant enzymes · Oxidative damage · Ageing

Introduction

It has been established for several decades now that the insulin/insulin-like growth factor-1 (IGF1) signaling (IIS) pathway induces pleiotropic effects on development, growth, reproduction, and metabolism of multicellular animals (Taguchi and White 2008). However, it has only been more recently that this pathway has also been shown to play a conserved role in lifespan determination (reviewed in (Piper et al. 2008; Taguchi and White 2008; Broughton and Partridge 2009)). For example, IIS regulates adult life span in both *Caenorhabditis elegans*, e.g., (Dorman et al. 1995; Lin et al. 1997) and *Drosophila melanogaster*, e.g., (Clancy et al. 2001; Tatar et al. 2001; Alic et al. 2011). IIS also plays a key role in mammalian aging, with global (Holzenberger et al. 2003; Selman et al. 2008, 2011) and tissue-specific (Bluhner et al. 2003;

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Kappeler et al. 2008; Taguchi et al. 2007) deletion of IIS genes extending lifespan in mice. In addition, this lifespan extension has been shown to be associated, in some models (Selman et al. 2008), with a greater period of adult life free from disease (see Selman and Withers 2011). Attenuated IIS may also underlie the longevity of growth hormone (GH)/GH receptor-deficient dwarf mice, i.e., Ames (*Prop1^{df/df}*), Snell (*Pit1^{dw/dw}*), Little (*Ghrhr^{lit/lit}*), growth hormone receptor knockout (GHRKO) mice (Masternak et al. 2009; Bartke 2011). Polymorphisms of several IIS genes are also correlated with longevity in humans (Bonafe et al. 2003; Pawlikowska et al. 2009).

While the evidence supporting a connection between reduced IIS and longevity is robust, what remains to be elucidated are the precise molecular mechanisms underlying this observation. One popular suggestion is that long-lived animals have enhanced protection and repair mechanisms that prevent against cellular damage induced by reactive oxygen species (Curtis et al. 2007; Kregel and Zhang 2007; Beckman and Ames 1998). While there is currently considerable debate on the exact role of ROS-induced oxidative stress in ageing (Gems and Doonan 2009; Perez et al. 2009; Speakman and Selman 2011), evidence from long-lived invertebrate IIS mutants suggests that increased antioxidant protection and/or reduced oxidative stress can be associated with longevity. For example, some long-lived IIS mutant *C. elegans* have been shown to have higher basal levels of antioxidant enzymes (Vanfleteren 1993; Honda and Honda 1999; Brys et al. 2007; Spanier et al. 2010) and reduced oxidative damage (Ishii et al. 2002; Brys et al. 2007) compared to WT controls. Similarly, protein and activity levels of superoxide dismutase (SOD) were elevated in long-lived *Drosophila* IIS mutants (Clancy et al. 2001; Tatar et al. 2001; Kabil et al. 2007). In mice, there is a paucity of data to support the premise that long-lived IIS mutants have enhanced basal activities of antioxidants and reduced oxidative damage. Indeed, currently only one study has reported antioxidant levels in long-lived IIS mutants, and this was limited to a single antioxidant enzyme in a single tissue. Taguchi et al. (2007) reported that long-lived brain-specific insulin receptor substrate 2 heterozygous (*bIrs2^{+/-}*) and homozygous (*bIrs2^{-/-}*) knockout mice were protected against an age-associated decrease in postprandial manganese superoxide dismutase (MnSOD) levels. However, in

contrast, a large amount of data on antioxidant enzyme status and oxidative damage has been reported for long-lived GH-deficient dwarf mice. Elevated catalase (CAT) and glutathione peroxidase (GPx) activities were reported in liver and heart from Ames and Snell dwarf mice, respectively (Brown-Borg et al. 1999; Brown-Borg and Rakoczy 2000; Page et al. 2010). However, these and other studies have also reported no change or reduced antioxidant enzyme activities in other tissues from long-lived mice (Brown-Borg et al. 1999; Hauck et al. 2002; Romanick et al. 2004; Page et al. 2010), suggesting that antioxidant enzyme activities in long-lived GH mutants are specific to particular tissues and/or particular antioxidants.

In this study, we present a comprehensive examination of the basal levels of intracellular antioxidants and oxidative damage in a long-lived IIS mutant, the insulin receptor substrate 1 (*Irs1^{-/-}*) null mouse (Selman et al. 2008, 2011). We determined the activities of copper zinc SOD (CuZnSOD; cytosolic), manganese SOD (MnSOD; mitochondrial), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and reduced glutathione (GSH) within brain, skeletal muscle, and liver of female *Irs1^{-/-}* and WT mice at 80, 450, and 700 days of age. Furthermore, we additionally determined protein carbonyl content, 4-hydroxynonenal (4-HNE) levels and the GSH/GSSG ratio as indicators of oxidative damage. Our data indicates that the lifespan extension of *Irs1^{-/-}* mice is not associated with greater basal levels of antioxidants or reduced oxidative damage within brain, skeletal muscle, or liver.

Experimental procedures

Animals

Genotyping of insulin receptor substrate 1 (IRS1) mice followed previously described protocols (Withers et al. 1999, 1998). Mice were housed and maintained as described previously (Selman et al. 2008, 2011). Briefly, mice were kept in groups of three to eight on a 12L:12D photoperiod (lights out from 1900 to 0700 h). Mice had ad libitum access to water and standard chow (2018 Teklad Global (5% fat, 18% protein, 57% carbohydrate, and 20% other components) Rodent Diet; Harlan Teklad, Bicester,

Oxfordshire, UK). All experiments were carried out following local ethical review, under a licence from the UK Home Office and followed the “principles of laboratory animal care” (NIH Publication No. 86–23, revised 1985). Following an overnight fast, mice at 80, 450, or 700 days of age were culled by cervical dislocation. Brain, hind limb skeletal muscle, and liver were dissected out and immediately flash frozen in liquid nitrogen. Female mice were used for all experiments described in this study. At 80 days of age 4–7 *Irs1*^{+/+} (wild type, WT) and 4–8 *Irs1*^{−/−} null mice were used, at 450 days of age 5–6 WT and 3–6 *Irs1*^{−/−} mice were used, and at 700 days of age 4–5 WT and 4–5 *Irs1*^{−/−} mice were used.

Materials

Chemicals were obtained from Sigma Aldrich (Dorset, UK) and Fisher Scientific (Leicestershire, UK). Bio-Rad protein dye and bovine gamma globulin standards were obtained from BioRad (Hertfordshire, UK). Protein carbonyl kits were purchased from Caymen Chemical Company (Cambridge Bioscience, Cambridge, UK), and 4-Hydroxynonenal ELISA kits obtained from Cell Biolabs, Inc (Cambridge Bioscience, Cambridge, UK).

Tissue homogenisation for antioxidant enzyme and oxidative stress assays

Brain, skeletal muscle, and liver were homogenised and prepared as previously described (Page et al. 2010). Protein concentration was determined by using the Bradford technique and protein supernatants were stored at −80°C.

In-gel superoxide dismutase assay

SOD isoform activities were assayed as in Page et al. (2009a). SOD activity of individual protein bands was analysed by scanning the gels on an Epson Expression 1460XL scanner (Hertfordshire, UK) and band intensity was quantified using BioRad's Quantity One® software (Hertfordshire, UK). MnSOD localises to the mitochondrial matrix, therefore citrate synthase activity was determined as a marker of mitochondrial abundance, as previously described (Page et al. 2009b). It should be noted that MnSOD activity was

below the threshold of detection in skeletal muscle using our assay.

Additional antioxidant and metabolic enzyme assays

Catalase, glutathione peroxidase, glutathione reductase, and citrate synthase activity assays were undertaken as previously described by Page et al. (2009b). Catalase activity was not detected in either brain or skeletal muscle. All measurements were made in duplicate. Enzymatic assays were performed at 30°C using a spectrophotometer (SpectraMax Plus 384, Molecular Devices, Berkshire, UK).

Reduced (GSH) and oxidised (GSSG) glutathione assays

Tissue samples were homogenised as previously (Rahman et al. 2006). GSH and GSSG levels, run in duplicate, were determined in a 96-well microtiter plate as in Rahman et al. (2006). For GSH measurements, 20 µl of each sample was incubated with a 5, 5'-dithiobis-(2-nitrobenzoic acid):glutathione reductase (DTNB:GR) mixture for 30 s to allow for the conversion of GSSG to GSH. The reaction was initiated with 1.6 mM β-NADPH and the change in absorbance over 2 min was measured at 412 nm. GSH concentration was calculated using linear regression from values obtained from a GSH standard curve. For GSSG measurements, 2-vinylpyridine was incubated with 100 µl tissue homogenate for 1 h at room temperature to derivatise GSH. Triethanolamine was added to each sample and incubated for 10 min to promote GSSG formation. The reaction was measured as above and GSSG concentration for the unknowns was calculated using a linear regression obtained from GSSG standards treated with 2-vinylpyridine and triethanolamine. Protein concentration for GSH and GSSG assays were measured as above using the Bradford technique.

Oxidative stress assays

Protein carbonyls were measured following the manufacturer's protocol (#10005020, Cayman Chemical Company, Michigan, USA). 4-Hydroxynonenal (HNE)-protein adducts were determined using an anti-HNE-His mouse IgG protein binding plate and

by following the manufacturer's protocol (#STA-334, Cell Biolabs, Inc, California, USA).

Statistical analyses

Statistical analyses were performed using SPSS (SPSS Inc., USA, version 19) and GraphPad Prism (GraphPad Inc., USA, version 5) software. Data were analysed using a general linear model (GLM) with genotype (*Irs1*^{-/-} or WT) and age (80, 450, or 700 days) introduced as fixed factors. All non-significant interaction effects ($p > 0.05$) were removed to obtain the best-fit model in each case. Differences between the means, for the age comparison, were analysed using post-hoc Tukey tests. Results are mean $\pm$ standard error of the mean (SEM), with $p < 0.05$ regarded as statistically significant.

Results

Genotype had no effect on CuZnSOD activity in brain (Fig. 1a; $p = 0.132$), skeletal muscle (Fig. 1b; $p = 0.620$), or liver (Fig. 1c; $p = 0.812$). However, age significantly affected CuZnSOD activity in brain ($p = 0.002$), with post-hoc analysis revealing a significant reduction in CuZnSOD activity in 450-day-old animals relative to the other two ages ($p = 0.004$ between 80 and 450 days; $p = 0.015$ between 450 and 700 days). In contrast, no significant effect of age on CuZnSOD activity was detected in either skeletal muscle ($p = 0.380$) or liver ($p = 0.831$). MnSOD activity in brain ($p = 0.633$) and liver ($p = 0.383$) was unaffected by genotype (Fig. 2a, b). MnSOD activity within brain decreased in an age-dependent manner ($p = 0.024$ between 80- and 700-day-old mice). However, age did not affect MnSOD activity in liver tissue ($p = 0.792$; Fig. 2b).

Hepatic CAT activity was significantly higher in *Irs1*^{-/-} mice (Fig. 3) compared to WT controls ($p = 0.043$), and elevated in 450-day-old mice ($p < 0.001$) compared to the two other time points. Neither GPx nor GR activity levels (Table 1) were affected by genotype in any tissue (brain: GPx, $p = 0.809$; GR, $p = 0.234$; skeletal muscle: GPx, $p = 0.415$; GR, $p = 0.174$; liver: GPx, $p = 0.968$; GR, $p = 0.106$). However, a significant age-related decline in GPx activity was observed across all three tissues (brain, $p = 0.004$; skeletal muscle, $p < 0.001$; liver, $p = 0.025$). In contrast,

Fig. 1 CuZnSOD activity was not altered in long-lived *Irs1*^{-/-} mice compared to wild type (WT) controls. CuZnSOD activity measured within **a** brain, **b** skeletal muscle, and **c** liver from female *Irs1*^{-/-} and WT mice at 80, 450, and 700 days of age. Open bars represent WT controls and filled bars represent *Irs1*^{-/-} mice. Statistical comparisons between age means were measured by GLM with post-hoc Tukey test. Similar letters are non-significant (age effect); a and b are significantly different $p < 0.05$. Values represent means $\pm$ SEM. $N = 4$ –8 mice per group

brain GR activity significantly increased with age ($p = 0.005$), whereas no age effect was seen in either skeletal muscle ($p = 0.494$) or liver ($p = 0.386$). A significant genotype $\times$ age interaction on hepatic GR activity was detected ($p = 0.026$), being highest in *Irs1*^{-/-} mice at 450 days of age but lowest in WT controls at this same time point (Table 2). No difference in GSH levels in brain ($p = 0.326$), skeletal muscle ($p = 0.973$), or liver ($p = 0.287$) were seen between

Fig. 2 MnSOD activity (corrected for citrate synthase (CS)) was not altered in long-lived *IrsI*^{-/-} mice compared to wild type (WT) controls. MnSOD activity measured within **a** brain and **b** liver tissue from *IrsI*^{-/-} and WT mice at 80, 450, and 700 days of age. MnSOD activity within brain tissue was altered by age (**a**). Identity of bars and statistical analysis are as in Fig. 1. Similar letters are non-significant (age effect); *a* and *b* are significantly different $p < 0.05$. Values represent means $\pm$ SEM. $N = 4$ –8 mice per group

IrsI^{-/-} and WT mice. Similarly, no effect of age on GSH levels (Table 1) was observed in brain ($p = 0.053$) or skeletal muscle ($p = 0.650$), although a significant age effect ($p = 0.044$) and a significant genotype $\times$ age interaction in hepatic GSH levels was found with levels increasing with age in *IrsI*^{-/-} mice but decreasing with age in WT animals (Table 1).

To determine levels of oxidative damage in *IrsI*^{-/-} mice relative to WT controls, we measured protein carbonyl content, 4-hydroxynonenal (4-HNE) levels, and GSH/GSSG ratios in brain, skeletal muscle, and liver. Protein carbonyl content (Fig. 4a–c) was similar between *IrsI*^{-/-} and WT mice in all tissues (brain, $p = 0.647$; skeletal muscle, $p = 0.994$; liver, $p = 0.405$). However, protein carbonyls decreased in both brain ($p = 0.012$) and skeletal muscle ($p = 0.023$) but increased in liver ($p = 0.004$), with advancing age. Genotype had no effect on 4-HNE levels in brain ($p =$

Fig. 3 Hepatic CAT activity was increased in long-lived *IrsI*^{-/-} mice compared to wild type (WT) controls. CAT activity was measured within liver tissue from *IrsI*^{-/-} and WT mice at 80, 450, and 700 days of age. Identity of bars and statistical analysis are as in Fig. 1. Letters represent an age effect; similar letters are non-significant; *a* and *b* are significantly different $p < 0.001$. Asterisk represents genotype effect, $p < 0.05$. Values represent means $\pm$ SEM. $N = 5$ –6 mice per group

0.649), skeletal muscle ($p = 0.767$), or liver ($p = 0.168$) (Fig. 5a–c), although levels increased significantly with advancing age across all three tissues (brain, $p = 0.005$; skeletal muscle, $p < 0.0001$; liver, $p < 0.0001$). The GSH/GSSG ratio was unaffected by genotype in brain ($p = 0.066$) and in skeletal muscle ($p = 0.407$) but was significantly reduced in the liver of *IrsI*^{-/-} mice ($p = 0.015$; Table 1). Finally, age had no effect on brain GSH/GSSG ratio ($p = 0.366$), but a significant age-related decline was observed in both skeletal muscle ($p < 0.0001$) and liver ($p < 0.0001$).

Discussion

The primary objective of this study was to determine whether long-lived *IrsI*^{-/-} mice possessed enhanced antioxidant protection or reduced oxidative damage relative to WT control mice. ROS-induced oxidative damage has been proposed as a key mechanism underlying the ageing process (Beckman and Ames 1998; Kregel and Zhang 2007), although this belief has recently been challenged (Gems and Doonan 2009; Perez et al. 2009; Speakman and Selman 2011). While there is some evidence to suggest that certain long-lived invertebrate IIS mutants have enhanced protection and reduced oxidative damage, whether this is the case, or not, in long-lived IIS mutant mice is currently unknown. In this study, we demonstrate that there is very little evidence to suggest

Table 1 The effects of *Irs1*^{-/-} and age on glutathione and glutathione enzymes within brain, skeletal muscle, and liver tissue

Antioxidant enzyme activity	80 days			450 days			700 days			Genotype		Age		Interaction	
	WT			<i>Irs1</i> ^{-/-}			WT			<i>F</i>	<i>P</i> value	<i>F</i>	<i>P</i> value	<i>F</i>	<i>P</i> value
GPx															
Brain	455.2±37.4	478.4±52.4	327.8±21.0	332.0±40.1	414.5±8.4	345.6±41.3	0.059	0.809	6.74	0.004	NS	NS	NS	NS	NS
Skeletal muscle	466.3±19.5	520.8±43.6	307.4±15.0	370.3±42.2	416.0±26.8	348.1±12.6	0.686	0.415	14.15	<0.001	NS	NS	NS	NS	NS
Liver	480.5±27.1	474.6±35.8	381.0±22.1	358.0±11.9	433.8±7.9	395.7±24.7	0.002	0.968	4.27	0.025	NS	NS	NS	NS	NS
GR															
Brain	8.5±0.98	7.7±0.52	8.5±0.43	9.1±1.05	12.2±0.53	9.6±0.86	1.467	0.234	6.25	0.005	NS	NS	NS	NS	NS
Skeletal muscle	3.6±0.47	4.7±0.79	3.8±0.61	5.7±0.92	5.5±1.15	4.9±0.75	1.950	0.174	0.72	0.494	NS	NS	NS	NS	NS
Liver	8.9±0.60	8.8±0.36	8.1±0.45	11.6±0.93	9.5±1.10	9.2±0.80	2.813	0.106	0.98	0.386	4.23	0.026	NS	NS	NS
GSH															
Brain	514.3±53.6	321.1±19.7	261.7±32.4	341.2±62.4	384.2±99.5	350.8±54.0	0.997	0.326	3.23	0.053	NS	NS	NS	NS	NS
Skeletal muscle	150.3±14.0	179.3±28.1	194.1±65.8	228.6±33.8	265.9±86.9	183.1±60.1	0.001	0.973	0.43	0.650	NS	NS	NS	NS	NS
Liver	384.8±22.9	315.9±31.5	350.5±37.3	312.4±110.3	358.6±56.2	660.6±124.2	1.185	0.287	3.55	0.044	3.89	0.034	NS	NS	NS
GSH/GSSG															
Brain	5.5±1.0	4.2±0.8	4.1±0.7	3.4±0.6	5.4±1.5	2.3±0.5	3.626	0.066	1.03	0.366	NS	NS	NS	NS	NS
Gastrocnemius	4.5±0.8	4.6±1.5	2.2±0.2	2.1±0.1	1.3±0.2	2.6±0.6	0.715	0.407	12.32	<0.001	NS	NS	NS	NS	NS
Liver	5.2±0.4	4.1±0.4	2.4±0.4	1.6±0.7	4.6±0.9	2.9±0.4	6.785	0.015	12.87	<0.001	NS	NS	NS	NS	NS

Values represent mean activity±SEM. GPx activity (nmol/min/mg) and GR activity (nmol/min/mg); 4–8 individual mice were used per experimental group. GSH (nM/mg) levels and GSH/GSSG ratios; 4–8 individual mice were used per experimental group. Statistical comparisons between age means were measured by GLM using post-hoc Tukey test

GPx glutathione peroxidase; GR glutathione reductase; GSH reduced glutathione; GSH/GSSG reduced to oxidised glutathione ratio; NS non-significant

Table 2 Antioxidants measured within brain, skeletal muscle, and liver of long-lived mice

Reference	Antioxidant											
	CuZnSOD			MnSOD			GPx			CAT		
	Brain	Muscle	Liver	Brain	Muscle	Liver	Brain	Muscle	Liver	Brain	Muscle	Liver
IIS												
This study	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
1				↔		↔ ^a	↔	↔	↔	↔	↔	↔
<i>Irs1</i> ^{-/-}												
<i>bhrs2</i> ^{-/-}												
GH dwarf												
2, 3, 4												
Ames (<i>Prop1</i> ^{dl/dl})												
5												
Snell (<i>Pit1</i> ^{dw/dw})												
6												
GHRKO												

References: 1, Taguchi et al. 2007; 2, Brown-Borg et al. 1999; 3, Hauck and Bartke 2000; 4, Romanick et al. 2004; Page et al. 2010; Hauck et al. 2002

IIS insulin/IGF-1 signalling pathway mutant*Irs1*^{-/-} insulin receptor substrate-1 knockout*bhrs1*^{-/-} brain-specific insulin receptor substrate-1 knockout*GH* growth hormone*GHRKO* growth hormone receptor/binding protein knockout*M* male and *F* female

ND Not determined

^a Measured in hypothalamic tissue, age-related postprandial decrease in MnSOD protein levels^b Measured in hypothalamic tissue, significant differences at 3–4 and 9–10 months but not at 19–23 months^c Significant differences at young (3 months) and middle-age (12 months) but no difference at old age (18 months)^d Significant difference at young (3 months) but no differences at middle-age (12 months) or old age (18 months)^e Total SOD activity was measured

Fig. 4 Protein carbonyl content was not altered in long-lived *Irs1*^{-/-} mice compared to wild type (WT) controls. Protein carbonyls were measured within **a** brain, **b** skeletal muscle, and **c** liver tissue from *Irs1*^{-/-} and WT mice at 80, 450, and 700 days of age. Protein carbonyl content was affected by age within **a** brain, **b** skeletal muscle, and **c** liver tissue. Identity of bars and statistical analysis are as in Fig. 1. Similar letters are non-significant (age effect). For **a** brain and **b** skeletal muscle, *a* and *b* are significantly different $p < 0.05$; for **c** liver, *a* and *b* are significantly different $p < 0.005$. Values represent means $\pm$ SEM. $N = 4$ –8 mice per group

that the longevity of *Irs1*^{-/-} mice (Selman et al. 2008, 2011) is associated with a consistent increase in antioxidant protection or decrease in oxidative damage in brain, skeletal muscle, and liver.

CuZnSOD (localised to the cytosol) and MnSOD (found within the mitochondrial matrix) both function to convert superoxide anions to hydrogen peroxide (H₂O₂). CuZnSOD activity was unaltered in brain,

Fig. 5 4-HNE levels were not altered in long-lived *Irs1*^{-/-} mice compared to wild type (WT) controls. 4-HNE levels were measured within **a** brain, **b** skeletal muscle, and **c** liver tissue from *Irs1*^{-/-} and WT mice at 80, 450, and 700 days of age. 4-HNE levels were affected by age within **a** brain, **b** skeletal, and **c** liver tissue. Identity of bars and statistical analysis are as in Fig. 1. Similar letters are non-significant (age effect). For **a** brain, *a* and *b* are significantly different $p < 0.05$; for **b** skeletal and **c** liver, *a* and *b* are significantly different $p < 0.001$. Values represent means $\pm$ SEM. $N = 3$ –6 mice per group

skeletal muscle, and liver of *Irs1*^{-/-} mice relative to WT controls, in agreement with previous reports in the brain and heart of Snell dwarf mice (Page et al. 2010). In contrast with these findings, hypothalamic CuZnSOD activity was elevated in Ames dwarf mice (Hauck and Bartke 2000). These differences may be explained by the use of whole brains within the current study rather

than examining specific brain regions, as it is suggested that different brain regions are more/less susceptible to oxidative stress and possess differences in antioxidant enzymes (Homi et al. 2002). In common with our findings for CuZnSOD, the activity of MnSOD, was unchanged in brain and liver of *Irs1*^{-/-} mice. MnSOD activity has been shown to be reduced in brains from Snell dwarf mice (Page et al. 2010) and MnSOD protein levels reduced in aortic preparation from Ames dwarf mice (Csiszar et al. 2008) compared to WT controls. Our data indicate that lifespan extension in *Irs1*^{-/-} mice is not associated with enhanced CuZnSOD or MnSOD activity. These findings support recent studies showing that global overexpression of either CuZnSOD or MnSOD had no impact on longevity in mice (Jang et al. 2009; Perez et al. 2009).

CAT, which is localised to peroxisomes and neutralises H₂O₂ to water and oxygen, was significantly elevated in the livers of *Irs1*^{-/-} mice. Elevated hepatic CAT activity was also reported in Ames dwarf mice across a range of ages (Brown-Borg and Rakoczy 2000), and we previously showed (Selman et al. 2008) that an age-related decrease in hepatic CAT mRNA levels was attenuated in *Irs1*^{-/-} mice. Recently, it was reported that

mice over-expressing CAT showed no lifespan effect (Perez et al. 2009), although mice carrying a human CAT transgene specifically targeted to mitochondria were reported as long-lived (Schriner et al. 2005). In addition, CAT activity within the brain correlates positively with maximum lifespan across 14 mammalian and avian species (Page et al. 2010). GPx and GR, functioning within the cytosol and mitochondria, are metabolically similar to CAT, in that they function together to neutralise H₂O₂. CAT, however neutralises H₂O₂ present in low cellular concentrations, whereas GPx does so when H₂O₂ concentrations are increased. Neither GPx nor GR were altered within brain, skeletal muscle, or liver of *Irs1*^{-/-} mice. This could suggest that H₂O₂ is present in low concentrations and therefore only CAT up-regulation is required. GPx activity was elevated in heart from Snell dwarf mice (Page et al. 2010), although significantly reduced in both liver (Brown-Borg et al. 1999) and skeletal muscle (Romanick et al. 2004) of Ames dwarf mice. GSH has multiple functions and can act as a potent antioxidant, reducing disulfide bonds formed following oxidative damage to proteins (Hayes and McLellan 1999). Genotype did not influence GSH activity in brain, skeletal muscle or liver. GSH

Table 3 Oxidative damage levels measured in brain, skeletal muscle, and liver of long-lived mice

Reference		Oxidative damage								
		Protein carbonyl			4-HNE			GSH/GSSG		
		Brain	Muscle	Liver	Brain	Muscle	Liver	Brain	Muscle	Liver
This study	IIS									
	<i>Irs1</i> ^{-/-}	↔	↔	↔	↔	↔	↔	↔	↔	↔
	GH dwarf									
1	Ames (<i>Prop1</i> ^{df/df})	↓ ^a		↓ ^b			↑ ^c			
2	Snell (<i>Pit1</i> ^{dw/dw})			↓						
3	GHRKO			↔						
				M, F						

References; 1, Brown-Borg et al. 2001; 2, Brooks et al. 2007; 3, Hauck et al. 2002

IIS insulin/IGF-1 signalling pathway mutant

Irs1^{-/-}; insulin receptor substrate-1 knockout

GH growth hormone

GHRKO growth hormone receptor/binding protein knockout

M male and F female

^aSignificant difference at young (3 months) but no difference at old age (24 months)

^bSignificant difference at old age (24 month) but no difference at young age (3 months)

^cMDA+4-HNE; significant differences at middle-age (12 months) and old age (24 months) but no differences at young age (3 months)

levels have previously been shown to be increased in brain, liver, and muscle tissue of Ames dwarf mice (Brown-Borg and Rakoczy 2003), as well as in dermal fibroblasts of Snell dwarf mice (Leiser and Miller 2010).

Perhaps surprisingly, we observed no difference in protein (carbonyls) or lipid (4-HNE) oxidative damage in brain, skeletal muscle, or liver between *Irs1*^{−/−} and WT mice. In contrast, significant reduction in protein carbonyl content were reported in brain and liver of Ames and Snell dwarf mice compared to WT controls (Brown-Borg et al. 2001; Brooks et al. 2007). Discrepancies between *Irs1*^{−/−} and GH deficient mice may be due to differences in GH signalling, genetic background and/or insulin sensitivity (Selman et al. 2008; Bartke 2011). In contrast, the GSH/GSSG ratio was significantly lower in livers of *Irs1*^{−/−} relative to WT mice, suggesting an oxidized hepatic environment. Cardiovascular ROS production has been shown to be elevated within the long-lived Ames dwarf mice (Csizsar et al. 2008), suggesting that elevated ROS levels may not be detrimental and may serve as essential signalling molecules to promote extended lifespan (reviewed by Van Raamsdonk and Hekimi 2010; Ristow and Schmeisser 2011). For example, increased ROS production associated with exercise has been shown to attenuate insulin resistance and enhance adaptive antioxidant capacity within humans (Ristow et al. 2009).

Our experimental design also enabled us to examine the effect of age on antioxidant protection and oxidative damage in both *Irs1*^{−/−} and WT mice. While limited antioxidant parameters were affected by age, we did see age-related changes in oxidative damage (i.e., protein carbonyls and 4-HNE), although the direction of the age-related changes was not consistent between different tissues and different markers (Jacobson et al. 2010). Protein carbonyl content increased with advancing age in brains of Ames dwarf mice, whereas age had no effect on hepatic protein carbonyls (Brown-Borg et al. 2001). This differs from the age-related effects we observed in the current study using *Irs1*^{−/−} mice, with decreased protein carbonyl content within brain and increased content within liver. 4-HNE levels on the other hand increased with age in brain, skeletal muscle, and liver tissue, which agree with findings in various tissues and in various model organisms reviewed by (Negre-Salvayre et al. 2010).

In summary, our findings demonstrate that longevity in long-lived IIS mutant mice is not correlated with a general increase in basal antioxidant activities across

brain, skeletal muscle, and liver. Indeed, only one antioxidants (liver CAT activity) out of 15 measured was higher in *Irs1*^{−/−} mice (Table 2). Consistent with this finding, we found no evidence of reduced oxidative damage in *Irs1*^{−/−} mice using a panel of oxidative stress markers (Table 3). It should be noted that all our studies were measured in tissues isolated from non-stressed mice, and perhaps it is more important to longevity how exactly specific tissues and cells respond to chemical insult. For example, long-lived GHRKO mice (Hauck et al. 2002), Ames dwarf (Bokov et al. 2009), and female insulin-like growth factor-1 receptor heterozygous (*Igf1R*^{+/-}) mice (Holzenberger et al. 2003) all showed increased survival following oxidative challenge, relative to WT controls, although this was not seen in long-lived brain-specific *Igf1R*^{+/-} mice (Kappeler et al. 2008). While basal antioxidant activities and oxidative stress is generally unaltered in long-lived *Irs1*^{−/−} mice, we suggest that the challenge now is to determine whether IIS mutant mice have enhanced cellular stress resistance, as this will determine whether reduced IIS alone is sufficient to enhance stress resistance.

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