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Alternate Day Fasting Impacts the Brain Insulin Signaling Pathway of Young Adult Male C57BL/6 Mice

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Abstract

Dietary restriction (DR) has recognized health benefits that may extend to brain. We examined how DR affects bioenergetics-relevant enzymes and signaling pathways in the brains of C57BL/6 mice. Five month-old male mice were placed in ad libitum (AL) or one of two repeated fasting and refeeding (RFR) groups, an alternate day (intermittent fed; IF) or alternate day plus antioxidants (blueberry, pomegranate, and green tea extracts) (IF+AO) fed group. During the 24 hour fast blood glucose levels initially fell but stabilized within 6 hours of starting the fast, thus avoiding frank hypoglycemia. DR in general appeared to enhance insulin sensitivity. After six weeks brain AKT and GSK3 β phosphorylation were lower in the RFR mice, suggesting RFR reduced brain insulin signaling pathway activity. Pathways that mediate mitochondrial biogenesis were not activated; AMPK phosphorylation, SIRT1 phosphorylation, PGC1 α levels, and COX4 levels did not change. ATP levels also did not decline, which suggests the RFR protocols did not directly impact brain bioenergetics. Antioxidant supplementation did not affect the brain parameters we evaluated. Our data indicate in young adult male C57BL/6 mice, RFR primarily affects brain energy metabolism by reducing brain insulin signaling, which potentially results indirectly as a consequence of reduced peripheral insulin production.

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The authors report no conflicts of interest.

Keywords

antioxidants; brain; caloric restriction; dietary restriction; insulin; mitochondrial biogenesis

INTRODUCTION

Dietary restriction (DR) conditions exist when animals cannot feed ad libitum (AL). DR is accomplished by limiting caloric intake over defined periods (caloric restriction; CR) or by repeated fasting and refeeding (RFR). Popular mouse DR protocols include 30-50% CR and alternate day RFR (Ingram *et al.* 2007, Varady & Hellerstein 2007, Yamamoto *et al.* 2009). Mice maintained under CR or RFR conditions outlive their AL-fed counterparts (Weindruch & Sohal 1997).

Some believe DR could benefit human health. While it is still not known whether DR enhances human longevity, DR appears to reduce blood pressure, cholesterol, and insulin resistance (Fontana 2008, Rae 2004). DR may also affect the brain (Duan & Mattson 1999, Yu & Mattson 1999), and it was recently reported that 30% CR improved memory test performance in a human cohort (Witte *et al.* 2009). Mechanistic-oriented studies also suggest DR affects brain physiology. CR-treated rats have increased brain mitochondrial DNA (mtDNA) levels (Cassano *et al.* 2006). In one study, compared to AL mice the brains of alternate day fed mice showed increased oxygen consumption and mitochondrial biogenesis (Nisoli *et al.* 2005). CR reduced brain amyloidosis in tg2576 mice that express a mutated human amyloid precursor protein gene (Wang *et al.* 2005).

Bioenergetic manipulation is increasingly being considered for treating human diseases in general and brain diseases specifically (Civitarese *et al.* 2007, Henderson 2008, Swerdlow *et al.* 1989). While the effects of DR on liver, muscle, and fat bioenergetics are relatively well studied (Chang *et al.* 2007, Civitarese *et al.* 2007), studies evaluating the effects of DR on brain bioenergetics are limited (Nisoli *et al.* 2005). To help address this knowledge gap, we randomized young male C57BL/6 mice to AL, an RFR protocol with alternating 24 hour fasting and feeding cycles (intermittent fasting; IF), and an RFR protocol that was supplemented with antioxidants (IF+AO). After six weeks we evaluated bioenergetics-relevant enzymes and signaling pathways in the brains of these mice.

MATERIALS AND METHODS

Manipulations of Living Mice

The vertebrate animals work described in this manuscript was approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Most but not all studies were performed using fifteen male, five-month old C57BL/6 mice that were obtained from the Jackson Laboratory. All mice were maintained on an AL diet for one week while they accommodated to our vivarium. The individual mice were housed in separate clear plastic cages on a 12:12 hour light:dark schedule. After the accommodation period the mice were placed into 3 groups: AL-fed mice with unlimited food access (n=5), IF mice with 24 hours of food access followed by 24 hours of no access (n=5), and IF mice with antioxidant-supplemented chow (IF+AO mice) (n=5). The AL and IF groups consumed an AIN-93G chow (TD.94045, Harlan-Teklad). The IF+AO mice consumed TD.07226 (Harlan Teklad), which is TD.94045 modified to contain 2% blueberries, 115 ppm Sunphenon (epigallocatechin gallate; EGCG), and 0.3% pomegranate powder at the expense of corn starch. All mice had unlimited access to drinking water.

Blood glucose levels in these mice were measured on tail vein blood using a One-Touch Ultra Blood Glucose Monitoring System (LifeScan, Milpitas, CA). For mice in the AL group blood glucose was measured during a period of food access (non-fasting), and after a 6 hour fast (regular fasting blood glucose). For the mice in the two DR groups (the IF and the IF+AO mice) blood glucose was measured at the end of a 24 hour feast period (non-fasting), after a 6 hour fast (regular fasting blood glucose), and at the end of the 24 hour fast period (prolonged fasting blood glucose). For each mouse, the different categories of blood glucose were independently measured 2-4 times and the average of the different independent measurements for each mouse was calculated. The average value from each mouse constituted the data point used for the statistical analysis. After six weeks on either an AL, IF, or IF+AO diet mice were sacrificed by isoflurane anesthesia immediately followed by cerebral microwave irradiation using a Microwave Animal Fixation System model GA5013 (Gerling Applied Engineering, Inc.; Modesto, CA) with the settings on low power, 2000 watts, for 1 second. IF and IF+AO mice were sacrificed at the end of a 24 hour fast period.

Glucose tolerance testing (GTT) and insulin tolerance testing (ITT) were performed on a different set of C57BL/6 mice that were similarly randomized to one of the three diets also starting at approximately five months of age; these mice were part of a different study evaluating longevity and they were approximately 2 years of age when the GTT and ITT tests were performed. GTT tests were performed at the end of a 14 hour fast. Each mouse was injected with 1g of glucose per body weight (kg), and blood glucose levels were determined over a two hour period. ITT tests were also performed at the end of a 14 hour fast. Each mouse was injected with 0.75 U per body weight (kg) of insulin (Novolin T, Novo Nordisk, Princeton, NJ) and blood glucose levels were determined over a two hour period. Several of the aged mice that were used for GTT and ITT testing were also sacrificed by decapitation. In these mice the brain was rapidly dissected and frozen. The time from decapitation to brain freezing took less than two minutes.

Immunohistochemistry

Protein lysates were prepared by separating the right cerebral hemisphere from the left cerebral hemisphere, cerebellum, and brainstem and homogenizing it in Mammalian Protein Extraction Buffer (MPER; Pierce-Thermo Scientific, Rockford, IL). The homogenates were subsequently sonicated three times, for 5 seconds each time, at setting 4 using an F60 Sonic Dismembrator (Fisher Scientific).

Several proteins that participate in insulin signaling, influence aerobic metabolism, or are influenced by energy status were analyzed by Western blot. Primary antibodies to the following proteins were used: phospho-ser473 AKT (catalogue number 4060, 1:1000 dilution; Cell Signaling Technology); AKT (catalogue number 4691, 1:1000 dilution; Cell Signaling Technology); phospho-ser9 GSK3 β (catalogue number 9336, 1:1000 dilution; Cell Signaling Technology); GSK3 β (catalogue number 9315, 1:1000 dilution; Cell Signaling Technology); phospho-thr172 AMPK (catalogue number 2531, 1:1000 dilution; Cell Signaling Technology); AMPK (catalogue number 2603, 1:1000 dilution; Cell Signaling Technology); phospho-ser47 SIRT1 (catalogue number 2314, 1:1000 dilution; Cell Signaling Technology); SIRT1 (catalogue number 2028, 1:1000 dilution; Cell Signaling Technology); PGC1 α (catalogue number SC-13067, 1:1000 dilution; Santa Cruz Biotechnology); and cytochrome oxidase subunit 4 (A21348, 1:2000; Invitrogen). An antibody to GAPDH (catalogue number 2118, 1:1000 dilution; Cell Signaling Technology) was used to assess protein loading.

Primary antibody binding was revealed using horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution; Cell Signaling Technology) and Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Densitometry was performed using a

ChemiDoc XRS with Quantity One software (BioRad, Hercules, CA). To ensure equivalent protein loading, blots were subsequently stained with the GAPDH antibody. In addition to normalizing the density of each protein band to the GAPDH density from the respective blot, for phosphorylated proteins band densities were also normalized to the density of the corresponding total protein band. Normalized densities are therefore reported as relative density values. Relative density values for each protein analyzed were summarized by average and standard error. Means were compared by one-way analysis of variance (ANOVA) followed by a Least Square Difference post hoc multiple comparisons test (PASW Statistics version 18). P values less than 0.05 were considered significant.

ATP Measurements

The left cerebral hemisphere from each mouse was homogenized in 2.5 ml of 10% perchloric acid (Khan 2003). After centrifuging at 10,000 g for 10 minutes, 500 μ l of the supernatant was neutralized with 320 μ l of 2.5 M KOH. The precipitate was removed by a second centrifugation at 10,000 g for 10 minutes. 60 μ l of this supernatant was transferred to a fresh tube, on ice, and 240 μ l of Tris-HCL/EDTA buffer (pH 7.75) were added. Brain homogenate ATP levels were measured using an ATP Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Luminescence values were determined using a Tecan Infinite M200 plate reader. Values from individual samples belonging to a group were summarized by average and standard error. Means were compared by one-way ANOVA. ATP levels are expressed as pmol/mg tissue.

RESULTS

We determined glucose levels from the tail vein blood of mice maintained under different diet conditions (Figure 1). After a 6-hour fasting period, blood glucose levels were almost one third lower in the DR groups than in the AL group. An additional 18 hours of fasting, though, did not further lower the DR mice blood glucose levels. Interestingly, after 24 hour food access, blood glucose levels in DR mice were, at least in the IF group, lower than they were in the AL mice. This could occur if DR mice were more insulin-sensitive than AL mice. As indirect support, we generated GTT and ITT data from a different set of male C57BL/6 mice maintained on the same AL, IF, and IF+AO diets for a considerably longer period (approximately 19 months). The GTTs showed the IF blood glucose was lower at 120 minutes than it was in the AL mice. The ITTs showed more robust inter-group differences, as the blood glucose levels in the IF and IF+AO groups were lower than those of the AL group throughout the entire 120-minute test period (Figure 2).

The effects of DR on several proteins and pathways that sense cell nutrient and bioenergetic status were assessed in mouse brain protein lysates. To minimize the effects of post-mortem changes, the mice were anesthetized and sacrificed by microwave irradiation of the whole brain. For mice in the DR groups, protein lysates were prepared from mice at the end of a 24 hour fasting cycle.

We evaluated the status of the insulin signaling pathway. AKT is an insulin-signaling pathway serine/threonine kinase that plays a role in metabolic signaling, contributes to glucose homeostasis, and is activated via phosphorylation (Manning & Cantley 2007). A Western blot performed using an AKT-Ser473P specific primary antibody showed reduced AKT phosphorylation at serine 473 in both DR groups (Figure 3a). Reduced AKT-Ser473P was still evident after normalizing to total AKT (which was unchanged by DR) and to GAPDH (Figure 3b-d). A Western blot performed using an antibody specific for GSK3 β phosphorylation at Ser9 also found reduced GSK3 β phosphorylation at that site (Figure 4a). Reduced phosphorylation of GSK3 β -Ser9 was still evident after normalizing to total GSK3 β (which was unchanged by DR) and to GAPDH (Figure 4b-d).

We also used a Western blot immunochemistry approach to investigate the status of proteins that sense cell energy supplies, influence bioenergetic metabolism, and are influenced by cell bioenergetic demands. The AMPK enzyme is activated by an increase in the cytosolic AMP to ATP ratio (Hardie 2007). High ratios are associated with AMPK phosphorylation at Thr172 (Scharf *et al.* 2008), and this AMPK phosphorylation is often used as a surrogate measure of its activation status. Very little AMPK-Thr172P was seen in our brain homogenates, and between the groups there was no difference in AMPK-Thr172 (Figure 5a). AMPK-Thr172P levels were still equivalent after normalizing to total AMPK and GAPDH (Figure 5b-d). Consistent with the low level of AMPK-Thr172P observed, we measured brain ATP levels in perchloric acid-prepared brain homogenates and detected relatively high and quantitatively equivalent levels across groups (Figure 6). In other experiments in which AL and DR mice were sacrificed by decapitation, brain homogenates showed more robust, quantitatively equivalent AMPK-Thr172P staining and nearly undetectable ATP levels (data not shown).

SIRT1 is an NAD⁺-dependent histone deacetylase that is believed to mediate some of the benefits of DR, and also to upregulate aerobic metabolism (Guarente 2007, Nemoto *et al.* 2005). It is reported that SIRT1 phosphorylation as indicated by the phosphorylation-specific Ser47 SIRT1 antibody is a marker of SIRT1 activation (Sasaki *et al.* 2008). In our DR experiments SIRT1 phosphorylation was found to be equivalent between groups (Figure 7a). Total SIRT1 levels were also equivalent between the groups (Figure 7b). Normalizing SIRT1-P to either SIRT1 or GAPDH also did not reveal a DR effect (Figure 7c-d).

SIRT1 and AMPK reportedly activate PGC1 α , a “master regulator” of mitochondrial mass and aerobic metabolism (Handschin & Spiegelman 2006, Finck & Kelly 2006, Houten & Auwerx 2004). This transcriptional co-activator promotes both its own expression and mitochondrial biogenesis (Mootha *et al.* 2004, Schreiber *et al.* 2003). Total PGC1 α levels were equivalent between groups, which suggests our DR regimen did not induce PGC1 α activation in the brains of young male C57BL/6 mice (Figure 8). Consistent with this, levels of COX4 protein, a nuclear encoded protein that constitutes part of cytochrome oxidase and which is often used as a marker of mitochondrial mass, were unchanged by DR (Figure 9).

Since prior studies have found an RFR schedule induced brain mitochondrial biogenesis and respiration (Nisoli *et al.* 2005), and increased mitochondrial respiration could cause oxidative stress that mediates this mitochondrial biogenesis (Schulz *et al.* 2007), we included a group of IF mice whose chow was supplemented with a mix of antioxidants (IF+AO mice). The antioxidant cocktail included pomegranate, blueberry, and green tea extracts. Compared to the IF mice, the IF+AO mice may have been slightly less insulin sensitive (Figures 1 and 2), but if a real difference did in fact exist that difference was subtle. In our brain studies, IF+AO mice resembled IF mice on all measured parameters (Figures 3-9).

DISCUSSION

DR alters metabolic physiology in multiple tissues and most alterations are judged to benefit organism health and survival. DR benefits also appear to extend to the nervous system, as evidenced by increased resistance to injuries, toxic stresses, and enhanced cognitive function (Witte *et al.* 2009, Duan & Mattson 1999, Wang *et al.* 2005, Yu & Mattson 1999). In this study we specifically assessed how 24 hour RFR DR affected metabolism-relevant parameters in the brains of young, adult male C57BL/6 mice. The first main finding of our study was that the experimental protocol induced a detectable reduction in insulin signaling pathway activity within the brain. The second main finding of our study was that this protocol did not induce detectable levels of AMPK activation, SIRT1 activation, PGC1 α

activation, or mitochondrial biogenesis. We conclude that at least in young male C57BL/6 mice, DR's effect on brain function and brain health is likely mediated through reductions in the insulin signaling pathway.

Studies by others report DR profoundly affects liver metabolism. Liver effects include increased gluconeogenesis, mitochondrial proliferation, and oxygen consumption (Civitarese et al. 2007, Hagopian et al. 2003, Rodgers et al. 2005). Severe DR also results in liver ketone body production (Owen et al. 1969, Owen et al. 1967). These adaptive changes ensure the brain receives an uninterrupted supply of energy substrates, either in the form of glucose or as a combination of glucose and the ketone bodies betahydroxybutyrate and acetoacetate. In our study the greatest degree of nutrient deprivation occurred at the end of a 24 hour fast, at which point blood glucose levels were lower than they were in non-fasting mice. Although blood glucose levels declined with fasting, for mice repeatedly cycling through a 24 hour fast period this decline was maximal at 6 hours. During the entire 24 hour fast period frank hypoglycemia was avoided. Although we did not specifically test the mechanisms that stabilized glucose levels in our fasting mice, it seems likely this stability reflects the ability of liver gluconeogenesis to maintain a fairly constant blood glucose level over the course of a 24 hour fast.

Overall reductions in nutrient intake also affect insulin production by the pancreas. DR is associated with reduced insulin release and increased muscle, liver, and adipose insulin sensitivity (Kemnitz et al. 1994). Blood glucose levels were lower in our DR mice than in our control mice during periods where both groups had unlimited access to food, and also when both groups were subjected to a six hour fast. This suggests DR enhanced insulin sensitivity in the mice studied in these experiments. Also, aged C57BL/6 mice maintained on an otherwise identical IF diet showed enhanced recovery from a GTT glucose challenge, and aged IF and IF+AO mice showed greater ITT responses to an insulin challenge than aged AL mice. While it remains important to note we did not directly measure blood insulin levels or perform glucose and insulin tolerance tests in our young mice, literature data clearly indicate DR reduces insulin secretion (Merry 2002). It therefore seems reasonable to assume insulin levels in our DR mice were likely lower than they were in our AL mice. In future studies we would like to directly address this by measuring blood insulin levels.

Reduced insulin signaling in the brains of our DR mice could reflect either brain insulin resistance or reduced insulin in the brain. As there is no reason to expect DR would increase insulin resistance in any tissue the latter possibility seems more likely. Reduced pancreas insulin secretion leading to reduced plasma insulin should also predictably lower the brain insulin level.

While AKT phosphorylation is not solely determined by the insulin signaling pathway, we suspect reduced insulin signaling in DR-treated mouse brains accounts for the observed reduction in their AKT ser473 phosphorylation. We were also able to show phosphorylation of GSK3 β , a target of the AKT kinase, was decreased. Reduced GSK3 β phosphorylation is associated with increased GSK3 β activity (Grimes & Jope 2001). In neurons, increased GSK3 β activity promotes tau phosphorylation (Bhat & Budd 2002). A prior study of C57BL/6 mice found 24-72 hour fasts increased brain tau phosphorylation (Yanagisawa et al. 1999). Our finding that GSK3 β phosphorylation is decreased, most likely as a consequence of reduced AKT phosphorylation, is consistent with the results of that study.

The 6 week DR protocol we used did not appear to induce brain mitochondrial biogenesis in young male C57BL/6 mice. Other studies, though, have reported DR potentially induces brain mitochondrial biogenesis. A study done in rats found mtDNA content was increased by a DR protocol (Cassano et al. 2006). Of more direct relevance is the study of Nisoli et al,

who did report evidence of brain mitochondrial biogenesis in mice fed on alternate days (Nisoli et al. 2005). Specifically, increased brain PGC1a mRNA expression, increased SIRT1 protein, increased COX4 protein, and increased oxygen consumption were detected. We did not measure brain PGC1a transcript levels nor oxygen consumption. We did not find increased SIRT1 or COX4 protein, but a number of methodologic issues may account for differences between that and our study. The mice in the Nisoli et al study were strain hybrids, they were older than our mice, they were sacrificed at the end of a feeding cycle, and they were maintained on RFR diets for longer periods (3-12 months). It is possible our small sample sizes may have caused a type II error. It is also clear other technical issues can influence relevant measurements, as evidenced by the fact that we detected very low ATP levels in the brains of mice sacrificed by decapitation, and much higher ATP levels in the brains of mice sacrificed by microwave irradiation. Other investigators have also experienced this, and some report microwave irradiation likely permits a more accurate assessment of brain ATP levels (Scharf et al. 2008). In our study neither approach revealed inter-group differences in brain ATP levels. Regardless, differences between the models and protocols used by us and other investigators preclude gross generalizations as to whether DR can or cannot induce mitochondrial biogenesis.

Because of the study of Nisoli et al, which found RFR does induce brain mitochondrial biogenesis in mice (Nisoli et al. 2005), and the study of Schulz et al, which found antioxidants mitigate increased respiration in 2-deoxyglucose-treated *C. elegans* (Schulz et al. 2007), we included a DR group that was co-treated with an antioxidant cocktail. We were, however, unable to critically test whether antioxidant supplementation reduced brain mitochondrial biogenesis in our RFR mice because even without antioxidant supplements we did not detect evidence of brain mitochondrial biogenesis. It is worth commenting, though, on the nature of our antioxidant cocktail. Blueberry, pomegranate, and EGCG extracts reportedly constitute antioxidants that reduce morbidity (Seeram 2008, Khan & Mukhtar 2007). We wanted to avoid antioxidants that may induce morbidity, such as might be the case with high dose vitamin E supplementation (Bjelakovic et al. 2007, Miller et al. 2005). In retrospect, blueberry, pomegranate, and EGCG extracts may have actions that extend beyond their ability to reduce oxidative stress (Singh et al. 2008), and it could be argued more traditional antioxidants should have been selected.

While it is possible other brain-specific compensatory mechanisms may have occurred to maintain brain ATP levels during DR, the fact that frank hypoglycemia did not develop during the course of a 24 hour fast suggests the brains of our DR mice were at no point deprived of energy substrates. If this is correct, a need to increase brain ATP levels by upregulating aerobic metabolism may not have occurred. The ability of liver gluconeogenesis to generate adequate glucose to fuel the brain over a 24 hour period may have cushioned the direct brain effects of the DR, and mitigated any need to activate AMPK or SIRT1 within the brain. This could potentially explain why we observed neither an increase in the brain PGC1a level nor an increase in COX4 protein.

Evidence from others nevertheless indicates DR does affect the brain. Humans maintained for 6 months on a 30% calorie restricted diet show improved performance on memory tests (Witte et al. 2009). A 30% calorie restriction reduces amyloid plaque deposition in transgenic mice that over-express a mutant version of the human amyloid precursor protein gene (Wang et al. 2005). Although not a test of DR per se, mice with brain-restricted IRS2 knock out also show lifespan prolongation (Taguchi et al. 2007). Despite its limitations (small number of animals in each group, a high degree of intra-group variation with some measurements, no direct measurements of brain insulin levels, only one particular DR protocol studied) our results suggest that at least in young, adult male C57BL/6 mice, DR brain effects are mostly mediated indirectly, through reductions in the brain insulin signaling

pathway, and that reduced brain insulin signaling occurs because DR reduces circulating insulin levels.

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Abbreviations used

AL	ad libitum
AMPK	AMP kinase
ANOVA	analysis of variance
CR	caloric restriction
COX4	cytochrome oxidase subunit 4
DR	dietary restriction
EGCG	epigallocatechin gallate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GTT	Glucose tolerance testing
GSK3β	glycogen synthase kinase 3 beta
IF	intermittent fed
IF+AO	intermittent fed plus antioxidants
IRS2	Insulin Receptor Signaling Protein 2
ITT	insulin tolerance testing
mtDNA	mitochondrial DNA
PGC1α	peroxisomal proliferator-activated receptor-gamma coactivator 1 alpha
RFR	repeated fasting and refeeding
SIRT1	silent information regulator 2.

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Figure 1.

Effect of DR on blood glucose levels. The blood glucose units are in mg/dL. Error bars are $\pm$ SEM. The 6 hour fasting glucose levels in the IF and IF+AO mice were lower than that of the AL mice, which is consistent with increased insulin sensitivity in the IF and IF+AO mice. Over the next 18 hours the blood glucose levels in the IF and IF+AO mice did not drop further, indicating that after the initial fasting-induced decline in the blood glucose occurred gluconeogenesis was able to maintain the blood glucose at a lower but constant level. The AL mice were not exposed to a 24 hour fast and therefore no 24 hour fast data are available for the AL mice. At the end of a 24 hour period in which there was unrestricted access to food, the blood glucose level was lower in the IF mice than it was in the AL mice, which is also consistent with increased insulin sensitivity in the IF mice. Although there was also a trend towards a lower blood glucose level in the IF+AO mice versus the AL mice, this difference was not significant.

Figure 2.

Glucose tolerance testing (GTT) and insulin tolerance testing (ITT) from aged male C57BL/6 mice on three different diets. For each mouse, the glucose or insulin value obtained at any given time was divided by the baseline value from that mouse in order to provide a quantitative measure of its relative change from the baseline. In (A), at 120 minutes after the GTT glucose injection the blood glucose level was lower in the IF mice than it was in the AL control mice, which is also with increased insulin sensitivity in the IF mice. Although there was a trend towards a lower blood glucose level in the IF+AO mice versus the AL control mice, this difference was not significant. In (B), the blood glucose levels in the IF and IF+AO mice declined soon after insulin was injected, while the insulin injection did not reduce the blood glucose levels in the AL control mice. At each time point after the baseline the blood glucose levels were statistically lower in the IF and IF+AO mice than they were in the AL control mice.

Figure 3.

Effect of DR on brain AKT-Ser473 phosphorylation and AKT total levels. Relative to AL control mice, AKT-Ser473 phosphorylation was reduced in IF and IF+AO mice that were sacrificed at the end of a 24 hour fasting period. (A) shows the mean density readings for each group when equivalent amounts of protein were added to each well of the gel but corrections for total AKT or a housekeeping protein were not performed. Levels were statistically lower in the IF and IF+AO groups than they were in the AL control group. (B) shows the total AKT mean densities for each group. Total AKT levels are comparable between the groups. (C) shows the phosphorylated AKT to total AKT ratio for each group. Compared to the AL control group, after correcting the phosphorylated AKT densities by the total AKT densities the phosphorylated AKT was still reduced in the IF and IF+AO groups. (D) shows the phosphorylated AKT to GAPDH ratio for each group. Compared to the AL control group, after correcting the phosphorylated AKT densities by the GAPDH densities the phosphorylated AKT was still reduced in the IF and IF+AO groups.

Figure 4.

Effect of DR on brain GSK3B-Ser9 phosphorylation and GSK3B total levels. Relative to AL control mice, GSK-Ser9 phosphorylation was reduced in IF and IF+AO mice that were sacrificed at the end of a 24 hour fasting period. (A) shows the mean density readings for each group when equivalent amounts of protein were added to each well of the gel but corrections for total GSK3B or a housekeeping protein were not performed. Levels were statistically lower in the IF and IF+AO groups than they were in the AL control group. (B) shows the total GSK3B mean densities for each group. Total GSK3B levels are comparable between the groups. (C) shows the phosphorylated GSK3B to total GSK3B ratio for each group. Compared to the AL control group, after correcting the phosphorylated GSK3B densities by the total GSK3B densities the phosphorylated GSK3B was still reduced in the IF and IF+AO groups. (D) shows the phosphorylated GSK3B to GAPDH ratio for each group. Compared to the AL control group, after correcting the phosphorylated GSK3B densities by the GAPDH densities the phosphorylated GSK3B was still reduced in the IF and IF+AO groups.

Figure 5.

Effect of DR on brain AMPK-Thr172 phosphorylation and AMPK total levels. The RFR protocols did not affect AMPK phosphorylation. (A) The mean AMPK-Thr172 density readings were equivalent between the AL control group, the IF group that was sacrificed at the end of a 24 hour fast, and the IF+AO group that was sacrificed at the end of a 24 hour fast. (B) The total AMPK density readings were equivalent between the AL control group, the IF group that was sacrificed at the end of a 24 hour fast, and the IF+AO group that was sacrificed at the end of a 24 hour fast. (C) shows the phosphorylated AMPK to total AMPK ratio for each group. These ratios were comparable between the groups. (D) shows the phosphorylated AMPK to GAPDH ratio for each group. These ratios were comparable between the groups.

Figure 6.

Effect of DR on brain ATP levels. ATP levels are expressed as pmol/mg tissue. Brain ATP levels were equivalent between the the AL control group, the IF group that was sacrificed at the end of a 24 hour fast, and the IF+AO group that was sacrificed at the end of a 24 hour fast.

Figure 7.

Effect of DR on brain SIRT1-Ser47 phosphorylation and SIRT1 total levels. Substantial variation was seen within each group and no differences were seen between the groups. (A) The mean SIRT1-Ser47 density readings were equivalent between the AL control group, the IF group that was sacrificed at the end of a 24 hour fast, and the IF+AO group that was sacrificed at the end of a 24 hour fast. (B) The total SIRT1 density readings were equivalent between the AL control group, the IF group that was sacrificed at the end of a 24 hour fast, and the IF+AO group that was sacrificed at the end of a 24 hour fast. (C) shows the phosphorylated SIRT1 to total SIRT1 ratio for each group. These ratios were comparable between the groups. (D) shows the phosphorylated SIRT1 to GAPDH ratio for each group. These ratios were comparable between the groups.

Figure 8.

Effect of DR on PGC1a levels. (A) The mean PGC1a density readings were equivalent between the AL control group, the IF group that was sacrificed at the end of a 24 hour fast, and the IF+AO group that was sacrificed at the end of a 24 hour fast. (B) Correcting the PGC1a density readings by GAPDH did not alter these relationships.

Figure 9.

Effect of DR on COX4 levels. (A) The mean COX4 density readings were equivalent between the AL control group, the IF group that was sacrificed at the end of a 24 hour fast, and the IF+AO group that was sacrificed at the end of a 24 hour fast. (B) Correcting the COX4 density readings by GAPDH did not alter these relationships.