5'AMP-activated protein kinase activity is increased in adipose tissue of northern elephant seal pups during prolonged fasting-induced insulin resistance

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Abstract

Northern elephant seals endure a 2- to 3-month fast characterized by sustained hyperglycemia, hypoinsulinemia, and increased plasma cortisol and free fatty acids, conditions often seen in insulin-resistant humans. We had previously shown that adipose Glut4 expression and 5'AMP-activated protein kinase (AMPK) activity increase and plasma glucose decreases in fasting seals suggesting that AMPK activity contributes to glucose regulation during insulin-resistant conditions. To address the hypothesis that AMPK activity increases during fasting-induced insulin resistance, we performed glucose tolerance tests (GTT) on early ($n=5$) and late ($n=8$)-fasted seal pups and compared adipose tissue expression of insulin signaling proteins, peroxisome proliferator-activated receptor γ (PPAR γ), and AMPK, in addition to plasma adiponectin, leptin, cortisol, insulin, and non-esterified fatty acid (NEFA) levels. Fasting was associated with decreased glucose clearance, plasma insulin

and adiponectin, and intracellular insulin signaling, as well as increased plasma cortisol and NEFAs, supporting the suggestion that seals develop insulin resistance late in the fast. The expression of Glut4 and VAMP2 increased (52 and 63% respectively) with fasting but did not change significantly during the GTT. PPAR γ and phosphorylated AMPK did not change in the early fasted seals, but increased significantly (73 and 50% respectively) in the late-fasted seals during the GTT. Increased AMPK activity along with the reduction in the activity of insulin-signaling proteins supports our hypothesis that AMPK activity is increased following the onset of insulin resistance. The association between increased AMPK activity and Glut4 expression suggests that AMPK plays a greater role in regulating glucose metabolism in mammals adapted to prolonged fasting than in non-fasting mammals.

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Introduction

Many factors contribute to the development of insulin resistance including impaired hormone secretion and/or activity, reductions in the expression of key signaling proteins, and reduced protein activity through inhibition (Mothe & van Obberghen 1996, Perseghin *et al.* 2003, Cornier *et al.* 2006). As insulin regulates numerous critical physiological functions, such as cellular glucose uptake, the pancreas compensates for diminished insulin sensitivity by secreting more insulin (Czech 1995, Leney & Tavaré 2009). Once the pancreas reaches a point where it can no longer produce sufficient insulin to elicit a cellular effect in insulin-responsive tissues, glucose clearance becomes impaired and persistent hyperglycemia occurs (i.e. type 2 diabetes mellitus) (Campbell 2009, Lin & Sun 2010). Various hormones and proteins, such as adiponectin, glucagon-like peptide-1 (GLP-1), insulin-like growth factor 1 (IGF1), and peroxisome proliferator-activated

receptor γ (PPAR γ), are insulinotropic and facilitate insulin sensitivity and function in peripheral tissues (Ahima & Lazar 2008, Arafat *et al.* 2010, Cefalu 2010, Hsiao *et al.* 2011). However, the circulating concentrations of these factors, or their intracellular expression, are usually decreased in diabetic patients (Gregoire *et al.* 1998, Kahn & Flier 2000, Knop *et al.* 2007, Koudhi *et al.* 2010). At the same time, cortisol, which inhibits insulin secretion from the pancreas and stimulates hepatic gluconeogenesis (the generation of glucose from non-carbohydrate substrates), and free fatty acids, which decrease insulin sensitivity by inhibiting intracellular insulin signaling and glucose uptake, are increased in the circulation, contributing to continuation of the diabetic state (Khan *et al.* 1992, Epps-Fung *et al.* 1997, Khani & Tayek 2001, Lam *et al.* 2003, Macfarlane *et al.* 2008, van Raalte 2009).

Similar conditions are often observed in mammals chronically deprived of food, including humans, as stress-induced increases in plasma cortisol, paired with the mobilization of

limited metabolic substrates, lead to dysregulation of carbohydrate and lipid metabolism (Saboureaux *et al.* 1980, Scheen *et al.* 1988, Krassas 2003). This results in reductions in the concentrations of insulinotropic hormones and eventually leads to loss of sensitivity to insulin (Scheen *et al.* 1988, Krassas 2003). Although prolonged fasting-induced insulin resistance may be detrimental to most mammals, elephant seals have evolved robust physiological mechanisms that have allowed them to adapt to a fasting lifestyle and endure months without food or exogenous water.

The prolonged, fast of the elephant seal is characterized by hyperglycemia, along with increasing plasma cortisol and free fatty acids (Costa & Ortiz 1982, Castellini *et al.* 1987, Keith & Ortiz 1988, Ortiz *et al.* 2001, Champagne *et al.* 2005). Insulin signaling, plasma adiponectin, and the expression of adipose tissue PPAR γ decrease during the fasting of elephant seals, suggesting that the seal becomes insulin resistant late in the fast (Viscarrá *et al.* 2011). However, expression of Glut4 and phosphorylation of 5'AMP-activated protein kinase (AMPK) in adipose are increased (Viscarrá *et al.* 2011). Increased AMPK activity can stimulate Glut4 translocation (Russell *et al.* 1999), suggesting that glucose uptake is not compromised late in the seal's fast. Apart from the contributions that can be made to our understanding of the regulation of metabolic substrates during prolonged fasting, because elephant seals do not appear to be as susceptible to insulin resistance as humans (the health detriments associated with insulin resistance have not been observed in the seal (Castellini & Costa 1990, Adams & Costa 1993, Champagne *et al.* 2005)), the elephant seal provides a unique opportunity to promote and enhance our knowledge of the effects of, as well as the adaptation to, insulin resistance.

The goal of this study was to examine the intracellular changes associated with insulin signaling and carbohydrate metabolism that occur as a result of the hormonal and biochemical flux that elephant seals experience during prolonged fasting. We had previously shown that AMPK activity is increased in late-fasted elephant seals, suggesting that it may contribute to increased Glut4 translocation. To examine our hypothesis that AMPK activity in adipose tissue increases during reduced insulin signaling, we performed a glucose tolerance test (GTT) on early- and late-fasted elephant seals. The GTT allowed us to 1) gauge the sensitivity of the pancreas and adipose to glucose and 2) to examine the response of the insulin signaling pathway and facilitating proteins in adipose tissue to better ascertain the mechanisms induced following the onset of insulin resistance.

Materials and Methods

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee's of both the University of California Merced and the Sonoma State University. All work was conducted under the National Marine Fisheries Service marine mammal permit #87-1743.

GTT and sample collection

In total, 13 northern elephant seal (*Mirounga angustirostris*) pups were studied at the Año Nuevo State Reserve (30 km north of Santa Cruz, CA, USA) during their natural post-weaning fast while they are still on land. The pups were sampled at two time periods: early (2–3 week post-weaning (Feb 2010); $n=5$) and late (6–8 week post-weaning (March 2010); $n=8$) fasting. The elephant seal pups were isolated during the procedures to avoid interruption by the much larger adults. The body mass was measured using a hanging-load cell suspended from a tripod. After weighing, the pups were initially sedated with 1 mg/kg Telazol (tiletamine/zolazepam HCl, Fort Dodge Labs, Ft Dodge, IA, USA) administered intramuscularly. Once immobilized, an 18 gauge, 3.5-inch spinal needle was inserted into the extradural spinal vein. Blood glucose was measured using a commercially available blood glucose monitor (OneTouch Ultra2; LifeScan, Inc., Milpitas, CA, USA). Pre-infusion blood samples were collected in chilled, EDTA-treated vacutainer sample tubes containing a protease inhibitor cocktail (PIC; Sigma–Aldrich) and kept on ice until they could be centrifuged. A pre-infusion adipose tissue biopsy was collected by first cleaning a small region in the flank of the animal near the hind flipper, with alternating wipes of isopropyl alcohol and betadine, followed by a s.c. injection of 2–3 ml lidocaine (Henry Schein, Melville, NY, USA). A small (<1.5 cm) incision was made using a sterile scalpel, and a blubber biopsy (ca. 100–200 mg) was collected with a sterile biopsy punch needle (Henry Schein). The biopsy samples were rinsed with cold, sterile saline, placed in cryogenic vials, immediately frozen by immersion in liquid nitrogen, and stored at -80°C until later analyses. Following initial sample collection, animals were infused with a mass-specific dose of glucose (0.5 g/kg) administered through the spinal needle over a 2 min period (Kirby & Ortiz 1994, Fowler *et al.* 2008). Continued immobilization was maintained with ~ 100 mg bolus i.v. injections of ketamine as needed. Subsequent blood samples were collected at 5, 10, 15, 20, 30, 45, 60, 90, 120, and 150 min post-infusion, measured for glucose, and placed on ice until they could be processed. Subsequent adipose biopsies were collected at 60 and 150 min post-infusion and stored as described above.

Sample preparation

As described previously (Viscarrá *et al.* 2011), all blood samples were centrifuged for 15 min at 3000 g at 4°C , and the plasma was transferred to cryo-vials, frozen by immersion in liquid nitrogen, and immediately stored at -80°C . Frozen tissue samples were homogenized in 500 μl hypotonic buffer containing a PIC and phosphatase inhibitor cocktail (Halt PIC; Thermo, Waltham, MA, USA). The homogenate was then centrifuged at 16 100 g for 15 min and the aqueous layer was aliquoted into a separate tube. TBS (500 μl) containing 1% v/v Triton X-100, 1% w/v SDS, and 1% v/v PPIC was

Table 1 Morphological and biochemical parameters measured prior to the glucose tolerance tests early and late in the fast

Parameter	Early	Late
Body mass (kg)	122 ± 5	83 ± 7 [†]
Blood glucose (mM)	9.6 ± 0.7	7.1 ± 0.5*
NEFA (mM)	0.85 ± 0.10	1.55 ± 0.14 [†]
Cortisol (nM)	264 ± 35	477 ± 49 [†]
Adiponectin (ng/ml)	386 ± 35	296 ± 15*
GLP-1 (pM)	21.2 ± 4.0	70.9 ± 9.0 [†]
IGF1 (nM)	18.3 ± 3.4	6.1 ± 1.0 [†]
Insulin (μU/ml)	2.5 ± 0.3	1.2 ± 0.1 [†]
Leptin (nM)	0.19 ± 0.02	0.19 ± 0.01

*, †, and ‡ denote significant difference from early fasting at $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively.

added to the pellet and sonicated, the resulting suspension was then centrifuged at 16 100 *g* for 15 min, and the aqueous layer was again transferred to a separate tube. Total protein content in both cytosolic and membrane bound fractions was measured by Bradford assay (Bio-Rad Laboratories).

Western blot

Total protein (30 μg) was resolved in 4–15% Tris–HCl SDS gradient gels. Proteins < 100 kDa were electroblotted using the Bio-Rad Trans Blot SD semi-dry cell onto 0.45 μm nitrocellulose membranes. Proteins > 100 kDa were electroblotted using the Bio-Rad Mini Protean Transfer apparatus onto 0.45 μm nitrocellulose membrane. The membranes were blocked with 3% BSA in PBS containing 0.05% v/v of Tween 20 (PBS-T) and incubated overnight with primary antibodies (diluted 1:500–1:5000) against p-insulin receptor (IR)-β, p-IRS-1/IRS-1, PI3k, AKT2, Glut4, PPARγ, AMPK/p-AMPK, lamin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), IR-β, p-AKT2, β-actin (Assay Designs, Ann Arbor, MI, USA), and GAPDH (Enzo Life Sciences, Farmingdale, NY, USA). The membranes were washed, incubated with HRP-conjugated secondary antibodies (Pierce, Rockford, IL, USA), re-washed, and developed using the Immun-Star Western C kit (Bio-Rad). The blots were visualized using a Chemi-Doc XRS system (Bio-Rad) and quantified with Bio-Rad's Quantity One software. In addition to consistently loading the same amount of total protein per well (30 μg), densitometry values were further normalized by correcting the densitometry values of a constitutively expressed protein (β-actin, GAPDH, or lamin).

Plasma analyses

The plasma concentrations of total adiponectin (canine; Linco, St Charles, MO, USA), cortisol (Siemens, Los Angeles, CA, USA), GLP-1 (Millipore, Billerica, MA, USA), IGF1 (DSL, Inc., Webster, TX, USA), insulin (guinea pig anti-porcine; Linco), leptin (guinea pig; Millipore), and

non-esterified fatty acids (NEFA; Wako Chemicals, Richmond, VA, USA) were measured with commercially available RIA or enzyme immunoassay kits, previously validated for use with elephant seal plasma (Ortiz *et al.* 2001, 2003, Champagne *et al.* 2005, Viscarra *et al.* 2011). All samples were analyzed in duplicate and run in a single assay with intra-assay, the intra-assay percent coefficients of variability of < 10% for all assays.

Statistical analysis

The baseline (or T0) measurements (plasma or tissue protein content) of the early and late groups were used to assess the changes in variables as a function of fasting duration and these means (± S.E.M.) were compared by one-way ANOVA using a Fisher's PLSD *post hoc* test. The rate of glucose disappearance (*K*) was calculated assuming the 20 min post-infusion sample represented complete dilution of the injected glucose within the total body pool (Champagne *et al.* 2005, Fowler *et al.* 2008). *K* was then calculated by using the linear regression as the negative slope of glucose concentrations from 20 to 150 min post-infusion (Fowler *et al.* 2008). Mean (± S.E.M.) area-under-the-curve (AUC) was calculated for each variable measured during the GTTs and used to determine integrated changes between early and late fasting groups. Variables that changed as a result of fasting (i.e. different baseline levels) were then corrected by calculating the percent change from baseline. Mean percent changes were calculated for each time point and compared by repeated measures by ANOVA to identify the changes in response to the GTT using a Fisher's PLSD *post hoc* test. The changes were considered significantly different at $P < 0.05$. Adipose protein content was normalized by percent change and presented as ratios of either phosphorylated protein to total protein, or protein to constitutively expressed protein (actin, lamin, and GAPDH) and compared by ANOVA to determine the changes in response to the GTT. Statistical analyses were performed with StatView5 software (SAS Institute, Inc., Cary, NC, USA).

Table 2 Ratios of proteins measured in adipose of early- and late-fasted seals prior to the glucose tolerance tests

Proteins	Early	Late
p-IR:IR	0.98 ± 0.03	0.52 ± 0.04 [§]
p-IRS-1:IRS-1	1.01 ± 0.05	0.69 ± 0.01 [†]
PI3k:β-actin	1.05 ± 0.03	0.82 ± 0.01*
p-AKT2:AKT2	0.99 ± 0.01	0.62 ± 0.05 [†]
p-AMPK:AMPK	1.01 ± 0.07	1.33 ± 0.08 [†]
PPARγ:lamin	0.95 ± 0.03	0.71 ± 0.03 [†]
VAMP2:GAPDH	0.99 ± 0.07	1.63 ± 0.04 [§]
Glut4:GAPDH	0.95 ± 0.06	1.52 ± 0.05 [§]

*, †, ‡, and § denote significant difference from early fasting at $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$ respectively.

Figure 1 Mean ($\pm$ S.E.M.) percent change from baseline of blood glucose during the GTT in early ($n=5$; 2–3 week post-weaning) and late ($n=8$; 6–8 week post-weaning)-fasted elephant seal pups. +, *, #, and ‡ denote significant difference from baseline (time 0) at $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$ respectively. a, b, and c denote significant difference from early fasting at $P<0.05$, $P<0.01$, and $P<0.001$ respectively.

Results

Fasting-associated changes

Elephant seal pups were, on average, 32% smaller ($P<0.01$) in the late fasting period compared with the early fasting period (Table 1). Mean blood glucose and plasma adiponectin, insulin, and IGF1 decreased 35% ($P<0.05$), 23% ($P<0.05$), 52% ($P<0.001$), and 66% ($P<0.01$) respectively, with fasting (Table 1). Mean plasma cortisol, NEFA, and GLP-1 increased ($P<0.01$) 80, 82, and 233% respectively, with fasting (Table 1). Mean plasma leptin did not change (Table 1).

The phosphorylation ratios of IR, IRS-1, and AKT2 decreased 46% ($P<0.0001$), 39% ($P<0.001$), and 37% ($P<0.001$) respectively, with fasting (Table 2). The content of PI3k and PPAR γ decreased 21% ($P<0.05$) and 25% ($P<0.001$) respectively, with fasting (Table 2). The phosphorylation ratio of p-AMPK increased 38% ($P<0.01$) with fasting (Table 2). The content of Glut4 and VAMP2 increased ($P<0.0001$) 60 and 64% respectively, with fasting (Table 2).

Response to GTT

Glucose The rate of glucose disappearance (indicated by the negative slope of the line fitted to glucose concentrations) following the GTTs decreased ($P<0.05$) late in the fast (0.81 ± 0.06 vs 0.50 ± 0.03 mg/dl per min). This resulted in mean blood glucose returning to baseline by 120 min in early fasted seals versus more than 150 min in late-fasted seals (Fig. 1) consistent with impaired clearance of infused glucose in the latter. The mean AUC_{glucose} was almost doubled in response to the GTT and glucose remained significantly elevated in late-fasted animals (Fig. 1 insert), the findings that coincide with the slower clearance of glucose.

Hormones Mean percent change in insulin significantly increased at 15 min post-infusion in both groups; however, the increase in mean insulin at 30 min was 2.5-fold greater in early fasted seals (Fig. 2A). In contrast to early fasted animals, which returned to baseline after 120 min, the mean insulin remained elevated throughout the sampling period in late-fasted animals, coinciding with the elevated blood glucose at the same time point in the late group, suggesting that the impaired clearance of glucose maintained chronic stimulation of insulin (Fig. 2A). Mean AUC_{insulin} did not differ between the two groups (Fig. 2A insert). GTTs induced similar suppressive effects on plasma cortisol during the early and late periods, with the mean percent change in plasma cortisol decreasing ($P<0.05$) until 120 min post-infusion in both early- and late-fasted animals (Fig. 2B). Mean AUC_{cortisol} was not different between the two groups suggesting that the adrenal responsiveness to a glucose challenge was not altered with fasting duration (Fig. 2B insert). Both early- and late-fasted seals also showed similar responses to GTT with respect to plasma adiponectin, which decreased ($P<0.05$) and did not fully recover until after 150 min (Fig. 3). Plasma GLP-1, IGF1, and leptin did not change in response to the GTT.

Figure 2 Mean ($\pm$ S.E.M.) percent change from baseline of (A) plasma insulin and (B) plasma cortisol during the GTT in early ($n=5$; 2–3 week post-weaning) and late ($n=8$; 6–8 week post-weaning)-fasted elephant seal pups. +, *, #, and ‡ denote significant difference from baseline (time 0) at $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$ respectively. a, and d denote significant difference from early fasting at $P<0.05$ and $P<0.0001$ respectively.

Figure 3 Mean ($\pm$ S.E.M.) percent change from baseline of plasma adiponectin during the GTT in early ($n=5$; 2–3 week post-weaning) and late ($n=8$; 6–8 week post-weaning)-fasted elephant seal pups. + and # denote significant difference from baseline (time 0) at $P<0.05$ and $P<0.0001$ respectively.

Non-esterified fatty acids Plasma NEFAs decreased ($P<0.0001$) in response to the GTT early in the fast and remain suppressed until 150 min post-infusion (Fig. 4). In contrast, plasma NEFAs increased ($P<0.0001$) in response to GTTs in late-fast animals and levels remained elevated until 120 min post-infusion (Fig. 4).

Intracellular proteins No significant changes were observed in the expression of total IR, IRS-1, AKT2, AMPK, Glut4, or VAMP2 in response to the GTT during either period. Phosphorylated IR increased about twofold ($P<0.0001$) at 60 min but returned to basal levels by 150 min in the early fasted animals (Fig. 5A). In the late-fast seals, phosphorylated IR increased 22% ($P<0.01$) and not until 150 min in response to glucose infusion (Fig. 5A). Tyrosine phosphorylated IRS-1 increased 80% ($P<0.0001$) by 60 min and returned to basal levels by 150 min early in the fast (Fig. 5B). Tyrosine phosphorylated IRS-1 increased 20% ($P<0.05$) at 150 min (Fig. 5B). PI3k expression did not change in the early fasted animals but increased 20% ($P<0.05$) at 150 min in the late-fast animals (Fig. 5C). Phosphorylated AKT2 increased 50% ($P<0.001$) in response to the GTT in early fasted animals; however, the recovery at 150 min overcompensated and levels were reduced 24% ($P<0.01$) compared with basal levels (Fig. 5C). Phosphorylated AKT2 increased 40% ($P<0.001$) in late-fast seals at 150 min (Fig. 4C) and levels were greater ($P<0.0001$) than early at the same time point (Fig. 5D). Phosphorylated AMPK did not change in response to GTT early in the fast but increased 60% ($P<0.001$) at 60 min and remained elevated ($P<0.0001$) at 150 min in the late-fast animals (Fig. 6A). Similar to AMPK, PPAR γ expression did not change as a result of GTT in the early fasted seals, but PPAR γ expression increased 75% ($P<0.0001$) at 60 min and remained elevated ($P<0.001$) at 150 min in the late-fast seals (Fig. 6B).

Discussion

Insulin plays a critical role in regulating the availability of metabolic substrates, and defects in insulin signaling in one tissue can have profound effects on whole-body insulin sensitivity. In this study, we found significant reductions in plasma insulin and adipose tissue insulin signaling, leading to an impaired response to a GTT in late-fast elephant seal pups. The impaired response, associated with an increase in adipose tissue AMPK activity, suggests that AMPK 1) is activated following the onset of insulin resistance and 2) may be compensating for the reduction in cellular insulin signaling. Because the contributions of AMPK activation to the regulation of glucose during prolonged fasting and insulin resistance are not well defined, this study enhances our understanding of the cellular signaling events and mechanisms used by prolonged-fast mammals to counteract the deleterious effects of fasting-induced insulin resistance. In addition, this study provides a unique opportunity to assess both the cellular and the systemic responses to a GTT in a mammal adapted to insulin resistance.

Although the GTT stimulated activation of the insulin signaling pathway at both periods, the critical difference is the timing of the events. In early fasted animals, GTT stimulated phosphorylation of insulin receptor, IRS-1, and AKT2 at 60 min post-infusion, but phosphorylation of these insulin signaling proteins is not increased until 150 min in late-fast seals, suggesting that delayed phosphorylation of these proteins contributes to the insulin-resistant condition observed. In addition to the delayed phosphorylation of these signaling proteins, the magnitude of the phosphorylation in late-fast animals is much less than in early fasted animals, suggesting that, in addition to the timing of phosphorylation, blunted phosphorylation also contributes to the observed insulin resistance.

Figure 4 Mean ($\pm$ S.E.M.) percent change from baseline of plasma NEFA during the GTT in early ($n=5$; 2–3 week post-weaning) and late ($n=8$; 6–8 week post-weaning)-fasted elephant seal pups. + and # denote significant difference from baseline (time 0) at $P<0.05$ and $P<0.0001$ respectively. d denotes significant difference from early fasting at $P<0.0001$.

Figure 5 Mean ($\pm$ S.E.M.) ratios and representative western blots of (A) p-IR to IR, (B) p-IRS-1 to IRS-1, (C) PI3k to β -actin, and (D) p-AKT2 to AKT2 in adipose tissue of early ($n=5$; 2–3 week post-weaning) and late ($n=8$; 6–8 week post-weaning)-fasted elephant seal pups at 0, 60, and 150 min post-infusion. +, *, #, and † denote significant difference from time 0 at $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$ respectively. d denotes significant difference from early fasting at $P<0.0001$.

The similarities in AUC_{insulin} following the GTT would suggest that glucose-stimulated pancreatic secretion of insulin is not impaired over the measurement period (150 min); however, the difference in the secretion pattern over time is indicative of differential pancreatic responsiveness that is likely a residual and characteristic effect of fasting duration. In early fasted animals, the patterns of insulin secretion and signaling and glucose clearance are more characteristic of healthy, non-insulin-resistant mammals. Unlike early fasted animals in which plasma insulin returned to baseline levels (at 120 min), plasma insulin plateaued after the peak secretion at 30 min, and stayed elevated over the measurement period, coinciding with reduced glucose clearance suggesting that the chronically elevated blood glucose levels in late-fast animals maintained chronic, however blunted, stimulation of the pancreas. This pancreatic response is a unique observation in that insulin-resistant mammals (e.g. humans, rodents, and canines) usually maintain chronically elevated plasma insulin at much higher concentrations (McGuinness *et al.* 1990, Fernandez-Veledo *et al.* 2008, Shanik *et al.* 2008) and also suggests that pancreatic sensitivity to glucose is impaired in late-fast animals.

Glucocorticoids inhibit pancreatic insulin secretion, inhibit glucose uptake in peripheral tissues, and stimulate hepatic gluconeogenesis and free fatty acid release from adipocytes (Billaudel & Sutter 1979, Khan *et al.* 1992, Lambilotte *et al.* 1997, Khani & Tayek 2001, Macfarlane *et al.* 2008, van Raalte 2009). This is generally assumed to represent an adaptive response in animals during stressful conditions (e.g. nutritional stress and sustained exercise) to ensure that a pool of metabolic substrates is maintained in circulation and available to supply increased energetic demands. Although plasma cortisol increased nearly twofold late in the fast, GTTs

suppressed plasma cortisol similarly between both the periods suggesting that 1) the adrenal gland (zona fasciculata) is similarly responsive to negative feedback regulation of glucocorticoid secretion and 2) fasting duration has not altered the adrenal's sensitivity to glucose (substrate availability). Furthermore, the integrated (AUC_{insulin}) GTT-induced increase of insulin was similar between both the periods and coincided with similar degrees of suppression in cortisol during the GTTs, suggesting that cortisol contributes to the suppression of insulin secretion during prolonged fasting.

In addition to elevated cortisol, free fatty acids can also impair insulin signaling in peripheral tissues and may be the link by which dyslipidemia contributes to insulin resistance (Golay *et al.* 1987, Epps-Fung *et al.* 1997, Kashyap *et al.* 2003). Furthermore, data regarding the response of NEFAs to GTTs is scarce (Sumner *et al.* 2004) and not well described under prolonged fasting conditions in models of insulin resistance. A unique observation of this study was the differential response of NEFAs to a glucose infusion represented by

Figure 6 Mean ($\pm$ S.E.M.) ratios and representative western blots of (A) p-AMPK to AMPK and (B) PPAR γ to lamin in adipose tissue of early ($n=5$; 2–3 week post-weaning) and late ($n=8$; 6–8 week post-weaning)-fasted elephant seal pups at 0, 60, and 150 min post-infusion. # and † denote significant difference from time 0 at $P<0.001$ and $P<0.0001$ respectively. c and d denote significant difference from early fasting at $P<0.001$ and $P<0.0001$ respectively.

suppression of NEFAs early and stimulation late. This differential NEFA response to GTTs is likely an evolutionary adaptation associated with resource allocation of substrates necessary to maintain energetic demands associated with prolonged fasting (conservation of lipid stores early in the fast, mobilization of lipid stores late in the fast when metabolism has shifted to oxidation of fat). Adiponectin promotes insulin sensitivity in peripheral tissues by stimulating the removal of free fatty acids (FFA) from circulation and thus its decrease is another commonly used measure to gauge insulin resistance (Punyadeera *et al.* 2005, Ahima 2006, Ahima & Lazar 2008). Though the precise mechanisms have not been elucidated, we propose that suppressed adiponectin resulting from the GTTs, along with the increased availability of glucose, suppressed fatty acid oxidation and increased lipolysis, thus increasing plasma NEFAs. Moreover, the increase in the expression of PPAR γ following the infusion in late-fasted animals reinforces the suggestion that lipolysis was increased (Benton *et al.* 2010, Hsiao *et al.* 2011), contributing to the increased FFA release.

Though Glut4 is considered to be an insulin-responsive glucose transporter, AMPK has been shown to stimulate Glut4 translocation in a number of tissues, including adipose tissue (Hardie 2003, Yamaguchi *et al.* 2005). Despite the reduced phosphorylation of insulin receptor, IRS-1, and AKT late in the fast, the phosphorylation of AMPK increased and may have compensated for the suppressed insulin signaling. The observed increase in the expression of Glut4 and VAMP2 late in the fast may be adaptive to compensate for the elevated NEFAs associated with late fasting and necessary to maintain proper regulation of glucose uptake in adipose tissue. Increased Glut4 expression may have resulted from increased fatty acid oxidation late in the seal's fast as ligands (such as palmitoyl L-carnitine) derived from mitochondrial fatty acid import have been shown to increase *Glut4* mRNA expression (Griesel *et al.* 2010). If the increased availability of glucose inhibited fatty acid oxidation, it could have also triggered the activation of AMPK to compensate for the suppression of insulin signaling by FFA and increase Glut4 turnover, thus helping to restore proper energy balance in adipocytes.

In conclusion, the sustained elevation of blood glucose (compared to its baseline concentration) following the infusion late in the seal's fast demonstrates that glucose clearance is impaired and is indicative of insulin resistance. The nearly twofold increase in plasma cortisol with fasting supports the idea that glucocorticoids suppress insulin secretion late in the seal's fast and potentially contributed to the decreased clearance of blood glucose. The suppression of adiponectin paired with a prominent increase in NEFA following the infusion in late fasted animals further reinforces the idea that prolonged fasting induces insulin resistance in the seal. Because FFAs inhibit insulin-mediated glucose uptake, and therefore decrease insulin sensitivity, increased plasma NEFAs in response to GTTs provide a probable cause for the diminished insulin signaling in

late-fasted seals. Determining how the seal is able to maintain such high levels of FFA and cortisol, without any apparent detriment to its health, would be of great benefit to our understanding of the conditions that manifest insulin resistance and type 2 diabetes mellitus (T2DM). In addition, increased AMPK activity following the glucose infusion supports the suggestion that AMPK stimulated Glut4 translocation, in the absence of normal insulin signaling, to restore energy balance in adipocytes. If AMPK functions in the manner that we suggest, determining how it can be readily activated in the elephant seal will improve our understanding of the mechanisms possessed by mammals adapted to chronic food deprivation to regulate metabolism during prolonged fasting and may also lead to improvements to the current treatments for insulin resistance and T2DM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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