Published in final edited form as:
Cell Metab. 2010 May 5; 11(5): . doi:10.1016/j.cmet.2010.04.004.

Regulation of Skeletal Muscle Oxidative Capacity and Insulin Signaling by the Mitochondrial Rhomboid Protease PARL

Anthony E. Civitarese[1,2,9], **Paul S. MacLean**[3], **Stacy Carling**[1], **Lyndal Kerr-Bayles**[4], **Ryan P. McMillan**[5], **Anson Pierce**[6], **Thomas C. Becker**[7], **Cedric Moro**[1], **Jean Finlayson**[2], **Natalie Lefort**[2], **Christopher B. Newgard**[7], **Lawrence Mandarino**[2,9], **William Cefalu**[1], **Ken Walder**[4], **Greg R. Collier**[4,8], **Matthew W. Hulver**[5], **Steven R. Smith**[1], and **Eric Ravussin**[1]

[1]Pennington Biomedical Research Center, Baton Rouge, USA

[2]Arizona State University, USA

[3]University of Colorado Denver, Aurora, USA

[4]Deakin University, Geelong, Australia

[5]Virginia Polytechnic Institute and State University, Blacksburg, USA

[6]Barshop Institute for Longevity and Aging Studies, San Antonio, USA

[7]Duke University, Durham, USA.

[8]ChemGenex Pharmaceuticals, Geelong, Australia

[9]Mayo Clinic, Department of Medicine, Scottsdale, Arizona, USA

SUMMARY

Type 2 diabetes Mellitus (T2DM) and aging are characterized by insulin resistance, lower mitochondrial density and function and increased production of reactive oxygen species (ROS). In lower organisms continuous remodeling critically maintains the function and life cycle of mitochondria, in part by the protease *pcp1* (PARL ortholog). We therefore examined whether variation in PARL protein content is associated with mitochondrial abnormalities and insulin resistance. Relative to healthy, young individuals (23±1y), PARL mRNA and mitochondrial mass were both reduced in elderly subjects (64.4±1.2 y; 51% and 44% respectively) and in subjects with T2DM (51.8±3 y; 31% and 41% respectively; all $p<0.05$). Muscle knock-down of PARL in mice resulted in lower mitochondrial content (−31±3%, $p<0.05$), lower OPA1 and PGC1α protein levels and impaired insulin signaling. Furthermore, mitochondrial cristae were malformed and resulted in elevated *in vivo* oxidative stress. Adenoviral suppression of PARL protein in healthy myotubes lowered mitochondrial mass (−33±8%), insulin stimulated glycogen synthesis (−33±9%) and increased ROS production (2-fold) (all $p<0.05$). We propose that lower PARL expression may contribute to the mitochondrial abnormalities seen in aging and T2DM.

Address correspondence and reprint requests to: Anthony E. Civitarese Pennington Biomedical Research Center 6400 Perkins Rd Baton Rouge, LA 70808 civitaae@pbrc.edu.

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is the most common metabolic disease in the elderly (King et al., 1998) and is one of the leading causes of death in the US (Marx, 2002). T2DM is projected to afflict more than 300 million individuals worldwide by the year 2025 (Zimmet et al., 2001). Although the primary cause of T2DM is unknown, it is clear that insulin resistance in skeletal muscle and liver plays a primary role in its pathogenesis before the progressive failure of pancreatic beta cells (Lillioja et al., 1993). Studies of the etiology of insulin resistance in skeletal muscle have implicated numerous factors including reduced mitochondrial mass (Kelley et al., 2002), lower mitochondrial oxidative phosphorylation (Befroy et al., 2007; Petersen et al., 2005), blunted lipid oxidation (Zhang et al., 2007) and elevated reactive oxygen species (ROS) production (Civitarese et al., 2006; Patti et al., 2003; Petersen et al., 2004; Ukropcova et al., 2007). However, the molecular mechanism(s) governing mitochondrial dysfunction and insulin resistance in skeletal muscle are not completely understood.

Mitochondria carry out a vast array of cellular functions including ATP production, biosynthesis of amino acids and lipids, cytosolic calcium transport (Frazier et al., 2006) and amplification of apoptotic stimuli (Cipolat et al., 2004). Part of the functional diversity and variability in mitochondrion ultra-structure is mediated by the interaction with the cytoskeleton (Hollenbeck and Saxton, 2005) and the balance between the forces of mitochondrial fusion and fission. In lower organisms, mitochondrial fusion is regulated in part by the evolutionary conserved GTPase protein *mgm* (Herlan et al., 2003), the human ortholog of which is optic atrophy 1 (OPA1). Mutations in the OPA1 gene are the most common cause of autosomal dominant optic atrophy in humans (Alexander et al., 2000), a progressive disease of the retinal ganglion. *Mgm1*/OPA1 participates in several biological processes, most notably fusion of the inner mitochondrial membrane, remodeling of the mitochondrial cristae (Cipolat et al., 2004), inhibition of cytochrome c release, and apoptosis (Cipolat et al., 2006). In budding yeast (McQuibban et al., 2003) and in mice (Cipolat et al., 2006) *mgm1*, is cleaved by the inner mitochondrial membrane rhomboid protease, *pcp1*. Yeast strains lacking *pcp1* exhibit decreased oxidative capacity, impaired growth and fragmented mitochondria (McQuibban et al., 2003; Sesaki et al., 2003). Importantly, insertion of the human “presenilin-associated rhomboid-like” (PARL) gene (the *pcp1* human ortholog) into *pcp1*-deficient yeast rescues growth and restores mitochondrial morphology, suggesting that PARL may play a similar role in humans (McQuibban et al., 2003). We have previously shown that PARL mRNA is reduced in skeletal muscle of obese-diabetic *Psammomys obesus* and is positively correlated with insulin sensitivity in humans (Walder et al., 2005). Collectively, the large body of accumulating evidence over the past decade demonstrating impaired mitochondrial energetics in states of insulin resistance, we hypothesized that dysregulation of PARL contributes to the defective lipid and glucose metabolism frequently seen in T2DM and aging.

RESULTS

PARL mRNA is associated with reduced mitochondrial mass in T2DM and aging

To investigate whether variation in PARL mRNA expression was associated with mitochondrial dysfunction and insulin resistance in humans, we studied healthy, young individuals (23±1y, n=11) and older subjects (64.4±1.2 y, n=11) matched for body composition, plasma free fatty acids and glucose concentrations as well as physical activity levels (Suppl. Table 1). Relative to younger participants, elderly individuals had elevated plasma insulin ($p<0.01$), lower insulin sensitivity ($p<0.01$) and a 35% reduction in resting metabolic rate (RMR; $p<0.01$; Suppl. Table 1). Analysis of the skeletal muscle transcriptome from muscle biopsies collected under fasting conditions revealed lower

mRNA content of peroxisome proliferator-activated receptor gamma coactivator-β (PGC1β), nuclear respiratory factor (NRF1) and mitochondrial transcription factor A (TFAM) in elderly individuals compared to young controls, suggesting a reduction in mitochondrial number and function (Suppl. Table 2). Consistent with these observations, PARL mRNA, mtDNA (Fig 1a-b) and citrate synthase activity (Suppl. Table 2) (the latter two being markers of mitochondrial content (Wang et al., 1999)) were reduced in elderly subjects by 51%, 48% and 33% respectively (all, $p<0.05$). In addition, PARL mRNA was strongly correlated with TFAM mRNA ($r=0.87$, $p<0.001$) and mtDNA content (r-=0.61, $p=0.001$), suggesting PARL may be involved in the regulation of mitochondrial mass.

Functional studies by Petersen et al (Petersen et al., 2003) and Conley et al. (Conley et al., 2007) have observed reduced resting rates of ATP synthesis in elderly subjects with insulin resistance, suggesting that mitochondrial dysfunction with aging may be relevant to mitochondrial dysfunction seen in T2DM (Petersen et al., 2005). We therefore next identified that PARL mRNA and mtDNA content were reduced in subjects with T2DM relative to lean healthy controls (Fig 1a-b, both $p<0.05$), and in Pima Indians with a family history of T2DM (FH+; n=14) compared to those without a family history (FH−; n=7) (50±6 vs. 86±15 PARL mRNA/cyclophilin mRNA, $p=0.05$). Collectively, these observations establish that PARL mRNA and mitochondrial content in skeletal muscle are reduced in subjects with a genetic predisposition to develop T2DM, those presenting with T2DM, and in insulin resistant elderly individuals.

mCK-CAR-PARL(−) transgenic mice have reduced mitochondrial mass in muscle

Tissues with high demand for aerobic respiration such as the brain, skeletal muscle and heart, have the most prominent requirement for mitochondrial reticulum networks (Bach et al., 2003; Kirkwood et al., 1986). Accordingly, in *Psammomys obesus*, PARL protein was highly expressed in the brain, heart and skeletal muscle and predominantly in oxidative muscle fibers of lean healthy animals (Fig 1c). Similarly, in humans PARL mRNA is mainly expressed in skeletal muscle, brain, placenta, testis and heart (Suppl. Fig 1a). Given that skeletal muscle is the major site of insulin-stimulated glucose disposal and therefore a key tissue in the development of whole-body insulin resistance (Petersen et al., 2005), we set out to examine the functional consequences of reduced PARL protein on mitochondrial function and glucose metabolism in the skeletal muscle of a transgenic rodent model.

Mice expressing the human coxsackie /adenovirus receptor (CAR) under the control of the muscle creatine kinase promoter (mCK) were used to create mCK-CARPARL(−) mice muscle, herein referred to as PARL(−) mice. mCK-CAR mice have a dramatic enhancement of skeletal muscle transduction with site-specific injections of AdV-shuttle because they transgenetically overexpress CAR on mature myofibers (Nalbantoglu et al., 2001). We treated mCK-CAR mice (16 weeks of age, *n=7*) with a single intramuscular transverse injection into each lobe of the gastrocnemius (gastroc) and tibialis anterior/extensor digitorum longus (TA/EDL) muscle groups with AdV PARL(−) siRNA injected into the left leg and an “empty” vector with a GFP expression cassette in the right leg to serve as a collateral control (Supple Fig 1b). Analysis of PARL expression in the TA/EDL muscle of the PARL(−) leg revealed lower PARL protein that was associated with reduced PGC1α and CS protein levels relative to the control leg (mCK-GFP) (Fig 2a). Similar reductions in PARL protein was observed in gastroc muscle (data not shown). MtDNA (Fig 2d; $p=0.03$) content and CS activity (Fig 2e; $p=0.007$) were lower in PARL(−) muscle relative to GFP control muscle. Thus, it appears that mitochondrial content is affected by PARL protein content in muscle.

The key regulators of mitochondrial fusion, mitofusin 1 and 2 (Mfn1/2) and OPA1 (long and short isoform of OPA1 (Guillery et al., 2007)) were examined next. Surprisingly, MFN2 and

OPA1 were lower in PARL(−) muscle, whereas, MFN1 was unchanged (Fig 2a). The reductions in OPA1 protein are consistent with previous reports demonstrating enhanced degradation of OPA1 protein (constitutive cleavage) and increased susceptibility to apoptosis (Cipolat et al., 2006; Griparic et al., 2007) with reduced PARL and DRP-1 (Mopert et al., 2009) expression. Therefore, lower OPA1 protein may result from reduced mitochondrial mass or dysfunction as observed in PARL-loss of function myotubes (see below). OPA1 functions include mitochondrial fusion and mitochondrial cristae remodeling (Cipolat et al., 2006; Griparic et al., 2007). To determine the effects of PARL depletion on cellular morphology, we examined mCK-CAR mice muscle by transmission electron microscopy. Electron micrographs demonstrated normal mitochondrial distribution (Fig 3 a-b) in both PARL(−) and GFP-mice muscle, however in PARL(−) (Fig 3 d) muscle mitochondrial cristae appeared “disorganized”, with some mitochondria totally lacking clearly defined cristae. This mitochondrial phenotype is characteristic of HeLa cells (Griparic et al., 2007) and “class II” remodeled mitochondria from mouse embryonic fibroblasts depleted of OPA1 (Frezza et al., 2006). We also observed increased activity of the pro-apoptopic protein Caspase-9 in PARL(−) TA/EDL muscle (1.8±0.4 vs 2.9±0.6 rfu/mg protein; $p=0.048$), consistent with a role for PARL in the anti-apoptotic function of OPA1 (Frezza et al., 2006). Interestingly, global PARL knockout mice display normal intrauterine development but from the fourth postnatal week undergo progressive multi-systemic atrophy leading to accelerated cellular apoptosis and cachectic death (Cipolat et al., 2006). Skeletal muscle loss and the development of sarcopenia is a hallmark feature of the aging process and is associated with elevated levels of apoptosis (Whitman et al., 2005), suggesting that lower PARL protein may be a contributing factor to muscle atrophy during aging.

The inner mitochondrial membrane cristae are easily damaged by ROS as a large portion of protein complexes associated with oxidative phosphorylation are embedded in this membrane (Gilkerson et al., 2003). Analysis of cysteine oxidation was performed by measuring protein disulfides and TBARS concentration (marker of lipid peroxidation) in PARL(−) muscle and revealed elevated oxidative damage relative to the control muscle (Fig 2f-g; both $p<0.05$). Superoxide dismutase (SOD) catalyses the dismutation of superoxide radicals into hydrogen peroxide and oxygen and is considered to be one of the primary antioxidant responses to elevated ROS production (Luo et al., 2006). SOD protein was elevated in the muscle of PARL(−) muscle when compared to control muscle, whereas there was no difference in glutathione peroxidase content (Fig 2a). Thus, PARL protein appears to influence total OPA1 protein levels and a low level of PARL protein expression is associated with increased oxidative damage in skeletal muscle. Importantly, reduced muscle PARL protein content results in abnormal mitochondrial morphology consistent with the mitochondrial structural abnormalities observed in skeletal muscle of subjects with T2DM (Kelley et al., 2002; Ritov et al., 2005).

PARL(−) mouse muscle has elevated lipid content and impaired insulin-signaling

Insulin resistant individuals have excess triglyceride and intramyocellular lipid (IMCL) accumulation (Forouhi et al., 1999; Jacob et al., 1999; Krssak et al., 1999; Perseghin et al., 1999) in skeletal muscle (Goodpaster et al., 2000a; Goodpaster et al., 2000b; Pan et al., 1997) that has been postulated to accumulate in part, due to deficient mitochondrial oxidative capacity (Befroy et al., 2007; McGarry, 1992; Morino et al., 2005; Petersen et al., 2003; Petersen et al., 2005; Zhang et al., 2007). We next measured the triglyceride content in PARL(−) mice muscle homogenates. PARL(−) muscle had elevated triglyceride content relative to the GFP-null muscle, although this did not reach significance (65±15 vs. 48±9 μM/mg protein; $p=0.077$). Several groups have shown the accretion of long chain fatty acyl-CoAs (LCFCoA), diacylglycerols (DAG) and ceramides (Adams et al., 2004; Adams et al.,

2009; Chavez and Summers, 2003; Yu et al., 2002) is more closely associated with impaired insulin signaling than triglycerides or IMCL *per se.* Among these bioactive lipid intermediates, there is evidence to support a direct role for fatty acyl CoAs in the resistance to insulin action (Adams et al., 2004; Chavez and Summers, 2003; Yu et al., 2002). Consistently, knock down of PARL protein in skeletal muscle resulted in increased concentration of all fatty acyl CoAs examined (Fig 2h-total fatty aycl CoAs). In particular, the saturated long-chain myristoyl-CoA (14:0; p=0.031), palmitoyl-CoA (16:0; p=0.016) and stearoyl-CoA (18:0; p=0.004) were all elevated in PARL(−) muscle relative to the collateral GFP control.

We then tested whether elevated LCFCoA concentration in PARL(−) muscle was associated with impaired insulin signaling. Animal models and *in vitro* experiments have shown that the phosphorylation of serine residues ($^{307, 636, 639, 1101}$) of insulin-receptor substrate-1 (IRS-1) blocks the activity of IRS-1 on critical tyrosine sites that are required for phosphatidylinositol 3-kinase (PI3K) activation (at phosphorylation site PI3K p85-Tyr^{458} (Luo et al., 2007; Yi et al., 2007). Downstream of PI3K, Akt phosphorylation at Ser^{473} has been shown to be a key step for the activation of glucose transport in skeletal muscle (Morino et al., 2005). In fasting conditions, we observed reduced basal phosphorylation of $PI3K^{-Tyr458}$ and AKt-Ser^{473} in PARL(−) TA/EDL muscle relative to control muscle (Fig 2b). In the insulin-stimulated state, phosphorylation at IRS-$1^{Ser\text{-}1101}$ was elevated in PARL(−) muscle relative to control, in parallel with lower insulin-stimulated $Akt^{-Ser473}$ phosporylation (Fig 2b). We next isolated the cytosolic and plasma membrane fraction of PARL(−) and AdV-GFP red (oxidative) gastrocnemius muscle under insulin-stimulated conditions. No change was observed in the plasma membrane content of the insulin receptor-β between PARL(−) and GFP control muscle(Fig 2c). However, reductions in muscle PARL protein resulted in lower insulin-stimulated translocation of GLUT4 protein to the plasma membrane (Fig 2c). Together, these data indicate that skeletal muscle-specific reduction of PARL protein results in elevated saturated long chain fatty acyl CoAs, impaired insulin signaling and reduced GLUT4 translocation to the plasma membrane.

HFD lowers muscle PARL and PGC1α mRNA along with glucose tolerance in mice

Impairments in mitochondria transcription and function (Civitarese et al., 2006; Patti et al., 2003; Petersen et al., 2004) along with lower PARL mRNA (see above) are present in the pre-diabetic state, suggesting that PARL and mitochondrial abnormalities may be primary factors in the development of skeletal muscle insulin resistance. Because a high fat diet (HFD) causes insulin resistance and a decrease in the expression of multiple genes involved in mitochondrial biogenesis and oxidative phosphorylation (Koves et al., 2008; Sparks et al., 2006; Sparks et al., 2005; Tunstall and Cameron-Smith, 2005), we examined whether the regulation of PARL transcription, precedes or coincides with changes in glucose tolerance, mtDNA, fusion and fission gene expression in C57Bl6/J mice fed a HFD for 4 or 10 wk. After 4 wk of the HFD, there was no difference in fasting glucose levels between groups, however, blood glucose levels were significantly elevated in mice fed a HFD for 10wk (Suppl. Table 1, <0.05). By 4 wk, HFD-mice had glucose intolerance and resistance to the glucose lowering effects of insulin that worsened by the end of the study (Suppl. Table 3). Glucose homeostasis remained normal in control diet mice.

Despite the insulin resistance in HFD mice, there was no change in the expression of the majority of genes examined at 4 wk (Suppl. Table 3). DRP1 was the only gene modified, with an up regulation in HFD group (Suppl. Table 3, $p<0.05$). The increase in DRP1 expression in the HFD mice may relate to the role of DRP1 in the tethering and biogenesis of peroxisomes (Koch et al., 2003), that participate in the β-oxidation of fatty acids and the adaptation to nutritional stimuli that elevates lipid flux (Reddy and Mannaerts, 1994). After 10wk of HFD, there was a down regulation in mtDNA that was mirrored by a suppression of

PARL and PGC1α mRNA (Suppl. Table 3, $p<0.05$). No other significant changes were observed, although there was a trend for elevated PGC1β and OPA1 mRNA (Suppl. Table 1). Together, these data suggest that in the progression of insulin resistance to T2DM, the suppression of PARL and PGC1α parallel the impairment in mitochondrial biogenesis.

Abnormal fusion and fission protein expression in primary cultures of human muscle

To address the biological significance of reduced PARL protein expression in human muscle, we first investigated mitochondrial content and PARL protein expression and key proteins of mitochondrial energetics, fusion and fission in primary human skeletal muscle cell model derived from *vastus lateralis* samples from our clinical cohorts. Consistent with our mRNA analysis in whole skeletal muscle, PARL mRNA (Suppl. Fig 2a) and protein levels (Fig 4a), were strikingly reduced in primary myotubes derived from both T2DM and elderly subjects vs. myotubes from control subjects (both $p<0.001$). In mammals, the separation of adjoined mitochondria by the fission process is chiefly controlled by the cytosolic dynamin-related GTPase protein 1 (DRP1) that is recruited to the mitochondrial outer membrane by FIS1 (Stojanovski et al., 2004). FIS1 protein was lowest in myotubes from T2DM individuals relative to myotubes from elderly ($p=0.01$), which in turn had lower FIS1 protein than in controls ($p=0.005$) (Fig 4a). DRP1 was not different between the three cohorts (Fig 4a) possibly reflecting high cytosolic abundance of DRP1 (Smirnova et al., 1998). The fusion of mitochondria requires MFN-1 and -2 to conjoin the “tethered” outer membranes of juxtaposed mitochondria (Bach et al., 2003; Pellegrini and Scorrano, 2007), whereas OPA1 unifies the inner mitochondria membranes (Frezza et al., 2006). However, despite lower protein content of MFN2 ($p=0.04$) and OPA1 (Fig 4a, $p<0.001$) in myotubes from T2DM/elderly subjects compared to those of control subjects, there was no significant difference in MFN1 (Fig 4a) between the 3 groups. Similarly, reduced MFN2 protein was observed in skeletal muscle samples from obese humans (Bach et al., 2003). Thus, we show for the first time that primary skeletal muscle cells derived from elderly and T2DM subjects are characterized by low expression of PARL, and mitochondrial fusion and fission proteins (FIS1, MFN2, and OPA1).

Low PARL protein expression was also associated with impaired muscle energetics, with lower levels of mtDNA (Fig 4b), CS protein (Fig 3a) and impaired insulin-stimulated glycogen synthesis (Fig 4c) in insulin resistant groups vs controls. However, we observed no difference in PGC1α and TFAM mRNAs (Suppl. Fig 2a) between the three cohorts. Despite the fact that sirtuin-1 (SIRT1) activates PGC1α and mitochondrial biogenesis (Nemoto et al., 2005; Rodgers et al., 2005), we observed a surprising up-regulation of SIRT1 mRNA (Suppl. Fig 2a). However, SIRT1 protein was lower in myotubes of T2DM participants ($p=0.04$) and elderly individuals ($p=0.03$) relative to healthy controls (Fig 4a). The mitochondrial fractions derived from myotubes of insulin resistant subjects (T2DM and elderly) demonstrated reduced levels of DTT-reducible cysteines (measure of irreversible cysteine oxidation; Fig 4d) and SOD2 activity (Fig 4e) compared with controls. Together, these data show myotubes derived from insulin resistant subjects are characterized by low expression of PARL, FIS1, MFN2, and OPA1 protein, reduced mitochondrial mass, non-oxidative glucose metabolism and elevated oxidative stress. Furthermore, the reductions in mitochondrial mass in elderly and T2DM myotubes were independent of PGC1α, a known regulator of mitochondrial mass.

PARL protein is a determinant of mitochondrial mass in primary human muscle cells

To investigate whether reduced PARL may be causative, rather than a consequence, of perturbed mitochondrial function in human-derived muscle cells, we treated myotubes from the lean-young cohort with AdV-PARL(−)-siRNA and AdV-GFP control shuttles. Myotubes transduced with AdV-PARL-siRNA began to senesce (detachment of adherent cells) on day

7 of differentiation (data not shown) in conjunction with lower OPA1 protein and elevated protein expression of the stress-induced, pro-apoptotic protein BAX (Fig 5a) suggesting an elevated rate of apoptosis (Frezza et al., 2006). No obvious morphological defects were observed between the two types of myotubes (decreased PARL vs normal PARL). Mitochondrial mass ($p<0.001$) and mtDNA ($p=0.04$) were both reduced in PARL depleted myotubes (Fig 5b).

We next examined potential mechanisms by which PARL may regulate mitochondrial biogenesis. Regulated intramembrane proteolysis (RIP) is a signaling mechanism that involves the generation of biologically active peptides from membrane-tethered precursor proteins (Sik et al., 2004). PARL is cleaved by RIP to generate a smaller 33KDa inner mitochondrial membrane protein termed PARL-C terminal product of β-cleavage (PACT) and in turn liberates a nuclear targeted 25-amino acid peptide named PARL-β (Pβ) (Sik et al., 2004). Fluorescent microscopy analysis of healthy human myotubes transfected with a synthetic Pβ-construct with a GFP (Pβ-GFP) flag in the 5′position was used to study Pβ subcellular localization. Image overlay analysis demonstrated a small fraction of Pβ-GFP localized within the nucleus (white arrows), with the majority of Pβ-GFP in the perinuclear region (pound sign), with mitochondria evenly distributed through out the myotubes (as indicated by Mitotracker Red) (Supple. Fig 2b, i-iv). It has been hypothesized that Pβ may mediate mitochondria-to-nucleus cross-talk and regulate transcription factor(s) to alter the expression of mitochondrial specific genes (Sik et al., 2004). We next treated myotubes with naive synthetic Pβ with no GFP flag. Consistently, Pβ (0.1μg) robustly increased the mRNA expression of PARL and genes involved in mitochondrial biogenesis (PGC1β and NRF1) and mitochondrial fusion (MFN-1 & -2; Supple Table 4 and OPA1 protein expression (Fig 6a). No changes were observed in PGC1α, NRF2, and RPLPO (internal control) mRNA (Suppl. Table 4). Importantly, Pβ increased SIRT1 protein expression (Fig 6 a,d) in conjunction with elevated mitochondrial biogenesis (Fig 6b), suggesting a potential Pβ-SIRT1 regulation of mitochondrial mass. To confirm Pβ effects on cellular energetics and SIRT1 protein content we treated RMS-13 human myoblasts with Pβ-GFP plasmid containing the wild type Pβ (PβWt) sequence (amino acids 53-77 of human PARL gene), Pβ-GFP plasmid with coded mutation of the nuclear localization sequence (PβNLSM), the backbone pEGFPN1 plasmid as control and synthetic naïve-Pβ peptide (no GFP-flag). Expression of PβWt and transfection of naïve-Pβ peptide increased cellular oxygen consumption above that of PβNLSM (Fig 6e) and coincided with an increase in SIRT1 protein (Fig 6d). Consistent with the ability of SIRT1 to regulate mitochondrial mass (Lagouge et al., 2006), PβWt in RMS13 cells robustly increased mitochondrial biogenesis (Fig 6b) however, the expression of PARL cDNA in myoblasts did not alter mitochondrial mass (data not shown). Conversely, expression of PARL in myoblasts increased cellular oxygen consumption (Fig 6f). This suggests that Pβ is able to modulate mitochondrial energetics by increasing the transcriptional machinery involved in regulating mitochondrial mass, whereas PARL effect on cellular oxygen consumption may relate to its effects on cristae remodeling or direct interaction with OXPHOS protein(s).

PARL protein depletion in myotubes lower energetics and increases ROS production

Consistent with lower mitochondrial mass, PARL(−) myotubes displayed lower rates of oxygen consumption (Fig 5c; $p<0.01$), diminished COX II activity (Fig 5d, $p<0.01$), and lower rates of palmitate oxidation (Fig 5e, $p=0.04$), indicating a reduction in oxidative capacity in PARL(−) myotubes. PARL(−) myotubes were also characterized by impairments in non-oxidative glucose disposal with reduced rates of insulin-stimulated glycogen synthesis (Fig 4c) and AKT-phosphorylation (Fig 4c-*upper panel*). The effects on oxygen consumption and glucose metabolism is similar to lower cellular respiration and glucose oxidation in mammalian fibroblasts deficient in MFN2 protein (Bach et al., 2003; Pellegrini

and Scorrano, 2007). Analysis of mitochondrial morphology by electron microscopy in PARL(−) myotubes showed mitochondria to be uniformly distributed throughout the myotubes (Fig 3e-f). Confocal microscopy reconfirmed distribution results with mitochondria contained within reticulum networks (Fig 3g-h). Therefore, PARL protein depletion in primary human muscle cells results in reduced mitochondrial mass, impaired oxidative capacity and lower glycogen storage. However, PARL does not appear to play a role in mitochondrial distribution in human muscle cells-consistant with recent reports in other mammalian models (Cipolat et al., 2006; Griparic et al., 2007). Therefore, PARL appears to be involved in mitochondrial bioenergetics by modulating cristae remodeling and/or participation in the regulation of mitochondrial mass or cellular apoptosis.

Disturbance in mitochondrial cristae morphology can result in elevated ROS production (Yu et al., 2006) as a significant proportion of the oxidative phosphorylation machinery is embedded in the inner mitochondrial membrane. Analysis of ROS production using carboxy-dichloro-fluorescence (Civitarese et al., 2006) in PARL(−) myotubes revealed a 84% increase in H_20_2 levels ($p=0.02$) (Fig 5f), along with elevated lipid peroxidation (Fig 5g), cysteine oxidation and SOD2 activity (Fig 4d-e; all $p<0.05$) relative to AdV-GFP. In addition, acute (3 hour) exposure of PARL(−) myotubes to 20 mM glucose resulted in a 96% increase in H_2O_2 production compared with basal levels ($p=0.04$, data not shown). Together, this indicates that reduced PARL protein may explain a proportion of the elevated oxidative stress observed in myotubes from elderly and T2DM subjects.

In summary, we have shown that lowering PARL protein in human muscle cells results in lower mitochondrial oxidative capacity, coupled with reduced mitochondrial mass, increased protein oxidation and ROS production and impaired insulin signaling — all known metabolic defects in T2DM and aging (Conley et al., 2007; Imoto et al., 2006; Kelley et al., 2002; Morino et al., 2005; Petersen et al., 2003; Petersen et al., 2005; Ritov et al., 2005).

DISCUSSION

There is an abundance of indirect evidence demonstrating diminished oxidative phosphorylation and lipid oxidation in T2DM and aging (Civitarese et al., 2006; Kelley et al., 2002; Morino et al., 2005; Petersen et al., 2003; Petersen et al., 2005; Ritov et al., 2005; Ukropcova et al., 2007). This has led to the widely held hypothesis that mitochondrial dysfunction can lead to insulin resistance. Elevated ROS production resulting from impaired mitochondrial metabolism and the accumulation of ectopic lipid intermediates (LI) in skeletal muscle are linked to the activation of serine kinases that inhibit insulin signaling (Adams et al., 2004; Chavez and Summers, 2003; Yu et al., 2002). Early functional studies postulated that in combination with dyslipidemia, the elevation of the LI (i.e. diacylglycerol, cerimides and fatty acyl CoA) was driven by the inability of skeletal muscle and mitochondria to appropriately shift between carbohydrate and lipid as metabolic substrates – a defect referred to as “metabolic inflexibility” (Kelley and Mandarino, 2000). Later, in vitro studies demonstrated that increased PGC-1α activity elevated mitochondrial mass and lipid oxidation in isolated mitochondria and, in parallel, increased insulin-stimulated glucose transport (Benton et al., 2008). Taken together, these data indicated that mitochondrial abnormalities in insulin resistance can result from either lower oxidative capacity, and therefore is reflective of a functional impairment, or lower mitochondrial number, or a combination of both these defects. Some studies have questioned whether decreased mitochondrial markers from insulin resistant muscle are merely the result of less active muscle (Holloszy and Coyle, 1984; Terjung et al., 2002) and therefore reflective of the diminished energy requirement of the muscle.

To address this, investigators' have performed direct experiments to determine the ability of isolated mitochondria from healthy and insulin resistant muscle to respire and synthesize ATP with contradictory results. Hojlund *et al.* demonstrated the rates of state 3 respiration in isolated mitochondria is lower in the skeletal muscle from subjects with T2DM relative to obese controls (Mogensen et al., 2007). In accordance with this study, a 35% lower ADP-stimulated respiration in muscle fibers from subjects type 2 diabetes has been described (Phielix et al., 2008). Contradictory to these studies, Boushel *et al.* using permeabilised human skeletal muscle fibers showed no difference in either NADH nor $FADH_2$-linked respiration in patients with T2DM compared to lean healthy subjects (Boushel et al., 2007). In fact, metabolic control theory modeling from ^{31}P NMR spectroscopy experiments in resting skeletal muscle (the condition all the above studies were performed) indicates metabolic control lies exclusively at ATP utilization and demand and not at sites of ATP production (Jeneson et al., 2000). On the other had, reduced demand for energy supply does not exclude the coexistence of a functional abnormality in mitochondria, and in fact the two could be related. Given that reduced mitochondrial mass and function (lower oxygen consumption, lipid oxidation and elevated ROS production) and impaired insulin signaling was observed in PARL (−) muscle and myotubes, we propose that PARL protein, insulin sensitivity status, and mitochondrial energetics (mass and substrate oxidation) are directly linked and may account for a portion of the energetic defects observed in insulin resistant muscle. Recent evidence in β-cells demonstrates that regulation of mitochondrial remodeling, assembly and distribution are important in modulating both mitochondrial number and energetic status (Twig et al., 2008).

The mechanism which governs the transcriptional interplay between mitochondrial function in insulin resistance is still unclear. Although it is well accepted that PGC1α is one of the chief regulators of mitochondrial biogenesis and multiple studies report lower PGC1α expression in the skeletal muscle of insulin resistant subjects with or without T2DM (Civitarese et al., 2006; Mootha et al., 2003; Patti et al., 2003), lower PGC1a mRNA levels can result from chronic physical inactivity (Scarpulla, 2008) and genetic models of PGC1α and PGC1β deletion do not show muscle insulin resistance (Leone et al., 2005; Lin et al., 2004; Sonoda et al., 2007). Similarly, global PARL knock out mice do not develop T2DM, although failure to develop this phenotype may relate to premature mortality, as PARL null mice die before the end of the 3rd month from cachexia (Cipolat et al., 2006). Although, mice fed a HFD in this study had a progressive decline in glucose tolerance that was mirrored by reduced PARL mRNA and mtDNA content in skeletal muscle indicating the suppression of PARL mRNA tracks with lower mtDNA and a worsening of glucose tolerance. PPARδ knock mice develop both mitochondrial dysfunction and T2DM, with mitochondrial abnormalities predating insulin resistance (Schuler et al., 2006). This suggests that additional genetic "players" may be involved in mitochondrial dysfunction and that insulin resistance can occur in concert or independent of the PGC1 system. Mice fed a HFD in this study had a progressive decline in glucose tolerance that was mirrored reduced PARL mRNA and mtDNA content in skeletal muscle. We propose an alternative pathway for the regulation of mitochondrial mass may also include PARL-Pβ. Reduced PARL protein would be expected to result in reduced ability of Pβ to provide a "retrograde" signal to the nucleus to stimulate mitochondrial mass via SIRT1.

In most mammalian cells, mitochondria exist as "branched-chain" reticulum networks which enable mitochondrial matrix lumens to merge and the exchange of metabolites and mitochondrial nucleoids to occur (Civitarese and Ravussin, 2008). Cells can then adapt to varying energy requirements by coupling the mitochondrial electrochemical gradient between mitochondria and to distal cellular locations where ATP demand is highest (Bach et al., 2003; Skulachev, 2001). This *in situ* "energy sensing" (Amchenkova et al., 1988; Frazier et al., 2006) by mitochondria is highly dependent on OPA1-mediated remodeling of the

cristae scaffold. OPA1 activity in the inter-membrane mitochondrial space is controlled in part, by the proteolytic cleavage of OPA1 by PARL (Cipolat et al., 2006). Such activity can explain our electron microscopy data showing altered mitochondrial cristae in mCK-PARL(−) mice muscle as well as the lower energetic capacity and elevated ROS production in AdV-PARL(−) myotubes. These data provide a rationale whereby mitochondrial cristae remodeling and mitochondrial function in skeletal muscle are mediated in part by PARL, and lower PARL protein content results in lower energetic efficiency of the cells and contributes to cellular damage and aging (Figure 7).

Pβ was able to up regulate fusion specific expression (MFN1/2 and OPA1) which would favor the energy and metabolite transfer between mitochondria (Civitarese et al., 2007) or clearance of dysfunctional mitochondria (Twig et al., 2008). Recent studies indicate that mitochondrial fusion and fission and OPA1 levels constitute a quality control “check point” that allows for the sorting and elimination of functionally impaired mitochondria by autophagic clearance (Twig et al., 2008). Given that mitochondrial mass is in constant flux and that steady-state levels of mitochondrial mass is the product of mitochondrial biogenesis and degradation (chiefly through macroautophagy); defects in the regulators of this balance may contribute to mitochondrial dysfunction in insulin resistance. A balanced level of mitochondrial autophagy is required to maintain the quality of cellular and mitochondrial function (i.e. to eliminate the cell of damaged/aging organelles) (Meijer and Codogno, 2007). Therefore, an imbalance in fusion or fission or an over active autophagy machinery would be expected to result is altered mitochondrial energetics or lower mitochondrial mass. This hypothesis is consistent with the lower levels of PARL, OPA1, FIS1 and MFN2 protein content measured in insulin resistant myotubes from this study and by others (Bach et al., 2003). Collectively, these data further support our hypothesis that 1) altered PARL function may contribute to worsening mitochondrial function, mass and insulin resistance and 2) changes in PARL protein and the balance between fusion and fission may be involved in changes in mitochondrial energetics and mass in insulin resistance.

In summary, PARL shares commonalities with OPA1 by regulating cristae shape (Cipolat et al, 2006; Frezza et al., 2006). However, unlike *bona fide* fusion and fission proteins, PARL has the ability to induce the transcription of both the fusion/fission genes (including OPA1) and mitochondrial mass vis-à-vis Pβ. In addition, the suppression of PARL mRNA tracks with lower mtDNA and a worsening of glucose tolerance in mice fed a HFD for 10 weeks. These studies demonstrate that PARL mRNA suppression predates the suppression of FIS1, MFN2 and OPA1 in insulin resistance. Taken together, this indicates that lower PARL expression is an early defect altering mitochondrial function and insulin resistance in response to a metabolic challenge. As insulin resistance worsens, declines in FIS1, MFN2 and OPA1 protein probably occur at later stages (as observed in insulin resistant elderly and T2DM subjects) contributing to a further decline in mitochondrial function. We hypothesize that impaired PARL function is an important risk factor for the development of insulin resistance in skeletal muscle by decreasing mitochondrial mass and energetics, and increasing oxidative stress, thus contributing to impaired glucose metabolism. As insulin resistance continues to develop, mitochondrial function, oxidative damage and PARL activity may decline further, leading to a vicious cycle that eventually contributes to the development of T2DM or other age-associated diseases including sarcopenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The mCK-CAR mice were made available by Dr. Josephine Nalbantoglu and Dr. Paul Holland at McGill University. We appreciate the technical assistance of Ginger Johnson, Brooke Fleming, Elder, Jeremy Ravussin, Phirom Saly and Tianna Stubblefield. The authors would also like to thank Dr Clifton Bogardus and the NIDDK for their generosity in supplying muscle samples from the Pima Indians. This work was supported in part by: RO1 AG20478 (E.R.), P30 DK072476 (E.R.), DK67403 (P.S.M), RO1 DK 078765 (M.W.H), Pennington Institutional grant from the Health & Performance Enhancement Division (A.E.C.) and the John S McIlhenny Foundation (A.E.C).

REFERENCE

Adams JM 2nd, Pratipanawatr T, Berria R, Wang E, DeFronzo RA, Sullards MC, Mandarino LJ. Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. Diabetes. 2004; 53:25–31. [PubMed: 14693694]

Adams SH, Hoppel CL, Lok KH, Zhao L, Wong SW, Minkler PE, Hwang DH, Newman JW, Garvey WT. Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. J Nutr. 2009; 139:1073–1081. [PubMed: 19369366]

Alexander C, Votruba M, Pesch UE, Thiselton DL, Mayer S, Moore A, Rodriguez M, Kellner U, Leo-Kottler B, Auburger G, Bhattacharya SS, Wissinger B. OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nat Genet. 2000; 26:211–215. [PubMed: 11017080]

Bach D, Pich S, Soriano FX, Vega N, Baumgartner B, Oriola J, Daugaard JR, Lloberas J, Camps M, Zierath JR, Rabasa-Lhoret R, Wallberg-Henriksson H, Laville M, Palacin M, Vidal H, Rivera F, Brand M, Zorzano A. Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. J Biol Chem. 2003; 278:17190–17197. [PubMed: 12598526]

Befroy DE, Petersen KF, Dufour S, Mason GF, de Graaf RA, Rothman DL, Shulman GI. Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. Diabetes. 2007; 56:1376–1381. [PubMed: 17287462]

Benton CR, Nickerson JG, Lally J, Han XX, Holloway GP, Glatz JF, Luiken JJ, Graham TE, Heikkila JJ, Bonen A. Modest PGC-1alpha overexpression in muscle in vivo is sufficient to increase insulin sensitivity and palmitate oxidation in subsarcolemmal, not intermyofibrillar, mitochondria. J Biol Chem. 2008; 283:4228–4240. [PubMed: 18079123]

Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. Scand J Clin Lab Invest. 1975; 35:609–616. [PubMed: 1108172]

Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsoe R, Dela F. Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. Diabetologia. 2007; 50:790–796. [PubMed: 17334651]

Bruning JC, Winnay J, Cheatham B, Kahn CR. Differential signaling by insulin receptor substrate 1 (IRS-1) and IRS-2 in IRS-1-deficient cells. Mol Cell Biol. 1997; 17:1513–1521. [PubMed: 9032279]

Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. Arch Biochem Biophys. 2003; 419:101–109. [PubMed: 14592453]

Cipolat S, Martins de Brito O, Dal Zilio B, Scorrano L. OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc Natl Acad Sci U S A. 2004; 101:15927–15932. [PubMed: 15509649]

Cipolat S, Rudka T, Hartmann D, Costa V, Serneels L, Craessaerts K, Metzger K, Frezza C, Annaert W, D'Adamio L, Derks C, Dejaegere T, Pellegrini L, D'Hooge R, Scorrano L, De Strooper B. Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. Cell. 2006; 126:163–175. [PubMed: 16839884]

Civitarese AE, Jenkinson CP, Richardson D, Bajaj M, Cusi K, Kashyap S, Berria R, Belfort R, DeFronzo RA, Mandarino LJ, Ravussin E. Adiponectin receptors gene expression and insulin sensitivity in non-diabetic Mexican Americans with or without a family history of Type 2 diabetes. Diabetologia. 2004; 47:816–820. [PubMed: 15105989]

Civitarese AE, Ravussin E. Mitochondrial energetics and insulin resistance. Endocrinology. 2008; 149:950–954. [PubMed: 18202132]

Civitarese AE, Smith SR, Ravussin E. Diet, energy metabolism and mitochondrial biogenesis. Curr Opin Clin Nutr Metab Care. 2007; 10:679–687. [PubMed: 18089947]

Civitarese AE, Ukropcova B, Carling S, Hulver M, Defronzo RA, Mandarino L, Ravussin E, Smith SR. Role of adiponectin in human skeletal muscle bioenergetics. Cell Metab. 2006; 4:75–87. [PubMed: 16814734]

Conley KE, Amara CE, Jubrias SA, Marcinek DJ. Mitochondrial function, fibre types and ageing: new insights from human muscle in vivo. Exp Physiol. 2007; 92:333–339. [PubMed: 17170059]

Forouhi NG, Jenkinson G, Thomas EL, Mullick S, Mierisova S, Bhonsle U, McKeigue PM, Bell JD. Relation of triglyceride stores in skeletal muscle cells to central obesity and insulin sensitivity in European and South Asian men. Diabetologia. 1999; 42:932–935. [PubMed: 10491752]

Frazier AE, Kiu C, Stojanovski D, Hoogenraad NJ, Ryan MT. Mitochondrial morphology and distribution in mammalian cells. Biol Chem. 2006; 387:1551–1558. [PubMed: 17132100]

Frezza C, Cipolat S, Martins de Brito O, Micaroni M, Beznoussenko GV, Rudka T, Bartoli D, Polishuck RS, Danial NN, De Strooper B, Scorrano L. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell. 2006; 126:177–189. [PubMed: 16839885]

Gilkerson RW, Selker JM, Capaldi RA. The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. FEBS Lett. 2003; 546:355–358. [PubMed: 12832068]

Goodpaster BH, Thaete FL, Kelley DE. Thigh adipose tissue distribution is associated with insulin resistance in obesity and in type 2 diabetes mellitus. Am J Clin Nutr. 2000a; 71:885–892. [PubMed: 10731493]

Goodpaster BH, Theriault R, Watkins SC, Kelley DE. Intramuscular lipid content is increased in obesity and decreased by weight loss. Metabolism. 2000b; 49:467–472. [PubMed: 10778870]

Griparic L, Kanazawa T, van der Bliek AM. Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. J Cell Biol. 2007; 178:757–764. [PubMed: 17709430]

Guillery O, Malka F, Landes T, Guillou E, Blackstone C, Lombes A, Belenguer P, Arnoult D, Rojo M. Metalloprotease-mediated OPA1 processing is modulated by the mitochondrial membrane potential. Biol Cell. 2007

Herlan M, Vogel F, Bornhovd C, Neupert W, Reichert AS. Processing of Mgm1 by the rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA. J Biol Chem. 2003; 278:27781–27788. [PubMed: 12707284]

Hollenbeck PJ, Saxton WM. The axonal transport of mitochondria. J Cell Sci. 2005; 118:5411–5419. [PubMed: 16306220]

Holloszy JO, Coyle EF. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J Appl Physiol. 1984; 56:831–838. [PubMed: 6373687]

Hulver MW, Berggren JR, Carper MJ, Miyazaki M, Ntambi JM, Hoffman EP, Thyfault JP, Stevens R, Dohm GL, Houmard JA, Muoio DM. Elevated stearoyl-CoA desaturase-1 expression in skeletal muscle contributes to abnormal fatty acid partitioning in obese humans. Cell Metab. 2005; 2:251–261. [PubMed: 16213227]

Imoto K, Kukidome D, Nishikawa T, Matsuhisa T, Sonoda K, Fujisawa K, Yano M, Motoshima H, Taguchi T, Tsuruzoe K, Matsumura T, Ichijo H, Araki E. Impact of mitochondrial reactive oxygen species and apoptosis signal-regulating kinase 1 on insulin signaling. Diabetes. 2006; 55:1197–1204. [PubMed: 16644673]

Jacob S, Machann J, Rett K, Brechtel K, Volk A, Renn W, Maerker E, Matthaei S, Schick F, Claussen CD, Haring HU. Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. Diabetes. 1999; 48:1113–1119. [PubMed: 10331418]

Jeneson JA, Westerhoff HV, Kushmerick MJ. A metabolic control analysis of kinetic controls in ATP free energy metabolism in contracting skeletal muscle. Am J Physiol Cell Physiol. 2000; 279:C813–832. [PubMed: 10942732]

Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes. 2002; 51:2944–2950. [PubMed: 12351431]

Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabetes. 2000; 49:677–683. [PubMed: 10905472]

King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care. 1998; 21:1414–1431. [PubMed: 9727886]

Koch A, Thiemann M, Grabenbauer M, Yoon Y, McNiven MA, Schrader M. Dynamin-like protein 1 is involved in peroxisomal fission. J Biol Chem. 2003; 278:8597–8605. [PubMed: 12499366]

Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, Muoio DM. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 2008; 7:45–56. [PubMed: 18177724]

Krssak M, Falk Petersen F, Dresner A, DiPietro L, Vogel SM, Rothman DL, Shulman GI, Roden M. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study (Rapid communication). Diabetologia. 1999; 42:113–116. [PubMed: 10027589]

Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, Auwerx J. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell. 2006; 127:1109–1122. [PubMed: 17112576]

Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C, Chen Z, Holloszy JO, Medeiros DM, Schmidt RE, Saffitz JE, Abel ED, Semenkovich CF, Kelly DP. PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. PLoS Biol. 2005; 3:e101. [PubMed: 15760270]

Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E, Knowler WC, Bennett PH, Bogardus C. Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians. N Engl J Med. 1993; 329:1988–1992. [PubMed: 8247074]

Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, Zhang CY, Mootha VK, Jager S, Vianna CR, Reznick RM, Cui L, Manieri M, Donovan MX, Wu Z, Cooper MP, Fan MC, Rohas LM, Zavacki AM, Cinti S, Shulman GI, Lowell BB, Krainc D, Spiegelman BM. Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. Cell. 2004; 119:121–135. [PubMed: 15454086]

Luo L, Chen H, Trush MA, Show MD, Anway MD, Zirkin BR. Aging and the brown norway rat leydig cell antioxidant defense system. J Androl. 2006; 27:240–247. [PubMed: 16304208]

Luo M, Langlais P, Yi Z, Lefort N, De Filippis EA, Hwang H, Christ-Roberts CY, Mandarino LJ. Phosphorylation of human insulin receptor substrate-1 at Serine 629 plays a positive role in insulin signaling. Endocrinology. 2007; 148:4895–4905. [PubMed: 17640984]

MacLellan JD, Gerrits MF, Gowing A, Smith PJ, Wheeler MB, Harper ME. Physiological increases in uncoupling protein 3 augment fatty acid oxidation and decrease reactive oxygen species production without uncoupling respiration in muscle cells. Diabetes. 2005; 54:2343–2350. [PubMed: 16046300]

Marx J. Unraveling the causes of diabetes. Science. 2002; 296:686–689. [PubMed: 11976439]

McGarry JD. What if Minkowski had been ageusic? An alternative angle on diabetes. Science. 1992; 258:766–770. [PubMed: 1439783]

McQuibban GA, Saurya S, Freeman M. Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. Nature. 2003; 423:537–541. [PubMed: 12774122]

Meijer AJ, Codogno P. Macroautophagy: protector in the diabetes drama? Autophagy. 2007; 3:523–526. [PubMed: 17534140]

Mogensen M, Sahlin K, Fernstrom M, Glintborg D, Vind BF, Beck-Nielsen H, Hojlund K. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes. 2007

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. PGC-1alpha-responsive genes

involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 2003; 34:267–273. [PubMed: 12808457]

Mopert K, Hajek P, Frank S, Chen C, Kaufmann J, Santel A. Loss of Drp1 function alters OPA1 processing and changes mitochondrial membrane organization. Exp Cell Res. 2009

Morabito D, Montessuit C, Rosenblatt-Velin N, Lerch R, Vallotton MB, Lang U. Impaired glucose metabolism in the heart of obese Zucker rats after treatment with phorbol ester. Int J Obes Relat Metab Disord. 2002; 26:327–334. [PubMed: 11896487]

Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, Neschen S, White MF, Bilz S, Sono S, Pypaert M, Shulman GI. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J Clin Invest. 2005; 115:3587–3593. [PubMed: 16284649]

Nalbantoglu J, Larochelle N, Wolf E, Karpati G, Lochmuller H, Holland PC. Muscle-specific overexpression of the adenovirus primary receptor CAR overcomes low efficiency of gene transfer to mature skeletal muscle. Journal of virology. 2001; 75:4276–4282. [PubMed: 11287577]

Nemoto S, Fergusson MM, Finkel T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. J Biol Chem. 2005; 280:16456–16460. [PubMed: 15716268]

Pan D, Lillioja S, Kriketos A, Milner M, Baur L, Bogardus C, Jenkins A, Storlien L. Skeletal muscle triglyceride levels are inversely related to insulin action. Diabetes. 1997; 46:983–988. [PubMed: 9166669]

Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A. 2003; 100:8466–8471. [PubMed: 12832613]

Pellegrini L, Scorrano L. A cut short to death: Parl and Opa1 in the regulation of mitochondrial morphology and apoptosis. Cell Death Differ. 2007; 14:1275–1284. [PubMed: 17464328]

Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Maschio A, Luzi L. Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. Diabetes. 1999; 48:1600–1606. [PubMed: 10426379]

Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science. 2003; 300:1140–1142. [PubMed: 12750520]

Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med. 2004; 350:664–671. [PubMed: 14960743]

Petersen KF, Dufour S, Shulman GI. Decreased insulin-stimulated ATP synthesis and phosphate transport in muscle of insulin-resistant offspring of type 2 diabetic parents. PLoS Med. 2005; 2:e233. [PubMed: 16089501]

Phielix E, Schrauwen-Hinderling VB, Mensink M, Lenaers E, Meex R, Hoeks J, Kooi ME, Moonen-Kornips E, Sels JP, Hesselink MK, Schrauwen P. Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. Diabetes. 2008; 57:2943–2949. [PubMed: 18678616]

Pierce AP, de Waal E, McManus LM, Shireman PK, Chaudhuri AR. Oxidation and structural perturbation of redox-sensitive enzymes in injured skeletal muscle. Free Radic Biol Med. 2007; 43:1584–1593. [PubMed: 18037124]

Reddy JK, Mannaerts GP. Peroxisomal lipid metabolism. Annu Rev Nutr. 1994; 14:343–370. [PubMed: 7946524]

Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE. Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabetes. 2005; 54:8–14. [PubMed: 15616005]

Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature. 2005; 434:113–118. [PubMed: 15744310]

Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev. 2008; 88:611–638. [PubMed: 18391175]

Schuler M, Ali F, Chambon C, Duteil D, Bornert JM, Tardivel A, Desvergne B, Wahli W, Chambon P, Metzger D. PGC1alpha expression is controlled in skeletal muscles by PPARbeta, whose ablation results in fiber-type switching, obesity, and type 2 diabetes. Cell Metab. 2006; 4:407–414. [PubMed: 17084713]

Sesaki H, Southard SM, Hobbs AE, Jensen RE. Cells lacking Pcp1p/Ugo2p, a rhomboid-like protease required for Mgm1p processing, lose mtDNA and mitochondrial structure in a Dnm1p-dependent manner, but remain competent for mitochondrial fusion. Biochem Biophys Res Commun. 2003; 308:276–283. [PubMed: 12901865]

Sik A, Passer BJ, Koonin EV, Pellegrini L. Self-regulated cleavage of the mitochondrial intramembrane-cleaving protease PARL yields Pbeta, a nuclear-targeted peptide. J Biol Chem. 2004; 279:15323–15329. [PubMed: 14732705]

Skulachev VP. Mitochondrial filaments and clusters as intracellular power-transmitting cables. Trends Biochem Sci. 2001; 26:23–29. [PubMed: 11165513]

Smirnova E, Shurland DL, Ryazantsev SN, van der Bliek AM. A human dynamin-related protein controls the distribution of mitochondria. J Cell Biol. 1998; 143:351–358. [PubMed: 9786947]

Sonoda J, Mehl IR, Chong LW, Nofsinger RR, Evans RM. PGC-1beta controls mitochondrial metabolism to modulate circadian activity, adaptive thermogenesis, and hepatic steatosis. Proc Natl Acad Sci U S A. 2007; 104:5223–5228. [PubMed: 17360356]

Sparks LM, Xie H, Koza RA, Mynatt R, Bray GA, Smith SR. High-fat/low-carbohydrate diets regulate glucose metabolism via a long-term transcriptional loop. Metabolism. 2006; 55:1457–1463. [PubMed: 17046547]

Sparks LM, Xie H, Koza RA, Mynatt R, Hulver MW, Bray GA, Smith SR. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes. 2005; 54:1926–1933. [PubMed: 15983191]

Stojanovski D, Koutsopoulos OS, Okamoto K, Ryan MT. Levels of human Fis1 at the mitochondrial outer membrane regulate mitochondrial morphology. J Cell Sci. 2004; 117:1201–1210. [PubMed: 14996942]

Terjung RL, Zarzeczny R, Yang HT. Muscle blood flow and mitochondrial function: influence of aging. Int J Sport Nutr Exerc Metab. 2002; 12:368–378. [PubMed: 12432180]

Thiberge S, Nechushtan A, Sprinzak D, Gileadi O, Behar V, Zik O, Chowers Y, Michaeli S, Schlessinger J, Moses E. Scanning electron microscopy of cells and tissues under fully hydrated conditions. Proc Natl Acad Sci U S A. 2004; 101:3346–3351. [PubMed: 14988502]

Tunstall RJ, Cameron-Smith D. Effect of elevated lipid concentrations on human skeletal muscle gene expression. Metabolism. 2005; 54:952–959. [PubMed: 15988707]

Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh SE, Katz S, Las G, Alroy J, Wu M, Py BF, Yuan J, Deeney JT, Corkey BE, Shirihai OS. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 2008; 27:433–446. [PubMed: 18200046]

Ukropcova B, McNeil M, Sereda O, de Jonge L, Xie H, Bray GA, Smith SR. Dynamic changes in fat oxidation in human primary myocytes mirror metabolic characteristics of the donor. J Clin Invest. 2005; 115:1934–1941. [PubMed: 16007256]

Ukropcova B, Sereda O, de Jonge L, Bogacka I, Nguyen T, Xie H, Bray GA, Smith SR. Family history of diabetes links impaired substrate switching and reduced mitochondrial content in skeletal muscle. Diabetes. 2007; 56:720–727. [PubMed: 17327442]

Walder K, Kerr-Bayles L, Civitarese A, Jowett J, Curran J, Elliott K, Trevaskis J, Bishara N, Zimmet P, Mandarino L, Ravussin E, Blangero J, Kissebah A, Collier GR. The mitochondrial rhomboid protease PSARL is a new candidate gene for type 2 diabetes. Diabetologia. 2005; 48:459–468. [PubMed: 15729572]

Wang H, Hiatt WR, Barstow TJ, Brass EP. Relationships between muscle mitochondrial DNA content, mitochondrial enzyme activity and oxidative capacity in man: alterations with disease. Eur J Appl Physiol Occup Physiol. 1999; 80:22–27. [PubMed: 10367719]

Whitman SA, Wacker MJ, Richmond SR, Godard MP. Contributions of the ubiquitinproteasome pathway and apoptosis to human skeletal muscle wasting with age. Pflugers Arch. 2005; 450:437–446. [PubMed: 15952031]

Yi Z, Langlais P, De Filippis EA, Luo M, Flynn CR, Schroeder S, Weintraub ST, Mapes R, Mandarino LJ. Global assessment of regulation of phosphorylation of insulin receptor substrate-1 by insulin in vivo in human muscle. Diabetes. 2007; 56:1508–1516. [PubMed: 17360977]

Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J Biol Chem. 2002; 277:50230–50236. [PubMed: 12006582]

Yu T, Robotham JL, Yoon Y. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci U S A. 2006; 103:2653–2658. [PubMed: 16477035]

Zhang D, Liu ZX, Choi CS, Tian L, Kibbey R, Dong J, Cline GW, Wood PA, Shulman GI. Mitochondrial dysfunction due to long-chain Acyl-CoA dehydrogenase deficiency causes hepatic steatosis and hepatic insulin resistance. Proc Natl Acad Sci U S A. 2007

Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature. 2001; 414:782–787. [PubMed: 11742409]

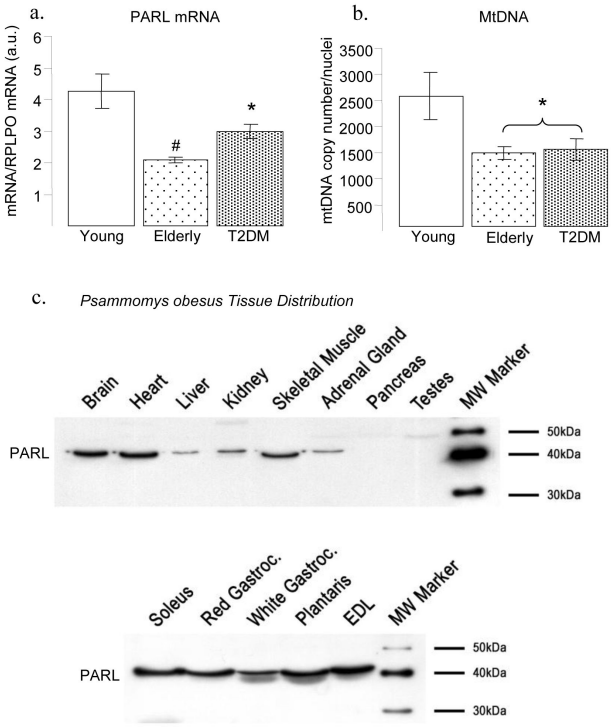

Figure 1. Human mRNA Expression and Rodent Tissue Distribution

Presenillin-associated rhomboid-like (PARL) mRNA and mitochondrial content by mtDNA copy number (mtDNA) (**a-b**) in Lean-young, Lean-insulin resistant elderly subjects (n=11, both groups) and participants with Type 2 Diabetes Mellitus (T2DM) (n=32). Data represented mean ±S.E.M. (**c**) Western analyses with affinity purified anti-PARL antibodies in *Psammomys obesus*. PARL protein expression is most abundant in tissues that rely heavily on oxidative metabolism (*upper panel*). PARL protein expression is greater in muscle groups, including soleus and the red gastrocnemius, that contain a higher proportion of oxidative fibers (*lower panel*). Polyclonal antisera were generated using a KLH-conjugated peptide corresponding to amino acids 53-77 of human PARL. PARL specific antibodies were affinity purified using GST-PARL (53-77).

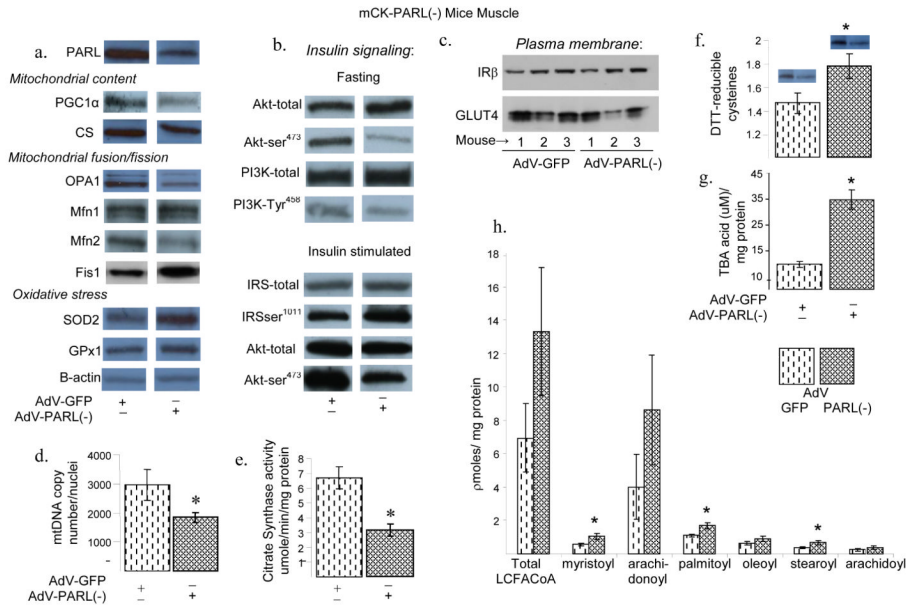

Figure 2. mCK-PARL(−) Mice Muscle

Molecular characterization of 12-week old mice expressing the human coxsackie / adenovirus (CAR) receptor under the control of the muscle creatine kinase promoter (mCK) treated with either adenovirus (AdV)-GFP-empty cassette control in the left grastrocnemius muscle and AdV-PARL-siRNA in the collateral leg to create mCK-CARPARL(−) muscle. (**a**). Total amount of PARL, peroxisome proliferative activated receptor gamma coactivator 1-α (PGC1α), citrate synthase (CS), optic atrophy 1 (OPA1), Mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), fission 1 (Fis1), superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPx1) and β-actin protein in the gastrocnemius muscle of AdV-GFP and PARL(−) muscles. (**b**) Phosphorylation and total amount of protein of key insulin signaling cascade proteins in AdV-GFP and PARL(−) muscles in the fasting (upper panel) and insulin stimulated condition (lower panel). (**c**) Total amount of insulin receptor-β (IRβ) and GLUT4 protein in the plasma membrane fraction of AdV-GFP and PARL(−) muscles. (**d**) MtDNA and citrate synthase (CS) activity (**e**) in AdV-GFP and PARL(−) muscles. (**f**) Measurements of irreversible cysteine oxidation using dithiothreitol-reducible cysteines with 6-iodoacetimido-fluorescein. A low value equates to elevated rates of irreversible cysteine oxidation whereas, a high value implies a very low level of cysteine oxidation. (**g**) Measurement of thiobarbituric acid (TBA) reactive substances for estimate of muscle lipid peroxidation. (**h**) Measurement of long chain fatty acyl-CoA using high performance liquid chromatography. Total amount of saturated long chain fatty acyl-CoAs (p=0.16), myristoyl-CoA (14:0; p=0.031), arachidonoyl-CoA (20:4, p=0.25), palmitoyl-CoA (16:0; p=0.016), oleoyl-CoA (18:1, p=0.17), stearoyl-CoA (18:0; p=0.004) and arachidoyl-CoA (20:0, p=0.4) in GFP and PARL(−) muscle. (**a-e, h;** *n=6*). (**f-g**, *n=5).* (**d-g**, data represented mean ±S.E.M). *, different to control $p<0.05$.

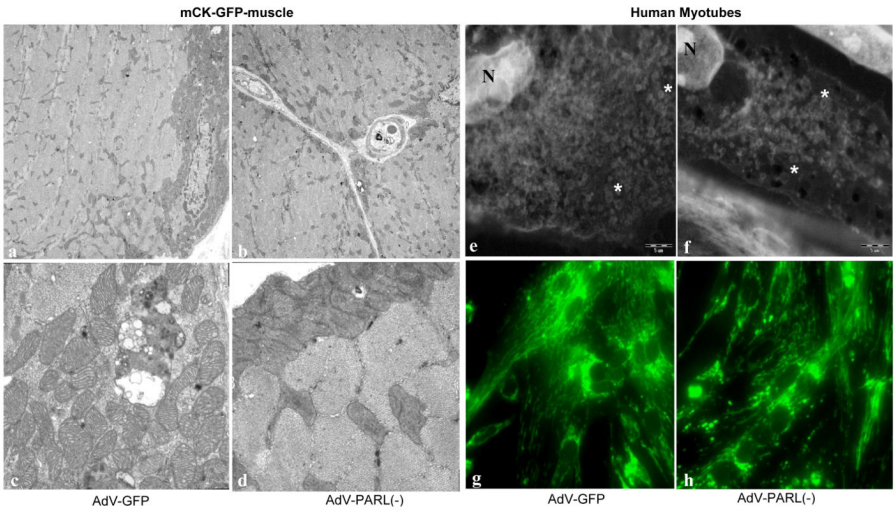

Figure 3.

Representative transmission electron microscopy of cross-sectional slice of AdV-GFP (**a,c**) and PARL(−) (**b,d**) muscles at low (x4000-top two images) and high (x20,000-bottom 2 images) magnification are shown. (**e-h**) Effects of PARL protein depletion on mitochondrial distribution in primary skeletal muscle cells derived from Lean-young subjects and transduced with AdV-GFP control and AdV-PARL-siRNA. (**ef**) Wet-scanning electron microscopy analysis of AdV-GFP (**e**) and AdV-PARL(−) (**f**) depleted myotubes. N=nucleus. (line = 5μm). (**g-h**) Mitochondrial distribution in AdV GFP (**g**) and AdV-PARL(−) (**h**) myotubes measured using MTG. Respiring myotubes were incubated with 100 nM MTG for 30 min. Images were acquired within 5 minutes using a conventional wide-field microscope fitted with an x40 Nikon plan-apo objective. *n=4*.

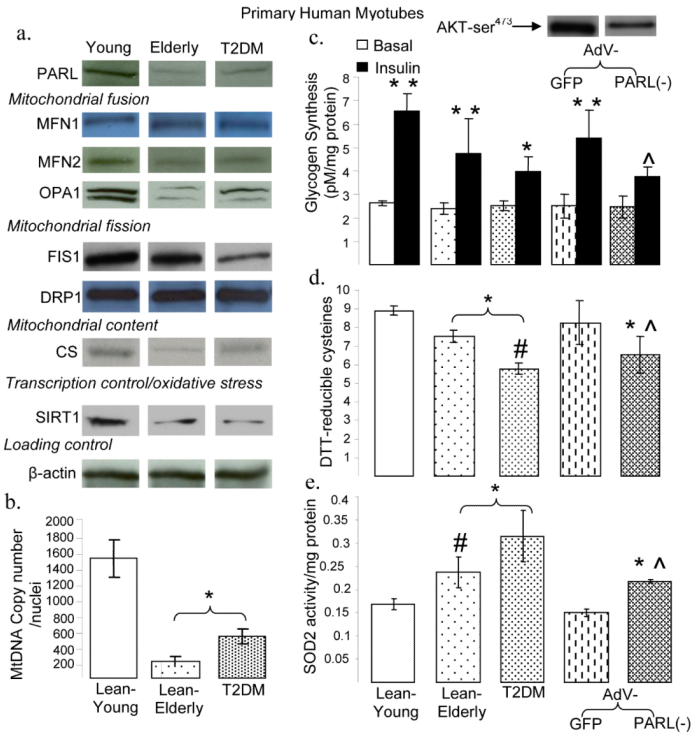

Figure 4. Human Myotubes

(**a**) PARL protein expression and key proteins of mitochondrial fusion (MFN -1 & -2, OPA1); mitochondrial fission, human fission 1 (FIS1) and dynamin-related GTPase 1 (DRP1) and mitochondrial energetics, citrate synthase (CS) and silent mating type information regulation 2 homolog-1 (SIRT1). (**b**) mitochondrial content assessed by mtDNA copy number. (**c**) insulin-stimulated glycogen synthesis (*top insert*, AKT-ser^{473} phosphorylation) in AdV-GFP and AdV-PARL(−) myotubes). (**d**) cysteine oxidation and (**e**) superoxide dismutase 2 (SOD2) activity in primary skeletal muscle cells derived from *vastus lateralis* muscle from Lean-young, Lean-insulin-resistant elderly subjects and participants with T2DM. Lean-young myotubes where also transduced with (AdV)-GFP-empty cassette and AdV-PARL-siRNA. *n=5*. (**b-e**, data represented mean ±S.E.M). *, different to control. #, different to subjects with T2DM. ^, Different to AdV-GFP. **, Different to basal (non-insulin-stimulated). $p<0.05$.

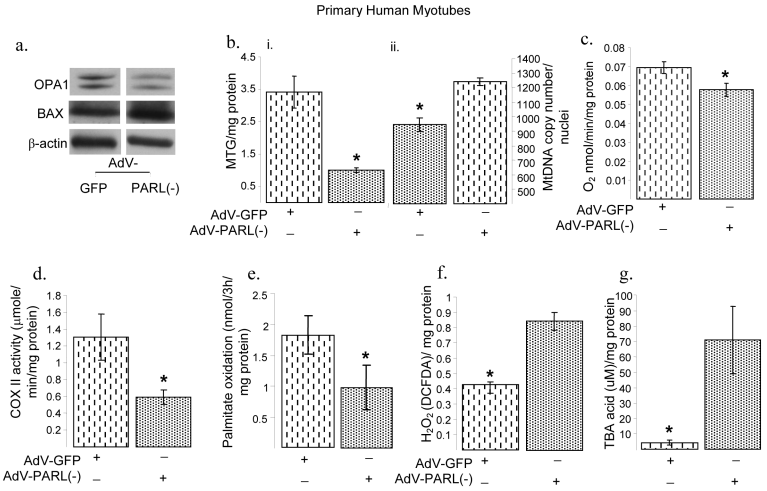

Figure 5. Mitochondrial Energetics in Human Myotubes with PARL(−)

OPA1 and BAX protein expression (**a**) and mitochondrial mass using MitoTracker Green™ (**b-i**) and mtDNA copy number (**b-ii**) in primary skeletal muscle cells from Lean-young subjects transduced with AdV-GFP control and AdV-PARL-siRNA shuttles. Mitochondrial energetics (**c-f**). Oxygen consumption (**c**), cytochrome c oxidase 2 activity (**d**), lipid oxidation (**e**), hydrogen peroxide (H_20_2) production (using 1 μmol/l of the H_2O_2-sensitive fluorescent probe 1 carboxy-H_2 [5-(and-6)-carboxy-2′, 7′-dichlorohydofluorescein diacetate] (DCFDA)) (**f**) and lipid peroxidation (TBA acid) (**g**) in Lean-young myotubes transduced with AdV-GFP control and AdV-PARL-siRNA shuttles. (**b-g**, data represented mean ±S.E.M). *n=5 all groups*. *, different to AdV-GFP control. $p<0.05$.

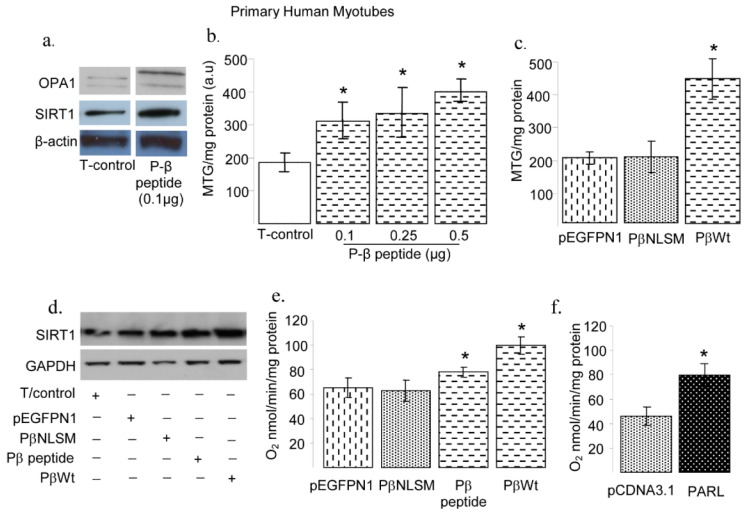

Figure 6. PARL-β

OPA1 and SIRT1 protein expression (**a**) in human myotubes treated with 0.1μg of naïve-Pβ. Mitochondrial mass (**b-c**). Myotubes from Lean-young subjects were transfected with (**b**) 0.1, 0.25 and 0.5 μg of naïve-Parl-β (Pβ)-peptide (synthetic peptide without a 5′-GFP tag (FRKAPRKVEPRRSDPGTSGEAYKRS)); GenePorter transfection reagent (Genetherapy Systems) was used as tranfection control (T-control) and (**c**), with the backbone pEGFPN1 plasmid as control, Pβ-GFP plasmid with coded mutation in the nuclear localization sequence (PβNLSM) and Pβ-GFP plasmid containing the wild type Pβ (PβWt) sequence (amino acids 53-77 of human PARL gene). (**e-f**) Cellular oxygen consumption analysis. RMS-13 myoblasts (**e**) were treated with Pβ-GFP plasmid containing the wild type Pβ (PβWt) sequence (amino acids 53-77 of human PARL gene), Pβ-GFP plasmid with coded mutation of the nuclear localization sequence (PβNLSM), the backbone pEGFPN1 plasmid as control and synthetic naïve-Pβ peptide (no GFP-flag). In separate experiments (**f**), RMS-13 myoblasts were treated with control vector (pCDNA3.1(+)) and pCDNA3.1-PARL (PARL). (**b-f**, data represented mean ±S.E.M). (**c, d; e, f***n=6;* (**c, d**), *n=3).*. *, different to control. $p<0.05$.

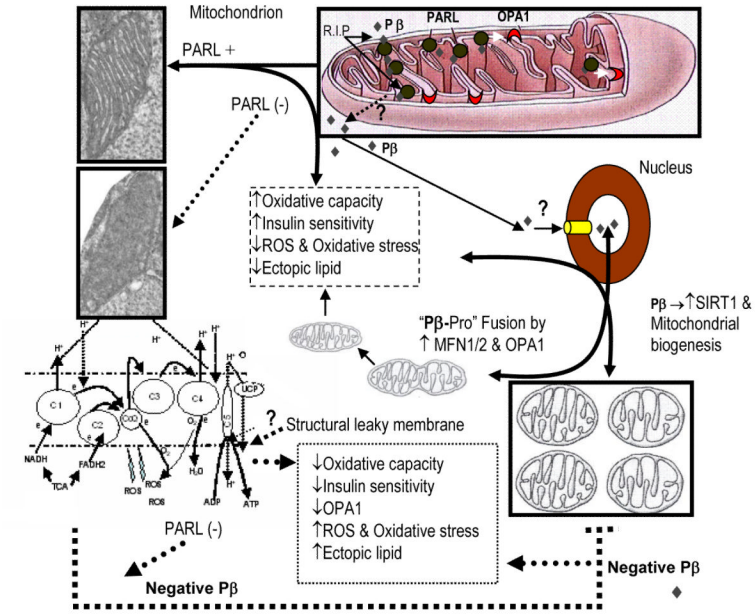

Figure 7.

Overview of PARL function and steps that contribute to the formation of ROS and insulin resistance. PARL-mediated cristae remodeling results in maintenance of 'normal' mitochondrial structure, oxidative capacity (both oxidative phosphorylation and β-oxidation) and insulin sensitivity. Improvement in mitochondrial energetics are further sustain by the ability of PARL-β (Pβ) to induce SIRT-1 protein and mitochondrial biogenesis. Inhibition of PARL-mediated cristae remodeling results in damaged inner membrane structure, loss of mtDNA and reduced oxidative capacity. Under hyperglycemic conditions the increased production of NADH and FADH2, coupled with lower mitochondrial mass and a 'Structural-leaky' inner membrane would favor electron transfer to oxygen creating reactive oxygen species (ROS) and damaging of the mitochondrial membrane. If mitochondrial damage is not sufficiently compensated by increased mitochondrial biogenesis Pβ, mitochondrial fusion/fission complementation and ROS defense mechanism, oxidative capacity would decline further and eventually leading to a vicious cycle of increasing muscle insulin resistance and oxidative damage.