Decoding insulin resistance and metabolic syndrome for promising therapeutic intervention

Shaodong Guo1,2,3

1Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A&M University Health Science Center, 1901 South 1st Street, Building 205, Temple, Texas 76504, USA
2Scott & White, Temple, Texas 76504, USA
3Central Texas Veterans Health Care System, Temple, Texas 76504, USA

Correspondence should be addressed to S Guo
Email sguo@medicine.tamhsc.edu

The metabolic syndrome, also known as insulin resistance syndrome, has become a major public health problem worldwide. It consists of obesity, hyperglycemia, hyperinsulinemia, dyslipidemia, and hypertension. The metabolic syndrome is a major risk factor for the development of type 2 diabetes mellitus, which currently afflicts 22 million Americans and over 100 million Chinese (Alberti et al. 2005, Cornier et al. 2008, Roger et al. 2011). Importantly, the metabolic syndrome is also a significant risk factor for the development of cardiovascular disease, and two-thirds of patients with diabetes mellitus die of heart failure (Roger et al. 2011). In 2011, an estimated 366 million people around the world had diabetes, and this number is predicted to rise to 522 million by 2030 (Whiting et al. 2011). The estimated costs of diagnosed diabetes in the USA have risen from $174 billion in 2007 to $245 billion in 2012 (Herman 2013). Obviously, understanding and controlling the metabolic syndrome and associated cardiovascular disorders have far-reaching impacts on our healthcare and economic systems that affect the quality of our daily life.

As a first step, understanding the mechanisms responsible for insulin action and resistance is critical for developing therapeutic interventions and, thereby controlling the metabolic syndrome. In this special issue of Journal of Endocrinology, we provide four thematic review articles, which combine discussion of the disease mechanism behind the metabolic syndrome with the most recent research from cell-based and animal studies. These reviews address how insulin resistance in different organs contributes to the metabolic syndrome at the molecular, biochemical, and physiological levels. In particular, we largely focus on studies using genetically engineered mouse models, which have provided detailed information with respect to the inactivation of the insulin signaling cascade in the brain, adipose tissue, pancreas, muscle, and liver, as well as other tissues. Thus, we can determine the insulin resistance contribution of each organ to the clinical features of the metabolic syndrome.

In the first review of the series, Dr S Guo updates our understanding of the insulin signaling cascade, with an emphasis on the role of phosphatidylinositol-3-kinase (PI3K) in metabolic control (Guo 2014). A key action of insulin on metabolic regulation involves activation of PI3K by association with the insulin receptor substrate 1 (IRS1) and IRS2, and subsequent phosphorylation of Akt/Foxo1. This pathway has a central role in the control of nutrient homeostasis and organ survival. A large amount of evidence suggests that inactivation of Akt and subsequent phosphorylation of Akt→Foxo1. This pathway has a central role in the control of nutrient homeostasis and organ survival. A large amount of evidence suggests that inactivation of Akt and subsequent activation of the forkhead/winged helix family transcription factor Foxo1, through the suppression of IRS1 and IRS2 in organs following hyperinsulinemia, metabolic inflammation, and overnutrition, may form key mechanisms for the metabolic syndrome in humans. Thus, targeting the IRS→Akt→Foxo1 signaling cascade will probably provide a strategy for therapeutic intervention in the treatment of type 2 diabetes mellitus and its complications. Recent studies carried out in Dr Guo’s laboratory have further demonstrated that suppression of IRS1 and IRS2 expression and functionality occurs in the liver and heart of animals with insulin resistance and/or type 2 diabetes, suggesting that loss of IRS1 and IRS2 not only contributes to the occurrence of hyperglycemia, but also promotes heart failure and death.
in rodents (Qi et al. 2013). Clearly, an impaired and/or biased signaling cascade resulting from the loss of IRS1 and IRS2 forms a common mechanism leading to the deactivation of the endogenous protein kinase Akt and activation of Foxo1 in association with the development of type 2 diabetes mellitus and cardiac dysfunction.

Body weight and appetite are tightly controlled by insulin signaling as a result of its interaction with other factors through a complex and multi-level integration process in the central nervous system. Dr Schneeberger and colleagues provide a concise and up-to-date overview of energy homeostasis control by hypothalamic and brainstem neurons (Schneeberger et al. 2014). Insulin and/or leptin signaling in hypothalamic neurons, including the AgRP and POMC neurons, has long-term and central roles in suppression of appetite and obesity, as well as in the maintenance of nutrient homeostasis. However, gastrointestinal hormones, such as ghrelin and GLP1, and vagal afferents are also responsible for short-term regulatory mechanisms involved in the suppression of appetite and obesity. This indicates that alternative hormones and/or pathways can be targeted to achieve body weight and appetite control, in addition to pancreas-secreted insulin and the adipocyte-released hormone leptin.

Obesity results from an excessive proliferation and expansion of adipocytes. Dr Cao provides a compelling review on how adipose tissue can secrete an array of hormones (adipokines or adipose secretome) that signal key organs to maintain systemic metabolic homeostasis and how dysregulation of this system has been causally linked to a wide range of metabolic diseases (Cao 2014). Obesity induces the production of inflammatory cytokines and the infiltration of immune cells into adipose tissue, creating a state of chronic low-grade inflammation, termed metabolic inflammation related to a broad spectrum of pathological conditions, including insulin resistance.

The accumulation of lipids in adipose tissue, muscle, and other organs, via excess energy intake, also provides mechanisms for the build-up of bioactive lipid species that interfere with the insulin signaling cascade. Dr Turner and colleagues report that fatty acids, the essential elements of all cells, not only serve as components of cellular structure and fuel substrates, but also act as signaling molecules that activate intracellular protein kinases, thereby inhibiting the action of insulin on metabolic regulation in muscle. Moreover, an excess amount of the metabolic intermediate acetyl-CoA derived from fatty acid oxidation has a profound effect on gene post-translational modifications, such as the protein acetylation that epigenetically regulates energy homeostasis.

Overall, it is clear that insulin resistance in each organ contributes differently to the features of the metabolic syndrome: obesity results from insulin resistance in the brain; hyperglycemia from insulin resistance in the brain, pancreas, liver, and adipose tissue; hyperlipidemia from insulin resistance in adipose tissue and brain; and hypertension from insulin resistance in, at least, the vascular endothelial cells. We are hopeful that readers will find the four thematic reviews to be of high interest and that many will be prompted to decipher the mechanism of the metabolic syndrome and further develop therapeutic intervention. Although most of the studies discussed herein are based on rodents, the important mediators and concepts of insulin signaling remain to be validated in humans. With extensive collaborations among basic scientists in academia, clinical investigators in healthcare systems, and R&D researchers in the biopharmaceutical industry, more rational strategies need to be employed for the development of new therapeutics, as well as for better disease control in the future.

Declaration of interest
The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by the American Diabetes Association (IF-7-07-27), American Heart Association (BGIA-7880040), Faculty Start-up funds from Texas A&M University Health Science Center, and National Institutes of Health (RO1 DK095118). The Central Texas Veterans Health Care System, Temple, Texas, USA, also supported this work by providing resources and allowing the use of its facilities.

References
Herman WH 2013 The economic costs of diabetes: is it time for a new treatment paradigm? Diabetes Care 36 775–776. (doi:10.2337/dc13-0270)


Received in final form 6 December 2013
Accepted 2 January 2014
Insulin signaling, resistance, and the metabolic syndrome: insights from mouse models into disease mechanisms

Shaodong Guo
Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A&M University Health Science Center, Scott & White, Central Texas Veterans Health Care System, 1901 South 1st Street, Bldg. 205, Temple, Texas 76504, USA

Abstract
Insulin resistance is a major underlying mechanism responsible for the ‘metabolic syndrome’, which is also known as insulin resistance syndrome. The incidence of the metabolic syndrome is increasing at an alarming rate, becoming a major public and clinical problem worldwide. The metabolic syndrome is represented by a group of interrelated disorders, including obesity, hyperglycemia, hyperlipidemia, and hypertension. It is also a significant risk factor for cardiovascular disease and increased morbidity and mortality. Animal studies have demonstrated that insulin and its signaling cascade normally control cell growth, metabolism, and survival through the activation of MAPKs and activation of phosphatidylinositide-3-kinase (PI3K), in which the activation of PI3K associated with insulin receptor substrate 1 (IRS1) and IRS2 and subsequent Akt → Foxo1 phosphorylation cascade has a central role in the control of nutrient homeostasis and organ survival. The inactivation of Akt and activation of Foxo1, through the suppression IRS1 and IRS2 in different organs following hyperinsulinemia, metabolic inflammation, and overnutrition, may act as the underlying mechanisms for the metabolic syndrome in humans. Targeting the IRS → Akt → Foxo1 signaling cascade will probably provide a strategy for therapeutic intervention in the treatment of type 2 diabetes and its complications. This review discusses the basis of insulin signaling, insulin resistance in different mouse models, and how a deficiency of insulin signaling components in different organs contributes to the features of the metabolic syndrome. Emphasis is placed on the role of IRS1, IRS2, and associated signaling pathways that are coupled to Akt and the forkhead/winged helix transcription factor Foxo1.

Introduction
Obesity, hyperglycemia, hyperlipidemia, and hypertension clustered together have been described as ‘insulin resistance syndrome’ or ‘syndrome X’ by Reaven et al. (Reaven 1988, Moller & Kaufman 2005). The constellation of metabolic abnormalities tightly correlates with cardiovascular dysfunction, resulting in high morbidity and mortality rates (Reaven 2005a). The term ‘metabolic syndrome’ has been adopted (Reaven 1988, DeFronzo & Ferrannini 1991, Kahn et al. 2005) and the clinical features of the syndrome have been established...

Patients with type 1 diabetes suffer from insulin deficiency, owing to pancreatic β-cell failure, and insulin is a primary and effective therapy to decrease hyperglycemia and reduce the risk of cardiovascular dysfunction, as demonstrated by the Diabetes Control and Complications Trial (DCCT) (Nathan et al. 2005, Wilson 2011). However, patients with type 2 diabetes are non-insulin-dependent, in these patients intensive insulin therapy lowers blood glucose levels, but increases body weight and cardiovascular risk, as demonstrated in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial (Wilson 2011). Intensive insulin therapy does not provide much cardioprotective benefit in adults, and two-thirds of patients with type 2 diabetes die of heart failure. Understanding the action of insulin and finding an effective management strategy for the metabolic syndrome, type 2 diabetes mellitus, and associated cardiovascular dysfunction have important clinical implications.

Hyperinsulinemia, a major characteristic of the metabolic syndrome, results from the oversecretion of insulin from pancreatic β-cells and is recognized as a primary contributor to the development of type 2 diabetes and cardiovascular dysfunction (Reaven 2005b, Battiprolu et al. 2010, Cao et al. 2010, Qi et al. 2013). Understanding the mechanisms responsible for insulin action and resistance will be critical for the management of the metabolic syndrome and development of therapeutic interventions to prevent or treat type 2 diabetes. In this review, we provide mechanistic insights from animal studies as to how insulin resistance in different organs contributes to the metabolic syndrome at the molecular, biochemical, and physiological levels.

**Part 1: molecular basis of insulin signaling**

Insulin and signal transduction studies have resulted in breakthroughs in the area of diabetes and biomedical research. Innovative attempts at insulin purification from the pancreas of animals, DNA and protein sequencing, crystallography, and RIA have been made by Banting, Sanger, Hodgkin, and Yalow, who all received Nobel prizes in 1923, 1958, 1969, and 1977 respectively (Yalow & Berson 1960). With the advent of molecular cloning technology in 1980, the genes encoding insulin receptor (IR (INSR)) and IR substrate (IRS) proteins were identified and sequenced (Kasuga et al. 1983, White et al. 1985, Sun et al. 1991, White & Kahn 1994).

**IRS1 and IRS2**

IR, a glycoprotein consisting of an extracellular α-subunit (135 kDa) and a transmembrane β-subunit (95 kDa), is an allosteric enzyme in which the α-subunit inhibits tyrosine kinase activity of the β-subunits. Insulin binding to the α-subunit results in the dimerization of the receptor to form the α2β2 complex in the cell membrane and autophosphorylation of the β-subunit at Tyr1158,
Try^{1162}, and Tyr^{1163}, the first step in the activation of IR. The activation of IR tyrosine kinase recruits and phosphorylates several substrates, including IRS1–4, SHC, Grb-2-associated protein (GAB1), DOCK1, CBL, and APS adaptor proteins, all of which provide specific docking sites for the recruitment of other downstream signaling proteins, leading to the activation of both the Ras→MAPKs and phosphatidylinositide-3-kinase (PI3K)→Akt signaling cascade (White 2003).

IR and its homologous insulin-like growth factor 1 receptor (IGF1R) can also form heterodimers (IR/IGF1R) that modulate the selectivity and affinity for insulin and IGF1 in the activation of downstream signaling molecules (White 2003). Moreover, a recent report has indicated that IR forms a hybrid complex with Met, a transmembrane tyrosine kinase cell-surface receptor for hepatocyte growth factor (HGF) and structurally related to IR (Fafalios et al. 2011). The IR/Met hybrid complex results in robust signal output, by activating IR downstream signaling cascades, and mediates the metabolic effects of insulin (Fafalios et al. 2011).

IRS proteins and the docking proteins for IR provide interfaces by which insulin, IGF1, or HGF signaling propagates and engages with similar intracellular signaling components. IRS proteins are characterized by the presence of a NH₂-terminal pleckstrin homology (PH) domain adjacent to a phosphotyrosine-binding (PTB) domain, followed by a COOH-terminal tail that contains numerous tyrosine and serine/threonine phosphorylation sites (Copps & White 2012). The PH domain mediates cell membrane interactions and the PTB domain binds to the phosphorylated NPXpY motif (Asn-Pro-Xaa-Tyr (pi); X, any amino acid and pi, inorganic phosphate) of the phosphorylated NPXpY motif (Asn-Pro-Xaa-Tyr (pi); X, any amino acid and pi, inorganic phosphate) of the activated IR. The COOH terminal of each IRS protein has about 20 potential tyrosine phosphorylation sites that act as on/off switches to transduce insulin action, recruiting downstream signaling proteins, including PI3K subunit, phosphotyrosine phosphatase SHP2, and adaptor molecules such as GRB2, SOCS3, NCK, CRK, SH2B, and other molecules (White 2003, Sun & Liu 2009).

The activation of Ras→MAPKs mediates the effect of insulin on mitogenesis and cell growth; however, the activation of PI3K generates phosphatidylinositol (3,4,5)-triphosphate (PIP₃), a second messenger activating 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PDK2, which mediate the effect of insulin on metabolism and pro-survival. PDK1 and PDK2, in turn, activate the protein kinase Akt (PKB), by inducing phosphorylation at T^{308} and S^{473} respectively, and both PDK1 and PDK2 are crucial for the activation of Akt (Fig. 1).

PDK1 and TORC2→Akt→TORC1 signaling cascades

Although PDK1 phosphorylates T^{308} of Akt resulting in the activation of Akt and has a profound effect on cell survival and metabolism (Alessi et al. 1997, Williams et al. 2000, Kikani et al. 2005), the action of PDK2 remains more of an enigma (Dong & Liu 2005). Mammalian target of rapamycin complex 2 (mTORC2), which interacts with rictor adaptor protein, is a rapamycin-insensitive companion of mTOR and has been identified to be PDK2 that phosphorylates the S^{473} of Akt (Alessi et al. 1997, Sarbassov et al. 2005, 2006). mTOR is a highly conserved protein kinase that controls cell growth and metabolism in response to nutrients, growth factors, and energy status and exists as two distinct complexes called complex 1 (mTORC1) and mTORC2 (Sengupta et al. 2010).

mTORC2 phosphorylates and activates Akt and other protein kinases, such as protein kinase C (PKC), controlling cell survival and energy homeostasis (Sarbassov et al. 2006, Hagiwara et al. 2012). mTORC2, through Akt, promotes the expression and activation of the sterol regulatory element-binding protein 1 (SREBP1) transcription factor, a family member of the SREBPs that promote lipid and cholesterol synthesis (Yecies et al. 2011). Moreover, mTORC2 and PDK1 suppress the Foxo1 forkhead transcription factor that promotes gluconeogenesis, mediating the effect of insulin on the suppression of hepatic glucose production (Hagiwara et al. 2012; Fig. 1).

mTORC1 is the mTOR interacting with the raptor adaptor protein, which is rapamycin-sensitive and is activated by Ras homolog enriched in brain GTPase (RhebGTPase), via the suppression of tuberous sclerosis protein 2 (TSC2) following the activation of Akt (Sengupta et al. 2010). mTORC1, which is not required for hepatic gluconeogenesis (Li et al. 2010), has as its substrates ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP), both of which control protein synthesis. Recent data indicate that mTORC1 promotes lipogenesis via the phosphorylation of a phosphatidic acid phosphatase Lipin 1 and nuclear translocation of Lipin 1, stimulating SREBP1c and lipogenesis (Li et al. 2010, Peterson et al. 2011). S6K is required for the stimulation of SREBP1c in rat hepatocytes (Owen et al. 2012). Additionally, mTORC1 is also activated by nutrients, such as amino acids, suppressing cellular autophagy. Autophagy is a basic catabolic mechanism that involves the degradation of unnecessary or dysfunctional cellular components through lysosomal machinery and expression of a number of autophagy genes (Klionsky 2007). The breakdown of cellular components ensures cell survival during starvation by...
Figure 1
Insulin signaling cascade and interaction with intracellular signaling components from nutrients and cytokines involved in the control of cell metabolism, including the synthesis of glucose, glycogen, lipids and proteins, as well as other biological responses, such as autophagy, apoptosis, mitochondrial biogenesis, food intake, antioxidation, calcium handling, bone growth, and vascular dilation. PKA, protein kinase A; IR, insulin receptor; IRS, IR substrate; PI3K, phosphatidylinositol (PI)-3-kinase; PDK1, phosphoinositide-dependent protein kinase 1; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; CRTC2, CREB-regulated coactivator 2; Foxo1, forkhead/winged helix transcription factor O class member 1; SREBP1, sterol response element-binding protein 1; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; CRT2, CREB-regulated coactivator 2; Foxo1, forkhead/winged helix transcription factor O class member 1; SREBP1, sterol response element-binding protein 1; Insig2, insulin induced gene 2; S6K, ribosome protein p70 S6 kinase; Gsk3, glycogen synthase kinase 3; GS, glycogen synthase; mTORC, mammalian target of rapamycin complex 1/2; Rheb, Ras homolog enriched in brain; pPKC, atypical protein kinase C; AS160, Akt substrate 160 kDa protein; Bad, BCL2-associated agonist of cell death; PDK4, pyruvate dehydrogenase kinase 4; ACC, acetyl-CoA carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; 6Pase, glucose-6-phosphatase; FAS, fatty acid synthase; MnSOD, manganese superoxide dismutase; TLR, Toll-like receptor; FFA, free fatty acids; ChREBP, carbohydrate-responsive element-binding protein; AMPK, AMP-dependent protein kinase; pY, phosphorylated tyrosine; TNFα, tumor necrosis factor α; pS/T, phosphorylated serine or threonine; Pomc, pro-opiomelanocortin; Agrp, Agouti-related peptide; Gpr 17, G-protein-coupled receptor 17; Serca2a (Atp2a2), sarco/endoplasmic reticulum Ca2+-ATPase; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1α; Homx1, heme oxygenase 1; ATG8, autophagy-regulated gene 8; LC3 (MAP1L3A), microtubule-associated protein 1A/1B-light chain 3; eNOS, endothelial nitric oxide synthase; Glut, glucose transporter; JNK, c-Jun N-terminal kinase; IKKβ, inhibitor of NFκB kinase.
maintaining cellular energy levels (Liu et al. 2009b). Thus, TORC1 and TORC2 serve as sensors and mediators for the action of both nutrients and hormones in cells.

**Targets of Akt in metabolic control**

Akt phosphorylates a number of downstream targets, including the inhibitors of macromolecular synthesis as follows: i) it phosphorylates and inhibits glycogen synthase kinase 3β (Gsk3b), which, in turn, dephosphorylates and activates glycogen synthase (GS) and ii) it inhibits TSC2, thereby activating RhebGTPase for the activation of mTORC1 and S6K, which promote protein synthesis (Inoki et al. 2002). Akt also phosphorylates many other mediators involved in the control of numerous biological responses, including AS160 for Rab10GTPase activation and Glut4 translocation; Bad for apoptosis inhibition; and PDE3B for cAMP degradation. Akt phosphorylates and inhibits CAMP response element-binding protein (CREB)-regulated transcription coactivator 2 (CRTC2), a CREB coactivator that increases hepatic gluconeogenesis (Wang et al. 2010). Most importantly, Akt regulates metabolism and survival by controlling the expression of a number of genes through transcription factors, such as SREBP1c and Foxo1.

Akt phosphorylates and stimulates Sreb1c, promoting liver lipogenesis through the suppression of INSIG2, a protein of the endoplasmic reticulum that blocks the activation of SREBP1c by binding to SREBP cleavage-activating protein (SCAP) and preventing it from escorting SREBPs to the Golgi (Yabe et al. 2002). In contrast, Akt phosphorylates Foxo1 at S256 and inhibits Foxo1 transcriptional activity, suppressing glucose production in the liver and promoting cell survival in the heart (Guo et al. 1999, Hannenhalli et al. 2006, Matsumoto et al. 2007, Evans-Anderson et al. 2008, Battiprolu et al. 2012, Zhang et al. 2012). Many of these phosphorylation events are indicators of insulin signaling, and Akt→Foxo1 phosphorylation serves as a powerful indicator of insulin sensitivity in metabolic regulation in a variety of cells and tissues (Guo et al. 2006, 2009, Gonzalez et al. 2011, Qi et al. 2013; Fig. 1).

**Forkhead transcription factor Foxo1 signaling**

Foxo1, a member of the O class of forkhead/winged helix transcription factors (Foxo), was first identified as an Akt substrate in insulin signaling (Guo et al. 1999, Rena et al. 1999). Insulin suppresses the gene expression of IGF-binding protein 1 (IGFBP1) through a conserved insulin response element (IRE: CAAACAAA), located on the IGFBP1 promoter region (Cichy et al. 1998, Guo et al. 1999). A similar sequence is present in the promoter regions of a number of genes, including phosphoenolpyruvate carboxykinase (Pepck (Pck1)) and glucose-6-phosphatase (G6pcase (G6p)), two rate-limiting enzymes for gluconeogenesis (Schmoll et al. 2000, Yeagley et al. 2001). We demonstrated that Foxo1 serves as the endogenous transcription factor interacting with the IRE for the activation of target gene expression (Guo et al. 1999, Zhang et al. 2012). Foxo1 has three Akt phosphorylation sites at T24, S256, and S319 (Rena et al. 1999), and the phosphorylation of these residues, by insulin, promotes Foxo1 cytoplasmic translocation from the nucleus and interaction with SKIP2, a subunit of the SKIP1 (TRIB1)/CUL1/-F-box protein for Foxo1 ubiquitination and inhibits Foxo1-mediated gene transcription, by removing Foxo1 from gene transcriptional machinery (Biggs et al. 1999, Nakae et al. 1999, Rena et al. 2001, Woods et al. 2001, Rena et al. 2002, Matsuzaki et al. 2003, Huang et al. 2005). This provides a molecular link by which Foxo1 integrates cell-surface receptor signaling with gene transcriptional activity (Guo et al. 1999). Other members of the O class of forkhead family include Foxo3, Foxo4, and Foxo6, sharing the conserved Akt phosphorylation motif – RRXRX/S/T (R, arginine; X, any amino acid; and S/T, Akt phosphorylation site of serine or threonine). Mice lacking Foxo1 displayed embryonic lethality and failed to complete embryonic angiogenesis, while mice lacking Foxo3 or Foxo4 survived beyond parturition (Hosaka et al. 2004). Mice lacking hepatic Foxo1, rather than Foxo3 or Foxo4, exhibited lower hepatic glucose production and blood glucose levels, and mice lacking both Foxo1 and Foxo3 or Foxo1, Foxo3, and Foxo4 exhibited a further reduction in blood glucose levels (Haeusler et al. 2010, Estall 2012, Zhang et al. 2012). Similarly, mice lacking Foxo6 also exhibited impaired hepatic glucose production (Kim et al. 2011, 2013). Thus, each of the members of the Foxo family has redundant as well as distinct roles in the regulation of physiological functions, the mechanisms of which are incompletely understood, but the inhibition of Foxo transcription factors mediates many of the metabolic effects of insulin (Fig. 1).

**Part 2: mechanisms for insulin resistance**

During the postprandial state, insulin secretion from the pancreatic β-cells controls systemic nutrient homeostasis by promoting anabolic processes in a variety of tissues. Insulin stimulates glucose influx into the muscle and adipose tissue, protein and glycogen synthesis in the
muscle and liver, and lipid synthesis and storage in the liver and adipose tissue, while it inhibits fatty acid oxidation, glycogenolysis, and gluconeogenesis, as well as apoptosis and autophagy in insulin-responsive tissues. During the fasting state, insulin secretion decreases, and tissues coordinate with counter-regulatory hormones, such as glucagon in the liver and adipose tissue, in favor of using fatty acids largely derived from adipocyte lipolysis for the generation of ATP and maintenance of glucose homeostasis. The substrate preferences for metabolic adaptation, during the transit from the fasting to the postprandial state, are tightly controlled by insulin under physiological conditions (Randle et al. 1963). This adaptive transition reflects the action of insulin in insulin-responsive organs, while it is largely blunted in organs with insulin resistance preceding the development of type 2 diabetes (Johnson & Olefsky 2013).

**Loss of Irs1 and Irs2 results in insulin resistance**

Gene knockout experiments in mice have helped to elucidate the role of IR, IRS1, and IRS2 in the control of growth and nutrient homeostasis (Guo 2013). Mice lacking the *I* gene were born with slight growth retardation, but rapidly developed hyperglycemia and hyperinsulinemia, followed by diabetic ketoacidosis and early postnatal death (Accili et al. 1996, Joshi et al. 1996). Although both *Irs1* and *Irs2* null mice displayed embryonic lethality (Withers et al. 1999), systemic *Irs1* null mice displayed growth retardation and peripheral resistance to insulin and IGF1, mainly in the skeletal muscle, but did not develop diabetes because of IRS2-dependent pancreatic β-cell growth and compensatory insulin secretion (Araki et al. 1994). Systemic *Irs2* null mice displayed metabolic defects in the liver, muscle, and adipose tissue, but developed diabetes secondary to pancreatic β-cell failure (Withers et al. 1998).

Tissue-specific gene knockout studies in mice provided new insights into the action of IR and control of glucose homeostasis and body weight (Nandi et al. 2004, Biddinger & Kahn 2006, Rask-Madsen & Kahn 2012). Mice lacking *I* in the liver, pancreatic β-cells, adipose tissue, or brain developed hyperglycemia, hyperlipidemia, hyperinsulinemia, and obesity (Kulkarni et al. 1999, Bruning et al. 2000, Michael et al. 2000, Boucher & Kahn 2013). The deficiency of *I* in the skeletal muscle also impaired glucose tolerance, even though circulating blood glucose levels were normal (Bruning et al. 1998, Kulkarni et al. 1999, Katic et al. 2007). Moreover, reconstitution of IR in the liver, β-cells, and brain prevented diabetes in mice lacking *I* and prevented premature postnatal death (Okamoto et al. 2004, Lin & Accili 2011), suggesting that the liver, pancreatic β-cells, and brain are crucial for the maintenance of glucose homeostasis.

Recently, we have demonstrated that the deletion of both *Irs1* and *Irs2* genes in the liver of mice, designated as L-DKO mice (liver double *Irs1* and *Irs2* gene knockout mice), prevented the activation of hepatic Akt—Foxo1 phosphorylation and resulted in the development of hyperglycemia, hyperinsulinemia, insulin resistance, and hypolipidemia (Dong et al. 2008, Guo et al. 2009). The deletion of both *Irs1* and *Irs2* in the cardiac muscle diminished the phosphorylation of Akt (T308 and S473) and Foxo1 (S253) and caused sudden death of male animals at the age of 6–8 weeks (Qi et al. 2013; Table 2). These results indicate that the loss of *Irs1* and *Irs2* may serve as a key component for insulin resistance and cardiac failure.

**Loss of Irs1 and Irs2 is linked to the inactivation of PI3K and Akt**

IRS1 and IRS2 are associated tightly with PI3K and Akt activation and minimally with MAPK activity. The deficiency of *Irs1* and *Irs2* causes biased PI3K inactivation and sustained MAPK activation in the liver and heart of mice (Dong et al. 2008, Guo et al. 2009, Qi et al. 2013). Differential PI3K inactivation and MAPK activation by the loss of *Irs1* and *Irs2* in vivo may act as a fundamental mechanism to elucidate the prevalence of insulin resistance and association with type 2 diabetes, obesity, and cardiovascular dysfunction. The inhibition of IRS1 and IRS2 inactivates PI3K, disrupting nutrient homeostasis, and prolongs the activation of MAPKs (ERK1/2, p38, and JNK), promoting mitogenesis and overgrowth, resulting in obesity. Supporting this concept, mice lacking either the PI3K catalytic subunit or Akt2 exhibited insulin resistance and type 2 diabetes (Cho et al. 2001, Brachmann et al. 2005), while in mice lacking *Erk1* (*Mapk3*), the growth of adipocytes was prevented and insulin resistance was improved following high-fat diet (HFD) treatment (Bost et al. 2005). Furthermore, in mice lacking *Gab1*, which is an ERK activator, insulin sensitivity was enhanced with elevated hepatic Akt activity (Bard-Chapeau et al. 2005).

**Inactivation of PI3K → Akt → Foxo1 signaling causes diabetes and heart failure**

The activation of PI3K and Akt plays a central role in metabolic regulation, which is supported by studies in animals and humans. Hepatic inactivation of PI3K,
PKD1, mTORC2, or both Akt1 and Akt2 is sufficient for the induction of hyperglycemia, hyperinsulinemia, and hyperlipidemia (Miyake et al. 2002, Mora et al. 2005, Hagiwara et al. 2012, Lu et al. 2012). Mice lacking Akt2 developed type 2 diabetes mellitus (Cho et al. 2001), and AKT2 mutation has also been described in patients with type 2 diabetes mellitus (George et al. 2004). The expression of constitutively active Foxo1, when three Akt sites were mutated to alanine, blocked phosphorylation in either the liver, causing insulin resistance (Zhang et al. 2002), or the heart, resulting in embryonic lethality in mice (Evans-Anderson et al. 2008). Conversely, the

**Table 2** Phenotypes of conditional Irs knockout and Foxo knockout mice using the Cre-LoxP genetic approaches

<table>
<thead>
<tr>
<th>Tissue-specific Irs or Foxo null mouse genotype</th>
<th>Phenotype</th>
<th>Cre-mice</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamic and β-cell Irs2−/−</td>
<td>Obesity; hyperglycemia; insulin resistance</td>
<td>RIP-cre</td>
<td>Lin et al. (2004)</td>
</tr>
<tr>
<td>Hypothalamic (AGRP neuron) Foxo1−/−</td>
<td>Leanness; reduced food intake; increased insulin and leptin sensitivity</td>
<td>Agrp-cre</td>
<td>Ren et al. (2012)</td>
</tr>
<tr>
<td>Hypothalamic (POMC neuron) Foxo1−/−</td>
<td>Leanness; reduced food intake; increased insulin and leptin sensitivity</td>
<td>Pomc-cre</td>
<td>Plum et al. (2009)</td>
</tr>
<tr>
<td>Leptin receptor neuron Irs2−/−</td>
<td>Obesity; hyperglycemia; insulin resistance</td>
<td>Lep-R-cre</td>
<td>Sadagurski et al. (2010, 2012)</td>
</tr>
<tr>
<td>Leptin receptor neuron Foxo1−/−:Irs2−/−</td>
<td>Leanness; prevented obesity and hyperglycemia from Irs2 deficiency</td>
<td>Lep-R-cre</td>
<td>Sadagurski et al. (2010, 2012)</td>
</tr>
<tr>
<td>Liver Irs1−/−</td>
<td>Normal glucose levels; severe insulin resistance on a high-fat diet</td>
<td>Alb-cre</td>
<td>Guo (2013)</td>
</tr>
<tr>
<td>Liver Irs1−/−::Irs2−/−</td>
<td>Normal glucose levels</td>
<td>Alb-cre</td>
<td>Guo et al. (1999, 2006, 2009)</td>
</tr>
<tr>
<td>Liver Foxo1−/−</td>
<td>Reduced blood glucose levels</td>
<td>Alb-cre</td>
<td>Guo et al. (1999, 2006, 2009) and Kubota et al. (2008, 2011)</td>
</tr>
<tr>
<td>Liver Foxo3−/−</td>
<td>Normal glucose levels</td>
<td>Alb-cre</td>
<td>Kubota et al. (2008, 2011)</td>
</tr>
<tr>
<td>Liver Foxo4−/−</td>
<td>Normal glucose levels</td>
<td>Alb-cre</td>
<td>Kubota et al. (2008, 2011)</td>
</tr>
<tr>
<td>Liver Foxo1−/−::Foxo3−/−::Foxo4−/−</td>
<td>Reduced blood glucose levels; increased triglyceride levels; hepatic steatosis</td>
<td>Alb-cre</td>
<td>Kubota et al. (2008, 2011)</td>
</tr>
<tr>
<td>Liver Foxo1−/−::Irs1−/−::Irs2−/−</td>
<td>Prevented hyperglycemia from hepatic Irs1 and Irs2 deficiency</td>
<td>Alb-cre</td>
<td>Dong et al. (2008)</td>
</tr>
<tr>
<td>Skeletal and cardiac muscle Irs1−/−::Irs2−/−</td>
<td>Normal glucose levels; normal insulin levels; die 2 weeks after birth</td>
<td>MCK-cre</td>
<td>Long et al. (2011)</td>
</tr>
<tr>
<td>Cardiac Irs1−/−::Irs2−/−</td>
<td>Males die of heart failure at the age of 7 weeks; hyperlipidemia</td>
<td>αMhc-cre</td>
<td>Qi et al. (2013)</td>
</tr>
<tr>
<td>Cardiac Foxo1−/−</td>
<td>Prevented heart failure from a high-fat diet</td>
<td>αMhc-cre</td>
<td>Battiprolu et al. (2010, 2012)</td>
</tr>
<tr>
<td>Cardiac Foxo3−/−</td>
<td>Did not prevent heart failure from a high-fat diet</td>
<td>αMhc-cre</td>
<td>Battiprolu et al. (2010, 2012)</td>
</tr>
<tr>
<td>Pancreatic β-cell Foxo1−/−</td>
<td>Reduced β-cell regeneration; β-cells dedifferentiate into progenitor-like cells or α-cells; hyperglucagonemia; hyperglycemia</td>
<td>Ins2-cre</td>
<td>Talchai et al. (2012)</td>
</tr>
<tr>
<td>Endothelium Irs1−/−::Irs2−/−</td>
<td>Reduced Akt and eNOS phosphorylation; impaired skeletal muscle glucose uptake; insulin resistance on a high-fat diet</td>
<td>Tie2-cre</td>
<td>Kubota et al. (2011)</td>
</tr>
<tr>
<td>Endothelium Foxo1−/−::Foxo3−/−::Foxo4−/−</td>
<td>Increased eNOS phosphorylation; reduced inflammation and oxidative stress of endothelium; prevented atherosclerosis</td>
<td>Tie2-cre</td>
<td>Tsuchiya et al. (2012)</td>
</tr>
<tr>
<td>Bone osteoblast Foxo1−/−</td>
<td>Increased osteocalcin and insulin production; reduced blood glucose concentration</td>
<td>Collagen</td>
<td>Rachied et al. (2010)</td>
</tr>
</tbody>
</table>

Abbreviation of promoters driving Cre expression: RIP, rat insulin promoter; Agrp, Agouti-regulated peptide; Pomc, pro-opiomelanocortin; Lep-R, leptin receptor; Alb, albumin; MCK, muscle creatine kinase; αMhc, myosin heavy chain α; Ins2, insulin 2; Tie2, angiopoietin 2 receptor.
inactivation of Foxo1 in either the liver of mice with type 2 diabetes reversing hyperglycemia (Lu et al. 2012) or the heart of animals with type 2 diabetes preventing heart failure (Battiprolu et al. 2012) indicates that the activation of Foxo1 is both sufficient and necessary for the induction of hyperglycemia and organ failure following insulin resistance or type 2 diabetes.

Mechanism of insulin resistance by hyperinsulinemia

Insulin resistance occurs at multiple levels in cells, from the cell surface to the nucleus, including desensitization of IR, suppression of IRS proteins and functionality, inhibition of PI3K cascades, and failure to restrain Foxo1-activated gene transcriptional profiling, all of which can result from the inhibition of IRS1 and IRS2.

IRS1 and IRS2 each contain 40 potential serine/threonine sites, which are phosphorylated by p38α MAPK, JNK, mTOR, and PKC, stimulating IRS protein degradation or inhibiting IRS-associated PI3K activation under pathological conditions (Sun & Liu 2009, Copps & White 2012, Guo 2013, Qi et al. 2013). Even under physiological conditions, there is a 50% reduction in hepatic IRS2 protein levels under feeding conditions, compared with fasting conditions (Ide et al. 2004). This observation suggests that the liver is probably more insulin resistant during a feeding state than during a fasting state, in which serine/threonine phosphorylation of IRS2 may decrease the expression and function of IRS2 protein. It is of note that PI3K→Akt signaling serves as a common platform for multiple hormone and growth factor signaling events (Hirsch et al. 2007, Sussman et al. 2011). Our recent studies have demonstrated that IRS1 and IRS2 are the major endogenous mediators activating the PI3K→Akt signaling cascade in the liver and heart of animals (Guo et al. 2009, Qi et al. 2013). Normal expression and functionality of IRS activating the PI3K→Akt signaling pathway are essential for animals to maintain nutrient homeostasis and cardiac function, while many factors can result in insulin resistance.

Hyperinsulinemia has profound effects on the induction of insulin resistance, which is supported by several lines of recent evidence: i) prolonged insulin treatment is sufficient for preventing the acute action of insulin on Foxo1 phosphorylation or Glut4 cellular membrane trafficking in myocardium and adipocytes (Gonzalez et al. 2011, Qi et al. 2013). ii) Insulin inhibits Irs2 gene transcription in the liver (Zhang et al. 2001) and promotes IRS2 ubiquitination or degradation in murine embryonic fibroblasts (Rui et al. 2001, Guo et al. 2006).

The activation of mTORC1 following insulin stimulation is a major pathway that results in IRS2 ubiquitination and the mTORC1 inhibitor rapamycin completely prevents insulin- or IGF1-induced IRS2 degradation (Rui et al. 2001, Guo et al. 2006). Moreover, the deletion of hepatic S6k (Rps6k), a downstream target of mTORC1, improved insulin resistance, enhancing Irs1 and Irs2 gene expression and preventing diabetes in mice (Um et al. 2004, Bae et al. 2012). In contrast, the deletion of Torc2 in the liver of mice resulted in a diabetic phenotype, similar to that of L-DKO mice lacking both Irs1 and Irs2 in the liver (Guo et al. 2009, Hagiwara et al. 2012). It is of note that long-term treatment with rapamycin blocks mTORC2-mediated Akt phosphorylation/activation and the use of rapamycin for the treatment type 2 diabetes is a clinical challenge (Sarbassov et al. 2005). iii) Hyperinsulinemic treatment induces insulin resistance and is associated with oxidative stress and mitochondrial dysfunction in the skeletal muscle and liver of mice with type 1 diabetes (Liu et al. 2009a). iv) Decreased IRS1 and IRS2 expression levels are observed in the tissues of animals and patients with hyperinsulinemia or type 2 diabetes (Kerouz et al. 1997, Rondinone et al. 1997, Qi et al. 2013). v) The activation of p38α MAPK following prolonged insulin treatment in cardiomyocytes mediates insulin resistance by increasing IRS1 and IRS2 serine/threonine phosphorylation and degradation, as demonstrated in our recent studies (Qi et al. 2013). vi) p38 MAPK also mediates the induction of inflammatory cytokines that promote insulin resistance (Li et al. 2005, Shoelson et al. 2006). vii) Many, if not all, MAPKs can induce IRS serine/threonine phosphorylation and degradation, particularly when animals are fed a HFD. The activation of JNK induces IRS1 phosphorylation at S253 and desensitizes insulin action in the liver and other tissues, acting as a mechanism for insulin resistance (Lee et al. 2003). The deletion of Jnk1 (Mapk8), in mice, reduced blood glucose levels and improved insulin sensitivity following HFD treatment (Tuncman et al. 2006). Although ERK1/2 was thought to have a minor effect on metabolic regulation (Gabbay et al. 1996), recent data indicate that ERK1/2-mediated upstream MEK activation, reduced hepatic Akt phosphorylation, and contributed to insulin resistance (Jager et al. 2011, Jiao et al. 2013). It is likely that the activation of MAPK phosphatase 3 (MKP3) or phosphatase 2A (PP2A) following ERK1/2 activation may result in Foxo1 dephosphorylation at S253, promoting gluconeogenesis. Indeed, either MKP3 or PP2A interacts with Foxo1 and contributes to Foxo1 dephosphorylation at S253 and activation (Yan et al. 2008, Wu et al. 2010).
Additionally, some PKC isoforms, such as PKCδ and PCK9, also have important roles in the induction of IRS serine/threonine phosphorylation, resulting in insulin resistance in tissues following HFD treatment (Gao et al. 2007, Bezy et al. 2011). Currently, there are about 1100 protein kinases found in mouse or human genome sequences. It is important to identify these kinases and activation mechanisms under different cellular and environmental conditions for the induction of IRS serine/threonine phosphorylation and inactivation of insulin signaling.

Foxo1 activation following insulin resistance

During the development of insulin resistance and diabetes mellitus, following the loss of Irs and inactivation of the PI3K→Akt signaling pathway, the inhibitory mechanism of Foxo1 by the activation of Akt upon feeding or insulin stimulation is uncontrolled. Thus, the dephosphorylation of Foxo1 at the conserved Akt phosphorylation sites (T24, S256, and S319) enhances Foxo1 stability and transcriptional activity, stimulating gluconeogenesis and resulting in hyperglycemia. An increase in nuclear dephosphorylated Foxo1-S253 levels was detected in the liver and heart of animals with type 2 diabetes (Altomonte et al. 2003, Battiprolu et al. 2012). The deletion of Foxo1 in the liver of L-DKO mice and db/db mice reduced hepatic glucose production and ameliorated diabetes (Dong et al. 2008, Zhang et al. 2012), and the deletion of Foxo1 in the heart of HFD mice prevented heart failure (Battiprolu et al. 2012). These results indicate that IRS→Akt→Foxo1 signaling cascades are critical to nutrient homeostasis and organ survival.

The aberrant activation of Foxo1 disrupts metabolic homeostasis and promotes organ failure, by regulating the expression of a target genes (Fig. 1). Foxo1 promotes hepatic glucose production via the expression of Pepck and G6pase and inhibits lipogenesis, resulting from the suppression of Srebp1c and glucokinase and fatty acid synthase (Zhang et al. 2006, Zhang et al. 2012, Deng et al. 2013). Recently, we have identified a novel Foxo1 target gene – hemoxynogenase 1 (Hmox1), an enzyme catalyzing the degradation of heme to produce biliverdin, iron, and carbon monoxide. Heme is a component of the mitochondrial electron transport chain complexes III and IV, and constitutive Foxo1 activation, following the loss of Irs1 and Irs2, is a key component for heme degradation and impairment of mitochondrial biosynthesis and function (Cheng et al. 2009, Qi et al. 2013). This impairment results in reduced fatty acid oxidation and ATP generation, significantly contributing to triglyceride accumulation, resulting in organ steatosis or energy deficiency, as often observed in type 2 diabetes mellitus.

Activation of Foxo1 by multiple signaling mechanisms

The phosphorylation of Foxo1 at S253 by Akt promotes Foxo1 cytoplasmic retention and ubiquitination, which serve as a central mechanism controlling Foxo1 stability and activity (Guo 2013). However, Foxo1 can also be phosphorylated at different serine or threonine residues by other protein kinases, enhancing transcriptional activity. For example, mammalian sterile 20-like kinase 1 (MST1) promotes Foxo1 phosphorylation at S212, which promotes neuronal cell apoptosis (Yuan et al. 2009) or anti-oxidative stress responses, extending lifespan in Caenorhabditis elegans (Lehtinen et al. 2006). In addition to the phosphorylation-based pathway, the activity of Foxo1 can also be regulated by other post-translational modifications, including methylation, glycosylation, and acetylation (Fig. 2).

The methylation of Foxo1 at arginine R251 and R253 by protein arginine methyltransferase 1 (PRMT1) at the Akt consensus motif RXRXXS/T blocks Akt-mediated phosphorylation of Foxo1 at S253, resulting in long-lasting Foxo1 retention in the nucleus and activation of Foxo1 transcriptional activity (Yamagata et al. 2008, Takahashi et al. 2011). However, whether PRMT1 expression and Foxo1 methylation are altered in diabetics is unclear.

The glycosylation of Foxo1 at threonine T317 via O-GlcNac modification in response to glucose increased Foxo1 transcriptional activity for the expression of gluconeogenic genes (Pepck and G6pase) and anti-oxidative stress genes (Mnsod (Sod2) and catalase) (Housley et al. 2008). The flux of glucose through the hexosamine biosynthetic pathway provides a substrate for the glucosamine-6-phosphate forming UDP-GlcNac (UDP-N-acetylglucosamine). O-GlcNac modification of proteins results in an enzymatic addition of the N-acetyl glucosamine (GlcNAc) moiety of UDP-GlcNac on the hydroxyl oxygen of serines and threonines (Kuo et al. 2008). Foxo1-T317 is GlcNacylated in the liver and it is a modification that is increased in diabetic animals (Housley et al. 2008), indicating that hyperglycemia further enhances Foxo1 activity in the absence of Foxo1-S253 phosphorylation following insulin resistance.

The acetylation of Foxo1 at several lysine residues has been identified, including at K242, K245, and K262, and the reversible acetylation is regulated by histone acetyltransferase CBP/p300 and NAD+-dependent histone
deacetylase SIRT2 (Matsuzaki et al. 2005). Early studies indicate that p300 acetylates Foxo1 and enhances Foxo1-induced transcription (Perrot & Rechler 2005), which may also involve histone acetylation by p300 for the activation of basal transcriptional machinery, while the deacetylation of Foxo1 by SIRT1 represses Foxo1 (Motta et al. 2004, Yang et al. 2005). In contrast, recent studies indicate that the acetylation of Foxo1 suppresses Foxo1 activity, while deacetylation by SIRT1 increases it (Matsuzaki et al. 2005, Jing et al. 2007), which is supported by a report that mutations of the lysines to glutamines (Q) in Foxo1, mimicking acetylation, resulted in the loss of Foxo1 function and embryonic lethality, while mutations of the lysines to arginines (R) prevented acetylation and potentiated Foxo1 activity (Banks et al. 2011). Moreover, Foxo1 is deacetylated and activated by class IIa histone deacetylases (HDACs), promoting hepatic glucose production (Mihaylova et al. 2011). Nuclear HDAC4, HDAC5, and HDAC7 are phosphorylated and excluded from the nucleus by AMP-dependent protein kinase (AMPK), but fasting hormone glucagon rapidly dephosphorylates and translocates the HDACs to the nucleus, where they associate with the promoters of gluconeogenic enzymes, such as Pepck and G6pase. In turn, HDAC4 and HDAC5 recruit HDAC3, which results in acute transcriptional induction of these genes via the deacetylation and activation of Foxo transcription factors. The loss of class IIa HDACs in murine liver results in the inhibition of Foxo target genes and lowers blood glucose levels (Mihaylova et al. 2011). Thus, the suppression of class IIa HDACs in mouse models of type 2 diabetes ameliorates hyperglycemia, indicating that the inhibitors of class I/II HDACs may serve as a potential therapeutic modality for the metabolic syndrome. Moreover, with food intake, cells accumulate acetyl-CoA from glucose oxidation, providing substrate for the acetylation of Foxo1 and suppression of Foxo1 activity, in addition to insulin-induced inhibitory phosphorylation. Thus, Foxo1 merges the nutritional and hormonal signaling into a well-controlled metabolic regulation (Fig. 2).

It is of note that Foxo1 stimulates the expression of manganese superoxide dismutase (MnSOD) and catalase and enhances antioxidant responses. In rodents, the activation of Foxo1 following Irs2 deficiency in the brain enhanced longevity, but promoted obesity and diabetes (Taguchi et al. 2007). Also, the activation of Foxo1 enhanced myocardial survival upon the induction of oxidative stress (Sengupta et al. 2009, 2011, 2012) and autophagy for the control of cell size following serum starvation (Sengupta et al. 2009). Mice lacking systemic Foxo1 display embryonic lethality, since Foxo1 is required for endothelial cell lineage during cardiovascular development (Hosaka et al. 2004, Sengupta et al. 2012). In C. elegans, the Foxo1 ortholog Daf-16 enhances longevity when IR/IGF1R signaling is inactivated and potentially increases the expression of anti-oxidative genes (MnSOD) and also stimulates lipid droplet accumulation (Ogg et al. 1997). Together, these data indicate that the activation of Foxo1 is required for the maintenance of the life cycle under stressful conditions, such as prolonged fasting, in
the liver for hepatic glucose production and activation of anti-oxidative mechanisms promoting survival in C. elegans. However, Foxo1 is activated through multiple layers of regulatory mechanisms, contributing to the development of type 2 diabetes mellitus and organ failure, following insulin resistance.

Part 3: insulin resistance differentially contributes to the metabolic syndrome phenotype

CNS insulin resistance causes obesity

Human appetite is tightly controlled by the action of insulin in the CNS. The hypothalamus at the base of the forebrain comprises numerous small nuclei, each with distinct connections and neurochemistry, which regulate food intake, hormone release, sleep and wake cycles, and other biological functions. When an action potential, traveling along an axon, arrives at a neuronal synapse, it causes neurotransmitter release triggering biological responses in target cells (Myers & Olson 2012). A low dose of insulin delivery by i.c.v. infusion decreased both food intake and hepatic glucose production, effects which were blocked by PI3K inhibitors (Woods et al. 1979, Obici et al. 2002). Combined with evidence that mice with neuron-specific Ir deletion are overweight and insulin resistant (Bruning et al. 2000), current data indicate that neuronal insulin signaling is required for both body weight control and glucose homeostasis.

The functional significance of brain insulin signaling is further evidenced by the deletion of Irs2 in the hypothalamus resulting in hyperglycemia and obesity in mice (Lin et al. 2004, Taguchi et al. 2007). The deletion of Irs1 in the hypothalamus did not disrupt glucose homeostasis and obesity did not develop in young mice (Table 2; Guo & White, unpublished data 2009). Similar to the action of leptin, an adipocyte-derived hormone that inhibits food intake through CNS leptin receptor neurons activating the Jak2→Stat3 signaling cascade (Bates et al. 2003, Myers & Olson 2012), brain insulin signaling reduced food intake by the activation of PI3K via IRS2 and inactivation of Foxo1, which can be independent of the Jak2→Stat3 pathway (Taguchi et al. 2007). However, both leptin and insulin promoted IRS2 tyrosine phosphorylation and PI3K activation in the brain (Warne et al. 2011), and the deletion of Irs2 in leptin receptor-expressing neurons caused diabetes and obesity, in which the inactivation of Foxo1 completely reversed the metabolic dysfunction (Sadagurski et al. 2012).

Hypothalamic neurons expressing Agouti-regulated peptide (Agrp) stimulate food intake (orexigenic: appetite stimulant) during the fasting state. Foxo1 stimulates orexigenic Agrp expression, an effect reversed by leptin delivery, in which the activation of Stat3 abrogates Foxo1 occupancy on the Agrp promoter region (Kitamura et al. 2006). The deletion of Foxo1 in AGRP neurons of mice resulted in reduced food intake, leanness, and decreased hepatic glucose production, involving the suppression of a G-protein-coupled receptor Gpr17, a Foxo1 target gene in AGRP neurons (Ren et al. 2012). By antagonizing the effect of Agrp, hypothalamic neurons expressing pro-opiomelanocortin (Pomc) inhibit food intake during the feeding state (anorexic: lack of appetite). The deletion of Foxo1 in POMC neurons resulted in reduced food intake and body weight, by increasing the expression of obesity susceptibility gene, carboxypeptidase E (Cpe), and subsequent production of β-endorphin, which mediates anorexigenic effects in mice (Plum et al. 2009).

Insulin resistance in adipose tissue, hyperlipidemia, and the role of inflammation

A key feature of the metabolic syndrome is hyperlipidemia, which probably results from insulin resistance in adipose tissue. Insulin promotes fat cell differentiation, enhances adipocyte glucose uptake, and inhibits adipocyte lipolysis. Mice lacking adipocyte Torc2 exhibited hyperglycemia, hyperinsulinemia, failure to suppress lipolysis in response to insulin, elevated circulating fatty acid and glycerol levels, and insulin resistance in the skeletal muscle and liver (Kumar et al. 2010). Recent studies have shown that mice lacking Ir in adipose tissue, created by the adiponectin promoter-driven Cre/LoxP system, developed severe lipoatrophic diabetes, a 95% reduction of white adipose tissue, hyperglycemia, hyperinsulinemia, hyperlipidemia, and liver steatosis (Boucher & Kahn 2013). These data indicate that when insulin action fails in the adipose tissue, adipocyte development is retarded and lipids are unable to convert from carbohydrates for storage. Thus, both glucose and lipids will redistribute into the circulation and organs, resulting in hyperlipidemia and fatty organs. These studies significantly underscore the contribution of insulin resistance in adipose tissue, via the inactivation of Akt signaling, to the control of systemic nutrient homeostasis.

Adipose tissue is also an endocrine organ secreting cytokines and hormones, including TNFα (TNF), IL6, leptin, adiponectin, and many other factors, influencing food intake, systemic insulin sensitivity, and nutrient...
homeostasis. However, obesity from fat expansion disrupts a proper balance of cytokine and hormone generation, promoting insulin resistance. For example, TNFα, IL6, and leptin are pro-inflammatory factors and their levels are markedly increased in obesity, where the levels of adiponectin, which has anti-inflammatory effects on the enhancement of insulin sensitivity, are markedly reduced (Hotamisligil et al. 1993, Shoelson et al. 2006, Hotamisligil & Erbay 2008, Romeo et al. 2012). The overexpression of IKKb for the activation of NFκB (a key player in the control of pro-inflammatory responses) in the liver of mice is sufficient for inducing insulin resistance and type 2 diabetes (Cai et al. 2005). TNFα reduces IRS1 protein levels by the activation of JNK or the liver of mice is sufficient for inducing insulin resistance (Gao et al. 2002, Zhang et al. 2008). Thus, the suppression of inflammation increases insulin sensitivity and reduces metabolic dysfunction in type 2 diabetes mellitus (Hotamisligil et al. 1996). However, the outcome of anti-inflammatory therapy in treating insulin resistance deserves a cautionary note for several reasons, which are as follows: i) inflammation is involved in the deployment and mobilization of immune cell leukocytes to defend against infections or toxins. Many inflammatory actors, such as TNFα, reduce body weight and increase energy expenditure (Ye & McGuinness 2013). The overexpression of IL6, in the liver, increased energy expenditure and insulin sensitivity in mice (Sadagurski et al. 2010). ii) During physical exercise, inflammatory factors, such as TNFα and IL6, are secreted resulting in the inhibition of anabolic metabolism (insulin action) and promoting catabolic metabolism (fat lipolysis) to meet the fuel requirements of the muscle. iii) NFκB is essential for hepatocyte proliferation and survival, and mice lacking the p65 subunit of NFκB die of liver failure (Geisler et al. 2007, Malato et al. 2012). iv) Inflammation not only triggers pro-inflammatory responses, but also activates anti-inflammatory processes. Together, these data indicate that a balance between inflammation and anti-inflammation is required for proper insulin actions and nutrient homeostasis. Thus, correcting the imbalance of hormones, nutrients, and inflammation may provide opportunities and challenges for the prevention and treatment of the metabolic syndrome and type 2 diabetes.

In general, excess energy storage in tissues, particularly lipids, is now believed to be a primary factor contributing to the metabolic syndrome (Reaven 2005a). Free fatty acids derived from nutritional intake or conversion from carbohydrates not only act as an important energy source, but also act as signaling molecules in the modulation of intracellular protein kinases (PKC, JNK, etc.) for the inactivation of insulin signaling (Oh et al. 2010, Holz et al. 2011). Excess lipid accumulation in several organs, including adipose tissue, liver, muscle, heart, and blood vessels, results in insulin resistance and triggers metabolic inflammation, a low-grade and chronic inflammatory response (Samuel et al. 2010, Samuel & Shulman 2012). An acute lipid or fatty acid infusion or chronic HFD directly induces insulin resistance in mice via the activation of PKCδ (Griffin et al. 1999, Boden 2011). Saturated fatty acids also interact with a liver-secreted glycoprotein fetuin A that binds and activates Toll-like receptor 4, resulting in NFκB activation (Pal et al. 2012) and c-SRC recruitment for the activation of JNK and inhibition of insulin action (Holz et al. 2011). Moreover, saturated fatty acids induce apoptosis in hepatocytes and pancreatic β-cells, by activating PKCζ, JNK, and oxidative stress, inhibiting IRS1/2 tyrosine phosphorylation, and blocking insulin signaling (Fig. 1; Wrede et al. 2002, Malhi et al. 2006, Wong et al. 2009, Galbo et al. 2013). In contrast, unsaturated fatty acids interact with the G-protein-coupled receptor GRP120, inhibiting inflammation and obesity and increasing insulin sensitivity (Ichimura et al. 2012). In the liver, lipid accumulation (hepatic steatosis) is a risk factor for non-alcoholic steatohepatitis, fibrosis, cirrhosis, and liver cancer (Kumashiro et al. 2011, Samuel & Shulman 2012).

Hepatic insulin resistance results in hyperglycemia

Hyperglycemia is caused by insulin resistance not only in the brain and adipose tissue, but also in the liver, which is a central organ controlling blood glucose and lipid homeostasis. Insulin promotes the synthesis of the macromolecules glycogen, lipids and protein in the liver and suppresses hepatic glucose production by inhibiting gluconeogenesis. The deletion of either Irs1 or Irs2 in the liver maintained glucose homeostasis, but the deletion of both Irs1 and Irs2 (L-DKO mice) blocked the induction of Akt and Foxo1 phosphorylation by insulin or feeding and resulted in unrestrained gluconeogenesis for hepatic glucose production, resulting in hyperglycemia, with a reduction in hepatic lipogenesis and blood lipid levels (Kubota et al. 2008, Guo et al. 2009). Moreover, a HFD severely impaired IRS2 expression and tyrosine phosphorylation in the hepatocytes of liver-specific Irs1 null mice and the mice developed severe diabetes (Guo et al. 2009). Overnutrition or a HFD can modify intracellular signaling, affecting IRS2 expression and functionality, altering metabolic gene expression, and impairing glucose homeostasis.
Hepatic insulin resistance also results in insulin resistance in other tissues, which is demonstrated in L-DKO mice. The L-DKO mice exhibited not only inhibition of the hepatic Akt signaling cascade, but also blunted brain i.c.v. insulin action on the reduction of hepatic glucose production in i.c.v. clamp experiments (Guo et al. 2009). Moreover, L-DKO mice exhibited features of heart failure, probably secondary to hyperinsulinemia, resulting in cardiac IRS1 and IRS2 suppression (Qi et al. 2013). Similarly, mice lacking hepatic Ir displayed pro-atherogenic lipoprotein profiles with reduced HDL cholesterol and VLDL particles, and within 12 weeks of being placed on an atherogenic diet, they developed severe hypercholesterolemia (Biddinger et al. 2008). These data indicate that hepatic insulin resistance is sufficient to produce dyslipidemia and increased risk of atherosclerosis and cardiac dysfunction.

The role of Foxo1 activation in the control of the development of diabetes is supported by findings in L-TKO mice, which lack Irs1, Irs2, and Foxo1 genes in the liver. L-TKO mice demonstrated a significant reversal of elevated blood glucose levels, glucose intolerance, and the fasting–feeding effect on hepatic gene expression, which were observed in L-DKO mice (Dong et al. 2008). Similarly, mice lacking both Akt1 and Akt2 in the liver (Akt-DLKO) or lacking Pdk1 or Mtorc2 (which blocks Akt activation) developed a similar diabetic phenotype to that seen in L-DKO mice (Mora et al. 2005, Guo et al. 2009, Hagiwara et al. 2012, Lu et al. 2012). Moreover, mice lacking Akt1, Akt2, and Foxo1 (TLKO) rescued diabetes in the Akt-DLKO mice (Lu et al. 2012). It is of interest that, L-TKO and TLKO mice had normal glucose tolerance and responses to the fasting–feeding challenge and suppressed Pepck and G6Pase gene expression to a degree similar to that of control mice (Chai et al. 2008, Lu et al. 2012), indicating that there is an Akt and Foxo1-independent pathway regulating blood glucose homeostasis, the mechanism of which is unclear. It is likely that hepatic Foxo1 deletion may sensitise brain insulin signaling to reduce hepatic glucose production, even though Akt activity is not controlled.

Cardiac insulin resistance promotes heart failure

The loss of Irs1 and Irs2 in the liver and brain resulted in hyperglycemia, while loss in other tissues, such as the heart and pancreas, resulted in organ failure. Thus, it is likely that diabetes may serve as a link to the development of heart failure via the loss of IRS proteins. The heart is an insulin-responsive and energy-consuming organ that requires a constant fuel supply to maintain intracellular ATP levels for myocardial contraction. The deletion of both cardiac Irs1 and Irs2 (H-DKO mice: heart-specific double Irs1 and Irs2 gene knockout) diminished cardiac Akt and Foxo1 phosphorylation and resulted in heart failure and death of male animals at 7–8 weeks of age (Qi et al. 2013). The deletion of both Irs1 and Irs2 in the skeletal and cardiac muscle caused heart failure and diminished Akt and Foxo1 phosphorylation in the skeletal muscle, but the mice had normal blood glucose levels and insulin sensitivity (Long et al. 2011), indicating that insulin resistance in the skeletal muscle is not necessary for the disruption of glucose homeostasis in mice. In contrast, cardiac muscle requires either IRS1 or IRS2 for the maintenance of endogenous Akt activity and Foxo1 inactivation to promote cardiac function and survival. The overexpression of cardiac Foxo1, which caused heart failure in mice (Evans-Anderson et al. 2008), was also observed in failing human hearts (Hannenhalli et al. 2006).

The loss of Irs1 and Irs2 following chronic insulin stimulation and p38 MAK activation contributes to insulin resistance in the heart (Qi et al. 2013). Based on our recent studies, we proposed that the regulation of IRS1 and IRS2 has a major role in the control of cardiac homeostasis, metabolism, and function. This concept was based on the following observations: i) metabolic adaptation during physiological conditions (phase I); ii) metabolic remodeling following the development of insulin resistance and mild cardiac dysfunction (phase II); and iii) maladaptive metabolic and cardiac remodeling, leading to cardiac failure and sudden death (phase III).

During phase I in the postprandial setting, insulin stimulates glucose transport and oxidation, resulting in effective cardiac utilization of glucose as a substrate for the supply of ATP. A 20–40% reduction in IRS2 protein levels was found in mouse liver and heart, compared with those in the fasting state (Guo et al. 2009). In phase II when insulin resistance occurs, the heart undergoes adaptive responses to limit glucose utilization (insulin-dependent) and responds to lipid oxidation (less insulin-dependent). The heart is capable of generating ATP for myocardial contraction and changes in gene expression patterns, with unaltered cardiac morphology. During this period, the metabolic adaptation or remodeling compensates for cardiac energy demand, even without overt indications of heart failure. With continued insulin resistance resulting from hyperinsulinemia and/or other metabolic and mechanical stresses, cardiac dysfunction develops, as exhibited by L-DKO mice, which have a 60–70% reduction in cardiac IRS1 and IRS2 levels in the heart in association with cardiac dysfunction (Qi et al. 2013). During phase III
in H-DKO mice, when maladaptive metabolic remodeling occurs, there is a lack of compensation for cardiac energy demand, secondary to the loss of Irs1 and Irs2, with Akt inactivation, utilization of both glucose and fatty acids being restrained, resulting in hyperlipidemia and cardiac ATP deficiency and sudden death (Qi et al. 2013). In this phase, the failing heart may exhibit a loss of mitochondrial biogenesis, a process required for fatty acid and glucose utilization via mitochondrial oxidative phosphorylation. In addition, unknown myocardial factors, which are derived from the loss of Irs1 and Irs2 and released to cardiofibroblasts, may also contribute to the onset of interstitial fibrosis. Thus, sensitizing myocardial Akt→Foxo1 signaling, by integrating insulin therapy and blocking the p38→IRS1/2 signaling cascade, may serve as a new treatment modality for heart failure, during insulin resistance, type 2 diabetes mellitus, and other chronic physiological stresses (Guo 2013, Qi et al. 2013).

**Insulin resistance in pancreas impairs β-cell regeneration**

Pancreatic β-cell failure is essential for the development of hyperglycemia in type 1 diabetes, but β-cell failure is also observed in patients with type 2 diabetes (Rhodes 2005, Rhodes et al. 2013). The β-cells secrete insulin, reducing blood glucose levels, and the α-cells secret glucagon, increasing blood glucose levels to meet bodily metabolic requirements. Recent studies have shown that insulin enhances glucose-stimulated insulin secretion in healthy humans (Bouche et al. 2010) and mice lacking Ir in β-cells exhibit impaired insulin secretion (Kulkarni et al. 1999). However, whether insulin has a direct autocrine action on β-cells in promoting insulin secretion is unclear (Rhodes et al. 2013).

The deletion of whole-body Irs2 in mice resulted in diabetes owing to pancreatic β-cell failure (Withers et al. 1998), while the inactivation of Foxo1 in Irs2 null mice prevented β-cell apoptosis and diabetes (Nakae et al. 2002), indicating that IRS2→Foxo1 signaling or Foxo1 inactivation is required for β-cell survival. On the other hand, the deletion of Irs2 in β-cells triggered β-cell repopulation or regeneration, leading to a restoration of insulin secretion and resolution of diabetes in aged mice (Lin et al. 2004), indicating that Foxo1 activation following IRS2 inactivation in β-cells promotes β-cell regeneration or differentiation. Conversely, the inactivation of Foxo1 in β-cells resulted in reduced β-cell mass, hyperglycemia, and hyperglucagonemia, owing to the dedifferentiation of β-cells into progenitor-like cells or pancreatic α-cells (Talchai et al. 2012, Kitamura 2013).

Insulin resistance and/or hyperinsulinemia is the main cause of type 2 diabetes, but more recently, there has been evidence for a failure of functional β-cell mass to meet metabolic demand, the mechanism of which is unclear (Rhodes 2005, Kahn et al. 2006). On the other hand, antagonizing glucagon receptor action in type 1 diabetes induced by streptozotocin and type 2 diabetes mellitus in mice markedly reduced blood glucose levels and completely prevented diabetes (Liang et al. 2004, Sorensen et al. 2006, Ali & Drucker 2009, Lee et al. 2011). Thus, an abnormality at the level of the pancreas is critical for the development of diabetes, and the correction of the imbalance of hormones between insulin (β-cells) and glucagon (α-cells) may provide a potential strategy to prevent diabetes.

**Insulin resistance in skeletal muscle shortens lifespan**

Skeletal muscle is an important fuel storage tissue for glucose uptake, converting it to glycogen and triglycerides, a process stimulated by insulin. Skeletal muscle demonstrates remarkable metabolic flexibility to consume and store glucose and lipids. Mice lacking muscular Ir display elevated fat mass, serum triglyceride levels, and free fatty acid levels, but blood glucose levels, serum insulin levels, and glucose tolerance are normal. Thus, insulin resistance in muscle contributes to the altered fat metabolism associated with type 2 diabetes, but tissues other than muscle appear to be more involved in insulin-regulated glucose disposal than previously recognized (Bruning et al. 1998). Mice lacking Mtorc2 exhibited decreased insulin-stimulated phosphorylation of Akt-S473 and glucose uptake and mild glucose intolerance (Kumar et al. 2008), while mice lacking Mtorc1 displayed dystrophic muscle, mild glucose intolerance, and shortened lifespan (Bentzinger et al. 2008). Mice lacking both Irs1 and Irs2 in the skeletal and cardiac muscle died at 3 weeks of age, and had a much shorter lifespan than mice lacking both Irs1 and Irs2 in only the cardiac muscle (H-DKO mice), which died at 7 weeks of age (Qi et al. 2013), indicating that insulin action in skeletal muscle has a key and unrecognized role in the control of lifespan and mTORC1 may also contribute to this observed effect.

Mice lacking both Irs1 and Irs2 in the skeletal and cardiac muscle did not develop hyperglycemia or hyperinsulinemia, though insulin-induced glucose uptake was diminished. However, AMP levels were elevated in the skeletal muscle, resulting in the activation of AMPK (Long et al. 2011). AMPK stimulates glucose uptake in an insulin-independent manner, by phosphorylating and

---

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-13-0327
© 2014 Society for Endocrinology
Printed in Great Britain

Published by Bioscientifica Ltd
activating the Rab GAP family member AS160, which promotes Glut4 translocation (Taylor et al. 2008, Pehmoller et al. 2009). AMPK also induces acetyl-CoA carboxylase (ACC) phosphorylation and inhibits ACC activity, preventing the conversion of acetyl-CoA to malonyl-CoA, disrupting lipid synthesis, and enhancing fatty acid oxidation (Hoehn et al. 2010). Together, these studies underscore the flexibility of skeletal muscle in the control of glucose homeostasis and longevity. Since skeletal muscle actively secretes hormones (myokines), such as irisin, a hormone that systemically regulates glucose homeostasis and obesity (Bostrom et al. 2012, Muoio & Neufer 2012), it would be of interest to determine whether a skeletal muscle-derived hormone affects longevity in animals.

Insulin resistance in vascular endothelium promotes hypertension and disrupts glucose homeostasis

Vasodilator actions of insulin are mediated by PI3K-dependent signaling pathways that stimulate the production of nitric oxide from vascular endothelium (Muniyappa et al. 2008, Xu & Zou 2009). Insulin resistance in vascular endothelium stimulates vasoconstriction, promotes hypertension and atherosclerosis, and impairs systemic insulin sensitivity and glucose homeostasis. The inactivation of IR in vascular endothelium diminished insulin-induced eNOS phosphorylation and blunted aortic vasorelaxant responses to acetylcholine and calcium ionophore in normal mice (Duncan et al. 2008) and accelerated atherosclerosis in apolipoprotein E null mice (Rask-Madsen et al. 2010). Vascular endothelium deficient in Irs2 or both Irs1 and Irs2 reduced endothelial Akt and eNOS phosphorylation and impaired skeletal muscle glucose uptake, resulting in systemic insulin resistance (Kubota et al. 2011). The activation of Foxo following the deficiency of Irs2 or both Irs1 and Irs2 may play a key role in the stimulation of endothelial cell dysfunction. In fact, the deletion of Foxo1, Foxo3, and Foxo4 in the endothelium enhanced eNOS phosphorylation, reduced inflammation and oxidative stress of endothelial cells, and prevented atherosclerosis in HFD or LDL receptor null mice (Tsuchiya et al. 2012). Endothelium-targeted deletion of Ir or Foxo genes in mice barely disrupted glucose homeostasis (Duncan et al. 2008, Rask-Madsen et al. 2010, Tsuchiya et al. 2012); however, we have recently shown that endothelium-targeted deletion of the transcription factor-related transcriptional enhancer factor 1 (Rtef1, known as Tead4) increased blood glucose levels and insulin resistance. Rtef1 has the potential to interact with the IRE and Foxo1 in cells (Messmer-Blust et al. 2012). Thus, vascular endothelium serves as an organ that potentially regulates glucose homeostasis.

Insulin resistance in bone impairs glucose homeostasis

Insulin promotes the formation of bone and differentiation of osteoblasts that synthesize osteocalcin, a bone-derived insulin secretagogue that regulates pancreatic insulin secretion and systemically controls glucose homeostasis. Mice lacking Ir in osteoblasts exhibited reduced bone formation, increased peripheral adiposity, and insulin resistance, primarily by reduced gene expression and activity of osteocalcin (Ferron et al. 2010, Fulzele et al. 2010). The results of these studies indicate that in osteoblasts insulin may stimulate osteocalcin by suppressing Foxo1, which affects bone remodeling and glucose homeostasis control. Foxo1 inhibits osteocalcin expression and activity by increasing the expression of ESP, a protein tyrosine phosphatase that inhibits the bioactivity of osteocalcin by favoring its carboxylation. Moreover, osteoblast-specific Foxo1 null mice exhibit increased osteocalcin expression and insulin production and reduced blood glucose levels (Rached et al. 2010). Collectively, these data indicate that bone serves as an endocrine organ involved in the control of glucose homeostasis, through bone–pancreas crosstalk, in which Foxo1 plays a key role in insulin action regulating osteocalcin expression and activity in osteoblasts.

Part 4: other considerations

Mouse models

A large body of evidence related to the mechanisms of diabetes, obesity, and cardiovascular diseases has been derived from mouse studies. However, mice have a high heart rate: 600 vs 70 beats/min in humans; brain glucose intake in mice is much less than that in humans, 15 vs 65% respectively; and mice are nocturnal animals and inactive during daytime when many data are often collected for analyses. Also, experimental mice have immune gene transcriptional programs that are divergent from those of humans (Shay et al. 2013). Humans live in a mobile environment. Recent studies have indicated that gastrointestinal microbiota may trigger inflammation and insulin resistance (Kau et al. 2011, Nicholson et al. 2012, Johnson & Olefsky 2013) and increased levels of circulating bacteria or bacterial products derived from microbiota, such as
lipopolysaccharides, can initiate infection and metabolic inflammation that induce insulin resistance and promote the metabolic syndrome (Burcelin 2012).

Genetic approaches often rely on the Cre/LoxP system. Since tissue-specific deletion of a gene of interest is dependent on the tissue specificity and intensity of Cre-recombinase expression, a tissue-specific promoter that drives Cre-recombinase is critical to achieve a partial or complete deletion of the target gene to affect the phenotype observed in animals. For example, myosin heavy chain-Cre-driven Irs1 and Irs2 deletion is almost complete and the heart failure phenotype striking, while myocyte enhancer factor-Cre-driven Irs1 and Irs2 deletion is partial and there is no observed phenotype. Similarly, adiponectin-Cre-driven Ir gene deletion is much stronger than aP2-Cre-driven Irs gene deletion and a diabetic phenotype is evident. The interpretation of the role of insulin in adipose tissue and contribution to nutrient homeostasis may be affected. For example, RIP-cre is a rat insulin promoter-driven Cre transgenic mouse model, but Cre exhibits leaky expression in the hypothalamus of the brain (Lin et al. 2004). Thus, the deletion of Irs2 by the RIP-Cre system resulted in a phenotype that is derived not only from pancreatic β-cells, but also from the brain hypothalamus (Rhodes et al. 2013). Thus, tissue specificity and intensity of Cre-recombinase expression, though advancing our understanding of mouse genetic engineering, also have a significant role in the analysis of gene function.

**Integrative physiology of insulin resistance and hyperlipidemia**

Insulin inhibits hepatic glucose production and stimulates lipid synthesis, and the deletion of Ir or both Irs1 and Irs2 in the liver of mice results in hyperglycemia, hyperinsulinemia, and hypolipidemia (Michael et al. 2000, Guo et al. 2009). A valid question is whether the mouse disease models created by genetic engineering accurately reflect the clinical features of the metabolic syndrome and type 2 diabetes. Many patients with the metabolic syndrome and type 2 diabetes have hyperglycemia, hyperinsulinemia, and hypolipidemia (Brown & Goldstein 2008). Given that the IRS→PI3K→PDK1/2→Akt→Fox01 branch of the insulin signaling pathway has a central role in the control of glucose homeostasis and organ survival, suppression will result in unchecked hepatic glucose production and hyperglycemia. Although the inhibition of this signaling branch also limits hepatic TOCR2 or Akt-stimulated lipogenesis, suppression in adipose tissue may block the insulin inhibitory effect on fat lipolysis, contributing to hyperlipidemia in patients with type 2 diabetes mellitus, in whom other alternative pathways promoting lipogenesis remain active. For example, insulin-independent mTORC1 activation and carbohydrate-activated lipogenic gene expression profiles via Chrebp and AMPK facilitate the progression of lipogenesis in patients with the metabolic syndrome and type 2 diabetes mellitus (Fig. 1). The identification of these and other novel mediators in the control of lipid homeostasis is important for understanding disease mechanisms and developing interventions for the control of the metabolic syndrome, type 2 diabetes mellitus, and their complications.

**Bariatric and metabolic surgery**

More than 60% of patients with type 2 diabetes are obese; thus, body weight loss is an attractive but challenging therapeutic option (Zimmet et al. 2011, Dixon et al. 2012). Bariatric surgery, designed to achieve and sustain substantial weight loss and reduce food intake, effectively prevents and remediates type 2 diabetes (Sjostrom et al. 2012). Moreover, gastric bypass surgery reduces adverse cardiovascular events, not only in obese adults (Sjostrom et al. 2012), but also in patients suffering from type 2 diabetes without severe obesity (Cohen et al. 2012). The actions of metabolic surgery on metabolic control are unclear (Rubino et al. 2010), but it is likely that the surgery resets metabolic parameters in a balanced way, such that energy intake and expenditure are controlled.

**Part 5: conclusion**

Mouse studies have demonstrated that Akt inactivation and Foxo1 activation following the suppression of IRS1 and IRS2 act as a fundamental mechanism for insulin resistance, which occurs in insulin-responsive tissues, impairing systemic glucose and lipid homeostasis and body weight control and serving as an important mechanism for the development of the metabolic syndrome. The metabolic syndrome includes insulin resistance in different organs of the body, such as the brain, liver, pancreas, adipose tissue, muscle, and the cardiovascular system. The IRS→Akt→Fox01 signaling cascade and its regulatory network require further exploration under different cellular and environmental contexts. Hyperinsulinemia, pro-inflammation, and overnutrition are important environmental factors that affect this system, contributing to type 2 diabetes and cardiovascular dysfunction.
Although genome-wide association analyses have identified a number of genes that control the development of diabetes and obesity (Doria et al. 2008, Wagner et al. 2013), the metabolic syndrome is a result of complex interactions between genetic and environmental factors, among which are protein modifications by environmental stimuli, such as overnutrition through phosphorylation (hormones), ubiquitination, acetylation (excess acetyl-CoA), and glycosylation (hyperglycemia), all of which modify the IRS→Akt→Foxo1 branch. Current anti-diabetic therapeutics, such as glucagon-like peptide, pioglitazone, and metformin, as well as metabolic surgery, may affect this pathway directly or indirectly, helping to correct the imbalance of hormones, nutrients, and inflammation. Targeting IRS1 and IRS2 by activating the Akt→Foxo1 signaling cascade, associated protein kinases, and gene expression profiles may provide important therapeutic modalities in the pursuit of a balanced action at the level of hormones, nutrients, and inflammation, for the treatment or prevention of the metabolic syndrome, type 2 diabetes mellitus, and cardiovascular dysfunction.

Declaration of interest
The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

Funding
This research was supported by grants from the American Diabetes Association (IF-7-07-27), American Heart Association (BGIA-7880040), Faculty Start-up from Texas A&M University Health Science Center College of Medicine, and National Institutes of Health (RO1 DK095118). This research was also supported by resources and the use of facilities at the Central Texas Veterans Health Care System, Temple, Texas, USA.

Acknowledgements
The author thanks Drs Kenneth M Baker and Yajuan Qi for reading/editing the manuscript.

References
Boden G 2011 Obesity, insulin resistance and free fatty acids. Current Opinion in Endocrinology, Diabetes, and Obesity 18 139–143. (doi:10.1097/MED.0b013e3283444b09)


Holzer RG, Park EJ, Li N, Tran H, Chen M, Choi C, Solinas G & Kastin M 2011 Saturated fatty acids induce c-Src clustering within membrane


Kerouz NJ, Horsch D, Pons S & Kahn CR 1997 Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (db/db) mouse. Journal of Clinical Investigation 100 3164–3172. (doi:10.1172/JCI19872)


Li HY, Han J, Cao SY, Hong T, Han J, Liu Z & Cao W 2009a Insulin is a stronger inducer of insulin resistance than hyperglycemia in mice with type 1 diabetes mellitus (T1DM). Journal of Biological Chemistry 284 27909–27910. (doi:10.1074/jbc.M109.16675)


Nathan DM, Cleary PA, Backlund JY, Gennuth SM, Lachin JM, Orchard TJ, Raskin P & Zinman B 2013 Myocardial loss of IRS1 and IRS2 causes heart failure and is controlled by p38a MAPK during insulin resistance. Diabetes 62 3887–3900. (doi:10.2337/db13-0095)


Rena G, Prescott AR, Guo S, Cohen P & Unterman TG 2001 Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in...
Thematic Review

S. GUO

Mouse models for metabolic syndrome mechanisms

220:2

T23


White MF, Maron R & Kahn CR 1985 Insulin rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells. Nature 318 183–186. (doi:10.1038/318183a0)


Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI et al. 1998 Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391 900–904. (doi:10.1038/36116)

Withers DJ, Burks DJ, Towery H, Altamuro SL, Flint CL & White MF 1999 IRS-2 coordinates Igf-1 receptor-mediated peripheral insulin signalling. Nature Genetics 33 32–40. (doi:10.1038/12631)


Zhang J, Ou J, Bashmakov Y, Horton JD, Brown MS & Goldstein JL 2001 Insulin inhibits transcription of IRS-2 gene in rat liver through an insulin response element (IRE) that resembles IREs of other insulin-repressed genes. PNAS 98 3756–3761. (doi:10.1073/pnas.071054598)


Received in final form 30 October 2013
Accepted 22 November 2013
Accepted Preprint published online 26 November 2013
Hypothalamic and brainstem neuronal circuits controlling homeostatic energy balance

Marc Schneeberger1,2,3, Ramon Gomis1,2,3 and Marc Claret1,3

1Diabetes and Obesity Research Laboratory, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain
2Department of Endocrinology and Nutrition, School of Medicine, Hospital Clinic, University of Barcelona, 08036 Barcelona, Spain
3Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), 08036 Barcelona, Spain

Abstract

Alterations in adequate energy balance maintenance result in serious metabolic disturbances such as obesity. In mammals, this complex process is orchestrated by multiple and distributed neuronal circuits. Hypothalamic and brainstem neuronal circuits are critically involved in the sensing of circulating and local factors conveying information about the energy status of the organism. The integration of these signals culminates in the generation of specific and coordinated physiological responses aimed at regulating energy balance through the modulation of appetite and energy expenditure. In this article, we review current knowledge on the homeostatic regulation of energy balance, emphasizing recent advances in mouse genetics, electrophysiology, and optogenetic techniques that have greatly contributed to improving our understanding of this central process.

Key Words

- CNS
- ghrelin
- leptin
- neuroendocrinology
- obesity

Introduction

The regulation of appetite and body weight is an intricate process controlled by redundant and distributed neural systems that integrate a myriad of cognitive, hedonic, emotional, and homeostatic cues to precisely regulate systemic energy balance through behavioral, autonomic, and endocrine outputs. These sophisticated biological programs are influenced by multiple factors, including environmental, genetic, and epigenetic mechanisms. The immense complexity of these systems illustrates the biological importance of adequate nutrient and energy balance, a process that has been evolutionarily conserved and refined to guarantee appropriate adiposity levels. Despite the precision of these systems in matching energy demand with energy expenditure, contemporary, and lifestyle factors are the main causes of the prevailing obesity epidemics. The present review attempts to summarize current understanding of the anatomy, neurochemistry, functions, and interactions of relevant neural circuits involved in the homeostatic regulation of energy balance.

The homeostatic system: hypothalamus and brainstem

The hypothalamus: neuronal anatomy, nuclei, and neuropeptides

Seminal lesioning studies conducted in rodents during the 1940s and 1950s highlighted the importance of the hypothalamus in the regulation of body weight. Since then, extensive experimental evidence and extraordinary
progress in understanding the neurobiology of obesity have firmly established the mediobasal hypothalamus as a fundamental nexus in the neuronal hierarchy controlling whole-body energy balance. The hypothalamus is constituted by distinct hypothalamic nuclei including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the dorsomedial nucleus (DMN), and the ventromedial nucleus (VMN).

**Arcuate nucleus** The ARC is a very important area of the CNS involved in the control of energy homeostasis. It is located below the VMN, on both sides of the third ventricle, and immediately adjacent to the median eminence (ME). This area has a semi-permeable blood–brain barrier (BBB; Broadwell & Brightman 1976), and thus it is strategically positioned to sense hormonal and nutrient fluctuations in the bloodstream. In the ARC, there are at least two major populations of neurons controlling appetite and energy expenditure: i) a subset of neurons that coexpress orexigenic neuropeptide Y (NPY) and agouti-related peptide (AGRP) and ii) a population of neurons that coexpress the anorexigenic neuropeptides cocaine- and amphetamine-regulated transcript (CART (CARTPT)) and α-melanocyte-stimulating hormone (α-MSH, a product of proopiomelanocortin (POMC) processing). These two populations of neurons (hereafter referred to as AgRP and POMC respectively), together with downstream target neurons expressing the melanocortin receptor 4 (MC4R) and MC3R, constitute the central melanocortin system. This neuronal circuit is crucial for sensing and integrating a number of peripheral signals allowing for a precise control of food intake and energy expenditure (see section ‘ARC neuronal circuits: POMC, AgRP, and RIPCre neurons’).

NPY is widely expressed throughout the CNS, but it is most densely localized in the ARC in the hypothalamus (Gehlert et al. 1987). The expression and release of ARC NPY respond to changes in energy status, being reduced under feeding conditions and increased under fasting conditions (Beck et al. 1990, Kalra et al. 1991). Increasing NPY tone pharmacologically results in hyperphagia and reduced thermogenesis of brown adipose tissue (BAT), associated with diminished activity of the thyroid axis (Clark et al. 1984, Stanley et al. 1986, Egawa et al. 1991). Although NPY acts at five different receptors (Y1, Y2, Y3, Y4, and Y6), genetic and pharmacological studies suggest that postsynaptic Y1 and Y5 receptors mediate the effects of NPY on positive energy balance (Nguyen et al. 2012, Sohn et al. 2013).

AGRP is also an orexigenic neuropeptide that is exclusively expressed in the ARC, where it colocalizes with NPY and the neurotransmitter γ-aminobutyric acid (GABA; Broberger et al. 1998, Cowley et al. 2001). The central administration of AGRP or its genetic over-expression stimulates food intake, reduces energy expenditure, and causes obesity (Graham et al. 1997, Ollmann et al. 1997, Small et al. 2003). Interestingly, lasting orexigenic effects (over days) after AGRP delivery have been reported (Hagan et al. 2000).

AGRP neurons express receptors for peripheral hormonal signals such as insulin (Marks et al. 1990), leptin (Elmquist et al. 1998), and ghrelin (Willesen et al. 1999). These neurons send projections mainly into the PVN, DMN, and LHA. Despite the well-documented effects of NPY and AGRP as positive modulators of energy balance, genetic studies have yielded conflicting results. For example, Agrp- and Npy-knockout (KO) mice failed to exhibit alterations in body weight or feeding behavior (Palmiter et al. 1998, Qian et al. 2002, Corander et al. 2011). However, the ablation of AgRP neurons in adults leads to uncontrolled anorexia but is well tolerated in neonates, indicating the existence of developmental compensations (Bewick et al. 2005, Gropp et al. 2005, Luquet et al. 2005).

CART is widely expressed in the brain, but it is particularly abundant in the hypothalamus, and it colocalizes (>95%) with POMC in the ARC (Elias et al. 1998). Its expression is enhanced under feeding conditions and reduced under fasting conditions (Kristensen et al. 1998), and it has been shown that i.c.v. infusion of CART inhibits food intake, while antibodies against CART reverse this effect (Kristensen et al. 1998). Furthermore, CART also stimulates the thermogenesis of BAT (Kotz et al. 2000). However, Cartpt-deficient mice exhibit no alterations in food intake or body weight when fed with a standard diet, but develop obesity after being fed with a high-fat diet (HFD; Asnicar et al. 2001). Interestingly, and contrary to the prevailing anorexigenic view, other studies have shown that under certain experimental conditions CART may stimulate food intake (Abbott et al. 2003, Kong et al. 2003). Collectively, results regarding the effects of CART on feeding behavior are inconclusive and indicate anatomically divergent roles for this neuropeptide.

POMC is a prohormone precursor that is cleaved into several bioactive peptides in the hypothalamus, including α-MSH, which exerts potent anorexigenic effects by binding to MC3R and MC4R (Mercer et al. 2013). The levels of Pomc transcripts and α-MSH are increased under feeding conditions and decreased under fasting conditions (Schwartz et al. 1997). The i.c.v. administration of α-MSH or its delivery into the PVN suppresses food intake and
reduces body weight (Poggioli et al. 1986, Wirth et al. 2001). Genetic manipulation of the Pomc gene leading to the overexpression of α-MSH has been shown to cause anti-obesity effects in genetic and diet-induced obesity (DIO) models (Mizuno et al. 2003, Savontaus et al. 2004, Lee et al. 2007). A key role for POMC in whole-body energy homeostasis is evident, as mice lacking Pomc, melanocortin peptides, or POMC neurons develop obesity (Yaswen et al. 1999, Gropp et al. 2005, Xu et al. 2005a, Smart et al. 2006). Furthermore, mutations in the POMC gene have been reported to be associated with morbid obesity in humans (Krude et al. 1998, Lee et al. 2006). GABAergic and glutamatergic subpopulations of POMC neurons have been described, although their functional roles are unclear (Mercer et al. 2013).

Paraventricular nucleus The PVN is located in the anterior hypothalamus, just above the third ventricle, and expresses high levels of MC3R/MC4R. It receives innervation not only from the AgRP and POMC neurons of the ARC but also from extrahypothalamic regions such as the nucleus of the tractus solitarius (NTS). The PVN is an important integration site involved in whole-body energy homeostasis, as shown by the diverse afferent inputs and its high sensitivity to the administration of endogenous neuropeptides involved in the regulation of food intake such as NPY, AGRP, and α-MSH, among others (Stanley et al. 1986, Kim et al. 2000). Part of these effects are mediated by a subset of neurons that express thyrotropin-releasing hormone (TRH), which are activated by α-MSH and inhibited by AGRP (Fekete et al. 2000, 2004). Another relevant subset of neurons express corticotrophin-releasing hormone (CRH), which are directly involved in the control of energy balance through AGRP innervation or indirectly through the regulation of adrenal glucocorticoids controlling the expression of POMC (Richard & Baraboi 2004).

Lateral hypothalamus area The LHA plays a critical role in the mediation of orexigenic responses, a function that can be significantly attributed to orexin and melanin-concentrating hormone (MCH) neurons. Orexin neurons produce orexin A and orexin B from prepro-orexin, the expression of which is increased under fasting conditions (Sakurai et al. 1998). The central administration of orexins not only increases food intake (Sakurai et al. 1998, Dube et al. 1999), but also promotes behavioral responses to food reward and increases arousal (Cason et al. 2010). Orexin neurons project not only within the LHA, ARC, PVN, and NTS, but also into other regions involved in additional physiological functions such as body temperature and wakefulness control, among others (Peyron et al. 1998). Similarly, fasting enhances the expression of Mch (Pmch) mRNA and its i.c.v. administration or genetic overexpression causes an orexigenic output (Qu et al. 1996, Ludwig et al. 2001). Conversely, mice with reduced MCH tone or disruption of the MCH1 receptor are lean (Marsh et al. 2002).

Dorsomedial nucleus The DMN is involved in a range of physiological processes, including appetite, thermoregulation, stress, and circadian rhythms. It receives projections from most of the hypothalamic nuclei, especially the ARC, and sends projections into the PVN and LHA. A number of neuropeptides (such as NPY and CRH) as well as receptors for peptides involved in the control of appetite and energy balance are expressed within the DMN. Increased expression of NPY in the DMN has been reported in several rodent models of obesity (Guan et al. 1998, Bi et al. 2001), and it may play a significant role in the regulation of thermogenesis and the development of DIO (Chao et al. 2011).

Ventromedial nucleus The AgRP and POMC neurons of the ARC project into the VMN. In turn, VMN neurons project into hypothalamic and extrahypothalamic areas such as the brainstem (Cheung et al. 2013). Laser-microdissection studies have identified a number of VMN-enriched genes (Segal et al. 2005), including steroidogenic factor 1 (Sf1 (Nr5a1)), which has been directly implicated in the development of the VMN (Parker et al. 2002, Davis et al. 2004). Sf1-expressing neurons play significant roles in the control of energy balance, as demonstrated by the metabolic phenotypes of conditional KO mice (Bingham et al. 2008, Zhang et al. 2008, Kim et al. 2011). Another abundantly expressed protein in the VMN is the brain-derived neurotrophic factor (BDNF). The lack of BDNF or its receptor (TRKB (NTRK2)) leads to hyperphagia and obesity in humans and mice (Lyons et al. 1999, Yeo et al. 2004). In contrast, the central or peripheral administration of BDNF results in the loss of body weight and reduction in food intake through MC4R signaling (Xu et al. 2003). The VMN also plays a key role in the regulation of thermogenesis (Lopez et al. 2010, Kim et al. 2011, Martinez de Morentin et al. 2012, Whittle et al. 2012).

The brainstem Brainstem neurons make key contributions to the control of energy balance by processing energy status information...
Neuronal circuits and energy balance

M SCHNEEBERGER and others

Thematic Review

Neuronal circuits and energy balance

DOI: 10.1530/JOE-13-0398
Printed in Great Britain

Published by Bioscientifica Ltd

at four different levels: i) by sensing circulating metabolites and hormones released by peripheral organs; ii) by receiving vagal inputs from the gastrointestinal (GI) tract; iii) by receiving neuronal inputs from midbrain and forebrain nuclei that also detect and integrate energy-related signals; and iv) by projecting into local brainstem circuits and other regions of the brain to provide information that will be integrated by these neurons to control energy balance. Within the brainstem, the dorsal vagal complex (DVC) is a key module for the integration of energy-related cues by relying peripheral signals through vagal afferents and projecting into the hypothalamus and other relevant areas. The DVC comprises the dorsal motor nucleus of the vagus, the NTS, and the area postrema (AP), which has an incomplete BBB and therefore it is accessible to peripheral signals.

The brainstem is constituted by heterogeneous populations of neurons, with distinct biophysical and neurochemical properties, that express appetite-modulatory neuropeptides such as tyrosine hydroxylase (TH), pro-glucagon, CART, GABA, NPY, BDNE, and POMC, among others. These neurons also express a variety of receptors mediating the effects of some of the aforementioned neuropeptides, indicating the existence of local circuits that contribute to the regulation of ingestive behaviors. In addition, receptors for a number of circulating hormones such as leptin, ghrelin, glucagon-like peptide 1 (GLP1), and cholecystokinin (CCK) have been described in brainstem neurons or in vagal afferent projections to brainstem areas.

Vagal signaling from the GI tract is an important afferent to the NTS, conveying information about luminal distension, nutritional content, and locally produced peptides via glutamate neurotransmission (Travagli et al. 2006). This vagal sensory and hormonal information will be assimilated by second-order NTS neurons that project into the hypothalamus and other basal forebrain areas to elaborate precise outputs. The significance of the vagus nerve transmission has been demonstrated through a number of manipulations to eliminate or enhance its activity. For example, chronic or acute vagus nerve stimulation in rats leads to a reduction in body weight and food intake, indicating that direct vagal afferent interventions influence feeding behavior (Krolczyk et al. 2001, Gil et al. 2011). Vagal signaling also plays important roles in the regulation of meal size and duration (Schwartz et al. 1999).

The NTS receives inputs from descending projections from the hypothalamus. In particular, ARC POMC neurons project into the NTS, where high expression levels of MC4R have been reported (Kishi et al. 2003). In addition to the release of α-MSH from ARC POMC neurons, the NTS also receives melanocortin agonist signals from a local population of ~300 POMC neurons (around 10% of the total number of POMC neurons; Palkovits & Eskay 1987). Recent pharmacogenetic studies have shown different functions and time scale effects of ARC and NTS POMC neurons on food intake and metabolism (Zhan et al. 2013). The importance of this neuronal circuit is further demonstrated by hindbrain MC4R agonist delivery, which leads to a reduction in food intake and an increase in energy expenditure, whereas MC4R antagonism drives the opposite effect (Williams et al. 2000, Skibicka & Grill 2009b). MC4Rs in the NTS seem to mediate not only the satiation effects of CCK (Fan et al. 2004), but also the anorexigenic effects of hypothalamic and brainstem leptin signaling (Skibicka & Grill 2009a, Zheng et al. 2010).

The NTS also receives descending projections from orexin and MCH neurons located in the LHA (Ciriello et al. 2003), and the delivery of orexin A into the hindbrain increases food intake (Parise et al. 2011). The orexigenic nature of the LHA and the anatomical connection with the NTS indicated that this system may serve as a mechanism to limit the satiety signals from the GI tract.

Another hypothalamic nucleus sending projections into the NTS is the PVN (Sawchenko & Swanson 1982, Luiten et al. 1985). The PVN–brainstem pathway plays a significant role in the regulation of energy balance, as contralateral disruption of PVN output and NTS input causes hyperphagic obesity (Kirchgessner & Sclafani 1988). Different areas of the brainstem show TRH-positive fibers, and evidence indicates that TRH is involved in the brainstem regulation of energy homeostasis by integrating endocrine and vagal–sympathetic responses (Ao et al. 2006, Zhao et al. 2013).

Hormonal signals involved in energy homeostasis control

Peripheral adiposity signals: leptin and insulin

The discovery of leptin, the product of the Ob gene, in 1994 (Zhang et al. 1994) opened a new dimension in the field of the central regulation of energy balance. Leptin is an anorexigenic adipose tissue-derived hormone that circulates in proportion to fat mass (Considine et al. 1996). It reaches the CNS through a saturable transport system and conveys information about the energy status of the organism. There are multiple leptin receptor (LEPR) isoforms, with the long form (LEPRb) being essential for
the effects of leptin. The lack of leptin or LEPRb in both rodents and humans causes a phenotype characterized by hyperphagia, reduced energy expenditure, and severe obesity (Halaas et al. 1995, Chen et al. 1996, Montague et al. 1997, Clement et al. 1998). Most obese patients exhibit a state of leptin resistance, which is the inability of high circulating leptin levels to exert central anorexigenic actions, which precludes the use of leptin as a therapeutic approach.

LEPRb is highly expressed in different hypothalamic nuclei and other CNS regions involved in the control of energy balance (Elmquist et al. 1998). In the ARC, the POMC, and AgRP neurons are the direct targets of leptin (Cheung et al. 1997, Elias et al. 1999, Cowley et al. 2001). The ablation of LEPRb in POMC neurons, AgRP neurons, or both populations of neurons causes increased body weight, emphasizing the importance of leptin signaling (Table 1). However, the magnitude of these changes is smaller than that observed in mice globally lacking Lepr, indicating the existence of additional subsets of neurons mediating the effects of leptin on food intake and body weight. Leptin binds to LEPRb and activates JAK2, which, in turn, phosphorylates several tyrosine residues on the intracellular domain of the LEPRb. This results in the activation, dimerization, and nuclear translocation of STAT3 (Robertson et al. 2008). In the nucleus, STAT3 enhances Pomc gene expression and inhibits Agrp gene expression (Munzberg et al. 2003, Kitamura et al. 2006). Accordingly, Stat3 deficiency in POMC neurons results in overweight and Pomc gene transcriptional defects in females (Table 1). This signaling cascade is negatively regulated by the suppressor of cytokine signaling 3 (SOCS3), the expression of which is also regulated by STAT3 and protein tyrosine phosphatase 1B (PTP1B) (Robertson et al. 2008). Consistent with this, deletion of either Socs3 or Ptp1b (Ptpn1) in POMC neurons leads to reduced adiposity, improved leptin sensitivity, and increased energy expenditure under HFD conditions (Table 1). In addition, leptin also activates the phosphatidylinositol-3-kinase (PI3K) pathway. A variety of genetic mouse models targeting the catalytic or regulatory subunits of PI3K in specific subsets of neurons have been reported with divergent results (Table 1). Overall, these studies indicate that PI3K is required for leptin-mediated regulation of energy balance and that, contrary to the prevailing view, the catalytic p110α subunit in ARC neurons may play a more prominent role than p110ζ. PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP3) and activates downstream targets such as phosphoinositide-dependent kinase 1 (PDK1) and AKT (also known as protein kinase B), which consecutively phosphorylates the transcription factor forkhead box protein O1 (FOXO1). Upon phosphorylation, FOXO1 is excluded from the nucleus, allowing STAT3 to bind to Pomc and Agrp promoters, thereby stimulating and inhibiting respectively the expression of these neuropeptides (Kitamura et al. 2006). These findings are consistent with the effects of genetic manipulations in vivo (Table 1). PI3K signaling is counterbalanced by phosphatase and tensin homolog (PTEN), which specifically dephosphorylates PIP3. The loss of Pten in POMC neurons results in increased PIP3 signaling and diet-sensitive obesity via KATP channel modulation, suggesting a role for the PI3K pathway in the regulation of the activity of this channel (Table 1). Overall, leptin stimulates Pomc transcription, depolarizes POMC neurons, and also increases α-MSH processing and secretion (Cowley et al. 2001, Munzberg et al. 2003, Guo et al. 2004) while attenuating the expression and release of orexigenic NPY and AGRP neuropeptides (Stephens et al. 1995, Mizuno & Mobbs 1999).

Insulin, produced by pancreatic β-cells, has traditionally been associated with glucose metabolism, but compelling evidence indicates that insulin also acts as an anorectic signal within the CNS. Glucose-induced insulin is secreted into the bloodstream in proportion to fat stores (Bagdade et al. 1967) and enters the brain through a saturable transport mechanism (Baura et al. 1993). The i.c.v. or intrahypothalamic administration of insulin to primates and rodents reduces food intake (Woods et al. 1979, McGowan et al. 1993, Air et al. 2002). Insulin receptor (IR (INSR)), as well as its downstream signaling machinery, is expressed in hypothalamic areas involved in the control of appetite (Havrankova et al. 1978, Corp et al. 1986) and colocalizes with AgRP and POMC neurons (Benoit et al. 2002). Surprisingly, the loss of Insr in either POMC or AgRP neurons does not lead to alterations in energy balance (Table 1), although hepatic glucose production defects have been observed in mice lacking Ir in AgRP neurons (Konner et al. 2007). Neuron-specific IR reconstitution in L1 mice (which have >90% reduction of IR levels in the ARC) confirmed that insulin signaling in AgRP and POMC neurons controls glucose metabolism and energy expenditure respectively (Table 1). Insulin binding to IR leads to the autophosphorylation of the receptor and the consequent recruitment of IRS proteins, which converge with the leptin pathway at the PI3K level (Xu et al. 2005b). Negative regulators of the LEPR, such as SOCS3 and PTP1B, also directly inhibit the IR and its signaling cascade acting on IRS1. The activation of the
<table>
<thead>
<tr>
<th>Genetic manipulation</th>
<th>Neuronal cell type</th>
<th>BW</th>
<th>Adiposity</th>
<th>Food intake</th>
<th>Energy expenditure</th>
<th>Diet</th>
<th>Other features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepr deletion</td>
<td>POMC</td>
<td>+</td>
<td>+</td>
<td>=</td>
<td>=</td>
<td>Chow</td>
<td>Altered neuropeptide expression</td>
<td>Balthasar et al. (2004)</td>
</tr>
<tr>
<td>Lepr deletion</td>
<td>AgRP</td>
<td>+</td>
<td>+</td>
<td>=</td>
<td>=</td>
<td>Chow</td>
<td>Reduced locomotor activity</td>
<td>van de Wall et al. (2008)</td>
</tr>
<tr>
<td>Lepr deletion</td>
<td>POMC and AgRP</td>
<td>+</td>
<td>+</td>
<td>Transient +</td>
<td>-</td>
<td>Chow</td>
<td>Increased respiratory exchange ratio</td>
<td>van de Wall et al. (2008)</td>
</tr>
<tr>
<td>Ir deletion</td>
<td>POMC</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>ND</td>
<td>Chow and HFD</td>
<td>Enhanced hepatic glucose production</td>
<td>Konner et al. (2007)</td>
</tr>
<tr>
<td>Ir deletion</td>
<td>AgRP</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>ND</td>
<td>Chow and HFD</td>
<td>Insulin resistance</td>
<td>Konner et al. (2007)</td>
</tr>
<tr>
<td>Ir re-expression in L1 mice</td>
<td>POMC</td>
<td>-</td>
<td>=</td>
<td>+</td>
<td>+</td>
<td>Chow</td>
<td>Rescued hepatic glucose production Insulin resistance</td>
<td>Lin et al. (2010)</td>
</tr>
<tr>
<td>Ir re-expression in L1 mice</td>
<td>AgRP</td>
<td>-</td>
<td>=</td>
<td>=</td>
<td>+</td>
<td>Chow</td>
<td>Rescued hepatic glucose production Insulin resistance and reduced fertility in females</td>
<td>Lin et al. (2010)</td>
</tr>
<tr>
<td>Lepr and Ir deletion</td>
<td>POMC</td>
<td>+</td>
<td>=</td>
<td>=</td>
<td>-</td>
<td>Chow</td>
<td>Insulin resistance and reduced fertility in females</td>
<td>Hill et al. (2010)</td>
</tr>
<tr>
<td>Irs2 deletion</td>
<td>POMC</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>Chow</td>
<td>Normal insulin and leptin levels</td>
<td>Choudhury et al. (2005)</td>
</tr>
<tr>
<td>Ptp1b deletion</td>
<td>POMC</td>
<td>-</td>
<td>-</td>
<td>=</td>
<td>+</td>
<td>HFD</td>
<td>Improved leptin sensitivity</td>
<td>Banno et al. (2010)</td>
</tr>
<tr>
<td>Stat3 deletion</td>
<td>POMC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>Chow</td>
<td>Normal phenotype in male mice</td>
<td>Xu et al. (2007)</td>
</tr>
<tr>
<td>Stat3 constitutive active form</td>
<td>AgRP</td>
<td>-</td>
<td>-</td>
<td>=</td>
<td>+</td>
<td>Chow</td>
<td>Hyporesponsive to leptin</td>
<td>Gong et al. (2008)</td>
</tr>
<tr>
<td>Stat3 constitutive active form</td>
<td>POMC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>Chow</td>
<td>No additional effect on HFD administration</td>
<td>Ernst et al. (2009)</td>
</tr>
<tr>
<td>Pdk1 deletion</td>
<td>POMC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Chow and HFD</td>
<td>+</td>
<td>Increased locomotor activity</td>
<td>Mesaros et al. (2008)</td>
</tr>
<tr>
<td>Pdk1 deletion</td>
<td>AgRP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Chow</td>
<td>Decreased Pomc gene expression</td>
<td>Iskandar et al. (2010)</td>
</tr>
<tr>
<td>Pdk1 deletion</td>
<td>POMC</td>
<td>Transient</td>
<td>Transient</td>
<td>Transient +</td>
<td>ND</td>
<td>Chow and HFD</td>
<td>Belgardt et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Foxo1 deletion</td>
<td>POMC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>=</td>
<td>Chow</td>
<td>Increased Cpe expression and α-MSH levels</td>
<td>Plum et al. (2009)</td>
</tr>
<tr>
<td>Foxo1 constitutive active form</td>
<td>POMC</td>
<td>+ (Females)</td>
<td>+ (Females)</td>
<td>+ (Females)</td>
<td>=</td>
<td>Chow</td>
<td>Decreased Pomc gene expression</td>
<td>Iskandar et al. (2010)</td>
</tr>
<tr>
<td>Foxo1 deletion</td>
<td>AgRP</td>
<td>=</td>
<td>-</td>
<td>-</td>
<td>=</td>
<td>Chow</td>
<td>Resistant to HFD</td>
<td>Ren et al. (2012)</td>
</tr>
<tr>
<td>Soc3 deletion</td>
<td>POMC</td>
<td>-</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>HFD</td>
<td>No body weight phenotype on chow diet</td>
<td>Kievit et al. (2006)</td>
</tr>
<tr>
<td>Soc3 overexpression</td>
<td>POMC</td>
<td>+</td>
<td>+</td>
<td>=</td>
<td>-</td>
<td>Chow</td>
<td>Leptin resistance</td>
<td>Reed et al. (2010)</td>
</tr>
<tr>
<td>Soc3 overexpression</td>
<td>AgRP</td>
<td>=</td>
<td>=</td>
<td>+</td>
<td>+</td>
<td>Chow</td>
<td>Altered glucose metabolism</td>
<td>Olofsson et al. (2013)</td>
</tr>
<tr>
<td>Pten deletion</td>
<td>POMC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>=</td>
<td>Chow</td>
<td>Gender dimorphism on HFD administration</td>
<td>Plum et al. (2009)</td>
</tr>
</tbody>
</table>
IR signaling pathway results in reduced expression of NPY and increased levels of POMC in the ARC, thus stimulating an anorexigenic effect (Schwartz et al. 1992, Sipols et al. 1995, Benoit et al. 2002).

Leptin and insulin also regulate the activity of AMPK, an evolutionarily conserved cellular and organismal energy sensor that plays a central role in the hypothalamic regulation of energy homeostasis (Minokoshi et al. 2004, Claret et al. 2007). In particular, both hormones inhibit AMPK and its downstream targets in the hypothalamus (Minokoshi et al. 2004). A recent study has reported that leptin-mediated inhibition of AMPK is achieved through phosphorylation on serine491 by mTOR/p70S6K, an event that is necessary for the action of leptin on food intake and body weight (Dagon et al. 2012).

The molecular significance and detailed mechanisms of the different components of the aforementioned signaling pathways have become better understood, thanks to the advent of the Cre/Lox technology. Table 1 summarizes the phenotypes of several conditional mouse models that provided valuable information in this regard.

**GI hormones**

Ghrelin is a 28-amino acid acylated hormone, mainly produced by the stomach, which exerts its biological actions on energy balance through the growth hormone secretagogue receptor (GHSR; Kojima et al. 1999, Sun et al. 2004). Circulating ghrelin levels are increased under fasting conditions and reduced after refeeding (Tschop et al. 2000). The central and peripheral administration of ghrelin in rodents has been shown to robustly promote feed intake, adiposity, and body weight gain (Tschop et al. 2000, Nakazato et al. 2001). Likewise, ghrelin also enhances appetite in humans (Wren et al. 2001). GHSR is expressed in AgRP neurons of the ARC (Willesen et al. 1999), and this population of neurons is essential for the mediation of the orexigenic effects of ghrelin (Chen et al. 2004). Ghrelin is able to stimulate the transcription of Npy and Agrp, and it also increases the number of stimulatory synapses on AgRP neurons while increasing the number of inhibitory synapses on POMC neurons (Kamegai et al. 2001, Nakazato et al. 2001, Cowley et al. 2003). However, neuronal activation and positive energy balance have also been reported after ghrelin administration in the PVN, LHA, and hindbrain and in the mesolimbic reward pathway (Faulconbridge et al. 2003, Naleid et al. 2005).

Peptide tyrosine tyrosine (PYY) is mainly released from the L-cells of the intestinal epithelium in response to nutrient ingestion (Tatemoto & Mutt 1980,
Adrian et al. 1985). Circulating PYY levels are proportional to calorie intake and are reduced under fasting conditions (Adrian et al. 1985). Two endogenous forms, PYY1–36 and PYY3–36, are synthesized and secreted. The latter form is the most abundant in the bloodstream and exerts a direct action in the ARC. This has been demonstrated by peripheral and intra-ARC administration of PYY3–36, which increases neuronal activity in this region and reduces appetite and body weight in a dose-dependent manner (Batterham et al. 2002, Challis et al. 2003). These anorexigenic effects are mediated via the inhibition of ARC Y2 receptors, as demonstrated by pharmacological (Abbott et al. 2005, Scott et al. 2005) and genetic (Batterham et al. 2002) studies, which eventually leads to increased z-MSH and reduced NPY release (Batterham et al. 2002). The effects of PYY3–36 in the brainstem and the vagal–brainstem circuit have also been confirmed, as the peripheral delivery of this peptide has been shown to increase neuronal activity in NTS and AP neurons and stimulate vagal afferent firing (Koda et al. 2005, Blevins et al. 2008). Consistent with a role for PYY in the regulation of appetite and body weight, transgenic mice globally lacking or overexpressing Pyy exhibit opposite alterations in energy balance control (Batterham et al. 2006, Boey et al. 2008).

GLP1, the cleavage product of proglucagon in the intestine and brain, is mainly secreted from intestinal L-cells. Similar to PYY, circulating GLP1 levels are high following a meal and are low under fasting conditions. This hormone exerts a strong incretin effect, via the GLP1 receptor (GLP1R) expressed in pancreatic islets, enhancing insulin secretion after carbohydrate ingestion (Kreymann et al. 1987). GLP1R is also expressed in key CNS areas involved in the control of energy balance, such as the hypothalamus and brainstem (Merchenthaler et al. 1999). A number of studies have shown that the central or site-specific administration of GLP1 or GLP1 analogs inhibits food intake in rodents (Tang-Christensen et al. 1996, Turton et al. 1996, McMahon & Wellman 1998, Hayes et al. 2008). Interestingly, neurons expressing the proglucagon gene are present in the NTS, suggesting the existence of a local circuit involved in the control of appetite (Merchenthaler et al. 1999). In fact, recent studies have provided evidence for a dual (peripheral and central) role of GLP1 in the suppression of appetite mediated by local vagal afferents and a gut–brain feedback mechanism (Barrera et al. 2011).

CCK is postprandially secreted from I-cells from the small intestine and its systemic delivery suppresses food intake in both animal models and humans (Gibbs et al. 1973, Gibbs & Smith 1977, Kissileff et al. 1981). CCK1 and CCK2 receptors are expressed in the brainstem and hypothalamus, but the anorectic effects of CCK are critically mediated by vagal sensory neurons that project into the NTS (Moran et al. 1997). Interestingly, NTS POMC neurons are activated by CCK and brainstem MC4R signaling is required for CCK-induced suppression of appetite (Fan et al. 2004). It has also been reported that ghrelin attenuates and leptin synergistically potentiates the effects of CCK on appetite (Barrachina et al. 1997, Lee et al. 2011).

**Neural circuits regulating homeostatic energy balance**

Certain physiological conditions, such as the prandial state, are associated with notable changes in the circulating concentration of metabolites and hormones involved in the regulation of whole-body energy homeostasis. For example, in a postabsorptive situation, circulating cues of energetic surfeit (leptin, insulin, GLP1, PYY, and glucose) are elevated, while cues of energetic deficit (ghrelin) are reduced. The opposite is true under fasting conditions. These hormones act in concert to engage specific neuronal circuits in different brain regions, including the hypothalamus and brainstem, establishing reciprocal and dynamic interactions to restore systemic energy balance. In this section, we summarize the main circuits and the neuronal responses engaged by leptin and ghrelin, as prototypical examples of anorexigenic and orexigenic signals respectively.

**ARC neuronal circuits: POMC, AgRP, and RIPCre neurons**

Melanocortin peptides and NPY are two basic components of a critical hypothalamic circuit involved in the convergence and integration of nutritional and hormonal cues aimed at regulating organismal energy balance. In the ARC, the POMC, and AgRP neurons are located in proximity to each other and project in parallel into similar brain areas expressing MCRs. Both POMC and AgRP neurons are able to sense a number of peripheral (leptin, insulin, and ghrelin) and central (NPY, GABA, serotonin, and melanocortin) signals, which are able to acutely modulate their electrical activity influencing the release of neuropeptides and neurotransmitters to ultimately regulate appetite, energy expenditure, and metabolism.

In general terms, POMC (anorexigenic) and AgRP (orexigenic) neurons have opposite physiological functions, which are largely the consequence of the
contrasting actions of α-MSH and AGRP peptides on MCRs: while α-MSH is an endogenous MCR agonist, AGRP is an inverse agonist (Haskell-Luevano & Monck 2001, Nijenhuis et al. 2001, Tolle & Low 2008). Indeed, substantial experimental evidence indicates that the agonism of MCRs attenuates appetite and enhances energy expenditure, whereas their antagonism has essentially the opposite effects (Fan et al. 1997, Harrold et al. 1999, Hwa et al. 2001). This is consistent with data showing that the loss of or mutations in MC3R and MC4R genes cause obesity both in rodents and in humans (Huszar et al. 1997, Butler et al. 2000, Farooqi 2008). In addition to the inhibition of MCR signaling, the orexigenic actions of AgRP neurons are also mediated by the release of NPY and GABA.

The anorexigenic effects of leptin are basically achieved by repressing AgRP neurons and activating POMC neurons (Fig. 1A). Leptin enhances POMC gene expression and processing into α-MSH (Schwartz et al. 1997, Thornton et al. 1997, Mizuno et al. 1998). Electrophysiological studies have demonstrated that locally applied leptin is able to depolarize (excite) POMC neurons (Cowley et al. 2001, Claret et al. 2007, 2011, Hill et al. 2008, Al-Qassab et al. 2009, Qiu et al. 2010) probably through TRPC channels (Qiu et al. 2010). In contrast, leptin inhibits the transcription of Npy and Agrp genes in the hypothalamus (Stephens et al. 1995, Schwartz et al. 1996, Mizuno & Mobbs 1999). Electrophysiological recordings have shown that leptin decreases the GABAergic-mediated tone induced by AgRP neurons onto neighboring POMC neurons, resulting in the disinhibition of POMC neuron activity (Cowley et al. 2001). The ability of leptin to directly hyperpolarize (inhibit) AgRP neurons is controversial (Cowley et al. 2001, Claret et al. 2007, Al-Qassab et al. 2009), but studies in rats have reported leptin-mediated inhibition of identified NPY neurons (van den Top et al. 2004). In addition, leptin also acts directly on presynaptic GABAergic neurons that do not express AGRP, reducing the inhibitory input to postsynaptic POMC neurons, thus further contributing to the maintenance of the anorexigenic actions mediated by this hormone (Fig. 1A; Vong et al. 2011).

On the other hand, under conditions of negative energy balance, circulating ghrelin levels are increased. The actions of ghrelin on food intake and energy balance are mediated by AgRP neurons, as mice lacking Agrp and Npy are insensitive to the orexigenic effects of external ghrelin (Chen et al. 2004, Luquet et al. 2007). In line with this, ghrelin increases the expression of Npy and Agrp transcripts (Kamegai et al. 2001, Nakazato et al. 2001) and depolarizes AgRP neurons while increasing the number of GABAergic inhibitory synapses on POMC neurons (Fig. 1B) (Cowley et al. 2003, van den Pol et al. 2009, Yang et al. 2011, Atasoy et al. 2012). The importance of these GABAergic stimuli in the control of energy balance has been substantially demonstrated (Horvath et al. 1997, Wu et al. 2009, 2012, Wu & Palmiter 2011), and conditional deletion of the vesicular GABA transporter in AgRP neurons blunts the inhibitory tone onto postsynaptic POMC neurons, leading to an enhanced melanocortigenic output and a lean phenotype (Tong et al. 2008). Moreover, AGRP and NPY directly hyperpolarize POMC neurons and decrease the production and release of α-MSH, further inhibiting the activity of this population of neurons (Roseberry et al. 2004, Smith et al. 2007, Cyr et al. 2013). Thus, AgRP neurons are able to negatively modulate the anorexigenic effects of POMC neurons by direct (GABAergic synapsis) and indirect (MCR antagonism) mechanisms (Fig. 1B).

In addition to changes in neuropeptide release, leptin and ghrelin also exert rapid and reversible effects on synaptic connections onto POMC and AgRP neurons. Seminal studies carried out at the Horvath laboratory have provided the first evidence for synaptic plasticity in hypothalamic energy balance circuits and established the basis for a new mechanism by which these hormones dynamically regulate circuit responsiveness to control energy homeostasis (Pinto et al. 2004). The role of synaptic remodeling in neuronal circuits regulating metabolism has recently been reviewed in detail (Zeltser et al. 2012, Dietrich & Horvath 2013).

A novel subpopulation of ARC neurons involved in the control of energy balance (defined by virtue of Cre-mediated expression of rat insulin II promoter-Cre transgene and called RIPCre neurons) has recently been described. Comparative electrophysiological and histological studies indicate that RIPCre neurons constitute a distinct population from POMC or AgRP neurons (Choudhury et al. 2005). However, close apposition of these neuronal subsets suggests that RIPCre neurons may be the targets of POMC and/or AgRP neurons. Indeed, bath application of a melanocortin agonist has been found to cause direct long-lasting depolarization and increased firing in ARC RIPCre neurons (Choudhury et al. 2005). Interestingly, insulin has also been found to depolarize these neurons, while leptin has been found to not cause any electrophysiological effect (Choudhury et al. 2005).

Although a number of mouse genetic studies indicate that ARC RIPCre neurons regulate systemic energy balance (Cui et al. 2004, Choudhury et al. 2005), this interpretation
is called into question by the fact that the RIPcre transgene is also expressed in other regions of the brain and pancreatic β-cells. However, recent data show that the acute and selective ablation of ARC RIPCre neurons leads to hypophagia, reduced food intake, and adiposity through compensatory increase in the number of anorexigenic neurons in the PVN (Rother et al. 2012). Consistent with the anorexigenic nature of RIPCre neurons, a combination of genetic and pharmacogenetic approaches has shown that the synaptic release of GABA, but not of glutamate, from this subset of neurons increases the thermogenic function of BAT without affecting food intake (Kong et al. 2012). The effects of leptin on RIPCre neurons are complex, as suggested by heterogeneous electrophysiological recordings demonstrating subsets of neurons being depolarized, hyperpolarized, or silent (Choudhury et al. 2005, Kong et al. 2012). Nevertheless, the ability of leptin to increase energy expenditure is impaired in mice lacking vesicular GABA transporter in RIPCre neurons, indicating a functional effect of this hormone on these neurons (Kong et al. 2012).

Taken together, current evidence indicates that a local ARC circuit constituted by the ‘first-order’ POMC, AgRP, and RIPCre neurons plays a key role in the integration of humoral signals reporting on energy conditions. This is achieved by a sophisticated and multilevel

---

**Figure 1**
Schematic representation of the main neuronal circuits engaged by leptin and ghrelin. (A) Leptin is released in proportion to fat stores and stimulates the activity of anorexigenic POMC neurons in the ARC while inhibiting neighboring AgRP neurons. This results in increased release of α-MSH and the activation of downstream second-order neurons expressing MC4R in hypothalamic and extrahypothalamic regions. POMC neurons also express MC4R, indicating the existence of an autoregulatory mechanism induced by α-MSH. Leptin also acts on GABAergic presynaptic neurons attenuating its inhibitory effect on POMC neurons. Overall, these effects result in reduced food intake and increased energy expenditure. (B) Ghrelin exerts its orexigenic effects through AgRP neurons. Ghrelin increases inhibitory GABAergic projections onto POMC neurons and enhances the expression and release of NPY and AgRP. In the PVN, AgRP acts as a MC4R inverse agonist, while NPY binds to Y1 and Y5 receptors. Collectively, these events lead to increased orexigenic output. Red arrows and synapses, inhibitory effect and green arrows, activation effect. WAT, white adipose tissue.
organizational structure that allows the accurate regulation of orexigenic and anorexigenic outputs through direct and indirect mechanisms.

**Downstream neurocircuitry engaged by hypothalamic neuron activity**

Given that POMC and AgRP neurons are the sole source of MCR ligands in the brain, a fine balance between α-MSH and AGRP is necessary to precisely regulate their mediated physiological outputs on MC4Rs in target areas. These receptors are localized in many nuclei involved in the regulation of energy balance where POMC and AgRP neurons send axon projections. MC4Rs are G-protein-coupled receptors that stimulate adenylyl cyclase, thereby increasing intracellular cAMP levels (Florijn et al. 1993). A series of elegant studies using a cell-specific MC4R re-expression strategy indicate that MC4Rs in the PVN are mainly involved in the control of food intake (Balthasar et al. 2005), while MC4Rs in autonomic preganglionic neurons regulate energy expenditure and hepatic glucose production (Rossi et al. 2011) (Fig. 1A). Furthermore, and contrary to the prevailing view, a recent report has shown that POMC neurons also express MC4Rs that contribute to the regulation of body weight and composition through changes in both feeding behavior and energy expenditure (do Carmo et al. 2013). This autoregulatory mechanism, induced by α-MSH released from the same cell and/or neighboring POMC neurons, could represent an additional layer of regulation within a widely segregated network of melanocortin receptors involved in the regulation of homeostatic (appetite) and autonomic (thermogenesis, hepatic metabolism, and insulin release) functions (Fig. 1A).

NPY receptors are Gi/o-protein-coupled receptors that reduce cAMP production, leading to the activation of G-protein-gated inwardly rectifying K+ (GIRK) channels and inhibition of voltage-dependent Ca2+ channels (Sohn et al. 2013). The precise roles of NPY receptors and their contribution to the mediation of the orexigenic effects of NPY have been difficult to delineate due to the paradoxical phenotypes of receptor KO mouse models. This is probably the consequence of receptor redundancies and compensatory mechanisms exhibited after the application of germ-line deletion strategies. Despite these limitations, pharmacological and genetic studies indicate that the orexigenic actions of NPY are mediated by postsynaptic Y1 and Y5 within the PVN (Nguyen et al. 2012, Sohn et al. 2013; Fig. 1B). Notably NPY from ARC neurons acts through PVN Y1, resulting in a functional inhibition of TH tonus and BAT thermogenesis (Shi et al. 2013). Furthermore, NPY may also decrease pro-TRH transcription and proconvertase 2-mediated pro-TRH processing in the PVN through Y1/Y5 receptors (Cyr et al. 2013). Taken together, abundant amounts of evidence suggest that the effects of ARC NPY on energy balance are principally mediated by the PVN. However, it is important to note that other sources of NPY may also play a role in the regulation of energy balance.

**Correlating neuronal circuit activity with behavioral responses by pharmacogenetic and optogenetic techniques**

Most of the experimental findings that have allowed researchers to outline the models suggested so far are largely the result of circumstantial evidence. However, the recent development of pharmacogenetic and optogenetic techniques has provided a way to exert temporally and spatially precise control over the activity of defined circuit elements. This permits the establishment of causal connections between circuit activity and behavioral responses (Sternson 2013).

Using an elegant combination of optogenetics and mouse genetic approaches, Aponte et al. (2011) have confirmed that the selective activation of AgRP neurons is sufficient to evoke voracious feeding behavior in mice, without previous training and independent of melanocortin signaling. The level of neuronal activation has been found to correlate with the magnitude, dynamics, and duration of the induced behavioral response. Furthermore, continuous photostimulation is required to maintain evoked feeding behaviour, indicating that the activation of AgRP neurons does not initiate a sustained propagating effect (Aponte et al. 2011). In contrast, prolonged (but not brief) optogenetic stimulation of POMC neurons has been shown to result in reduced food intake and body weight gain, which requires downstream MC4R activity (Aponte et al. 2011).

The behavioral effects on food intake caused by AgRP or POMC neuron activation have been further supported by studies using pharmacogenetic (designer receptors exclusively activated by designer drugs (DREADDs)) technology. Pharmacogenetic activation of AgRP neurons rapidly induces feeding and food-seeking behaviors associated with decreased energy expenditure and enhanced adiposity (Krashes et al. 2011). Consistent with the optogenetic data (Aponte et al. 2011), long-term stimulation of ARC POMC neurons is necessary to reduce appetite. Interestingly, the acute stimulation of NTS POMC neurons has been shown to generate an immediate suppression of food intake (Zhan et al. 2013).
In a subsequent study, the Sternson group performed a series of experiments to determine which brain regions and cell types mediate evoked feeding behavior triggered by activated AgRP neurons. The authors used optogenetic approaches to map synaptic connections downstream of AgRP neurons and assessed their role in terms of ingestive behavior by perturbing electrical activity in presynaptic and postsynaptic neuronal types (Atasoy et al. 2012). Notably the authors found that ARC AgRP neurons induce evoked feeding behavior through inhibitory input onto oxytocin neurons in the PVN, while ARC POMC neurons are involved in the long-term control of appetite and energy balance (Atasoy et al. 2012).

Collectively, these results emphasize the previously unrecognized importance of the temporal and spatial activation of POMC and AgRP neurons. Thus, ARC AgRP and NTS POMC neurons could be involved in the regulation of acute feeding behavior while ARC POMC neurons may be involved in long-term responses. This demonstrates the existence of multiple, distinct behavioral and anatomical modules that act in synchrony to regulate whole-body energy balance. The use of these tools in the field of central control of energy balance has provided novel valuable information and has confirmed previous findings. However, it has also generated some controversial observations. Further research needs to be conducted to precisely define the importance of these factors and to reconcile these observations with previous evidence (Mercer et al. 2013). Nevertheless, these reports demonstrate that optogenetics and pharmacogenetics are exceptionally useful tools to study the interrelationships between synaptology, neuronal circuit activity, and behavioral outputs.

New players in energy balance control

Non-neuronal cell types: macroglia and microglia

Glial cells have traditionally been considered satellite neuronal partners with supportive and structural roles. However, in recent years, glial cells have acquired a new rank and are now regarded as active players in many physiological functions including energy balance control.

Astrocytes are star-shaped cells that are involved in a number of functions, such as metabolic support to neurons and transmitter uptake and release as well as synaptic remodeling (Sofroniew & Vinters 2010). Astrocytes express LEPR (Cheunsuang & Morris 2005, Hsuchou et al. 2009b), and modifications in circulating leptin levels alter hypothalamic astrocyte expression of structural proteins as well as glutamate and glucose transporters (Garcia-Caceres et al. 2011, Fuente-Martin et al. 2012). This may cause changes in the synaptic plasticity and excitability of surrounding neurons, leading to metabolic adaptations. In fact, HFD administration in rodents is associated with increased glial coverage of POMC neuron perikarya (Horvath et al. 2010). It has also been reported that DIO mice exhibit increased expression of functional astrocytic LEPR in the hypothalamic region, an effect that may play a role in the development of leptin resistance (Hsuchou et al. 2009a). Indeed, loss of astrocytic Lepr under HFD conditions provides partial protection against developing disturbances in neuronal leptin signaling (Jyaram et al. 2013).

Obesity and lipid overload induce chronic low-grade inflammation in the hypothalamus (Thaler et al. 2010). This is regarded as a protective effect, which is mainly promoted by microglial cells that have immunitary actions in the CNS. HFD feeding selectively and rapidly activates microglia in the hypothalamus and increases the production of proinflammatory cytokines (De Souza et al. 2005, Milanski et al. 2009, Thaler et al. 2012). Interestingly, it has been demonstrated that moderate physical activity reduces hypothalamic microglial activation independently of body mass (Yi et al. 2012). Enhanced hypothalamic microglial activation has also been reported in rodents and primates after nutritional manipulations during the prenatal or perinatal period (Grayson et al. 2010, Tapia-Gonzalez et al. 2011).

Tanycytes have recently emerged as novel modulators of the hypothalamic networks that control energy balance. They contact the cerebrospinal fluid and send processes that come into proximity with neurons into the ARC and VMN (Bolborea & Dale 2013). Although it is not known whether tanycytes are able to modulate the activity of hypothalamic neurons, several lines of evidence suggest that this particular cell type may be involved in the regulation of energy homeostasis. For example, tanycytes respond to fluctuations in glucose concentration (Frayling et al. 2011), express a number of genes related to energy homeostasis control (Bolborea & Dale 2013), and regulate the permeability properties of the fenestrated capillaries of the ME, which may constitute a way of modulating the access of metabolites into the ARC (Langlet et al. 2013). Intriguingly, tanycytes may be a novel population of adult neural stem cells in the hypothalamus. Tanycytes express stem cell markers, including nestin and SOX2 (Lee et al. 2012), and lineage-tracing studies have shown that they give rise to neurons in vivo with functional implications. While short-term HFD feeding promotes hypothalamic neurogenesis in pre-adult ages (Lee et al. 2012), chronic
HFD administration causes depletion of hypothalamic neural stem cells (Li et al. 2012). Furthermore, the manipulation of hypothalamic neurogenesis in adult mice has also produced divergent results. Selective inhibition of ME neurogenesis in adult mice fed a HFD resulted in reduced weight gain and adiposity due to enhanced energy expenditure (Lee et al. 2012). By contrast, genetic IKKβ/NF-kB activation in SOX2-positive hypothalamic cells led to overeating and weight gain (Li et al. 2012). It is important to note that these strategies did not exclusively target tanycytes and so these metabolic effects cannot be solely attributed to this cell type. Together, these results indicate that neurogenesis after short- or long-term HFD administration may have a compensatory or detrimental effect respectively on cell fate. These differences can also be the consequence of targeting distinct tanycyte populations (Bolborea & Dale 2013).

**Epigenetic mechanisms**

The interplay between genetic and environmental factors (nutrition, maternal health, chemicals, lifestyle, etc.) during the prenatal or perinatal period and their influence on the development of energy balance and metabolic alterations into adulthood have recently received substantial interest. In both humans and animal models, prenatal or perinatal nutritional manipulations lead to chronic metabolic disturbances in terms of feeding behavior, energy expenditure, leptin sensitivity, and glucose homeostasis. These metabolic defects may be partially the consequence of abnormal development of appetite-regulating neuronal circuits due to perinatal programming (Contreras et al. 2013). Epigenetic changes have been proposed as likely candidates to mediate, at least in part, these neuronal programming events, but a limited number of studies have explored this hypothesis. The epigenetic machinery that controls chromatin dynamics includes DNA methylation, posttranslational histone modifications, and non-coding RNAs. Neonatal overfeeding in rats, which results in overweight and the metabolic syndrome, is associated with the hypermethylation of the Pomc gene promoter (Plagemann et al. 2009). The extent of this DNA methylation is negatively correlated with the expression of POMC in relation to leptin and insulin levels, indicating the functionality of acquired epigenomic alterations (Plagemann et al. 2009). In the same overnutrition model, Plagemann et al. (2010) also found increased methylation of the Insr promoter in the hypothalamus. Similarly, epigenetic remodeling of hypothalamic genes induced by mild maternal undernutrition (Stevens et al. 2010, Begum et al. 2012) or stress (Paternain et al. 2012) has also been reported to be associated with altered energy balance and metabolism in experimental animal models. In humans, different methylation patterns of POMC and NPY promoter regions in leukocytes have been proposed as biomarkers to predict weight regain after an energy restriction program (Crujeiras et al. 2013). Collectively, this evidence supports the hypothesis that early prenatal or postnatal environmental perturbations cause chronic metabolic alterations that are partially the consequence of epigenetic changes in key genes and areas of the CNS involved in the control of energy balance. Nevertheless, further research is warranted to address the significance of these epigenetic events.

MicroRNAs (miRNAs), a class of small, non-coding RNAs that regulate gene expression at the posttranscriptional level, have recently been suggested to be involved in the hypothalamic control of energy balance. It has been demonstrated that the expression of Dicer1, an essential endoribonuclease for miRNA maturation, is regulated by nutrient availability and excess in the hypothalamus (Schneeberger et al. 2013). Furthermore, we have also shown that deletion of Dicer1 in POMC neurons leads to an obese phenotype characterized by increased adiposity, hyperleptinemia, defective glucose metabolism, and alterations in the pituitary-adrenal axis. This phenotype is associated with a progressive POMC neuron degeneration, indicating a key role for miRNAs in the survival of this population of neurons (Greenman et al. 2013, Schneeberger et al. 2013). High-throughput sequencing studies in ARC and PVN of rats have shown a specific miRNA enrichment pattern that could be used to define a prototypic profile in these brain regions. These miRNAs include seven of the eight genes of the let-7 family, the two miR-7 genes, miR-9 gene, and 5′ copy of the three miR-30 loci (Amar et al. 2012). Moreover, in situ hybridization experiments have revealed a limited and distinct expression of miR-7a in the hypothalamus, preferentially colocalizing with AgRP neurons (Herzer et al. 2012). Despite these efforts in describing the miRNA transcriptome and patterns of expression in the hypothalamus, the role of specific miRNAs in particular neuronal circuits in the regulation of whole-body energy balance still remains unknown.

**Concluding remarks: neuronal circuitry integration and physiological responses**

As has been outlined above, organismal energy balance is regulated by many factors through complex and
multi-level integration processes that involve multiple neuronal circuits. The homeostatic system is basically influenced by long-term (leptin and insulin) and short-term (GI hormones and vagal inputs) signals that act in concert to engage specific neuronal circuits in the hypothalamus and brainstem aimed at fulfilling whole-body metabolic needs. In addition to this homeostatic module, the corticolimbic and mesolimbic centers (which include the ventral tegmental area, nucleus accumbens, prefrontal cortex, hippocampus, and amygdala) integrate cognitive, hedonic, and emotional stimuli in a non-homeostatic process (Berthoud 2011). Circulating energy balance signals, such as leptin and ghrelin, also target hedonic networks to modulate appetite. However, this system may override homeostatic control and cause energy imbalance (Berthoud 2011). In fact, striking similarities between food reward and drug addiction mechanisms have been reported (DiLeone et al. 2012). Therefore, these complex interactions between the homeostatic and non-homeostatic systems culminate in coordinated appetite and energy balance regulation through the modulation of endocrine, autonomic, and behavioral outputs (Fig. 2). The precise integrative mechanisms of these different levels of regulation and the generation of specific physiological outputs are among the main unsolved enigmas of the central regulation of energy balance.

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

**Funding**
This work was supported by RecerCaixa grant number 2010ACUP_00275; EFSD/Lilly Fellowship Award; Ministerio de Ciencia e Innovación (MICINN), Instituto de Salud Carlos III (ISCIII) grant number PI10/01074; and MICINN grant number SAF2010-19527 (R G). M S is a recipient of an undergraduate grant from the University of Barcelona. M C is a recipient of a Miguel Servet contract (CP09/00233) from MICINN-ISCIII. Some of these grants are co-financed by the European Regional Development Fund ‘A way to build Europe’. This work was carried out in part at the Esther Koplowitz Centre, Barcelona.
References
Bagdade JD, Bierman EL & Porte D Jr 1967 The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic humans. Journal of Clinical Investigation 46 1721–1730. (doi:10.1172/JCI39620)
Butler AA, Kesterson RA, Khong K, Cullen MJ, Pellemounter MA, Dekoning J, Baetscher M & Cone RD 2000 A unique metabolic


Thematic Review

M Schneebberger and others

Neuronal circuits and energy balance

220:2 T41


Lee YS, Jensen PB, Madsen OD, Vrang N et al. 1998 Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393 72–76. (doi:10.1038/29993)


Mizuno TM & Mobbs CV 1999 Hypothalamic agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. Endocrinology 140 814–817. (doi:10.1210/en.140.2.814)

Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA & Mobbs CV 1998 Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and in db/db and ob/db mice, but is stimulated by leptin. Diabetes 47 294–297. (doi:10.2337/dbi7.4.2.294)


Olofsson LE, Unger EK, Cheung CC & Xu AW 2013 Modulation of AgRP-neuronal function by SOCS3 as an initiating event in diet-induced...
Thematic Review

M SCHNEEBERGER and others

Neuronal circuits and energy balance

Journal of Endocrinology

220:2

T44


Sawchenko PE & Swanson LW 1982 Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. Journal of Comparative Neurology 205 260–272. (doi:10.1002/cne.9020510306)


Received in final form 29 October 2013
Accepted 7 November 2013
Adipocytokines in obesity and metabolic disease

Haiming Cao
National Heart, Lung and Blood Institute, National Institutes of Health, Center for Molecular Medicine, 10 Center Drive, Building 10, BN109, MSC 1760, Bethesda, Maryland 20892, USA

Abstract

The current global obesity pandemic is the leading cause for the soaring rates of metabolic diseases, especially diabetes, cardiovascular disease, hypertension, and non-alcoholic hepatosteatosis. Efforts devoted to find cures for obesity and associated disorders in the past two decades have prompted intensive interest in adipocyte biology, and have led to major advances in the mechanistic understanding of adipose tissue as an essential endocrine organ. Adipose tissue secretes an array of hormones (adipokines) that signal key organs to maintain metabolic homeostasis, and their dysfunction has been causally linked to a wide range of metabolic diseases. In addition, obesity induces production of inflammatory cytokines (often referred to together with adipokines as adipocytokines) and infiltration of immune cells into adipose tissue, which creates a state of chronic low-grade inflammation. Metabolic inflammation has been increasingly recognized as a unifying mechanism linking obesity to a broad spectrum of pathological conditions. This review focuses on classic examples of adipocytokines that have helped to form the basis of the endocrine and inflammatory roles of adipose tissue, and it also details a few newly characterized adipocytokines that provide fresh insights into adipose biology. Studies of adipocytokines in clinical settings and their therapeutic potential are also discussed.

Key Words
- adipocytokine
- obesity
- adipokine
- metabolic inflammation
- adipocyte

Introduction

In the past two decades, the world has seen a sustained increase in obesity, and the levels of overweight and obese persons worldwide have reached epidemic proportions (Finucane et al. 2011). It is well established that obesity induces all major metabolic disorders, especially diabetes, cardiovascular disease, hypertension, and fatty liver disease (Eckel et al. 2005). Mounting evidence also links obesity to a growing list of debilitating disorders including neurodegenerative disease, airway disorders, and cancer, all of which contribute to the staggering morbidity and mortality associated with obesity. Aimed at developing effective therapies for obesity and its associated disorders, scientists worldwide have intensified their efforts to elucidate the pathophysiological mechanisms by which obesity induces or amplifies its major adverse consequences. The concept of an adipocytokine was developed in this process and dysfunction of adipocytokine pathways has been recognized as a key etiological factor of obesity-induced disorders. Furthermore, the rational manipulation of adipocytokines is becoming a promising avenue of therapy for obesity and associated metabolic abnormalities.

Endocrine function of adipose tissue and adipokines

Obesity is the expansion of white adipose tissue (WAT), the most effective lipid storage organ in the body. In obese
subjects, white adipocytes in WAT have increased release of free fatty acids (FFAs) through lipolysis process leading to elevated serum fatty acid levels. This overflow of lipids from obese adipose depots has been considered a key reason for obesity-associated insulin resistance and hepatosteatosis for several decades (Randle et al. 1963, Samuel et al. 2010). But fatty acids in this setting have often been considered as a whole, and studies examining the distinct impact of individual lipid species have provided intriguing insights into the specificities of adipose-secreted lipids (Cao et al. 2008). In 1994, leptin was identified as an adipose-secreted hormone (adipokine) that exhibits potent anorexigenic effects, and this finding redefined WAT as an endocrine organ (Zhang et al. 1994). In the following two decades, several more adipokines were identified as critical regulators of systemic lipid and glucose homeostasis, and the list continues to grow (Fig. 1). Adipokines mediate the crosstalk between adipose tissue and other key metabolic organs, especially the liver, muscle, and pancreas, as well as the CNS (Rosen & Spiegelman 2006). Consistent with this notion, dysfunctions in adipokine pathways often result in impaired organ communications and metabolic abnormalities in multiple tissues thereby constituting a critical pathological component in the development of metabolic disease (Trujillo & Scherer 2006).

Metabolic inflammation and adipokytines

Hotamisligil et al. (1993) showed that adipose tissue in obese mice secretes tumor necrosis factor α (TNFα), a proinflammatory cytokine typically produced by immune cells, and also demonstrated that adipocyte-derived TNFα plays a direct role in obesity-induced insulin resistance. This was the first functional link between obesity and inflammation, and over the years it has evolved into the concept of metabolic inflammation (Fig. 1), which has been widely accepted as an important mechanistic connection between obesity and its complications (Hotamisligil 2006). After TNFα, it was demonstrated that adipose tissue produces an array of cytokines and chemokines such as IL6 and MCP1, which either positively or negatively regulate systemic glucose and lipid metabolism. Interestingly some adipokines also exhibit features within adipose tissue and also travel to remote organs to regulate systemic energy metabolism. The level and action of adipokytines are often altered in obese subjects, which contribute to obesity-induced disorders.
of cytokines or regulate inflammatory responses, and so these two groups of adipose-derived factors are often collectively referred to as ‘adipocytokines’ (Fig. 1). In 2003, two studies simultaneously reported that obesity induces macrophage infiltration of adipose tissue in both mice and humans (Weisberg 2003, Xu et al. 2003b), which not only provided an explanation for the source of adipose-derived cytokines but also demonstrated for the first time the close juxtaposition between immune and metabolic cells in a metabolic organ. Adipose-resident macrophages are classified into two very distinct subtypes, M1, or classically activated, and M2, or alternatively activated. M1 macrophages secrete proinflammatory cytokines, such as TNFα and IL6, produce iNOS and reactive oxygen species (ROS), and cause insulin resistance. M2 macrophages produce IL10 and IL1 receptor antagonists and arginase-1 and have been implicated in tissue remodeling (Gordon 2003). Obesity causes a shift of macrophage subtypes in adipose tissue from M2 to M1 activation, leading to increased levels of proinflammatory cytokines and ROS, which induce insulin resistance (Lumeng et al. 2007). Meanwhile, the loss of certain beneficial effects associated with M2 macrophages might also contribute to the metabolic deterioration in obesity. For examples, M2 macrophages produce catecholamines that sustain adaptive thermogenesis (Nguyen et al. 2011), and lipolysis during fasting recruits macrophages that buffer local lipid increase and protect adipose function (Kosteli et al. 2010). Following macrophages, nearly every major type of immune cell has been identified in adipose tissue in recent years (Feuerer et al. 2009, Liu et al. 2009, Winer et al. 2009, Wu et al. 2011) and is actively involved in the endocrine function of adipose tissue in systemic metabolic regulation. Furthermore, the close physical and signaling interactions between immune and metabolic cells also exist in all major metabolic organs of obese subjects especially the liver, muscle, and pancreas, indicating that metabolic inflammation is a universal feature and a pathological basis for obesity-induced metabolic dysfunction.

There are a number of potential underlying causes for obesity-induced adipose inflammation. Adipose tissue expansion in the development of obesity can cause hypoxia which induce compensatory angiogenesis. Macrophages are recruited to the site to facilitate the vascularization process (Pang et al. 2008). Similar function of immune cells was also demonstrated in other metabolic tissues such as liver where Kupffer cell-secreted TNFα and IL6 in mouse liver are required for efficient liver regeneration (Abshagen et al. 2007). Infiltrated macrophages in adipose tissues have also been proposed to be a mechanism to remove apoptotic cells (Cinti et al. 2005, Strissel et al. 2007). In addition, endotoxemia associated with altered gut permeability and obesity might potentiate adipose inflammation (Cani et al. 2007). Although accumulating evidence supports an overall negative effect of adipose inflammation on energy metabolism, it should bear in mind that not all metabolic inflammation is detrimental to metabolic homeostasis. Inflammation associated with adipose expansion or repair might be necessary for the body to adapt to the excess energy and maintain metabolic homeostasis (Ye & McGuinness 2013). In the same vein, certain cytokines stimulate energy expenditure and reduce food intake which might help to curtail obesity (Ye & Keller 2010). Therefore, the metabolic outcomes of adipose inflammation should always be considered in the context of their physiological underpinnings, and more studies are needed to fully understand the extent and mechanism of beneficial inflammatory responses associated with different stages of obesity.

**Key adipocytokines in metabolic regulation and obesity-induced metabolic disorders**

**Leptin**

Leptin was identified through positional cloning by Zhang et al. (1994), and is one of most potent adipocytokines in metabolic regulation. Leptin regulates body weight by signaling nutritional status to other organs especially the hypothalamus, which produces neuropeptides and neurotransmitters that modulate food intake and energy expenditure (Friedman & Halaas 1998). Leptin also has anti-diabetic effects independent of its regulation of body weight and energy intake (Kamohara et al. 1997). Leptin regulates hepatic lipogenesis by suppressing the expression of key enzymes in the fatty acid synthesis pathway (Cohen et al. 2002) and enhances muscle fatty acid oxidation by activating a critical energy sensor AMPK (Minozoshi et al. 2002).

At the signaling level, leptin activates the leptin receptor, which has multiple splicing isoforms, although the long isoform mediates all known leptin actions (Lee et al. 1996). There are multiple pathways downstream of the leptin receptor, each of which mediates different aspects of leptin activities (St-Pierre & Tremblay 2012). The main signaling branch of leptin is the JAK–STAT pathway, which regulates expression of anorexic neuropeptides (Baumann et al. 1996). This pathway is essential...
for leptin regulation of energy balance but not its effects on reproduction (Bates et al. 2003). The anti-diabetic effect of leptin is mediated by centrally activating the phosphatidylinositol-3-kinase (PI3K)/AKT pathway that stimulates insulin sensitivity in the peripheral tissues (Morton et al. 2005).

In the light of the significance of metabolic inflammation in the pathogenesis of metabolic disease, it is worth mentioning that leptin bears striking similarity to cytokines and modulates immune responses (De Rosa et al. 2007). Leptin is structurally similar to Class I helical cytokines and shares the same JAK–STAT pathway down-stream of its receptor. Leptin expression can be induced by endotoxin or cytokine TNFα (Grunfeld et al. 1996). Conversely, leptin increases thymic secretion of acute-phase reactants and TNFα and promotes T helper 1 cell differentiation (La Cava & Matarese 2004). Leptin acts on T cell, macrophages, and other immune cells to stimulate the production of a wide spectrum of cytokines (La Cava & Matarese 2004). In light of the role of several cytokines in enhancing energy expenditure and suppressing food intake (Ye & Keller 2010), this proinflammatory action of leptin might contribute to its overall effects in body weight regulation. Interestingly, inflammation induced by metabolic stress also negatively regulates leptin signaling in a manner similar to insulin receptor signaling (Zhang et al. 2008). In addition, leptin has been implicated in a number of immune dysfunctions. For examples, leptin is able to reverse starvation-induced immunosuppression (Lord et al. 1998) and has been proposed to be a metabolic link to multiple sclerosis (Matarese et al. 2010).

Despite the thorough understanding of leptin’s actions and numerous attempts to target leptin for obesity and metabolic disorders (Coppari & Bjorbaek 2012), leptin’s clinical applications have been very limited. Leptin is used to treat genetically obese subjects carrying leptin mutations, but such mutations are extremely rare (Faroqui et al. 1999). Leptin is largely ineffective for treating regular obese patients due to leptin resistance caused by hyperleptinemia, and leptin administration into these individuals does not generate anorexic effects (Heymsfield et al. 1999). Leptin is successfully used to treat insulin resistance and hepatic steatosis in patients with congenital severe lipodystrophy who have very low levels of circulating leptin (Oral et al. 2002, Petersen et al. 2002). With increased mechanistic understanding of leptin resistance (St-Pierre & Tremblay 2012), it is still possible that approaches to enhance leptin sensitivity could help to revive some of stalled attempts to target leptin for anti-obesity and anti-diabetic therapies.

Adiponectin

Several research groups identified adiponectin almost simultaneously as an abundantly secreted adipokine (Scherer et al. 1995, Hu et al. 1996, Maeda et al. 1996, Nakano et al. 1996). Recombinant adiponectin can enhance insulin action and partially reverse insulin resistance in obese mice (Berg et al. 2001, Yamauchi et al. 2001). Consistently, multiple groups have reported that adiponectin-deficient mice develop insulin resistance associated with high level of TNFα in adipose tissue and reduced responsiveness to PPARα (Maeda et al. 2002, Nawrocki et al. 2006), although an independently generated adiponectin knockout mouse line has no change in insulin sensitivity (Ma et al. 2002). Adiponectin has also been reported to have antiatherogenic effects (Funahashi et al. 1999, Ouchi et al. 1999). In addition, adiponectin exhibits cardioprotective activity in ischemic heart disease through AMPK and cyclooxygenase 2 pathways (Shibata et al. 2005).

Adiponectin signaling is mediated by two adiponectin receptors, adipor1 and adipor2 (Yamauchi et al. 2003). Adipor1 is ubiquitously expressed whereas adipor2 is enriched in the liver tissue. Knockout of adipor1 and adipor2 abrogates adiponectin binding and causes lipid accumulation, inflammation, and insulin resistance (Yamauchi et al. 2007). Activation of adipor1 in the liver and muscle tissues increases AMPK activity, which mediate the insulin sensitizing effect of adiponectin and also enhances fatty acid oxidation (Yamauchi et al. 2002). The adipor2 pathway in the liver increases PPARα and expression of its target genes, which also results in increased fatty acid oxidation (Yamauchi et al. 2007). Recently, it has been reported that a variety of down-stream effects of the adiponectin receptor are mediated by ceramidase activity associated with adipor1 and adipor2 (Holland et al. 2011). Adiponectin also has anti-inflammatory effects that contribute to its protective role against metabolic stress in obesity. Adiponectin suppresses TNFα production in obese mice (Xu et al. 2003a), and adiponectin-deficient mice have high levels of TNFα in adipose tissue (Maeda et al. 2002). Low levels of plasma adiponectin are associated with C-reactive protein in humans (Ouchi et al. 2003). Adiponectin enhances the clearance of apoptotic cells by facilitating their opsonization and uptake by macrophages (Takemura et al. 2001). Some of the anti-atherogenic effects of adiponectin are also mediated by its role in the suppression of inflammatory responses. Adiponectin inhibits nuclear factor-κB (NFκB) activity and its downstream adhesion molecules.
leading to reduced monocyte adhesion to endothelial cells (Ouchi et al. 1999, Okamoto et al. 2002). In addition, adiponectin confers vascular-protective activities by suppressing the apoptosis of endothelial cell (Kobayashi et al. 2004).

Clinical observations support the idea that plasma adiponectin levels are associated with obesity-induced disorders, especially diabetes. Plasma adiponectin levels are decreased in type 2 diabetic patients, and higher adiponectin levels are associated with low risk of diabetes (Li et al. 2009). Adiponectin levels are also negatively associated with adiposity and fasting glucose (Ryo et al. 2004). A multi-ethnic meta-analysis of a large cohort also demonstrated that numerous genetic loci associated with adiponectin levels influence risk of insulin resistance and type 2 diabetes (Dastani et al. 2012). Currently, several strategies to boost adiponectin levels or adiponectin receptor activities are being explored for the treatment of obesity-induced inflammation and insulin resistance (Yamauchi & Kadowaki 2008).

**Tumor necrosis factor α**

TNFα was the first cytokine identified in the adipose tissue of obese mice, marking the start of the metabolic inflammation concept (Hotamisligil et al. 1993). The direct involvement of TNFα in obesity-induced insulin resistance was confirmed by observations that TNFα treatment interferes with insulin signaling and blocks insulin actions (Hotamisligil et al. 1994). Mice lacking the functions of TNFα or its receptors are protected from obesity-induced insulin resistance and hyperglycemia (Uysal et al. 1997, 1998). It was initially thought that adipose-derived TNFα was produced mainly by adipocytes, but the parallel trend of macrophage infiltration and TNFα expression in adipose tissue of obese mice suggests that a significant portion of the adipose TNFα pool might be derived from macrophages and other immune cells. Interesting, FFA strongly stimulates TNFα production in macrophages (Nguyen et al. 2005) and in turn, TNFα stimulates lipolysis to increase fatty acid release from adipocytes (Wang et al. 2008). This FFA-cytokine cycle suggests that metabolic inflammation, once started, can use this self-perpetuating mechanism to further its inhibitory effects on insulin signaling and energy metabolism. In addition, TNFα directly stimulates hepatic lipogenesis *in vivo* (Feingold & Grunfeld 1987), and adipose-derived TNFα is also a major mechanistic link between obesity and cancer (Park et al. 2010).

TNFα exerts its effects through two distinct receptors, p55 and p75, which further activate JNK1 and inhibit IκB kinase(IKK)/NFκB pathways (Baud & Karin 2001). JNK1 can directly inhibit insulin signaling by phosphorylating insulin receptor substrate 1 (IRS1) on serine residues (Aguirre et al. 2002) and can also potentiate fatty acid-induced cytokine production (Nguyen et al. 2005). Consistent with these observations, JNK1 knockout mice are protected from obesity and insulin resistance (Hirosumi et al. 2002). IKK can also directly inhibit IRS1 function through serine phosphorylation in a manner similar to JNK1 (Gao et al. 2002) and also activate NFκB to produce inflammatory cytokines both in metabolic organs and myeloid cells. It has been demonstrated that systemic or selective inhibition of IKK in either hepatocytes or myeloid cells improves glucose metabolism in mice (Yuan et al. 2001, Arkan et al. 2005, Cai et al. 2005). TNFα also induces the expression of cytokine signaling 3 (SOCS3) suppressor, which inhibits insulin signaling by increasing ubiquitin-mediated IRS1 and IRS2 degradation (Emanuelli et al. 2001, Rui et al. 2002). Recently, a report has demonstrated that TNFα increase leptin receptor expression, raising an interesting possibility that TNFα might enhance leptin action (Gan et al. 2012), although the physiological relevance of this connection needs to be confirmed in an *in vivo* setting.

Numerous studies in humans have demonstrated strong associations between circulating TNFα and insulin resistance (Hivert et al. 2008) or other obesity-associated metabolic complications (Berg & Scherer 2005). However, attempts to block TNFα function in patients have not yet produced consistent metabolic outcomes. For example, neutralization of TNFα with an engineered antibody did not improve insulin sensitivity in type 2 diabetes patients (Ofei et al. 1996), whereas blockade of TNFα in patients with rheumatoid arthritis or psoriasis indeed improved their insulin resistance (Gonzalez-Gay et al. 2006, Lo et al. 2007). Considering the wide spectrum of inflammatory cytokines that are elevated in obesity, targeting TNFα alone might not have sufficient efficacy to improve systemic metabolic responses and might need to be considered in the context of managing the overall metabolic inflammation.

**Resistin**

Resistin was initially identified in a screen for adipocyte genes that are suppressed by insulin-sensitizing drugs in rodents (Steppan et al. 2001). Depletion of circulating resistin by a neutralizing antibody improves insulin action.
in obese mice, suggesting that resistin is an adipokine linking obesity to insulin resistance (Steppan et al. 2001). Subsequently, it was shown that resistin knockout mice on a high-fat diet have improved glucose metabolism mainly due to reduced glucose production in the liver (Banerjee et al. 2004). Resistin also expresses the expressions of cytokines and adhesion molecules in murine vascular endothelial cells and contributes to atherogenesis (Burnett et al. 2005). Resistin circulates in two distinct assembly states, which exhibit differential activities in metabolic regulation (Patel et al. 2004). However, the relevance of resistin to human disease is complicated by the fact that rodent resistin is produced in adipocytes and human resistin is produced mostly in macrophages. Human and rodent resistin only shares 59% identity at the amino acid level, which is relatively low compared with other hormones (Ghosh et al. 2003). But interestingly, human resistin, when expressed in mouse macrophages, also induces insulin resistance (Qatanani et al. 2009) suggesting that human and mouse resistin might have similar function despite their different sites of production.

In humans, experimental endotoxemia induced elevated resistin and produced an insulin-resistant state (Lehrke et al. 2004). Epidemiological studies have associated elevated circulating resistin with increased risk for type 2 diabetes, inflammatory markers, myocardial infarction, and atherosclerosis (Burnett et al. 2005, 2006, Reilly et al. 2005, Heidemann et al. 2008, Chen et al. 2009). These studies support the idea that resistin levels could serve as an informative marker for metabolic disease in humans, and it will be of great interest to determine the therapeutic potential of resistin inhibition in future studies.

**IL6**

IL6 is one of the major pro-inflammatory cytokines whose expression level increases in the adipose tissue of obese mice and patients, but its role in glucose metabolism has not been fully resolved. IL6 depletion in obese mice with a neutralizing antibody improves hepatic insulin action (Klover et al. 2005) while chronic infusion of IL6 causes insulin resistance in the liver of mice (Klover et al. 2003). Conversely, mice with targeted ablation of IL6 develop obesity and insulin resistance, which can be reversed by centrally delivered exogenous IL6 (Wallenius et al. 2002) suggesting that IL6 is required for the maintenance of whole-body glucose metabolism and metabolic homeostasis. An independently generated IL6-targeted mutation mouse line, however, does not develop obesity or insulin resistance and only exhibits elevated glucose level in a glucose tolerance test (Di Gregorio et al. 2004). In a mouse model with adipose-specific ablation of JNK1, increased secretion of IL6 was proposed to be the primary reason for systemic insulin resistance (Sabio et al. 2008). There are several potential explanations for the seemingly contradictory data regarding IL6 in insulin action and glucose metabolism. Effects of acute vs chronic treatments need to be differentiated and dose and site of action of IL6 need to be carefully considered. In addition, IL6 produced by different organs might also contribute to its complex effects on metabolic regulation.

During exercise, IL6 is mainly released from working skeletal muscle. IL6 release from contracting skeletal muscle might mediate the beneficial effects associated with exercise, including increased glucose uptake and fatty acid oxidation (Febbraio & Pedersen 2002). It appears that activation of AMPK by IL6 mediates these effects (Al-Khalili et al. 2006). In addition, transgenically expressed human IL6 in mice increases leptin sensitivity and prevents diet-induced obesity (Sadagurski et al. 2010). However, the function of muscle-derived IL6 might also vary depending on its context. In a mouse model with muscle-specific disruption of PPARγ coactivator 1α (PGC1α), muscle-secreted IL6 causes impaired insulin production from pancreatic islets and glucose intolerance (Handschin et al. 2007).

In patient studies, increased serum IL6 correlates with obesity and insulin resistance (Vozarova et al. 2001, Bastard et al. 2002, Spranger et al. 2003). The IL6 174G>C single nucleotide polymorphism (SNP) is associated with insulin resistance and metabolic syndrome (Fernandez-Real et al. 2000, Stephens et al. 2007). However, the mechanism of action of IL6 in human metabolism needs to be further studied to understand the therapeutic potential of IL6, partly due to the fact that there is low similarity between human and mouse IL6, and thus information generated from mouse studies cannot be readily applied to humans. To add to the complexity of IL6 signaling in human metabolism, two reports showed that MAB against the IL6 receptor, Tocilizumab, either increases or has no effects on insulin sensitivity in patients with rheumatoid arthritis (Schultz et al. 2010, Ogata et al. 2011, Ye & McGuinness 2013).

**Rbp4**

Rbp4 is a transport protein for retinol in systemic circulation, and is mainly produced by the liver but also expressed in white adipocytes. Rbp4 was first characterized as an adipokine based on the finding that Rbp4 is highly
Secreted frizzled-related protein 5

Secreted frizzled-related protein 5 (Sfrp5) was recently identified as an anti-inflammatory adipokine (Ouchi et al. 2010). Sfrp5 is highly expressed in adipose tissue of lean mice but downregulated in obese mice. Targeted mutation of Sfrp5 in mice caused insulin resistance, glucose intolerance, and hepatosteatosis when the animals were fed a high-fat diet (Ouchi et al. 2010). Mechanistically, Sfrp5 activates JNK1 through noncanonical Wnt signaling to increase the levels of inflammatory cytokines and block insulin action (Ouchi et al. 2010). However, a second independently generated Sfrp5 mutation mouse line was reported to have different phenotypes, and accordingly the authors proposed a very different mechanism of actions for Sfrp5. In this study, Sfrp5-deficient mice were resistant to diet-induced obesity due to enhanced mitochondrial activities (Mori et al. 2012). Sfrp5 deficiency increased the expression of PGC1 and mitochondrial transcription factor A (Tfam), leading to increased mitochondrial biogenesis. Lack of Sfrp5 also stimulated mitochondrial respiration and gene expression through Wnt3a activity (Mori et al. 2012). The cause of these discrepancies is unclear. Human studies regarding Sfrp5 in metabolic disease have also given rise to conflicting data (Carstensen et al. 2013, Hu et al. 2013). Regardless, further studies about the function of Sfrp5 in metabolic regulation could provide important insights into adipose biology. Sfrp5 regulates multiple Wnt proteins that play a crucial role in adipogenesis (Cristancho & Lazar 2011). Dissecting the Sfrp5/Wnt network in adipose tissue could also help to explain the autocrine/paracrine mechanism of metabolic inflammation, which is still poorly understood.

aP2, a lipid-activated adipokine

The identification of aP2 as a lipid-activated adipokine is a surprising and exciting finding considering it has been extensively studied for over two decades as an essential intracellular regulator of lipid metabolism and inflammation in metabolic disease. AP2 is a member of fatty acid-binding protein (FABP) family and was initially thought to be exclusively expressed in adipocytes. In fact, the aP2 promoter has been widely used to specially drive transgene expression in adipose tissue. AP2-deficient mice have normal adiposity and gain more weight than controls when fed with high-fat diet, but they were partially protected from obesity-induced insulin resistance (Hotamisligil et al. 1996). The mild effect of aP2 deficiency could be due to the upregulation of mal1, a related FABP (Maeda et al. 2005). Therefore, mice deficient in both FABPs were produced to study the full impact of adipose FABP deficiency. The double-knockout mice have reduced adiposity, enhanced insulin sensitivity, and reduced hepatosteatosis (Maeda et al. 2005). It appears that some of the beneficial effects of FABP deficiency are mediated by robust upregulation of the fatty acid species, palmitoleate (C16:1n7), in the adipose tissue and its secretion into circulation (Fig. 2). Palmitoleate enhances insulin action in the muscle and suppresses de novo lipogenesis in the liver (Cao et al. 2008).

Yet the molecular mechanism for the pronounced reduction in gluconeogenesis in FABP-deficient mice remained elusive until it was found that aP2 is in fact actively secreted from adipocytes to control liver glucose metabolism (Cao et al. 2013; Fig. 2). Secretion of aP2 from adipocytes is regulated by lipolysis, which might be the reason that circulating aP2 levels are markedly elevated in obesity. Recombinant aP2 stimulates hepatic glucose production whereas neutralization of secreted aP2 reduces glucose production and corrects the diabetic phenotype of obese mice (Cao et al. 2013).

aP2 is the first adipokine whose secretion is strongly regulated by lipolysis-released fatty acids, suggesting that aP2 might function as a lipid sensor in adipocytes and might also carry specific lipids in plasma to specific organs or cells. Therefore, like other well-studied adipocytokines, it is conceivable that secreted aP2 could potentially act on other key organs such as the CNS or heart to regulate other aspects of metabolic homeostasis (Fig. 2) and these
questions need to be addressed in future studies. Another interesting question is whether secreted aP2 is also involved in metabolic inflammation. Despite long having been considered an adipocyte-specific protein, aP2 was found to be expressed in the macrophages (Makowski et al. 2001) and can be quickly induced by endotoxin (Kazemi et al. 2005). Mice with aP2 deficiency in macrophages are protected from atherosclerosis partly because of activated PPARγ and reduced inflammatory responses (Makowski et al. 2005). The proinflammatory action of aP2 was also demonstrated in an asthma mouse model, in which aP2 deficiency protects mice from airway inflammation (Shum et al. 2006). It will be interesting to investigate whether aP2 is also secreted from macrophages and whether secreted aP2 regulates inflammatory responses in metabolic diseases (Fig. 2).

Accumulating evidence suggests that circulating aP2 is implicated in human metabolic syndrome. Plasma aP2 levels are closely associated with obesity and metabolic syndrome in cohorts of multiple ethnicities (Stejskal & Karpisek 2006, Xu et al. 2006, Simon et al. 2009). In addition, circulating aP2 has also been linked to carotid atherosclerosis in humans (Yeung et al. 2007) and non-alcoholic fatty liver disease (NAFLD; Koh et al. 2009). In NAFLD patients, elevated plasma aP2 levels independently predict inflammation and fibrosis (Milner et al. 2009). Neutralizing secreted aP2 robustly improves glucose metabolism (Cao et al. 2013), indicating that plasma aP2 could constitute a potential therapeutic target for diabetes, NAFLD, and cardiovascular disease.

**Conclusion and future perspective**

There is overwhelming evidence that adipocytokines play a pivotal role in metabolic homeostasis of healthy subjects, and that deficiencies in these factors, caused by excess adiposity and adipocyte dysfunction, are a central component in the pathogenesis of the constellation of diseases surrounding obesity. Therefore, it will be fruitful to fully define the secretome of adipose tissue; novel adipocytokines identified in this process will, no doubt, provide critical insights into the functions of adipose tissue as an essential metabolic regulator. Identifying receptors for existing adipocytokines and mapping their downstream signaling pathways, especially in the context of metabolic disorders, is another area of research that could generate fresh therapeutic targets for managing adipocytokines to treat metabolic diseases. Due to the intertwined nature of metabolic and immune cells in major metabolic organs, further mechanistic
investigations are required to understand how adipocytokines integrate metabolic and inflammatory responses in each site and the pathological significance of these responses in metabolic disorders. It is particularly important to differentiate the detrimental effects of metabolic inflammation inflicted by nutritional stress and those beneficial ones underlying the physiological tissue expansion when designing anti-inflammation therapies for metabolic disorders (Ye & McGuinness 2013). Following the example of adipocytokines, numerous muscle- and hepatocyte-secreted hormones (myokine and hepatokine) have been identified as essential metabolic regulators. Therefore, it is very likely that a comprehensive endocrine network of organ communications in nutrient sensing and metabolic homeostasis could be established in the foreseeable future. Such a blueprint of organ crosstalk would have far-reaching impact on the development of effective therapies against obesity and metabolic disease.

---

**Declaration of interest**

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

**Funding**

H.C is funded by the Division of Intramural Research of the National Heart, Lung and Blood Institute (HL006103-02) of the NIH, USA. The author expresses his apology for not being able to cite all worthy papers owing to space limitation.

**References**


Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE & White MF 2002 Hepatocyte-secreted hormones (myokine and hepatokine) have been identified as essential metabolic regulators. *Nature Medicine* 7 947–953. (doi:10.1038/nm992)


Burnett MS, Devaney JM, Adenika RJ, Lindsay R & Howard BV 2006 Cross-sectional associations of resistin, coronary heart disease, and insulin resistance. *Journal of Clinical Endocrinology and Metabolism* 91 64–68. (doi:10.1210/jc.2005-1653)


H C is funded by the Division of Intramural Research of the National Heart, Lung and Blood Institute (HL006103-02) of the NIH, USA. The author expresses his apology for not being able to cite all worthy papers owing to space limitation.


Hotamisligil GS, Murray DL, Choy LN & Spiegelman BM 1994 Tumor necrosis factor-α inhibits signaling from the insulin receptor. PNAS 91 4854–4858. (doi:10.1073/pnas.91.11.4854)


macrophages produce catecholamines to sustain adaptive thermogenesis. Nature 480 104–108. (doi:10.1038/nature10653)


Fatty acid metabolism, energy expenditure and insulin resistance in muscle

Nigel Turner1,2, Gregory J Cooney3,4, Edward W Kraegen2,3 and Clinton R Bruce5

1Department of Pharmacology and 2School of Medical Sciences, University of New South Wales, Sydney, New South Wales, Australia
3Diabetes and Obesity Division, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, New South Wales 2010, Australia
4St Vincent’s Clinical School, University of New South Wales, Sydney, New South Wales, Australia
5Department of Physiology, Monash University, Clayton, Victoria 3800, Australia

Correspondence should be addressed to G J Cooney
Email g.cooney@garvan.org.au

Abstract

Fatty acids (FAs) are essential elements of all cells and have significant roles as energy substrates, components of cellular structure and signalling molecules. The storage of excess energy intake as fat in adipose tissue is an evolutionary advantage aimed at protecting against starvation, but in much of today’s world, humans are faced with an unlimited availability of food, and the excessive accumulation of fat is now a major risk for human health, especially the development of type 2 diabetes (T2D). Since the first recognition of the association between fat accumulation, reduced insulin action and increased risk of T2D, several mechanisms have been proposed to link excess FA availability to reduced insulin action, with some of them being competing or contradictory. This review summarises the evidence for these mechanisms in the context of excess dietary FAs generating insulin resistance in muscle, the major tissue involved in insulin-stimulated disposal of blood glucose. It also outlines potential problems with models and measurements that may hinder as well as help improve our understanding of the links between FAs and insulin action.

Key Words

- fatty acid metabolism
- fatty acids and energy expenditure
- muscle insulin resistance

Overview

Fatty acids (FAs) are organic acids largely defined by the length and saturation of the aliphatic side chain attached to a carboxylic acid. In animals, these side chains normally contain an even number of carbon atoms and FAs are grouped into short chain (2–6 carbon atoms), medium chain (8–12 carbon atoms), long chain (14–18 carbon atoms) and very long chain (20–26 carbon atoms). The major types of FAs in the circulation and in the tissues of mammals are the long-chain and very-long-chain FAs with varying degrees of saturation. These include palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6) and, particularly in smaller mammals, arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3). These FAs are the major components of storage triglycerides and cellular membranes, and although C16–C18 FAs are also components of some of the FA-derived signalling molecules (diacylglycerols (DAGs) and ceramides), many of the major lipid signalling molecules (prostaglandins and leukotrienes) are synthesised from very-long-chain, unsaturated FAs (e.g. arachidonic and docosahexaenoic acids) (Kruger et al. 2010).

In the context of the links between excessive lipid storage (obesity) and reduced insulin action (insulin
resistance) in muscle, this article will deal with FAs as an alternative energy substrate to glucose, the relevance of this substrate competition to overall energy expenditure and an assessment of the various mechanisms by which excess FA availability is thought to reduce insulin action in muscle and predispose to metabolic diseases.

Fuel for energy production

All three of the major types of macromolecules that make up organic material (carbohydrates, proteins and fats) can be broken down and oxidised to provide energy for the maintenance, growth and reproduction of biological systems. In animals, all proteins have a cellular function (e.g. as enzymes, or with structural or carrier function), and there is no identifiable depot of proteins specifically manufactured and stored solely for future use in energy production. On the other hand, carbohydrates and fats in various forms have specific and important functional roles in cells, but are also present in animal tissues as energy storage depots of glucose polymers (glycogen) and lipid droplets (triglycerides). Although glycogen and triglyceride stores can be found in nearly all tissues, glycogen stored in the liver is critical for the maintenance of blood glucose levels when glucose is not being absorbed from the gut and triglycerides stored in adipose tissue act as an alternative, more reduced and higher energy-yielding substrate (in terms of energy per gram) for energy production in tissues with a capacity for fat oxidation. Although excess protein intake can be converted to glucose and FAs for energy storage and glucose can also be converted to fat for energy storage or amino acids for protein synthesis, it is one of the maxims of energy metabolism that fat cannot be quantitatively converted to carbohydrate or protein. Essentially, this means that FAs stored in adipose tissue can only be used as an energy source to support cellular functions or to provide specific precursors that are needed to replace or expand the structure or signalling functions of FAs.

The contribution of different tissues to fuel oxidation and energy expenditure

Some tissues have an obligatory need for glucose (brain, red blood cells and retinal cells), while most tissues have the capacity to switch between glucose and FAs. The contribution of different fuels to energy production in specific tissues and the contribution of different tissues to the overall energy production and utilisation in the whole body vary quite markedly. Because of its relative size in man and most animals, muscle is considered to be a major tissue for the disposal of both glucose (James et al. 1985, Shulman et al. 1990) and FAs (Furler et al. 2000). Because of the ability of muscle to substantially increase energy expenditure during exercise (Bangsbo 2000), this tissue is also very flexible in its capacity to act as a sink for energy substrates. Other tissues such as the heart have a similar capacity to increase both the amount and type of substrate oxidation depending on demand, but because of the relative size of heart to muscle in the body, the overall contribution of the heart to whole-body substrate oxidation is only 5–10% (Rolfe & Brown 1997). The liver has a significant role in the disposal of glucose after a meal and in the provision of glucose to the circulation to maintain blood glucose levels when nutrients are not being absorbed from the gut. The liver also has the ability to take up FAs, oxidise them or package them in lipoproteins for export and storage in other tissues and is therefore central to lipid and glucose homoeostasis (Postic et al. 2004, Moore et al. 2012). Adipose tissue can, particularly in obese individuals, be the tissue contributing most to whole-body mass, but per unit mass it does not have a major impact on whole-body glucose disposal (Kraegen et al. 1985, Ng et al. 2012). White adipose tissue also has little impact on the whole-body oxidation of FAs, although there is significant current research interest in investigating whether white adipocytes can acquire a more oxidative brown adipocyte phenotype with a greater contribution to whole-body substrate oxidation and energy expenditure (Wu et al. 2013).

The effect of fibre composition and exercise on substrate utilisation by muscle

Although the musculature as a whole is a major contributor to total body glucose and FA metabolism (Ng et al. 2012), individual muscles may contribute differently depending on their fibre composition. Type 1 red muscle fibres are considered more insulin sensitive, with a greater oxidative capacity for glucose and FAs, while type II white muscle fibres contain less mitochondria, are considered less insulin sensitive and contribute less to FA oxidation (Nyholm et al. 1997, Pearen et al. 2012). Therefore, a higher composition of type 1 red fibres in muscle has been reported to be associated with increased insulin responsiveness (Stuart et al. 2013). This view has been challenged by some recent studies where genetically manipulated mice (Izumiya et al. 2008, Meng et al. 2013) and pharmacological approaches (Akpan et al. 2009) suggest that altering the fibre composition of muscles towards
glycolytic type II fibres improves glucose homoeostasis and insulin action in the whole animal. It does seem important to consider that the contribution of the skeletal musculature to whole-body energy metabolism and substrate oxidation should not be based on the assessment of these parameters in a single muscle type. Acute exercise and exercise training also have a significant impact on substrate preference and utilisation at a whole-body and muscle level (Spriet & Watt 2003, Kiens 2006). Some of these effects correlate with observed shifts in muscle size and fibre type that occur with training (Shaw et al. 2012, Stuart et al. 2013), but other adaptations in muscle metabolism and body organs could also contribute to changes in energy metabolism and substrate utilisation associated with exercise (Laughlin & Roseguini 2008).

Linking substrate oxidation to energy conservation and energy expenditure

The pathways by which different fuels are oxidised to support tissue and cellular energy demands in animals are thoroughly dealt with in major textbooks and summarised in Fig. 1. Through the glycolytic pathway, pyruvate dehydrogenase (PDH) and the tricarboxylic acid (TCA) cycle, glucose can be completely oxidised to CO2 and the energy released (as reducing equivalents) harnessed in the form of NADH and FADH2. NADH+H+ and FADH2 are reoxidised by the electron transport chain (ETC) and the reducing equivalents used to reduce atomic oxygen to water. The electrochemical (proton) gradient generated by the ETC then drives ATP synthesis via ATP synthase (Fig. 1). The oxidation of FAs by the mitochondrial β-oxidation pathway also produces NADH+H+ and FADH2 for the ETC and acetyl CoA that can also be completely oxidised in the TCA cycle. Because FAs are chemically more reduced molecules than carbohydrates, FAs are theoretically able to produce more energy when completely oxidised than an equivalent carbohydrate molecule. In other words, the complete oxidation of six-carbon glucose consumes six oxygen molecules and produces six carbon dioxide molecules accompanied by the synthesis of 36 ATP molecules. On the other hand, the complete oxidation of six-carbon hexanoic acid consumes eight oxygen molecules and produces six carbon dioxide molecules for 44 ATP molecules. Such calculations are based on a fixed stoichiometry between NADH+H+ and FADH2 oxidation and ATP synthesis and can lead to the conclusion that the oxidation of FAs produces ATP at a cost of greater oxygen consumption or lower efficiency. Therefore, a switch to the oxidation of FAs as the major energy substrate should result in less efficient ATP production and an increase in whole-body energy expenditure that could lead to a loss of fat mass if energy intake remains constant (Clapham 2004a, b, Leverve et al. 2007).

Indirect calorimetry is often used in human and animal studies to determine total energy expenditure (indirectly by the measurement of oxygen consumption and carbon dioxide production), and the measurement of oxygen consumption and carbon dioxide production can also be used to calculate the relative use of glucose and FAs to support that energy expenditure (respiratory exchange ratio, RER), assuming that any contribution of protein oxidation is relatively small and constant (Ferrannini 1988, Arch et al. 2006). Based on the assumption that there is a direct stoichiometry between NADH+H+ and FADH2 oxidation, proton translocation and ATP synthesis, calculations have been made suggesting that a complete shift from glucose to FAs as a source of energy would increase oxygen consumption by 7%. However, in practice, it is unlikely that such theoretical calculations can be applied to the regulation of energy balance with any certainty. For instance, rarely does the measured RER shift from complete glucose oxidation (1.0) to complete FA oxidation (0.7), even with prolonged exercise (Gimenez et al. 2013) and starvation (Hoeks et al. 2010) or without some change in protein oxidation. More importantly, although there is a direct stoichiometry between the oxidation of a substrate molecule and the production of NADH+H+ and FADH2, NADH+H+ can be reoxidised in reactions other than Complex I of the ETC and the proton motive force generated by the ETC can be dissipated by processes other than ATP synthesis (e.g. counter-ion transport and uncoupling protein activity) (Mazat et al. 2013). The concepts of efficiency and plasticity in the coupling of substrate oxidation to energy conservation (ATP synthesis) have been expanded on in several authoritative review articles (Harper et al. 2008, Mazat et al. 2013). These review articles have highlighted the presence of a significant and variable basal proton leak in mitochondria (20–25%) of most tissues and reported that in perfused rat muscle systems futile proton cycling may contribute as much as 50% to the respiration rate (Rolfe & Brand 1996), although other methodologies have suggested that this could be as little as 10% (Marcinek et al. 2004, Conley et al. 2007).

Irrespective of the exact mechanisms of proton leak and mitochondrial coupling of substrate oxidation to ATP production and oxygen consumption, it seems clear that using strict stoichiometric relationships (three ATPs per
NADH + H⁺ and two ATPs per FADH₂ to calculate whole-body oxygen consumption and energy expenditure from the measurements of relative substrate oxidation is unlikely to reflect the actual measurements of energy expenditure. In reality, coupling efficiency can vary significantly depending on changes in proton leak or ATP demand, but in cell systems at least, changes in substrate oxidation do not appear to influence the relationship between oxygen consumption and ATP synthesis (Brand et al. 1993).

**Can switching substrate alter energy expenditure?**

The above discussion clearly leads to the conclusion that the cost of generating mitochondrial ATP in terms of ETC activity and oxygen consumption can vary significantly and is not affected to any large extent by the substrate being oxidised to provide the reducing equivalents for electron transport. Despite this, it is not uncommon to read about studies in whole animal systems (particularly genetically modified mice) where differences in fat mass are often mechanistically related to changes in the mRNA levels of FA metabolism genes in a variety of tissues without appropriate consideration of the contribution of these tissues to whole-body energy expenditure (Abu-Elheiga et al. 2001, 2003, Lee et al. 2009, Hu et al. 2012, Ronis et al. 2013). In the context of investigations of energy balance in lean and obese mice, there are excellent recent reviews pointing out potential problems with assessing differences in food intake and energy expenditure using indirect calorimetry systems and extrapolating any differences to explain gain or loss of fat mass (Butler & Kozak 2010, Tschop et al. 2012). For example, expression of oxygen consumption or heat production on a kilogram body weight basis can be misleading if animals have significantly different amounts of fat tissue, because the metabolic rate of fat per gram is much lower in tissues such
as muscle and liver (Frayn et al. 1995). Similarly, the difference in daily food intake needed to contribute to a significant gain of body fat over several weeks in mice can be so small as to be undetectable unless large numbers of mice (200–300) are used for the comparison (Tschop et al. 2012). Changes in the body weight and body fat of groups of adult mice with different genotypes on different diets should reflect cumulative differences in energy intake and energy expenditure. However, any differences might not be easily detected if animals are assessed for food intake and energy expenditure individually in indirect calorimetry systems, away from their home cage and communal environment for only a 24–48-h period of the several weeks over which body weight and fat mass have been monitored.

**AMPK activation, FA oxidation and energy expenditure**

AMP-activated protein kinase (AMPK) is recognised as a master regulator of energy metabolism, particularly in times of energy stress such as exercise, hypoxia and starvation (Hardie et al. 2012). The activation of AMPK has been shown to acutely increase FA and glucose uptake and metabolism in a variety of experimental situations including in vitro and in vivo experiments in muscle (Iglesias et al. 2004, Smith et al. 2005). The long-term effects of AMPK activation in muscle lead to the activation of gene transcription pathways that increase mitochondrial biogenesis and proteins of oxidative metabolism (Winder et al. 2000, Hardie et al. 2012). The acute regulation of FA oxidation by AMPK is largely through the phosphorylation and inactivation of the enzyme acetyl CoA carboxylase 2 (ACC2). ACC2 produces malonyl CoA, an allosteric inhibitor of the key enzyme carnitine palmitoyltransferase 1 (CPT1), which controls the entry of FAs into the mitochondria for oxidation (Hardie & Pan 2002).

The pharmacological activation of AMPK has been shown to produce changes in muscle metabolic pathway capacity similar to those produced by exercise training (O’Neill et al. 2013); however, there is considerable controversy as to whether AMPK activation can drive energy expenditure in the absence of exercise. A series of studies employing genetic deletion of Acc2 (Acacb) have reported reduced fat depots in association with increased FA oxidation in isolated muscle (Abu-Elheiga et al. 2001, 2003) and have subsequently reported increased energy expenditure (although not increased FA oxidation) in Acc2-knockout mice with less fat and less lean mass (Choi et al. 2007). These results suggest that the inhibition of ACC2 by the activation of AMPK or development of ACC2 inhibitors might promote FA oxidation and produce fat loss. Subsequent studies using independently generated Acc2-knockout mice did not reproduce these effects, reporting that although these mice exhibited increased FA oxidation at the whole-body and isolated muscle level, there was no measurable difference in energy expenditure, fat mass or food intake (Hoehn et al. 2010). However, there was an increased glycogen content in muscle, an effect of AMPK activation noted previously (Winder et al. 2000, Buhl et al. 2001), which is consistent with AMPK activation and ACC2 inhibition promoting FA oxidation and channelling glucose taken up by muscle into storage as glycogen (Vitzel et al. 2013). Another study using independently generated genetically manipulated mice has reported no difference in body weight, food intake or fat mass in global or muscle-specific Acc2 gene-deleted mice (Olson et al. 2010), adding to the conclusion that altering FA oxidation in the absence of any change in energy expenditure or energy intake is insufficient to have a significant impact on whole-body fat mass.

Therefore, it would appear that apart from theoretical calculations suggesting that increasing fat oxidation will drive increased energy expenditure, there is little experimental evidence to support the idea that energy expenditure can be increased simply by increasing substrate availability or by switching to oxidise FAs.

**Insulin regulation of energy metabolism**

From an energy metabolism point of view, the flow of different substrates to tissues for oxidation or storage is largely under the control of the circulating hormone insulin. After a meal, direct stimulation of the β-cells of the islets of Langerhans of the pancreas by nutrients (glucose, FAs and amino acids) increases insulin release into the circulation. Certain gut hormones (GLP1 and G-1-P) can also augment insulin secretion, as can neural signals from the brain (Thorens 2011). Insulin has many stimulatory and inhibitory actions in different tissues mediated by a complex intracellular signalling pathway, but for the purpose of this discussion, the actions of insulin to stimulate glucose uptake and metabolism in muscle and regulate FA metabolism will be a major focus. The failure of insulin to appropriately regulate glucose and FA metabolism is termed insulin resistance, and this condition is most frequently observed in the muscle and liver of overweight or obese individuals (Eckardt et al. 2011). Insulin resistance is considered a significant predisposing
factor for the development of type 2 diabetes (T2D) and therefore there is considerable research effort put into determining the mechanistic relationship between excess lipid accumulation (obesity) and insulin resistance, particularly in muscle. Studies from over 20 years ago first showed that triglyceride accumulation in the muscle of high-fat diet-fed rats coincided with insulin resistance (Storlien et al. 1987, Kraegen et al. 1991), thereby establishing the hypothesis that insulin resistance is causally related to triglyceride accumulation in muscle. Since then, the relationship between muscle lipid accumulation and insulin resistance has also been established in humans, and many mechanisms have been put forward to explain how lipid accumulation could generate insulin resistance (Bosma et al. 2012, Samuel & Shulman 2012). Over the last decade, the major challenge has been determining whether these proposed mechanisms are universal or specific to the model of lipid-induced insulin resistance being studied. It is also possible that different mechanisms are important at different times during the development of insulin resistance and that some proposed mechanisms depend on the experimental methods used to assess insulin action.

Methods for assessing insulin action in muscle

All discussions of the relationship between increased fat metabolism and insulin action are dependent on the methodology used to assess insulin resistance and the assumptions associated with different methodologies. As has been mentioned previously, nearly all investigations of lipid-induced insulin resistance in rodent models utilise high-fat diet feeding to increase adiposity, but the methods of assessing insulin action can be quite varied and rely on glucose tolerance tests or insulin tolerance tests and less frequently (because of the technical difficulty) on hyperinsulinaemic–euglycaemic clamps. Various technical considerations of glucose and insulin tolerance tests must be considered when discussing the metabolic implications of these tests for muscle insulin action. The timing and route of administration of glucose and duration of fast before glucose administration influence the results of glucose tolerance tests (Andrikopoulos et al. 2008, McGuinness et al. 2009), and our recent studies suggest that changes in glucose tolerance may reflect changes in lipid content and insulin action in the liver more than insulin action in muscle, especially in the initial stages of fat accumulation after starting a high-fat diet (Montgomery et al. 2013, Turner et al. 2013). Insulin tolerance tests were devised largely to assess the effectiveness of counter-regulatory mechanisms in response to insulin-induced hypoglycaemia and therefore the utility of these tests to assess peripheral insulin action is debatable. This is particularly the case when conclusions about insulin effectiveness are related to glucose measurements in the later half of the test (30–90 min) when the injected insulin has largely been cleared or when there is a difference in basal glycaemia and results are expressed as % basal (McGuinness et al. 2009). Neither glucose tolerance nor insulin tolerance tests give specific data regarding insulin effectiveness in muscle, although several methodological variations have used concurrent injection of radioactive tracers to assess glucose clearance into muscle during a glucose tolerance or insulin tolerance test (Crosson et al. 2003, Cooney et al. 2004).

The hyperinsulinaemic–euglycaemic clamp with glucose tracer administration gives the most reproducible assessment of muscle glucose clearance in response to constant insulin stimulation and constant glucose availability (Ayala et al. 2006, Wasserman et al. 2011). This technique relies on plasma insulin levels (not insulin infusion rates) during the comparison of the clamp being matched between the groups. In many studies, plasma insulin levels during the clamp are not reported, making the assessment of muscle insulin action difficult (Chapman et al. 2010, Laskewitz et al. 2010, Parlevliet et al. 2010). In vitro assessment of insulin effectiveness in isolated soleus or extensor digitorum longus muscle is also often used to demonstrate the effects of FA exposure (Thompson et al. 2000, Alkhateeb et al. 2007), and although this methodology provides reproducible comparisons between control and treatment muscle, it is subject to all the assumptions of comparing the in vitro situation with the in vivo situation (e.g. reliance on diffusion and not on perfusion). While all the above methods can give useful information about the effects of muscle lipid accumulation on insulin action, this information can be specific for the test employed. Even the data obtained from hyperinsulinaemic–euglycaemic clamp studies describe fluxes measured after at least an hour of exposure to constant insulin stimulation and constant glucose availability, a situation that is unlikely to ever exist in the normal 24-h feeding–fasting cycle. Therefore, it would seem important to consider the method used to demonstrate a difference in insulin action with lipid accumulation, when assessing the relevance of various mechanisms to reduced glucose metabolism in muscle when no restrictive experimental conditions (e.g. in vitro assessment, constant infusion and i.p. delivery) have been applied to the ‘free-living’ system.
Linking intramyocellular triglyceride content and insulin action

As has been mentioned above, the association between intramyocellular triglyceride (IMTG) content and insulin resistance is now well established in animals and obese humans, and most studies investigating the mechanisms of insulin resistance in muscle use high-fat diet rodent models. It has also become standard practice in assessing the phenotype of genetically manipulated mice to place them on high-fat diets to investigate whether there is any impact (favourable or detrimental) of gene manipulation on glucose and energy homeostasis. There is a reasonable assumption that, independent of genetic background in animals or humans, overconsumption of energy-dense diets plays a major role in the accumulation of fats and development of metabolic derangements in muscle. In humans, overconsumption of energy-dense diets for a few weeks is enough to increase fat mass and have detrimental effects on whole-body insulin action (Samocha-Bonet et al. 2010). In mice, high-fat feeding for as little as a few days can impair glucose tolerance (Turner et al. 2013) and 2–3 weeks of exposure to a high-fat diet is enough to observe significant insulin resistance in muscle using in vitro (Thompson et al. 2000) or in vivo (Turner et al. 2013) assessment. Apart from the well-documented ‘athlete’s paradox’ where increased IMTG content is associated with improved insulin action (Coen & Goodpaster 2012), most interventions that change insulin action are associated with reciprocal changes in IMTG content. Studies that improved insulin sensitivity by low-calorie diets in patients with T2D were accompanied by a reduction in IMTG content (Jazet et al. 2008, Lara-Castro et al. 2008). Insulin resistance associated with ageing (Nakagawa et al. 2007), growth hormone administration (Krag et al. 2007) and post-burn trauma (Cree & Wolfe 2008) has been reported to be associated with increased IMTG content. Current opinion is reasonably clear on the fact that IMTG is a useful marker of the level of cytosolic lipid accumulation, but it is more likely that active lipid metabolites such as LCACoAs, DAGs and ceramides or intermediates of FA oxidation pathways interfere with insulin action via a variety of potential mechanisms (Fig. 2). These mechanisms are largely based on the idea that insulin resistance in muscle is the result of reduced transduction of the insulin signal through the phosphorylation cascade leading to the translocation of the glucose transporter GLUT4 to the sarcolemmal membrane (Stockli et al. 2011). A significant body of work in the 1990s using nuclear magnetic resonance has identified glucose transport/phosphorylation and glycogen synthesis as major defects in FA-induced insulin resistance in humans (Shulman et al. 1990, Roden et al. 1996). Since that time, research into the molecular mechanism of FA-induced insulin resistance in muscle has mainly focused on linking excess FAs to defects in the insulin signalling pathways that regulate glucose uptake. However, there are some established and some more speculative mechanisms that also link increased FA metabolism with reduced insulin action, and these are discussed in the subsequent sections.

Lipid intermediates, inflammation and insulin resistance

IMTGs are considered to be relatively benign with regard to insulin resistance (Goodpaster et al. 2001), largely because they are packaged into discrete lipid droplets that are located within the cytoplasm and are thus unlikely to directly interfere with proximal insulin signalling (Fujimoto & Parton 2011). However, despite the general consensus that IMTGs are metabolically inert, it is possible that the expanded IMTG pool generates intermediates of lipid metabolism that are more likely to play a mechanistic role in the development of muscle insulin resistance. In this respect, the bioactive lipid metabolites DAG and ceramide are leading candidates. The levels of both DAG (Turinsky et al. 1990, Turpin et al. 2009) and ceramide (Holland et al. 2007, Bruce et al. 2013) are elevated in the muscle of obese insulin-resistant rodents, and the earliest detectable defect in muscle insulin sensitivity in high-fat diet-fed mice is associated with the accumulation of these lipids (Turner et al. 2013). While less is known about the role of these lipids in humans, it has been reported that acute lipid-induced insulin resistance is associated with muscle DAG accumulation (Itani et al. 2002) and that ceramide levels are elevated in the muscle of obese insulin-resistant individuals (Amati et al. 2011). Furthermore, interventions that enhance insulin action, such as exercise training, cause reductions in muscle DAG and ceramide content (Bruce et al. 2006).

Mechanistically, DAG and ceramide are potent signalling molecules that may cause insulin resistance by activating a cascade of serine/threonine kinases that ultimately impinge upon insulin signalling (Summers et al. 1998, Ruvolo 2003, Li et al. 2004). Specifically, DAG accumulation is thought to impair insulin action via the activation of novel protein kinase C (PKC) isoforms, which subsequently inhibits insulin signal transduction to glucose transport via serine phosphorylation of insulin.
receptor substrate 1 (IRS1; Yu et al. 2002, Li et al. 2004). Ceramide has been reported to cause insulin resistance by impairing insulin signalling at the level of Akt (Schmitz-Peiffer et al. 1999, Bruce et al. 2006, Holland et al. 2007). In addition, ceramide is a potent activator of inflammatory molecules, including c-Jun N-terminal kinase (JNK; Westwick et al. 1995) and nuclear factor κB/inducer of κ kinase (IKK) (Wang et al. 1999), which have been reported to be associated with the development of muscle insulin resistance (Itani et al. 2002, Sriwijitkamol et al. 2006, Henstridge et al. 2012). However, while inflammation has been proposed as a critical factor causing insulin resistance, studies carried out by our group and other groups suggest that inflammation is not involved in the initiation of lipid-induced insulin resistance, but may be more important in the exacerbation and maintenance of insulin resistance once obesity is established (Lee et al. 2011, Turner et al. 2013).

Although there is mounting evidence supporting a role for DAG and ceramide in the regulation of insulin sensitivity, it is important to highlight that the accumulation of these lipids is not always associated with insulin resistance. In fact, a recent study has found that total DAG content is actually elevated in the muscle of highly insulin-sensitive endurance-trained athletes compared with the skeletal muscle of obese individuals (Amati et al. 2011). Furthermore, a positive correlation between total muscle ceramide content and insulin sensitivity has been reported (Skovbro et al. 2008). These data suggest a more complex role for DAG and ceramide in the regulation of insulin action (Amati et al. 2011) and emphasise the importance of not only determining the total content of these lipids but also examining specific molecular species as well as their subcellular localisation, as these are likely to be critical factors that influence the relationship between lipids, insulin signalling and muscle insulin sensitivity (Bergman et al. 2012).

While the bioactive lipid hypothesis has gained strong support, an alternative concept linking the accumulation of intermediates of mitochondrial FA oxidation with muscle insulin resistance has gained attention (Koves et al. 2008). This model proposes that lipid oversupply drives an increase in mitochondrial β-oxidation that exceeds the capacity of the Krebs cycle, leading to the accumulation of by-products of FA oxidation (Koves et al. 2008). This is supported by studies demonstrating an
increase in incomplete FA oxidation and an accompanying increase in intramuscular acylcarnitine levels in obese rodents (Koves et al. 2008). While data in humans are currently limited, there is evidence that acylcarnitine does accumulate in the muscle of humans in response to a high-fat diet (Putman et al. 2003). However, it is not clear whether acylcarnitine plays a direct role in the modulation of skeletal muscle insulin sensitivity by disrupting signalling processes or whether it simply reflects a state of mitochondrial stress. Unravelling the role of acylcarnitine in muscle insulin sensitivity will no doubt be a focus of future research.

**Mitochondrial dysfunction, reactive oxygen species and insulin resistance**

Another prominent theory on the aetiology of insulin resistance implicates abnormalities in mitochondrial function as a major causative factor leading to reductions in insulin sensitivity. More specifically, defects in mitochondrial metabolism have been suggested to lead to inadequate substrate oxidation, precipitating a build-up of intracellular lipid metabolites, impaired insulin signalling and the subsequent development of insulin resistance (Lowell and Shulman 2005, Kim and the subsequent development of insulin resistance (Vianna et al. 2006, Wredenberg et al. 2006, Handschin et al. 2007, Pospisilik et al. 2007). Collectively, all these studies suggest that at some level, mitochondria in insulin-resistant individuals are not as effective at burning fuel substrates in muscle and this compromises insulin action.

Despite the large body of evidence described above, this area is controversial, as many studies report a dissociation between insulin resistance and mitochondrial dysfunction. For example, providing rodents with excess fat in their diet leads to an enhancement of mitochondrial oxidative capacity in muscle while at the same time inducing insulin resistance (Turner et al. 2007, Hancock et al. 2008, Stephenson et al. 2012). Several lines of mice with genetic manipulations that cause compromised mitochondrial function in muscle do not exhibit insulin resistance (Vianna et al. 2006, Wredenberg et al. 2006, Handschin et al. 2007, Pospisilik et al. 2007). Conversely, two separate lines of muscle-specific Pgc1α (Ppargc1α) transgenic mice displayed a significant enhancement in the markers of mitochondrial content and yet were insulin resistant due to excessive FA delivery and reduced GLUT4 (SLC2A4) expression in muscle (Miura et al. 2003, Choi et al. 2008). A growing number of studies in humans have also reported intact mitochondrial function in various insulin-resistant populations (De Feyter et al. 2008, Trenell et al. 2008, Lefort et al. 2010, van Tienen et al. 2012, Fisher-Wellman et al. 2013). Collectively, these studies suggest that mitochondrial dysfunction in muscle is not an obligatory factor required for the accumulation of intramuscular lipids and the development of insulin resistance. Furthermore, it has also been argued that as muscle has such a high amount of ‘spare’ capacity to elevate substrate oxidation over basal levels (Bangsbo 2000), it is questionable whether mitochondrial deficiencies of the magnitude reported in some insulin-resistant subjects would have any impact on the rate of FA oxidation (and lipid accumulation) when energy requirements are relatively low (e.g. normal free-living conditions) (Hancock et al. 2008).

In addition to their role as major sites for energy transduction, mitochondria are also known to be a major source of reactive oxygen species (ROS), which are
produced as a by-product of normal metabolic reactions (Andreyev et al. 2005). ROS have the capacity to damage macromolecules, and when the production of these reactive species is in excess of the antioxidant defences, a state of oxidative stress results. FA catabolism is known to promote mitochondrial ROS production (St-Pierre et al. 2002, Anderson et al. 2009, Seifert et al. 2010), and studies carried out by several groups have shown that in cultured cell models, genetic or diet-induced obese rodents, and in human subjects fed a high-fat diet, there is increased mitochondrial ROS production in muscle in association with insulin resistance (Houstis et al. 2006, Anderson et al. 2009, Hoehn et al. 2009, Hey-Mogensen et al. 2012, Fisher-Wellman et al. 2013). Importantly, many studies have shown that insulin action is improved when mitochondrial ROS production is attenuated (Houstis et al. 2006, Anderson et al. 2009, Boden et al. 2012), indicating a potentially important role for reactive species generation in this organelle in insulin resistance. While the exact mechanism linking mitochondrial ROS with insulin resistance is not resolved, it has been proposed that insulin resistance may be caused by ROS-dependent changes in stress-sensitive Ser/Thr kinases, leading to perturbed insulin signalling, although this requires verification (Fisher-Wellman & Neufer 2012).

Substrate competition and reduced insulin action

Before the elucidation of the insulin signalling pathway and recognition of the complex processes involved in the translocation of GLUT4 from intracellular vesicles to sarcolemmal membrane, there was a large amount of experimental data pointing to significant FA regulation of glucose metabolism at the level of PDH (Randle et al. 1963, Randle 1998). If humans, animals or in vitro preparations of muscle are exposed to an increased availability of FAs in the presence of glucose, the oxidation of FAs increases and the oxidation and uptake of glucose decrease (Boden et al. 1994, Vaag et al. 1994). On the other hand, reduction of the availability of FAs by inhibiting lipolysis (Vaag et al. 1991, Lim et al. 2011) and blocking FA entry into the mitochondria reduces FA oxidation and increases glucose uptake and oxidation (Oakes et al. 1997, Timmers et al. 2012, Keung et al. 2013), although there are some reports that prolonged inhibition of FA oxidation can lead to reduced glucose uptake (Dobbins et al. 2001). Although the initial observations of Randle and colleagues on the reciprocal relationship between glucose and FA metabolism were made 50 years ago, the idea that increasing or reducing FA availability will reciprocally affect glucose utilisation is no less valid today. Therefore, in the context FA-induced insulin resistance, a role for substrate competition and regulation at the level of PDH should not be overlooked.

Reassessment of the role of insulin signalling in FA-induced insulin resistance

As outlined in other sections of this review, the current dogma suggests that the major mechanisms for FA-induced insulin resistance in muscle involve active lipid species interfering with insulin signalling via the activation of various serine kinases (Fig. 2). The canonical insulin signalling cascade comprises scaffolding proteins (e.g. IRS1) and enzymes (e.g. PI3 kinase, Akt and GSK3), and the activity of these proteins is modulated by tyrosine and/or serine phosphorylation. DAG via the activation of PKC and inflammatory factors via the activation of the serine kinases JNK and IKK are thought to serine phosphorylate and reduce the insulin receptor-mediated tyrosine phosphorylation of IRS1 (Samuel & Shulman 2012). Mitochondrial insufficiency and ROS are also thought to feedback and impinge on the efficiency of insulin signalling via the activation of regulatory kinases. While there are many studies showing clear differences in the phosphorylation status of various insulin signalling proteins after insulin stimulation in control and FA-exposed or obese or high-fat diet-fed muscle, these changes are not always consistent. For example, a change in Akt phosphorylation is not always accompanied by a detectable change in downstream GSK3 or AS160 phosphorylation or upstream changes in IRS1 phosphorylation (Frangiadakis & Cooney 2008, Hoehn et al. 2008, Tonks et al. 2013). There are a number of studies reporting that insulin-stimulated Akt activation is in fact not impaired in the muscle of obese individuals with insulin resistance, of glucose-intolerant first-degree relatives of patients with T2D and of patients with T2D (Kim et al. 1999, Storgaard et al. 2004). Furthermore, in rats made insulin resistant by 5 h of hyperlipidemia/hyperinsulinaemia (Hoy et al. 2009) or in isolated soleus muscle made insulin resistant by palmitate incubation (Alkhateeb et al. 2007), no defect in insulin-stimulated Akt phosphorylation was reported. Finally, reduction of IRS1 levels in muscle by 60% by direct in vivo genetic manipulation did not result in impaired insulin action (Cleasby et al. 2007).

This dissociation between measured changes in insulin-stimulated glucose flux and insulin effects on signalling proteins has a number of implications. First, it
might highlight the technical difficulties of obtaining reliable, quantitative data on protein modification using the essentially non-quantitative technique of immuno blotting. The ability to detect differences with this methodology can also depend on the affinity of individual antibodies, and the amount of phosphorylation does not necessarily correlate linearly with the activity of the signalling protein. A good example of this is provided by two studies showing that in adipocytes maximal insulin-stimulated glucose transport and GLUT4 translocation are achieved when only 10–20% of the total IRS1 and Akt is phosphorylated (Whitehead et al. 2001, Hoehn et al. 2008). If a similar situation exists in muscle, the physiological importance of statistically significant differences of 10–20% in the phosphorylation of signalling intermediates could be difficult to assess. The introduction of mass spectrometry techniques to analyse changes in global protein phosphorylation in response to insulin, as has been applied in adipocytes (Humphrey et al. 2013), could be helpful in this regard. Another possibility is that phosphorylation is not the only post-translational modification of proteins involved in the generation of lipid-induced insulin resistance. Recently, the emergence of nitrosative modifications (White et al. 2010), reversible acetylation, malonylation and succinylation of proteins in central metabolic pathways has revealed new possibilities by which increased FA metabolism could influence metabolic fluxes (Newman et al. 2012, Park et al. 2013). Similarly, reversible modification of proteins by O-linked N-acetylglucosamine has been proposed to have a significant impact on metabolism in response to nutrient levels (Bond & Hanover 2013, Ruan et al. 2013).

Circadian metabolism and insulin resistance

Another area of research that is increasingly realised to have a significant impact on metabolic disease is circadian biology. Daily patterns of activity and rest are historically aligned with feeding and fasting and changes in energy metabolism are intrinsically linked to the light/dark cycle (Bass 2012). The suprachiasmatic nucleus in the brain is considered to be the master regulator of circadian behaviour because of its ability to coordinate inputs from the environment (light, food, exercise and temperature), but it is now clear that every tissue has the molecular components that comprise the clock, raising the possibility that circadian processes in tissues could be regulated directly by some inputs. Some mouse models with genetic manipulations of core clock genes have altered circadian rhythms and are more prone to developing obesity (Turek et al. 2005, Kennaway et al. 2007, Paschos et al. 2012), and manipulation of feeding schedules in mice and rats has been shown to have significant effects on adiposity, energy expenditure and glucose homeostasis (Bray et al. 2013, Coomans et al. 2013, Reznick et al. 2013). If there is an underlying rhythm to metabolism in muscle driven by the molecular clock (Lefta et al. 2011), the timing of experiments over the normal 24-h period might be critical to a proper understanding of how repeated daily exposure to a high-fat diet leads to lipid accumulation and insulin resistance in muscle. In fact, a recent report has suggested that the time of day can have a significant effect on the data obtained from euglycaemic-hyperinsulinaemic clamps in mice (Shi et al. 2013), and as rodents have an phase opposite to that of humans with regard to activity and sleep and feeding and fasting, the relevance of daylight experiments in nocturnal animals to human physiology requires renewed debate.

Summary and perspective

The correlation between increased FA availability and reduced insulin-stimulated glucose metabolism is well established. Despite this clear relationship, to date, there has been no unifying mechanism that explains lipid-induced reductions in insulin action under all circumstances. The most described mechanisms are that toxic lipid intermediates and/or activation of inflammatory and stress signalling pathways act to decrease the phosphorylation and function of proteins in the insulin signalling pathway, and this explains the decreased insulin-stimulated glucose uptake observed with lipid accumulation. However, there are an increasing number of experimental situations where reduced effects of insulin in muscle have been observed without significant changes in the phosphorylation of signalling proteins or where differences in phosphorylation are only observed with stimulation by supraphysiological insulin concentrations. This suggests that other control mechanisms or other forms of protein modification may predominate depending on the exact experimental conditions used to examine insulin resistance (e.g. bolus insulin injections, hyperinsulinaemic clamps and glucose or lipid infusion).

Figure 3 summarises some of the key control points other than insulin signalling for GLUT4 translocation that could alter the balance between glucose and FA metabolism and affect insulin-stimulated glucose disposal. For example, utilisation of glucose and FAs is dependent on their availability in the circulation and delivery to the muscle tissue, and changes in microvasculature occur with
obesity and contribute to muscle insulin resistance (St-Pierre et al. 2010, Premilovac et al. 2013). Other work (Furler et al. 1991, Wasserman 2009) has established that glucose transport into muscle is not rate limiting for glucose metabolism under all conditions. The phosphorylation of glucose by hexokinase and the pathway for conversion of glucose-6-phosphate to glycogen are subject to regulation by glucose-6-phosphate and glycogen respectively, and decreased glucose phosphorylation and glycogen synthesis will affect glucose uptake (Fueger et al. 2007, Bouskila et al. 2010). Another well-documented node regulating the metabolism of glucose is centred on the activity of PDH. The activity of this enzyme complex is inhibited by phosphorylation via PDH kinase 4 (PDK4). Interestingly, the amount of PDK4 in muscle is significantly increased in high-fat diet-fed, insulin-resistant animals and PDK4 is activated by acetyl CoA, providing evidence that this regulatory node could significantly affect glucose metabolism in muscle as hypothesised by Newsholme and Randle many years ago (Randle et al. 1963) and many others since (Holness & Sugden 2003, Hue & Taegtmeyer 2009).

FA metabolism in muscle can also be regulated at the membrane by transporter proteins (such as CD36), and at activation to acyl CoA by acyl CoA synthase (Glatz et al. 2010). The partitioning of FAs towards triglyceride storage or mitochondrial oxidation may depend on the activity of key enzymes such as glycerol phosphate acyltransferase and adipose triglyceride lipase (Greenberg et al. 2011, Watt & Hoy 2012). The entry of long-chain FAs into the mitochondria for oxidation is thought to be largely regulated by the activity of CPT1. The activity of CPT1 is modulated allosterically by malonyl CoA, and numerous studies, including our recently published papers using genetic and pharmacological interventions (Bruce et al. 2009, Hoehn et al. 2010), have manipulated CPT1B, AMPK

Figure 3
Nodes of control of glucose metabolism other than insulin-stimulated translocation of GLUT4 that could be influenced by the excess availability of FAs. Utilisation of glucose and FAs is dependent on their availability in the circulation and delivery to the muscle tissue. The phosphorylation of glucose and conversion to glycogen are regulated by substrate availability and G-6-P concentration. PDH is a critical regulator balancing glucose use and FA oxidation to support energy requirements. The regulation of FA sequestration in, or release from, muscle fat droplets can control the level of bioactive lipid species. The regulation of FA metabolism at the AMPK–ACC2–malonyl CoA–CPT1 axis also has a significant impact on the balance between FA and glucose metabolism. There are a number of newly recognised post-translational modifications that can occur on key metabolic or signalling proteins and would be expected to be influenced by changes in the availability and metabolism of FAs.
and ACC activity to increase FA oxidation. Depending on the experimental design used, acutely increasing fatty oxidation in muscle can decrease glucose utilisation (Hoehn et al. 2010), while chronically increasing FA oxidation in muscle via CPT1 (CPT1B) overexpression can subsequently improve insulin-stimulated glucose uptake in fat-fed animals (Bruce et al. 2009). Interestingly, acute blockade of FA oxidation increases insulin-stimulated glucose uptake (Oakes et al. 1997), while chronic blockade of FA oxidation has been shown to be associated with decreased insulin sensitivity (Dobbins et al. 2001). These differences in acute and chronic responses when substrate metabolism is manipulated may be reconciled by considering the fact that energy metabolism is not constant in animals and humans, but has a substantial diurnal variation that is highly relevant to designing appropriate experiments to investigate lipid-induced insulin resistance.

In conclusion, it may be unrealistic to expect that a unifying mechanism may explain all situations where there is reduced glucose metabolism in muscle in response to insulin, as multiple factors may contribute to the establishment and long-term maintenance of insulin resistance in this tissue. With the emergence of powerful techniques for determining global changes in gene expression, protein modifications and metabolite profiles, it will hopefully become possible to gain a more comprehensive idea of the factors and pathways that may contribute to the aetiology of lipid-induced insulin resistance in muscle.

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

Funding
The work carried out in the laboratories of the authors is supported by Program and Project grant funding from the National Health and Medical Research Council of Australia (NHMRC), the Australian Research Council (ARC) and the Diabetes Australia Research Trust. NT is supported by an ARC Future Fellowship. G J C and E W K hold research fellowships from the NHMRC and C R B has received a career development award from the NHMRC.

References

Abu-Elheiga L, Oh W, Kordari P & Wakil SJ 2003 Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets. PNAS 100 10207–10212. (doi:10.1073/pnas.1733877100)


Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunuson R & Delf A 2007 Patients with type 2 diabetes have normal mitochondrial function in


Ferrannini E 19926–19931. (doi:10.1007/978-1-4471-39105)


Moseley OP, Ayala JE, Laughlin MR & Wasserman DH 2009 NIH experiment in centralized mouse phenotyping: the Vanderbilt experi-


Nyholm B, Qu Z, Kaal A, Pedersen SB, Gravholt CH, Andersen JL, Saltin B & Schmitz O 1997 Evidence of an increased number of type IIb muscle fibers in insulin-resistant first-degree relatives of patients with NIDDM. Diabetes 46 1822–1828. (doi:10.2337/diab.46.11.1822)


Olson DP, Pulinilunkunnil T, Cline GW, Shulman GI & Lowell BB 2010 Gene knockout of Acox2 has little effect on body weight, fat mass, or food intake. PNAS 107 7598–7603. (doi:10.1073/pnas.0913492107)


Randall PJ 1998 Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. Diabetes/Metabolism Reviews 14 263–283. (doi:10.1002/smr.09810990895(199812)14:4%3C:263%3E;AID-DMR233%3E;3.0.CO;2-C)


Schmitz-Peiffer C, Craig DL & Biden TJ 1999 Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB phosphorylation in myotubes. Journal of Biological Chemistry. 274 24202–24210. (doi:10.1074/jbc.274.34.24202)


Thematic Review

N. Turner and others

Fatty acids and insulin action

220:2  T78


concentrations. European Journal Endocrinology 130 70–79. (doi:10.1530/eje.0.1300070)


Vitzel KF, Bikopoulos G, Hung S, Pistor KE, Patterson JD, Curi R & Ceddia RB 2013 Chronic treatment with the AMP-kinase activator AICAR increases glycogen storage and fatty acid oxidation in skeletal muscles but does not reduce hyperglucagonemia and hyperglycemia in insulin deficient rats. PLoS ONE 8 e62190. (doi:10.1371/journal.pone.0062190)


Received in final form 27 November 2013
Accepted 9 December 2013
Accepted Preprint published online 9 December 2013