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MOLECULAR PHYSIOPATHOLOGY OF OBESITY-RELATED DISEASES: MULTI-ORGAN INTEGRATION BY GRK2

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ABSTRACT

Obesity is a worldwide problem that has reached epidemic proportions both in developed and developing countries. The excessive accumulation of fat poses a risk to health since it favours the development of metabolic alterations including insulin resistance and tissue inflammation, which further contribute to the progress of the complex pathological scenario observed in the obese. In this review we put together the different outcomes of fat accumulation and insulin resistance in the main insulin-responsive tissues, and discuss the role of some of the key molecular routes that control disease progression both in an organ-specific and also in a more systemic manner. Particularly, we focus on the importance of studying the integrated regulation of different organs and pathways that contribute to the global pathophysiology of this condition with a specific emphasis on the role of emerging key molecular nodes such as the G protein-coupled receptor kinase 2 (GRK2) signalling hub.

INTRODUCTION

Obesity is a pathological condition in which there is an excessive accumulation of body fat that contributes to a myriad of pathological effects. Over the last decade, the prevalence of obesity has reached epidemic proportions. The onset of obesity is linked to an imbalance between energy intake and energy expenditure (EE) (Weiser, 1997). However, given the complex nature of the regulation of fat mass content and the multiple compensatory mechanisms involved, counteracting obesity stands as a difficult challenge. Of note, visceral obesity is recognized as a key risk factor for the development of hypertension, dyslipidaemia, impaired glucose tolerance and insulin resistance (IR) (Kopelman, 2007). Insulin signalling regulates glucose and lipid metabolism as well as energy homeostasis by acting on different insulin-target tissues including liver, skeletal muscle, adipose tissue, heart and vessels (Figure

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1). Therefore, the consequences of IR underlie the development of a wide variety of metabolic disorders, including cardiovascular disease, non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes (T2D). Considering the multi-organ effects of insulin and the integration of the effects of obesity and IR in the different tissues (Figure 1), it is crucial to understand how this physiopathological network operates in order to design suitable therapeutic approaches for the treatment of these disorders.

The functional defects associated to IR partially arise from impaired insulin signalling through the PI3K/AKT axis in the different insulin-target tissues. In this regard, insulin exerts its actions via complex signalling networks with positive and negative modulators acting at different stages of the transduction cascade, which tightly control the biological response to this messenger. Alterations in any of these modulators may lead to the development of IR. These alterations include changes in protein functionality as well as mutations or aberrant posttranslational modifications in the components of the insulin signalling pathway (Biddinger and Kahn, 2006, Gesta, 2006).

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Insulin signalling and IR

Insulin binding stimulates tyrosine auto-phosphorylation in the insulin receptor β subunits which activates its kinase activity and recruits and phosphorylates members of a family of adaptor proteins called insulin receptor substrates 1–6 (IRS1–6) (reviewed in (Taniguchi, 2006)). IRS proteins act as scaffolds coupling IR stimulation to downstream effectors, recruiting and activating various SH2 domain–containing proteins, including the phosphatidylinositol 3-kinase (PI3K). The activation of PI3K produces PI3,4P2 and PI3,4,5P3, which recruit PDK1 and AKT to the plasma membrane. AKT is activated via

phosphorylation at T308 by PDK1 and at S473 by mTOR in complex with Rictor, this complex known as mTORC2 and also termed PDK2. AKT phosphorylates many cellular proteins, allowing the metabolic and transcriptional reprogramming of the cell (White, 2003) (Figure 2). In many cases AKT substrates directly controlling metabolic steps have been identified, such as glycogen synthase kinase 3 (GSK3) (Cross, 1995), the transcription factor Forkhead box O (FoxO) (Biggs, 1999), the phosphodiesterase PDE3B (Kitamura, 1999) or TBC1D4/AS160, a Rab GTPase-activating protein (GAP) (Kane, 2002), which plays an important role in insulin-stimulated translocation to the plasma membrane of the glucose transporter GLUT4. Additionally, AKT activation can activate mTOR in complex with Raptor, also known as mTORC1, which in turn activates S6K1 promoting the serine phosphorylation of IRS1 and reducing its stability in an auto-regulatory negative feedback loop (Harrington, 2005). Indeed, in contrast to tyrosine phosphorylation, the multi-site serine/threonine phosphorylation of IRS has classically been described to inhibit the interaction between its PTB domain and the phosphorylated receptor causing their dissociation and decreasing tyrosine phosphorylation (reviewed in (Copps and White, 2012)), although some studies have demonstrated that certain phosphorylations on serine residues have a positive effect on insulin signalling (Copps, 2010).

IR is a pathological condition in which cells fail to respond to the actions of insulin. Given that IR is a complex metabolic disorder that challenges a key signalling pathway, defining the precise mechanisms of IR in peripheral tissues is difficult while vital for the development of new and more effective therapies for T2D. In this regard, several studies have demonstrated that hyperinsulinemia, hyperlipidaemia, mitochondrial dysfunction and oxidative stress routes lead to attenuated insulin signalling and decreased cellular responsiveness to insulin. Moreover, accumulation of lipid metabolites, activation of the unfolded protein response

(UPR) and activation of proinflammatory signalling cascades have also been related to the pathogenesis of this disorder (reviewed in (Samuel and Shulman, 2012)).

Inflammation and IR

Remarkably, obesity itself has been described to result in a chronic low grade inflammatory state, contributing to IR and metabolic dysfunction (Gregor and Hotamisligil, 2011). In fact, the proinflammatory cytokine TNFα has long been considered as a link between obesity and IR (Fernandez-Veledo, 2009a), and the roles of this cytokine in other pathologies associated with obesity have been examined in several experimental systems including obese mice with homozygous null mutations at the TNFα or TNF receptor loci (Hotamisligil, 2000, Xu, 2002). Several studies of this inflammatory process underlying the IR and metabolic dysfunction that precedes T2D, have identified components of both the innate and adaptive immune response as key players in regulating metabolic homeostasis (reviewed in (Brestoff and Artis, 2015)).

TNFα and other proinflammatory cytokines activate MAP kinases (Tanti, 2012) thus interfering with insulin signalling. In this regard, the activity of IκB-kinase β (IKKβ) and c-Jun NH(2)-terminal kinase (JNK), both activated by TNFα, is elevated in different tissues during obesity, and several studies in mice have established the importance of these kinases in the development of IR (Tanti, 2012, Hirosumi, 2002). Heterozygous IKKβ mice are partially protected against diet-induced IR (Kim, 2001a), and disruption of JNK1 function protects mice against obesity-induced IR (Hirosumi, 2002, Sabio and Davis, 2010). JNK2 may also play a role in IR, but to a lesser extent (Tuncman, 2006). At the molecular level, one mechanism by which these kinases impair insulin signalling is the phosphorylation of IRS proteins on inhibitory serine sites, either directly or indirectly (Hirosumi, 2002) (Figure 2). In the same line, activation of the mTORC1 node by TNFα (Gao, 2003) is known to feedback negatively on IRS1 via S6K, thus decreasing insulin sensitivity (Figure 2). Accordingly, mice

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with adipose-specific knockout of the Raptor component of mTORC1 are lean, resistant to diet-induced obesity (Polak, 2008) and display improved glucose tolerance and insulin sensitivity.

On the other hand, activation of the innate immune system in the course of obesity may also be mediated by metabolic signals, such as increased circulating free fatty acids (FFAs), that may be recognized by receptors involved in the immune response, and thereby lead to stimulation of inflammatory signalling cascades, including IκBα kinase/nuclear factor-κB (IKK/NF-κB) or endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR), that may interfere with insulin signalling (reviewed in (Ringseis, 2015)).

Alternative molecular mechanisms leading to IR

While distinct signalling mechanisms specific for each tissue will be described later in individual paragraphs, an overview of the general mechanisms leading to IR is summarized in this section (and in Figure 2).

Emerging data are pointing at endoplasmic reticulum (ER) stress as an important player in the pathophysiology of obesity, IR and T2D (Ozcan, 2004). In obesity the capacity of the ER is surpassed, thus leading to the accumulation of misfolded/unfolded proteins, a condition termed ER stress. Accordingly, interventions that suppress ER stress can improve diabetes and associated comorbidities (Ozcan, 2006). In addition, diacylglycerol (DAG)-mediated IR has been suggested as a further unifying hypothesis to explain the most common forms of IR associated with obesity and T2D. Thereby, an increase in intracellular DAG content, due to an imbalance between fatty acid delivery and intracellular fatty acid oxidation and storage, would lead to activation of new protein kinase C (PKC) isoforms that may in turn inhibit insulin action in liver and skeletal muscle (reviewed in (Erion and Shulman, 2010)).

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Another mechanism potentially responsible for the insulin signalling defects found in obesity is the increased expression and/or activity of protein tyrosine phosphatases (PTPs), able to terminate signals propagated through tyrosine phosphorylation events. For instance, protein tyrosine phosphatase 1B (PTP1B) has been described to directly modulate insulin action *in vitro* and *in vivo* (Figure 2) (Goldstein, 2001). This phosphatase is increased in expression and/or activity in muscle and adipose tissue of obese humans and rodents (Goldstein, 2001), and the activation of the negative regulator of insulin signalling IKKβ/NFκB pathway increases its levels (Zabolotny, 2008). Accordingly, PTP1B-deficient mice show increased insulin sensitivity and resistance to diet-induced obesity, partly by an increased energy expenditure (Goldstein, 2001). Interestingly, the improved insulin sensitivity is present in muscle and liver but not in adipocytes (Goldstein, 2001). PTP1B inhibitors have shown beneficial effects to enhance insulin sensitivity, but structural homologies in the catalytic domain of PTP1B with other PTPs present a challenging task to achieve selectivity (Tamrakar, 2014).

Along the same line, the lipid phosphatase and tensin homologue (PTEN) has also been widely implicated as a negative regulator of insulin/PI3K signalling (Sasaoka, 2006) (Figure 2), apart from its role as a tumour suppressor. Several studies have shown that specific down-regulation of PTEN in different insulin target tissues protects against insulin resistance and diabetes (Carracedo and Pandolfi, 2008). On the contrary, transgenic mice overexpressing PTEN have also been reported to display enhanced insulin sensitivity, suggesting that moderate overexpression of PTEN results in improved insulin signalling and in protection from the damaging effects of high-fat diet (HFD). This effect, apparently paradoxical, is explained by the concomitant reduction in the negative feedback routes that emanate from the insulin signalling pathway (Ortega-Molina, 2012).

In the context of insulin signalling modulators, recent lines of research support the identification of G protein-coupled receptor kinase 2 (GRK2) as a novel and important negative regulator of insulin effects (reviewed in (Mayor, 2011)). GRK2 is a Ser/Thr kinase that has been classically named and widely studied for its role in the regulation and desensitization of G protein-coupled receptors (GPCRs). Besides such canonical role, recent data indicate that GRK2 is also able to phosphorylate a variety of non-GPCR substrates (Penela, 2010, Ribas, 2007). In addition, changes in GRK2 expression and activity have been identified in several relevant inflammatory, metabolic, cardiovascular or cancer diseases, suggesting that those alterations may contribute to the onset or development of these pathologies (Gurevich, 2012). In particular, recent data from our laboratory suggest that GRK2 levels play a relevant role in insulin signalling and resistance as well as in fat mass accretion (Garcia-Guerra, 2010). In fact, GRK2 expression levels are increased in insulin-resistant human adipocytes, in muscle and adipose tissue from TNFα, aging or HFD-induced insulin-resistant murine models and also in peripheral blood cells from metabolic syndrome patients (Garcia-Guerra, 2010). Accordingly, GRK2 haploinsufficient ($GRK2^{+/-}$) mice maintain glucose tolerance and insulin signalling in the major insulin-responsive tissues under different conditions of insulin resistance (Garcia-Guerra, 2010), suggesting that enhanced GRK2 expression impairs insulin sensitivity (Figure 2) and that a moderate decrease in GRK2 levels/activity could be a new and feasible therapeutic strategy to tackle T2D. In keeping with these findings, peptide GRK2 inhibitors result in improved glucose homeostasis in different animal models of diabetes (Anis, 2004). Moreover, a recent work from our laboratory has shown that decreasing GRK2 levels in a tamoxifen-inducible mice model is not only able to prevent, but also to reverse a pre-established insulin-resistant and obese phenotype (Vila-Bedmar, 2015). In this context, several tissue-specific processes seem to contribute to the beneficial effects of a reduction in GRK2 during a high fat feeding: improved insulin

signalling, enhanced lipolysis, increased expression of fatty acid oxidation and thermogenesis markers, and reduced hepatic steatosis and inflammation (Vila-Bedmar, 2015). At the molecular level, enhanced GRK2 expression appears to impair insulin-mediated AKT stimulation and insulin-induced glucose uptake by different mechanisms in specific tissues: directly interacting with IRS1 in adipocytes and myocytes (Garcia-Guerra, 2010); directly phosphorylating IRS1 at Ser 307 in cardiomyocytes (Ciccarelli, 2011); and by mediating endothelin-1-induced IR in 3T3-L1 preadipocytes via the inhibition of both Galphaq/11 and IRS1 pathways (Usui, 2005). Thus, the precise mechanisms modulating GRK2-mediated IR development in different tissues and the potential implication of phosphorylation events on GRK2-mediated IRS1 modulation are key issues for future research. Moreover, these findings are in agreement with the fact that IRS proteins represent a major node key to the development of IR (Copps and White, 2012).

INSULIN TARGET TISSUES

Insulin target tissues are able to function in response to insulin, given they express the insulin receptor and hold the appropriated intracellular enzymatic machinery able to regulate lipid and glucose metabolism. Under obesity or T2D conditions, these tissues are resistant to many of the actions of insulin. Thus, unravelling the different outcomes of obesity and IR in the main insulin-responsive tissues, as well as the physiopathological mechanisms involved, is crucial to understand the overall implications of an obese and insulin-resistant phenotype.

In the next sections we will next summarize the most important obesity-induced alterations in the normal physiological functions of the different insulin target tissues, and relate these

alterations to the key molecular mechanisms implicated in each case, with a clear focus on the integration of the different mechanisms contributing to the global IR phenotype.

Adipose Tissue

The adipose tissue can be considered a multi-depot organ since it consists of several "stores" located in two compartments: subcutaneous and visceral (Cinti, 2005). Likewise, adipose depots are commonly classified following their appearance in white and brown adipose tissue, although some groups also refer to 'beige' (Ishibashi and Seale, 2010) or 'brite' (brown in white) (Petrovic, 2010) adipocytes, meaning regions of white adipose tissue containing brown or brown-like adipocytes.

White adipose tissue

White adipose tissue (WAT) has been classically considered as a mere energy store and generally regarded as a connective tissue lacking specific anatomy. However, accumulating data support the idea that WAT forms a large organ with discrete anatomy, specific vascular and nerve supplies, high physiological plasticity (Cinti, 2012) and important endocrine and homeostatic functions (Trayhurn and Beattie, 2001). Accordingly, the structural and/or functional changes or distribution of adipose tissue as well as its excessive fat load, rather than obesity itself, are the key features correlating with metabolic alterations (Primeau, 2011, McLaughlin, 2012). In this regard, many researchers have reported that the intra-abdominal (visceral) adipose depot is a major contributor to metabolic risk, whereas the subcutaneous depot may have a protective role (McLaughlin, 2011). In fact, contrary to the subcutaneous WAT, excess of fat in visceral depots drives adipocyte hypertrophy rather than adipogenesis, a form of storage associated with more important metabolic alterations and inflammation

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(McLaughlin, 2014). Nevertheless, when adipogenesis is impaired, IR and inflammation develop also in the subcutaneous fat, so that this depot could also participate in the pathogenesis of the metabolic syndrome (Weyer, 2000). Remarkably, obese mice and humans without adipocyte hypertrophy (hyperplastic obesity) are insulin-sensitive (Cinti, 2005, Hoffstedt, 2010).

Along this line, macrophages seem to play an important role in the development of the metabolic disorders associated with obesity. Classical resident macrophages in WAT harbour a M2 or alternatively activated phenotype and produce anti-inflammatory cytokines. By contrast, obesity induces the recruitment of macrophages prone to M1 priming (Lumeng, 2007) which produce pro-inflammatory cytokines that interfere with insulin signalling pathways (Gregor and Hotamisligil, 2011). Other immune cell populations are also modified in the obese such as $CD8^+$ and $CD4^+T_H1$ lymphocytes which are increased and probably contribute to M1 macrophage recruitment and polarization (Nishimura, 2009, Winer, 2009). However, macrophages are generally considered as the effector cells contributing to inflammation-mediated IR, and it is macrophage infiltration what positively correlates with the size of adipocytes both in visceral and in subcutaneous fat (Cinti, 2005). Nevertheless, the number of macrophages is much higher in visceral fat, both in diet- and in genetically-induced obesity (Strissel, 2007, Murano, 2008). This fact is in agreement with the more deleterious consequences of visceral obesity (compared to the subcutaneous type) on insulin action, and with the depot-specific effect of TNFα inducing IR through the activation of JNK1/2 and serine phosphorylation of IRS1 in human visceral but not in subcutaneous adipocytes (Fernandez-Veledo, 2009b).

Molecular keys in WAT

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The production of adipokines (bioactive molecules released from adipose tissues) by overloaded and inflamed adipose depots is altered during obesity. This deregulated adipokine secretion pattern further contributes to adipose inflammation and altered lipid homeostasis (Jung and Choi, 2014), which promotes IR and fat accumulation in ectopic tissues (DeFronzo, 2004) leading to the stimulation of stress-induced kinases such as JNK and IKK-β. Moreover, activation of these kinases within the adipose tissue further contributes to the production of pro-inflammatory cytokines, such as IL-6, that lead to the development of IR in other tissues, including the liver (Sabio, 2008), and also *in vitro* in human myocytes (Fernandez-Veledo, 2008). FFAs also exacerbate pro-inflammatory cytokine secretion from M1 macrophages through activation of toll like receptors (TLRs). Interestingly, stress kinases may also be directly induced by FFA or endotoxemia through activation of TLR2 and 4 (Shoelson, 2007), thus providing an additional mechanism for the direct induction of IR and inflammation by dietary and gut-derived products. On the contrary, exercise reduces TLR4 expression, suppressing M1 macrophage infiltration in adipose tissue and/or promoting phenotypic switching from pro-inflammatory M1 to anti-inflammatory M2 macrophages (Ringseis, 2015). Along the same line, increasing fatty acid oxidation in adipocytes and macrophages has been suggested to decrease TG content and inflammation in adipocytes and reduce ER stress and oxidative damage in macrophages as well as improving insulin sensitivity (Malandrino, 2015).

Importantly, insulin is a critical regulator of several aspects of adipocyte biology. This hormone promotes adipocyte triglyceride storage by several mechanisms, including promoting adipocyte differentiation, and stimulating glucose uptake and triglyceride synthesis while inhibiting lipolysis. Insulin also enhances the uptake of FA from circulating lipoproteins by stimulating lipoprotein lipase (LPL) activity in WAT. Thus, maintaining the integrity of insulin signalling in adipocytes is crucial for the proper functioning of this tissue.

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Contrary to skeletal muscle, during obesity and T2D the expression of the glucose transporter GLUT4 is selectively decreased in adipocytes, and adipose-specific knockout or overexpression of GLUT4 alters systemic insulin sensitivity (Abel, 2001, Yang, 2005, Carvalho, 2005). Another important molecule, the phosphatase PTP1B, positively correlates with adiposity and contributes to IR. However, the role of PTP1B specifically in adipocytes is unclear, with studies demonstrating beneficial, detrimental or no effect(s) of adipose PTP1B deficiency on whole body mass and IR. Nevertheless, in a recent study using adipocyte-specific PTP1B knockout mice, no benefit of this deletion was found in terms of glucose homeostasis, lipid metabolism or adipokine secretion, suggesting that PTP1B does not appear to be the major negative regulator of IR in adipocytes (Owen, 2012).

On the other hand, mice lacking PTEN specifically in adipose tissue show improved systemic glucose tolerance and insulin sensitivity, in association with decreased fasting insulin levels, even when no differences in adiposity or plasma FFA were found (Kurlawalla-Martinez, 2005). Moreover, and in contrast to the increased global insulin sensitivity of transgenic mice overexpressing PTEN, the WAT of these mice showed reduced levels of phosphorylated AKT, but also decreased levels of IRS1 in serine inhibitory sites (Ortega-Molina, 2012).

Finally, in our laboratory we have put forward GRK2 not only as a novel regulator of insulin signalling in different insulin-target tissues including WAT, but also as a modulator of overall adiposity and fat mass accretion (reviewed in (Mayor, 2011)). In fact, GRK2 can act as an inhibitor of insulin-mediated glucose uptake in 3T3L1 adipocytes by interfering with Gαq/11 (Usui, 2005), and by interacting with IRS1 independently of its kinase activity (Garcia-Guerra, 2010). Furthermore, WAT from GRK2$^{+/-}$ mice presents a reduced expression of enzymes involved in lipogenesis (Vila-Bedmar, 2012) consistent with the decreased size of white adipocytes in aged or HFD-fed mice compared with WT littermates (Garcia-Guerra,

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2010). This reduced size is not related to alterations in the differentiation capacity of white adipocytes, since inducing GRK2 loss during a HFD also decreases epididymal fat mass and adipocyte size, in association with an enhanced lipolytic capacity in WAT (Vila-Bedmar, 2015).

Brown adipose tissue

Brown adipose tissue (BAT) represents a natural target for increasing energy expenditure (EE) since it is the main organ involved in heat production through a process known as non-shivering adaptive thermogenesis, an energy-dissipating process key to maintaining body temperature. This process depends on the specific expression of the uncoupling protein (UCP)1 in the mitochondrial inner membrane of brown adipocytes, which allows to dissipate the excess of energy in the form of heat (reviewed in (Cannon and Nedergaard, 2004)). Therefore, although insulin regulates metabolism in both brown and white adipocytes, the role of both tissues in energy storage and utilization is quite different. Unlike WAT, BAT accumulates lipids not as a store for excess of energy but as a source of FA to be oxidized in mitochondria when thermogenesis is activated to produce heat at the expense of coupled ATP production, although an increase in the uncoupling machinery of brown adipocytes has also been described to be followed by an enhancement of proteins involved in ATP synthesis (Guillen, 2013). BAT was classically considered of metabolic significance only in small mammals and human newborns, since it was thought to disappear rapidly after birth in humans. However, several pieces of evidence have put forward the role of this tissue in the regulation of energy balance in humans (reviewed in (Vila-Bedmar and Fernandez-Veledo, 2011, Cypess and Kahn, 2010)). As a result, much interest has focused on BAT as a target for pharmacotherapy of obesity-associated disorders. In fact, alterations in this tissue have been related to adiposity control, IR and T2D (Lowell, 1993, Feldmann, 2009).

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Given the role of BAT as a sink for draining and oxidation of glucose and triglycerides from blood, promotion of BAT development/activity or even remodelling of WAT depots in order to promote browning of this tissue offers the possibility of increasing EE, contributing to the reduction of body weight and the improvement of glucose tolerance. In keeping with this notion, the inherent plasticity of the adipose organ offers the possibility to manipulate this tissue, pointing at the enhancement of BAT activity as an important therapeutic strategy in the protection against obesity and metabolic syndrome. In fact, animals with higher amount of BAT are more resistant to obesity and T2D (Kopecky, 1995, Collins, 2004, Almind, 2007) whereas, animals without functional BAT are prone to these conditions (Lowell, 1993, Feldmann, 2009). Most interestingly, these observations are also valid for humans (Vila-Bedmar and Fernandez-Veledo, 2011, Cypess and Kahn, 2010).

Molecular keys in BAT

While sympathetic innervation of BAT plays an essential role in its thermogenic function, several studies *in vitro* and *in vivo* have revealed that brown adipocytes are also target cells for insulin action, since insulin stimulation increases glucose uptake and GLUT4 translocation to the plasma membrane in foetal brown adipocytes (Lorenzo, 2002, Valverde, 2005). Interestingly, IR in BAT has been associated with impaired thermogenesis in obese mutant rodents (Mercer and Trayhurn, 1984). However, the mechanism by which insulin affects glucose transport in brown fat appears to be unrelated to the activation of thermogenesis. In fact, transgenic mice moderately overexpressing PTEN present BAT hyperactivity mediated by the inhibition of PI3K/AKT/Foxo signalling pathway and associated with increased EE and with lower adiposity and body weight. These effects run in parallel to a number of systemic beneficial consequences, including improved insulin sensitivity and protection from HFD-induced IR and steatosis (Ortega-Molina, 2012). Additionally, in the absence of insulin,

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physiological concentrations of norepinephrine stimulate glucose transport in brown adipocytes via β-adrenergic pathways (Dallner, 2006, Shimizu, 1991).

One key mediator of IR in brown adipocytes, as described in WAT, are proinflammatory cytokines such as TNFα. The molecular mechanisms involved in TNFα-induced IR depend on *de novo* ceramide production (Fernandez-Veledo, 2006a) and on the activation of stress kinases and potential Ser-Thr phosphorylation of IRS2 (Hernandez, 2004). A significant enhancement of PTP1B expression and activity is observed in TNFα-treated brown adipocytes, and the lack of this phosphatase conferred protection against TNFα-induced impairment of glucose uptake or insulin signalling (Fernandez-Veledo, 2006b). Interestingly, this cytokine not only stimulates IR in BAT, but also acts as a negative regulator of adipogenic and thermogenic differentiation in brown adipocytes (Lorenzo, 2008). In addition,

Besides glucose uptake, *de novo* lipid synthesis also occurs in BAT with insulin as an essential lipogenic regulator. In this regard, lack of IRS1 leads to IR in brown adipocytes at the level of thermogenic gene expression (Valverde, 2003) and lipid synthesis (Valverde, 1999). Thus, besides its essential role in adipogenesis, IRS1 is a key molecule in mediating insulin-induced thermogenic gene expression in foetal brown adipocytes.

Finally, GRK2 also appears to play an important role in BAT function and architecture, as well as in brown adipocyte differentiation (Vila-Bedmar, 2012). In this regard, the decreased weight observed in 9-month old $GRK2^{+/-}$ mice seems to be due, at least in part, to an increased function of BAT in these animals. Accordingly, GRK2 hemizygous mice display higher EE and lower respiratory exchange ratio, which correlates with an improved morphology of BAT in $GRK2^{+/-}$ adult mice (Vila-Bedmar, 2012). Along the same line, inducing GRK2 depletion with tamoxifen during a HFD also preserved the morphology of BAT that shows an enhanced lipolytic response to adrenergic stimulation. These features

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were associated to an increased expression of thermogenic and FA oxidation markers, such as uncoupling protein 1 (UCP1) and carnitine palmitoyltransferase 1 (CPT1), respectively, which may underlie an increased capacity for fatty acid metabolism and thermogenesis in BAT upon GRK2 downmodulation. Accordingly, these changes would allow BAT to act as an efficient "metabolic sink" for the extra free fatty acids produced in WAT, thereby avoiding the establishment of lipotoxicity (Vila-Bedmar, 2015). Thus, our data point toward GRK2 inhibition as a potential tool for the enhancement of brown fat activity in addition to the reported function of this kinase in the regulation of insulin signalling.

Liver

The liver plays a central role in the maintenance of glucose homeostasis. During fasting, the liver is responsible for the synthesis of glucose from non-carbohydrate sources, in a process called gluconeogenesis. After feeding, when the glucose levels rise, the liver acts as a glucose sensor inactivating the endogenous production of glucose and storing the excess of monocarbohydrates as glycogen. These two processes are mainly regulated by insulin and in physiological conditions restore the normoglycaemia after a meal. However, in the obesity-related diabetic or pre-diabetic state, the liver is an end-organ for the effects of IR. Hepatic IR causes an increased glucose output and a decreased glucose clearance leading to sustained hyperglycaemia.

One of the most important complications related to obesity-induced hepatic IR is non-alcoholic fatty liver disease (NAFLD). NAFLD expands different stages, from simple steatosis to non-alcoholic steato-hepatitis (NASH) involving the development of inflammation, varying degrees of fibrosis and, ultimately, cirrhosis, end-stage liver failure and

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hepatocellular carcinoma (Malaguarnera, 2009). IR promotes hepatic triglyceride accumulation as a result of increased peripheral adipose lipolysis and the influx of free FA released from dysfunctional and insulin-resistant adipocytes. IR also upregulates the levels of hepatic lipogenic transcription factors (Smith and Adams, 2011) causing hepatic steatosis, a condition often present in metabolic syndrome patients. In the steatotic liver the lipotoxicity caused by FFA accumulation and triglyceride-derived toxic metabolites (such as DAGs and ceramides) triggers the activation of inflammatory pathways, reactive oxygen species (ROS)-induced cellular dysfunction and ER stress. Altogether, these mechanisms lead to hepatic inflammation and worsening of hepatic IR (Smith and Adams, 2011, Malaguarnera, 2009, Cusi, 2012).

Moreover, the liver of subjects with NAFLD might release a variety of proatherogenic, proinflammatory, and diabetogenic mediators (Anstee, 2013), which play an important role in the pathogenesis of systemic inflammation and may contribute to the development of IR and cardiovascular disease (Stefan and Haring, 2013). In this regard, the discovery of proteins that are exclusively or predominantly secreted from the liver and that directly regulate inflammation as well as glucose and lipid metabolism, such as fibroblast growth factor 21 (FGF21), has boosted the investigation into this field. In parallel to the proteins released from adipose tissue these liver-derived proteins are known as hepatokines. Therefore, the liver not only functions as a target organ of inflammatory and lipolytic reactions occurring within dysfunctional adipose tissue, but as an inducer of systemic inflammation.

Molecular keys in the liver

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Cumulative evidence suggests that IRS2 is the major effector of the actions of insulin in cultured hepatocytes. A distinctive role for IRS1 and IRS2 have been suggested on the basis that individual knockouts of IRS1 or IRS2 have only modest effects on murine nutrient metabolism, while the combined knockouts result in diabetes (Dong, 2006, Haeusler and Accili, 2008, Gonzalez-Rodriguez, 2010). Importantly, IRS2 is highly regulated by nutrient signals in this tissue (Dong, 2006, Ide, 2004), and the accumulation of DAG is described to activate PKCϵ and/or JNK1, which can lead to impaired IRS2 tyrosine phosphorylation thus contributing to hepatic IR (Erion and Shulman, 2010).

In line with this notion, recent studies have introduced the peroxisome proliferator-activated receptor α (PPARα)-FGF21 hormone axis as a target for hepatic JNK-mediated IR. This study also demonstrates how hepatic ablation of both genes (*Jnk1* and *Jnk2*) and not hepatic JNK1 deficiency protects against HFD-induced IR (Vernia, 2014).

The specific role of phosphatases that switch off the insulin signalling pathway has also been investigated in the liver. In this regard, reducing PTP1B levels increase hepatic insulin signalling, decrease expression of gluconeogenic genes, enhancing insulin-induced suppression of hepatic glucose production, and improves glucose tolerance (Klaman, 2000). Accordingly, PTP1B antisense oligonucleotides that specifically downregulate PTP1B levels in liver and fat enhance insulin signalling and decrease adiposity in ob/ob and db/db mice in parallel with a reduced expression of lipogenic genes in these tissues (Waring, 2003, Zinker, 2002). Liver-specific PTP1B knockout led to improved systemic glucose and lipid homeostasis and diminished expression of lipogenic genes, also protecting against HFD-induced ER stress response in the liver (Delibegovic, 2009). Moreover, recent studies using inducible liver-specific PTP1B knockdown mice have shown that PTP1B downregulation

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reverses glucose intolerance and improves lipid homeostasis decreasing lipid deposition in the liver in HFD-fed obese and insulin-resistant adult mice (Owen, 2013).

Also, a liver-specific deletion of PTEN led to increased insulin sensitivity in this tissue, increased skeletal muscle insulin signalling and glucose uptake and improved overall glucose tolerance (Stiles, 2004). Moreover, WAT depots were reduced and had characteristics of browning in these mice (Peyrou, 2015). This systemic insulin-sensitizing effect of a liver-specific deletion of PTEN has been explained through the modulation of the expression of liver-derived circulating factors that regulate muscle insulin sensitivity and WAT homeostasis, such as FGF21 (Peyrou, 2015). However, PTEN expression has been reported to be downregulated in steatotic rat and human livers (Vinciguerra, 2008), and PTEN liver-specific KO mice develop fatty liver through increased FA uptake and *de novo* lipogenesis, together with decreased VLDL export (Qiu, 2008), whereas transgenic mice overexpressing PTEN are protected against HFD-induced steatosis (Ortega-Molina, 2012). Accordingly, studies in hepatocytes showed that unsaturated fatty acids down-regulate PTEN levels via activation of a complex formed by mTOR and NFκB (Vinciguerra, 2008). The paradoxical results found in the liver of PTEN liver-specific KO mice suggest a bifurcation in the effects of PTEN on insulin signalling cascade within the liver, producing on the one hand insulin hypersensitivity and in the other hand inducing the accumulation of lipids in this tissue. One possible explanation is based on the fact that mTORC1 is required for insulin-induced stimulation of lipogenesis, but not for other insulin-mediated hepatic effects (Li, 2010).

Notably, our group has shown that GRK2 levels were increased in the liver of mice fed a HFD (Garcia-Guerra, 2010), and decreasing GRK2 protected against HFD-induced hepatic insulin resistance and impaired lipid accumulation in tamoxifen-induced $GRK2^{-/-}$ mice (Vila-Bedmar, 2015). Along the same lines, Sprague-Dawley rats fed a HFD for two weeks

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presented increased hepatic plasma membrane GRK2 (Charbonneau, 2007). Moreover, strong insulin-mediated AKT phosphorylation was detected in the liver of $GRK2^{+/-}$ mice under different IR-inducing conditions (Garcia-Guerra, 2010). *In vitro* results in mouse liver FL83B cells demonstrated that GRK2 negatively regulates basal and insulin-stimulated glycogen synthesis via a post-IR signalling mechanism, and that GRK2 may contribute to reduced IR expression and function during chronic insulin exposure. Mechanistically, GRK2 seems to affect phosphorylation of Ser307 on IRS1, reducing insulin receptor-IRS1 interaction and thus insulin receptor-mediated phosphorylation of Tyr612 on IRS1 (Shahid and Hussain, 2007). A similar increase of IRS1 phosphorylation at Tyr612 after IGF-1 treatment was found in HepG2 cells with reduced GRK2 protein levels (Wei, 2013). Preliminary results from our laboratory also reveal a role for GRK2 in the regulation of steatohepatitis (Cruces-Sande M, unpublished observation), what warrants further investigation on the control of hepatic glucose and lipid metabolism by this kinase.

Muscle

Skeletal muscle is a critical tissue for glycaemic control since it is the quantitatively major site of insulin-stimulated glucose clearance in the postprandial state, accounting for approximately 80% of glucose disposal under insulin-stimulated conditions (DeFronzo and Tripathy, 2009), and it also represents the largest glycogen storage organ (almost quadrupling the capacity of the liver). Thus, IR and metabolic dysfunction in skeletal muscle play a major role in the development of the metabolic syndrome and T2D (DeFronzo and Tripathy, 2009).

The multiple alterations in adipose tissue homeostasis occurring during obesity, including an altered secretion profile of adipokines as well as increased lipolysis, lead to the redistribution

of FFA to non-adipose tissues such as skeletal muscle (DeFronzo, 2004). As a consequence, increased amounts of ectopic lipid stores are found in muscle from obese patients, which have been reported to induce IR in skeletal muscle by directly inhibiting insulin-stimulated glucose transport (reviewed in (Erion and Shulman, 2010)). However, some studies suggest that the connection between the amount of intra-myocellular lipids and IR might not be straightforward, since other factors, such as lipid droplet size or training status, may also play a role (Eckardt, 2011). Intra-myocellular lipids provide a source for the generation of metabolites including DAGs and ceramides, which have been widely described to impair insulin signalling. Abnormal accumulation of these metabolites activates different isoforms of PKC, including PKCθ, as well as IKKβ and JNK kinases, reducing or even abrogating insulin-mediated glucose uptake. Lipid infusion studies in humans have confirmed these findings, although PKCβII and PKCδ, rather than PKCθ, were found to be the relevant PKC isoforms implicated. Additional studies with different mouse models have provided further insight into the mechanisms that links intracellular DAG accumulation to IR (reviewed in (Erion and Shulman, 2010)). Furthermore, IKKβ is able to activate NFκB, which in turn regulates the production of pro-inflammatory cytokines such as IL-6 (Shoelson, 2003) which has been reported to have a dual effect on IR in muscle: stimulating insulin actions after an acute IL-6 challenge, but precluding insulin signalling upon a chronic exposure (Nieto-Vazquez, 2008).

The accumulation of lipid metabolites may be due to an imbalance between FFA delivery and intracellular fatty acid oxidation and storage. Accordingly, several studies have reported that fatty acid oxidation is reduced in both T2D and obese insulin-resistant individuals (reviewed in (Kelley, 2005)), suggesting that muscular mitochondrial oxidative capacity is impaired in these disorders and putting forward a role for mitochondrial dysfunction as a cause of muscle IR. In addition, several studies performed in obese humans and rats have revealed an increase

in the FFA transporter CD36 at the plasma membrane of skeletal muscle cells as well as an enhanced transport of FFA into this tissue (Eckardt, 2011), together with a reduction in intracellular lipid content after specific inhibition of this transporter in myotubes obtained from obese patients (Aguer, 2010). Nevertheless, it is still unclear whether it is mitochondrial dysfunction that leads to increased intra-myocellular lipid content and IR or rather if an increased muscle lipid content secondary to elevated plasma FFA levels is what leads to mitochondrial dysfunction and IR. Despite discrepancies in the exact mechanisms, it is clear that an acute exposure to FFAs and excess dietary lipid intake may lead to lipid-induced IR, which is a major trigger for IR in muscle in obesity. Notably, recent studies have reported impaired regenerative capacity of skeletal muscle following injury in obese mice, pointing to the possibility that muscle satellite cell function is also compromised under conditions of lipid overload. In fact, toxic lipid metabolites would also contribute to decreased regenerative capacity of skeletal muscle in obese animals impairing the potential for satellite cell-mediated repair (reviewed in (Akhmedov and Berdeaux, 2013)).

On the other hand, nowadays, skeletal muscle is also recognized as an endocrine organ, and proteins released from this tissue have been termed myokines, in analogy to adipokines secreted by adipose tissue. Similarly to adipokines, these recently discovered myokines are likewise able to mediate metabolic homeostasis, contributing to the regulation of glucose and fatty acid metabolism as well as modulating inflammation (reviewed in (Eckardt, 2014)).

Molecular keys in muscle

In morbid obesity, the expression and/or activation of some components of the insulin signalling cascade is altered in skeletal muscle, including IRS1 phosphorylation as well as PI3K activity (Goodyear, 1995), although no alterations have been reported in GLUT4 expression levels in skeletal muscle of obese and diabetic humans (reviewed in (Shepherd and

Kahn, 1999)). In fact, defective glucose transport has been suggested to be due to impaired translocation, docking, or fusion of GLUT4-containg vesicles with the plasma membrane (Bogan, 2012). However, inactivation of GLUT4 in skeletal muscle leads to IR (Kim, 2001b) and muscle-specific overexpression of GLUT4 improves glucose disposal in animal models of IR (Leturque, 1996). These reports suggest that maintaining high levels of GLUT4 in skeletal muscle might be a potential strategy for treatment of IR. In contrast, transgenic overexpression of PTP1B in muscle decreased glucose uptake (Zabolotny, 2004), whereas, ablation of PTP1B specifically in this tissue improved systemic insulin sensitivity in mice fed a HFD (Delibegovic, 2007). Furthermore, PTP1B is upregulated by the proinflammatory cytokine TNF-α and myocytes lacking PTP1B are protected against TNF-α-induced IR (Nieto-Vazquez, 2007), and the lack of PTP1B has also been reported to confer protection against long term IL-6 treatment-induced IR in skeletal muscle *in vitro* and *in vivo* (Nieto-Vazquez, 2008). Moreover, despite the fact that JNK1 deficiency does not enhance muscle glucose metabolism in lean mice (Witczak, 2006), the saturated fatty acid palmitate has been reported to induce PTP1B expression in skeletal muscle cells through a mechanism involving the activation of ceramide, JNK and NFκB pathways (MohammadTaghvaei, 2012).

Interestingly, many chronic diseases, including obesity and IR, have been associated with reduced muscle mass and strength, so enhancing skeletal muscle may also offer great benefit in improving glucose homeostasis. In this regard, $GRK2^{+/-}$ mice contained hypertrophied skeletal muscle fibres than WT littermates (Garcia-Guerra, 2014), but whether this fact contributes to the improved glucose homeostasis observed in hemizygous GRK2 mice is still unknown. In any case, tamoxifen-induced $GRK2^{-/-}$ mice display enhanced insulin signalling in muscle (Vila-Bedmar, 2015), according to the suggested negative role for GRK2 in the regulation of insulin signalling in skeletal muscle both in cultured myocytes and *in vivo* in $GRK2^{+/-}$ mice (Garcia-Guerra, 2010), most probably by mechanisms independent of kinase

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activity and involving the formation of dynamic GRK2/IRS1 complexes (Garcia-Guerra, 2010). In addition, overexpression of GRK2 in C2C12 myoblasts impairs cell differentiation, a process known to depend on different protein kinases including AKT (Garcia-Guerra, 2014).

Heart

Although the heart is not a canonical metabolism-regulatory organ, IR and associated reductions in cardiac insulin signalling is emerging as a major factor for the development of heart failure. The insulin cascade is crucial to maintaining cardiac functionality since the heart is a constitutive energy-demanding organ. Glucose and long chain FFA are major substrates in cardiac tissue although it is highly flexible and able to use other substrates when available, this metabolic plasticity being key to normal cardiac physiology. Notably, increased long chain FFA supply to cardiomyocytes, as found during obesity, will evoke persistent localization of its transporter CD36 in the sarcolemma, thus leading to chronically elevated long chain FFA uptake and lipid accumulation what finally results in IR (Muoio, 2014). This IR further prevents the cardiac metabolic flexibility required to supply the vast amounts of ATP needed for continuous cardiac contraction. Moreover, abundant evidence shows that obesity-related disorders are associated with structural and functional changes in the heart both in humans and in animal models. In fact, heart failure and overt cardiovascular disease is the leading cause of death worldwide and obesity and its related comorbidities are well-established risk factors considered to dramatically increase their incidence (Dzau, 2006). Under unhealthy lifestyle habits, the heart undergoes initially compensatory changes that, when maintained, finally initiate heart failure. The main features of obesity-related cardiac remodelling are an increase in total blood volume and thus increased cardiac output, left

ventricular hypertrophy, left ventricular systolic and diastolic dysfunction, and fat accumulation (Abel, 2008). The aetiology of cardiac hypertrophy is complex and may have as underlying triggers the expansion of plasma volume and the activation of the sympathetic nervous system. While left ventricular hypertrophy is regarded as the main characteristic of obesity-induced cardiac remodelling both in humans and animal models (Abel, 2008), other emerging features such as accumulation of intramyocelullar triglycerides in the heart also appear to be important. Intra-myocyte fat accumulation has been reported in ob/ob and db/db mice (Buchanan, 2005), Zucker obese rats (Olsen, 2013) and following HFD (Torre-Villalvazo, 2009). This cardiac steatosis has been related with increased levels of ceramides and cardiomyocyte apoptosis (Zhou, 2000, van de Weijer, 2011) leading to lipotoxic cardiomyopathy. However, lipid accumulation in the myocardium is not necessary damaging as it could be a marker of the adaptation of the heart upon disproportionate dietary fat and/or cardiac IR thereby helping avoid an excessive flux towards mitochondrial oxidative phosphorylation and ROS production. In any case, this intramyocardial lipid accumulation has been reported to alter cardiac functionality in obese animal models, with mild impaired diastolic function in adult animals in the absence or presence of mild systolic dysfunction (Christoffersen, 2003). Moreover in some cases interstitial (Zhou, 2000) or perivascular fibrosis (Zaman, 2004) have been reported in some animal models of obesity.

Molecular keys in the heart

At a molecular level, the mechanisms that contribute to structural and functional changes in the heart of obese animals are: impaired insulin signalling and changes in cardiac metabolism (characteristic of diabetic cardiomyopathy), mitochondrial dysfunction and oxidative stress, neurohumoral activation and volume/pressure overload, inflammation, fibrosis and apoptosis (Abel, 2008). Two main signalling pathways are involved in cardiac remodelling and

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dysfunction: the insulin and the renin-angiotensin systems. In fact, inhibition of negative modulators of the insulin signalling pathway such as PTP1B (Kandadi, 2014) or the use of inhibitors of the angiotensin-converting enzyme (Duarte, 1999, Nevelsteen, 2013) or the use of antagonists of the angiotensin II receptor (Oliveira Junior, 2013) restore, at least in part, obesity-induced alterations in cardiac tissue.

Interestingly, GRK2 is a negative modulator of both insulin and angiotensin II pathways (Mayor, 2011, Lucas, 2014b, Avendano, 2014). In fact, GRK2 is a key player in the crosstalk between insulin receptor signalling and other GPCRs critical for cardiac contractility and physiology, such as the β-adrenergic receptors (β-AR). Indeed, its inhibition or genetic deletion in several animal models of HF has shown that GRK2 targeting improves different parameters of the failing heart (reviewed in (Sato, 2015, Cannavo, 2013). Along the same line, paroxetine, which selectively inhibits GRK2 (Homan and Tesmer, 2015), has been recently reported to block or even reverse heart damage after myocardial infarction in a mouse model (Schumacher, 2015). In addition, non-βAR properties of GRK2 appear to also contribute to its pathological effects. In this regard, GRK2 also desensitizes adiponectin receptor 1 in failing cardiomyocytes, contributing to and HF progression (Wang, 2015). Thus, its inhibition will likely complement existing therapies such as βAR blockade.

At the molecular level, it has been suggested that increased GRK2 levels upon chronic β-AR stimulation can directly interact and phosphorylate IRS1 and underlie the excessive sympathetic nervous system activity-triggered IR in HEK-293 cells (Cipolletta, 2009). A similar mechanism occurs in cardiomyocytes and has been related to the pathogenesis of the injured heart (Ciccarelli, 2011). On the other hand, it was shown that insulin can promote β_2-AR phosphorylation directly impairing β-AR-regulated cardiac contractility. The insulin-induced phosphorylation of the β-AR seems to be dependent on IRS1 and IRS2 and involve

PKA and GRK2 activity (Fu, 2014). So, GRK2 would link cardiac remodelling and IR to impaired contractility and cardiac dysfunction, features that are present in obese individuals and may lead to heart failure. In fact, our group has recently described that the levels of GRK2 are increased in the cardiac tissue of ob/ob mice and after HFD feeding (Lucas, 2014b). On the contrary, exercise, which is critical for the prevention and treatment of obesity and has been described to reduce IR in cardiac muscle of HFD-fed rats (Medeiros, 2011), is able to decrease myocardial GRK2 levels (MacDonnell, 2005) highlighting the cardioprotective role of lowering GRK2 levels for the obese heart. Moreover, unpublished results from our and other groups suggest that lower levels of GRK2 protect the heart from obesity-induced cardiac remodelling (Lucas E, in preparation). Altogether, our findings suggest that pathological inputs of different aetiology such as increased catecholamine levels or high dietary fat converge in common important nodes. One such key connecting hub is GRK2 whose enhanced cardiac expression would be responsible for fuelling dysfunctionality both in the GPCR and insulin signalling cascades, thus allowing progression towards maladaptive remodelling (Lucas, 2014a).

Vasculature

Obesity also has a key impact on vascular function, structure and on the mechanical properties of the vessels by several important means: it decreases endothelium-dependent vasodilation, increases intima-media ratio and vascular media thickness and enhances vessel stiffness (Briones, 2014, Prieto, 2014). These vascular alterations might impair tissue perfusion and contribute to the damage in different target organs observed in this pathology (Briones, 2014, Prieto, 2014). Mechanisms responsible for the vascular alterations associated to obesity include indirect phenomena associated to IR and also the presence of concomitant

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risk factors such as hypertension, diabetes or dyslipidaemia as well as direct mechanisms associated with effects of adipokines, FFA and inflammation on the vascular wall (Briones, 2014, Prieto, 2014).

Molecular keys in the vasculature

Particularly important mediators in the vascular dysfunction associated to obesity are effectors of the renin-angiotensin-aldosterone system such as AngII and aldosterone, but also endothelin and prostanoids that can signal through specific GPCRs (Briones, 2014, Prieto, 2014, Even, 2014). These mediators stimulate vascular smooth muscle cells (VSMC) proliferation or migration, change extracellular matrix deposition, and counteract the vasodilator actions of nitric oxide (NO) through different mechanisms including increased ROS that reduce NO bioavailability. NO deficiency during obesity can also be ascribed to altered expression/activity of the endothelial NO synthase (eNOS) or eNOS uncoupling due to substrate or cofactor deficiency (Briones, 2014, Prieto, 2014, Miao and Li, 2012, Iantorno, 2014, Paneni, 2013). In addition, recent evidences demonstrate that the diminished NO production in obesity is triggered by alterations in eNOS activation through the phosphorylation at Ser1177 by different kinases (Mount, 2007, Prieto, 2014). More specifically, defective eNOS phosphorylation and NO production by the PI3K/AKT pathway have been described in obesity in response to different stimuli including insulin (reviewed in (Prieto, 2014)). In this regard, IR in endothelial cells impairs the production of NO, favours the production of endothelin-1 though the MAPK axis and thus the vasoconstrictive and mitogenic responses on the vascular wall (Muniyappa and Quon, 2007) leading to endothelial dysfunction.

Earlier studies demonstrated that AKT physically interacts with GRK2 and this interaction inhibits AKT activity and NO production (Liu, 2005). In addition, different mediators

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involved in vascular damage in obesity/diabetes upregulate GRK2 expression. Thus, the increased GRK2 expression observed in vessels from diabetic mice (Taguchi, 2011a, Taguchi, 2011b, Taguchi, 2012a, Taguchi, 2012b) is abolished by AngII type I receptor blockade (Taguchi, 2011b) suggesting that AngII increases GRK2 expression as confirmed recently by our group (Avendano, 2014). Moreover, in cultured endothelial cells high glucose/high insulin milieu upregulates GRK2 by an still unknown mechanism leading to inhibition of the insulin/AKT/eNOS pathway (Taguchi, 2014). In diabetes, the increased levels of GRK2 seem to prevent the translocation of β-arrestin2 to the membrane which acts as a scaffold molecule for AKT to the insulin receptor (Luan, 2009), thereby contributing to impaired AKT/eNOS/NO production in response to insulin and other agonists (Taguchi, 2011a, Taguchi, 2012a, Taguchi, 2012b, Taguchi, 2014). More importantly, GRK2 inhibition or partial GRK2 deletion improved the endothelial dysfunction observed in obese/diabetic (Taguchi, 2011b, Taguchi, 2012b, Taguchi, 2013) or hypertensive (Avendano, 2014) animal models by restoring the impaired AKT/eNOS pathway and NO availability.

Vessels from obese patients or animal models of obesity generally display hypertrophic remodelling with particular differences depending of the vascular bed (Briones, 2014). This vascular remodelling seems to be influenced by hemodynamic factors such as hypertension and also by metabolic factors such as insulin or adipokines and it is partially reversed by pharmacological blockade of different components of the renin- angiotensin-aldosterone or endothelin systems (Briones, 2014). To date, the role of GRK2 in the structural and mechanical alterations observed in obesity is unknown, however, partial GRK2 deletion prevented vascular hypertrophy and increased vessel stiffness induced by AngII (Avendano, 2014), suggesting that GRK2 might have a key role in vascular alterations in obesity-associated hypertension. The mechanisms responsible of these vascular effects of GRK2 *in vivo* are unknown, and *in vitro* studies have revealed conflicting results. Thus, GRK2 and

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arrestin seem to be essential for agonist-stimulated VSMC migration through different mechanisms including activation of proliferative and promigratory MAPK such as ERK1/2 (Morris, 2012). However, a protective role for GRK2 in VSMC proliferation has also been shown (Peppel, 2000, Guo, 2009). In sum the results presented in this section suggest that GRK2 is a newly identified key contributor of the endothelial dysfunction and other vascular alterations observed during obesity.

CONCLUSIONS AND FUTURE DIRECTIONS

Given the multi-organ effects of insulin and the key role for the integration of these tissue-specific effects, it becomes crucial to understand how this physiopathological network operates in order to globally explain the outcomes of obesity and IR (Figure 1).

In this review we put together the different outcomes of fat accumulation and IR in the main insulin-responsive tissues, highlighting the role of the most important molecular routes that participate in the control of disease progression both in an organ-specific and also in a more systemic manner (Figure 2). We believe that a multi-organ approach to the study of the onset and progression of the insulin-resistant and obese condition is, currently, the most valid approximation if we aim to understand in depth the different pathophysiological outcomes and the distinct molecular basis of the disease. In particular, this type of approach becomes essential if we intend to interpret the consequences of our experimental findings in a particular cell type in a more global whole body context. From the analysis of cell line and tissue-specific studies, important molecular mechanisms are identified and its cellular consequences can be described, but they will only be validated if introduced into a more

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complex picture including a network of inter-organ communication and regulatory activities that help explain the final outcomes observed in the obese condition.

We believe that certain signalling nodes, in particular those having specific roles in different organs and cell types such as PTP1B, PTEN or GRK2 among others (Figure 2), are bound to emerge as key pharmacological targets given the fact that they can more actively help integrate the response of different tissues and efficiently fight the pathological consequences of obesity and insulin resistance in a multi-organ manner.

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DECLARATION OF INTEREST

The authors report no declarations of interest.

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FIGURE LEGENDS

Figure 1. Physiological roles of the different insulin-target tissues and integration of the multi-organ effects of obesity in the physiopathology of insulin resistance. In healthy individuals, insulin-target tissues function coordinately in order to finely tune metabolism and energy homeostasis. During obesity, the specific deregulation of each tissue contributes to the progress of the complex pathological scenario observed in the obese, including the development of a low grade chronic inflammatory state and the appearance of insulin resistance in a systemic manner. Moreover, when the fat storage capacity in the adipose tissue is surpassed, fat is accumulated in other organs, boosting tissue inflammation, shifting substrate utilization and altering endocrine function, further contributing to the systemic insulin resistance and subsequent metabolic deregulation.

Figure 2. Key molecular nodes regulating insulin signalling and resistance. Insulin activates the insulin receptor (IR), which phosphorylates and recruits different substrate adaptors such as the IRS protein family. Tyrosine-phosphorylated IRS proteins act then as scaffolds coupling IR stimulation to the activation of the phosphatidylinositol 3-kinase (PI3K), which has a major role in insulin function, mainly via the activation of AKT, which phosphorylates many cellular proteins, thus regulating several processes that lead to the control of physiopathological effects in different organs and tissues. Insulin signalling is controlled by negative modulators that tightly control the biological response to this hormone and act at different stages of the transduction cascade. Pathological alterations in any of these modulators, as observed during obesity or inflammation, may lead to the development of IR. These signalling nodes, in particular those having specific roles in different organs and at various levels, such as JNK and IKK as well as mTORC1, PTP1B, PTEN or GRK2 emerge as key potential pharmacological targets.

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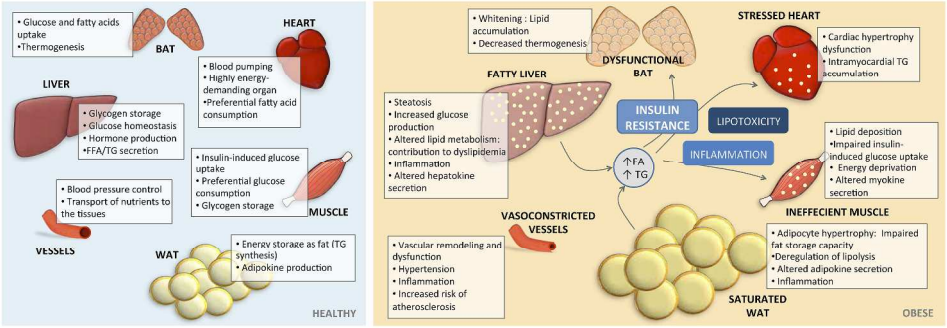

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268x92mm (300 x 300 DPI)

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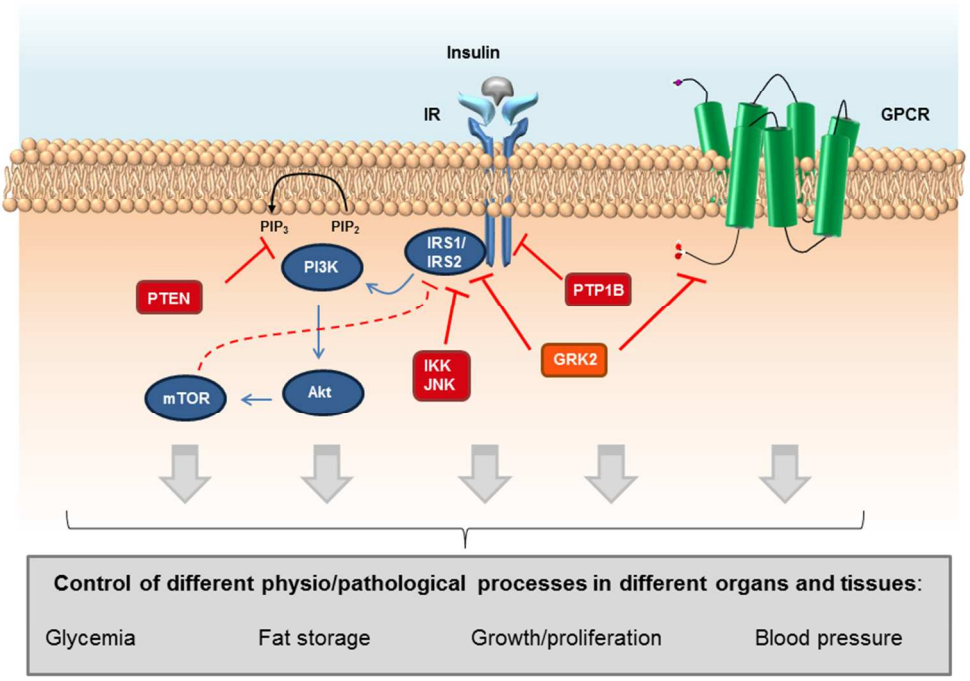

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254x190mm (96 x 96 DPI)