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REVIEW ARTICLE

Targeting the NO/superoxide ratio in adipose tissue: relevance to obesity and diabetes management

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Insulin sensitivity and metabolic homeostasis depend on the capacity of adipose tissue to take up and utilize excess glucose and fatty acids. The key aspects that determine the fuel-buffering capacity of adipose tissue depend on the physiological levels of the small redox molecule, nitric oxide (NO). In addition to impairment of NO synthesis, excessive formation of the superoxide anion ($O_2^{\bullet-}$) in adipose tissue may be an important interfering factor diverting the signalling of NO and other reactive oxygen and nitrogen species in obesity, resulting in metabolic dysfunction of adipose tissue over time. Besides its role in relief from superoxide burst, enhanced NO signalling may be responsible for the therapeutic benefits of different superoxide dismutase mimetics, in obesity and experimental diabetes models. This review summarizes the role of NO in adipose tissue and highlights the effects of NO/$O_2^{\bullet-}$ ratio 'teetering' as a promising pharmacological target in the metabolic syndrome.

LINKED ARTICLES

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Abbreviations

AMPK, AMP-activated protein kinase; BH_4, tetrahydrobiopterin; CcOx, cytochrome c oxidase; ETC, electron transport chain; IR, insulin receptor; IRS-1, insulin receptor substrate 1; MnSOD, manganese superoxide dismutase; NOX, NADPH oxidase; OXPHOS, oxidative phosphorylation; PGC-1α, PPARγ coactivator 1α; PEPCK-C, cytosolic phosphoenolpyruvate carboxykinase; PTP, protein tyrosine phosphatase; RNS, reactive nitrogen species; sGC, soluble guanylate cyclase; SNO, *S*-nitrosothiol; TAG, triacylglycerol; Trx, thioredoxin; UCP, uncoupling protein; XO, xanthine oxidoreductase

DOI:10.1111/bph.13498

Tables of Links

TARGETS	
Enzymes[a]	**Catalytic receptors**[b]
AMPK	Insulin receptor
eNOS	**Nuclear hormone receptors**[c]
sGC, soluble guanylyl cyclase	PPARγ
iNOS	**Other proteins**[d]
nNOS	Fatty acid binding protein 4
PDE3B	PGC-1α, PPARγ coactivator 1α
PKB/Akt	**Transporters**[e]
XO, xanthine oxidoreductase	UCP1, uncoupling protein 1, SLC25A7

LIGANDS
Adiponectin
BH_4, tetrahydrobiopterin
GSH
NO

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d,e] Alexander *et al.*, 2015a,b,c,d,e).

Introduction

The majority of type 2 diabetes cases (up to 90%) are related to insulin resistance and obesity (Anderson *et al.*, 2003), suggesting a causal connection between these conditions (Berger, 1992; Daly, 1994). According to the dynamic nature of energy balance maintenance (and long-term mechanisms of adiposity regulation), increase in energy uptake is paralleled by energy expenditure by peripheral tissues, including adipose tissue, and obesity develops when fuel intake chronically exceeds expenditure (Hill and Peters, 1998; Kashyap and Defronzo, 2007). The failure of adipose tissue expansion (not obesity *per se*) is the essential factor linking positive energy balance, diabetes and other cardiometabolic diseases (Lafontan, 2013). Thus, when the inflows of nutrients into adipose tissues exceed the capability of adipocytes to handle nutrient excess and the capacity of adipose tissue to expand by hyperplasia, more adipocytes become hypertrophic and defend themselves by developing insulin insensitivity (Gray and Vidal-Puig, 2007). Improper lipolytic and lipogenic response of adipose tissue during fasting/feeding conditions in either case results in overflow and ectopic deposition of lipids in non-adipose organs (Frayn, 2002). According to the lipid overflow hypothesis of systemic insulin resistance development (Unger, 2003), fat-specific insulin resistance is the earliest risk factor for systemic insulin resistance and type 2 diabetes pathogenesis. Thus, clarification of the molecular mechanisms underlying the lipid buffering capacity of adipose tissue, that is, oxidative capacity and insulin sensitivity, may aid in the improvement of therapeutic options for obesity and diabetes prevention.

Nitric oxide (NO) has emerged as a central regulator of energy metabolism and body composition that acts mainly by modulating the oxidative capacity and insulin sensitivity of adipose tissue (see Jobgen *et al.*, 2006; Dai *et al.*, 2013; Bernlohr, 2014; Sansbury and Hill, 2014). Supraphysiological production of NO by inducible NO synthase (iNOS) in adipocytes (Merial *et al.*, 2000) and macrophages (Weisberg *et al.*, 2003; Lumeng *et al.*, 2007) and lack of endothelial NOS (eNOS)-mediated NO synthesis (Valerio *et al.*, 2006) in adipose tissue are important risk factors leading to obesity. Moreover, uncontrolled flux of superoxide anion ($O_2^{\bullet-}$) from the electron transport chain (ETC) in mitochondria and NADPH oxidase (NOX) under persistent nutrient inflow into adipocytes may divert the role of NO in hypertrophic adipocytes, leading to local and ultimately systemic insulin resistance and type 2 diabetes (see Maritim *et al.*, 2003; Bashan *et al.*, 2009; Afanas'ev, 2010). The current review discusses the underlying mechanisms and consequences of impairment of physiological signalling of reactive oxygen and nitrogen species (ROS/RNS) in adipose tissue, their effects on lipid buffer function, and implications in the development of obesity and obesity-related type 2 diabetes.

Adipose organ and roles in metabolic homeostasis: healthy and unhealthy expansion

The function of adipose tissue in buffering daily lipid flux is traditionally understood as hydrolysis of triacylglycerol (TAG) and (re)esterification (Frayn, 2002). Compared with the other insulin-sensitive tissues, such as liver and muscle, adipose tissue is less important for postprandial glucose clearance, and the energy for cellular functions in adipocytes is primarily obtained via glycolytic ATP production. However, it appears that glucose uptake and metabolism as well as the oxidative capacity of adipocytes underlie metabolic flexibility and healthy expansion of adipose tissue during overnutrition and influence its role in protecting non-adipose tissues against lipotoxicity. The metabolic pathways facilitating adipocyte energy storage and release during fasting/fed transitions have been recently reviewed in detail by Rutkowski *et al.* (2015) and briefly presented in Figure 1.

Fat cells (white adipocytes) compose the largest adipocyte population of human adipose tissue (Figure 2). Adipocytes may additionally be brown or brite/beige according to their origin (endothelial or myogenic) and ultrastructure. The

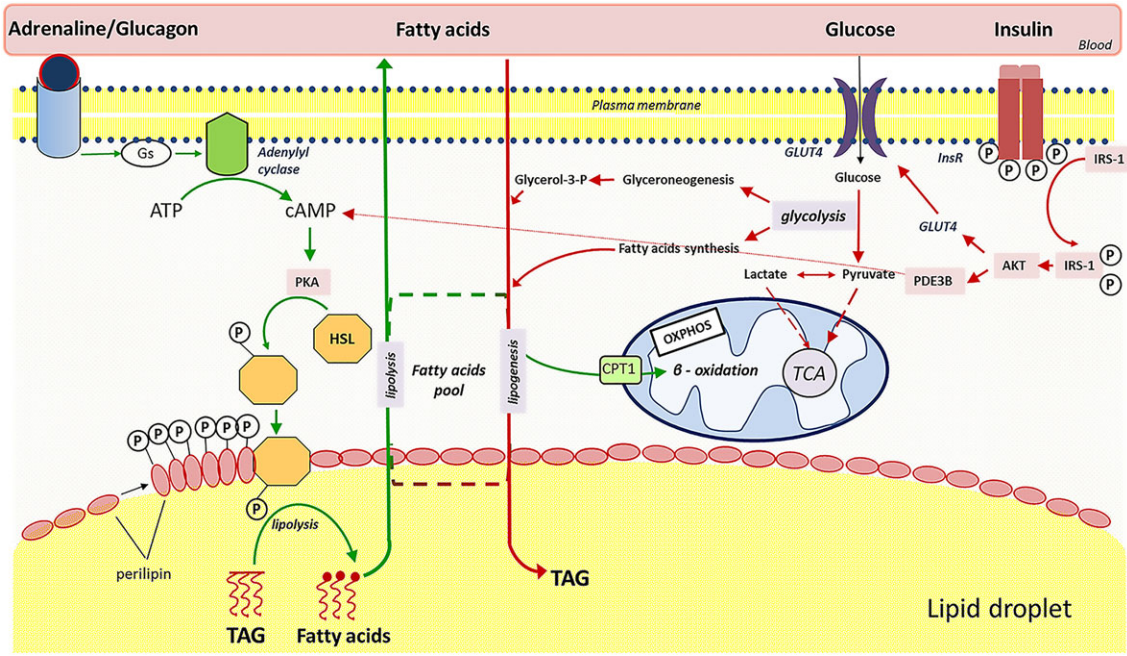

Figure 1

Adipocytes export fatty acids during times of energy deficit (involved pathways are marked with green arrows). The rise in cAMP, a sign of increased glucagon or adrenergic stimulation, and low insulin stimulate hydrolysis of triglycerides into glycerol and fatty acids. The increased cAMP pool activates PKA that, in turn, phosphorylates hormone-sensitive lipase (HSL) and perilipins to increase lipolysis. Glycerol and fatty acids are mostly exported into the circulation for systemic utilization. A proportion of fatty acids is re-esterified within the adipocytes, while another part may, after activation to form acetyl-CoA, enter the mitochondria for β-oxidation through carnitine palmitoyl transferase-1 (CPT-1). This rate-limiting enzyme is inhibited by malonyl-CoA, an intermediate of *de novo* lipogenesis regulated by acetyl carboxylase (ACC). ACC prevents the oxidation of fatty acids when adipocytes are in a lipogenic state. Inhibition of ACC by AMPK relieves this inhibition for β-oxidation. Under positive energy balance, insulin regulates glucose and fatty acid uptake in adipose tissue, and expression and activity of enzymes involved in their metabolism and deposition into TAG, that is, lipogenesis (marked by red arrows). In short, insulin through binding to its cell surface receptor stimulates tyrosine kinase activity, which phosphorylates key residues on several 'docking proteins', IRS proteins. Assembly of a stable complex leads to the regulation (in most cases, activation) of downstream signalling pathways. Recruited proteins include the p85 regulatory subunit of PI3-kinase, which stimulates signalling pathways ultimately leading to PI3-kinase-dependent serine/threonine PKAkt/PKB activation. Phosphorylation of Akt1 at two regulatory residues, Ser^{473} and Thr^{308}, is critical for complete Akt/PKB activation in adipose tissue. Akt stimulates the translocation of the glucose transporter, GLUT4, to the plasma membrane, thereby promoting uptake of glucose into the cell. A high level of circulating insulin also stimulates PDE3B, promoting cAMP hydrolysis, lowering PKA activity and PKA-dependent HSL phosphorylation, activation and lipolysis. Chronic insulin signalling enhances cAMP production through β-adrenoceptor activation in adipocytes but also disrupts the signalling pathway between β-adrenoceptors and PKA. Imported as well as *de novo* synthesized fatty acids from excess glucose combine with CoA, and after successive esterification, form TAG. The (re)-esterification process requires production of glycerol-3-phosphate as a substrate for fatty acid re-esterification into TAG. Glycerol-3-phosphate is mostly derived from glucose in the fed state (glycolytic intermediates). Because the glucose supply to the tissue is limited in the fasting state (lipolytic stimulation) and adipocytes have no significant glycerol kinase activity, glycerol-3-phosphate is acquired from lactate or pyruvate.

appearance and distinct origins of adipocytes reflect their specialization in energy partitioning. All adipocytes store excess calories as TAG, while brown and brite/beige cells are highly specialized for calorie combustion, specifically metabo-regulatory and/or thermo-regulatory thermogenesis. The metabolic inefficiency of these adipocytes is attributed to high mitochondrial content and the uncoupling protein 1 (UCP1), a protonophore that uncouples oxidative phosphorylation (OXPHOS) from respiration, generating heat instead of ATP (Cannon and Nedergaard, 2004). Generally, the number of brown and beige adipocytes found in white areas varies with age, strain and environmental conditions (Cinti, 2000). The number of brown adipocytes in humans is inversely correlated with body mass index and body fat mass (van Marken Lichtenbelt *et al.*, 2009) as well as fasting glycaemia (Cypess *et al.*, 2009) and positively correlated with resting metabolic rate (van Marken Lichtenbelt *et al.*, 2009). The real significance of brown adipose tissue in overall human metabolism and energy expenditure is yet to be established (Kozak *et al.*, 2010; Schlögl *et al.*, 2013; Halpern *et al.*, 2014), but there are indications that this tissue mediates cold as well as diet-induced thermogenesis in humans (as in mice and rats) (Vosselman *et al.*, 2013a, b), and its lack or unresponsiveness under conditions of hypercaloric diet and overfeeding may explain a propensity towards easy weight gain (Stock, 1999; Vosselman *et al.*, 2013b).

Oxidative capacity and uncoupling also have significant implications for white, unilocular adipocytes, especially in obesity, in terms of regulation of metabolic function, and glucose and fatty acid partitioning during both fasting and feed

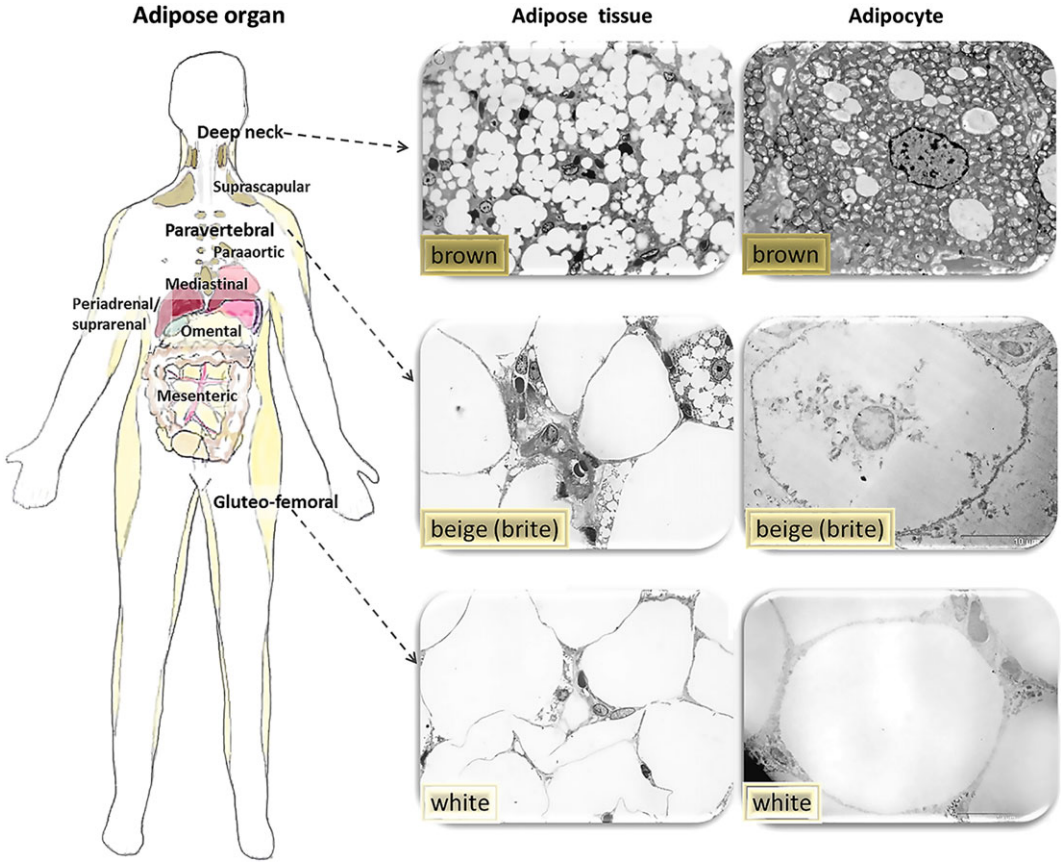

Figure 2

Adipose tissue is loose connective tissue composed of adipocytes and stromal-vascular cells, anatomically organized in distinct adipose tissue depots. In terms of energy balance, adipose tissue depots may appear as predominantly white – energy saving, brown – energy dissipating, or beige (brite or convertible), depending on the relative amount of white, beige or brown adipocytes. All adipose tissue depots in the body are functionally integrated in a highly dynamic multidepot adipose organ. The main brown (deep neck), beige (paravertebral) and white (gluteo-femoral) adipose tissue depots of the adult human adipose organ are presented.

periods. During the fasting response, stimulation of β-oxidation in adipocytes may restrict high fatty acid export into the circulation and prevent or delay obesity development (Horowitz, 2001). In the fed state, adipocytes shift to glycolytic ATP production. Consequently, the levels of all metabolic products of glucose (CO_2 and pyruvate/lactate) increase up to 10-fold, along with adipocyte size (DiGirolamo *et al.*, 1992). Intensified flux through the glycolysis and pentose phosphate pathway provides energy for adipocyte activity and directs excess metabolic substrates into TAG synthesis (lipogenesis) (DiGirolamo *et al.*, 1992). Earlier, Rossmeisl *et al.* (2000) demonstrated that total uncoupling of OXPHOS in 3T3-L1 adipocytes induced by 2,4-dinitrophenol or ectopic UCP1 in white fat of transgenic aP2-UCP1 mice restricts *in situ* lipogenesis. Subsequent studies revealed that mitochondrial OXPHOS supports high energy-consuming processes, such as fatty acid storage, adipokine synthesis/secretion (Koh *et al.*, 2007), insulin signalling and glucose uptake (Shi *et al.*, 2008) and adipogenesis (Ryu *et al.*, 2013), in differentiating 3T3-L1 adipocytes. Moreover, increased glucose and fatty acid flux/cycling through adipocytes occurring after treatment of adipocytes with insulin, corticosteroids, pro-inflammatory cytokines, lipid (Hoehn *et al.*, 2009) or lactate (Carriere *et al.*, 2014) or exposure of rats to the cold (Jankovic *et al.*, 2015a), promote mitochondrial biogenesis and uncoupling. Besides increased energy dissipation and thermogenesis, these processes could be considered an adaptive stress response of adipocytes to the increased inflow of reducing equivalents to the mitochondrial ETC (Jeanson *et al.*, 2015; Jankovic *et al.*, 2015b). Thus, proper capacity for oxidation of glucose and fatty acids, OXPHOS, uncoupling and biogenesis of new mitochondria are indispensable in the daily metabolic regulation of adipocytes and long-term adipose tissue function and healthy expansion (Wilson-Fritch *et al.*, 2004; De Pauw *et al.*, 2009; Hao *et al.*, 2010; Lu *et al.*, 2010). In diabetes, the capacity of adipose tissue in energetic remodeling is impaired (Choo *et al.*, 2006; Keller and Attie, 2010).

In addition to metabolic and cellular plasticity, healthy adipose tissue expansion requires normal blood flow and vascularity (Frayn *et al.*, 2003) as well as hyperplastic potential, because these factors account for greater lipid-buffering capacity (Rutkowski *et al.*, 2015). Conversely, pathological (unhealthy) expansion of adipose tissue is characterized by changes in blood flow and the presence of enlarged, dysfunctional, that is, insulin-resistant, adipocytes (see Lafontan,

2013). Adipose tissue-specific insulin resistance (impaired glucose and fatty acid uptake and utilization) appears to be an early and irreversible defect that explains the causal relationship between adipocyte dysfunction and systemic insulin resistance (Iozzo, 2009; Lafontan, 2013). Hitherto, studies on animal models (mutant mice, diet-induced obesity) and cultures of mouse and human adipocytes have provided strong support for the involvement of hypoxia, inflammatory signalling, endoplasmic reticulum stress and unfolded protein response, autophagy, dysfunction of mitochondria/impaired mitochondrial biogenesis, and oxidative stress in the development of fat-specific insulin resistance (see Wood *et al.*, 2009; Blüher, 2009; Netzer *et al.*, 2015).

Temporal progression of oxidative damage in the pathogenesis of obesity and associated metabolic disorders is poorly understood, because the classical 'markers' of oxidative damage (oxidation products of lipids, DNA and proteins), in contrast to levels in plasma, urine and various non-adipose tissues, are only minimally increased in mouse (Furukawa *et al.*, 2004; Garcia-Diaz *et al.*, 2007; Grimsrud *et al.*, 2007) and human (Frohnert *et al.*, 2011; Jankovic *et al.*, 2014) adipose tissues in obesity. The data suggest that the increase in superoxide and ROS/RNS levels in the mitochondria of expanding adipose tissue precedes adipocyte dysfunction and progression of obesity-related metabolic disorders in metabolically healthy control mice (Houstis *et al.*, 2006; Matsuzawa-Nagata *et al.*, 2008) and individuals (Jankovic *et al.*, 2014). Notably, increased $O_2^{\bullet-}$ is a critical interfering factor in signalling of NO and other ROS/RNS essential for the lipid buffering function of adipose tissue, thus differentiating unhealthy from healthy expansion, that is, insulin-resistant from insulin-sensitive obesity (Sansbury and Hill, 2014).

Biological effects of NO in adipose tissue

NO, a gaseous signalling molecule similar to CO_2, CO, H_2S and O_2, is toxic at high levels but essential in the regulation of biological processes when endogenously produced in nM concentrations. The multifaceted role of NO in adipose tissue corresponds to the extremely complex mechanisms of biological effects that depend on its (i) site and level of production, determined by enzymes involved in NO synthesis and availability of their substrates and cofactors, and (ii) interactions with different cellular biotargets, primarily ferrous iron (haem and non-haem), thiyl radicals, molecular oxygen and superoxide (Beckman and Koppenol, 1996; Wink and Mitchell, 1998).

Endogenous production of NO in adipose tissue

In virtually all cell types, NO is synthesized via NOS-catalysed oxidation of L-arginine (Moncada *et al.*, 1989). Basal eNOS (NOS3) and iNOS (NOS2) expressions have been reported in rat and human adipose tissue and adipocytes (Ribiere *et al.*, 1996; Elizalde *et al.*, 2000; Gaudiot *et al.*, 2000). Neuronal NOS (nNOS, NOS1) protein does not appear to be present in significant amounts (Engeli *et al.*, 2004), although some studies have provided evidence for protein expression of nNOS in the cytoplasm (Fu *et al.*, 2005; Jobgen *et al.*, 2006) and mitochondria (Finocchietto *et al.*, 2011) of adipocytes. eNOS is mostly membrane bound, while iNOS is localized in the cytoplasm of adipocytes and macrophages (Jobgen *et al.*, 2006). However, following post-translational modification and protein/protein interactions, NOS proteins translocate into different cellular compartments, leading to localized biological effects of NO and regulation of different signalling pathways (Giordano *et al.*, 2002; Villanueva and Giulivi, 2010). Under most physiological conditions, eNOS and nNOS synthesize low levels of NO (nM), whereas iNOS expression is up-regulated by LPS, TNF-α and interferon-γ, leading to the generation of high levels of NO (μM) lasting for several hours or days (Stamler and Meissner, 2001; Pilon *et al.*, 2004).

The reductive pathways, that is, synthesis of NO from nitrite (NO_2^-) catalysed by several transition metal-containing proteins, such as xanthine oxidoreductase (XO) (Zhang *et al.*, 1998), deoxymyoglobin/deoxyhaemoglobin (Nagababu *et al.*, 2003), cytochrome *c* (Basu *et al.*, 2008), complex III (Nohl *et al.*, 2000), cytochrome *c* oxidase (CcOx) (Castello *et al.*, 2006) or NOS (Vanin *et al.*, 2007), represent a relevant NO source in hypoxic and acidic intracellular micro-environments (Shiva, 2013; Sparacino-Watkins *et al.*, 2014) after exercise (Cosby *et al.*, 2003) or ischaemia/reperfusion injury and myocardial ischaemic conditioning (Shiva *et al.*, 2007; also see Andreadou *et al.*, 2015). A recent study by Roberts *et al.* (2015 showed that exposure of rats *in vivo* and primary adipocytes *ex vivo* to hypoxia augments nitrate-mediated NO production with subsequent up-regulation of brown adipocyte-associated genes. These authors suggested that augmentation of the nitrate-stimulated browning response during hypoxia represents a physiological adaptation of adipocytes undergoing hypertrophy in obesity. This NO-producing pathway may be exploited therapeutically to maintain oxidative capacity of adipocytes to metabolize fatty acids and to counteract the obesity-related pathological metabolic state of adipose tissue (Roberts, 2015).

Interaction of NO with soluble guanylate cyclase (sGC) and cGMP-mediated signalling in adipocytes

NO was initially identified as the first gaseous messenger molecule that acts through a completely novel mechanism, that is, binding to ferrous haem of sGC, leading to increased levels of the second messenger cGMP (Ignarro, 1990a, b). This NO-mediated mechanism covers a range of downstream signalling pathways in fundamental processes of virtually all cell types (Murad, 1988; Murad *et al.*, 1990; Krumenacker and Murad, 2006), including adipocytes (Hemmrich *et al.*, 2010). In adipose tissue, the reaction between NO and sGC, occurring at nM concentrations of NO (Bellamy and Garthwaite 2001; Rodríguez-Juárez *et al.*, 2007), is mediated by constitutive eNOS. Recent data suggest that NO produced by the nitrite reductase activity of XO also acts through the sGC/cGMP signalling pathway (Roberts *et al.*, 2015).

Physiological levels of NO produced in adipose tissue regulate blood flow and vascularization in a sGC/cGMP-dependent manner, promoting substrate uptake and product removal via the circulation, thereby matching energy inflow/outflow with tissue perfusion (Jobgen *et al.*, 2006). Additionally, endogenous NO directly mediates the metabolic response of adipocytes (see Jobgen *et al.*, 2006, McKnight *et al.*, 2010). First, physiological levels of NO stimulate the insulin-triggered (Roy *et al.*, 1998) and (probably) insulin-independent uptake and oxidation of glucose (Tanaka

et al., 2003; Jobgen *et al.*, 2006). The underlying molecular mechanisms of the latter involve sGC/cGMP-dependent stimulation of AMP-activated protein kinase (AMPK) through increasing gene expression and PKG-dependent AMPK phosphorylation (Jobgen *et al.*, 2006). Second, NO stimulates lipid degradation (lipolysis) and β-oxidation through both AMPK-dependent and AMPK-independent mechanisms.

Upon activation by NO, AMPK phosphorylates and inactivates acetyl-CoA carboxylase, thereby reducing the conversion of acetyl-CoA to malonyl-CoA, which suppresses *de novo* fatty acid synthesis and activates carnitine palmitoyltransferase I, facilitating the transport and oxidation of fatty acids in mitochondria. NO also increases OXPHOS and mitochondriogenesis through an AMPK-mediated increase in PPARγ coactivator 1α (PGC-1α) expression (Tedesco *et al.*, 2010). PGC-1α is the principal regulator of mitochondrial biogenesis and function. In combination with PPARγ, PGC-1α increases mtDNA replication, OXPHOS, mitochondrial fatty acid β-oxidation, UCP1 and mitochondrial antioxidant defence primarily in brown adipocytes (Bossy-Wetzel and Lipton, 2003; Kelly and Scarpulla, 2004) and possibly white adipocytes (Clementi and Nisoli, 2005; Tedesco *et al.*, 2010). Through these pathways, NO supports healthy adipose tissue function, that is, long-term insulin sensitivity (Fu *et al.*, 2005; Wu *et al.*, 2007; Jobgen *et al.*, 2009).

The groups of Fu *et al.*, (2005) and Wu *et al.*, (2007) showed that dietary supplementation with L-arginine, a substrate for NO synthesis, reduces body weight and fat mass in Zucker diabetic fatty rats. L-arginine induced a marked increase in expression of nNOS, haem oxygenase 3, AMPK and PGC-1α in white adipose tissue of Zucker diabetic fatty rats (Fu *et al.*, 2005). In addition to stimulation of eNOS activity by high-dose L-arginine via direct administration, L-arginine can outcompete binding of the endogenous inhibitor of eNOS, asymmetric dimethyl-L-arginine, and force its removal from endothelial cells by driving the cationic amino acid transporter (Closs *et al.*, 2012). Effects of NO on reducing fat mass may be attributed, in part, to the appearance of brown/brite adipocytes or brown-like phenotype (UCP1) in white adipocytes, that is, browning (Nisoli and Carruba 2006; Joffin *et al.*, 2015; Roberts *et al.*, 2015). We recently showed that L-arginine enhances the cold exposure-induced UCP1 level in mitochondria of unilocular white adipocytes in rats. Moreover, a similar increase in UCP1 protein expression was observed in rats maintained at room temperature (presented in Figure 3).

The essential role of eNOS-synthesized NO at nM levels in the metabolic plasticity of adipose tissue was confirmed in studies showing that eNOS knockout mice exhibit decreased UCP1 and PPARγ expression, reduced number of mitochondria, defective energy expenditure, increased body weight, insulin resistance and hypertension (Nisoli *et al.*, 2007). $eNOS^{-/-}$ mice displayed exaggerated high-fat diet-induced weight gain (Shankar *et al.*, 2000; Duplain *et al.*, 2001; Cook *et al.*, 2003). Furthermore, eNOS expression was remarkably diminished in fat tissue in obese rodents (Valerio *et al.*, 2006) and humans (Perez-Matute *et al.*, 2009; Georgescu *et al.*, 2011), while its overexpression prevented diet-induced obesity, increased metabolic activity and promoted brown adipose tissue-like phenotype in white adipose tissue in mice (Sansbury *et al.*, 2012). Similarly, constitutive activation of eNOS by knocking in a phosphomimetic point mutation at Ser^{1176} was shown to promote resistance to diet-induced weight gain (Kashiwagi *et al.*, 2013).

NO activates AMPK and its downstream pathways in a cGMP/PKG-dependent manner (Jobgen *et al.*, 2006). Conversely, eNOS itself may be activated by AMPK. In endothelial cells, AMPK activates eNOS via phosphorylation at Ser^{1177} and Ser^{633} (Chen *et al.*, 1999, 2009). The latter phosphorylation event is slower and Ca independent and serves to maintain NO synthesis after the initial increase in NO induced by

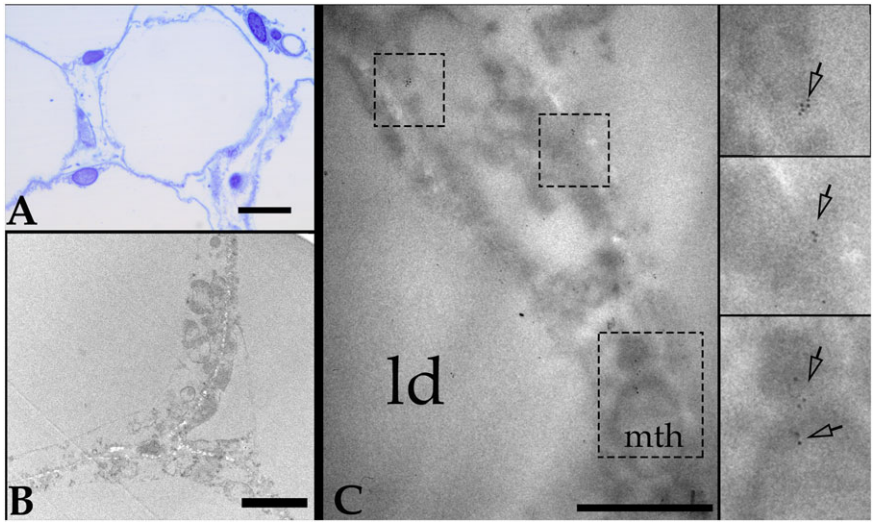

Figure 3

Appearance of UCP1 in the mitochondria of unilocular, white adipocyte in retroperitoneal white adipose tissue of rats maintained at room temperature after 3 days of L-arginine treatment. Light (A), electron (B) microscopy and immunogold (C) revealed the presence of UCP1 (arrows) in white adipocytes mitochondria. Bars: (A) 20, (B) 2 and (C) 1 μm.

Ser^{1177} phosphorylation, owing to a positive feedback loop (Schulz *et al.*, 2009). On the other hand, AMPK-mediated post-translational phosphorylation of iNOS inhibits its activity and enhances insulin sensitivity in adipose tissue (Pilon *et al.*, 2004). Thus, regulation of AMPK by NO may play an important role in controlling the relative eNOS and iNOS activities, NO levels and effects on adipose tissue.

Interaction of NO with CcOx: a potential key metabo-regulatory role in the postprandial response of adipocytes?

In addition to the ferrous iron in sGC, NO interacts directly with several ferrous haem proteins in near diffusion-limited reactions ($k \times 10^7$–10^8 $M^{-1} \cdot s^{-1}$). The most important of these is the reaction with CcOx, the terminal complex of the mitochondrial ETC. The NO–CcOx system helps to fine-tune cellular respiration (Brown, 1995) and metabolism (Semenza, 1999).

Increased expression of mitochondrial NOS (potentially nNOS) in adipocytes of ob/ob mice led to reduced oxygen uptake via inhibition of CcOx (Finocchietto *et al.*, 2011). The presence of mitochondrial NOS in adipocytes (as in other cells) is disputable, but other NOS stimulated by insulin may have similar mitochondrial effects (Jezek *et al.*, 2010), because in adipocytes, insulin rapidly enhances NOS activity (Ribière *et al.*, 2002; Engeli *et al.*, 2004) as in endothelial cells (Dimmeler *et al.*, 1999). Moreover, eNOS relocalizes upon post-transcriptional modification, that is, attaching to the outer mitochondrial membrane, at least in neurons and endothelial cells (Henrich *et al.*, 2002; Gao *et al.*, 2004). This finding indicates that eNOS regulates mitochondrial function, and conversely, mitochondria regulate eNOS activity (Nisoli and Carruba, 2006). Accordingly, NO synthesized by eNOS may transiently bind to CcOx and inhibit respiration under appropriate stimulation (e.g. by insulin) (Brown, 2001). This may induce a type of 'metabolic hypoxia', a phenomenon that restricts oxygen utilization (Moncada and Erusalimsky, 2002), that is, glucose oxidation. In postprandial adipocytes, 'metabolic hypoxia' may switch the cell to the glycolytic mode of ATP production and redirect glucose into lipids, similar to mtNOS-derived NO in muscle cells, in response to insulin (Finocchietto *et al.*, 2008). Overexpression of nNOS or iNOS may contribute to ETC inhibition, oxygen uptake inhibition, mitochondrial dysregulation and insulin resistance progression in prediabetic states via the same mechanism (see Jezek *et al.*, 2010).

Interaction of NO with oxygen and thiols

NO also binds to oxygen and thiyl radicals, especially at μM levels. Reaction of NO with O_2, resulting in N_2O_3 or NO_2, may further cause *S*-oxidation and *S*-nitrosylation (*S*-nitrosation) of protein side chains (Stamler *et al.*, 1992; Stamler, 1995; Beltrán *et al.*, 2000; Hess *et al.*, 2001). Moreover, these reactions may occur by direct covalent binding of NO with cysteinyl thiols of GSH and proteins upon transfer of NO^+ from *S*-nitrosothiol (SNO). *S*-nitrosoglutathione contributes to preservation of cellular NO activity as a delayed donor of NO. *S*-nitrosoglutathione and protein CysNO may subsequently react with GSH to generate a mixed disulphide and promote the release of HNO, another vasodilator. Such *trans*-*S*-nitrosation reactions within cells facilitate redistribution of NO among different -SNO pools (Liu *et al.*, 1998; Yang and Loscalzo, 2005).

Protein Cys residues are capable of oscillating between reduced (−SH) and different oxidized states, including thiolate anion ($-S^-$), sulphenate ($-SO^-$), disulphide (−S-S-), sulphinate ($-SO_2^-$) or sulphonate ($-SO_3^-$). Among these, the latter two are considered irreversible oxidative modifications in mammalian cells (Jones, 2008). The redox state of the sulphur atom within Cys in the catalytic (regulatory or binding) domain of proteins may alter activity. In the biological context, oxidation and nitrosation reactions are compared with phosphorylation/dephosphorylation as the second prototypic post-translation mechanisms leading to redox regulation of protein activity/function (Lane *et al.*, 2001).

In adipocytes, as in most cells, *S*-nitrosation of mitochondrial ETC thiol groups (especially complexes I and II) may slow down respiration (Moncada and Erusalimsky, 2002), acting synergistically in the transient inhibition of CcOx via NO binding. Both processes play an important role in the regulation of glycolytic (i.e. lipogenic) mode of postprandial adipocytes (Jezek *et al.*, 2010).

Moreover, transient *S*-oxidation/*S*-nitrosation is indispensable in the maintenance of normal insulin signalling in adipocytes. Under normal (unstimulated or basal) circumstances, most protein Cys residues are maintained in the reduced state due to high levels and overall presence (cytoplasmic and mitochondrial) of GSH and thioredoxins (Trx), peroxiredoxins and the corresponding NADPH-coupled reductase systems (Fisher-Wellman and Neufer, 2012). This sets up a 'basal' phosphatase tone in unstimulated adipocytes, because protein phosphatases, particularly protein tyrosine phosphatases (PTPs) and the phosphoprotein phosphatase family of Ser/Thr phosphatases, are active under reduced conditions (Chiarugi, 2005; Wright *et al.*, 2009; Fisher-Wellman and Neufer, 2012). However, a small shift in the redox state to pro-oxidative conditions, for example, due to insulin-mediated increase in ROS/RNS, promotes *S*-oxidation/*S*-nitrosylation of Cys in different proteins, such as protein kinases and phosphatases involved in insulin signalling. ROS/RNS play a superimposing role by controlling the phosphorylation state of proteins in the insulin signalling cascade via reversible *S*-oxidation/*S*-nitrosylation of PTPs, and their reactivity may therefore lead to changes in insulin sensitivity in adipose tissue. Moreover, NO-induced *S*-nitrosylation at the active site Cys residue of PTPs (SHP-1, SHP-2 and PTP1B), concomitant with eNOS-mediated NO burst in response to insulin action, supports NO-dependent regulation of tyrosine phosphorylation of the insulin receptor and its downstream effector kinases, insulin receptor substrate 1 (IRS-1) and PKB/Akt, at least in mouse endothelial MS-1 cells (Hsu and Meng, 2010). It is tempting to speculate that NO plays important permissive roles for transmission of the insulin signal in adipocytes also, via similar mechanisms.

Interaction of NO with superoxide

Within all the molecular targets of NO in the cell, superoxide is the most critical intervening factor of its biological effects, especially in the presence of high levels of NO. At equimolar

concentrations, these two reactive species combine at a diffusion-limited rate (k approximately 10^9 to 10^{10} $M^{-1}{\cdot}s^{-1}$) that exceeds the reactions of NO with all other biotargets (iron, thiols and oxygen) and the reaction velocity upon combination with superoxide dismutase (SOD) (k approximately 10^9 $M^{-1}{\cdot}s^{-1}$), changing the biological action of NO and other ROS/RNS under oxidative stress conditions (Koppenol *et al.*, 1992; Koppenol, 2001). The balance between local concentrations of NO, $O_2^{\bullet-}$ and SOD is important in determining the effects of the NO/superoxide radical pair (Beckman and Koppenol, 1996; Rubbo *et al.*, 1996). No significant competition occurs between NO and SOD for $O_2^{\bullet-}$ under normal physiological conditions, because NO is in the nM range, compared with μM levels of SOD in cells. Under physiological conditions, reaction of $O_2^{\bullet-}$ with NO constitutes only a minor part of the dismutation reaction of $O_2^{\bullet-}$ by SOD. As a result, very little peroxynitrite ($ONOO^-$) is formed (Ferdinandy and Schulz, 2003). However, at increased rates of NO production (possibly via iNOS expression), when the levels of NO are ≥1 μM, $ONOO^-$ formation predominates over dismutation of $O_2^{\bullet-}$ (Depre and Hue, 1994; Csonka *et al.*, 1999; Ferdinandy *et al.*, 2000). Additionally, the increase in $O_2^{\bullet-}$ levels due to increased production and/or decreased dismutation may lead to an effective increase in the probability of $ONOO^-$ formation. Both prerequisites, iNOS-mediated NO production and increase in superoxide levels, occur in adipose tissue under conditions of obesity.

Adipose tissue in the obese state: the paradigm of NO/superoxide interactions

As mentioned previously, decreased NO bioavailability is a sign of early risk of fat-specific insulin resistance development in obesity, and increased superoxide level in accumulating adipose tissue is the key factor in the initiation and progression of fat-specific insulin resistance. *Inter alia*, high $O_2^{\bullet-}$ and NO levels are characteristic of adipose tissue in obesity. $O_2^{\bullet-}$ antagonizes the direct effects of NO, and $ONOO^-$ formation and/or derived ROS/RNS can alter cell signalling, in part, by promoting oxidation, nitrosylation or nitration of a broad range of proteins, including enzymes of intermediary metabolism, mitochondrial complexes and insulin signalling.

Sources of high superoxide in obesity: antagonists of physiological NO functions

Superoxide in adipose tissue is primarily generated by overloading the mitochondrial OXPHOS system with metabolites from glucose, fatty acids and NOXs (Han *et al.*, 2012). In recent years, eNOS uncoupling has been recognized as an important superoxide source in various tissues in obesity (Margaritis *et al.*, 2013; Yu *et al.*, 2014). XO, an enzyme that catalyses the conversion of hypoxanthine to uric acid and $O_2^{\bullet-}$, has been proposed to play a critical role in superoxide production in mature adipocytes (Furukawa *et al.*, 2004). Although hyperuricaemia has been linked to metabolic syndrome, the role of XO in adipose tissue as the source of superoxide remains poorly understood. The enzyme may be of importance, especially in impairment of adipose tissue blood flow, because pro-inflammatory cytokines irreversibly convert endothelial xanthine dehydrogenase to the oxidase form, XO (Vorbach *et al.*, 2003).

Enhanced production of superoxide by mitochondria. During respiration, a significant proportion of oxygen molecules are incompletely converted to H_2O and end up as $O_2^{\bullet-}$ via a non-enzymatic pathway, owing to electron leak from complexes I and III of the ETC (Turrens and Boveris, 1980). Mitochondrial $O_2^{\bullet-}$ is linked to hyperglycaemia-induced metabolic dysfunction in endothelial cell systems (Brownlee, 2001) and inflammation in adipocytes (Lin *et al.*, 2005). A recent study showed that overfeeding (and/or high-calorie intake) increases the reduced state of electron carriers in the ETC and subsequently the probability of electron extraction by high reduction potential molecules, such as molecular oxygen (to create $O_2^{\bullet-}$) (Matsuzawa-Nagata *et al.*, 2008). This series of events is more evident in hyperglycaemia. When the elevated carbohydrate fuel supply exceeds the metabolic needs of the cell, reducing equivalents (NADH) are overproduced (Frizzell *et al.*, 2012). A dramatic increase in NADH exceeds the electron acceptor capacity of ETC complexes, thereby perpetuating electron leak and subsequent $O_2^{\bullet-}$ generation.

NOX. Superoxide/H_2O_2 originating from NOX produced transiently in response to insulin stimulation acts as a second messenger for insulin signalling in adipocytes (Krieger-Brauer and Kather, 1992, 1995), while excessive and long-term activation of NOX reduces insulin sensitivity (Furukawa *et al.*, 2004). NOX is a membrane-associated multimeric oxidoreductase that transports electrons preferentially from cytosolic NADPH (although nonphagocytic NOX, especially the NOX1 isoform, may also use NADH as substrate) down to the electrochemical gradient through the membrane to oxygen, generating $O_2^{\bullet-}$ that is rapidly converted to H_2O_2 (Griendling *et al.*, 2000). Among the seven isoforms of the catalytic subunit $gp91^{phox}$ of NOX, phagocytic NOX2 and NOX4 are present in adipose tissue, especially in resident macrophages and adipocytes (Mouche *et al.*, 2007). NOXs are significantly increased in adipose tissue in different genetic and diet-induced models of obesity in rats (Furukawa *et al.*, 2004) and humans (Jankovic *et al.*, 2014). Moreover, a higher $gp91^{phox}$ protein level in visceral fat characterizes subjects with increased risk factors for metabolic syndrome, compared with metabolically healthy weight-matched subjects (Jankovic *et al.*, 2014).

NOX4 generates H_2O_2 even under basal conditions (via constitutive expression) (Nisimoto *et al.*, 2010). Insulin and some anabolic hormones induce a several-fold increase in H_2O_2 levels and expression of NOX4 (Goldstein *et al.*, 2005).

Increased oxidative state can lead to immune cell activation (Schulz *et al.*, 2014; Kröller-Schön *et al.*, 2014). In particular, excess glucose and palmitate generate ROS via a mechanism that involves translocation of NOX4 into lipid rafts of adipocytes, leading to expression of monocyte chemotactic factors (Yeop Han *et al.*, 2010; Han *et al.*, 2012). Conversely, inflammatory cytokines and NOX2-mediated ROS originating from activated macrophages may be involved in augmentation of adipocyte NOX4 and consequent ROS production in obese adipose tissue (Furukawa *et al.*, 2004). Possibly,

complex crosstalk of adipocyte and macrophage NOXs establishes a vicious cycle that augments superoxide production in adipose tissue in obesity and diabetes (recently reviewed in Karbach *et al.*, 2014, and Jankovic *et al.*, 2015b).

Enhanced production of superoxide through eNOS uncoupling. In the absence of the coenzyme tetrahydrobiopterin (BH_4), NOS reduces molecular oxygen, rather than L-arginine, resulting in production of superoxide rather than NO, a phenomenon known as 'NOS uncoupling' (Münzel *et al.*, 2008). Several hypotheses have been proposed for intracellular BH_4 depletion, including $ONOO^-$-mediated oxidation. Moreover, activation of the superoxide source (uncoupled eNOS) may stimulate the formation of superoxide and/or $ONOO^-$ in a positive feedback manner, in turn, oxidising BH_4 to the BH_3 radical, leading to further ROS/RNS formation (Kuzkaya *et al.*, 2003). eNOS dysfunction was observed under conditions of oxidative stress and inflammation in perivascular adipose tissue (Filip *et al.*, 2012; Margaritis *et al.*, 2013). Increased superoxide production by uncoupling of eNOS is recognized as an important factor that decreases the vasodilatory role of perivascular adipose tissue in obesity. During early diet-induced obesity, adaptive overproduction of NO occurs in perivascular adipose tissue, while in established obesity, perivascular adipose tissue loses its vasodilatory properties via an increase in 'contractile' superoxide, leading to endothelial dysfunction and vascular disease (Fernandez-Alfonso *et al.*, 2013). Increased nitro-oxidative pressure seen in perivascular adipose tissue in obesity may also occur in other adipose tissue areas. In fact, this may be critical for the impairment of blood flow, tissue oxygen and substrate supply in adipose tissues in obesity (see Alemany, 2012).

Lower antioxidant defence mechanisms. To prevent ROS/RNS excess, cells are equipped with antioxidant enzymes, such as manganese SOD (MnSOD), in the mitochondrial matrix and copper, zinc SOD in the cytosol and intermembrane space of mitochondria, which convert $O_2^{\bullet-}$ into H_2O_2 (Fridovich, 1995). H_2O_2 is further reduced to H_2O by catalase, GSH peroxidase and Trx/peroxiredoxin systems. GSH and Trx reduce peroxide concentrations and protein disulphide, subsequently producing oxidized GSH and Trx respectively. Oxidized GSH and Trx are converted back to reduced GSH, that is, Trx, via NADPH-dependent reductases, respectively, thus maintaining the reduced cellular redox state (Halliwell and Gutteridge, 2007). NADPH, the reducing power for GSH (Cys) and Trx recycling, is provided by the pentose phosphate pathway and malic enzyme.

Accordingly, lower SOD levels may contribute to increased superoxide and thus higher probability of $ONOO^-$ formation in the metabolic syndrome. Likewise, the mitochondrial antioxidant enzymes, MnSOD and GSH peroxidase, show decreased activity in adipose tissue, with the most pronounced decline in obese type 2 diabetic subjects and, to a lesser degree, non-obese type 2 diabetic or non-diabetic obese subjects (Chattopadhyay *et al.*, 2015). Consistent with these findings, our group showed that SOD levels were not significantly decreased in obese, insulin-sensitive subjects, while a significant decrease in SOD activity and MnSOD protein expression, in addition to lower levels of GSH, characterized visceral adipose tissue of subjects with increased cardiometabolic risk factors (Jankovic *et al.*, 2014).

Besides conventional antioxidant mechanisms, uncoupling capacity plays a significant role in determining superoxide production from ETC. Uncoupling decreases the pressure on ETC complexes and increases electron transfer capability, thereby suppressing the leak of electrons and subsequent production of $O_2^{\bullet-}$. *UCP1* gene expression is undetectable in adipogenic precursor cells isolated from lean and obese individuals from subcutaneous abdominal white adipose tissue biopsies. However, after adipocyte differentiation, both gene expression and protein content of UCP1 are increased. UCP1 levels are significantly greater in cultures from lean, compared with obese individuals (Carey *et al.*, 2014). Moreover, morbidly obese subjects express significantly lower levels of UCP mRNA than lean controls (Oberkofler *et al.*, 1997). In parallel, fat mitochondria from obese type 2 diabetic subjects produce considerably more ROS, compared with those of controls and non-diabetic subjects. In the majority of cells, including adipocytes, the antioxidant role is mainly attributed to UCP2. However, recent data indicate that a short-term UCP1 increase may buffer the reductive pressure on mitochondrial ETC and consequent oxidative pressure in white adipocytes (Jankovic *et al.*, 2015b). In addition, both uncoupling agents and MnSOD mimics that alleviate the $O_2^{\bullet-}$ level in the ETC rapidly restore the insulin sensitivity of adipocytes (Hoehn *et al.*, 2009). Thus, the antioxidant role of mitochondrial UCPs is important during adipocyte differentiation and their response to overfeeding, and its impairment may contribute to higher mitochondrial superoxide release. Moreover, reduction in mitochondrial number without concomitant reduction in nutrient uptake leads to an increase in net substrate flux through the remaining mitochondria and subsequent $O_2^{\bullet-}$ production (Hoehn *et al.*, 2009). Thus, lower or impaired mitochondriogenic potential may also contribute to increased superoxide levels in persistent overfeeding.

iNOS in obesity and insulin resistance

$iNOS^{-/-}$ mice are protected from high-fat diet-induced insulin resistance. While wild-type and $iNOS^{-/-}$ mice on a high-fat diet develop obesity, obese $iNOS^{-/-}$ mice exhibit improved glucose tolerance, normal insulin sensitivity *in vivo* and normal insulin-stimulated glucose uptake in muscles. iNOS is increased in fat of genetic and dietary (high-fat feeding) models of obesity (Perreault and Marette, 2001). Chronic NO synthesis by iNOS represents an important contributory factor to nitrosative stress and development of fat-specific insulin resistance (Kaneki *et al.*, 2007). In adipose tissue, the majority of iNOS is derived from phenotypic transformation of anti-inflammatory 'alternatively activated' macrophages to a more pro-inflammatory 'classically activated' form (Weisberg *et al.*, 2003; Lumeng *et al.*, 2007). In addition, high levels of TNF-α in adipocytes induce increased iNOS expression (Merial *et al.*, 2000). Almost any factor that

promotes insulin resistance may trigger iNOS expression (see Kaneki *et al.*, 2007).

Effects of interaction of NO with superoxide in adipose tissue in obesity

Interactions between NO and superoxide and the consequent effects *in vivo* are very complex, because they depend not only on the relative levels of reactants and kinetics but also on spatial (where) and temporal (at the same time) prerequisites. In contrast to NO that may act as either an intracellular or intercellular messenger, the half-life of superoxide is mainly restricted by SODs, and therefore, interactions most likely occur at the sites of superoxide production. In adipocytes, mitochondria and NOX4 enzyme localized in the cellular plasma membrane and intracellular membranous structures represent the major sites of NO/superoxide interactions (Bedard and Krause, 2007). This may also explain why the major burden of *S*-nitrosated/nitrated proteins is found in these compartments.

Finally, $ONOO^-$, the product of NO/superoxide interactions, exists as peroxynitrous acid (ONOOH) at physiological pH that is prone to proton-catalysed and carbon dioxide-catalysed homolysis, generating even more potent oxidants, such as hydroxyl ($^{\bullet}OH$), nitrogen dioxide ($NO_2^{\bullet}$) and carbonate anion ($CO_3^{\bullet-}$) radicals, which induce oxidation, nitrosation or nitration of protein side chains (see Toledo and Augusto, 2012). Protein modification by tyrosine nitration is mainly attributable to $ONOO^-$ (Ischiropoulos *et al.*, 1992). The reversibility of tyrosine nitration by denitrases (Kamisaki *et al.*, 1998; Irie *et al.*, 2003) and proteolysis (and re-synthesis of nitrated proteins) (Souza *et al.*, 2000) has altered the mainly negative perception of nitration and $ONOO^-$. Accumulating data indicate that this protein modification plays an adaptive role in specific settings (Ferdinandy and Schulz, 2003), and the target proteins and extent of modification determine the resulting patho/physiological effects (Koeck *et al.*, 2005, 2009). Extensive nitration of tyrosine residues in proteins is the fingerprint of different pathophysiological conditions, including the diabetic state (Koeck *et al.*, 2009; Zhou *et al.*, 2009; Charbonneau and Marette, 2010; Pilon *et al.*, 2010).

All aspects of NO and superoxide interactions should be taken into account to explain why the higher production rate of NO and superoxide usually contributes to pathological conditions but also plays an adaptive role. This is very challenging when considering adipose tissue in obesity whose expansion is associated with pathologies but *per se* represents the adaptive response of tissue (schematically presented in Figure 4).

The literature shows that the level of endogenously formed $ONOO^-$ increases in adipose tissue mainly as a result of hyperglycaemia, that is, established diabetes (Koeck *et al.*, 2009). A similar increase in the $ONOO^-$ level in diabetic hearts has been reported (Pechánová *et al.*, 2015; Varga *et al.*, 2015). In these circumstances, increased signalling through advanced glycation end products or their corresponding receptors (RAGE) may additionally lead to excessive superoxide formation and $ONOO^-$ increase. Once formed, $ONOO^-$ triggers a vicious cycle, further decreasing NO bioavailability and increasing nitro-oxidative stress. Using different mechanisms, $ONOO^-$ may oxidize and decrease eNOS-mediated NO production and divert direct sGC-mediated and CcOx-mediated bioeffects of NO in adipocytes, including insulin sensitivity and mitochondrial number and function (through low PGC-1α activation and/or expression – biogenic, OXPHOS and antioxidant capacity of mitochondria). In addition, $ONOO^-$ may increase superoxide generation (again via eNOS and/or iNOS uncoupling, although the latter has not been confirmed for adipose tissue) and decrease superoxide neutralization (via nitration of MnSOD) and the pool of GSH. The latter parameters are initially increased in obesity as a compensatory response to increased levels of superoxide, SNO and $ONOO^-$. The diminished antioxidant capacity of adipocytes (initially mitochondrial and subsequently cytosolic) renders more proteins susceptible to oxidation and nitration, resulting in inactivation or dysfunction. However, the important issue of which of these multiple effects represents an early event in $ONOO^-$-mediated toxicity at a time point prior to the onset of irreversible functional changes and insulin resistance in adipocytes remains to be resolved.

Nitro-oxidative state and insulin signalling/sensitivity

A number of researchers are currently investigating the pathways with potential $ONOO^-$ involvement in relation to insulin signalling and resistance. Growing evidence suggests that tyrosine nitration can alter protein function by preventing functional phosphorylation (Mondoro *et al.*, 1997; Rawlingson *et al.*, 2003; Kaneki *et al.*, 2007; Stadler, 2011). Hepatic insulin resistance in lipid-challenged mice results from $ONOO^-$-mediated tyrosine nitration of insulin receptor (IR)β and IRS-1/-2 (which promotes inhibitory serine phosphorylation of IRS proteins) and Akt, directly inhibiting insulin signalling (Charbonneau and Marette, 2010). In addition, $ONOO^-$ mediates muscle insulin resistance via nitration of IRβ/IRS-1 and Akt (Zhou and Huang, 2009), while targeted disruption of iNOS reverses high-fat diet-induced impairment of the insulin-stimulated tyrosine phosphorylation of IR, IRS-1 and IRS-1-associated PI3K activity, Akt and insulin resistance in muscle of mice (Perreault and Marette, 2001). Yasukawa *et al.* (2005) reported that a similar mechanism of $ONOO^-$-mediated nitration may contribute to insulin resistance in adipocyte tissue. The group showed that pretreatment of cultured mouse 3T3-L1 adipocytes with the NO donor, *S*-nitroso-*N*-acetylpenicillamine, inhibits insulin-stimulated Akt/PKB activation. Activation of the renin–angiotensin–aldosterone system plays an important role in cardiovascular complications in metabolic syndrome, and angiotensin II is a strong trigger of tyrosine nitration and inactivation of kinases involved in insulin signalling (Csibi *et al.*, 2010).

The data of Nomiyama *et al.* (2004) showed that constitutive production of $ONOO^-$ by the $NO/O_2^{\bullet-}$ donor – 3-(4-morpholinyl) sydnonimine hydrochloride (SIN-1) – dose-dependently inhibited insulin-stimulated glucose uptake in rat-1 fibroblasts expressing human insulin receptors. SIN-1 reduced the IRS-1 protein level, and IRS-1 associated PI3K activity through tyrosine nitration of at least four tyrosine residues, including Tyr^{939}, which is critical for the association of IRS-1 with PI3K.

A continuous consumption of surplus of nutrients also increased tyrosine nitration in adipocytes (Koeck *et al.*, 2009). Moreover, the extent of tyrosine nitration and the cellular nitroproteome profile in adipocytes reflects rise in the glucose

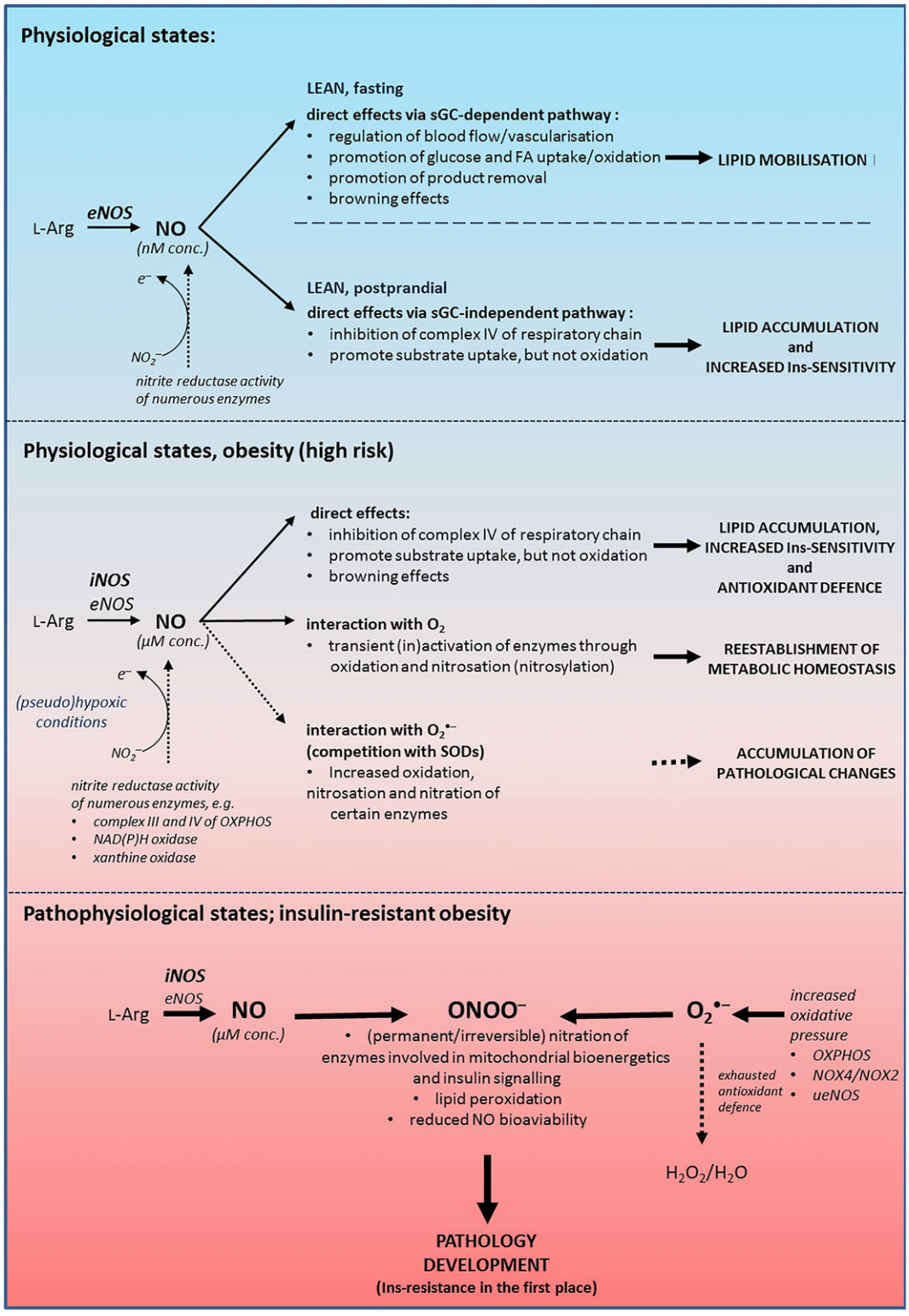

Figure 4

Interaction of NO and superoxide in the physiology and pathophysiology of adipose tissue. FA, fatty acid.

concentration during continuous exposure, as well as during the periodic fluctuation in the more physiological glucose concentration (Koeck *et al.*, 2009). The nitrated proteins in glucose-overloaded adipocytes are primarily those involved in glucose metabolism – glycolysis pathway and citric acid cycle (aldolase A, glyceraldehyde 3-phosphate dehydrogenase,

phosphoglycerate kinase, malate dehydrogenase, aconitase). Besides, increased nitration of some of the enzymes involved in lipid trafficking/oxidation contributes to insulin resistance in adipocytes. For example, both functionally important tyrosine residues (Tyr^{19} and Tyr^{128}) in fatty acid binding protein 4 are found to nitrate in adipocytes exposed to high metabolic load (Koeck *et al.*, 2009). The nitration of fatty acid binding protein 4 may affect its interaction with hormone-sensitive lipase (Adida and Spener, 2006), translocation into the nucleus and interaction with PPARγ (Smith *et al.*, 2007), which, in turn, may additionally down-regulate all PPARγ-mediated metabolic effects in adipose tissue, including insulin sensitivity (Koeck *et al.*, 2009).

In contrast to these *in vitro* data, the evidence of high endogenous $ONOO^-$ levels and activity, primarily tyrosine nitration, in adipose tissue in obese or prediabetic animal models or humans is rather scarce. The reason may be that tyrosine nitration is a highly selective process limited to specific tyrosine residues on a surprisingly small number of proteins (Aulak *et al.*, 2001; Gow *et al.*, 2004; Kanski *et al.*, 2005) and is thus detectable only at high $ONOO^-$ levels that mainly characterize already established diabetic conditions, as stated above.

Nevertheless, increased total protein *S*-nitrosylation, an alternative sign of $ONOO^-$ acting, has been detected in intra-abdominal adipose tissue of obese humans and high fat-fed or leptin-deficient *ob/ob* mice (Ovadia *et al.*, 2011). Importantly, Yin *et al.* (2015) recently revealed the role of increased *S*-nitrosylation as the mechanism that links obesity-associated inflammation, lower PPARγ activity and insulin resistance in adipose tissue. They found that iNOS-mediated increase of nitro-oxidative stress translates from macrophages to adipocytes leading to *S*-nitrosylation of PPARγ on Cys^{168}. This in turn potentiates proteasomal degradation of PPARγ, decreasing its transcriptional function. Moreover, expression level of the main PPARγ-regulated insulin-sensitizing adipokine, adiponectin, was also lower as a result of increased *S*-nitrosylation of PPARγ (Yin *et al.*, 2015). Similar results were observed *in vivo*, in visceral adipose tissue of obese diabetic *db/db* mice (Yin *et al.*, 2015).

Nitro-oxidative state and mitochondrial dysfunction

Disruption of mitochondrial function is implicated in fat-specific insulin resistance. Many mitochondrial proteins, including the enzymes involved in fat oxidation and energy supply, could be oxidized/nitrated under increased nitro-oxidative pressure, leading to depletion of ATP and impairment of lipid-buffering capacity in diabetic adipose tissue in obesity. The half-life of $ONOO^-$ under cellular conditions is short (~less than 1 s), but sufficient to cross biological membranes. Thus, $ONOO^-$ or peroxynitrous acid may enter mitochondria from the cytosol or be directly produced within mitochondria. Indeed, persistent hyperinsulinaemia and nNOS/mtNOS activation may contribute to mitochondrial dysfunction and insulin resistance of adipocytes (Jezek *et al.*, 2010; Finocchietto *et al.*, 2011). A remarkable increase in nNOS activity and NO level in mitochondria of *ob/ob* adipocytes inhibits CcOx and cause intense nitration at complex I impeding subsequent electron transfer from NADH (Finocchietto *et al.*, 2011). Recurrent nitrosative stress of progressively higher intensity and duration would lead to parallel oxidative stress due to corresponding shifts in respiratory chain redox states that stimulate $O_2^{\bullet-}$ formation. In fact, according to the 'Poderoso hypothesis', accumulating oxidative/nitrosative stress in mitochondria of fat cells could lead to retrograde modulation of the insulin signalling pathway and therefore represents the basis of type 2 diabetes aetiology (Jezek *et al.*, 2010).

Nitro-oxidative state and lipolysis/re-esterification dysregulation

As stated, nM levels of NO stimulate cAMP and PKA/hormone-sensitive lipase signalling, that is, lipolysis (Gaudiot *et al.*, 2000; Jobgen *et al.*, 2006), and decreased eNOS-mediated lipolysis could favour the development of obesity through retention of TAG within adipocytes. However, in obesity, diminished lipolysis can also be viewed as a mechanism limiting excessive fatty acid release and alleviating the development of insulin resistance and metabolic abnormalities (Girousse *et al.*, 2013). Notably, impaired anti-lipolytic effects of insulin in the postprandial period could restrict fatty acid esterification in adipocytes, exposing non-adipose tissue to lipotoxicity. Recent studies have revealed that reduction in lipolysis improves glucose incorporation into adipocyte lipids without increasing fat mass (Girousse *et al.*, 2013). Because high NO levels inhibit basal as well as catecholamine-stimulated lipolysis (Gaudiot *et al.*, 1998; Andersson *et al.*, 1999; Klatt *et al.*, 2000), iNOS-mediated increase in NO in adipose tissue under insulin stimulation is considered an important regulator of the lipogenic function of postprandial adipocytes (Engeli *et al.*, 2004). However, recent data indicate that, in obesity, iNOS may, in fact, impair anti-lipolytic regulation in postprandial adipocytes, possibly via increased nitro-oxidative pressure. Specifically, iNOS increases *S*-nitrosylation of PKB/Akt and cAMP PDE (PDE3B). *S*-nitrosylation of PDE3B at Cys^{768} and Cys^{1040} (Ovadia *et al.*, 2011) inhibits its cAMP hydrolytic activity (Zmuda-Trzebiatowska *et al.*, 2007). The main metabolic consequence of increased *S*-nitrosylation and inactivation of Akt/PDE3B axis in adipose tissue in obesity is impairment of insulin-induced anti-lipolysis (Ovadia *et al.*, 2011).

In addition to uncontrolled lipolysis, a high fatty acid output from adipose tissue leading to lipotoxicity and insulin resistance may result from impaired fatty acids re-esterification. This metabolic pathway requires glycerol-3P synthesis, a pathway relaying on the activity of cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C). Niang *et al.* (2011) and Jaubert *et al.* (2012) showed that long-term leptin treatment inhibits PEPCK-C by increasing iNOS-mediated PEPCK-C nitration, therefore limiting fatty acid -re-esterification in rat adipocytes. The increased nitration of PEPCK-C by leptin may initially play beneficial role in obesity setting, by increasing energy expenditure (Jaubert *et al.*, 2012). Nevertheless, in the long-standing hyperleptinaemic obesity increased nitration of PEPCK-C may restrict glyceroneogenesis, essential in the lipid buffering function of adipose tissue, ultimately leading to lipotoxicity.

Nitro-oxidative state and endoplasmic reticulum stress

The endoplasmic reticulum is essential for the folding and trafficking of proteins that enter the secretory pathway. The accumulation of misfolded or unfolded proteins in this organelle, known as endoplasmic reticulum stress, is a common pathogenic molecular mechanism underlying adipocyte-specific insulin resistance (Kawasaki *et al.*, 2012; Boden *et al.*, 2014). It has been recently shown in both genetic (*ob/ob*) and dietary (high-fat diet-induced) models of obesity that, in the setting of obesity, inflammatory input through increased iNOS-mediated *S*-nitrosylation and impaired splicing activity of a key regulator of adaptive unfolded protein response – inositol-requiring protein 1 – leads to impaired endoplasmic reticulum function (Yang *et al.*, 2015). These data were obtained with liver tissue, but it could be assumed that increased *S*-nitrosylation of the important proteins mediating unfolded protein response exists in adipose tissue in obesity. In support of this, injection of mice with a SOD mimetic (Mn (iii)tetrakis(4-benzoic acid) porphyrin) attenuated the induction of the unfolded protein response (Malhotra *et al.*, 2008), while inhibition of iNOS reversed palmitate-induced endoplasmic reticulum stress response in 3T3-L1 adipocytes (Jeon *et al.*, 2012).

Nitro-oxidative stress versus endogenous antioxidant defence

In addition to protein-bound thiols, $ONOO^-$ can oxidize GSH (Villa *et al.*, 1994; Mayer *et al.*, 1995; Prendergast *et al.*, 1997). GSH is an efficient endogenous scavenger of $ONOO^-$ and plays a major role in cellular defence against this species. Thus, in the long term, exhaustion of GSH may

Table 1

Potential therapeutic strategies to improve fat-specific insulin sensitivity by targeting NO/superoxide ratio in adipose tissue

Strategy (treatment)	Molecular mechanisms	Target cells and effects	References
Exercise training and hypoglycemic agents	↓Uncoupling of ↑eNOS, ↑SOD, ↓NOX activity	Endothelial cells; ↑mitochondrial function	Shen, 2010
Caloric restriction mimics (resveratrol)	↑eNOS, ↑mitochondrial biogenesis factors	Endothelial cells; ↑mitochondrial function	Rivera *et al.*, 2009; Csiszar *et al.*, 2009
Statins, angiotensin-converting enzyme inhibitors, AT_1 receptor blockers or β-blocker - nebivolol	↑NO production, ↓superoxide production	Endothelial cells and adipocytes; ↑mitochondrial function	Münzel and Gori, 2009; Münzel *et al.*, 2010; Huang *et al.*, 2013
BH_4, BH_4 precursor (sepiapterin)	↓Uncoupling of eNOS, ↑NO production, ↓superoxide	Endothelial cells; ↑mitochondrial function and insulin sensitivity	Heitzer *et al.*, 2000; Schulz *et al.*, 2008
Folate	↓Uncoupling of eNOS, ↑NO production, ↓superoxide	Endothelial cells; ↑mitochondrial function and insulin sensitivity	Verhaar *et al.*, 2002
Coenzyme Q10	↓Superoxide production	Adipocytes and other cells; ↑mitochondrial function, ↓inflammatory process and lipid-metabolizing effects	Alam and Rahman, 2014
Curcumin	↓Superoxide and NO overproduction	Adipocytes and other cells; protecting mitochondria and ↓inflammation	Martinez-Morua *et al.*, 2013; Priyanka *et al.*, 2014a
Bilobalide	↓Superoxide production, ↑SOD	Adipocytes; protecting mitochondria and ↓inflammation	Priyanka *et al.*, 2014b
Mitochondria-targeting SOD mimetics	↓mitochondrial superoxide production	Adipocytes; ↑insulin sensitivity	Hoehn *et al.*, 2009
Lipoamide or lipoic acid	↑eNOS–cGMP–PKG pathway; biogenesis of mitochondria	Adipocytes; ↑mitochondrial function and insulin sensitivity	Shen *et al.*, 2011
ω-3 fatty acids (GPR120, a functional receptor of the ω-3 fatty acids and GPR120-selective agonist)	↓Nitrosylation of Akt	Adipocytes; ↓inflammation and insulin-sensitizing effects	Oh *et al.*, 2014
L-arginine or citrulline	↑eNOS–cGMP–PKG pathway; ↓superoxide by UCP up-regulation, biogenesis of mitochondria; ↑glutamate-cysteine ligase expression and GSH levels	Adipocytes and other cells; ↑mitochondrial function, ↑ insulin sensitivity, ↓ fat mass	Jobgen *et al.*, 2006; Lucotti *et al.*, 2006; Petrović *et al.*, 2009; Joffin *et al.*, 2015
Dietary polyphenols	↑SOD expression	Adipocytes; ↑mitochondrial function, ↓inflammation	Baret *et al.*, 2013; Hatia *et al.*, 2014; Marimoutou *et al.*, 2015

render adipose tissue more susceptible to $ONOO^-$-mediated nitro-oxidative damage (Pacher *et al.*, 2007). In addition, lower reduced GSH/Trx levels may decrease de-nitrosylation of SNO moieties and further increase the *S*-nitrosylated protein level. Overall, changes in intracellular GSH level correspond to changes in lipogenesis and adipogenesis (Galinier *et al.*, 2006). However, the GSH level decreases in adipose tissues in subjects with long-lasting obesity (Jankovic *et al.*, 2014). This may represent an early sign of redox dysbalance that, if not compensated by other redox molecules like the Trx system, leads to increased vulnerability to oxidant insult and decreased fat storage buffering capacity of adipocytes in obese subjects (Jankovic *et al.*, 2014). Interestingly, subcutaneous, compared with visceral, adipose tissue characterizes greater adaptive capacity of antioxidant defence on GSH depletion (Jankovic *et al.*, 2014).

Perspectives – does targeting of the NO/superoxide ratio in adipose tissue hold promise?

Targeting of the $NO/O_2^{\bullet-}$ ratio in adipose tissue may have relevance in the elucidation and management of obesity and diabetes pathogenesis. Although the use of antioxidant supplements may have beneficial effects on the overall health of diabetic patients, classical antioxidant therapy with vitamins failed to show benefits in curbing fat-specific insulin resistance, diabetes and related cardiovascular diseases (Münzel *et al.*, 2010; Abdali *et al.*, 2015). Adipose tissue in obesity is possibly a paradigm of *parahormesis* (Forman *et al.*, 2014), whereas parallel increases in electrophiles, oxidants (superoxide, $ONOO^-$), nucleophiles and antioxidants (GSH, Trx and NADPH), in concert with increased nutrient metabolism in adipocytes, play important signalling roles, enabling adaptive expansion of adipose tissue in increasing nutrient intake (Jankovic *et al.*, 2015b). Thus, antioxidant as well as the hormetic approach (to increase oxidants that would strengthen endogenous antioxidants) may interfere with the endogenous steady-state redox level in adipose tissue in obesity, thereby restricting its metabolic plasticity. The ideal approach is to increase NO bioavailability and/or simultaneously limit excessive superoxide formation (briefly summarized in Table 1). Currently, alternative antioxidant-promoting methods, such as L-arginine supplementation (Lucotti *et al.*, 2006; McKnight *et al.*, 2010), caloric restriction (Schulz *et al.*, 2008) or use of SOD mimetics (Hoehn *et al.*, 2009), are considered promising therapeutic strategies.

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Conflict of interest

The authors declare no conflicts of interest.

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