THE ROLE OF REACTIVE OXYGEN SPECIES IN ADIPOGENIC DIFFERENTIATION

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ABSTRACT

Interest in reactive oxygen species and adipocyte differentiation / adipose tissue function is steadily increasing. This is due in part to a search for alternative avenues for combating obesity, which results from the excess accumulation of adipose tissue. Obesity is a major risk factor for complex disorders such as cancer, type 2 diabetes and cardiovascular diseases. The ability of mesenchymal stromal/stem cells (MSCs) to differentiate into adipocytes is often used as a model for studying adipogenesis *in vitro*. A key focus is the effect of both intra- and extracellular reactive oxygen species (ROS) on adipogenesis. The consensus from the majority of studies is that ROS, irrespective of the source, promote adipogenesis.

The effect of ROS on adipogenesis is suppressed by antioxidants or ROS scavengers. Reactive oxygen species are generated during the process of adipocyte differentiation as well as by other cell metabolic processes. Despite many studies in this field, it is still not possible to state with certainty whether ROS measured during adipocyte differentiation are a cause or consequence of this process. In addition, it is still unclear what the exact sources are of the ROS that initiate and/or drive adipogenic differentiation in MSCs *in vivo*. This review provides an overview of our understanding of the role of ROS in adipocyte differentiation as well as how certain ROS scavengers and antioxidants might affect this process.

Key words

Reactive oxygen species, adipogenic differentiation, ROS scavengers, adipose-derived stromal cells, mesenchymal stem/stromal cells

1. Introduction

Adipogenesis can be described as the differentiation of stem cells to form fully differentiated lipid-filled mature adipocytes (Lefterova and Lazar 2009). It is a complex and well-orchestrated process that occurs through different stages involving numerous transcription factors, cell-cycle proteins, extracellular signals, hormones, and small molecules which mediate the up- and downregulation of a well-defined cascade of genes essential for the differentiation and maturation of adipocytes (Ali et al. 2013; Jiang et al. 2012).

Adipogenic differentiation has primarily been studied *in vitro* using murine preadipocyte cell lines such as 3T3-L1, but some studies have also used primary or immortalized murine embryonic fibroblasts (MEFs), murine embryonic stem cells (mESCs) and murine mesenchymal stem/stromal cells (mMSCs). A disadvantage of using murine cell lines is that the translation of the findings to the human setting is uncertain and challenging. As an example, the same repertoire of gene expression that occurs during human adipogenesis may not necessarily be present when murine cells are used. Another key difference between mouse and human adipose tissue is that the distribution of white and brown fat differs between the two species. Heterogeneity in the cellular composition of adipose tissue and inter-patient variability are some of the other main differences observed between humans and murine experimental models (Ambele et al. 2016). A human primary cell model would therefore provide better insight into adipose differentiation and metabolism, thus increasing our understanding of obesity and its related comorbidities in humans.

Reactive oxygen species (ROS) are known to play a role in promoting adipogenic differentiation of mouse, rat and human preadipocytes as well as immortalized preadipocyte cell lines (Tormos et al. 2011; Kanda et al. 2011; Schroder et al. 2009; Lee et al. 2009a). Excess accumulation of ROS in cells, as a result of limited scavenging activity by antioxidant systems, induces oxidative stress, which may lead to cellular damage of proteins, lipids and nucleic acids (Atashi et al. 2015b). This review will discuss the effect of ROS on adipogenesis using immortalized cell lines and primary cells of both human and murine origin.

2. Brief history of redox biology and ROS involvement in biological processes

The accumulation of atmospheric oxygen (O_2) began around 2.3 billion (Ga) years ago (Bekker et al. 2004). The rise in atmospheric O_2 levels and the need for O_2 in aerobic life forms for cellular

respiration is the fundamental tenet underlying redox biology. Oxygen can be converted through electron-transferring chemical reactions or exposure to environmental stresses such as ultraviolet radiation and heat, into a variety of highly reactive chemical forms that are collectively known as reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2) was the first ROS discovered by Thénard in 1818, but the production of biological ROS was only described in 1954 (Commoner et al. 1954). Initially, ROS were simply considered to be by-products of aerobic metabolism that were toxic in high levels to biological systems through oxidation and nitration of macromolecules such as proteins, lipids and nucleic acids. A few years later these damaging properties of ROS would be associated with aging (Harman 1956).

Subsequently, it was shown that the generation of ROS does not only have negative consequences, but constitutes an important intracellular signaling system that plays an essential role in biological processes such as differentiation, proliferation, cell death and senescence (Liu et al. 2012a; D'Autreaux and Toledano 2007; Atashi et al. 2015b; Kawagishi and Finkel 2014). An example is phagocytosis that requires a dramatic increase in O_2 uptake due to the demand for metabolic energy through glycolysis during the phagocytic process (Sbarra and Karnovsky 1959). Other immune cells, such as neutrophils and macrophages, have also been shown to produce high levels of ROS through an oxidative burst that contributes to innate host defense (Chen and Junger 2012; Slauch 2011).

Initially the mechanisms involved in ROS production were largely unknown. In 1967, Hinkle and colleagues discovered that the electron transport chain (ETC) of mitochondria produces H_2O_2 (Hinkle et al. 1967). This discovery was followed by the discovery in 1969 of the superoxide dismutase (SOD) enzyme and its ability to convert (dismutate) chemically generated superoxide (O_2^-) into O_2 and H_2O_2 (McCord and Fridovich 1969). In 1974, Loschen and colleagues discovered that O_2^- is a reactive precursor of mitochondrial H_2O_2 (Loschen et al. 1974). In 1961, Iyer and colleagues suggested that the phagocyte respiratory burst results in the generation of H_2O_2, but the mechanism involved was unknown (Bedard and Krause 2007; Jiang et al. 2011; Iyer et al. 1961). In 1964, Rossi and Zatti, discovered that an NADPH oxidase (NOX) was responsible for the respiratory burst in phagocytes (Rossi and Zatti 1964). A few years later, in 1973, Babior and colleagues showed that the initial product of the respiratory burst is O_2^- and not H_2O_2 as had originally been proposed (Babior et al. 1973).

In 1991, Meier and colleagues discovered NOX enzyme systems in human fibroblasts (Meier et al. 1991). The discovery of these systems in non-phagocytic cells was a pivotal event in redox biology,

as ROS now appeared to have functions other than just in host defense. Subsequently, gp91^{phox}, the catalytic subunit of the phagocyte NOX and various other components of the NOX enzyme complex, including p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox} and others were characterized (Bedard and Krause 2007; Jiang et al. 2011). The most important historical events in the discovery of ROS are summarized in Figure 1.

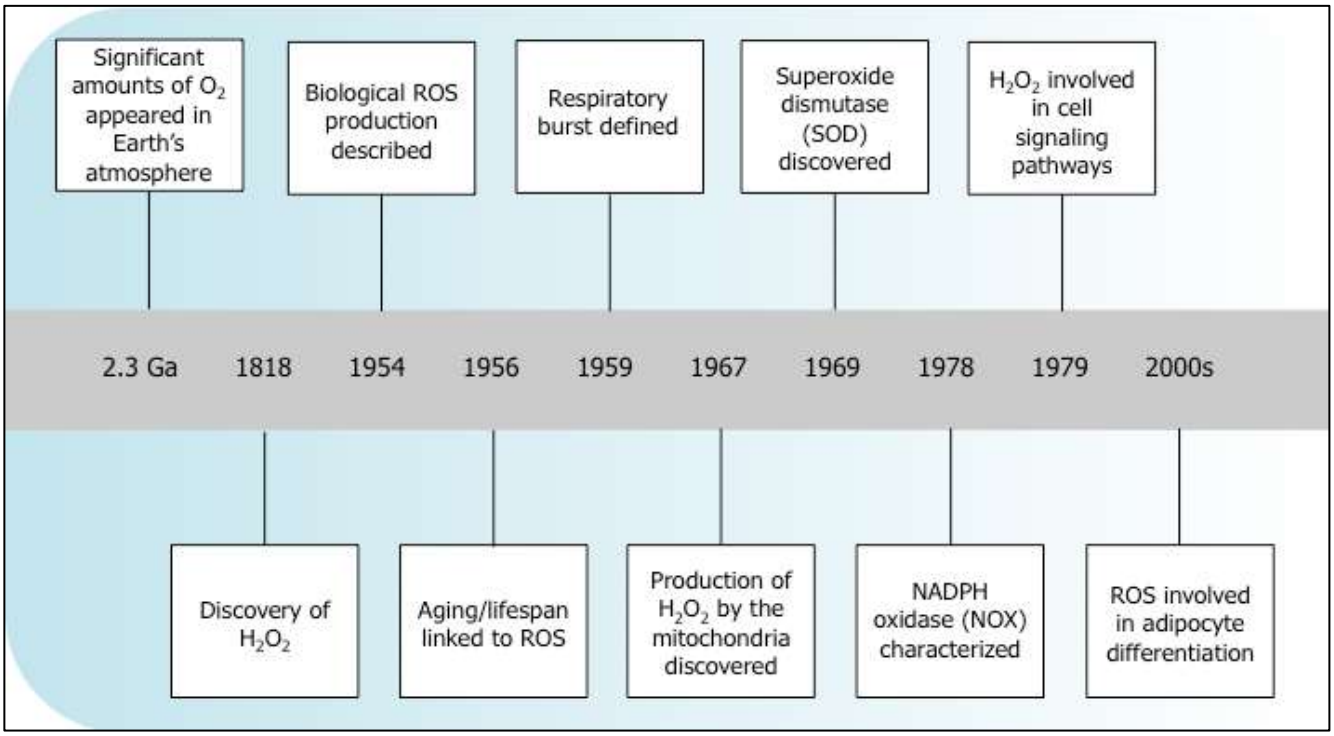

Fig. 1: A summary of the history of redox biology

3. Sources of ROS and their mechanism of production

The production of cellular ROS originates from many different sources including the mitochondrial ETC, NOX, xanthine oxidase**,** cytochrome p450**,** uncoupled nitric oxide synthase (NOS), myeloperoxidase and others. However, the mitochondrial ETC and NOX enzymes remain the major sources of cellular ROS production.

3.1 Mitochondria

Mitochondria play an important role in the cellular metabolism of aerobes by facilitating the conversion of O_2 into adenosine triphosphate (ATP) and water (H_2O) (Holzerova and Prokisch 2015). Low levels of O_2^- are naturally produced via the ETC during this process (Holzerova and Prokisch 2015). Electron transport chain sites that are involved in ROS generation include Complexes I

(NADH:ubiquinone oxidoreductase), II (succinate dehydrogenase) and III (cytochrome *bc1* complex) (Tahara et al. 2009; Sabharwal and Schumacker 2014). Complexes I and III are generally regarded as the main sites of O_2^- production in mitochondria (Drose and Brandt 2008). Although implicated in O_2^- generation, Complex II seems to be involved to a lesser extent when compared to Complexes I and III (Ryu et al. 2015). However, other studies suggest that Complex II is able to generate O_2^- at similar levels to those produced by Complex I and III (Quinlan et al. 2012). Complex IV accepts an electron from cytochrome *c* (Cyt c) and passes it to O_2, the final electron acceptor in this chain, to form H_2O. Water is formed by the reduction of O_2 with a series of four electrons, consecutively. Energy is released by electron transfer, which is used to pump protons (H^+) out of the matrix, through Complex I, III, and IV into the intermembrane space, building up a significant H^+ concentration gradient. The H^+ gradient that is generated provides the energy needed to produce ATP by ATP synthase (Complex V). Leakage of electrons to O_2 forming the one-electron reduction of O_2 to O_2^- and thus, its derivative ROS, occurs mainly at Complex I and III (Figure 2). Superoxide is inherently unstable and is, soon after being produced, dismutated into the more stable H_2O_2. The dismutation of O_2^- can occur spontaneously or through the action of antioxidant enzymes such as Cu-, Mn-, and ZnSOD found in the mitochondrial intermembrane space (Turrens 2003; Finkel 2011). Hydrogen peroxide is then converted to H_2O and O_2 by glutathione peroxidase (GPx). Hydrogen peroxide can be scavenged by antioxidants such as catalase (CAT), GPx, and peroxiredoxin 3 (Prx3) or act as a signaling molecule in different signaling pathways including redox balance, energy metabolism, cell cycle, and the stress response (Atashi et al. 2015b; Droge 2002; Finkel 2011). The production of ROS is summarized in Figure 3.

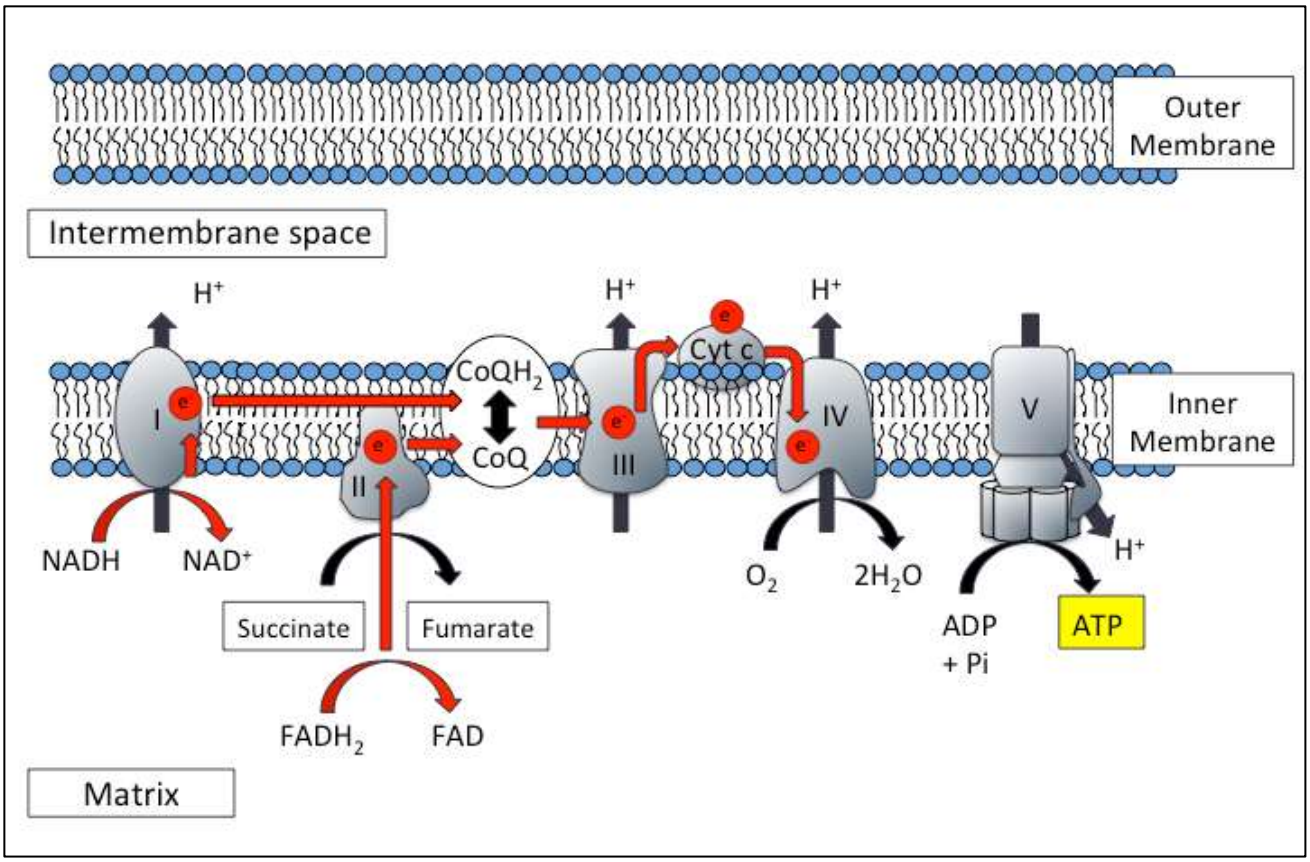

Fig. 2: The mitochondrial electron transport chain (ETC). Electrons are transferred through Complex I to IV resulting in the leakage of electrons and finally the addition of electrons to oxygen (O_2). The proton (H^+) gradient generated by the electron transfer provides the energy needed to generate ATP (adenosine triphosphate) by the final Complex V or ATP synthase. CoQ = oxidised ubiquinone; $CoQH_2$ = reduced ubiquinol; e^- = electrons

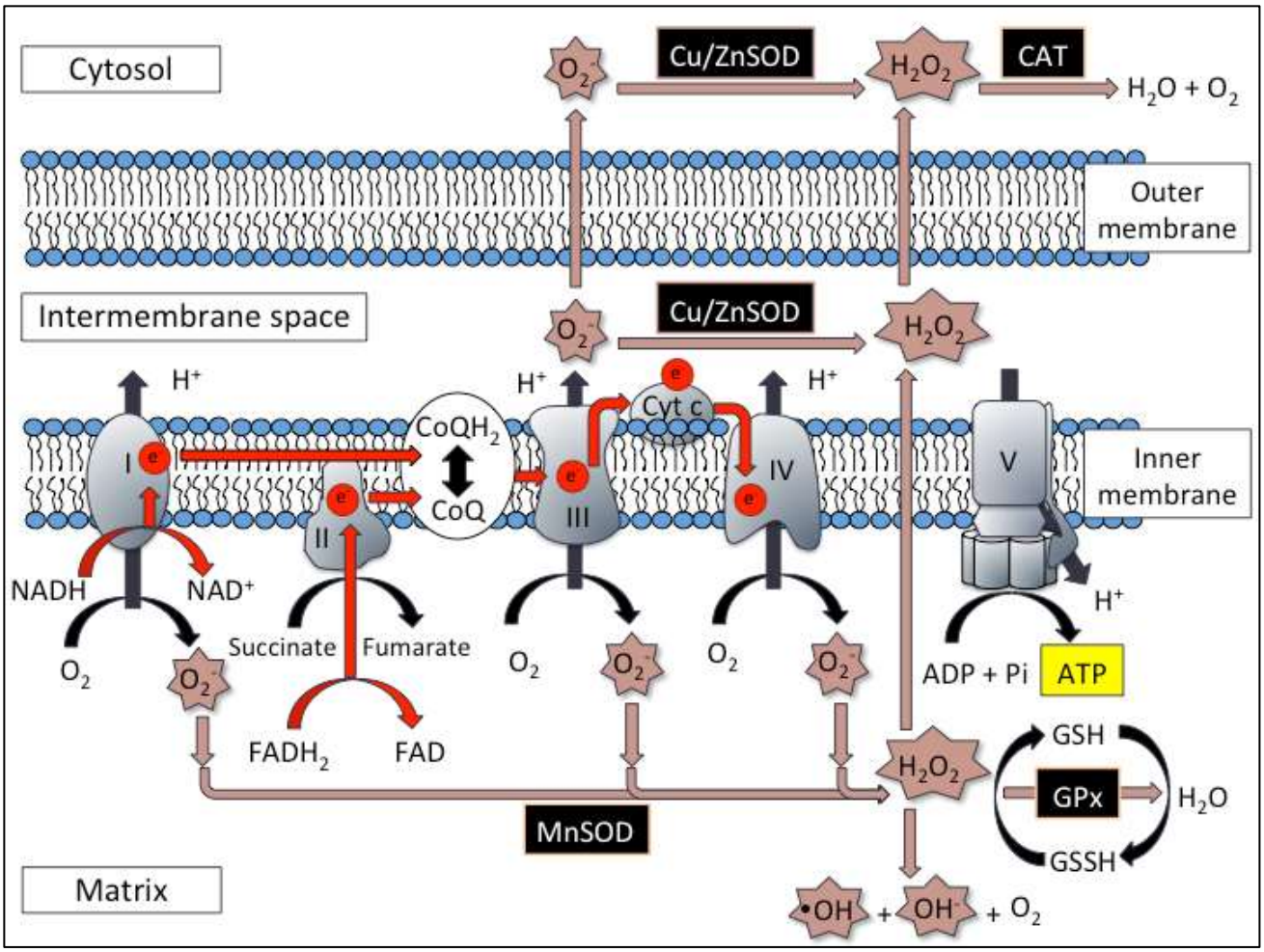

Fig. 3: An overview of the production of reactive oxygen species (ROS). Molecular O_2 may be reduced to superoxide anion radical (O_2^-), which can be further reduced to hydrogen peroxide (H_2O_2) either spontaneously, or through the action of superoxide dismutase (SOD) enzymes (MnSOD and Cu/ZnSOD). The transition metals such as Fe^{2+} and Cu^+ catalyze the conversion of H_2O_2 to the hydroxyl radical (•OH) via the Fenton and Harber–Weiss reactions. The enzymes catalase (CAT) or glutathione peroxidase (GPx) detoxify H_2O_2 by converting it to water (H_2O). GSSG = oxidized glutathione; GSH = reduced glutathione

Although the ETC has been identified as the main source of mitochondrial ROS generation, various other enzymes located within mitochondria play a role in ROS production. These enzymes include monoamine oxidase, α-ketoglutarate dehydrogenase, cytochrome b5 reductase, glycerol-3-phosphate dehydrogenase, various P450 enzymes, aconitase, pyruvate dehydrogenase and others (Finkel 2011).

3.2 NADPH oxidase

The NOX family is a group of NADPH oxidases that are responsible for transferring electrons, generating O_2^- in the process (Bedard and Krause 2007; Panday et al. 2015) (Figure 4). This dedicated function of NOX differentiates this group of enzymes from other oxidoreductases, such as

cyclooxygenase, lipoxygenase, cytochrome P450 enzymes, NOS, xanthine oxidase, mitochondrial NADPH:ubiquinone oxidoreductase (Complex I) and others, which produce ROS only as by-products during their catalytic action in their various pathways (Jiang et al. 2011).

The membrane protein, cytochrome b558 consists of two subunits, a large subunit $gp91^{phox}$ (NOX2) and a smaller subunit $p22^{phox}$, and forms the backbone of NOX. During cell activation, two regulatory subunits in the cytoplasm, $p47^{phox}$ and $p67^{phox}$, as well as a small G protein, Rac, translocate to the cell membrane and associate with the two subunits of cytochrome b558 to generate O_2^- (Bedard and Krause 2007). The discovery of NOX in non-phagocytic cells led to the identification of six additional NOX isoforms (NOX1, 3-5, DUOX1, DUOX2) as well as two subunit isoforms, NOXO1 ($p47^{phox}$ isoform) and NOXA1 ($p67^{phox}$ isoform). The expression patterns of these isoforms are distinct and seem to be tissue-specific (Jiang et al. 2011). Interestingly, the NOX enzyme systems are reportedly only found in multicellular organisms (eukaryotes) (Panday et al. 2015; Lalucque and Silar 2003). While most of the NOX enzymes generate O_2^-, only NOX4, DUOX1, and DUOX2 generate H_2O_2 (MacFie et al. 2014; Yoshihara et al. 2012). The physiological function of NOX is the modulation of multiple redox-sensitive intracellular signaling pathways (Jiang et al. 2011).

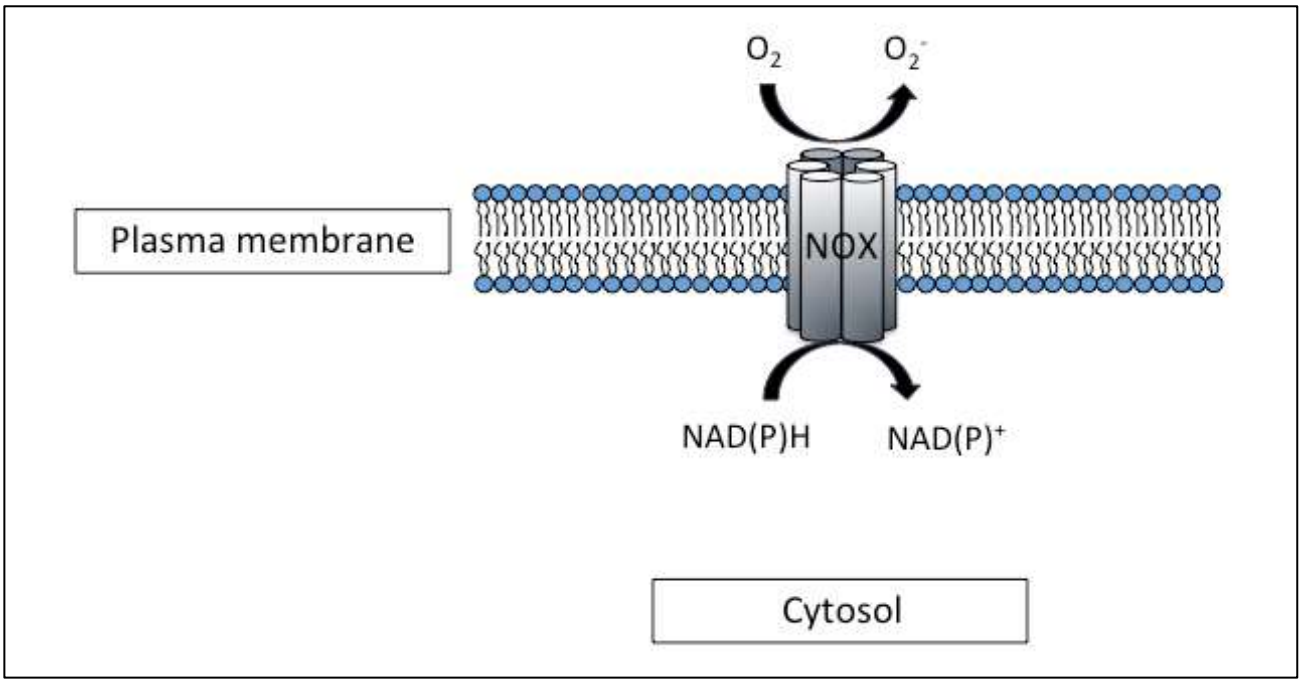

Fig. 4: NADPH oxidase (NOX). The typical NADPH oxidase members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen (O_2) to superoxide (O_2^-).

4. ROS in adipocyte differentiation

4.1 Introduction

Elevated levels of ROS are associated with cancer, diabetes, cardiovascular and other diseases (Alfadda and Sallam 2012a). Inflammation in chronic inflammatory diseases such as rheumatoid arthritis is also associated with ROS overproduction. The mechanisms of ROS production in these conditions are still uncertain. Obesity is also associated with chronic inflammation of adipose tissue and is strongly linked to other non-communicable diseases including cancer, type 2 diabetes and cardiovascular disease (Alfadda and Sallam 2012b; Roy et al. 2017). The worldwide prevalence of obesity and its comorbidities has reached alarming levels. This has led to an increase in studies designed to understand the biology of adipose tissue, the process of adipogenesis as well as the investigation into molecules such as ROS, which could modulate the process. A better understanding of the mechanisms involved in obesity may not only provide insight into the development of novel treatment strategies to combat obesity, but also the pathogenesis of other non-communicable diseases.

4.2 Adipose derived stromal cells and their ability to differentiate into adipocytes

Adipose tissue consists of various cell types, including adipocytes, preadipocytes, adipose-derived stem/stromal cells (ASCs), endothelial precursors, T regulatory cells, macrophages, smooth muscle cells and pericytes (Riordan et al. 2009; Nguyen et al. 2016). Adipogenesis is the process by which MSCs differentiate into adipocytes, which forms the major cellular component of adipose tissue (Ambele et al. 2016; Ambele and Pepper 2017). Mesenchymal stem/stromal cells harvested from adipose tissue are referred to as adipose-derived stem/stromal cells (ASCs) (Bassi et al. 2012). The ability of ASCs to differentiate into adipocytes *in vitro* is often used as a model to study adipogenesis.

To understand the role of adipose tissue in obesity it will be necessary to understand the process of adipogenesis in its entirety. However, the full process of adipogenesis is not well defined. In particular, there is a deficit in our understanding at a molecular and cellular level of the intermediate adipocyte sub-populations that exist from the stem cell to the mature adipocyte (Durandt et al. 2016b; Rosen and MacDougald 2006). In an attempt to address the lack in knowledge regarding adipocyte sub-populations during adipocyte differentiation, Durandt *et al.* (2016) showed in a recent study that the cell surface expression of the fatty acid translocase (CD36/FAT) precedes lipid accumulation allowing the identification of at least three adipocyte sub-populations during adipocyte differentiation (Durandt et al. 2016a). Adipogenesis is most often described to occur in two steps, namely a determination phase and a terminal differentiation phase. The former consists of MSC commitment to differentiate into

preadipocytes. These precursor cells do not possess defining morphological features or specific gene expression patterns that are distinct from their predecessors (Rosen and MacDougald 2006; Otto and Lane 2005), but have lost their ability to differentiate into other cell types and are committed to the adipocyte lineage (Otto and Lane 2005; Ali et al. 2013).

Several studies suggest that it is a prerequisite for preadipocytes to undergo growth arrest at the commitment phase for adipogenesis to proceed. Growth arrest, not necessarily cell-cell contact or confluence, is essential for differentiation to occur (Gregoire et al. 1998). Following growth arrest, upon reaching confluence and upon hormonal induction, it is reported that some cell lines, e.g. 3T3-L1, Ob17 and 3T3 F442A, undergo a mitotic clonal expansion phase prior to adipocyte differentiation. During the mitotic clonal expansion phase the cells undergo one or two rounds of cell division prior to differentiation. On the other hand, studies using mouse CH3H10T1/2 cells and human preadipocytes have shown that the cells differentiate without post confluence mitosis (Gregoire et al. 1998).

The second phase in adipocyte differentiation is terminal differentiation into functional mature adipocytes with the accumulation of intracellular lipid droplets (Gregoire et al. 1998). Initially, lipid droplets appear in the cytoplasm near the periphery of preadipocytes, followed by the aggregation and enlargement of lipid droplets through fusion. The enlarged lipid droplets migrate more centrally in preadipocytes. A mature adipocyte is a terminally differentiated cell and contains a large unilocular lipid droplet. Lipid droplets are constitutively formed in adipocytes. However, non-adipocytes may also contain small mobile lipid droplets (Murphy et al. 2009).

Adipogenesis can be thought of as a transition through the up- and downregulation of thousands of different genes (Satish et al. 2015; Ambele et al. 2016). This makes it difficult to accurately discuss the progression of events in chronological order. Various positive and negative regulators are involved and adipogenesis is a consequence of an equilibrium between these factors (Ambele and Pepper 2017). Positive regulators of adipogenesis include proliferator-activated receptor-gamma (PPARγ), CCAAT enhancer-binding protein (C/EBP) transcription factors, the Kruppel-like factor (KLF) family of C2H2 zinc-finger proteins *e.g*. KLF15, KLF5, KLF6, KLF9, KROX20, sterol regulatory element binding transcription factor 1 (SREBP1c), cyclic AMP response element-binding protein (CREB), the zinc finger protein 423 (ZFP423), signal transducer and activator of transcription-5a (STAT5a), endothelial PAS domain Protein 1 (EPAS1), and brain and muscle ARNT-like Protein 1 (BMAL1) among others (Rosen and MacDougald 2006; Moseti et al. 2016; Stephens 2012). Peroxisome proliferator-activated receptor-gamma (PPARγ) and C/EBP transcription factors play a key role in regulating terminal

differentiation (Ambele et al. 2016; Ambele and Pepper 2017). There are three C/EBP transcription factors (C/EBPα, C/EBPβ, and C/EBPδ) that can form heterodimers with each other and that bind to C/EBP regulatory elements within promoters (Otto and Lane 2005). The first transcription factors to be induced *in vitro* upon exposure to glucocorticoids or insulin are C/EBPβ and C/EBPδ, which together activate C/EBPα expression. CCAAT enhancer-binding protein alpha is thought to play an important role in establishing terminally differentiated adipocytes (Samuelsson et al. 1991). Both C/EBPβ and C/EBPδ induce the upregulation of PPARγ, which together with C/EBPα coordinate the expression of various adipogenic-specific genes leading to the mature adipocyte phenotype (Figure 5).

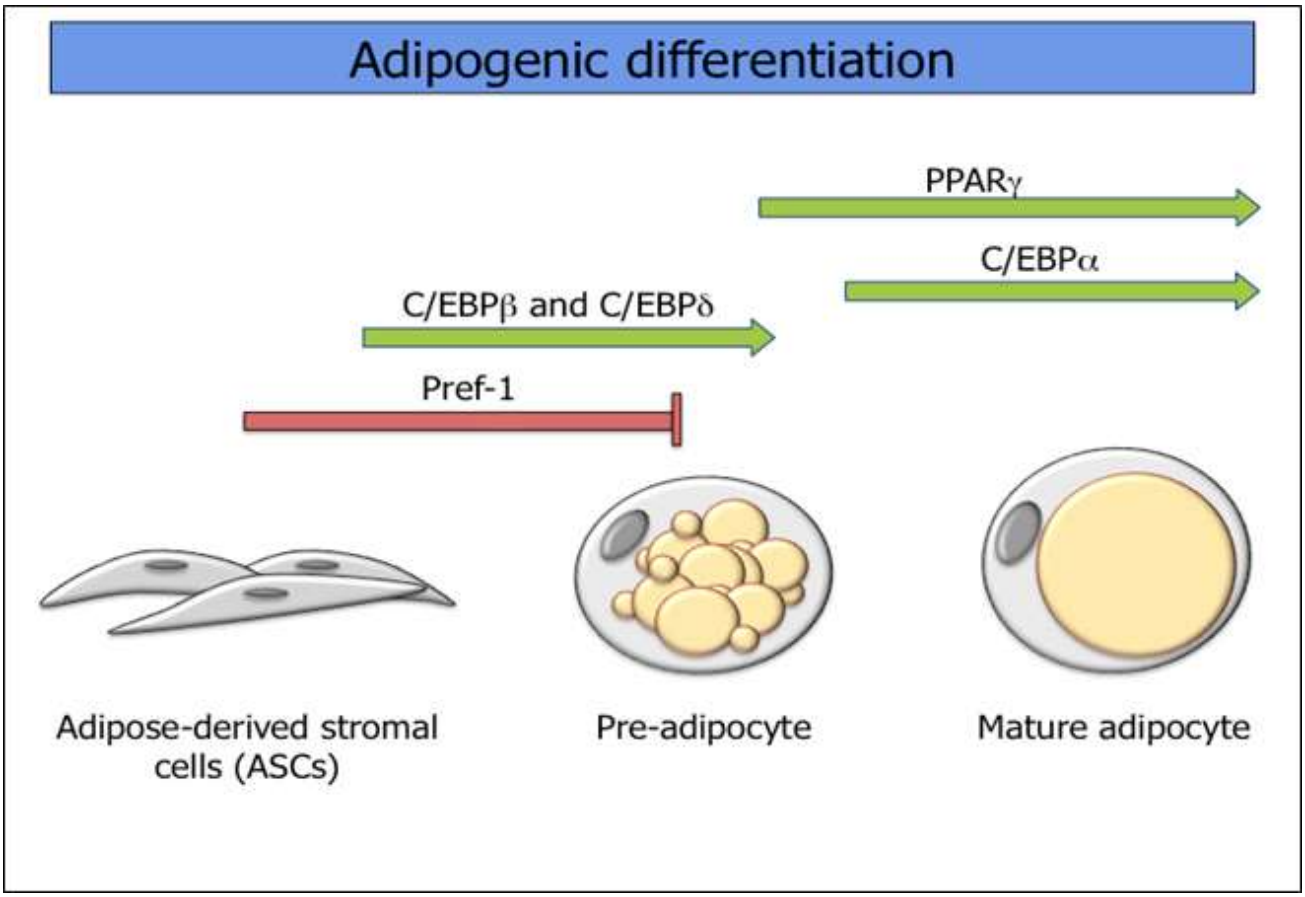

Fig. 5: Adipogenic differentiation. Adipose-derived stem/stromal cells (ASCs) can be committed to the adipogenic lineage and thus converted to preadipocytes, which further terminally differentiate into mature adipocytes. During the conversion of preadipocytes to adipocytes, growth arrest occurs. The subsequent activation of adipocyte genes by the transcription factors CCAAT/enhancer-binding protein (C/EBPα) and peroxisome proliferator-activated receptor-gamma (PPARγ) drives adipogenesis. Changes in morphology include the acquisition of a more spherical shape and the accumulation of lipid droplets.

Among the repressors of adipogenesis is preadipocyte factor-1 (Pref-1). Downregulation of Pref-1 occurs during adipocyte differentiation (Wang et al. 2010a; Wang et al. 2010b). Other negative regulators include KLF2, KLF7, GATA binding protein 2 and 3 (GATA2 and GATA3), Forkhead Box O1 (FOXO1), Forkhead Box A2 (FOXA2), transcriptional-coactivator with PDZ-binding motif (TAZ), and histone deacetylase Sirtuin 1 (SIRT1). The hedgehog, bone morphogenic protein (BMP) and the β-catenin dependent Wnt signaling pathways have also been implicated as negative regulators of adipogenesis (Stephens 2012; Rosen and MacDougald 2006; Moseti et al. 2016).

4.3 The role of ROS in adipocyte differentiation

Several studies have demonstrated the important role of exogenous and intracellular generated ROS during adipogenesis using different experimental models including preadipocyte cell lines, primary bone-marrow derived MSCs (BM-MSCs) and ASCs isolated from mouse, rat and humans. The consensus from the majority of these studies is that ROS promote adipogenesis (Atashi et al. 2015b; Wang et al. 2015; Lee et al. 2009a). However, it is still not fully understood how molecules such as ROS affect this process. A better understanding of ROS during adipogenic differentiation could potentially lead to the development of new therapies for obesity and related comorbidities.

The *in vitro* adipogenic differentiation of 3T3-L1 cells has been shown to increase in the presence of exogenous H_2O_2 (Lee et al. 2009b). The H_2O_2 associated increase in adipogenic differentiation was accompanied by increases in C/EBPβ and PPARγ expression. CCAAT enhancer-binding protein beta is thought to initiate mitotic clonal expansion of preadipocytes as well as cell cycle progression from the S to G2/M phase. Addition of antioxidants causes S phase arrest, thus preventing mitotic clonal expansion during differentiation. It was therefore suggested that ROS facilitate adipogenic differentiation by accelerating mitotic clonal expansion in preadipocytes (Lee et al. 2009b). *In vitro*, a standard adipogenic induction cocktail, consisting of insulin, dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine (cocktail abbreviated as IDII), induced adipocyte differentiation in human ASCs and this was accompanied by an increase in ROS production. IDII-induced adipogenic differentiation was inhibited by ROS scavengers such as N-acetyl-L-cysteine (NAC), SOD and CAT (Higuchi et al. 2013). Interestingly, several antioxidant enzymes, including superoxide dismutase 2 (SOD2), CAT and GPx were also found to be upregulated during induction of adipocyte differentiation using IDII. This IDII-induced upregulation of antioxidant expression was inhibited in FOXO1 transcription factor knock-out experiments. FOXO transcription factors play an important role in maintaining cellular redox homeostasis and have been found to be significantly downregulated during IDII-induced adipogenesis (Higuchi et al. 2013). These findings suggest that a delicate balance

between ROS production and endogenous antioxidant generation is maintained during adipogenesis. Several other studies, using mouse 3T3 preadipocyte cultures, also suggest that FOXO transcriptional factors are involved in the regulation of adipogenesis, but the findings are inconsistent and need further investigation. Nakae and colleagues (2003) showed that the overexpression of an activated form of FOXO1 inhibits adipogenesis by preventing clonal expansion of progenitor cells (Nakae et al. 2003). Contrary to these findings, Munekata and Sakamoto (2009) reported that FOXO1 silencing results in a decrease in adipogenic differentiation (Munekata and Sakamoto 2009).

Obesity is associated with increased infiltration of classical pro-inflammatory (M1) macrophages in the adipose tissue (Weisberg et al. 2003; Thomas and Apovian 2017). One of the key functions of M1 macrophages is the production of ROS and nitric oxide (NO) by NOX and inducible nitric oxide synthase (iNOS), respectively. The overproduction of ROS and NO in turn mediates the release of pro-inflammatory cytokines. This highights another potential link between ROS and adipocyte differentiation and suggests that several active cellular mediators such as chemokines, cytokines and adipokines contribute to the chronic inflammatory state of adipose tissue. This in turn contributes to the overproduction of ROS, causing systemic oxidative stress (Alfadda and Sallam 2012b). Figure 6 summarises how ROS production is increased in adipose tissue.

Various studies suggest that NOX is one of the main sources of ROS production in adipose tissue. Two independent studies using high-fat diet fed murine experimental models have shown that obesity is associated with the activation of NOX (Chen and Stinnett 2008; Thomas and Apovian 2017). Treating obese mice with the NOX inhibitor Apocynin resulted in a significant decrease in ROS production in adipose tissue as well as a decrease in the expression of the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) (Furukawa et al. 2004). Several *in vitro* studies also suggest that preadipocytes and adipocytes produce ROS through insulin-induced NOX-associated pathways (Liu et al. 2012a; Wang et al. 2015; Krieger-Brauer et al. 1997). ROS generated during the adipogenic differentiation of 3T3-L1 preadipocytes and ASCs were suppressed by NOX inhibitors (Liu et al. 2012a; Kanda et al. 2011). Other studies have shown that gene silencing of NOX4 inhibited insulin-induced terminal differentiation of 3T3-L1 cells, suggesting a positive role for NOX4 in promoting adipogenesis through insulin signaling (Schroder et al. 2009; Mahadev et al. 2004; Kanda et al. 2011; Mitchell et al. 2003; Mouche et al. 2007). NADPH oxidase 4 impairs insulin-induced proliferation but facilitates insulin-induced differentiation of preadipocytes by controlling the expression of MAP kinase phosphatase-1 (MKP-1), which limits ERK1/2 signaling, thereby acting as a switch from proliferation to differentiation in response to insulin (Schroder et al. 2009). Furthermore,

overexpression of NOX4 in ASCs and exogenous application of H_2O_2, enhanced adipogenic differentiation (Higuchi et al. 2013), thereby supporting the idea of a positive influence of ROS in adipogenesis. Mouche and colleagues (2007) showed that NOX4 is highly expressed in primary preadipocytes isolated from murine brown and white adipose tissue (BAT and WAT). As confirmation, these investigators also showed that NOX4 is located *in vivo* in the stromal vascular fraction of adipose tissue rather than in the mature adipocyte fraction. In addition, systemic administration of a low molecular weight O_2^- scavenging agent, Tempol, in C3H mice through either drinking H_2O or food, prevented mice from becoming obese and also reduced their leptin levels (Mitchell et al. 2003). This complements *in vitro* findings on a positive role for ROS in adipogenesis. Contrary to the above-mentioned findings, Li and colleagues (2005) reported that mice deficient in NOX4 showed accelerated development of obesity and insulin resistance (Li et al. 2012), which brings into question the exact role of NOX4 in adipogenesis *in vivo*. Kanda and colleagues (2011) suggested in their study that ROS production by NOX4 promotes adipocyte differentiation through CREB (Kanda et al. 2011). These investigators showed that H_2O_2 induced the activation of CREB and that treatment of the 10T1/2 multipotent mouse embryonic cell line with the pharmacological antioxidant NAC blocked the expression of CREB and consequently inhibited adipogenic differentiation [6]. Cyclic AMP response element-binding protein is an early regulator of adipogenesis and is reported to regulate C/EBPβ expression, which when upregulated leads to the upregulation of C/EBPα and PPARγ, two key regulatory transcriptional factors in the adipogenic process. Recently, both isoforms of NOS, endothelial nitric oxide (eNOS) and iNOS, were detected in differentiating preadipocytes derived from rat WAT, with an approximately 50% increase in NOS over basal level observed in preadipocytes on the first two days of adipogenic differentiation (Yan et al. 2002). In this study, eNOS was shown to be the major isoform promoting adipogenesis as iNOS was found to have little effect on total NO production and preadipocyte differentiation. Currently, the role of other NOX isoforms (NOX1, NOX2, NOX3 and NOX5) in adipogenesis is unknown and needs to be investigated.

Although most studies suggest that NOX is mainly responsible for ROS overproduction during adipogenesis, a few studies have also investigated the role of mitochondrial ROS in this context. In one of the first studies that investigated the effect of mitochondrial ROS on adipogenesis, Carriere and colleagues showed that the inhibition of mitochondrial ETC Complex I and V leads to an increase in mitochondrial ROS production which prevents 3T3-L1 proliferation (Carriere et al. 2003). A few years later, Tormos and colleagues showed, using primary BM-MSCs, that an increase in mitochondrial metabolism is essential for and promotes adipocyte differentiation through the overproduction of ROS via mitochondrial Complex III (Tormos et al. 2011). Mitochondrial Complex III-derived ROS

promotes adipogenic differentiation via the upregulation of C/EBPα and PPARγ (Tormos et al. 2011). This finding was confirmed using ASCs in which partial suppression of adipogenic differentiation was observed after treatment with Rotenone (Liu et al. 2012a). Rotenone prevents mitochondrial ROS production by inhibiting the electron transport function of Complex I (Lindahl and Oberg 1960; Li et al. 2003). These findings suggest a complex role of mitochondria-derived ROS in adipogenesis.

It is suggested that ROS promote adipogenesis via two closely related mechanisms: (a) the promotion of adipocyte differentiation; and (b) intracellular lipid accumulation. The enhancement of adipogenesis with increased NOS production was evidenced by increased lipoprotein lipase (LPL) expression and accelerated triacylglycerol (TG) accumulation in differentiating adipocytes (Yan et al. 2002). The accumulation of intracellular ROS (O_2^-) and intracellular lipid droplets in OP9 mouse stromal preadipocytes was shown to increase with adipogenic differentiation when compared to undifferentiated cells. The presence of an antioxidant led to a decrease in differentiation-dependent lipid droplets and intracellular ROS accumulation in OP9 cells during adipocyte differentiation (Saitoh et al. 2010). Not surprisingly, these studies reveal a positive correlation between increased endogenous ROS production and lipid droplet accumulation during adipocyte differentiation.

Irrespective of the intracellular source of ROS, studies using different experimentally designed approaches including manipulation of gene expression, treatment with antioxidant agents as well as the direct measurement of intracellular ROS levels have all confirmed a close link between adipocyte differentiation and ROS (Younce and Kolattukudy 2012; Liu et al. 2012b; Nam et al. 2012; Hou et al. 2012; Imhoff and Hansen 2011; Saitoh et al. 2010; Samuni et al. 2010; Vigilanza et al. 2011; Atashi et al. 2015a).

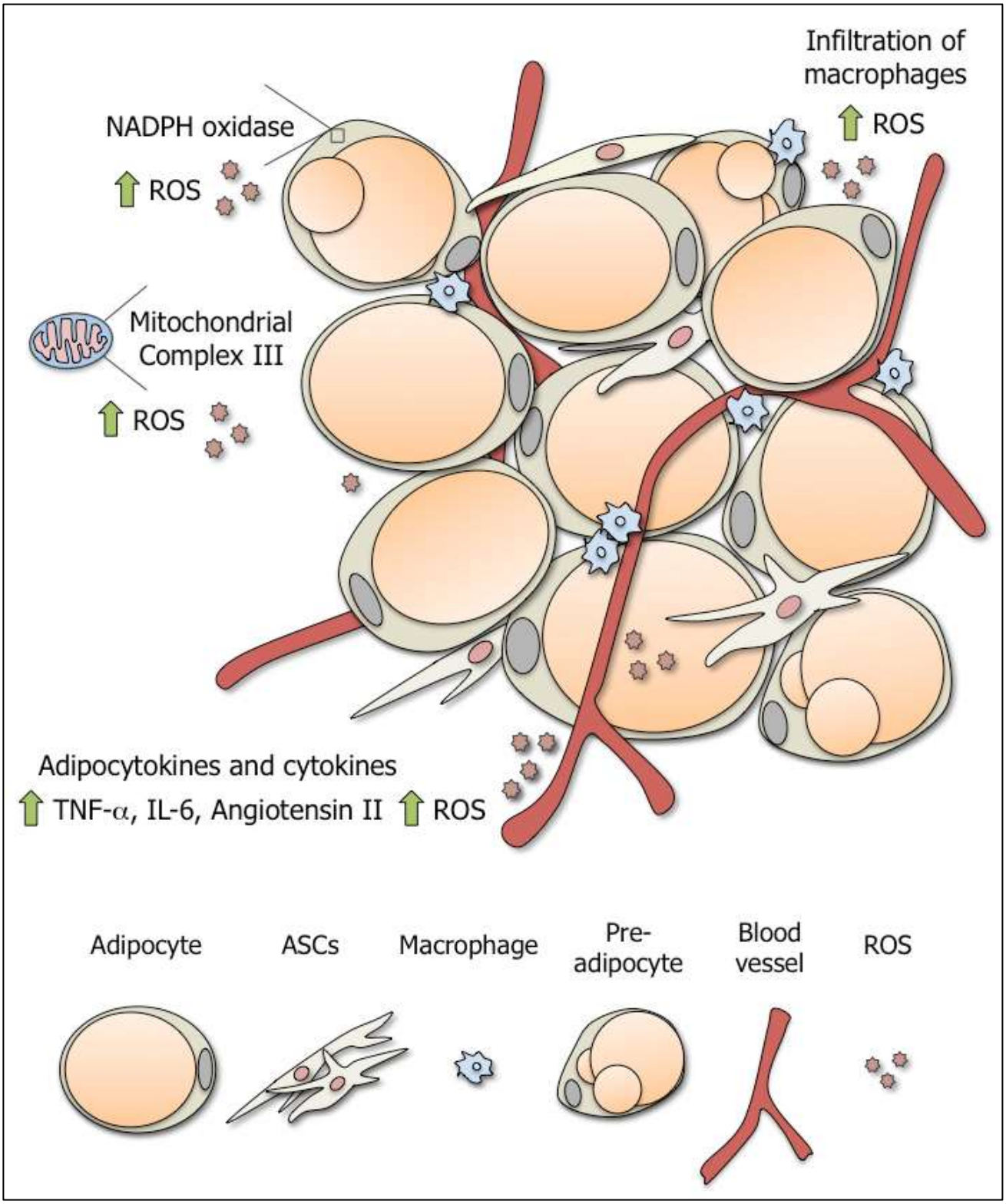

Fig. 6: Schematic illustrating how ROS production is increased in accumulating adipose tissue and how this ROS may contribute to adipogenesis. Reactive oxygen species are derived from multiple sources such as NADPH oxidase (NOX) and mitochondrial Complex III.

4.4 ROS scavengers, adipocytes and adipogenesis

Under normal physiological conditions, ROS production in cells is countered by antioxidant defense mechanisms (ROS scavengers), which can either be enzymatic or non-enzymatic. Reactive oxygen species production during the process of adipogenesis is also accompanied by induction of endogenous antioxidant defense mechanisms. These antioxidant mechanisms scavenge excess ROS to prevent oxidative stress and to maintain ROS levels necessary for cellular homeostasis.

An increase in ROS production during the first seven days of human ASC adipogenic differentiation was accompanied by an increase in the activities of SOD and CAT antioxidant enzymatic systems and a reduction in non-protein thiols such as glutathione, thereby indicating the presence of a ROS scavenging effect during adipogenesis (Drehmer et al. 2016). Adipose tissue samples from obese rats and mice showed activity of SOD, CAT and GPx antioxidant enzymatic systems. The decrease in the redox state of glutathione by antioxidant treatment was shown to promote TG accumulation in preadipocytes *in vitro* (Galinier et al. 2006). The markedly increased ROS production during 3T3-L1 cell differentiation into adipocytes was suppressed by diphenyleneiodonium (DPI) and apocynin (a NOX specific inhibitor), which are two structurally unrelated NOX inhibitors, and by the general antioxidant NAC (Furukawa et al. 2004). Six weeks of apocynin treatment significantly reduced lipid peroxidation and H_2O_2 production in WAT in a KKAy mouse model, thereby counteracting the positive effect of NOX-specific ROS production on adipogenesis (Furukawa et al. 2004). In addition, the downregulation of glutathione S-transferase A4 (GSTA4) (enzyme responsible for lipid aldehyde detoxification) in cultured 3T3-L1 adipocytes resulted in increased mitochondrial ROS production, lipolysis and protein carbonylation (Curtis et al. 2010) indicating the significance of GSTA4 as a ROS scavenger in adipocytes.

Treatment of 3T3-L1 cells with the flavonoid, quercetin, with reported antioxidant properties, using a serum concentration range of 0.1 to 10 µM, inhibited TG accumulation in developing adipocytes and reduced the TG content in mature adipocytes. Furthermore, this treatment downregulated the expression of C/EBPβ, PPARγ, SREBP1c and LPL genes, thus inhibiting adipogenesis (Eseberri et al. 2015). This is in accordance with another study that reported that quercetin as well as resveratrol at 25 µM individually inhibited lipid accumulation in 3T3-L1 preadipocytes (Yang et al. 2008). Interestingly, when quercetin and resveratrol were combined at 25 µM, the expression of key adipogenic transcription factors such as PPARγ1, PPARγ2 and C/EBPα was significantly suppressed (Yang et al. 2008). This suggests that ROS scavengers can work synergistically to inhibit adipogenesis. However, individually, quercetin and resveratrol had no effect on PPARγ2 and C/EBPα but reduced

the expression of PPARγ1 (Yang et al. 2008). Genistein is an isoflavone that has been shown to inhibit the accumulation of lipid as well as to decrease the nonesterified fatty acid (NEFA) content of 3T3-L1 preadipocytes on day 6 of adipogenic differentiation (Zhang et al. 2009). The suppression of 3T3-L1 differentiation by genistein is suggested to be through multiple signaling pathways, which includes the janus-activated kinase and p38 pathways (Zhang et al. 2009). Catechins, which are antioxidant flavonoids from tea, have been shown to inhibit adipogenesis in 3T3-L1 adipocytes by inhibiting intracellular lipid accumulation and glycerol-3-phosphate dehydrogenase activity in the differentiating cells. This is accompanied by the downregulation of PPARγ2, C/EBPα, and GLUT4 (Furuyashiki et al. 2004). The antioxidant glutathione precursor, NAC, at 5 mM significantly decreased the accumulation of triglyceride in differentiating mouse embryonic fibroblasts. N-acetyl-L-cysteine was also reported to significantly inhibit the mitotic clonal expansion of these cells (Pieralisi et al. 2016), thereby negatively affecting adipogenesis. Bixin, β-carotene and lycopene have been shown to inhibit intracellular lipid accumulation in 3T3-L1 cells during adipogenic differentiation, which is accompanied by the downregulation of PPARγ, fatty acid binding protein 4 (FABP4) and leptin protein expression. β-carotene had the strongest inhibitory effect (Zhao et al. 2017).

5. Clinical implications

The incidence of obesity and its related metabolic disorders is on a steady increase worldwide. The development of any preventive or therapeutic strategy requires a greater understanding of its etiology. The existence of a state of oxidative stress in obesity has been linked to some of its related pathophysiological conditions. This has contributed to the understanding of some associated metabolic disorders and their attenuation by pharmaceuticals in rodent studies (Le Lay et al. 2014). Oxidative stress in adipose tissue results from an imbalance between ROS production and neutralization by antioxidants, and has been linked to adipocyte dysfunction. Reactive oxygen species such as NOX4-generated H_2O_2 are important modulators of adipogenesis and adipose tissue function, which is key in health and disease (Tormos et al. 2011; Kanda et al. 2011; Higuchi et al. 2013) since impairment of adipocyte differentiation and metabolism is said to precede WAT dysfunction. Studies on C57BL/6 mice in which NOX4 had been pecifically deleted sin adipocytes reveal a role for NOX4-derived ROS in the onset of insulin resistance and adipose tissue inflammation (Den Hartigh et al. 2016). Between 10-20% of type 2 diabetics are considered to have a familial component (Drong et al. 2012), while obesity remains the common and main risk factor for the development type 2 diabetes (Ambele et al. 2016). The growth of adipocytes beyond a critical volume (the tolerated adipocyte volume which does not compromise its functions, depends on individual parameters that are not yet fully understood) is

linked to functional impairment and an increased risk of developing type 2 diabetes (Guilherme et al. 2008; Cotillard et al. 2014). The link between adipocyte dysfunction and type 2 diabetes has been called adipose tissue expandability, which states that it is the failure of adipose tissue expansion capacity, rather than obesity *per se*, which is the key factor linking positive energy balance and type 2 diabetes (Virtue and Vidal-Puig 2010). This suggests an important role for adipocyte formation/adipogenesis, which is under the influence of ROS, in the development of type 2 diabetes. In addition, increased lipolysis, impairment of insulin-stimulated glucose uptake, leptin secretion and increased secretion of pro-inflammatory cytokines such as interleukin 6 (IL-6), IL-8, TNFα, and reduced secretion of adiponectin and IL-10, have been associated with adipocyte hypertrophy (Castro et al. 2016). The secretome of dysfunctional WAT has been shown to contribute locally and systemically to a chronic pro-inflammatory state, which increases insulin resistance through an imbalanced secretion of insulin sensitizing (adiponectin) and inhibiting (TNFα, C-C motif chemokine 2 precursor; CCL2) adipokines (Castro et al. 2016). Macrophage infiltration has been observed in low-grade inflammation of WAT in obese subjects, which correlates with adipocyte mean size and body mass (Bremer and Jialal 2013; Weisberg et al. 2003). Han *et al.* (2016) have suggested that NOX4-derived ROS from adipocytes in the early stages of obesity provoke the onset of insulin resistance and initiate the recruitment of immune cells in adipose tissue (Han 2016). NOX4-derived ROS from infiltrating immune cells such as macrophages worsen insulin resistance and adipose tissue inflammation at the intermediate stages of obesity, while mitochondria-derived ROS maintain insulin resistance and adipose tissue inflammation at the late stages of obesity (Han 2016).

Overall, ROS is important for the differentiation of preadipocytes into adipocytes; however, an increase in ROS in mature adipocytes leads to adipocyte dysfunction, which causes oxidative stress and inflammation within and around WAT, thereby contributing to the development of type 2 diabetes and other metabolic disorders. Therefore, the pathophysiological role of ROS in biological processes, including adipogenesis, adipose tissue function, obesity, obesity-related inflammation and obesity-related comorbidities such as type 2 diabetes, highlights the importance of ROS as clinically relevant molecules. Reactive oxygen species could either serve as biomarkers or targets in combating these related diseases.

6. Conclusion

Recent advances in research on ROS and adipogenesis has elicited much interest in the fields of obesity and other metabolic diseases. Understanding how ROS influence adipogenic differentiation and

adipose tissue function *in vivo* nonetheless remains a challenge. Both extra- and intracellular ROS promote adipocyte differentiation at multiple stages. However, to date, research findings are unable to fully explain the exact source(s) of ROS that initiate and/or drive adipogenic differentiation *in vivo*. Clarity on the mechanistic interplay between the source/type of ROS and ROS inhibitors regulating adipocyte differentiation *in vivo* is likely to open new avenues for combating obesity and other related metabolic disease processes.

Conflicts of interest statement

The authors have no conflicts of interest to declare.

Author contributions

Danielle de Villiers drafted the first version of the manuscript with input from Marnie Potgieter and Michael Pepper. Melvin Ambele, Chrisna Durandt and Ladislaus Adam provided intellectual input and contributed to the writing of the manuscript. All authors vetted and approved the final version of the manuscript. Michael Pepper conceived the project.

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