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Review

Reactive oxygen species (ROS) in macrophage activation and function in diabetes

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ABSTRACT

In a diabetic milieu high levels of reactive oxygen species (ROS) are induced. This contributes to the vascular complications of diabetes. Recent studies have shown that ROS formation is exacerbated in diabetic monocytes and macrophages due to a glycolytic metabolic shift. Macrophages are important players in the progression of diabetes and promote inflammation through the release of pro-inflammatory cytokines and proteases. Because ROS is an important mediator for the activation of pro-inflammatory signaling pathways, obesity and hyperglycemia-induced ROS production may favor induction of M1-like pro-inflammatory macrophages during diabetes onset and progression. ROS induces MAPK, STAT1, STAT6 and NFκB signaling, and interferes with macrophage differentiation via epigenetic (re)programming. Therefore, a comprehensive understanding of the impact of ROS on macrophage phenotype and function is needed in order to improve treatment of diabetes and its vascular complications. In the current comprehensive review, we dissect the role of ROS in macrophage polarization, and analyze how ROS production links metabolism and inflammation in diabetes and its complications. Finally, we discuss the contribution of ROS to the crosstalk between macrophages and endothelial cells in diabetic complications.

1. Introduction

Diabetes mellitus is a major public health problem worldwide which seriously impairs the quality of life of the patients. The Global Diabetes Report released by World Health Organization states that the number of diabetics has increased from 108 million in 1980 to 422 million in 2014. The global disease prevalence has grown from 4.7% to 8.5% in adult population, with higher prevalence in low to middle-income countries (Anon, 2018). Chronic untreated diabetes causes various macro- and micro-vascular complications, such as atherosclerosis, diabetic nephropathy, diabetic retinopathy, neural damage while poor perfusion of extremities often requires their amputation (Brownlee, 2001). Diabetic retinopathy is an important cause of blindness (Kowluru and Chan, 2007), while diabetic nephropathy is one of the most serious complications of diabetes as well as leading causes of end-stage renal disease (Kowluru and Chan, 2007; Saran et al., 2015). Diabetes and its associated complications are a serious burden for the

patients and their families both morally and economically, and weighs down the national economics (Anon, 2018). Therefore, effective medical intervention to treat this disease is of critical importance.

Recent studies show that oxidative stress is a major mediator that underlies diabetic complications (Pitocco et al., 2013; Giacco, 2011). In hyperglycemia-sensitive cells, such as endothelial cells, the excessive load of glucose triggers the formation of ROS in mitochondria which in turn impairs mitochondrial function (Brownlee, 2001; Pitocco et al., 2013; Giacco, 2011). Due to its high reactivity, the ROS reacts with various cellular constituents, including DNA, lipids and proteins causing cellular damage. Furthermore, excessive ROS activates pro-inflammatory transcription factors such as NFκB and AP-1 that upregulate expression of pro-inflammatory chemokines/cytokines and adhesion molecules. Activated endothelial cells attract monocytes that further augment inflammation which promotes macrovascular and microvascular injury (Pitocco et al., 2013). ROS has also been implicated in epigenetic modifications involved in the maintenance of pro-

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inflammatory response (El-Osta, 2012a).

In addition to endothelial cells, diabetic conditions also trigger ROS formation in macrophages (Tesch, 2007). The diabetes-induced secretion of monocyte chemoattractant protein CCL2 (MCP1) by endothelium attracts monocytes whereas upregulated endothelial surface expression of adhesion molecule VCAM promotes their adhesion and diapedesis (Tesch, 2007). Transmigrated monocytes differentiate into macrophages and further exacerbate inflammation by mediating tissue injury and secreting pro-inflammatory cytokines and proteases as well as producing increased ROS levels in the tissues (Tesch, 2007). Hyperglycemia causes mitochondrial dysfunction in endothelial cells and macrophages and aberrant activation of cytoplasmic NADPH oxidases (NOX) that together exacerbate ROS production (Widlansky et al., 2011; Huang et al., 2011). In contrast to well-regulated ROS production in anti-microbial response, metabolically-generated ROS in diabetic macrophages is more erratic and dysregulated. Its production is associated with promotion of M1-like macrophage phenotype that favors the progression of diabetic complications (Kumar et al., 2016). Here, we review ROS biology in diabetes and the influence of ROS on macrophage polarization. The second part discusses the function of macrophages in diabetic disease progression and the role of macrophage-produced ROS as a detrimental factor in diabetic complications.

2. Biology of ROS

Oxidative stress is implicated in the pathogenesis of severe diseases such as cancer, diabetes, auto-immune and neuro-degenerative diseases. Oxidative stress is caused by intracellular presence of reactive oxygen species (ROS) which overcomes the natural anti-oxidant defense of the cell (Schieber and Chandel, 2014). The strong oxidative properties of ROS result in damage of intracellular constituents such as proteins, lipids and nucleic acids. The resulting modifications alter the structure and therefore the function of these molecules. ROS is mostly produced as a by-product of various cellular processes in multiple forms including superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^{\cdot}). In general the balance between ROS formation and its elimination is tilted under stressed conditions such as diabetes which cause upregulated ROS formation (Schieber and Chandel, 2014; Holmström and Finkel, 2014; Birben et al., 2012).

2.1. Sources of ROS

There are at least four major sources of ROS in cells: mitochondria, ROS-generating NADPH oxidases (NOXs), endoplasmic reticulum (ER) and peroxisomes (Holmström and Finkel, 2014; Birben et al., 2012). Mitochondria are major contributors of ROS in the form of superoxide. Superoxide is produced as a by-product of the mitochondrial electron transport chain (ETC) during the generation of ATP (Reczek and Chandel, 2015; Li et al., 2013). In order to generate ATP, an electron is transported along the protein complexes of respiratory chain to generate an H^+ gradient in the inner membrane of mitochondria. The gradient of H^+ then fuels ATP synthase to produce ATP. This process requires an oxygen molecule to be reduced into water. However, in complex IV, the oxygen molecule can also interact with the electron forming superoxide anion (Holmström and Finkel, 2014; Reczek and Chandel, 2015; Li et al., 2013). In addition, ROS can be produced in mitochondria during electron leakage that occurs while the electron flows down the ETC. This leaking electron reduces molecular oxygen leading to superoxide anion causing an accumulation of mitochondrial superoxide (Reczek and Chandel, 2015; Li et al., 2013; Brownlee, 2005a). Produced superoxide is rapidly converted into H_2O_2 by superoxide dismutase (SOD) 1 and 2, making it more membrane permeable to diffuse into cytosol (Schieber and Chandel, 2014).

Another major source of intracellular ROS is NADPH oxidase (NOX). NOX is a family of cytoplasmic enzymes, consisting of seven different isoforms which comprise cytosolic and membrane subunits. Upon

stimulation, such as viral and bacterial infection, both cytosolic and membrane subunits cooperate to produce ROS. In phagocytes such as macrophages and neutrophils, NOX is important for oxidative burst which allows phagocytes to generate high levels of ROS to kill microorganisms (Panday et al., 2015; Lambeth, 2004). Similar to mitochondria, NOX also produces ROS in the form of superoxide which is further converted by SOD1 into H_2O_2 (Reczek and Chandel, 2015). In addition to NOX, during respiratory burst in phagocytes, ROS is also produced through inducible nitric oxide synthase (iNOS) by catalyzing the formation of robust nitric oxide which reacts with superoxide anion resulting in a very reactive oxidant peroxynitrite (Lo Faro et al., 2014; Fang, 2004). In contrast, in vascular endothelial cells, endothelial nitric oxide synthase (eNOS) is a major source of nitric oxide (Fish and Marsden, 2006). In the absence of its cofactor tetrahydrobiopterin (BH4), uncoupled eNOS switches to NADPH-dependent superoxide anion production, which contributes to endothelial dysfunction in diabetes and cardiovascular diseases (Karbach et al., 2014; Luo et al., 2014).

Peroxisomes generate ROS in the form of H_2O_2 as a by-product of long chain fatty acid oxidation while the endoplasmic reticulum generates ROS as by-product of protein oxidation. Besides these four mechanisms, a broad range of enzymes is also able to generate ROS such as cytochrome P450 in mitochondria and ER, and lipoxygenases in cell membrane and cytosol (Holmström and Finkel, 2014). The contribution of these sources to oxidative stress varies depending on the stimuli by which ROS production is triggered, such as hyperglycemia, free fatty acids, LPS induction as well as a broad range of cytokines (Reczek and Chandel, 2015). A common denominator of these additional ROS generating mechanisms is that all of them are associated with metabolic pathways.

2.2. ROS in cell signaling

Although high levels of ROS are deleterious, intermediate levels of ROS serve as an important short-lived second messenger in cell signaling by oxidizing the thiol group in cysteine residues of a protein. In general, oxidized cysteine residues alter the structure and consequently the function of proteins. The role of ROS in protein oxidation is tightly regulated by reducing systems such as thioredoxin (TRX) and peroxiredoxin (PRX) (Reczek and Chandel, 2015; Ray et al., 2012).

Mitogen-activated protein kinase (MAPK) signaling is redox sensitive and regulates a variety of cellular processes such as cell differentiation, survival and proliferation (Schieber and Chandel, 2014). Endogenous ROS activates apoptosis signal-regulated kinase 1 (ASK1) and promotes p38 MAPK signaling cascades in various cell types such as cardiac myoblasts and fibroblasts (Ray et al., 2012). ROS also indirectly maintains the activity of this cascade by deactivation of JNK-inhibiting phosphatase (Ray et al., 2012). Besides MAPK, phosphoinositide-3 kinase (PI3K) is also regulated by ROS which serves as a second messenger upon stimulation by growth factors, hormones and cytokines (Schieber and Chandel, 2014; Holmström and Finkel, 2014; Ray et al., 2012). ROS indirectly maintains the activation of PI3K through inhibition of its negative regulator, PTEN (Ray et al., 2012; Lee et al., 2002). MAPK and PI3K pathways are only two examples from the list of cellular pathways affected by ROS. Detailed overview about redox-sensitive pathways is provided by Ray et al (Ray et al., 2012). Interestingly, during pro-inflammatory stimulation of murine macrophages ROS generation is maintained via mutual cross-talk between TGF- β -associated kinase (TAK1 or MAP3K7), which is activated by ROS (Wang et al., 2015) and which activates NF κ B. By itself, NF κ B promotes ROS generation via activation of ROS producing proteins such as NOXes (Morgan and Liu, 2011; Sakurai, 2012).

2.3. Regulation of ROS production in the cells

The balance between damage and physiological signaling by ROS is

maintained by cells with an array of anti-oxidant enzymes i.e. ROS scavengers. These include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSX). Each anti-oxidant enzyme targets a specific oxidant, for instance, SOD scavenges superoxide, while CAT disintegrates H_2O_2 (Holmström and Finkel, 2014; Birben et al., 2012). Due to their specific targets, these enzymes are not only anti-oxidants, but also transducers of redox-sensitive cell signaling. Functionally, thioredoxin (TRX) and peroxiredoxin (PRX) reduce oxidized proteins to their native form. In addition, GSX catalyzes H_2O_2 and hydroperoxide to form water by oxidizing reduced glutathione (GSH) into glutathione disulfide (GSSH). To maintain the level of reduced GSH, GSSH is then reduced by NADPH which forms during glucose metabolism and plays an important role in the ROS production through the NOX system. Given the growing evidence of interplay between metabolism and oxidative stress, GSH might be the molecular linker between these two processes (Holmström and Finkel, 2014; Birben et al., 2012; Reczek and Chandel, 2015).

2.4. Oxidative stress

In general, oxidative stress occurs when the ROS level overwhelms the anti-oxidant defense in cells. In high concentrations, H_2O_2 , which is normally neutralized by anti-oxidant enzymes, reacts with metal cations such as Fe^{2+} and Cu^{2+} to form hydroxyl radicals that are highly reactive towards cellular macromolecules like proteins, lipids and DNA which causes irreversible intracellular damage (Schieber and Chandel, 2014). In addition, oxidative stress is also caused indirectly by pro-inflammatory signaling in endothelial cells in response to stress stimuli (Sakurai, 2012). These include danger signals such as pathogen (PAMP) and damage associated molecular patterns (DAMP) that are bound by Toll-like receptors (TLRs). These pattern recognition receptors signal through interaction of their cytoplasmic domain with MyD88 and Toll-interleukin receptor (TIR)-domain-containing adapter-inducing interferon- β (TRIF) (Sakurai, 2012). TLR interaction with MyD88 activates the IRAK-TRAF6-TAK-1 axis and further downstream activates NF κ B and activating protein-1 (AP-1) transcription factors (Sakurai, 2012); while interaction with TRIF activates interferon-regulatory factor-3 (IRF-3). Together, ligand activated TLR causes pro-inflammatory activation (Lugrin et al., 2014). Besides pro-inflammatory responses, this signaling cascade also results in the upregulation of ROS through activation of NOX, iNOS as well as mitochondria, which accumulates intracellular ROS (Lugrin et al., 2014). Vice versa, the high level of ROS stimulates dimerization and localization of TLR into lipid rafts which enhances the density of TLR in the cell membrane and augments pro-inflammatory signaling, further sustaining high ROS levels (Lugrin et al., 2014).

3. ROS in macrophage polarization

3.1. Macrophage polarization

Macrophages are innate immune cells with broad and dynamic phenotype plasticity (Stout, 2004; Gratchev et al., 2006; Gordon and Mantovani, 2011). Traditionally, macrophages with pro-inflammatory and bactericidal activity are classified as M1 macrophages whereas M2 macrophages are involved in the resolution of inflammation, wound healing, tumor growth and produce anti-inflammatory mediators and growth factors (Mantovani et al., 2013; Riabov et al., 2014). *In vitro*, prototypic human M1 and M2 macrophages are differentiated from peripheral blood monocytes with IFN- γ and IL-4 respectively (Murray et al., 2014). At the level of signal transduction and molecular phenotype, M1 macrophages are characterized by induction of STAT1 and NF κ B transcription factors as well as production of pro-inflammatory cytokines including TNF α , IL-1 β , IL-6, IL-12 among others (Van Ginderachter et al., 2006). M2 macrophages are characterized by activation of transcription factor STAT6, elevated expression of mannose

receptor (CD206) and production of cytokines such as TGF- β , CCL18 and IL-1Ra (Gordon and Martinez, 2010). Recently, the concept of macrophage polarization was revisited due to the observation that in fact a plethora of molecular stimuli induces distinct and only partially overlapping transcriptional programs in macrophages that substantially differ from prototypic M1 and M2 phenotypes (Murray et al., 2014; Xue et al., 2014). Macrophage phenotype is considered as a dynamic continuum that quickly adapts to changes in microenvironmental cues. However, the traditional M1/M2 nomenclature is still utilized to reflect macrophage ability to exist in more pro-inflammatory (M1-like) or anti-inflammatory/immunosuppressive (M2-like) functional states.

ROS have long been considered as important antimicrobial mediators produced by macrophages. Upon phagocytosis, membrane-bound NADPH oxidase complex NOX2 is assembled on the membrane of phagolysosome and pumps electrons into the lumen of phagolysosome generating superoxide anions ($O_2^{\cdot-}$) (Slauch, 2011). In addition to NOX2, mitochondrial ROS (mtROS) also participates in respiratory burst. Although the mechanism is still poorly understood, it is reported that mitochondria produce superoxide and deliver it to phagosomes by active translocation (Hall et al., 2014; West et al., 2011). Interestingly, another study showed that the mtROS are produced by utilization of fatty acid beta-oxidation by mitochondria which is regulated by a gene called immunoresponsive gene-1 (Hall et al., 2013). Besides the classical antimicrobial role of ROS, an increasing number of studies suggests the ability of ROS to function as secondary messengers implicated in cell signaling pathways and regulation of M1/M2 macrophage polarization (summarized in Table 1) (Kohchi et al., 2009; Formentini et al., 2017; Padgett et al., 2015a; Zhang et al., 2013).

3.2. ROS and M1 macrophage polarization

ROS are essential for the induction and maintenance of M1 type of macrophage polarization. Several studies reported the role of ROS in activation of NF κ B as well as p38 MAPK signaling pathways, which promote pro-inflammatory gene expression in macrophages (Table 1) (Kohchi et al., 2009; Padgett et al., 2015a; Zhang et al., 2013; Padgett et al., 2015b; Pawate et al., 2004). One of the proposed mechanisms involves activation of NOX and SOD, which results in H_2O_2 generation upon TLR4 stimulation (Kohchi et al., 2009). In HEK293 cells it was shown that produced H_2O_2 diffuses out of the phagosomes and causes dissociation of apoptosis signal-regulating kinase (ASK1) inhibitor thioredoxin from ASK1-trx complexes inducing ASK1 phosphorylation and activation of downstream p38 MAPK (Kohchi et al., 2009; Liu et al., 2000). In addition, ROS enhance phosphorylation of NF κ B inhibitor I κ B leading to NF κ B pathway activation in a human breast cancer cells overexpressing GSHPx (Kohchi et al., 2009). In rat microglia stimulated by IFN- γ and LPS, inhibition of peroxide accumulation by NADPH oxidase inhibitors and catalase diminished activation of p38 MAPK, STAT1 and NF κ B pathways followed by decrease in expression of M1-associated markers iNOS, TNF α and IL-6 (Pawate et al., 2004). In non-obese diabetic (NOD) mice deficient for NOX expression, macrophages infiltrating pancreatic islets as well as bone marrow-derived macrophages had a reduced expression of M1 markers with concomitant increase of M2 markers (Padgett et al., 2015b). These phenotypic changes prevented the progression of type 1 diabetes. In this study, NOX-derived superoxide was at least partially responsible for activation of M1-associated transcription factors STAT1 and IRF5 in macrophages (Padgett et al., 2015b). In high-fat diet-induced obese (DIO) mice, dipeptidyl peptidase-4 (DPP-4) expressed by macrophages was found to be essential for their recruitment into adipose tissue, ROS production in response to cytokine stimulation and concomitant M1-like polarization resulting in glucose intolerance and insulin resistance (Zhuge et al., 2016). An induction of arginase II (ArgII) in macrophage cell lines and mouse primary macrophages by LPS and high fat diet led to generation of mitochondrial $O_2^{\cdot-}$ and H_2O_2 resulting in ROS-dependent expression of pro-inflammatory factors TNF α , IL-6 and CCL2 (Ming et al., 2012).

Table 1
The involvement of ROS in macrophage polarization.

Model	Method of phenotype characterization	M1 phenotype markers	M2 phenotype markers	ROS source	Mechanism of ROS action	Functional consequence	Reference
Rat microglia stimulated by IFN- γ and LPS	Immunoblot for iNOS expression, RT-PCR for cytokines expression	iNOS, TNF α , IL-6	Not tested	NOX	Induction of pro-inflammatory cytokines; p38 MAPK, STAT1 and NF κ B pathways activation	Not tested	(Pawate et al., 2004)
Pancreas-infiltrating macrophages and BMDM from NOD.Nef1m1J mice	Flow cytometry, ELISA	TNF α , IL-1 β , IL12p70, CCL5	Arg-1, CCL17	NOX	Induction of pro-inflammatory cytokines; STAT1, IRF5 induction	Contribution to T1D development	(Padgett et al., 2015b)
BMDM from Arg-II - / - mice	qRT-PCR	TNF α , IL-6, CCL2	Not tested	Mt	Induction of pro-inflammatory cytokines	Not directly tested. May contribute to high fat induced insulin resistance and atherosclerosis development.	(Ming et al., 2012)
Human primary monocytes, mouse BMDM	Flow cytometry, qRT-PCR, Western blotting		CD163, IL-10, CCL17, CCL18, CCL24, Arg-1, Ym-1	NOX	Induction of anti-inflammatory factors; Late-phase activation of ERK signaling	Reduction of tumor-associated macrophage infiltration and tumor burden in lung and breast cancer mouse models	(Zhang et al., 2013)
Mouse RAW 264.7 cell line	RT-PCR, qRT-PCR	IL-12p40	Arg-1	Mt	Induction of M2-like phenotype. Presumable involvement of ROS/ERK signaling pathway	Not directly tested	(Shan et al., 2017)
Human and mouse alveolar macrophages	qRT-PCR, TNF- α reporter assay	TNF- α , iNOS	Ym-1, FIZZ1	Mt	Induction of M2-like phenotype through direct activation of STAT6	Not directly tested. Presumably favors the development of pulmonary fibrosis	(He et al., 2013)
Mouse colon macrophages	Immunofluorescence	iNOS	Not tested	Mt	Induction of anti-inflammatory phenotype through NF κ B activation	Amelioration of DSS-induced colitis	(Formentini et al., 2017)

However, the mechanism of ArgII-induced ROS induction and associated signaling cascades was not elucidated.

In addition, ROS is a prerequisite for the activation of NLRP3 inflammasome (Abais et al., 2015; He et al., 2016; Jo et al., 2016). NLRP3 inflammasome initiates caspase-1-dependent cleavage of pro-IL-1 β and pro-IL-18 upon stimulation with DAMPs, PAMPs as well as ATP, pore-forming toxins, and various particulates (e.g. asbestos, silica, uric acid crystals etc.) (Jo et al., 2016). Both intracellular (NOX-derived) and mitochondria-derived ROS trigger activation of NLRP3 in macrophages (Abais et al., 2015). However, mtROS likely has a dominant role in NLRP3 inflammasome activation (Bulua et al., 2011). For instance, genetic deletion of NOX subunits gp91phox and p22phox was dispensable for NLRP3 activation and IL-1 β and IL-18 secretion. In agreement with this study, the inhibition of mtROS in macrophages using antioxidant MitoQ, a coenzyme Q derivate, ameliorated activation of NLRP3 which in turn decreased IL-1 β and IL-18 secretion (Dashdorj et al., 2013; Kelly et al., 2015). The role of ROS in NLRP3 activation is acknowledged and well-described in recent reviews (Abais et al., 2015; He et al., 2016; Jo et al., 2016).

3.3. ROS and M2 macrophage polarization

Unexpectedly, ROS are involved in both pro- and anti-inflammatory control of macrophage phenotype in a context-dependent fashion (Table 1). Zhang et al showed that inhibition of NOX-derived O₂^{•-} production in human primary monocytes prevented their M2-like polarization in the presence of M-CSF and subsequent response to M2-polarizing cytokine IL-4 as assessed by reduction in expression of M2-associated genes CD163, IL-10, CCL17, CCL18 and CCL24 (Zhang et al., 2013). In these settings ROS was involved in late-phase activation of ERK signaling required for M2 differentiation (Zhang et al., 2013). In RAW 264.7 cells inhibition of mtROS decreased the expression of M2 marker Arg I and increased the expression of M1 marker IL12p40 (Shan et al., 2017). In human and mouse alveolar macrophages, mitochondria-localized Cu, Zn-superoxide dismutase caused the generation of H₂O₂. This ROS molecule directly oxidized specific cysteine residues in the STAT6 transcription factor which stimulated expression of the M2-associated genes Arg I and FIZZ1 (He et al., 2013). In a mouse DSS-induced colitis model, moderate increase in mtROS production induced anti-inflammatory M2-polarized macrophages that coincided with their reduced expression of M1 marker iNOS (Formentini et al., 2017). Paradoxically, in this study NF κ B activation by mtROS was linked to an anti-inflammatory response, while the administration of mitochondria-targeted antioxidant MitoQ exacerbated colon inflammation (Formentini et al., 2017). This shows the context-dependent function of ROS in macrophage activation.

Despite evident implication of ROS in the regulation of macrophage phenotype, the influence of NOX-derived ROS on macrophage activation and function is currently ambiguous. For instance, blocking NOX-derived O₂^{•-} reduces the expression of M2-associated markers whereas M2 markers were increased in NOX-deficient NOD mice (Table 1) (Zhang et al., 2013; Padgett et al., 2015b). These discrepancies could be observed due to the differences in experimental approaches used to inhibit ROS production. Overall, signaling pathways responsible for the establishment of both M1 and M2 macrophage phenotypes are affected by NOX-derived ROS and mtROS. However, it is still unclear in which situation ROS will preferentially activate M1- and M2-associated signaling pathways. The stage of cell differentiation and levels of intracellular/mitochondrial ROS likely influence the induction of M1 or M2 polarization. The extent of ROS impact on macrophage phenotype as well as conditions that precede preferential activation of M1- vs M2-signaling pathways upon ROS generation need to be further elucidated.

4. Immunometabolism and macrophage activation in diabetes: the role of ROS

Macrophages are professional producers of ROS that act both in pathogen destruction and as secondary messengers affecting cell activation. Given the importance of ROS in antimicrobial defense of pro-inflammatory macrophages, ROS is also a key player that bridges metabolic dysregulation and inflammation in diabetes pathophysiology.

4.1. Hyperglycemia-induced macrophage-mediated inflammation contributes to diabetes progression

Diabetic complications result from an abnormal metabolic environment lauded by chronic hyperglycemia (HG). HG typically comprises fasting plasma glucose levels ≥ 7.0 mmol/L and levels of glycated haemoglobin (HbA1c) $\geq 6.5\%$ (Ho-Pham et al., 2017). In Type 1 diabetes (T1D) a genetically determined susceptibility may explain why not all individuals with severe HG or vice versa some individuals with only modest HG develop complications (Reichard, 1995). In type 2 Diabetes (T2D) the patient is often diagnosed only at advanced disease stage and long-term complications may be already present at the time of diagnosis. The Diabetes Control and Complications Trial (DCCT) as well as the United Kingdom Prospective Diabetes Study (UKPDS) are landmark studies showing that intensive treatment to reduce (chronic) HG slows the progression of diabetic microvascular complications in T1D and T2D (Control and Group, 1993; Group UKPDS, 1998). However, fluctuations in glucose levels may counteract the therapeutic benefit of lowering fasting plasma glucose and HbA1c (Hanefeld and Temelkova-Kurktschiev, 2002). In addition, HG may compound over time suggesting the progression to be proportional to the number of complications already present at onset or other mechanisms involved (for the Diabetes TWT, 2002). We have recently demonstrated the effect of HG on the production of prototypic M1 and M2 cytokines in cultured human primary macrophages. HG was found to stimulate acute production of TNF- α and long-term production of IL-1 β during macrophage differentiation (Moganti et al., 2016). Similarly, mouse peritoneal macrophages treated with hyperglycemic medium showed an increase in expression of pro-inflammatory cytokines including IL-1 β , IL-6, IL-12, IL-18, and TNF- α in a time and dose-dependent manner (Wen et al., 2006). Induction of T1D in mice also increased IL-12 expression in peritoneal macrophages via activation of protein kinase C (PKC), p38 MAPK and JNK pathways (Wen et al., 2006). Furthermore, monocytes isolated from T1D patients secreted IL-6 and IL-1 β (Devaraj et al., 2006). Circulating monocytes of pre-diabetic patients (fasting blood glucose levels about 6 mM) expressed higher levels of activation marker CD11c that was associated with obesity and insulin resistance (Torres-Castro et al., 2016). The IL-10 concentration in serum samples of pre-diabetic patients as well as the IL-10 production by HG treated human monocyte-derived macrophages was decreased (Torres-Castro et al., 2016). In addition, RAW264.7 macrophages and mouse bone marrow-derived macrophages (BMDM) exposed to HG were resistant to anti-inflammatory action of IL-10 due to deficient STAT3 activation (Barry et al., 2016). Therefore, in a number of studies HG conditions caused pro-inflammatory M1-like programming of monocytes/macrophages that may lead to the development of diabetes-associated inflammation and consequent vascular complications.

4.2. Interplay between obesity and hyperglycemia promotes metabolic changes and ROS formation in macrophages

The tight link between T2D, obesity and metabolic syndrome renders it an immunometabolic disease, in other words a disease in which a dysfunctional metabolism is strongly linked to the immunological status of the patient (Donath and Shoelson, 2011). Inflammation has been postulated to propel metabolic syndrome into T2D (Donath and

Shoelson, 2011), while obese individuals have increased systemic inflammation, circulating pro-inflammatory cytokines and macrophage accumulation in the adipose tissue (AT) (Weisberg et al., 2003). Macrophages, especially adipose tissue macrophages (ATM) are involved in a large spectrum of mechanisms that drive diabetic pathology. AT-infiltrating subpopulations of ATM, such as CD9 + ATM, are held responsible for obesity-associated inflammation (Hill et al., 2018). The increased expression of TLRs (e.g. TLR7 and TLR10) by ATM in obese individuals correlates with increased expression of inflammatory markers in AT (Hill et al., 2018; Sindhu et al., 2015). In obese AT local pro-inflammatory microenvironment is characterized by the infiltration of Th1, Th17 cells and neutrophils, the presence of free fatty acids (FFAs), saturated fatty acids and cytokines that drive activation of adipocytes and favor differentiation of M1-like macrophages (Castoldi et al., 2016). Adipocytes are important for the control of macrophage phenotype in AT (Lihn et al., 2005; Ouchi et al., 2010). In healthy individuals, adiponectin secreted by adipocytes stimulates an anti-inflammatory phenotype of ATM by inducing M2-like polarization, suppression of ROS and other ROS-related genes (Ohashi et al., 2010). In obesity, decrease in adiponectin production may favor prevalence of M1-like polarized macrophages (Goh et al., 2016; Arita et al., 1999). Of note, M1 macrophages upregulate expression of glucose transporter GLUT1 that enhances glucose consumption (Nishizawa et al., 2014; Freerman et al., 2014). The expression of GLUT1 is one of hallmarks of metabolic disruption in ATM which correlates with macrophage infiltration in epididymal fat in obese mice fed with a high fat diet (HFD) (Freerman et al., 2014). Overexpression of GLUT1 in RAW 264.7 cells caused a metabolic shift marked by increase in glucose metabolism and decrease of mitochondrial respiration (Freerman et al., 2014). This coincided with increased production of plasminogen activator inhibitor-1 (PAI-1), a pro-inflammatory mediator and a target of NFkB. Importantly, GLUT1-overexpressing macrophages also exhibited increased ROS formation both in cytosol and mitochondria, and inhibition of ROS with NAC treatment attenuated PAI-1 expression, presumably through the suppression of redox-dependent NFkB activation (Freerman et al., 2014). The upregulation of GLUT1 in obesity indicates an increase in glucose consumption in macrophages which appears to enhance pro-inflammatory phenotype of these cells (Langston et al., 2017). Overall, obesity-associated factors alter activation of ATM and skew them towards glycolytic metabolism. At the same time, obesity-induced HG facilitates oxidative stress and mitochondrial dysfunction that further supports ROS production, glycolytic metabolism and release of pro-inflammatory mediators by macrophages (Fig. 1). These changes underlie macrophage function and highlight their role in the development of T2D. Therefore, more attention is paid to changes in metabolic reprogramming that occur with macrophage activation in order to find new targets for the treatment of metabolic syndrome as altering metabolic status can control inflammation (Appari et al., 2017a).

Another hallmark of increased glucose metabolism is an upregulation of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that participates in pentose phosphate pathway. Besides HG, a variety of obesity-associated factors such as FFAs, cholesterol and LPS increase G6PDH levels (Ham et al., 2013). Importantly, G6PDH plays roles in both glucose metabolism through pentose phosphate pathway and redox regulation (Appari et al., 2017b), therefore its upregulation in obesity might potentiate glucose metabolism, making obese people more sensitive towards HG. Furthermore, the interplay between diet and gut microbiota was suggested to play a role in the pathology of obesity. Disbalanced microbiota due to high fat diet increases gut permeability for LPS and its entrance into circulation (Hersoug et al., 2018). LPS serum levels are higher in obese individuals (Trøseid et al., 2013). It was hypothesized that upon LPS sequestration in lipoproteins such as HDL, it can be delivered into AT and contribute to M1 macrophage phenotype shift in the AT (Hersoug et al., 2018).

Metabolic changes induced by high glycolytic flux ultimately result

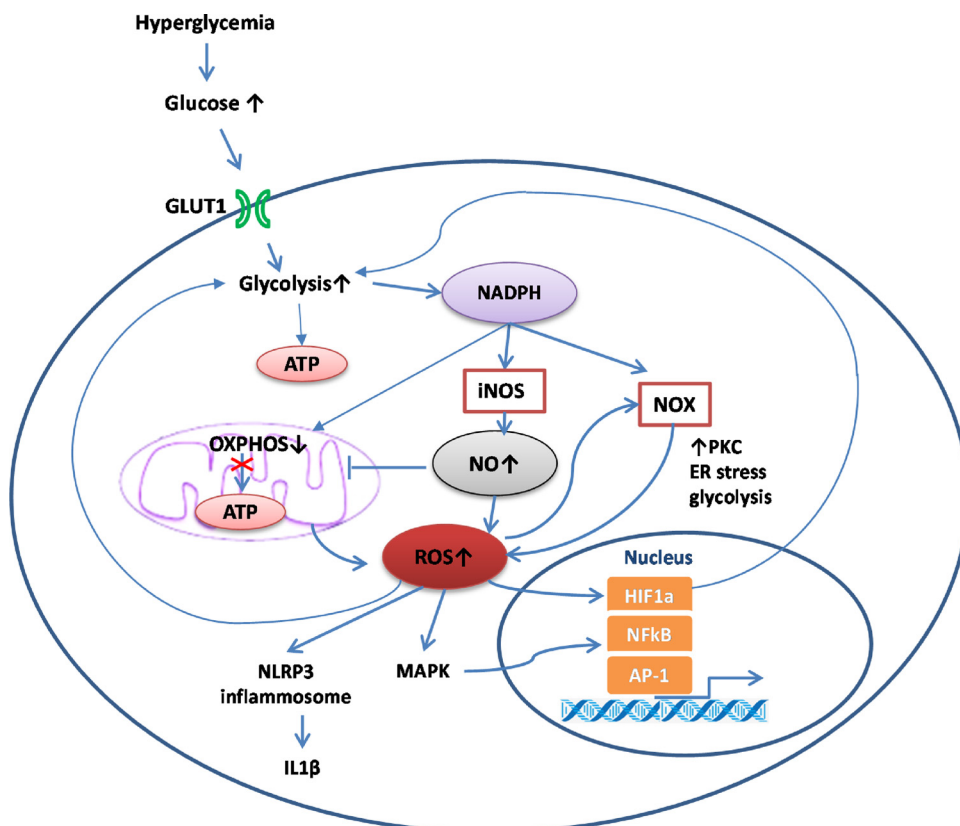


Fig. 1. Metabolic shift in macrophages under hyperglycemic conditions. Excess of glucose is metabolized through glycolysis and TCA cycle in order to yield electron donors for ETC in mitochondria during OXPHOS. However, HG results in robust electron donors entering ETC thus causing electron leakage and formation of ROS. Concomitantly, the production of ATP through OXPHOS is abrogated and mitochondrial function is disrupted. Ultimately, the metabolism is switched from OXPHOS towards aerobic glycolysis which allows ATP generation in shorter period of time under HG condition. This metabolic switch results in the increase of NADPH levels which can be used as a substrate for NOX and iNOS to generate ROS and NO respectively. Concomitantly, produced NO further down-regulates OXPHOS and increases intracellular ROS formation. The accumulated ROS subsequently activates NLRP3 inflammasome to synthesize IL-1 β , induces signaling cascade of MAPK and facilitates nuclear localization of transcription factors, such as AP-1, HIF-1 α and NF κ B, which results in expression of pro-inflammatory cytokines, chemokines and receptors. The activation of HIF-1 α further favors glycolysis, forming a cycle of metabolic switch, ROS production and pro-inflammatory response.

in ROS formation, the process that involves multiple mechanisms and serves as a linker between metabolism and pro-inflammatory signaling (Fig. 1). Expression of G6PDH was significantly increased in ATM and AT of obese and diabetic mice (high fat diet (HFD)-fed and *db/db* mice), and correlated with insulin resistance (Ham et al., 2013). Over-expression of G6PDH upregulated NOX and thus activated NF κ B and p38 MAPK that induced pro-inflammatory gene expression as well as excessive ROS formation in macrophages (Ham et al., 2013). Mitochondria are one of the sources of ROS in monocytes isolated from diabetic patients. High levels of mitochondrial ROS were observed in mononuclear cells of these patients along with hyperpolarized and more spherical mitochondria indicating mitochondrial dysfunction (Widlansky et al., 2011). Mitochondrial dysfunction not only elevates ROS production, but also promotes glycolytic metabolism to keep up with ATP demand (Widlansky et al., 2011). The cytoplasmic NOxes are another important source of ROS in diabetic macrophages. It is noteworthy that glucose is required for ROS formation by NOX as the absence of glucose in the culture medium of human peripheral blood monocytes impairs the electron translocation in NOX system (Musset et al., 2012). Due to the switch of metabolism towards glycolysis, the synthesis of NADPH is increased and triggers the activation of NOX and iNOS to catalyze formation of ROS and NO respectively (Kelly and O'Neill, 2015) as depicted in Fig. 1. NO reacts with superoxide to form peroxynitrite which causes accumulation of ROS (Kelly and O'Neill, 2015). NO also suppresses OXPHOS in mitochondria, while ROS stabilizes HIF-1 α , which further enhances glycolysis (Kelly and O'Neill, 2015; Calvani et al., 2012; Nishi et al., 2008). Increased intracellular ROS levels induced by elevated glucose in murine macrophages lead to DNA damage, upregulation of pro-inflammatory cytokines TNF- α , IL-1 α , ICAM and CCL-8 through activation of Akt and ERK pathways (Kumar et al., 2016). HG is also known to increase NOX2 activity by up-regulation of the PKC pathway, p47phox activation and p22phox in diabetic monocytes (Huang et al., 2011). In RAW 264.7 macrophages, HG-induced NOX2 activation and associated ROS production causes

downregulation of Lethe, an anti-inflammatory long non-coding RNA that acts as an inhibitor of NF κ B by binding to p65 subunit (Simar et al., 2015; Zgheib et al., 2017). Conversely, overexpression of Lethe attenuates ROS production and NOX2 activity by blocking p65 NF κ B translocation to the nucleus (Zgheib et al., 2017). ROS produced by NOX is also important in T1D because the onset of T1D is delayed in the absence of NOX-derived superoxide such as through inhibition of M1 polarization in NOD mice (Padgett et al., 2015a). In addition, endoplasmic reticulum stress may contribute to NOX-derived ROS production in T2D since treatment with ER-stress inhibitor successfully decreased ROS level in THP-1 cells treated with plasma from T2D patients. This was accompanied by decreased expression of genes encoding NOX subunit, indicating the interaction of NOX and ER in ROS production (Restaino et al., 2017). Taken together, diabetic conditions favor metabolic changes resulting in ROS formation in monocytes and macrophages through multiple sources including mitochondria, NOX and ER. In these settings, ROS act as signaling messengers, which link altered metabolism with phenotypic changes and production of pro-inflammatory mediators.

It should also be noted that besides metabolic reprogramming diabetic conditions influence macrophage phenotype via epigenetic changes during hematopoietic stem cell (HSC) differentiation in a ROS-dependent way (Yan et al., 2018). Hematopoietic stem cells obtained from HFD or *db/db* mice showed significantly higher level of oxidative stress and NOX2-dependent increase of DNA methyltransferase-1 (Dnmt-1) expression that led to hyper-methylation of Notch1, PU.1 and Klf4 which are essential transcription factors for HSC differentiation towards monocytes/macrophages (Yan et al., 2018). These epigenetic changes repressed the expression of the transcription factors resulting in reduced differentiation of HSC towards macrophages and predominance of M1-like phenotype (Yan et al., 2018). Eventually, higher number of M1-like macrophages significantly slowed down wound healing in *db/db* mice (Yan et al., 2018; Khanna et al., 2010; Gallagher et al., 2015).

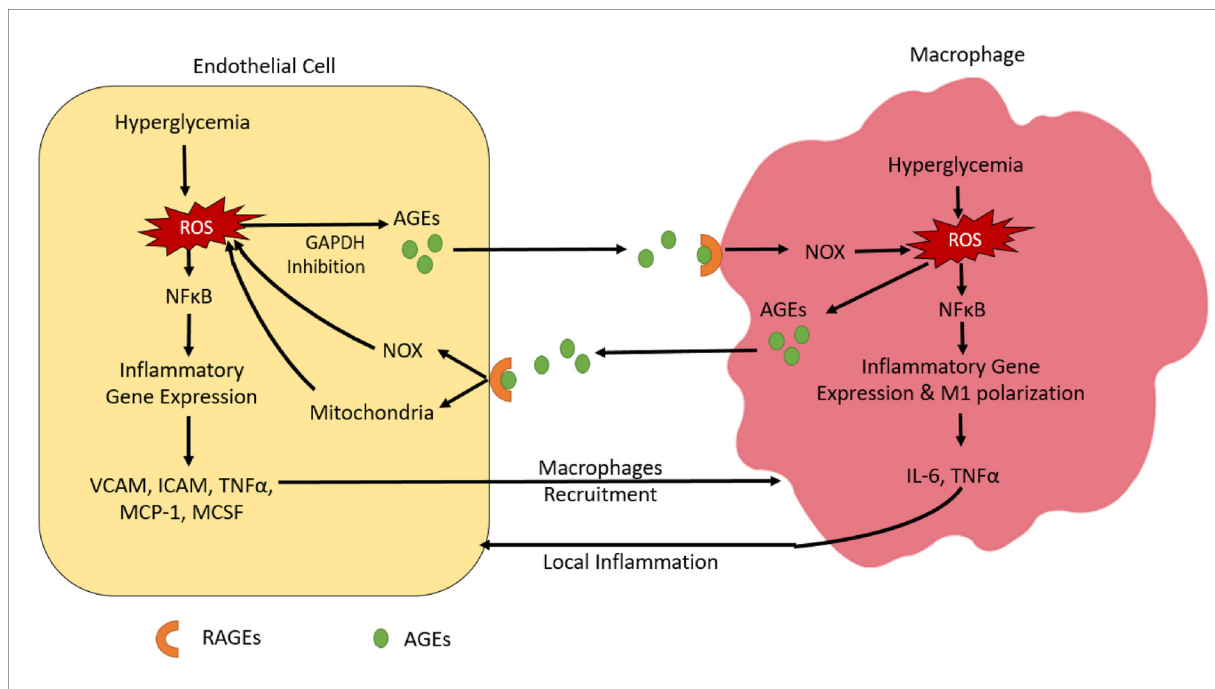


Fig. 2. Endothelial cell/macrophage cross talk in diabetic complications is triggered and maintained by overproduction of ROS. HG enhances the activation of NFκB via ROS, resulting in the expression of inflammatory genes such as VCAM, ICAM, TNFα, MCP-1 and MCSF by endothelial cells. The secretion of chemoattractants and cytokines by endothelium induces macrophage recruitment into the lesion. Furthermore, elevated levels of ROS increase the formation of AGEs and their precursors, such as peroxidized lipids and MGO, through GAPDH inhibition in both cell types. AGEs can be released extracellularly and bind to RAGE in autocrine and paracrine way. The interaction of AGEs and RAGE further facilitates ROS formation through NOX and mitochondria leading to the activation of ROS-sensitive downstream inflammatory pathways. In macrophages, AGEs/RAGE interaction promotes M1 polarization by inducing secretion of IL-6 and TNFα which augments macrophage recruitment and inflammation in endothelium.

4.3. Altered lipid metabolism and autophagy dysfunction facilitate macrophage ROS production and insulin resistance in obesity

In contrast to glucose metabolism, lipid metabolism in macrophages is downregulated in obesity-induced insulin resistance. Exposure of macrophages to saturated fatty acids was shown to downregulate lipid hydrolysis which is indicated by suppression of comparative gene identification-58 (CGI-58), a lipid droplet-associated protein that enhances the activity of adipose triglyceride lipase and is responsible for intracellular fat breakdown (Miao et al., 2014). Macrophage-specific deletion of CGI-58 in HFD-fed mice enhanced macrophage infiltration in epididymal fat and insulin resistance through activation of stress MAP kinase JNK (an inhibitor of insulin signaling). The underlying mechanism of macrophage pro-inflammatory response in the absence of CGI-58 was ROS-dependent and involved activation of NLRP3 which increased caspase-1 activity and in turn IL-1β synthesis (Miao et al., 2014). Accordingly, NAC treatment decreased caspase-1 activity, IL-1β synthesis and JNK phosphorylation, confirming the role of ROS in inflammation and insulin resistance (Miao et al., 2014; Vieira-Potter, 2014).

Interestingly, insulin resistance correlates with mitochondrial damage and epigenetic modification of the mitochondrial DNA in obese human (Zheng et al., 2015). Mitochondrial damages aggravate ROS formation which potentially augments inflammation in obesity (Zhou et al., 2011).

Normally, damaged mitochondria are eliminated by autophagy or mitophagy to prevent further oxidative stress (Strzyz, 2018). However, in diabetes autophagy is dysfunctional, while damaged mitochondria accumulate and eventually activate NLRP3 inflammasomes in a ROS-dependent manner (Nakahira et al., 2011). The down regulation of autophagy was observed in peritoneal macrophages of HFD-fed mice which showed low level of microtubule-associated protein 1 A/1B-light chain 3 (LC3)-II and high level of protein complex p62 (Kang et al.,

2016). During autophagy, the cytosolic form of LC3-I is recruited into the autophagosomal membrane where it undergoes proteolysis and lipidation to form LC3-II, while p62 is a protein complex which tags cellular waste to be degraded through autophagy; therefore inhibition of autophagy causes accumulation of p62 (Schläfli et al., 2015; Tanida et al., 2008). Furthermore, a mouse model (Atg7-KO mice) lacking macrophage autophagy-related protein-7 (ATG7) expression, a protein accountable for formation of autophagosomal membrane, developed insulin resistance and glucose intolerance under HFD (Kang et al., 2016). Peritoneal macrophages isolated from Atg7-KO mice showed higher intracellular ROS along with increased JNK signaling compared to WT mice, indicating that autophagy blockade induced ROS-related signaling in macrophages. Moreover, conditioned medium harvested from Atg-KO mice-derived peritoneal macrophages was able to suppress insulin signaling in 3T3-L1 adipocytes. In contrast, NAC treatment rescued the insulin signaling indicating the importance of ROS in insulin resistance during autophagy defect (Kang et al., 2016). Autophagy defects and ER stress have also been implicated in the failure of efferocytosis of arterial wall macrophage-derived foam cells in diabetes patients which contributes to atherosclerosis (Brophy et al., 2017). Although the involvement of ROS in this process was not reported, its role in the inhibition of efferocytosis cannot be excluded. Taken together, the changes of microenvironment due to obesity alter ATM metabolism leading to the increase of ROS formation and insulin resistance which is maintained and/or exacerbated by mitochondrial dysfunction and down regulation of autophagy.

5. ROS on the interface of macrophage and endothelial cell cross talk in diabetic complications

Crosstalk between macrophages and endothelial cells has been implicated in the progression of diabetic complications (Giacco, 2011). HG-activated endothelium recruits monocytes resulting in the release of

pro-inflammatory cytokines and promotion of vascular damage (Miao et al., 2014; Vieira-Potter, 2014; Zheng et al., 2015; Zhou et al., 2011). It is noteworthy, that ROS production in diabetes is increased in both endothelial cells and monocytes/macrophages resulting in activation of pro-inflammatory pathways and reciprocal macrophage/endothelial cell interactions (Giacco and Brownlee, 2010).

ROS in endothelial cells induces the activation of NF κ B and inhibition of endothelial NOS and causes their pro-inflammatory activation (Fig. 2) (Tabit et al., 2010). As a result, activated endothelial cells increase the expression of adhesion molecules such as vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM); pro-inflammatory cytokines such as TNF- α and macrophage colony stimulating factor (M-CSF), and chemotactic factors such as macrophage chemoattractant protein-1 (MCP-1) (Tabit et al., 2010). Upregulation of adhesion molecules and chemokines promotes macrophage recruitment to the endothelium and their transmigration which augments and maintains the inflammation in diabetic vascular dysfunction (Tabit et al., 2010). Macrophage infiltration in diabetic nephropathy of db/db mice correlated with expression of MCP-1, macrophage migration inhibitory factor (MIF) and M-CSF in glomeruli (Chow et al., 2004). The expression of ICAM-1 was upregulated in diabetic rat glomeruli and associated with hyperfiltration which is one of the signs of early stage diabetic nephropathy (Sugimoto et al., 2018). Deletion of MCP-1, monocyte/macrophage chemokine receptor CCR2 and ICAM in various diabetes mouse models, such as db/db, Ins2^{Akita} and streptozotocin-induced mice respectively, resulted in reduction of macrophage infiltration and kidney damage in diabetic nephropathy (Chow et al., 2007, 2005; Awad et al., 2011). As described in earlier sections, HG promotes polarization towards pro-inflammatory M1-like macrophages which contribute to pathophysiology and tissue damage. Thus, ablation of MCP-1 prevents macrophage influx and their deleterious aftermath.

In addition to described above effects of HG on cellular metabolism, glucose can chemically crosslink proteins, lipids and other (macro) molecules resulting in accelerated formation of advanced glycation end products (AGEs). The macrophage/endothelial cell cross talk is sensitive to and mediated by AGEs (Fig. 2) (Brownlee, 2005a). Via Maillard reaction, glucose plays a primary role in enhanced glycation and cross-linking of proteins in diabetic tissues (Brownlee et al., 1984), whereas intermediate metabolites of glycolytic and polyol pathways such as triose phosphate and glyceraldehyde have been shown to be potent glycation agents (Hamada et al., 1996). Their biological effects include inactivation of enzymes, crosslinking and trapping of proteins as well as decreased susceptibility to proteolysis (Brownlee et al., 1984).

Glycolysis is also the most important source of methylglyoxal (MGO) through non-enzymatic phosphate elimination from two intermediates of glycolysis, glyceraldehyde phosphate and dihydroxyacetone phosphate (Cooper, 1984).

Diabetic patients have increased plasma MGO levels (Maessen et al., 2015) which contributes to increased AGE-ing of macromolecules and increased risk of diabetes-related vascular complications (Hanssen et al., 2018). Furthermore, AGEs locally diffused from the cells can modify extracellular matrix molecules. Resulting changes in the cell/matrix interaction can cause endothelial cell dysfunction (Brownlee, 2005a, b).

When AGEs precursors diffuse out of the cell they can modify circulating proteins in the blood e.g. albumin, and in turn activate receptor for AGEs (RAGE), which expression is enhanced in HG conditions and correlates with ROS and MGO formation (Brownlee, 2005a). Endothelial cells and macrophages are the source of both AGEs and RAGE, thus the cross talk between these cell types is reciprocal (Fig. 2).

Formation of AGEs is enhanced in HG conditions through the increased levels of mitochondrial superoxide, which leads to the decreased activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Along with AGEs formation, GAPDH inhibition also induces other upstream pathways such as polyol pathway flux, hexosamine and

protein kinase C (PKC) pathway that favor development of diabetic complications (Brownlee, 2005a, b). The mechanism by which GAPDH inhibition activates these pathways is thoroughly reviewed by Brownlee and colleagues (Brownlee, 2005a). The engagement of AGEs and RAGE augments the production of ROS (Yan et al., 1994), especially hydrogen peroxide which serves as a mediator in pro-inflammatory response in endothelial cells and macrophages (Schieber and Chandel, 2014; Ray et al., 2012; Basta et al., 2005). In endothelial cells, NOX was activated following AGEs-RAGE interaction in PKC- α dependent manner (Thallas-bonke et al., 2008) and increased the expression of VCAM-1 as well as PAI-1, an important factor in diabetic microvascular complication in T2D (Wautier et al., 2001). The increase in both factors was attenuated by the treatment with diphenyliodonium, an inhibitor of NOX (Wautier et al., 2001). Concomitantly, the effect of AGEs-RAGE interaction on VCAM-1 and tissue factor expression was absent in macrophages lacking a NOX subunit, gp91phox, but not in WT macrophages, implicating NOX as the source of ROS in the AGEs-mediated inflammation (Wautier et al., 2001). In addition to NOX, AGEs also induce ROS production in mitochondria (Basta et al., 2005). ROS from both sources can trigger the activation of NF κ B leading to the upregulation of VCAM-1 in endothelial cells (Basta et al., 2005). AGEs-RAGE binding in macrophages induces oxidative stress and NF κ B activation through p21ras and MAPK signaling pathway, leading to M1 polarization and secretion of IL-6 and TNF- α (Jin et al., 2015; Singh et al., 2014) (Fig. 2). Of note, the downstream inflammatory pathway can be stimulated upon the activation of RAGE by other ligands, such as calgranulins S100 and high mobility group box-1 (HMGB-1) which expression correlates with ROS and MGO formation (Yao and Brownlee, 2010).

Although endothelial cell/macrophage interplay is triggered by HG via ROS production, diabetic progression is sustained even after the normalization of blood glucose levels indicating the involvement of epigenetic modification (El-Osta, 2012b; Ceriello et al., 2009; Paneni et al., 2013; Keating, 2015). HG-induced ROS overproduction is associated with epigenetic remodeling, although the causal relationship between ROS and epigenetic changes remains enigmatic (El-Osta, 2012b; Ceriello et al., 2009). Mammalian methyltransferase SET7 binds at the promoter region of NF κ B subunit p65 causing the mono-methylation of histone 3 at lysine 4 (H3K4me) amino residue in HG-treated endothelial cells. The chromatin remodeling in NF κ B promoter thus favors the persistent transcription of NF κ B gene, despite restoration of normoglycemic conditions, and maintains the overexpression of MCP-1 and VCAM-1, important molecules for endothelial cell/macrophage cross talk. Interestingly, suppression of mitochondrial superoxide ameliorates SET7-induced chromatin remodeling in NF κ B promoter region implying the role of mitochondrial ROS in SET7 modulation in bovine aortic endothelial cells (El-Osta et al., 2008; Keating et al., 2016). SET7 modulates the NF κ B-induced ROS production through regulation of mitochondrial function and Nrf-2 activity, a transcription factor responsible for anti-oxidant defense in Beas-2B cell line (He et al., 2015). Furthermore, glycemic memory is demonstrated through the persistent activation of p66^{Shc}, a mitochondrial adapter protein that is accountable for ROS metabolism and apoptosis (El-Osta, 2012a; Paneni et al., 2012). In endothelial cells, HG-induced upregulation of p66^{Shc}-enhanced ROS production in PKC- β II-dependent manner. P66^{Shc} overexpression was regulated on epigenetic levels by promoter hypomethylation and histone 3 acetylation (H3ace) and was maintained despite glucose normalization (Paneni et al., 2012). Silencing of p66^{Shc} diminished ROS production, MGO formation and ameliorated apoptosis by inhibition of cytochrome C release (Paneni et al., 2012). Given the strong implication of ROS and epigenetic remodeling in persistent vasculature damages, intervening ROS and epigenetic enzymes might be beneficial to attenuate macrophage/endothelial cell cross talk and the concomitant inflammation.

6. The effects of approved anti-diabetic drugs on ROS production in macrophages

In this review, we have discussed the effects of antioxidants on macrophage activation and function in diabetes via their direct effects on ROS production and redox sensitive pathways in these cells. However, it becomes clear that different types of therapeutic agents used in the management of diabetes influence ROS production in macrophages beyond direct anti-oxidant effects. Drugs used in the management of diabetes include hypoglycemic agents that improve insulin sensitivity and limit or reduce glucose absorption, and agents that correct metabolic disturbances often found in T2D e.g., dyslipidemia and hypertension. Main aspects of antioxidant properties of T2D drugs, including anti-oxidant status in patients and studies on the efficacy of oral anti-oxidant supplementation, are covered by recent comprehensive review (Choi and Ho, 2018). Although many drugs exert anti-oxidant functions via lowering the systemic glucose burden, some of them have direct effects on ROS production in macrophages. Metformin, one of the most frequently prescribed biguanides, improves insulin sensitivity of cells and thus reduces oxidative stress. In addition, metformin directly inhibits complex I of the electron transport chain resulting in decreased NADH oxidation. Although this effect was reported in hepatocytes and many other cell types, it was not yet assessed in macrophages. However, metformin as well as ROS exposure results in inhibition of I κ B α phosphorylation and subsequent nuclear translocation of p50-p65 NF κ B heterodimer in macrophages, and might therefore attenuate inflammatory responses via inhibition of mitochondrial ROS (Schuveling et al., 2018). In addition, it was reported that metformin decreased expression of NADPH oxidase and increased the activities of SOD and GSH-Px in LPS-stimulated human macrophages (Bułdak et al., 2014). Sulfonylureas like Glipizide, Glibenclamide and Gliclazide are the ligands of sulfonylurea receptor on the membrane of pancreatic β -cells which stimulate secretion of pro-insulin. In Raw 264.7 macrophages, Glibenclamide decreased intracellular ROS by inhibiting mitochondrial activity and blocking mitochondrial KATP channels (Li et al., 2014). Thiazolidinediones (TZD) increase insulin sensitivity of cells by binding PPAR γ and concomitantly influence mitochondria function (Feinstein et al., 2005). Sodium glucose cotransporter-2 (SGLT2) inhibitors such as Empagliflozin and Dapagliflozin promote urinary glucose secretion. Moreover, reduced macrophage infiltration and secretion of IL-1 β via the ROS-NLRP3-caspase-1 pathway, but no direct anti-oxidant effects were found in atherosclerotic lesions of ApoE $^{-/-}$ Streptozotocin-induced diabetic mice (Leng et al., 2016). Glucagon-like peptide-1 (GLP-1) agonists (Liraglutide, Dulaglutide, Exenatide, Semaglutide) reduce ER stress by directly activating Nrf2, major transcription factor involved in the regulation of antioxidant pathways, and restore eNOS induced ROS production in ECs (Erdogdu et al., 2013). Statins are lipid lowering drugs with pleiotropic effects. Simvastatin modestly decreased ROS production in J774 A.1 macrophages, and authors suggested this might be due to increased GSH content or upregulated PON2 expression (Rosenblat et al., 2013). In contrast, fluvastatin and lovastatin induced ROS production and could be linked to the observed decrease in geranylgeranyl pyrophosphate (GGPP) synthase level, ATP release and P2 \times 7 activation in LPS-primed THP-1 cells (Liao et al., 2013).

Overall, accumulating evidence suggests that anti-diabetic drugs may exert their therapeutic effects at least in part through regulation of ROS production in macrophages, which should be taken into consideration in further studies on pharmacodynamics of anti-diabetic medicines. Although the effects of diabetic drugs on ROS production have been explored, only a few of them have been shown modulating ROS levels in macrophages. This might partially explain why most of anti-oxidant-based drugs do not work efficiently in patients despite of promising results in preclinical tests (Pickering et al., 2018).

7. Concluding remarks and future perspectives

An increasing number of reports indicate that macrophages play an important role in diabetes progression and contribute to the development of diabetic complications in concert with endothelial cells and adipocytes. Macrophage contribution to the promotion of inflammation is exacerbated by metabolic stress due to cooperative action of obesity and HG. Metabolic imbalance in these conditions is characterized by enhanced glycolytic flux. Consequently, this metabolic shift drives a pro-inflammatory macrophage phenotype which is mediated by ROS as a second messenger. Both mitochondria and cytoplasmic NOXes are main producers of ROS in macrophages. ROS activates multiple pro-inflammatory pathways such as TAK1, MAPK, NLRP3 and NF κ B and causes epigenetic modifications in HG-exposed cells. In addition to metabolic changes, crosstalk between endothelial cells and macrophages in diabetic conditions further stimulates and maintains pro-inflammatory pathways in macrophages by upregulating ROS formation. Taken together, ROS is an important player in induction of a pro-inflammatory macrophage phenotype that favors insulin resistance and diabetic complications. Thus, targeting macrophage-generated ROS might be beneficial to suppress inflammation in diabetes.

Future research should focus on better understanding the ROS – macrophage – diabetes axis. Although recent studies showed the importance of macrophage-generated ROS in promoting M1-like macrophage phenotype, the role of macrophage-generated ROS in diabetic complications is still underappreciated. Recent studies targeting oxidative stress in vascular complications only assessed ROS in other cell types or tissues, such as endothelial and epithelial cells (Sancar-Bas et al., 2015; Xiao et al., 2014; Qi et al., 2017; Nishikawa et al., 2015). Although *in vitro* studies using anti-oxidants, such as vitamin C, showed promising results, their translation into clinical practice is not satisfactory. The lack of efficacy of anti-oxidant administration could be due to limited uptake by organs, the degree of diabetic complications and other interferences such as medications, genetic and environmental factors (Pickering et al., 2018). The lack of targeting of monocytes and macrophages in anti-ROS therapies may hamper the expected therapeutic benefit in diabetics. Furthermore, the incomprehensive understanding of the mechanisms that contribute to macrophage ROS production in diabetic complications is an important factor that impairs the efficacy of current anti-oxidant treatments (Schmidt et al., 2015). Indeed, suppressing ROS in inflammatory lesions is important, however, given the fact that circulating monocytes of diabetic patients upregulate ROS production, targeting monocyte- and macrophage-derived ROS warrants special attention. Knocking down or modulating the expression of specific genes in monocytes/macrophages, in particular those accountable for ROS formation, such as NOX in diabetic obese animals, might provide further insights into the role of macrophage-produced ROS in diabetic complications. The effect of systemic anti-oxidant treatment in diabetic animal studies should also assess the effect on macrophage-produced ROS. Moreover, ROS-induced epigenetic remodeling should also be considered as an emerging target for novel therapeutic approaches as it might be responsible for maintenance of high ROS levels, long-term metabolic changes and pro-inflammatory activation of macrophages after restoration of normoglycemic conditions. A better understanding of macrophage-derived ROS functions in diabetic complications may provide alternative approaches for clinical interventions in diabetes.

Conflict of interest

None.

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