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REVIEW

Metformin and its redox-related mechanisms of action in type 2 diabetes

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Abstract

Type 2 diabetes (T2D) is a long-term metabolic disease characterized by progressive β-cell functional decline and insulin resistance, which increases the risk of cardiovascular complications as well as associated-morbidity and mortality. Evidence suggests a strong relationship between hyperglycaemia, oxidative stress and the development and progression of T2D. Indeed, a hyperglycaemic state can reduce the activity of antioxidant enzymes and increase lipid peroxidation and protein oxidation products, as well as DNA damage. At present, metformin is the recommended first-line glucose-lowering agent in patients with T2D. Despite the vast clinical experience gained over several decades of use, several mechanisms of action of metformin have yet to be fully elucidated. This review provides an overview of the existing literature concerning the complicated interplay between oxidative stress and T2D and the molecular mechanisms underlying the redox-related mechanisms of action of metformin, which include (but are not limited to) interaction with AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms, inhibition of gluconeogenesis and action on leukocyte–endothelium interactions.

Keywords

- metformin
- oxidative stress
- diabetes
- cardiovascular complications
- mitochondria

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Introduction

Type 2 diabetes (T2D), characterized by hyperglycaemia, is associated with increased generation of reactive oxygen species (ROS) and, thus, oxidative stress (Apostolova *et al.* 2020). There are different endogenous sources of free radicals (endoplasmic reticulum, peroxisomes, phagocytic cells, etc.), although the mitochondria are the main ones (Murphy 2009). Mitochondrial ROS production is enhanced under hyperglycaemic conditions, therefore inducing oxidative stress, mitochondrial dysfunction, and tissue damage. Mitochondrial dysfunction is also linked to insulin resistance (IR) and impaired glucose uptake in target tissues, including adipose tissue, skeletal muscle and liver (Petersen *et al.* 2003). Furthermore, mitochondrial dysfunction has also been related to cardiovascular diseases (CVD) associated with diabetes.

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In addition, the redox state is tightly modulated by mitochondrial physiology, and the excess of ROS can induce other comorbidities, such as microvascular complications, including neuropathy, retinopathy and nephropathy, or macrovascular complications including myocardial ischaemia or stroke. Therefore, maintaining adequate mitochondrial function is essential to control or avoid the appearance of IR and, consequently, T2D and associated comorbidities. It is clear that different mechanisms, including mitochondrial dynamics, biogenesis, and mitophagy, among others, can regulate mitochondrial homeostasis.

Thus, mitochondria are not static organelle. They have great metabolic and plastic versatility due to their dynamic and constant change in size, shape and location in cells. Furthermore, mitophagy is a crucial pathway for maintaining cellular homeostasis. This function eliminates impaired mitochondria that are engulfed by autophagosomes and eventually degraded within lysosomes (Ding & Yin 2012). An 'adaptive' increase in mitophagy may modulate and delay progression to T2D by maintaining β-cell function in prediabetic individuals, the preliminary stage to disease development (Bhansali *et al.* 2017).

Diabetes is a chronic disease in which blood glucose levels rise because the body cannot produce enough insulin or is unable to use the insulin it produces effectively to modulate glucose levels. Most cases fall into two categories: type 1 diabetes (T1D) and T2D. T1D is caused by an absolute deficiency in insulin secretion due to an autoimmune and pathological process occurring in the pancreas. Whereas T2D is due to a combination of resistance to insulin action and an inadequate compensatory capacity for insulin secretion. T1D accounts for 5–10% of cases, being the most common type of diabetes in children and although its aetiology is not yet fully understood, it is related to genetic and environmental risk factors. On the other hand, T2D is much more common, accounting for more than 90% of cases, and is a health problem that is expanding worldwide and is closely linked to the obesity epidemic (WHO 2023)

It has been demonstrated in different clinical trials that good glycaemic control is critical in decreasing cardiovascular comorbidities associated with diabetes (UKPDS study). In this sense, multiple reports have shown that metformin and other hypoglycaemic drugs can prevent the development of endothelium injury and atherosclerosis by regulating glucose levels (Mather *et al.* 2001). It is important to highlight that the hypoglycaemic effects of dimethylbiguanide or metformin in the management of T2D in humans were provided a long time ago by the French medical doctor Jean Sterne (Bailey 2017). Nowadays, metformin is the gold standard drug for the treatment of T2D and other conditions with IR. However, further research is needed to clarify the molecular mechanisms underlying its beneficial effects.

Mitochondrial dysfunction and oxidative stress in T2D

Mitochondria are the powerhouse of the cell, thus play an essential role in metabolism and glucose homeostasis (Wang *et al.* 2013). Different mitochondrial diseases, such as MERRF (myoclonic epilepsy with ragged red fibres) or MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) syndrome, have been linked to T2D (Wang *et al.* 2013) suggesting mitochondria as a key factor in the pathophysiology of diabetes (Fig. 1). Unfortunately, several factors contribute to mitochondrial dysfunction (Pieczenik & Neustadt 2007) and its underlying mechanisms are very complex and remain unclear.

Mitochondrial dysfunction is characterized by decreased mitochondrial number and biogenesis, altered respiration, membrane potential and adenosine triphosphate (ATP) production and modified activities of oxidative proteins. All these factors have been found in different studies including experimental animal diabetes models as well as diabetic patients. Mutations in both mitochondrial DNA (mtDNA) and nuclear DNA that encode mitochondrial proteins or regulators involved in mitochondrial activity (e.g. NDUFB6, PGC-1α and tRNAs) are associated with T2D (Kim *et al.* 2008). Furthermore, alterations in mitochondrial morphology and mitophagy proteins have been previously described in leukocytes from T2D patients (de Maranon *et al.* 2022). Oxidative stress is certainly an inducer of mitochondrial dysfunction as well. It is defined as an imbalance between ROS production and antioxidant defenses. Mitochondria are responsible for the control of the redox state in the cell, and they are targets of oxidative stress, specially under glucose and fatty acid overflow, which increases ROS production and accumulation (de Maranon *et al.* 2020). This leads to the aforementioned morphological changes in mitochondria but can also damage proteins, lipids and DNA. In addition, leukocytes from T2D patients have

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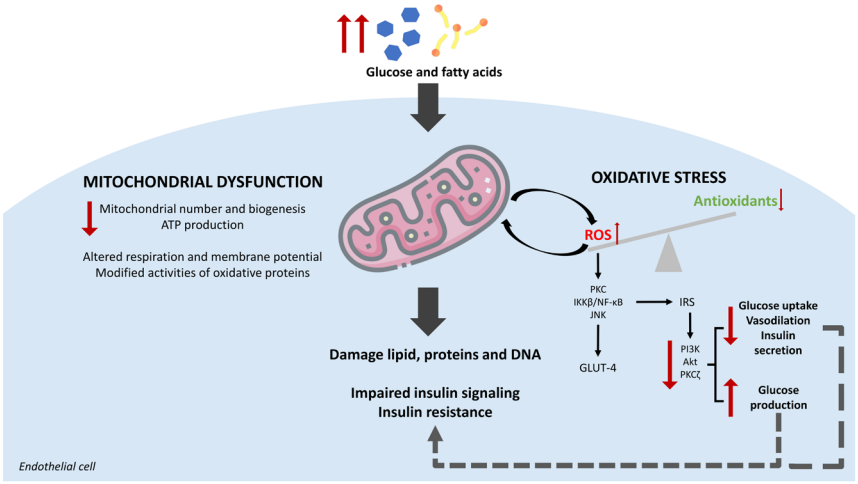

Figure 1
Disorders characterized by high levels of glucose and fatty acids, as in T2D, lead to mitochondrial alterations. On the one hand, mitochondrial dysfunction has been found in diabetes compiling a reduction in mitochondrial number and biogenesis, ATP production along with altered respiration and membrane potential and modified activity of different oxidative proteins. On the other hand, oxidative stress is also triggered. Mitochondria are not only affected by ROS but also produce them to a large extent. At the same time, antioxidant levels decrease causing a imbalance in oxidants and antioxidants that further activates different signalling pathways (PKC, IKKβ/NF-κB and JNK). This results in changes in GLUT-4 location and expression, as well as in IRS phosphorylation, degradation and location, the latter downregulating the downstream signalling (PI3K, Akt and PKCζ). Consequently, there is a reduction in glucose uptake, vasodilation and insulin secretion and an increase in glucose production. All these changes in the mitochondria functioning and their related signalling pathways ultimately result in lipid, protein and DNA damage, together with impaired insulin signalling and resistance. Akt, protein kinase B; ATP, adenosine triphosphate; GLUT-4, glucose transporter 4; IKKβ/NF-κB, inhibitor nuclear factor κB kinase complex; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; PI3K, phosphoinositide 3 kinase; PKC, protein kinase C; ROS, reactive oxygen species.

been described to have higher levels of total and mitochondrial ROS (de Maranon *et al.* 2022). Moreover, in hyperglycaemic conditions related to diabetes, there is a reduction in antioxidant activity. For instance, superoxide dismutase, erythrocyte catalase and α-lipoic acid are decreased in uncontrolled diabetic patients (Evans *et al.* 2002). Lower NADH:O_2 oxidoreductase and mitochondrial size have also been observed in patients with IR and/or T2D (Kelley *et al.* 2002).

Although it is not clear whether mitochondrial dysfunction and oxidative stress cause IR or vice versa, these processes have been strongly associated. In fact, there is a reciprocal relationship between insulin and mitochondria. Excessively high levels of ROS and impaired mitochondrial function culminate in impaired insulin signalling and IR (Wang *et al.* 2010). For instance, ROS overproduction interferes with acetyl CoA oxidation, increasing lipid and diacylglycerol accumulation. At molecular level, this accumulation of metabolites and ROS leads to the activation of different kinases and pathways, such as PKC, IKKβ/NF-κB and JNK, which are involved in IRS phosphorylation (on serine^{307}), degradation and location, or GLUT-4 translocation and expression, among others (Yaribeygi *et al.* 2019). Activation of IRS proteins downregulates the downstream insulin signalling pathways (PI3K, AKT and PKCζ) PKCζ and replace with the correct one.which leads to decreased glucose uptake, increased glucose production and reduced vasodilation and insulin secretion (Kim *et al.* 2008). Furthermore, a positive correlation between NF-κB and poor glycaemic control has been described in peripheral blood mononuclear cells from diabetic patients (Evans *et al.* 2002). Interestingly, insulin is also critical for mitochondrial function and mitochondrial free radicals at physiological levels induce insulin sensitivity (Yaribeygi *et al.* 2019). Therefore, it is difficult to establish whether mitochondrial dysfunction results in IR or vice versa depending on the ROS concentration, and additional studies are needed to clarify cause and effect.

Pharmacological or molecular interventions of the redox state with different molecules, such as resveratrol or alpha-lipoic acid, have been found to decrease ROS production and thus improve mitochondrial function and insulin sensitivity (Kim *et al.* 2008). Nonetheless, previous studies have revealed that the reduction of ROS levels could modulate the development of IR in mice but without complete prevention, suggesting either incomplete restoration of normal ROS levels or parallel implication of other pathways (Houstis *et al.* 2006).

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Metformin: the first line in T2D

Since its authorization to treat diabetes by the United States Food and Drug Administration (FDA) in 1994 (Bailey 2017), metformin has been used as first-line therapy in T2D patients. Most guideline committees recommend metformin as the first-choice treatment for individuals who are unable to reach their desired blood glucose levels through diet and lifestyle changes.

An early seminal study by DeFronzo *et al.* revealed that, as early as 2 weeks of metformin treatment in obese diabetic patients, a significant reduction in fasting plasma glucose was achieved (DeFronzo & Goodman 1995). By week 29, the fasting plasma glucose concentration had decreased by 52 mg/dL. Moreover, at 9 weeks of treatment, a significant decrease in glycated haemoglobin (HbA1c) levels, the retrospective marker of glucose control, was observed, achieving a dramatic decrease of 1.4% in the metformin-treated patients at week 29. Metformin not only improves glucose control, patients given metformin had statistically significant decreases in triglyceride and free fatty acid circulating levels, as well as low-density lipoprotein and plasma total cholesterol (DeFronzo & Goodman 1995). Furthermore, not only after a T2D diagnosis will patients benefit from metformin treatment. Even before the onset of T2D, metformin treatment has demonstrated protection against developing the disease in high-risk subjects (Lachin *et al.* 2007).

Metformin use in combination with other anti-diabetic oral drugs is very effective in improving glycaemic control. In fact, a combination of metformin and glibenclamide was significantly more effective than either metformin or glibenclamide alone in lowering HbA1c (Tosi *et al.* 2003).

Metformin use has been proven to be safe and effective at reducing glucose levels in gestational diabetes (Rowan *et al.* 2008). However, some caution is warned by the fact that metformin closes the placenta and may have unknown effects on the fetus. When compared to other oral glucose-lowering agents such as glyburide, weight gain during pregnancy is lower in the metformin-treated women than in the glyburide group. Therefore, metformin is considered the treatment of choice where weight gain is an issue during pregnancy.

Importantly, metformin has demonstrated CVD-related improvements. In this regard, treatment with metformin, either as monotherapy or in combination with sulfonylurea, led to a decrease in several CVD risk factors in T2D individuals, such as free fatty acids, triglycerides and soluble vascular cellular adhesion molecule-1 (VCAM-1) levels (Abbasi *et al.* 2004). Metformin can exert direct effects on the endothelium that can reduce/reverse the impact of hyperglycaemia on endothelial function, via mechanisms linked to increased phosphorylation of Akt and eNOS (Ghosh *et al.* 2015). This drug markedly improves oxidative stress, nitric oxide (NO) bioavailability and glycation in normal and T2D rats, thus ameliorating endothelial function. Research in recent years suggests a beneficial effect of metformin use for the prevention or treatment of CVD (Sanchez-Rangel & Inzucchi 2017). Metformin administration is safe in heart failure patients with and without T2D (Chowdhury *et al.* 2023) and it has demonstrated to lower the risk of death and cardiovascular events in individuals with stage 3 chronic kidney disease. However, the efficacy of metformin in preventing CVD associated with the development of diabetes is controversial. A recent analysis of the reduced DPPOS (Diabetes Prevention Program Outcomes Study) and DPP (Diabetes Prevention Program) trial to assess the incidence of major cardiovascular events over a mean follow-up of 21 years of metformin-treated participants failed to detect a reduction in major cardiovascular risks (Goldberg *et al.* 2022).

Overall, numerous studies strongly support the use of metformin as first-line treatment in T2D, alone or in combination with other glucose-lowering agents.

Molecular pathways underlying metformin's actions: news and views

The molecular mechanisms underlying metformin's actions are complex and not yet fully understood. They include AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms, as well as the inhibition of mitochondrial glycerophosphate dehydrogenase and mitochondrial respiration, among others. However, the effects of this biguanide vary depending on the doses and duration of the administration, with clear differences between chronic and acute treatment.

Gluconeogenesis

Gluconeogenesis (GNG) is a metabolic pathway that leads to glucose production from certain non-carbohydrate carbon precursors, such as glucogenic amino acids, pyruvate, lactate and glycerol. For over half a century, metformin has been used to treat T2D, since it specifically

reduces hepatic GNG through the sensitization of peripheral tissues to insulin and an antilipolytic effect, thus decreasing the amount of fatty acids available for GNG (Natali & Ferrannini 2006). The underlying mechanisms by which metformin exerts these effects are multiple and, at the cellular level, mitochondria seem to play a key role.

Accumulating data suggest that metformin inhibits mitochondrial glycerophosphate dehydrogenase, an enzyme that catalyses the conversion of glycerol phosphate to dihydroxyacetone phosphate (Madiraju *et al.* 2014). Consequently, there is a decrease in cytosolic concentration of nicotin-amide adenine dinucleotide (NAD) along with an increase in the reduced NADH/NAD ratio and, secondarily, a decrease in this ratio in the mitochondrion, which in turn restricts the conversion of lactate to pyruvate, thus preventing the use of these substrates for GNG. In this way, glucose generation reduces and excess glycerol and lactate is released into the plasma (Ferrannini 2014).

Another identified mechanism of action is the inactivation of the mitochondrial complex I of the electron chain, which reduces NADH oxidation and thus the mitochondrial ATP generation and increases the availability of adenosine monophosphate (AMP) and adenosine diphosphate (ADP). To equalize the low-energy generation, the cell suppresses ATP-requiring mechanisms, such as GNG. This process is also presumably slowed by high levels of AMP, which suppresses the activity of fructose 1,6-bisphosphatase, a regulatory enzyme of GNG (Hundal *et al.* 2000, Ferrannini 2014, LaMoia & Shulman 2021).

At the same time, metformin behaves as an insulin sensitizing agent through several mechanisms. It has a positive action on insulin receptor expression, mediated by modulation of tyrosine phosphorylation of the insulin receptor beta subunit (LKB1, liver kinase B1 or STK11, serine/threonine kinase), secondary to AMPK activation by the mechanisms described earlier (Viollet *et al.* 2012). Moreover, it increases plasma levels of incretin, glucagon-like peptide (GLP1) (Wu *et al.* 2014), a protein produced in intestinal L-cells under food stimulus, whose role is to activate insulin production, but at the same time, improving of peripheral tissues sensitivity to insulin also slows gastric emptying and suppresses glucose release and suppression of glucagon release; animal studies suggest that GLP1 also stimulates cell regeneration and increased cell mass (Farilla *et al.* 2002). GLP1 binds to its specific receptor in peripheral tissues and is rapidly degraded in plasma by the enzyme dipeptidylpeptidase-4, which appears to reduce its blood concentration with metformin treatment, increasing the half-life of GLP1, thereby lowering peripheral IR (Wu *et al.* 2014).

It is important to note that the inhibition of hepatic glucose production promoted by metformin has been recently associated with changes in microbiota composition. Specifically, Barroso *et al.* (2023) showed that metformin increases the expression of growth differentiation factor 15 (a cytokine that sustain full AMPK activation) and the secretion of GLP1 and suppresses GNG as well as intestinal glucose uptake by affecting the microbiome. Similarly, studies performed in both humans and animals showed that metformin treatment significantly increases the abundance of bacteria producing the short-chain fatty acids butyrate and propionate, which are involved in glucose homeostasis (Shin *et al.* 2014, Wu *et al.* 2017, Vallianou *et al.* 2019). However further investigation is needed to better understand the relationship between metformin and gut microbiota.

AMPK/SIRT3/NRF2 pathway

Since the beginning of evolution, all organisms have faced the challenge of adapting efficiently to changing environmental conditions. Among these, many situations involve the appearance of imbalances at the energetic or metabolic level, which in many cases lead to oxidative stress. Interestingly, eukaryotes have developed multiple stress response systems focused on two main targets: chromatin and mitochondria. Over the last decade, sirtuins have been suggested as an essential element in coordinating and detecting stress responses, targeting chromatin and mitochondria. These signalling proteins are involved in different cellular functions such as mitochondrial functioning, regulation of transcriptional expression and DNA repair. Sirtuins were originally described in yeast as a new class (III) of histone deacetylases (HDACs). HDACs are enzymes that regulate chromatin-associated functions by removing acetyl groups from key lysine residues, mainly in the N-terminal region of histones H3 and H4. Histone deacetylation promotes decompaction of these chromatin regions, allowing accessibility of transcriptional activity to the promoter regions of genes and thus their expression (Vaquero *et al.* 2007). However, unlike the other HDACs, sirtuins require the presence of a coenzyme in their enzymatic reaction, NAD^+ (Imai *et al.* 2000), to modulate acetylation/deacetylation status of the proteins.

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In recent years, accumulating data has highlighted the role and functions of sirtuins in diabetes and their potential as a therapeutic target for the prevention and management of this disorder. Although the mammalian sirtuins family consist of seven members (SIRT1–SIRT7), which differ in their substrate selectivity and subcellular localization, it seems that SIRT3 has a prominent role in metabolic disorders such as diabetes.

SIRT3 localizes primarily to the mitochondrial matrix and has been implicated in regulating mitochondrial metabolism, including amino acid metabolism, fatty acid oxidation, redox homeostasis, ketogenesis, the tricarboxylic acid cycle and urea cycle (Giralt & Villarroya 2012). Therefore, it is not surprising that SIRT3 is also a key regulator of insulin signalling in IR and diabetes states (Jing *et al.* 2010, Kitada *et al.* 2019). Specifically, Jing *et al.* showed that reduced levels of SIRT3 in the skeletal muscle of high-fat diet (HFD)-induced obese mice and streptozotocin (STZ)-induced diabetic mice were a critical element in the pathogenesis of T2D. The same authors also revealed that C2C12 myoblasts with a *Sirt3* knockdown displayed significant IR with reduced IRS-1 tyrosine phosphorylation, which was accompanied by a decrease in downstream events, such as MAPK and Akt phosphorylation.

In addition, Gao *et al.* give a breakthrough in this field by highlighting that continuous repression of SIRT3 caused by histone modification is a critical player in inflammation and hepatic steatosis, which favour hypertension and cardiac dysfunction under high salt intake (Gao *et al.* 2022). Interestingly, they found that drugs that improve liver metabolism, such as metformin, can ameliorate the cardiovascular injury caused by salt. Specifically, they used mice fed with high salt or normal diet for 8 weeks treated or not with salt withdrawal and metformin and analysed the hepatic mitochondrial activity, whose dysfunction is a key factor leading to liver inflammation (Begriche *et al.* 2013). The results obtained revealed that metformin therapy activated AMPK, which inhibited salt-induced cardiovascular damage and hepatic inflammation by decreasing the H3K27ac level in the SIRT3 promoter and increased the binding of NF-E2-related factor 2 (NRF2), a positive regulator of SIRT3. This study showed the critical role of hepatic inflammation and steatosis in salt-induced hypertension and examined the possibility of treatment and/or prevention of hypertension by ameliorating liver metabolism in an experimental model (Gao *et al.* 2022). However, further investigations are needed to clarify the molecular mechanisms underlying these processes.

AMPK/autophagy

Autophagy is a crucial survival mechanism for cellular homeostasis that tightly regulates intracellular degradation processes by removing damaged organelles and aggregated proteins. Several studies suggest that dysregulation of autophagy can lead to many diseases, including T2D and associated CVD (Rocha *et al.* 2020, Apostolova *et al.* 2023).

Xie Z *et al.* demonstrated that diabetic mice present reduced cardiac autophagy as well as decreased AMPK activity, which constitute key mechanism in the onset of diabetic cardiomyopathy. Surprisingly, chronic AMPK stimulation by metformin (200 mg/kg per day for 4 weeks) upregulated autophagy activity, thus improving cardiac functions in diabetic animals (Xie *et al.* 2011). Similarly, Efentakis *et al.* reported that metformin has a positive effect against carfilzomib-induced cardiotoxicity in rodents through stimulation of the AMPK/autophagy pathway. In particular, carfilzomib, a proteasome inhibitor used in patients with relapsed or refractory multiple myeloma, inhibited AMPK and the downstream signalling related to autophagy, thus promoting cardiac dysfunction during treatment. Restoration of the inhibited AMPK/autophagy pathway by metformin (140 mg/kg daily) preserves cardiac dilation in mice (Efentakis *et al.* 2019).

More recently, data by Robichaud *et al.* evidenced that this biguanide anti-hyperglycaemic agent also protects against atherosclerosis in rodents. As is known, atherosclerosis is characterized by excessive cholesterol uptake and buildup of cytosolic lipid droplets that lead to foam cells accumulation within the arterial wall. The authors revealed that autophagy-mediated cholesterol efflux is significantly decreased in vascular smooth muscle cell-derived (VSMC) foam cells compared with macrophage-derived foam cells in atherosclerotic lesions. Of note, metformin ameliorates autophagy-mediated cholesterol efflux in VSMC-derived foam cells, a major cell type present at all stages of an atherosclerotic plaque (Robichaud *et al.* 2022). This important advance could result in a novel therapeutic strategy to resolve atherosclerotic plaque. However, further investigation is warranted.

Vascular protection, inflammation and leukocyte–endothelium interactions

Another mechanism by which metformin provides additional benefits in patients with diabetes mellitus

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is vascular protection. As is known, a hyperglycaemic environment favours the production of fibronectin and collagen in endothelial cells, increasing the thickness of the vessel wall. This finding was described in endothelial cells of human umbilical veins, where high glucose levels induce the overexpression of fibronectin (Ceriello *et al.* 2009), which was not easily reversible after exposure to normal carbohydrate levels, further generating a prolonged induction of PKC-β (protein kinase C-β), NADPH oxidase, collagen and fibronectin.

Interestingly, metformin appears to modify this effect by inhibiting transforming growth factor beta-1 (TGFβ1) (Park *et al.* 2014) by which myofibroblast differentiation is blocked and synthesis and accumulation of extracellular matrix (ECM) components, as well as the expression of cell surface receptors for ECM components, is inhibited.

Additionally, metformin modifies atherogenic processes such as endothelial injury and inflammation. Endothelial injury occurs because the AMPK increases in response to stress, such as in acute heart attack myocardium, arterial hypertension and diabetes and then phosphorylates endothelial nitric oxide synthase stimulating NO production (Takahashi *et al.* 2015), which ultimately entails vascular proliferation and vasodilatation (Takahashi *et al.* 2015). Regarding the anti-inflammatory effect, metformin regulates this process by inhibiting the expression of adhesion molecules such as ICAM-1 and VCAM-1, via AMPK, decreasing NF-KB expression.

On the other hand, metformin reduces the expression of von Willebrand factor, the tissue activator inhibitor-1 plasminogen (de Jager *et al.* 2014) and factors VII and XIII, which confers an antithrombotic effect (Bailey 2008). In addition, it regulates the increase in prostaglandin F2α (Pitocco *et al.* 2013) and free fatty acids and maintains an adequate oxidation–reduction balance vascular with increased expression of superoxide dismutase, an enzyme of the system NADPH oxidase, finally generating a better vasodilator function. Consistent with these findings, and in order to have an additional advantage, Tousoulis *et al.* evidenced that combining metformin 850 mg/day with atorvastatin 10 mg/day for 6 weeks improved endothelium-dependent dilation (Tousoulis *et al.* 2010).

Likewise, it is widely known that hyperglycaemia and IR can increase ROS generation by peripheral blood leukocytes and activation of leukocyte–endothelium interactions. Specifically, in the early stages of atherosclerosis, activated leukocytes interact with the endothelial vessel wall, rolling and adhering to it and thus transmigrating towards the inflammatory focus. During this process, leukocyte trafficking is mediated by different adhesion molecules, such as selectins, ICAM-1 and VCAM-1, expressed on either endothelial cells or leukocytes (Galkina & Ley 2007).

Interestingly, metformin appears to directly affect the endothelium by ameliorating endothelium-dependent vascular responses. In this regard, Mather *et al.* demonstrated for the first time that, compared with placebo, 12-week metformin treatment markedly ameliorated endothelium-dependent (acetylcholine) vasodilation in T2D subjects (Mather *et al.* 2001). In line with these data, results from our group evidenced that T2D patients treated with metformin (for at least 12 months at a dose of 1700 mg per day) showed an increase in several antioxidant mRNA levels, such as those of *SIRT3* and *GPX1*, accompanied by a reduction in mitochondrial ROS production, leukocyte–endothelium interactions, as well as P-selectin and ICAM-1 levels (Diaz-Morales *et al.* 2017) (Fig. 2). These beneficial effects might prevent the vascular damage and onset of an atherogenic process in T2D. However, more investigation is carried to clarify whether treatment with insulin sensitizers, such as metformin, can improve cardiovascular function in patients with T2D.

Modulation of the redox status (complex IV/ubiquinone/GPD2 pathway)

As discussed earlier, metformin reduces hepatic glucose production by targeting both complex I of the mitochondrial electron transport chain. Inhibition of complex I leads to reduced ATP generation, thus increasing AMP levels, which suppress hepatic GNG through activation of AMPK (Foretz *et al.* 2014). Interestingly, several reports suggest that the glucose-lowering effect of metformin may also occur through pathways independent of AMPK activation. Data from Madiraju *et al.* demonstrated that this agent is able to non-competitively inhibit the redox enzyme – mitochondrial glycerol-3-phosphate dehydrogenase (GPD2), thus showing a cellular redox mechanism for the antihyperglycaemic effect of metformin. Furthermore, other experiments by the same authors indicated that chronic oral administration acute intraportal or acute intravenous administration in diabetic and awake normal rats suppressed GNG from glycerol and lactate

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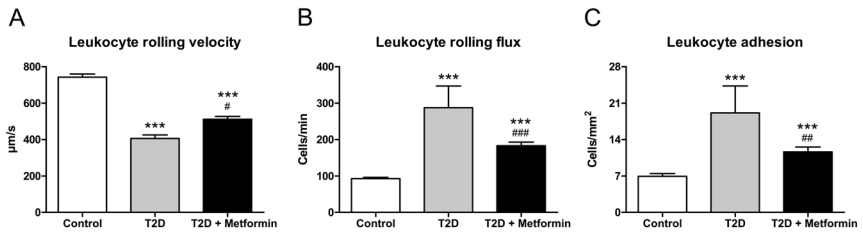

Figure 2
Assessment of leukocyte–endothelium interactions in T2D patients with and without metformin treatment and healthy control subjects. n = 25 subjects per group. (A) Leukocyte rolling velocity, (B) leukocyte rolling flux and (C) leukocyte adhesion. $^{***}P < 0.001$ with respect to control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ with respect to non-metformin-treated T2D, by using one-way ANOVA with a Student-Newman-Keuls multiple-comparison post hoc test. Data from a previous study in our lab, by Díaz-Morales *et al.* (2017). The study complied with the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the University Hospital Dr. Peset. Written informed consent was obtained from all the participants.

but not from alanine and pyruvate, revealing an elevated cytosolic redox state in facilitating the glucose-lowering action of metformin (Madiraju *et al.* 2018). Recently, LaMoia *et al.* have shed light on how metformin treatment causes GPD2 inhibition. In this sense, they demonstrated that metformin inhibited mitochondrial complex IV rather than complex I, thus reducing glucose production independently of AMPK activation and altering cellular redox state (LaMoia *et al.* 2022). Furthermore, LaMoia *et al.* suggest that metformin binds to and inhibits mitochondrial complex IV, causing a backlog of the mitochondrial electron transport and reducing the ubiquinone pool, which indirectly inhibits GPD2. However, further investigations are needed to confirm this preliminary observation and profoundly define this potential 'complex IV/ubiquinone/GPD2' pathway.

Conclusion

Despite the long-standing history of metformin discovery and use, its mechanisms of action remain uncertain, and advances in this field are vital to clarify the multiple beneficial effects of this molecule. Currently, it is the most commonly prescribed anti-hyperglycaemic agent in T2D therapy, also showing additional benefits by modulating mitochondrial function, oxidative stress, autophagy and endothelium–leukocyte interactions. Although many recent works highlight that metformin activates AMPK, thus inhibiting hepatic GNG and acting as an insulin sensitizer, evidence supports that it exerts crucial roles independent of AMPK (Fig. 3). However, future research is needed to explore the mechanisms underlying the actions of this agent more in depth.

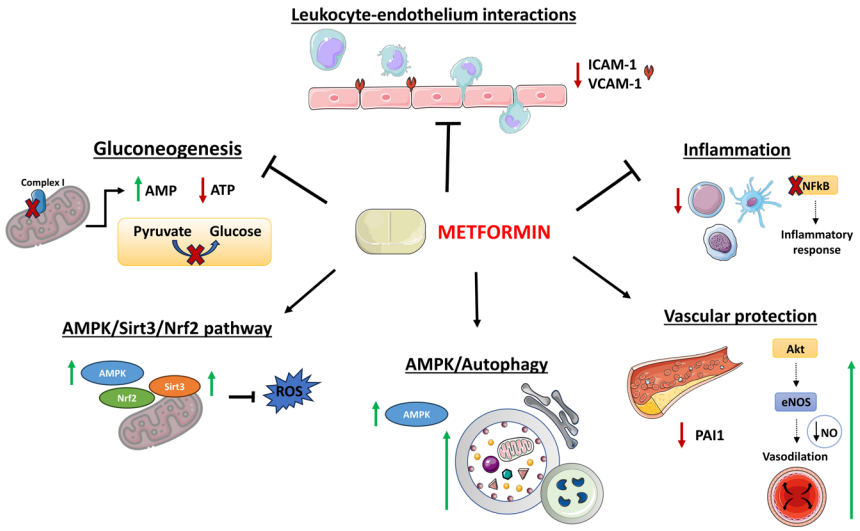

Figure 3
Schematic representation of the redox-related mechanisms of action of metformin, which include (but are not limited to) interaction with AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms, inhibition of GNG and action on leukocyte–endothelium interactions. Akt, protein kinase B; AMP, adenosine monophosphate; ATP, adenosine triphosphate; eNOS, endothelial nitric oxide synthase; ICAM-1, intercellular adhesion molecule-1; NFKB, Nuclear Factor Kappa B; NO, nitric oxide; NRF2, NF-E2-related factor 2; PAI1, activator inhibitor-1 plasminogen; ROS, reactive oxygen species; Sirt3, sirtuin-3; VCAM-1, vascular cellular adhesion molecule-1.

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Declaration of interest

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

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Author contribution statement

Conceptualization: TV, CLM, SRL, VMV; methodology: TV, CLM, SRL, VMV; investigation,: TV, CLM, SRL, VMV; data curation: TV, CLM, SRL, VMV; writing – original draft preparation: TV, CLM, SRL, VMV; writing – review and editing: TV, CLM, SRL, VMV; visualization; supervision; project administration; funding acquisition. All authors have read and agreed to the published version of the manuscript.

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