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REVIEW ARTICLE

Metformin: Revisiting The Old And Opening New Horizon

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INTRODUCTION

Improvements in healthcare and nutrition have remarkable impact on increasing the life expectancy worldwide. This is one of the greatest achievements of the modern world yet, it also presents a grave challenge: as more people survive into later life, they also experience the diseases of old age, including type 2 diabetes (T2D), cardiovascular diseases (CVD) and cancer. Developing new ways to improve health in the elderly is therefore a top priority for biomedical research. Although our understanding of the molecular basis of these morbidities has advanced rapidly, effective novel treatments are still lacking. Alternative drug development strategies are now being explored, such as the repurposing of existing drugs used to treat other diseases. This can save a considerable amount of time and money since the pharmacokinetics, pharmacodynamics and safety profiles of these drugs are already established, effectively enabling preclinical studies to be bypassed. Metformin (1, 1-dimethylbiguanide hydrochloride) has been widely used to treat type 2 diabetes since the 1950s[1], and is currently the drug of choice recommended by the American Diabetes Association and the European Association for the Study of Diabetes[2]. Although the detailed mechanisms underlying the metabolic effects of metformin have not been completely elucidated, the most commonly accepted mechanism is activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK[3,4]). AMPK is a highly conserved serine/threonine protein kinase composed of a catalytic a subunit and two regulatory *b* and *c* subunits, and is activated by an increased AMP: adenosine triphosphate (ATP) ratio in metabolic stress conditions, such as hypoxia or glucose deprivation[5]. Thus, AMPK can act as a sensor of cellular energy levels. However, recent studies have also suggested AMPK-independent pathways as important mechanisms of action of metformin[6].

The present review provides a thorough and detailed account of our current understanding of the molecular pharmacology and signalling mechanisms underlying biguanide–protein interactions. It also focuses on the key role of the microbiota in regulating age-associated morbidities and a potential role for metformin to modulate its function. Research in this area holds the key to solving many of the mysteries of our current understanding of drug action and concerted effects to provide sustained and long-life health.

METFORMIN AND TYPE 2 DIABETES MELLITUS (T2DM)

According to the latest global estimates, there are 382 million people who are currently living with diabetes[7]. It is a disorder with a complex aetiology involving interactions between multiple genetic and environmental factors. Strong predictors include family history, increased body-mass index,

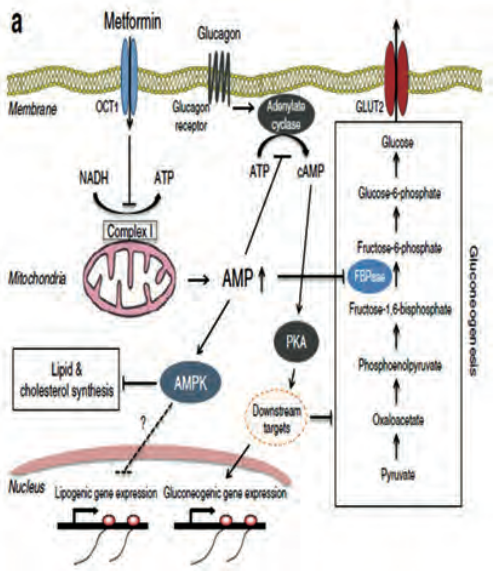
high blood pressure, physical inactivity, poor diet and advancing age[8]. In the long-term, T2D can give rise to several disabling and life-threatening complications such as CVD, neuropathy, retinopathy and nephropathy. The onset of these complications can be prevented or significantly delayed by effective management of blood glucose levels, which is achieved through lifestyle modifications and, in many cases, the use of oral antihyperglycaemic agents such as metformin. The drug's anti-hyperglycaemic effect has been partly attributed to increased hepatic insulin sensitivity and elevated uptake of glucose in peripheral tissues; however, it is now widely accepted that metformin acts predominantly via suppression of hepatic gluconeogenesis. It has been reported that metformin can lower the rate of gluconeogenesis by as much as 36% in diabetic patients[9].

MOLECULAR TARGET OF METFORMIN

Metformin is unable to passively diffuse through the cell membrane due to its unusually hydrophilic nature, and therefore, must rely on members of the organic cation transporter (OCT) family for uptake into hepatocytes. Specifically, OCT1 has been shown to be essential for the therapeutic efficacy of metformin and it has been suggested that genetic polymorphisms in human OCT1 may contribute to variation in clinical response to the drug[10]. Once it has entered the hepatocyte, metformin accumulates within the mitochondrial matrix. It is likely that this uptake results from the positively-charged molecule being driven by the membrane potential of energized mitochondria[11]. Additionally, interactions may take place between metformin's apolar hydrocarbon side chain and the hydrophobic phospholipids of the mitochondrial membrane[12]. It is generally agreed that complex I of the mitochondrial respiratory chain is a key target of metformin. This stems from the work of two independent research groups who reported that metformin selectively inhibits the oxidation of complex I substrates but not complex II or IV substrates[13]. Although these findings were first observed in isolated rat hepatocytes, similar results have since been obtained in numerous cell models including primary human hepatocytes[14].

AMPK-DEPENDENT MECHANISM

AMPK is a master regulator of cellular energy homoeostasis that is activated by the binding of an ADP or AMP molecule to a site on its regulatory γ –subunit[15,16]. This enables the cell to respond to falling energy status by transforming it from an ATP consuming anabolic state into an ATP-producing catabolic state. It was suggested that this LKB1/AMPK pathway, activated by metformin, alters the cell's gluconeogenic programme via inhibition of cAMP response element-binding protein (CREB) - regulated transcription coactivator 2 (CRTC2), a pivotal regulator of gluconeogenic gene expression[17].



Despite showing initial promise, AMPK's status as the major mediator of metformin's action was seriously undermined following the publication of work carried out by Foretz et al[18].

The prevailing hypothesis is that metformin activates AMPK by increasing ADP/AMP through inhibition of mitochondrial respiration; however, alternative models have been proposed. For example, it has been argued that the metabolic alterations induced by metformin in isolated skeletal muscle cells are not consistent with the interruption of mitochondrial energy supply; instead, they better reflect direct inhibition of the enzyme AMP deaminase[19]. This too would result in increased AMP levels and activation of AMPK. However, the results

of the present study have been called into question due to the very high concentrations of metformin used[16]. It has also been claimed that AMPK itself is a direct target of metformin.

AMPK-INDEPENDENT ACTIONS

With doubts being cast on AMPK's status as the central mediator of metformin action, several AMPK-independent mechanisms have been proposed. One alternative explanation is that the associated change in cellular energy charge directly modulates glucose output. Gluconeogenesis is an energetically demanding process requiring six ATP equivalents for every molecule of glucose synthesized[20]. Since metformin treatment causes ATP levels to fall, hepatocytes must respond by reducing glucose production accordingly. Indeed, Foretz et al[18] demonstrated that reduction in ATP content and inhibition of glucose production were strongly correlated in mouse primary hepatocytes incubated with metformin, underscoring the close link between hepatic energy status and glucose output.

Additionally, metformin-induced changes in cellular energy status may suppress gluconeogenesis via the allosteric inhibition of essential enzymes. For example, AMP is capable of synergizing with fructose 2,6-bisphosphate to inhibit the key gluconeogenic enzyme fructose 1,6-bisphosphatase.

An alternative AMPK-independent mechanism of metformin action has recently been put forward by Miller et al[21] that involves antagonism of glucagon signalling. The results of a series of in vivo and in vitro experiments on mouse primary hepatocytes have shown that metformin and the related biguanide phenformin block the glucagon-induced activation of adenylate cyclase leading to a reduction in cAMP synthesis[21]. This in turn lowers protein kinase A (PKA) activity, abrogating phosphorylation of critical substrates that enhance gluconeogenesis such as 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1, CREB-1 and inositol trisphosphate receptor. It has been proposed that the metformin-associated rise in cellular AMP levels is responsible for the inhibition of adenylate cyclase, possibly due to the direct binding AMP's adenine moiety to an inhibitory 'P-site'[21].

NOVEL PATHWAYS OF METFORMIN ACTIONS

- Mitochondria
- Intestinal microbiota
- Skeletal muscles
- Glucagon signalling
- Endoplasmic reticulum
- Anti-cancer action

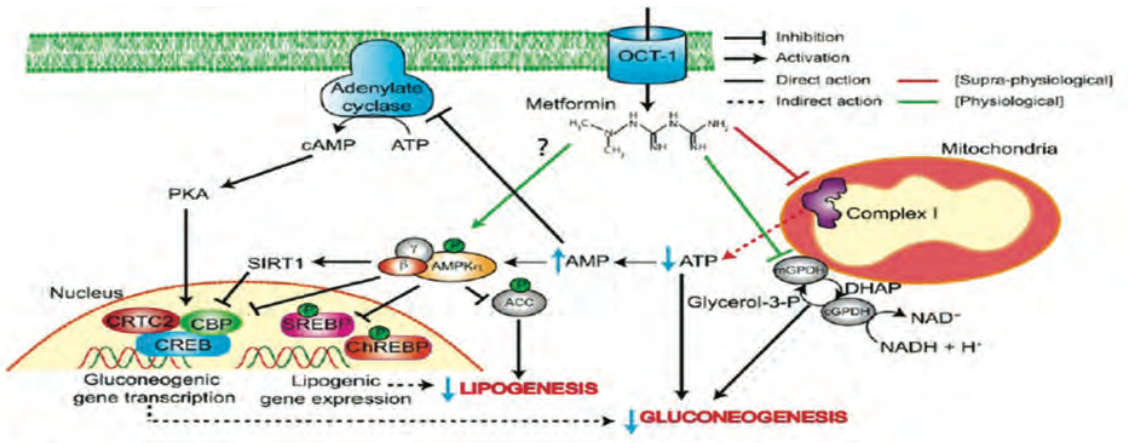


Figure 1 Proposed mechanisms of metformin action in T2D

Metformin enters the hepatocyte through OCT1 and accumulates in the mitochondria where it inhibits complex I. This leads to a reduction in ATP and concomitant rise in AMP. Elevated AMP levels lead to activation of AMPK, although metformin may also promote AMPK activation in a direct manner. AMPK inhibits gluconeogenic gene transcription by preventing formation of the CREB–CBP–CRTC2 complex, both directly and via SIRT1. Furthermore, AMPK inhibits lipogenesis through ACC, ChREBP and SREBP phosphorylation, which helps to improve insulin sensitivity. Several AMPK-independent mechanisms of metformin action also exist. The reduction in cellular energy status can directly inhibit gluconeogenic flux. Additionally, increased AMP has an inhibitory effect on adenylate cyclase leading to decreased cAMP production. This in turn reduces the activity of PKA and its downstream targets, which include CREB. Metformin also inhibits mGPD. This prevents glycerol from contributing to gluconeogenesis and increases the cytosolic redox state, which in turn makes the conversion of lactate to pyruvate unfavourable thus limiting the use of lactate as a gluconeogenic substrate.

MITOCHONDRIAL STRESS AND METFORMIN

The inhibition of mitochondrial complex activity by metformin might be a mechanism of metformin-induced AMPK activation[22], as intracellular ATP levels are decreased by the inhibition of mitochondrial complex activity and AMP levels are increased by the action of adenylate kinase converting two molecules of adenosine diphosphate (ADP) to ATP and AMP (Figure 1). AMP molecules can then bind to the c subunit of AMPK and activate AMPK activity directly or by inhibiting dephosphorylation of AMPK phosphorylated by liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase-b (CAMKKb)[23].

Mitochondrial stress can affect tissue metabolism independent of AMPK. Specifically, mitochondrial stress has been shown to initiate an integrated stress response (ISR)[24]11 through activating transcription factor 4 (ATF4) to induce fibroblast metabolic profile associated with obesity or lipid injury as a 'mitokine'[25] 12. A recent investigation examined whether metformin could induce a similar ISR by inducing mitochondrial stress. As hypothesized, metformin was able to induce the expression of FGF21 through the double-stranded ribonucleic acid-activated protein kinase-like endoplasmic reticulum (ER) kinase (PERK)-eukaryotic initiation factor 2a–ATF4 axis in hepatocytes, which was attributed to the inhibition of mitochondrial complex I activity[26] 13. Metformin-induced FGF21 expression was still observed in AMPKa1-dominant negative transfectants or AMPKa1a2-null mouse embryonic fibroblast cells, suggesting an AMPK-independent ISR leading to FGF21 induction. Treatment with (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride monohydrate (MitoTempo), a mitochondrial reactive oxygen species (ROS)-specific quencher not only reversed mitochondrial ROS production by metformin, but also attenuated FGF21 induction after metformin treatment, supporting the role of mitochondrial stress or mitochondrial ROS in the induction of FGF21. Serum levels of FGF21 were increased by in vivo administration of metformin in mice, suggesting the contribution of FGF2 in the metabolic effect of metformin administration in vivo. Finally, serum FGF21 levels were increased in patients with type 2 diabetes after metformin therapy for 6 months, supporting the possible role of FGF21 induction in metabolic improvement by metformin administration to human patients with diabetes[26].

MITOCHONDRIAL SHUTTLE AND METFORMIN

One of the main metabolic features of metformin is its ability to reduce hepatic glucose production[27]. A recent study suggested that inhibition of mitochondrial glycerophosphate dehydrogenase (mGPD), a critical enzyme in the glycerophosphate shuttle, could be the primary mechanism of metformin induced inhibition of gluconeogenesis[27]. Specifically, the glycerophosphate shuttle together with the malate-aspartate shuttle allows a cytoplasmic reduced form of nicotinamide adenine dinucleotide (NADH) generated by glycolysis to enter mitochondria for production of ATP and regeneration of cytoplasmic NAD+. The inhibition of the mitochondrial shuttle leads to the increased cytosolic redox state and decreased mitochondrial redox state. Thus, an increased cytosolic redox state could impair conversion of lactate to pyruvate by lactate dehydrogenase, leading to decreased gluconeogenesis and accumulation of lactate. The latter effect is frequently observed in animals and humans treated with metformin, and could be the cause of lactic acidosis, a well-known side-effect of metformin. Gluconeogenesis from glycerol can also be impaired, as conversion from glycerol-3-phosphate to dihydroxyacetone phosphate by mGPD in the mitochondrial matrix, a necessary step for gluconeogenesis from glycerol, is inhibited by metformin[27]. This finding could represent a novel mechanism of metformin that can explain its ability to inhibit gluconeogenesis and lactate overproduction, although it is not clear whether the inhibition of the glycerophosphate shuttle, which represents only a small portion of ATP production, can lead to significant changes in the cellular redox state[28]. These results might also potentially contribute to the identification of new molecular targets for development of a novel class of antidiabetic agents.

GLUCAGON AND METFORMIN

Another novel mechanism explaining decreased gluconeogenesis by metformin was recently proposed. Metformin was shown to inhibit glucagon signal transduction by decreasing 30-50-cyclic adenosine monophosphate (cAMP) production in hepatocytes[29]. Decreased cAMP content leads to decreased activity of both cAMP-dependent protein kinase A, an important signal transducer of glucagon action and glucagon-induced gluconeogenesis. Decreased cAMP was attributed to the direct inhibition of adenylate cyclase by increased intracellular AMP content after metformin treatment rather than AMPK activation. Increased AMP content could be a result of the aforementioned inhibition of mitochondrial complex I activity and reduced hepatic energy charge by metformin treatment. Together, these results suggest a novel mechanism of metformin action related to glucagon signalling, and a potential role of adenylate cyclase as a new therapeutic target for the treatment of type 2 diabetes.

INTESTINAL MICROBIOTA AND METFORMIN

Existing data suggest that gut microbiota play an important role in the control of energy balance by extracting energy from ingested food[30]. Intestinal microbiota also plays a crucial role in the maturation of gut immunity and maintenance of immune homeostasis[31] . The human gut microbiota comprises 10–100 trillion microorganisms of more than 1,000 species[32, 33]. Furthermore, recent studies have shown that changes in gut microbiota could be important in the pathogenesis of the obese and diabetic phenotypes. For example, germ-free mice are protected against diet-induced obesity, which is accompanied by increased levels of AMPK activity in the liver or muscle tissue and derepression of fasting-induced adipose factor (Fiaf) [30, 34]. As Fiaf is an inhibitor of lipoprotein lipase, Fiaf could inhibit the storage of lipid in adipose tissue in germ-free mice. In addition, obesity and high-fat diets are associated with a significant increase in the relative abundance of the Firmicutes phylum and decrease in the Bacteroidetes phylum[35,36]. Furthermore, transplantation of gut microbiota from obese mice to germ-free mice leads to a significant increase in body fat content and insulin resistance compared with those from lean mice[37].

Previous studies have shown that the intestines play a significant role in the glucose-lowering effect of metformin by facilitating uptake and utilization of glucose[38, 39]. The concentration of metformin reaches a higher level in the intestinal mucosa compared with other tissues, which might be related to the adverse effects of metformin on the gastrointestinal tract. Based on the significant potential impact of metformin on the intestine, whether metformin affects the gut microbiota was investigated, and also if the metabolic effects of metformin are related to changes in the gut microbiota. When microbiota abundance was studied using 16S ribosomal ribonucleic acid pyrosequencing, marked changes in microbiota composition by metformin treatment were observed, particularly in high-fat diet (HFD)-fed conditions, suggesting a possible interaction between HFD, metformin and intestinal microbiota.

INCRETIN AND METFORMIN

Incretins are a group of gastrointestinal hormones that increase insulin release after food ingestion, and comprise glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide. Incretin-based therapies have recently been introduced in clinical practice, where they are used to achieve improved glycemic control without weight gain. Additionally, those therapies have potential long-term beneficial effects on islet b-cell mass and function[40, 41]. In particular, incretin + metformin combination has become a popular treatment. In this regard, a study exploring the relationship between the action mechanisms of metformin and incretin was undertaken[42], which was based on the previous observation of increased plasma GLP-1 levels in obese individuals and diabetic patients treated with metformin[43, 44]. That study confirmed that metformin administration increases plasma levels of GLP-1, but not that of gastric inhibitory peptide or peptide YY, which co-localizes with GLP-1 in intestinal L cells. Increased GLP-1 levels after metformin treatment were not related to the inhibition of dipeptidyl peptidase- 4 that

degrades incretins or induction of proglucagon gene expression. Furthermore, metformin might not be a direct secretagogue of GLP-1 from intestinal L cells[45] Intriguingly, metformin has been reported to increase GLP-1 receptor expression on islet cells, which was dependent on peroxisome proliferator-activated receptors pathway, but not on AMPK activation[42]. These results provide a theoretical basis for combination therapies using metformin and incretins (or dipeptidyl peptidase-4 inhibitors that increase incretin levels), as induction of GLP-1 receptor expression by metformin can have synergistic effects with administered incretins.

AUTOPHAGY AND METFORMIN

Metformin can enhance autophagy, as AMPK activation is known to upregulate autophagic activity through direct phosphorylation of unc-51-like kinase and Beclin 1, key molecules involved in the initiation of autophagy[46]. Autophagy is a process of subcellular membrane rearrangement to form a double-membraned auto-phagosome enclosing cytoplasmic constituents and organelles, which is expedited by nutrient deficiency[47]. Thus, autophagy is important for nutrient supply in the case of energy deficiency, and is also critical for the proper turnover and function of organelles, such as mitochondria and the ER. As mitochondria and the ER play critical roles in pancreatic b-cell physiology and insulin sensitivity[48, 49]. Autophagy has a significant impact on body metabolism. Although the effects of autophagy deficiency on the body metabolism are distinct, depending on the location and severity of autophagy deficiency[50], a global increase in autophagic activity is likely to improve the metabolic profile under metabolic stress conditions [51, 52] which might be related to attenuation of low-grade tissue inflammation associated with obesity by autophagy activation [53].

METFORMIN AND CANCER

Cancer is a leading cause of morbidity and mortality worldwide. A recent lifetime risk analysis of the British population found that over 50% of adults below the age of 65 will be diagnosed with the disease at some point in their life[54]. Sobering projections like this have stimulated an enormous amount of investment into cancer research and rational drug design. Despite our best efforts, only 5% of oncology drugs entering phase I trials eventually receive approval and it has been argued that so far, targeted therapies have provided only modest survival benefits[55]. Metformin garnered considerable interest within the field of oncology in 2005 following the publication of an epidemiological report highlighting a link between metformin treatment and reduced cancer risk in diabetic patients[56]. This finding has stimulated a great deal of further research and numerous observational studies have supported a protective role for metformin against a variety of cancer types including liver, colorectal, pancreas, stomach and oesophagus cancer in diabetics[57]. However, it is important to note that such studies are prone to bias and confounding factors and contrastingly, meta-analyses of randomized controlled trials do not appear to demonstrate a significant effect of metformin on cancer outcomes[57,58]. Furthermore, it is not clear whether or not any positive results could be extrapolated to non-diabetic individuals. Nevertheless, increasing importance is being placed on the role of altered metabolism in cancer and the ability of metformin to interact with several metabolic pathways suggests that it could be effective at preventing the development and progression of this disease.

It is possible that the systemic effects of metformin could be protective against cancer. Both experimental and epidemiological evidence suggests that insulin and insulin-like growth factor 1 (IGF-1) can promote tumorigenesis by stimulating the proliferation of epithelial cells[59]. Metformin may prevent such neoplastic activity by reducing hyperinsulinaemia and lowering levels of these signalling molecules. Metformin can also modify inflammatory processes known to play a role in cancer progression. For example, it has been reported that metformin blocks the activity of the transcription factor nuclear factor-Kb (NF-κB) resulting in decreased secretion of pro-inflammatory cytokines by senescent cells[60].

CONCLUSION

Metformin has been used as a safe and effective treatment for T2DM for over half a century. Yet the precise mechanism of action of this drug still remains elusive. The anti-hyperglycaemic properties of metformin are chiefly mediated by the suppression of hepatic gluconeogenesis and it is generally accepted that this is achieved via inhibition of complex I in the mitochondrial respiratory chain. The subsequent reduction in cellular energy status has been shown to directly impede gluconeogenic flux, interfere with glucagon signalling and promote activation of the major metabolic regulator AMPK, although which of these make the greatest contribution to metformin's therapeutic effects is a subject of debate. Moreover, it is becoming increasingly apparent that complex I is not the only molecular target of metformin; for example, it has recently been confirmed that metformin inhibits mGPD. It is highly probable that additional targets will be identified in the near future. Work on C. elegans serves as a reminder that it is necessary to consider the effect of drugs not only on the individual but also on their microbiota. Very little is currently known about the bacterial targets of metformin and it is possible that the microbiota could regulate some of its effects on host physiology via unknown mechanisms. This undoubtedly warrants further investigation. Currently, metformin is only approved as a treatment for T2D, however in recent years, a vast number of studies have highlighted the therapeutic potential of metformin in the context of other diseases. In particular, metformin has shown promise as a treatment for CVD and cancer. The various mechanisms proposed to account for these beneficial effects have been outlined in the present review, although there are a number of outstanding issues that must still be resolved. Ultimately, a greater understanding of the molecular pathways involved will help to guide novel applications of metformin.

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"I have never let my schooling interfere with my education."
— Mark Twain