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REVIEW

Molecular mechanisms of action of metformin: latest advances and therapeutic implications

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Abstract

Metformin is among the most widely used antidiabetic drugs. Studies over the past few years have identified multiple novel molecular targets and pathways that metformin acts on to exert its beneficial effects in treating type 2 diabetes as well as other disorders involving dysregulated inflammation and redox homeostasis. In this mini-review, we discuss the latest cutting-edge research discoveries on novel molecular targets of metformin in glycemic control, cardiovascular protection, cancer intervention, anti-inflammation, antiaging, and weight control. Identification of these novel targets and pathways not only deepens our understanding of the molecular mechanisms by which metformin exerts diverse beneficial biological effects, but also provides opportunities for developing new mechanistically based drugs for human diseases.

Keywords Cardiovascular disease · Diabetes · Inflammation · Metformin · Mitochondria · Redox signaling

Abbreviations

AMPK	Adenosine monophosphate-activated kinase
ERAD	Endoplasmic reticulum-associated protein degradation
GDF15	Growth differentiation factor 15
GPD2	Mitochondrial glycerol-3-phosphate dehydrogenase
HNF4α	Hepatocyte nuclear factor 4 alpha
IL	Interleukin
METC	Mitochondrial electron transport chain
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
ORAC	Calcium release-activated channel
ox-mtDNA	Oxidized mitochondrial DNA
PM	Particulate matter
ROS	Reactive oxygen species

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Overview

Metformin (1,1-dimethylbiguanide) (structure in Fig. 1) improves glucose tolerance in patients with type 2 diabetes mellitus (diabetes thereafter for simplicity), lowering both basal and postprandial plasma glucose. It decreases hepatic glucose production and intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. Owing to its efficacy in lowering blood sugar and favorable safety profile, metformin is among the most widely prescribed antidiabetic drugs and currently remains the first-line therapy for type 2 diabetes [1]. In addition to its role in glycemic control in type 2 diabetes, metformin also exerts beneficial effects in other diseases and conditions, including cardiovascular disorders, cancer, and aging [2–4]. The pleiotropic biological activities of metformin suggest that the drug may affect multiple cellular processes. Indeed, studies over the past decades have identified multiple cellular targets on which metformin acts to cause its pharmacological effects [2–4]. Among them, activation of adenosine monophosphate (AMP)-activated kinase (AMPK) pathway and modulation of mitochondrial metabolism have been widely considered the two primary mechanisms underlying the diverse beneficial effects of metformin, including

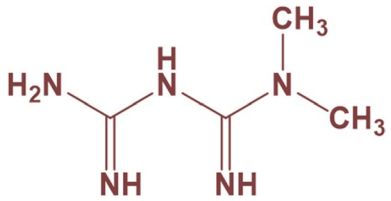

Fig. 1 Chemical structure of metformin (1,2-dimethylbiguanide)

glycemic control, cardiovascular protection, as well as anticancer and antiaging activities.

Despite the wide acceptance of AMPK activation and mitochondrial modulation as the major mechanisms of action of metformin, the detailed signaling pathways surrounding these two mechanistic components remain partly understood. Studies over the past few years have begun to delineate novel signaling events both upstream and downstream of AMPK activation and mitochondrial modulation. Moreover, the latest studies have also revealed additional novel molecular targets of metformin's action. These innovative studies provide important insights into the detailed molecular mechanisms of action of metformin, thereby expanding its therapeutic opportunities in the management of diverse pathological conditions. Accordingly, in this article, we provide a concise review of recent cutting-edge findings on novel molecular targets of metformin reported in influential journals rather than surveying all research findings in the literature.

Novel molecular targets involved in glycemic control

Inhibition of hepatic gluconeogenesis is a major action of metformin in lowering blood glucose. Two major mechanistic pathways have been identified for metformin's glucose-lowering efficacy: (1) AMPK activation and (2) cellular redox modulation. In addition, as discussed below, a microRNA-dependent pathway has recently been discovered.

AMPK activation

How metformin activates AMPK remains unclear. A widely recognized theory is that metformin activates AMPK via inhibiting the complex I of the mitochondrial electron transport chain (METC). Inhibition of METC complex I decreases the production of adenosine triphosphate (ATP), thereby increasing AMP levels. The elevated AMP levels activate AMPK, leading to the downregulation of gluconeogenic enzymes and the consequent inhibition of hepatic gluconeogenesis [2]. A major challenge to the above "METC complex I/AMP/AMPK" axis is the requirement of millimolar concentrations of metformin for inhibiting the METC complex I. Such millimolar concentrations are unlikely to be achieved with clinical doses of metformin in most cell types [5] except enterocytes, where millimolar concentrations of metformin are observed following oral or intravenous administration of clinical doses of metformin [6, 7].

Recently, using clinically relevant concentrations (or doses) of metformin, Ma et al. demonstrated that activation of AMPK by metformin occurs via inhibiting lysosomal proton pump v-ATPase [8]. Ma et al. further identified PEN2, a subunit of γ-secretase7, as a binding partner of metformin. They showed that metformin-bound PEN2 forms a complex with ATP6AP1, a subunit of the v-ATPase, leading to the inhibition of v-ATPase and the activation of AMPK [8]. Notably, using in vivo models, Ma et al. demonstrated that liver-specific knockout of PEN2 abolishes metformin-mediated reduction of hepatic fat content and glucose tolerance, whereas intestine-specific knockout of PEN2 impairs the glucose-lowering effects of metformin in high-fat diet-induced obese mice [8]. These findings identify PEN2-dependent AMPK activation in the gut as an important mechanism for metformin's glucose-lowering activity. This notion is also in line with an early study showing that metformin activates a duodenal AMPK-dependent pathway to lower hepatic glucose production in rats [9]. In addition, Ma et al. showed knockdown of PEN2 in *C. elegans* also abrogates metformin-induced extension of lifespan [8].

Taken together, the above novel findings by Ma et al. [8] revealed PEN2 as a direct molecular target for metformin to eventually activate AMPK and exert its clinical benefits in treating diabetes and possibly aging intervention as well ("Antiaging activity" section for more discussion on metformin's antiaging activity). This newly discovered lysosomal axis of "metformin/PEN2/ATP6AP1/v-ATPase/AMPK", on the one hand, delineates a novel AMP-independent mechanism for AMPK activation. On the other hand, it also provides a novel target for developing new drugs for diabetes as well as other pathological conditions impacted by AMPK signaling.

Cellular redox modulation, an AMPK-independent mechanism

Metformin's glucose-lowering activity may also occur via mechanisms independent of AMPK activation. An early study by Madiraju et al. [10] showed that metformin, at clinically relevant concentrations, non-competitively inhibits the redox shuttle enzyme—mitochondrial glycerol-3-phosphate dehydrogenase (GPD2). This results in an altered hepatocellular redox state, reduced conversion of lactate and glycerol to glucose, and decreased hepatic gluconeogenesis. Antisense oligonucleotide-mediated knockdown of hepatic

mitochondrial GPD2 in rats results in a phenotype akin to chronic metformin treatment, and abrogates metformin-induced increases in cytosolic redox state, decreases in plasma glucose concentrations, and inhibition of endogenous glucose production. Likewise, similar changes are observed in GPD2-knockout mice [10]. Hence, the findings by Madiraju et al. suggest a cellular redox mechanism for metformin's glucose-lowering effect.

In a subsequent study using ^{13}C positional isotopomer tracer analyses [11], Madiraju et al. further showed that clinically relevant concentrations of plasma metformin achieved by acute intravenous, acute intraportal, or chronic oral administration in awake normal and diabetic rats inhibit gluconeogenesis from lactate and glycerol, but not from pyruvate and alanine, reaffirming an elevated cytosolic redox state (as reflected by an increased ratio of cytosolic lactate to pyruvate) in mediating metformin's antihyperglycemic effect. Notably, the study showed that the above metformin-mediated effects occur independent of METC complex I inhibition as evidenced by unaltered hepatic energy charge (ratios of ATP/ADP and ATP/AMP) and citrate synthase flux [11].

The above studies conclusively demonstrate an important role for GPD2-dependent cytosolic redox state in metformin's inhibition of hepatic glucose production. In a more recent study [12], LaMoia et al. determined how metformin treatment causes GPD2 inhibition. They showed that metformin at clinically relevant concentrations reduces GPD2 activity via inhibiting METC complex IV instead of complex I. Specifically, metformin directly interacts with METC complex IV to reduce its enzymatic activity, leading to indirect inhibition of GPD2, increased cytosolic redox, and reduced glycerol-derived gluconeogenesis both in vitro and in vivo. Notably, selective inhibition of METC complex I with pieridicin A does not replicate any of the above effects in vitro or in vivo, thus making METC complex I unlikely a clinically meaningful target for metformin action [12]. While how exactly inhibition of METC complex IV by metformin reduces GPD2 activity remains to be defined, LaMoia et al. proposed that metformin binds to and inhibits METC complex IV, and this causes a backlog of the METC, leading to a decrease in the ubiquinone pool. As ubiquinone serves as the electron acceptor for GPD2, decreased ubiquinone results in indirect inhibition of GPD2 [12]. Further investigation is warranted to delineate this novel "complex IV/ubiquinone/GPD2" pathway and determine the glucose-lowering activities of other agents that inhibit METC complex IV.

MicroRNA modulation, another AMPK-independent mechanism

The evolutionarily conserved transcription factor hepatocyte nuclear factor 4 alpha (HNF4α) plays an important role in promoting hepatic glucose production and regulating energy balance [13]. In a previous study, Huang and associates reported that the DNA demethylase TET3 binds to and demethylates the P2 promoter of HNF4α, leading to increased transcription of the P2 isoform of HNF4α (known as HNF4α-P2) and a consequently abnormally augmented hepatic glucose production that underlies the chronic hyperglycemia in type 2 diabetes [14]. More recently, the same group, using mouse models and human primary hepatocytes, demonstrated that metformin, at clinically relevant doses, suppresses hepatic glucose production by activating a conserved regulatory pathway encompassing let-7, TET3, and HNF4α-P2 [15]. Specifically, metformin upregulates microRNA let-7, which in turn downregulates TET3. TET3 downregulation suppresses the TET3/HNF4α-P2 axis, thereby leading to decreased hepatic glucose production [15]. Notably, hepatic delivery of let-7 ameliorates hyperglycemia and improves glucose homeostasis in diabetic mice, whereas liver-specific inhibition of let-7 abrogates these beneficial effects of metformin. Furthermore, let-7 overexpression decreases glucose production by primary hepatocytes isolated from obese humans [15].

Collectively, the above novel findings identify another AMPK-independent, microRNA-mediated mechanism by which metformin acts to control hyperglycemia in type 2 diabetes. How metformin upregulates let-7 expression remains to be defined. Preliminary evidence suggests that metformin may alter the ratio of the cytosolic redox couples of glutathione (GSH) and glutathione disulfide (GSSG) [15]. As let-7 shows a promising efficacy in improving glucose homeostasis, further studies on the upregulation of let-7 expression by other redox-active drugs may advance the treatment of metabolic disorders involving dysregulated glucose metabolism.

In addition to the above described novel signaling pathways both upstream and downstream of metformin-mediated AMPK activation, a well-established downstream target of AMPK signaling is the mammalian target of rapamycin (mTOR), which is inhibited following metformin-mediated activation of AMPK [16]. mTOR is a major nutrient-sensitive regulator of growth in animals and plays important roles in diverse biological processes, including glucose homeostasis [17]. Early studies in rodents suggested that inhibition of small intestinal mTOR signaling either by administering rapamycin or via a genetic approach decreases glucose production and lowers blood glucose levels. On the other hand, molecular activation of the small intestinal mTOR signaling blunts the blood glucose-lowering effect of metformin [18]. However, it remains to be determined if mTOR inhibition is also a significant mechanism underlying metformin's glucose-lowering efficacy in treating diabetes.

Novel molecular targets involved in cardiovascular protection

Extensive studies in animal models suggested an important role for metformin in protecting against diverse cardiovascular disorders, including atherosclerosis, hypertension, myocardial ischemic injury, and heart failure (reviewed in [2]), as well as cardiovascular toxicity induced by drugs and environmental pollutants [19, 20]. Findings from multiple observational studies and randomized clinical trials also suggested a cardiovascular protective benefit of metformin in both diabetic and non-diabetic patients [21–23].

As noted earlier, metformin is a multitasking drug and affects numerous cellular targets and pathways to exert its glucose-lowering effects as well as other metabolic effects, such as immune modulation, suppression of proinflammatory responses, inhibition of oxidative stress, and attenuation of mTOR-mediated cell hypertrophy [2]. These beneficial effects all likely contribute to metformin's efficacy in protecting against cardiovascular and related metabolic disorders. Mechanistically, the cardiovascular protective effects of metformin are believed to result primarily from its ability to activate the AMPK-dependent signaling pathway [2]. Indeed, AMPK signaling plays key roles in maintaining physiological homeostasis of cardiovascular system via controlling inflammation and oxidative stress, as well as mTOR signaling, among others [24].

Recent studies have identified several novel AMPK-dependent and AMPK-independent signaling pathways underlying metformin's cardiovascular protection. Among them, the most notable are (1) AMPK/Sirt3/Nrf2, (2) AMPK/autophagy, and (3) METC ROS/CRAC/IL-6 axes. The sections below discuss the latest research studies leading to the discoveries of the above molecular targets.

AMPK/Sirt3/Nrf2 signaling

Sirtuins are a family of signaling proteins involved in metabolic regulation, and mammalian sirtuins include 7 members: Sirt 1–7. Sirtuins are protective molecules in metabolic and aging-related pathologies, including cardiovascular disorders [25].

An early study by Tang et al. showed that Sirt2 represses aging-related and stress-induced cardiac hypertrophy in mice, at least in part, by maintaining signaling through the liver kinase B1 (LKB1)/AMPK pathway. Notably, they found that Sirt2-knockout attenuates metformin-induced activation of AMPK signaling and, consequently, the cardioprotective effects of metformin in cardiac hypertrophy [26]. Likewise, Sirt3/AMPK activation by metformin also improves hyperglycemia and normalizes pulmonary hypertension associated with heart failure with preserved ejection fraction in rats [27]. These findings suggest that both Sirt2 and Sirt3 may mediate, at least partly, metformin-induced AMPK activation and cardioprotection.

More recently, using a mouse model of salt-induced hepatic inflammation and accompanied hypertension and cardiac dysfunction, Gao et al. delineated a novel mechanism involving Nrf2-regulated Sirt3 expression in metformin's cardiovascular protection [28]. Specifically, Gao et al. showed that persistent hepatic steatosis and inflammation are critical for hypertension and cardiac dysfunction in response to long-term high-salt diet in mice. The high-salt diet increases acetylated histone 3 lysine 27 (H3K27ac) on Sirt3 promoter in hepatocytes, thus inhibiting the binding of Nrf2, and resulting in the sustained inhibition of Sirt3 expression. Notably, the study demonstrated that treatment with metformin ameliorates high-salt diet-induced hepatic inflammation and cardiovascular damage. Mechanistically, activation of AMPK by metformin decreases H3K27ac level on Sirt3 promoter and thereby increases Nrf2 binding ability to activate Sirt3 expression. This Nrf2-augmented Sirt3 expression is critical for metformin's cardiovascular protection [28]. Hence, the study by Gao et al. identifies AMPK/Sirt3/Nrf2 as an important mechanism underlying metformin's ability to protect against cardiovascular damage resulting from high-salt diet-induced persistent hepatic inflammation. The study also for the first time shows an important role of Nrf2 signaling in metformin's cardiovascular protection. These findings further strengthen the notion that sirtuins are intrinsic cardiovascular protective factors and activation of these molecules may represent an important strategy for cardiovascular protection.

AMPK/autophagy axis

Autophagy is a crucial cellular machinery for removal of aggregated proteins and damaged organelles, and dysregulated autophagy is implicated in many disease states, including cardiovascular disorders [29–31]. An early study in diabetic mice showed that decreased AMPK activity and subsequent reduction in cardiac autophagy are important events in the development of diabetic cardiomyopathy [32]. Importantly, chronic AMPK activation by metformin (200 mg/kg per day for 4 weeks) prevents cardiomyopathy by upregulating autophagy activity in diabetic mice [32].

A recent study showed that metformin also protects against carfilzomib-induced cardiotoxicity in mice via activating the AMPK/autophagy pathway [20]. Specifically, carfilzomib, a proteasome-inhibiting drug for treating multiple myeloma, induces left ventricular dysfunction by inhibition of AMPK/autophagy pathway, and restoration of the suppressed AMPK/autophagy axis by metformin (140 mg/kg daily) preserves left ventricular function in mice [20].

Metformin suppresses atherosclerosis in animal models via AMPK activation [33, 34]. More recently, Robichaud et al. reported a novel autophagy-dependent mechanism for metformin's protection against atherosclerosis in a mouse model [35]. They found that autophagy-mediated cholesterol efflux is markedly reduced in vascular smooth muscle cell (VSMC)-derived foam cells compared with macrophage-derived foam cells in atherosclerotic lesions. Notably, treatment with metformin improves autophagy-mediated cholesterol efflux in VSMC-derived foam cells, but not in macrophage-derived foam cells [35]. As VSMC-derived foam cells are the predominant foam cell type in advanced atherosclerotic plaques [36], the above finding by Robichaud et al. yields important insight into the molecular mechanisms underlying metformin's atherosclerotic protection.

METC ROS/CRAC/IL-6 pathway

As described earlier, both complexes I and IV of METC are potential targets of metformin action in reducing hepatic glucose production. While complex I inhibition is believed to cause increased AMP levels and subsequent activation of AMPK, inhibition of complex IV by metformin alters cellular redox state and reduces glucose production independent of AMPK activation [12]. Likewise, an AMPK-independent, METC- mediated pathway has been identified to underly metformin's protection against air particulate matter (PM)-induced thrombosis in mice [19]. Mechanistically, urban PM promotes arterial thrombosis via stimulating interleukin-6 (IL-6) production from alveolar macrophages in mice. Treatment of mice with a clinically relevant dose (100 mg/kg/day) of metformin or exposure of murine or human alveolar macrophages to metformin prevents the PM-induced generation of METC complex III-derived reactive oxygen species (ROS). Such ROS production is necessary for the opening of calcium release-activated channels (CRAC) and release of IL-6. Notably, targeted genetic deletion of METC or CRAC channels in alveolar macrophages in mice prevents PM-induced acceleration of arterial thrombosis [19].

Hence, the above findings reveal a novel AMPK-independent signaling pathway on which metformin target to exert its protection against vascular thrombosis. As air PM is a significant cause of human atherosclerotic cardiovascular disease [37], delineation of this novel mechanism provides a unique avenue for using metformin or related compounds to intervene air pollution-mediated cardiovascular injury.

Novel molecular targets involved in anticancer activities

Direct and indirect mechanisms

Numerous studies in animal models and multiple cohort studies in humans show an anticancer activity for metformin (reviewed in [2, 3]). Two overall mechanisms are widely recognized to explain metformin's anticancer action: indirect mechanisms and direct mechanisms. In the indirect mechanisms, metformin-mediated AMPK activation, reduced gluconeogenesis, and decreased hyperinsulinemia are believed to contribute to reduced cancer cell growth and progression following metformin treatment. Indeed, AMPK downregulation and hyperinsulinemia are important mechanisms of tumorigenesis [38–40]. On the other hand, in the direct mechanisms, it is thought that metformin, by suppressing mitochondrial activity, reduces the availability of ATP and biosynthetic precursors required for tumor cell growth [2, 3, 41]. Moreover, metformin also inhibits mTOR signaling, leading to decreased cell proliferation and tumorigenesis [42–44]. Indeed, mTOR is a critical regulator of cell anabolism and growth, and dysregulated mTOR signaling plays an important role in cancer development and progression [45].

In addition to the aforementioned mechanisms of metformin's anticancer activity, multiple recent studies have identified a novel target underlying metformin's anticancer action: the PD-L1/PD-1 axis. Modulation of this axis by metformin may occur via AMPK-dependent and AMPK-independent mechanisms (see below).

Overview of the PD-L1/PD-1 axis

Programmed death ligand-1 (PD-L1) is a crucial immune checkpoint molecule involved in immune regulation. Normally, PD-L1 is expressed in antigen-presenting cells (e.g., macrophages) and binds to cell death protein-1 (PD-1) on activated cytotoxic T cells to downregulate their activity. PD-L1 is upregulated in cancer cells and is exploited by cancer cells to evade host's immune surveillance. When PD-L1 on cancer cells binds to PD-1 on activated cytotoxic T cells infiltrating into tumors, PD-L1-induced inhibitory signal shuts down the antitumor activity of the activated T cells. As such, blocking the PD-L1/PD-1 axis has been recognized as an effective strategy for cancer immunotherapy [46, 47]. Indeed, multiple monoclonal antibody drugs targeting the PD-L1/PD-1 axis have been approved by the U.S. Food and Drug Administration (FDA) to treat various types of cancers [48]. Recent efforts are also focusing on developing small molecule drugs (including novel compounds or existing drugs) to target the PD-L1/PD-1 axis to treat cancers [46, 47, 49].

AMPK-dependent modulation of the PD-L1/PD-1 axis

Metformin is known to promote the activity of $CD8^+$ tumor-infiltrating T cells [50, 51]. As described below, multiple recent studies show that metformin promotes antitumor immunity via downregulating the PD-L1/PD-1 axis [49, 52–54]. Cha et al. first reported that metformin promotes antitumor immunity via endoplasmic-reticulum-associated degradation of PD-L1 [52]. Using in vitro and in vivo models, Cha et al. showed that metformin (5 mM in vitro and 200 mg/kg/day in vivo) increases cytotoxic T cell activity by reducing the stability and membrane localization of PD-L1. Mechanistically, metformin activates AMPK which directly phosphorylates S195 of PD-L1. S195 phosphorylation induces abnormal PD-L1 glycosylation, resulting in its accumulation in the endoplasmic reticulum (ER) and ER-associated protein degradation (ERAD). Importantly, blocking the inhibitory signal of PD-L1 by metformin enhances cytotoxic T cell activity against cancer cells. Notably, Cha et al. also found that tumor tissues from metformin-treated breast cancer patients (0.5 g/day for one week followed by 2 g/day for another week) exhibit reduced PD-L1 levels with AMPK activation, indicating the clinical relevance of their findings in preclinical models. Moreover, combination of metformin and CTLA4 (another immune checkpoint) blockage (via an anti-CTLA4 antibody) results in synergistic suppression of tumor growth in vivo [52]. These findings reveal modulation of the AMPK/PD-L1/PD-1 axis as a novel mechanism of metformin's anticancer action.

AMPK-independent modulation of the PD-L1/PD-1 axis

In addition to the above AMPK-dependent ERAD-mediated pathway, PD-L1 degradation may also occur via direct electrostatic disruption by metformin, as demonstrated by Wen et al. in a more recent study [55]. PD-L1 is a type I transmembrane protein consisting of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The cytosolic domain regulates PD-L1 degradation and stability; specifically, interaction of the basic residues of the cytosolic domain with the acidic phospholipid-enriched inner leaflet of the plasma membrane leads to decreased degradation and thereby increased stabilization of PD-L1 [56]. Wen et al. using NMR and biochemical techniques, showed that the membrane association of PD-L1 cytosolic domain is mediated by electrostatic interaction between acidic phospholipids of the inner leaflet of the plasma membrane and basic arginine residues in the N-terminal region of the cytosolic domain, and removal of such electrostatic interaction via basic-to-acidic amino acid mutations of the cytosolic domain decreases cellular levels of PD-L1. Notably, Wen et al. found that, metformin (2–5 mM), a positively charged molecule, promotes the membrane dissociation of PD-L1 cytosolic domain by disrupting the electrostatic interaction, thereby decreasing the cellular abundance of PD-L1 [56]. The study by Wen et al. shows a unique AMPK-independent chemical mechanism by which metformin downregulates the PD-L1 levels in cancer cells. This discovery may pave the way for developing new drugs targeting the above electrostatic interaction to improve the efficacy of PD-L1-based cancer immunotherapy.

Novel molecular targets involved in other biological activities

The preceding sections have discussed the novel molecular targets of metformin's actions in glycemic control, cardiovascular protection, and suppression of tumorigenesis. This section summarizes recent discoveries of novel molecular mechanisms underlying metformin's other biological activities, including anti-inflammation, antiaging, and weight control. It should be noted that the various molecular targets and mechanistic pathways described above and next are not necessarily mutually exclusive. In fact, many of them are intimately related to one another. For instance, the metformin's anti-inflammatory action to be discussed below is also involved in its protection against diverse disease states and conditions as inflammation is considered a common final pathway of major human diseases, including metabolic disorders, cardiovascular diseases, and cancers, as well as neurodegeneration and aging [57].

Anti-inflammation

The anti-inflammatory activities of metformin have been observed in various animal models and human subjects [58–60]. However, the exact mechanisms by which metformin exerts its anti-inflammatory effects remain unknown. Early studies showed that metformin inhibits STAT3-dependent proinflammatory cytokine gene expression in an AMPK/mTOR-dependent manner both in cultured cells and in vivo [61]. Recently, Xian et al. reported a novel mechanism involving the suppression of activation of NLRP3 inflammasome by oxidized mitochondrial DNA (ox-mtDNA) [62]. Specifically, Xian et al. showed that metformin at a clinically relevant concentration (0.5 mM) inhibits mitochondrial electron transport chain, causing decreased ATP-dependent mtDNA synthesis and reduced

production of cytoplasmic ox-mtDNA. The decreased ox-mtDNA (an NLRP3 ligand) in turn results in suppression of NLRP3 inflammasome activation in macrophages and consequent decreased IL-1β release following lipopolysaccharide (LPS) exposure. This novel effect of metformin is independent of AMPK activation or NF-κB. Notably, suppression of the "ATP/mtDNA/ox-mtDNA/NLRP3/IL-1β" axis by metformin at a clinically relevant dose (50 mg/kg/day) inhibits LPS-induced acute respiratory distress syndrome (ARDS) in mice [62]. Myeloid-specific ablation of cytidine monophosphate kinase 2 (CMPK2), the LPS-inducible rate-limiting enzyme required for activation of the salvage pathway of mtDNA synthesis [63], recapitulates metformin's protective effects in LPS-induced ARDS in mice. Likewise, neither NF-κB nor AMPK is involved in the ARDS-protective activity of metformin. Notably, metformin treatment (50 mg/kg/day) also attenuates pulmonary inflammation in SARS-CoV2-infected human ACE2-transgenic mice [62]. Collectively, the study by Xian et al. reveals a novel mtDNA/NLRP3-dependent mechanism underlying metformin's anti-inflammatory effects.

Antiaging activity

Aging is characterized by a progressive loss of physiological integrity, leading to impaired function and increased vulnerability to developing chronic diseases, such as cardiovascular disorders, neurodegenerative diseases, and some types of cancers, among others. As such, these chronic diseases are frequently referred to as aging-related morbidities. López-Otín et al. proposed 9 hallmarks of aging in different organisms, especially mammals: (1) genomic instability, (2) telomere attrition, (3) epigenetic alterations, (4) loss of proteostasis, (5) deregulated nutrient sensing, (6) mitochondrial dysfunction, (7) cellular senescence, (8) stem cell exhaustion, and (9) altered intercellular communication [64].

In a recent review, Kulkami et al. surveyed research findings in experimental models, showing that metformin exerts beneficial effects on all the 9 hallmarks of aging [4]. The relative contribution of each of the above mechanisms to metformin's antiaging activities likely varies with different experimental models (e.g., cultured cells, mice, or *C. elegans*). Nevertheless, substantial evidence supports metformin as an effective antiaging drug in mammals [65] and potentially in humans as well [66, 67]. Notably, through cytokine profiling and bioinformatic analyses of $CD4^+$ T cells from older (60 years of age) and younger (30 years of age) people, a recent study by Bharath et al. showed that metformin enhances autophagy and normalizes mitochondrial function to alleviate aging-associated inflammation [68]. As aging-associated inflammation (also known as inflammaging) is an important contributor to the development of major chronic diseases [69], inhibition of inflammaging may be a novel mechanism by which metformin promotes healthy aging.

Weight control and energy balance

It is well established that metformin treatment is associated with weight loss in diabetic and non-diabetic people [70–72]. However, the molecular mechanisms by which metformin reduces body weight remain unknown. Recent studies suggest a critical role for growth differentiation factor 15 (GDF15) in mediating metformin's effects on body weight and energy balance [73, 74]. GDF15 is a member of the TGF-β superfamily, and its expression is upregulated in response to cellular stresses. Elevated GDF15 reduces food intake and body mass in animal models via its binding to glial cell-derived neurotrophic factor (GDNF) family receptor alpha-like (GFRAL) and the recruitment of the receptor tyrosine kinase RET in the hindbrain [75, 76].

Gerstein et al. first showed increased serum levels of GDF15 in metformin-treated patients as compared with control subjects, and that blood GDF15 levels are a novel biomarker for metformin therapy in people with dysglycemia and its concentration reflects the dose of metformin [77]. Subsequently, a study by Day et al. reported that in primary mouse hepatocytes, metformin stimulates the secretion of GDF15 by increasing the expression of activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) [73]. In wild-type mice fed a high-fat diet, oral treatment with metformin increases serum GDF15 levels and reduces food intake, body mass, fasting insulin, and glucose intolerance. Notably, the above metformin's beneficial effects are eliminated in GDF15-knockout mice, supporting a causal role for GDF15 in mediating metformin's effects. Importantly, Day et al. showed an increase in serum GDF15 is also associated with weight loss in patients with type 2 diabetes who take metformin [73], indicating the clinical relevance of their findings in mice.

More recently, by analyzing blood samples from individuals in two independent randomized controlled trials, Coll et al. reported that metformin treatment increases circulating levels of GDF15 [74], hence confirming early findings by others [73, 77]. With mouse models, Coll et al. found that in wild-type mice, oral metformin treatment increases circulating GDF15 levels and augments GDF15 mRNA expression predominantly in the distal intestine and the kidney, though GDF15 expression is inducible by metformin in other cell types, including hepatocytes. Consistent with early findings by others, metformin treatment prevents weight gain in response to a high-fat diet in wild-type mice, but not in mice lacking GDF15 or its receptor, GFRAL [74]. In obese mice on a high-fat diet, metformin's ability to reduce body weight is reversed by a GFRAL-antagonist antibody.

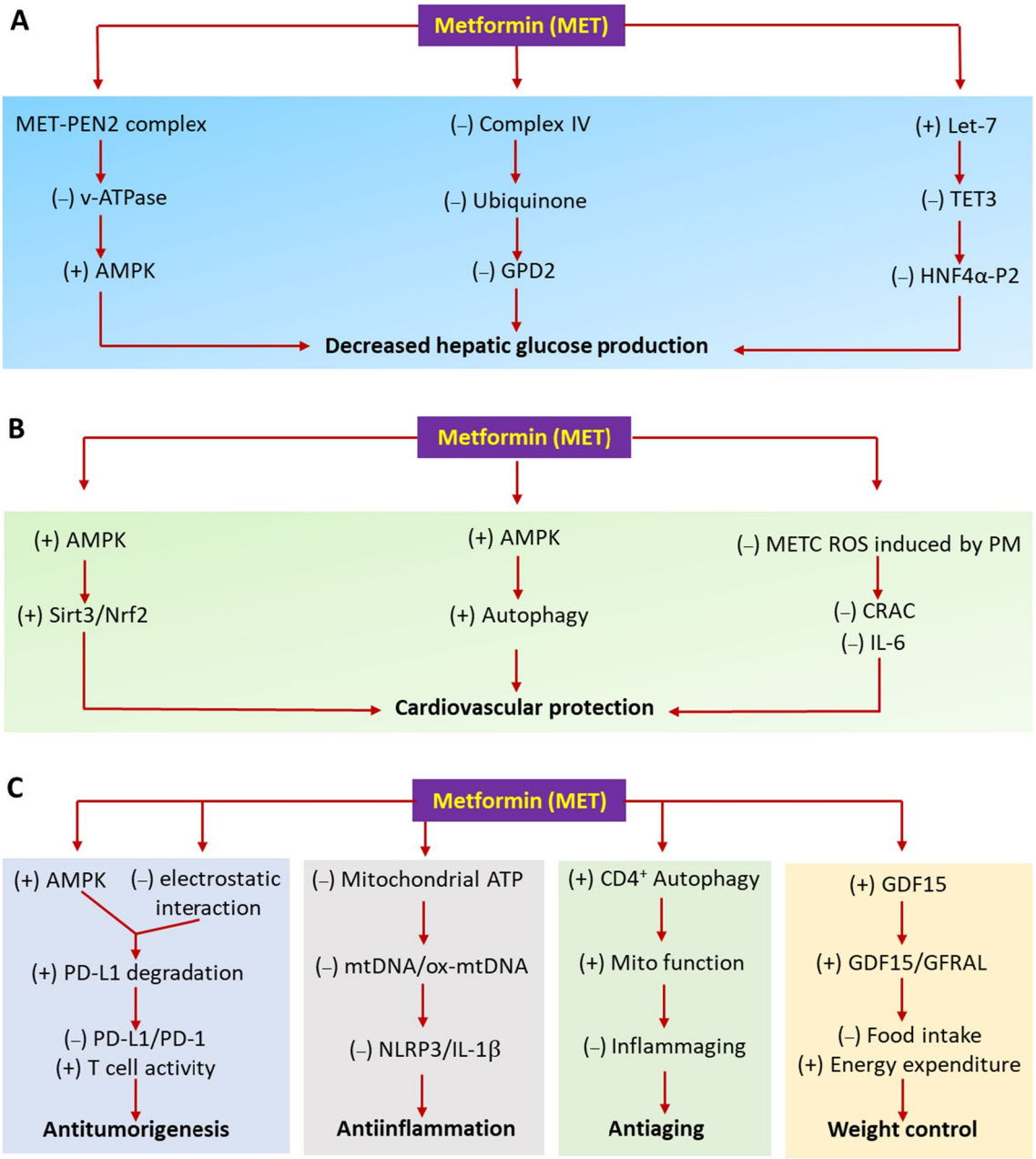

Fig. 2 Novel molecular targets of metformin. As illustrated, multiple novel molecular targets and signaling pathways have recently been discovered that underly metformin's actions in glycemic control (panel **A**), cardiovascular protection (panel **B**), and other beneficial effects including antitumorigenesis, anti-inflammation, antiaging, and weight control (panel **C**). See text for detailed description. (+) denotes activation or increase; (−) denotes inhibition or decrease. PM, particulate matter

Notably, metformin not only reduces energy intake but also increases energy expenditure, and both effects are dependent on GDF15. On the other hand, in the absence of GDF15, metformin retains its ability to lower blood glucose [74], suggesting the specific involvement of GDF15 in metformin's effects on food intake and energy balance.

Conclusion and perspectives

In conclusion, multiple novel molecular targets of metformin action have been discovered over the past few years (Fig. 2). These cutting-edge discoveries reported primarily in highly influential journals further advance our understanding of the molecular mechanisms by which metformin exerts its diverse biological activities, including improved glucose

homeostasis, cardiovascular protection, anti-tumorigenesis, anti-inflammation, antiaging, and enhanced energy balance and weight loss. As noted in the Introduction section, this mini-review is not intended to provide a comprehensive survey of current research findings reported in all scientific journals, but instead, to focus on novel discoveries from high-quality research studies. It is hoped that this focused discussion of only cutting-edge research findings will shed light on the direction of future innovative research. In this context, future research efforts should be devoted to translating these novel basic science discoveries to effective therapies for not only diabetes, but also other common human diseases. Indeed, several major clinical trials, based on cutting-edge basic research findings on metformin, either have been recently completed or are currently underway to determine the effectiveness of metformin in the management of major human diseases as well as aging. It is of note that the "Targeting Aging with Metformin (TAME)" trial (to be launched soon) is the first large clinical trial in modern medicine to test if human aging can be treated with a drug (metformin) (https://www.afar.org/tame-trial). Likewise, advanced understanding of the molecular signaling machineries involved in metformin's diverse biological activities also provides opportunities for developing more effective metformin analogs or new drugs to target these novel molecular axes for human disease intervention.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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