

Current understanding of metformin effect on the control of hyperglycemia in diabetes

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Abstract

Metformin is a first-line oral anti-diabetic agent that has been used clinically to treat patients with type 2 diabetes for over 60 years. Due to its efficacy in therapy and affordable price, metformin is taken by more than 150 million people each year. Metformin improves hyperglycemia mainly through the suppression of hepatic gluconeogenesis along with the improvement of insulin signaling. However, its mechanism of action remains partially understood and controversial, especially in regard to the role of AMPK in metformin's action and the mechanism of AMPK activation. In this review, we discuss recent advances in the understanding of metformin's suppression of hepatic glucose production and the mechanism related to the improvement of insulin signaling.

Key Words

- ▶ glucose metabolism
- ▶ insulin action
- ▶ metabolism
- ▶ liver

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Metformin, a time-proven effective agent for the treatment of patients with type 2 diabetes

In the medieval Europe, the *Gallega officinalis* plant was used as a folk medicine to treat diabetes. In the late 1800s, the plant was found to be rich in guanidine, then a subsequent animal study in 1918 showed that guanidine had a blood glucose-lowering effect (Watanabe 1918). However, guanidine proved to be too toxic for clinical use. In 1929, the dimethylbiguanide, including phenformin and metformin, was synthesized. These compounds preserve the glucose-lowering effect of their parent compound with reduced toxicity. Phenformin is more potent than metformin in improving hyperglycemia, but the high occurrence of lactic acidosis, a serious side effect, led to the withdrawal of phenformin from the market in the 1970s.

After over 60 years of clinical use for the treatment of type 2 diabetes (T2D), metformin has proven to be safe along with affordable, now making it the most commonly

prescribed oral anti-diabetic agent worldwide, taken by more than 150 million people each year. The guidelines for the treatment of T2D, published by the American Diabetes Association and the European Association for the Study of Diabetes in 2012, recommended metformin as the initial drug for T2D treatment (Inzucchi *et al.* 2012). Moreover, many studies have shown a reduction in cancer incidence in patients with T2D treated with metformin (Evans *et al.* 2005, Landman *et al.* 2010).

Metformin alleviates hyperglycemia in T2D mainly through the direct suppression of hepatic glucose production

T2D accounts for more than 90% of diabetes cases (Centers for Disease Control and Prevention 2011), and increased hepatic glucose production is the major cause of fasting hyperglycemia in these patients (Magnusson *et al.* 1992, Saltiel & Kahn 2001, Wajngot *et al.* 2001). It is now clear

that metformin improves hyperglycemia mainly through the suppression of gluconeogenesis in the liver. In a human study, treatment of patients with T2D with metformin led to a decrease in hepatic glucose output via the suppression of gluconeogenesis by 37% (Stumvoll *et al.* 1995). In another human study, patients with diabetes exhibited a twofold increase in the rate of gluconeogenesis, but metformin administration led to a decrease in gluconeogenesis by 33% (Hundal *et al.* 2000). In animal studies, metformin reduced hepatic glucose production by over 60% in a euglycemic-clamp study (Takashima *et al.* 2010) and led to 50% suppression of endogenous glucose production in high-fat-diet-fed rats (Song *et al.* 2001). Furthermore, we have found that metformin suppressed cAMP-stimulated glucose production by 35% in primary hepatocytes (Cao *et al.* 2014). These *in vivo* and *in vitro* data suggest that metformin acts mainly through the suppression of hepatic gluconeogenesis.

Metformin suppresses hepatic glucose production through AMPK activation

AMPK: a master regulator of cellular energy homeostasis

AMPK was first named and reported in 1989 for its AMP-activation property and its role in regulating three key enzymes in lipid metabolism (Brown *et al.* 1975, Hardie *et al.* 1989). Over the past decades, numerous studies have confirmed the function of AMPK as a principal energy sensor of the cell and its role in regulating cellular and whole-body energy balance by sensing and responding to changes in the AMP/ADP concentration relative to ATP (Hardie *et al.* 2012). AMPK is activated when cellular energy levels are low, prompting a switch from ATP-consuming anabolic pathways to ATP-producing catabolic pathways by stimulating glucose uptake and utilization and fatty acid oxidation together with suppression of hepatic glucose production.

Phylogenetically, AMPK is a highly conserved serine/threonine kinase and presents in virtually all eukaryotes. Functional AMPK is a heterotrimeric complex comprised of an α catalytic subunit and regulatory $\beta\gamma$ non-catalytic subunits, with each subunit having several isoforms ($\alpha 1, \alpha 2, \beta 1, \beta 2, \gamma 1, \gamma 2, \gamma 3$, encoded by separated genes) (Hardie *et al.* 2012). The catalytic α -subunit consists of an N-terminal protein kinase domain (conventional Ser/Thr kinase domain), followed by an auto-inhibitory domain, a linker domain, and a C-terminal regulatory domain (α -CTD) (Chen *et al.* 2009, Hardie *et al.* 2012). The β -subunits contain a carbohydrate-binding module (CBM) and a C-terminal domain (β -CTD). The CBM domain allows

AMPK to act as a glycogen sensor, while the β -CTD works as a scaffold, interacting with α -CTD and the γ -subunit, thus forming the core of the heterotrimeric complex. The γ -subunit has a tandem of four cystathionine- β -synthase domains, functioning as the adenine nucleotide-binding region (Hardie *et al.* 2012). When the cellular energy status changes, an increase in the AMP/ATP or ADP/ATP ratio leads to AMP or ADP binding to the γ -subunit. This results in an allosteric change in AMPK and augments α -subunit phosphorylation at T172 by an upstream kinase or prevents dephosphorylation by a protein phosphatase (Corton *et al.* 1994, Xiao *et al.* 2011). Phosphorylation of the α -subunit at T172 leads to an increase in AMPK enzymatic activity of over several hundredfold. In comparison, AMP only causes a two- to fivefold allosteric activation of AMPK, which also depends on the nature of the isoforms present in the AMPK complex (Xiao *et al.* 2011).

In mammals, the upstream kinases for AMPK α -subunit phosphorylation at T172 are the LKB1–STRAD–MO25 complex (Lizcano *et al.* 2004), the Ca^{2+} /calmodulin-activated protein kinases, especially CaMKK β (also termed as CaMKK2) (Hawley *et al.* 2005), or the transforming growth factor- β activated protein kinase-1 (Momcilovic *et al.* 2006). The LKB1/AMPK pathway plays a major role in metabolic regulation.

The role of AMPK in mediating metformin action

After oral administration, metformin is absorbed into enterocytes and then delivered directly to the liver through the portal vein. Plasma concentrations in the portal vein are between 40 and 70 μM in animals after a therapeutic dose (Wilcock & Bailey 1994, He & Wondisford 2015). Over a decade ago, Zhou *et al.* (2001) reported that metformin at concentrations found in the portal vein ($\leq 80 \mu\text{M}$) activates AMPK by increasing α -subunit phosphorylation at T172. A subsequent study reported that hepatic knockout of LKB1, an upstream kinase for AMPK α -subunit phosphorylation at T172, abrogated the effect of metformin on the alleviation of hyperglycemia in mice fed a high-fat diet (Shaw *et al.* 2005). In agreement with this result, our study showed that depletion of AMPK catalytic α -subunits abolished the suppression of cAMP- or glucagon-stimulated glucose production by metformin at concentrations typically found in the portal vein ($\leq 80 \mu\text{M}$) (Cao *et al.* 2014). Furthermore, metformin-mediated activation of AMPK increases CBP phosphorylation at S436 via atypical protein kinase ι/λ resulting in the disassembly of the CREB co-activator complex, inhibition of gluconeogenic gene expression,

and a reduction in glucose production (He *et al.* 2009). A growing body of evidence supports the notion that metformin suppresses mRNA or protein levels of rate-limited gluconeogenic gene expression in the liver of mice and in primary hepatocytes (Cheng *et al.* 2006, Heishi *et al.* 2008, He *et al.* 2009, Martin-Montalvo *et al.* 2013). Corroborating our findings, a P300G422S knock-in mouse model, which harbors a reconstituted metformin phosphorylation site found in CBP, exhibits exaggerated hypoglycemia in a metformin tolerance test (He *et al.* 2014). The previous evidence supports a mechanism for metformin suppression of hepatic glucose production via activation of the LKB–AMPK pathway.

Activation of AMPK by low metformin concentrations ($\leq 80 \mu\text{M}$) via the promotion of the formation of the AMPK $\alpha\beta\gamma$ heterotrimeric complex

Metformin has no direct effect on AMPK α enzymatic activity (Meng *et al.* 2015). But, metformin is able to increase AMPK enzymatic activity by stimulating the phosphorylation of the α subunit at T172 (Zhou *et al.* 2001). Considering that the upstream kinase LKB1 is a constitutively active kinase (Lizcano *et al.* 2004), we asked whether metformin could affect the formation of functional AMPK heterotrimeric complex, consisting of an α catalytic subunit and $\beta\gamma$ non-catalytic subunits, to increase the phosphorylation of the α subunit at T172 by upstream kinase (Davies *et al.* 1994, Mitchelhill *et al.* 1994, Stapleton *et al.* 1996). However, previous studies showed that AMPK $\alpha 1\beta 1\gamma 1$ or $\alpha 2\beta 1\gamma 1$ subunits exist in a 1:1:1 molar ratio (Davies *et al.* 1994, Stapleton *et al.* 1996), and it is generally thought that the heterotrimeric complex assembles immediately following translation in order to keep the subunits stable. Free subunits may not be stable and will therefore degrade rapidly (Neumann *et al.* 2003). Interestingly, we were able to express AMPK- $\alpha 1, \beta 1, \gamma 1$ individually in both primary hepatocytes and Hepa1-6 cells (Cao *et al.* 2014, Meng *et al.* 2015). Purified AMPK subunits did not associate with other subunits in a coomassie stained gel, indicating that free AMPK subunits can exist and are stable. Furthermore, we examined the protein levels of the AMPK subunits in different mouse tissues and found that the protein levels of different AMPK subunits vary from tissue to tissue (Meng *et al.* 2015), suggesting that cellular protein levels of AMPK subunits are unlikely to exist in a 1:1:1 molar ratio (Davies *et al.* 1994, Stapleton *et al.* 1996).

To assess the cellular ratio of endogenous AMPK subunits, we generated and purified FLAG-tagged $\alpha 1,$

$\beta 1, \gamma 1$ since they account for over 90% of AMPK activity in hepatocytes (Davies *et al.*, 1994, Stapleton *et al.* 1996). We first determined the amounts of purified FLAG-tagged $\alpha 1, \beta 1, \gamma 1$ subunits that were equivalent to the corresponding protein levels in the same amount of cellular lysates. Subsequently, these amounts of purified FLAG-tagged $\alpha 1, \beta 1,$ and $\gamma 1$ subunits were used and immunoblotted with anti-FLAG antibody. Our data reveal that the $\alpha 1, \beta 1,$ and $\gamma 1$ subunits exist approximately in a 1:5:0.6 molar ratio in Hepa1-6 cells and in a 1:31:0.5 molar ratio in mouse liver (Meng *et al.* 2015). Our data suggest that the $\beta 1$ subunit is much more abundant than $\alpha 1$ and $\gamma 1$ subunits in both Hepa1-6 cells and liver.

The unequal expression of endogenous $\alpha 1, \beta 1,$ and $\gamma 1$ protein levels suggest that not all of the $\alpha 1, \beta 1,$ and $\gamma 1$ are in the heterotrimeric complex. Therefore, we reasoned that metformin might be able to affect the assembly of the AMPK heterotrimeric complex. In immunoprecipitation studies, we found that both α and γ subunits were co-immunoprecipitated when using $\beta 1/2$ antibodies in metformin-treated Hepa1-6 cells. In contrast, in untreated Hepa1-6 cells, the $\gamma 1$ but not the α subunit co-immunoprecipitated with $\beta 1$ subunit. These data suggest that $\beta 1$ and $\gamma 1$ subunits are preassembled, and metformin promotes the association of the $\alpha 1$ subunit with $\beta 1$ and $\gamma 1$ complex to form the functional heterotrimeric complex (Meng *et al.* 2015). In agreement with these results, we found that mice fed a high-fat diet were given metformin in the drinking water exhibited an increase in α subunit phosphorylation at T172 as well as the association of the α subunit with the β subunit in the liver (Meng *et al.* 2015). Furthermore, low concentrations of metformin also increased the formation of AMPK $\alpha\beta\gamma$ heterotrimeric complex in *in vitro* assembly assay. Interestingly, neither AMP/ADP, nor phenformin affected the heterotrimeric assembly, suggesting that they activate AMPK through different mechanisms.

As we found that metformin was able to promote the formation of the AMPK $\alpha\beta\gamma$ heterotrimeric complex, we further tested whether the formation of the AMPK $\alpha\beta\gamma$ heterotrimeric complex had any effect on the phosphorylation of the $\alpha 1$ subunit at T172 by an upstream kinase. In *in vitro* phosphorylation assays, the phosphorylation of the $\alpha 1$ subunit at T172 by LKB1 is tenfold greater in the presence of $\beta 1$ and $\gamma 1$ subunits than $\alpha 1$ subunit alone. The presence of the $\gamma 1$ subunit is essential for metformin's effect on the phosphorylation of the $\alpha 1$ subunit at T172 by LKB1, as metformin's effect was lost when the $\gamma 1$ subunit was absent in the reaction. A previous study showed that cells expressing a mutant AMPK γ subunit (R531G) were

resistant to metformin activation of AMPK (Hawley *et al.* 2010), suggesting that R531 in the γ subunit is critical for metformin binding. Furthermore, low metformin concentrations ($<100 \mu\text{M}$) prevented the dephosphorylation of the catalytic $\alpha 1$ subunit by PP2C in the presence of $\beta 1$ and $\gamma 1$ subunits, and this effect was lost in the absence of $\beta 1$ and $\gamma 1$ subunits. Taken together, our data support that metformin binds directly to AMPK subunits, resulting in increased assembly of the AMPK $\alpha\beta\gamma$ heterotrimeric complex as well as allosteric changes, making it more accessible for upstream kinases, such as LKB1, to phosphorylate the $\alpha 1$ subunit at T172 (Fig. 1). The formation of the AMPK $\alpha\beta\gamma$ heterotrimeric complex also hinders the dephosphorylation of the $\alpha 1$ subunit at T172 by protein phosphatase (Meng *et al.* 2015). Overall, the collective effects of metformin action lead to the activation of AMPK.

Metformin suppresses hepatic glucose production through the inhibition of mitochondrial respiratory chain complex 1 and AMP deaminase

Mitochondria respiratory-chain complex 1, also termed NADH:ubiquinone oxidoreductase, is the first membrane-embedded supramolecular complex in the electron transport chain. Complex 1 catalyzes the transfer of a hydride ion from NADH and a proton from the matrix to ubiquinone. Ubiquinol (QH₂), a lipid-soluble carrier ubiquinone, can diffuse quickly in the inner mitochondrial membrane from complex 1 to complex 3. Complex 1 also transfers four protons from the matrix to the intermembrane space to generate a high electron gradient, which is the energy source in ATP synthesis. In 1955, it was documented that guanidine can inhibit the oxygen uptake of mitochondria. This was the first study that showed that guanidine may have an effect on the mitochondrial respiratory chain (Hollunger 1955). In 2000, two studies showed that metformin's action is through the disruption of mitochondrial complex I (El-Mir *et al.* 2000, Owen *et al.* 2000), leading to a decrease in ATP production. More recently, Foretz *et al.* (2010) suggested that the change in the AMP/ATP or ADP/ATP ratio after metformin's inhibition of the mitochondrial complex is primarily responsible for metformin's effect and that this occurs through an AMPK-independent pathway. It was also shown that metformin increases AMP levels via the inhibition of AMP deaminase (Fig. 2, Ouyang *et al.* 2011). In addition, elevated AMP levels after metformin treatment led to the inhibition of adenylate

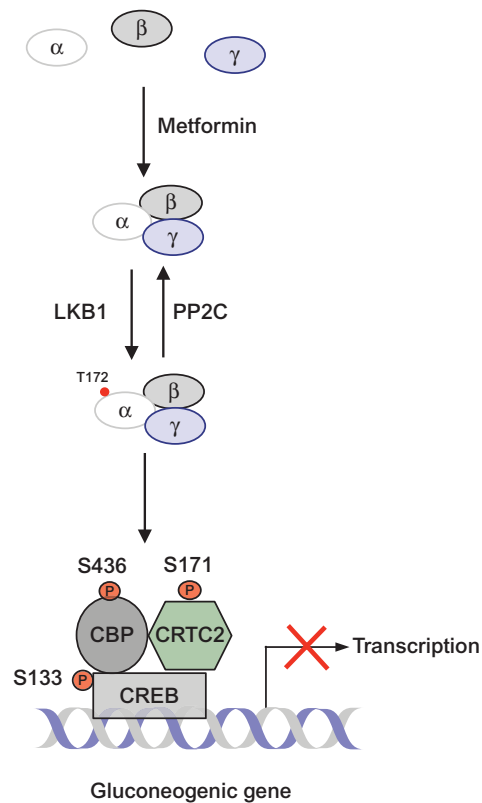
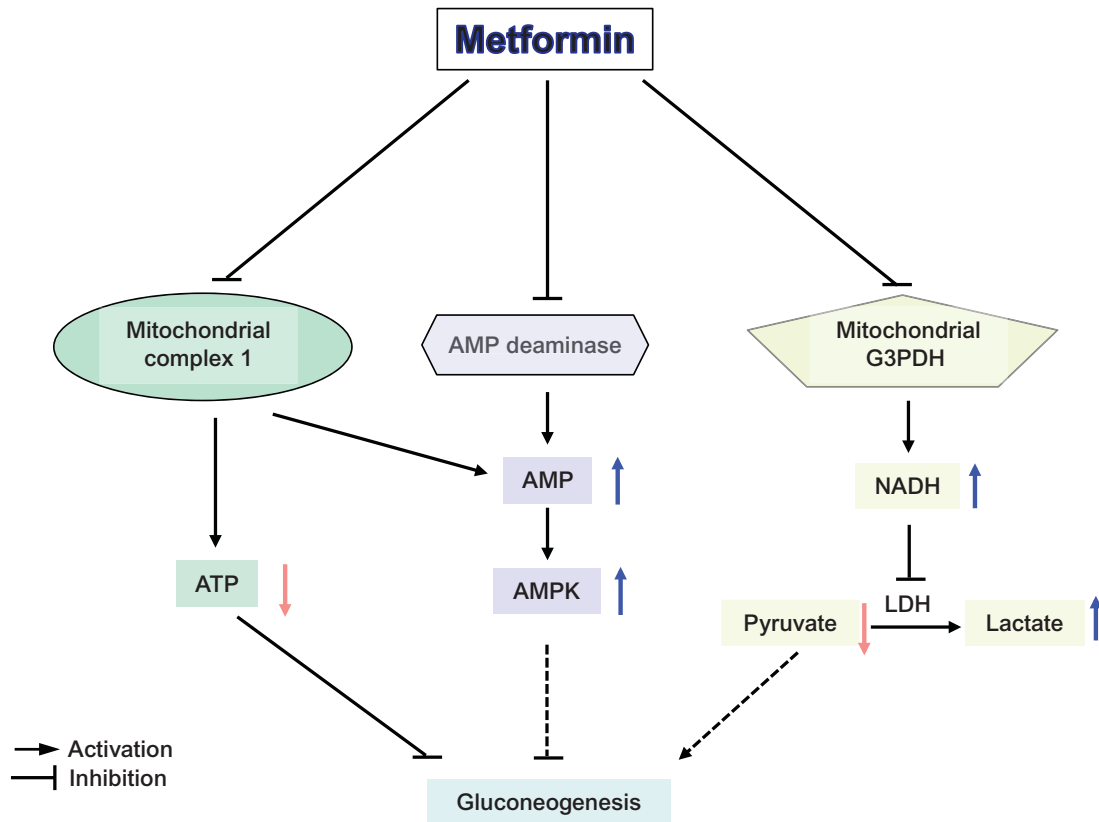


Figure 1

Metformin suppression of hepatic glucose production by promoting the formation of the AMPK $\alpha\beta\gamma$ heterotrimeric complex. Metformin increases the phosphorylation of AMPK by promoting the formation of the AMPK heterotrimeric complex. Activated AMPK leads to the phosphorylation of CREB co-activators and the inhibition of gluconeogenic gene expression.

cyclase and a decrease in cAMP levels, resulting in the suppression of the cAMP-PKA pathway, and the inhibition of gluconeogenesis was proposed (Miller *et al.* 2013). We also found that metformin is able to decrease cellular ATP levels and increase the ratios of AMP/ATP or ADP/ATP (Cao *et al.* 2014). Since gluconeogenesis is an energy-demanding process in which synthesis of one molecule of glucose from lactate or pyruvate requires four molecules of ATP and two molecules of GTP, reduction of cellular ATP levels by metformin will lead to the suppression of hepatic gluconeogenesis (Fig. 2).

However, metformin's inhibition of mitochondrial complex I or AMP deaminase activity occurred at concentrations $\geq 5 \text{ mM}$ in hepatocytes (El-Mir *et al.* 2000, Owen *et al.* 2000, Ouyang *et al.* 2011). The concentrations of metformin required to affect the ratios of AMP/ATP or ADP/ATP are $\geq 250 \mu\text{M}$ (Foretz *et al.* 2010, Cao *et al.* 2014). These metformin concentrations are unreachable in the portal vein after a therapeutic dose

**Figure 2**

Direct suppression of hepatic glucose production by metformin through the inhibition of mitochondrial respiratory chain complex 1, AMP deaminase, and mitochondrial glycerol 3-phosphate dehydrogenase. Inhibition of mitochondrial complex 1 also leads to an increase in AMP levels or the AMP/ATP ratio, as does the inhibition of AMP deaminase, resulting in the activation of AMPK. However, high metformin

concentrations are needed to inhibit the mitochondrial complex and AMP deaminase. Metformin inhibition of mitochondrial glycerol 3-phosphate dehydrogenase (G3PDH) will increase NADH levels in the cytoplasm and suppress the conversion of lactate from pyruvate. This mechanism of metformin action is important for patients with high levels of serum lactate.

(Wilcock & Bailey 1994, He & Wondisford 2015). Interestingly, using typical pharmacologic dosing in mice, metformin administration did not decrease, but significantly increased mitochondrial complex I activity in the liver (Martin-Montalvo *et al.* 2013). We also found that a low metformin concentration (80 μ M) significantly increased ATP levels in primary hepatocytes treated with cAMP. This may be due to the inhibition of gluconeogenesis and activation of fatty acid oxidation (Cao *et al.* 2014), but metformin still significantly activated AMPK (Zhou *et al.* 2001, Cao *et al.* 2014) and the suppression of glucose production relative to cAMP treatment alone in primary hepatocytes (Cao *et al.* 2014). These data indicate that inhibition of mitochondrial complex I activity is unlikely at pharmacologic metformin concentrations found in the portal vein, and that the inhibition of mitochondrial complex I is unnecessary

for metformin's suppression of gluconeogenesis. Since very high metformin concentrations are required to inhibit mitochondrial complex I and perhaps, the AMP deaminase activity in hepatocytes as well (El-Mir *et al.* 2000, Ouyang *et al.* 2011), we suggest the effects of metformin seen at high concentrations might be either an off-target phenomena or signals of cell toxicity and are therefore clinically irrelevant.

Metformin suppresses hepatic glucose production through the inhibition of mitochondrial glycerol 3-phosphate dehydrogenase and through affecting gut-brain communication

It has been reported that metformin suppresses hepatic glucose production by inhibiting the enzymatic activity of

mitochondrial glycerol 3-phosphate dehydrogenase (mG3PDH), which blocks the transport of NADH from the cytoplasm into mitochondria (Madiraju *et al.* 2014). This mechanism of metformin action may be important for the suppression of hepatic glucose production in patients with diabetes who have high levels of serum lactate, as the conversion of lactate to pyruvate leads to the generation of NADH. The inhibition of mG3PDH will result in an increase in NADH levels and a decrease in NAD⁺ levels, resulting in no NAD⁺ available for converting lactate to pyruvate. Subsequently, this action will halt glucose production from lactate (Fig. 2, right panel). Interestingly, AMPK negatively regulates the activity of yeast glycerol 3-phosphate dehydrogenase 2 (an analog of mammalian mG3PDH) by phosphorylating S72 (Lee *et al.* 2012). However, it remains to be determined whether AMPK can affect mG3PDH enzymatic activity by phosphorylation.

Moreover, intestinal nutrients can regulate glucose homeostasis in a manner involving the gut–brain–liver axis (Wang *et al.* 2008). Affecting gut–brain communication will lead to a change in hepatic glucose production. Duca *et al.* (2015) tested whether metformin has any effect on the gut–brain–liver axis and found that metformin suppression of hepatic glucose production occurs in part through the gut–brain–liver axis. Gut–brain communication is critical for acute metformin action, as metformin suppression of hepatic glucose production was negated in rats with blockade of the gut–brain–liver axis. The acute metformin effect is AMPK-dependent, involving the activation of PKA by GLP-1 in duodenal enterocytes (Duca *et al.* 2015). However, the importance of the gut–brain–liver axis in chronic metformin action remains to be determined.

Metformin alleviates hyperglycemia in T2D through the improvement of insulin signaling: an indirect pathway of metformin action

Even though metformin improves hyperglycemia mainly through the suppression of gluconeogenesis in the liver, it has also been found to be able to increase insulin sensitivity. This effect would improve insulin-mediated suppression of hepatic glucose production and enhance insulin-stimulated glucose disposal in skeletal muscle (Stumvoll *et al.* 1995, Inzucchi *et al.* 1998, Hundal *et al.* 2000). The potential mechanism of a metformin-mediated increase in insulin sensitivity will be discussed.

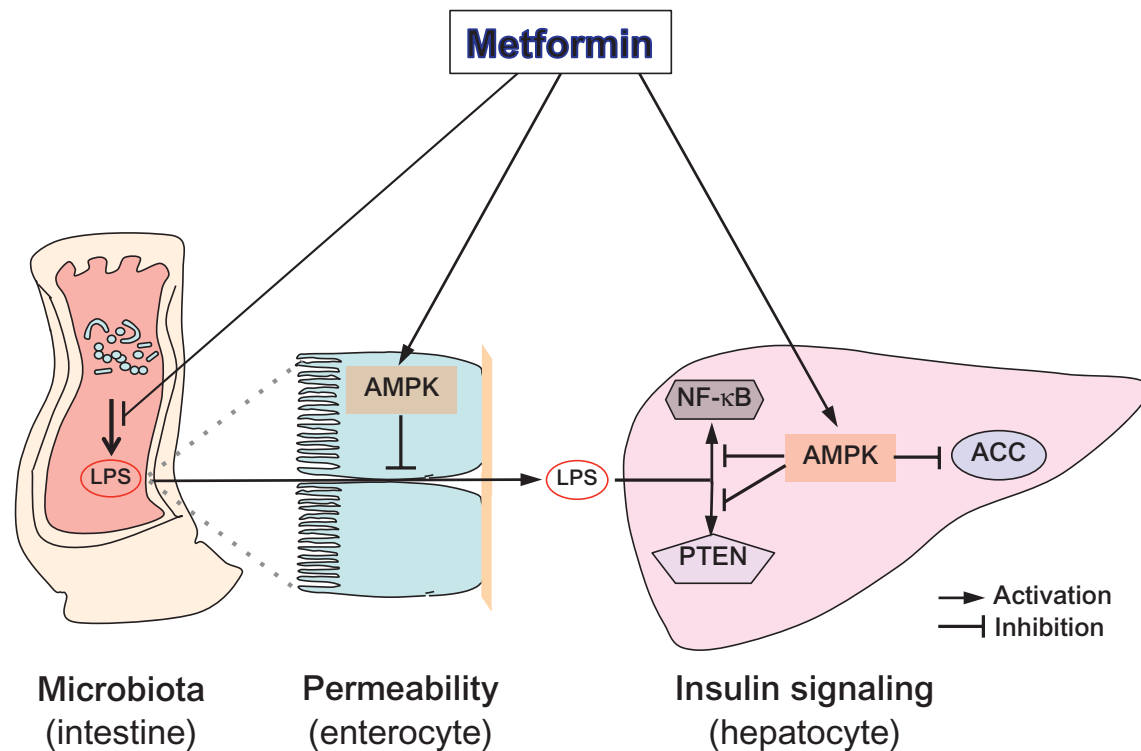
Metformin alters gut microbiota and maintains the integrity of the intestinal barrier

Obese individuals have a different composition of gut microbiota, and in a rodent model, obesity is associated with altered gut microbiota (Ley *et al.* 2005, 2006, Ridaura *et al.* 2013). High-fat-diet feeding leads to a similar change in gut microbiota composition and to a two- to threefold increase in blood lipopolysaccharide (LPS) (endotoxin) levels in mice (Cani *et al.* 2007, 2008). In addition, a single oral administration of lipids in human or mice acutely increased serum LPS concentration (Cani *et al.* 2007, Laugerette *et al.* 2011). Germ-free mice resisted high-fat-diet induced insulin resistance (Backhed *et al.* 2007). A low-dose LPS infusion mimicked high-fat-diet feeding and induced fasting hyperglycemia and hepatic insulin resistance in a mouse model (Ley *et al.* 2005). This line of evidence suggests that LPS from the gut is a critical etiologic factor for the development of insulin resistance. Increased LPS leakage from the gut has emerged as one of the most appealing mechanisms to explain the connection between changes in the intestinal microbiota and insulin resistance (Cani *et al.* 2007).

Recently, metformin has been shown to modulate the composition of gut microbiota in high-fat diet-fed and diabetic mice and decreases serum LPS levels (Shin *et al.* 2014). In addition, metformin can activate mucosal AMPK in the intestine (Duca *et al.* 2015). An earlier study showed that high-fat-diet feeding increases the intestinal permeability of LPS (Cani *et al.* 2007, 2008, Creely *et al.* 2007, Oliveira *et al.* 2011), and AMPK plays a critical role in maintaining intestinal barrier integrity (Peng *et al.* 2009, Elamin *et al.* 2013). Therefore, metformin-mediated activation of AMPK should decrease LPS leakage from the gut (Fig. 3).

Metformin blocks the activation of the NF- κ B pathway and affects the expression of PTEN

The liver is the major organ responsible for LPS clearance. After being delivered to the liver, LPS binds to the multireceptor complex constituted of CD14, TLR4 and MD2 within the lipid rafts of hepatocytes and triggers the signal activation of IRAK, and subsequently, its downstream target, the NF- κ B pathway (Scott & Billiar 2008). NF- κ B first drew attention in the development of insulin resistance and T2D after the landmark finding that the anti-inflammatory agent aspirin inhibits NF- κ B. Activation of NF- κ B leads to insulin resistance (Arkan *et al.* 2005), and inactivation of this pathway protects against

**Figure 3**

Metformin improves insulin signaling in the liver. Metformin can alter the microbiota in the intestine, resulting in a reduction in LPS production and translocation across the intestinal barrier. Activation of AMPK by

metformin also blocks LPS-mediated activation of the NF- κ B signaling pathway and PTEN induction.

the development of insulin resistance, as evident in mice with NF- κ B (p50) knockout (Gao *et al.* 2009). This mouse model exhibited increased insulin sensitivity in the liver and produced significantly less glucose in a hyperinsulinemic–euglycemic clamp. Furthermore, inhibition of the NF- κ B pathway improved insulin resistance in db/db mice (Kim *et al.* 2013). Of particular interest is the finding that the activation of AMPK by AICAR inhibited the NF- κ B pathway (Cacicedo *et al.* 2004). Aligning with this, metformin-mediated AMPK activation attenuates the activation of the NF- κ B pathway (Hattori *et al.* 2006, Huang *et al.* 2009). Therefore, the inhibition of the NF- κ B pathway by metformin-mediated AMPK activation would lead to an improvement in hepatic insulin signaling (Fig. 3).

Phosphatase and tensin homolog (PTEN), a tumor suppressor, can reverse PI3K (Phosphatidylinositol-4, 5-bisphosphate 3-kinase) function by dephosphorylating the PI(3,4,5)P₃ to PI(4,5)P₂, therefore, suppressing the PI3K-PKB/AKT pathway (Myers *et al.* 1998, Stiles *et al.* 2004). Intriguingly, LPS can induce the expression of PTEN (Okamura *et al.* 2007), and metformin can suppress

PTEN expression in pre-adipocyte 3T3 cells (Okamura *et al.* 2007, Lee *et al.* 2011). This metformin action is AMPK dependent, as the metformin effect is lost in cells treated with Compound C (an AMPK inhibitor) or with AMPK depletion by shRNA. This report showed that PTEN is a downstream regulator of AMPK and that the AMPK–PTEN pathway plays a critical role in regulating inflammatory response (Fig. 3). However, further studies will be needed to demonstrate conclusively how metformin's effect on PTEN occurs in the liver as well as muscle and determines how activated AMPK suppresses PTEN expression.

Perspective

Since the maximum metformin dose prescribed to patients with diabetes is ~ 2.5 g/day, this high therapeutic dose might affect multiple targets. As an oral agent, metformin can change the composition of gut microbiota (Shin *et al.* 2014) and activate mucosal AMPK (Duca *et al.* 2015) that will maintain intestinal barrier integrity (Peng *et al.* 2009, Elamin *et al.* 2013). Together, these metformin effects will decrease LPS levels in the

circulation as well as hepatic LPS levels, since LPS is delivered directly to the liver through the portal vein. In addition, the activation of duodenal AMPK by acute metformin treatment will stimulate the gut–brain–liver axis to regulate hepatic glucose production (Duca *et al.* 2015). After being delivered to the liver from the intestines, metformin can inhibit gluconeogenesis through different mechanisms. First, it can activate hepatic AMPK, which will then inhibit gluconeogenesis by phosphorylating the critical co-activators CBP and CRTC2 (He *et al.* 2009, Fig. 1). Second, high metformin concentrations can suppress hepatic gluconeogenesis by inhibiting mitochondrial respiratory chain complex 1 to reduce cellular ATP levels and increase the AMP/ATP ratio (El-Mir *et al.* 2000, Owen *et al.* 2000, Foretz *et al.* 2010) due to the high-energy demand of gluconeogenesis. We found that depletion of AMPK α 1/2 decreased the suppression of glucose production in primary hepatocytes at high metformin concentrations (Cao *et al.* 2014). Thus, the increased ratio of AMP/ATP or ADP/ATP should activate AMPK (Oakhill *et al.* 2011, Xiao *et al.* 2011, Gowans *et al.* 2013) to augment the inhibition of gluconeogenesis. Third, inhibition of mG3PDH enzymatic activity by metformin will affect transport of NADH from the cytoplasm into mitochondria (Madiraju *et al.* 2014), suppressing gluconeogenesis process from lactate.

Reducing LPS leakage from the gut and blocking the activation of the NF- κ B pathway, in concert with down-regulating the expression of PTEN, will result in an increase in insulin sensitivity. Moreover, metformin administration will activate AMPK to increase fatty acid oxidation by phosphorylating acetyl-CoA carboxylase and consequently improve insulin sensitivity in the liver (Fullerton *et al.* 2013). Collectively, suppression of gluconeogenesis by metformin's direct effects and the indirect improvement of insulin signaling in the liver will lead to the amelioration of hyperglycemia in patients with T2D. Even though our studies demonstrate that suppression of gluconeogenesis by low metformin concentrations is through the activation of AMPK by promoting the formation of the AMPK $\alpha\beta\gamma$ heterotrimeric complex in both *in vivo* and *in vitro* assays (Cao *et al.* 2014, Meng *et al.* 2015), and the γ subunit plays a critical role in the phosphorylation of the α subunit at T172 by upstream kinase LKB1. However, the exact metformin binding site(s) on AMPK subunits and the detailed mechanism leading to the improvement of insulin signaling by metformin still remain unknown and need to be unraveled in future studies.

Declaration of interest

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

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