implications

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Running title: Epigenetic effects of metformin

Abstract

There is a growing body of evidence that links epigenetic modifications to type 2 diabetes. Researchers have more recently investigated effects of commonly used medications, including those prescribed for diabetes, on epigenetic processes. This work reviews the influence of the widely used antidiabetic drug metformin on epigenomics, microRNA levels and subsequent gene expression and potential clinical implications. Metformin may influence the activity of numerous epigenetic modifying enzymes, mostly via modulating the activation of AMP-activated protein kinase (AMPK). Activated AMPK can phosphorylate numerous substrates, including epigenetic enzymes such as histone acetyltransferases (HATs), class II histone deacetylases (HDACs) and DNA methyltransferases (DNMTs), generally resulting in their inhibition, although HAT1 activity may be increased. Metformin has also been reported to decrease expression of multiple histone methyltransferases, increase the activity of the class III HDAC SIRT1 and to decrease the influence of DNMT inhibitors. There is evidence that these alterations influence the epigenome and gene expression, and may contribute to the antidiabetic properties of metformin and potentially protect against cancer, cardiovascular disease, cognitive decline and aging. The expression levels of numerous microRNAs are also reportedly influenced by metformin treatment and may confer antidiabetic and anticancer

activities. However, as the reported effects of metformin on epigenetic enzymes act to both increase and decrease histone acetylation, histone and DNA methylation, and gene expression, a significant degree of uncertainty exists on the overall effect of metformin on the epigenome, gene expression and subsequent effect on the health of metformin users.

Introduction

Epigenetics is a rapidly growing field in medical research. Epigenetic modifications, changes to DNA structure that alter gene expression without altering the base nucleotide code, are crucial in the development of organisms and the differentiation and function of specific cell types. For example, epigenetic modifications permit the expression of the insulin gene in pancreatic β cells, while silencing it in other cells.[1] However, epigenetic changes have also been associated with numerous disorders, including type 2 diabetes (T2D), CVD and cancer.[2,3] Epigenetic processes can be altered by environmental factors including diet, exercise and exposure to toxins, with the foetal environment playing a particularly important role in influencing epigenetic modifications that impact metabolism in adult life; both maternal nutrient deficiency and maternal obesity have been linked to epigenetic changes and the development of T2D in offspring.[4] There is growing evidence that pharmaceuticals also alter epigenetic processes, which may contribute to the beneficial and deleterious effects of widely-used medications.[5]

As a result of the morbidities and growing prevalence of T2D, the use of medications designed to lower blood glucose is widespread and growing. The biguanide drug metformin is the most widely prescribed antidiabetic drug and is considered the gold standard for the treatment of T2D.[6] In 2012, over 60 million prescriptions were filled for metformin in the United States, an increase of 97% from 2003.[7] Therefore, any epigenetic effects of metformin may affect the health of a significant number of individuals. Furthermore, as metformin can treat gestational diabetes, this may alter the foetal environment and thus affect the health of offspring. This review explores the epigenetic effects of metformin, including how they may modulate its glucose lowering and off-target activity, including potential protection from cardiovascular disease, cancer, cognitive decline and aging.

Epigenetic modifications

Epigenetic modifications fall under two main categories: histone modifications and DNA methylation. Although they do not directly interact with DNA, microRNAs are also often included as having epigenetic-like effects as they alter protein expression through the suppression of mRNA translation.

Histone modifications

DNA is wrapped around histone cores composed of two each of histone proteins H2A, H2B, H3 and H4; this structure is termed a nucleosome and comprises the fundamental unit of chromatin. DNA can be loosely packed and amenable to transcription as in euchromatin, or highly condensed and silenced as in heterochromatin; the state of organisation being determined by post-translational modifications to histone amino-terminal tails, most notably acetylation, phosphorylation and methylation.

Acetylation of histone lysine residues, particularly of H3 and H4, neutralises their positive charge and subsequently promotes an open chromatin structure.[8] Histone hyperacetylation is thus considered a signature of active transcription. Histone acetylation is highly dynamic and regulated by two opposing families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs add acetyl groups to lysine residues using acetyl-CoA as a cofactor. Of the two classes of HATs, Type-A HATs, including the CBP/p300 family, modify multiple sites in histone tails and act as transcriptional co-activators, whereas Type-B HATs, including HAT1, are cytoplasmic and acetylate newly formed histone proteins but not those already complexed to DNA.[8] HDACs are divided into 4 classes; class I HDACs, including HDAC 1, 2, 3 and 8, are nucleic whereas class II HDACs, including HDAC 4, 5, 6, 7, 9 and 10, move between the nucleus and cytoplasm.[9] Class III HDACs, also known as sirtuins, include SIRTs 1 to 7 and are associated with longevity and decreased disorders of aging, including T2D.[10] HDAC11 is the only class IV HDAC, of which little is known. Unlike class I, II and IV HDACs, which are zinc dependent, sirtuins rely on NAD+ for their deacetylating activity.

Similar to acetylation, histone phosphorylation neutralises the positive charge of the histone.[8] Serine, threonine and tyrosine residues serve as the phosphorylation/dephosphorylation sites for protein kinases and phosphatases respectively, in a process considered important for transcription, and therefore gene expression, as well as DNA repair, mitosis and apoptosis.[11]

Unlike acetylation and phosphorylation, methylation of histone lysine or arginine residues does not alter the charge of the histone and can have varying influences on transcription depending on the specific residue methylated (denoted by the histone protein and the lysine [K] or arginine [R] that is methylated) and the degree of methylation (mono-, di- or trimethylation). Histone methylation involves the transfer of methyl groups from S-adenosylmethionine (SAM) to histone residues, catalysed by histone methyltransferases (HMT). Methylation of histone lysines H3K4, H3K36, and H3K79 is generally associated with active transcription, whereas methylation of H3K9, H3K27, and H4K20 is more commonly found on transcriptionally silent genes.[12] Most HMTs are specific to a certain histone residue, for example enhancer of zeste homolog 2 (EZH2) trimethylates H3K27. Histone methylation is not as dynamic as acetylation and was thought to be a stable event until discovery of the first histone demethylase was reported in 2004.[13]

Numerous other post-translational modifications to histones have been discovered, including ubiquitination, sumoylation, O-GlcNAcylation, and ADP-ribosylation, although it is less clear how these modifications impact gene expression and other processes affected by chromatin configuration.

DNA methylation

DNA methylation occurs when a methyl group is transferred from SAM to nucleotides by DNA methyltransferases (DNMTs). In vertebrates, it is thought that methylation only occurs to cytosine

bases in CpG dinucleotides.[14] CpG dinucleotides are rare throughout the genome, accounting for only 1% of cytosines, but occur at a high frequency in the majority (approximately 70%) of gene promoters.[15] These regions of CpG clusters are termed CpG islands. Most cytosines in CpG islands are unmethylated, with hypermethylation of CpG islands generally resulting in transcriptional silencing due to decreased transcription factor binding and increased binding of methyl-CpG binding proteins, which may initiate histone modification and subsequent chromatin condensation.[14]

Four DNMTs with DNA methyltransfase activity have been identified, DNMT1, DNMT3a, DNMT3b and DNMT3c, while DNMT3L lacks methyltransferase activity and instead acts as a cofactor to *de novo* methyltransferases and DNMT2 methylates tRNA.[16] DNMT3a and DNMT3b are the *de novo* methyltransferases; they establish methylation in previously unmethylated cytosines, whereas DNMT1 maintains a state of methylation, for example in daughter cells following replication.[17] The recently described DNMT3c has been reported to silence retrotransposons in the germ cells of male mice.[18]

MicroRNAs

MiRNAs are short, non-coding RNAs that regulate gene expression through interaction with mRNA as part of the miRNA-induced silencing complex (miRISC). Due to their small size (approximately 21-24 nucleotides) and ability to bind even when complementarity is not perfect, each miRNA can target hundreds of mRNAs. Subsequently, translation is repressed by the blocking of translation initiation factors and the degradation of target mRNAs through the recruitment of deadenylating and decapping enzymes, followed by nucleases.[19]

Metformin

Metformin reduces hyperglycaemia by decreasing hepatic gluconeogenesis and increasing insulin sensitivity.[20] It is also used in the treatment of polycystic ovary syndrome (PCOS) to induce ovulation and regulate menstruation,[21] and may have additional benefits with meta-analyses reporting a reduced risk of cardiovascular mortality[22] and cancer[23] in individuals on metformin therapy. Metformin may also be neuroprotective, with placebo-controlled randomized clinical trials reporting metformin improves cognitive function in the cognitively impaired, such as those with Alzheimer's disease.[24,25] Metformin was also suggested as an antiaging drug as early as the 1970s,[26] and is currently being investigated in randomised controlled trials such as the Targeting Aging with Metformin (TAME) trial.[27] Metformin is generally well tolerated with gastrointestinal disturbances, particularly diarrhoea, being the most common side effect; less frequently liver damage and lactic acidosis have also been reported.[28] While the exact mechanisms of action are not completely understood, metformin's ability

to promote the phosphorylation and hence the activation of AMP activated protein kinase (AMPK) is considered to be central to its mode of action and to result in the inhibition of gluconeogenic genes.[20] Activation of AMPK, considered a major regulator of cellular metabolism, also impacts numerous pathways, not only those involved in glucose metabolism but also lipid metabolism, mitochondrial biogenesis, autophagy, cell growth and circadian rhythm.[29]

There is emerging evidence that metformin-induced modulation of AMPK activity influences epigenetic processes. Given metformin's widespread use, including in gestational diabetes and PCOS, this may be of concern. Studies exploring the effect of maternal metformin on offspring have been limited to short-term studies, and therefore the outcome regarding long-term metabolic effects is not yet known. Follow-up of the Metformin in Gestational diabetes (MiG) trial found offspring exposed prenatally to metformin had a preferable pattern of fat distribution at 2 years,[30] while a Finnish study found children exposed to metformin and supplemental insulin *in utero* were heavier at 18 months than children exposed to insulin alone.[31] Similarly, a trial of metformin use for PCOS during pregnancy found infants whose mothers took metformin were heavier at one year.[32] Animal studies report mixed results; with one study by Salomaki et al.[33] reporting increased weight gain and impaired glucose tolerance in mice on a high fat diet (HFD) exposed to metformin *in utero*, and a later study by the same group finding prenatal metformin exposure protected mice from HFD-induced weight gain and impaired glucose tolerance.[34]

Metformin and epigenetic aging

While the clinical effects of metformin as a pharmacological intervention to promote healthy aging and longevity are currently being investigated in clinical trials,[27,35] few studies have attempted to do so in the context of epigenetics. Epigenetic markers of aging accumulate with hyperglycaemia, hyperinsulinaemia and metabolic syndrome,[36,37] and correction of these metabolic abnormalities with metformin may ameliorate epigenetic aging. Metformin may also influence epigenetic aging through interaction with epigenetic modifying enzymes such as SIRT1, the HDAC associated with longevity. The clinical effects of metformin are similar to those of calorie restriction, an intervention known to prolong life,[37] and there is a growing body of evidence that SIRT1 plays an important role in mediating the anti-aging effects of calorie restriction, including antidiabetic actions.[10] In a randomised controlled trial, two months of metformin altered levels of several effectors associated with longevity, including increased SIRT1 expression in peripheral blood mononuclear cells.[35]

In a recent observational study by Quach, et al.[36], the degree of DNA methylation in peripheral blood (the 'epigenetic clock') was used to assess the effect of a variety of lifestyle factors on the rate of epigenetic aging, known as epigenetic age acceleration. Metformin did not significantly alter extrinsic

or intrinsic epigenetic age acceleration, although several other dietary and behavioural factors did. However, while epigenetic age acceleration has been found to correlate closely with expected longevity, it has not been validated as a means of assessing specific pharmacological effects, but rather is considered to reflect the 'cumulative effect of an epigenetic maintenance system.'[38] Furthermore, as pointed out by the authors, lack of power and the observational design of this study may mask a real effect, and randomised controlled trials may provide a more convincing result.

Metformin and histone modifications

Metformin-induced activation of AMPK has been reported to impact histone modifications via multiple mechanisms, including modifications that both increase and decrease gene expression (Figure 1). This includes phosphorylation of HATs, increased SIRT1 activity, inhibition of class II HDACs, and potentially phosphorylation of histone residues. There is also evidence that metformin may influence HAT expression through AMPK-independent mechanisms, and inhibit HMT expression and histone ubiquitination.

HATs

The effect of AMPK activation on HATs varies, increasing the activity of some HATs and decreasing others, and thus metformin may likewise have varying effects. For example, Marin, et al.[39] reported, using a mouse embryonic fibroblast model, that via AMPK activation, metformin induced HAT1 phosphorylation, increasing its activity. Conversely, in multiple other studies, the phosphorylation of the HATs p300 and CREB-binding protein (CBP) by AMPK reduced their activity.[40-43]

P300 and CBP are important transcriptional co-activators of multiple genes involved in inflammation and gluconeogenesis. He, et al.[40] demonstrated that metformin induced phosphorylation of CBP at Ser436. This prevented the formation of the CREB-CBP-TORC2 transcription complex and decreased CBP occupancy at the promoter regions of gluconeogenic genes including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC1α*) and phosphoenolpyruvate carboxykinase 1 (*Pck1*). Metformin significantly decreased blood glucose levels in wild-type mice but did not in mice with mutant CBP (S436A), suggesting this mechanism may play a key role in the antidiabetic activity of metformin.

Similarly, metformin-induced AMPK activation results in p300 phosphorylation at Ser89, decreasing its enzymatic activity.[42] AMPK-mediated p300 phosphorylation was found to reduce its interaction with nuclear receptors including PPARγ,[43] and consequently may reduce the transcription of PPARγ target genes, including genes involved in lipid and glucose metabolism. As PPARγ is considered

beneficial in T2D, with PPARγ agonists such as thiazolidinediones prescribed as insulin-sensitising agents, it is unclear how this influences the effects of metformin on glucose metabolism.

AMPK may also promote the proteasomal degradation of p300; Lim, et al.[41] demonstrated that metformin and AICAR, an unrelated AMPK agonist, reduced p300 protein, but not mRNA, levels in hepatic stellate cells, with proteasome inhibition restoring protein levels.

Metformin also increased mRNA and protein levels of Gcn5, a type A HAT, in diabetic mice and in a hepatocyte cell line.[44] However, this was not influenced by an AMPK inhibitor, suggesting metformin alters Gcn5 expression through an unknown, AMPK-independent mechanism.

HDACs

As with HATs, the effect of metformin-induced AMPK activation has differing effects on the activity of different HDAC classes, decreasing the deacetylation of class II HDACs while increasing the activity of the class III HDAC SIRT1. Khan et al.[45] demonstrated both an inhibitory effect on HDAC activity in rat liver and a consequent increase in global H3 acetylation following metformin treatment. HDAC inhibition with metformin has also been demonstrated in cancer cells.[46] This may be a result of HDAC phosphorylation; it has been demonstrated that AMPK phosphorylates HDAC4, 5 and 7 at Ser259 and Ser498,[47] and that such phosphorylation of HDAC5 results in its export from the nucleus and subsequently de-repression of target genes.[48] This may contribute to the antidiabetic activity of metformin; a previous study reported that AMPK activation decreased HDAC5 association with the glucose transporter type 4 (*GLUT4*) gene, resulting in increased GLUT4 expression in human primary myotubes, suggesting a direct mechanism by which metformin could ameliorate insulin resistance in muscle cells.[49]

As opposed to the inhibitory effect on class II HDACs, AMPK activation increases SIRT1 activity.[44] This is thought to be a result of NAD+ generation, as both metformin and overexpression of a constitutively active AMPK were reported to increase the NAD+/NADH ratio in myotubes.[50] Metformin may also increase SIRT1 gene expression; in a randomised controlled trial, two months of metformin treatment increased SIRT1 mRNA and protein levels in peripheral blood mononuclear cells, although no increase in SIRT1 deacetylase activity was detected.[35] Effects may differ in cancer cells, where metformin has been shown to reduce SIRT1 protein expression.[51] This is mediated through effects on miR-34a, as discussed in a later section.

In animal models, increased SIRT1 activity with metformin has been associated with inhibition of gluconeogenesis in the liver, implicating SIRT1 with the antidiabetic actions of metformin. Caton, et al.[44] found metformin treatment increased the NAD+/NADH ratio and SIRT1 activity in diabetic mice while decreasing plasma insulin and glucose as well as expression of the gluconeogenic enzyme *Pck1*.

Co-incubation of hepatocytes with inhibitors of either AMPK or SIRT1 reduced the antidiabetic effects of metformin, specifically increasing cellular glucose levels and *Pck1* expression and activity.

There is also evidence that metformin may reduce cellular aging and improve cardiovascular health through SIRT1, in particular by protecting vascular endothelial cells from cellular stress. In human umbilical vascular endothelial cells, treatment with metformin increased SIRT1 expression and activity and reduced signs of cellular aging and production of reactive oxygen species.[52] These effects were prevented when AMPK was silenced by siRNA. Similarly, Arunachalam, et al.[53] found metformin increased SIRT1 expression and conferred protective effects in cultured mouse microvascular endothelial cells exposed to high glucose, including increased levels of the anti-apoptotic Bcl-2 protein and reduced cellular senescence. Silencing SIRT1 prevented these protective effects.

SIRT1 has also been associated with neuroprotection in cell-based and animal models of aging and T2D and controlled clinical trials with resveratrol, a SIRT1 activator, have shown improved cognitive function.[54] In mice on a high fat diet, metformin increased hippocampal SIRT1 gene expression.[55] Although this study did not find significant changes in memory or learning with metformin treatment, this mechanism could potentially contribute to the reported neuroprotective effects of metformin.

Histone methylation

There is evidence that metformin can influence the methylation of numerous histone lysine residues. While both increases and decreases of lysine methylation have been reported, all these modifications are consistent with an increase in gene expression due to the previously mentioned fact that methylation of lysine residues can either activate (H3K4, H3K36) or repress (H3K9, H3K27) transcription. In particular, as these studies were conducted using cancer cells to investigate the potential anticancer activity of metformin, changes in histone methylation due to metformin treatment may lead to increased expression of tumour suppressor genes. For example, Banerjee, et al.[46] reported that metformin treatment decreased H3K9 and H3K27 methylation, as well as increased H3K4 methylation in breast cancer cells, both globally and specifically at the promoter of the tumour suppressor gene *E-cadherin*. These modifications may be a result of inhibition of HMTs; reductions of mRNA and protein expression of SUV39H1[56] and MMSET,[57] HMTs that influence the methylation of H3K9 and H3K27 respectively, have been reported in metformin treated prostate cancer cells. The mechanism by which metformin may inhibit HMTs is unknown as these studies did not examine whether AMPK is directly responsible for the reported effects.

Conversely, one study has reported that AMPK can increase histone demethylase activity, although this study did not use metformin treatment. Tanaka, et al.[58] demonstrated that AMPK can reduce H3K36 methylation through lysine-specific demethylase 2A (KDM2A) activity. AMPK activation by

AICAR reduced H3K36 dimethylation at the rDNA promoter with subsequent reduced transcription of rDNA in breast cancer cells. KDM2A knockdown prevented these effects.

Other histone modifications

The direct influence of metformin on histone phosphorylation has not been studied, however Bungard, et al.[59] found that AMPK activation by a related biguanide, phenformin, resulted in the phosphorylation of H2B at Ser36 in mouse embryonic fibroblasts. Abrogation of phosphorylation by Ser36>Ala36 substitution reduced the transcription of AMPK target genes. Furthermore, a study of Sertoli cells found AMPK knockout resulted in a reduction of phosphorylated H2B.[60]

AMPK activation has also been associated with a decrease in ubiquitination and O-GlcNAcylation of residues on H2B. Xu, et al.[61] reported that activation of AMPK by AICAR in mouse embryonic fibroblasts resulted in the phosphorylation of O-linked β-N-acetylglucosamine (O-GlcNAc) transferase, the enzyme responsible for histone O-GlcNAcylation, i.e. the addition of O-linked N-acetylglucosamine (O-GlcNAc). This decreased the affinity of the enzyme for chromatin and thus reduced O-GlcNAcylation of H2B at Ser112, both globally and at the region of several target genes associated with repression of these genes. As O-GlcNAcylation is thought to promote histone ubiquitination, they also found reduced ubiquitination at H2BK120. Although this study used a different AMPK activator, metformin has been proven to decrease H2BK120 ubiquitination in breast cancer cells.[62] These studies both associated ubiquitination and O-GlcNAcylation with increased gene expression, although both modifications have also been associated with gene silencing and linked to a variety of other histone modifications that may alter chromatin structure.[61,63]

Metformin and DNA methylation

Reported effects of metformin on DNA methylation include both hypo and hypermethylation at the promoters of different genes.[46,64-66] Furthermore, it has been reported that AMPK directly inhibits DNA methyltransferase activity, while metformin may indirectly reduce the activity of endogenous DNMT inhibitors. As a result, the overall effect of metformin on DNA methylation and subsequent gene expression is unclear.

Reduced DNA methylation with metformin treatment has been reported at the insulin gene promoter in a β cell line cultured using high glucose concentrations[65] and at the promoter of the tumour suppressor gene *E-cadherin* in both cancer cell lines and in white blood cells from diabetics,[46] leading to increased expression of the respective genes. These results thus implicate DNA demethylation in the antidiabetic and potential anti-cancer actions of metformin. Additionally, reduced methylation of transporter genes *SLC22A1*, *SLC22A3*, and *SLC47A1*, all involved in hepatic transport of metformin,

was reported in the livers of diabetics receiving metformin compared to those not receiving any antidiabetic medication.[66] Interestingly, these changes were not apparent in patients receiving metformin plus insulin. These changes in methylation may be a result of reduced DNMT activity; activation of AMPK by metformin has been reported to phosphorylate DNMT1 at Ser730 and consequently inhibit its methyltransferase activity.[39] This was associated with a decrease in promoter methylation of six target genes in wild type mouse embryonic fibroblasts, but not in AMPK knockout cells or cells with DNMT1 Ser730>Ala730 substitution. Furthermore, metformin treatment reduced DNMT1 protein expression in several human lung cancer cell lines.[67]

Conversely, several studies indicate that metformin may induce DNA hypermethylation via increased activity of S-adenosylhomocysteine hydrolase (SAHH). SAHH hydrolyses and thus inactivates S-adenosylhomocysteine (SAH), a feedback inhibitor of DNMT activity. Recently, it was reported that metformin decreased SAH levels in non-malignant breast epithelial cells and increased global DNA methylation in a variety of malignant and non-malignant cells.[68] AMPK knockout cells were resistant to these changes. Zhong, et al.[64] demonstrated that metformin increases SAHH activity and alters the methylation of numerous gene promoters in endometrial cancer cells. A SAHH inhibitor blocked the metformin-induced hypermethylation of five genes. Interestingly, the metformin-induced increase in SAHH activity may itself be due to DNA hypermethylation. The long noncoding RNA H19 has been demonstrated to bind and thus inhibit SAHH,[64] and Yan, et al.[69] reported increased methylation of the *H19* promoter and subsequent reduced H19 expression with metformin treatment in endometrial and ovarian cancer cells.

Metformin and microRNAs

Metformin has been reported to alter the expression of numerous miRNAs. Alterations in miRNA expression by metformin may be partially explained by an increase in DICER, one of the key enzymes in miRNA processing. Increases in DICER protein levels have been reported in metformin treated diabetic humans and mice,[70] and in cancer cells.[71] This increase was also reported in cells treated with AICAR, suggesting AMPK activation as a mechanism.[71]

Metformin-altered miRNAs in cancer

The potential anti-cancer properties of metformin have been linked to the regulation of numerous miRNAs (see Table 1). MiRNAs that may be important in the potential anti-cancer activity of metformin include upregulation of the let-7 family, miR-26a and miR-34a and downregulation of miR-181a, miR-221 and miR-222. Let-7 miRNAs are downregulated in many cancers and target mRNA of oncogenes including Ras and c-Myc,[72] while also inhibiting expression of the SAHH

inhibitor H19 and thus impacting DNA methylation (see previous section).[64] MiR-26a is also downregulated in many tumours and has been found to inhibit proliferation and induce apoptosis through the suppression of a number of oncogenes including EZH2, Oct4 and Notch-1.[73] MiR-34a is a transcriptional target of the tumour suppressor p53 and inhibits a number of oncogenes including c-Myc, c-MET and Notch, in addition to the anti-apoptotic Bcl-2 and the HDAC SIRT1.[74] The oncogenic miR-181a induces and maintains stem cell phenotypes in cancer cells by targeting transcription factors involved in cell differentiation,[75] while miR-221 and miR-222 promote cancer proliferation through the suppression of cell cycle inhibitors such as p27 and p57.[76,77]

Metformin-altered miRNAs in diabetes and diabetes complications

MiRNAs may contribute to the beneficial effects of metformin in diabetics, particularly in reducing the risk of diabetes-associated conditions such as cardiovascular disease, liver disease and diabetic nephropathy.

In one study, Santovito, et al.[78] reported 25 miRNAs with altered expression in diabetics, including downregulation of let-7 family miRNAs. Twelve months of metformin treatment combined with lifestyle changes significantly increased the expression of let-7a and let-7f. Although overexpression of let-7 has been associated with reduced insulin secretion and insulin sensitivity in mice,[79] a recent study by Brennan, et al.[80] suggests let-7 miRNAs may protect against inflammation in diabetic atherosclerotic plaques. Additionally, the internal mammary arteries of diabetics on metformin were found to have lower levels of miR-221 and miR-222 compared to diabetics not on metformin.[81] Inhibition and transfection of these miRNAs respectively reduced and increased the proliferation of isolated vascular smooth muscle cells, leading the authors to suggest that this mechanism may protect against intimal thickening in diabetes. Metformin may also improve impaired angiogenesis in diabetic vascular disease through miR-34a-mediated regulation of SIRT1 expression. Arunachalam, et al.[82] found metformin treatment lowered miR-34a expression, increased SIRT1 expression, increased expression of vascular growth factor Ang1 and subsequently increased tube formation in mouse microvascular endothelial cells. This downregulation of miR-34a is contradictory to the results found by multiple experiments in cancer cells and indicates metformin may have differing effects on miRNA expression in cancer cells compared to non-malignant cells.

Downregulation of miR-34a has also been found in livers of metformin treated mice. In a mouse model of liver disease, metformin protected mice from liver fibrosis, inflammation and steatosis and resulted in the downregulation of miR-34a, as well as miR-376a, miR-127, miR-300 and miR-342-3p, and the upregulation of miR-122, miR-194, miR-101b, and miR-705.[83] MiR-122 is the prominent miRNA in the liver and mice lacking the miR-122 gene develop liver disease and hepatocellular carcinoma,[84] while increased expression of miR-127 was found to inhibit the proliferation of rat

hepatocytes and thus may impede regeneration following injury.[85] Meng, et al.[86] found metformin treatment decreased levels of miR-291b-3p in the livers of mice on a high fat diet, with levels of this miRNA correlating with lipid accumulation. Overexpression of miR-291b-3p increased hepatic lipid accumulation in mice fed a normal diet, as well as AST and ALT levels, common biomarkers of liver damage. Interestingly, miR-291b-3p was shown to target AMPKα1, the catalytic subunit of AMPK, leading the authors to suggest that miR-291b-3p suppression contributes to the increase of AMPK activity by metformin, and thus may mediate numerous downstream effects.

Low expression of miR-192 has been associated with the progression of diabetic nephropathy and in one study circulating miR-192 levels inversely correlated with markers of impaired renal function (plasma urea and creatinine) in healthy subjects.[87] In this study, three months of metformin treatment increased plasma miR-192 concentration in diabetics by nearly 50%, although it did not investigate if this resulted in a mitigation of nephropathy.

Limitations of studies

The majority of studies reporting epigenetic effects of metformin have been conducted using mouse models or cell culture, with a few exceptions using human metformin users (increased SIRT1 expression,[35] DNA hypomethylation of certain genes[46,66] and changes in miRNA expression).[78,87] There are major limitations to these approaches due to differences in physiology between mice and humans and between transformed cell lines *in vitro* and human tissue *in vivo*. Furthermore, many studies used suprapharmacological concentrations of metformin in the millimolar concentration range, well above the 40-70 µM and 1-40 µM plasma concentrations reported in the portal vein and systemic circulation respectively following therapeutic doses.[88]

This may account for some of the disparate results of the studies. For example, studies reporting DNA hypomethylation with metformin were conducted on diabetics taking therapeutic doses of metformin,[46,66] or used micromolar doses of 500 µM[65] or 20 µM.[67] Conversely, studies associating metformin with DNA hypermethylation used suprapharmacological concentrations between 1 and 10 mM.[64,68-69] For a summary of the published studies, including the metformin dose and the model used, see Supplementary Table 1.

Additionally, the reported studies have largely investigated epigenetics in the context of potential beneficial effects of metformin, namely diabetes, cancer, atherosclerosis, cognitive function and aging, and have not investigated if these epigenetic modifications could have detrimental side effects. Finally, it is yet to be reported if metformin use during pregnancy, in conditions such as gestational diabetes or PCOS, may imprint epigenetic signatures onto the offspring that may program long-term metabolic changes.

Conclusion

Epigenetics is a rapidly growing field that has been linked with numerous disorders, including T2D. The widely used antidiabetic drug metformin can reportedly modulate numerous epigenetic processes that may contribute to its primary hypoglycaemic action, but may also result in off-target effects, both beneficial and potentially deleterious. Metformin can alter the activity of many different epigenetic modifying enzymes, primarily through the phosphorylating actions of AMPK. However, these include modifications that act to both increase and decrease gene expression. Furthermore, the literature suggests that metformin has opposing effects within the major groups of epigenetic modifying enzymes. Generally, it decreases the activity of most classes of HATs, HDACs and DNMTs through AMPK-mediated phosphorylation, and at the same time reportedly increases HAT1 and SIRT1 activity, and decreases the influence of DNMT inhibitors. This, together with the previously mentioned limitations of published studies, means it is difficult to make generalisations regarding the effects of metformin on the epigenome, subsequent gene expression and what impact this may have on the health of the millions of metformin users worldwide.

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Table 1: Effect of metformin on miRNAs in cancer cells

Cell/tissue source	Metformin dose	miRNAs altered	Observed effects	Reference
Breast cancer cell lines MCF-7, BT-474 and SUM-159	500 µM	MiR-33a upregulated	MiR-33a downregulated c-Myc and IRS-2	Blandino, et al.[71]
Breast cancer cell line MDA-MB-231	10 mM	MiR-26a upregulated	MiR-26a downregulated PTEN, decreased viability	Cabello, et al.[89]
Breast cancer cell line MCF-7	1 mM	Let-7a, miR-32, miR-96 upregulated MiR-183 downregulated	Metformin inhibited formation of mammospheres	Oliveras Ferraros, et al.[75]
Breast cancer cell line MCF-7	From 1 mM	MiR-34a upregulated	MiR-34a downregulated SIRT1 Metformin increased susceptibility to oxidative stress	Do, et al.[51]
Breast cancer cells lines MCF7, MDA-MB, BT-549, HCC70	10 mM	MiR-193a and miR-193b upregulated	MiR-193b downregulated fatty acid synthase (FASN) protein and induced apoptosis in BT-549 cancer cells but not non-cancerous MCF10A cells	Wahdan-Alaswad, et al.[90]
Breast cancer cell line MCF7, endometrial cancer cell line ARK2	2 mM	Let-7 upregulated	Let-7 and AICAR downregulated H19	Zhong, et al.[64]
Cholangiocarcinoma cell lines HCCC-9810, RBE, SSP25 and Hucct1	20-40 mM	MiR-124, miR-182, miR-27b and let-7b upregulated MiR-221 and miR-181a downregulated	MiR-124 downregulated CDK2, CDK4, CyclinD1 and CyclinE1 MiR-182 and miR-27b downregulated CDK2 and CyclinD1 Let-7b downregulated CyclinD1 MiR-124, miR-182, miR-27b and let-7b decreased proliferation Mir-221 and miR-181a upregulated p27 and increased proliferation	Jiang, et al.[91]
Hepatocellular carcinoma cell line Huh7	10 mM	33 upregulated including let-7a, let-7b, let-7e, miR-34a, miR-26a, miR-181a. 18 downregulated	Metformin decreased proliferation, downregulated angiogenin, cyclin D1, Cdk4 and cyclin E,	Miyoshi, et al.[72]
Hepatocellular carcinoma cell line HepG2	2.5 µM	MiR-23a upregulated	MiR-23a downregulated FOXA1, induced apoptosis	Sun, et al.[92]
Gastric cancer cell line MKN74	10 mM	30 miRNAs upregulated, including let-7 family. 21 downregulated	Metformin decreased proliferation, downregulated cyclinD1	Kato, et al.[93]
Lung cancer cell lines A549 and NCI-H358	10 mM	MiR-222 downregulated	Metformin upregulated p27, p57 and PTEN and decreased proliferation	Wang, et al.[76]
Pancreatic cancer cell lines AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR	20 mM	Let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c upregulated	MiR-26a downregulated EZH2, Oct4, Notch-1, and EpCAM MiR-26a and let-7b decreased growth of pancreatospheres	Bao, et al.[73]
Pancreatic cancer cell line Panc02	0.5 mM	MiR-34a upregulated	MiR-34a downregulated Notch. Metformin decreased tumour sphere formation	Cifarelli, et al.[94]
Pancreatic cancer cell lines Panc1 and Sw1990	5 mM	MiR-26a, miR-192, and let-7c upregulated	MiR-26a downregulated HMGA1, reduced cell proliferation and migration, and induced apoptosis	Li, et al.[95]
Pancreatic cancer cell line Panc1	10 mM	78 upregulated including let-7 family and miR-150 51 downregulated	Metformin downregulated cyclinD1 and Cdk4 and decreased proliferation	Kato, et al.[96]

		including miR-7		
Pancreatic cancer cell line Panc1	20 mM	MiR-221 downregulated	MiR-221 downregulated p27 and decreased G1-phase arrest	Tanaka, et al.[77]
Prostate cancer cell line PC-3	1-5 mM	10 upregulated 12 downregulated including miR-181a	Metformin decreased proliferation	Avci, et al.[97]
Prostate cancer cell line Vcap	5 mM	MiR-30a, miR-143 and miR-196b upregulated	MiR-30a downregulated SOX4, decreased proliferation, migration and invasion of cancer cells	Zhang, et al.[98]
Renal cancer cell line 786-O	10 mM	MiR-26 upregulated	MiR-26a downregulated Blc-2 and CyclinD1, decreased proliferation	Yang, et al.[99]

Legends to figure

Figure 1: Postulated mechanisms by which metformin may modify histones via AMPK activation.

Metformin phosphorylates (P) and activates AMPK, which subsequently has been found to activate gene expression through the phosphorylation and inactivation of HDACs and activation of HAT1, leading to increased acetylation (Ac) of histone (H) tails. AMPK may also increase gene expression through phosphorylation of histone H2B. Conversely, AMPK may also suppress gene transcription via increasing cellular NAD+ levels and thus increasing SIRT1 deacetylation activity and via phosphorylation and inactivation of HATs p300 and CBP.

Figure 1