Metformin As a Geroprotector

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Abstract

Geroprotectors are drugs that decrease the rate of aging and therefore extend life span. Metformin has been described as a geroprotector, and several studies have shown that metformin can slow down the rate of aging. The mechanisms behind the geroprotective effect of metformin are less established. The goal of this review is to investigate the evidence for the geroprotective effect of metformin and to describe the possible mechanisms behind it.

Introduction

EVERY DAY APPROXIMATELY 100,000 people die from age-related diseases,[1] and millions worldwide suffer from age-related frailty and disabilities. It is clear that geroprotectors, drugs that slow down the aging process, have a huge potential to improve the quality and increase the quantity of people's lives. Research in model organisms has uncovered several possible candidate drugs, such as ethoxysuximide,[2,3] lithium,[4] rapamycin,[5–7] resveratrol,[8–10] and nordihydroguaiaretic acid (NDGA).[11] Calorie restriction (CR) is the best researched intervention in aging and has been shown to extend the life span of yeast, flies, nematodes, rotifers, water striders, grasshoppers, water fleas, spiders, mice, rats, hamsters, guinea pigs, fish, and dogs,[12–14] and has recently been shown to increase survival in primates,[15] raising the hope that CR might be evolutionarily conserved in humans. Short-term investigations have discovered similar changes in biomarkers in humans on CR, as in model organisms,[16] indicating a strong possibility that CR will extend the human life span.

Metformin (*N,N*-dimethylbiguanide) (Fig. 1) is an antiglycemic, biguanide class drug used in the treatment of diabetes mellitus and polycystic ovarian syndrome.[17,18] It has been proposed that metformin could act as a CR mimetic.[19,20] Phenformin and buformin are closely related biguanides that have also been used to treat diabetes mellitus, but their use has been discontinued due to the high risk of lactic acidosis.[18,21] For this reason, metformin, and not buformin or phenformin, has been chosen as the focus of this review. However, references to related biguanides will be made, and it is important to keep in mind that their affinity, pharmacokinetics, and removal from the body might be different from that of metformin.

Metformin As a Geroprotector

Life span tests

In 1980 Dilman and Anisimov studied the effect of phenformin, diphenylhydantoin, and L-3,4-dihydroxyphenylalanine (L-DOPA) on life span and tumor incidence in female C3H/Sn mice. They discovered that phenformin (2 mg/day; 5 days/week) treatment increased the mean life span (21.1%) and the maximum life span (26%) as well as the mean life span of the last 10% of survivors (28.4%); at the time of death of the last mice in the control group, 42% of the phenformin-treated mice were still alive, and the incidence of mammary adenocarcinomas dropped 3.8-fold compared to the control.[22,23] The population aging rate decreased by 31.2%, the mortality rate doubling time increased 1.45-fold, and the survival curve shifted to the right.[23] The administration of phenformin (5 mg/rat per day) to female outbred LIO rats had no effect on the mean life span, but it did increase the maximum life span (9.8%) and the mean life span of the last 10% survivors (10.1%).[23,24] The influence of phenformin treatment on population aging rate, mortality rate doubling time, and the spontaneous tumor incidence were all statistically insignificant.[23] Buformin (5 mg/rat/day) was also tested in female LIO rats and was shown to extend the mean life span (7.3%), maximum life span (5.5%), as well as the mean life span of the last 10% of survivors (12%) and decreased tumor incidence 1.49-fold.[23,25] The population aging rate was decreased by 18.1%, the mortality rate doubling time increased 1.22-fold, and the survival curve was slightly shifted to the right.[23]

Anisimov and colleagues have published two reviews about their research on the geroprotective effects of biguanides.[23,26] Metformin (100 mg/kg) was shown to increase the mean life span (8%) and maximum life span (13.1%) of HER-2/neu mice.[27,28] However, this is most likely caused by
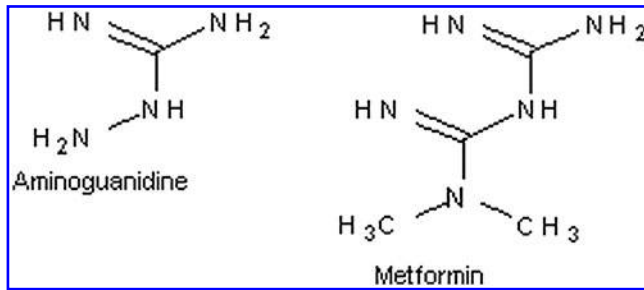
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FIG. 1. Structural relationship between metformin an aminoguanidine.

decreasing the incidence and size of mammary tumors rather than an effect on the rate of aging. Interestingly, a growing body of evidence shows the potential of metformin as an anticancer drug.[26,29,30] Metformin (100 mg/kg) increased the mean life span (37.8%), maximum life span (10.3%), and the mean life span of the last 10% of survivors (20.8%) but did not influence spontaneous tumor incidence in female SHR mice.[31]

In a more recent study, the age at which metformin treatment (100 mg/kg) was started in the female SHR mice was varied (3, 9, and 15 months of age). Treatment started at 3 and 9 months of age and increased mean life span by 14.1% and 6.1%, respectively. Maximum life span was only increased in the mice started at 3 months of age.[32] Metformin increased the mean life span (8%), maximum life span (16.2%), and the mean life span of the last 10% of survivors (13.1%) in female FVB/N mice.[27] A follow-up study using female mice of the same mice model (FVB/N) found 6.7% increase in mean life span, but a 11.1% and 9.3% reduction mean life span of last 10% survivors and maximum life span, respectively[30] However, this mouse model is known to develop renal lesions,[33] which are a contraindication for metformin use due to the risk of lactic acidosis.

A recent study[34] using 300 mg/kg per rat per day metformin in male Fischer-344 rats showed no significant effect on mean and maximum life span as well as mean life span of the last 10% survivors' life span. However, the same study also found no effect of CR on these parameters, whereas it was earlier repeatedly found that CR extended the life span in this strain of rats.[35,36] Another possible explanation is that the dosage used was too high. In a new study by Anisimov et al., the mean (5.1%) and maximum (3.9%) life span of female 129/Sv mice was slightly increased by metformin supplementation whereas the mean life span of male mice was decreased by 13.4%.[37] However, this study also found a higher blood glucose level (almost 1 mM) in metformin-treated animals versus controls.

Given the long history of clinical use of metformin as a blood glucose–lowering drug, this result raises severe questions on the validity of the data. Preliminary results suggest that the mean life span of B6C3F1 mice, a long-lived F_1 hybrid, is increased significantly by treatment with metformin at about the same concentrations shown by Anisimov and his colleagues[27,28,31] to extend the life span of shorter-lived strains of mice (Spindler and Mote, unpublished results). Higher doses appear to be less effective (Spindler and Mote, unpublished results). Metformin was also shown to increase the life span (20.1%) in a mouse model of Huntington disease.[38] Recently it was shown that metformin (50 mM) ex-

tends the mean but not the maximum life span of the nematode worm *Caenorhabditis elegans*.[39] Higher or lower dosages (10 or 100 mM, respectively) did not show any significant effect on life span.

In all, 75% of the gene expression changes induced by long-term CR were reproduced by the supplementation with metformin (8 weeks), whereas short-term (8 weeks) CR only reproduced 71% of the gene expression changes.[40] It is interesting to note that metformin decreases body temperature in female SHR mice[32] just as CR does in mice.[13] However, there was no significant difference in the body temperature of metformin-treated versus control in female HER-2/neu mice.[27] In female 129/Sv mice, the body temperature was higher in the metformin-treated group at 15 months but lower at 24 months.[37] In conclusion, no consistent effect of metformin on body temperature can be observed; however, CR has consistently been shown to reduce body temperature in mice, primates, and humans.[13,41,42] Another similarity between CR and metformin is that both of them decrease the incidence of cancer.[12,13,23,26] It is interesting to note that total tumor incidence was equal between metformin-treated and control female 129/Sv mice, but the number of malignant tumors was significantly (3.5 times) lower in the metformin group.[36,37]

In another study, there was no difference in total or malignant tumor incidence, but metformin treatment increased the life span of tumor-bearing mice.[32] It remains possible that the effect of metformin on life span and its CR-mimicking action is an artifact caused by a decrease in food consumption. Indeed, several studies found a decrease in either body weight or food consumption in the biguanide-treated group.[24,25,27] This is in line with human data that show that metformin functions as an appetite suppressor and decreases body weight in obese patients.[18] However, not all mice studies found a decrease in food consumption.[32] As will be discussed below, there are good indications that metformin influences the "CR pathway" through 5′ adenosine monophosphate (AMP)-activated protein kinase (AMPK) activation independently of its effect on food intake. More studies about the effects of metformin supplementation on life span in "normal" mice and rat strains, and especially in male animals (since most studies to this date have only investigated the effect in female animals), are needed to reach a definitive conclusion. The food intake should also be carefully controlled before a definitive conclusion on metformin's effects will be possible. A further interesting question would be if metformin is able to provide a further increase in life span in calorie-restricted animals.

Effect on glycation

Advanced glycation end products (AGEs) are nonenzymatic modifications of proteins by sugars or reactive degradation products of sugars and fats such as methylglyoxal, glyoxal, and 3-deoxyglucosone.[43–45] The formation of AGEs and protein cross-links has been suggested as a cause of aging[46,47] and surely contributes to a range of age-related diseases.[45,48,49] Aminoguanidine has a long history of research as an inhibitor of the formation of AGEs.[50–52] The strong structural similarity between metformin and aminoguanidine (Fig. 1) suggests that metformin could function as an inhibitor of glycation. Ruggiero-Lopez et al. incubated globin-free bovine serum albumin (BSA; 100 μM) with either

methylglyoxal or glyoxal (1 mM) in the presence or absence of metformin or aminoguanidine (1 mM) for 6 days at 37°C. They found that incubation with metformin or aminoguanidine markedly inhibited the characteristic fluorescence for AGEs (370/440 nm). Metformin and aminoguanidine decreased fluorescence of the glyoxal incubation by 37% and 85%, respectively, and in the methylglyoxal incubation by 45% and 58%, respectively.[53] These data indicate that metformin inhibits the formation of AGEs, although less efficiently than aminoguanidine. However, compared to aminoguanidine,[54–56] metformin does not give rise to dangerous long-term side effects.

In an experiment by Kiho et al., buformin inhibited crosslinking of ribonuclease (RNase) to the same extent as aminoguanidine and was more effective than metformin. The inhibition of cross-linking by metformin and buformin was dose dependent for both.[57] Tanaka et al. found that metformin exhibited a dose-dependent inhibitory effect on the formation of N^6(carboxymethyl)lysine (CML) measured by a polyclonal antibody against CML as well as on AGEs in general, as measured by a polyclonal antibody against nonspecific AGEs.[58] However, the effect only started at a concentration of 1 mM, which is higher than the plasma levels that can be obtained pharmacologically. Mean steady-state plasma levers are about 6 μM, and maximal concentrations during clinical trials never reached more than 24 μM.[59] Bailey et al. describe 10 μM as the maximal plasma concentration 1–2 hr after oral intake of 500–1,000 mg.[60] Beisswenger et al. observed a decrease in methylglyoxal levels in patients treated with metformin (>1 gram/day).[61] In women with polycystic ovary syndrome (PCOS), treatment with metformin (1,700 mg/day) for 6 months resulted in a reduction of serum AGEs.[62]

Although it is often suggested that metformin blocks glycation by scavenging reactive intermediates by reacting with them, other possible mechanisms have been suggested.

Metformin could chelate metals and thus prevent metal-catalyzed glyoxidation.[53] Beisswenger et al. speculated, based on an observed increase in D-lactate (the major end product of methylglyoxal detoxification of the glyoxalase pathway), that metformin might prevent glycation by increasing the detoxification of methylglyoxal.[61] Metformin also reduces the expression of the receptor for advanced glycation end products (RAGE) and lectin-like oxidized receptor 1 (LOX-1), both of which are activated by AGEs, in endothelial cells.[63] The binding of AGEs to RAGE has been suggested to activate proinflammatory genes, and could contribute to hyperglycemia[64] and diabetic cardiovascular complications.[65]

Mechanisms of Action

The mechanisms by which CR delays aging are largely debated. Two main targets have been proposed, Sir2p and target of rapamycin (TOR)[66]; however, these may not be independent of each other. Because metformin has very similar effects on gene expression as CR,[40] it would be appealing to suggest that metformin works by the same mechanisms. It should be noted that metformin decreases plasma glucose levels and improves insulin sensitivity.[17] Both of these are associated with greater longevity.[67]

AMPK signaling

AMPK is a serine/threonine protein kinase that belongs to the energy-sensing kinase family. It is a heterotrimeric complex that consists of a catalytic α-subunit (α1 or α2), a regulatory β-subunit (β1 or β2), and a regulatory γ-subunit (γ1, γ2, or γ3).[68–71] Homologs of all three subunits have been found in every eukaryotic species ever examined, including fungi, plants, and animals.[70,71] AMPK activity is regulated by many upstream effectors, including hormones such as insulin, leptin, ghrelin, and thyroid hormones (Fig. 2). AMPK
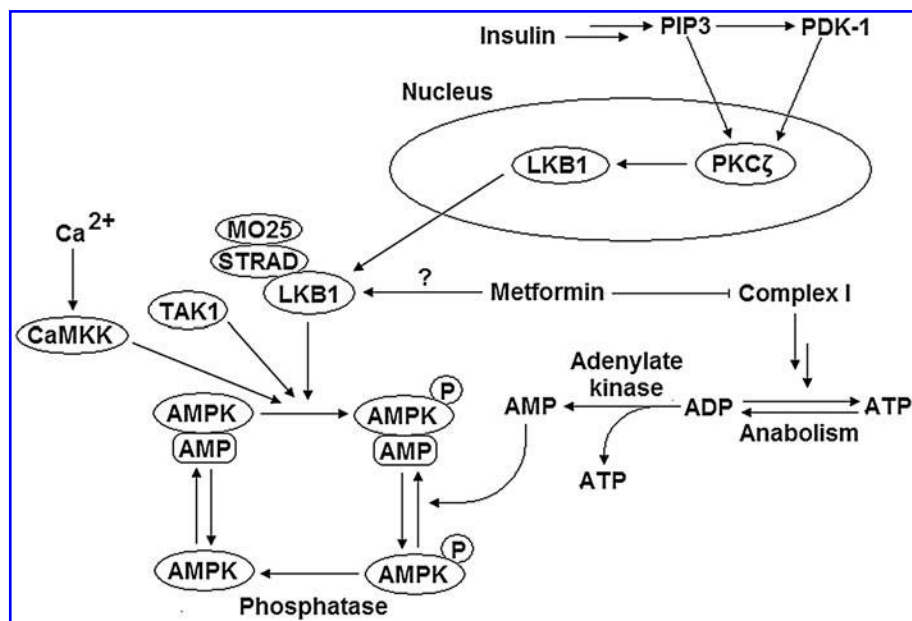

FIG. 2. Regulation of 5′ adenosine monophosphate (AMP)-activated protein kinase (AMPK) activity. The P indicates a phosphate group.

contains multiple autophosphorylation sites that are implicated in its activation,[68] but the most critical step is phosphorylation by AMPK kinases (AMPKKs) of the key regulatory site (Thr172) in the activation loop of the catalytic α-subunit, leading to an at least 100-fold increase in activity.[70,71] The activity of AMPK can be enhanced by 5'-AMP binding to each of the two tandem Bateman domains in the γ-subunit of AMPK. Four distinct mechanisms to explain how AMPK activation by AMP works have been proposed: (1) Allosteric activation of AMPKK; (2) binding of 5'-AMP to AMPK and thereby protecting it from phosphatases; (3) binding to AMPK, thereby making it a better substrate for AMPKK; and (4) allosteric activation of AMPK.[71,72]

However, Sanders et al. have shown that AMP does not increase the phosphorylation of Thr172 by two upstream AMPKKs, but that it does prevent the dephosphorylation of AMPK by protein phosphatase 2Cα (PP2Cα).[73] Allosteric activation, however, only results in a five-fold increase in activity.[74] The ratio of ADP:ATP increases during energy depletion. Adenylate kinase converts two molecules of ADP into one ATP and one AMP molecule. This results in a much bigger increase in the AMP:ATP ratio versus the ADP:ATP ratio.[70] High ATP levels antagonize activation of AMPK by all three allosteric mechanisms by competing with 5'-AMP for binding to the Bateman domains.[70,71] In this way, AMPK is able to sense cellular energy levels. In response to energy deficiency, AMPK activates ATP-producing processes such as fatty acid oxidation[74,75] and mitochondrial biogenesis,[76] glucose uptake (GLUT4 translocation), glucose oxidation, and glycolysis while switching off energy-consuming processes like fatty acid, glycogen, and sterol synthesis.[77,78] The β-subunit also binds to glycogen, and it is thus possible that AMPK senses medium-term energy availability in the form of glycogen. New evidence shows that ubiquitin-mediated degradation is another mechanism for AMPK activity control.[79]

Activation and inhibition of AMPK

Insulin antagonizes the activation of AMPK in certain tissues such as cardiac muscle through the activation of Akt and subsequent phosphorylation of Ser485 or Ser491 on the α1- or α2-subunit of AMPK, respectively.[71] Infusion of aminoimidazole carboxamide ribonucleotide (AICAR) into muscles, exercise, and β-guanadinopropionic acid (β-GPA) feeding, all of which work by either increasing AMP availability or by molecular mimicking of the AMP molecule (AICAR is converted to AICA-riboside monophosphate [ZMP], which is structurally similar to AMP) all activated AMPK-α2 in young but not old mice.[80] Furthermore, also phosphorylation at Thr172 and Ser79, phosphorylation of the downstream AMPK target ACC2, Pgc-1α mRNA expression, mitochondrial density, δ- aminolevulinate synthase (ALAS) mRNA expression, and cytochrome c content, all markers of AMPK activity, were altered in treated young but not old rats. The overexpression of the catalytic AMPK subunit AAK-2 extends the life span of Caenorhabditis elegans by about 13%.[81]

Metformin has been shown to activate AMPK in the hepatocytes,[82] skeletal muscle,[83] heart,[84] microglial,[85] endothelial,[86,87] β-cells,[88] and cortical neurons.[89] Metformin failed to activate AMPK in lung tissue. This study also showed that lungs have a very low expression of OCT1 (17 times lower than in liver), a receptor responsible for metformin uptake in cells.[90] One study found that metformin treatment did not increase AMPK in hypothalamic neurons,[91] but another one came to the opposite conclusion.[92] Such different effects have been observed for either AMPK activators; for example, leptin inhibits AMPK activity in the hypothalamus while activating it in nonneural tissues.[78] 2-Deoxy-D-glucose[93] and resveratrol,[94] two other suggested CR mimetics, have also been shown to activate AMPK.

Because AMPK activators fail in older rats,[80] it seems questionable that metformin administration to people who are already middle-aged will have any geroprotective effect. It should, however, be noted that only interventions that mimic or increase AMP levels had been used while metformin probably works by stimulating the phosphorylation of AMPK on Thr172 (see below). However, as discussed above, metformin failed to extend the life span when administered to 15-month-old mice.[32]

CR, on the other hand, has no effect on AMPK activity nor AMPK phosphorylation status in the heart, skeletal muscle, or liver of mice, and fasting only increases AMPK activity in the liver.[95] This is challenged by another study that found an approximate four-fold increase in Thr172 phosphorylation in gastrocnemius muscle after 6 hr of fasting.[96] Another study found that CR downregulates the phosphorylated (at Thr172) form of AMPK in the liver.[97] However, other studies find that CR increases Thr172 phosphorylation.[98,99] The reason for this may be that the energy stress (AMP levels) caused by CR or 24 hr of fasting is not severe enough to activate AMPK. This is supported by the finding that the ATP concentration is kept within normal levels during a fast at the expense of the phosphocreatine pool.[100] Furthermore, CR does not reduce ATP synthesis by the mitochondria.[101] Munday et al. have found that AMPK activity was significantly increased after 48 hr of fasting.[102] This supports the idea that a more severe stress than short-term fasting or CR is needed to activate AMPK.

There are at least 12 isoforms (excluding the alternative splice variants and/or alternative transcription start sites) of AMPK,[70] expressed in different tissues, and we can expect these to have different levels of sensitivity for various inputs such as hormonal signaling. This, together with the observation that certain interventions can extend the life span when done in just one type of tissue (e.g., fat-specific insulin receptor knockout),[103] leads to the possibility that AMPK might after all be part of the CR pathway. In this mechanism, CR would activate AMPK in specific tissues (most likely the nervous system), probably through hormonal signaling (ghrelin, agouti-related peptide, and adiponectin).[78] These tissues would then either send a pro-longevity signal or inhibit a pro-aging signal that is send to other tissues. Signals from one tissue that influence a longevity pathway in another tissue have already been found in C. elegans. Here the germ line generates a steroid signal that inhibits nuclear localization of DAF-16 in the intestine and thereby shortens life span.[104]

Metformin might increase AMPK activity and thereby metformin supplementation might reinforce life span extension by CR. It should however be noted that Dagon et al. discovered that while mild AMPK activation by CR improves cognition and prevents neuronal apoptosis, whereas

severe AMPK "overactivation" decreased cognitive abilities and increased neuronal apoptosis.[98] Thus, overactivation might be harmful.

How metformin activates AMPK

It has been suggested that metformin activates AMPK by decreasing cellular energy due to its inhibitory action on complex I of the electron transport chain (Fig. 3).[105,106] Indeed, an increase in AMP/adenosine triphosphate (ATP) ratio has been found in liver tissue of metformin-treated animals[107]; however, it is not clear if metformin changes AMP/ATP levels in other tissues. However in other studies it was shown that metformin activates AMPK without affecting the 5′-AMP/ATP ratio.[108,109] Two AMPKKs, the serine/threonine-protein kinase 11 (STK11), better known as liver kinase B1 (LKB1),[110,111] and calcium/calmodulin-dependent kinase kinase-β (CaMKKβ),[112] have been identified; and a third, transforming growth factor-β–activated kinase (TAK1),[113] has been proposed. CaMKKβ is only present in a small number of tissues (brain, testis, thymus, and T cells); therefore, AMPK activation by CaMKKβ will be limited to these tissues.[70] LKB1 is a 1:1:1 heterotrimeric complex that consists of LKB1, mouse protein 25 (MO25), and Ste20-related adaptor (STRAD).[114,115] LKB1 is primarily localized in the nucleus, due to the nuclear localization signal in the amino-terminal noncatalytic region, and it has to be transported to the cytosol for AMPK activation.[115] Phosphorylation of LKB1 serine 428 is needed for AMPK activation,[115] and the phosphorylation on serine 307 by protein kinase C zeta (PKCζ) is needed for transport to the cytosol.[116] Activation of LKB1 does not happen by the usual phosphorylation like other kinases but by binding to STRAD.[114] This heterodimerization also traps LKB1 in the cytoplasm. The LKB1:STRAD complex is stabilized by binding to MO25.[115] Exactly how metformin activates LKB1 is not known.

Metformin and AMPK in the CR pathway

We can conclude that metformin activates AMPK, but that AMPK is not a part of the CR pathway. However, AMPK or one of its downstream targets intersects with the CR pathway, and this explains why AMPK activation mimics CR. Therefore, I have termed metformin an "indirect" CR mimetic. This is
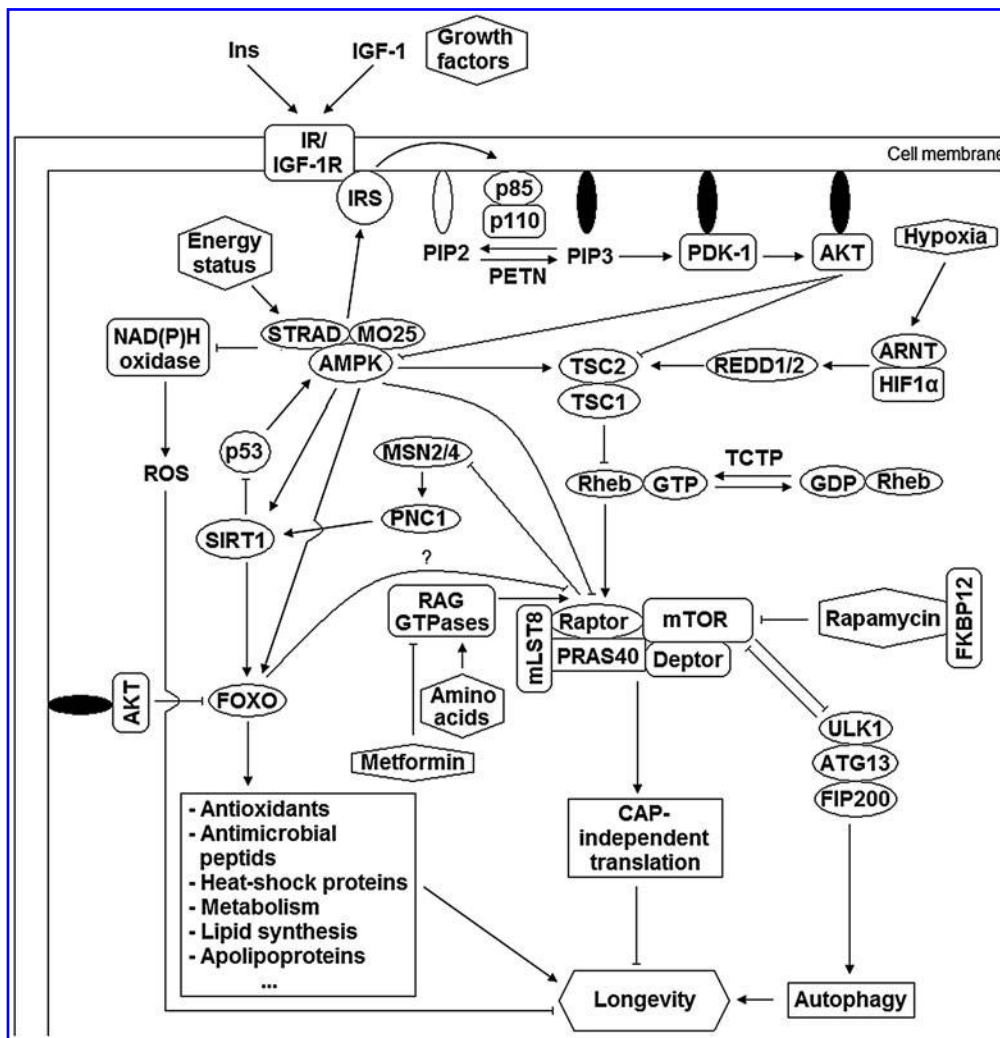

FIG. 3. Role of 5′ adenosine monophosphate (AMP)-activated protein kinase (AMPK) and mTORC1 signaling in the prolongevity effects of metformin. The input and output signals are depicted in hexagonal boxes.

perfectly in line with the observation that gene expression changes induced by metformin show a 71% similarity with those of CR,[40] given that the proteins involved in the signaling pathway that connects metformin with the CR pathway must have some other CR-independent targets (Fig. 4).

Downstream effectors of AMPK

Lack of space prevents the complete discussion of the downstream effectors in this review. Furthermore, the exact downstream effectors that mediate the antiaging effects of metformin are not known. This discussion is limited to the main mediators that activate downstream signaling. AMPK has recently been shown to activate SIRT-1,[117] the mammalian homolog of the silent information regulator 2 (Sir2p), connecting metformin and CR by their common effect on Sir2p. However, the role of Sir2p in CR has been questioned.[118] Furthermore, AMPK phosphorylates, and thus activates, DAF-16 (the mammalian ortholog is FOXO) in *C. elegans*.[119] The forkhead transcription factor FOXO undergoes nuclear localization after activation where it (in *C. elegans*) targets genes involved in antioxidant and antimicrobial protection, detoxification, steroid and lipid synthesis, and heat shock.[120]

Whether or not this mechanism is evolutionarily conserved is not clear. McElwee et al. have shown that, although the influence of insulin/insulin-like growth factor-1 (IGF-1) on life span is conserved from nematodes to rodents, no conservation exists between the genes that regulate that response. However, in all three investigated species, the same gene categories (detoxification, protein synthesis, energy metabolism) appear to be involved.[121] DAF-16, but not sir2, is required for life span extension in Ins/IGF-1 mutants,[104] and FOXO variants have been linked to human longevity.[122] SIRT-1 deacetylates FOXO proteins in response to oxidative stress, and this could result in their activation,[122] suggesting that sirtuins influence life span extension by DAF-16 independently of Ins/IGF-1 signaling.[122] SIRT-1 can either activate or decrease the activity of the tumor suppressor p53.[14] The sirtuin activator resveratrol has been shown to increase life span in some studies,[8–10] but not in all.[6,122]

Recently, doubt has been raised regarding the resveratrol/SIRT-1 connection.[123] AMPK also has complex effects on apoptosis, stimulating it in some situations while preventing it in others. A possible explanation for this is that AMPK activation might allow cells to recover from energy depletion and thus avoid apoptosis while stimulating apoptosis when the energy deficiency has become too severe.[98] The final downstream target is TOR, which will be discussed in the next section. PGC-1α (downstream target of AMPK), pha-4/FOXA, and the NF-E2-related transcription factors Nrf1 and Nrf2 have all been suggested to play a role in CR,[14] and thus might also be involved in the life span extension by metformin, but will not be discussed in this review.

mTOR signaling

Mammalian target of rapamycin (mTOR) is a 289-kD serine-threonine kinase that belongs to the phosphoinoside 3-kinase (PI3K)-related kinase family that is conserved from yeast to mammals.[124,125] Its action depends on the interplay between oxygen availability, amino acids, Wnt signaling, growth factors (such as insulin and IGF-1), and energetic status.[124—127] mTOR forms two district protein complexes, named TORC1 and TORC2, with distinct cellular functions. TORC1 is built from mTOR, rapamycin-sensitive adaptor protein of mTOR (Raptor), Pras40, mLST8 (GβL), and Deptor, whereas in TORC2 Raptor has been replaced by rapamycin-insensitive companion of mTOR (Rictor), Pras40 is missing, and two additional proteins (Protor-1 and mSin1) are included.[124,127] TORC1 controls translation initation and elongation,[128] mitochondrial biogenesis, ribosome biogenesis,[124] cell-cycle progression,[126] stem cell differentiation,[129] and lipid synthesis, while inhibiting autophagy.[130]

mTOR in aging

Inhibition of mTOR by RNAi has been shown to extend the life span of *C. elegans*[131] and *Drosophila*.[132] Furthermore, a heterozygous knockout mutant of *daf-15*, the nematode ortholog of Raptor, significantly extends the life span of
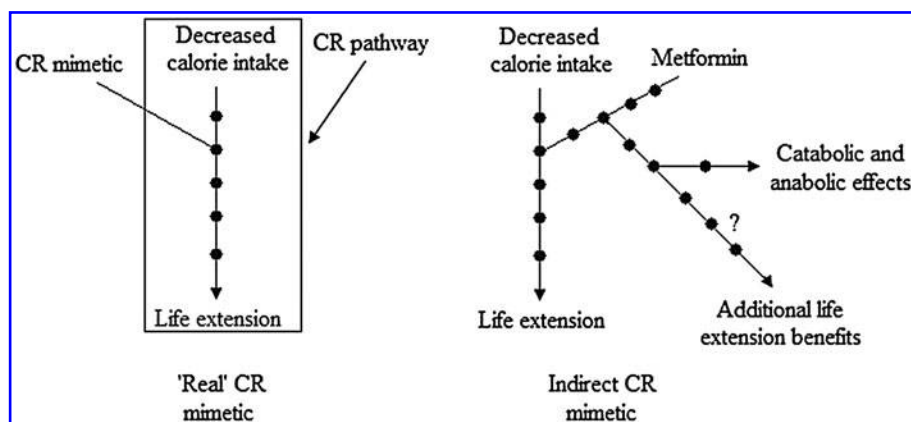
FIG. 4. The indirect versus the direct calorie restriction (CR) pathway. The rectangle encloses the part of metabolism that is needed for CR to function, called the CR pathway. A direct CR mimetic directly stimulates one member of this pathway resulting in a downstream effect indistinguishable from calorie restriction. An indirect CR mimetic (such as metformin), on the other hand, activates a pathway that intersects with the CR pathway at some point. This results in a CR-like action but with additional either not life span-related effects (not in the figure) or additional life extension benefits. The question mark indicates that there might be, but not necessarily are, other life extension benefits.

C. elegans.[133] Heterozygotic knockout of *let-363*, the nematode CeTOR, did not influence life span, and a homozygotic knockout resulted in nematodes that were stuck in a kind of dauer-like stage. The authors suggest that a heterozygous knockout might not reduce the TOR activity enough to increase the life span.[133] CeTOR inhibition does not further extend the life span of *daf-2* hypomorph worms. Indeed, *daf-16*, which lies downstream of *daf-2*, downregulates *daf-15*. It should also be noted that RNA interference (RNAi), as was used to downregulate *let-363*, can also in some cases downregulate genes downstream of the target gene. Given the fact that a gene encoding a mitochondrial subunit, whose downregulation might extend life span, lies downstream of *let-363* makes it impossible to draw definitive conclusions at this moment.[14] The effect of TOR inhibitions is sex dependent. For example, rapamycin resulted in a more moderate increase in life span in male versus female flies, and the same was also observed in mice.[5,7] Sex-specific effects are also observed in other longevity interventions.[134,135]

As illustrated above (see the section Metformin As a Geroprotector), metformin exhibited sex-specific effects on life span in some species. The reason for this sex specificity is not known. CR has consistently showed to inhibit the mTOR pathway.[136,137] The life span extension by TOR inhibition is in *C elegans* at least partially independent of DAF-16/FOXO.[104] It appears that TOR inhibition lengthens the life span in a *daf-16*-independent manner when the inhibition is throughout development but *daf-16*-dependent when initiated during adult life.[122] This conclusion is strengthened by the findings of Jia et al. that show that loss of *daf-16* suppressed the effect of *daf-15* on life span. They also found that DAF-16 negatively regulates the expression of *daf-15*.[133] It should be noted that *C. elegans* lacks a functional Tsc1/Tsc2 ortholog, and thus the connection between *daf-15* and *daf-16* might represent a private mechanism in the worm to connect the TOR pathway to growth factor signaling.

TOR inhibition has been suggested as a mechanism for life span extension by CR.[66] However, inhibition of *let-363* (the TOR homolog in *C. elegans*) worked synergistically with *eat-2* (genetic model of CR) to increase the life span.[121] However, other studies found no effect.[137] This might be explained by noting that the level of CR induced by *eat-2* mutants might not be optimal for life span extension.[14] Interestingly, TOR inhibition by rapamycin (see below) further increases median and maximum life span of flies on CR.[7] In *C. elegans*, on the other hand, CR fails to extend the life span of TOR RNAi worms.[14,136]

How might TOR activity cause aging? First, TOR phosphorylates insulin receptor substrate-1 (IRS-1) and this inhibits insulin signaling, leading to insulin resistance and eventually type 2 diabetes.[138] Second, growth signals in damaged cells, where the cell cycle is arrested, can cause these cells to become senescent.[139] Third, TORC1 inhibits autophagy, and authophagy is needed for life span extension by CR and in Ins/IGF-1 mutants.[104] Fourth, TORC1 stimulates protein synthesis,[140] and downregulating protein synthesis leads to life span extension for unknown reasons. However, it seems that mTOR does not regulate global protein synthesis but rather downregulates cap-dependent translation while stimulating cap-independent translation, resulting in a switch to the synthesis of stress protective proteins such as heat shock proteins.[7] And indeed, a shift to cap-independent translation is needed for adaptation to starvation in budding yeast.[141] Interestingly, the life span–extending effect of TOR inhibition was abrogated in both 4E-BP and Atg5 flies.[7] Fifth, it has been showed that TOR activation in yeast keeps nutrient-regulated transcription factors such as MSN2, MSN4, and GLN3 out of the nucleus.[142] It might be that similar transcription factors are sequestered in the cytoplasm after TOR activation in mammals. Blagosklonny has extensively reviewed the role of TOR signaling and the benefits of suppressing it in age-related diseases (such as type 2 diabetes, cancer, age-related macular degeneration, hypertension, and osteoporosis).[139]

Pharmacologic inhibition of mTOR

Rapamycin (Sirolimus®), an immunosuppressive drug used to prevent rejection in transplantation patients. It inhibits TORC1, but not TORC2 (although long-term treatment can indirectly cause inhibition of TORC2 in some cell lines[142,143]), and extends the life span of mice[5,6], yeast,[71] nematodes,[39] and *Drosophila*.[7] Furthermore, rapamycin-pretreated flies exhibit an increased survival under stress (starvation or paraquat) conditions.[7] Rapamycin binds to FK506-binding protein (FKBP-12) and the formed complex interacts with mTOR near its Raptor binding domain, causing the disruption of the TORC1 complex and thereby inhibiting it. The side effects of rapamycin are small, as expected, based on the fact that it only has one molecular target.[139,144] Indeed, in normal dosages (2–3 mg/day), the side effects are minimal.[144,145] The most common side effects are anemia, thrombocytopenia, leukopenia, hypercholesterolemia, and hyperlipidemia.[144] However, excessive blood clotting is a common problem in old people and thus mild thrombocytopenia might be beneficial. Similarly, hypercholesterolemia and hyperlipidemia might be beneficial effects of increased lipolysis in adipose tissue and a decrease in lipid accumulation in tissues such as the vascular wall.[144,146] However, in concentrations of 2 or 2.5 mg/kg per day, rapamycin and analogs (rapalogs) cause hyperlipidemia, hypercholesterolemia, hyperglycemia, glucose intolerance, and diabetes in rodents.[147–150] Together with its immunosuppressive effect,[151,152] these are major drawbacks in its use as a geroprotector.

The immunosuppressive action of rapamycin is the result of the antiproliferative effect of mTOR inhibition on the clonal expansion of activated lymphocytes.[153] However, in recent years it has become clear that rapamycin (or mTOR inhibition in general) should be viewed as an immune modulator instead of as a pure suppressant.[154] Clinical reports in disease settings show that rapalogs are well tolerated and show no immunosuppressive side effects.[155] Interestingly, metformin has also been shown to inhibit mTOR,[156] and, unlike rapamycin, it has no immunosuppressive action. In contrast, biguanides prevent age-related metabolic immunodepression.[157] Metformin also increases insulin sensitivity, lowers blood glucose levels, and improves lipoprotein profiles.[17,18] Metformin decreases plasma triglyceride, total cholesterol, low-density lipoprotein (LDL), lipoprotein(a) (Lp[a]), free fatty acids, and C-reactive protein, and reduces postprandial hyperlipemia; high-density lipoprotein (HDL) might be slightly increased.[18]

Thus, it appears that metformin has similar benefits as rapamycin but without the side effects. It is interesting to

note that metformin significantly decreased intestimal acyl-coenzyme A (CoA) cholesterol acyltransferase (ACAT) activity in normal rats; 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) was markedly reduced in diabetic rats after metformin administration but not in normal rats.[158] A combination of metformin and rapamycin has been suggested as a first-generation antiaging drug.[144]

Mechanism of mTOR inhibition by AMPK

Three mechanisms have been discovered through which AMPK can inhibit mTOR. First, AMPK can phosphorylate TSC-2 at Ser1345, which is expected to stimulate its Rheb-GAP activity. This renders Rheb inactive, thereby preventing it from stimulating TOR signaling. Phosphorylation of TSC2 by AKT inhibits its GAP activity[159] and thus prevents the deactivation of Rheb, which results in higher TORC1 activity. The second mechanism by which AMPK can inhibit TORC1 is the direct phoshorylation of the raptor subunit on serine 792. The opposite happens in insulin/IGF-1 signaling. It has been shown that AMPK activation antagonizes TORC1 activation by growth factors and amino acids.[160–162]

A very recent study[163] found yet another way in which metformin can inhibit TORC1 signaling. Metformin appears to inhibit the Rag GTPases (positive stimulators of TORC1 activity) in an AMPK-independent way. Indeed, in a recent study, it was shown that metformin inhibited mTOR in lung tumors in an AMPK-independent way.[90] Interestingly, the Ragulator-Rag complex also activates mTORC1 in response to amino acid availability.[164] Protein restriction increases life span in most studies,[12] and this might thus be related to a decrease in mTORC1 activity. Recently, it was shown that SIRT-1 inhibits TOR.[165] In a study by Jones et al., it was shown that glucose-deprived primary mouse embryonic fibroblasts (MEFs) exhibit an increase in cell size compared to MEFs cultured in standard medium, and this increase could be prevented by rapamycin. Similar results were obtained with the AMPK activator AICAR (0.5 mM), and it was further shown that at a higher AICAR concentration (2 mM) no increase in cell size occurred.[166] This result seems to indicate that moderate AMPK activation does not inhibit TOR signaling.

Interaction between mTOR and sirtuins

Interestingly, TOR inhibition by rapamycin seems to activate Sir2p and probably also other yeast sirtuins such as HST2.[66] TOR stimulates the cytoplasmic localization of *MSN2/4* transcription factor, which regulates the expression of nicotinamidase PNC1. PNC1 is the rate-limiting step in the degradation of nicotinamide. Medvedik et al. suggested that the mechanism for life span extension by CR in the yeast *Saccharomyces cerevisiae* works by inhibition of TOR and subsequent activation of Sir2p and HST2.[66]

Effects of metformin on insulin and IGF-1

Metformin also seems to lower the fasting and postprandial insulin levels in nondiabetic patients.[167] Metformin has been shown to decrease plasma IGF-1 levels in normal-weight subjects.[167] Leo et al. showed that the IGF-1 concentration was not significantly changed after metformin therapy in women with PCOS, but IGF-I/insulin-like growth

factor binding protein-1 (IGFBP-I) was significantly reduced.[168] These results are confirmed by another study in women with PCOS in which the total IGF-1 level was not changed but the level of IGFBP-1 was increased by 38%.[169] A new study has shown that metformin decreased tumor burden by 72%, decreased tumor volume by 50%, and tumor multiplicity by 66% in a nitrosamine 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung cancer mouse model. It was also shown that this decrease was due to mTOR inhibition by a downregulation in Ins/IGF-1 signaling. Oral and intraperitoneal injection of metformin led to a decrease in insulin of $\approx 20\%$ and $\approx 35\%$, respectively. Oral metformin also decreased IGF-1 by $\approx 20\%$, but intraperitoneal injection had no effect, probably because the stress of the injection increased IGF-1 levels.[163] The reduction in insulin and free IGF-1 levels would be expected to contribute to increased longevity.[103]

Effects of metformin on free reactive oxygen species generation

Another mechanism by which metformin might increase life span is the decrease in reactive oxygen species (ROS) production by several mechanisms. First, metformin inhibits complex I of the electron transport chain (ETC).[104,105] This reduces the electron flow and prevents electrons from being stalled at downstream parts of the ETC, which could increase the rate of ROS production.[170] A second mechanism is the inhibition of nicotinamide adenine dinucleotide phosphate (NAD[P]H)-oxidase by metformin. Piwkowska et al. have shown that metformin inhibits ROS production (in both normo- and hyperglycemic conditions) by NAD(P)H oxidase in a AMPK-dependent way.[171] Third, it has been shown that metformin increases the amount of antioxidant enzymes in newly diagnosed obese diabetic patients.[172] It was shown that metformin reduces superoxide production in platelets of diabetic patients.[173] This study, however, did not investigate the mechanism behind metformin's effect on superoxide generation.

Metformin: A CR Mimetic?

There appear to be some differences between metformin and CR, making it difficult to conclude definitely that metformin is a CR mimetic. As discussed above, there is an inconsistency in the results from studies investigating the effects of CR on AMPK activation. Metformin studies, on the other hand, show a consistent increase in AMPK activation. As noted before, AMPK downregulates fatty acid synthesis while CR increases fatty acid synthesis.[174] Metformin decreases HMG-CoA reductase (AMPK activation downregulates sterol synthesis), whereas CR activates HMG-CoA reductase.[13] Metformin also causes a downregulation of gluconeogenesis,[17] whereas CR results in an activation.[174] An obvious explanation for these results would be that anabolic pathways can be uncoupled from the life extension pathways. In other words, eating a restricted diet gives a prolongevity signal but also results in shortages of membrane building blocks (lipids and steroids) and reduces blood sugar below necessary limits unless the organism responds by increasing the synthesis of these compounds. In this model, the synthesis of these compounds would happen independently from the prolongevity signal exerted by a shortage in the first place.

Thus, it could be that metformin activates the same prolongevity pathway as CR but has other independent effects on anabolic and catabolic pathways. In this case, the activation of the prolongevity (CR pathway) would happen in an indirect way (Fig. 4). This would explain why it has so similar effects to CR on the one hand, whereas on the other hand it displays some other effects not seen in CR. Some of these non-CR related effects could even have additional life extension benefits. As has been discussed above, metformin does not activate AMPK directly, but through a still incompletely understood mechanism that involves LKB1.

Conclusion

Metformin is a promising life extension drug, with a reasonable good safety status and a long history of clinical use in humans. Metformin is currently the most widely prescribed drug against diabetes type 2 in the United States.[18] This makes metformin an attractive drug that could be used in the near future for life extension purposes. Some questions remain however. Does it extend the life span of male animals? Will it work in humans? If so, what is the optimal dose? Will it extend the life span when started late in life? Metformin is probably an "indirect" CR mimetic. The likely mechanism is activation of AMPK, which affects known downstream targets that control aging, such as TORC1, SIRT1, and FOXO. We conclude that metformin is currently one of the most promising geroprotective agents.

Note Added in Proof

One recent study did not detect an AMPK activation in cortical neurons after metformin or phenformin treatment.[175] But another recent study did find an AMPK activation after metformin treatment during neuronal development.[176]

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Author Disclosure Statement

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