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Review

Review

Biochemical mechanism underlying the pathogenesis of diabetic retinopathy and other diabetic complications in humans: the methanol-formaldehyde-formic acid hypothesis

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Abstract

Hyperglycemia in diabetic patients is associated with abnormally-elevated cellular glucose levels. It is hypothesized that increased cellular glucose will lead to increased formation of endogenous methanol and/or formaldehyde, both of which are then metabolically converted to formic acid. These one-carbon metabolites are known to be present naturally in humans, and their levels are increased under diabetic conditions. Mechanistically, while formaldehyde is a cross-linking agent capable of causing extensive cytotoxicity, formic acid is an inhibitor of mitochondrial cytochrome oxidase, capable of inducing histotoxic hypoxia, ATP deficiency and cytotoxicity. Chronic increase in the production and accumulation of these toxic one-carbon metabolites in diabetic patients can drive the pathogenesis of ocular as well as other diabetic complications. This hypothesis is supported by a large body of experimental and clinical observations scattered in the literature. For instance, methanol is known to have organ- and species-selective toxicities, including the characteristic ocular lesions commonly seen in humans and non-human primates, but not in rodents. Similarly, some of the diabetic complications (such as ocular lesions) also have a characteristic species-selective pattern, closely resembling methanol intoxication. Moreover, while alcohol consumption or combined use of folic acid plus vitamin $B_{6/12}$ is beneficial for mitigating acute methanol toxicity in humans, their use also improves the outcomes of diabetic complications. In addition, there is also a large body of evidence from biochemical and cellular studies. Together, there is considerable experimental support for the proposed hypothesis that increased metabolic formation of toxic one-carbon metabolites in diabetic patients contributes importantly to the development of various clinical complications.

Key words hyperglycemia, one-carbon metabolites, diabetes mellitus, pathogenic mechanism

Introduction

Diabetes mellitus (DM), one of the world's oldest diseases [1,2], has become a major epidemic of this century [3]. Its incidence continues to increase rapidly worldwide [4]. The International Diabetes Federation estimated that there were 382 million people aged between 20 and 79 years with DM in 2013, and this number is expected to increase to 592 million by 2035 [5]. Common complications associated with long-standing DM include microangiopathy, retinopathy, nephropathy, neuropathy and myocardiopathy [6]. The complications occurring at late stages of DM are among the major causes of morbidity and mortality worldwide. Understanding the mechanistic basis of these clinical complications has been a subject of intense research for decades. Most of the available experimental and clinical evidence suggests that diabetic complications are the consequence of metabolic derangements, mainly hyperglycemia. In support of this general concept, studies have shown that when the kidneys from non-DM donors were transplanted into DM recipients, they developed characteristic lesions of diabetic nephropathy within 2–5 years after transplantation [7,8]; conversely, kidneys with characteristic lesions of diabetic nephropathy showed a reversal of the lesions after transplantation into normal recipients [9,10]. Many clinical studies have also shown that the progression

of diabetic nephropathy can be delayed through better hyperglycemic control [11].

A number of mechanisms linking hyperglycemia to diabetic complications have been explored in the past. For instance, one of the well-known mechanisms is based on elevated glycation of various proteins as a pathogenic basis for certain diabetic complications [12–14]. It is known that glucose can form chemically-reversible glycation products with the amino groups of proteins without the aid of enzymes (a chemical reaction called Schiff base formation), and the intermediary products may rearrange to form more stable Amadori-type early glycation products. It is postulated that the early glycation products from collagens and other long-lived proteins in interstitial tissues and blood vessel walls can undergo a series of chemical rearrangements to form irreversible advanced glycation end products (AGEs), which might accumulate over a long period of time in the vessel walls. AGEs have a number of chemical and biological properties which are potentially pathogenic. Increased nonenzymatic glycation of proteins has long been suggested to be a pathogenic mechanism for certain diabetic complications, including microangiopathy, atherosclerosis, and nephropathy (reviewed in [12–14]). In addition, the levels of glycated hemoglobin HbA1c in the blood are widely used nowadays as an indicator in DM management [15].

In addition to increased formation of AGEs, a number of other metabolic pathways have also been suggested to be involved in the pathogenesis of diabetic complications [16]. These pathways include increased glucose flux through the aldose reductase pathway [17], increased flux through the hexosamine pathway [18,19], and activation of the protein kinase C isoforms [20]. While all these mechanistic hypotheses offer partial explanations for some of the pathogenic complications associated with advanced DM, their relative clinical importance is still unclear. For instance, a number of specific inhibitors of the above-mentioned metabolic pathways have been developed in the past, but their clinical effectiveness was not as expected [21–23]. In addition, there were also many clinical and experimental observations that could not be adequately explained by the existing mechanisms. For instance, the experimental rodent models of DM usually develop very severe hyperglycemia but they fail to develop the more severe forms of diabetic complications commonly seen in humans.

In this communication, a new hypothesis is proposed, which suggests that the pathogenesis of many DM-associated complications likely is contributed, at least in part, by the increased metabolic formation of methanol and/or its metabolites formaldehyde and formic acid inside the human body, particularly in those tissues and/or cells that have very high levels of cellular glucose, and these toxic one-carbon metabolites then drive the pathogenic processes associated with many human diabetic complications.

A General Hypothesis

A key component of the proposed hypothesis is that hyperglycemia-associated increase in cellular glucose levels in DM patients would lead to increased metabolic formation of endogenous methanol and/or formaldehyde, both of which are further metabolically converted to formic acid (Figure 1). Although the exact metabolic pathways as well as the enzymes involved are not clear at present, there is clear evidence showing that methanol, formaldehyde and formic acid are endogenously-formed metabolites present in humans, and that increased levels of these toxic one-carbon metabolites are present in DM patients (discussed in detail in later sections).

Mechanistically, while methanol *per se* has very weak cytotoxicity, its metabolites formaldehyde and formic acid are highly cytotoxic. Although formaldehyde usually does not accumulate in the body to high levels, it can covalently modify many nitrogen-containing compounds, *e.g.*, DNA, RNA, proteins and amino acids. For proteins, formaldehyde can react with their amine groups to form Schiff bases and cross-link proteins by forming methylene bridges between the amine groups (Figure 2) [24,25]. It is hypothesized that the ability of formaldehyde to covalently cross-link intracellular as well as extracellular components (mostly macromolecules) may contribute critically to the thickening of the basement membrane structures in certain tissues or organs that have high levels of blood supply or perfusion, such as the capillaries of the eye and brain and the glomeruli of the kidney in DM patients.

For formic acid, it can quite readily accumulate in humans and non-human primates to high levels [26–30], and can cause cytotoxicity by directly inhibiting the cytochrome oxidase complex of the mitochondrial respiratory chain [31,32], thereby reducing the cellular ATP production. Depending on the levels of formic acid being formed and present inside a cell, the degree of inhibition of the mitochondrial oxidative phosphorylation would vary considerably. In severe cases, high levels of formic acid are expected to cause histotoxic hypoxia (which is commonly seen during acute methanol poisoning) and drastic reduction in cellular ATP production. In addition, formic acid can cause metabolic acidosis and alter intracellular calcium homeostasis, both of which also contribute to its cytotoxicity.

It is readily understood that a relatively mild inhibition of the mitochondrial oxidative phosphorylation by endogenously-formed formic acid would result in activation of the anerobic glycolysis, which requires no oxygen for glucose metabolism for ATP production and serves as an alternative compensatory metabolic pathway. However, glycolysis is not efficient, producing far fewer ATP molecules compared to oxidative phosphorylation. Therefore, at early stages of DM, it is expected that the DM patients would suffer a relatively mild state of energy shortage in many tissues or cells, especially in those cells which have a high requirement for oxygen and energy supply.

However, when DM progresses and the hyperglycemia-associated formic acid production and accumulation reach higher levels, formic acid would pose a stronger inhibition of the mitochondrial respiratory chain, which would also result in elevated mitochondrial superoxide accumulation. High levels of superoxide would indirectly lead to inhibition of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity [33], thereby suppressing the anerobic glycolytic pathway of glucose metabolism, further aggravating the situation of cellular energy shortage. In the meanwhile, the upstream glucose metabolites are forced to be diverted into other metabolic pathways, such as the polyol pathway and the hexosamine pathway, resulting in increased formation of AGEs and over-activation of protein kinase C (PKC), all of which would contribute to the pathogenesis of diabetic complications [34].

According to the proposed methanol-formaldehyde-formic acid (MFF) hypothesis, therefore, the mitochondrial energy production in many tissues and cells of DM patients would be impaired to varying degrees, depending on the length of exposure to the elevated levels of formic acid (together with its highly-toxic metabolic precursor formaldehyde). It is believed that formic acid-induced cellular ATP shortage or deficiency is an important causative factor

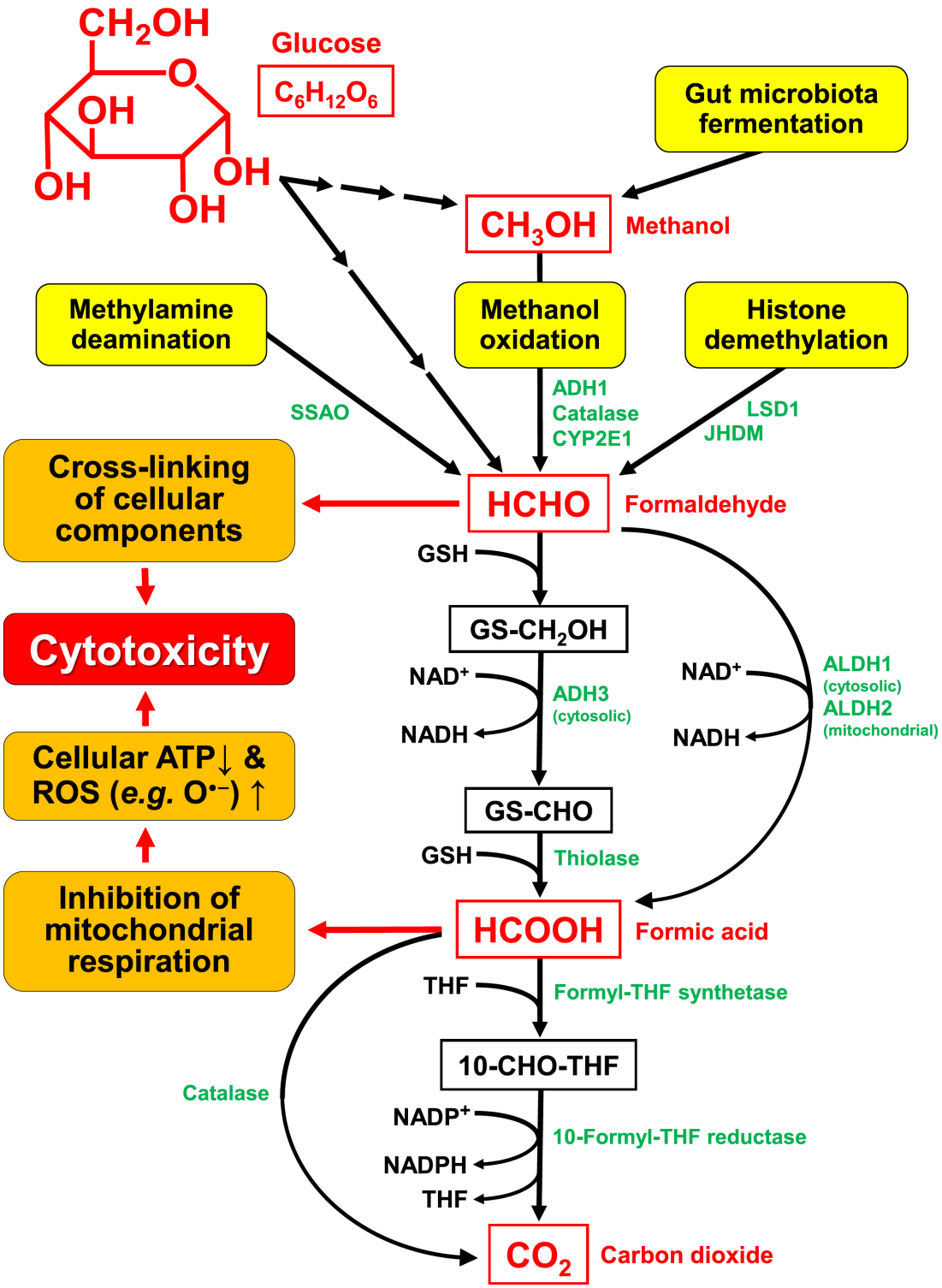

Figure 1. Proposed metabolic formation and biotransformation of the toxic one-carbon metabolites (*i.e.*, methanol, formaldehyde and formic acid) in the body as well as their cytotoxicity Chronic increase in cellular glucose levels would lead to increased metabolic formation of endogenous methanol and/or formaldehyde, both of which are further metabolically converted to formic acid. The enzymes involved in the metabolic conversion of glucose into methanol and/or formaldehyde are not clear at present. Some of the presently-known enymes involved in the endogenous formation of methanol and formaldehyde as well as their further metabolic biotransformation are shown in green (refer to Metabolic biotransformation of methanol for description). While methanol per se has very weak cytotoxicity, its metabolites (formaldehyde and formic acid) are highly cytotoxic. Formaldehyde can cause cytotoxicity by covalently modifying (cross-linking) many nitrogen-containing macromolecules (*e.g.*, DNA, RNA, proteins), and formic acid can directly inhibit the cytochrome oxidase complex of the mitochondrial respiratory chain, thereby reducing cellular ATP production and simultaneously causing cellular ROS (*e.g.*, mitochondrial superoxide) accumulation. Hence, formaldehyde and formic acid can jointly contribute to the development of strong cytotoxicity. Abbreviations: ADH1 and ADH3: alcohol dehydrogenase 1 and 3, respectively; ALDH1 and ALDH2: aldehyde dehydrogenase 1 and 2, respectively; JHDM: JmjC domain-containing histone demethylases; LSD1: lysine-specific demethylase 1; SSAO: semicarbazide-sensitive amine oxidases; THF: tetrahydrofolate; CYP2E1: cytochrome P450 2E1.

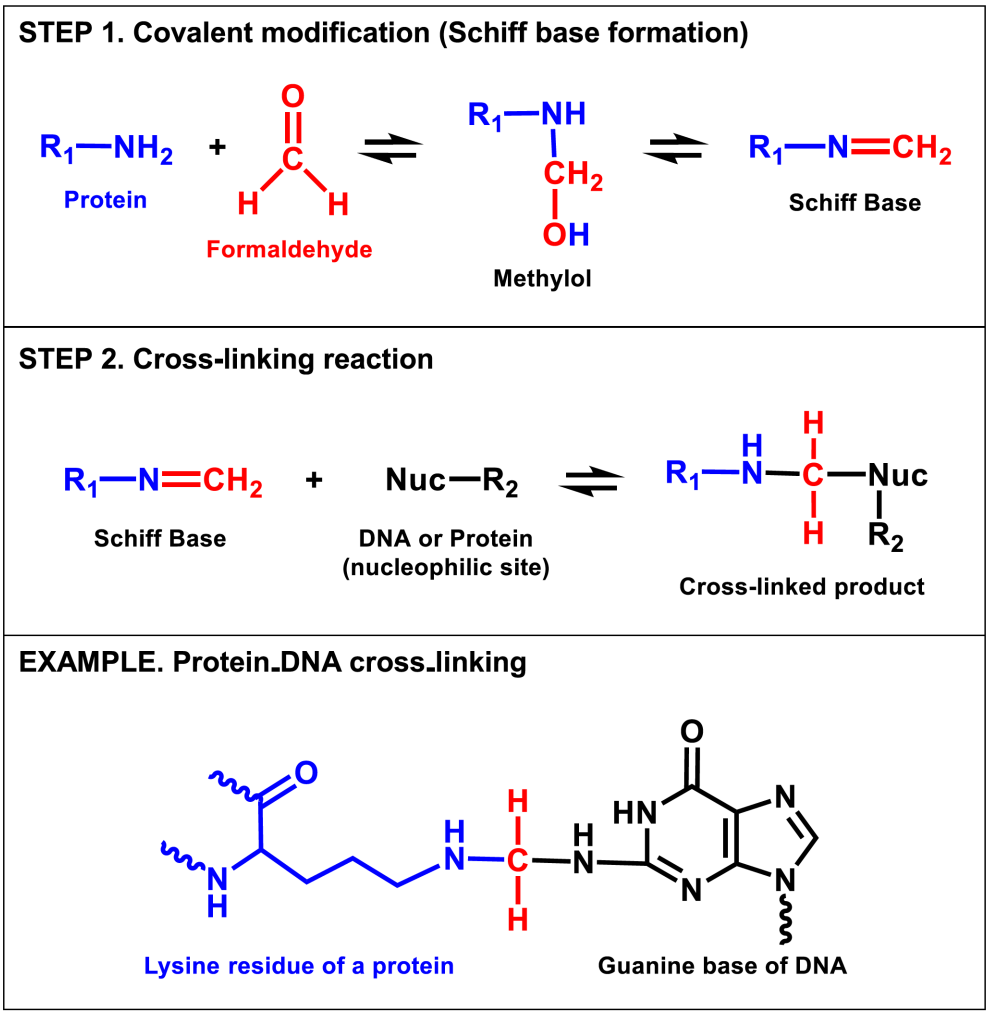

Figure 2. Chemical reactions underlying the cross-linking of molecules by formaldehyde in the body Formaldehyde can cause covalent cross-linking of molecules (usually macromolecules) in the body in two major chemical steps. First, formaldehyde reacts with a strong nucleophile contained in a biomolecule, such as a lysine ε-amino group. This reaction forms a methylol intermediate that subsequently will lose a water molecule to form a Schiff base (an imine). Second, the Schiff base reacts with another nucleophile, possibly an amine group contained in a protein or a nucleotide in the DNA to generate a cross-linked product. The protein-DNA cross-linked product as shown is only one of the possible products. This figure is modified after Hoffman *et al.* [24].

that drives the processes of many DM-associated lesions and complications in humans. Further, it is suggested that tissues and organs with a high level of oxygen and energy requirement (*e.g.*, optic neurons and their nerve fibers, brain, heart and kidney) would be particularly sensitive and vulnerabale to formic acid-induced histotoxic hypoxia, which is usually accompanied by varying degrees of cellular oxidative damage and even cell death.

The MFF hypothesis is supported by a large number of scattered observations from both clinical and experimental studies. For instance, methanol is known to have characteristic organ- and species-selective toxicities, including the characteristic ocular lesions (including blindness), which are commonly seen in humans and non-human primates, but this ocular toxicity is not seen in rodents. Interestingly, some of the diabetic complications (such as ocular lesions) also have a characteristic species-selective pattern, closely resembling what is seen in methanol toxicity. Moreover, alcohol and folic acid (together with vitamin $B_{6/12}$) are commonly used clinically to alleviate acute methanol toxicity; interestingly, long-term moderate alcohol consumption or joint use of folic acid and vitamin $B_{6/12}$ is also associated with an improved outcome in diabetic complications. A comparison of the pathological characteristics of the ocular changes in methanol toxicity *vs.* in advanced DM also reveals a high degree of similarity, which clearly suggests a shared role of formic acid in these two pathogenic processes. In addition, as discussed later, there is a large body of experimental evidence from clinical, animal and cellular/biochemical studies that jointly offer strong support for the proposed MFF-based pathogenic mechanism.

Provided below is a detailed discussion of each aspect of the proposed MFF hypothesis along with a critical analysis of the available supporting evidence, most of which is scattered in the literature in bits and pieces. In addition, efforts are made trying to apply the proposed MFF hypothesis to offer a tentative explanation for some of the well-known pathophysiological and pathogenic processes associated with human diabetic complications.

Sources of Methanol and Its Metabolites in Humans

Many years ago, a small amount of methanol was unexpectedly

detected by chromatography in the breath of normal, healthy human subjects [35–37]. This finding was confirmed later by mass spectrometry showing that methanol was indeed present in the exhaled breath of healthy volunteers [38–41]. Later, it was also observed that when individuals drank wine or distilled spirits with very low or undetectable methanol content, their endogenous methanol levels still increased sharply [36,42]. Similarly, the blood formaldehyde levels also rose significantly after ethanol ingestion, but with some delay [42]. These observations indicated the existence of an endogenous source of metabolically-formed methanol, and inhibition of the catalytic activity of liver aldehyde dehydrogenase (ADH) by ethanol was postulated to be responsible for the increased accumulation of metabolically-formed methanol in the body. Interestingly, it is of note that earlier studies reported that an average person also had endogenously-formed ethanol in their blood [43] and breath [40,41].

Based on the Mayo Clinic Laboratories in the U.S., the normal range of formic acid in the plasma (test definition: FORAC; determined by gas chromatography/mass spectrometry) is 1–9 μg/mL (*i.e.*, 21.7–195 μM), and during pregnancy, its normal range is 0.5–44 μg/mL (*i.e.*, 10.8–956.5 μM).

Provided below is a brief discussion of the exogenous and endogenous sources of methanol and its metabolites formaldehyde and formic acid.

Exogenous sources of methanol and its metabolites in humans

Certain food (*e.g.*, fresh fruits, vegetables and fermented beverages) is an important exogenous source of methanol present in humans [44,45]. Alcoholic beverages might contain methanol as a contaminant and thus would directly increase methanol levels in individuals after ingestion. Aspartame, a dietary sweetener, can be partially converted into methanol (about 10%) inside the human body [46–48].

Production of methanol and its metabolites by known endogenous metabolic pathways

Semicarbazide-sensitive amine oxidases (SSAOs)

SSAOs represent a group of copper-containing amine oxidases that are inhibited by semicarbazide [49–52]. The SSAOs-mediated oxidative deamination of methylamine produces formaldehyde together with ammonia and hydrogen peroxide [50,52]. In mammals, SSAOs is present in a membrane-associated form or in a soluble form in circulation [49].

Notably, an increased enzymatic activity of SSAOs has been observed in DM, and it has been suggested that inhibition of SSAOs is of therapeutic benefits in DM [53]. In addition, participation of the brain SSAOs in formaldehyde production has been shown in a mouse model of Alzheimer's disease [54,55].

Protein carboxymethylase and demethylases

s-Adenosyl-*l*-methionine (AdoMet) is a universal endogenous methyl donor and a limiting factor in various methylation reactions, including protein carboxymethylation [56]. Protein carboxymethylase is highly expressed in certain tissues of mammals [57,58]. It has been reported that the AdoMet-dependent protein carboxymethylation can result in the formation of methanol, formaldehyde and formic acid in tissue homogenates prepared from rat brain striatum [56].

Earlier studies also reported that formaldehyde could be formed as a by-product of the reactions catalyzed by the lysine-specific demethylase 1 and the JmjC domain-containing histone demethylases [59–62].

Methanol production through fermentation by gut microbiota

It was suggested earlier that methanol might be produced through microbial fermentation of undigested carbohydrates and fibers from vegetables and fruits in the intestines of animals and humans [35,63–67]. This would be an important source of endogenously-produced methanol in humans, which also provides an explanation for the suggestion that the gut microbiota might be a potential risk factor for type 2 DM and its complications. More studies are needed in the future to characeterize which groups of gut microbiome are involved in producing methanol and other toxic one-carbon metabolites, and how to effectively reduce their production through beneficial manipulation of the human gut microbiome composition.

Other potential sources of endogenous methanol and its metabolites

It is hypothesized that abnormally-elevated cellular glucose levels in DM patients would lead to increased metabolic formation of methanol and its toxic one-carbon metabolites formaldehyde and formic acid. Although the exact metabolic pathways and enzymes involved in converting excess glucose to methanol and/or its metabolites are not clear at present, there was unequivocal clinical evidence showing that markedly-elevated levels of methanol and formaldehyde were detected in the exhaled breath of advanced DM patients [35,40,68,69].

As mentioned earlier, the plasma levels of formic acid in a "normal, healthy" pregnant woman can reach as high as approximately 1 mM, which is a rather high concentration. Based on recent experiments in our laboratory (unpublished data), long-term exposure to sodium formate (a salt form of formic acid) at this concentration can pose a small but detectable reduction of cell viability in some of the cell lines tested. It is speculated that the marked increase in plasma formic acid levels in some pregnant women might be due to the possibility that some of them may actually acquire a mild form of gestational DM, which could be an important potential cause for the sharp rise in blood formic acid levels. It will be of interest to experimentally examine this possibility in the future.

Section summary

It is known that methanol and formaldehyde are naturally-occurring compounds in normal, healthy human subjects [42]. A few endogenous metabolic processes and enzymes are known to produce methanol and formaldehyde. Anerobic fermentation by gut bacteria might be another important source of endogenous methanol. Lastly, it is hypothesized that hyperglycemia would lead to abnormally-elevated cellular glucose levels and subsequently an increased metabolic conversion of glucose into methanol and/or its toxic one-carbon metabolites. The exact metabolic pathways and enzymes involved in their formation are not clear at present.

Methanol Metabolism in Humans and Experimental Animals

Methanol itself has a rather low cytotoxicity [70]. For example, animal cells in culture can tolerate high concentrations of methanol [71,72]. The toxic effects of methanol are mostly produced by its

metabolites formaldehyde and formic acid. Hence, differences in the rate of metabolic conversion of methanol to formaldehyde and formic acid would be important determinants of methanol toxicity. Provided below is a brief discussion of the different metabolic pathways and enzymes involved in biotransformation of methanol in humans and animal models as well as their major differences.

Metabolic biotransformation of methanol

Conversion of methanol to formaldehyde

In humans, oxidation of methanol to formaldehyde is primarily catalyzed by two metabolic pathways. The first pathway involves ADH1, catalyzing up to 90% of methanol (and ethanol) in the human liver [73–76]. As ADH1 has a strong preference for ethanol over methanol as substrate, this makes ethanol a powerful competitive inhibitor of this enzyme. Another pathway involves CYP2E1 (and probably also CYP1A2) [77–82], which accounts for approximately 9% of methanol oxidation to formaldehyde in the human liver. Non-human primates share similar metabolic pathways of methanol oxidation as humans.

Different from humans and non-human primates, ADH1 contributes minimally to methanol oxidation in rodents [83], but the catalase-peroxidase system plays a far more important role in methanol oxidation to formaldehyde in rodents [83–88]. Humans and non-human primates do not oxidize methanol through the catalase-dependent system [89], because of the low availability of peroxides [90].

It is of note that while formaldehyde is primarily produced through enzymatic oxidation of methanol, other enzymatic reactions can also directly produce formaldehyde in humans (already discussed in section "Production of methanol and its metabolites by known endogenous metabolic pathways").

Conversion of formaldehyde to formic acid

Metabolic conversion of formaldehyde to formic acid is catalyzed by multiple enzymes [91–93]. Glutathione-dependent formaldehyde dehydrogenase (ADH3) is considered the major pathway for formaldehyde metabolism in both humans and rodents [94]. Other pathways that can also contribute to the oxidation of formaldehyde to formic acid include the cytosolic and mitochondrial aldehyde dehydrogenases (ALDH1 and ALDH2, respectively) [76,94–98].

Owing to the multiple metabolic pathways involved in formaldehyde metabolism, formaldehyde usually would be rapidly metabolized in humans. An earlier investigation of a single human case of formaldehyde poisoning revealed that it was indeed so [99].

Conversion of formic acid to carbon dioxide

In humans and rodents, formic acid is further oxidized to carbon dioxide catalyzed by the tetrahydrofolate (THF)-dependent pathway, which detoxifies formic acid. Specifically, formic acid is first converted into 10-formyl-THF by formyl-THF synthetase, which is then further converted into carbon dioxide and water by 10-formyl-THF reductase [100]. In addition, rodents also use catalase to oxidize formic acid to carbon dioxide and water [101], but this metabolic process does not occur in humans.

It is of note that in humans and non-human primates, the hepatic THF level is only half of that in rats [100], and the 10-formyl-THF dehydrogenase activity is only 20–25% of that in rats [102]. The combination of the low hepatic THF level and the low hepatic 10-formyl-THF dehydrogenase activity in humans and non-human primates jointly accounts for the very slow metabolic oxidation of formic acid, leading to formic acid accumulation [103]. Experimental studies in non-human primates showed that the THF-dependent oxidation of formic acid became saturated when methanol doses exceeded 300 mg/kg body weight [104]. In contrast, the THF-dependent pathway was not capacity-limited in rodents, and formic acid essentially did not accumulate in rodents even after methanol overdose [105].

Accumulation of formaldehyde and formic acid in the body

Because formaldehyde is rapidly oxidized to formic acid or forms covalent adducts with many molecules in the body (including both small and large molecules) [24,25,106–108], it would not readily accumulate to high levels in body fluids or tissues [93]. Studies using monkeys revealed that even after intravenous infusion of formaldehyde, its apparent clearance from the blood occurred very rapidly [109]. In healthy human individuals, the formaldehyde concentration in the blood was reported to be at relatively low levels (0.01–0.08 mM) under physiological conditions, but the levels could significantly increase during ageing (over 65 years old) and under pathological conditions [110–113].

In contrast, formic acid can readily accumulate in humans and non-human primates to far higher levels following administration of methanol [26–30]. For instance, an earlier study reported that when formate (the salt form of formic acid) was infused into monkeys, its accumulation in blood lasted 10 h, and after that, its blood level was still at 10–30 mM [30,114]. In another study, when the plasma pharmacokinetics of formic acid in monkeys, mice and rabbits were compared, it was found that formic acid accumulation in primates within the first 6 h was 43-fold and 5-fold higher than that in mice and rabbits, respectively [115,116].

Section summary

Formaldehyde only has a very short half-life and usually does not accumulate to high levels in humans and animals. In comparison, formic acid can more readily accumulate in humans and non-human primates. Rodents have very different metabolic pathways for methanol and its toxic metabolites (formaldehyde and formic acid) from humans and non-human primates, and as a result, formic acid accumulation usually does not occur in rodents, unless under special conditions in which the metabolic disposition of methanol and its metabolites is selectively inhibited [117].

General Toxicity and Mechanism of Action of One-Carbon Metabolites

Methanol

The clinical presentation of methanol poisoning varies considerably from patient to patient. Methanol initially has a similar narcotic effect as ethanol, followed by a latent period of 6–24 h, depending on the absorbed amount [118]. This latent period (mostly asymptomatic) corresponds to the time required for the metabolism of methanol to formaldehyde and formic acid and the subsequent accumulation of formic acid in the body. Following this period, the patients may have a wide range of clinical symptoms and manifestations [118–120], depending on the severity of methanol-associated damage in various vital organs/tissues in the body, which commonly involve the eye, brain, kidney, heart, and other organs.

One of the characteristics of methanol or formic acid intoxication is the development of metabolic acidosis [26–28,118]. Mechanistically, acidosis is thought to be partly caused by anerobic metabolism (likely also includes ketogenesis) which produces large

amount of lactic acid (and ketone bodies), but not by the direct release of protons from formic acid [26–28,118].

Some of the organs or tissues (such as the neuroretina, brain, kidney and heart) are more sensitive to the toxicity of methanol and its toxic metabolites. A brief discussion of the general toxicity of methanol (along with its metabolites) in these organs of humans and animals is provided below.

Eye

It is widely accepted that the ocular lesion in methanol poisoning essentially represents a toxic optic neuropathy, which is accompanied with varying degrees of retinal edema. Moreover, the ocular toxicity associated with methanol poisoning is caused by formic acid but not by methanol or acidosis [30]. Humans and non-human primates are uniquely sensitive to the ocular toxicity of formic acid because of their limited capacity to oxidize and thus detoxify formic acid. A detailed discussion and comparison of the ocular lesions of methanol (and formic acid) in human subjects and other species is provided later; so it is only briefly mentioned here.

Brain

In addition to the eye, methanol poisoning also causes extensive pathological changes in the brain [30,121–123]. Based on clinical MRI and CT imaging analyses [124–131], the common brain damage in methanol-intoxicated patients includes lesions of the putamen, globus pallidus and brainstem, hemorrhage in various parts of the brain, and calcium deposits in white matter with a primary subcortical localization. Consistent with findings from clinical imaging studies, post-mortem pathological analysis of brains from methanol-intoxicated patients showed similar findings [132]. The lesions include cerebral edema and hemorrhage in many parts of the brain, hemorrhagic necrosis in the thalamus, putamen, globus pallidus, cerebral cortex, and areas around the basal ganglia [132].

It is of note that the observed brain lesions are usually non-specific, and may occur under other conditions of severe brain hypoxia (or ischemia), thus suggesting that histotoxic hypoxia plays a critical role in methanol intoxication-associated brain damage (this subject will be discussed in more detail later). Consistent with the pathological findings of lesions in the basal ganglia in methanol-intoxicated patients, there were survivors who later indeed developed Parkinsonism.

It is interesting that the basal ganglia is one of the brain regions that is particularly sensitive to the toxic effect of methanol. Mechanistically, it is postulated that neurons in this region contain very high levels of the neurotransmitter dopamine and thus are associated with a high basal level of intrinsic oxidative stress due to dopamine's well-known chemical property to undergo facile auto-oxidation and redox cycling. This preexisting condition would make these neurons particularly sensitive to methanol's toxicity as mitochondrial inhibition by formic acid or formaldehyde (toxic metabolites of methanol) would further increase mitochondrial ROS production and accumulation, which would aggravate ATP depletion, mitochondrial oxidative stress and damage, and ultimately neuronal death.

Animal studies showed that alterations in brain specimens from rabbits exposed to formic acid [122] were far less grave than changes seen in monkeys [30], which was consistent with the different rates of metabolic disposition of formic acid in these two animal species (discussed in section "Methanol Metabolism in Humans and Experimental Animals"). The observed calcium deposits in the cerebral hemispheres and hippocampus of rabbits were in good agreement with the intracerebral calcifications which is commonly associated with renal tubular acidosis [133].

Kidney

The kidney, due to its high oxygen requirement for cellular functions, is often on the verge of hypoxia even under normal conditions [134,135]. Renal mitochondrial respiration and cellular calcium content are severely altered under ischemic or hypoxic conditions [136]. Therefore, it is expected that the kidney would be highly susceptible to methanol poisoning. Indeed, acute kidney injury had been widely reported after methanol poisoning [123,131–139]. According to Verhelst's study [139], the causes of methanol-induced nephrotoxicity was largely due to a combination of both direct factors, such as high blood levels of methanol and formic acid, and indirect factors, such as hemolysis and myoglobinuria [139]. Histopathological evaluation of the kidneys from patients of acute methanol intoxication suggested an initial hydropic changes in the proximal tubular epithelial cells, without significant lesions in the glomeruli [138,139].

The mechanisms of methanol nephrotoxicity are multifactorial. The most important direct factor is the cytotoxic effect of formic acid on proximal tubular epithelial cells. There is a significant correlation between formic acid levels and the development of acute renal injury in the proximal tubules [139]. Because methanol and its metabolites are small water-soluble molecules and can be readily filtered through glomeruli, the clinical observations are consistent with the fact that the renal proximal tubules have a very high level of exposure to methanol and its toxic metabolites. Since formic acid is an inhibitor of mitochondrial cytochrome oxidase (discussed in detail later), it would inhibit mitochondrial respiration and ATP synthesis, resulting in hypoxia and injury in renal proximal tubular epithelial cells. In addition, the influx of calcium into cells is another contributing factor in methanol (formic acid)-induced cytotoxicity in renal tubular epithelial cells [32]. Earlier studies reported that formate increased the intracellular levels of sodium chloride in the proximal tubule [140,141]. Similarly, in a rat model of renal proximal tubule microperfusion model, infusion of formate led to increased proximal tubular cell volume (likely due to swelling) [141,142]. It is likely that all these changes are the direct result of formic acid-induced ATP deficiency in renal tubular cells.

It is known that formic acid in the kidney can interfere with parathyroid hormone (PTH)-controlled Ca^{2+} reabsorption in proximal tubules [143], which is an ATP-dependent active transport process. Consistent with this observation, a linear relationship between the urinary calcium levels and the formic acid concentrations in the air has been observed in workers chronically exposed to either methanol or formic acid [143].

Earlier studies have shown that the kidneys of rats and rabbits are far less sensitive to the toxicity of formaldehyde and formic acid (toxic metabolites of methanol) [122,144]. Major pathological changes observed in their kidneys mostly included swelling (with occasional focal degeneration) in proximal tubular epithelial cells [144].

Heart

Cardiac muscle cells have a very high demand for oxygen and energy supply. In adult rabbits injected intravenously with buffered formic acid once daily for 5 days, the maximum concentrations of formic acid in the brain, kidney and heart are similar to the IC_{50} values for inhibiting the cytochrome oxidase activity *in vitro* [122]. Histological analysis clearly demonstrated hypoxic changes at the

cellular levels in cardiac muscle cells in these animals, along with formation of calcium aggregates. The formic acid-induced acidosis exacerbates spontaneous sarcoplasmic reticulum Ca^{2+} release in the myocardium, which is also potentially arrhythmogenic [145].

Formaldehyde

Formaldehyde is the simplest aldehyde and highly reactive. It can undergo hydration and form hemiacetals with alcohols or thiohemiacetals with thiols. It can also covalently cross-link many nitrogen-containing compounds (*e.g.*, DNA, RNA, proteins and amino acids) [24,25,106,107]. In case of proteins, formaldehyde can react with amines to form Schiff bases and cross-link proteins by forming methylene bridges between the two amine groups [24,25,146]. Due to the extensive protein cross-linking ability of formaldehyde, formalin (a water solution containing 40% formaldehyde) is widely used for tissue and cell preservation and fixation in medicine and biological research [147].

Formaldehyde is highly toxic in humans when intoxicated. For instance, ingestion of formalin as a result of accidental, homicidal or suicidal attempts can rapidly cause deleterious effects in every part of the body, such as causing gastrointestinal hemorrhage, cardiovascular collapse, unconsciousness or convulsions, severe metabolic acidosis, acute respiratory distress syndrome, and others [148,149]. Similar toxic effects in different systems and organs have also been observed in animal models [150].

In cultured cells, formaldehyde is highly cytotoxic [151]. The cytotoxicity of formaldehyde appears to be associated with its ability to form adducts with DNA and proteins [152]. At the subcellular levels, formaldehyde reduces mitochondrial membrane potential and inhibits mitochondrial respiration, which is accompanied by mitochondrial ROS accumulation [94]. Formaldehyde can also induce lipid peroxidation and cell death [94]. In addition, exposure of cultured brain cells to high levels of formaldehyde causes intracellular GSH depletion and extracellular GSH accumulation [153,154]. As GSH is involved in many important cellular functions like protection against reactive oxygen species (ROS) and metabolic detoxification of chemically-reactive endobiotics and xenobiotics [155,156], GSH depletion is an important cause for severe oxidative stress, which usually occurs after prolonged exposure to elevated levels of formaldehyde [154].

Because formaldehyde can chemically react with many molecules (small and large) in the body plus it is rapidly metabolized, generally this chemical does not accumulate to high levels in tissues or circulation. Nevertheless, it has also been noted that the tissue levels of formaldehyde can still vary considerably depending on the tissues and under different (patho)physiological conditions. Therefore, it has been speculated that the endogenously-formed formaldehyde might be involved in certain (patho)physiological processes. For instance, a number of studies have reported that there was a direct correlation between chronically-elevated formaldehyde levels in the brain and memory impairment in animal models [54,55,62,112,113,157,158]. A similar correlation has been observed between brain formaldehyde levels and neurodegeneration in humans [55,110,113,159,160]. Interestingly, increased expression/activity of the formaldehyde-generating enzymes has also been observed in human DM [161–163]. These studies suggest that increases in endogenous metabolic formation of formaldehyde may contribute to the pathogenesis of certain degenerative conditions in humans.

Lastly, it is of note that because of its ability to covalently modify DNA, formaldehyde is believed to be mutagenic [164] and also potentially carcinogenic [165,166]. Human epidemiological studies have also indicated that there is sufficient evidence for the carcinogenicity of formaldehyde in humans [167–170].

Formic acid

General toxicity

Different from formaldehyde, formic acid can more readily accumulate in humans and non-human primates following methanol poisoning [26,30]. In methanol poisoning, formic acid accumulation in the body is believed to be largely responsible for the acute toxic effects such as metabolic acidosis, serious visual impairment (including blindness), damage to vital organs in the body (such as brain, kidney and heart), and even death [82,83]. The toxicity of formic acid was mostly investigated in connection with methanol studies; in fact, there were very few studies specifically studying the toxic effects of formic acid itself.

As discussed earlier, methanol poisoning is characterized by a severe metabolic acidosis, and acidosis is thought to be partly caused by anerobic metabolism (likely also includes ketogenesis) which produces large amount of lactic acid (and ketone bodies) [26–28,118].

Acute and severe formic acid intoxication usually has a high mortality rate [171]. Clinical features include the gastrointestinal tract (ulceration of the oral and pharyngeal mucosa), abdominal pain (accompanied by hematemesis and dysphagia), and inhalation pneumonitis which may proceed to respiratory infection and ultimately respiratory failure. The changes in the cardiovascular system are usually nonspecific, frequently accompanied by profound vascular hypotension, hematuria and acute renal failure. Renal biopsy indicated toxic, tubular necrosis. Patients usually succumb to severe vascular hypotension and respiratory arrest, acute renal failure and gastrointestinal hemorrhage.

Mechanism of cytotoxicity

Based on earlier studies, it has been suggested that the mitochondrial cytochrome oxidase is an important target of formic acid cytotoxicity [31,32,172]. The cytochrome oxidase is a terminal member of the eukaryotic mitochondrial electron transport chain and an integral protein complex of the inner mitochondrial membrane. This enzyme is involved in the four-electron reduction process of the oxygen molecule resulting in the formation of ATP and water. It was reported earlier that formic acid can inhibit the activity of cytochrome oxidase by binding at the sixth coordination position of its ferric heme iron [173], resulting in inhibition of the cytochrome oxidase complex of the mitochondrial respiratory chain [31,173]. The estimated K_i for the inhibition of the cytochrome oxidase activity by formate is around 1–5 mM [31]. This concentration of formic acid is readily achievable in liver and other organs under certain pathogenic conditions. This inhibition by formic acid would reduce cellular ATP synthesis [31,32,174], which might be accompanied by increased anerobic glycolysis and potentially lactic acid accumulation. Glycolysis is not efficient, producing far fewer ATP molecules compared to oxidative phosphorylation.

Inhibition of the mitochondrial respiratory chain by formic acid would also cause mitochondrial superoxide accumulation, which may result in lipid peroxidation, mitochondrial damage and even oxidative cell death [175–177]. In partial support of the involvement of ROS in methanol cytotoxicity, increased lipid peroxidation products have been detected in different tissues and urine samples from

methanol intoxication animal models [178,179].

Here it is of note that accumulation of mitochondrial superoxide could lead to inhibition of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity [33] and thereby suppress the anerobic glycolytic pathway of glucose metabolism, further aggravating the situation of cellular energy shortage. In the meanwhile, the upstream glucose metabolites might be forced to enter other metabolic pathways, such as the polyol pathway and the hexosamine pathway.

From a mechanistic point of view, formic acid-induced hypoxia and cytotoxic effect [180] are somewhat similar to the toxic effect of cyanide, albeit with a far milder cytotoxic effect [180–182]. Based on this mechanistic understanding, it is understood that those organs which have a high rate of oxygen consumption (such as the neuroretina, brain, kidney and heart) would be highly sensitive to the toxicity of formic acid (and formaldehyde); a brief discussion of the toxicity in these organs has already been provided in earlier section "Methanol".

Lastly, it is of note that formic acid may alter cellular calcium levels through induction of cellular hypoxia [122,183–185]. Calcium accumulation in ischemic and hypoxic conditions has been reported earlier in renal cortex [136,186] and cardiomyocytes [177]. An increase in free cytosolic calcium can activate calcium-dependent phospholipases, resulting in the breakdown of cell membranes and accumulation of free fatty acids and lysophospholipids which are toxic to cells [177]. It has been suggested that mitochondrial calcium overload may also contribute to mitochondrial dysfunction [177].

Section summary

The toxicity of methanol mostly comes from its oxidative metabolites, formaldehyde and formic acid [62]. Following methanol intoxication, formic acid would accumulate, and is largely responsible for methanol's toxic effects, including the induction of metabolic acidosis, ocular impairment (including blindness), damage to vital organs in the body, and even death. There is a clear correlation between the *in vivo* concentrations of formic acid (but not methanol) and various complications [187].

While humans and non-human primates are highly sensitive to the toxicities of methanol and formic acid, rodents are highly resistant to their toxicities [119,120]. The species difference in methanol toxicity is directly associated with the different levels of accumulation of formic acid in the body [26–30], which is determined by the different pathways involved in the metabolic activation and disposition of methanol [114,188,189].

Mechanistically, formic acid can cause cytotoxicity by directly inhibiting the cytochrome oxidase complex of the mitochondrial respiratory chain, which eventually results in hypoxia and decreased ATP production in tissues or cells that have a high requirement for oxygen and energy supply.

Lastly, it is of note that because formic acid is a weak acid (pK_a = 3.78), a pH drop of 0.3 would mean a doubling of the undissociated formic acid level. Since it is the undissociated formic acid that can cross the plasma membrane, a decrease in cellular pH theoretically would mean a stronger inhibition of cytochrome oxidase by formic acid. Therefore, the induction of acidosis by formic acid may further aggravate the inhibition of mitochondrial cytochrome oxidase.

Ocular Lesions of Methanol (and Formic Acid) in Humans and Experimental Animals

A comparison of the ocular lesions seen in humans and laboratory animal models following exposure to methanol or formic acid is provided below.

Methanol poisoning-induced ocular lesions in humans

In general, humans are very sensitive to methanol's ocular toxicity; even a very low dose of pure methanol ingestion may cause blindness in human subjects [117,190]. An early study suggested that the ocular lesion in acute methanol poisoning is mostly produced by its metabolite formic acid [30].

The clinical symptoms of acute methanol ocular toxicity in humans range from blurred vision to "snowfield vision" or total blindness in severe cases [104,191,192]. On visual field testing, central scotoma, hyperemia, pallor of the optic disc, papilledema, and an afferent papillary defect may be present. Electroretinography usually shows a diminished β-wave [193–195], a marker of bipolar cell dysfunction. Additionally, optical coherence tomography, which is similar to ultrasound but uses reflected light waves to image translucent tissues, often shows peripapillary nerve fiber swelling and intraretinal fluid accumulation [195].

Consistent with the clinical observations, pathological analysis also revealed that the ocular lesions in methanol-intoxicated patients essentially represent a toxic optic neuropathy, with retinal edema as an accompanying change [30,191,196–203]. In fatal cases, the optic nerve shows central necrosis in the distal (orbital) portion while the central optic tracts remain intact. Therefore, it is generally accepted that methanol poisoning causes eye impairments mostly through destruction of the optic nerves.

It has been noted that the retinal pigmented epithelial and optic nerve cells are uniquely susceptible to methanol toxicity [194,204,205]. The changes in the retinal ganglion cells was thought to be the result of retrograde degeneration of optic nerve axons, while myelin change in the retrolaminal part of the nerve was also observed [196–203].

An earlier study of methanol intoxication reported that the optic nerve has an axonal necrosis in the center part of the nerve, with a sharp border where they met the normally appearing axons in the periphery [206]. The selective bilateral damage of the orbital part of the optic nerve is interesting. It is apparent that the differential blood supply is an important factor. The orbital part is supplied by the pial plexus, with branches from the ophthalmic artery extending perpendicularly into the nerve, providing a generous perfusion of the optic nerve at the periphery [207]. This may explain why the central part of the nerve is more vulnerable [208,209]. Notably, similar changes had also been reported earlier for a case of bilateral ischemic optic neuropathy [210].

Similarly, earlier studies of chronic methanol vapor exposure among workers also revealed that the major changes included varying degrees of injury to the optic nerve [211,212].

Methanol (and formic acid)-induced ocular lesions in experimental animals

Ocular lesions in non-human primates

Administration of methanol in monkeys could induce nearly identical ocular changes as seen in humans, which included optic nerve lesions and pupillary reflex changes, with optic disc edema or hyperedema as accompanying changes [30,213]. Notably, the axonal necrosis was mostly seen in the central part of the optic nerve [214]. Electron microscopic examination found lesions in the optic nerve heads, retrolaminar optic nerve and intraorbital optic nerve, con-

sisting of swelling and clustering of the mitochondria, disruption of the neurotubules, formation of vesicles, and swelling of glial cells.

The ocular toxicity of intravenously-infused formic acid had also been studied in monkeys. Marked optic disc edema was observed 24–48 h after formic acid administration. Loss of pupillary reflexes occurred in most animals within 48 h. The symptoms produced by formic acid in monkeys were similar to the clinical features observed in monkeys with methanol administration [30]. As expected, it was observed that the onset of ocular toxicity occurred more rapidly in monkeys with formic acid administration than with methanol administration because the metabolic conversion of methanol to formic acid and the subsequent accumulation of the latter would take some time.

Ocular lesions in rodents and rabbits

A number of studies have reported that methanol-intoxicated rodents would not develop the same kind of clinical symptoms that are commonly seen in humans and non-human primates. Poon *et al.* [215,216] have investigated the toxic effect of methanol in rats that were exposed to vapors of methanol (300, 2500 and 3000 ppm) for 6 h a day, 5 days per week for 4 weeks. No eye disturbance was observed in these animals. A similar experiment had also been conducted in CD rats [217], and there were no treatment- or dose-related effects observed during ophthalmoscopic and histopathological examinations in all animals. Functional and morphological studies of methanol-intoxicated rats revealed that formic acid acts as a mitochondrial toxicant in the retina and optic nerve [204].

Like rodents, most studies found that rabbits were similarly insensitive to the ocular toxicity induced by methanol or formic acid [218]. It was reported that the rabbits after exposure to methanol vapors for 6 months only developed rather mild ocular toxicity, including ultrastructural changes in the photoreceptor cells and Müller cells [218].

Here it is of note that an earlier study demonstrated that the retinal and optic nerve toxicity of methanol could be readily and equally demonstrated in rats when their folate-dependent oxidation of formic acid was selectively inhibited by nitrous oxide [117]. Under this experimental condition, methanol-intoxicated rats developed nearly the same degree of visual toxicity along with formic acid accumulation. Moreover, the temporal relationship between methanol administration and the development of ocular toxicity and formic acid accumulation in rats [117] was remarkably similar to what was seen in human methanol intoxication [32]. These results clearly demonstrated that the drastically-different sensitivity between humans (and non-human primates) and rodents to methanol and formic acid toxicity is principally due to the differences in their metabolic disposition of formic acid.

Mechanisms of methanol (and formic acid)-induced ocular lesions

An earlier study showed that formic acid *per se* could directly cause damage to the optic nerve and disc, in the absence of acidosis [30]. Moreover, the ocular effects of methanol poisoning were also similarly associated with formic acid-induced damage to the optic nerve and disc [32].

It has been suggested earlier that formic acid has an apparent affinity for the retrolaminar optic nerve and optic disc, which would partially account for its preferential retinal toxicity in susceptible species (*e.g.*, humans and non-human primates). Mechanistically, as discussed earlier, formic acid can inhibit cytochrome oxidase in the mitochondria, which would result in decreased ATP production. Cellular ATP deficiency would impair the normal physiological functions of the optic nerve (*i.e.*, the transmission of the action potentials along the nerve), which subsequently may produce severe damage to optic nerve fibers and neurons.

Lastly, it is of note that formaldehyde is a cross-linking agent, and its carbonyl atom is a strong electrophilic site, making it react facilely with nucleophilic sites in proteins and DNA. Although formaldehyde usually does not accumulate to high levels in the body, its exceptional chemical reactivity may still contribute significantly to certain aspects of the methanol toxicity, including ocular lesions.

Section summary

Based on most observations, it is generally accepted that the ocular lesion in methanol poisoning essentially represents a toxic optic neuropathy, with retinal edema as an accompanying change. Moreover, the ocular toxicity associated with methanol poisoning is caused by formic acid but not by methanol or acidosis [30]. As expected, formic acid acts as a strong mitochondrial toxicant in the retina and optic nerves [204].

Humans and non-human primates are uniquely sensitive to the ocular and neurotoxicity of formic acid because they have a limited capacity to oxidize and thus detoxify formic acid. By contrast, rodents (such as rats and mice) are highly resistant to methanol and formic acid toxicity (including the ocular lesions), which is purely due to their exceptional ability to oxidize formic acid to carbon dioxide.

Diabetic Ocular Lesions in Humans and Experimental Animals

Clinical pathology of diabetic ocular lesions in humans

Nearly all type 1 DM patients and approximately 80% of insulin-dependent type 2 DM patients will have retinopathy 20 years after the disease diagnosis [219,220]. Diabetic retinopathy often includes altered vascular permeability, capillary microaneurysms, capillary degeneration, and excessive formation of new blood vessels (neovascularization). The neuroretina is also dysfunctional with neuronal cell death, which alters retinal electrophysiology as well as visual functions.

Diabetic retinopathy is traditionally classified into two main clinical forms: proliferative diabetic retinopathy (PDR) and NPDR (non-PDR). In the early stages, hyperglycemia can lead to thickening of the basement membrane and intramural pericyte death, which jointly contribute to changes in the integrity of blood vessels within the retina and alter the blood-retinal barrier and vascular permeability [221]. In this initial stage of NPDR, people usually do not notice significant visual impairment. Degeneration or occlusion of the retinal capillaries is strongly associated with a worsening prognosis [222] and often followed by the release of angiogenic factors including those related to hypoxia. This progresses the disease into the proliferative phase where neovascularization and accumulation of fluid within the retina, which is commonly referred to as “macula edema”, contribute to visual impairment. In more severe cases, retinal bleeding associated with distorting of the retinal architecture is often seen and this change can lead to retinal detachment [221].

The prevalence of the sight-threatening forms of retinopathy is typified by PDR and DME (diabetic macular edema) [223]. In ad-

dition to diabetic retinopathy, another sight-threatening ocular lesion that is associated with DM is cataract.

A new hypothesis on the pathogenic mechanism of human diabetic retinopathy

Traditionally, the understanding of the etiology and pathogenesis of diabetic retinopathy focuses on vascular dysfunction and loss of perfusion, which are hallmarks of diabetic retinopathy. However, it is still far from certain whether these changes are the fundamental causes that drive the cascade of diabetic retinopathy or there might be other more fundamental changes that we do not quite understand at present. It should be noted that there are clear clinical indications that the neuroretinal functions are compromised in DM patients, well before the onset of overt microvascular changes [224]. For instance, electrophysiological study of DM patients has revealed alterations in the neuroretina, including diminished color vision [225], reduced contrast sensitivity [226], and abnormal electroretinogram before the onset of overt retinopathy [227]. In agreement with these clinical observations, a number of studies using DM animal models as well as human subjects have also reported the early loss of inner retinal neurons and neuronal projections [228,229], degeneration of the pigment epithelium [230], and dysfunction of Müller cells and astrocytes [224,231–236].

Here, a new mechanistic hypothesis for the pathogenesis of diabetic retinopathy is proposed. This hypothesis is based on the premise that there is an increased metabolic formation and accumulation of formic acid (along with formaldehyde) in DM patients. The retina, in particular the neuroretina, probably requires the highest level of oxygen and energy supply among all the tissues in the human body, exceeding even that of the brain [237–241]. As such, the retina is richly vascularized for blood supply. It is hypothesized that hyperglycemia-associated increase in formic acid and formaldehyde levels in different retinal cells, in particular neuroretinal cells, can inhibit mitochondrial respiration, resulting in a state of hypoxia and reduced ATP synthesis [31,173,178]. This reduction in mitochondrial respiration may subsequently lead to increased anerobic glycolysis as a compensation. However, glycolysis is not efficient, producing far fewer ATP molecules compared to oxidative phosphorylation.

As already mentioned earlier, inhibition of the mitochondrial respiration may lead to accumulation of the mitochondrial superoxide, which would lead to inhibition of the GAPDH activity [33] and thereby suppress the anerobic glycolysis, likely further aggravating the situation of cellular energy shortage. In the meanwhile, the upstream glucose metabolites might be forced to enter other metabolic pathways, such as the polyol pathway and the hexosamine pathway. Therefore, it is expected that at late stages of DM, the patients are usually associated with a state of severe energy deficiency in many tissues, especially in those tissues that normally have a very high demand for oxygen and energy supply (such as the neuroretina).

In addition, the formic acid-induced mitochondrial superoxide accumulation may further cause mitochondrial damage and even oxidative cell death [175–177]. On the other hand, formaldehyde may contribute to mitochondrial damage and cell death in DM patients by causing extensive cross-linking of the mitochondrial and cellular proteins, in addition to its conversion to formic acid. In support of the proposed mechanistic explanation, mitochondrial dysfunction (such as dysregulation of the mitochondrial uncoupling proteins) [242–245] has been detected at the sites of diabetic complications, and these pathogenic changes are removed by overexpression of the manganese superoxide dismutase [246], which effectively helps to remove mitochondrial superoxide.

Based on the proposed MFF hypothesis, a tentative explanation of some of the biochemical, histopathological and clinical changes of diabetic retinopathy commonly seen in DM patients and/or animal models is provided below.

Hypoxic neuroretinal degeneration is a critical early change in diabetic retinopathy

As discussed in "Ocular Lesions of Methanol (and Formic Acid) in Humans and Experimental Animals", formic acid can preferentially induce hypoxic lesions (even death) in optical nerve and disc. According to the proposed MFF hypothesis, it is speculated that accumulation of formic acid in advanced DM patients would first result in hypoxia in the neuroretina, as this tissue has a very high demand for oxygen and energy supply. There are many earlier studies offering support for this suggestion. For instance, studies have shown that abnormalities in neuroretinal dysfunction were readily seen in DM patients in the absence of overt microvascular abnormalities [247–251]. Furthermore, apoptosis of retinal ganglion cells (RGCs) and reactive gliosis, which are hallmarks of retinal neurodegeneration, were also detected in diabetic donors without the appearance of microcirculatory abnormalities [252–254]. This loss of neuroretinal cells, which reduces the thickness of the retinal neuropile, was observed in DM patients with minimal or no diabetic retinopathy by using scanning laser polarimetry or optical coherence tomography (OCT) [255].

Pathogenic changes in retinal vasculature

As discussed above, neuroretinal degeneration is an early event in diabetic retinopathy, occurring before the onset of microvascular changes, including changes in retinal blood flow, thickening of the capillary basement membrane, breakdown of the blood-retinal barrier, and the impairment of neurovascular coupling [247,256–258]. Therefore, it is hypothesized that the neuroretinal degeneration caused by glucose-derived toxic one-carbon metabolites may drive the retinal vascular changes. A brief discussion of the supporting evidence is provided below.

Thickening of the capillary basement membrane. One of the early histopathological changes observed in DM patients is the thickening of the capillary basement membrane (BM) [36]. Mechanistically, it has long been suspected that accumulation of AGEs is an important cause for the cross-linking of various proteins in vascular BM [259–262]. In my personal view, the relative importance of AGE accumulation in the thickening of the capillary BM might be smaller than usually thought, because in the rodent DM models, the blood glucose levels are usually far higher than those seen in human DM, and yet DM rodents rarely develop the same level of capillary changes (including BM thickening).

Based on the proposed MFF hypothesis, the production of formaldehyde, which is a cross-linking agent, may contribute, in addition to AGEs, to the thickening of the capillary BM. The extensive cross-linking of capillary BM proteins by formaldehyde is expected to lead to increased synthesis of these components (*e.g.*, collagen IV, fibronectin and laminin) and reduced degradation by catabolic enzymes [263,264]. Indeed, earlier studies [259–264] have found that the synthesis of collagen IV, fibronectin and laminin in capillary BM is increased in DM whereas their degradation by catabolic enzymes is reduced. Such matrix modifications may cause impaired en-

dothelial-pericyte communication and inappropriate interaction between these cells with constituent BM proteins [199,259,265–267].

Alteration of retinal blood flow. It is known that the retinal vasculature lacks autonomic innervation, and regulation of blood flow to the retinal neuropile largely depends on local signaling mechanisms, carried out by Müller cells (discussed later), in a process commonly referred to as "neurovascular coupling". It is hypothesized that due to formic acid-induced hypoxia in diabetic neuroretina and nerve fibers, which, through the functional neurovascular coupling mechanism, would lead to activation of the Müller cells, and the activated Müller cells then release substances that cause dilation of the retinal blood vessels to improve the blood flow and oxygen supply to the neuroretina.

Consistent with this suggestion, it was noted, as early as in the 1930s, that the retinal blood flow was indeed markedly altered in DM patients [268]. Additional studies have also noted that the caliber of retinal vessels was consistently increased in DM patients [269,270]. As DM progresses, the degree of retinal arteriole dilation and blood flow would increase in proportion to the severity of retinopathy [271]. Understandably, these changes only represent a temporary fix to the existing problems, and if hyperglycemia is not improved, the heightened retinal vasodilation and increased blood flow likely would hasten the future progression of diabetic retinopathy [272–274].

In late stages of diabetic retinopathy, retinal capillary non-perfusion is the result of focal degeneration of the neuroretina (discussed in more detail in the next section). Retinal capillary non-perfusion, coupled with the existing mitochondrial respiratory inhibition by formic acid and formaldehyde, would lead to severe retinal ischemia and hypoxia, which would further heighten neovascularization, likely as a desperate last resort to improve the situation. As a result, neovascularization, accumulation of fluid within the retina, and associated vitreous hemorrhage, which are characteristic of the proliferative stage of diabetic retinopathy, would jointly worsen diabetic ocular impairments [6]. Here it is of note that the conditions of fluid accumulation within the retina and the associated vitreous hemorrhage are expected to be more severe in DM patients with hypertension, as clinical studies have consistently shown that high blood pressure is associated with a worsening outcome [275].

Lastly, it is of interest to point out that some of the presently-available treatment options for diabetic retinopathy also offer support for the proposed pathogenic mechanism of diabetic retinopathy. For instance, panretinal photocoagulation is commonly used as a treatment for PDR and DME [276,277]. The procedure involves creating thermal burns in the peripheral retina leading to tissue coagulation. In this way, the blood and oxygen requirement by the large peripheral areas of the neuroretina will be essentially removed, and as a result, the relative oxygen and energy supply to the remaining neuroretinal tissue (which is the central part of the retina with highest visual activities) would be significantly improved, and so are the clinical symptoms [278].

Capillary degeneration and microaneurysms. Capillary degeneration is another important feature of diabetic retinopathy, with the appearance of acellular capillaries which are usually non-perfused naked BM tubes [279]. Severe capillary closures are often linked with cotton wool spots in the neural retina and also the occurrence of intraretinal microvascular abnormalities (IRMAs) [280,281]. The large-caliber vessels in IRMAs traverse hypoxic retina with direct communication between pre-capillary arterioles and post-capillary venules and probably represent shunt vessels in a desperate attempt to re-vascularize the hypoxic neuropile [282,283].

Microaneurysms represent another hallmark lesion of diabetic retinopathy. Clinically, many microaneurysms are sclerosed and non-perfused, whereas others can be observed as fully or partially perfused during fluorescein angiography [280,284]. Microaneurysms occur largely on the arteriolar side of the circulation where they often occur immediately upstream of large areas of capillary acellularity [279].

It is hypothesized that the above-described retinal vascular degenerations in DM are the secondary changes to neuroretinal degeneration which is caused by chronic retinal accumulation of formic acid (and formaldehyde) and subsequent hypoxia and ATP deficiency. When neurons in certain parts of the retina degenerate, they would no longer be able to activate the nearby Müller cells to release growth factors to stimulate retinal angiogenesis. As a result, the capillary pericytes in certain regions of the retina would no longer regenerate and may not even survive for too long in the absence of proper growth factors. It is understandable that the calibers of these acellular capillaries might enlarge due to the weak vessel wall structure (in the absence of capillary pericytes) plus the action of vascular static pressure.

In support of the above explanation, it has been reported that one of the earliest features of microaneurysms in human diabetic retina is the loss of pericytes [285] and vascular smooth muscle cells [286,287]. In addition, endothelial cells also become dysfunctional, with exhausted replicative potential [288,289]. Retinal pericytes and smooth muscle cells are known to be heavily reliant on certain growth factors for regeneration and survival [290,291]. In DM, there is a selective depletion of these growth factors [292] (as a direct result of neuroretinal degeneration), which then leads to acellular capillary formation [282].

Dysregulation of Müller cell functions

Müller cells, the principal glial cell type found in the retina, span the entire width of the retina and have contact with almost every cell type in the retina [283]. The Müller cells perform many important physiological functions in the retina, including recycling of neurotransmitters, retinoic acids and certain ions (such as potassium); regulation of the retinal nutrient supply, energy metabolism and blood flow; and maintenance of the blood-retinal-barrier [283].

It is known that Müller cells live primarily from glycolysis [293]. In so doing, Müller cells can spare oxygen for retinal neurons and other cell types that use oxidative phosphorylation for ATP production. Also because of this unique property, it is expected that Müller cells would be less sensitive to the toxicity of formic acid and formaldehyde.

It is hypothesized that retinal accumulation of formic acid (along with formaldehyde) is the main cause for neuroretinal degeneration, which subsequently leads to Müller cell activation. The growth factors, cytokines and chemokines released from activated Müller cells serve as important biomediators between neuroretinal degeneration and retinal microangiopathy. As briefly discussed below, this speculation is supported by some experimental observations.

First, one of the most important functions of Müller cells is to serve as a mediator of the "neurovascular coupling" to regulate retinal blood flow and nutrient supplies, through the release of biomediators that control the constriction and dilation of the retinal

blood vessels [294,295]. It is hypothesized that due to formic acid (and formaldehyde)-induced hypoxia and ATP deficiency in diabetic neuroretina and nerve fibers, Müller cells are activated, which then release biomediators to dilate retinal blood vessels as a feedback regulation to improve blood flow to the neuroretina.

Second, another important function of Müller cells is their contribution to maintaining the integrity of the blood-retinal-barrier [296–298], through secretion of factors such as pigment epithelium-derived factor and thrombospondin-1 which increase the tightness of the endothelial barrier [299,300]. When the retinal blood vessels dilate and blood flow increase as a result of Müller cell activation caused by the hypoxic neuroretina, a potential side effect is the reduced integrity of the blood retinal barrier. Accordingly, it is speculated that the activated Müller cells would attempt to increase the secretion of relevant regulatory factors that would help improve the tightness of the endothelial barrier [299,300].

Third, many studies have shown that Müller cells become activated in DM [231,233,301–304]. One of the best-known indicators of Müller cell activation in diabetic retinopathy is the increased expression of glial fibrillary acidic protein (GFAP) [302,305,306]. Under healthy conditions, Müller cells generally do not express GFAP [307,308]. Studies have shown that high levels of glucose can induce Müller cell activation to release growth factors, cytokines and chemokines [308–314].

While many studies have focused on the detrimental effects of Müller cell-derived VEGF on the microvasculature in the diabetic retina [307,315–317]), it should be noted that the real intent of the growth factor production and release by Müller cells likely is to protect the neuroretina from chronic hypoxia and ATP deficiency caused by hyperglycemia-associated formic acid/formaldehyde accumulation. This notion is supported by a study in mice which carry a disrupted VEGF receptor 2 specifically in Müller cells. Loss of this receptor was found to reduce the Müller cell density, decrease the scotopic and photopic electroretinography amplitudes, and accelerate the loss of photoreceptors and neuroretinal cells in the retina [318].

Besides growth factors, Müller cells also release a number of cytokines and chemokines under hyperglycemic conditions, such as interleukin-1β [308,313,319–322]. Vascular endothelial cells are susceptible to interleukin-1β and rapidly progress to cell death in response to this pro-inflammatory cytokine [323], along with the formation of acellular capillaries [324]. Besides interleukin-1β, Müller cells also produce other pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-6 [325–329]. Interleukin-6 has detrimental effects associated with vascular dysfunction [330–332]. It is speculated that the release of cytokines and chemokines by Müller cells under hyperglycemic conditions is the result of severe neuroretinal degeneration caused by formic acid accumulation. It is expected that following neuroretinal degeneration, the retinal vessels would regress, and eventually the population of Müller cells would also reduce.

In summary, it was widely considered in the past that during the development of diabetic retinopathy, “dysregulation” of Müller cell function is a primary cause for retinal dysfunction through production of pro-angiogenic factors leading to neovascularization and eventually the creation of a chronic inflammatory retinal environment, which results in cell death. In light of the proposed MFF hypothesis, it is suggested that Müller cells are a key player that maintains a healthy retinal environment. Once this microenvironment is disrupted by accumulation of formic acid and formaldehyde in DM patients which subsequently causes histotoxic hypoxia and ATP deficiency in retinal neurons and nerve fibers, Müller cells would become activated to counter-regulate in an attempt to “repair” the disrupted retinal environment. While Müller cell-released growth factors and cytokines are closely associated with vascular dysfunction and angiogenesis, this is only part of the picture. Based on the new MFF hypothesis, it is postulated that the release of the regulatory factors (in particular growth factors) by Müller cells is intended to alleviate hyperglycemia-associated damage to the retinal neurons and nerve fibers, and these factors are only turned into self-damaging components in diabetic retinopathy when the initial causative factors (*i.e.*, hyperglycemia-associated, formic acid-induced neuroretinal hypoxia and ATP deficiency) are not effectively corrected. When the Müller cell-derived pro-inflammatory cytokines/chemokines are released, it signals that the pathogenic process is reaching a late phase where severe retinal neurodegeneration has already occurred.

Reconciliation with other pathogenic mechanisms of diabetic retinopathy

In the past, a number of independent hypotheses have been proposed to explain the role of the increased polyol pathway flux, the increased hexosamine pathway flux, the increased formation of AGEs, and the over-activation of protein kinase C in the pathogenesis of diabetic retinopathy [333]. These mechanisms should not be viewed as independent phenomena. Based on the proposed MFF hypothesis, it appears that they are the result of hyperglycemia-associated, formic acid/formaldehyde-mediated inhibition of the mitochondrial electron transport chain, which is associated with accumulation of mitochondrial ROS such as superoxide. Superoxide can indirectly cause inhibition of the glycolytic enzyme GAPDH (glyceraldehyde phosphate dehydrogenase) [33], thereby diverting upstream glucose metabolites from glycolysis into other metabolic pathways mentioned above.

The contribution of these metabolic derangements to the pathogenesis of diabetic retinal lesions (as well as other diabetic complications) has been discussed in many earlier papers. While a detailed discussion of these pathways is beyond the scope of this paper, a summary of selected major metabolic pathways is provided in Table 1 and Figure 3 for ease of reference.

It is of note that a number of pharmacological inhibitors have been designed and tested for their potential beneficial effects in alleviating diabetic complications. For instance, elevated aldose reductase activity is linked to the early-onset severe diabetic retinopathy [333], and pharmacological inhibition of the aldose reductase activity ameliorates the pathogenic process in certain animal models [334,335]. However, clinical trials with aldose reductase inhibitors have shown little or no effect in preventing human diabetic retinopathy. Ruboxistaurin, an oral protein kinase C-β inhibitor, was shown to ameliorate retinal microvascular abnormalities in animal models [336], but it did not prevent diabetic retinopathy progression in human subjects with moderately severe to very severe NPDR [337]. Lastly, inhibition of AGE formation, blockade of the AGE-RAGE interaction, and attenuation of the downstream signaling pathways were postulated to be of benefit in preventing diabetic retinopathy. However, aminoguanidine, the first AGE inhibitor tested in diabetic patients, was suspended due to its adverse effects [338].

It is important to note that the above-described inhibitors, while they are partially effective in certain animal models, are generally without significant benefits in DM patients. One of the main reasons

Table 1. Summary of glucose metabolic pathways that are activated by hyperglycemia [34,334] (Figure 3)

Pathway	Characteristic changes
The polyol pathway	Aldose reductase (AR) reduces glucose to sorbitol, and sorbitol dehydrogenase (SDH) oxidizes sorbitol to fructose. Both enzymes are abundantly expressed in tissues that are prone to develop diabetic complications. Hyperglycemia activates the AR pathway partly by mass action. It is expected that increased flux through the AR pathway could increase the intracellular sorbitol, a relative intracellular hypertonic state, and this change may result in a compensatory efflux of other osmolytes such as myoinositol and taurine. In addition, the consumption of NADPH during AR-mediated glucose reduction to sorbitol would reduce cellular NADPH content, this change would suppress the regeneration of GSH, thereby contributing to heightened oxidative stress.
The hexosamine pathway	Fructose-6-phosphate is a metabolic intermediate of glycolysis. When glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inhibited, some fructose-6-phosphate is shunted from the glycolytic pathway to the hexosamine pathway where it is converted to glucosamine-6-phosphate by glutamine fructose-6-phosphate amidotransferase (GFAT). Glucosamine-6-phosphate is then converted to uridine diphosphate-*N*-acetyl glucosamine (UDP-GlcNAc), a molecule that attaches to the serine and threonine residues of transcription factors. In addition, hyperglycemic conditions can also directly activate the conversion of glucose-6-phosphate to fructose-6-phosphate, which creates additional flux through the hexosamine pathway, ultimately resulting in excess GlcNAc and abnormal modification of gene expression.
The PKC pathway	Accumulation of glyceraldehydes-3-phosphate due to inhibition of GAPDH in hyperglycemic condition would lead to elevated level of dihydroxyacetone-3-phosphate (DHAP). DHAP is subsequently reduced to α-glycerol-3-phosphate which in turn combines with fatty acids to drive the *de novo* synthesis of diaclyglycerol (DAG), which in turn activates protein kinase C (PKC). Increased production of PKC β has been implicated in overexpression of the angiogenic protein vascular endothelial growth factor (VEGF), plasminogen activator inhibitor-1 (PAI-1), nuclear factor kappa B (NF-κB), transforming growth factor-β (TGF-β) and may contribute to the pathogenesis of some diabetic complications. Overexpression of PKC isoforms may also be involved in the induction of insulin resistance. Elevated activities of the PKC pathway may also stimulate ROS-generating enzymes such as NADPH oxidases and lipooxygenses which all together exacerbate cellular oxidative environment.
The AGE pathway	Non-enzymatic reactions between the reducing sugars or oxaldehydes and proteins/lipids would result in the formation of the advanced glycation endproducts (AGEs). Three main pathways are responsible for the formation of reactive dicarbonyls (AGE precursors): 1) oxidation of glucose to form glyoxal; 2) degradation of the Amadori products (fructose-lysine adducts); and 3) aberrant metabolism of glycolytic intermediates to methylglyoxal. Extracellular protein AGEs such as those formed with plasma and matrix proteins may interfere with cellular adhesion and activate the receptor for AGEs (RAGE). AGE-RAGE interaction may lead to activation of the transcription factor NF-κB. Also, activation of the RAGE in some tissues may induce oxidative stress through the NADPH oxidase activity.

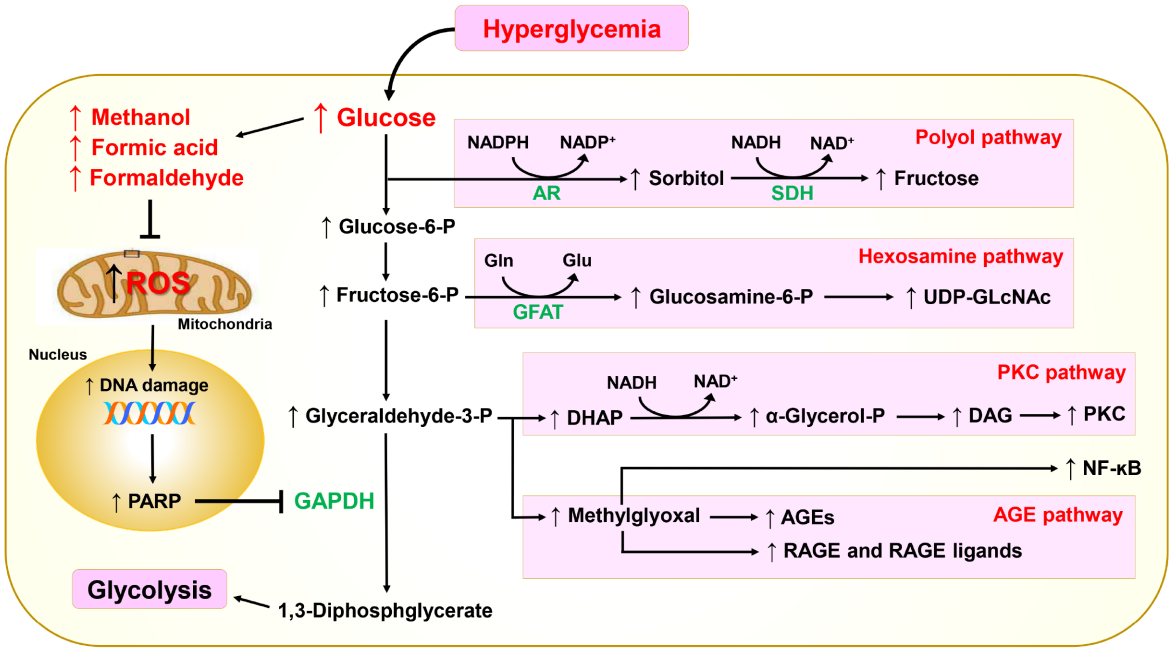

Figure 3. Effect of hyperglycemia-associated accumulation of toxic one-carbon metabolites on glucose metabolic pathways in DM It is hypothesized that hyperglycemia would result in increased cellular glucose levels in DM patients, and this increase would further lead to increased metabolic formation of toxic one-carbon metabolites (methanol, formaldehyde and formic acid). Accumulation of formic acid (along with formaldehyde) would strongly inhibit mitochondrial respiration and superoxide production. High levels of superoxide would indirectly lead to inhibition of GAPDH, thereby suppressing anerobic glycolysis. As a result, the upstream glucose metabolites are forced to be diverted into other metabolic pathways, such as the polyol pathway, the hexosamine pathway, the PKC pathway, and the AGE pathway. It has been well documented that activation of these metabolic pathways contributes to the development of diabetic complications. Abbreviations: AR: aldose reductase; SDH: sorbitol dehydorgenase; GFAT: glutamine fructose-6-phosphate amidotransferase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NF-κB: nuclear factor kappa B; PARP: poly(ADP-ribose) polymerase; DHAP: dihydroxyacetone-3-phosphate; DAG: diacylglycerols; PKC: protein kinase C; AGE: advanced glycation endproducts; RAGE: receptor of AGE; ROS: reactive oxygen species; UDPGlcNAc: UDP-*N*-acetylglucosamine.

for the discrepancy may lie in the fact that formic acid (along with formaldehyde) only plays a very minor role in the pathogenesis of diabetic complications in the commonly-used animal models of DM as opposed to their proposed pathogenic role in DM patients. Since these inhibitors likely would not significantly reduce formic acid and formaldehyde accumulation and their neuroretinal toxicity in DM patients, it is thus not surprising that they are without significant benefits in humans. On the other hand, the observed discrepancy as mentioned above also indirectly reflects the potential importance of formic acid (along with formaldehyde) in the pathogenesis of diabetic retinopathy in humans.

Pathology of diabetic ocular lesions in animal models

Diabetic ocular lesions in non-human primates

Studies have reported that monkeys treated with a single dose of streptozotocin (STZ) develop DM, and ischemic/hypoxic retinopathy with cotton-wool spots and hyper-fluorescent spots developed after 10 years [339,340]. In another study, monkeys were found to develop insulin dependency and hyperglycemia following pancreatectomy, and the blood-retinal barrier leakage was observed within 1 year following the onset of hyperglycemia. However, these monkeys still did not develop proliferative diabetic retinopathy (PDR) 10 years later [341].

Diabetic ocular lesions in rodents

In general, the rodent models of DM would only develop early stages of diabetic retinopathy, such as degeneration of retinal capillaries, but preretinal (intravitreal) neovascularization and degeneration of retinal neurons were not observed in diabetic rats and mice [6,228,342]. Moreover, the rodent models did not develop measurable visual impairment or blindness due to DM.

For instance, in STZ-induced DM rats, their blood glucose levels were increased to ~700 mg/dL in 4 weeks, but these animals would not develop severe ocular impairment (including blindness) [343]. An earlier study [344] compared the ocular lesions in alloxan-induced diabetic rats or in rats fed a 30% or 50% galactose diet for 1.5–2 years. It was concluded that both DM rat models only developed microvascular lesions that are consistent with relatively earlier stages of diabetic retinopathy. Similar observations were also made in many other studies [339,345-347].

While the inability to develop late-stage diabetic retinopathy in DM rodent models might be due to the short life span of rodents relative to humans, another important reason might be associated with the low sensitivity of rodents to formic acid/formaldehyde-induced ocular toxicity.

Section summary

According to the proposed MFF hypothesis, energy production in the neuroretinal cells under DM conditions would be reduced, initially resulting from an inhibition of mitochondrial respiration by formic acid (along with formaldehyde); this change may lead to activation of the anerobic glycolysis [34,348], which produces far fewer ATP molecules compared to oxidative phosphorylation. However, when DM progresses, elevated mitochondrial superoxide production may lead to suppression of the anerobic glycolysis through inhibition of GAPDH [33], further aggravating cellular energy shortage. It is expected that at late stages of DM, formic acid (and formaldehyde)-induced mitochondrial dysfunction and damage, coupled with severe energy deficiency in the neuroretina, would jointly drive the pathogenic processes of diabetic retinopathy.

As discussed in earlier sections, rodents are highly resistant to the ocular toxicity of methanol and formic acid whereas humans (and non-human primates) are highly sensitive to their toxicity. Interestingly, the severe diabetic ocular lesions that are seen in DM patients (and non-human primates) are seldom seen in rodent models. These intriguing similarities, along with many other observations discussed above, offer support for the proposed hypothesis that the hyperglycemia-associated formation and accumulation of methanol and/or its toxic one-carbon metabolites in ocular tissues of diabetic human subjects may contribute importantly to the development of diabetic retinopathy.

Mechanisms Underlying the Pathogenesis of Other Diabetic Complications

Besides retinopathy, there are also several other well-known diabetic complications, such as microangiopathy/angiopathy, nephropathy, neuropathy, myocardiopathy, and others [6]. In addition, insulin resistance is a common clinical manifestation of type 2 DM. Traditionally, microvascular complications were thought to be an important underlying cause for many of the diabetic complications (except insulin resistance). As already discussed in earlier sections regarding retinopathy, the microvascular changes in the retina likely are the secondary changes in response to neuroretinal damage caused by formic acid (and formaldehyde), which may play a more important role in driving the pathogenic process of diabetic retinopathy. In addition to the neuroretina, the brain, kidney, heart and muscles are other organs or tissues in the body that also have a very high requirement for oxygen and energy supply, and accordingly, these organs are also expected to be highly susceptible to formic acid (and formaldehyde)-induced hypoxic damage. It is suggested that each of these diabetic complications might be viewed as a tissue-specific manifestation of a somewhat similar formic acid (and formaldehyde)-induced pathogenic process. Provided below is a discussion of the potential roles of formic acid and formaldehyde in the pathogenesis of diabetic complications in other vital organs, including the brain, kidney, and heart. In addition, a brief discussion of the potential contributing roles of formic acid and formaldehyde in the development of insulin resistance, diabetic ketoacidosis, cataract and cancer in DM patients is also provided.

Vascular complications

Hyperglycemia-associated vascular complications are usually grouped under “microvascular disease” (microangiopathy, due to damage to small blood vessels) and “macrovascular disease” (angiopathy, due to damage to arteries).

In diabetic microangiopathy, one of the most consistent morphological changes is the diffusive thickening of the capillary basement membrane [349,350]. The thickening is most evident in the capillaries of the retina, renal glomeruli and medulla, skeletal muscles, brain and skin, giving rise to characteristic diabetic microangiopathy in these organs or tissues. Notably, thickening of the basement membrane may also occur in some nonvascular structures such as renal tubules, Bowman’s capsules, peripheral nerves, and placentas. Biochemically, a number of characteristic changes in the basement membrane have been commonly observed in diabetic microangiopathy, which typically include increased levels and synthesis of collagen type IV plus decreases in proteoglycans.

Diabetic macroangiopathy contributes to the pathogenesis of coronary heart disease, peripheral vascular disease, and cerebrovascular disease. Cardiovascular disease remains a major cause of morbidity and mortality worldwide [351,352].

Pathogenic explanation

Traditionally, it has been suggested that increased formation of AGEs under hyperglycemic conditions plays a critical role in the pathogenesis of DM-associated vascular changes [13,14]. However, it should be noted that while increased formation of AGEs contributes to chronic vascular damage in a given organ or tissue, the real extent of its contribution to the overall pathogenic process might be smaller than thought. In STZ-induced DM rodent models, for instance, the animals generally lack advanced clinical characteristics of microvascular complications although their blood glucose level is usually very high, far higher than what is commonly seen in human DM patients. This notion has also been noted in the preceding section regarding diabetic retinopathy.

The vascular endothelial cells in the body are prone to suffer from hyperglycemia-associated cellular damage, because these cells lack the ability to effectively regulate the rate of glucose influx to prevent excessive accumulation of glucose inside the cells [334,353]. When the glucose concentration inside the vascular endothelial cells becomes too high, it may lead to increased formation and accumulation of formic acid and formaldehyde in these cells. While formaldehyde can cause direct damage to vascular endothelial cells through chemical cross-linking, it can also be readily converted to formic acid. Formic acid accumulation in these cells can induce hypoxic cytotoxicity, which is usually coupled with endothelial hyperplasia. It is hypothesized that the cytotoxic effects of formic acid and formaldehyde may jointly contribute to the damages to vascular endothelial cells seen in chronically hyperglycemic patients. Offering support for the above mechanistic explanation, studies have shown that diabetic microvascular abnormalities, including capillary basement membrane thickening and endothelial hyperplasia, are often associated with diminished oxygen tension and particularly hypoxia in vascular endothelial cells [354].

Diabetic neuropathy

Over 50% of DM patients eventually develop neuropathy [355]. Diabetic neuropathy represents a complex group of clinical conditions which include lesions in the somatic and autonomic peripheral nervous systems, the spinal cord [356] and brain [357]. The most common DM neuropathy is the symmetric peripheral neuropathy affecting both motor and sensory nerves of the lower extremities. In advanced stages, diabetic neuropathy may result in nerve fiber deterioration which is characterized by altered sensitivities to vibrations and thermal thresholds and ultimately progresses to loss of sensory perception. Pathologically, it is characterized by Schwann cell injury, myelin degeneration, and axonal damage.

Besides peripheral nerve injury, people with DM often also have neurocognitive changes that include declines in cognitive function together with impairment of psychomotor speed, cognitive flexibility, attention, visual perception, sexual function and others [358,359]. In addition to the neuro-functional changes, advanced DM patients usually also have pathological degenerative changes in the brain and other parts of the nervous system [360–365].

Pathogenic explanation

The exact cause and mechanism of diabetic neuropathy is still poorly understood. Traditionally, diabetic neuropathy was considered to be closely associated with microvascular abnormalities, such as capillary basement membrane thickening and vascular endothelial hyperplasia. This view, however, has been challenged by some investigators because there is evidence showing that diabetic neuropathy preferentially targets sensory and autonomic neurons over motor neurons, initially with little or no vascular involvement. For instance, the loss of epidermal and corneal innervation was observed in DM patients before overt vascular changes were detected [366–370].

It is of note that diabetic neuropathy usually develops in an axon length-dependent manner, *i.e.*, it first develops in long peripheral nerve fibers, and then it progresses in a distal-to-proximal manner. This feature suggests that the neuronal damage is initiated in the distal axons, such as those at the finger or toe tips. The axons are known to contain a high density of mitochondria, which are required for neural functions (such as neurotransmission) by providing the necessary ATP. However, for long nerve fibers, it would be more difficult for the nerve terminals to effectively repair and recover from chemically-induced hypoxic damage, as they are farther away from the neuronal cell body and it would be more difficult to transport the required elements from the neuronal cell body to the nerve endings. Moreover, the distal parts of a long axon (such as those at the finger and toe tips) usually have very poor blood circulation and oxygen/nutrient supply compared to the proximal regions of an axon. It is, therefore, hypothesized that a combination of the above two factors would predispose the distal parts of a long axon (usually of a large neuron) particularly vulnerable to hyperglycemia-associated, formic acid (and formaldehyde)-induced inhibition of their mitochondrial respiration [371], which may result in reduced ATP production, mitochondrial ROS accumulation, and mitochondrial dysfunction [372,373].

Notably, the mitochondria-produced superoxide is normally removed by cellular detoxification agents such as superoxide dismutase, catalase, and glutathione [373]. Hyperglycemia-associated, formic acid-induced over-production of superoxide may lead to inhibition of the GAPDH activity and subsequently the glycolytic pathway, thus further aggravating cellular ATP deficiency. Moreover, suppression of the glycolytic pathway would result in accumulation of the upstream glycolytic intermediates, which would then activate other glucose metabolic pathways (Table 1 and Figure 3). These metabolic derangements would further aggravate hyperglycemia-associated neural injury.

At late-stages of DM, it is expected that excessive superoxide production as a result of hyperglycemia-associated formic acid and formaldehyde accumulation eventually would overload the antioxidant capacity of neuronal cells and their nerve fibers, which would exacerbate mitochondrial dysfunction and cellular ATP deficiency. These changes, coupled with the naturally low blood flow to the distal regions of the nerve fibers, would damage the distal axons, and over time, it might induce the characteristic pathological process of the distal axons gradually dying back toward their neuronal cell bodies [374,375].

It is of note that in DM rodent models, the animals only display rather mild complications in the nervous system, which mostly include segmental demyelination accompanied with axon and fiber loss [376]. A longer duration of DM appears to precipitate discernible nerve pathology in some diabetic rodent models [377-380], with partial loss of skin sensory fiber terminals [359,360] in addition to sensory loss. The relatively short life span of rodents and their

physically-shorter axons compared to humans may be the partial reasons for the lack of severe degenerative neuropathy in DM rodent models. However, it should also be noted that the insensitivity of rodents to the toxicities of methanol and its toxic one-carbon metabolites may be another, pherhaps more important, underlying factor for the apparent lack of severe diabetic neuropathy in DM rodent models.

Compared to astrocytes, neurons are more vulnerable to formic acid-mediated inhibition of mitochondrial respiration and ATP depletion because neurons are unable to increase glycolysis to supply ATP due to the lack of activity of the glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (isoform 3). In comparison, astrocytes, which are apt to utilize glycolytically-produced ATP to maintain their mitochondrial membrane potential, are expected to be more resistant to the damaging effect of formic acid in the mitochondria.

In addition to formic acid, it is postulated that increased tissue levels of formaldehyde in DM patients may also play an important role in mediating diabetic neuropathy. In partial support of this hypothesis, many earlier studies have shown that elevated levels of formaldehyde in certain brain regions is closely associated with neurodegeneration in animal models [54,55,62,112,113,157,158] as well as in human subjects [55,110,159,160]. Interestingly, there were also studies reporting an association of the increased expression/activity of the formaldehyde-generating enzymes with human DM [101,162,163,381,382].

In summary, formic acid (along with formaldehyde)-induced production and accumulation of mitochondrial ROS may drive a feed-forward cycle in which oxidative stress impairs cellular antioxidative defense mechanisms, which then leads to progressive nerve fiber dysfunction, damage, and loss in diabetic neuropathy. At late stages of peripheral neuropathy, severe ATP deficiency would ensue, and this would lead to injuries to the peripheral nerve fibers, which is usually accompanied with axons dying back toward their cell bodies [374,375].

Diabetic nephropathy

The kidneys are among the most severely-damaged organs in advanced DM patients. Renal failure as a result of long-lasting diabetic nephropathy accounts for many DM-associated deaths in both juveniles and adults [383]. Diabetic nephropathy is characterized clinically by the development of proteinuria (mostly albuminuria) with a subsequent decline in glomerular filtration rate, which progresses over a long period of time, usually over 10–20 years. Importantly, diabetic kidney disease is also a major risk factor for the development of macrovascular complications such as heart attacks and strokes [384].

The pathogenesis of diabetic nephropathy is highly complex given the diversity of cell populations present in the kidney and the various physiological functions this organ performs. It is commonly accepted that hyperglycemia can induce certain cellular changes, which then lead to functional changes in various resident cells in the kidney, such as endothelial cells, podocytes, mesangial cells, cells of the tubular system and inflammatory cells, and these changes jointly contribute to the pathogenesis of diabetic nephropathy.

Pathogenic explanation

The kidney is among the most highly-perfused organs in the body and yet it is still highly susceptible to ischemia or hypoxia [385] because the renal tubular cells are mitochondria-rich and have very high ATP requirement for normal functioning. In fact, it has been suggested that the kidney is nearly always on the verge of hypoxia even under normal conditions [134,385]. In line with this suggestion, the kidney is known to be highly susceptible to ischemia/hypoxia-induced acute and chronic injuries [385]. It is hypothesized that diabetic nephropathy (diabetic kidney disease) can be viewed as the renal manifestation of a similar hyperglycemia-associated, formic acid/formaldehyde-dominated pathogenic process. This general hypothesis is partially supported by earlier observations indicating that dysfunctional renal mitochondria are key pathological mediators of diabetic nephropathy [386]. Furthermore, some of the pathological characteristics of formic acid-induced kidney injuries closely resemble those of the diabetic nephropathy. A discussion of the proposed formaldehyde/formic acid-dominated changes in renal cellular and non-cellular structures as well as functions is provided below.

Changes in glomerular basement membrane and capillary functions. The glomerular basement membrane (GBM), a thin (250–400 nm) meshwork of extracellular matrix proteins, is an integral part of the glomerular filtration barrier. GBM has a similar composition as other basement membranes [387], which are synthesized jointly by podocytes and endothelial cells [388]. GBM is mostly situated between glomerular endothelial cells and podocytes as part of the peripheral capillary wall [389], and serves as a size- and charge-selective filtration barrier for circulating proteins [390–392].

One of the earliest and most characteristic glomerular changes in DM is the thickening of GBM [393,394], and more-pronounced changes are observed later when diabetic kidney disease becomes more evident [395]. It is hypothesized that the increased formation of formaldehyde in hyperglycemic patients would markedly increase the cross-linking of GBM proteins, in addition to the formation of AGEs. It is of note that GBM is the region in the body which has the highest perfusion rate and thus would have the highest exposure to all water-soluble small molecules in circulation, including glucose, formaldehyde and formic acid. Extensive cross-linking of GBM compositions by formaldehyde, in addition to covalent modifications by glucose, would contribute to GBM thickening (due to reduced degradation of the cross-linked protein components) as well as changes in GBM charge or architecture, both of which would contribute partially to the development of albuminuria in DM patients.

It is known that the GBM plays a pivotal role in maintaining the glomerular tuft and filtration function. Abnormal thickening and stiffening of GBM as a result of extensive cross-linking of the GBM proteins by formaldehyde and the covalent modifications by sugar molecules are expected to reduce the distensibility of the pericapillary walls, thus facilitating glomerular injury through hemodynamic mechanisms [396].

Changes in glomerular cellular components. It has been suggested that the early pathogenic changes in renal glomerular cells are critical for the subsequent development of kidney damage, such as glomerulosclerosis and nephron dropout. The renal glomerulus mostly consists of glomerular endothelial cells, podocytes and mesangial cells, and these three types of cells cooperate with each other to carry out effective glomerular filtration. Podocytes are a group of highly-specialized, terminally-differentiated cells that cover the urinary side of the GBM. Intercellular signaling between glomerular endothelial cells and podocytes has been well established [397]. These two groups of cells work jointly for the maintenance of GBM and its charge barrier as well as the shape and

integrity of the glomerular capillaries [398].

In diabetic glomerulus, it is hypothesized that the glomerular endothelial cells and podocytes are each chronically exposed to elevated levels of formic acid and formaldehyde, and as a result, these cells are chronically under severe hypoxic conditions, which would compromise their regular functions. It is further hypothesized that chronic exposure to elevated levels of formic acid (and formaldehyde) may be partly responsible for the induction of a number of patho-adaptive changes in podocytes, such as reduced motility and hypertrophy [399–401]. These changes in podocytes are an important contributing factor in the development of diabetic nephropathy. In support of this suggestion, an earlier study showed that podocyte-specific injuries alone can induce DM-like phenotypes of glomerulosclerosis and tubulointerstitial fibrosis, even in the absence of hyperglycemia [402].

In addition to endothelial cells and podocytes, the mesangial cells are another major group of cells in the glomerulus. These cells are responsible for constructing and maintaining the architecture of the glomerular capillary network through production of the mesangial matrix. These cells also regulate the functions of podocytes to affect glomerular filtration [403]. It is hypothesized that in DM patients, chronic hyperglycemia would lead to chronic injury to mesangial cells through formation and accumulation of formic acid/formaldehyde in these cells, which would then lead to mesangiolysis (focal degeneration of mesangial cells and the mesangial matrix) and the development of abnormal glomerular capillary structures as well as compensatory mesangial expansion (an increase in the fractional volume of the glomerulus occupied by the mesangium). This hypothesis is partially supported by the following experimental observations: First, selective injury to mesangial cells can lead to enlarged glomerular capillary loops, including balloon-like glomeruli (formed by the fusion of glomerular capillaries), as commonly seen in diabetic nephropathy [404]. Second, mesangial cell injury can alter the functions of podocytes and affect glomerular filtration by inducing a change in the phosphorylation of slit diaphragm proteins [403]. Third, mesangial cell injury can stimulate compensatory cell growth of its own along with increased synthesis of mesangial matrix, jointly contributing to mesangial expansion [405,406].

Glomerular hypertrophy and proximal tubular changes. The early-stage diabetic kidney undergoes significant hypertrophy within the glomeruli, which is accompanied by thickening of GBM and mesangial expansion [407]. The main cellular constituents in glomerular mesangium are mesangial cells, which account for approximately 30–40% of the total cells in the glomerulus. Earlier studies showed that under hyperglycemic conditions, mesangial cells underwent proliferation and hypertrophy along with increased production of matrix proteins [408]. Mechanistically, it is hypothesized that mesangial hypertrophy in diabetic patients is mostly due to hyperglycemia-associated, formic acid/formaldehyde-induced hypoxic damage to glomerular mesangial cells, which causes focal mesangiolysis [409], followed by compensatory over-growth of mesangial cells and increased production of extracellular matrix, leading to mesangial expansion.

It is of note that mesangial expansion would reduce the total glomerular capillary surface area and consequently, reduce glomerular filtration [410]. When glomerular filtration is significantly compromised as a result of mesangial expansion, it is expected that compensatory intra-glomerular hypertension (and sometimes systemic hypertension as well) may ensue in some DM patients, which may hasten the progression toward the late-stage diabetic nephropathy depending on the severity of the compensatory hypertension. In partial support of this notion, some studies have indicated that there was a clear link between mesangial matrix expansion and the progression toward glomerulosclerosis [393,394,411,412].

In addition to the glomeruli, the renal tubules are also adversely affected by DM. It is known that DM can cause significant growth and enlargement of renal proximal tubules at relatively early stages of diabetic nephropathy [413,414]. The following two reasons may jointly contribute to the pathogenic change. First, it is known that the proximal tubular cells have a very high metabolic rate and energy requirement imposed by the active reabsorption of a number of glomerular filtrates at this site. Coupled with the metabolic needs, the proximal tubular cells also contain a high density of mitochondria for the oxidative phosphorylation of glucose for ATP synthesis. However, the cortical region of the kidney has a relatively limited oxygen delivery due to the high density of peritubular capillaries. These factors jointly make the proximal tubular cells particularly vulnerable to hypoxia caused by formic acid (along with formaldehyde), which would cause varying degrees of ATP deficiency in these cells. Second, the proximal tubules are actively involved in ATP-dependent reabsorption of a number of filtrates. For instance, it is known that a fraction (10–15%) of calcium transport which is regulated by parathyroid hormone (PTH) and calcitonin is carried out by an ATP-dependent active transport system. An earlier study showed that this active transport system could be readily inhibited by formic acid in proximal tubules [143]. Based on this information, it is, therefore, hypothesized that the hyperglycemia-associated, formic acid-induced ATP deficiency in proximal tubular cells would cause significant functional inadequacy in the active reabsorption of glomerular filtrates at this site. This functional inadequacy would then lead to compensatory proliferation of the proximal tubular epithelial cells through feedback regulations.

As the tubules grow, it is very likely that more of the glomerular filtrates (including sodium and glucose) are reabsorbed in a passive, energy-independent manner, and consequently, sodium delivery to the *macula densa* is reduced and tubule-glomerular feedback is activated [415], which would lead to increased intra-glomerular pressure and hyperfiltration to increase the glomerular filtration rate (GFR) [415]. These compensatory changes usually occur at relatively early stages of diabetic nephropathy. As DM advances, chronic renal exposure to elevated levels of formic acid and formaldehyde is expected to cause progressive and cumulative injuries to tubular epithelial cells, even cell death. These changes eventually would result in atrophic or dilated tubules, and in some cases, disappearance of tubules [416]. These tubular dysfunctions would contribute to the worsening of albuminuria [417]. In addition, these pathogenic tubular changes are usually accompanied by progressive tubulointerstitial fibrosis, which is considered the 'final common pathway' for loss of renal function in diabetic kidney disease [418].

In partial support of the above explanation, it is of interest to note that an earlier study [419] showed that when rats were chronically administered with trichloroethanol (a major metabolite of trichloroethylene), the animals excreted large amounts of formic acid in their urine. These animals developed kidney lesions that shared characteristic changes of diabetic nephropathy, such as protein

excretion, hypoxic tubular damage along with compensatory tubular epithelial over-growth, and focal formation of abnormal tubules [419]. The pathological similarities between trichloroethanol-induced kidney damage and diabetic nephropathy offer further support for the proposed MFF hypothesis on the pathogenesis of diabetic nephropathy.

In addition to renal proximal tubules, other renal tubular cells are also affected, to varying degrees, in DM patients. It is expected that the degree and extent of pathogenic changes in other tubular cells would be jointly determined by a number of factors, such as the levels of the intracellular formic acid and formaldehyde, the cellular mitochondrial density and the level of cellular demand for oxygen and energy supplies.

Renal functional changes. Changes in hemodynamics, associated with intraglomerular and/or systemic blood pressure increase, have been reported to occur early in DM and are characterized by glomerular hyperfiltration. Glomerular hyperfiltration was widely viewed as a significant contributing factor to the damage to glomeruli and preglomerular vessels [420]. It is hypothesized that these hemodynamic changes take place largely in response to accumulation of formic acid (along with formaldehyde) in renal tubular cells, which causes hypoxia and ATP deficiency in these cells. As a result, through feedback regulations of hemodynamic changes, the intra-glomerular pressure and sometimes systematic blood pressure would be increased. It is assumed that these compensatory changes in pressure likely are intended to improve the hypoxic conditions of many renal cells (tubular cells in particular). Providing partial support for this suggestion, there were clinical data suggesting that DM individuals who maintained normal glomerular filtration or hyperfiltration are often better protected against the progression to end-stage diabetic kidney disease [421]. However, it should also be noted that the increase in the glomerular filtration pressure could also become detrimental if it is abnormally elevated to too high levels.

Proteinuria, with the protein albumin as a major component, often is a clinical manifestation of the disruption of the integrity of the glomerular filtration barrier, which jointly involves pathological changes in the structure and function of GBM and the functions of glomerular epithelial cells and podocytes. In addition, degenerative changes of the renal tubules also contribute importantly to the worsening of proteinuria in late-stage diabetic nephropathy.

Involvement of inflammatory cells. It is hypothesized that the flux of inflammatory cells into the diabetic kidney is mostly due to formic acid/formaldehyde-induced renal hypoxic damage and cell death. These inflammatory responses are generally believed to serve as an important mediator of late-stage diabetic nephropathy [422,423]. In partial support of this general concept, animal studies have shown that inhibition of leukocyte recruitment and accumulation in diabetic kidney can help slow down the progressive renal damage [424,425], including alleviating albuminuria.

Observations made in diabetic rodent models. Results from animal studies have also offered some support for the mechanistic explanation based on the MFF hypothesis. In the most widely-used rodent DM models induced by multiple low-dose STZ injections, animals displayed a range of early functional and structural changes reminiscent of human diabetic nephropathy [426,427]. These included kidney hypertrophy, elevations in glomerular filtration, progressive leeching of albumin (albuminuria) and proteins into the urine, and ultrastructural changes such as GBM thickening and mesangial expansion. However, the STZ-induced DM in rodents usually did not progress to more advanced renal disease commonly seen in humans which is characterized by a loss of glomerular filtration, overt proteinuria, and advanced structural lesions [426,427]. This may be partly attributed to the fact that rodents are generally insensitive to the toxicities of metabolically-formed endogenous methanol and its toxic one-carbon metabolites as these chemicals would not readily accumulate in rodents.

In summary, diabetic nephropathy can be viewed as the renal manifestation of a similar hyperglycemia-associated, formic acid/formaldehyde-dominated pathogenic process. It is hypothesized that the formic acid/formaldehyde-induced chronic renal hypoxia and ATP deficiency would induce a number of compensatory, but ultimately maladaptive, changes in the kidney [428–430], eventually leading to the pathogenesis of diabetic nephropathy. In partial support of the proposed pathogenic explanation of diabetic nephropathy, it should be noted that earlier studies have already shown that the kidneys are vulnerable to formic acid-induced damage [31,122,431,432]. Pathological analysis revealed that the renal proximal tubular cells of rats underwent hypoxic edematous degeneration after the animals were exposed to formaldehyde or formic acid [144]. These changes are characteristic of severe hypoxia and ATP deficiency, and are also similar to tubular cell injuries commonly seen in diabetic nephropathy.

Here it is of note that while poor glycemic control [433] and hypertension [434] frequently precede overt diabetic nephropathy, there are also a subset of patients who develop nephropathy despite good glycemic control [435] and normal blood pressure. This discrepancy underscores the relative importance of other pathogenic factors. Based on the proposed MFF hypothesis, it is likely that the endogenous formation and accumulation of formic acid and formaldehyde in DM patients is a more important determinant in diabetic kidney disease, which, sometimes, may overwrite the influence of other modulating factors, such as glycemic control and blood pressure management.

Diabetic cardiomyopathy

Diabetic cardiomyopathy often refers to hyperglycemia-associated direct damage to the myocardium in DM patients in the absence of hypertension and coronary artery disease [436,437]. A direct damaging effect of DM to the heart was first proposed in 1972 according to postmortem findings of heart failure in DM patients free of coronary artery disease or other known cardiac risk factors [437]. Additional studies reporting similar findings offered strong additional support for the existence of a specific cardiomyopathy with origins in diabetic heart muscle cells [438,439].

Clinically, cardiomyopathy is characterized by diastolic dysfunction [440], which is an apparent inability of the heart muscle to undergo adequate relaxation and then refilling during the diastolic part of a normal cardiac contract–relaxation cycle. Diastolic dysfunction actually is a common clinical manifestation, observed in up to 40–60% of all heart failure cases [441-443], with diabetic individuals over-represented [444].

Pathogenic explanation

The pathogenic mechanism of diabetic cardiomyopathy is still not quite understood. It has been suggested that diastolic dysfunction may be the result of a number of pathological processes, including stiffening of the myocardium, hypertrophy of cardiac muscle, and neuronal abnormalities [433]. At the cellular level, it has been

postulated that oxidative stress of cardiomyocytes is an important pathogenic factor [445,446]. According to the MFF hypothesis, it is suggested that DM-associated hyperglycemia will lead to elevated levels of formic acid in circulation as well as in cardiomyocytes, which then impairs cadiomyocyte energy metabolism and eventually leads to cardiac ATP deficiency and dysfunction. It is understood that under normal conditions, cardiac muscle cells prefer to use fatty acids over glucose as the energy source (although they can also use glucose for ATP production) [446]. Elevated levels of formic acid in cardiomyocytes will cause the same inhibition of the mitochondrial ATP synthesis as in other cells. Because of the unique physiological functions the heart performs, the cardiac muscle cells are equipped with a very high density of mitochondria and require high levels of oxygen and energy supply nearly all the time. It is postulated that the impaired mitochondrial ATP production in diabetic cardiomyocytes due to the presence of elevated levels of formic acid would lead to chronic ATP deficiency, which is believed to be an important cause for diastolic dysfunction in DM patients. In addition, the formic acid-induced severe hypoxia in cardiomyocytes is expected to increase cellular ROS levels (*i.e.*, oxidative stress) and calcium levels [183], both of which would also contribute to the development of diastolic dysfunction.

Aldehyde dehydrogenase 2 (ALDH2) is a non-cytochrome P450 mitochondrial aldehyde oxidizing enzyme, and is best known for its role in the metabolism of aldehydes, including formaldehyde (Figure 1). Surprisingly, earlier studies have shown that ALDH2-deficiency rendered the mice highly susceptible to develop STZ-induced diastolic dysfunction [447]. These animals showed a characteristic reduced energy reserve (phosphocreatine/adenosine triphosphate ratio) [447]. It is hypothesized that the reduced ATP reserve is due to the fact that deficiency of mitochondrial ALDH2 would slow down the metabolic conversion of formaldehyde to formic acid inside the mitochondria, thus faciliating mitochondrial accumulation of formaldehyde. Formaldehyde usually has a far shorter half-life and does not accumulate inside the cells or cellular organelles such as the mitochondria, but when it does accumulate, formaldehyde would be far more cytotoxic than formic acid because of its cross-linking ability, which can covalently damage various mitochondrial and cellular proteins and also cause increased stiffening of cardiac muscle cells. In addition, formaldehyde can be readily converted to formic acid. It is expected that the elevated levels of formaldehyde (a part of it is formed inside the mitochondria) and formic acid (formed mostly in the cytosolic compartment) may jointly pose an inhibition of the mitochondrial ATP synthesis, contributing to the pathogenesis of diastolic dysfunction. Here it is of note that patients with ALDH2 mutations were also reported to be associated with more severe diastolic dysfunction [448]. Taken together, these results suggest that ATP deficiency in cardiomyocytes resulting from formic acid/formaldehyde-induced inhibition of the mitochondrial oxidative phosphorylation is an important cause for diastolic dysfunction in DM patients.

Lastly, it is of note that, in addition to formic acid/formaldehyde-induced ATP deficiency in diabetic cardiomyocytes, diastolic dysfunction is also known to be associated with the stiffening of the cardiomyocytes. It is hypothesized that the hyperglycemia-associated formation of formaldehyde in DM patients, which can cause extensive cross-linking of proteins inside and outside cardiac muscle cells, might cause stiffening of the cardiac muscle fibers and thus partially contribute to the development of diastolic dysfunction in DM patients, in particular in those patients with ALDH2 mutations. In this context, it is of note that there are ~560 million East Asians who carry a common ALDH2-deficient variant (which causes the well-known alcohol flushing syndrome); it will be of interest to determine whether diabetic patients carrying an ALDH2-deficient variant are more susceptible to develop diastolic dysfunction.

Insulin resistance

One of the most important functions of insulin in the body is to regulate blood glucose levels through stimulation of glucose uptake into major storage organs or tissues, such as the liver, skeletal muscle and fat. The main regulator of insulin secretion is the plasma glucose levels. Under normal conditions, higher plasma levels of glucose, whether from exogenous (dietary) or endogenous (through hepatic glucose production) sources, would usually stimulate more insulin secretion, assuming that the insulin-producing islet β cells are functionally preserved. However, the ability of insulin to stimulate glucose uptake may vary widely under different conditions. In DM (in particular type 2 DM), the patients often experience a markedly reduced sensitivity to insulin's actions, which is commonly referred to as "insulin resistance" [449]. From a mechanistic point of view, the degree of insulin resistance is closely associated with the severity of type 2 DM. Provided below is a brief explanation of the pathogenic processes leading to the development of insulin resistance, with a focus on the potential roles of the toxic one-carbon metabolites.

Pathogenic explanation

The major insulin-responsive tissues related to glucose metabolism are the liver, skeletal muscle and fat tissue. In a healthy individual under fasting conditions, the basal insulin levels are usually low, and the muscle uptake of glucose from the blood would be at a basal minimal level. Through feedback regulations (such as the release of glucagon), the adipose tissue would be activated to provide free fatty acids via lipolysis for hepatic glucose production, which helps maintain euglycemia and provide feul for important glucose-consuming cell types, such as neurons, red blood cells and renal medullary cells [450]. In comparison, when the plasma insulin level is elevated (such as shortly after a meal), the adipocyte lipolysis and hepatic glucose production are suppressed, whereas muscle glucose uptake and lipogenesis are stimulated. These effects of insulin are partly achieved by suppressing gluconeogenesis and glycogen breakdown in the liver and by activating the activity of the glucose transporter-4 in the muscle.

From a more systemic point of views, the development of insulin resistance and type 2 DM is caused by the following two closely-related pathogenic processes. First, the combination of the daily excessive calorie intake (*i.e.*, eating too much food, in particular carbohydrates and fats) and a lack of adequate physical activities (*i.e.*, energy expenditures) constitutes the initial main cause for the development of insulin resistance and type 2 DM. When a healthy individual has an excessive calorie intake during a meal, it is certain that a higher plasma insulin level would be needed in order to exert a stronger stimulation of the liver, muscle and adipose tissues to uptake more sugar molecules into these storage sites. If this type of excessive calorie intake is only an occasional event, it is believed that the body could quite readily and effectively deal with the situation with no major long-term negative consequences. However, if an initially-healthy individual always has a daily calorie intake that exceeds (or far exceeds) the actual needs of the body, then it is

expected that the islet β cells would always need to secrete more insulin after each meal in order to stimulate the storage sites (*i.e.*, the liver, muscle and adipose tissues) to uptake more sugar molecules. It is not difficult to understand that if this situation continues day after day for some time, not long the body would no longer have too much additional storage capacity left to handle the excess sugar molecules that are taken up into the circulation after each meal. Under this situation, the much-elevated plasma glucose levels might cause a stronger stimulation of the islet β cells, and more insulin molecules might be released into the circulation. However, due to the limited (or lack of) storage capacity left in the storage sites (*i.e.*, the liver, muscle and adipose tissues), even when much higher levels of insulin are present, these tissues may still not be able to respond to insulin's stimulation with the same sensitivity and intensity as they did initially, thus gradually manifesting the characteristics of insulin resistance. If this vicious circle of excessive calorie intake–hyperglycemia–hyperinsulinemia continues, understandably not long the excess sugar molecules present in circulation may not have any good place to go in the body, except being filtered out by the renal glomeruli and passively released into urine for disposal, which would be a characteristic clinical manifestation of type 2 DM. Therefore, the occurrence of type 2 DM is almost always intimately coupled with the development of insulin resistance.

In addition to the excessive calorie intake coupled with low energy expenditures, it is hypothesized that the increased formation of formic acid and formaldehyde is another important contributing factor in the development of insulin resistance. According to the MFF hypothesis, hyperglycemia in diabetic patients is associated with increases in cellular glucose levels, which would lead to increased formation of endogenous methanol and/or formaldehyde, both of which are then metabolically converted to formic acid. Formic acid and formaldehyde are inhibitors of mitochondrial cytochrome oxidase, capable of inducing histotoxic hypoxia. Mitochondrial inhibition by formic acid and formaldehyde would lead to reduced cellular ATP levels, and as such, the cells (in particular brain cells) would falsely "sense" a lack of cellular fuel (glucose) supply. Therefore, through feedback regulations (although the mechanism is still not completely clear), the body likely would stimulate the release of counter-regulatory hormones (such as glucagon and catecholamines) and increase hepatic gluconeogenesis and simultaneously suppress the insulin-stimulated glucose uptake by the liver, muscle and adipose tissues. These effects would jointly lead to higher blood glucose levels, thereby promoting the situation of insulin resistance.

In addition, the inhibition of the mitochondrial respiration by formic acid and formaldehyde in pancreatic islet β cells would result in a reduction in the ATP/ADP ratio, which is expected to decrease the sensitivity of islet β cells to postprandial glucose surge-induced insulin secretion. This effect would cause further increases in plasma glucose levels, accompanied by a reduced rate of insulin release by the islet β cells. The change in sensitivity of islet β cells to glucose may also partially explain the marked reduction in insulin secretion when type 2 DM progresses to late stages. The above explanation is supported by studies showing that there is a severe inability of islet β cells to compensate for the reduced insulin activity in insulin-resistant DM patients [451].

As discussed earlier, excess daily intake of carbohydrates is an important causal factor for the development of insulin resistance and type 2 DM. Similarly, it is also readily understood that obesity is a strong risk factor for type 2 DM because it is expected that a higher percentage of the obese individuals will have an excessive daily calorie intake compared to the normal-weight individuals. Now let's look at a more complex hypothetical scenario here: Assume that two initially-healthy normal adult males (with the same starting body weight and age) are being placed on the same carbohydrate-rich meal plan which provides excessive daily calorie that far exceeds what the body actually needs to maintain its normal functions. Also, we assume that these two individuals will keep the same daily physical activities (*i.e.*, the same level of energy expenditure). After one year, one person becomes obese (*i.e.*, gained a lot of weight), whereas the other person remains lean-looking (*i.e.*, with very little weight gain). Undoubtedly, if these two individuals continue with their daily excessive calorie intake, both would, sooner or later, develop insulin resistance and type 2 DM. Theoretically, it is expected that the obese individual likely would have a significantly slower initial progression of his diabetic conditions compared to the lean-looking individual. The main reason is because the fat tissue in the obese person has a far greater storage capacity (*i.e.*, "the cushion effect") for the excess carbohydrates that are being absorbed and present in circulation after every meal. In comparison, under the same conditions, the lean-looking person is expected to develop DM more quickly, and the disease conditions would be more severe as most of the excess glucose molecules would have no place to go except staying in the circulation and being filtered through the glomeruli and removed from the body through urine. Also, it is expected that a higher fraction of the excess free glucose molecules would be enzymatically converted into the toxic one-carbon metabolites in the body, thereby accelerating the development of insulin resistance as well as various diabetic complications.

During the development of insulin resistance, the sensitivity to insulin's action may vary considerably in different insulin-responsive organs in a DM patient. For example, if there is an increased insulin resistance in skeletal muscle, greater insulin concentrations will be needed to induce muscle glucose uptake. As such, it would result in relative hyperinsulinemia to which all other tissues and organs in the body will also be exposed, and some of the metabolic/signaling pathways regulated by insulin but are not related to glucose homeostasis would be activated in excess, which may have pathogenic consequences. For example, exposure of certain brain regions to elevated insulin levels results in increased sympathetic discharge, usually manifesting in the form of elevated blood pressure [452,453]. Similarly, in the liver, elevated insulin levels, would excessively activate the insulin-responsive lipogenesis pathway (which is still sensitive to insulin), resulting in increased production of VLDL and reduced production HDL and manifesting as increased plasma triglycerides and reduced HDL-cholesterol levels [454,455].

It is generally accepted that diabetic patients with significant insulin resistance would be predisposed to the development of a cluster of abnormalities, including varying degrees of glucose intolerance, increased plasma triglyceride levels and decreased high-density lipoprotein cholesterol levels, elevated blood pressure, hyperuricemia, and others. The cluster of changes associated with insulin resistance is usually referred to as syndrome X, which is closely associated with increased risk of coronary heart disease [456].

The non-nutritive sweeteners (such as aspartame) which have

zero-to-negligible caloric load have been suggested for clinical use in the obese and diabetic individuals to aid in controlling their carbohydrate intake and blood glucose levels. However, a number of clinical studies have led to the suggestion that the intake of the non-nutritive sweeteners, aspartame in particular, may actually cause impaired blood glucose tolerance in DM patients, often accompanied by increased risk of weight gain rather than weight loss [457,458]. This intriguing clinical finding is actually not surprising in the light of the proposed MFF hypothesis. It is known that aspartame can be partially converted into methanol (about 10%) in humans [46–48,459]. Therefore, long-term intake of aspartame would significantly increase the formation of methanol together with formaldehyde and formic acid in the body [460]. Increased levels of these toxic one-carbon metabolites would worsen the situation of hyperglycemia and insulin resistance. Regarding the potential mechanism underlying the increased risk for weight gain in patients chronically receiving aspartame, it is likely due to an increased appetite stemming from the reduced intracellular fuel source and especially ATP levels.

Lastly, it should also be noted that metformin, the most commonly-prescribed first-line therapy for type 2 DM worldwide, was reported to decrease hepatic glucose output and increase the body's sensitivity to insulin [461]. It is speculated that some of the beneficial effects of metformin in DM may be related to its ability to reduce the endogenous formation and/or accumulation of the toxic one-carbon metabolites. This intriguing possibility merits experimental testing in the future.

Diabetic ketoacidosis

Diabetic ketoacidosis is one of the most serious and potentially life-threatening complications of DM. Diabetic ketoacidosis often results from a severe deficiency of insulin associated with increased secretion of counter-regulatory hormones, such as glucagon, epinephrine, cortisol, and growth hormone. Provided below is a discussion of the pathophysiology of diabetic ketoacidosis with a focus on the potential contribution of formic acid/formaldehyde-induced metabolic changes.

Pathogenic explanation

When glucose is available intracellularly, many types of cells in the body would prefer to use glucose for ATP synthesis, but these cells can also switch to the use of ketones for ATP synthesis during starvation, extensive exercise, or low insulin states. Therefore, under conditions of cellular glucose deficiency, most of the ketone bodies which are produced in liver are transported to extrahepatic tissues (such as the brain, heart and skeletal muscle) for catabolism in their mitochondria to form acetyl-CoA, which then goes into the Kreb's cycle for terminal mitochondrial oxidation and ATP production [462–465].

As aforementioned, diabetic ketoacidosis is triggered by a severe deficiency of insulin plus heightened activity of counter-regulatory hormones. One of the most important functions of insulin is to stimulate the influx of glucose into various cells for use (*i.e.*, metabolic processing) and storage, and during insulin deficiency, many cells in the body would strongly sense a lack of cellular fuel (glucose) for ATP synthesis. As a result, the vital organs of the body (such as the brain and heart) likely would, through feedback regulations, activate the secretion of counter-regulatory hormones, especially glucagon and epinephrine. These hormones would lead to strong fat mobilization, *i.e.*, activation of the hormone-sensitive lipase in adipose tissue followed by breakdown of triglycerides into glycerol and free fatty acids. In the liver, free fatty acids are catabolized into ketone bodies [466], a process predominantly stimulated by glucagon. Ketogenesis mostly occurs in the mitochondria of liver cells which produces two main ketone bodies: acetoacetate and 3β-hydroxybutyrate from acetyl-CoA. Both chemicals are short-chain four carbon carboxylic acids with high water solubility and can serve as an energy source for tissues such as the brain, kidney, heart, and skeletal muscle [467,468]. Acetoacetate and 3β-hydroxybutyrate are released from the liver in their protonated form, which may cause a reduction in blood pH, *i.e.*, acidosis.

It is hypothesized that diabetic ketoacidosis might have an underlying cause which is the increased formation and accumulation of endogenous methanol and its metabolites under ketotic conditions. Increased levels of formic acid (and formaldehyde) would inhibit the oxidative metabolism of ketone bodies inside the mitochondria for ATP synthesis in various organs and tissues in the body. As a result, the ketone bodies would not be utilized efficiently in catabolism for ATP production, and thus would accumulate in the body, and the state of severe ATP deficiency in many organs and tissues would persist. ATP deficiency would then lead to a vicious cycle of feedback regulations, resulting in even stronger fat mobilization and heightened ketogenesis in the liver, and ultimately more severe ketoacidosis.

The above mechanistic explanation is somewhat consistent with the following two indirect observations: First, an earlier clinical study reported that a patient with type 1 DM would be more readily to develop severe metabolic ketoacidosis if the patient is also intoxicated with methanol [469]. Second, it is known that in STZ-induced DM rodent models, the animals usually develop more severe conditions of hyperglycemia plus insulin deficiency, and yet they usually do not develop the same degree of diabetic ketoacidosis. The reason likely is because rodents generally are insensitive to the toxicities of methanol and its metabolites as these chemicals are rapidly metabolized and do not accumulate in rodents.

In addition to increased fat mobilization which results in ketogenesis and ketoacidosis, insulin deficiency (plus the presence of high levels of counter-regulatory hormones) would also lead to increased hepatic gluconeogenesis and glycogenolysis plus impaired glucose utilization by peripheral tissues. The joint consequence of these metabolic derangements would be a rapid and dangerous surge in blood glucose levels. Hyperglycemia would then lead to glycosuria and osmotic diuresis, subsequently resulting in severe dehydration, hypovolemia and reduction in glomerular filtration, and eventually impeding further glucose excretion [470].

It is of note that there are also cases of ketoacidosis occurring in DM patients without severe hyperglycemia [471–473], commonly referred to as "euglycemic ketoacidosis". In euglycemic ketoacidosis, insulin deficiency and insulin resistance are often milder, thereby limiting the drastic surge in blood glucose levels. Therefore, glucose over-production is lesser than in regular hyperglycemic ketoacidosis. It should be noted, however, while euglycemic ketoacidosis often only displays moderate hyperglycemia, it usually has very severe ketonemia and acidosis. It is speculated that the formic acid levels in DM patients with euglycemic ketoacidosis might actually be higher than in other DM patients, and thus it would produce a stronger inhibition of the mitochondrial function,

and ultimately a more severe state of cellular ATP deficiency. As a result, some of the vital organs in the body (such as the brain) would have a stronger "false sense" of cellular fuel (glucose) deficiency, and thus would, through feedback regulations, more powerfully stimulate the secretion of counter-regulatory hormones, resulting in excessive lipolysis and ketogenesis, and ultimately more severe ketoacidosis, which often becomes life-threatening if the underlying causes for severe cellular ATP deficiency in vital organs of the body persist or get worse.

Cataract

Besides diabetic retinopathy, cataract formation, *i.e.*, the opacification of the eye lens, is another leading cause of human blindness worldwide, accounting for 47.8% of all causes of blindness [474,475]. While age is an important risk factor for cataract formation, DM is also recognized as a significant risk factor [475,476]. A number of studies have shown that glycemic control can significantly improve cataract outcome [477–484].

Cataracts result from the deposition of aggregated proteins in the eye lens and damage to the plasma membrane of lens fiber cells, which cause clouding of the lens, light scattering, and obstruction of vision. It is known that ROS can induce damages in the lens cells, including oxidation of proteins, DNA damage, and/or lipid peroxidation, and these ROS-induced changes have been implicated in cataractogenesis [482–484].

Pathogenic explanation

It is known that more than 95% of oxygen produced during normal metabolism is generated by the electron transport chain in the inner mitochondrial membrane. Research has shown that dysfunctional mitochondria are the primary site of ROS production. Mitochondria are also a major target of ROS-induced cellular injury. It is hypothesized that the increased accumulation of formaldehyde and formic acid in eye lens of DM patients contributes significantly in the pathogenesis of cataract. The locally-formed formaldehyde would pose a more significant pathogenic effect, through cross-linking of the protein components in the eye lens. In addition, the hyperglycemia-associated formic acid accumulation in eye lens cells would increase mitochondrial superoxide production, and the elevated cellular superoxide level is known to cause perturbations in the polyol and oxidative stress pathways as well as in the unfolded protein response. These changes have been suggested earlier to be important contributing factors in the pathogenesis of cataract in advanced DM patients [482–485]. Consistent with the suggested role of ROS in cataract formation, animal and human studies have each shown that supplementation with antioxidants targeting the mitochondrial superoxide is beneficial for the prevention and treatment of cataract.

DM and cancer risk

The association between DM and cancer has recently received considerable attention partly due to the alarming increase in the prevalence of type 2 DM. Many studies have shown that individuals with DM are at a greater risk of developing and dying from multiple types of cancers [486–492]. Mechanistically, a number of potential links between DM and cancer have been proposed. Attention in recent years has been placed on addressing the potential roles of the insulin receptor-mediated signaling, gut microbiomes and AGEs in the pathogenesis of cancer.

Formaldehyde is a strong cross-linking agent, and is known to cause covalent DNA modifications (described earlier). Formaldehyde is also a known mutagen and carcinogen [164–166]. Human epidemiological studies have indicated that there is sufficient evidence for the carcinogenicity of formaldehyde in humans [167–170]. According to the proposed MFF hypothesis, it is postulated that covalent modifications of DNA by formaldehyde may be an important causal factor that increases the risk and formation of different types of cancers in DM patients.

Comparison of the Clinical Treatments for Methanol Toxicity and Diabetic Complications

As briefly discussed below, some of the commonly-used treatments that help mitigate methanol toxicity in humans are also of benefit for improving diabetic complications.

Folate can help reduce both methanol/formic acid toxicity and diabetic complications

Methanol and formic acid intoxication

Folic acid is a necessary cofactor for enzyme-mediated conversion of formic acid to carbon dioxide [493]. During methanol poisoning, folate (a salt form of folic acid) is usually given to methanol-intoxicated patients to enhance the metabolic conversion of formic acid to carbon dioxide and water. Results from animal experiments and clinical case reports all suggest that administration of folate or its derivatives (such as folinic acid, a reduced form of folate) is therapeutically beneficial in mitigating methanol toxicity [494–499]. On the other hand, folate-deficient rats are more susceptible than normal rats to methanol toxicity [189].

Folate administration can also help mitigate the toxicity of formic acid. For instance, in a clinical case, a 36-year-old female intentionally ingested about 250 mL Ataka cleaning fluid (a 44% formic acid solution), which was estimated to contain 110 g of formic acid. Folinic acid therapy was initiated at 1 mg/kg every 4 h for the first 24 h. Serial analysis of formic acid concentrations showed low levels of formic acid during folinic acid treatment but a sharp rise in formic acid concentration was observed when folinic acid therapy was discontinued [499].

Diabetic complications

Nutritional folate deficiency is known to accelerate the progression of DM complications, and the use of folate is beneficial for slowing down the progression of these complications. For instance, it was reported that combined supplementation of folate, B_6 and B_{12} improved retinal edema and light sensitivity in individuals with diabetic retinopathy [500]. In another study, a six-month supplementation of vitamin B_6 showed a decrease in retinal edema and an improvement in light sensitivity in diabetic patients with nonproliferative retinopathy [500]. On the other hand, vitamin B_{12} deficiency was found to be a potential risk factor for other diabetic complications [501].

Here it is of note that earlier clinical studies have shown that joint supplementation of vitamins B_6, B_9 and B_{12} (in the absence of folate or folic acid) which was intended for the reduction of homocysteine levels exacerbated the decline of renal function in DM patients and increased the risk of vascular disease in patients with diabetic nephropathy [502]. Similar observations were also made in another clinical study [503]. It is speculated that the lack of a beneficial effect of vitamins $B_{6/9/12}$ supplementation alone (in the absence of folate or folic acid) might be due to the possibility that some of these vitamins (most likely vitamin B_9) might be involved (likely serving

as an enzyme cofactor) in the metabolic conversion of glucose into methanol and/or its toxic one-carbon metabolites in DM patients. It will be of interest to experimentally examine this intriguing possibility in the future.

Ethanol can help reduce methanol toxicity and diabetic complications

Methanol poisoning

Ethanol has been recognized as an antidote for ethylene glycol poisoning since the 1960's because ADH1 has a higher affinity for ethanol than for ethylene glycol and the presence of ethanol inhibits the conversion of ethylene glycol to its toxic aldehyde. Similarly, ADH1 has a much higher affinity (10 to 20-fold higher) for ethanol than for methanol [504]. Ethanol would competitively inhibit the metabolism of methanol to its toxic metabolite, formaldehyde, by occupying ADH1's substrate-binding site. Therefore, it is readily understood that ethanol, like fomepizole (an ADH inhibitor), can help reduce methanol toxicity. In clinical practice, ethanol is commonly used as an inexpensive, readily-available antidote to delay methanol metabolism until methanol is mostly eliminated from the patient's system either naturally or via dialysis [505,506].

Diabetic complications

There is a large body of epidemiological evidence from cross-sectional studies which indicates that regular mild-to-moderate alcohol intake is associated with improvements in insulin sensitivity and diabetic complications. For instance, Knott *et al*. [507] conducted a dose-response meta-analysis including 38 observational studies. Results showed that reductions in the risk of type 2 DM were observed at all levels (< 63 g/day) of alcohol intake, with a peak risk reduction seen at 10–14 g/day. Similarly, Li and colleagues conducted a meta-analysis of 26 studies on the association between alcohol consumption and the subsequent risk of type 2 DM [508]. A nonlinear relationship between alcohol consumption and the risk of type 2 DM was identified in all cohorts. Light and moderate alcohol consumptions were associated with a reduced risk of type 2 DM, while heavy alcohol consumption had little or no beneficial effect. The mechanism of this phenomenon is not completely understood. A possible explanation is based on the ability of ethanol to inhibit the metabolic conversion of endogenously-formed methanol to formaldehyde and formic acid. It is known that the hepatic ADH1 catalyzes the metabolism of methanol and ethanol to their respective aldehydes (see Figure 1; also discussed in "Metabolic biotransformation of methanol"). It is expected that moderate ethanol consumption can competitively inhibit the conversion of methanol to formaldehyde, thus slowing down the production of endogenous formaldehyde (and also formic acid) in the organs. However, when the alcohol level in the body becomes too high, it might adversely affect the biotransformation of formaldehyde and formic acid, favoring their accumulation in the body.

In summary, while the use of folate, folic acid or folinic acid alone or in combination with vitamins $B_{6/12}$ is beneficial for reducing the complications of human methanol or formic acid intoxication, their use is also similarly beneficial for reducing human diabetic complications. In a similar manner, while alcohol is commonly used as an effective antidote for acute methanol toxicity, clinical studies have shown that long-term moderate alcohol consumption is associated with improved outcomes of diabetic complications. These correlations provide partial support for the proposed hypothesis that increased metabolic formation of endogenous methanol and/or its toxic one-carbon metabolites in chronic DM patients may contribute importantly to the pathogenesis of diabetic complications.

Concluding Remarks

A new hypothesis is proposed to explain the pathogenic mechanism of diabetic complications in humans. It is hypothesized that hyperglycemia-associated increase in cellular glucose levels in DM patients may lead to increased metabolic formation of endogenous methanol and/or formaldehyde, both of which are further metabolically converted into formic acid. Chronic increase in metabolic production and accumulation of formaldehyde and formic acid would jointly drive the pathogenic processes of ocular lesions as well as other complications in DM patients.

As discussed in detail in earlier sections, the proposed MFF hypothesis provides a plausible mechanistic explanation for the pathogenesis of many hyperglycemia-associated complications in humans. In addition, this hypothesis is consistent with many clinical and experimental observations. For instance, methanol is known to have organ- and species-selective toxicities, including the characteristic ocular lesions (including blindness) which are commonly seen in humans and non-human primates, but are not seen in rodents. Similarly, diabetic ocular lesions also have a similar characteristic organ- and species-selective pattern, closely resembling what is seen in methanol toxicity. Moreover, while alcohol consumption or combined use of folate and vitamin $B_{6/12}$ is beneficial for reducing acute methanol/formic acid toxicity in humans, their long-term use is also associated with improved outcomes in human diabetic complications. The pathological and clinical similarities between human methanol toxicity and diabetic complications point to a shared role of these toxic one-carbon metabolites in the pathogenic processes.

In addition to the clinical observations, there is also a large body of supporting evidence from biochemical and cellular studies. Many earlier studies have shown that the mitochondria are an important site of damage in DM patients [509–515]. However, the exact causes for mitochondrial dysfunction and damage are not clear. The proposed MFF hypothesis provides a mechanistic basis for the observed mitochondrial damage and dysfunction in DM patients, which is due to hyperglycemia-associated production and accumulation of the endogenous formaldehyde and formic acid. These toxic one-carbon metabolites would inhibit mitochondrial respiration and ATP synthesis, along with mitochondrial superoxide overproduction.

Superoxide, a by-product of electron transport, is continuously produced in mitochondria. Many earlier studies have suggested that accumulation of mitochondrial superoxide plays a critical role in the pathogenesis of diabetic complications [16,246,516,517]. In addition, there is evidence showing that accumulation of mitochondrial superoxide can subsequently lead to inhibition of GAPDH as well as activation of other pathological pathways, such as protein kinase C, the aldose reductase pathway, and formation of AGEs, all of which were believed to be important contributing factors in the development of diabetic complications [246,518].

According to the proposed MFF hypothesis, hyperglycemia-associated formation and accumulation of formaldehyde and formic acid would impair energy production in many cells of DM patients. Therefore, it is expected that many tissues and cells in the late-stage DM patients would be in a state of severe energy deficiency, and this would be particularly so in those tissues and cells that normally

require a very high level of oxygen and energy supply for maintaining their physiological functions. Indeed, abnormalities in ATP production have also been suggested in many earlier studies as an important contributor to the development of diabetic complications [519–524]. It is hypothesized that the eventual loss of cellular ATP production would be the principal cause for the wide-spread cell death and drastic functional decline often seen in patients with end-stage diabetic complications.

Based on the proposed MFF hypothesis, it is proposed that the testing of blood levels of toxic one-carbon metabolites should also be routinely included for all diabetic patients as these biochemical parameters are more directly related to diabetic injuries to cells and tissues than the traditional parameters of hyperglycemia and H1bAc, and the levels of toxic one-carbon metabolites would also be more directly indicative of the pathogenic process of diabetic complications as well as their progression. It is proposed that the effective strategies for prevention and treatment of human diabetic complications should focus squarely on reducing abnormally-elevated glucose levels in the blood and inside the cells, and on reducing the endogenous production and accumulation of toxic one-carbon metabolites (mostly formaldehyde and formic acid). To achieve these goals, it is critically important to reduce the daily intake of total carbohydrates and fats and simultaneously increase the daily energy expenditure through increased physical activities. The combination of these two basic approaches is believed to be of fundamental importance in helping restore normal insulin sensitivity and glucose homeostasis, which subsequently will help curb and even revert the progression of various human diabetic complications. At present, there are no known drugs that can selectively target the enzymes involved in the endogenous metabolic conversion of excess glucose molecules to toxic one carbon metabolites. Should such selective agents become available some day in the future for clinical use, they may be of considerable benefit to help slow down the development and progression of diabetic complications. Nevertheless, at present, the joint use of folic acid (or folate) and vitamins $B_{6/12}$ is highly recommended as an important part of drug treatment as they can help the human body to more effectively detoxify the toxic one-carbon metabolites. In addition, the use of effective antioxidants that can target cellular and mitochondrial superoxide production and accumulation may also be of some benefit (as part of the adjuvant therapy) to help slow down the progression of diabetic complications.

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Conflict of Interest

The author declares that he has no conflict of interest.

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