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Commentary

Gut Microbiota Metabolite Trimethylamine N-oxide Reduced Pancreatic β Cell Calcium Transients and Function

Lijuan Kong[1-3], Pingping Li[1-3,*]

[1]State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

[2]Diabetes Research Center of Chinese Academy of Medical Sciences, Beijing, China

[3]CAMS Key Laboratory of Molecular Mechanism and Target Discovery of Metabolic Disorders and Tumorigenesis, Beijing, China

[*]Correspondence should be addressed to Pingping Li, lipp@imm.ac.cn

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Abstract

Pancreatic β cell dysfunction is an important cause of type 2 diabetes (T2D). Previous studies show trimethylamine N-oxide (TMAO), a gut microbiota-related metabolite, promotes cardiovascular disease and insulin resistance. However, the effect and mechanism of TMAO at pathological concentrations on β cell function remain unknown. The recently published work demonstrates TMAO reduces pancreatic β cell function and glucose homeostasis. Furthermore, TMAO inhibits calcium transients through NLR family pyrin domain containing 3 (NLRP3) inflammasome activation and induced Serca2 loss. Additionally, long-term TMAO exposure promotes β cell endoplasmic reticulum (ER) stress, dedifferentiation and apoptosis. Here, we review the impaired effect of TMAO on β cell function and comment on the implications of this study for understanding the role and mechanism of TMAO in the development of T2D. Further work should focus on screening flavin-containing monooxygenase 3 (FMO3) inhibitors for antidiabetes.

Keywords: Trimethylamine N-oxide, Pancreatic β cell function, Calcium transients, Serca2, Antidiabetes

Commentary

Diabetes is a chronic metabolic disease that seriously harms human health. The main type of diabetes is type 2 diabetes (T2D). T2D is caused by insulin resistance and insufficient insulin secretion resulting from β cell dysfunction and loss. Pancreatic β cell dysfunction and loss play central roles in the initiation and progression of T2D [1]. A decrease in β cell function is characterized by a decrease in glucose-stimulated insulin secretion (GSIS). However, the molecular pathways involved in β cell dysfunction are not fully understood.

Trimethylamine N-oxide (TMAO) is a gut microbiota-dependent metabolite and is related to several metabolic diseases. The dietary precursors choline, carnitine, and phosphatidylcholine are metabolized by the gut microbiota to the intermediate compound trimethylamine (TMA), which is then oxidized to TMAO by hepatic flavin monooxygenase 3 (FMO3) [2]. Importantly, the end product of this nutrient metabolism pathway, TMAO, is linked to cardiovascular disease (CVD) and can be used to predict CVD risk [3]. TMAO promotes atherosclerosis and enhances platelet hyperreactivity and thrombosis risk by enhancing Ca^{2+} release from platelet intracellular stores [4-6]. High TMAO plasma levels have been linked to diabetes in both mice and humans [7-10]. In mice fed a high-fat diet (HFD), dietary TMAO (~ 17 μM in serum) was shown to exacerbate insulin resistance [11]. The knockdown or inhibition of Fmo3, the TMAO-producing enzyme, prevents insulin resistance in liver insulin receptor knockout insulin-resistant mice and obesity in HFD-fed mice [12,13]. However, the relationship between pathological concentrations of TMAO and β cell dysfunction remains largely unknown.

In this study, the role and mechanism of TMAO in pancreatic β cell function was elucidated [14]. Insulin resistance alone does not lead to hyperglycemia since pancreatic β cells compensate by secreting much more insulin to overcome insulin resistance. It has been reported that β cell function is reduced to 50 to 80% of normal at the time of T2D onset [15]. Therefore, to elucidate the role of TMAO in the pathogenesis of T2D, understanding its effects on β cell function is necessary. This study is the first to show that a pathological dose of TMAO inhibits β cell GSIS and promotes β cell dedifferentiation and apoptosis, revealing a new mechanism for the pathogenesis of diabetes.

In this study, plasma TMAO levels in control and type 2 diabetic mice (*db/db* mice) or subjects were assessed, revealing that T2D was associated with high circulating levels of TMAO. Hepatic Fmo3, a TMAO-producing enzyme, was more highly expressed in *db/db* mice. Furthermore, TMAO at a similar concentration to that found in diabetes, inhibited GSIS in a mouse-derived insulin-secreting β cell line MIN6 cells, in a dose-dependent manner, with no change in cell viability or insulin synthesis. Similar to its effect on MIN6 cells, TMAO also inhibited GSIS in mouse and human primary islets. These results demonstrated that *in vitro*, TMAO reduced the ability of β cells to secrete insulin in response to glucose stimulation. This finding was consistent with several meta-analyses suggesting that elevated TMAO levels are associated with a greater risk of type 2 diabetes [7-9,16-21].

Choline is an important source of TMAO. Therefore, a choline-supplemented chow-based diet (choline diet) is believed to produce more TMAO [3]. For gain-of-function studies, a choline diet was used to increase TMAO levels in C57BL/6J mice. Elevated plasma TMAO levels in mice led to impaired glucose tolerance and decreased insulin and C-peptide levels after intraperitoneal glucose injection. Hyperglycemic clamp confirmed that a choline diet significantly decreased first- and second-phase insulin secretion. The GSIS of isolated primary islets was also inhibited after choline diet feeding. The choline diet impaired islet morphology in mice and reduced the proportion of β cells in the islets and the β cell mass. For loss-of-function studies, genetic knockdown of Fmo3 was performed in choline diet-fed mice to inhibit TMAO production. Fmo3 knockdown increased glucose tolerance and normalized insulin and C-peptide levels after intraperitoneal glucose injection and first-phase insulin secretion in the hyperglycemic clamp test. Staining showed that the proportion of β cells in islets and the β cell mass increased after Fmo3 knockdown. These data showed that TMAO inhibited GSIS *in vivo* and confirmed the *in vitro* results.

Previous research has indicated that mitochondrial respiration and cytosolic calcium transients play key roles in GSIS. Endoplasmic reticulum (ER) calcium release is an important contributor to cytosolic calcium transients and depends on the ER calcium recovery channel Serca and the ER release channels Ip3r and Ryr. The results of this study indicated that TMAO reduced ATP content and the ATP/ADP ratio by inhibiting oxidative phosphorylation and increasing glycolysis and interfered with glucose-stimulated calcium transients. RNA-seq revealed that Serca2 is the key protein involved in TMAO-mediated inhibition of GSIS *in vitro*. qPCR, digital PCR and western blotting confirmed that TMAO reduced Serca2 expression. In the islets of *db/db* mice, the *Serca2* mRNA and protein levels were significantly reduced. TMAO decreased Serca activity and glucose-stimulated ER calcium release, which confirmed that TMAO affects Serca2 function. A Serca2 agonist reversed the TMAO-mediated inhibition of GSIS *in vitro* and *in vivo*. Based on the literature, it seems that TMAO has broad and different effects on specific target cells or tissues. Wang *et al.* suggested that within macrophages, TMAO contributes to the development of atherosclerosis in part by promoting cholesterol accumulation, perhaps by inducing scavenger receptors such as CD36 and SRA1, both of which are involved in the uptake of modified lipoproteins [3]. Zhu *et al.* showed that the direct exposure of platelets to TMAO enhanced submaximal stimulus-dependent platelet activation induced by multiple agonists through augmented Ca^{2+} release from intracellular stores [6]. Chen *et al.* reported that in hepatocytes, the ER stress kinase PERK is the receptor for TMAO and that TMAO selectively activates the PERK branch of the unfolded protein response, induces the transcription factor FoxO1, and leads to insulin resistance [22]. Wu *et al.* revealed that TMAO-induced allogenic graft-versus-host disease (GVHD) progression results from Th1 and Th17 differentiation, which is mediated by polarized M1 macrophages requiring NLRP3 inflammasome activation [23]. Wang *et al.* reported that TMAO induces pyroptosis in tumor cells by activating the endoplasmic reticulum stress kinase PERK and thus enhances $CD8^+$ T cell-mediated antitumor immunity in triple-negative breast cancer (TNBC) *in vivo* [24]. The effect of TMAO on β cells does not occur through PERK since the pathological dose of TMAO did not activate the PERK pathway in β cells. Serca2 is the key mechanism by which TMAO inhibits GSIS. This is the first study to connect TMAO with Serca2.

In β cells, the proinflammatory cytokine IL-1β leads to a loss in Serca2 expression through peroxisome proliferator-activated receptor (PPAR)-γ activation [25]. In macrophages, TMAO activates the NLRP3 inflammasome and increases bioactive IL-1β through mitochondrial ROS and NF-κB [23]. RNA-seq revealed that the ROS pathway was enriched in the TMAO-treated MIN6 cells under high-glucose conditions. The results of this study indicated that TMAO increased mitochondrial ROS and activated the NLRP3 inflammasome to reduce Serca2 expression in β cells.

β-cell loss is another reason for decreased insulin secretion and mainly manifests as β cell dedifferentiation and apoptosis. By evaluating the expression of β cell dedifferentiation markers, β cell apoptosis markers and β cell transcription factors, long-term TMAO treatment was shown to promote

β cell dedifferentiation and apoptosis and inhibit β cell transcription factor expression. It has been reported that ER stress drives β cell dedifferentiation and apoptosis [26,27]. Long-term TMAO treatment significantly increases ER stress-related protein levels in β cells and mouse islets. It has been reported that TMAO induces the osteoblast-like transdifferentiation of vascular smooth muscle cells [28] and the innate immune cell transdifferentiation of human aortic endothelial cells [29]. In this study, the choline diet decreased β cell mass and increased α cell mass. This suggests that TMAO may also lead to β cell transdifferentiation to α cells in islets.

In *db/db* model mice, the inhibition of plasma TMAO production through antisense oligonucleotides to knock down Fmo3 restored Serca2 expression and increased glucose tolerance and GSIS. Fmo3 knockdown in *db/db* mice also increased the β cell mass, inhibited β cell dedifferentiation and apoptosis, and restored β cell identity.

In insulin-resistant and diabetic *db/db* mice, TMAO knockdown by Fmo3 antisense oligonucleotides markedly improved β cell function and increased insulin sensitivity. These findings suggest that FMO3 is a promising target for treating diabetes. We are currently developing an FMO3 inhibitor screening method.

Some studies have shown that TMAO (100 mM or 2.78 mM in combination with a 0.25 μl/h infusion through subcutaneous osmotic minipumps) attenuates ER stress and could be beneficial [30,31], which contradicts our results. However, the dosages used in the aforementioned studies are far greater than those used *in vivo*. An extremely high dose of TMAO functions as a chemical chaperone and corrects protein folding defects indirectly by affecting hydrogen bonds within water molecules [32]. Protein stability is the result of a balance between the intramolecular interactions of protein functional groups and their interactions with the solvent environment. TMAO can enhance the strength of water-water hydrogen bonds, which eventually stabilizes the hydrogen bonds between amide groups in a protein and favors protein folding [33]. This may be one of the reasons for the beneficial effect of TMAO.

This study revealed that the gut microbiota metabolite TMAO plays an important role in the regulation of pancreatic β cell function both *in vitro* and *in vivo*. Mechanistically, short-term TMAO treatment inhibits glucose-stimulated calcium transients through NLRP3 inflammasome-related cytokines and Serca2 loss. Long-term TMAO treatment promotes β cell ER stress and dedifferentiation. The inhibition of TMAO/FMO3 might be a potential therapeutic strategy for β cell protection and antidiabetes treatment.

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