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Does Citrulline Sit at the Nexus of Metformin's Pleotropic Effects on Metabolism and Mediate Its Salutatory Effects in Individuals With Type 2 Diabetes?

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For more than two decades, physicians have prescribed metformin as the frontline pharmacological therapy to treat type 2 diabetes (T2D) despite knowing little about its underlying mechanisms of action (1). Metformin reduces hepatic glucose production (1) and has pleotropic effects on multiple tissues, including liver (1), muscle (2), and adipose (3) tissue, as well as on immune cells (4). Clinically, metformin also reduces the risk for cardiovascular diseases, cancers, and other diseases (5,6). Pharmacometabolomics provides an opportunity to measure changes in absolute and/or relative concentrations of hundreds to thousands of small molecules in biofluids and tissue extracts to determine the response and variations in response to therapy (7). Pharmacometabolomics could facilitate identification of metabolic pathways and mechanisms of action that contribute to metformin's widespread salutatory effects (7). In this issue of *Diabetes*, Adam et al. (8) utilize this approach to "uncover" metformin's effect on citrulline metabolism in individuals with and without T2D participating in the KORA (Cooperative Health Research in the Region of Augsburg) study.

Adam et al. should be commended on conducting a thorough investigation of metformin's impact on serum metabolites in individuals with T2D using nontargeted, semiquantitative liquid chromatography–tandem mass spectrometry coupled with rigorous statistical and bioinformatic analyses. The cross-sectional "human discovery" study revealed that out of a total of 353 identified serum metabolites, the concentrations of citrulline were significantly lower and X-21365 significantly higher in metformin-treated participants with T2D compared with nontreated participants. The longitudinal "human validation" study further demonstrated that the initiation of metformin treatment during the 7-year follow-up significantly reduced citrulline and increased X-21365 serum concentrations. Subsequently, the "translational" study showed that daily treatment with metformin in *db/db* diabetic mice decreased plasma, skeletal muscle, and epididymal adipose tissue but not liver concentrations of citrulline.

The study by Adam et al. (8) provides convincing evidence that metformin reduces serum citrulline concentrations in humans with T2D and may reduce tissue citrulline concentrations in multiple metabolically active tissues. Of note, metformin also reduced serum concentrations of arginine in their unadjusted model. These results corroborate our prior findings that 3 months of metformin plus pioglitazone therapy reduced plasma concentrations of citrulline and arginine in overweight/obese adults with impaired fasting glucose (9). Likewise, acutely administering metformin reduced plasma citrulline concentrations in African Americans without diabetes (10). The mechanism(s) responsible for reductions in plasma/serum citrulline and arginine concentrations remain unclear. However, both amino acids play prominent roles in nitric oxide (NO) biosynthesis and the urea cycle.

The physiological relevance of metformin-induced reductions in serum/plasma and tissue concentrations of citrulline and arginine with respect to NO biosynthesis is intriguing. NO synthase (NOS) synthesizes NO in equimolar amounts with citrulline from its precursor arginine (arginine→citrulline+NO). Endothelial (eNOS) and neuronal (nNOS) NOS are the primary NOS that are expressed in the tissues measured in the "translational" study by Adam et al. (8). Metformin activates NOS and increases

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COMMENTARY

NO bioactivity by promoting the association between NOS and heat shock protein 90 (11). Although not directly measured in the current study, elevations in NO biosynthesis should result in relative reductions in arginine with concomitant elevations in citrulline. However, in the present and prior studies metformin therapy reduced both arginine and citrulline serum/plasma concentrations (9,10). The authors suggest that increased urinary excretion of citrulline could potentially explain this discrepancy. De novo synthesized citrulline could also be converted back to arginine in the kidneys by argininosuccinate synthase and argininosuccinate lyase (12) in an attempt to stabilize circulating and tissue concentrations of arginine. Reductions in citrulline could also simply reflect reductions in citrulline's precursor (arginine), with equivalent reductions in NO biosynthesis.

Further studies are necessary to elucidate the underlying mechanisms driving the metformin-induced reductions in citrulline and arginine and their overall relevance to NO biosynthesis. With respect to the latter, the impact of metformin monotherapy on whole-body NO biosynthesis could be tested using stable isotope methodologies. Specifically, NO biosynthesis could be assessed using an infusion of ^{15}N-arginine with simultaneous measurements of ^{15}N-arginine and [^{15}N]nitrate enrichment in whole blood as previously described (13). Moreover, measurements of circulating concentrations of nitrites/nitrates may also serve as useful biomarkers for NO biosynthesis (14)

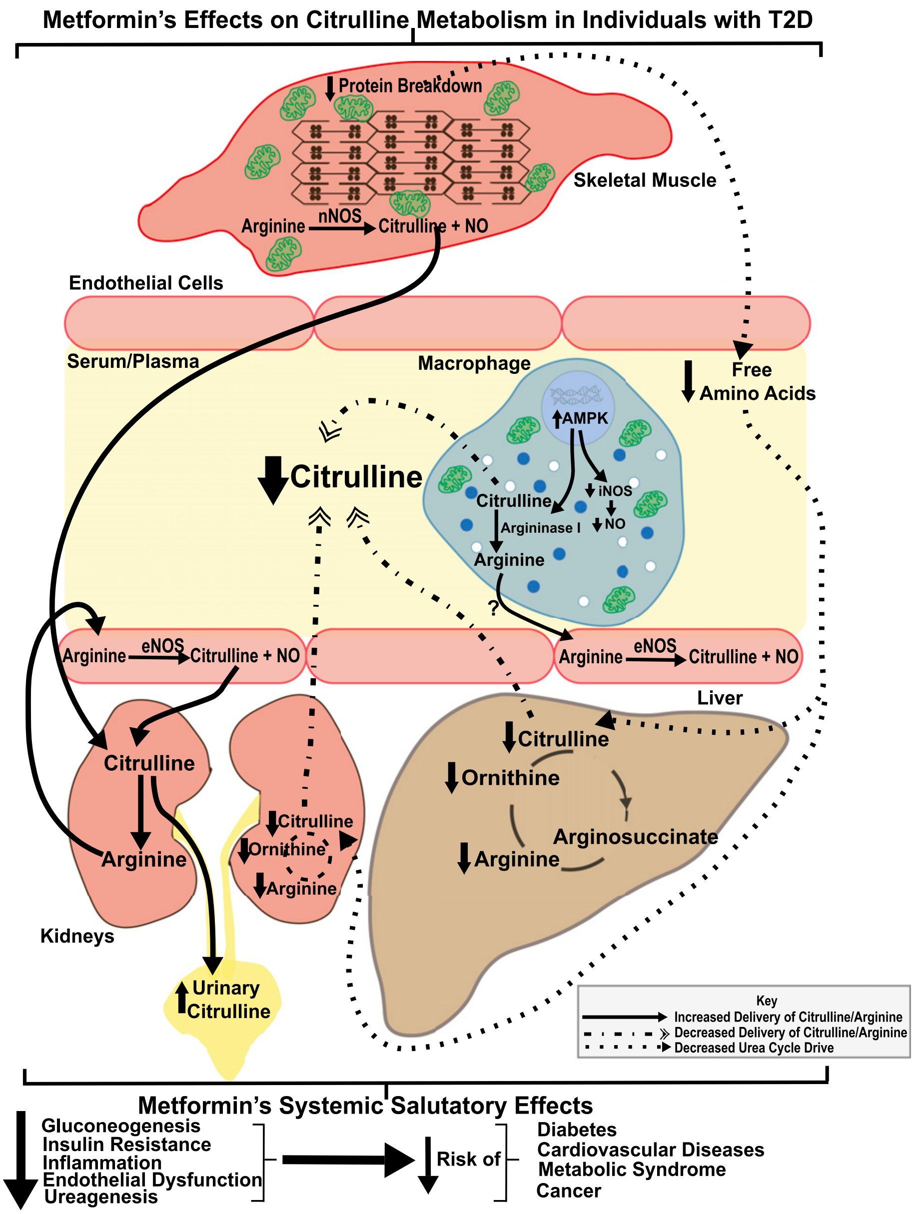

Figure 1—Proposed effects of metformin on citrulline metabolism in individuals with T2D, linking metformin-induced alterations to cellular and systemic NO and urea biosynthesis to metformin's systemic salutatory effects.

and its clinically associated improvements endothelial function (15).

Although the current study did not evaluate the effect of metformin treatment on immune cells, innate immune cells, in particular macrophages and dendritic cells, represent another well-documented source of NO via inducible NOS (iNOS) activation. As such, NO biosynthesis plays a crucial part in the innate immune response against bacterial infections and tumor development and progression, and it is heavily regulated by both AMPK phosphorylation and the immune cells activation status. While metformin-induced AMPK activation enhances iNOS-mediated NO production in resting macrophages and dendritic cells, metformin appears to drastically reduce NO production of activated cells (4). Consequently, we hypothesize that metformin treatment in patients affected by low-grade inflammation, as often seen in individuals with insulin resistance and T2D, could lead to a reduction in iNOS-mediated NO biosynthesis by chronically activated innate immune cells, thus exerting anti-inflammatory properties and lowering serum citrulline concentrations.

Lipopolysaccharide-activated macrophages have also been shown to transform citrulline into arginine (16), which is further degraded into urea and ornithine by the lipopolysaccharide-activated arginase (17). Animal studies have demonstrated that metformin increases arginase I expression in various innate immune cells, such as microglia and activated macrophages, thus exhibiting anti-inflammatory effects while fueling the urea and ornithine cycle (18), as suggested by Adam et al. (8). These anti-inflammatory effects are not limited to innate immune cells, as metformin treatment reduces inflammation in murine models of various chronic diseases by promoting regulatory T-cell formation (19), modulating memory CD8 T-cell metabolism (20) and impeding tumor cell growth (21). These effects of metformin on immune cells appear as strong advocates for further mechanistic immune-related studies.

Emerging evidence also suggests that metformin may play a role in ammonia detoxification (10). A hallmark finding of metformin therapy includes reductions in urea cycle metabolites citrulline, arginine, and ornithine (8–10). Indeed, in the "human discovery" study, serum concentrations of citrulline, arginine, ornithine, and urea were all lower (based on the unadjusted model) in the metformin-treated versus untreated participants with T2D (8). Metformin-induced reductions in these metabolites likely reflect reductions in urea biosynthesis, secondary to reductions in gluconeogenesis (9). Reductions in serum/plasma amino acids may also result in reduced pressure for the liver and kidneys to perform high rates of urea detoxification secondary to reductions in protein breakdown. Using targeted metabolomics, we previously reported significant reductions in several amino acids and their metabolites following 3 months of metformin plus pioglitizone therapy. Moreover, several amino acids and their metabolites were also lower (crude model) in the metformin-treated versus untreated participants with T2D in the present "human discovery" study (8). Further studies are needed to elucidate the underlying mechanisms driving metformin-induced reductions urea biosynthesis and detoxification. The impact of metformin on whole-body urea biosynthesis could be tested using stable isotope methodologies (22). For example, in vivo synthesis of [1-^{13}C]urea following administration of [1-^{13}C]acetate has been reported as a reliable method for measuring in vivo urea biosynthesis (22). Of interest, in the present "translational" study, metformin did not affect citrulline concentrations in the *db/db* diabetic mouse liver and metformin's effects on other urea cycle metabolites was not evaluated. In contrast, in a prior study (10), metformin treatment significantly reduced ornithine concentrations in the livers of diabetic (MKR) mice. Therefore, further investigations are necessary to determine the impact of metformin on ureagenesis in the liver and in the kidneys.

In conclusion, the elegant study conducted by Adam et al. (8) provides valuable insights on the potential mechanism behind metformin's action on citrulline metabolism while opening the door for further studies on the role metformin plays in cellular and systemic NO and/or urea biosynthesis in individuals with T2D (Fig. 1). Such mechanistic studies will be required before a wider clinical implementation of metformin can be observed.

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