

Metabolic and cardiovascular effects of chronic mild hyperuricemia in rodents

Sun K Park,¹ Tara R Rosenthal,² Jessica S Williams,² John M Shelton,^{2,3} Masaya Takahashi,^{4,5} Shanrong Zhang,⁵ Ion Alexandru Bobulescu^{1,2}

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¹The Charles and Jane Pak Center for Mineral Metabolism and Clinical Research, Dallas, Texas, USA

²Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, USA

³Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

⁴Department of Radiology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

⁵The Advanced Imaging Research Center, University of Texas Southwestern Medical Center, Dallas, Texas, USA

Correspondence to

Dr Ion Alexandru Bobulescu, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA; alexandru.bobulescu@utsouthwestern.edu

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ABSTRACT

Mildly elevated serum uric acid levels are common in people with metabolic syndrome and type 2 diabetes mellitus (T2DM), but whether elevated uric acid has a causal role in the pathogenesis of diabetes remains uncertain. We tested whether chronic mild hyperuricemia in rodents under controlled laboratory conditions can cause glucose intolerance in otherwise healthy animals, or whether it can worsen glucometabolic control in animals that are genetically predisposed to T2DM. We used an established model of experimental hyperuricemia in rodents with potassium oxonate dietary supplementation, which led to sustained, approximately two-fold elevation of uric acid compared with control animals. We also reversed the hyperuricemic effect of oxonate in some animals by treatment with a xanthine oxidase inhibitor. Manipulation of serum uric acid levels in Sprague-Dawley rats for up to 18 weeks did not affect fasting glucose and glucose tolerance. Blood pressure was also not affected by hyperuricemia in rats fed a Western-type diet. We next sought to determine whether uric acid may aggravate or accelerate the onset of glucometabolic abnormalities in rats already predisposed to T2DM. Chronic oxonate treatment in Zucker diabetic fatty (ZDF) and lean control rats for up to 6 weeks did not affect fasting glucose, insulin, and glucose tolerance in ZDF rats. Taken together, these findings indicate that elevated uric acid does not directly contribute to the pathogenesis of glucose intolerance and T2DM in rodents.

INTRODUCTION

More than 29 million people in the USA are estimated to have diabetes mellitus, either diagnosed or undiagnosed (2012 estimates),¹ with the vast majority of cases classifiable as type 2 diabetes (T2DM).

A number of clinical studies in cohorts with diverse characteristics have suggested that increased serum uric acid levels even within the normal range, or mild hyperuricemia, may independently predict incident T2DM, after adjustment for other known diabetes risk factors.^{2–7} However, a pathogenic link between uric acid and T2DM has not been supported by other studies,^{8–11} and conclusion may be affected by positive-results publication bias.¹²

Significance of this study

What is already known about this subject?

- Multiple studies have shown that mild hyperuricemia in people without gout is associated with type 2 diabetes mellitus (T2DM) and may predict incident diabetes even after adjustment for other known diabetes risk factors. However, other studies do not support a link between hyperuricemia and diabetes.
- Whether mild hyperuricemia can directly cause or precipitate the development of diabetes is unknown.
- Hyperuricemia has also been associated with high blood pressure and cardiac dysfunction.

What are the new findings?

- Mild hyperuricemia does not cause impaired glucose tolerance in healthy rodents fed a Western-type (high-salt and high-fat) diet.
- In rodents predisposed to T2DM, mild hyperuricemia does not accelerate or worsen the disease.
- Mild hyperuricemia does not affect blood pressure and cardiac function in rodents fed a Western-type diet.

How might these results change the focus of research or clinical practice?

- This study's findings do not support an independent causal or contributory role for uric acid in the pathogenesis of T2DM.

Experimental hyperuricemia has also been shown to result in features of the metabolic syndrome in laboratory animals.^{13–15} However, almost 70 years after the first reports of 'uric acid diabetes' in rabbits exposed to massive doses of uric acid,^{16 17} whether mild hyperuricemia can independently cause or contribute to the pathogenesis of T2DM remains unclear.¹⁸

The critical experiments to test the causal relationship between serum uric acid and T2DM rest in animal studies where uric acid levels can be manipulated under tightly controlled conditions and the contribution of potential confounding factors can be minimized



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or eliminated. To address the present knowledge gap, we set out to either prove or refute a direct causal relationship between chronic mild hyperuricemia and glucose tolerance in otherwise healthy rats, as well as in rats genetically predisposed to T2DM.

MATERIALS AND METHODS

Animals

All experimental procedures were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at University of Texas Southwestern Medical Center. Male Sprague-Dawley rats, 6–7 weeks old, were purchased from Harlan Laboratories (Hayward, California, USA). Male leptin receptor-deficient Zucker diabetic fatty (ZDF, *fa/fa*) and wild-type littermates, 5–6 weeks old, were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA). All animals were acclimatized for 1 week on standard rodent chow prior to commencing the study and kept on a 12 hours light–dark cycle with free access to food and water throughout the study.

Manipulation of uric acid levels in rats

After acclimatization, animals from each strain/genotype described above were fed ad libitum a Western-type diet (Teklad TD.88137, Envigo, Indianapolis, Indiana, USA) custom modified (TestDiet, St. Louis, Missouri, USA) to contain 2% potassium oxonate (Sigma-Aldrich, St. Louis, Missouri, USA), or a calorie-matched and nutrient-matched control diet (with KCl used to match for the K⁺ content of potassium oxonate) for 6–18 weeks. Dietary manipulation with 2% potassium oxonate, in the absence of dietary uric acid or purine supplements, has been previously shown to cause mild hyperuricemia (serum uric acid increased 1.5-fold to 2-fold vs normal levels), with no overt renal disease or intrarenal urate crystal deposition.¹⁹ In addition, the xanthine oxidase inhibitor febuxostat (Takeda Pharmaceuticals U.S.A., Deerfield, Illinois, USA) was administered in the drinking water at a calculated dose of 6 mg/kg/day as previously described²⁰ to lower uric acid levels in subgroups of rats, as detailed in the Results section. In separate experiments aimed to achieve a greater contrast in serum uric acid levels, rats were fed a diet supplemented with 2% potassium oxonate and 2% uric acid, or a control diet calorie-matched and nutrient-matched as above. All diets were custom modifications of the TestDiet Western Diet 5342 (Test Diet, Richmond, Indiana, USA).

Urine and serum analysis

For urine collection, animals were housed in metabolic cages and 24 hours urine samples were collected in tubes containing thymol crystals to inhibit bacterial growth. Samples were analyzed for creatinine (Sekisui Diagnostics, Lexington, Massachusetts, USA), Na⁺, and K⁺ (Vitros Chemistry, Ortho-Clinical Diagnostics, Piscataway, New Jersey, USA). Blood samples were collected from the tail vein after fasting for 12 hours to determine glucose (OneTouch Ultra2, LifeScan, Milpitas, California, USA), insulin (Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden), uric acid, and creatinine levels (Vitros Chemistry, Ortho-Clinical Diagnostics).

Oral glucose tolerance testing

For oral glucose tolerance testing (OGTT), rats were fasted for 12 hours and blood was drawn from the tail vein before and at 15, 30, 60, 90, and 120 min after an oral glucose load (1 g/kg body weight) as previously described.²¹ Blood glucose concentration was measured using a glucose meter (OneTouch Ultra2, LifeScan) approved by the US Food and Drug Administration. Glucose area under the curve (AUC) was calculated using the trapezoidal rule.²²

Blood pressure measurements

Blood pressure was measured in conscious animals by the tail-cuff method using a computerized CODA Blood Pressure Analyzer (Kent Scientific Corporation, Torrington, Connecticut, USA). Rats were trained daily for the procedure for 1 week prior to the actual blood pressure measurement. All blood pressure measurements were performed by an experienced technician and data analysis was performed by a different investigator who was blinded to study conditions and groups.

Cardiac MRI

Rat cardiac function was evaluated using a 9.4 T small animal MR scanner (Agilent Technologies, Santa Clara, California, USA) and a 55 mm surface radiofrequency coil while the animals were anesthetized with 1.5–3% isoflurane in O₂. ECG electrodes and respiratory sensors were attached to the chest, and the images were acquired using cardiac and respiratory triggering with a heart rate of 270–330 bpm. A series of gradient echo scout images was first generated to determine the long axis of the heart. Cine images were acquired at 12 phases per cardiac cycle, with a stack of 2 mm contiguous true short-axis slices to cover the left ventricle (echo time 1.10 ms, R-R interval on electrocardiogram (RR)/repetition time (TR) 180–200/15–19 ms, flip angle 45°, number of excitations (NEX)=6, matrix size 128×128, and field view 64×64 mm). Image analysis was performed using ImageJ (NIH Image v1.47j).²³ Left ventricular end-systolic volume (LVESV) and left ventricular end-diastolic volume (LVEDV) were calculated by manually tracing the endocardial borders of the left ventricle on each slice, and then summing all respective slice volumes.²⁴ Left ventricular (LV) wall thickness was measured at the mid-ventricular short axis at just below papillary muscle level. Image acquisition and measurements were performed by an experienced technician who was blinded to study conditions and groups. Stroke volume (SV) was calculated as LVEDV–LVESV. Cardiac output was calculated as SV x heart rate. LV ejection fraction (EF) was calculated as (LVEDV–LVESV)/LVEDV x 100.

Heart histology

Following euthanasia, the hearts were excised, weighed, and rinsed in phosphate buffered saline (PBS) before fixation in 4% paraformaldehyde for 2 days. Whole hearts were dehydrated, cleared, and paraffin embedded using standard procedures.^{25 26} Resulting embeds were sectioned coronally to matching planes of section as determined by atrial appendages and the aortic valve. Sections were stained with H&E for routine histopathology and

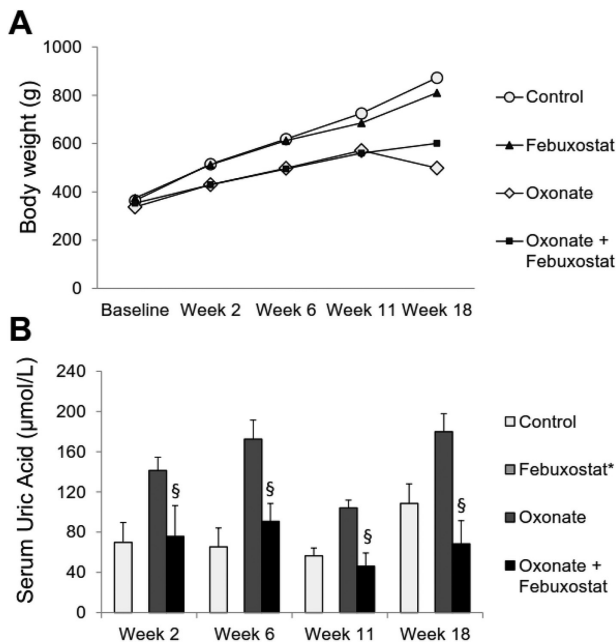


Figure 1 (A) Body weight and (B) serum uric acid in Sprague-Dawley rats treated with vehicle (control), potassium oxonate, febuxostat, or both for 18 weeks. Data are shown as means or means and SDs. *All animals in the febuxostat group had undetectable serum uric acid. $\$P < 0.01$ oxonate+febuxostat versus oxonate alone. $n=4$ /group (16 rats total).

with Masson's trichrome for detection of fibrosis as previously described.²⁷

RESULTS

Manipulation of serum uric acid levels in Sprague-Dawley rats

Sprague-Dawley rats were randomized to four treatment groups: (i) oxonate, to cause hyperuricemia; (ii) oxonate plus febuxostat, to normalize the uric acid and thus control for a potential direct effect of oxonate on glucose homeostasis, independent of hyperuricemia; (iii) febuxostat alone; and (iv) control. The two groups of rats given oxonate gained weight at a slightly slower rate than rats given febuxostat alone or the control diet (figure 1A), with the difference reaching statistical significance by study end. This is likely attributable to a modest decrease in food intake in the oxonate groups (data not shown). As shown in figure 1B, treatment with oxonate led to a persistent and significant rise in serum uric acid levels, while rats given oxonate +febuxostat had serum uric acid levels similar to control animals. In rats given febuxostat alone, serum uric acid levels were below the detection limit of the assay. There were no significant differences in creatinine clearance between the four groups at 10 weeks and at 16 weeks (data not shown).

Fasting glucose and OGTT in Sprague-Dawley rats

OGTT was performed in each study animal at 6 weeks (not shown), 11 weeks (not shown), and 18 weeks (figure 2) after the start of the experiment. There were no differences at any experimental time point between the four groups in

fasting plasma glucose levels, as well as in plasma glucose levels at 15, 30, 60, 90, and 120 min after the oral glucose load. There were also no differences in plasma glucose total AUC and plasma glucose AUC from baseline.

Blood pressure

There were no differences between the four groups in systolic and diastolic blood pressures measured at baseline and at 4, 7, and 10 weeks after the start of the experiment (online supplementary figure 1).

Renal crystal deposition and mortality with higher serum uric acid levels

To test whether further elevation of serum uric acid levels may affect glucose intolerance, we conducted separate experiments in which Sprague-Dawley were given 2% oxonate and 2% uric acid dietary supplementation, achieving approximately fivefold serum uric acid elevation compared with control animals (data not shown). However, this resulted in high mortality during the experiment (all treated animals died or had to be euthanized within the first 2 weeks of treatment), with massive renal crystal deposition noted on postmortem examination, similar to renal findings previously described in urate oxidase (uricase) knockout mice.²⁸

Hyperuricemia in ZDF and lean control rats

We next sought to test whether hyperuricemia can either accelerate the onset or further impair glucose tolerance in a rat model that is already genetically predisposed to T2DM. ZDF and lean control rats were started on the experimental diets at 6 weeks of age, when ZDF rats were not yet diabetic, and were kept on the diets until after development of diabetes in ZDF rats. Body weights were similar in the four groups (ZDF, lean, ZDF +oxonate, and lean +oxonate) at the beginning of the experiment, but diverged as expected after 6 weeks, with ZDF rats gaining significantly more weight (figure 3A). There was no effect of oxonate on body weight. Serum uric acid was significantly increased in oxonate-treated versus vehicle-treated lean controls and oxonate-treated versus vehicle-treated ZDF rats (figure 3B).

Fasting glucose and OGTT in ZDF and lean rats

OGTT was performed in each study animal at 2 weeks and 6 weeks after the start of the experiment. At 2 weeks (figure 4), ZDF rats had significantly increased fasting glucose and plasma glucose total AUC compared with lean rats, but oxonate treatment did not affect these parameters within each genotype. At 6 weeks (figure 5), ZDF rats had significantly increased fasting glucose, glucose total AUC as well as AUC from baseline compared with lean rats, but again oxonate treatment did not affect these parameters within each genotype. As shown in figure 6, fasting plasma insulin levels were not different between the four groups at baseline, were significantly increased in ZDF rats after 2 and 6 weeks, and were not affected by oxonate treatment within each genotype.

Cardiac function, morphology, and fibrosis

Cardiac functional MRI (data not shown) did not reveal differences in EF, end-diastolic volume, end-systolic

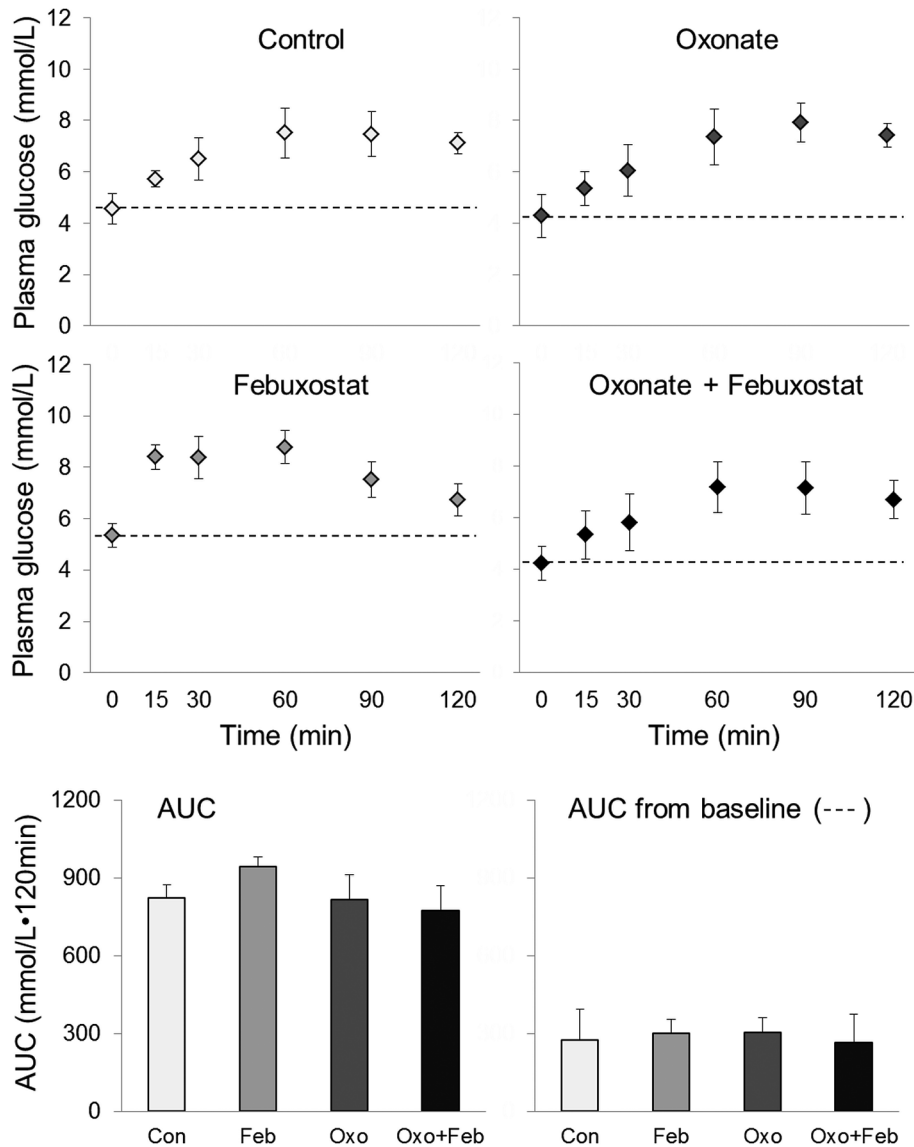


Figure 2 Oral glucose tolerance testing in Sprague-Dawley rats treated with vehicle (control), potassium oxonate, febuxostat, or both for 18 weeks. Data are shown as means \pm SD. n=4/group (16 rats total). AUC, area under the glucose curve.

volume, SV, and LV mass in ZDF rats treated with vehicle or oxonate for 6 weeks. No histopathological evidence of disease was noted in hearts from any of the study animals, and no qualitative differences in cardiac morphology and fibrosis (trichrome staining) were noted between groups (online supplementary figure 2).

DISCUSSION

Hyperuricemia and diabetes in rodents

This study used an established model of mild hyperuricemia in rodents by oral treatment with potassium oxonate¹⁹ to test the hypothesis that chronically elevated uric acid levels may cause, or contribute to, the development of glucose intolerance and T2DM in rodents. In spite of sustained hyperuricemia, Sprague-Dawley rats randomized to the oxonate diet had similar fasting glucose levels and oral glucose tolerance profiles as rats randomized to control diet for up to 18 weeks. Treatment with febuxostat lowered

uric acid levels and effectively counteracted the hyperuricemic effect of oxonate, but did not affect fasting glucose or glucose tolerance. These findings suggest that chronic elevation of uric acid does not cause glucose intolerance in this animal model.

We also considered the possibility that uric acid is not sufficient to cause glucose intolerance in otherwise healthy animals (Sprague-Dawley rats), but may contribute to or accelerate the development of glucose intolerance in an animal model that is already predisposed to T2DM. We explored this possibility in ZDF rats, a widely used model of T2DM. ZDF rats developed diabetes during the experiment as expected, with higher fasting glucose and insulin and impaired glucose tolerance compared with lean control rats. However, experimental hyperuricemia clearly did not worsen or accelerate these defects.

Taken together, these findings in two different animal models suggest that chronic mild hyperuricemia is not a

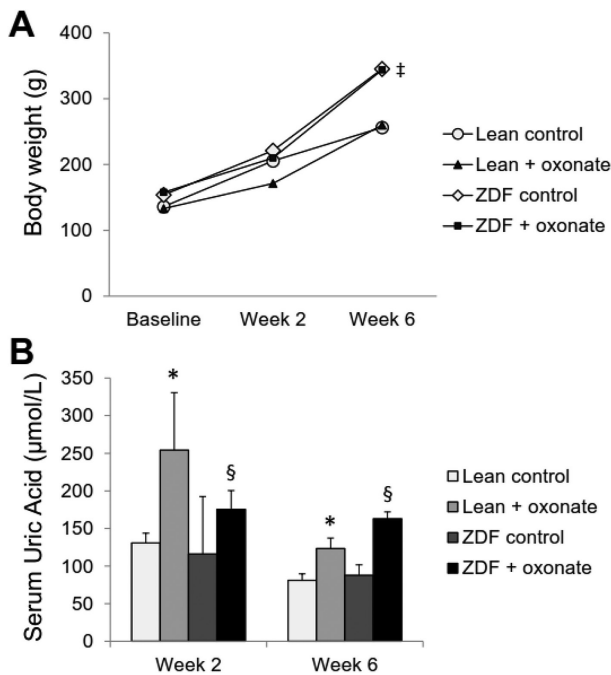


Figure 3 (A) Body weight and (B) serum uric acid in Zucker diabetic fatty (ZDF, fa/fa) rats and lean littermates treated with vehicle (control) or potassium oxonate for 6 weeks. Data are shown as means or means and SD. # $P < 0.001$ ZDF versus lean. * $P < 0.01$ lean+oxonate versus lean control. § $P < 0.01$ ZDF+oxonate versus ZDF control. $n = 4/\text{group}$ (16 rats total).

causal contributing factor for the development of glucose intolerance and T2DM in rodents.

Study findings in context

Clinical observational studies have suggested that uric acid levels independently predict incident T2DM, even after adjustment for known diabetes risk factors. For example, serum uric acid in 4536 initially non-diabetic subjects followed for a mean of 10 years in the Rotterdam Study was an independent predictor of diabetes, with the HR for incident diabetes during follow-up in the highest compared with the lowest quartile of baseline serum uric acid of 1.68 [95% CI 1.22 to 2.30] after multiple adjustments.² In analyses of the Framingham Heart Study original and offspring cohorts, the adjusted relative risks of incident diabetes per mg/dL increase in serum uric acid were 1.20 (95% CI 1.11 to 1.28) for the original cohort and 1.15 (95% CI 1.06 to 1.23) for the offspring cohort.³ Qualitatively similar results were reported by other studies including populations with diverse characteristics from the USA, China, and Italy.⁴⁻⁷ Several large meta-analyses including retrospective and prospective cohort studies also supported the notion that uric acid may increase the risk for incident diabetes,^{12 29 30} with a 1 mg/dL increment in serum uric acid deemed roughly comparable with a 1 kg/m² increment in body mass index in increasing T2DM risk.¹²

In contrast, higher plasma uric acid was not associated with increased risk for T2DM in 6356 Japanese men followed for 5–16 years in the Osaka Health Survey,⁸ and baseline uric acid levels in 475 individuals at high risk for

T2DM followed for an average of 4 years in the Finnish Diabetes Prevention Study did not predict incident diabetes after adjustment.⁹ Finally, two recent studies using Mendelian randomization in a total of over 40,000 individuals found no evidence for a link between uric acid and T2DM.^{10 11} When analyses of epidemiological data oppose each other, one needs to directly test causality in a highly controlled experimental design where hyperuricemia is discreetly introduced in isolation and its effects tested.

In laboratory animals, a potential direct role of uric acid in the pathogenesis of diabetes was proposed almost 70 years ago, with some of the earliest experiments describing a transient diabetes-like phenotype in rabbits injected intraperitoneally with large successive doses of uric acid.^{16 17} These results were subsequently reproduced using various experimental designs, such as in a recent study of mice with acute hyperuricemia induced by intraperitoneal injection of potassium oxonate and simultaneous intragastric administration of hypoxanthine.¹³ The key common denominator of these studies is experimental induction of massive acute hyperuricemia, with unclear pathophysiological relevance for humans with chronic mild hyperuricemia. Milder, sustained elevations of serum uric acid were implicated in the pathogenesis of metabolic syndrome features induced in rats by high-fructose feeding.^{14 15} In addition, mice lacking the intestinal uric acid transporter SLC2A9 (Glut9) have mild hyperuricemia and develop a number of metabolic disturbances that are mitigated in part by treatment with allopurinol, but these do not include glucose intolerance.³¹

Finally, a recent urate oxidase (uricase) knockout mouse model that achieved stable, twofold to threefold elevation in uric acid was associated with impaired glucose tolerance in male animals.³² There are several potential explanations for the apparent discrepancy between these findings and our results in oxonate-treated rats. First, knockout mice are subjected to hyperuricemia early in life. In contrast, oxonate treatment was started in our experiments in mature rats (6–8 weeks of age) to better mimic human hyperuricemia, which in most cases is diagnosed in adulthood. Second, hyperuricemia in knockout mice was more severe than in our oxonate-treated rats. Third, only approximately 40% of knockout mice survived beyond 10 weeks of age, and glucose tolerance experiments were only performed in surviving animals. While this heterogeneity in survival has yet to be explained, surviving mice were likely selected for factors that helped them better cope with hyperuricemia, and it is conceivable that these same factors could have also affected their glucose homeostasis. Fourth, one cannot exclude the possibility of species differences in the pathophysiology of hyperuricemia between mice and rats. Further studies are required to elucidate the underlying reasons for these experimental discrepancies and to definitively prove or refute a pathogenic link between hyperuricemia and glucometabolic abnormalities in humans.

Hyperuricemia, blood pressure, and the heart

In a parallel effort, but using the same animals, we examined the effects of chronic mild hyperuricemia on blood pressure in Sprague-Dawley rats, as well as cardiac function and fibrosis in ZDF rats. Our results suggest that chronic mild hyperuricemia in rodents has no discernible effects on blood

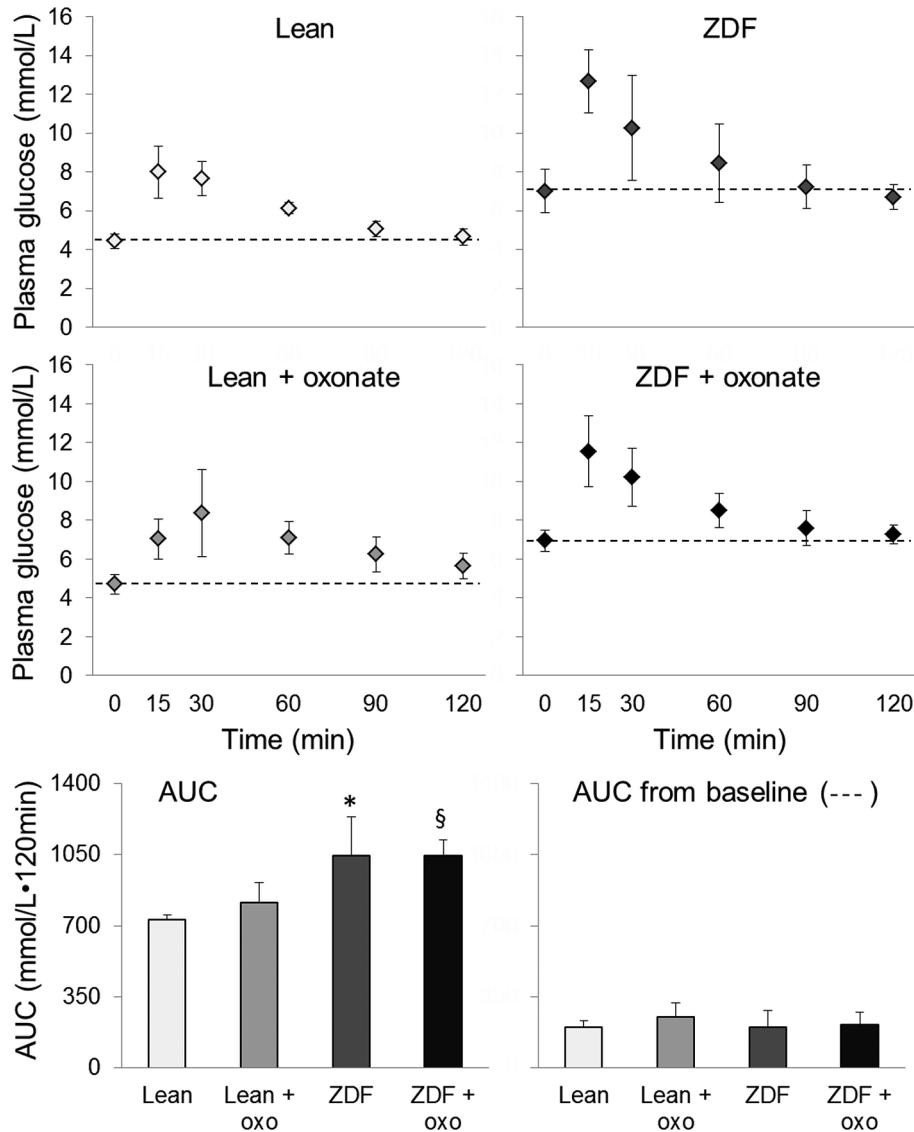


Figure 4 Oral glucose tolerance testing in Zucker diabetic fatty (ZDF, *fa/fa*) rats and lean littermates treated with vehicle (control) or potassium oxonate for 2 weeks. Data are shown as means \pm SD. * $P < 0.01$ lean+oxonate versus lean control. § $P < 0.01$ ZDF+oxonate versus ZDF control. $n = 4$ /group (16 rats total). AUC, area under the glucose curve.

pressure, cardiac function, or cardiac fibrosis in rodents. Of note, the lack of effect of uric acid on blood pressure in this study is in apparent contradiction with previous findings in rodents with oxonate-induced hyperuricemia.¹⁹ However, hyperuricemia in that study had a very modest effect on blood pressure when animals were fed a normal salt diet, and the effect was amplified by low-salt feeding.¹⁹ Since rats in our study were fed a high-salt Western-type diet (a widely used model of the typical American diet), these findings are in fact not contradictory.

Study limitations

The fact that Sprague-Dawley rats treated with oxonate weighed less than non-oxonate-treated rats by the end of the experiment could have theoretically masked differences in glucose metabolism between these groups. However, the body weights of rats in the oxonate and oxonate +febuxostat groups did not differ while uric acid levels differed

markedly, and fasting glucose and OGTT profiles were indistinguishable between these two groups. In addition, oxonate treatment did not affect body weight in ZDF and lean rats and also did not affect fasting glucose, OGTT, and insulin levels. The validity of our findings is unlikely to have been confounded by the observed differences in body weight between Sprague-Dawley rats treated with versus without oxonate. Another limitation of the study is the sample size projected to provide 90% power to detect a significant ($\alpha = 0.05$) change of 25% in fasting glucose and OGTT profiles with a SD of 10%. Although the study was not powered to detect more subtle changes (less than 25%), it is worth noting that SD values for fasting glucose and OGTT AUC were largely consistent with (and in many cases lower than) the SD assumptions used in our power calculation, and we did not observe any distinguishable trends or 'borderline significant' differences between groups treated with versus without oxonate. However, because of

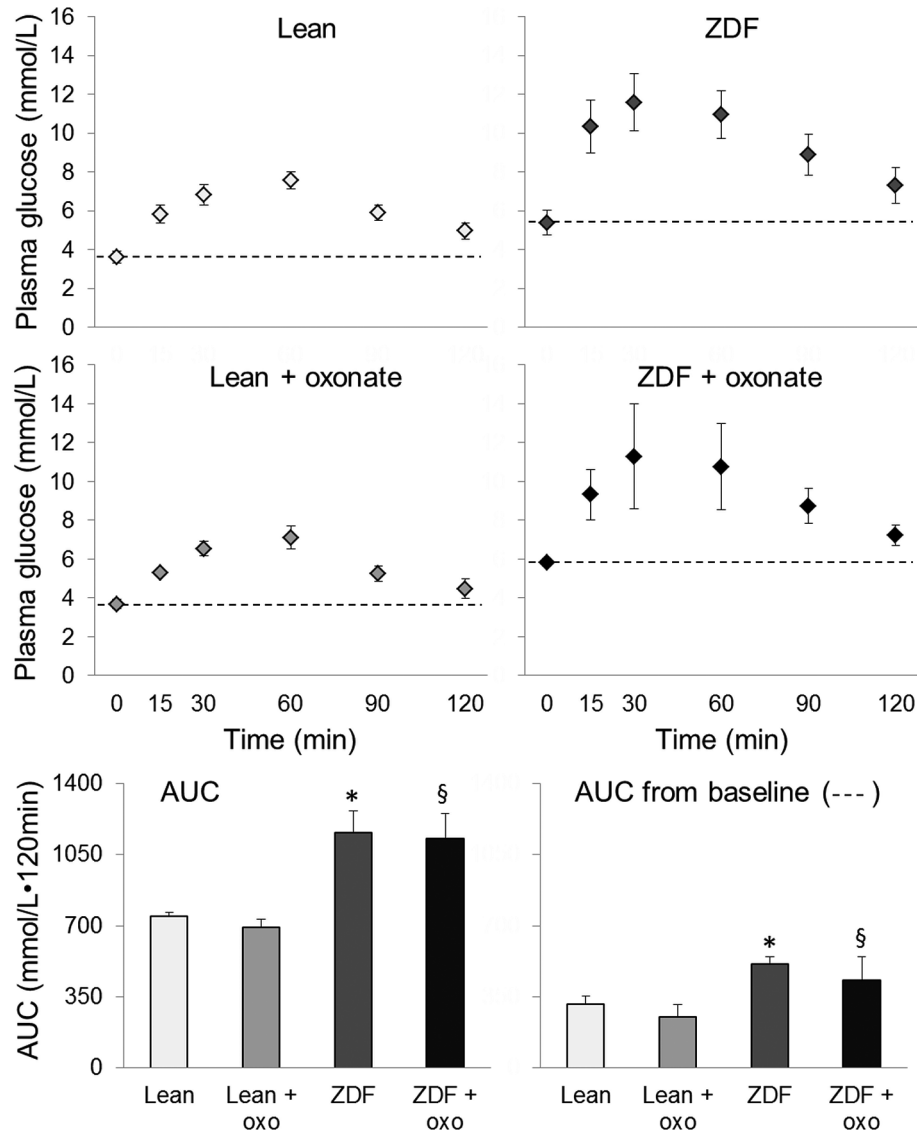


Figure 5 Oral glucose tolerance testing in Zucker diabetic fatty (ZDF, *fa/fa*) rats and lean littermates treated with vehicle (control) or potassium oxonate for 6 weeks. Data are shown as means±SD. * $P < 0.01$ lean+oxonate versus lean control. § $P < 0.01$ ZDF+oxonate versus ZDF control. $n = 4$ /group (16 rats total). AUC, area under the glucose curve.

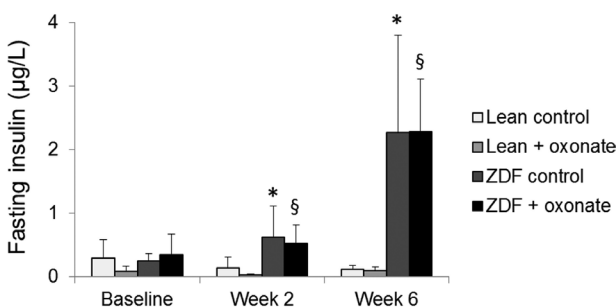


Figure 6 Fasting insulin in Zucker diabetic fatty (ZDF, *fa/fa*) rats and lean littermates treated with vehicle (control) or potassium oxonate for 6 weeks. Data are shown as means and SDs. * $P < 0.01$ ZDF control versus lean control. § $P < 0.01$ ZDF+oxonate versus lean+oxonate. $n = 4$ /group (16 rats total).

the slightly greater within-group variability of our data for blood pressure and cardiac parameters, we cannot exclude the possibility of type II statistical error with regard to the effects of uric acid on blood pressure and cardiac function. Finally, uric acid physiology in humans differs from rodents because humans have lost urate oxidase (uricase) gene function, resulting in multiple adaptations to physiologically higher serum uric acid levels.³³

CONCLUSIONS

Chronic pharmacological elevation of uric acid levels in otherwise healthy rodents fed a Western diet does not affect glucose tolerance, and experimental hyperuricemia in rodents with genetic predisposition to T2DM does not worsen or accelerate the onset of T2DM. Taken together, these findings do not support an independent causal or contributory role for uric acid in the pathogenesis of T2DM in rodents.

Contributors Conceived and designed experiments: IAB. Performed experiments: SKP, TRR, JSW, SZ and IAB. Analyzed the data: SKP, TRR, MT, SZ and IAB. Interpreted results, prepared figures and drafted manuscript: SKP and IAB. Edited and revised manuscript: SKP, JSW, JMS, MT and IAB. Approved the final version: SKP, TRR, JSW, JMS, SZ, MT and IAB.

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Patient consent Not required.

Ethics approval Institutional Animal Care and Use Committee (IACUC) at University of Texas Southwestern Medical Center.

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Data sharing statement All data are available upon request.

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