Tetrathiomolybdate Is Partially Protective Against Hyperglycemia in Rodent Models of Diabetes

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The objective was to evaluate whether copper lowering therapy with tetrathiomolybdate (TM) affected blood sugar levels in three rodent models of diabetes, streptozotocin (STZ) treated rats and mice, and the db/db mouse model. STZ was administered to rats and mice, and blood sugar levels were followed over a protracted time in these and non-STZ control animals. TM was administered by oral gavage (rats) or in the drinking water (mice) to a portion of the rats and mice to observe effects on blood sugar. Mice with genetically determined diabetes (db/db) were studied by giving half the mice TM in the drinking water and following blood sugar. The results show that TM caused a significant reduction in blood glucose in both STZ treated rats and mice, but no effect on blood glucose in db/db mice. However, TM caused a significant reduction in proteinuria in db/db animals. The results are discussed around the likelihood that TM is inhibiting ongoing inflammatory damage in the pancreas from STZ. A metabolic effect of TM on blood glucose is possible but seems less likely. TM is also likely inhibiting inflammatory and/or fibrogenic effects in the kidneys of db/db mice. Exp Biol Med 233:1021–1025, 2008

Key words: diabetes; tetrathiomolybdate; copper; streptozotocin

Introduction

Tetrathiomolybdate (TM) is an anticopper drug, defined as a drug that reduces copper levels in the body, gradually developed over many decades of experimentation (1–9). Its structure is shown in Figure 1. It has a unique mechanism of action, forming a tight three-way complex between protein (albumin in the blood), copper, and itself (6, 8–10). This complex is not absorbed by the intestine, nor is the copper in the complex available for cellular uptake if the complex forms in the blood. Because of its mechanism of action, TM can very quickly gain control over copper toxicity. Its first use was as a dramatically effective treatment for copper poisoned sheep (10). Its first extended clinical use is as a treatment for the neurological presentation of Wilson's disease (11–15), an inherited disease of copper toxicity.

Lowering copper levels with TM seems to decrease the synthesis or activity of a wide variety of cytokines. The ability of TM to inhibit angiogenic cytokines was first made use of as an antiangiogenic agent in a number of preclinical cancer models (16–20) and in a mouse model of the retinopathy of prematurity (21), leading to clinical trials in cancer (22). Later, TM was found to be an antifibrotic agent in mouse models of pulmonary fibrosis (23, 24) and cirrhosis (25), perhaps through inhibition of the transforming growth factor beta (TGF_{β}) cytokine pathway. This is leading to clinical trials in idiopathic pulmonary fibrosis and primary biliary cirrhosis.

Therapy with TM has also proven to be effective in animal models in a wide variety of toxicities from exogenous agents, such as bleomycin (23, 24), carbon tetrachloride (25), acetaminophen (26), and doxorubicin (27), toxicities from immune modulated damaging agents, such as concanavalin A (25) and bovine collagen arthritis (28), and from autoimmune disease such as systemic lupus and type 1 diabetes in the non-obese diabetic (NOD) mouse (29). The mechanism of these latter effects may involve inhibition of cytotoxic inflammatory cytokines such as tumor necrosis factor alpha (TNFα), and interleukin-1b ($IL\text{-}1_{\beta}$), and/or immune modulating cytokines such as interleukin-2 (IL-2) and nuclear factor kappa B (NFκB), although other mechanisms are possible, such as a direct effect on immune cells.

In the NOD mouse, which we studied to see if there

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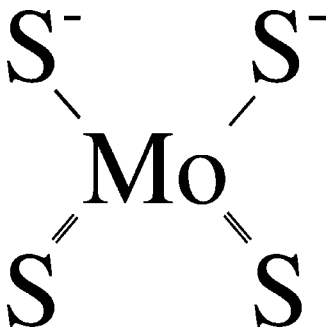

Figure 1. The structure of tetrathiomolybdate is shown.

was an effect of TM on development of immune-mediated diabetes, we saw a delay in onset of diabetes as a result of TM therapy, and a trend toward fewer animals developing diabetes (29). To further study the effects of TM in diabetes models, in this study we examine the effect of TM treatment on streptozotocin (STZ)-induced diabetes in rats and mice and on the diabetes of the db/db mouse model of diabetes. We hypothesize that TM will improve hyperglycemia in STZ-treated animals because of its known ability to protect organs against the type of damage caused by STZ. The db/db animals are studied to see if any effect on blood glucose in the STZ models is specific to STZ damage.

Materials and Methods

STZ Rats. Male Sprague Dawley rats were obtained from Harlan Laboratories at about 8 weeks of age and weighing approximately 250 g. They were housed at 21 ± 2°C in a 12 h light:dark cycle in polycarbonate cages containing corn cob bedding. They were provided Purina rat chow and tap water ad libitum.

Diabetes Induction with STZ. Ten rats received STZ and ten did not. STZ, freshly made up in citrate buffer (pH 5.5), was injected intraperitoneally in a single dose of 50 mg/kg body weight. Rats that did not receive STZ were injected with buffer only. STZ-treated rats were given water bottles with 10% sucrose for 48 h (to prevent severe hypoglycemia). Rats were weighed and fasting blood glucose checked regularly (three times the first week, then weekly for 2 weeks, then monthly). Glucose was checked with Genuine One Touch strips with a One Touch Profile system (Johnson and Johnson Co., New Brunswick, NJ)

TM Treatment. Five of the STZ rats and five of the non-STZ rats were treated with TM beginning on day 3 after STZ treatment. TM was given in a dose of 5 mg twice a day for 2 days, 5 mg once a day for 4 days, and then 2 mg once a day by oral gavage. Minor adjustments in TM dose were subsequently made to maintain ceruloplasmin in a range of 20–60% of baseline.

Supplementary Study. As the above STZ rat experiment progressed, it was obvious that TM treated STZ rats had lower blood glucose levels than STZ controls. We were concerned that TM started 3 days after STZ might have partially protected the pancreas against STZ damage. To evaluate this, we treated another group of 5 rats with STZ as in the above experiment, and waited 8 days before starting TM treatment, as described above, in this group of 5 rats.

STZ Mice. Male C57BL/6 mice were obtained from Jackson Laboratory at 7 weeks of age. They were housed at 21 ± 2°C on a 12h light:dark cycle in polycarbonate cages containing corn cob bedding. They were provided chow containing 2 mg/kg copper and 10 mg/kg zinc and tap water ad libitum.

Diabetes Induction. STZ was dissolved in citric acid buffer (PH 4.5) at a concentration of 0.4% and injected within 5 min after preparation. STZ was administrated by a single intraperitoneal injection at the dose of 100 mg/kg body weight (BW) to all mice. In STZ mouse project 2 (see below), 20 of the 27 mice did not develop diabetes (non-fasting blood sugar of 300 mg/dl or higher), and 2 weeks after the first dose, these mice received a second dose of STZ of 80 mg/Kg body weight. These mice were evenly divided among the 3 groups of mice of this project (TM-STZ, Late TM-STZ, and STZ controls). To measure blood glucose, non-fasting blood from the tail vein was collected weekly and glucose measured using the Hemocue Glucose 20 Analyzer (Hemocue AB, Sweden).

Mice to be treated with TM were pretreated by TM for 3 weeks from 8 weeks of age until ceruloplasmin (Cp) reached 20–60% of baseline, then received STZ. TM treatment continued during and after STZ injection. TM treatment generally involved 0.015 to 0.02 mg of TM/ml of drinking water. The concentration of TM in the drinking water was adjusted periodically to try to maintain Cp in the target range (20–60% of baseline). TM in the drinking water was renewed every two days.

STZ Mouse Project 1. In this project, ten mice received STZ, and five of these were treated with TM (TM-STZ animals).

STZ Mouse Project 2. In this project, 27 mice received STZ, and 20 of them received a second dose of STZ because blood sugar was below 300 mg/dl. Nine of the mice received TM treatment initially (TM-STZ animals). Later (20 days after the second dose of STZ), the 18 STZ control animals were broken into two equal groups, and one of these groups received TM (Late TM-STZ animals).

db/db Mice. db/db Mice were purchase from Jackson Laboratories at 6 weeks of age. These mice were housed and fed as described above for STZ mice. Some of these mice received TM, which was given as described above for STZ mice. Blood glucose was monitored as described above for STZ mice.

Urine for evaluation of protein content was collected in metabolic cages housing individual mice. Protein was measured by the Bradford method (30), with the reagent purchased from Sigma Chemical Co. (St. Louis, MO) and also reported in relation to creatinine content, which was measured by a direct colorimetric method (31).

All animals were housed in the University of Michigan

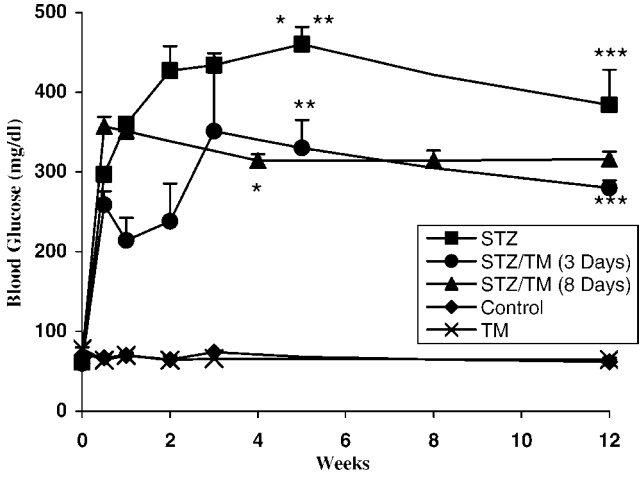

Figure 2. Shown are mean fasting blood glucose values in the five groups of rats ($n = 5$ in each group). At 4 to 5 weeks after treatment, both STZ/TM treated groups were significantly different than the STZ controls (*$P = 0.0001$ for 8 day STZ/TM vs STZ; **$P = 0.01$ for 3 day STZ/TM vs STZ). At 12 weeks, ***$P = 0.03$ for either 8 day or 3 day STZ/TM vs STZ.

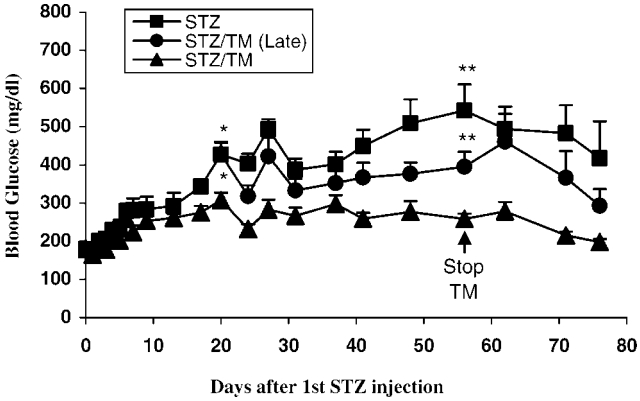

Figure 4. Shown are the mean non-fasting blood glucose values of STZ treated mice, project 2, 9 of whom received TM before STZ. At day 20 the mean blood glucose of these 9 was significantly lower than the 18 STZ controls (*$P = 0.003$). At day 20 the 18 STZ animals were split into two groups of 9 each, and one of these groups was started on TM (late STZ/TM group). At day 56, the mean blood sugar of the late STZ/TM group was significantly lower than the STZ group (**$P = 0.008$). Stopping TM in the original STZ/TM group at day 56 did not affect subsequent glucose levels.

Unit for Laboratory Animal Medicine facility and maintained in accord with a protocol approved by the University of Michigan Institutional Animal Care and Use Committee.

Statistical Methods. Differences between means were evaluated by *t* test. When comparing changes over time in the same animals the paired *t* test was used. In multiple group comparisons of means, analysis of variance was used.

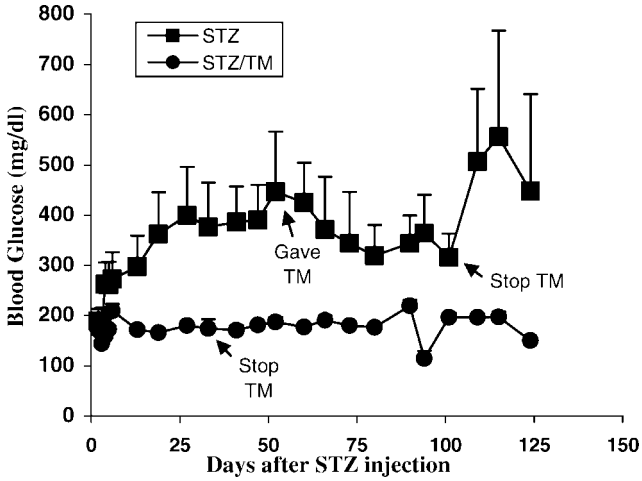

Figure 3. Shown are mean non-fasting blood glucose values in STZ treated mice, project 1, who received TM before STZ ($n = 5$ in each group). The TM treated animals had lower blood glucose but this didn't quite reach statistical significance ($P = 0.085$ at day 25). Stopping TM at day 32 in the TM-treated mice did not seem to affect glucose levels. TM was started at day 51 in STZ controls, and mean blood sugar decreased but didn't reach statistical significance (day 101 vs day 115, $P = 0.087$). TM stopped at this point, and mean blood sugar increased, but the increase did not reach statistical significance ($P = 0.125$).

Results

STZ Studies in Rats. It can be seen from Figure 2 that TM significantly ameliorated the hyperglycemia from STZ, irrespective of whether the TM was started 3 or 8 days after STZ treatment. This effect was substantial. For example at 5 weeks, the mean blood glucose was more than 100 mg/dl lower in the TM treated groups (Fig. 2).

STZ Studies in Mice. In STZ mouse project 1, the observations in rats were repeated except that the TM-treated group was on TM before STZ was given. Again we saw a marked difference in hyperglycemia, with TM-STZ animals having a much lower mean blood glucose than STZ animals (Fig. 3). Because of the small number of animals in each group and relatively large variances, these differences didn't quite reach statistical significance ($P = 0.085$) when comparing the means of single points, although the trend for TM-treated animals to have a lower blood glucose is clear. At day 32, TM was stopped in the TM-STZ animals. The protective effect against hyperglycemia seemed to be a permanent effect of prior TM treatment, because blood sugar was stable at relatively low levels over the next 90 days (Fig. 3). At day 51, TM was started in the STZ mice, and over the next 50 days blood sugars declined, but the mean drop didn't quite reach statistical significance by paired *t* test ($P = 0.087$). At this point (day 101), TM was stopped in this group of mice, and mean blood sugar rose markedly, but again the increase escaped statistical significance ($P = 0.125$).

In STZ mouse project 2, a larger number of animals were used to increase chances of statistical significance. Again, TM-STZ animals received TM prior to receiving STZ. At day 20 (Fig. 4), mean blood glucose was markedly and significantly lower in the TM treated group ($P = 0.003$). At day 56, TM was stopped in the TM-STZ group, and

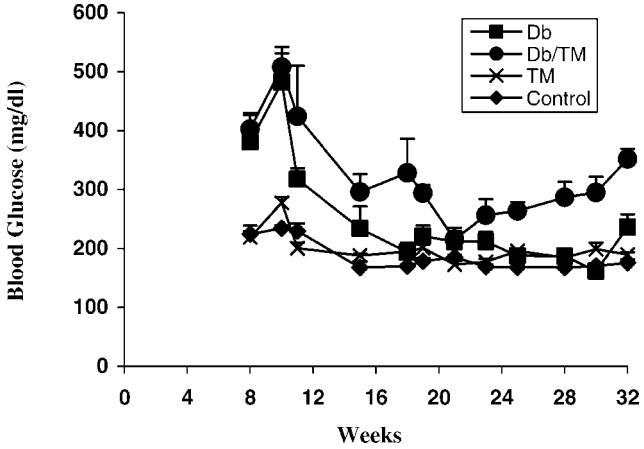

Figure 5. Shown are the mean non-fasting blood glucose values of db/db animals and controls. Half of the db/db animals received TM and there was no resulting decrease in blood glucose compared to db/db controls.

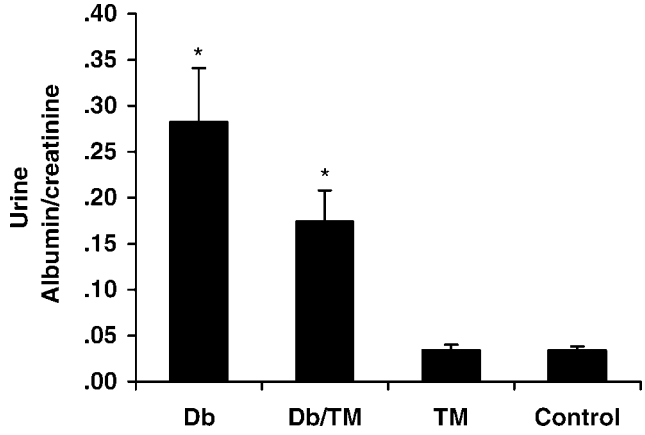

Figure 6. Shown are the mean and SEs of urine albumin/creatinine ratios in the mice of the db/db project. The db/db animals had about a 10-fold higher ratio than control animals. TM therapy in db/db animals significantly lowered this ratio compared with db/db controls ($P = 0.04$).

blood sugar was stable for the next 20 days (Fig. 4). At day 20, the STZ group was split into two equal groups, one to continue as STZ controls, and the other to now begin on TM (Late TM-STZ). At day 56, the mean blood sugar of the Late TM-STZ group was markedly and significantly lower than STZ controls ($P = 0.008$).

db/db Studies in Mice. *Effects of TM on Blood Glucose.* In the db/db mouse project, the TM-treated mice showed no differences in blood glucose over many weeks of measurement compared with db/db controls (Fig. 5).

Effects of TM on Urine Protein. In the db/db mouse project, 24 hour urine protein studies were done and related to creatinine content to standardize for dilution. db/db Control animals had about a 10-fold higher urine protein excretion than non-db/db control animals, and TM treatment significantly ($P < 0.04$) reduced protein excretion in db/db animals (Fig. 6).

Discussion

It is clear that in STZ studies of diabetes in both rats and mice that TM has a significant effect on lowering the blood sugar. This does not seem to be due to a direct interaction between TM and STZ, that is, an immediate protective effect against STZ damage, because delaying the start of TM therapy by as long as 8 days after STZ in rats gave an equivalent lowering of blood sugar (Fig. 2). On the other hand, the effect of TM on blood sugar seemed to be a permanent effect after a few weeks of TM therapy. Stopping the TM in STZ-treated mice resulted in a continuation of the lowered blood sugar effect in both mouse studies (Figs. 3 and 4). Further, starting the TM as late as 20 days after STZ resulted in a lower blood sugar than STZ controls (Fig. 4).

One possible explanation for much of what we see in these STZ studies is that the process initiated by STZ in the pancreas is ongoing in terms of continued inflammatory damage, and TM inhibits the inflammation. This is consistent with numerous studies we have conducted that show that TM has a strong anti-inflammatory effect (23–29). If continuing inflammation through the first few weeks after STZ was further destroying pancreatic insulin secretion, and TM inhibited the inflammatory damage, it would explain much of what we see in Figures 2–4. Given the results in Figure 3 of a stable blood sugar after stopping TM at day 32, one would have to postulate that this kind of damage was essentially over in mice by day 32. And, given the beneficial effects of starting TM at day 20 (Fig. 4), one would have to postulate that it is still going on for awhile after day 20. Blinded examination by a pathologist of the pancreas tissue of the three groups of mice in mouse project 2, all of whom received STZ, was not helpful. Some of the specimens had a few scattered chronic inflammatory cells around blood vessels or in association with islets, but there was no difference between those that received TM and those that did not. Similarly, the number and size of islets were not different among groups.

This narrow time frame for when such postulated damage is continuing and then stopping is perhaps against this explanation. Alternative explanations such as a metabolic effect of TM, such as increasing insulin secretion, increasing insulin sensitivity, inhibiting glucagon secretion, or inhibiting gluconeogenesis are not supported by the stability of blood glucose after TM is stopped. They are supported, a little, by the changes in blood glucose in STZ mice of Figure 3, when starting TM caused a trend downward, and stopping it caused a trend upward, but these results didn't reach statistical significance. It is, of course, possible that both events are occurring, some protection against the inflammatory pancreatic damage set in motion by STZ, and some metabolic effect. Additional experiments are required to sort these possibilities out.

We did not see an effect of TM on blood glucose of db/db mice. On the other hand, there was a significant effect of TM on inhibiting the proteinuria of db/db mice (Fig. 6). We

postulate that this is due to TM inhibition of the inflammatory or fibrogenic damage occurring in the kidney of these mice, consistent with the anti-inflammatory (23–29) and antifibrotic (23–25) effects of TM we have seen in other models, but we did not examine the kidneys histologically.

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