The Effect of Troglitazone on Plasma Homocysteine, Hepatic and Red Blood Cell *S*-Adenosyl Methionine, and *S*-Adenosyl Homocysteine and Enzymes in Homocysteine Metabolism in Zucker Rats

Vivian Fonseca, Mary Keebler, Aliza Dicker–Brown, Cyrus DeSouza, Lionel A. Poirier, S.N. Murthy, and Dennis B. McNamara

We studied the effect of troglitazone on the plasma concentrations of homocysteine (tHcy), the erythrocyte and hepatic concentrations of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH), and the hepatic activities of cystathionine-β-synthase (CβS) and methylenetetrahydrofolate reductase (MTHFR) in lean and fatty Zucker rats (a model of insulin resistance). Four groups of female Zucker rats were studied. Troglitazone (200 mg/kg) was administered by gavage daily for 3 weeks to lean and fatty Zucker rats. The other 2 groups served as controls. The blood parameters were determined at days 0, 10, and 21. The hepatic SAM and SAH concentrations and MTHFR and CβS were measured in the 3-week liver samples. Plasma homocysteine fell significantly in all troglitazone-treated animals from a mean ± SD of 7.6 ± 1.5 μmol /L to 4.5 ± 1.1 μmol /L ($P < .02$) but not in control animals (5.7 ±1.8 μmol /L to 5.9 ± 1.8 μmol /L). The decreases induced by troglitazone in homocysteine were seen in both the lean and the fatty Zucker rats. This was accompanied by significant rises in the hepatic concentrations of SAH and SAM + SAH. In addition, a significant decline in the hepatic SAM/SAH ratio was observed. The mean ± SD hepatic CβS (expressed as nmol of cystathionine formed at 37°C) in the troglitazone-treated rats was 1,226 ± 47 nmol/h/mg protein, which was significantly higher than that in the control group (964 ± 64 nmol/h/mg protein; $P = .03$). We conclude that troglitazone lowers plasma homocysteine in insulin-resistant animals. The homocysteine-lowering effects of troglitazone may be mediated in part by a shift in the concentrations of tHcy and its related metabolites from the blood to the liver as well as by an upregulation of hepatic CβS activity. These data support the hypothesis that insulin may regulate homocysteine metabolism through regulation of hepatic CβS activity, although activity of other hepatic enzymes not studied here may also contribute to these observations.

AN ELEVATED plasma homocysteine (THCY) is recognized as a risk factor for cardiovascular disease (CVD).[1] Recent studies have demonstrated that plasma tHcy is an important risk factor for CVD and mortality in patients with type 2 diabetes.[2-4] Plasma tHcy is elevated in patients with type 2 diabetes who have coexistent CVD.[5]

Many factors have been found to be important in determining plasma homocysteine concentrations.[6,7] Genetic mutations have been described, which result in changes in the function of enzymes such as methylenetetrahydrofolate reductase (MTHFR) and cystathionine-β-synthase (CβS). Nutritional factors are important, and include plasma concentrations and intake of folic acid and vitamin B_{12}, which serve as cofactors for enzymes that regulate fasting tHcy, and pyridoxine, which is a cofactor for enzymes that regulate plasma tHcy following a methoinine load. Although vitamin status and renal function are the most important determinants of plasma tHcy, data suggest that a variety of drugs and hormones play a role.[8]

Insulin resistance is also a risk factor for CVD and is an early abnormality in the natural history of type 2 diabetes[9]. Thiazolidinediones are a new class of antidiabetic agents that directly target insulin resistance and have a variety of effects on cardiovascular risk factors.[10]

Because of the association between tHcy and CVD risk in diabetes, it is important to determine whether insulin resistance is associated with changes in plasma tHcy. Data on plasma tHcy in patients with diabetes is somewhat confusing due to confounding variables including complications of diabetes, medications, and lack of controlled nutritional status. However, several studies have demonstrated a relationship between plasma insulin and tHcy in well-nourished subjects with normal renal function.[11,12] Furthermore, acute hyperinsulinemia lowers plasma homocysteine concentrations in normal subjects, but not in insulin-resistant patients with type 2 diabetes.[13]

Animal and in vitro studies also suggest a role for insulin and / or insulin resistance in determining plasma homocysteine.[14,15] We have demonstrated that plasma tHcy concentrations were elevated in rats fed a high-fat and -sucrose diet to induce insulin resistance.[14] A decrease in the activity of CβS, a key enzyme in the trans-sulfuration pathway of homocysteine metabolism, accompanied this elevation in homocysteine. Furthermore, plasma insulin correlated positively with plasma tHcy. Studies in cultured hepatocyes also support a role for the effect of insulin in regulating homocysteine metabolism.[16]

To further elucidate the role of insulin/insulin resistance in the regulation of plasma homocysteine, we measured tHcy concentrations and the activity of these enzymes in a genetic model of obesity and insulin resistance, the Zucker fatty rat.

From the Tulane University School of Medicine, New Orleans LA; Univeristy of Arkansas for Medical Sciences, Little Rock, AR; and the National Center for Toxicological Research, Jefferson, AR.

Submitted September 10, 2001; accepted December 31, 2001.

Supported by a grant from Parke Davis Pharmaceuticals, Morris Plains, NJ. Research in Diabetes and Cardiovascular Disease at Tulane University Medical Center is supported in part by the John C. Cudd Memorial Fund and the Tullis Tulane Alumni Chair in Diabetes. V.F. and S.N.M. are supported in part by the American Heart Association, Southeastern Affiliate.

Address reprint requests to Vivian A. Fonseca, MD, Department of Medicine, Section of Endocrinology, Tulane University School of Medicine, 1430 Tulane Ave SL-53, New Orleans, LA 70112-2699.

0026-0495/02/5106-0013$35.00/0

doi:10.1053/meta.2002.32731

The treatment with insulin sensitizers such as with the thiazolidinedione (troglitazone) have been shown to reduce insulin resistance in this animal model. We therefore studied homocysteine metabolism in animals treated with troglitazone for a period of 3 weeks.

MATERIALS AND METHODS

Female Zucker fatty rats (fa/fa; Genetic Models Inc, Indianapolis, IN) were randomized to receive either troglitazone (Parke Davis, Ann Arbor, MI) CβS (n = 7) or no treatment (n = 7). We also studied lean Zucker rats with either troglitazone (n = 7) or no treatment (n = 7). All animals were 10 weeks old, and nondiabetic. Animals were fed with a standard laboratory rodent diet (Labdiet; PMI Nutrition International, Richmond, IN). This diet contains approximately 28% of calories from protein, 12% from fat, and 60% from carbohydrate. The diet contains multiple vitamins including folic acid 5.9 ppm, pyridoxine 6.0 ppm, and vitamin B_{12} 22 μg/kg.

Troglitazone, 200 mg/kg/d, was administered by gavage for 3 weeks. Although much higher than the dose used in humans, this dose has been shown to alleviate insulin resistance in zucker rats. After 3 weeks of treatment, the animals were killed, and blood and liver tissue was obtained and immediately frozen for later analysis.

Hepatic CβS and MTHFR activity was measured by previously described methods.[14] We also measured the hepatic and erythrocyte concentrations of the body's chief physiological methyl donor *S*-adenosylmethionine (SAM) and of its metabolite and inhibitor *S*-adenosylhomocysteine (SAH). The analyses of SAM and SAH in the blood and liver extracts were conducted by high-performance liquid chromatography (HPLC).[17-19] Plasma homocysteine was measured before and after troglitazone treatment using the Bio-Rad homocysteine analysis kit (Bio-Rad Diagnostics Group, Hercules, CA).[20]

Statistical analysis was performed using Sigmastat software (HALLOGRAM, Aurora, CO). Comparisons between groups were made using the 1–way analysis of variance (ANOVA) and *t* tests. Data are expressed as the mean ± SD.

RESULTS

At the time of death the mean ± SD body weight in the fatty rats was 334 ± 25 g and was significantly less in the lean rats, 182 ± 14 g ($P < .001$). The plasma tHcy in the Zucker fatty rats did not differ significantly from that in lean animals (6.2 ± 1.9 μmol /L *v* 6.5 ±1.6 μmol /L, respectively). Since there was no difference in plasma tHcy between groups, we pooled data from both groups to determine the effect of troglitazone treatment.

Plasma homocysteine fell significantly in all troglitazone-treated animals from 7.6 ± 1.5 μmol/L to 4.5 ± 1.1 μmol/L ($P < .02$), but not in control animals (5.7 ±1.8 μmol/L to 5.9 ± 1.8 μmol/L). The effect was more marked in the Zucker fatty rats in which plasma tHcy fell significantly from 6.2 ± 0.5 μmol/L to 3.9 ± 0.5 μmol/L in troglitazone-treated animals ($P = .02$). The effect of treatment on plasma tHcy is illustrated in Fig 1.

The hepatic CβS activity (expressed as nmol of cystathionine formed at 37°C) in the troglitazone-treated rats was 1,226 ± 47 nmol/h/mg protein, which was significantly higher than that in the control group (964 ± 64 nmol/h/mg protein; $P = .03$).

The mean plasma insulin was 186.9 ± 42 pmol/L in the fatty rats and fell significantly with troglitazone to 114.5 ± 21 pmol/L ($P < .001$). In the lean rats, plasma insulin was 61.4 ± 8 pmol/L and fell to 35.8 ± 19 pmol/L ($P = .018$). There was no change in the plasma insulin in the control rats over the 3-week period.

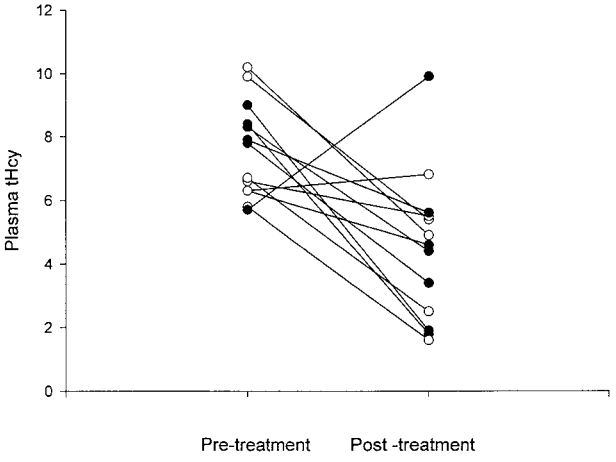

Fig 1. Effect of treatment with troglitazone on plasma homocysteine (μmol/L) in Zucker rats.

The mean hepatic MTHFR activity in the troglitazone-treated rats was 3.5 ± 1.4 nmol/h/mg, which was not significantly different compared to that in the control group (3.2 ± 1.2 nmol/h/mg).

The erythrocyte concentrations of SAM and SAH did not differ between the fatty and lean rats and changed during the course of these studies (Table 1). Table 1 indicates that the concentrations of SAM increased and that those of SAH decreased during the course of the 3-week experiment. This resulted in significant increases in the erythrocyte SAM/SAH ratios. While the time-dependent changes in SAM, SAH and SAM/SAH ratios were not invariably seen by pairwise comparisons between the groups in Table 1, regression analysis of combined groups against time showed that the changes seen in the following groups were all significant ($P < .01$): Zucker lean combined, Zucker fat combined, troglitazone-treated, and combined fat + lean controls. No significant effect of troglitazone treatment or of fat status on the time-dependent changes in erythrocyte SAM, SAH, or SAM/SAH ratios was observed. No other consistent effect of treatment or group was observed on erythrocyte SAM and SAH.

Hepatic SAM did not differ between fatty and lean rats (24.3 ± 1.1 *v* 21.5 ± 1.2 μg/g, respectively) nor was there any difference between treated and untreated rats (24.5 ± 1.1 *v* 21.5 ± 1.2 μg/g. Similarly, hepatic SAH did not vary significantly between the fatty and the lean animals (Table 1). However, in rats treated with troglitazone, hepatic SAH was significantly higher than control rats (Table 1). When data from the lean and fatty groups were combined, the hepatic SAM/SAH ratio in the troglitazone-treated group was 2.7 ± 0.1, which was significantly lower than that in untreated rats, 3.4 ± 0.2. Similarly, the SAM+SAH concentrations were higher in the livers of the troglitazone-treated animals than they were in the corresponding controls (84.2 ± 3.6 *v* 69.9 *v* 3.5 nmol/g for the troglitazone-treated animals and the controls, respectively).

To determine whether there was a relationship between

Table 1. Hepatic and Erythrocyte SAM and SAH in Zucker Rats

Tissue	Parameter	Time	Group			
			Fat + Troglitazone	Fat Only	Lean + Troglitazone	Lean Only
RBC	SAM (μg/g)	0	5.0 ± 0.2	4.3 ± 0.4	5.3 ± 0.5	5.1 ± 0.2
		1	6.1 ± 0.4‡	5.4 ± 0.2*	6.3 ± 0.2	6.4 ± 0.3‡
		2	6.1 ± 0.4‡	5.5 ± 0.4	5.8 ± 0.4	6.6 ± 0.6‡
	SAH (μg/g)	0	0.74 ± 0.09	0.79 ± 0.08	0.83 ± 0.06	0.86 ± 0.03
		1	0.62 ± 0.06	0.53 ± 0.03*	0.57 ± 0.04‡	0.68 ± 0.05‡
		2	0.32 ± 0.03‡	0.31 ± 0.03	0.33 ± 0.05‡	0.43 ± 0.05‡
	SAM/SAH	0	8.1 ± 1.3	5.8 ± 0.8	6.54 ± 0.7	6.0 ± 0.4
		1	10.3 ± 1.4	10.5 ± 0.6‡	11.5 ± 1.1‡	9.6 ± 0.8‡
		2	20.2 ± 2.1	19.2 ± 2.9‡	19.0 ± 2.8‡	16.6 ± 1.8‡
Liver	SAM (μg/g)	2	24.9 ± 1.4	23.7 ± 1.8	24.1 ± 1.8†	19.4 ± 1.3
	SAH (μg/g)	2	9.5 ± 0.9†	6.0 ± 0.4	8.0 ± 0.3†	6.2 ± 0.5
	SAM/SAH	2	2.58 ± 0.16†	3.84 ± 0.30*	2.90 ± 0.19	3.03 ± 0.18
Liver	SAM + SAH (μmol/L)	2	87 ± 5	75 ± 5	81 ± 5	65 ± 5

*Significantly different from corresponding lean ($P < .05$).

†Significantly different from corresponding controls not receiving troglitazone ($P < .05$).

‡Significantly different from corresponding O-time controls ($P < .05$).

Abbreviations: time 0, start of experiment; time 1, 2 weeks; time 2, 3 weeks.

changes in plasma tHcy and tissue concentrations of the metabolites we carried out regression analysis between the concentrations of tHcy in blood and those of SAM and SAH in liver and erythrocytes. An inverse linear correlation was noted between the concentrations of tHcy and hepatic SAM ($P < .01$). Similarly, as the concentrations of erythrocyte (red blood cell [RBC]) SAM + SAH declined, those of hepatic SAM + SAH increased ($P < .05$). These results are consistent with a tHcy-lowering effect of troglitazone mediated by the increased utilization or retention of homocysteine by the liver.

DISCUSSION

Our data demonstrate that troglitazone treatment led to a significant decrease in plasma tHcy and plasma insulin in Zucker rats. Our data suggest 2 possible mechanisms for this effect: (1) an increase in in the activity of CβS, and (2) increased utilization or retention of homocysteine by the liver.

Plasma homocysteine concentrations in Zucker fatty rats were no different from lean Zucker rats. These results contrast with our previous findings in rats made obese through high-fat and sucrose feeding.[14] This difference may relate to the genetic differences between the rat model and also subtle changes in nutritional status that may have been induced during the relatively long period of high-fat feeding in our previous experiments. Troglitazone treatment led to a significant reduction in plasma homocysteine in both lean and fatty rats, indicating a direct effect of the drug independent of the degree of obesity or insulin resistance.

Data from other studies indicate that the activity of CβS is modulated by insulin.[15] In an insulin-deficient animal CβS activity is decreased and is increased with insulin replacement, whereas in high-fat and -sucrose-fed animals insulin resistance is associated with decreased CβS activity. Thus, it is possible that troglitazone enhances any effect that insulin may have on this enzyme. No effect of troglitazone was seen on hepatic MTHFR activity. Since troglitazone works by enhancing insulin action these data support a role for insulin in homocysteine metabolism in general and in the regulation of CβS activity in particular.

With treatment, the erythrocyte SAM + SAH concentrations declined, while those of hepatic SAM + SAH increased. These findings are consistent with the tHcy-lowering effect of troglitazone. A recent study using rats fed an atherogenic, homocystine-containing diet has described similar findings.[21] Compared to animals fed a homocystine-supplemented, folate-deficient diet, rats fed the corresponding diet containing methionine and folate, like those treated with troglitazone in the present investigation, showed decreased tHcy and increased hepatic SAM + SAH concentrations.[21] Thus, troglitazone treatment resembled that of the dietary methyl donors in elevating the hepatic concentrations of the adenosylated derivatives of methionine and homocysteine. RBC SAH declined significantly with time with a concomitant increase in SAM that was significant only in the untreated animals. As a result there is a reduction in a time-dependent increase in the SAM/ SAH ratio with troglitazone. At the time of death, this ratio was significantly lower in the liver in the troglitazone-treated animals. The significance of this change in ratio is unclear but may represent a change in methylation status. We have previously demonstrated abnormalities in the relationship between these metabolites and their feedback regulation of enzyme activity in patients with diabetes.[22]

The effects of troglitazone on the absolute concentrations of SAM and SAH are relatively minor. It has been suggested that these metabolites, particularly SAM, regulate the activity of enzymes in tHcy metabolism. The lack of a major change in both hepatic and erythrocyte SAM in the face of changes in the activity of CβS suggest that other factors regulate CβS activity. These data support the hypothesis that insulin and/or insulin resistance may modulate plasma tHcy concentrations through changes in CβS activity.[8,14,15] We have not investigated the possibility that the changes seen in our study relate to the effects of the drug on other enzymes in the complex metabolic pathways involved in metabolism of homocysteine.[23] We have selected the 2 enzymes previously shown to be affected by

insulin and also known to be important in the pathogenesis of cardiovascular disease associated with hyperhomocysteinemia. Further research is needed to determine whether other enzymes are involved in this process.

Thiazolidinediones act through activation of peroxisome-proliferator activator receptors, a family of nuclear receptors, which serve as transcription factors for several genes. These receptors are ubiquitous in various tissues. Thiazolidinediones have been shown to have many effects independent of their glucose-lowering effect.[10] Our data add to the growing body of literature supporting their potential for favorable effects on cardiovascular risk factors associated with insulin resistance. Further work is needed to establish whether other thiazolidinediones have similar effects and whether these effects are also seen in human subjects with insulin resistance and type 2 diabetes.

ACKNOWLEDGMENT

The exceptional technical ability of Carolyn Wise of the National Center for Toxicological Research and Kimberly Poole at Tulane University is gratefully acknowledged.

REFERENCES

1. Eikelboom JW, Lonn E, Genest JJ, et al: Homocyst(e)ine and cardiovascular disease: A critical review of the epidemiologic evidence. Ann Intern Med 131:363-375, 1999

2. Hoogeveen EK, Kostense PJ, Jakobs C, et al: Hyperhomocysteinemia increases risk of death, especially in type 2 diabetes: 5-year follow-up of the Hoorn Study. Circulation 101:1506-1511, 2000

3. Stehouwer CD, Gall MA, Hougaard P, et al: Plasma homocysteine concentration predicts mortality in non-insulin-dependent diabetic patients with and without albuminuria. Kidney Int 55:308-314, 1999

4. Kark JD, Selhub J, Bostom A, et al: Plasma homocysteine and all-cause mortality in diabetes. Lancet 353:1936-1937, 1999 (letter)

5. Munshi MN, Stone A, Fink L, et al: Hyperhomocysteinemia following a methionine load in patients with non-insulin-dependent diabetes mellitus and macrovascular disease. Metabolism 45:133-135, 1996

6. Lussier-Cacan S, Xhignesse M, Piolot A, et al: Plasma total homocysteine in healthy subjects: sex-specific relation with biological traits. Am J Clin Nutr 64:587-593, 1996

7. Jacobsen DW: Determinants of hyperhomocysteinemia: A matter of nature and nurture. Am J Clin Nutr 64:641-642, 1996 (editorial)

8. Fonseca V, Guba SC, Fink LM: Hyperhomocysteinemia and the endocrine system: implications for atherosclerosis and thrombosis. Endocr Rev 20:738-759, 1999

9. Reaven GM: The role of insulin resistance and hyperinsulinemia in coronary heart disease. Metabolism 41:16-19, 1992

10. Parulkar AA, Pendergrass ML, Granda-Ayala R, et al: Nonhypoglycemic effects of thiazolidinediones. Ann Intern Med 134:61-71, 2001

11. Meigs JB, Jacques PF, Selhub J, et al: Fasting plasma homocysteine levels in the insulin resistance syndrome: The Framingham offspring study. Diabetes Care 24:1403-1410, 2001

12. Emoto M, Kanda H, Shoji T, et al: Impact of insulin resistance and nephropathy on homocysteine in type 2 diabetes. Diabetes Care 24:533-538, 2001

13. Fonseca VA, Mudaliar S, Schmidt B, et al: Plasma homocysteine concentrations are regulated by acute hyperinsulinemia in nondiabetic but not type 2 diabetic subjects. Metabolism 47:686-689, 1998

14. Fonseca V, Dicker-Brown A, Ranganathan S, et al: Effects of a high-fat-sucrose diet on enzymes in homocysteine metabolism in the rat. Metabolism 49:736-741, 2000

15. Jacobs RL, House JD, Brosnan ME, et al: Effects of streptozotocin-induced diabetes and of insulin treatment on homocysteine metabolism in the rat. Diabetes 47:1967-1970, 1998

16. Dicker-Brown A, Fonseca VA, Fink LM, et al: The effect of glucose and insulin on the activity of methylene tetrahydrofolate reductase and cystathionine-beta-synthase: Studies in hepatocytes. Atherosclerosis 158:297-301, 2001

17. Cooney CA, Wise CK, Poirier LA: An improved sample preparation method for the quantitative hplc determination of 5-methyldeoxycytidine in animal tissue DNA. J Liquid Chromatogr Rel Technol 20:1279-1293, 1997

18. Wise CK, Fullerton FR: Analytical procedure for determination of S-adenosylmethionine, S-adenosylhomocysteine, and S-adenosylethionine in the same isocratic HPLC run, with a procedure for preparation and analysis of the analog S-adenosylhomocysteine sulfoxide. J Liquid Chromatogr 18:2005-2017, 1995

19. Halsted CH, Villanueva J, Chandler CJ, et al: Ethanol feeding of micropigs alters methionine metabolism and increases hepatocellular apoptosis and proliferation. Hepatology 23:497-505, 1996

20. Jacobsen DW, Gatautis VJ, Green R, et al: Rapid HPLC determination of total homocysteine and other thiols in serum and plasma: Sex differences and correlation with cobalamin and folate concentrations in healthy subjects. Clin Chem 40:873-881, 1994

21. Southern F, Eidt J, Drouilhet J, et al: Increasing levels of dietary homocystine with carotid endarterectomy produced proportionate increases in plasma homocysteine and intimal hyperplasia. Atherosclerosis 158:129-138, 2001

22. Poirier LA, Brown AT, Fink LM, et al: Blood S-adenosylmethionine concentrations and lymphocyte methylenetetrahydrofolate reductase activity in diabetes mellitus and diabetic nephropathy. Metabolism 50:1014-1018, 2001

23. Finkelstein JD: Pathways and regulation of homocysteine metabolism in mammals. Semin Thromb Hemost 2000;26:219-225, 2001