Troglitazone Reduces Reactive Oxygen Species Generation by Leukocytes and Lipid Peroxidation and Improves Flow-Mediated Vasodilatation in Obese Subjects

Rajesh Garg, Yuvraj Kumbkarni, Ahmad Aljada, Priya Mohanty, Husam Ghanim, Wael Hamouda, Paresh Dandona

Abstract—Because troglitazone has been shown to have antioxidant properties, we investigated whether troglitazone administration to obese subjects causes a reduction in (1) reactive oxygen species (ROS) generation by polymorphonuclear leukocytes (PMNLs) and mononuclear cells (MNCs) and (2) lipid peroxidation as reflected in the plasma concentrations of 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE). Seven obese subjects were given 400 mg/d troglitazone for 4 weeks. Blood samples were obtained before troglitazone administration and at weekly intervals thereafter. Insulin concentrations fell significantly at week 1 and remained low at weeks 2 and 4 (P<0.001). ROS generation by PMNLs fell to 77.6±25.1% of the basal at week 1 and 47.9±41.1% at week 4 (P<0.001). ROS generation by MNCs fell to 59.8±15.7% of the basal at week 1 and 35.1±17.6% at week 4 (P<0.001). 9-HODE and 13-HODE concentrations fell significantly from 787.4±52.4 and 713.1±44.7 pg/mL to 720.4±66.7 (P<0.004) and 675.2±65.0 pg/mL (P<0.01) after 4 weeks, respectively. Postischemic dilatation of the brachial artery was measured by ultrasonography. The mean percent dilatation after forearm ischemia before and after troglitazone was 5.5±3.01% and 8.75±3.37% (P<0.001), respectively. The percent increase in diameter after nitroglycerin was 17.08±1.18% before troglitazone, whereas it was 18.9±1.91% (P<0.02) after troglitazone. We conclude that troglitazone has a potent and rapid biological inhibitory effect on ROS generation by PMNLs and MNCs and that it inhibits lipid peroxidation significantly. These changes are associated with a significant improvement in postischemic flow-mediated vasodilation in the brachial artery over a relatively short period of 4 weeks. (Hypertension. 2000;36:430-435.)

Key Words: troglitazone ■ antioxidants ■ vasodilatation

Obesity is now considered a major independent risk factor for coronary heart disease and atherosclerosis. The mechanism underlying increased atherogenesis is unclear. Several mechanisms may be responsible, and these issues need careful investigation.

Lipid peroxidation, including the conversion of LDL to oxidized LDL, is cardinal in the process of formation of the fatty streak, the initial lesion of atherosclerosis. We have recently demonstrated that in obese subjects, the enzyme generating the superoxide (O2−) radical in the mononuclear cells (MNCs), NADPH oxidase, is relatively nonsuppressible in the obese when incubated with the specific inhibitor diphenyleneiodonium chloride. However, there is no difference in the basal reactive oxygen species (ROS) generation by the MNCs or by the polymorphonuclear leukocytes (PMNLs) in the lean and obese subjects from our previous work. Superoxide radical generation by the MNCs thus is not inhibited adequately in the obese. Because ROS generation and superoxide generation (O2−) in particular may be responsible for the oxidative conversion of LDL to oxidized LDL, it is important to assess the effect of weight loss and antioxidants on ROS generation by PMNLs and MNCs. Our previous study showed that PMNLs and MNCs are suppressed by glucocorticoids. This led us to investigate the effect of troglitazone on PMNLs, although these cells have not been shown to have peroxisome proliferator–activated receptor-γ (PPARγ) receptors.

Although thiobarbituric acid–reactive substances (TBARS) are the long-standing indices of lipid peroxidation, they are not specific, and now more specific indices of 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), the products of ROS-induced oxidation of linoleic acid, are being used. Similarly, carbonylated proteins reflect oxidative damage of proteins. Ortho-tyrosine (o-tyrosine) and meta-tyrosine (m-tyrosine) are the products of ROS attack on phenylalanine and are accepted as indices of amino acid oxidation.
It is possible that the oxidative damage to proteins may be reduced with reduction in ROS generation.

Troglitazone, a thiazolidinedione bound to an α-tocopherol moiety, has antioxidant properties and therefore may be expected to reduce oxidative damage. We have previously demonstrated that troglitazone has a potent antioxidant activity in vitro, and it was recently shown that troglitazone may prevent lipid peroxidation of LDL, in vitro. It has also recently been demonstrated that vitamin E (tocopherol) given to humans causes a reduction in ROS generation by leukocytes. We have therefore embarked on an investigation to determine (1) whether troglitazone exerts an antioxidant effect in the obese by inhibiting ROS generation by PMNLs and MNCs; (2) whether troglitazone reduces lipid peroxidation, as reflected by TBARs, 9-HODE, 13-HODE, and amino acid oxidation, as reflected in α-tirosine and m-tirosine; and (3) whether troglitazone improves vascular reactivity, as reflected in postischemic vasodilatation of the brachial artery in the forearm of subjects known to have an increased oxidative load.

Methods

Subjects
Seven obese subjects (age 32 to 52 years, mean 40.6 ± 8.0 years) with a body mass index >37 kg/m² (body weight range 91.4 to 157.3 kg, mean 127.8 ± 27.0 kg; body mass index range 37.3 to 60.9, mean 46.1 ± 8.7 kg/m²) were included in this study (Table 1). Subjects included 6 women and 1 man. There were 6 whites and 1 black. All subjects had a fasting venous plasma glucose of <110 mg/dL. None of the obese subjects were on vitamin E or C or any other antioxidant therapy. The subjects were not advised any special diet, and none of them were actively trying to lose weight during the duration of the study. There was no significant change in weight and blood pressure at the end of the study. The drugs that the patients were taking were not altered for the duration of this study; all patients had been on these drugs for 3 months at the current dose level.

The Institutional Review Board of the Millard Fillmore Hospital at the State University of New York at Buffalo approved the study. Written informed consent was obtained from all subjects.

Troglitazone Treatment and Follow-Up
Baseline liver function tests were performed in each patient. The patients were then given 400 mg/d troglitazone for 4 weeks. A weekly follow-up was performed to note any side effects of the drug and to collect fasting blood samples at each weekly visit. Tablet count were counted at the end of every week to verify compliance. Liver function tests were repeated at the end of 4 weeks. Brachial artery reactivity with ultrasonography was performed before and after 4 weeks of troglitazone administration.

Preparation of PMNLs and MNCs
Blood samples were collected with Na-EDTA as an anticoagulant. Three and one half milliliters of the anticoagulated blood sample was carefully layered over 3.5 mL of PMN medium (Robbins Scientific Corp) in a 5-mL centrifuge tube. The samples were centrifuged at 450g in a swing-out rotor for 30 minutes at 22°C. At the end of centrifugation, 2 bands separated out at the top of the RBC pellet. The top band consisted of MNCs, and the bottom band consisted of PMNLs. The bands were harvested with a Pasteur pipette. The harvested cells were repeatedly washed with Hanks’ balanced salt solution and were reconstituted to a concentration of 4 × 10⁶ cells/mL in the solution. This method yields >95% pure PMNLs and MNCs suspensions.

Assay of ROS Generation
ROS generation was measured by our method, which was developed independently; this method is similar to that published by Tosi and Hamedani. In this assay system, the release of superoxide radical, as measured by chemiluminescence, has been shown to be linearly correlated with that measured by the ferricytochrome c method and to be inhibited by diphenylpyrylum chloride.

Assay of TBARS
TBARS were assayed by the method described by Ohkawa et al.

Assay of 13-HODE and 9-HODE
Hydroxy polyunsaturated fatty acids were measured by a modification of the HPLC-based method of Browne and Armstrong. Total lipid extracts were made from 0.5 mL of EDTA plasma according to a modification of Hara and Radin with hexane isopropanol 3:2. Extracts were then saponified in 0.5 mol/L ethanolic NaOH according to Thomas and Jackson to release the free acids.

Assay of α-Tirosine and m-Tirosine
α-Tirosine and m-tirosine determinations in serum were performed with HPLC-fluorometric detection as described by Ishimitsu et al with modification.

Measurement of Brachial Artery Reactivity
All vascular imaging studies were conducted in an environmentally controlled laboratory at a constant temperature of 21°C. Participants were made comfortable in the supine position, at which point a sphygmomanometer cuff was placed on the forearm and a 3-lead ECG was set in the normal fashion. An Acuson 128XP/10c high-resolution ultrasonograph with a 7.5-MHz linear array transducer was used. The brachial artery diameter was measured at baseline. The forearm was compressed 40 mm above the systolic blood pressure for 5 minutes, and brachial artery diameter was recorded at 15 seconds and again at 45 to 60 seconds. Once the baseline was reached, ultrasound imaging was performed to determine the extent of brachial artery vasodilatation.
achieved, the subject was given 0.3 mg of nitroglycerin sublingually, and the brachial artery diameter was measured after 5 minutes. Details of the methodology for vascular reactivity have previously been described in detail.18

Results

Plasma Glucose, Insulin, Lipid, and Blood Pressure Levels

Plasma glucose at baseline was 96 ± 15 mg/dL (5.28 ± 0.83 mmol/L). It was 94 ± 6 ± 125 mg/dL (5.17 ± 0.49 mmol/L) at week 1, 94.6 ± 8.9 mg/dL (5.17 ± 0.49 mmol/L) at week 1, 94.6 ± 8.9 mg/dL (5.17 ± 0.49 mmol/L) at week 2, 87.9 ± 7.7 mg/dL (4.83 ± 0.42 mmol/L) at week 3, and 93.3 ± 6 ± 10.2 mg/dL (5.13 ± 0.62 mmol/L) at week 4. Plasma insulin concentrations fell significantly from 31.2 ± 26.9 mU/mL at baseline to 14.2 ± 12.5 mU/mL at week 1, 6.9 ± 2.8 mU/mL at week 2, and 7.3 ± 4.9 mU/mL at week 4 (P < 0.001). Serum lipid concentrations were as follows: triglycerides 118 ± 74 mg/dL (1.35 ± 0.84 mmol/L), cholesterol 189 ± 31 mg/dL (4.91 ± 0.81 mmol/L), HDL 46 ± 11 mg/dL (1.2 ± 0.29 mmol/L), and LDL 120 ± 28 mg/dL (3.12 ± 0.73 mmol/L). Two patients had elevated triglyceride concentrations (>1.71 mmol/L). The mean concentrations of these indices remained unchanged after troglitazone. Triglyceride concentrations fell in 5 patients, whereas they increased in 2 patients. The increase occurred in patients whose triglyceride concentrations were normal. Blood pressure did not change after troglitazone in the 4-week period. Systolic blood pressure fell in 3 subjects, did not change in 3 subjects, and increased in 1 subject. Diastolic blood pressure increased in 4 subjects, whereas it did not change in 3 subjects.

ROS Generation

ROS generation decreased significantly after treatment with troglitazone. This decrease was evident after 1 week and continued until week 4. ROS generation by PMNLs was 324.3 ± 312.7 mV at baseline (100%). It fell to 77.6 ± 25.1% of the basal at week 1, 52.7 ± 29.5% of the basal at week 2, 42.6 ± 14.2% of the basal at week 3, and 47.9 ± 41.1% of the basal at week 4 (F = 5.99; P = 0.001) (Figure 1). ROS generation by MNCs was 316.4 ± 55.7 mV at baseline (100%). It fell to 59.8 ± 15.7% of the basal at week 1, 53.7 ± 27.4% of the basal at week 2, 36.6 ± 18.5% of the basal at week 3, and 35.1 ± 17.6% of the basal at week 4 (F = 14.65; P < 0.001) (Figure 2). The most impressive fall was at week 1 for both PMNLs and MNCs.

Lipid Peroxidation

Plasma TBARS concentration fell from 1.14 ± 0.22 to 0.99 ± 0.18 mmol/L at 4 weeks, which was not significant. Plasma 9-HODE concentrations fell from 787.4 ± 52.4 to 720.4 ± 66.7 pg/mL at 4 weeks (P < 0.004). Also, plasma 13-HODE concentrations fell from 713.1 ± 44.7 to 675.2 ± 65.0 pg/mL at 4 weeks (P < 0.01) (Figure 3). Linoleic acid concentration did not change significantly.

Plasma o-Tyrosine and m-Tyrosine Concentrations

Plasma m-tyrosine concentrations fell from 6.26 ± 1.4 to 6.15 ± 1.52 ng/mL (NS) and o-tyrosine concentrations fell from 5.94 ± 1.27 to 5.83 ± 1.3 ng/mL (NS) at 4 weeks.
Endothelium-Dependent Vascular Reactivity

The mean basal diameter of the brachial artery before troglitazone therapy was 3.37 ± 0.39 mm, and the mean basal diameter after the troglitazone was 3.31 ± 0.40 mm (NS). The postischemic diameter before troglitazone was 3.56 ± 0.43 mm, and the postischemic diameter after troglitazone was 3.60 ± 0.42 mm. Thus, the mean percent dilatation after forearm ischemia was 5.5 ± 3.01%, whereas the mean percent dilatation after troglitazone was 8.75 ± 3.37% (P = 0.02). The mean postnitroglycerin (NTG) diameter before troglitazone was 4.01 ± 0.42 mm, whereas the mean post-NTG diameter after troglitazone was 3.96 ± 0.45 mm. The percent increase in diameter after NTG was 17.08 ± 1.18% before troglitazone, whereas it was 18.9 ± 1.91% after troglitazone (P < 0.02) (Figure 4 and Table 2).

Discussion

Our data show clearly for the first time that the administration of troglitazone to obese subjects results in a progressive reduction in ROS generation by both PMNLs and MNCs. A significant fall in ROS generation was observed at 1 week, which steadily became more marked over the course of 4 weeks. The reduction in ROS generation after troglitazone therapy was more marked than that observed with vitamin E given at a dose of 800 IU.11 This amount of vitamin E is greater than the vitamin E contained in 400 mg of troglitazone (≈200 IU). This ROS-suppressive effect of troglitazone is probably caused by the thiazolidinedione moiety of this molecule in addition to the α-tocopherol moiety. Because the lipid concentrations and the blood pressure did not change during the study, the changes in ROS generation are independent of these variables. It should be mentioned that in diabetic patients, the administration of 400 mg of troglitazone has been shown to produce a significant reduction in triglyceride concentrations.19 It is possible that in the obese, a longer period of troglitazone is necessary induce a fall in triglyceride concentrations.

Troglitazone has the ability to bind to both PPARα and PPARγ receptors.20 In addition, circulating monocytes are known to have both PPARα and PPARγ receptors,21 to which troglitazone is known to bind. PPARα-mediated effects are known to increase fatty acid metabolism and to exert an anti-inflammatory effect.21 It is through PPARγ receptors that thiazolidinediones are known to exert their insulin-sensitizing and -synergizing effect in adipocytes and possibly in the skeletal muscle.22 It is possible that in the monocyte, ROS generation is modulated by PPARα, PPARγ, or a combination of both. The fact that even PMNLs respond to troglitazone by reducing ROS generation by a magnitude similar to that observed in MNCs implies that PMNLs may also have PPARα and/or PPARγ receptors. This area requires further investigation.

An increase in ROS generation, especially O2•−, reduces the bioavailability of nitric oxide and therefore has an effect in limiting vasodilatation and is thus proconstrictor in nature. A reduction in ROS generation would have a potential vasodilatory influence. Our observations on brachial artery vasodilatation support this concept: postischemic vasodilatation of the brachial artery was enhanced significantly after troglitaza-
zone treatment. It is also of interest that there was a small, but significant, increase in the vasodilatory response to NTG (glyceryl trinitrate) after troglitazone. This suggests that even the vasodilatory response of the brachial artery to exogenous nitrate may be modulated by ROS. There was a small decline in the baseline diameter of the brachial artery after 4 weeks, but it was not significant. The fact that the basal diameter was smaller after troglitazone may have contributed to the increase in post–NTG–(glyceryl trinitrate) vasodilatation; this is a limitation in our data.

Postischemic vasodilatation of the brachial artery has recently been shown to increase from 4.5% to 6.5% of the basal diameters after troglitazone treatment in patients with coronary heart disease.23 These patients also reported a fall in the frequency and duration of anginal episodes and nitrate usage.23 Clearly, troglitazone has a vascular effect of some clinical relevance. Our observations on postischemic vasodilatation in the obese may also have a potential role in the development of macrovascular disease in the obese. In addition to the beneficial effect on endothelial function in the obese, the reduction of ROS generation may also reduce oxidative damage of lipids as reflected in diminution in plasma TBARS, 9-HODE, and 13-HODE concentrations.

ROS generation in our assay is a measure of superoxide production. $\alpha$-Tyrosine and $\mu$-tyrosine are produced by the attack of the hydroxyl radical to the phenylalanine ring. Hence, it is possible that the $\alpha$-tyrosine and $\mu$-tyrosine did not change despite a significant fall in ROS generation. Recently, it has also been demonstrated that the oxidizability of LDL by divalent cations, in vitro, is inhibited after treatment of type II diabetics with troglitazone.9 There are several other studies

![Figure 3. Percent fall in plasma concentration of (A) 9-HODE and (B) 13-HODE after 400 mg of troglitazone. There was a significant fall in the 2 indices at week 4.](image)

![Figure 4. Percent change before and after troglitazone administration in brachial artery diameter after (A) ischemia and (B) nitroglycerin.](image)

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<th>TABLE 2. Endothelium-Dependent Vascular Reactivity After Troglitazone Treatment for 4 Weeks</th>
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that report a beneficial affect of troglitazone on blood vessels. Thus, progression of atherosclerosis in the carotid artery was reversed with intimal medial thickness as an index.\textsuperscript{24}

In conclusion, troglitazone has a powerful inhibitory effect on ROS generation by PMNLs and MNCs, which may be more potent than that of vitamin E. There is also a decrease in lipid peroxidation during this period. This is associated with an increase in postischemic vasodilatation of the brachial artery, which is consistent with an improvement in endothelial function and an increase in nitric oxide bioavailability.

Acknowledgments
The authors thank the William G. McGowan Charitable Fund and Parke-Davis for financial support.

References
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Hypertension. 2000;36:430-435
doi: 10.1161/01.HYP.36.3.430

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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