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Troglitazone Has a Scavenging Effect on Reactive Oxygen Species

Ikuo Inoue,[1] Shigehiro Katayama, Keiichi Takahashi, Kiyohiko Negishi, Takashi Miyazaki,* Masaru Sonoda,* and Tsugikazu Komoda*
*The Fourth Department of Internal Medicine, and *the First Department of Biochemistry, Saitama Medical School, 38 Moro-Hongo, Moroyama, Iruma, Saitama, Japan*

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Troglitazone (CS-045), a newly developed antidiabetic thiazolidinedione that enhances insulin sensitivity, is similar in structure to several antioxidants, including α-tocopherol and probucol. The *in vitro* antioxidant activity of troglitazone has been demonstrated in alloxan-induced hyperlipoperoxidemic and hyperlipidemic mice. In this study, we found that troglitazone had a scavenging effect on reactive oxygen produced by xanthine-xanthine oxidase and generated by stimulated neutrophils and tends to be the radical form. Our results suggest that troglitazone is an antioxidant similar to α-tocopherol. However, under the same conditions, pioglitazone, another thiazolidinedione drug, did not have a scavenging effect. The antioxidant action of troglitazone, which is attributable to the similarity of its molecular structure to that of α-tocopherol, may be of benefit in preventing diabetic vascular complications, in addition to having hypoglycemic and hypolipidemic effects.

Oxidatively modified lipoproteins, especially low-density lipoproteins (LDL), are believed to be involved in atherogenesis. Patients with diabetes mellitus have increased plasma levels of glycated LDL, which is more susceptible to oxidization than native LDL. Therefore, an antidiabetic drug that has both an antioxidant activity and an antihyperlipidemic effect may be of value in the prevention of atherosclerotic disease in diabetes.

Troglitazone (CS-045), one of the thiazolidinedione group, is a newly developed antidiabetic drug (Figure 1) that enhances insulin sensitivity and improves hyperglycemia and hyperlipidemia in obese and non-obese patients with diabetes mellitus (1,2), and also in fructose-fed rats (3, 4).

Troglitazone has been reported to reduce the serum lipid peroxide concentration in mice with alloxan-induced hyperlipoperoxidemia and hyperlipidemia (5), although this reduction may be secondary to a decrease in total serum triglyceride (TG) or cholesterol (TC) (6). However, it is possible that this effect of troglitazone may be due to its molecular structure, which is similar to that of α-tocopherol, an established antioxidant. Although Nagasaka *et al.* reported that troglitazone has an inhibitory effect on peroxidation in human LDL (7), the exact mechanism remains to be clarified.

In this study, we investigated whether troglitazone has an *in vitro* action to scavenge reactive oxygen species produced by xanthine-xanthine oxidase or generated by stimulated neutrophils. In addition, we compared the scavenging effect of troglitazone with that of α-tocopherol and pioglitazone, another thiazolidinedione, whose molecular is unlike that of α-tocopherol (Figure 1). And electron spin resonance (ESR) spectra of troglitazone, α-tocopherol, and pioglitazone radicals were also studied.

MATERIALS AND METHODS

Materials. Xanthine-xanthine oxidase, α-tocopherol, phorbol myristate acetate (PMA), human recombinant Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD), hydrogen peroxide, ammonium iron (II) sulfate, and cytochrome *c* were obtained from Wako Pure Chemicals, Osaka, Japan. Troglitazone was a generous gift from Sankyo Co., Tokyo, Japan, and pioglitazone a generous gift from Takeda Co., Osaka, Japan.

Reactive oxygen species assay. The scavenging activity of troglitazone, α-tocopherol, and pioglitazone was estimated from their inhibitory effect on superoxide-induced reduction of cytochrome *c.* Superoxide was generated by the xanthine-xanthine oxidase reaction (8) or by PMA-stimulated neutrophils (9), as described below. Cytochrome *c* reduction was measured by the increase in absorbance at 550 nm.

Troglitazone, α-tocopherol, and pioglitazone, used as scavengers, were dissolved in 8% dimethylsulfoxide (DMSO). The reaction mixture comprised 60 μl of xanthine (68 μmol/l), cytochrome *c* (30 μmol/l), potassium phosphate buffer (50 mmol/l, pH 7.8) containing EDTA (1 mmol/l), and 300 μl of scavenger solution (0, 50, 100, 200, 400, 600 or 1000 μmol/l containing 8% DMSO). The temperature was maintained at 25°C, and after the reaction had reached a plateau,

[1] To whom correspondence should be addressed. Fax: 81492-94-9752.

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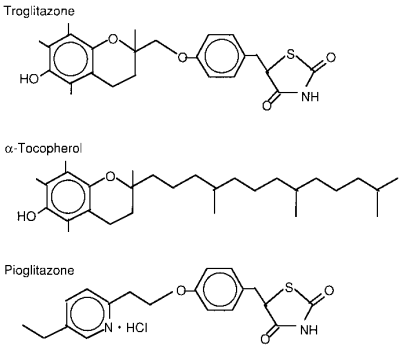

FIG. 1. Structures of troglitazone, α-tocopherol, and pioglitazoane.

20 μl of xanthine oxidase (4.4 mU/l) was added. The reaction was monitored spectrophotometrically for 5 min, and each experiment was performed 20 times.

To generate the reactive oxygen species from stimulated neutrophils, healthy human neutrophils were isolated by Ficoll-Hypaque centrifugation (MONO-POLY RESOLVING MEDIUM, Flow Laboratories Japan Co. Ltd., Tokyo, Japan) and suspended in PBS (pH 7.3) at 6×10^6/ml. To 250 μl of neutrophils, 25 μl of cytochrome *c* (53 μmol/l) and 250 μl of scavenger solution (0, 200, 400, 800 or 1000 μmol/l, containing 2% DMSO) were added and mixed at 37°C. After the reaction had reached a plateau, 250 μl of PMA (0.32 μmol/l) was added to stimulate the neutrophils. The reaction was monitored spectrophotometrically for 5 min, and each experiment was performed 20 times.

Detection of α-tocopherol, troglitazone, and pioglitazone radical in Fenton-type reaction system. The Fenton-type reaction system was used accordingly to slightly modification of Nikolai and others (10-12). This system consisted of 1.0 mM hydrogen peroxide, 0.1 mM ammonium iron (II) sulfate and 1.0 mM α-tocopherol, 1.0 mM troglitazone, or 1.0 mM pioglitazone.

ESR measurements. ESR measurements were performed on JOEL JES-REIX spectrometer (Tokyo, Japan) equipped with a ESPRIT computer system at room temperature (22°C). The spectrometer conditions were : center field 335.0 mT, modulation amplitude 0.25 mT, scan range 5 mT, time constant 30 msec, microwave power 10 mW, accumulation count 20. Samples were placed into a standard quartz cell (60 × 10 × 0.5 mm, Labotec, Co., Tokyo, Japan).

Statistical analysis. All data were expressed as mean ± standard deviation. Differences were assessed by Student's *t* test, and considered to be significant at $P<0.05$.

RESULTS

Figure 2 shows that DMSO at 5%, 8% and 10%, but not at 1% or 2%, generated the reactive oxygen species,

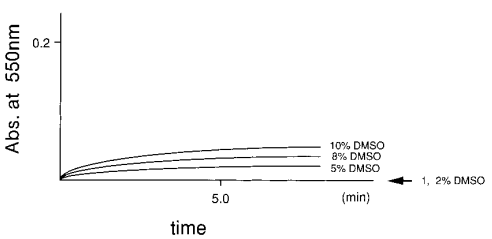

FIG. 2. Effect of DMSO concentration (1%, 2%, 5%, 8%, 10%) on cytochrome *c* reduction.

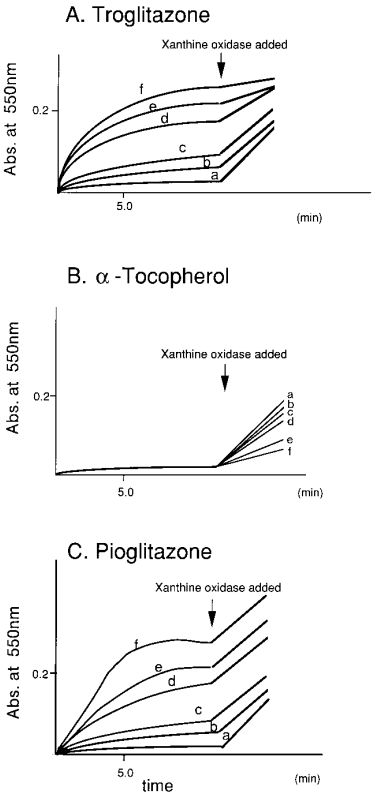

FIG. 3. Effect of troglitazone (A), α-tocopherol (B), or pioglitazone concentration (C) (a: 0 μmol/l, b: 200 μmol/l, c: 400 μmol/l, d: 600 μmol/l, e: 800 μmol/l, or f: 1000 μmol/l) on 8% DMSO-mediated cytochrome *c* reduction with superoxide generated by xanthine-xanthine oxidase. After the reaction had reached a plateau, xanthine oxidase was added (↓).

as reported by other workers (13). Therefore, DMSO itself promoted the reduction of cytochrome *c*.

A typical experiment using troglitazone and α-tocopherol is illustrated in Figure 3. Before addition of xanthine oxidase, troglitazone, but not α-tocopherol, reduced cytochrome *c* dose-dependently (Figure 3). An increase in absorbance at 550 nm means that the drugs promote reduction of cytochrome *c*. When xanthine oxidase is added after the reaction has reached a plateau, the superoxide generated by xanthine-xanthine oxidase will be able to reduce cytochrome *c* in the absence of scavenger, resulting in a further increase in absorbance at 550 nm. The reaction was linear up to at least 5 min (Figure 3-A-a, B-a). When a scavenger such as superoxide dismutase (SOD) is present, the slope of the reduction rate is decreased. In fact, a certain amount of reactive oxygen species was completely scavenged by 15 unit/ml SOD, and the slope was 0.0022 Abs/min in the presence of 7.5 unit/ml.

Both troglitazone (Figure 3-A) and α-tocopherol (Figure 3-B) decreased the slope of the reduction rate dose-dependently, indicating their scavenging effect on the reactive oxygen species generated by xanthine-xan-

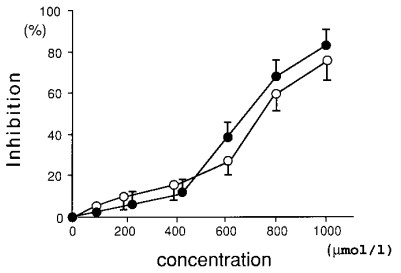

FIG. 4. Inhibition of cytochrome *c* reduction in terms of percentage at various concentrations of troglitazone (●) and vitamin E (○). Each value indicates the mean ± SD.

thine oxidase (troglitazone, from 0.0333 ± 0.0006 Abs/min at 0 μmol/l to 0.0060 ± 0.0006 Abs/min at 1000 μmol/l, $p<0.05$; α-tocopherol, from 0.0331 ± 0.0006 Abs/min at 0 μmol/l to 0.0090 ± 0.0010 Abs/min at 1000 μmol/l, $p<0.05$). However, pioglitazone did not decrease the slope of the reduction rate (Figure 3-C), indicating that the drug did not scavenge reactive oxygen species. The scavenging effect of α-tocopherol and troglitazone in 8% DMSO was dependent on their concentrations, and the difference between them was not significant (Figure 4).

Figure 5 shows that troglitazone dose-dependently scavenged the reactive oxygen species generated by PMA-stimulated neutrophils. Troglitazone at 1000 μmol/l completely scavenged the reactive oxygen species.

Figure 6 shows ESR spectra of α-tocopherol, troglitazone, and pioglitazone in Fenton-type reaction system. The typical ESR signal of α-tocopherol radical was observed (Figure 6-A). The signal of troglitazone radical was also observed, but the intensities were lower than those of α-tocopherol (Figure 6-B). On the other hand, the signal of pioglitazone radical could not be observed (Figure 6-C).

DISCUSSION

Reactive free radicals generated from oxygen are potent mediators of tissue injury associated with many pathological conditions such as inflammatory and ischemic states. Reactive oxygen species such as the superoxide and hydroxyl radicals can act on membrane lipids or lipids in lipoprotein particles, and have been implicated in the development of atherosclerosis. To prevent injury by the reactive oxygen species, two approaches can be adapted: scavenging the reactive oxygen species, and inhibiting reactive oxygen species. Although no drug has been developed specifically as an inhibitor of superoxide production, there are various superoxide scavengers including SOD (14) and hindered phenol agents (5).

Recently, several thiazolidinedione compounds, such as troglitazone, pioglitazone, ciglitazone, and englitazone have been developed as antidiabetic drugs. Of interest is that troglitazone possesses structural similarity to certain antioxidants including α-tocopherol. By contrast, pioglitazone has a different structure to α-tocopherol (Figure 1). In fact, Nagasaka *et al.* have reported that troglitazone has an inhibitory effect on peroxidation of human LDL (7). Our data indicate that troglitazone and α-tocopherol had similar scavenging effects in our xanthine-xanthine oxidase system (Figure 4). Troglitazone and α-tocopherol are both hydrophobic, and may be deposited in tissues. α-tocopherol reacts with free radicals, leading to the production of α-tocopherol radicals (15), which are then immediately recoverted to α-tocopherol by ascorbic acid (16), glutathione (17), uric acid and other substances in the bloodstream. Because α-tocopherol is so efficiently recycled, it is maintained in the body in an unoxidized state. Because of its molecular similarity to α-tocopherol (Figure 1), troglitazone in the circulation might

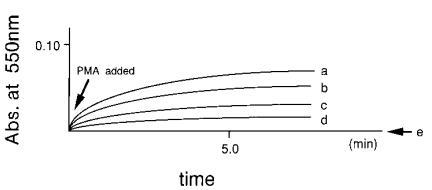

FIG. 5. Effect of troglitazone concentration (a: 0 μmol/l, b: 200 μmol/l, c: 400 μmol/l, d: 800 μmol/l, e: 1000 μmol/l) on 2% DMSO-mediated cytochrome *c* reduction with superoxide generated by phorbol myristate acetate (PMA)-stimulated neutrophils. After the reaction had reached a plateau, PMA was added (↓).

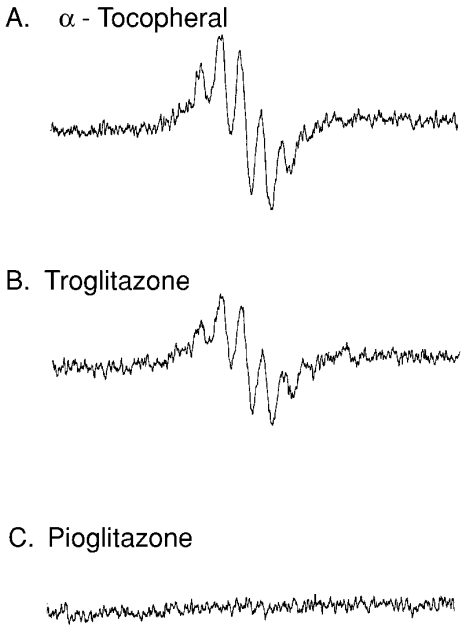

FIG. 6. The electro spin resonance spectra of α-tocopherol (A), troglitazone (B), and pioglitazone (C) in Fenton-type reaction system.

also be regenerated in the same way, and maintained in the body in an unoxidized state. The plasma concentration of troglitazone has been reported to be around 2.26 μmol/l, when administered as a single dose (800 mg) to humans. In the present study, troglitazone at less than 100 μmol/l also had a scavenging effect which inhibited the reduction of cytochrome *c*, but this was not significant in comparison with that in the absence of troglitazone solution. Although the effective and significant minimun concentration in the present study (100 μmol/l) was much higher with respect to the plasma concentration of troglitazone, the troglitazone accumulated in tissues, resulting in a higher level that might have reacted with free radicals.

In addition, our data indicate that troglitazone has scavenging effects on the reactive oxygen species generated by PMA-stimulated neutrophils (Figure 5). In inflammatory states, the active free radicals may be generated by many cells such as macrophages, neutrophils and endothelial cells. Troglitazone might thus act as an anti-inflammatory agent. In addition, as demonstrated in this study, troglitazone can dose-dependently transfer electrons directly to cytochrome *c*. Thus, both troglitazone and cytochrome *c* can compete for electrons from superoxide in the cytochrome reduction assay. Therefore, the investigation should be done to determine whether troglitazone can act as a scavenger of free radicals using lucigenin-enhanced chemiluminescence to detect superoxide (18). In fact, the ESR signal of troglitazone radical was observed, suggested that this drug could act as a scavenger of free radicals.

The observation that troglitazone, but not pioglitazone, has antioxidant activity is of interest, since it complements the drug's hypoglycemic and hypolipidemic effects in diabetic patients. This unique antioxidant characteristic may confer some benefit for preventing vascular complications in these patients.

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