



Mitochondrial Dysfunction as a Mechanism for Pioglitazone-Induced Injury toward HepG2 Cell Line

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ABSTRACT

Background: Thiazolidinediones (TZDs) are widely used for treatment of type II diabetes mellitus in humans. The first drug of this class, troglitazone, was withdrawn from the market due to a high incidence of hepatotoxicity. Several cases of liver injury induced by other TZDs such as pioglitazone is also reported. The mechanism of cellular injury induced by pioglitazone is not recognized precisely so far. Mitochondria is a postulated target for TZDs to induce injury. **Methods:** This investigation was designed to evaluate the cytotoxic effects of pioglitazone in cultured HepG2 cells. Cell death, glutathione level, occurrence of lipid peroxidation, and finally cellular adenosine triphosphate (ATP) content, as an index of mitochondrial functionality, were monitored in HepG2 cells after pioglitazone administration. **Results:** It was found that pioglitazone was toxic towards HepG2 cells concentration dependently and 1 mM of pioglitazone reduced cellular viability to less than 60% in 48 hours of incubation. Pioglitazone administration reduced ATP content of the cells, but did not induce oxidative stress in this cell line, as no lipid peroxidation and/or decrease in cellular glutathione were detected. Administration of ATP suppliers dihydroxyacetone (5 and 10 mM) and glyceraldehyde (1 and 5 mM), inhibited drop in cellular ATP induced by pioglitazone and prevented drug-induced cell death. **Conclusion:** These results suggest that pioglitazone affect cellular mitochondria and might cause cytotoxicity by prevention of ATP production.

Introduction

Drug-induced liver injury is a major complication for pharmaceutical industry and drug development.¹⁻³ Thiazolidinediones (TZDs) are the most widely used antidiabetic agents. A wide range of other pharmacological effects of TZDs are also reported.⁴⁻⁶ However, some serious adverse events including hepatic injury is associated with TZDs administration.^{7,8} Troglitazone was introduced as a promising antidiabetic drug but had to be withdrawn from market within a few years because of serious liver injury accompanied with its administration.⁹ It appears that its sulfate conjugate inhibits bile salt transport from hepatocytes, leading to severe idiosyncratic hepatotoxicity.¹⁰ The other TZD drug, rosiglitazone, was withdrawn from the market due to cardiotoxicity.¹¹ Development of darglitazone and ciglitazone was discontinued, because of cataractogenic potential in rats.¹² Hence, finding the mechanisms of drug-induced cellular injury will help to develop safer TZDs. Several cases of pioglitazone-induced liver injury have been reported.^{13,14} In clinical trials high serum levels of alanine aminotransferase (ALT) was seen after pioglitazone and rosiglitazone administration.¹⁵ Although the precise mechanism of liver injury induced

by TZDs is not recognized yet, but reactive metabolite formation^{16,17}, oxidative stress induction,¹⁶ and intracellular organelles dysfunction¹⁸ are proposed to be involved in TZDs-induced liver injury.

It has been shown that troglitazone is cytotoxic to HepG2 cells and hepatocytes.¹⁹ Pioglitazone has a reactive ring-opened product which is trapped by high performance liquid chromatography. These metabolites were identified in rat and human liver microsomes and in suspensions of freshly isolated rat hepatocytes, but not in human cells.²⁰

Mitochondrial impairment is involved in the etiology of hepatotoxicity, myopathy, cardiopathy, rhabdomyolysis and other serious side effects of many drugs.²¹⁻²³ Mitochondrial dysfunction caused by some of the TZDs, fibrates, statins and many other drugs have been previously reported based on studies of membrane potential, mitochondrial swelling and assays of the respiratory chain in isolated mitochondria.²⁴⁻²⁷ It has been shown that troglitazone caused a drop in membrane potential in HepG2 cells and also a decrease in ATP levels.²⁸

The purpose of this study was to investigate the mechanisms involving in pioglitazone toxicity and its possible effect on cell mitochondria in an *in vitro* model of cultured HepG2 cells. Furthermore, the protective effects of some ATP suppliers such as glyceraldehyde and dihydroxyacetone were evaluated.

Materials and methods

Chemicals

Pioglitazone hydrochloride, 5-diphenyltetrazolium bromide (MTT), glyceraldehyde, and dihydroxyacetone were purchased from Acros (New Jersey, USA). Fructose, glucose, α -ketoglutarate, 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB), Trichloroacetic acid (TCA), Ethyleneglycol-bis (2-aminoethylether)-N, N, N', N'-tetra acetic acid (EGTA), and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Thiobarbituric acid (TBA) was obtained from SERVA (Heidenberg, New York). Fetal bovine serum (FBS) was from Gibco (Gibco, Germany). All salts used for preparing buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

Cell line and culture

HepG2 is a perpetual cell line widely used as an *in vitro* system to investigate the effect of xenobiotics on liver. HepG2 cells were obtained from the Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran. The cells were grown in an atmosphere with 5% CO₂ at 37 °C in RPMI 1640 medium[®] supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (50 µg/ml), and L-glutamine (2 mM). After trypsinization and seeding at low density, isolated clones were selected and used in the cytotoxicity assay.

Drug treatment

Different concentrations of pioglitazone (10, 100, 500 and 1000 µM) were added into the culture media and all toxicity markers were evaluated 48 hours after drug administration. Dihydroxyacetone (1, 5 and 10 mM) and glyceraldehyde (1 and 5 mM) were co-administered with pioglitazone as ATP suppliers.

Cell viability assessment

Cell viability was measured by MTT test (methyl tetrazolium assay). Cells (3×10^6) were washed with phosphate buffer saline (PBS, pH=7.4) and added to 96-well plate. Then 10 µl of MTT (0.5 mg/ml) was added and the incubation continued for 4 h at 37 °C. The formazan dye accumulated in living cells was dissolved in 100 µl of dimethyl sulfoxide (DMSO), and then quantified by optical density (OD) measured at 570 nm with a microplate reader.²⁹

Measurement of ATP

Cells were washed with pre-warmed (37 °C) phosphate buffer saline (PBS, pH=7.4) to remove the culture medium. One milliliter of ice-cold perchloric acid (0.3 M) containing Na-EDTA (1 mM) was added to cells. Cell lysate was transferred to a test tube, and centrifuged at 9000×g (5 min at 4°C). The supernatant was neutralized with equimolar KOH (2 M base). After re-centrifugation (9000×g for 5 min), the extract was stored at -70 °C for subsequent analysis. Cellular ATP levels were analyzed by an isocratic reversed-phase high performance liquid chromatography (HPLC) method, using a Knauer[®] HPLC system, coupled with a UV detector. Ammonium dihydrogen phosphate (0.05 M, pH=6.0) was used as the mobile phase with a flow rate of 1 ml/min. The absorbance of nucleotides was recorded at 254 nm and the run time of 20 min. The concentration of ATP was determined in terms of micromole nucleotide per mg of protein.³⁰

Measurement of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) assay was used to determine the amount of lipid peroxidation in cell culture. Briefly, at the end of the incubation period, cells were collected and washed with PBS (pH=7.4). Cells were centrifuged and resuspended in a mixture of 2 ml reagent consisted of trichloroacetic acid (20%), thiobarbituric acid (0.8%) and HCl (0.5 N). The cell suspension was heated in boiling water for 60 minutes and then centrifuged (10 minutes, 5000 rpm). The absorbance of developed color was assessed in 532 nm using a UV spectrophotometer.^{26,31}

Analysis of cellular reduced and oxidized glutathione (GSH/GSSG)

GSH analysis was performed spectrophotometrically.³² Cells were washed with PBS, then 200 µl of trichloroacetic acid (20%) was added and vortexed. After centrifugation, 0.5 ml of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) (0.01 M), 2 ml NaHPO₄ (0.3 M) and 1ml NaBH₄ (5%) was added to the supernatant. The absorbance of developed color was recorded at 412 nm using a UV spectrophotometer.^{33,34} In order to analyze the GSSG amount. After addition of NaBH₄, GSH was measured again. The difference between these two values was reported as the GSSG amount.

Statistical Analysis

Results represent the Mean±SD. Statistical significance of difference between control and treatment groups was determined using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. The minimal level of significance was P < 0.05.

Results

Pioglitazone caused cell injury in a concentration-dependent manner (Table 1). It was found that, incubation of HepG2 cells with 500 µM and 1 mM of

pioglitazone for 48 hours reduced cell viability to about 84% and 39% of control respectively (Table 1). Lower concentrations of pioglitazone didn't show significant cytotoxicity (Table 1). The ATP content of the cells were dropped in accordance with decreasing in cell viability (Table 2). Administration of 1 mM of pioglitazone significantly decreased cellular ATP content (Table 2). Dihydroxyacetone (5 and 10 mM) and glyceraldehyde (1 and 5 mM) administration

significantly prevented ATP depletion and cell death induced by pioglitazone (Table 2). However, fructose, glucose and α -ketoglutarate were unable to prevent cytotoxicity or ATP depletion induced by pioglitazone (data not shown). Pioglitazone administration didn't affect cellular lipid peroxidation, or reduced (GSH) and oxidized (GSSG) glutathione content of HepG2 cells (Table 3).

Table 1. HepG2 cells injury induced by different concentrations of pioglitazone as assessed after 48 hours of incubation.

| Incubate | Cytotoxicity (MTT assay , % of control) |
|----------------------------|--|
| Control (only HepG2 cells) | 100±1.52 |
| + Pioglitazone 10 μ M | 99.25±1.91 |
| + Pioglitazone 100 μ M | 96.78±1.9 |
| + Pioglitazone 500 μ M | 84.03±1.58 ^a |
| + Pioglitazone 1 mM | 38.82±3.25 ^b |

Data are expressed as Mean±SD for at least four independent experiments.

^a Indicates significantly different from control ($P<0.5$).

^b Indicates significantly different from control ($P<0.01$).

Table 2. Effect of ATP suppliers on the cell injury and ATP content in pioglitazone-treated cells (Incubation time = 48 hours).

| Incubate | Cytotoxicity (MTT assay , % of control) | Cell ATP content (μ mol/ 3×10^6 cells) |
|----------------------------|--|--|
| Control (only HepG2 cells) | 100.03±0.9 | 22±3 |
| + Pioglitazone 1mM | 26.73±3.48 ^a | 14±2 ^a |
| + Dihydroxyacetone 1 mM | 38.98±4.49 | 16±3 |
| + Dihydroxyacetone 5 mM | 66.23±15.14 ^b | 17±5 |
| + Dihydroxyacetone 10 mM | 89.00±10.35 ^b | 23±5 ^b |
| + Glyceraldehyde 1 mM | 55.08±7.87 ^b | 24±4 ^b |
| + Glyceraldehyde 5 mM | 91.73±10.35 ^b | 25±3 ^b |

Data are given as Mean±SEM for at least four independent experiments.

^a Significantly lower as compared with control (only HepG2 cells) ($P<0.05$).

^b Significantly higher than pioglitazone-treated cells ($P<0.05$).

Table 3. Effects of pioglitazone on lipid peroxidation and glutathione content of HepG2 cells (Incubation time = 48 hours).

| | TBARS (nmol/ 3×10^6 cells) | GSH (μ mol/ 3×10^6 cells) | GSSG (μ mol/ 3×10^6 cells) |
|----------------------|--|---|--|
| Control (only HepG2) | 0.193±0.070 | 5.58±0.089 | 2.993±0.046 |
| + Pioglitazone 1 mM | 0.067±0.032 | 5.323±0.299 | 3.163±0.090 |

Data are expressed as Mean±SD for at least four independent experiments. TBARS; thiobarbituric acid reactive substances, GSH; reduced glutathione, GSSG; oxidized glutathione.

Pioglitazone did not significantly affect lipid peroxidation and GSH/GSSG content of HepG2 cells.

Discussion

Pioglitazone caused injury toward HepG2 cells concentration dependently. Cellular injury caused by this drug was accompanied with ATP depletion in cells but no significant lipid peroxidation or decrease in cellular glutathione reservoirs were found.

Mitochondria seems to be a potential target for thiazolidinediones (TZDs)-induced cytotoxicity. Tirmenstein et al. showed that troglitazone caused a drop in mitochondrial membrane potential in HepG2

cells and also decreased ATP levels.²⁸ In the present study, we showed that pioglitazone decreased ATP synthesis by mitochondria. Dihydroxyacetone and glyceraldehyde reduced Pioglitazone-induced mitochondrial injury and prevented ATP depletion and cytotoxicity. Therefore, our study suggest that Pioglitazone-induced toxicity toward HepG2 cells is partly mediated by ATP depletion possibly through the inhibition of mitochondrial respiration.

Different factors might affect the mechanisms by which xenobiotics induce liver injury.³⁵ Inherited mitochondrial dysfunction in human population^{36,37} might affect the cytotoxicity of different drugs including pioglitazone. Hence, some patients might be more susceptible to pioglitazone-induced liver injury. Pioglitazone is metabolized by CYP2C8 and CYP3A4.³⁸ Pioglitazone and/or its reactive metabolites

might affect mitochondrial function (Figure 1), which finally might lead to cell death. Dihydroxyacetone and glyceraldehyde are produced in glycolysis pathway and finally produce ATP in the cell.³⁹ Our data suggest that cellular ATP suppliers are protective agents against pioglitazone-induced cell injury (Table 2). Hence, these agents might be considered as potential therapeutic options against TZDs-induced liver injury.

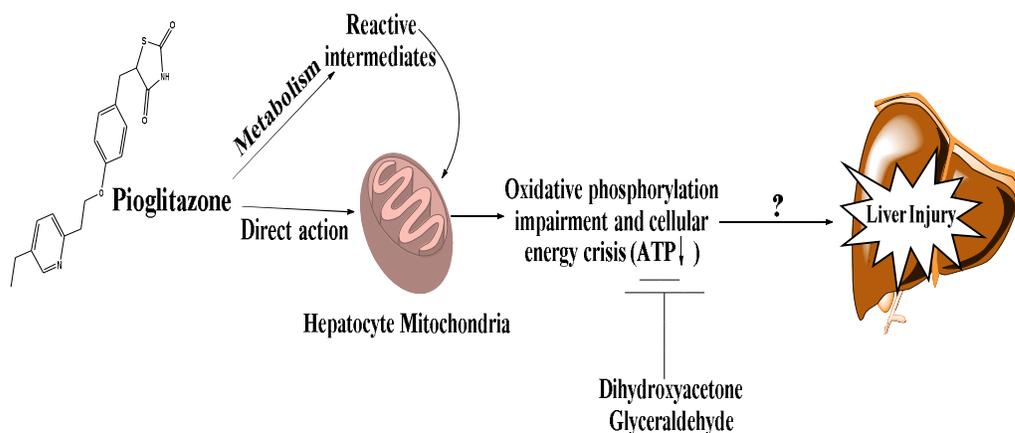


Figure 1. The proposed mechanism of cell injury induced by pioglitazone and the cytoprotective effect of ATP suppliers.

Oxidative stress and GSH depletion are postulated mechanisms for pioglitazone-induced cell injury. However, pioglitazone did not induce lipid peroxidation or GSH depletion in HePG2 cells in our experiments (Table 3). Some previous studies have shown that pioglitazone has a reactive ring-opened product identified in microsomes taken from rat and human hepatocytes.^{20,40} This metabolite is trapped by glutathione and.^{20,40} It is possible that HePG2 cell lines do not metabolize this compound to the mentioned ring-opened metabolite, as we did not observe any change in GSH content of the cells. On the other hands, although we have shown that pioglitazone caused cell injury through the mitochondrial dysfunction in HepG2 cell line, the *in vivo* conditions might be different for this drug to induce cytotoxicity.

Collectively, our data suggest that cellular toxicity induced by pioglitazone in HePG2 cells is mainly mediated by ATP depletion (Figure 1). These findings need to be confirmed by other experimental models.

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Conflict of interests

None to be declare.

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