CIGLITAZONE-INDUCED LENTICULAR OPACITIES IN RATS: IN VIVO AND WHOLE LENS EXPLANT CULTURE EVALUATION

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RUNNING TITLE: Cataractogenic Effects of Ciglitazone In Vivo and In Vitro

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Manuscript statistics:

Number of text pages: 32

Number of tables: 2

Number of figures: 4

Number of references: 35; 40 maximum

Number of words in abstract: 250; 250 word limit

Number of words in introduction: 622; 750 word maximum

Number of words in discussion: 1232, 1500 word maximum

ABBREVIATIONS: ANOVA, analysis of variance; ATP, adenosine triphosphate; CIG, ciglitazone (sodium salt); CI-II, calpain inhibitor II; Cmax, concentration of drug at peak serum or plasma levels; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, glutathione disulfide; LE, Long Evans; LSD, least significant difference; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SD, Sprague-Dawley; S.D., standard deviation; S.E.M., standard error of the mean; SORB, sorbinil; RR, ruthenium red.
ABSTRACT

The cataractogenic potential of the thiazolidinedione ciglitazone (CIG) was investigated in vivo and in vitro. In the rat CIG caused a dose-dependent (30-300 mg/kg/day) increase in incidence and severity of nuclear cataract formation during a three-month nonclinical safety assessment study. Potential mechanisms of toxicity were surveyed using whole rat lens explants exposed to CIG with/without various inhibitors of cataract formation. In vitro, CIG caused a concentration- (0.375-30 µM) and time-dependent (3-24 hr) change in biochemical (adenosine triphosphate (ATP) content or mitochondrial reduction of a tetrazolium dye (MTT), and reduced glutathione (GSH) content) and morphometric (lens wet weight and clarity) markers of damage. Within 3 hr of exposure, 7.5 µM CIG decreased lens ATP content 37±7% (% difference from control, p<0.05). After 24 hr of exposure lens ATP content, MTT reduction, and GSH content declined 57±5%, 30±28%, and 42±8%, respectively. Lens wet weight increased 17±4% with a concomitant decrement in lens clarity. Pretreating lenses with the mitochondrial calcium uniport inhibitor ruthenium red (RR) partially/fully protected lenses from toxicity. In contrast, the antioxidant dithiothreitol, the aldose reductase inhibitor sorbinil, and selective cell permeable calpain inhibitors (calpain II inhibitor and E64d) were ineffective in providing protection under the present testing conditions. Early and selective changes in lenticular ATP content and the partial/full protective effect of RR suggest that alterations in lens bioenergetics may play an important role in CIG-induced cataract formation. Lens explant cultures were successfully used to select two thiazolidinediones that lacked cataractogenic activity when evaluated in three-month rat safety assessment studies.
Thiazolidinediones represent a major new therapy in the treatment of noninsulin-dependent diabetes. However, there is new interest in exploring the use of thiazolidinediones in the treatment of ophthalmic diseases (Murata et al., 2000; Aoun et al., 2003). Although the safety profile of these agents is well established based on oral exposure, such new indications and routes of exposure could potentially alter the safety profile of marketed drugs and would influence the selection of new drug candidates. This may occur since intravitreal or topical ocular administration may lead to higher intraocular drug concentrations and/or the potential for a longer half-life of the compound in ocular tissue compared to oral exposure. Of even more relevance to the exploration and potential use of thiazolidinediones for ophthalmic indications is the relatively unknown safety profile of ciglitazone (CIG), an early drug candidate in this chemical class. Upon oral administration CIG was associated with the formation of lenticular opacities during nonclinical safety assessment studies conducted in rats by the Upjohn Company (Kalamazoo, MI). This adverse finding limited further development of CIG. Years later based on anecdotal information we independently used CIG as a positive control to survey basic mechanisms of cataract formation (Aleo et al., 2000). Although these independently produced investigations were not reported in the peer-reviewed literature at that time, we believe it is appropriate to document and disseminate the ocular safety profile of CIG based on these earlier findings because of the current interest in developing thiazolidinediones for treating ophthalmic diseases.

Lenticular opacities are caused by compounds that either penetrate the lens and directly disrupt normal cellular processes important in maintaining lens transparency or interact indirectly with the lens surface through the generation of reactive oxygen species (Bhuyan et al., 1973; Bhuyan and Bhuyan, 1979). CIG appears to interact directly with the lens since it has been shown to distribute to the rat eye following oral administration (Torii et al., 1984). Cataractogenic compounds that penetrate the lens have an intrinsic ability to adversely perturb the lenticular environment resulting in opacity formation. In some cases, such as acetaminophen, the compound that penetrates the lens and causes the damage is a reactive
metabolite that is formed via the cytochrome P-450 system found in the ciliary body of the eye (Zhao and Shichi, 1995) or liver (Lubek et al., 1988). In other cases, such as $S$-(1,2-dichlorovinyl)-L-cysteine (Walsh Clang and Aleo, 1997), naphthalene (Lou et al., 1996) or glucose (Yeh et al., 1986; 1987; 1990), the compound appears to require metabolic activation within the lens itself.

Lenticular opacities are caused by at least three broad and sometimes overlapping mechanisms: A) oxidative stress (Spector, 2000) and/or Ca$^{2+}$-dependent activation of calpains (Azuma et al., 2003), B) osmotic stress (Chung et al., 2003) caused by the accumulation of active osmolytes like glucose metabolites (polyols) within the lens or C) disregulation of normal bioenergetic processes (Walsh Clang and Aleo, 1997; Martynkina et al., 2002; Belusko et al., 2003) or thiol status (Lou, 2003).

Here we report the cataractogenic effects of CIG in rats. Using rat lens explants in organ culture as a short-term, mechanistically-based ex vivo model, we examined the relative contributions of either perturbation of mitochondrial function, osmotic stress, oxidative stress, and/or calpain activation in the mechanism of CIG toxicity to the rat lens. The effects of CIG on rat lens explants in culture were assessed by analysis of selective biochemical markers (lenticular ATP content and/or mitochondrial MTT reduction and GSH content) and morphometric markers (lens weight and daily observations of changes in lens clarity) of damage to the crystalline lens. The results of experiments using whole lens explants in culture were used to propose a mechanism for cataract formation by CIG and to screen other drug candidates for cataractogenic potential in vitro.
MATERIALS AND METHODS

CIG was the generous gift of Dr. David Clark (Pfizer Global Research and Development, Groton, CT). Dithiothreitol (DTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), monochloroacetic acid, sodium octyl sulfate, and ethylenediamine-tetraacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Ruthenium red (RR) was acquired from ICN (Cleveland, OH). Media 199, glutamine, penicillin and streptomycin were obtained from Gibco (Grand Island, NY). E64d and calpain inhibitor-II (CI-II) were obtained from Calbiochem (La Jolla, CA). All other reagents were at least analytical or high pressure liquid chromatography grade.

Animal care and use were conducted in accordance with all applicable state and federal regulations and guidelines and were in full compliance with national legislation (Animal Welfare Act Regulation, Title 9 Code of Federal Regulations parts 1 to 3 and the Association for Assessment and Accreditation of Laboratory Animal Care, International Standards as set forth by the Guide for the Care and Use of Laboratory Animals (1996 National Academy Press, Washington, D.C.)) and approved by the Animal Care and Use Committee of this research site.

Three month safety assessment studies of CIG in rodents: Male and female Sprague-Dawley Rats (Upj:TUC(SD)spf) were obtained from the Upjohn Company (Kalamazoo, MI). Animals ranged in age between 6-7 weeks for males and 7-9 weeks for females. Animals were housed individually and fed Purina certified rat chow (#5002) and water ad libitum. Drug administration occurred once a day (15 animals/dose/gender) via the oral route using a suspension of ciglitazone (0.1% sorbic acid, 0.25% methylcellulose, 0.5% polysorbate 80, 2.0% Avicel RC-591®, and purified water).

Three-month safety assessment studies of CIG in canines: Male and female purebred beagles were obtained from Laboratory Research Enterprises (Kalamazoo, MI) and White Eagle Laboratories, respectively. Animals ranged in age between 10-12 months for males and 18-20 months for females. Animals were housed individually and fed Purina certified canine diet
Drug administration occurred once a day (4 animals/dose/gender) via the oral route using gelatin capsules containing ciglitazone.

**Lens Explant Studies:** Male Sprague-Dawley (SD) rats (120-200 g) from Charles River (Raleigh, NC) were housed individually in wire rack cages with free access to standard lab chow and water in an environmentally controlled room on a 12 hr light/dark cycle. The rats were euthanized by CO₂ asphyxiation. After enucleation, lenses were extracted from the globes using a posterior approach and placed in a 20 ml glass scintillation vial with a 2 mm hole in the cap. The vial contained 4 ml of warmed bicarbonate based Media 199 (without phenol red) supplemented with L-glutamine (2 mM), penicillin (50,000 U/L), and streptomycin (50,000 mg/L). Final osmolality of the base medium was adjusted to 295-300 mOsmol/kg. The medium was bubbled for 10 min with 95:5% air/CO₂, filter sterilized and stored at 4°C. The lenses were incubated on an orbital shaker in an humidified 37°C incubator in a 95:5% air/CO₂ environment. Lenses were harvested and cultured as previously described (Walsh Clang and Aleo, 1997) and after 24 hr were inspected visually for damage according to Shearer et al. (2000). Only lenses without procedure-induced damage (i.e. partial opacification of the lens or rupture of the capsule) were retained for further experimentation. Lenses were incubated and appearance assessed at least daily in treated medium containing a combination of vehicle, CIG, and/or other test compound(s) for 3, 6, 24, 48, or 96 hr. Ethanol was used as a vehicle at a final concentration of 0.2%. Lens appearance was assessed and graded at least daily by visual inspection. Lenses were graded in the following manner: STAGE 0 = clear; STAGE 1 = hazy cortical region; STAGE 2 = haziness characterized by a visible demarcation between nuclear and cortical regions. Grading system is based on a modification from Dickerson et al. (1997). For multi-day exposure experiments treated medium was exchanged every 24 hr for four days as indicated. At the end of the incubation period, lenses were rinsed with ice-cold saline, blotted and weighed prior to processing for biochemical analysis. Lenticular ATP content, mitochondrial MTT reduction, and GSH content were determined as previously described (Walsh Clang and Aleo, 1997).
Three month safety assessment studies of englitazone in rats: Male and female Long-Evans (LE) (Crl(LE)BR) rats were obtained from Charles River (Wilmington, MA). Animals (120-200 g) were housed individually and fed Agway Prolab RMH 3200 diet (Agway Country Foods Inc., Syracuse, NY) and water ad libitum. Drug administration occurred once a day (15 animals/dose/gender) via the oral route (15, 45 and 100 mg free base/kg/day) using a suspension of englitazone in its sodium salt form (0.1% methylcellulose).

Three month safety assessment studies of darglitazone in rats: Male and female Long-Evans (Crl(LE)BR) rats were obtained, housed, fed, and dosed as previously described (Aleo et al., 2003).

Statistical Analysis
Comparisons were made using a one-way analysis of variance (ANOVA). When necessary, the data were transformed to natural logarithms to meet better the assumptions of the ANOVA. Multiple means were compared using Fisher’s Protected Least Significant Difference (LSD) Test. Statements of significance were based on a p < 0.05. All results are expressed as mean ± S.E.M unless noted otherwise.
RESULTS

**In Vivo Safety Assessment of CIG in Rodents and Canines:** CIG caused a dose-related increase in the incidence of opacity formation during the conduct of a three month nonclinical safety assessment study in SD rats (15 animals/sex/group) at daily oral doses of 30, 100, and 300 mg/kg/day (Table 1). Opacities were first noted in some animals on day 34 of study. An ophthalmic exam conducted on day 49 revealed 4 animals (male and female) in the 100 and 14 animals in the 300 mg/kg/day group with varying degrees of opacity formation (bilateral changes ranging from stress lines to stress lines with early cataracts). After 86 days of daily exposure 4, 9, and 16 animals receiving 30, 100, and 300 mg/kg/day were diagnosed with varying degrees of opacity formation (bilateral changes ranging from posterior cortical capsular cataracts with or without nuclear cataracts to mature complete cataracts). There was no evidence of opacity formation identified in a three-month canine study at daily doses up to 300 mg/kg/day (data not shown). Drug exposure data was not obtained during the in-life portion of either the rat or the canine three-month safety assessment study.

**Preliminary Exposure and Lens Penetration Studies:** In separate animal experiments lens drug levels after a single dose of CIG (25 mg/kg p.o.) approached 0.15 and 0.26 µg/g lens weight after 2 and 6 hr of exposure, respectively. This dose resulted in plasma levels that ranged between 8.8 and 12.6 µg/ml (26-38 µM). After a single dose of 300 mg/kg, lens levels approached 0.18 and 0.70 µg/g lens weight after 2 and 6 hr of exposure, respectively. Plasma levels of drug at this dose ranged between 22 and 30 µg/ml (65-90 µM). Based on protein binding information (Torii et al., 1984) the free circulating concentration of CIG would range between 1.85 and 2.7 µM for these two doses.
**In Vitro Assessment of CIG in Rodent Whole Lens Explant Culture:** CIG adversely affected the transparent quality of explanted lenses obtained from SD rats within 24 hr of exposure in treated culture medium. The incidence and severity of these adverse changes in lens clarity were concentration-dependent (Fig. 1). While exposure to 0.375 and 3.75 µM CIG for 24 hr caused no discernible adverse effect on lens clarity, concentrations ≥7.5 µM caused considerable damage. Lenses exposed to 7.5 µM CIG for 24 hr showed a graded response. Of the lenses treated 23% showed no effect (Stage 0), while 50% were hazy in the cortical area (Stage 1), and 27% were hazy with a visible demarcation between the nuclear and cortical regions (Stage 2). Progression to Stage 2 graded opacities was also concentration-dependent with 27%, 68%, and 100% of all the lenses treated with 7.5, 15, and 30 µM CIG, respectively, being affected within 24 hr of exposure.

There was a correlation between adverse changes in the clarity of lenses exposed to CIG and several biochemical markers of toxicity. Lenses exposed to CIG for 24 hr showed concentration-dependent alterations in lens ATP content, mitochondrial MTT reduction, GSH content, and wet weight (Fig. 2). While lenticular GSH content increased after exposure to 3.75 µM CIG, progressive decrements in lenticular ATP content, MTT reduction, and GSH content occurred after exposure to concentrations ≥7.5 µM for 24 hr. Lenticular GSSG content was below the lower limit of quantitation (10 nmol/lens) in all samples tested (data not shown). Lens wet weight progressively increased as a function of concentration at exposure levels ≥7.5 µM. Changes in these representative biochemical markers of toxicity and lens weight indicate that 7.5 µM CIG was the threshold concentration at which both biochemical and morphological evidence of opacity formation became evident after 24 hr of exposure.
In separate experiments lenticular ATP content was the most sensitive biochemical marker of toxicity used in the present investigation. A decrease of $37 \pm 7\%$ in lenticular ATP content (control: $250 \pm 27$ vs treated: $157 \pm 39$ nmol ATP/lens, mean $\pm$ S.D., $p < 0.05$) occurred within 3 hr exposure to 7.5 $\mu$M CIG. Decrements in lenticular ATP content occurred before any other potential adverse changes in lenticular GSH content (control: $77 \pm 12$ vs treated: $79 \pm 9$ nmol GSH/lens, $p > 0.05$), wet weight (control: $31 \pm 2$ vs treated: $31 \pm 1$ mg/lens, mean $\pm$ S.D., N = 5 lenses per treatment, $p > 0.05$), and lens clarity were noted (data not shown).

The effects of lower CIG concentrations during longer exposure periods were also explored. Low concentrations of CIG (3.75 $\mu$M) decreased lenticular ATP content $20 \pm 6$, $57 \pm 6$, and $57 \pm 4\%$ after 24, 48, and 96 hr, respectively. Lenticular GSH content showed a biphasic response, increasing $33 \pm 8\%$ after 24 hr and then decreasing $39 \pm 6\%$ at 48 hr before returning to control values after 96 hr. Lens weight did not increase relative to controls during the 96 hr exposure period. Except for an isolated reduction in lens GSH content (48 hr) and lens weight (96 hr), there were no significant changes in any other measured biochemical markers of toxicity in lenses exposed to 0.375 $\mu$M CIG. These minimal and isolated effects at 0.375 $\mu$M CIG suggest it was a relatively non-toxic concentration in this short-term assay. There were no discernable changes in lens transparency compared to controls at any of the concentrations (0.375 and 3.75 $\mu$M) or exposure periods (24, 48, and 96 hr) tested (data not shown).

Since a decline in lenticular ATP content was noted before any other biochemical changes, the role of changes in lenticular mitochondrial function in the cataractogenic potential of CIG was further explored. Lenses pretreated with 100 $\mu$M RR, a mitochondrial calcium uniport inhibitor, 15 min prior to addition of CIG were partially protected from the adverse effects of 15 $\mu$M CIG after 24 hr of exposure (Fig. 3). Precipitous declines in lenticular ATP
and GSH content (up to 100% compared to control values) were partially (ATP) or completely (GSH) attenuated by pretreating lenses with RR. Pretreatment with RR also attenuated alterations in lens weight (Fig. 3) and lens clarity compared to CIG treatment alone (Stage 1 vs. Stage 2 grade opacities, respectively). In separate experiments using a lower concentration of CIG (7.5 µM) and RR (30 µM), RR completely prevented the loss of ATP caused by CIG (control: 76 ± 11a vs 30 µM RR treatment: 75 ± 4a vs 7.5 µM CIG treatment: 41 ± 6b vs 30 µM RR + 7.5 µM CIG treatment: 73 ± 8a nmol ATP/lens, mean ± S.D., N = 5 lenses per treatment, p < 0.05). In this experiment lens wet weight and transparency were not adversely affected by CIG relative to control (data not shown).

Because the general protective effects of RR suggest that CIG may perturb intracellular free calcium regulation we also explored whether cataract formation by CIG would be blocked with inhibitors of calcium-dependent calpain proteases. Pretreating lenses with the membrane permeant calpain inhibitors E64d (100 µM) and calpain inhibitor II (CI-II, 1mM) did not protect lenses from CIG toxicity after a 24 hr exposure period (Table 2). Aside from a statistically significant improvement on lens weight by CI-II, both calpain inhibitors did not protect lenses from decrements in lenticular ATP content (CI-II treatment tested) or mitochondrial MTT reduction (E64d treatment tested) and GSH content caused by exposure to 15 µM CIG for 24 hr. More importantly, these calpain inhibitors had no apparent protective effect on the adverse changes in lens transparency caused by CIG exposure (i.e. treated lenses were still graded as Stage 2 opacity). The Stage 1 opacity observed with CI-II treatment alone is a known effect at concentrations ≥ 250 µM (Lampi et al., 1992).
In order to determine whether CIG caused toxicity by oxidative stress we investigated whether antioxidants protected explanted lenses from the cataractogenic potential of CIG. Lenses were exposed to 15 µM CIG for 24 hr with or without the antioxidant and sulfhydryl reducing reagent dithiothreitol (DTT; 2 mM). As shown in Table 2, DTT had no protective effect on any of the biochemical (ATP content, GSH content, and lens wet weight) markers of toxicity. DTT also did not protect against adverse changes in the transparent quality of the lens caused by CIG. In contrast, DTT completely protected lenses from the oxidative damage caused by 300 and 400 µM H₂O₂, concentrations associated with either slight (300 µM) or substantial (400 µM) reductions in several biochemical markers of toxicity, equivalent increases in lens wet weight, and similar adverse changes in lens transparency.

Finally, because the hypoglycemic activity of CIG is related to its ability to facilitate translocation of glucose transporters from intracellular storage pools to the plasma membrane and isoforms of the transporter are differentially expressed in normal adult rat lenses (Merriman-Smith et al., 2003), an increase in lens weight may indicate osmotic stress caused by the abnormal accumulation of polyols (sugar metabolites). We examined whether the cataractogenic potential of CIG was associated with its pharmacologic mechanism of action. Under the culture conditions described, sorbinil (SORB), a specific inhibitor of aldose reductase, the enzyme responsible for converting glucose to its active osmolyte sorbitol, had no discernible protective effect on the biochemical or morphological indicators of CIG-mediated toxicity after a 24 hr exposure period (Table 2). Furthermore, independent work by Chang and colleagues at the Upjohn Company showed that CIG (50 or 100 mg/kg/day for 2 to 4 days, respectively) inhibits aldose reductase activity in an uncompetitive manner and lowers sorbitol accumulation in the lens (18-55% compared to control) and sciatic nerve (14-33% compared to
control) of streptozotocin-treated rats (70 mg/kg i.v.) fed a sucrose diet for 2 to 5 days (data on file Pfizer Global Research and Development, Kalamazoo, MI).

**In Vitro Ranking of Thiazolidinediones for Cataractogenic Potential:** In order to demonstrate the utility of the explant culture model system to distinguish cataractogenic compounds we rank ordered the thiazolidinediones: ciglitazone, darglitazone, and englitazone *in vitro*. Explanted lenses, obtained from SD rats, exposed to equivalent concentrations (15 µM) of all three thiazolidinediones for 48 hr showed marked differences in their clarity (Fig. 4). CIG-treated lenses were graded as Stage 2, while darglitazone and englitazone treated lenses were clear or very slightly hazy (barely gradable as Stage 1), respectively. Based on this single concentration comparison the rank order of cataractogenic potential based on visual assessment alone was ciglitazone>>englitazone>darglitazone. Visual assessment alone was sufficient to rank compounds for cataractogenic potential *in vitro*.

**In Vivo Safety Assessment of Englitazone and Darglitazone for Cataractogenic Potential:** Since both englitazone and darglitazone were less toxic to the lens compared to CIG, both compounds were selected for evaluation in subacute safety assessment studies in animals. Unlike CIG, neither darglitazone or englitazone caused lenticular opacities during the in-life portion of three month safety assessment studies in LE rats at consecutive daily oral doses of up to 50 mg/kg (darglitazone) and 100 mg/kg (englitazone), respectively. These doses were associated with serum concentrations at C_{max} that ranged from 24-60 µM (darglitazone) and 170-340 µM (englitazone) in male and female rats, respectively. With protein binding greater than 99% for both compounds the effective free concentrations were at the most 0.6 and 3.4 µM, respectively.
DISCUSSION

Rat and bovine lens explant cultures have long been used to effectively investigate mechanisms of cataract formation in animals induced by several xenobiotics like tamoxifen, naphthalene, lovastatin, simvastatin, and S-(1,2-dichlorovinyl)-L-cysteine (Mosley et al., 1989; Kalinowski et al., 1991; Xu et al., 1992; Zhang et al., 1994; Walsh Clang and Aleo, 1997). The present study is the first combined report of lenticular opacity formation by CIG in rodents in vivo and in vitro using rat lens explant culture. CIG was a potent cataractogenic agent in vitro, producing a concentration-dependent loss of lens transparency and formation of opacities (graded Stage 2) that were evident within 24 hr of exposure at concentrations ≥7.5 µM. Several biochemical markers (ATP and GSH content) were sensitive indicators of toxicity with changes in lenticular ATP content preceding any visually perceptive changes in lens transparency. For example, before discernible effects on lens transparency were noted, lenticular ATP content declined 37% within 3 hr of exposure to 7.5 µM CIG. Additionally, lenses exposed to lower concentrations of CIG (3.75 µM) for extended treatment periods (<96 hr) had perturbations in both lenticular ATP and GSH content before any adverse effect on lens transparency was observed. Increased lens wet weight was not a predictive or sensitive marker of toxicity since it occurred only after changes in lens clarity were evident by visual inspection and was not consistently reproducible between some experiments.

Although tissue distribution of CIG to the eye is low compared to other body tissues (Torii et al., 1984), concentrations used in vitro to cause lens opacification were consistent with in vivo exposure data. Peak plasma concentrations of CIG 2 hr after a 30 mg/kg or 6 hr after a 300 mg/kg oral dose approach 37 (Torii et al., 1984) and 90 µM in the plasma, respectively. This range of oral doses caused a dose-related degree and incidence of bilateral cataract formation in a three-month subacute safety assessment study in SD rats. Since CIG is approximately 95-97% bound to rat plasma proteins at these concentrations (Torii et al., 1984), the free circulating concentration of CIG at peak would range between 1.85-2.7 µM for these
two doses. Thus the concentrations used in the in vitro studies, especially results obtained at 3.75 µM are extremely relevant to discerning mechanisms of toxicity. In vitro exposure to 3.75 µM CIG decreased lenticular ATP content 56% after extended exposure periods of 48 and 96 hr. Based on exposure data and tissue dosimetry information in the eye after exposure in vivo and the toxic effects of this compound at similar in vitro concentrations, these data strongly suggest that investigations of various biochemical mechanisms of cataract formation in the present study are germane to the in vivo setting.

Changes in lenticular ATP content by CIG appear to be caused in part by mitochondrial dysfunction. RR, an inhibitor of the mitochondrial Ca²⁺ uniport, effectively blocked toxicity and opacity formation at concentrations of CIG that did not result in changes in lens weight (7.5 µM) and provided partial protection from the adverse effects of higher concentrations of CIG (15 µM). A protective effect of RR was also seen in this model system by us with another cataractogenic agent S-(1,2-dichlorovinyl)-L-cysteine (Walsh Clang and Aleo, 1997), a compound known to cause its primary toxic effects via disturbances in mitochondrial bioenergetics and mitochondrial calcium regulation. However, since certain portions of the lens obtain the majority of its ATP through anaerobic respiration it is possible that the dramatic decrease in lens ATP content also reflects inhibition of this pathway as well. In either case, periodic or sustained reductions in lenticular ATP content over a three month period caused by low concentrations of CIG could easily disrupt or impair crystallin formation, cholesterol biosynthesis or proper ion homeostasis within the lens. The functional integrity of all of these processes is necessary to maintain normal lens transparency.

Other mechanisms of cataract formation appear to be less likely given the lack of a protective effect of pharmacologically active agents. The sulphydryl reducing agent DTT and inhibitors of calpain activation were ineffective in preventing CIG toxicity to the lens despite their effectiveness in preventing ionophore- and sugar-induced cataract formation in explanted rodent lenses (Azuma et al., 1992; 1995). Future investigations should focus on direct determinations of pertubations in these pathways using GSH/GSSG ratio and lens protein
sulphhydryl status as markers of oxidative stress, tempol-H or other agents as a pure antioxidant (Zigler et al., 2003) and/or activation of calpains using casein zymography or lens protein proteolysis in order to understand the relative role of each of these mechanisms compared to early and significant reductions in lens ATP content.

More importantly for this class of compounds the cataractogenic potential of CIG does not appear to be related to its pharmacological mechanism of action. Although an increase of lens weight could indicate osmotic stress caused by polyol accumulation within lens tissue, the cataractogenic effects of CIG do not appear to be involved using the present approach. Despite the documented ability of the aldose reductase inhibitor SORB to effectively inhibit sugar-induced cataract formation *in vivo* (Kador et al., 1986; Yeh et al., 1987; Yeh and Ashton, 1990) and *in vitro* (Kador et al., 1986; Yeh et al., 1986), both in terms of adverse changes in lens weight and clarity and lenticular ATP and GSH content, SORB did not attenuate the toxicity of CIG to rat lenses either in terms of lens clarity or on the same biochemical markers of toxicity under the present culture conditions (see Table 2). The concentration of SORB used in this study was 5x higher than the concentration that has been shown to effectively inhibit aldose reductase (>90%) and sorbitol accumulation (>80%) in rat lenses exposed to 35 mM glucose (Yeh et al., 1986). The early work by Chang et al. in-house further indicates that CIG does not cause abnormal accumulation of lens polyols.

To our knowledge the cataractogenic potential of CIG appears to be unique among other thiazolidinediones. There have been no reports of cataract formation in rats with darglitazone up to three months of treatment at 50 mg/kg/day (Aleo et al., 2003) or troglitazone during subacute (13 weeks) and chronic studies (104 weeks) in rats at daily doses of up to 800 mg/kg (Herman et al., 1997; 2002). Troglitazone also did not cause lenticular opacities after 52 weeks of treatment in cynomolgus monkeys (Rothwell et al., 1997) at oral doses of up to 1200 mg/kg/day. Furthermore, troglitazone (20 µM) actually has a protective effect on polyol-induced cataract formation in rat lenses exposed to galactose *in vitro* (Yokoyama et al., 1999).
To our knowledge there have been no reports of lenticular opacities with currently marketed thiazolidinediones.

This study establishes the cataractogenic potential of CIG in rats, shows the utility of explanted lenses for investigating lenticular opacity formation, and ability to rank order compounds in vitro. Preliminary investigation into mechanisms of CIG toxicity in the rat lens suggests that mitochondrial dysfunction may play a significant role but more extensive investigative studies are warranted. The foundation of biochemical information presented here should be useful in establishing the basis for future investigations into mechanisms of cataract formation by CIG. Because of the potential for CIG to induce cataract formation in rats it is advisable to avoid the use of this compound to investigate ophthalmic indications for thiazolidinediones, especially since other drugs in this chemical class do not have this safety liability in animals.
ACKNOWLEDGMENT

The authors thank Kathy Bonnema for retrieving study information related to ciglitazone testing in rats and dogs generated by the late Dr. Henry D. Webster and colleagues from the former Upjohn Company. *In vitro* portions of this work were reported in abstract form at the 36th annual meeting of The Society of Toxicology in Cincinnati, Ohio (1997). Portions of this work have been reprinted from Aleo et al. (2000) with permission "Copyright 2000 New York Academy of Sciences, U.S.A."
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FIGURE LEGENDS

Figure 1: Concentration-dependent changes in the transparency of explanted Sprague-Dawley rat lenses treated with ciglitazone (CIG) for 24 hr. Gradation of shading represents percentage of lenses within each treatment group at the various stages of lens clarity indicated. KEY: (N)=total number of treated lenses; STAGE 0 = Clear; STAGE 1 = hazy cortical region; STAGE 2 = haziness characterized by a visible demarcation between nuclear and cortical regions. Grading system is based on a modification from Dickerson et al., 1997. Figure is based on the collective results of several independent experiments.

Figure 2: Concentration-dependent alterations in adenosine triphosphate (ATP) content, glutathione (GSH) content, mitochondrial tetrazolium dye (MTT) reduction, and wet weight of explanted Sprague-Dawley rat lenses treated with ciglitazone (CIG) for 24 hr. N=3-32 lenses per treatment, mean ± S.E.M. Values with different superscripts within a given parameter are significantly different from each other using one way ANOVA followed by Fisher’s LSD test, p < 0.05. Figure is based on the collective results of several independent experiments.

Figure 3: Protective effects of 100 µM ruthenium red (RR) on adenosine triphosphate (ATP) content, glutathione (GSH) content, and wet weight of explanted Sprague-Dawley rat lenses treated with 15 µM ciglitazone (CIG) for 24 hr. Lens clarity was significantly improved in RR+CIG treated lenses compared to CIG treatment alone (cloudy vs. cloudy with nuclear cataract formation, respectively). N=3-4 lenses per treatment, mean ± S.E.M. Values with different superscripts are significantly different from each other using one way ANOVA followed by Fisher’s LSD test, p < 0.05.
Figure 4: Visual appearance of rat lenses treated with the thiazolidinediones ciglitazone, englitazone, and darglitazone (15 μM) after 48 hr of exposure (representative examples). Note presence of Stage 2 opacity in ciglitazone-treated lens. Reprinted from Aleo et al. (2000) with permission "Copyright 2000 New York Academy of Sciences, U.S.A."
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>DAY 49</th>
<th>DAY 82</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mg/kg/day, p.o.)</td>
<td>Incidence</td>
<td>Description range</td>
</tr>
<tr>
<td>CONTROL (male)</td>
<td>0/14</td>
<td>Normal</td>
</tr>
<tr>
<td>CONTROL (female)</td>
<td>0/15</td>
<td>Normal</td>
</tr>
<tr>
<td>30 (male)</td>
<td>0/14</td>
<td>Normal</td>
</tr>
<tr>
<td>30 (female)</td>
<td>0/15</td>
<td>Normal</td>
</tr>
<tr>
<td>100 (male)</td>
<td>3/15</td>
<td>Stress lines (anterior + posterior)</td>
</tr>
<tr>
<td>100 (female)</td>
<td>1/15</td>
<td>Total cataract</td>
</tr>
<tr>
<td>300 (male)</td>
<td>6/14</td>
<td>Stress lines (anterior + posterior)</td>
</tr>
<tr>
<td>300 (female)</td>
<td>8/13</td>
<td>Stress lines (anterior + posterior)</td>
</tr>
</tbody>
</table>
TABLE 2

LACK OF PROTECTIVE EFFECTS OF DITHIOTREITOL (DTT), CALPAIN-INHIBITOR-II (CI-II),
E64d, and SORBINIL (SORB) ON THE TOXICITY OF CIGLITAZONE TO EXPLANTED SPRAGUE-DAWLEY RAT LENSES

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>ATP CONTENT</th>
<th>MTT REDUCTION</th>
<th>GSH CONTENT</th>
<th>LENS WEIGHT</th>
<th>LENS APPEARANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CALPAIN INHIBITORS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>0 ± 10a</td>
<td>ND</td>
<td>0 ± 5a</td>
<td>0 ± 3a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>CI-II (1 mM)</td>
<td>7 ± 8a</td>
<td>ND</td>
<td>-14 ± 3b</td>
<td>-2 ± 1a</td>
<td>STAGE 1</td>
</tr>
<tr>
<td>CIG (15 µM)</td>
<td>-74 ± 3b</td>
<td>ND</td>
<td>-53 ± 4c</td>
<td>31 ± 5c</td>
<td>STAGE 2</td>
</tr>
<tr>
<td>CI-II+CIG</td>
<td>-70 ± 6b</td>
<td>ND</td>
<td>-51 ± 6c</td>
<td>15 ± 2b</td>
<td>STAGE 2</td>
</tr>
<tr>
<td>CONTROL</td>
<td>ND</td>
<td>0 ± 3a</td>
<td>ND</td>
<td>0 ± 5a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>E64d (100 µM)</td>
<td>ND</td>
<td>25 ± 7b</td>
<td>ND</td>
<td>-2 ± 3a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>CIG (15 µM)</td>
<td>ND</td>
<td>-53 ± 4c</td>
<td>ND</td>
<td>43 ± 9b</td>
<td>STAGE 2</td>
</tr>
<tr>
<td>E64d+CIG</td>
<td>ND</td>
<td>-79 ± 1d</td>
<td>ND</td>
<td>27 ± 3b</td>
<td>STAGE 2</td>
</tr>
<tr>
<td><strong>ANTIOXIDANT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>0 ± 12a</td>
<td>ND</td>
<td>0 ± 10a</td>
<td>0 ± 1a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>DTT (2 mM)</td>
<td>-6 ± 5a</td>
<td>ND</td>
<td>5 ± 4a</td>
<td>-12 ± 2b</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>CIG (15 µM)</td>
<td>-70 ± 2b</td>
<td>ND</td>
<td>-67 ± 2b</td>
<td>30 ± 8c</td>
<td>STAGE 2</td>
</tr>
<tr>
<td>DTT+CIG</td>
<td>-77 ± 2b</td>
<td>ND</td>
<td>-75 ± 7b</td>
<td>29 ± 2c</td>
<td>STAGE 2</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0 ± 5a</td>
<td>ND</td>
<td>0 ± 4a</td>
<td>0 ± 2a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>DTT (2 mM)</td>
<td>-6 ± 5a,b</td>
<td>ND</td>
<td>5 ± 4a</td>
<td>-12 ± 2b</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>H₂O₂ (300 µM)</td>
<td>-12 ± 3b</td>
<td>ND</td>
<td>-29 ± 3b</td>
<td>10 ± 2c</td>
<td>STAGE 1</td>
</tr>
<tr>
<td>DTT+H₂O₂</td>
<td>-0 ± 4a</td>
<td>ND</td>
<td>9 ± 8a</td>
<td>-2 ± 2a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>CONTROL</td>
<td>ND</td>
<td>0 ± 3a</td>
<td>ND</td>
<td>0 ± 5a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>DTT (2 mM)</td>
<td>ND</td>
<td>-2 ± 3a</td>
<td>ND</td>
<td>-8 ± 1a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>H₂O₂ (400 µM)</td>
<td>ND</td>
<td>-73 ± 2b</td>
<td>ND</td>
<td>12 ± 3b</td>
<td>STAGE 1</td>
</tr>
<tr>
<td>DTT+H₂O₂</td>
<td>ND</td>
<td>4 ± 3a</td>
<td>ND</td>
<td>-5 ± 3a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td><strong>ALDOSE REDUCTASE INHIBITOR</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>ND</td>
<td>0 ± 3a</td>
<td>ND</td>
<td>0 ± 6a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>SORB (50 µM)</td>
<td>ND</td>
<td>7 ± 6a</td>
<td>ND</td>
<td>-4 ± 5a</td>
<td>STAGE 0</td>
</tr>
<tr>
<td>CIG (15 µM)</td>
<td>ND</td>
<td>-55 ± 1b</td>
<td>ND</td>
<td>40 ± 7b</td>
<td>STAGE 2</td>
</tr>
<tr>
<td>SORB+CIG</td>
<td>ND</td>
<td>-53 ± 7b</td>
<td>ND</td>
<td>42 ± 1b</td>
<td>STAGE 2</td>
</tr>
</tbody>
</table>
Results are expressed as % difference from control, mean ± S.E.M. of 3-5 lenses per treatment. Lenses were incubated in the presence of ciglitazone (CIG) for 24 hr using 0.2% ethanol as vehicle. Calpain inhibitor-II (CI-II) and E64d were added 15 min or 2 hr before CIG, respectively. Dithiothreitol (DTT) and sorbinil (SORB) were added to the treated medium at the same time as CIG. ND = Not determined; STAGE 0 = Clear; STAGE 1 = hazy cortical region; STAGE 2 = haziness characterized by a visible demarcation between nuclear and cortical regions. Grading system is based on a modification from Dickerson et al., 1997. Values with different superscripts within a given experiment are significantly different from each other, $p < 0.05$. 

\(^1\)
Figure 1

The diagram illustrates the percentage of affected lenses in different treatment groups, categorized by stages 0, 1, and 2.

- **CONTROL (46)**
- **0.375 μM CIG (8)**
- **3.75 μM CIG (15)**
- **7.5 μM CIG (22)**
- **15 μM CIG (31)**
- **30 μM CIG (8)**

The percentages for each stage are as follows:

- **Stage 0**
  - CONTROL: 100%
  - 0.375 μM CIG: 100%
  - 3.75 μM CIG: 100%
  - 7.5 μM CIG: 100%
  - 15 μM CIG: 100%
  - 30 μM CIG: 100%

- **Stage 1**
  - CONTROL: 0%
  - 0.375 μM CIG: 0%
  - 3.75 μM CIG: 0%
  - 7.5 μM CIG: 100%
  - 15 μM CIG: 100%
  - 30 μM CIG: 100%

- **Stage 2**
  - CONTROL: 0%
  - 0.375 μM CIG: 0%
  - 3.75 μM CIG: 0%
  - 7.5 μM CIG: 0%
  - 15 μM CIG: 100%
  - 30 μM CIG: 100%
Figure 2

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Figure 3

% DIFFERENCE FROM CONTROL

-100  -75  -50  -25  0  25

CONTROL RR CIG RR+CIG

Total ATP Total GSH Lens Weight

Figure 3
Ciglitazone  
Englitazone  
Darglitazone  

(15 µM after 48 hr)

Figure 4