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Specific Activation of the Nuclear Receptors PPARγ and RORA by the Antidiabetic Thiazolidinedione BRL 49653 and the Antiarthritic Thiazolidinedione Derivative CGP 52608

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ABSTRACT

The thiazolidinedione BRL 49653 and the thiazolidinedione derivative CGP 52608 are lead compounds of two pharmacologically different classes of compounds. BRL 49653 is a high affinity ligand of peroxisome proliferator-activated receptor γ (PPARγ) and a prototype of novel antidiabetic agents, whereas CGP 52608 activates retinoic acid receptor-related orphan receptor α (RORA) and exhibits potent antiarthritic activity. Both receptors belong to the superfamily of nuclear receptors and are structurally related transcription factors. We tested BRL 49653 and CGP 52608 for receptor specificity on PPARγ, RORA, and retinoic acid receptor α, a closely related receptor to RORA, and compared their pharmacological properties in *in vitro* and *in vivo* models in which these compounds have shown typical effects. BRL 49653 specifically induced PPARγ-mediated gene activation, whereas CGP 52608 specifically activated RORA in transiently transfected cells. Both compounds were active in nanomolar concentrations. Leptin production in differentiated adipocytes was inhibited by nanomolar concentrations of BRL 49653 but not by CGP 52608. BRL 49653 antagonized weight loss, elevated blood glucose levels, and elevated plasma triglyceride levels in an *in vivo* model of glucocorticoid-induced insulin resistance in rats, whereas CGP 52608 exhibited steroid-like effects on triglyceride levels and body weight in this model. In contrast, potent antiarthritic activity in rat adjuvant arthritis was shown for CGP 52608, whereas BRL 49653 was nearly inactive. Our results support the concept that transcriptional control mechanisms via the nuclear receptors PPARγ and RORA are responsible at least in part for the different pharmacological properties of BRL 49653 and CGP 52608. Both compounds are prototypes of interesting novel therapeutic agents for the treatment of non-insulin-dependent diabetes mellitus and rheumatoid arthritis.

BRL 49653 [(±)-5-([4-[2-methyl-2(pyridylamino)ethoxy]phenyl]methyl)2,4-thiazolidinedione)] is a novel hypoglycemic and hypolipidemic agent used in animal models of NIDDM (Oakes *et al.*, 1994). BRL 49653 and structurally related thiazolidinediones such as ciglitazone, pioglitazone, and troglitazone improve insulin resistance by enhancing insulin action in skeletal muscle, liver, and adipose tissue. Although the precise mechanism of action remains unknown, it has been recently shown that these thiazolidinediones, as well as the prostaglandin J_2 metabolite 15d-PGJ$_2$, are ligands of the PPARγ (Forman *et al.*, 1995; Lehmann *et al.*, 1995; Lambe and Tugwood, 1996). Thiazolidinedione-induced activation of PPARγ correlates with the antidiabetic actions *in vivo* (Berger *et al.*, 1996). PPARγ is a member of the nuclear receptor superfamily of transcription factors (for a review, see Schoonjans *et al.*, 1996). The three known PPAR

subtypes (α, β, and γ) exhibit typical tissue distribution in adult animals and during development (Braissant *et al.*, 1996). The expression of PPARγ is one of the earliest events during the differentiation of fibroblasts to adipocytes (Tontonoz *et al.*, 1994a), and ectopic expression of PPARγ promotes this conversion (Tontonoz *et al.*, 1994b). PPARγ regulates the transcription of several adipocyte-specific genes, including phospho*enol*pyruvate carboxykinase, adipocyte fatty acid binding protein aP2, and leptin (Schoonjans *et al.*, 1996). Antidiabetic thiazolidinediones and 15d-PGJ$_2$ promote adipocyte differentiation and suppress leptin gene expression in concentrations similar to their K_d values for binding to PPARγ (De Vos *et al.*, 1996; Kallen and Lazar, 1996). These findings suggest a pivotal role for PPARγ and its ligands in controlling adipocyte development and glucose homeostasis.

aspet

ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; ROR, retinoic acid receptor-related orphan receptor; RORA, retinoic acid receptor-related orphan receptor α, RAR, retinoic acid receptor; RXR, retinoid X receptor; RA, all-*trans* retinoic acid, GR, glucocorticoid receptor; CAT, chloramphenicol acetyltransferase; FCS, fetal calf serum; NIDDM, non-insulin-dependent diabetes mellitus; PGD$_2$, prostaglandin D$_2$; 15d-PGJ$_2$, 15-deoxy-Δ12,14-prostaglandin J$_2$.

CGP 52608 [1-[3-allyl-4-oxo-thiazolidine-2-ylidene]-4-methyl-thiosemicarbazone] is the lead compound of a structurally different class of thiazolidinedione derivatives with potent therapeutic effects in experimental arthritis models (Missbach et al., 1996). This compound has been shown to specifically activate RORA, another member of the nuclear receptor superfamily (Wiesenberg et al., 1995). RORA (RORα or RZRα) is one of three known subtypes (α, β, and γ) of the ROR. Each subtype shows a characteristic tissue expression pattern (for a review, see Carlberg and Wiesenberg, 1995). In searching for a natural ligand, the pineal gland hormone melatonin was found to specifically activate RORA and to compete with CGP 52608 for binding (Wiesenberg et al., 1995). Structure-activity relationship studies with CGP 52608 analogues revealed a striking correlation between activation of RORA and inhibition of rat adjuvant arthritis, suggesting a key role of this receptor in mediating the antiarthritic effects of these compounds (Missbach et al., 1996).

The identification of orphan receptor ligands is always an important step toward a better understanding of their regulatory functions during development and homeostasis. In the case of PPARs, structurally diverse compounds such as peroxisome proliferators, antidiabetic thiazolidinediones, fatty acids, prostaglandin and leukotriene derivatives, and the endogenous steroid dehydroepiandrosterone have been shown to activate PPARs, either directly as ligands or indirectly by as-yet-unknown mechanisms (Devchand et al., 1996; Peters et al., 1996; Forman et al., 1997; Kliewer et al., 1997). So far, known activators of RORs are the pineal gland hormone melatonin (Becker-Andre et al., 1994; Wiesenberg et al., 1995) and the antiarthritic thiazolidinedione derivatives (Missbach et al., 1996).

Given the diversity of compounds capable of activating PPARs, the aim of this study was to investigate the specificity of PPARγ and RORA activation by the antidiabetic thiazolidinedione BRL 49653 and the antiarthritic thiazolidinedione derivative CGP 52608 and to compare their effects in functional assays in which either compound had shown typical effects. The models used were leptin production in differentiated adipocytes and glucocorticoid-induced insulin resistance in rats (models for PPARγ ligands) and rat adjuvant arthritis, an *in vivo* model in which CGP 52608 and analogues have shown high activity.

Materials and Methods

Compounds. All thiazolidinediones and derivatives were synthesized in the Department of Chemical Research (Novartis Pharma AG, Basel, Switzerland). Th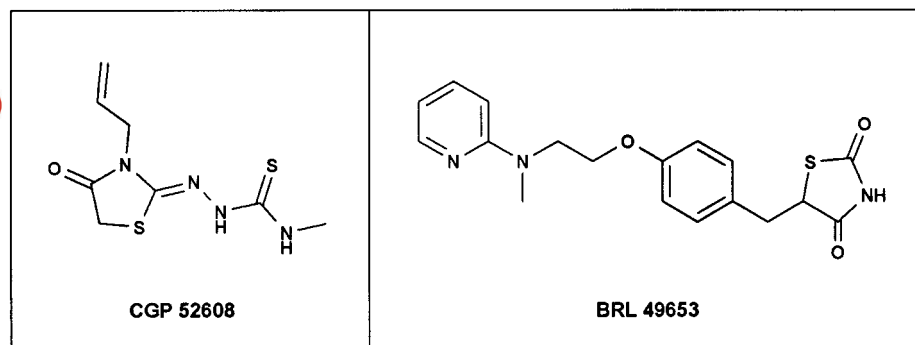e structures of CGP 52608 and BRL 49653 are given in Fig. 1. CGP 52608, CGP 55707, and CGP 55066 were synthesized as described by Missbach et al. (1996), and BRL 49653 (racemate) was synthesized as described by Cantello et al. (1994). Melatonin and PGD$_2$ were obtained from Fluka (Buchs, Switzerland), and 15d-PGJ$_2$ was from Cayman Chemical (Ann Arbor, MI). Thiazolidinedione derivatives and melatonin were dissolved in dimethylsulfoxide, and prostaglandins were dissolved in ethanol at 10 mM; dilutions were made in cell culture medium before use.

Preparative separation of the enantiomers of BRL 49653. The two enantiomers of BRL 49653 were separated from the racemate by chiral high performance liquid chromatography (Abbott et al., 1994). A 500 × 50-mm column loaded with Chiracel absorbance was used. The eluent was 15% ethanol in n-heptane, and the flow rate was 150 ml/min. The enantiomers were detected by UV (254 nm). Retention times were 14.6 min for the R-(+)-enantiomer and 37.6 min for the S-(−)-enantiomer, yielding 99.6% pure R-(+)-enantiomer and 77% pure S-(−)-enantiomer.

DNA constructs, transfection, and CAT assays. Materials and methods were described in detail by Wiesenberg et al. (1995). Briefly, we used the pBLCAT2-derived CAT reporter constructs, containing, in the XbaI site, natural response elements for ROR (CAAAATGGGTCA), identified in the human 5-lipoxygenase gene promoter (Steinhilber et al., 1995); for PPAR (AATGTAGGTAAT-AGTTCAATAGGTCA), found in the mouse bifunctional enzyme gene promoter (Bardot et al., 1993); or for RAR (AGGGTTCACCGAAAGT-TCA), from the human RARβ gene promoter (De The et al., 1990). The cDNAs of human RORA (Becker-Andre et al., 1993), Xenopus laevis PPARγ (Dreyer et al., 1992), human RXRα, and human RARα have been subcloned into the expression vector pSG5 (Stratagene, La Jolla, CA).

Drosophila SL-3 cells (2×10^6 cells per well in a six-well plate) were grown overnight in Schneider's medium (Life Technologies, Grand Island, NY) without FCS. Liposomes were formed by incubating 2 μg of the reporter plasmid, 1 μg of receptor expression vector, and 1 μg of the reference plasmid pCH110 (Pharmacia, Piscataway, NJ) with 15 μg of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (Boehringer-Mannheim, Mannheim, Germany) for 15 min at room temperature in a total volume of 100 μl. After dilution with 0.9 ml of Schneider's medium, the liposomes were added to the cells. Eight hours after transfection, 500 μl of Schneider's medium supplemented with the indicated ligand was added. After an additional 16 hr, the cells were harvested, and CAT assays were performed. The CAT activities were normalized to β-galactosidase activity, and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of mock-induced controls. Each condition was analyzed in triplicate, and data are shown as mean values with standard deviation.

Secretion of leptin by fully differentiated 3T3-F442A adipocytes. The clonal cell line 3T3-F442A (Green and Kehinde, 1976) was obtained through the courtesy of Dr. B. Fève (INSERM Crétail, France). Differentiation was carried out as described previously (Dani et al., 1989). Cells were plated onto 12-well tissue culture plates (Falcon) at a density of 1.5×10^3 cells/cm^2 in Dulbecco's

Fig. 1. Structures of CGP 52608 and BRL 49653.

CGP 52608

BRL 49653

modified Eagle's medium supplemented with 8% FCS, 200 units/ml penicillin, 50 μg/ml streptomycin, 33 μM biotin, and 17 μM pantothenate. Differentiation was initiated after the cells reached confluency by adding to the standard medium 2 nM triiodothyronine, 17 nM insulin, 100 nM dexamethasone, and 100 μM isobutylmethylxanthine (differentiation medium). After 3 days, the medium was replaced by differentiation medium without dexamethasone and isobutylmethylxanthine and changed thereafter every second day for 2 weeks until differentiation to adipocytes was complete.

For measurement of leptin secretion, fully differentiated adipocytes were incubated for 72 hr in fresh medium containing test compounds or the vehicle. Immunoreactive leptin was quantified in the supernatants by radioimmunoassay using rabbit polyclonal antibodies against recombinant mouse leptin (Rentsch *et al.*, 1995). Typically, 50-μl standards or samples were incubated for 18 hr at 4° with 50 μl of radiolabeled [^{125}I]leptin, 50 μl of antiserum (diluted 1:4000), and 50 μl of phosphate-buffered saline in the presence of 0.1% Tween-20 and 0.1% bovine serum albumin (final concentrations). Bound and free leptin were separated by centrifugation (30 min at 3000 × g at 4°) after the addition of 600 μl of 20% polyethylene glycol and 50 μl of γ-globulin (10 mg/ml). Pellets were counted in a gamma counter. The detection limit was 0.48 ng of leptin/ml. Experiments were performed in triplicate, and the results are given as mean values with standard deviation.

Glucocorticoid-induced insulin resistance. Male Lewis rats (LEW/TIF; specific pathogen free, 250–270 g body weight, five animals/group; Ciba Animal Farm, Sisseln, Switzerland) received either dexamethasone alone (0.15 mg/kg p.o.) or a combination of dexamethasone (0.15 mg/kg p.o.) and CGP 52608 or BRL 49653 (0.01–10 mg/kg p.o.) for 9 days. Test compounds were dissolved in 0.5 ml of DMSO plus 4.5 ml of 0.75% methylcellulose and were administered in a volume of 5 ml/kg. Control animals received the vehicle. After the animals were fasted overnight, blood samples were taken from the orbital vein in isoflurane narcosis on days 4 and 9. Blood glucose and plasma triglyceride levels were estimated using commercially available test kits (Boehringer-Mannheim). Differences between groups were statistically evaluated by Student's t test.

Rat adjuvant arthritis. Adjuvant arthritis was induced as described previously (Wiesenberg *et al.*, 1989). Briefly, male Lewis rats (LEW/TIF; SPF, 180–200 g of body weight) were immunized by an intraplantar injection of Freund's complete adjuvant (0.2 mg of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) in 0.05 ml of paraffin oil (Riedel de Haen, Seelze, Switzerland) into the left hind paw (day 0). This procedure induced arthritis in 100% of the animals. Disease progression was followed by plethysmographic edema measurements of the injected hind paw (primary lesion) and the noninjected hind paw (secondary lesion). CGP 52608, BRL 49653 and prednisolone (reference compound) were given orally to five animals/group between 8.00 and 10.00 a.m. from day 0 to 30 in 10 ml/kg of 0.75% methylcellulose. Normal ($n = 5$) and arthritic control animals ($n = 10$) received the vehicle. Differences between groups were statistically evaluated by Student's t test.

Results

Specific activation of PPARγ and RORA. PPARγ- and RORA-mediated transcriptional activation was investigated in transiently transfected *Drosophila* SL-3 cells under serum-free conditions to overcome the high constitutive activity of RORA in the presence of serum (Wiesenberg *et al.*, 1995; Missbach *et al.*, 1996). SL-3 cells were transfected with the expression vectors for *X. laevis* PPARγ and human RXRα or with human RORA and a thymidine kinase-CAT reporter construct containing either the PPAR response element found in the promoter of the mouse bifunctional enzyme (Bardot *et al.*, 1993) or the ROR response element of the human 5-lipoxygenase gene (Steinhilber *et al.*, 1995). To fur-

ther evaluate receptor specificity, all compounds were also tested on RARα/RXRα heterodimers on the RA response element of the human RARβ gene promoter. The structures of the lead compounds BRL 49653 and CGP 52608 are shown in Fig. 1, and the results of receptor-mediated gene activation are shown in Fig. 2.

The antidiabetic thiazolidinedione BRL 49653 (0.01–1 μM) specifically stimulated PPARγ-mediated gene activity. Surprisingly, only minor differences in potency were seen between the two enantiomers of BRL 49653. The racemate and both enantiomers induced a 2–7-fold gene activation in concentrations between 0.01 and 1 μM. The putative natural PPARγ ligand 15d-PGJ$_2$ was distinctly less potent and induced only a 1.4–2.6-fold gene induction in the same concentration range. PGD$_2$, a natural precursor of 15d-PGJ$_2$ (10 μM), as well as the antiarthritic thiazolidinedione derivative CGP 52608, two structural analogues (CGP 55066, CGP 55707), and the pineal gland hormone melatonin did not induce PPARγ-mediated gene activation at 10 μM.

RORA-mediated gene activation was shown only for the antiarthritic thiazolidinedione derivative CGP 52608 (0.01–1 μM, 3.1–4.9-fold) and the putative natural ROR ligand melatonin (0.01–1 μM, 2.4–4.6-fold). Neither the two pharmacologically inactive CGP 52608 analogues CGP 55066 and CGP 55707 (Missbach *et al.*, 1996) nor BRL 49653 or any other compound tested induced RORA-mediated gene activation at 10 μM.

Specific gene activation via RARα/RXRα was obtained only with the specific ligand RA (0.01–1 μM). All thiazolidinedione derivatives and other test compounds were inactive at 10 μM. The RAR was selected for specificity control (Carlberg *et al.*, 1994).

These results demonstrate PPARγ specificity for the antidiabetic thiazolidinedione BRL 49653 and 15d-PGJ$_2$ and RORA specificity for the antiarthritic thiazolidinedione derivative CGP 52608 and the pineal gland hormone melatonin.

Influence on leptin secretion by differentiated 3T3-F422A adipocytes. PPARγ regulates the activity of several adipocyte-specific genes, including leptin (Tontonoz *et al.*, 1994b; Schoonjans *et al.*, 1996), and antidiabetic thiazolidinediones suppress leptin gene expression in differentiated adipocytes (De Vos *et al.*, 1996; Kallen and Lazar, 1996). The effects of BRL 49653 and CGP 52608 on leptin production in fully differentiated 3T3-F422A adipocytes were investigated to compare these compounds in a cellular functional PPARγ-regulated assay. The results are shown in Table 1.

The antidiabetic thiazolidinedione BRL 49653 (0.002–0.2 μM) dose-dependently inhibited leptin secretion from differentiated adipocytes. As already shown for PPARγ-mediated transcriptional activation in SL-3 cells (Fig. 2), the two BRL 49653 enantiomers were not significantly different in their potency. A potential explanation might be a rapid racemization of the enantiomers under the cell culture conditions used. BRL 49653-induced PPARγ activation in transfected SL-3 cells and inhibition of leptin production in adipocytes were obtained with low nanomolar concentrations. The putative natural PPARγ ligand 15d-PGJ$_2$ (0.1–10 μM) also suppressed leptin secretion but was ≥100 times less potent than BRL 49653. The precursor PGD$_2$ (0.1–10 μM) was inactive.

In contrast to BRL 49653, the antiarthritic thiazolidinedi-

one derivative CGP 52608 and both analogues (10 μM), as well as melatonin (0.1–10 μM), were inactive.

Our data confirm published results for antidiabetic thiazolidinediones (Kallen and Lazar, 1996) and show that PPARγ ligands, but not RORA-activating compounds, inhibit leptin production in differentiated adipocytes.

Effects on glucocorticoid-induced insulin resistance. Glucocorticosteroids up-regulate leptin secretion in adipocyte cultures (Slieker *et al*., 1996), and pharmacological doses induce leptin gene expression within 24 hr in rat adipose tissue (De Vos *et al*., 1995). Glucocorticoids induce insulin resistance in rats (Inoue *et al*., 1996), whereas antidiabetic thiazolidinediones improve insulin responsiveness in animals and humans (Turner, 1996). To compare the *in vivo* profile of BRL 49653 and CGP 52608 in a model responding to PPARγ ligands, both compounds were tested in rats with glucocorticoid-induced insulin resistance.

Male rats received pharmacological doses of dexamethasone alone (0.15 mg/kg p.o.) or in combination with BRL 49653 or CGP 52608 (0.01–10 mg/kg p.o.) for 9 days, and changes in blood glucose, plasma triglycerides, and body weight were monitored on days 4 and 9 after overnight fasting. Additional test groups received BRL 49653 or CGP 52608 (0.01–10 mg/kg p.o.) without dexamethasone. The effects of BRL 49653 and CGP 52608 on dexamethasone-induced insulin resistance are shown in Fig. 3.

Blood glucose levels were significantly enhanced by dexamethasone after 4 and 9 days of treatment. BRL 49653 (0.01–10 mg/kg p.o.) dose-dependently reduced the elevated glucose levels after 4 days, but this reduction was transient and no longer seen after 9 days. CGP 52608 (0.01–10 mg/kg p.o.) did not lower the corticosteroid-induced elevated glucose levels during 9 days of treatment.

Plasma triglyceride levels were slightly but not signifi-
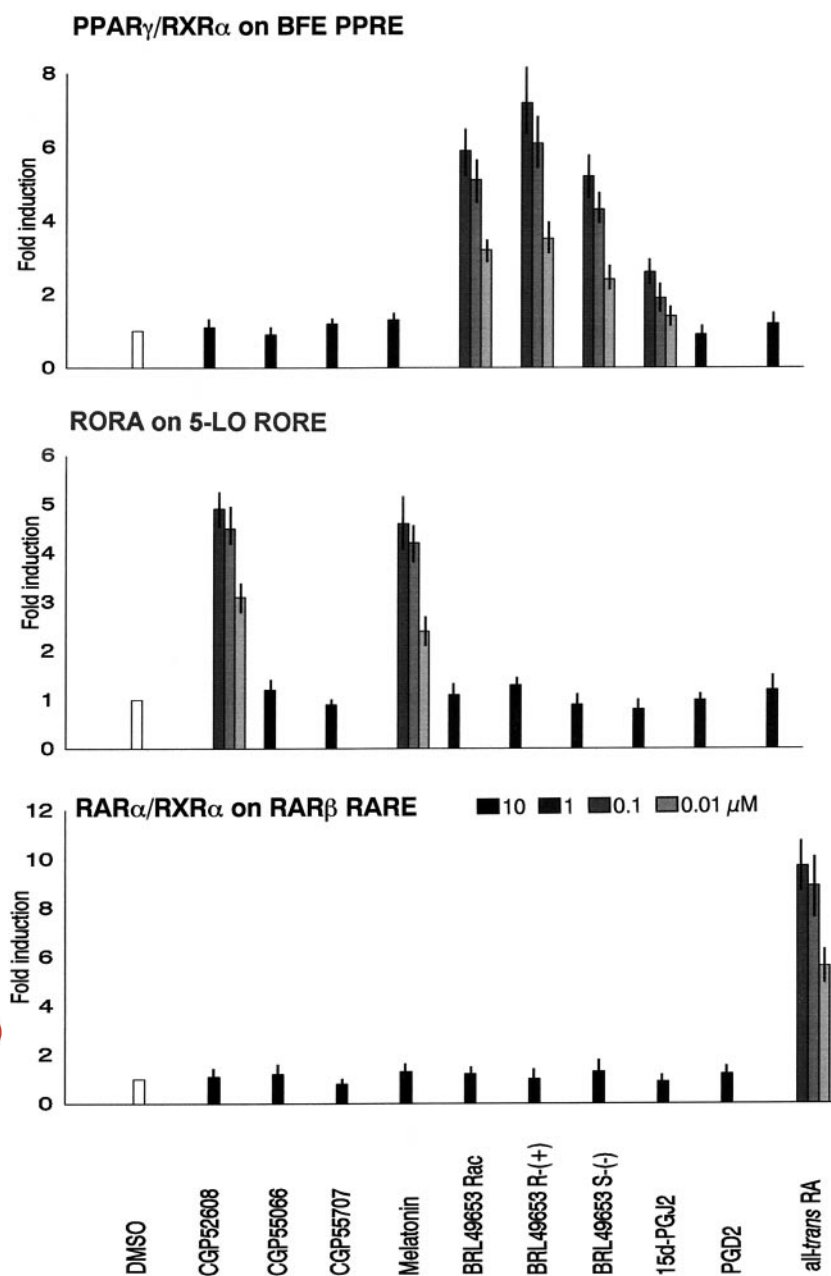
Fig. 2. Specificity of PPARγ- and RORA-induced gene activation. *Drosophila* SL-3 cells were transfected with the expression vector for human RORA, *X. laevis* PPARγ, or human RARα (the last two in combination with an expression vector for human RXRα) and the CAT reporter construct containing their specific response elements as indicated. The cells were treated with the potential ligands in the absence of FCS and were harvested 16 hr later. CAT activities were normalized to β-galactosidase activity, and induction factors were calculated. Each condition was analyzed in triplicate, and induction factors are shown as mean values with standard deviation.

cantly enhanced by dexamethasone. However, the combined treatment of dexamethasone and BRL 49653 resulted in significant triglyceride-lowering effects after 4 and 9 days. In contrast, combined treatment with CGP 52608 had no effect after 4 days, but the highest dose of CGP 52608 significantly enhanced triglyceride levels after 9 days of treatment.

A progressive decrease in body weight was seen after 4 and 9 days of treatment with dexamethasone, indicating that catabolic doses of the steroid were used. Combined treatment with BRL 49653 improved the body weight loss under dexamethasone, whereas CGP 52608 caused an additional weight loss.
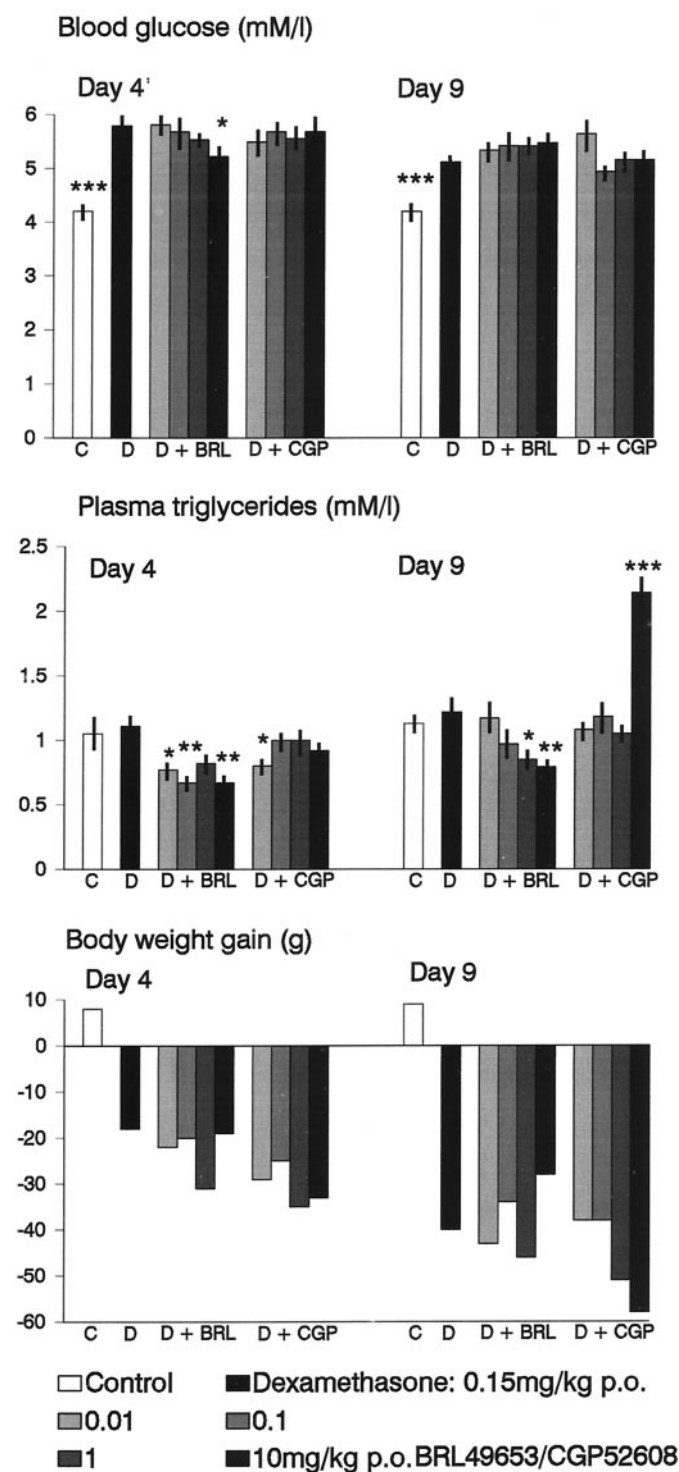
BRL 49653 and CGP 52608 (0.01–10 mg/kg p.o.) had only minor effects in normal rats (data not shown). BRL 49653 did not change blood glucose and triglyceride levels but slightly enhanced body weight gain in comparison with controls. CGP 52608 had no effects on plasma triglyceride levels, but the highest dose slightly but significantly lowered blood glucose and delayed body weight gain.

Taken together, although the effects of BRL 49653 on blood glucose and triglyceride levels in this model of induced insulin resistance were not as pronounced as those in models of genetic insulin resistance (Oakes *et al.*, 1994; Lehmann *et al.*, 1995), the results are qualitatively similar in that BRL 49653 improved dexamethasone-induced insulin resistance and weight loss. CGP 52608 exhibited a clearly different profile. Additional weight loss and enhanced triglyceride levels were seen during combined treatment with dexamethasone.

Antiarthritic activity in rat adjuvant arthritis. Rat adjuvant arthritis is a chronic T cell-dependent autoimmune disease with many similarities to rheumatoid arthritis, such as chronic inflammation, progressive joint destruction, enhanced T cell responses, and pronounced cytokine-mediated acute-phase reactions. CGP 52608 and structural analogues

exhibit potent inhibitory effects in this experimental autoimmune model (Missbach *et al.*, 1996).

To further compare the *in vivo* pharmacological profiles of

Fig. 3. Effects of BRL 49653 and CGP 52608 on glucocorticoid-induced insulin resistance in rats. Male Lewis rats received either dexamethasone alone (*D*) (0.15 mg/kg p.o.) or a combination of dexamethasone and BRL 49653 (*D + BRL*) or CGP 52608 (*D + CGP*) (0.01–10 mg/kg p.o.) for 9 days. Control animals received the vehicle (*C*). Blood glucose and plasma triglyceride levels and body weight were measured on days 4 and 9 after overnight fasting. Differences between groups were statistically evaluated by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

TABLE 1

Effect of test compounds on leptin secretion by differentiated 3T3-F422A adipocytes

Fully differentiated adipocytes were incubated for 72 hr with test compounds or vehicle, and cumulative leptin secretion was measured by radioimmunoassay. The leptin content in the supernatant of control cultures was 1.7 ± 0.22 ng/ml. Data are given as mean $\pm$ standard error for three determinations.

Compound	Concentration	Leptin secretion
	μM	% of Control
CGP 52608	10	103 ± 8
CGP 55066	10	88 ± 3
CGP 55707	10	90 ± 4
Melatonin	10	114 ± 14
	1	128 ± 4
	0.1	132 ± 10
BRL 49653	0.2	19 ± 4
(racemate)	0.05	25 ± 12
	0.01	31 ± 5
	0.002	117 ± 16
BRL 49653	0.2	30 ± 14
R-(+)	0.05	25 ± 7
	0.01	48 ± 9
	0.002	92 ± 14
BRL 49653	0.2	13 ± 6
S-(−)	0.05	27 ± 14
	0.01	23 ± 4
	0.002	102 ± 11
15d-PGJ$_2$	10	61 ± 24
	1	85 ± 34
	0.1	110 ± 27
PGD$_2$	10	82 ± 28
	1	135 ± 27
	0.1	115 ± 18

BRL 49653 and CGP 52608, we tested both compounds in rat adjuvant arthritis.

Daily oral treatment was started on the day of arthritis induction (day 0) and continued up to day 30. Groups of five arthritic rats received two or three different doses of CGP 52608, BRL 49653 (racemate), or the reference compound prednisolone. Fig. 4 shows the development of the inflammatory edema in the adjuvant-injected hind paw (primary lesion) and in the noninjected hind paw (secondary lesion) during drug treatment.

CGP 52608 inhibited edema development in both hind paws significantly and dose-dependently with very low doses (0.01 and 0.1 mg/kg). The higher dose of 0.1 mg/kg was at least as effective as prednisolone at 10 mg/kg and caused a nearly total suppression of the primary and secondary inflammatory edema after 3–4 weeks of treatment.

The same low doses of BRL 49653 (0.01 and 0.1 mg/kg) did not inhibit arthritis development and progression. A 10-fold higher dose (1.0 mg/kg) reduced the edema in the injected hind paw only slightly and transiently by 15–25% from day 18 to 28. The secondary lesion was not significantly inhibited.

Prednisolone (1–10 mg/kg), as expected, dose-dependently inhibited the inflammatory edema in both hind paws.

Our results show potent anti-inflammatory activity in rat
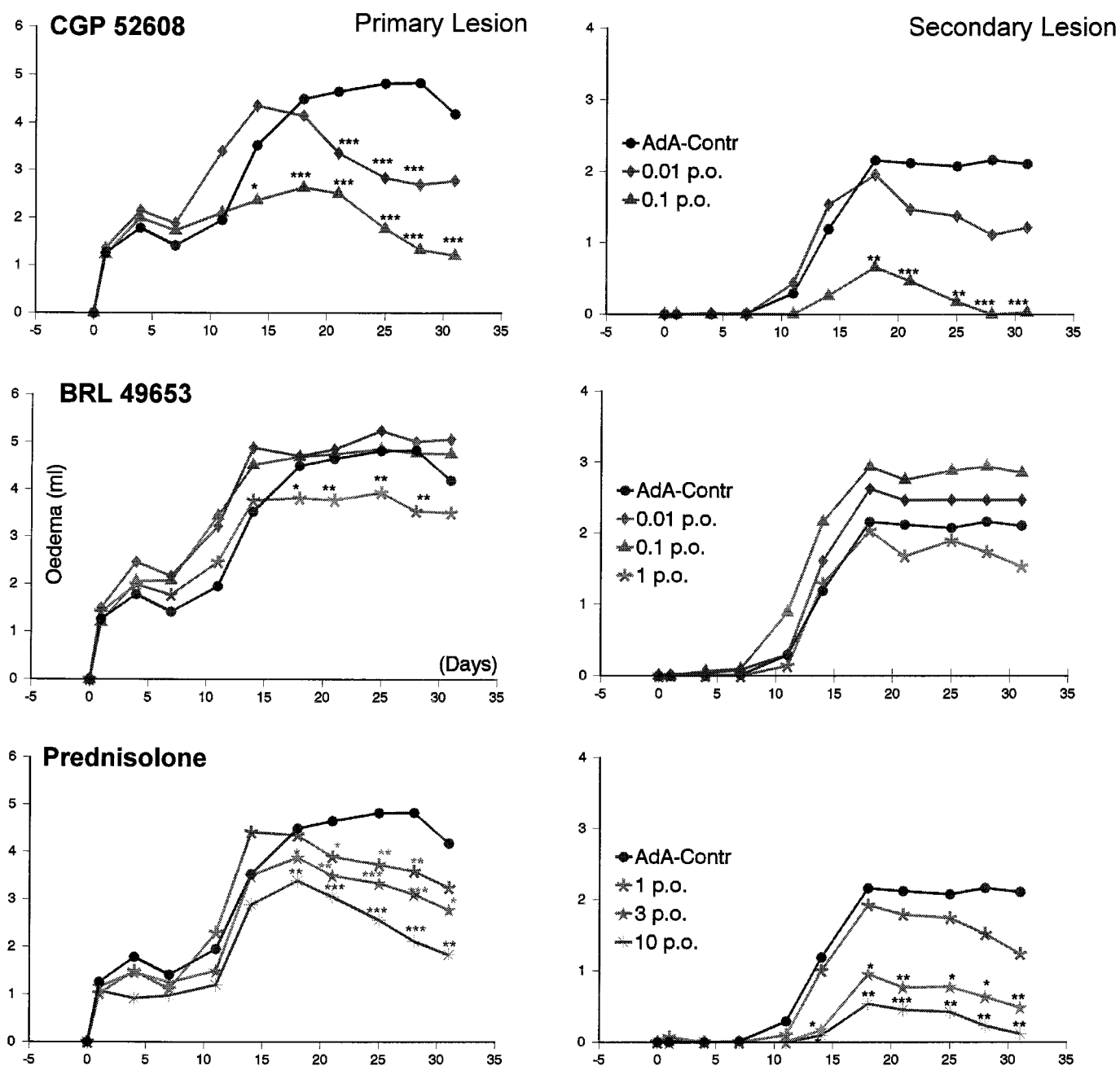
Fig. 4. Comparison of CGP 52608, BRL 49653 and prednisolone in rat adjuvant arthritis. Adjuvant arthritis was induced in male Lewis rats by an intraplantar injection of Freund's complete adjuvant. Disease progression was followed by edema measurements of the injected (primary lesion) and the noninjected (secondary lesion) hind paws. Compounds or vehicle (*AdA-Contr*) were orally administered from day 0 (arthritis induction) to day 30. Differences between arthritic controls and drug-treated animals were statistically evaluated by Student's *t* test. $*, p < 0.05$; $**, p < 0.01$; $***, p < 0.001$.

adjuvant arthritis for the RORA-activating thiazolidinedione derivative CGP 52608 and the GR ligand prednisolone, whereas the PPARγ ligand BRL 49653 did not exhibit significant effects in this chronic model for autoimmune diseases.

Discussion

Several lines of evidence indicate that the antidiabetic thiazolidinedione BRL 49653 (Berger *et al*., 1996; De Vos *et al*., 1996; Kallen and Lazar, 1996) and the antiarthritic thiazolidinedione derivative CGP 52608 (Wiesenberg *et al*., 1995; Missbach *et al*., 1996) exert their pharmacological effects at least in part via specific activation of the nuclear hormone receptors PPARγ and RORA. Our results obtained in transiently transfected *Drosophila* SL-3 cells confirm PPARγ-specific gene activation for BRL 49653 and the putative natural ligand 15d-PGJ$_2$ and RORA-specific gene activation for CGP 52608 and the putative natural ligand melatonin (Fig. 2).

Nuclear hormone receptors are involved in the regulation of many pathophysiological processes, and it is well known that there exists a complex cell- and tissue-specific interplay between different members of the receptor superfamily. To further investigate the functional role of PPARγ and RORA, we compared the pharmacological properties of BRL 49653 and CGP 52608 in *in vitro* and *in vivo* models that had been used in previous studies to demonstrate typical effects of these compounds.

The leptin (*ob*) gene is exclusively expressed in adipocytes. The *ob/ob* mice, which carry a nonsense mutation in the *ob* gene, do not produce leptin and develop a profound obesity, often accompanied by diabetes (Zhang *et al*., 1994). Although a functional PPARγ response element (PPRE) has not been identified in the promoter of the *ob* gene (De Vos *et al*., 1996), BRL 49653 and structurally related antidiabetic thiazolidinediones inhibit leptin mRNA synthesis *in vitro* and *in vivo* and increase food intake and adipose tissue weight in rats (De Vos *et al*., 1996; Kallen and Lazar, 1996). Our data show that low nanomolar concentrations of BRL 49653 cause a profound suppression of immunoreactive leptin in the supernatants of differentiated adipocytes, whereas CGP 52608 and structurally related thiazolidinedione derivatives were completely inactive at 10 μM (Table 1).

Pharmacological doses of glucocorticosteroids induce *ob* gene expression in rat adipose tissue within 24 hr (De Vos *et al*., 1995). This early event is followed by complex metabolic changes resulting in a decrease in food consumption, a reduction in body weight gain, and the development of insulin resistance with enhanced blood glucose and triglyceride levels. BRL 49653 counterregulated the dexamethasone-induced insulin resistance, whereas CGP 52608 exhibited steroid-like effects in this model (Fig. 3).

Finally, BRL 49653 and CGP 52608 were tested in a chronic autoimmune model, in rat adjuvant arthritis, and the GR ligand prednisolone was taken as reference compound. Glucocorticosteroids are potent immunosuppressive and anti-inflammatory drugs, and down-regulation of nuclear factor-κB-induced genes such as interleukin (IL)-1, IL-2, IL-3, IL-6, IL-8, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, interferon-γ, class I and II MHC, and cell adhesion molecules by transcriptional activation of the IκB gene has been recently discovered to be a key mech-

anism in mediating their therapeutic effects (Auphan *et al*., 1995). Fig. 4 shows that prednisolone (1–10 mg/kg) dose-dependently suppressed adjuvant arthritis and that CGP 52608 (0.01–0.1 mg/kg) exhibited a 10–100 times higher antiarthritic potency, indicating a highly specific and efficacious mechanism of action. On the other hand, BRL 49653 was nearly inactive in the dose range tested (0.01–1 mg/kg), demonstrating again the different pharmacological properties of CGP 52608 and BRL 49653.

Nuclear signaling via RORA is suggested to be a key mechanism in mediating the anti-inflammatory and antiarthritic effects of CGP 52608 and structurally related derivatives (Missbach *et al*., 1996) and may be a promising therapeutic supplement and/or alternative to glucocorticosteroids in the treatment of rheumatoid arthritis and related autoimmune diseases. The molecular mechanisms that occur after RORA activation and finally cause the therapeutic effects of CGP 52608 and analogues are currently unknown. First, RORA-regulated genes have been identified (Steinhilber *et al*., 1995; Schraeder *et al*., 1996); potentially interesting candidates are human 5-lipoxygenase, an important enzyme in the control of allergic and inflammatory reactions, and human and mouse p21$^{WAF1/CIP1}$, a cell cycle inhibitor that could play a role in suppressing autoimmune processes. However, it is most likely that other target genes central to the immunoinflammatory process also are involved.

Cross-talk between nuclear receptors resulting in either synergistic or antagonistic effects is a common regulatory principle in controlling gene transcription. PPAR interactions with thyroid hormone receptors, C/EBP receptors, and COUP-TF have been shown to regulate genes involved in lipid metabolism (Hunter *et al*., 1996). PPARγ-activating thiazolidinediones counteract glucocorticosteroid-induced metabolic changes and reverse glucocorticoid-induced insulin resistance (De Vos *et al*., 1995; Turner, 1996; current study), indicating that GR and PPARγ are major players with antagonistic properties in the regulation of carbohydrate and lipid homeostasis and energy balance. Whether the observed steroid-like effects of CGP 52608 on triglyceride levels and body weight are ROR-mediated effects of physiological relevance is unknown.

Recent progress has been made in the identification of PPAR ligands. The availability of radiolabeled thiazolidinediones (Lehmann *et al*., 1995; Berger *et al*., 1996) and the discovery of GW 2331, a fibrate derivative with high affinity for PPARα and PPARγ (Kliewer *et al*., 1997), facilitates the screening for PPAR subtype-selective ligands. First results are available showing that certain naturally occurring fatty acids and arachidonic acid metabolites (leukotriene B$_4$, 8-hydroxy-eicosatetraenoic acid, 15d-PGJ$_2$, prostacyclin) are either nonselective or subtype-specific ligands of PPARα and PPARγ (Devchand *et al*., 1996; Hertz *et al*., 1996; Forman *et al*., 1997; Kliewer *et al*., 1997). The fact that arachidonic acid metabolites that are produced in inflamed tissues via the cyclooxygenase pathway (15d-PGJ$_2$, prostacyclin) or the lipoxygenase pathway (leukotriene B$_4$, 8-hydroxy-eicosatetraenoic acid) are not only potent inflammatory mediators but also PPAR ligands has generated the interesting hypothesis that activation of PPARs might be involved in the control and limitation of inflammatory reactions (Devchand *et al*., 1996; Kliewer *et al*., 1997). Our results with the PPARγ ligand BRL 49653 show that this compound did not inhibit

inflammation and joint destruction in a chronic arthritis model. However, PPARα ligands might be more promising candidates, but we are not aware of systematic studies investigating their effects in inflammatory models. In contrast, GR ligands and RORA activating thiazolidinedione derivatives (Missbach et al., 1996; current study) are potent inhibitors in experimental arthritis and autoimmune models and may have synergistic therapeutic effects in rheumatoid arthritis and related autoimmune diseases.

In summary, our results with the thiazolidinedione BRL 49653 and the thiazolidinedione derivative CGP 52608 support the concept that specific activation of PPARγ or RORA results in distinctly different pharmacological effects. Both compounds are prototypes of novel therapeutic agents for the treatment of NIDDM or rheumatoid arthritis and related autoimmune diseases. They also are valuable tools for further investigation of the pathophysiological role of PPARγ and RORA and their potential interplay with other members of the superfamily of nuclear receptors.

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