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Thiazolidinediones reduce the LDL binding affinity of non-human primate vascular cell proteoglycans

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Abstract

Aims/hypothesis. Retention of atherogenic lipoproteins in the artery wall by proteoglycans is a key step in the development of atherosclerosis. Thiazolidinediones have been shown to reduce atherosclerosis in mouse models. The aim of this study was to determine whether thiazolidinediones modify vascular proteoglycan synthesis in a way that decreases LDL binding.
Methods. Primate aortic smooth muscle cells were exposed to troglitazone or rosiglitazone, or no stimulus at all for a 24-hour steady-state labelling period. Sulphate incorporation, size and LDL binding affinity of proteoglycans were determined. Proteoglycans secreted by cells in the presence or absence of troglitazone were separated into large and small classes by size exclusion chromatography, and LDL binding affinity was determined.
Results. Proteoglycans synthesised by cells exposed to troglitazone or rosiglitazone were smaller, with decreased sulphate incorporation and decreased LDL binding affinity. However, troglitazone had a greater effect than rosiglitazone. Troglitazone reduced the LDL binding affinities of both the large and small proteoglycans compared with control. The binding differences persisted when glycosaminoglycan chains released from proteoglycans were incubated with LDL, indicating that troglitazone affects the glycosaminoglycan synthetic machinery of these cells.
Conclusions/interpretation. Thiazolidinediones decrease the LDL binding affinity of the proteoglycans synthesised by primate aortic smooth muscle cells. This could, in part, account for the reduced atherosclerosis observed in animal models.

Keywords Atherosclerosis · Glycosaminoglycans · Lipoprotein retention · Proteoglycans · Rosiglitazone · Thiazolidinediones · Troglitazone.

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ · K_d, binding constant

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Introduction

Type 2 diabetes is characterised by hyperglycaemia, insulin resistance and often central obesity. Atherosclerotic complications continue to be a major cause of morbidity and mortality in persons with diabetes. Thiazolidinediones, a class of peroxisome proliferator-activated receptor γ (PPARγ) ligands, have been shown to decrease atherosclerosis in animal models [1, 2, 3, 4]. Thiazolidinediones have several potential anti-atherogenic properties, including inhibition of smooth muscle cell [5] and endothelial cell proliferation [6], improvements in fibrinolysis [7] and decreases in carotid artery intima-media thickness [8]. Although the effect of thiazolidinediones on clinical

cardiovascular events in individuals with Type 2 diabetes is unknown due to the short duration of availability, troglitazone was shown to inhibit the formation of atherosclerotic lesions in diabetic and non-diabetic male LDL-receptor-deficient mice [2]. The diabetic and non-diabetic mice differed in their glycaemic responses to troglitazone, yet both groups had decreased lesion formation suggesting a direct vascular effect of troglitazone separate from its metabolic effects [2]. Troglitazone supplementation also inhibited the formation of atherosclerotic lesions in male apolipoprotein-E-deficient mice [1]. Rosiglitazone was shown to decrease atherosclerosis development in male LDL-receptor-deficient mice [3] and in diabetic and non-diabetic male apolipoprotein-E-deficient mice [4]. Troglitazone and rosiglitazone were shown to decrease the development of glomerulosclerosis in rats [9, 10, 11]. In one of these studies, the kidneys were stained for the glomerular mesangial basement membrane chondroitin sulphate proteoglycans, and the data suggested that the inhibitory effect of troglitazone on diabetic glomerulosclerosis may be related to reduced proteoglycan synthesis [10].

Proteoglycans are thought to play a critical role in the development of atherosclerosis, as outlined in the response to retention hypothesis [12]. Proteoglycans are a heterogeneous group of complex molecules, composed of a core protein to which one or more glycosaminoglycan chains are attached [13]. Proteoglycans bind lipoproteins through ionic interactions between negatively charged sulphate and carboxyl groups on their glycosaminoglycan side chains and positively charged amino acid residues on the apolipoproteins, or through linking molecules such as lipoprotein lipase [14]. Proteoglycans are ubiquitous in the arterial wall, and are mainly synthesised by vascular smooth muscle cells. The major proteoglycans synthesised by vascular smooth muscle cells are the large chondroitin sulphate proteoglycan, versican, the small dermatan sulphate proteoglycans, biglycan and decorin, and the heparan sulphate proteoglycan, perlecan [15]. The critical role of proteoglycans in the pathogenesis of atherosclerosis was demonstrated in a study of mice that were transgenic for human apolipoprotein B100 and that expressed either proteoglycan-binding-defective apolipoprotein B100 or proteoglycan-binding-intact apolipoprotein B100. At equal cholesterol levels, the mice expressing proteoglycan-binding-defective apolipoprotein B100 had significantly less atherosclerosis [16]. This demonstrates the critical role of proteoglycan binding in the retention of atherogenic lipoproteins in the development of atherosclerosis.

In this paper we present data demonstrating that exposure of vascular smooth muscle cells to thiazolidinediones leads to the synthesis of proteoglycans with reduced binding affinity for LDL, which could, in part, account for the reduced atherosclerosis observed with the thiazolidinediones in animal models.

Materials and methods

Materials. Chemicals and reagents were obtained from Sigma (St. Louis, Mo., USA) unless otherwise specified. Radioactive sulphate was obtained from ICN (Costa Mesa, Calif., USA). Troglitazone (Parke-Davis Pharmaceutical Research, Ann Arbor, Mich., USA) and rosiglitazone (GlaxoSmithKline, Dandenong, Vic, Australia) were dissolved in DMSO, then added directly to the culture media.

Cell culture techniques. Monkey (*Macaca nemestrina*) arterial smooth muscle cells were cultured in DMEM containing 5.6 mmol/l glucose as described previously [17]. Confluent cells were made quiescent for 48 h by lowering the serum concentration to 0.1%, and were metabolically labelled with 1.85 to 3.7 mBq/ml [^{35}S]-SO_4 for 24 h in the presence or absence of troglitazone or rosiglitazone. Parallel dishes were treated identically except without [^{35}S]-SO_4 labelling, and either cells were washed with PBS, trypsinised and counted in a Coulter counter, or cell protein was determined by the method of Lowry [18].

Proteoglycan isolation and characterisation. Sulphate incorporation was assessed by cetylpyridinium chloride precipitation [17]. The relative sulphate incorporation was assessed as a percentage of sulphate incorporation into proteoglycans synthesised by control vascular smooth muscle cells (not exposed to thiazolidinediones) [19, 20, 21]. Metabolically labelled cell culture medium was collected in the presence of protease inhibitors as described previously [20, 22]. Secreted proteoglycans from the different experiments were concentrated and purified using separate diethylaminoethyl-sephacel mini-columns [20, 22]. Proteoglycans were separated into large (peak 1, containing predominantly the chondroitin sulphate proteoglycan, versican [20, 23]) and small (peak 2, containing a mixture of dermatan sulphate proteoglycans, biglycan and decorin [24]) size classes using size exclusion chromatography [14, 25]. Glycosaminoglycan chains were released from core proteins as described previously [20].

LDL binding assay. As described previously, LDL was isolated from the plasma of normal human volunteers by preparative ultracentrifugation, and purified by sequential density gradient ultracentrifugation [26, 27]. LDL was stored under nitrogen in the dark at 4 °C with 1 mmol/l EDTA. A modified gel mobility shift assay was used to evaluate the LDL binding affinity of proteoglycans or free glycosaminoglycan chains synthesised in the presence or absence of troglitazone as described previously [17, 28]. The percentage of proteoglycans or glycosaminoglycans bound was calculated as the proportion of radioactivity remaining at the gel's origin relative to the total radioactivity per lane. Binding constants (K_d) were calculated using the Software Architecture Analysis Method II modelling program (SAAM Institute, Seattle, Wash., USA) using the Michaelis-Menten equation.

Statistical analysis. Results are expressed as means ± SEM unless otherwise noted. Results from LDL binding experiments were analysed using paired *t* tests. All other comparisons were made using a two-tailed Student's *t* test, with a p value of less than 0.05 considered statistically significant.

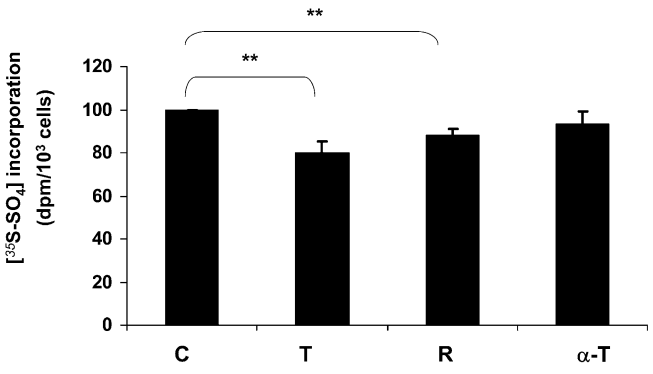

Fig. 1. Proteoglycan sulphate incorporation is reduced by treatment of vascular smooth muscle cells with troglitazone or rosiglitazone. Vascular smooth muscle cells were exposed to troglitazone (10 μmol/l), rosiglitazone (30 μmol/l) or α-tocopherol (10 μmol/l) for 24 h and metabolically labelled with [^{35}S]-SO_4. The sulphate incorporation of proteoglycans secreted into the culture medium was analysed by cetylpyridinium chloride precipitation, and is expressed as incorporated sulphate counts per 10^3 cells. Values are expressed as means ± SEM relative to unstimulated cells, which are expressed as 100%. Data are from two independent experiments performed in triplicate. C, control; T, troglitazone; R, rosiglitazone; α-T, α-tocopherol; $^{**}p<0.01$

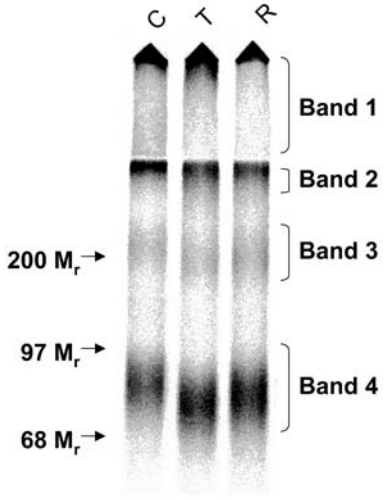

Fig. 2. SDS-PAGE (3.5% stacking gel with 4–12% gradient resolving gel) analysis of radiolabelled secreted proteoglycans synthesised in the absence or presence of troglitazone (10 μmol/l) or rosiglitazone (30 μmol/l) for 24 h. Proteoglycans synthesised by cells exposed to troglitazone or rosiglitazone are smaller than those synthesised by control cells. Lanes are loaded with 25,000 dpm. The gel shown is representative of seven independent experiments. C, control; T, troglitazone; R, rosiglitazone

Results

Dose–response experiments indicated that troglitazone had near-maximal effect on proteoglycan synthesis at 10 μmol/l, with toxicity developing at 30 μmol/l as shown by loss of cells. Rosiglitazone had maximal effect on proteoglycan synthesis at 30 μmol/l, with toxicity developing at 50 μmol/l.

The LDL binding affinities of total secreted proteoglycans synthesised in the presence of either troglitazone (K_d 0.029±0.006 mg/ml LDL, p=0.008 compared with control) or rosiglitazone (K_d 0.024±0.002 mg/ml LDL, p=0.01 compared with control) were less than that of control proteoglycans (K_d 0.016±0.004 mg/ml LDL). Troglitazone had a greater effect in reducing LDL binding affinity than rosiglitazone.

We and others have previously demonstrated that decreased LDL binding affinity is mediated via smaller proteoglycans and glycosaminoglycans [17, 29]. To determine whether the thiazolidinediones alter the steady-state levels of newly synthesised vascular proteoglycans, the incorporation of radiolabelled sulphate into proteoglycans was assessed. Troglitazone reduced the incorporation of sulphate to 80.1±5.3% of that of control (p<0.01; Fig. 1). Rosiglitazone also reduced sulphate incorporation, although the magnitude of reduction was not as great as that with troglitazone. Troglitazone is unique amongst thiazolidinediones in that it has an α-tocopherol moiety. To determine whether the greater reduction of sulphate incorporation by troglitazone compared with that by rosiglitazone was in part mediated by the α-tocopherol moiety, we also exposed cells to α-tocopherol alone in equimolar concentrations. α-Tocopherol did not affect sulphate incorporation (Fig. 2), nor did it have any additive effect when added to cells in combination with rosiglitazone.

The apparent molecular weight of proteoglycans synthesised in the presence and absence of thiazolidinediones was evaluated using SDS-PAGE. Vascular smooth muscle cells secrete four major proteoglycans that are seen on SDS-PAGE. Band 1 has previously been identified as the chondroitin sulphate proteoglycan, versican [20, 23]. Band 2 is a mixture of heparan sulphate and chondroitin sulphate proteoglycans that has not been described in detail. These bands do not enter the resolving gel, and thus the effect of troglitazone on their size cannot be assessed by this method. The other major proteoglycans, biglycan (band 3) and decorin (band 4) [24], synthesised in the presence of troglitazone and rosiglitazone, had increased mobility compared with control proteoglycans, indicating smaller apparent molecular weights and/or altered charge (Fig. 2). To determine whether the increased mobility of proteoglycans was due to smaller size, proteoglycans were separated according to hydrodynamic size. Given that troglitazone had a greater effect than rosiglitazone on size, sulphate incorporation and LDL binding affinity, further characterisation studies were performed comparing proteoglycans synthesised in the presence or absence of troglitazone. Analysis using molecular sieve chromatography demonstrated that peak 1 (containing predominantly versican) and peak 2

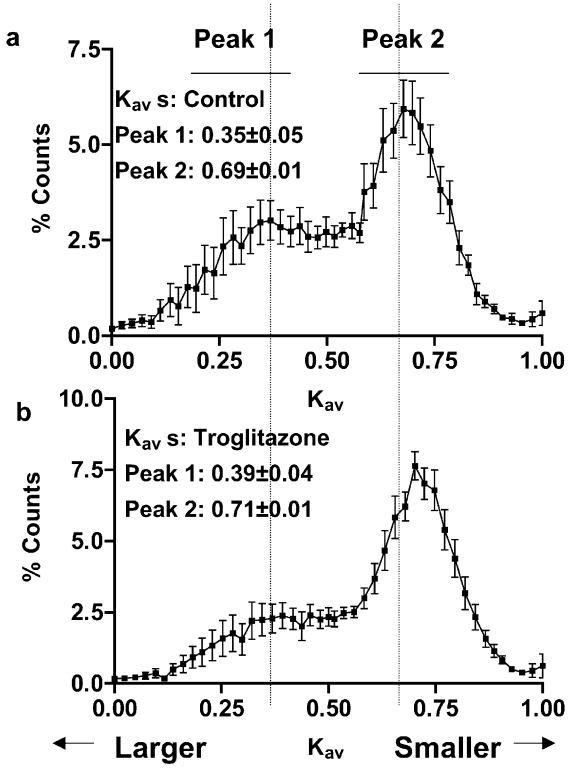

Fig. 3. Troglitazone decreases the size of both large and small proteoglycans. Secreted proteoglycans synthesised in the absence (**a**) or presence (**b**) of troglitazone were applied to Sepharose 2B columns for size analysis by molecular sieve chromatography. The curves shown are the means ± SEM of seven independent experiments. The dotted vertical lines indicate the mean K_{av}s for peak 1 and peak 2 proteoglycans synthesised by control cells. Fractions eluting under the horizontal bars as indicated were pooled separately into peak 1 (versican) and peak 2 (biglycan and decorin) for use in subsequent experiments

(containing predominantly biglycan and versican) [14, 25] proteoglycans synthesised in the presence of troglitazone were smaller than proteoglycans synthesised by control cells (peak 1, K_{av} 0.35±0.05 vs 0.39±0.04, n=6, p=0.04; peak 2 K_{av} 0.69±0.01 vs 0.71±0.009, n=6, p=0.05, in the absence and presence of troglitazone respectively; Fig. 3). Thus, troglitazone stimulation of vascular smooth muscle cells results in a decrease in size of all major secreted proteoglycans. Furthermore, troglitazone did not affect the relative synthesis of peak 1 and 2 proteoglycans, as areas under the peaks are not significantly different for peak 1 or peak 2 proteoglycans synthesised in the presence or absence of troglitazone. Thus, the decreased sulphate incorporation (Fig. 1) is probably distributed amongst all major secreted proteoglycans.

To determine whether troglitazone had different effects on the binding of the large chondroitin sulphate proteoglycan, versican, compared with the small dermatan sulphate proteoglycans, biglycan and decorin, LDL binding affinity was assessed on proteoglycans separated into large and small size classes by preparative size exclusion chromatography. Fractions eluting under each peak were pooled (Fig. 3), and LDL binding affinity was assessed by a modified gel mobility shift assay.

Peak 1 and peak 2 proteoglycans synthesised in the presence of troglitazone each demonstrated a lower LDL binding affinity than control (K_d 0.184±0.026 vs 0.047±0.005 mg/ml LDL for peak 1 proteoglycans, p<0.01; K_d 0.210±0.030 vs 0.123±0.014 mg/ml LDL for peak 2 proteoglycans, p=0.02, synthesised in the presence and absence of troglitazone respectively; Fig. 4), although there was a greater reduction of LDL binding affinity for peak 1 proteoglycans. To determine whether the effect of troglitazone (reducing LDL binding by proteoglycans) is mediated via their glycosaminoglycan chains, we assessed the LDL binding af-

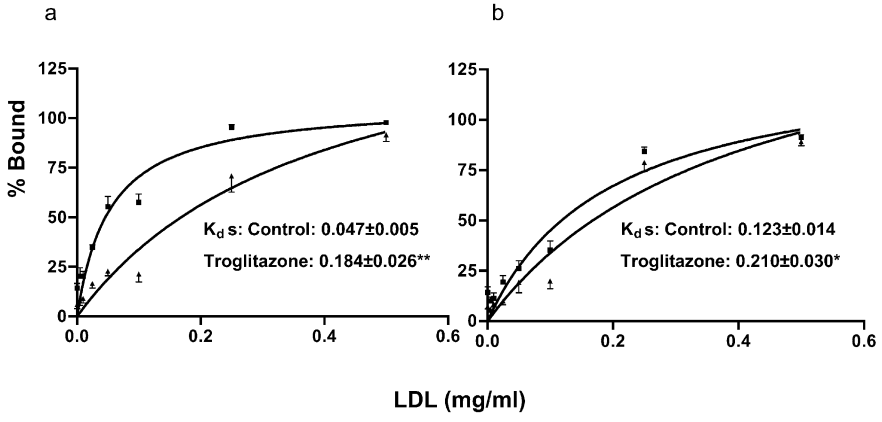

Fig. 4. Troglitazone decreases the LDL binding affinity of both large and small proteoglycans. Equal amounts of peak 1 proteoglycans (**a**) and peak 2 proteoglycans (**b**) synthesised by cells exposed to troglitazone (*triangles*) or no stimulus (control, *squares*) were used in a modified gel mobility shift assay. The percentage of proteoglycan bound was calculated as the proportion of radioactivity remaining at the origin of the gel relative to the total radioactivity per lane. The binding curves shown are the means ± SEM of two independent experiments, each performed in duplicate. **p<0.01; *p=0.02; K_d, binding constant

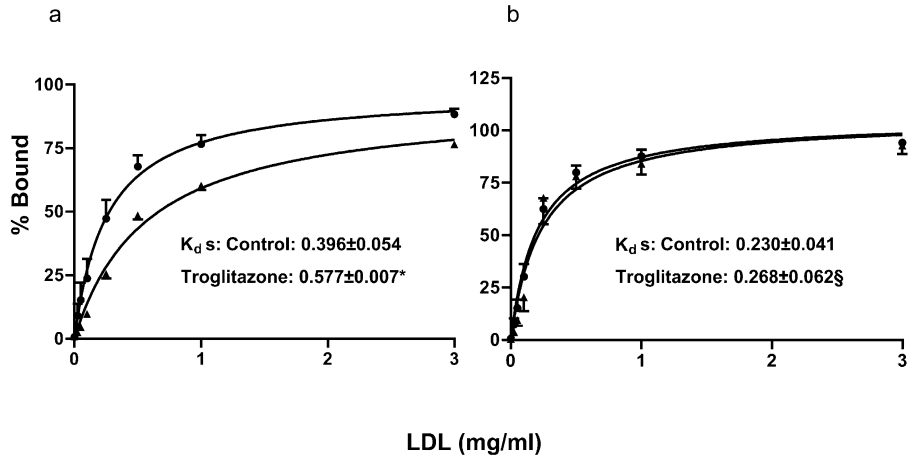

Fig. 5. Peak 1 glycosaminoglycans synthesised in the presence of troglitazone have reduced LDL binding affinity. Glycosaminoglycans cleaved from peak 1 (**a**) or peak 2 (**b**) proteoglycans synthesised by cells exposed to troglitazone (*triangles*) or no stimulus (control, *squares*) were used in a gel mobility shift assay. The percentage of glycosaminoglycan bound was calculated as the proportion of radioactivity remaining at the gel's origin relative to the total radioactivity per lane. The binding curves shown are the means ± SEM of two independent experiments each performed in duplicate. **p*=0.03; §, NS; K_d, binding constant

finity of peak 1 and peak 2 glycosaminoglycan chains. Glycosaminoglycan chains cleaved from peak 1 proteoglycans synthesised in the presence of troglitazone had lower binding affinity for LDL compared with glycosaminoglycans synthesised by unexposed cells (K_d 0.577±0.007 vs 0.396±0.054 mg/ml LDL respectively, *p*=0.03; Fig. 5a). However, we were unable to detect a significant alteration in LDL binding affinity for glycosaminoglycan chains cleaved from peak 2 proteoglycans synthesised in the presence or absence of troglitazone (K_d 0.268±0.062 vs 0.230±0.041 mg/ml LDL respectively, NS; Fig. 5b).

Discussion

The data presented here demonstrate that primate vascular smooth muscle cells exposed to troglitazone or rosiglitazone synthesise smaller proteoglycans with reduced LDL binding affinity, although troglitazone has greater effects than rosiglitazone on proteoglycan synthesis. The effect of troglitazone to reduce the LDL binding affinity of peak 1 proteoglycans is mediated through its glycosaminoglycan chains. However, we did not detect a significant difference in the LDL binding affinity of peak 2 glycosaminoglycan chains synthesised in the presence versus absence of troglitazone. Our inability to demonstrate decreased LDL binding affinity for peak 2 glycosaminoglycans synthesised in the presence of troglitazone could be due to a limitation of the assay, or to troglitazone having less of an effect on the LDL binding affinity of dermatan sulphate chains than on that of chondroitin sulphate chains. The LDL binding affinity of free glycosaminoglycan chains is lower than the binding affinity of intact proteoglycans, as previously described [17, 30].

Versican is the predominant component of peak 1 [20] and is composed of a core protein to which 15 to 20 chondroitin sulphate glycosaminoglycans are attached [13, 31]. Peak 2 is composed of biglycan and decorin [24] which have dermatan sulphate glycosaminoglycan chains. Biglycan has 2 glycosaminoglycans, while decorin has only a single glycosaminoglycan chain [13]. Our laboratory has previously demonstrated that purified versican has a greater binding capacity for LDL than purified biglycan [14], which is probably due to the greater number of glycosaminoglycan chains on versican than on biglycan.

We have previously demonstrated that LDL binding affinity of intact proteoglycans and glycosaminoglycan chains relates to the relative size and sulphation of the molecules [17, 30]. In the present experiments we confirm these previous observations, demonstrating that proteoglycans synthesised in the presence of troglitazone or rosiglitazone are smaller and have decreased LDL binding affinity. Decreased sulphate incorporation is usually due to a decreased size of proteoglycans, which in turn is due to decreased length of their glycosaminoglycan chains. The mechanism by which these thiazolidinediones exert their effects is not clear. Vascular smooth muscle cells have been shown to express PPARγ [5]. Therefore, these thiazolidinediones may exert their effects on glycosaminoglycan chains via this class of nuclear receptors. The synthesis of glycosaminoglycan chains is complex and involves multiple enzymes including sulphotransferases, xylosyltransferases, galactosyltransferases and others [13, 32, 33]. PPARγ has been shown to decrease extracellular signal-regulated kinase and mitogen-activated protein kinases, but to increase phosphatidylinositol-3 kinase signalling [34]. Further studies into the signalling pathways by which

troglitazone exerts its effects are underway. In addition, we have shown that thiazolidinediones decrease human vascular smooth muscle cell proliferation [35]. Their effect to inhibit proteoglycan synthesis may be related to their inhibitory effects on proliferation.

Although troglitazone is no longer used clinically due to hepatotoxicity, we demonstrate that rosiglitazone, but not α-tocopherol, has a similar effect: it decreases sulphate incorporation. Rosiglitazone also decreases the size and LDL binding affinity of the vascular proteoglycans, although to a lesser extent than troglitazone. This suggests that the observed effects of troglitazone on proteoglycan synthesis are due to its thiazolidinedione moiety, rather than to its α-tocopherol moiety. Several different thiazolidinediones have been reported to decrease atherosclerosis in animal models [1, 2, 3, 4]. Therefore, we suggest that the effects of troglitazone on proteoglycan synthesis described here are class effects. Although troglitazone is no longer clinically available and thus not a potential anti-atherosclerosis therapy, it is still useful as a tool to manipulate glycosaminoglycan synthesis. An increased understanding of glycosaminoglycan synthesis will allow identification of novel therapeutic targets and potential anti-atherosclerosis or atherosclerosis-preventing therapies. The critical role played by proteoglycan-mediated LDL retention in the development of atherosclerosis [16] suggests that strategies to modify arterial wall proteoglycans have potential clinical impact.

In summary, the data presented in this paper suggest a mechanism for the observed decrease in atherosclerosis seen in animal models treated with thiazolidinediones. A decrease in the binding affinity of vascular proteoglycans for atherogenic lipoproteins caused by troglitazone or rosiglitazone exposure could account for reduced atherosclerotic lesions described in the literature. Since the effects described here are probably class effects of thiazolidinediones, the potential clinical benefits of thiazolidinediones for reducing atherosclerosis development or progression are significant. Long-term clinical trials with cardiovascular outcomes are needed to confirm this hypothesis. However, the different thiazolidinediones may have differing effects on atherosclerosis that are independent of their relative affinities for PPARγ.

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