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Fenofibrate modifies human vascular smooth muscle proteoglycans and reduces lipoprotein binding

J. Nigro[1, 2] · M. L. Ballinger[1, 2] · R. J. Dilley[3] · G. L. R. Jennings[1, 2] · T. N. Wight[4] · P. J. Little[1, 2]

[1] Cell Biology of Diabetes Laboratory, Baker Heart Research Institute, Melbourne, Victoria, Australia
[2] Department of Medicine, Central and Eastern Clinical School, Alfred Hospital, Monash University, Prahran, Victoria, Australia
[3] Morphology Laboratory, Baker Heart Research Institute, Melbourne, Victoria, Australia
[4] Hope Heart Program, Benaroya Research Institute at Virginia Mason, Seattle, Washington, USA

Abstract

Aims/hypothesis. Vascular disease in type 2 diabetes is associated with an up-regulation of atherogenic growth factors, which stimulate matrix synthesis including proteoglycans. We have examined the direct actions of fenofibrate on human vascular smooth muscle cells (VSMCs) and have specifically investigated proteoglycan synthesis and binding to LDL.
Methods. Proteoglycans synthesised by human VSMCs treated with fenofibrate (30 µmol/l) were assessed for binding to human LDL using a gel mobility shift assay, metabolically labelled with $[^{35}S]$-sulphate and quantitated by cetylpyridinium chloride. They were then assessed for electrophoretic mobility by SDS-PAGE, for size by gel filtration, for sulphation pattern by fluorophore-assisted carbohydrate electrophoresis, and for glycosaminoglycan (GAG) composition by enzyme digestion.
Results. Proteoglycans synthesised in the presence of fenofibrate showed an increase in the half-maximum saturation concentration of LDL from 36.8±12.4 µg/ml to 77.7±17 µg/ml under basal conditions, from 24.9±4.6 µg/ml to 39.1±6.1 µg/ml in the presence of

TGF-β1, and from 9.5±4.4 µg/ml to 31.1±3.4 µg/ml in the presence of platelet-derived growth factor/insulin. Fenofibrate treatment in the presence of TGF-β1 inhibited the incorporation of $[^{35}S]$-sulphate into secreted and cell-associated proteoglycans synthesised by human VSMCs by 59.2% ($p<0.01$) and 39.8% ($p<0.01$) respectively. The changes in sulphate incorporation following treatment with fenofibrate were associated with a concentration-related increase in the electrophoretic mobility due to a reduction in GAG length. There was no change in the sulphation pattern; however, there was an alteration in the disaccharide composition of the GAGs.
Conclusions/interpretation. Fenofibrate modifies the structure of vascular proteoglycans by reducing the length of the GAG chains and GAG composition, resulting in reduced binding to human LDL, a mechanism which may lead to a reduction of atherosclerosis and cardiovascular disease in people with diabetes treated with fenofibrate.

Keywords Atherosclerosis · Fenofibrate · Glycosaminoglycan · Lipoprotein · Proteoglycans · Sulphation · Vascular smooth muscle.

P. J. Little (✉)
Cell Biology of Diabetes Laboratory,
Baker Heart Research Institute, St. Kilda Rd Central,
PO Box 6492, Melbourne, Victoria, 8008, Australia
E-mail: peter.little@baker.edu.au
Tel.: +61-3-85321203, Fax: +61-3-85321100

Introduction

Ever-expanding rates of obesity resulting from excess and poor quality energy intake and sedentary workplace and lifestyle practices are generating a demo-

Abbreviations: CPC, cetylpyridinium chloride · FACE, fluorophore-assisted carbohydrate electrophoresis · GAG, glycosaminoglycan · PDGF, platelet-derived growth factor · PPAR-α, peroxisome proliferator-activated receptor-α · VSMCs, vascular smooth muscle cells

graphically broadening epidemic of morbidity and mortality from cardiovascular disease. This is occurring in association with the insulin resistance syndrome and type 2 diabetes [1]. The major underlying pathology is inflammatory atherosclerotic vascular disease leading to ischaemic end organ damage. Clinical trials directed at cardiovascular risk factors such as blood pressure and lipid levels show positive results, but an element of 'residual' or apparently resistant disease is always present. Therapies directed at the vessel wall are the suggested pathway to eliminate this residual disease [2].

Recent evidence has strongly implicated vascular proteoglycans for their role in trapping LDL in the vessel wall leading to the formation of foam cells as a critical initiating event in atherogenesis and therefore a potential point of therapeutic intervention [3, 4, 5]. Glycosaminoglycan (GAG) elongation is stimulated by cell proliferation [6], platelet-derived growth factor (PDGF) [7], TGF-β1 [7], oxidised LDL [8], and NEFA [9]. There are fewer examples of inhibition of GAG elongation including calcium channel antagonists [10] and gemfibrozil [11]. We are investigating the pharmacological modulation of vascular proteoglycan biosynthesis and structure as a pathway to new therapeutic agents for the prevention and treatment of atherosclerosis and for the delineation of beneficial vascular actions of existing cardiovascular and oral hypoglycaemic drugs.

Fenofibrate is used for the treatment of hypertriglyceridaemia and is presently the subject of the worldwide Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial. The Diabetes Atherosclerosis Intervention Study (DAIS) is the only completed trial to date that has specifically looked at fibrate treatment in diabetes. The results showed that fenofibrate treatment significantly reduced the progression of coronary narrowing by 40% [12]. We have investigated whether fenofibrate displays anti-atherogenic activity in human vascular smooth muscle cells (VSMCs) through modification of proteoglycan biosynthesis and structure and a consequent reduction in binding to LDL. Our findings suggest that fenofibrate has pleotropic actions on the vascular wall, which may complement its metabolic actions in the treatment and prevention of atherosclerosis.

Materials and methods

Materials. Fenofibrate, DMSO, TGF-β1, sodium chlorate, PDGF, insulin, DEAE-Sephacel, Sepharose CL-2B, Sepharose CL-6B, chondroitinase ABC, chondroitinase AC and chondroitinase B were from Sigma (St. Louis, Mo., USA). FBS was from CSL (Parkville, Melbourne, Vic, Australia). Carrier-free sodium [35S]-sulphate (specific activity 155.9 TBq/mmol), [35S]-methionine/cysteine (specific activity 43.5 TBq/mmol), and cetylpyridinium chloride (CPC) were from ICN Biomedicals (Irvine, Calif., USA). 2-Aminoacridone was from Molecu-

lar Probes (Eugene, Ore., USA). Unsaturated dermato/hyalurodisaccharide standards were from Seikagaku (Tokyo, Japan).

Cell culture. Human VSMCs were isolated from the internal mammary artery and characterised as previously described [13, 14]. The Human Ethics Committee of the Alfred Hospital (Melbourne, Vic, Australia) approved the acquisition of the tissue. Experiments were conducted with cells (11th to 22nd passage) from the same donor.

Proteoglycan–LDL binding by gel mobility shift assay. Human VSMCs were cultured at 5×10^5 cells per 60-mm plate in DMEM (Invitrogen, Grand Island, N.Y., USA) containing 5 mmol/l glucose and 10% FBS, which was replenished the following day. Cells were grown to confluence and serum-deprived in DMEM containing 0.1% FBS (48 h). Cells were pretreated (6 h) with medium containing 0.1% FBS/0.1% DMSO or treatment medium with 30 μmol/l fenofibrate in 0.1% DMSO. The minimum preincubation period required to show an effect of fenofibrate on GAG length (data not shown) was 6 h. Cells were replenished with control medium or medium containing fenofibrate in the presence or absence of 2 ng/ml TGF-β1 and were metabolically labelled with 1.48 MBq/ml [35S]-methionine/cysteine to label proteoglycan core proteins [15]. LDL (0–500 μg/ml) isolated from human plasma [16] was incubated with proteoglycans (1500 cpm) for 1 h (37 °C). Samples were run on a 0.8% agarose gel as previously described [17]. Dried gels were exposed to a phosphorimaging plate (3 days) and scanned on a BAS-1000 MacBas phosphorimager (Fuji Photo Film, Tokyo, Japan). Computer analysis (MacBas Version 1.0) was used to quantify the bound and free proteoglycans [18]. Experiments were repeated with LDL from different plasma donors. In addition, an aliquot of each sample was used to assess core protein size by digesting the GAG chains with 2 U/ml chondroitinase ABC followed by SDS-PAGE of the core proteins [17].

Quantitation of proteoglycans by CPC precipitation assay. Cells were cultured at 5×10^4 cells per well in a 24-well plate and were established and treated in an identical manner to that described above. Cells were metabolically labelled with 1.85 MBq/ml [35S]-sulphate (16 h). Parallel plates were established without [35S]-sulphate to count cells [11]. Incorporation of [35S]-sulphate into secreted proteoglycans and cell-associated proteoglycans was quantitated using the CPC precipitation assay [11].

Assessment of proteoglycan/GAG length by SDS-PAGE and size exclusion chromatography. Human VSMCs were cultured at 5×10^4 cells per well in a 24-well plate. VSMCs that had reached confluence and had been serum-deprived (48 h) were treated in an identical manner to that described before with fenofibrate (0.3–50 μmol/l) in the presence and absence of TGF-β1 (2 ng/ml), and were metabolically labelled with [35S]-sulphate. Proteoglycans were isolated and concentrated from the culture medium using DEAE-Sephacel and were sized by SDS-PAGE as previously described [11, 17]. For the size exclusion chromatography VSMCs were cultured at 5×10^5 cells per 60-mm plate, and following serum deprivation (48 h), they were treated with fenofibrate (30 μmol/l) in the presence and absence of TGF-β1 (2 ng/ml), as described before. Cells were metabolically labelled with 1.85 MBq/ml [35S]-sulphate (16 h). Intact proteoglycans were separated on a 100-mm Sepharose CL-2B column, while GAG chains cleaved using the beta elimination reaction were sized on a 100-mm Sepharose CL-6B column and eluted with 4 mol/l guanidine [17]. The V_t (total volume) was assigned to the fraction with the highest exog-

enous free [^{35}S]-sulphate count. Each fraction was analysed on a liquid scintillation analyser (Packard, Meriden, Conn., USA).

Quantitation of proteoglycan core protein. VSMCs were established on 24-well plates as described above. VSMCs were treated with medium containing 0.1% DMSO or 30 μmol/l fenofibrate in 0.1% DMSO (16 h). The treatments were removed and replaced with fresh medium containing 0.1% FBS and 0.74 MBq/ml [^{35}S]-methionine/cysteine (4 h). The culture medium was harvested and proteoglycans were precipitated using the CPC precipitation assay.

*Fluorophore-assisted carbohydrate electrophoresis.* VSMCs were cultured on 90-mm plates at a density of 0.8 to 1×10^6 cells per plate. Cells were treated with 0.5 to 5 mmol/l sodium chlorate in the presence of TGF-β1 (2 ng/ml, 24 h). In separate experiments, cells were treated with fenofibrate (30 μmol/l) in the presence and absence of TGF-β1 (2 ng/ml, 24 h). Proteoglycans were isolated from the culture medium as described above and dialysed into high quality water. Samples were prepared for fluorophore-assisted carbohydrate electrophoresis (FACE) following the method described by others [19, 20]. Disaccharides were lyophilised and labelled with 2-amino-acridone (50 mmol/l, 16 h, 37 °C) in 0.5 mol/l cyanoborohydride (10 μl). Samples were mixed with 37.5% glycerol (10 μl) and an aliquot (3–5 μl) separated on 20% acrylamide gels. Fluorescently labelled disaccharide standards were run in separate lanes. Images were captured using the Glyko Imager (Prozyme, San Leandro, Calif., USA) and quantitation of bands was performed using the Glyko software.

Characterisation of GAG composition by enzyme digestion. Radiolabelled proteoglycans (20,000 cpm) were digested with 42 mU/ml chondroitinase AC, chondroitinase B or no enzyme (37 °C, 16 h). Following ethanol precipitation, proteoglycans were separated by SDS-PAGE and visualised using a phosphorimager. Computer analysis (MacBas Version 1.0) was used to quantify undigested material.

Statistical analysis. LDL binding data was assessed by two-way ANOVA with interactions comparing factor 1 (without fenofibrate) with factor 2 (with fenofibrate). CPC precipitation assay data was analysed by one-way ANOVA. Results were taken to be significant when the *p* value was below 0.05. Levels of significance are indicated in the text and figures.

Results

Fenofibrate treatment of VSMCs decreases proteoglycan–LDL binding. Human smooth muscle cells were treated with fenofibrate in the presence and absence of TGF-β1 (2 ng/ml) and PDGF (50 ng/ml) plus insulin (1 μmol/l), the latter treatment being a metabolic mod-
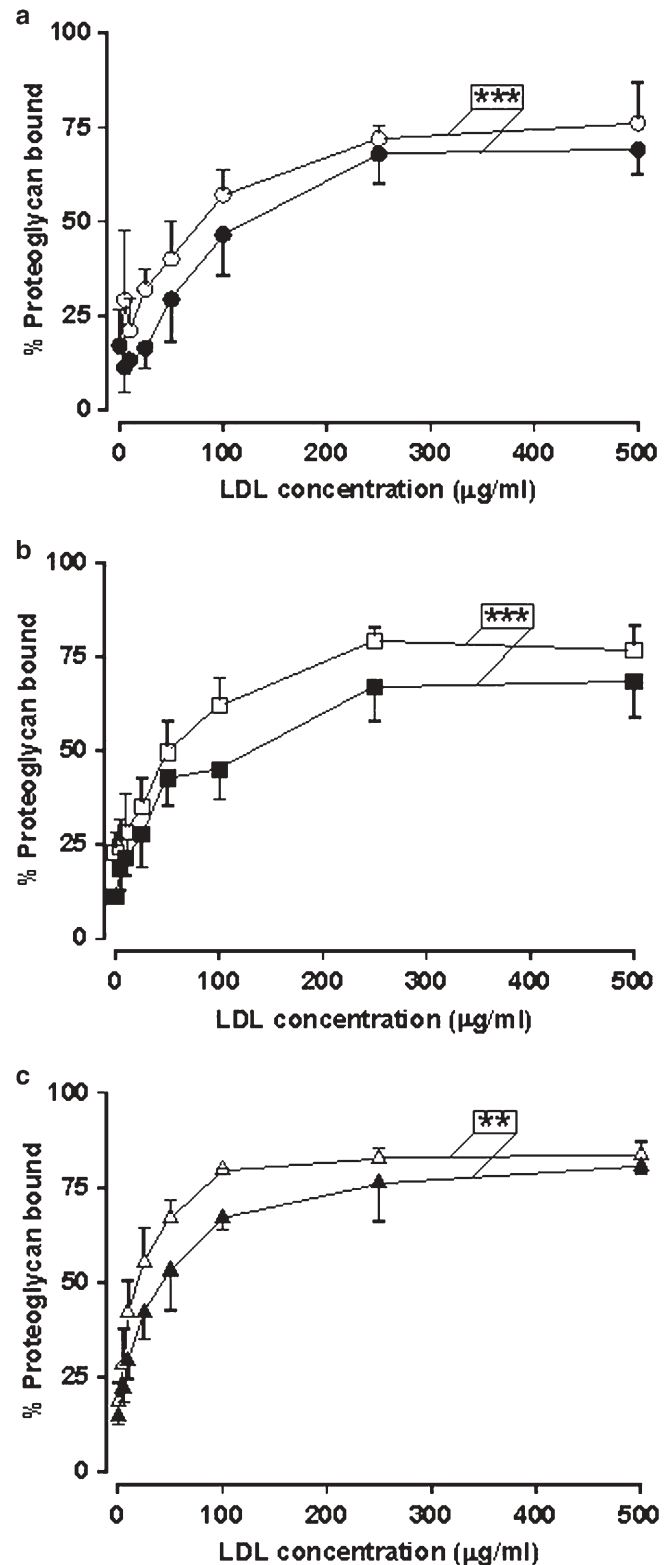
Fig. 1. Gel mobility shift assay for the analysis of LDL binding to proteoglycans synthesised in the presence of fenofibrate. **a.** Binding of LDL to proteoglycans synthesised under basal conditions (open circles) and proteoglycans synthesised in the presence of fenofibrate (30 μmol/l; closed circles). **b.** Binding of LDL to proteoglycans synthesised in the presence of TGF-β1 (2 ng/ml; open squares) and TGF-β1 with fenofibrate (30 μmol/l; closed squares). **c.** Binding of LDL to proteoglycans synthesised in the presence of PDGF (50 ng/ml) and insulin (1 μmol/l; open triangles) and PGDF/insulin in the presence of fenofibrate (30 μmol/l; closed triangles). Each figure is representative of two experiments performed in duplicate. The statistical analysis was performed using a two-way ANOVA with interactions as described in the Materials and methods, comparing factor 1 (without fenofibrate) to factor 2 (with fenofibrate). *** *p*<0.001 for the control curve vs fenofibrate curve, and the TGF-β1 curve vs TGF-β1 + fenofibrate curve; ** *p*<0.01 for the PDGF/insulin curve vs PDGF/insulin + fenofibrate curve
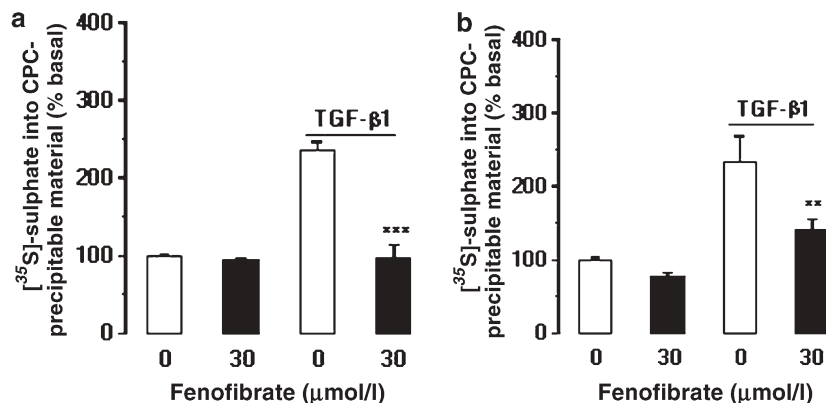
Fig. 2. Fenofibrate treatment of VSMCs decreases sulphate incorporation into GAGs. Incorporation of [^{35}S]-sulphate into GAGs was quantitated by CPC assay. The figure shows the amount of [^{35}S]-sulphate incorporated into (**a**) secreted proteoglycans and (**b**) cell-associated proteoglycans. Sulphate incorporation under basal conditions was designated as 100% (**a**=718±229 cpm/10^3 cells, **b**=306±34 cpm/10^3 cells). Each bar represents the mean ± SEM from three separate experiments (*n*=6). *** *p*<0.001, ** *p*<0.01 for TGF-β1 (2 ng/ml) vs TGF-β1 plus fenofibrate

el for type 2 diabetes [21, 22]. Proteoglycans were isolated and binding to LDL was assessed by gel mobility shift assay. Proteoglycans from VSMCs treated with fenofibrate (30 μmol/l) either with or without growth factors, exhibited a shift in the LDL binding curve to the right, relative to its respective control, an indication of reduced binding (Fig. 1a–c). The concentration of LDL required to bind 50% of the proteoglycans in this assay was increased by proteoglycans from fenofibrate-treated cells, from 36.8±12.4 μg/ml to 77.7±17.0 μg/ml (*p*<0.01), indicating that proteoglycans synthesised in the presence of fenofibrate had a reduced affinity for LDL (Fig. 1a). Proteoglycans synthesised in the presence of TGF-β1 had a half-maximum saturation concentration of 24.9±4.6 μg/ml LDL and this was increased by fenofibrate treatment to 39.1±6.1 μg/ml LDL; these proteoglycans showed a significantly different binding curve (*p*<0.001) from that of proteoglycans of cells stimulated with TGF-β1 alone (Fig. 1b). The binding curve of proteoglycans from smooth muscle cells treated with PDGF/insulin was similarly affected by fenofibrate (30 μmol/l) treatment; the half-maximum saturation concentration of LDL increased from 9.5±4.4 μg/ml to 31.1±3.4 μg/ml (Fig. 1c). The data suggest that fenofibrate treatment of VSMCs changes the proteoglycans produced, resulting in reduced binding to LDL.

Fenofibrate decreases [35S]-sulphate incorporation into GAGs on proteoglycans synthesised by human VSMCs. Fenofibrate treatment of VSMCs did not affect the incorporation of [^{35}S]-sulphate into proteoglycan GAGs; however, in the presence of TGF-β1,

fenofibrate treatment reduced [^{35}S]-sulphate incorporation into secreted proteoglycan GAGs by 59% (*p*<0.001, Fig. 2a), and cell-associated proteoglycans were decreased by 40% (*p*<0.01) compared with cells treated with TGF-β1 alone (Fig. 2b).

Fenofibrate treatment decreases GAG length. Radiolabelled bands at approximately 200 M$_r$ and approximately 100 M$_r$ on the electrophoretogram corresponding to the proteoglycans, biglycan and decorin, respectively [7], showed a fenofibrate-concentration-related increase in the electrophoretic mobility both in the presence and absence of TGF-β1 (Fig. 3a, b). Fenofibrate (30 μmol/l) treatment of smooth muscle cells in the presence and absence of TGF-β1 did not change the electrophoretic mobility of radiolabelled proteoglycan core proteins corresponding to biglycan and decorin (Fig. 3c). Taken together, these data indicate that fenofibrate reduces the apparent length and/or charge of the GAGs, independently of the core protein.

We used size exclusion chromatography to further analyse the molecular sizes of the proteoglycans and cleaved GAG chains following treatment of cells with fenofibrate (30 μmol/l). Cleaved GAG chains from proteoglycans of untreated cells had a peak K_{av} value of 0.470±0.006 (Table 1). Cleaved GAGs from VSMCs treated with TGF-β1 had a reduced K_{av} value (0.417±0.003) compared with the control (Table 1). Fenofibrate treatment in the presence of TGF-β1 reduced the size of the cleaved GAG chains having a K_{av} value of 0.430±0.006 compared with VSMCs treated with TGF-β1 alone (Table 1). The data of Wasteson [23] was used to convert the peak K_{av} values to molecular weight values (Table 1). The small changes in K_{av} observed with fenofibrate relate to a change in molecular weight of cleaved GAG chains from 32.0±0.8 M$_r$ to 30.0±1.3 M$_r$ from smooth muscle cells treated with TGF-β1 and TGF-β1 plus fenofibrate, respectively. This represents a 6.3% decrease in molecular weight or approximately eight to nine monosaccharides on a GAG chain following treatment of VSMCs with fenofibrate in the presence of TGF-β1. Thus, fenofibrate treatment of human VSMCs induced small but consistent reductions in the length of
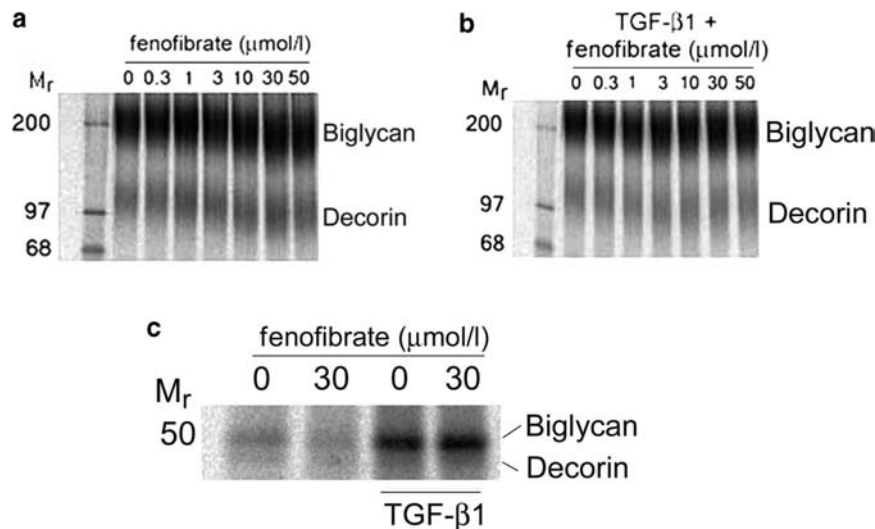
Fig. 3. Fenofibrate reduces the apparent molecular size of vascular GAGs. **a.** Mobility of [^{35}S]-sulphate-labelled proteoglycans on SDS-PAGE synthesised in the presence of increasing concentrations of fenofibrate (0.3–50 μmol/l). **b.** Mobility of [^{35}S]-sulphate-labelled proteoglycans on SDS-PAGE synthesised in the presence of increasing concentrations of fenofibrate (0.3–50 μmol/l) and TGF-β1 (2 ng/ml). **c.** Mobility of [^{35}S]-methionine/cysteine-labelled core proteins for biglycan and decorin following digestion of GAGs with chondroitinase ABC (42 U/ml) on SDS-PAGE following fenofibrate (30 μmol/l) treatment of VSMCs in the presence and absence of TGF-β1 (2 ng/ml)

amined proteoglycan core protein synthesis, following treatment of VSMCs with fenofibrate under basal conditions, by assessing [^{35}S]-methionine/cysteine incorporation into CPC-precipitable material. Fenofibrate (30 μmol/l) treatment of VSMCs increased [^{35}S]-methionine/cysteine incorporation into core proteins by 15.7% ($p<0.05$) compared with no treatment (79.0±10.4 cpm/10^3 cells). These data indicate that under basal conditions, treatment of VSMCs with fenofibrate activates proteoglycan core protein synthesis.

GAGs on vascular proteoglycans, suggesting that fenofibrate treatment may inhibit GAG elongation processes in smooth muscle cells.

Fenofibrate treatment increases basal core protein synthesis. Fenofibrate (30 μmol/l) treatment of VSMCs resulted in the synthesis of proteoglycans that showed increased electrophoretic mobility by SDS-PAGE in the absence of a reduction in total radiosulphate incorporation. One possible explanation for these results is that there is an increase in core protein synthesis which negates the decrease in GAG length when measuring [^{35}S]-sulphate incorporation. We ex-

Validation of FACE using sodium chlorate in human VSMCs. Fenofibrate treatment of VSMCs reduced [^{35}S]-sulphate incorporation into proteoglycans, which may result from a decrease in sulphation of GAGs leading to an alteration in the interaction with LDL. We used sodium chlorate, a *4*-sulphotransferase inhibitor in fibroblasts [24], to demonstrate that the FACE technique is capable of detecting changes in the sulphation pattern of GAGs. Sodium chlorate (0.5–5 mmol/l) treatment of VSMCs caused a concentration-dependent decrease in the Δdi4S, accompanied by a concentration-dependent increase in the Δdi0S (Fig. 4). These findings indicate that the mechanism of GAG synthesis in VSMCs is similar to GAG synthesis in other cells [24] and that the FACE technique

Table 1. Effect of fenofibrate on VSMC proteoglycan and GAG size

	Control	Fenofibrate (30 μmol/l)	TGF-β1	TGF-β1 + fenofibrate (30 μmol/l)
Intact proteoglycans on Sepharose CL-2B				
K_{av}	0.74±0.02	0.75±0.02	0.72±0.02	0.73±0.02
Cleaved GAG chains on Sepharose CL-6B				
K_{av}	0.470±0.006	0.467±0.003	0.417±0.003	0.430±0.006
M_r[a]	24.2±3.9	24.5±5.9	32.0±0.8	30.0±1.3

Values are means ± SEM; n=3. [a] Calculated average molecular weight using the Wasteson conversion of K_{av} to M_r for small GAGs on Sepharose CL-6B [23]
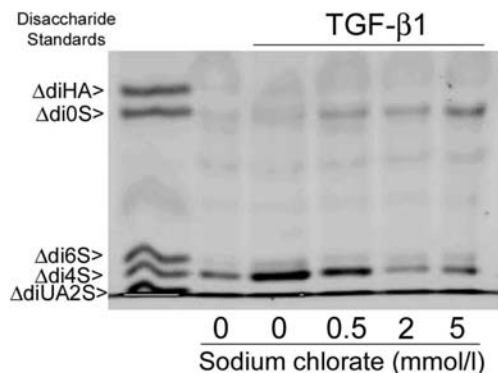
Fig. 4. Validation of the FACE technique in human VSMCs using sodium chlorate. Fluorophore-tagged disaccharides from VSMC GAGs synthesised in the presence of sodium chlorate (0.5–5 mmol/l) were separated by FACE. Sodium chlorate increases Δdi0S in a concentration-dependent manner. Sodium chlorate has a minimal effect on *6*-sulphation. Sodium chlorate inhibits *4*-sulphation in a concentration-dependent manner

is suitable for the analysis of potential pharmacologically induced changes in the sulphation pattern of GAGs [15]. We therefore used FACE to analyse the GAGs that had been synthesised by fenofibrate-treated VSMCs.

Fenofibrate treatment does not alter the sulphation pattern of GAGs. Glycosaminoglycans from untreated smooth muscle cells were composed of disaccharides with 7.7±1.4% Δdi0S (Fig. 5a), 18.0±3.7% Δdi6S (Fig. 5b) and 75.8±4.6% Δdi4S (Fig. 5c). A similar sulphation pattern was observed for disaccharides isolated from cells stimulated with TGF-β1 (Fig. 5a–c). The extent of the sulphation on the disaccharides did not alter when smooth muscle cells were treated with fenofibrate (30 μmol/l) alone or in the presence of TGF-β1 (Fig. 5a–c). These data indicate that the decrease in radiosulphate incorporation induced by fenofibrate in the presence of TGF-β1 results solely from the effect of decreasing GAG length and was not due to an alteration in the sulphation pattern.

Fenofibrate treatment alters GAG composition of vascular proteoglycans. The composition of GAGs on proteoglycans secreted by untreated smooth muscle cells, or those treated with fenofibrate, were characterised by digestion with chondroitinase AC which digests chondroitin sulphate chains, and chondroitinase B which digests dermatan sulphate chains. Subsequent
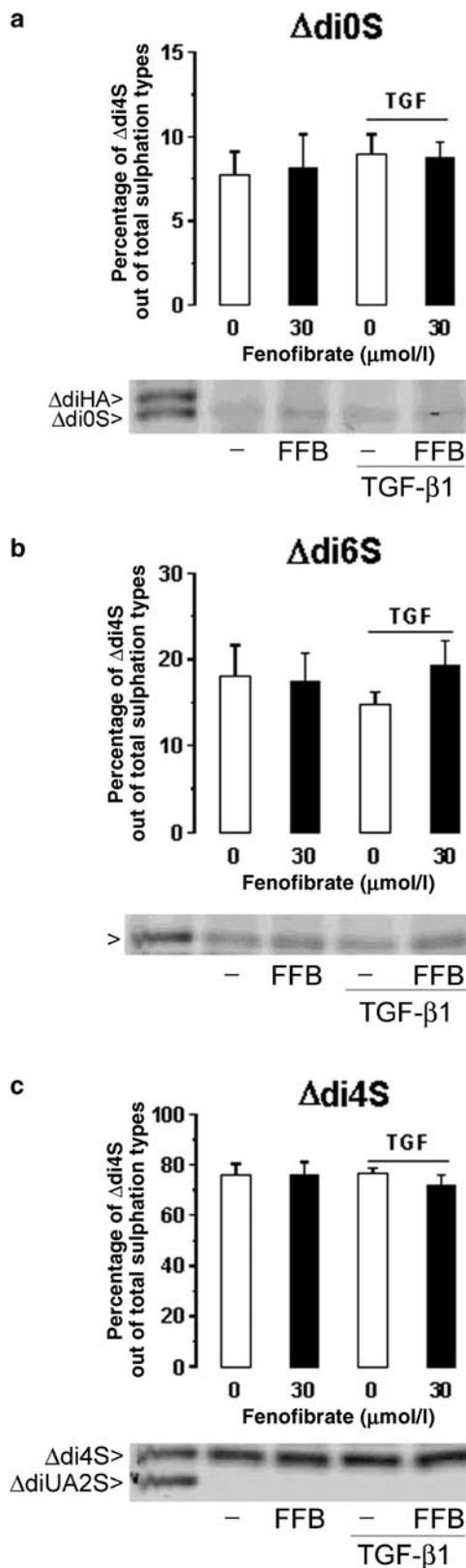
Fig. 5. Analysis of sulphation of vascular GAGs synthesised in the presence of fenofibrate (30 μmol/l) by VSMCs using FACE. Disaccharides (Δdi0S, Δdi6S and Δdi4S) were separated on a polyacrylamide gel from GAGs synthesised in the presence and absence of fenofibrate under basal conditions and in the presence of TGF-β1 (bottom panels of **a**, **b** and **c**). The figure shows the quantitation of (**a**) Δdi0S, (**b**) Δdi6S and (**c**) Δdi4S from GAGs synthesised in the presence and absence of fenofibrate under basal conditions and in the presence of TGF-β1. The density of the disaccharide band indicated is expressed as a percentage of the combined density of Δdi0S plus Δdi6S and Δdi4S. Each bar represents the mean ± SEM from three separate experiments performed in duplicate (*n*=5)

analysis by SDS-PAGE showed that the treatment of VSMCs with fenofibrate (30 µmol/l) increased the chondroitin sulphate component of the band corresponding to biglycan (Fig. 3a, b) compared with untreated cells from 80.7% to 88.0% chondroitin sulphate, and that the chondroitin sulphate in decorin was increased from 80.0% to 86.3%. Fenofibrate treatment of VSMCs increased the dermatan sulphate component of biglycan compared with untreated cells from 23.6% to 33.1%; however, in the decorin band, fenofibrate treatment reduced the dermatan sulphate component from 24.4% to 15.3%. The results indicate that there is an alteration in the chondroitin sulphate : dermatan sulphate ratio of vascular GAGs synthesised in the presence of fenofibrate, and this parameter may also contribute to changes in LDL binding.

Discussion

Fenofibrate treatment of VSMCs modifies the structure of proteoglycans and reduces binding to LDL under basal conditions and two types of stimulation by atherogenic growth factors. PDGF and TGF-β1 signal by distinct tyrosine and serine/threonine kinase receptors respectively, yet ultimately stimulate GAG elongation [7, 25]. Fenofibrate blocks GAG chain elongation with both growth factors, suggesting that its actions must be well downstream of either signalling pathway and closer to the GAG synthesising mechanisms. Fenofibrate treatment of VSMCs does not affect the sulphation pattern but does modify the carbohydrate composition of the GAGs, indicating that the reduction in LDL binding is due to the decrease in GAG length and possibly an alteration in the chondroitin sulphate/dermatan sulphate composition.

We have previously shown that gemfibrozil has similar actions on proteoglycan synthesis and GAG length to that reported here for fenofibrate; however, those studies did not evaluate the effects on LDL binding [11]. We now show that the structural changes in the GAGs lead to a reduction in LDL binding, so we can conclude that fibrates, as a class, show anti-atherogenic actions in this in vitro model.

One question that arises from these studies is whether or not the action of fenofibrate is through peroxisome proliferator-activated receptor-α (PPAR-α). Consistent with the data of Staels and colleagues [26], the smooth muscle cells used in this study express PPAR-α (data not shown). The effects of fenofibrate on vascular proteoglycans in the presence of atherogenic growth factors were not observed with a potent PPAR-α ligand, GW7647 (data not shown), a urea-substituted thioisobutyric acid with 5000 times higher potency for PPAR-α than fenofibrate [27]. At this stage we can conclude that the observed effects with fenofibrate are most likely a fibrate class effect, but it is uncertain whether this is mediated via PPAR-α.

Whether agents acting on nuclear receptors are actually working through their respective receptor is very difficult to assess. Most of the informative work relates to PPAR-γ studies. The PPAR-γ ligand troglitazone inhibits the proliferation of PPAR-γ$^{-/-}$ smooth muscle cells, leading to the conclusion that the inhibition is independent of PPAR-γ [28]. However, we have recently shown by molecular biological and pharmacological techniques that ligand activation of PPAR-γ can inhibit VSMC proliferation by cell-cycle-dependent mechanisms [29]. Furthermore, we have shown that the vascular effects of several thiazolidinedione PPAR-γ ligands correlate more closely with their lipophilicity rather than with their PPAR-γ receptor binding affinity [29]. Thus, the complex nature of signalling via heteromeric nuclear receptors such as PPARs suggests that the extent of the involvement of the receptor may depend upon concentrations of the ligand and levels of expression of the receptor [30].

We used the vascular growth factors TGF-β1 and PDGF as an atherogenic background to test the effects of fenofibrate on proteoglycan synthesis and structure. We included insulin with PDGF; however, it would also be interesting to stimulate VSMCs with agents that correlate with other metabolic abnormalities of diabetes, such as fatty acids [9, 31].

Data are emerging that strongly support the role of PPAR ligands (including PPAR-α) in inhibiting the vascular inflammatory mechanisms in atherogenesis, a process which accompanies lipid accumulation [32]. The mechanisms include repression of nuclear factor-κB-regulated inflammatory gene expression (PPAR-α and -γ ligands) [33], decreasing C-reactive protein (PPAR-γ ligands) [34] and monocyte chemoattractant protein 1 (PPAR-δ ligands) [35, 36]. Our data extend the vascular effects of PPAR-α ligands (fibrates) to an additional area of the initiation of atherogenesis, a reduction in proteoglycan-associated LDL retention [4, 5]. PPAR ligands (fibrates) may have multiple but complementary effects in the vasculature by modifying proteoglycan synthesis and inflammatory responses that may reduce LDL binding and inflammation respectively. If the effects of fibrate treatment on vascular proteoglycan synthesis observed in vitro are maintained in vivo, these changes would contribute to the reduction in macrovascular disease observed with administration of these agents [12, 37].

Few studies have investigated the pharmacological control of GAG sulphation [24], although regulation by therapeutic agents is potentially possible. Our data show that the actions of fenofibrate are specific for GAG elongation processes and carbohydrate composition with no observable effects on sulphation pattern. The reduced LDL binding partly arises from a reduction in GAG length and this is consistent with the effect of calcium channel antagonists [10] and glucosamine [38].

Overall, the characterisation of GAGs by enzyme digestion showed an alteration in the proportion of

chondroitin sulphate to dermatan sulphate in biglycan and decorin from fenofibrate-treated VSMCs, and this may affect LDL binding. Dermatan sulphate GAGs arise from the epimerisation of the chondroitin sulphate chain by the glucuronyl C5-epimerase in the *trans*-Golgi network [39]. The effect of fenofibrate in reducing dermatan sulphate containing GAGs on decorin may be due to an inhibition of the epimerase, as this is the limiting factor in dermatan sulphate biosynthesis [40]. The iduronic acid content and *4*-sulphation features of dermatan sulphate GAGs have been reported to give structural stability to the proteoglycan–LDL complex [41, 42]. In preparations of proteoglycans isolated from human arteries, those with a high affinity for LDL contained a higher proportion of dermatan sulphate than the proteoglycans with a low affinity for LDL [42]. The effect of fenofibrate in reducing the dermatan sulphate component of decorin, and thus epimerisation, may contribute to the reduced binding to LDL. Factors that regulate C5-epimerase expression/activity have yet to be identified and the promoter region has not yet been characterised. Whether or not fenofibrate and other PPAR-α ligands directly regulate C5-epimerase expression or activity in VSMCs is yet to be explored. Targeting this enzyme presents a mechanism for the reduction in dermatan sulphate GAG and reduced binding to LDL.

We demonstrate in this report that fenofibrate modifies the structure of proteoglycans produced by VSMCs by reducing the length of the GAG chains and modifying their carbohydrate composition resulting in reduced binding to human LDL. Fibrate therapy favourably modifies the proteoglycan binding properties of LDL in humans [11, 43], and thus fibrate treatment represents a "two-pronged" attack on reducing proteoglycan–LDL interactions by beneficial alterations in the biochemistry of both complex macromolecules [11, 43]. The studies in this report show direct vascular actions which may complement the metabolic actions of fibrates in reducing cardiovascular disease. We predict that this vascular mechanism underlies a favourable outcome for the FIELD trial and related studies, and advance the "response to retention" hypothesis as a rational model of atherogenesis for developing therapies based on targeting the vessel wall.

References

1. Mokdad AH, Ford ES, Bowman BA et al. (2003) Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. J Am Med Assoc 289:76–79
2. Staels B (2002) Cardiovascular biology: a cholesterol tether. Nature 417:699–701
3. Skalen K, Gustafsson M, Rydberg EK et al. (2002) Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature 417:750–754
4. Williams KJ, Tabas I (1995) The response-to-retention hypothesis of early atherogenesis. Arterioscler Thromb Vasc Biol 15:551–561
5. Williams KJ, Tabas I (1998) The response-to-retention hypothesis of atherogenesis reinforced. Curr Opin Lipidol 9:471–474
6. Camejo G, Fager G, Rosengren B, Hurt-Camejo E, Bondjers G (1993) Binding of low density lipoproteins by proteoglycans synthesized by proliferating and quiescent human arterial smooth muscle cells. J Biol Chem 268:14131–14137
7. Schonherr E, Jarvelainen HT, Kinsella MG, Sandell LJ, Wight TN (1993) Platelet-derived growth factor and transforming growth factor-beta 1 differentially affect the synthesis of biglycan and decorin by monkey arterial smooth muscle cells. Arterioscler Thromb 13:1026–1036
8. Chang MY, Potter-Perigo S, Tsoi C, Chait A, Wight TN (2000) Oxidized low density lipoproteins regulate synthesis of monkey aortic smooth muscle cell proteoglycans that have enhanced native low density lipoprotein binding properties. J Biol Chem 275:4766–4773
9. Olsson U, Bondjers G, Camejo G (1999) Fatty acids modulate the composition of extracellular matrix in cultured human arterial smooth muscle cells by altering the expression of genes for proteoglycan core proteins. Diabetes 48:616–622
10. Vijayagopal P, Subramaniam P (2001) Effect of calcium channel blockers on proteoglycan synthesis by vascular smooth muscle cells and low density lipoprotein-proteoglycan interaction. Atherosclerosis 157:353–360
11. Nigro J, Dilley RJ, Little PJ (2002) Differential effects of gemfibrozil on migration, proliferation and proteoglycan production in human vascular smooth muscle cells. Atherosclerosis 162:119–129
12. Diabetes Atherosclerosis Intervention Study Investigators (2001) Effect of fenofibrate on progression of coronary-artery disease in type 2 diabetes: the Diabetes Atherosclerosis Intervention Study, a randomised study. Lancet 357:905–910
13. Neylon CB, Little PJ, Cragoe EJ Jr, Bobik A (1990) Intracellular pH in human arterial smooth muscle. Regulation by Na+/H+ exchange and a novel 5-(N-ethyl-N-isopropyl)amiloride-sensitive Na(+)- and HCO3(-)-dependent mechanism. Circ Res 67:814–825
14. Sudhir K, Hashimura K, Bobik A, Dilley RJ, Jennings GL, Little PJ (2001) Mechanical strain stimulates a mitogenic response in coronary vascular smooth muscle cells via release of basic fibroblast growth factor. Am J Hypertens 14:1128–1134
15. Ballinger M, Nigro J, Frontanilla K, Dart A, Little P (2004) Regulation of glycosaminoglycan structure and atherogenesis. Cell Mol Life Sci 61:1296–1306
16. Heinecke JW, Baker L, Rosen H, Chait A (1986) Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. J Clin Invest 77:757–761
17. Little PJ, Tannock L, Olin KL, Chait A, Wight TN (2002) Proteoglycans synthesized by arterial smooth muscle cells

in the presence of transforming growth factor-beta1 exhibit increased binding to LDLs. Arterioscler Thromb Vasc Biol 22:55–60

18. Olsson U, Egnell AC, Lee MR et al. (2001) Changes in matrix proteoglycans induced by insulin and fatty acids in hepatic cells may contribute to dyslipidemia of insulin resistance. Diabetes 50:2126–2132

19. Calabro A, Benavides M, Tammi M, Hascall VC, Midura RJ (2000) Microanalysis of enzyme digests of hyaluronan and chondroitin/dermatan sulfate by fluorophore-assisted carbohydrate electrophoresis (FACE). Glycobiology 10:273–281

20. Calabro A, Hascall VC, Midura RJ (2000) Adaptation of FACE methodology for microanalysis of total hyaluronan and chondroitin sulfate composition from cartilage. Glycobiology 10:283–293

21. Wakino S, Kintscher U, Kim S, Yin F, Hsueh WA, Law RE (2000) Peroxisome proliferator-activated receptor gamma ligands inhibit retinoblastoma phosphorylation and G1→S transition in vascular smooth muscle cells. J Biol Chem 275:22435–22441

22. Chait A, Bierman E (1994) Pathogenesis of macrovascular disease in diabetes. In: Kahn C, Weir G (eds) Joslin's diabetes mellitus. Lea and Febiger, Philadelphia, pp 648–664

23. Wasteson A (1971) A method for the determination of the molecular weight and molecular-weight distribution of chondroitin sulphate. J Chromatogr 59:87–97

24. Greve H, Cully Z, Blumberg P, Kresse H (1988) Influence of chlorate on proteoglycan biosynthesis by cultured human fibroblasts. J Biol Chem 263:12886–12892

25. Schonherr E, Jarvelainen HT, Sandell LJ, Wight TN (1991) Effects of platelet-derived growth factor and transforming growth factor-beta 1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. J Biol Chem 266:17640–17647

26. Staels B, Koenig W, Habib A et al. (1998) Activation of human aortic smooth-muscle cells is inhibited by PPARalpha but not by PPARgamma activators. Nature 393:790–793

27. Brown PJ, Stuart LW, Hurley KP et al. (2001) Identification of a subtype selective human PPARalpha agonist through parallel-array synthesis. Bioorg Med Chem Lett 11:1225–1227

28. Bruemmer D, Berger JP, Liu J et al. (2003) A non-thiazolidinedione partial peroxisome proliferator-activated receptor gamma ligand inhibits vascular smooth muscle cell growth. Eur J Pharmacol 466:225–234

29. de Dios ST, Bruemmer D, Dilley RJ et al. (2003) Inhibitory activity of clinical thiazolidinedione peroxisome proliferator activating receptor-gamma ligands toward internal mammary artery, radial artery, and saphenous vein smooth muscle cell proliferation. Circulation 107:2548–2550

30. Bishop-Bailey D, Hla T, Warner TD (2002) Intimal smooth muscle cells as a target for peroxisome proliferator-activated receptor-gamma ligand therapy. Circ Res 91:210–217

31. Renard CB, Askari B, Suzuki LA, Kramer F, Bornfeldt KE (2003) Oleate, not ligands of the receptor for advanced glycation end-products, promotes proliferation of human arterial smooth muscle cells. Diabetologia 46:1676–1687

32. Ross R (1999) Atherosclerosis—an inflammatory disease. N Engl J Med 340:115–126

33. Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, Evans RM (2001) PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med 7:48–52

34. Haffner SM, Greenberg AS, Weston WM, Chen H, Williams K, Freed MI (2002) Effect of rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. Circulation 106:679–684

35. Lee CH, Chawla A, Urbiztondo N, Liao D, Boisvert WA, Evans RM (2003) Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. Science 302:453–457

36. Plutzky J (2003) Medicine. PPARs as therapeutic targets: reverse cardiology? Science 302:406–407

37. Rubins HB, Robins SJ, Collins D et al. (1999) Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. N Engl J Med 341:410–418

38. Tannock L, Little PJ, Wight TN, Chait A (2002) Arterial smooth muscle cell proteoglycans synthesized in the presence of glucosamine demonstrate reduced binding to LDL. J Lipid Res 43:149–157

39. Malmstrom A, Fransson LA (1975) Biosynthesis of dermatan sulfate. I. Formation of L-iduronic acid residues. J Biol Chem 250:3419–3425

40. Tiedemann K, Larsson T, Heinegard D, Malmstrom A (2001) The glucuronyl C5-epimerase activity is the limiting factor in the dermatan sulfate biosynthesis. Arch Biochem Biophys 391:65–71

41. Gigli M, Ghiselli G, Torri G, Naggi A, Rizzo V (1993) A comparative study of low-density lipoprotein interaction with glycosaminoglycans. Biochim Biophys Acta 1167:211–217

42. Cardoso LE, Mourao PA (1994) Glycosaminoglycan fractions from human arteries presenting diverse susceptibilities to atherosclerosis have different binding affinities to plasma LDL. Arterioscler Thromb 14:115–124

43. Wiklund O, Bondjers G, Wright I, Camejo G (1996) Insoluble complex formation between LDL and arterial proteoglycans in relation to serum lipid levels and effects of lipid lowering drugs. Atherosclerosis 119:57–67