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Reduction of arteriosclerotic nanoplaque formation and size by fluvastatin in a receptor-based biosensor model

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Abstract

Proteoheparan sulfate can be adsorbed onto a methylated silica surface in a monomolecular layer via its transmembrane hydrophobic protein core domain. Due to electrostatic repulsion, its anionic glycosaminoglycan side chains are stretched out into the blood substitute solution, thereby representing a receptor site for specific lipoprotein binding through basic amino acid-rich residues within their apolipoproteins. The binding process was studied by ellipsometric techniques suggesting that HDL has a high binding affinity and a protective effect on interfacial heparan sulfate proteoglycan layers with respect to LDL and Ca^{2+} complexation. LDL was found to be deposited strongly at the proteoheparan sulfate-coated surface, particularly in the presence of Ca^{2+}, apparently through complex formation 'proteoglycan–low density lipoprotein–calcium'. This ternary complex build-up may be interpreted as arteriosclerotic nanoplaque formation on the molecular level responsible for the arteriosclerotic primary lesion. In a receptor-based biosensor application, this system was tested on its reliability to unveil possible acute pleiotropic effects of the lipid lowering drug fluvastatin. The VLDL/IDL/LDL and VLDL/IDL/LDL/HDL plasma fractions from a high risk patient with dyslipoproteinaemia and type 2 diabetes mellitus showed the start of arteriosclerotic nanoplaque formation at a normal blood Ca^{2+} concentration, with a strong increase at higher Ca^{2+} concentrations. Nanoplaque formation and size of the HDL-containing lipid fraction remained well below that of the LDL-containing lipid fraction. Fluvastatin, whether applied acutely to the patient (one single 80 mg slow release matrix tablet) or in a 2-month medication regimen, markedly slowed down this process of ternary aggregational nanoplaque build-up and substantially inhibited nanoplaque size development at all Ca^{2+} concentrations used. The acute action gave no significant change in lipid concentrations of the patient. Furthermore, after nanoplaque generation, fluvastatin, similar to HDL, was able to reduce nanoplaque formation and size. These immediate effects of fluvastatin have to be taken into consideration when interpreting the clinical outcome of long-term studies.

Keywords: Atherosclerosis; CSE inhibitor; Ellipsometry; Lipoproteins; Proteoglycan receptor

1. Introduction

Nowadays, arteriosclerosis is not considered as a separate entity but in the context both of its multifarious history of origins and its multiplex irradiation into cardiovascular clinical pictures. Correspondingly, arteriosclerosis itself is an inflammatory and a fibroproliferative process that is probably initiated in response to a variety of vascular 'insults', including hypercholesterolaemia, hypertension, diabetes and smoking [1]. The process involves lipid accumulation, proliferation and migration of macrophages and smooth muscle cells and acquisition of a layer of connective tissue which covers the mature plaque. This mechanistic framework for atherogenesis is important for visualizing the role of dyslipidaemia and the other coronary heart disease (CHD) risk factors in creating a

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vascular milieu prone to disease, and increasingly the synergistic effects of multiple risk factors have been of interest. Bearing this in mind, it is not astonishing that powerful and beneficial substances against arteriosclerosis and its consequential diseases such as the statins have pleiotropic mechanisms of action which we are just unveiling. Statins inhibit a cholesterol synthesis enzyme (CSE) leading to a lowering of low-density lipoprotein (LDL) levels. This is the principal mechanism. Based on this alone, however, neither the great success of clinical trials in patients with stable CHD and in CHD risk individuals nor the effects on other symptoms and risk factors can be explained [2].

The present communication elucidates results from a clinical trial which led to the discovery of a further pleiotropic statin effect, that is the immediate intervention of fluvastatin into plaque formation and disintegration at the very earliest stages of arteriosclerosis development. Nanoplaque build-up and estimate of its size could be pursued by ellipsometric techniques using an established molecular model for arteriosclerosis [3–5], that was tested in a biosensor application with the lipid fractions of CSE-inhibitor-treated patients. Since the initial lipid deposition steps in atherogenesis are surface-related phenomena at endothelial cells and vascular matrices, we carried out this investigation on the adsorption of lipoproteins in order to learn more about their interfacial behaviour. Hydrophobic silica surfaces were modified through adsorption of proteoheparan sulfate, the latter substrate thus mimicking the surface receptors exposed to lipoproteins in the blood stream or on their paracellular pathway [4,6,7]. In order to obtain information on some biological relevance, an important aspect of the present investigations was to perform these experiments at close to in vivo conditions. The results of an acute intervention of fluvastatin, independent of any lipid lowering effect, into arteriosclerotic nanoplaque build-up using the plasma of a high risk patient with type 2 diabetes mellitus from a clinical trial are highlighted and discussed in the present study and contrasted with the results of long-term treatment of the same patient.

2. Methods

This was part of a double-blind, randomized, placebo-controlled, 12-week study comprising a 4-week dietary run-in phase followed by a study treatment period of 8 weeks, which was conducted at 10 centres in Germany. The study protocol was approved by the Ethics Committee of the University of Freiburg, Germany, and the institutional review boards at each study centre. All patients gave their informed, written consent.

2.1. Preparations and solutions

All experiments were carried out in a blood substitute solution. The normal blood substitute solution consisted of a Krebs solution simulating the extracellular ionic microenvironment of the proteoglycans and lipoproteins, and was of the following composition: Na^+ 151.16, K^+ 4.69, Ca^{2+} 2.52, Mg^{2+} 1.1, Cl^- 145.4, HCO_3^- 16.31, $H_2PO_4^-$ 1.38 mmol/l (25 °C, pH 7.27). Normally, the solution was aerated with a 95% O_2/5% CO_2 gas mixture (carbogen). Krebs solutions containing LDL were gassed by 93% N_2/7% CO_2 to hinder LDL oxidation [8].

Native proteoheparan sulfate (HS-PG) from the bovine aorta has a relative molecular mass of about 560 kDa, 11.6% of which is protein ($M_r \approx 400$ kDa). It has a few heparan sulfate (HS) side chains (88.4%) in covalent bonding (mol. wt. 35–38 kDa) with an average sulfate content of 0.5 mol sulfate/disaccharide unit [9,10].

Fasting blood samples from a high risk patient not yet treated with lipid-lowering agents in a clinical trial proving the efficacy of fluvastatin in hyperlipidemic patients with type 2 diabetes mellitus was taken via an EDTA-Sarstedt monovette in a medical practice. In the evening of the same day, this patient took one slow release matrix tablet of fluvastatin 80 mg (Novartis Pharma, Nürnberg, Germany) for the first time [11]. The next morning, after approximately 10 h, a blood specimen of the patient was obtained again in order to detect a possible immediate effect of fluvastatin after its processing in the body of the patient. A further blood sample was derived from the same patient after 2 months of continuous therapy in order to separate out a long-term from a short-term effect of the statin.

Blood samples were allowed to cool to room temperature for 10 min, centrifuged at 4000 rpm for 10 min, and plasma supernatants carefully pipetted into small polyethylene tubes. Thereafter, lipoproteins were isolated by sequential preparative ultracentrifugation essentially as described previously [12]. The following densities were used: $d<1.063$ kg/l for the preparation including very low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and LDL, and $d<1.21$ kg/l for the preparation including VLDL, IDL, LDL and high-density lipoproteins (HDL). All centrifugation steps were carried out at 18 °C using partially filled polycarbonate bottles (6 ml) in a 50 Ti rotor. Both preparations were then dialyzed against phosphate-buffered saline (PBS) to eliminate salt and EDTA. All samples were collected in small glass tubes (2 ml) and treated with a continuous flow of nitrogen before closure with a metallic screw cap in order to minimize lipid oxidation. Cholesterol and triglycerides were determined enzymatically with the CHOD-PAP and the GPO-PAP method (Wako Chemicals, Japan), respectively. Concentrations of apolipoproteins were determined by turbidimetry on an Olympus A640 analyzer (Olympus, Tokyo, Japan) using polyclonal antisera (Rolf Greiner Biochemica, Germany) specific for the respective antigens.

Prior to the ellipsometry investigations, the lipoprotein fractions were again run through a chromatography column

(Econo-Pac Column 732-2010, Bio-Rad, Hercules, CA, USA) filled with Krebs buffer solution and finally applied to the experiment in their natural patient blood concentrations. At the end of each experiment, fluvastatin (2.2 μmol/l) was administered to discover a possible disintegrating effect on ternary nanoplaque complexes.

2.2. Ellipsometry measurements

The adsorption of the lipoproteins and of proteoheparan sulfate was monitored by in situ ellipsometry, as detailed previously [13–15]. All measurements were carried out with a Rudolph 436 thin film ellipsometer at 401 nm. In summary, the optical response of the bare surfaces was first investigated, whereafter the proteoglycan or lipoprotein was added and changes in the state of polarization of the reflected light were monitored. The adsorbed amount was determined according to de Feijter using a refractive index increment of 0.18 cm^3/g [16]. A bulk concentration of approximately 0.1 mg/ml proteoheparan sulfate (0.192 mmol/l in disaccharide units) was used [5]. Furthermore, the measurements were carried out at different Ca^{2+} concentrations around that in the biological system. The normal blood substitute solution consisted of a Krebs solution. The pH was kept between 7.25 and 7.30 by the bicarbonate/phosphate buffer, and by the continuous aeration of the cuvette solution with a 95% O_2/5% CO_2 (lipoproteins: 93% N_2/7% CO_2) gas mixture (Aga, Sweden). The latter is included due to the necessity to keep the pH well regulated during these experiments in order to avoid both proteoglycan and lipoprotein degradation and calcium phosphate precipitation. The adsorption was carried out at 25 °C throughout. Further details can be found in previous publications [17,18].

3. Results

Proteoheparan sulfate deposition at a methylated silica surface is initiated by its transmembrane hydrophobic core domain [19,20]. The GAG side chains are stretched out into the blood substitute solution because of their negative fixed charges giving rise to electrostatic repulsion [5,19,21]. Lipoprotein particles or cations may interact with the GAG chains, e.g. on occasion of their positive amino acid residues or simply their positive charges. Thus, the situation reflects physiological conditions, since, for example in the endothelial cell membrane, the scenario differs only in one respect, that the short hydrophilic intracellular core domain is located in the cell interior. In this sense, the hydrophobic silica surface simulates the cell membrane overlaid with the glycocalyx quite perfectly. Furthermore, in this in vitro model, any drug can be tested with respect to its antiarteriosclerotic potential [5].

With the following experiments, we wanted to evaluate the applicability of the molecular model for nanoplaque formation in the clinical situation. Within a clinical study investigating the effectiveness of fluvastatin on lipoprotein subfractions in high-risk patients with type 2 diabetes mellitus, the blood of a patient was taken before, after 10 h therapy with a single dose of fluvastatin and after 2 months therapy with a single dose of fluvastatin daily (Table 1), and the in vivo-concentration of the VLDL/IDL/LDL-fraction applied in our assay [22,23]. The ternary complex build-up was then pursued through increasing the Ca^{2+} concentration. The sample from the patient treated once a day was used in the experiment after the usual preparation and double-dialysis in order to prove a possible acute effect of fluvastatin after its processing in the human body. At the end of the experiment, control and test samples were again treated for 30 min with fluvastatin (2.2 μmol/l) after formation of the ternary nanoplaque complex.

Shown in Fig. 1 is the adsorption of VLDL/IDL/LDL at a HS-PG coated surface. As can be seen, the adsorption is limited in the absence of Ca^{2+}, at least for a considerable time. Opposite to this limited adsorption, in the presence of the physiological serum concentration of Ca^{2+} (Ca1: 2.52 mmol/l), the deposition is strongly accelerated in the control experiment (by 67%) and so much the more with twice the normal Ca^{2+} concentration (by 103%), whereas upon fluvastatin medication, a moderate reduction in ternary complex deposition was measured (cf. Fig. 3A) [1 day fluvastatin treatment: −12% (2.52 mmol/l Ca^{2+}), −11% (5.04 mmol/l Ca^{2+}); 2 months fluvastatin treatment: −16% (2.52 mmol/l Ca^{2+}), −21% (5.04 mmol/l Ca^{2+})]. Already prior to the end of the 40 min Ca1-

Table 1
Lipoprotein and apolipoprotein plasma concentrations in a high risk patient with type 2 diabetes mellitus before, after 1 day and after 2 months treatment with fluvastatin (80 mg)

	Baseline concentration (mg/dl)	Concentration after 1 day fluvastatin (mg/dl)	Concentration after 2 months fluvastatin (mg/dl)
VLDL+IDL+LDL (d<1.063)	120.7	118.4	73.9
VLDL+IDL+LDL+HDL (d<1.210)	134.9	132.8	88.2
HDL	14.2	13.1	14.3
apoB	68.2	65.8	45.2
apoA-I	55.9	51.6	53.8

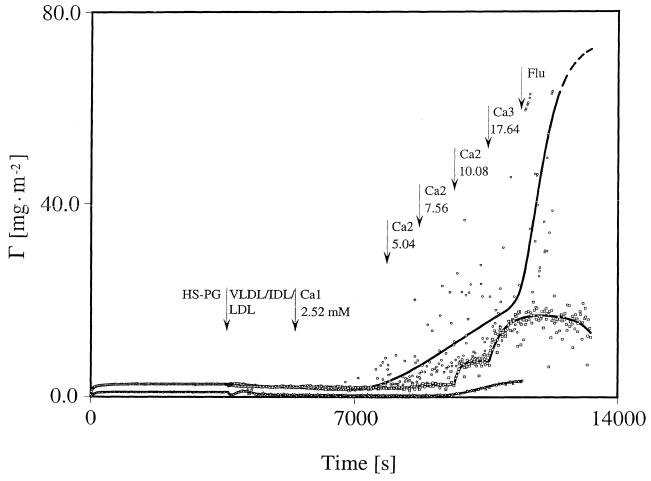

Fig. 1. Total adsorbed amount versus time. At time zero, HS-PG (0.1 mg/ml) was adsorbed on hydrophobic silica from a Ca^{2+}-free Krebs solution. The first arrow indicates the addition of the plasma VLDL/IDL/LDL fraction at its in vivo concentration from a cardiovascular high-risk patient either untreated (○), after 1-day treatment with a single 80-mg tablet of fluvastatin (□) or after 2 months treatment with daily 80-mg fluvastatin (△) (cf. Table 1). Total Ca^{2+} concentrations in solution are indicated at the arrows. At the end of each experiment, fluvastatin (2.2 μmol/l) was applied for another 0.5 h. The thick, solid lines were computed by an iterative parameter fit of the nonlinear allosteric-cooperative, simple-saturative or exponential kinetics to the experimental points using an algorithm for least-squares estimates. The pH was 7.33 (○), 7.31 (□) and 7.34 (△), respectively.

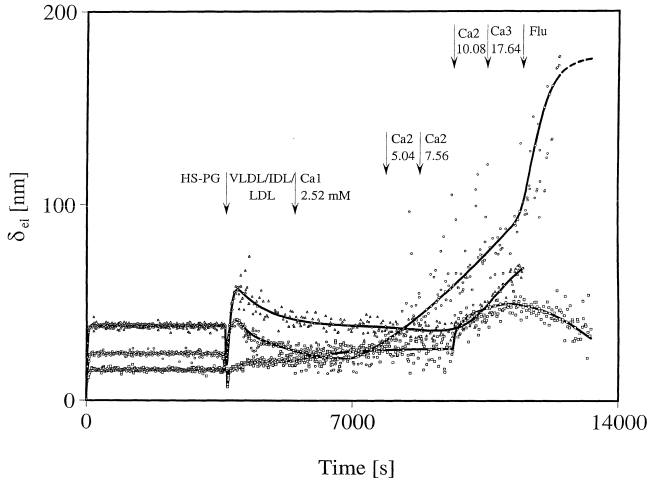

Fig. 2. Adsorbed layer thickness versus time for HS-PG (0.1 mg/ml) at methylated silica from Ca^{2+}-free Krebs solution. Same experiments and indications as in Fig. 1.

application, the steep increase in nanoplaque formation and deposition started, in parallel with a large scatter of the measuring points (Fig. 1). The scatter reflects the heterogeneity in particle size of the aggregational ternary complexes. The shape of the curve orients itself by the Γ-mean values at the respective Ca^{2+} concentrations, and was calculated using an algorithm for least-squares estimation of nonlinear parameters [24]. The acute effect of fluvastatin became apparent in a strong diminution of nanoplaque formation and a change in allosteric Hill kinetics towards simple saturation kinetics at all Ca^{2+} concentrations. After 2 months duration of treatment with fluvastatin, there was an impressive decrease in nanoplaque build-up for physiological Ca^{2+} concentrations and a comparatively minor increase only for very high Ca^{2+} concentrations. Noteworthy is the rise in ternary nanoplaque deposition upon first application of fluvastatin at the end of the control experiment, whereas in the test experiment with the lipid fraction exposed to a single dose of fluvastatin only, there occurred no increase worth mentioning (Fig. 1). On the contrary, there was a decrease in ternary proteoheparan sulfate–lipoprotein–calcium complex deposition after the interaction with fluvastatin for 15 min in the sample from the patient prepared after the intake of a single dose fluvastatin.

These findings were confirmed by measuring the thickness of the interfacial layer (Fig. 2). In the control experiment, the adsorbed layer thickness (δ_{el}) showed a decreasing trend after the application of VLDL/IDL/LDL upon Ca1 addition, but then reversed before the Ca2 addition and rose steeply with increasing Ca^{2+} concentrations. Fig. 3 demonstrates that beyond nanoplaque formation and deposition (A) also its dimensional build-up (B) is a Ca^{2+} driven process. Adsorbed layer thickness which is related to the molecular size of the ternary aggregational complexes did not markedly increase upon Ca1, but prominently upon Ca2 additions, in complete accordance with former experiments in the absence of proteoglycan coating of the silica surface [5] and with light scattering experiments [8], where the adsorbed amount and particle size, respectively, increased significantly with Ca2 incubation. In the case where the patient was treated with only one fluvastatin tablet for 10 h, the adsorbed layer thickness showed no essential augmentation until the addition of 10.08 mmol/l Ca^{2+}. Accordingly, no change in nanoplaque size in normal serum Ca^{2+} concentration is recognizable in Fig. 3B, and the increase in 5.04 and 7.56 mmol/l Ca^{2+} is rather limited. Altogether, the augmentation in adsorbed layer thickness with increasing Ca^{2+} concentration took a distinctly reduced course compared to the control curve. Furthermore, the dramatic reduction in nanoplaque size compared to the initial values is especially prominent after 2 months of therapy. This applies particularly to Ca^{2+} concentrations up to 10.08 mmol/l.

Finally, the strong rise in adsorbed layer thickness at the end of the control experiment during the first fluvastatin incubation ran parallel to an equal increase in total adsorbed amount. On the other hand, a more or less marked reduction in size of the ternary nanoplaque complex appeared in the lipid fraction processed by a single tablet in the patient.

The investigations with a lipoprotein fraction from a high-risk patient emphasized that short- and long-term treatment with fluvastatin can partly prevent both the

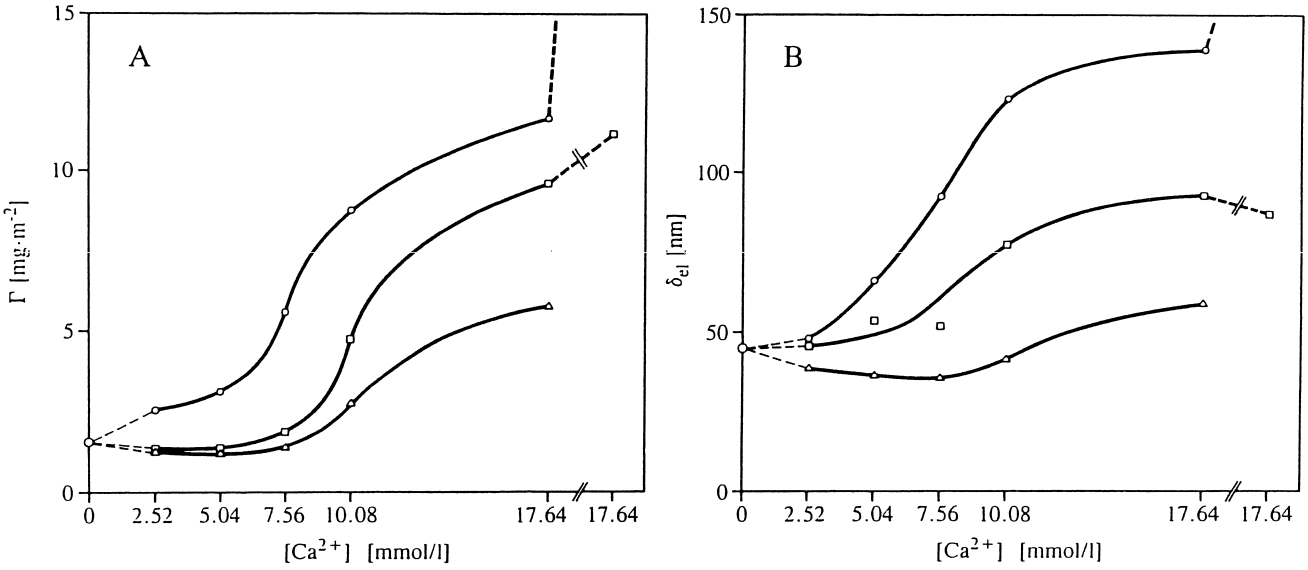

Fig. 3. Total adsorbed amount (A) and adsorbed layer thickness (B) as derived from the experiments in Figs. 1 and 2, dependent on the Ca^{2+} concentration of the Krebs solutions. Adsorbed amounts and layer thicknesses were normalized to a common mean value of the VLDL/IDL/LDL incubation periods (zero Ca^{2+} concentration). Symbols as indicated in Fig. 1 (○, □, △) reflect the mean values of all experimental points during the Ca^{2+} period in question. Broken lines represent the curve courses to their average end points under fluvastatin.

formation and the size development of ternary nanoplaques, and this even at high Ca^{2+} concentrations. Fig. 4 illustrates the extent of inhibition. There arose a similar curve profile for the reduction in adsorbed amount of ternary nanoplaques dependent on the Ca^{2+} concentration used, not only for the VLDL/IDL/LDL lipoprotein fraction processed by fluvastatin in the body of the patient on a long-term scale but also upon a 1-day exposure time. The degree of inhibition varied between 17 and 76%. Similar results were found for the reduction in adsorbed layer thickness, although nanoplaque size was only moderately diminished in normal serum Ca^{2+}. On the other hand, a continuous diminution of nanoplaque size was measured with increasing Ca^{2+} concentrations. Moreover, the peak inhibition seemed to be shifted to higher Ca^{2+} concentrations after long-term medication. In total, the inhibition ranged between 4 and 67%. Furthermore, it should be stressed that the addition of fluvastatin at the end of the experiments led to a drastic decline in adsorbed amount and layer thickness.

In order to corroborate the fluvastatin effect raised with the VLDL/IDL/LDL-fraction and to demonstrate both the competitive receptor binding between LDL and HDL and the protective effect of HDL against nanoplaque formation, the Ca^{2+} titration curves were repeated for the complete lipoprotein fraction of VLDL/IDL/LDL/HDL in its natural blood concentration. In the presence of HDL, total adsorbed amount, adsorbed layer thickness and scatter of experimental points were reduced substantially (Figs. 5–7). As one can see from Fig. 5, a complete Ca^{2+} titration curve was measured again. Fluvastatin was added at the end of the experiment to examine whether arteriosclerotic nanoplaques were resolvable after their build-up. This picture impressively shows that the steep increase in nanoplaque formation was shifted to higher Ca^{2+} concentrations upon a longer duration of treatment and that fluvastatin was able to dissolve freshly formed nanoplaques. When fluvastatin was added for the first time in the control curve, the ternary complex formation did not increase (cf. Fig. 1, VLDL/IDL/LDL: +210%) but decrease (cf. Fig. 5, VLDL/IDL/LDL/HDL: −15%) within 30 min. Thus, HDL and fluvastatin seem not only to hinder partially the process of nanoplaque formation but also to reverse it to some extent. Additionally, it appeared that the reaction of the docking process of the lipid fraction to the proteoglycan receptor followed HDL-type kinetics, that is an initial simple-exponential desorption [4]. These results are confirmed in Fig. 6, where the adsorbed layer thickness of the ternary aggregational complexes was recorded. Again, it is clear that there is a strong correlation between duration of statin treatment and Ca^{2+} concentration where nanoplaque size has been strongly promoted.

The graphs in Fig. 7 show nanoplaque formation (A) and size (B) as a function of Ca^{2+} concentration, where the initial amounts for the dimeric complex proteoglycan/lipoprotein in Ca^{2+}-free solution were normalized to common starting values. At first glance, one recognizes a clear stepwise reduction both in nanoplaque formation and size as a function of the duration of fluvastatin therapy for all Ca^{2+} concentrations used. Whereas plaque build-up increased with normal blood serum Ca^{2+} of 2.52 mmol/l (+33.2%), it was significantly reduced even after one single fluvastatin tablet (−17.9%) and underwent only a small further decrease after 2 months of medication (−22.6%). In plaque size, however, a further marked decrease took place after long-term therapy (−32.3%)

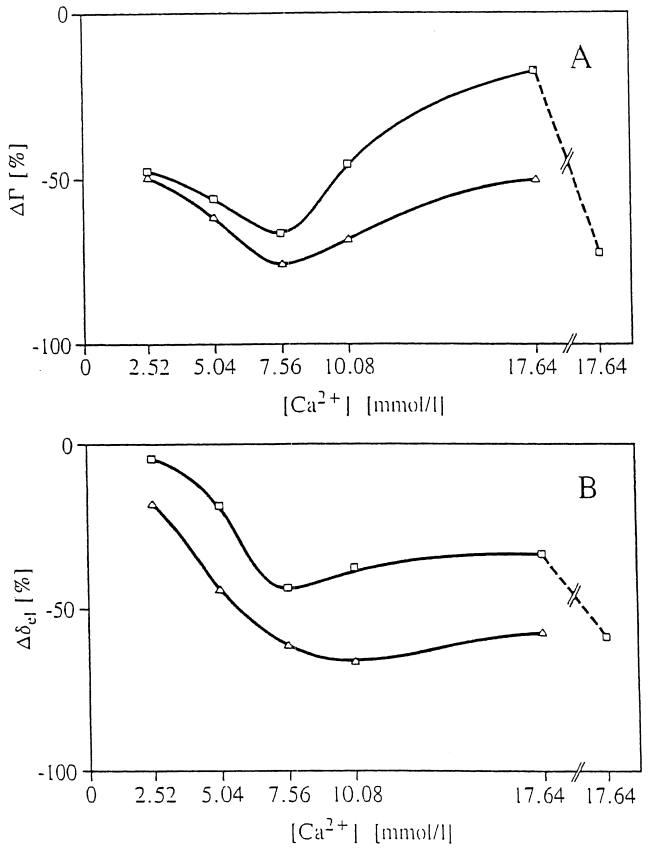

Fig. 4. Reduction in Ca^{2+}-induced changes in adsorbed amount (A) and layer thickness (B) as derived from the curves in Fig. 3. The reductions upon application of the VLDL/IDL/LDL fractions taken from the same patient treated with one fluvastatin tablet only (□) and for 2 months daily intake (△) were calculated as a ratio to the control values of the untreated patient. Broken lines represent the curve courses to their average end points under fluvastatin.

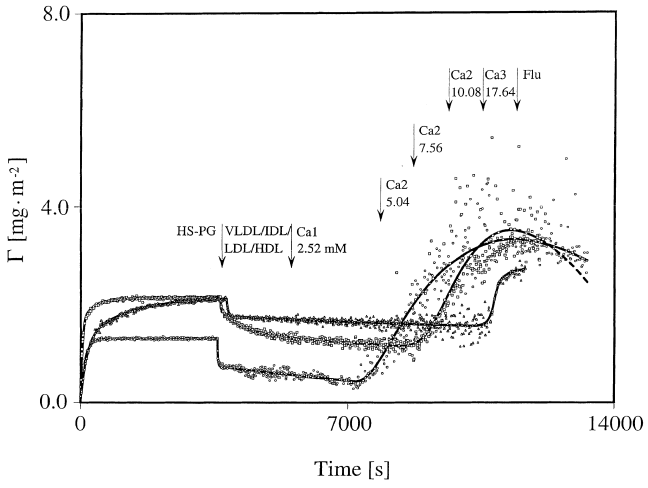

Fig. 5. Total adsorbed amount versus time. At time zero, HS-PG (0.1 mg/ml) was adsorbed on hydrophobic silica from a Ca^{2+}-free Krebs solution. The first arrow indicates the addition of the plasma VLDL/IDL/LDL/HDL fraction at its in vivo concentration from a cardiovascular high-risk patient either untreated (○), after 1-day treatment with a single 80-mg tablet of fluvastatin (□) or after 2 months treatment with daily 80 mg fluvastatin (△) (cf. Table 1). Total Ca^{2+} concentrations in solution are indicated at the arrows. At the end of each experiment, fluvastatin (2.2 μmol/l) was applied for another 0.5 h. The thick, solid lines were computed by an iterative parameter fit of the nonlinear allosteric-cooperative, simple-saturative or exponential kinetics to the experimental points using an algorithm for least-squares estimates. The pH was 7.27 (○), 7.15 (□) and 7.31 (△), respectively.

versus the short-term application of 1 day only (−17.5%). The longer the fluvastatin therapy lasted, the trend to a right shift of the steep rise in nanoplaque formation and size to higher Ca^{2+} concentrations was repeatedly emphasised.

The reductions as calculated as a ratio to the control values in question (Fig. 7, ○) are represented quantitatively in Fig. 8, i.e. for nanoplaque formation in the upper part, and for plaque size in the lower part. With normal Ca^{2+} concentration, new nanoplaques were formed to a roughly 40% less extent (1-day treatment: −38.3%; 2 months treatment: −41.9%), however, nanoplaque size was reduced by 9.9% after 1 day treatment and by 26.0% after 2 months medication. After this long-term therapy, the maxima in reductions of both amounts were shifted to a higher Ca^{2+} concentration of about 10 mmol/l. Here, it shall be mentioned that the effects of a 2-month therapy with fluvastatin collected from seven patients of the same high-risk patient collective showed the same tendency to retardation and partial inhibition of nanoplaque development compared to the control experiments. After 2 months of treatment, nanoplaque formation and size were diminished by 40.0±7.0% ($P \leq 0.018$, non-parametric Wilcoxon-test for paired samples; median 34.9%) and 35.6±8.2% ($P \leq 0.018$, non-parametric Wilcoxon-test for paired samples; median 26.0%), respectively, in 2.52 mmol/l Ca^{2+}-Krebs solution. Baseline concentrations of total cholesterol, LDL cholesterol and HDL cholesterol were 260.6±13.9, 127.4±7.8 and 44.7±3.7 mg/dl, respec-

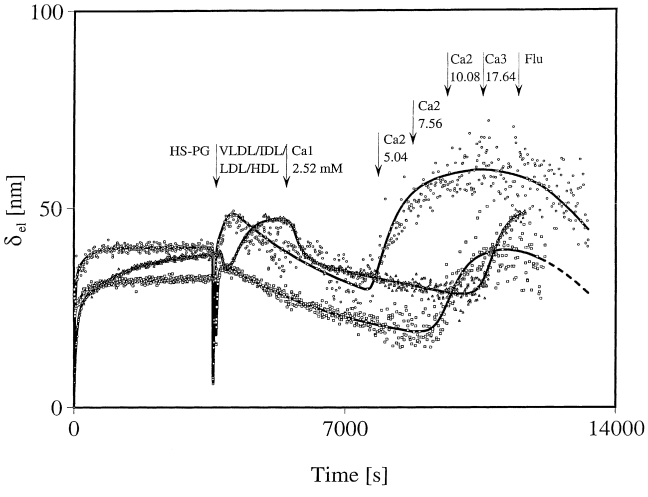

Fig. 6. Adsorbed layer thickness versus time for HS-PG (0.1 mg/ml) at methylated silica from Ca^{2+}-free Krebs solution. Same experiments and indications as in Fig. 5.

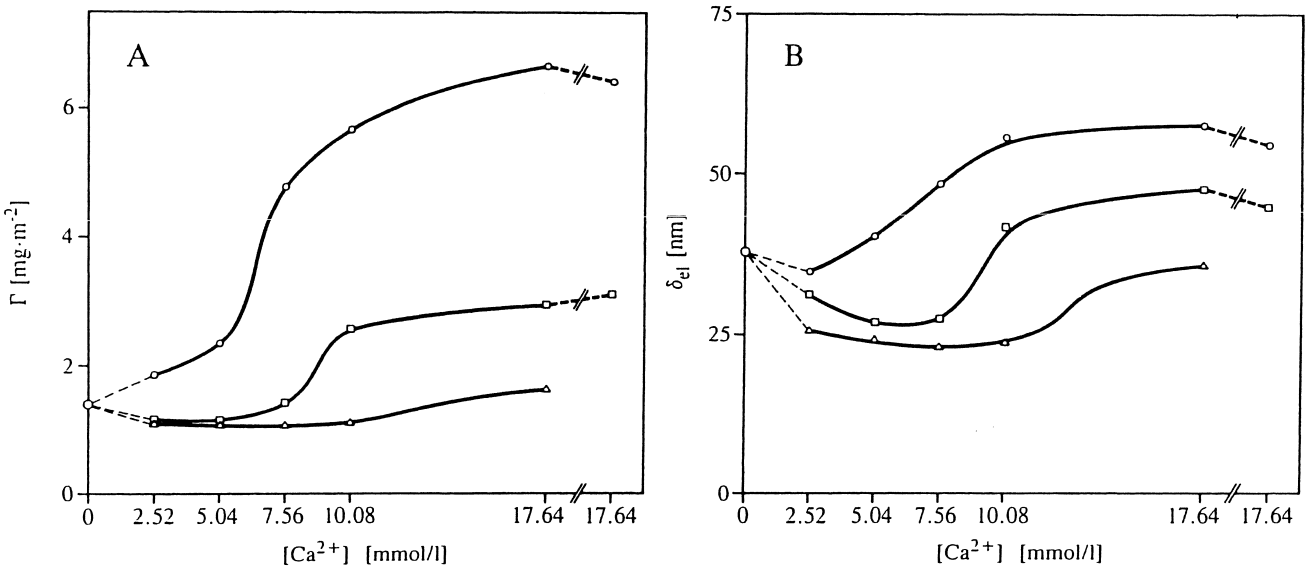

Fig. 7. Total adsorbed amount (A) and adsorbed layer thickness (B) as derived from the experiments in Figs. 5 and 6, dependent on the Ca^{2+} concentration of the Krebs solutions. Adsorbed amounts and layer thicknesses were normalized to a common mean value of the VLDL/IDL/LDL/HDL incubation periods (zero Ca^{2+} concentration). Symbols as indicated in Fig. 5 (○, □, △) reflect the mean values of all experimental points during the Ca^{2+} period in question. Broken lines represent the curve courses to their average end points under fluvastatin.

tively, and concentrations after 2 months of therapy 184.6±11.1, 81.6±4.4 and 45.6±4.8 mg/dl, respectively. In view of the different blood lipid values in the patients, these results, which will be presented in detail elsewhere, will underscore the general validity of the statements being derived from one patient.

4. Discussion

Stretches of basic amino acid-rich residues within apoB, apoA and apoE constituting the predominant protein moiety of LDL and HDL display a high electrostatic binding affinity to proteoglycans. In the proteoheparan sulfate perlecan, the large protein core carries three HS chains of 30–70 kDa which are 100–170 nm in length and covalently attached to Ser–Gly consensus sequences clustered at the N terminus [25]. This major HS-attachment domain I is considered to be a putative lipoprotein binding site for reasons of electrostatic attraction [26]. Immediately adjacent, domain II contains four copies of LDL receptor repeats and one immunoglobulin repeat (NCAM module) [27]. Also such motifs in perlecan are thought to mediate LDL binding for reasons of core protein receptor module–lipoprotein ligand interactions. Interestingly, the expression of the proteoglycan in endothelial cells is modulated by LDL [28]. Full-blown arteriosclerotic plaques as clinically detected by spiral computer tomography and magnetic resonance imaging basically contain lipoproteins associated with proteoglycans of high diversity and versatility and complexly aggregated with high amounts of calcium [29]. Therefore, the replica in nanoscale dimensions of the heterotrimeric aggregational complex consisting of proteoglycan receptor, lipoprotein and calcium presumably portrays the very earliest stages of an arteriosclerotic plaque, which remains undiscovered even with the most up to date clinical diagnostics. This so-called primary lesion impresses merely as endothelial dysfunction. The formation of the proteoglycan–lipoprotein–calcium complex (Figs. 1 and 5) may be initiated by cooperative Ca^{2+}-binding to the proteoglycanic GAG chain inducing a diminution of the axial rise of its left-handed 2_1-helix through a conformational change, so that the pitch of turns is compressed [10,21,30]. Simultaneously, the cooperativity of this dual complex may be amplified by allosteric lipoprotein docking to that shortened conformation supported by the higher charge density and fitting stereospecificity. Thus, the heterotrimeric complex would become rather stable. The adsorption to the silica surface may be promoted. This, however, is rather speculative and not known at present. It is equally possible that deposition through Ca^{2+}-dependent complex formation between the lipoproteins and HS-PG is likely to occur and contribute to the observed effects.

With respect to the lipoprotein fractions used in our experiments, the question arises whether these concentrations are relevant within the scenario studied. On the one hand, HS-PGs are integral constituents of the plasma membrane of endothelial cells (e.g. syndecans) and, on the other hand, of the extracellular matrices (e.g. perlecan, fibroglycan). In the first case, our model is adequately suited because we used the lipoprotein fractions at their natural patient blood concentration, and these could interact with the proteoglycan receptor. For the second case, it should be critically mentioned that the lipoprotein ratio applied probably does not correspond to the in vivo relations in the extracellular matrix of the vessel wall.

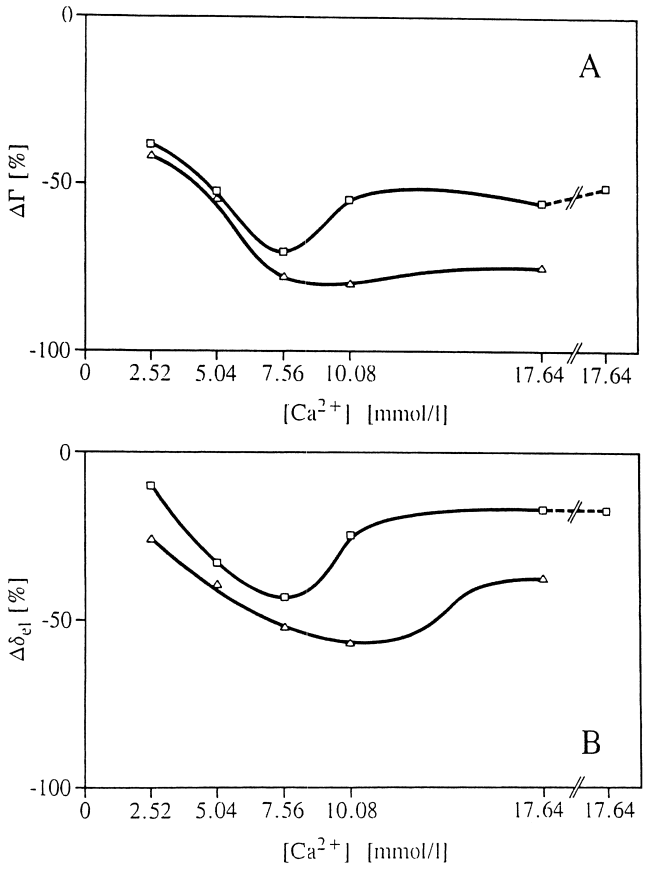

Fig. 8. Reduction in Ca^{2+}-induced changes in adsorbed amount (A) and layer thickness (B) as derived from the curves in Fig. 7. The reductions upon application of the VLDL/IDL/LDL/HDL fractions taken from the same patient treated with one fluvastatin tablet only (□) and for 2 months daily intake (△) were calculated as a ratio to the control values of the untreated patient. Broken lines represent the curve courses to their average end points under fluvastatin.

Based on different sizes of the lipoprotein particles and since molecular sieving takes place at the endothelial barrier, the lymph/plasma concentration ratios vary from 0.03 for VLDL sized particles to 0.3 for HDL sized particles [31]. These ratios indicate that blood concentrations are likely to be much higher than concentrations in the extracellular matrix of the vessel wall. Since we wanted to exemplarily show with our experiments that nanoplaque formation and size can be restricted by fluvastatin treatment, the results should be correlated with the in vivo situation.

The HDL effect as disclosed through comparison of the corresponding curves in Figs. 1 and 5, may be interpreted as an indication that HDL strongly counteracts primary lesion generation in the form of the ternary complex, proteoglycan–LDL–calcium. Furthermore, it was derived from earlier experiments that HDL has a higher affinity to its proteoglycan receptor by a factor of at least four compared to LDL [5]. Although the ratio (VLDL–IDL–LDL)/HDL before medication was 8.5 in the high-risk patient in our study, the protective efficacy of HDL can be deduced from Figs. 5–7 as a significantly diminished nanoplaque formation and size compared to the related curves without HDL, a further indication for the strong binding of HDL to the proteoglycan receptor. In order to be able to additionally compare the fluvastatin effect with the HDL effect, we had carried out all the experiments with the whole lipoprotein fraction of the patient and one devoid of HDL. The courses of the corresponding curves in each set of experiments behave very similar in principle, however, the absolute values of adsorbed amount and layer thickness of the VLDL/IDL/LDL fraction are in total approximately twice as high as for the HDL-containing fraction. Comparing the results of both experimental series for normal serum calcium with each other as well as with the fluvastatin effect in the HDL-containing experiments led to the surprising fact that fluvastatin was at least 3.5 times more powerful in our patient than his own HDL to prevent arteriosclerotic nanoplaque formation and growth, whereas both agents were about equally effective in nanoplaque size inhibition.

Taking together these findings on the HDL/LDL-interplay at their receptor binding site with the results from previous experiments illustrate that HDL is able not only to hinder the formation of the ternary complex HS-PG/LDL/Ca^{2+} but also to probably disintegrate such a primary lesion nanoplaque [5,32,33]. We had just worked out that in our high-risk patient, the potency of fluvastatin is in no way inferior to the efficacy of HDL and even exceeds the latter. Whereas we will deal with the mode of action of fluvastatin in the next paragraph, here a few thoughts on the cardiovascular protection through HDL.

Largely based on its higher affinity, compared with LDL, to the HS-PG receptor and dependent on its plasma concentration, the great majority of the peripheral proteoglycan receptors both on and in the arterial vascular wall are supposed to be occupied by HDL particles and thus LDL uptake limited. In addition to the three known antiatherogenic action principles of HDL, namely inhibition of monocyte adhesion and LDL oxidation as well as mediation of cholesterol efflux, a fourth beneficial forechecking mechanism is active which effectively controls cholesterol influx into the vascular wall via the transendothelial and paraendothelial pathways. Therefore, intercellular LDL diffusion into the subendothelial matrix spaces is neither an open flank within an ingenious control system nor the internalization of LDL as a prerequisite to the transendothelial transport. Both, cholesterol entry and exit, are powerfully checked via feedforward and feedback loops (competition at the receptor site; expression of the proteoglycan receptor; posttranslational modification of the GAG side chains). Only if LDL increasingly occupies proteoglycan receptors via a high plasma concentration (LDL/HDL≥4), then LDL-influx into and LDL-retention time within the vascular wall become enhanced, and thus an antiatherogenic barrier is broken through. This was the case in our high risk patient. Accordingly, in the complete

lipid fraction, the transition from the heterodimeric to the heterotrimeric nanoplaque complex, i.e. from the atheromatous to the calcified plaque, was promptly enhanced by 33.2% in normal serum calcium (Fig. 7). The HDL protection amounted to merely 20.3%. After a single dose of fluvastatin, this protection against nanoplaques could be elevated to 58.6%. Correlated to this, the formation of the ternary from the binary aggregational complex was diminished by 17.9% (Fig. 7), that is fluvastatin reduced the ternary compared to the binary nanoplaque building block by 51.1% altogether. These numbers impressively substantiate the cooperative action of HDL and fluvastatin in the prevention of primary lesion generation.

In order to test the applicability of the biosensor model more thoroughly, we investigated the efficacy of fluvastatin on arteriosclerotic nanoplaque formation with lipid fractions that originated from high risk patients with dyslipoproteinaemia and type 2 diabetes mellitus after a long-term treatment (2 months). Here, in addition, results were presented with the VLDL/IDL/LDL and VLDL/IDL/LDL/HDL fractions after acute treatment of a patient (1 day), whose cardiovascular risk consisted of diabetes mellitus and a low HDL plasma level and whose lipid concentrations had undergone no significant changes approximately 10 h after the intake of one fluvastatin 80 mg tablet only (cf. Table 1). Therefore, possible effects of fluvastatin could in no case be traced back to an influence on the lipid concentrations [11,34]. We could show that neither adsorbed amount nor layer thickness of the ternary versus the binary nanoplaque complex were elevated at a physiologic blood Ca^{2+} concentration of 2.5 mmol/l (a reduction was even the case) (Figs. 3 and 7) and that also, at all Ca^{2+} concentrations used, calcified atheromatous nanoplaques were reduced in formation and size in comparison to untreated controls of the same patient (Figs. 4 and 8). Including the results after 2 months treatment into these considerations, there was a surprising finding. In normal serum Ca^{2+} concentration, after 1 day of statin intake, an inhibitory effect on nanoplaque formation of on average 93.6% of the effect after 2 months therapy appeared in both lipid fractions. The hindrance on nanoplaque size, however, was only 30.6% on average (VLDL/IDL/LDL: −23.3%; VLDL/IDL/LDL/HDL: −37.9%). Obviously, blocking of plaque formation is an immediate effect, whereas plaque size can be reduced much better after long-term therapy. Furthermore, it could be argued that the altered lipid profile (VLDL-IDL-LDL)/HDL=5.2, especially the decrease in serum LDL concentration, contributes to the additional effect seen after 2 months of statin medication.

Since dialysis was repeatedly used during the reprocessing of the plasma and in addition ion exchanger columns were applied, the immediate statin effect can only be based upon fluvastatin bound still in or on the lipid particles, or upon a persistent conformational change in the apolipoprotein or some transition involving the lipid moiety of the particles initiated through fluvastatin [35]. We are about to unravel these questions, which however, is outside the scope of the present investigation. In agreement with several clinical studies in which, under statins, a decline in atheromatous plaques has been demonstrated [32,36,37], the dissolving effect of fluvastatin on nascent, even calcified, nanoplaques was illustrated at the end of the test period (Figs. 1, 2, 5 and 6). Based on the background of recent clinical studies whereupon the Ca^{2+}-content in the coronary vascular wall as measured by electron beam tomography is predictive of future cardiovascular events [38], this finding of a disintegration of calcified nanoplaques may have high clinical relevance.

Summarizing, it should be stated that the pleiotropic effects of statins described so far, such as plaque stabilization [36], antiinflammatory effects [39], reduction of thrombus formation [40] and removal of endothelial dysfunction [41], are joined by a further pleiotropic effect, that possibly has decisive prophylactic and therapeutic importance for the lowering of cardiovascular risk under fluvastatin with respect to myocardial infarction and stroke, independent of any decrease in LDL or increase in HDL concentrations [42,43]. According to our thesis, fluvastatin could acutely intervene in the deranged control circuit at the blood–endothelium–matrix interface and assist HDL in its vasculoprotective effect from an increased LDL uptake and formation of ternary proteoglycan receptor–lipoprotein–calcium nanoplaques as well as through their redissolving. The long-term effects of fluvastatin raised from the same high risk patient collective, which are accompanied by the usual changes in lipid concentrations and which will be presented in detail in another publication, have to be interpreted in the light of the immediate effects portrayed above.

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