Urokinase-receptor-mediated phenotypic changes in vascular smooth muscle cells require the involvement of membrane rafts

Julia KIYAN*†, Graham SMITH†, Hermann HALLER* and Inna DUMLER*

*Hannover Medical School, Carl-Neuberg Straße 1, D-30625 Hannover, Germany, and †AstraZeneca, Alderley Park, Macclesfield SK10 4TF, U.K.

The cholesterol-enriched membrane microdomains lipid rafts play a key role in cell activation by recruiting and excluding specific signalling components of cell-surface receptors upon receptor engagement. Our previous studies have demonstrated that the GPI (glycosylphosphatidylinositol)-linked uPAR [uPA (urokinase-type plasminogen activator) receptor], which can be found in lipid rafts and in non-raft fractions, can mediate the differentiation of VSMCs (vascular smooth muscle cells) towards a pathophysiological de-differentiated phenotype. However, the mechanism by which uPAR and its ligand uPA regulate VSMC phenotypic changes is not known. In the present study, we provide evidence that the molecular machinery of uPAR-mediated VSMC differentiation employs lipid rafts. We show that the disruption of rafts in VSMCs by membrane cholesterol depletion using MCD (methyl-β-cyclo-dextrin) or filipin leads to the up-regulation of uPAR and cell de-differentiation. uPAR silencing by means of interfering RNA resulted in an increased expression of contractile proteins. Consequently, disruption of lipid rafts impaired the expression of these proteins and transcriptional activity of related genes. We provide evidence that this effect was mediated by uPAR. Similar effects were observed in VSMCs isolated from Cav1−− (caveolin-1-deficient) mice. Despite the level of uPAR being significantly higher after the disruption of the rafts, uPA/uPAR-dependent cell migration was impaired. However, caveolin-1 deficiency impaired only uPAR-dependent cell proliferation, whereas cell migration was strongly up-regulated in these cells. Our results provide evidence that rafts are required in the regulation of uPAR-mediated VSMC phenotypic modulations. These findings suggest further that, in the context of uPA/uPAR-dependent processes, caveola-associated and non-associated rafts represent different signalling membrane domains.

Key words: caveolin, cell differentiation, lipid raft, platelet-derived growth factor-receptor (PDGFR), urokinase-type plasminogen activator receptor (uPAR), vascular smooth muscle cell.

INTRODUCTION

Cholesterol is an essential component of the membranes of mammalian cells and an intricate system has been developed to regulate cellular levels of this lipid. In part, cholesterol reaches cells via the circulation, packaged in LDL (low-density lipoprotein) particles. However, it is synthesized further by most cells using enzymes residing in the endoplasmic reticulum [1]. Cholesterol is a major component of eukaryotic cell membranes and is highly enriched in plasma membranes, but not in other cell organelles. It is believed that, in membranes, cholesterol plays a role in forming specific segregated microdomains, called lipid rafts, thought to regulate membrane protein sorting and construction of signalling complexes [2]. According to lipid raft models of plasma membrane organization, lipids of specific chemistry, namely cholesterol and sphingolipids, spontaneously associate with each other to form rigid platforms for the segregation of proteins such as GPI (glycosylphosphatidylinositol)-anchored proteins. Performing biochemical studies to investigate rafts is technically challenging. The main approaches used are cholesterol depletion using cholesterol-extracting drugs, such as MCD (methyl-β-cyclo-dextrin) [3], or cholesterol binding using filipin [4], and inhibition of intracellular cholesterol biosynthesis, using, for example, inhibitors of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) synthetase, namely statins.

VSMCs {vascular SMCs [SM (smooth muscle) cells]} play an important role in the pathology of vascular remodelling-associated diseases. During cellular remodelling following vascular injury and the development of atherosclerotic plaques, VSMCs change from their physiological contractile phenotype to the pathophysiological synthetic phenotype and migrate into the intima, where they proliferate and produce extracellular matrix [5,6]. uPA (urokinase-type plasminogen activator) and its specific receptor (uPAR) have been implicated in a broad spectrum of pathophysiological processes involved in cardiovascular diseases and vascular remodelling. The fibrinolytic uPA/uPAR system is induced after vascular injury and up-regulated in atherosclerotic lesions, contributing to neo-intima formation and early lesion development [7–9]. Beyond the regulation of cell-surface-associated proteolysis, uPAR orchestrates the signalling pathways underlying the functional changes in vascular cells, such as adhesion, migration and proliferation [10]. uPAR targeting in human organ cultures and in animal models leads to a strong inhibition of negative vascular remodelling [11,12]. As uPAR is linked to the outer membrane leaflet by a GPI anchor and is devoid of any known catalytic activity, all of its diverse biological functions are strictly dependent on its interactions with other proteins. Indeed, a large variety of proteins capable of interacting with uPAR in a cell-specific fashion have been identified over the last two decades. Our previous findings have demonstrated that, in human VSMCs, uPAR is

Abbreviations used: CTB, cholera toxin B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glycosylphosphatidylinositol; MCD, methyl-β-cyclo-dextrin; PDGFRβ, platelet-derived growth factor-receptor β; RT–PCR, reverse transcription–PCR; siRNA, small interfering RNA; SM, smooth muscle; SMA, SM α-actin; SMC, SM cell; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; uPARsi, uPAR siRNA; VSMC, vascular SMC; WT, wild-type.

1 To whom correspondence should be addressed (email kiyan.inouia@mh-hannover.de).
associated with PDGFRβ (platelet-derived growth factor-receptor β) to mediate intracellular signalling and cell functions [13]. uPA binding to uPAR induced rapid PDGFRβ activation, phosphorylation and dimerization. As a GPI-linked protein, uPAR has been found to localize to different membrane domains including lipid rafts [14,15]. Our recent studies have revealed an important role of lipid rafts in uPA/uPAR-directed signalling in human VSMCs [16]. However, whether this might have functional consequences for VSMC phenotypic modulations has remained unexplored.

Modulation of cholesterol content in the cell membrane leads to changes in cell functions and behaviour. In VSMCs, cholesterol might regulate cell phenotypic states. It has been shown previously that loading VSMCs with cholesterol rapidly induces cell transdifferentiation [18]. On the contrary, decreasing the cholesterol content represses VSMC de-differentiation in culture [19]. Together with the observation that increased cholesterol levels represent a risk factor for the development of cardiovascular diseases, these findings suggest that cholesterol is a powerful regulator of VSMC functions. We have demonstrated recently that VSMC inhibition of cholesterol biosynthesis by rosuvastatin leads to inhibition of cell proliferation in both ex vivo and in vitro models, and consequently exerts a positive effect on injury-induced vascular remodelling [20]. We found that this beneficial effect of rosuvastatin was mediated by uPAR-mediated promotion of the differentiated VSMC phenotype. However, the role of rosuvastatin, as well as other statins, in VSMC transdifferentiation has not yet been elucidated. One possibility is that deregulation of uPAR-directed cell signalling via membrane rafts leads to changes in cell behaviour and phenotype. Our present study was designed to verify this hypothesis.

**MATERIALS AND METHODS**

**Reagents and antibodies**

High-quality commercial grade chemicals were purchased from Sigma, Amersham Pharmacia Biotech and Merck. Monoclonal anti-h-caldesmon, anti-calponin and anti-SMA (SM α-actin) antibodies were from Sigma. Monoclonal anti-PDGFRβ and anti-uPAR antibodies were from R&D Systems. Monoclonal anti-caveolin-1 antibodies were from Transduction Laboratories (BD Biosciences). Polyclonal anti-caveolin-1 antibodies were from Cell Signaling. Fluorescent Alexa Fluor® 488- and Alexa Fluor® 594-conjugated secondary antibodies, and raft labelling kits were from Molecular Probes (Invitrogen). uPARsi [uPAR siRNA (small interfering RNA)] duplexes, caveolin-1 siRNA duplexes and control non-specific siRNA duplexes were from Santa Cruz Biotechnology. MCD and filipin were from Sigma. Duplexes and control non-specific siRNA duplexes were from Qiagen. Overexpression or down-regulation of proteins was proved by Western blot analysis 24–72 h after cell nucleofection and was stable for at least 4–5 days.

Chemiluminescent images were captured using VersaDoc-3000 and quantified using Quantity One software (Bio-Rad Laboratories).

**Quantitative RT (reverse transcription)–PCR analysis of uPAR in human VSMCs**

Total RNA was isolated from VSMCs using an RNasy miniprep kit (Qiagen), and real-time quantitative RT–PCR for uPAR mRNA was performed on a TaqMan ABI 7700 sequence detection system (Applied Biosystems). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a reference gene, as described previously [22].

**FACS analysis**

VSMCs treated with MCD or filipin were de-attached using 5 mM EDTA/PBS (pH 7.4) and labelled with anti-uPAR monoclonal antibodies (R&D Systems), according to the manufacturer’s recommendation, and Alexa Fluor® 488-conjugated secondary antibodies (Invitrogen). Analysis was performed using FACS CANTO system (BD Biosciences) using acquisition software FACS DIVA (BD Biosciences). Data were analysed using Summit software (Dako).

**Immunofluorescent confocal microscopy**

For immunocytochemical staining, cells were cultured on glass coverslips. Cells were fixed with 2% paraformaldehyde in PBS for 15 min at 4°C, permeabilized with 0.1% Triton X-100 in PBS for 3 min at 4°C, and blocked overnight at 4°C in 1% (w/v) BSA in PBS. Cells were labelled with primary antibody (2 h at 18–22°C) and fluorescently labelled secondary antibodies (1 h at room temperature). Lipid rafts were labelled using Alexa Fluor® 594-conjugated CTB (cholera toxin B) subunit (raft labelling kit; Molecular Probes), according to manufacturer’s instructions. After staining, cells were embedded in Aqua-Poly-Mount mounting medium (Polysciences). The fluorescence cell images were captured using a Leica TCS-SP2 AOBS confocal microscope. All of the images were taken with oil-immersed ×63 objective (numerical aperture = 1.4). Images were recorded with detection wavelengths range for Alexa Fluor® 488- and Alexa Fluor® 594-double staining of 505 to 550 nm and 605 to 675 nm respectively. All the images were acquired with a resolution of 1024 × 1024 pixels. The fluorograms were obtained by plotting each pixel of the overlay Alexa Fluor® 488/Alexa Fluor® 594 colour fluorescence cell image [13]. Pixel values of Alexa Fluor® 488 and Alexa Fluor® 594 fluorescence images represent horizontal and vertical axis values for each point respectively. The points of the fluorograms are coloured in accordance with pixel counts with specific Alexa Fluor® 488 and Alexa Fluor® 594 fluorescence intensities. The pixel count is shown in logarithmic scale.

**Isolation of lipid raft fractions**

A detergent-free raft isolation method employing centrifugation in OptiPrep gradient [23] was used with some modifications,
using HRP (horseradish peroxidase)-conjugated CTB. marker GM1 as determined using dot-blot analysis of fractions (fraction 7) are shown. Fraction 2 contained most of the raft marker GM1 as determined using dot-blot analysis of fractions using HRP (horseradish peroxidase)-conjugated CTB.

Chemotaxis assay, cell proliferation assay and luciferase assay
The chemotaxis assay was performed using a wound model on Cell Locate grid coverslips (Eppendorf). Briefly, cells were seeded on to grid coverslips reaching approx. 80% confluence after attachment. After cell starvation, wounding was performed under a microscope and the wound position documented. Cells were allowed to migrate in the presence of various stimuli for 12–24 h. Additionally, cell migration was assessed using a Boyden chamber, as described previously [13].

VSMC proliferation was quantified using the colorimetric cell proliferation ELISA kit (Roche Applied Bioscience) in accordance with the manufacturer’s instructions. The mean ± S.D. absorbance at 450 nm was calculated for three independent experiments is indicated.

Luciferase activity was measured using the Promega ONE-Glo™ luciferase assay system, according to manufacturer’s instructions.

Statistical analysis
All the experiments were performed in triplicate. Values are means ± S.E.M. Statistical significance analysis (P < 0.05) was performed using a Student’s t test.

RESULTS

**uPA induces raft enrichment in uPAR and PDGFRβ**

In human VSMCs, PDGFRβ serves as a transmembrane interactor of uPAR, mediating its cellular and functional effects [13]. The observation that both uPAR and PDGFRβ could be found in lipid rafts [14,24] persuaded us to test whether this localization is uPA-dependent. We applied an immunocytochemical approach and used Alexa Fluor® 594-conjugated CTB to indicate lipid rafts. As shown in Figures 1(A) and 1(B), co-localization of uPAR and PDGFRβ with fluorescently labelled CTB was up-regulated in response to uPA, thus pointing to the role of rafts in uPAR/PDGFRβ-dependent cellular responses.

These visual observations were confirmed by their quantification in fluorograms. Distribution of the points in the fluorograms confirmed increased co-localization of both receptors with lipid rafts in VSMCs stimulated with uPA for 20 min. These results are in agreement with our earlier observation showing increased association and co-localization of uPAR and PDGFRβ in response to uPA [13]. Furthermore, co-localization of uPAR with caveolin-1 was also increased after uPA stimulation of VSMCs (Figure 1C).

**Rafts regulate uPAR-mediated VSMC phenotypic changes**

Recently, we have demonstrated that uPAR contributes to vascular remodelling by promoting VSMC phenotypic modulations [20].

To elucidate the role of rafts in these processes, we first examined whether raft disruption might affect the abundance of uPAR, as this is critical for the propagation of trans-differentiation. We targeted membrane rafts by cholesterol depletion using the extracting drug MCD and filipin. As shown in Figure 2(A), both MCD and filipin treatment led to uPAR up-regulation. Immunocytochemical staining of the cells under non-permeabilizing conditions showed that uPAR was accumulated on the cell membrane (Figure 2B). In order to clarify whether uPAR up-regulation was transcriptional, we performed TaqMan analysis. Figure 2(C) shows that both MCD and filipin induced significant up-regulation of uPAR mRNA. Further FACS analysis of VSMCs treated with both substances confirmed that MCD treatment did indeed increase uPAR on the cell surface (Figure 2D). Filipin treatment, although increasing the total amount of uPAR (Figure 2A) and uPAR transcription (Figure 2C), led to only a small increase in uPAR on the cell surface (Figure 2D).

To investigate the consequences of this effect on uPAR-mediated VSMC phenotypic modulation, we examined the expression of SM marker proteins after uPAR silencing and raft disruption. Cells were nucleoected with uPARsi duplexes or control duplexes, and then the expression of SMA, calponin and SM high-molecular-mass caldesmon was analysed by Western blotting. Down-regulation of uPAR promoted the expression of contractile markers (Figure 3A). Conversely, cell treatment with MCD or filipin decreased the expression of contractile marker proteins (Figures 3B and 3C). We found that the down-regulation of uPAR using siRNA duplexes prevented the MCD-dependent decrease in contractile marker expression (Figure 3D), confirming further the role of uPAR in this process. To elucidate the functional consequences of this effect, we examined the possibility of a direct transcriptional activation of SM-specific genes in these cells. For this purpose, control and uPARsi VSMCs were transfected with luciferase reporter constructs driven by an SMC-specific promoter from the SMA gene. As shown in Figure 3(E), SMA promoter was activated in uPARsi VSMCs and the inhibitory effect of MCD was abolished in these but not in control cells.

Together, these findings indicate that the level of uPAR is an important regulator of VSMC phenotype. Thus a decrease in uPAR expression leads to cell differentiation, whereas accumulation of uPAR by cell treatment with raft-disrupting drugs leads to VSMC de-differentiation.

**uPAR-related cellular effects are differently regulated by caveolae-associated and non-associated membrane rafts**

Caveolae, a major specialized subclass of lipid rafts, are membrane invaginations enriched in particular lipids (e.g. cholesterol and glycosphingolipids) and are characterized by an abundance of palmitoylated scaffolding proteins (e.g. caveolins) that cause rafts to polymerize and interact with a wide variety of proteins. The number and distribution of caveolae are different in synthetic and contractile VSMC phenotypes [19]. By regulating cholesterol trafficking and multiple receptor-mediated signalling pathways, caveolae influence vascular function and cardiovascular disease development [25].

We observed uPA-dependent redistribution of caveolin-1, together with uPAR and PDGFRβ, to raft fractions in response to uPA (Figures 4A–4D). Therefore we investigated whether uPA stimulation might also regulate the uPAR/caveolin-1 association. As shown in Figure 4(E), these proteins had an increased association in response to uPA in co-immunoprecipitation experiments.

© The Authors Journal compilation © 2009 Biochemical Society
Caveolae-associated and non-associated rafts have been shown to differ, at least partially, in specific protein clustering and, consequently, to mediate different cellular responses [26]. To distinguish between the roles of caveolar and non-caveolar rafts in uPAR-dependent VSMC cellular functions, we investigated VSMCs after down-regulation of caveolin-1 using siRNA. We found that expression of SM markers was activated in these cells; consequently uPAR was down-regulated (Figure 4F).

Finally, we analysed the uPA-dependent functional responses of Cav1−/− murine VSMCs generated previously [27] and human
as membrane rafts, have become a central facet of signalling research, as the function of many receptors and their downstream effectors is dependent on rafts [2,28]. Numerous proteins may cluster in membrane rafts, including GPI-anchored proteins, G-protein-coupled receptors, receptor tyrosine kinases and phosphatases, thereby accounting for the potentially important role of rafts in cell signalling [29,30]. Lipid rafts may or may not be enriched in caveolin-1, the major structural protein found in the anatomic membrane structures referred to as caveolae. Raft-associated caveolin-1 may function in maintaining high cholesterol levels. Previous studies have implicated lipid rafts in various disease processes including vascular disease [31,32]. These studies have demonstrated that alteration in lipid raft expression and occupancy is decisive for cell–cell contact and cell signalling, and that these are abnormal in patients with vascular disease [33–35]. Moreover, translocation of some receptors to lipid rafts and associated signalling can differ between prognostically important groups of patients and serve as a prognostic marker [36].

uPAR, a multi-functional cell-surface receptor, is an important participant in the molecular machinery underlying vascular remodelling and cardiovascular diseases [9,37–39]. Our recent findings revealed a novel aspect of uPAR in these processes, which is linked to the regulation of VSMC trans-differentiation [20]. Although uPAR, as a GPI-anchored protein, has been identified previously in lipid rafts [14], little is known regarding the dependence on rafts for eliciting downstream signalling of uPAR and consequent biological outcomes in VSMCs.

In the present study, we have identified a novel role for membrane lipid rafts in uPA/uPAR-mediated promotion of VSMC functional responses, in particular in directing VSMC phenotypic modulations. The evidence we present in support of a role for lipid rafts in VSMC phenotypic changes is as follows: (i) raft disruption by depletion of cholesterol in the plasma membrane resulted in uPAR up-regulation; (ii) raft disruption led to decreased expression of contractile proteins and transcriptional activity of SM genes, an effect abolished in uPAR si VSMCs; (iii) uPAR and its transmembrane adaptor PDGFRβ were redistributed to lipid rafts in response to uPA; and (iv) raft disruption influenced some uPA-initiated cellular responses. Our findings provide the first evidence that VSMCs employ a cholesterol-rich membrane raft compartment for transmission of the uPAR-mediated differentiation signal. Since uPAR has been identified in rafts and non-raft fractions [14], our present findings suggest further that raft-associated uPAR represents a distinct and separately functioning pool of receptors. Our present study demonstrates that three-dimensional cell morphology and uPAR-directed signal transduction become integrated to regulate cell phenotype.

Different classes of rafts exist in mammalian cells. Some lipid rafts lack structural protein components, but when enriched with a specific protein raft morphology function can change. The first structural protein component to be identified was caveolin-1 [40]. Although incompletely understood, caveolin-1 appears to function as a scaffolding molecule that regulates the activities of signalling molecules clustered in caveolar domains. However, a growing body of evidence points to a clear distinction between signalling mechanisms and related functional responses in lipid rafts compared with caveolae [26]. Results of our experiments on VSMCs isolated from Cav1−/− mice support this concept. Thus the functional responses were different in VSMCs with disrupted rafts and in Cav1−/− VSMCs. We favour the hypothesis that certain components of the uPAR interactome may be regulated by caveolin-1 in the context of plasma-membrane-attached caveolae or perhaps by the recruitment of other regulatory molecules in the caveolae, but not to a raft. We can conclude that, at least in terms of

**DISCUSSION**

Signalling cascades activated by cell-surface receptors are tremendously complicated processes involving both acute- and longer-term signalling events. Although early studies did not generally address the membrane environment of receptors, a body of work has now demonstrated that heterogeneity exists in the composition of the plasma membrane and that distinct lipid environments play a role in receptor function. Cholesterol-rich microdomains, often referred to as lipid rafts and more recently

**Figure 2  Raft disruption increases uPAR expression in VSMCs**

(A) Expression of uPAR in VSMCs treated with 0.1% MCD or 5 μM filipin for 24 h was assessed by Western blotting. The positions of molecular-mass markers (in kDa) are indicated on the right. Lower panels, Western blotting with tubulin as a loading control. (B) VSMCs treated with 0.1% MCD (right-hand panel) were stained under non-permeabilizing conditions with anti-uPAR primary antibodies, then fixed, blocked overnight and stained with Alexa Fluor® 488-conjugated secondary antibodies. (C) Effect of VSMC treatment with 0.1% MCD or 5 μM filipin on uPAR expression as determined by TaqMan analysis. (D) Amount of surface uPAR after VSMC treatment with 0.1% MCD or 5 μM filipin assessed using FACS analysis, as described in the Material and methods section. N. control, negative (isotype IgG) control.

VSMCs treated with raft-disrupting drugs. As shown in Figure 5, caveolin-1 deficiency elicited effects on cell behaviour that were different from those of raft disruption. Thus raft targeting by rosuvastatin resulted in the down-regulation of uPA-induced cell migration, whereas cell proliferation was not significantly affected (Figure 5A). By contrast, only uPA-initiated cell proliferation was impaired in Cav1−/− VSMCs, whereas the migratory response of these cells was strongly up-regulated by uPA (Figure 5B). We can conclude, therefore, that, at least in the context of uPA-dependent processes, rafts and caveolae represent different signalling membrane domains.

VSMCs with disrupted rafts. As shown in Figure 5, caveolin-1 deficiency elicited effects on cell behaviour that were different from those of raft disruption. Thus raft targeting by rosuvastatin resulted in the down-regulation of uPA-induced cell migration, whereas cell proliferation was not significantly affected (Figure 5A). By contrast, only uPA-initiated cell proliferation was impaired in Cav1−/− VSMCs, whereas the migratory response of these cells was strongly up-regulated by uPA (Figure 5B). We can conclude, therefore, that, at least in the context of uPA-dependent processes, rafts and caveolae represent different signalling membrane domains.

VSMCs with disrupted rafts. As shown in Figure 5, caveolin-1 deficiency elicited effects on cell behaviour that were different from those of raft disruption. Thus raft targeting by rosuvastatin resulted in the down-regulation of uPA-induced cell migration, whereas cell proliferation was not significantly affected (Figure 5A). By contrast, only uPA-initiated cell proliferation was impaired in Cav1−/− VSMCs, whereas the migratory response of these cells was strongly up-regulated by uPA (Figure 5B). We can conclude, therefore, that, at least in the context of uPA-dependent processes, rafts and caveolae represent different signalling membrane domains.

VSMCs with disrupted rafts. As shown in Figure 5, caveolin-1 deficiency elicited effects on cell behaviour that were different from those of raft disruption. Thus raft targeting by rosuvastatin resulted in the down-regulation of uPA-induced cell migration, whereas cell proliferation was not significantly affected (Figure 5A). By contrast, only uPA-initiated cell proliferation was impaired in Cav1−/− VSMCs, whereas the migratory response of these cells was strongly up-regulated by uPA (Figure 5B). We can conclude, therefore, that, at least in the context of uPA-dependent processes, rafts and caveolae represent different signalling membrane domains.
uPAR-dependent processes, rafts and caveolae represent different signalling membrane domains.

One intriguing observation of our present study was the strong dependence of uPAR levels in VSMCs on the integrity of rafts. Raft disruption resulted in a strong uPAR up-regulation. This, in turn, initiated VSMC phenotypic transition to the de-differentiated pathophysiological phenotype. uPA stimulation induced translocation of uPAR and its transmembrane adaptor PDGFRβ to lipid rafts. We have reported recently that association between components of the uPAR interactome was increased after raft disruption, as well as basal phosphorylation levels of ERK (extracellular-signal-regulated kinase), SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase-2) and RhoA activity [16]. However, uPA-initiated functional responses, such as cell migration and proliferation, were abrogated in these cells. This raises the idea that the control of uPAR-related events in VSMCs provided by rafts may be of at least two different functional compartments. The first one is the strict regulation of uPAR cellular levels that determines cell phenotype. The second implies that rafts in VSMCs may function as the platform for a network of signalling molecules that start to assemble in response to external stimuli such as uPA. These molecules may work in concert with downstream targets known to regulate cytoskeletal organization, such as PI3K (phosphoinositide 3-kinase), Rho, Rac and Cdc42 [13,41]. Additionally, lipid rafts may also help to preserve the membrane uPAR capable of uPA binding and signalling, and have an impact on regulators for efficient signal transduction. What the molecular mechanism is underlying uPAR-directed VSMC phenotypic transition is an intriguing question and the answer is largely obscure and requires specific study. Our preliminary results suggest the involvement of myocardin (J. Kiyan and I. Dumler, unpublished work), a serum-response factor cofactor that is expressed specifically in SMC and cardiac muscle cell lineages and which plays an important role in VSMC differentiation in vivo.

Finally, our results suggest that cellular cholesterol levels and, in particular membrane lipids, regulate uPAR-directed VSMC phenotypic changes. They suggest further that uPAR signalling and cellular functions can be modified in VSMCs by statin treatment; in particular to shift cells to a physiologically differentiated phenotype. It is tempting to speculate that some of the pleiotropic effects of statins on the cardiovascular system could result from
Figure 4  uPA induces the redistribution of uPAR-associated signalling molecules to the lipid raft membrane fraction

(A) Typical GM1 distributions in fractions collected from the OptiPrep gradient.  (B) uPA-induced re-distribution of uPAR (B), PDGFRβ (C) and caveolin-1 (D) in the OptiPrep gradient fractions. Lower panels, quantification of proteins in the fractions. (E) Lysates of uPA-stimulated VSMCs were used to immunoprecipitate (IP) uPAR. The presence of caveolin-1 in the immunoprecipitates was assessed by immunoblotting (WB). Lower panel, immunoblotting with anti-uPAR antibodies to demonstrate the equal amount of immunoprecipitated uPAR. (F) VSMCs were nucleofected with control or caveolin-1 siRNA (Cav si) duplexes. At 48 h after nucleofection, silencing was verified. Expression of uPAR and contractile proteins was assessed by Western blotting. a.u., arbitrary units; Co, control.
changes in membrane lipids, at least in the context of uPAR-related vascular remodelling. These findings may have applications in the understanding and potential treatment of these diseases.

**AUTHOR CONTRIBUTION**

Julia Kiyan performed the experiments and prepared the manuscript. Graham Smith designed and optimized the experiments on the cell treatment with rosuvastatin, Hermann Haller approved the paper, and Inna Dumler, the scientific supervisor, prepared and edited the manuscript prior to submission.

**ACKNOWLEDGEMENTS**

We are grateful to Iris Kilian and Petra Wubbolt for excellent technical assistance, Professor Gary Owens for giving us the construct expressing luciferase under the SMA promoter, and Maren White for editing the manuscript prior to submission.

**FUNDING**

This work was supported by the Deutsche Forschungsgemeinschaft [grant numbers DU 344/1-4, DU 344/6-1] and an ERA-AGE FLARE grant financed by Bundesministerium für Bildung und Forschung [grant number 01 ET 0802].

**REFERENCES**

17 Reference deleted
Membrane rafts and uPAR in VSMC phenotypic modulations


Received 18 March 2009/16 July 2009; accepted 20 August 2009
Published as BJ Immediate Publication 20 August 2009, doi:10.1042/BJ20090447