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Lipid-modified proteins as biomarkers for cardiovascular disease: a review

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Abstract
Lipid-modified proteins are classified based on the identity of the attached lipid, a post- or co-translational modification required for their biological function. At least five different lipid modifications of cysteines, glycines and other residues on the COOH- and NH_2-terminal domains have been described. Cysteine residues may be modified by the addition of a 16-carbon saturated fatty acyl group by a labile thioester bond (palmitoylation) or by prenylation processes that catalyze the formation of thioether bond with mevalonate derived isoprenoids, farnesol and geranylgeraniol. The NH_2-terminal glycine residues may undergo a quite distinct process involving the formation of an amide bond with a 14-carbon saturated acyl group (myristoylation), while glycine residues in the COOH-terminal may be covalently attached with a cholesterol moiety by an ester bond. Finally, cell surface proteins can be anchored to the membrane through the addition of glycosylphosphatidylinositol moiety. Several lines of evidence suggest that lipid-modified proteins are directly involved in different steps of the development of lesions of atherosclerosis, from leukocyte recruitment to plaque rupture, and their expression or lipid modification are likely altered during atherogenesis. This review will briefly summarize the different enzymatic pathways of lipid modification and propose a series of lipid-modified proteins that can be used as biomarkers for cardiovascular disease.

Keywords: *Prenylated proteins, farnesol, geranylgeraniol, statin, atherosclerosis, mevalonate pathway*

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Introduction

The classic view of membrane-associated proteins is one in which proteins are inserted into the membrane bilayer so that they span the membrane. Such proteins are synthesized with a sorting signal at their NH_2-terminus, which results in their trafficking through the endoplasmic reticulum–Golgi apparatus pathway on their way to the cell surface. Additional pathways also exist for directing proteins to cell membranes that involve co- or post-translational modification by specific lipids. These modifications are required for a correct localization of proteins involved in cell-signalling events and for other biological activities including platelet aggregation, blood coagulation and extracellular matrix (ECM) remodelling. In eukaryotic systems, five major forms of lipid modification have been described: (1) the addition of palmitic acid to intracellular proteins; (2) the co-translational NH_2-terminal myristoylation

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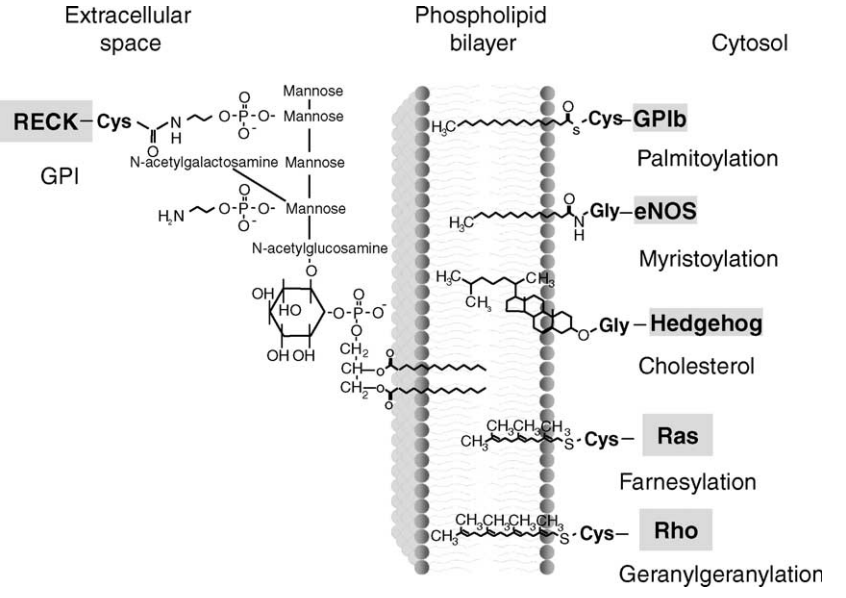

Figure 1. Class of lipid modified proteins. Schematic representation of five types of lipid anchors by which cellular proteins are attached to the plasma membrane. GPI-anchored proteins, which include RECK, are only found on the exoplasmic face, while myristate- (eNOS), palmitoylated- (GPIb), prenylated- (Ras and Rho) and cholesterol- (Hedgehog) modified proteins are in the cytosolic face.

of cytosolic proteins; (3) the modification of plasma membrane proteins with glycophosphatidyl inositol (GPI) moiety; (4) the addition of a cholesteryl adduct of Hedgehog and other intracellular proteins; and (5) the COOH-terminal isoprenylation of cytoplasmic proteins (Figure 1). Access to a comprehensive range of programs and databases of lipidated proteins is available in the ExPASy and Swiss-Prot web sites (http://www.expasy.org and www.expasy.org/sprot).

Palmitoylation

Palmitoylated proteins contain a 16-carbon saturated fatty acyl group attached by a labile thioester bond to cysteine residues. Although the vast majority of the cellular proteins palmitic acid is esterified to the free thiol of cysteine, the addition of other saturated (myristic and stearic) and unsaturated (oleic and arachidonic) fatty acids has also been observed (Hallak et al. 1994). Hence, S-acylation is the most appropriate term for this kind of protein modification (Bizzozero et al. 1994, Casey 1995).

Palmitoylated proteins synthesized on soluble ribosomes either undergo sequential modification with different lipids or are exclusively S-palmitoylated (Table I). The mechanism of palmitoylation is still poorly understood since attempts to isolate palmitoyl acyltarnsferases (PATs) have not been successful because of the extreme instability of PAT activity. However, what is now clear is that enzyme-mediated protein palmitoylation takes place post-translationally (Bonatti et al. 1989, Resh 1999).

A significant breakthrough has been recently been achieved with the identification of two enzyme activities in yeast that mediate COOH-terminal palmitoylation: the Erp2p-Erp4p complex and Akr1p (Roth et al. 2002). Both enzymes have been shown to mediate Ras2p palmitoylation in yeast, but a mammalian homologue has not been identified as yet.

Table I. Palmitoylated proteins.

Dually lipidated proteins	*Exclusively palmitoylated*
(A) Prenylation and S-palmitoylation:	(A) NH_2-terminal motifs:
Ras GTPases	GAP-43
H-Ras	PSD-95
N-Ras	SCG10
R-Ras	GAD65
Rho GTPases	$G_{q\alpha}$
Rho B	RGS4
	RGS16
(B) N-myristoylation and S-palmitoylation:	
	(B) COOH-terminal motif:
$p59^{fyn}$	
$p56^{lck}$	Yck2 (*S. cerevisiae*)
$G_{i\alpha 1}$	
eNOS	(C) Cysteine string motifs:
(C) N-palmitoylation and S-palmitoylation:	SNAP-25b
	Cysteine string protein 1
$G_{s\alpha}$	GAIP (RGS19)

Modified from Smotrys & Linder (2004).

PAT activity has been found in fractions containing plasma membrane, Golgi and mitochondrial membranes (Dunphy et al. 1996), and particularly enriched in sphingomyelin- and cholesterol-rich membrane microdomains: the so-called 'lipid rafts' (Bonatti et al. 1989). For these reasons, soluble proteins must interact with plasma membrane, at least transiently, in order to be palmitoylated. An excellent model has been advanced for the ability of palmitoylated proteins to be targeted in the plasma membrane. Proteins with a single lipophilic group (myristate or farnesyl) transiently interact with multiple intracellular membranes and after they reach the plasma membrane will be rapidly palmitoylated by a PAT and remained stably attached to the plasma membrane (Shahinian & Silvius 1995).

The functional significance of the palmitate moiety in fatty acylated proteins has primarily been inferred by mutating the modified cysteine residue(s) to serine or alanine and observing the behaviour of the mutant protein (Mumby 1997, Dunphy & Linder 1998). These studies have demonstrated that palmitoylation directly regulates trafficking of lipidated proteins from the early secretory pathway to the plasma membrane. In addition, modification with fatty acids impacts the lateral distribution of proteins on the plasma membrane by targeting them to 'lipid rafts' (Smotrys & Linder 2004). However, the function of palmitoylation goes beyond that of a simple membrane anchor. *In vitro* assays have in fact revealed the importance of palmitoylation in promoting or inhibiting protein–protein interactions and protein activities (Smotrys & Linder 2004).

Myristoylation

Myristoylated proteins contain a saturated acyl group of 14 carbons added by a quite distinct process involving co-translational modification of the NH_2-terminal glycine residue through amide bond formation (Casey 1995). In contrast to the palmitoylation

process, the enzyme responsible for myristoylation, N-myristoyltransferase, has been identified and is the subject of extensive investigations with regard to substrate specificities and properties (Johnson et al. 1994). To date, nearly a dozen N-myristoyltransferases from fungal and mammalian sources have been identified, and the consensus sequence for protein substrate has been characterized (Met-Gly-X-X-X-Ser/Thr). The initiating Met is removed by methionine amino peptidase during translation and Gly-2 becomes the NH_2-terminal amino acid. The requirement for Gly at the NH_2-terminus is absolute; no other amino acid will substitute. However, not all proteins with an N-terminal glycine are N-myristoylated and the ability to be recognized by N-myristoyltransferase depends on the downstream amino acid sequence (Resh 1999). A list of known N-myristoylated proteins is shown in Table II.

Myristoylation is generally regarded as a constitutive process resulting in a stably modified protein and usually the half-life of myristate on a protein is equivalent to the half-life of the polypeptide chain backbone (Wolven et al. 1997). The attachment of myristic moiety results in an increase of hydrophobicity that triggers membrane and protein association and, abrogation of myristoylation by mutation of Gly2 to Ala, generally results in reduction or loss of membrane binding (Cross et al. 1984, Kamps et al. 1985), and lower affinity of protein–protein interactions (Matsubara et al. 2003). Myristoylation can also serve as a structural moiety in the proteins (Zheng et al. 1993, Ames et al. 1994). For instance, in the catalytic subunit of protein kinase A, myristate is positioned within a hydrophobic pocket and is required for structural

Table II. Myristoylated proteins.

Protein kinases and phosphatases	*Ca^{2+} binding/EF hand proteins*
(A) Src family tyrosine kinases: Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Yrk, Blk	Recoverin, Neurocalcin, Aplycalcin, Visin-like protein 3, P22, NAP-22, Hippocalcin, GCAP1, GCAP2, S-modulin, Rem-1, NCS-1
(B) Abl tyrosine kinases: c-Abl, Arg	*Membrane- and cytoskeletal-bond structural proteins*
(C) Serine/threonine and anchoring proteins:	MARCKS, MAC-MARCKS, annexin XIII, Rapsyn, Pallidin, Hisactophilin 2
CAMP-dependent protein kinase, catalytic subunit alpha, beta1, VSP15, MPSK, AKAP18	*Miscellaneous* NADH cytochrome b5 reductase
(D) Phosphatases:	NO synthase Enteropeptidase
Calcineurin B	NADH-ubiquinone Oxidoreductase
Guanine nucleotide binding proteins:	BASP1 FRS2
(A) Gα proteins:	CPLA2-γ MARCKS
Gαi1, Gαo, Gαt, Gαx	
(B) ADP-ribosylation factors: Arf-1, Arf-3, Arf-5, Arf-6	

Modified from Resh (1999).

and thermo-stability of the enzyme (Zheng et al. 1993). Finally, N-myristoylation regulates Ca^{2+} binding with neuronal calcium sensor-1 (NCS-1) protein (Jeromin et al. 2004).

Myristic acid represents less than 1% of all fatty acids in cells, but its specific length provides the possibility for reversible interactions with other proteins or membranes in contrast to highly stable associations facilitated by other, more hydrophobic lipid modifications. Changes of the relative orientation of the myristoyl moiety and the protein to which is attached, a phenomenon known as the 'myristoyl switch', allows myristoylated proteins to reverse membrane binding (Resh 1999). Myristoylation is therefore required, but not necessarily sufficient, for membrane anchoring. In fact, a second signal within the N-myristoylated proteins is necessary for efficient protein localization into membrane bilayers (Resh 1994). This 'second signal' has been defined as either a polybasic cluster of amino acids or a palmitate moiety (Buser et al. 1994, Sigal et al. 1994, Zhou et al. 1994, Murray et al. 1998). It should also be noted that a similar 'two signal' model applies to membrane binding of farnesylated proteins, e.g. Ras (Hancock et al. 1990, Resh 1994).

Cholesterol modification

The Hedgehog family of molecules consists of secreted proteins that undergo several post-translational modifications to gain full activity. In a maturation process, they perform an autocatalytic cleavage, generating an NH_2-terminal polypeptide containing all the signalling functions (Porter et al. 1995, Roelink et al. 1995, Briscoe et al. 2000, Mullor et al. 2002). During this cleavage process, a cholesterol moiety is attached covalently by an ester function to the COOH-terminal glycine of the signalling domain (Porter et al. 1996). The hydrophobicity of the protein is further increased by the addition of a palmitic acid residue to the NH_2-terminus of the cleavage product.

Cholesterol alone anchors proteins to membranes with significant strength (Peters et al. 2004). Its membrane anchoring ability is comparable with dual lipidation motifs such as double geranylgeranylation or S-palmitoylation plus S-farnesylation found in other lipidated proteins (Peters et al. 2004). Importantly, many other cholesterol-modified proteins have been detected in mammalian cells that still need to be identified (Porter et al. 1996). This observation suggests that cholesterol modification of polypeptides, perhaps by esterification, may also be employed as a means of directing proteins, other than Hedgehog, to membranes or other hydrophobic targets.

GPI modification

GPI-anchored proteins constitute an exceptionally diverse family of membrane molecules with the only common feature in their attachment to the exoplasmic leaflet of the membrane (Figure 1 and Table III) (Englund 1993). As the name indicates, these proteins contain an entire phospholipid with associated sugars and ethanolamine. On average, about 0.5% of cellular proteins in eukariotes are GPI anchored (Eisenhaber et al. 2001). GPI anchors are diverse structures, but have a common core structure that is conserved among all species that have so far been investigated (Ikezawa 2002). Proteins are initially directed into the secretory pathway by means of an NH_2-terminal signal sequence, where they acquire the GPI moiety. The GPI

Table III. GPI-anchored proteins.

Enzymes	*Surface antigens*
Alkaline phosphatase	Thy-1
Acethylcholinesterase	Ly-6 (TAP)
5′ nucleotidase	Qa-2
MT4-MMP	Sca-2
MT6-MMP	CD24,
Lipoprotein lipase	CD48 (sgp-60)
	CD52 (CAMPATH-1)
Adhesion molecule	CD5 (DAF)
	CD59
LFA3	CD73
NCAM	Cerebroglycan
Lfa-3	Ceruloplasmin
ApCAM	Prion proteins
OBCAM	
	Miscellaneous
Receptors	
	RECK
Folate receptor	Thy-1
CNTFR-α	Qa-2
GDNFR-α	Ly-6a
uPAR	
CD14	
FcγRIIIb	

Modified from Chatterjee & Mayor (2001).

moiety is apparently pre-assembled on the cytoplasmic face of the endoplasmic reticulum and then transferred into the lumen, where it is attached to a COOH-terminal residue of the protein (Vidugiriene & Menon 1994, Mayor & Riezman 2004). The entire procedure, which involves proteolytic processing of the host protein to expose the GPI addition site, has been fully characterized and the gene products involved have been identified (Miyata et al. 1993). The striking conservation of the core structure of the GPI anchor from yeast to mammals suggests that this modification is of prime functional significance (Mayor & Riezman 2004).

The GPI anchor undoubtedly provides a more stable anchorage to proteins than other lipid-based anchors such as myristoyl, palmitoyl and prenyl groups, which allow only weak transient membrane anchorage (Mayor & Riezman 2004).

In mammalian cells, the GPI moiety, besides acting as a membrane anchor, has been shown to transduce signals across the bilayer resulting in several intracellular responses: oxidative burst, Ca^{2+} influx, protein tyrosine phosphorylation, cell proliferation or inhibition of cell growth (Robinson 1991, 1997). GPI-anchored proteins can also be released from the plasma membrane by the action of endogenous hydrolases including PI-phospholipase C (PI-PLC), PI-specific phospholipase D (PI-PLD) and GPI-specific PLC (Mayor & Riezman 2004).

Prenylation

Prenylated proteins contain one of two isoprenoid lipids, either 15-carbon farnesyl or the 20-carbon geranylgeranyl. The lipids are attached to cysteine residues at or near

Table IV. Prenylated proteins.

Molecular weight (kDa)	Protein
Farnesylated	
66–72	Nuclear lamin family
53–55	Unidentified proteins
41–46	Inositol triphosphate 5-phosphatase, 2′,3′-cyclic nucleotide 3′-phosphatase, prostacyclin receptor
37	Peroxisomal protein
21–28	*Ras*. Involved in cell proliferation and differentiation
Geranylgeranylated	
21–28	*Rho*/*Rac*/*Cdc42*. Involved in cytoskeletal assembly, superoxide generation, cell cycle progression
5–8	*Rab*. Involved in transport of vesicles
	Rap. Involved in cellular replication, platelet activation generation of oxygen radicals
	G proteins (γ subunit). Involved in signal transduction

Modified from Bellosta et al. (2000).

their COOH-terminus through stable thioether bonds in a post-traslational process (Schafer & Kattermann 1992). As with other lipid-modified proteins, this constitutive process results in a stably modified protein. Proteins containing a cystein residue fourth from the COOH-terminus (the so-called CAAX motif) can be modified by either farnesyl or geranylgeranyl moiety, depending on the identity of the COOH-terminal residue (the X) (Table IV) (Park et al. 1997, Long et al. 1998, Strickland et al. 1998, Sinensky 2000, Reid et al. 2004). The CAAX motifs are recognized by two closely related cytoplasmic enzymes, one specific for farnesylation (farnesyltransferase, FTase), the other for geranylgeranylation (geranylgeranyltransferase I, GGTaseI) (Moores et al. 1991, Reiss et al. 1991, Yokoyama et al. 1991, Roskoski & Ritchie 1998, Reid et al. 2004).

A second mechanism for prenylation exists for guanosine triphosphate (GTP)-binding proteins of the Rab family, which are involved in membrane trafficking in cells (Seabra et al. 1992). These proteins are geranylgeranylated at two cystein residues at or very near the COOH-terminus by a distinct enzyme, GGTase II or Rab GGTase (Farnsworth et al. 1994). Prenylated proteins undergo final post-translational modification through the removal of the last three amino acids from the protein (i.e. the -*AAX*), mediated by a prenylprotein-specific endoprotease, Ras-converting enzyme 1 (Rce1) and methylation of the newly exposed C-terminal isoprenylcysteine by isoprenylcysteine carboxyl methyltransferase (Icmt) (Dai et al. 1998, Sinensky 2000). Prenylation of CAAX proteins is vitally important to eukaryotic cells. In fact, between 0.5 and 1% of total cellular proteins undergo the geranylgeranylation process, which is modification absolutely required for their proper targeting to membrane surface and function (Casey & Seabra 1996).

Role of lipid modified proteins in cardiovascular disease

A plethora of lipid-modified proteins have been identified as pivotal players of different steps of development of lesion of atherosclerosis (Table V). Vascular cell adhesion molecule-1 (VCAM-1) binds monocyte and T-lymphocyte mediating their recruitment in nascent atheroma. VCAM-1 is usually expressed as a transmembrane

Table V. Lipid-modified proteins and their role in atherosclerosis.

Protein	Modification	Function	Source	References
TNF-α	palmitoylation, myristoylation	inflammatory cytokine	T-lymphocyte	Stevenson et al. (1992), Utsumi et al. (2001)
Splice variant of VCAM-1	GPI	adhesion molecule	plasma	Terry et al. (1993)
Ras	farnesylated, palmitoylated	cell proliferation	platelets	Chiang (1997)
Rho	geranylgeranylated	cell migration, platelet aggregation	platelets	Walther et al. (2003)
MT4-MMP	GPI	extracellular matrix remodelling	eosinophils	Gauthier et al. (2003)
RECK	GPI	extracellular matrix remodelling	macrophages	Van Lent et al. (2005)
GPIb	palmitoylation	platelet adhesion	platelets	Schick & Walker (1996)
GPIX	myristoylation	platelet adhesion	platelets	Schick & Walker (1996)
TF	palmitoylation	coagulation	monocyte	Drake et al. (1993)
eNOS	palmitoylation, myristoylation	vascular homeostasis, platelet aggregation	platelets	Sase & Michel (1995)
PGI2 receptor	prenylated, palmitoylated	anticoagulant	lymphocytes platelets	Oida et al. (1995)
Ecto-5′-nucleotidase	GPI	platelet aggregation	lymphocytes	Resta et al. (1993)
HDJ-2	farnesylated	heat shock protein	peripheral blood mononuclear cell	Lobell et al. (2002)
Rap	geranylgeranylated	platelet aggregation, cell proliferation	platelets	Peterson & Lapetina (1994)

molecule on the cell surface, but a splice variant containing a GPI membrane anchor has been also documented. Although this splice variant of VCAM-1 has not been fully characterized, it is likely that it may contribute to the early inflammatory response observed in atherogenesis (Terry et al. 1993).

Release of inflammatory cytokines, from recruited macrophages and T-lymphocytes, results in smooth muscle cell (SMC) migration and proliferation with a final accumulation of SMC within intimal lesions (Ross 1999). Tumour necrosis factor-α (TNF-α) is a pleiotropic pro-inflammatory cytokine synthesized as a 26-kDa precursor localized as a transmembrane molecule, which is cleaved by membrane-bound metalloproteinases, mainly the TNF-α converting enzyme (TACE) to yield the soluble form (Black et al. 1997, Moss et al. 1997). Transmembrane TNF-α is myristoylated in human monocytes on Lys-58 and Lys-57, located within the leader sequence of TNF-α (Stevenson et al. 1992). The functional significance of these modifications has been proposed to regulate TNF-α shedding and therefore its pro-inflammatory activity in atherogenesis (Utsumi et al. 2001, Canault et al. 2004).

Prenylated proteins have been implicated in several intracellular signalling transduction pathways that directly impact the uncontrolled smooth muscle proliferative and migratory activities (Corsini et al. 1993, Irani et al. 1994, Indolfi et al. 1995, Olson et al. 1995, Chemla et al. 1997, Cohen et al. 1999, Stark et al. 1998). Indeed, small G proteins of the Ras and Rho families are key intracellular transducers of a number of growth-signalling events induced by tyrosine kinase receptors, non-

receptor tyrosine kinases and G protein coupled seven-membrane-spanning receptors (Casey 1995, Olson et al. 1995, Coleman et al. 2004). Pharmacological modulation of protein prenylation by perillic acid and ajoene has been shown to inhibit SMC proliferation significantly, demonstrating that both geranylgeranylated and farnesylated proteins directly control cellular division (Ferri et al. 2001, 2003). In particular the Rho proteins have been identified as pivotal regulators of actin organization and the formation of lamellipodia and filopodia, cellular protrusions essential for cell migration (Ridley et al. 2003).

In advanced atherosclerotic lesions, the stability of the fibrous cup that covers the pro-thrombotic necrotic core is dependent on the integrity of interstitial type I collagen and elastin (Galis et al. 1994, Sukhova et al. 1999). Matrix metalloproteases (MMPs) are proteolytic enzymes capable of virtually degrading all ECM components (Sternlicht & Werb 2001). Membrane-type MMPs (MT-MMPs) are a sub-family of MMP expressed on the cell surface through the presence of a single-pass transmembrane domain. In contrast to the others MT-MMPs, MT4 and MT6-MMP are localized to the cell surface through a C-terminal hydrophobic region that acts as a GPI membrane-anchoring signal (Itoh et al. 1999, Kojima et al. 2000). GPI modification has also been identified in the new MMPs inhibitor reversion-inducing cysteine-rich protein with Kazal motifs (RECK) (Takahashi et al. 1998, Oh et al. 2001, Baker et al. 2002). Thus, GPI modification localizes several important proteolytic events to specific regions of the cellular surface, and may be involved in atherosclerotic plaque stability.

The coagulation and fibrinolytic systems contribute to the development and progression of atherosclerosis, as well as the incidence of atherosclerosis-related clinical events. Pivotal regulatory proteins of these enzymatic cascades, such as tissue factor (TF), glycoprotein Ib (GPIb) and IX (GPIX), undergo different acylation processes (Table V) (Schick & Walker 1996, Dorfleutner & Ruf 2003). Lipid-modified proteins have also been identified among anticoagulant effectors of endothelial cells, such as endothelial nitric oxide synthase (eNOS), prostacyclin receptor and adenosine triphosphate diphosphohydrolase (ATPDase) (Moncada 1982, Ignarro 1990, Misumi et al. 1990, Zimmermann 1992, 1996, Robinson et al. 1995, Shaul et al. 1996, Marcus et al. 1997, Feron et al. 1998). The prostacyclin receptor is isoprenylated and palmitoylated, a dual lipidation that does not affect agonist binding but which modulates receptor coupling to G_s- and G_q-regulated effectors systems and therefore receptor signalling (Hayes et al. 1999, Miggin et al. 2003). The target of eNOS to the specialized plasmalemmal microdomains named caveolae is mediated by dual acylation, an irreversible N-myristoylation at Gly-2, and reversible palmitoylation at Cys-15 and Cys-26 (Busconi & Michel 1993, Robinson & Michel 1995, Robinson et al. 1995, Feron et al. 1998, Ghosh et al. 1998, Blair et al. 1999). Finally, the enzyme activity of ecto-5′-nucleotidase correlates with its expression on the cell surface through a GPI moiety, an event regulated by the Rho-GTPase protein (Ledoux et al. 2002). Platelet aggregation is directly regulated by RhoA, Rac1, Cdc42 and Rab4, and other small GTPases that undergo geranylgeranylation processes (Azim et al. 2000, Shirakawa et al. 2000, Schoenwaelder et al. 2002).

Collectively, with the exemption of cholesterol-modified proteins, these observations suggest that myristoylated, palmitoylated, prenylated and GPI-modified proteins play an integral role in the initiation of atherosclerosis (characterized by accumulation of inflammatory cells) to its progression (SMC proliferation and migration) and the

end event (rupture of atherosclerotic plaque and activation of platelets resulting in formation of thrombus). The measure of protein expression or fraction modified by a particular lipid moiety may therefore be correlated with the progression of atherosclerotic lesions, and potentially used as a biomarker for cardiovascular diseases.

Inhibition of post-translational modification of Ras and the impairment of atherosclerotic lesion development

Despite the fact that intense research activities have accumulated a substantial body of knowledge about lipid-modified proteins, many questions concerning their implications in cardiovascular diseases remain open (Corsini et al. 1993, Irani et al. 1994, Indolfi et al. 1995, Olson et al. 1995, Chemla et al. 1997, Stark et al. 1998, Cohen et al. 1999). However, some emerging evidence supports the role of prenylated proteins in atherogenesis. The exogenous expression of H-Ras-dominant negative determined a significant reduction in SMC proliferation in response to vascular injury, demonstrating that Ras-mediated intracellular signalling significantly contributes to SMC accumulation (Indolfi et al. 1995, Work et al. 2001). Although the actual involvement of Ras farnesylation still needs to be addressed, reduced Ras levels in cell membranes by the inhibition of the enzyme prenylated protein methyltransferase (PPMTase) has shown to significantly affect the development of lesions of atherosclerosis in apolipoprotein E-deficient mice (Hancock et al. 1991a, George et al. 2002). More specifically, mice with reduced expression of the intracellular linker protein Grb2 in the signalling pathway mediated by Ras are resistant to the development of neointima formation in response to vascular injury (Zhang et al. 2003). From this evidence, it is tempting to speculate that post-translational farnesylation of Ras contributes to SMC accumulation in atherosclerosis. Cell membrane association of Ras is also dependent on the post-translational palmitoyl thioesterification, but its role in SMC recruitment in atherosclerotic lesions still needs to be addressed (Hancock et al. 1991b).

Interestingly, emerging evidence suggests that Ras may regulate directly the intracellular biosynthesis of the glycophosphatidyl inositol (Sobering et al. 2004). Therefore, alteration of Ras prenylation may affect the GPI modification of a set of proteins potentially expressed in atherosclerotic lesions, including ecto-5′-nucleotidase MMPs and MMP inhibitors. Moreover, the GPI-specific phospholipase D enzyme detected in macrophages in human atherosclerosis mediates release of GPI-anchored proteins from the cell surface (O'Brien et al. 1999).

Levels of lipidated circulating proteins and correlation with the extent of lesions of atherosclerosis

Quantification of the classical biomarkers for atherosclerosis, such as highly sensitive C-reactive protein and soluble adhesion molecules, is usually performed by an enzyme-linked immunosorbent assay (ELISA). Circulating levels of lipid-modified proteins might also be determined by ELISA. However, this assay does not discriminate between non-lipidated and lipidated proteins. Recent advances in protein analysis technology, in particular the availability of exquisitely accurate and sensitive mass spectrometers, are making possible a large-scale analysis of proteins and may potentially be used to discriminate between non-modified and modified fractions of

lipidated proteins (Mann & Talbo 1996, Roepstorff 1997). Proteins may be separated in parallel by two-dimensional electrophoresis techniques, which can array thousands of proteins in quantities sufficient for analysis (Sanchez et al. 1997). Robots can then excise protein spots from gels, place them into 96-well plates, subject them to endoproteinase digestion and prepare the resulting peptides for automated analysis on mass spectrometers (Traini et al. 1998). The feasibility of mass spectrometry analysis for identifying fatty acylation of particular proteins was demonstrated for Src family kinase Fyn, Fus1 and GAP-43/neuromodulin (Taniguchi et al. 1994, Liang et al. 2002, 2004, Uno et al. 2004). A recent trend towards the reduced cost for some mass spectrometers should make this technique more accessible to a greater number of investigators and clinicians.

Although mass spectrometry analysis is the most accurate and sensitive way to detect circulating protein levels, the evaluation of the fraction of proteins that are prenylated may also be performed by Western blot analysis (Adjei et al. 2000, Lobell et al. 2002). This analysis has been developed for preclinical trials of FTase-I and GGTase-I inhibitors, and Rap1A and HDJ2 have been used as markers for geranylgeranylation and farnesylation, respectively (Lobell et al. 2002). Moreover, analysis of protein fractions that undergo prenylation may be monitored by two-dimensional electrophoresis analysis (Cicha et al. 2004).

One of the essential characteristics for an 'ideal' biomarker is accessibility, and serum proteins represent the best source for biomarker discovery. Lipid-modified proteins are associated with the cytoplasmic membrane, therefore they are unlikely to be detectable in the serum, but blood cells often express a set of lipidated proteins involved in the development of lesion of atherosclerosis. For instance, eNOS, GPIb, Rap1, RhoA and Rab are expressed in platelets (Muszbek & Laposata 1989, Walther et al. 2003), HDJ2, TF and TNF-α can be detected in circulating monocytes (Ishibashi et al. 2003, Lobell et al. 2002), eosinophils express MT4-MMP (Gauthier et al. 2003), and ecto-5′-nucleotidase is present in lymphocytes (Resta et al. 1993) (Table V). Therefore, expression and modification of different lipidated proteins may theoretically be performed by a proteomic analysis from blood cells.

Prenylated proteins: potential biomarker for statin treatment in cardiovascular diseases

Farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate moiety are intracellular metabolites of the mevalonate (MVA) pathway (Figure 2). The exposure to 3-hydroxy-3-methyl-glutaryl coenzyme (HMG-CoA) reductase inhibitors (statins) blocks the biosynthesis not only of cholesterol, but also of farnesol and geranylgeraniol, and subsequently prenylation of intracellular proteins (Raiteri et al. 1997). Clinical trials have demonstrated that HMG-CoA reductase inhibitors, statins, can induce regression of vascular atherosclerosis as well as the reduction of cardiovascular-related morbidity and mortality in patients with and without coronary artery diseases (Lancet 1994, 1996). It is usually assumed that any beneficial effect of statins on coronary events is linked to their hypocholesterolemic properties, but their inhibitory action on protein prenylation results in pleiotropic effects. Hence, effects other than cholesterol reduction may help to explain the anti-atherosclerotic properties of statins. Several indications suggest that most of the cholesterol-independent effects of statins may be mediated by inhibition of liver HMG-CoA reductase, leading to subsequent

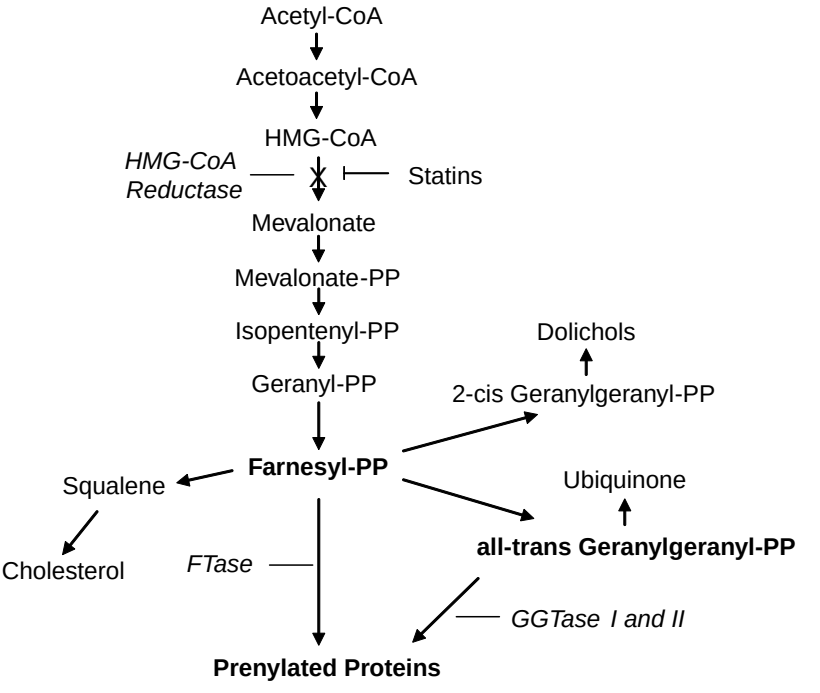

Figure 2. Mevalonate pathway. Statin treatment leads to the inhibition of HMG-CoA reductase enzymatic activity affecting not only cholesterol biosynthesis, but also the intracellular production of farnesyl-PP and geranylgeranyl-PP, substrates of protein prenyl transferases.

reduction in circulating isoprenoid levels, in particular farnesyl-pyrophosphate (Figure 2) (Corsini et al. 1999, Liao 2002). Therefore, isoprenoid plasma levels, as well as their availability for protein prenylation, are reduced. This evidence is also supported by the fact that a direct analytical evaluation, using high-performance liquid chromatography, of plasma farnesyl-pyrophosphate in dogs has been successfully performed, and statin treatment has been shown to reduce its plasma levels significantly (Saisho et al. 1997). This effect may explain why hydrophilic statins, such as pravastatin and rosuvastatin, can still exert cholesterol-independent benefits on the vessel wall without directly entering vascular wall cells. From this evidence, it is tempting to speculate that by measuring the levels of circulating prenylated proteins and their modification, it might be possible to monitor the pleiotropic effects or non-lipid related effects of statins.

Conclusions

Although there is considerable pathophysiological and clinical interest in the development of novel biomarkers for cardiovascular disease that may help in the detection of individuals at high risk for future vascular events, few new markers have demonstrated an ability to predict risk over the conventional cardiovascular risk factors (Ridker et al. 2004). The discovery of new biomarkers has been primarily focused for prognosis in primary intervention, but very little has been done for the development of biomarkers for specific pathological stages and therapeutic interventions.

Several lines of evidence suggest that lipid-modified proteins play a role in different steps of atherogenesis, from leukocyte recruitment to plaque rupture. The clear relationship between lipid/lipoprotein abnormalities and vascular disease has led to the hypothesis that the expression and modification of lipidated proteins may be altered in the pathogenesis of atherosclerosis. Similarly, therapeutic intervention with statins may also be monitored by measuring the profile of circulating lipidated proteins. Ongoing efforts in proteomic analysis will need to evaluate carefully the clinical applications and efficacy of circulating levels of lipidated proteins in patients with coronary heart disease.

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