Summary. Coronary heart disease and stroke, caused by rupture of atherosclerotic plaques in the arterial wall, are the major causes of death in industrialized countries. A key event in the pathogenesis of atherosclerosis is the transformation of smooth muscle cells and in particular of macrophages into foam cells, a result of massive accumulation of lipid droplets. It is well known that the formation of these lipid droplets is a result of the uninhibited uptake of modified lipoproteins by scavenger receptors. However, only more recently has it become apparent that a special set of lipid droplet associated proteins - the PAT protein family (perilipin, adipophilin, TIP47, S3-12 and OXPAT) - is fundamental to the formation, growth, stabilization and functions of lipid droplets. Here we review recent findings and assess the current state of knowledge on lipid droplets and their PAT proteins in atherogenesis.

Key words: Atherosclerosis, Lipid droplets, PAT proteins

Introduction

The essential features of atherosclerosis

Lipid droplet accumulation is one of the hallmarks of atherosclerosis, the inflammatory process by which plaques develop in the arterial lumen which eventually lead to the potentially fatal complications of ischemic heart disease and stroke. The initial step of atherogenesis is dysfunction of the endothelium which may arise from multiple factors such as hypertension and diabetes mellitus (Ross, 1993, 1999). The resultant enhanced permeability of the endothelium allows penetration of low density lipoproteins (LDL) into the intima. LDL is protected from oxidation in the blood but becomes enzymatically modified when bound to components of the extracellular matrix (Schwenke and Carew, 1989). Modified LDL, in particular oxidized LDL (oxLDL), stimulates the expression of chemotactic molecules by endothelial cells and attracts blood monocytes and other inflammatory cells (Dong et al., 1998). Migration of monocytes into the arterial wall leads to their differentiation into macrophages (MΦ). Intimal MΦ internalize the oxLDL and in so doing become engorged with lipid droplets, forming foam cells (Robenek and Severs, 1992). Foam cell accumulation characterizes the first visible stage of the atherosclerotic lesions, the fatty streak (Fig. 1). Expression and secretion of cytokines and growth factors by intimal MΦ and foam cells lead to the migration and proliferation of smooth muscle cells (SMC) in the intima. Intimal SMC actively synthesize collagen and other components of the extracellular matrix resulting in the development of a fibrous cap (Koyama et al., 1992). At this stage, a lipid core, derived from dead foam cells, becomes visible in the developing plaque. Many lesions remain asymptomatic because the fibrous cap is sufficiently robust to prevent disruption. Others, however, are at risk of rupture if the fibrous cap is too thin. Cap thinning may be promoted by decreased collagen synthesis caused by death of collagen-synthesizing cells and/or as a result of digestion of extracellular matrix molecules through matrix metalloproteinase secretion by MΦ (Galis et al., 1995). Rupture of these vulnerable plaques triggers acute thrombosis leading to unstable angina, acute myocardial infarction or stroke.

Foam cell formation

The removal of pro-inflammatory oxLDL by intimal
MΦ, with massive accumulation of cholesterol ester rich lipid droplets, results from uninhibited uptake of oxLDL by scavenger receptors. The importance of the scavenger receptor family in atherogenesis is clearly demonstrated in mice lacking the class A scavenger-receptor (SR-A) and apolipoprotein E (apoE) (SR-A<sup>-/-</sup>/apoE<sup>-/-</sup>-mice) or CD36<sup>-/-</sup>/apoE<sup>-/-</sup>-mice which show significantly reduced formation of atherosclerotic lesions (Suzuki et al., 1997; Febbraio et al., 2000).

OxLDL, taken into the cell via scavenger receptors, contains high amounts of cholesterol esters that are hydrolyzed in lysosomes. The resulting free cholesterol is used by the cell, for example for incorporation as an essential component of the plasma membrane. Increased free cholesterol in the plasma membrane leads to a reduction of cholesterol synthesis and LDL-receptor expression, but does not influence the uptake of oxLDL/cholesterol via scavenger receptors (Lindstedt et al., 1992; Brown and Goldstein, 1999). Excess free cholesterol is transferred to the endoplasmic reticulum (ER) where it is re-esterified by acyl coenzyme A: cholesterol acyltransferase (ACAT). These newly synthesized cholesterol esters are then incorporated into lipid droplets (Kruth et al., 2001; Soccio and Breslow, 2004).

Lipid droplets also contain triglycerides which originate from other lipoproteins - the very low density lipoproteins and chylomicrons - and are also synthesized directly from free fatty acids. The uptake of free fatty acids is mediated by fatty acid translocase or fatty acid transport proteins (Liken et al., 1999). At the plasma membrane, triglycerides are esterified by the acyl coenzyme A synthetase and are subsequently transferred to the ER (Stremmel et al., 2001). Incorporation of free fatty acids into triglycerides at the ER is mediated by a series of enzymes in which diacylglycerol acyl transferase (DGAT) catalyzes the terminal step. Finally, new synthesized triglycerides, together with cholesterol esters, are packaged into the lipid droplets of the MΦ (Doege and Stahl, 2006).

**Structure and biogenesis of lipid droplets**

Lipid droplets were for many years envisaged as simple storage organelles for lipids but are now considered multi-functional organelles with additional roles in lipid homeostasis, cell signaling, and intracellular vesicle trafficking (Wang et al., 1999; Liu et al., 2003; Umlauf et al., 2004). The essential structure of the lipid droplets is of a hydrophobic core of neutral lipids surrounded by a phospholipid monolayer. Freeze-fracture electron microscopy has demonstrated that the hydrophobic lipid core - once thought to be homogeneous - in fact often has an elaborate structure of lamellar stacks and/or concentrically arranged layers (Fig. 2) (Robenek et al., 2009).

The ER is the site of lipid droplet formation. A widely promoted idea is that cholesterol ester and triglycerides, synthesized by ACAT and DGAT, accumulate within the lipid bilayer of the ER membrane and, upon reaching a critical size, the accumulation is pinched off into the cytoplasm as a lipid droplet enveloped in a phospholipid monolayer formed from the cytoplasmic leaflet of the ER membrane (Brown, 2001; Murphy, 2001). A variation on this idea proposes that the intramembrane lipid accumulation is released with portions both of the cytoplasmic and luminal phospholipid monolayer leaflets of the ER membrane (Ploegh, 2007). However, lipid accumulations within the ER membrane have never been observed. Freeze-fracture electron microscopy demonstrates that growing lipid droplets are intimately associated with but lie

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**Fig. 1.**Thin-section electron micrographs of foam cells from a fatty streak from human femoral artery. **A.** Example of a smooth muscle cell-derived foam cell. Contractile features are reduced, abundant ER is present, and the cell is packed with large lipid droplets. **B.** Typical morphology of MΦ derived foam cells. Bars: 0.2 µm.
external to specialized cup-like sites of the ER membrane (Robenek et al., 2004, 2006). The images suggest that initial formation of the droplet may occur at these sites, but in this case the phospholipid monolayer would need to be synthesized in situ on the droplet surface.

Proteins of the phospholipid monolayer

An important feature of the surface phospholipid monolayer of the lipid droplet is the presence of a series of characteristic proteins implicated in its key functions (Tauchi-Sato et al., 2002; Ozeki et al., 2005). The first of these to be discovered was perilipin in adipocytes (Greenberg et al., 1991). Four additional related proteins were subsequently discovered: i) adipophilin (also known as adipose differentiation-related protein or perilipin 2), ii) TIP47 (tail interacting protein of 47 kDa or perilipin 3), iii) S3-12 (perilipin 4), and iv) OXPAT (also known as myocardial lipid droplet protein (MLDP), lipid storage droplet protein 5, or perilipin 5) (Brasaemle et al., 1997; Heid et al., 1998; Diaz and Pfeffer, 1998; Scherer et al., 1998; Wolins et al., 2001, 2003; Yamaguchi et al., 2006). These five proteins have high sequence homology and are collectively classified as the PAT protein family, named after perilipin, adipophilin, and TIP47.

The best characterized member of the PAT family is perilipin, characteristically expressed in white and brown adipose tissue and steroidogenic cells. Alternative splicing of the perilipin gene leads to four perilipin isoforms (perilipin A-D) (Greenberg et al., 1993; Lu et al., 2001). Perilipin A and B are expressed in adipocytes whereas perilipin C and D are found in steroidogenic cells. Under basal conditions, perilipin A stabilizes lipid droplets by inhibiting lipolysis of the lipid droplet core by the hormone sensitive lipase (HSL). Lipolysis is activated when perilipin A and the cytoplasmic HSL are phosphorylated by protein kinase A; binding of HSL to the lipid droplet surface ensues, leading to hydrolysis of neutral lipids of the lipid droplet core (Tansey et al., 2003; Zimmermann et al., 2004). Adipocyte triglyceride lipase (ATGL), which hydrolyses triglycerides of the lipid droplet, is also associated with the lipid droplet surface (Lass et al., 2006). Binding of CGI-58 to perilipin A allows hydrolysis of triglycerides by ATGL (Lass et al., 2006; Schweiger et al., 2006). Less is known of the functions of the other perilipins; perilipin B is proposed to protect triglycerides against hydrolysis within the plasma membrane (Aboulaich et al., 2006), perilipin C appears to be involved in hydrolysis of cholesterol esters by cholesterol ester hydrolase (Zhao et al., 2005) but the function of perilipin D is unclear.

Adipophilin was originally described as a protein associated with lipid droplets during differentiation of pre-adipocytes into adipocytes (Jiang et al., 1992; Brasaemle et al., 2000). Further studies revealed adipophilin to be the most abundant lipid droplet associated protein, found in a wide range of cell types, including macrophages (Brasaemle et al., 1997; Heid et al., 1998). Adipophilin binds fatty acids and cholesterol (Gao and Serrero, 1999; Serrero et al., 2000; Atshaves et al., 2001), stabilizes triglyceride content and its expression is correlated with that of intracellular neutral lipids (Heid et al., 1998).

The first hint of a role for TIP47 in lipid metabolism came from its detection at the lipid droplet surface of HeLa cells (Wolins et al., 2001). In contrast to perilipin and adipophilin, TIP47 was the first known member of the PAT family that, in addition to being localized at the lipid droplet surface, also exists in a soluble cytosolic form. Sequence analysis revealed that TIP47 features a four helix bundle that potentially opens for interactions with the hydrophobic surface of lipid droplets (Hickenbottom et al., 2004; Ohsaki et al., 2006). The localization of TIP47 predominantly on nascent lipid

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**Fig. 2.** A,B. Typical freeze-fracture views of cytoplasmic lipid droplets in THP-1 MΦ incubated with 50 µg/ml acetylated LDL (acLDL) for 24h at 37°C to stimulate lipid droplet accumulation. Note the stacks of concentric lipid lamellae which give the lipid droplet a multilayered onion-like appearance. C. Standard thin-section electron microscopy, in contrast to freeze fracture, reveals no information on lipid droplet substructure. Bars: 0.2 µm.
droplets implies a role in formation rather than in stabilization of the droplet (Wolins et al., 2006a). S3-12 is primarily expressed in white adipose tissue but is also detectable in skeletal muscle and heart. Similarly to TIP47, S3-12 is soluble in the cytoplasm but re-localizes to the lipid droplet surface under lipid loaded conditions (Scherer et al., 1998; Wolins et al., 2003). The last member of the PAT family, OXPAT, is expressed in heart, brown adipose tissue, liver, and skeletal muscle. These tissues exhibit a high capacity for fatty acid oxidation, so OXPAT may facilitate lipid storage for near-term utilization through oxidative pathways (Wolins et al., 2006b; Dalen et al., 2007).

**PAT proteins in macrophages and atherogenesis**

**Adipophilin**

Of all the PAT proteins, adipophilin is the most abundant and best characterized in human monocyte derived MΦ (HMDM), mouse peritoneal MΦ and other MΦ cell lines (Buechler et al., 2001; Chen et al., 2001; Larigauderie et al., 2004; Wei et al., 2005; Paul et al., 2008). Originally assumed to be confined to the lipid droplet surface (Fig. 3A), freeze-fracture electron microscopy combined with immunogold labeling has also clearly demonstrated the presence of adipophilin in the hydrophobic core (Fig. 3B) (Robenek et al., 2005a,b). How adipophilin enters the interior of lipid droplets is unknown but may be facilitated by fusion processes of lipid droplets or interactions between neutral lipids and adipophilin during lipid droplet growth.

Apart from the lipid droplet surface, membrane fractionation studies first suggested that adipophilin may also be present in the ER (Heid et al., 1996; Robenek et al., 2006). This localization was confirmed by the electron-microscopical technique of freeze-fracture replica immunolabeling. As introduced in Figure 3A, the beauty of this technique is that proteins can be localized at high resolution in en face views of the membrane. With this approach, adipophilin in MΦ is found specifically associated with the cytoplasmic half-membrane leaflet of the ER membrane (Figs. 4, 5), concentrated in clusters at the specialized sites in which the ER envelopes the lipid droplet (Robenek et al., 2006).

Apart from the ER, adipophilin is readily detectable by freeze-fracture replica immunolabeling in the plasma membrane of THP-1 MΦ. Under normal culture conditions, adipophilin is widely dispersed in the plasma membrane but when the cells are incubated with modified LDL, a remarkable clustering of adipophilin occurs in raised plasma membrane domains adjacent to underlying lipid droplets (Fig. 6) (Robenek et al., 2005c). Thus, far from being restricted to the lipid droplet as originally supposed, high concentrations of adipophilin occur both in ER and plasma membrane domains immediately adjacent to lipid droplets.

Adipophilin is the most extensively analyzed PAT protein in MΦ. Under basal cell culture conditions, MΦ exhibit tiny lipid droplets coated by adipophilin. Different forms of lipid loading result in increased lipids...
droplet formation and adipophilin expression (Larigauderie et al., 2004; Robenek et al., 2009). Conversely, additional expression of adipophilin elevates the lipid content during acLDL incubation whereas suppression of adipophilin reduces lipid accumulation (Larigauderie et al., 2004; Paul et al., 2008; Buers et al., 2009). Furthermore, peritoneal MΦ of adipophilin-deficient mice reveal a remarkable reduction of cytoplasmic lipid droplets during lipid incubation (Paul et al., 2008). Thus, adipophilin is centrally implicated in the development of foam cells and stabilization of their lipid droplets.

There are a number of clues as to how this effect is brought about. The specific clustering of adipophilin in ER domains that interact with lipid droplets points to a role in growth and perhaps in formation of the droplet. The ability of adipophilin to bind free fatty acids and cholesterol raises the possibility that adipophilin receives newly synthesized cholesterol esters from ACAT in the ER membrane and transfers them to adjacent lipid droplets. A similar role would explain the juxtaposition of the plasma membrane adipophilin-rich domains with lipid droplets. Clustering of adipophilin could promote the transfer of free fatty acids from the plasma membrane to underlying lipid droplets for incorporation into triglycerides.

Evidence for a role in the stabilization of the lipid droplet (i.e., inhibition of hydrolysis) comes from studies in which adipophilin is over-expressed in THP-1 MΦ; under these conditions, cholesterol efflux is repressed (Larigauderie et al., 2004). Correspondingly, absence of adipophilin leads to enhanced cholesterol efflux in mouse peritoneal MΦ (Paul et al., 2008). Adipophilin has further been implicated in decreased β-oxidation in THP-1 MΦ resulting in enhanced triglyceride content (Larigauderie et al., 2006a). Stabilization of lipid droplets by adipophilin may be brought about by both inhibition of the hydrolysis of triglycerides by ATGL/HSL (as shown for other cell types) and cholesterol ester by cholesterol ester hydrolase.

Given the roles that have emerged for adipophilin in the formation, growth and stabilization of lipid droplets in foam cells in vitro, what is the evidence for a corresponding role in atherogenesis in the intact artery?
Adipophilin is highly expressed in atherosclerotic lesions of human arteries (Fig. 7) (Nuotio et al., 2007) and advanced atherosclerotic lesions of apoE-deficient mice (Larigauderie et al., 2004). In all stages of atherogenesis, adipophilin is co-localized with intimal MΦ (Hofnagel et al., 2007; Nuotio et al., 2007; Paul et al., 2008). Whether adipophilin influences formation of atherosclerotic plaques was examined in adipophilin deficient mice with an atherosclerotic background (adipophilin<sup>−/−</sup>/apoE<sup>−/−</sup>-mice). These mice reveal significantly decreased lipid droplet formation in intimal MΦ. Furthermore, adipophilin<sup>−/−</sup>/apoE<sup>−/−</sup>-mice, and also mice with specific inhibition of adipophilin in bone marrow derived cells, show reduced formation of atherosclerotic lesions (Paul et al., 2008). Combined with the in vitro data, the evidence that adipophilin is a key player in lipid droplet formation and foam cell development during atherogenesis is now compelling.

In addition, evidence for an independent pro-inflammatory effect of adipophilin has emerged. Adipophilin expression is reported to correlate with the expression and secretion of tumor necrosis factor α (TNF-α), monocyte chemotactrant protein 1 (MCP-1), and interleukin 6 (IL-6) in THP-1 MΦ. Secretion of TNF-α, MCP-1, and IL-6 by intimal MΦ can directly attract blood monocytes and other inflammatory cells and finally results in their migration into the arterial wall (Chen et al., 2009). Thus, adipophilin may promote atherogenesis in two different ways: i) by promoting MΦ-derived foam cell formation and ii) by intensifying the inflammatory process through enhancement of cytokine expression.

**Perilipin**

Although it is widely held that perilipin expression is limited to adipocytes and steroidogenic cells, recent studies have revealed perilipin in MΦ (Faber et al., 2001; Forcheron et al., 2005; Zhao et al., 2005; Larigauderie et al., 2006b; Hofnagel et al., 2007; Persson et al., 2007). HMDM are reported to express both perilipin A and perilipin B (Larigauderie et al., 2006b; Hofnagel et al., 2007). In THP-1 MΦ, the presence of Perilipin C (Zhao et al., 2005), only perilipin A (Larigauderie et al., 2006b) and both perilipin A and B have been variously reported (Hofnagel et al., 2007). During the course of differentiation of human blood monocytes into HMDM, rising perilipin levels are observed whereas lipid loading of HMDM or suppression of adipophilin in THP-1 MΦ has no effect on perilipin expression levels (Larigauderie et al., 2006b). Over-expression of perilipin, however, leads to enhanced triglyceride levels in MΦ and apparently compensates for the absence of adipophilin (Larigauderie et al., 2006b). There thus appears to be some degree but not complete overlap in the functions of perilipin and adipophilin.

The first link between perilipin and atherosclerotic lesions came from detection of perilipin in foam cells of ruptured plaques (Faber et al., 2001). Subsequent studies...
Fig. 6: Adipophilin is present in the P-face of the plasma membrane of THP-1 Mφ. A. In the plasma membrane of the normal cultured Mφ adipophilin label is widely distributed throughout the membrane. B. Upon lipid loading, the adipophilin becomes clustered in elevated domains in the plasma membrane. Bars: 0.2 μm.
**Fig. 7.** Immunohistochemistry of human aorta in early atherosclerotic lesions. Adipophilin expression (A) co-localizes with intimal MΦ (B). I: intima layer, M: media layer. Bars: 100 µm.

**Fig. 8.** Serial sections of an advanced lesion from human coronary artery. Perilipin is revealed in the shoulder of atherosclerotic plaque (A) and is co-localized with MΦ (B). I: intima layer, M: media layer. Bars: 200 µm.
have confirmed readily detectable perilipin in intimal MΦ and foam cells of the advanced atherosclerotic lesion (Fig. 8) (Faber et al., 2001; Larigauderie et al., 2006b). Western-blot analysis reveals that perilipin A is the predominant isoform, with weak expression of perilipin B and no detectable perilipin C or D (Forcheron et al., 2005; Hofnagel et al., 2007). Taken together with the results of cell culture studies, the findings raise the possibility that perilipin A and possibly perilipin B protect lipid droplets against hydrolysis in advanced lesions.

An interesting question concerns the relationship between perilipin and adipophilin during atherogenesis. Distinct spatio-temporal patterns of perilipin and adipophilin expression are displayed during the differentiation process of pre-adipocytes into adipocytes. During this process perilipin replaces adipophilin within a few hours. Additionally, adipophilin is exclusively localized at smaller lipid droplets whereas perilipin coats the larger lipid droplets (Jiang et al., 1992; Brasaemle et al., 2000; Wolins et al., 2006). This distinct distribution pattern is ascribed to differentiation of the functions of the proteins. By extension to foam cell formation during atherogenesis, it might be speculated that adipophilin promotes lipid droplet formation of MΦ in early stages of atherogenesis whereas perilipin stabilizes the droplets in advanced lesions.

**TIP47**

In contrast to adipophilin and perilipin, TIP47 is mainly localized in the cytoplasm of THP-1 MΦ (Buers et al., 2009). Oleate incubation induces TIP47 association with the lipid droplet surface whereas incubation of the cells with acLDL results in no changes in the TIP47 distribution pattern (Buers et al., 2009). As with adipophilin, TIP47 is not only localized at the lipid droplet surface but also in the hydrophobic core (Robenek et al., 2005b), as well as in the plasma membrane. Again, as with adipophilin, TIP47 undergoes dramatic reorganization into plasma membrane clusters in lipid loaded MΦ (Robenek et al., 2005c) suggesting similar functions. Lipid loading, or the absence of adipophilin, do not influence the expression level of TIP47 in MΦ (Paul et al., 2008; Buers et al., 2009). However, the absence of adipophilin induces a remarkable redistribution of TIP47 from the cytoplasm to lipid droplets accompanied by elevated triglyceride content. Triglyceride level is also enhanced by additional expression of TIP47 in this cell type (Buers et al., 2009). Bearing in mind the similarity of localization of TIP47 in the plasma membrane, its influence on the triglyceride level and the high sequence homology to adipophilin, it appears that TIP47 may act as a transporter for free fatty acids from the plasma membrane to the lipid droplets. As a result of its structure, TIP47 is able to bind at the lipid droplet surface where it releases fatty acids. Thus, TIP47 appears to promote the growth of lipid droplets during foam cell formation rather than their stabilization. The expression pattern and the localization of TIP47 in atherosclerotic lesions are yet to be examined, but the *in vitro* studies imply roles for TIP47 in the development in atherosclerotic lesions.

**S3-12 and OXPAT**

In contrast to perilipin, adipophilin, and TIP47, S3-12 is not expressed in mouse MΦ or atherosclerotic lesions (Paul et al., 2008) and no data are available for OXPAT. Neither protein has yet been studied in human samples.

**Conclusion**

In conclusion, the PAT protein family is implicated in the formation, growth and stabilization of lipid droplets in foam cells and hence potentially placed to play a pivotal role in the development of atherosclerotic lesions. Despite their emerging relevance to atherogenesis, studies on their specific function in lipid metabolism of MΦ are still at an early stage. The different expression patterns and localization of PAT proteins in MΦ and lesions suggest multiple functions in atherogenesis. Loss of adipophilin protects against formation of lipid droplets in MΦ and atherogenesis, showing its specific role during lipid droplet formation and accumulation. Expression of perilipin exclusively in advanced atherosclerotic lesions raises the possibility that this PAT protein stabilizes MΦ lipid droplets. TIP47 appears to act as a transport protein for fatty acids in MΦ, thereby possibly promoting growth of lipid droplets. Among key questions to be addressed in the future are: 1) do PAT proteins stabilize lipid droplets by regulating hydrolysis of cholesterol ester in MΦ? 2) are perilipin and TIP47 involved in the development of atherosclerotic lesions? 3) do other (non PAT family members) lipid droplet associated proteins influence foam cell formation? and 4) could PAT proteins be used as a therapeutic target to protect against lipid droplet formation in MΦ.

**References**


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