A Macrophage Sterol-Responsive Network Linked to Atherogenesis

Lev Becker, Sina A. Gharib, Angela D. Irwin, Ellen Wijsman, Tomas Vaisar, John F. Oram, and Jay W. Heinecke

1Department of Medicine
2Department of Biostatistics
University of Washington, Seattle, WA 98195, USA
*Correspondence: heinecke@u.washington.edu
DOI 10.1016/j.cmet.2010.01.003

SUMMARY

Cholesteryl ester accumulation by macrophages is a critical early event in atherogenesis. To test the hypothesis that sterol loading promotes foam cell formation and vascular disease by perturbing a network of interacting proteins, we used a global approach to identify proteins that are differentially expressed when macrophages are loaded with cholesterol in vivo. Our analysis revealed a sterol-responsive network that is highly enriched in proteins with known physical interactions, established roles in vesicular transport, and demonstrated atherosclerotic phenotypes in mice. Pharmacologic intervention with a statin or rosiglitazone and use of mice deficient in LDL receptor or apolipoprotein E implicated the network in atherosclerosis. Biochemical fractionation revealed that most of the sterol-responsive proteins resided in microvesicles, providing a physical basis for the network’s functional and biochemical properties. These observations identify a highly integrated network of proteins whose expression is influenced by environmental, genetic, and pharmacological factors implicated in atherogenesis.

INTRODUCTION

Macrophages are essential effectors of innate and adaptive immunity (Gordon and Taylor, 2005). Via their scavenger receptors and other mechanisms, they clear bacterial pathogens and apoptotic cells (Suzuki et al., 1997) and release a wide range of cytokines and chemokines that orchestrate inflammation. Recent studies have also implicated macrophages in tumor progression, adipose tissue expansion, and insulin resistance (de Luca and Olefsky, 2008; Wellen and Hotamisligil, 2005). Moreover, these cells promote atherosclerosis by mediating the uptake of modified lipoproteins into the artery wall (Li and Glass, 2002).

When macrophages take up and degrade more lipoprotein-derived cholesterol than they excrete, they convert free cholesterol to cholesteryl ester, which accumulates as cytosolic lipid droplets that appear foamy under the light microscope (Brown and Goldstein, 1983). Genetic, biochemical, and clinical studies provide compelling evidence that macrophage foam cells are of central importance in atherogenesis (Li and Glass, 2002).

One key source of this excess sterol in humans is low-density lipoprotein (LDL). Moreover, mice with targeted disruption of the LDL receptor gene (Ldlr) have markedly elevated LDL levels when fed a western-type diet rich in cholesterol and saturated fat (though mice normally have low levels of LDL and are resistant to atherogenesis). Consequently, Ldlr−/− mice rapidly develop atherosclerosis (Ishibashi et al., 1994). Because macrophages are key players in atherosclerosis and many other conditions, it is likely that broad networks of interacting genes and proteins (Ghazalpour et al., 2004; Schadt and Lum, 2006; Lusis et al., 2008)—rather than simple linear pathways that affect only sterol balance—promote foam cell formation and atherogenesis. We therefore combined proteomics with bioinformatics to obtain a global view of macrophages’ roles in vascular disease. This approach uncovered a network of proteins that is regulated when macrophages become foam cells. Dietary studies, pharmacological interventions, and work with genetically engineered mice linked this highly interconnected sterol-responsive network to atherosclerosis.

RESULTS

Identification of Macrophage Sterol-Responsive Proteins

Previous studies have demonstrated that peritoneal macrophages of Ldlr−/− mice can be loaded with cholesteryl ester in vivo and that this model is relevant for studying the roles of foam cells in atherosclerosis (Li et al., 2004). We therefore placed Ldlr−/− mice on a chow (low-fat) or western-type (high-fat) diet for 14 weeks (Figure 1A) and then harvested macrophages from the peritoneal cavity. Compared with control cells, macrophages isolated from the mice on the western diet had markedly higher levels of cellular cholesteryl ester mass (Figure 1B), the biochemical hallmark of foam cells (Brown and Goldstein, 1986). Oil red O staining and light microscopy confirmed that these macrophages were loaded with neutral lipid (Figures 1C and 1D).

We used LC-ESI-MS/MS analysis to identify 777 proteins with high confidence in the medium of control and/or sterol-loaded macrophages (Table S1 available online). To determine which of those proteins changed their relative abundance when macrophages were sterol loaded in vivo, we used the G-test and t test to find significant differences in spectral counts (Fu et al., 2008; Liu et al., 2004; Old et al., 2005), a measure of relative protein...
concentration (Figure 1E). We estimated the false discovery rate (FDR) by using the same statistical tests with all possible permutations of the data. Permutation analysis revealed that \( G > 1.5 \) (\( G \)-test) and \( p < 0.04 \) (t test) yielded the most true-positive protein identifications, with an estimated FDR of 6.3% (data not shown).

Using these stringent statistical criteria (Figure 1E), we identified 46 proteins that were expressed at a different level in medium of macrophage foam cells than in medium of control cells isolated from \( \text{Ldlr}^{-/-} \) mice fed a chow diet (Figure 2 and Table S1). In contrast, only two proteins resided in this region of the graph in a representative random permutation analysis (Figure 1F), which is consistent with our estimated FDR.

We assessed the effectiveness of our analytical strategy by using commercially available antibodies to quantify the relative abundance of 7 of the 46 macrophage proteins that met our statistical criteria: apolipoprotein E (APOE), cystatin C (CYSC), complement factor C3 (C3), vimentin (VIM), \( \beta\)-actin (ACTB), LDL receptor-related protein 1 (LRP1), and adipophilin (ADFP). Immunoblot analysis of conditioned media of control and cholesteryl loaded macrophages (Figure 1G) verified significant differences in the levels of all seven proteins (two-tailed Student’s t test: APOE, \( p = 0.001 \); CYSC, \( p = 0.002 \); C3, \( p = 0.001 \); VIM, \( p = 0.008 \); ACTB, \( p = 0.0006 \); LRP1, \( p = 0.03 \); ADFP, \( p = 0.02 \)).

Differentially Expressed Proteins Form a Network

We call the 46 proteins that are differentially expressed by foam cells “sterol responsive” because their relative abundance changes significantly when \( \text{Ldlr}^{-/-} \) macrophages become loaded with cholesteryl ester in vivo. We assessed the potential functional significance of these proteins in three ways. First, we searched for known physical interactions among them, using Ingenuity systems (Calvano et al., 2005) and the BIND, DIP, MIPS, IntAct, BioGRID, and MINT databases. These analyses revealed a protein-protein interaction network of 26 proteins (nodes) and 30 interactions (edges) (Figure 3), predicting multiple interactions among many of the 46 sterol-responsive proteins.

Second, we used Gene Ontology annotations to organize the 46 proteins into functional modules (Figure 3). This approach identified significant enrichment (relative to the entire mouse genome) in three modules: cytoskeletal regulation (\( p = 0.001 \)), vesicle-mediated transport (\( p = 0.003 \)), and lipid binding.
Figure 2. Proteins Differentially Expressed by Macrophage Foam Cells
Proteins differing in relative abundance in the conditioned medium of control and foam cells were identified as described in the legend to Figure 1. A positive or negative value for the G-test indicates an increased or decreased level of protein expression relative to the control. See also Table S1.

(p = 0.01). Whereas alterations in lipid metabolism are known to regulate foam cells, the potential roles of the cytoskeleton and vesicular transport in foam cell formation have received little attention, though vesicular transport is thought to play a key role in the intracellular trafficking of lipids.

Third, we used an algorithm to systematically search PubMed for documented atherosclerotic phenotypes in mice with genetic manipulations (e.g., targeted mutagenesis, transgenic overexpression) of proteins we detected in macrophage-conditioned media. Remarkably, mice that overexpress or lack any of 10 of the 46 sterol-responsive proteins have atherosclerotic phenotypes. This greater than 8-fold enrichment of proteins associated with atherosclerosis in the sterol-responsive proteins was highly significant (p = 3 × 10^{-6}, Fisher’s exact test). Moreover, previous studies have shown that the atherosclerotic phenotypes of 6 of the 10 sterol-responsive proteins were recapitulated in mice by transplanting bone marrow from genetically engineered mice into wild-type mice. Thus, macrophage-specific expression of these proteins may contribute to atherogenesis.

Collectively, these observations identify a set of 46 proteins that respond to sterol loading of macrophages in vivo in a coordinated fashion. We term this set the “macrophage sterol-responsive network” (MSRN) (Figure 3A) because these proteins are: (1) coordinately regulated when macrophages become foam cells in vivo, (2) integrated through predicted protein-protein interactions, (3) functionally overrepresented in proteins involved in lipid binding, cytoskeletal regulation, and vesicle-mediated transport, and (4) highly enriched in proteins with causative roles in atherogenesis.

Most MSRN Proteins Localize to Vesicles
Shed microvesicles are enriched in cholesterol and proteins that are involved in vesicular transport and cytoskeletal regulation (Simpson et al., 2008), and these two functional categories are significantly enriched in the MSRN. To test the hypothesis that a subset of MSRN proteins resides in microvesicles, we centrifuged macrophage-conditioned medium at 100,000 × g and analyzed the pellet material by LC-ESI-MS/MS. The
Figure 3. The Macrophage Sterol-Responsive Network

(A) A protein-protein interaction network was constructed, using the 46 proteins that were differentially expressed (upregulated, red; downregulated, green) by macrophage foam cells isolated from \( \text{Ldlr}^{-/-} \) mice. GO analysis of the network revealed modules enriched in proteins implicated in lipid binding, cytoskeletal regulation, and vesicle-mediated transport (\( p = 0.01, 0.001, \) and \( 0.003, \) respectively; Fisher’s exact test with Benjamini-Hochberg correction). Proteins that associate with an atherosclerotic phenotype (circled in blue) in genetically engineered mice or with myeloid-specific expression/deletion as assessed by bone marrow transplantation (BMT, **) were identified with PubMatrix. Note that 10 of 16 sterol-responsive proteins that were not previously shown to interact physically or functionally with other MSRN proteins, termed previously unassigned, reside in the microvesicle fraction.

(B) Comparison of MSRN protein expression in media and isolated microvesicles. A positive or negative value for the \( G \)-test indicates an increased or decreased level of protein expression relative to the control.

See also Table S2.

Neither statin nor rosiglitazone therapy affected plasma cholesterol levels or plasma lipoprotein profiles (Figure S1), but they reduced macrophage cholesterol accumulation by \(-40\%\) (Figure 4A). In striking contrast, both interventions markedly altered the expression pattern of MSRN proteins in macrophages isolated from \( \text{Ldlr}^{-/-} \) mice. Indeed, only 2 (statin) and 4 (rosiglitazone) of the 46 MSRN proteins were still differentially expressed by macrophages isolated from \( \text{Ldlr}^{-/-} \) mice that had received the high-fat diet and one of the two interventions (Figures 4B and 4C). Neither intervention appreciably altered levels of non-MSRN proteins in macrophage-conditioned medium (Figure 4D), indicating that the effect on the MSRN was specific. These data demonstrate that two different pharmacological interventions, each of which inhibits atherosclerosis without affecting plasma cholesterol levels, specifically target the MSRN. Thus, dysregulation of this network might promote the development of atherogenesis in vivo, and pharmacologic interventions that retard atherosclerosis might act, in part, by normalizing expression of the MSRN.

Apolipoprotein E Is a Key Protein in the MSRN

Because simvastatin or rosiglitazone therapy markedly alters the expression pattern of MSRN proteins in macrophages isolated from \( \text{Ldlr}^{-/-} \) mice while only modestly affecting macrophage sterol levels (Figure 4A), cholesterol accumulation alone is
unlikely to explain MSRN regulation. Instead, a protein that interacts with many other proteins might exert widespread effects on its parent network if its expression changed (Chen et al., 2008). APOE, which is underexpressed in the MSRN and is highly antiatherogenic in mice (Bellosta et al., 1995; Linton et al., 1995), might be one such protein. To test the proposal that APOE regulates the MSRN, we harvested macrophages from Apoe\textsuperscript{-/-} mice fed a low-fat or western diet, incubated them for 6 hr, and analyzed the conditioned media with proteomic methods.

Diet-induced changes in plasma cholesterol and lipoproteins (Figure S1) were similar in the Apoe\textsuperscript{-/-} and Ldlr\textsuperscript{-/-} mice. Moreover, > 96% of the proteins detected in media conditioned by macrophages from the two mouse strains were the same. Thus, the overall pattern of protein expression was similar in the two genotypes. In contrast, Apoe\textsuperscript{-/-} macrophages isolated from mice on the western diet displayed significantly higher cholesteryl ester levels than Ldlr\textsuperscript{-/-} macrophages (Figure SA). This observation is consistent with APOE’s well-established role in promoting cholesterol efflux from macrophages (Langer et al., 2000).

Despite this difference in cholesteryl ester content, only six proteins responded to sterol accumulation in Apoe\textsuperscript{-/-} macrophages (Table S4; estimated FDR = 6.4%). Moreover, the extent of MSRN regulation imposed by cholesterol loading was substantially attenuated in Apoe\textsuperscript{-/-} mice (compare Figures 5B and 5C). These observations suggest that macrophages need to express APOE to modulate MSRN expression in vivo, raising the possibility that APOE is an important node in the network. To test this proposal, we compared protein expression levels in conditioned media of macrophages isolated from Apoe\textsuperscript{-/-} and Ldlr\textsuperscript{-/-} mice. The expression of many MSRN proteins in the Apoe\textsuperscript{-/-} macrophages from mice on the chow diet strongly resembled that in Ldlr\textsuperscript{-/-} macrophages loaded with cholesteryl ester (Figure 5D). Thus, levels of ~40% of the MSRN proteins
in Ldlr−/− foam cells and Apoe−/− control cells were statistically indistinguishable (p > 0.1, two-tailed Student’s t test) (Figure 5E and Table S5). This suggests that APOE deficiency regulates the expression—either stimulation or repression—of a subset of MSRN proteins even in the absence of cholesterol loading.

To further test the hypothesis that APOE regulates the MSRN in the absence of cholesterol loading, we isolated macrophages from wild-type C57BL/6J mice. We then acutely lowered the cells’ APOE level by treating them with siRNA duplexes and monitored changes in APOE, CTSL, CYSC, and CLTC levels in the medium. As predicted from the proteomic analysis of Apoe−/− macrophages, immunoblot analysis confirmed that CTSL and CYSC levels fell when APOE release into medium was suppressed, whereas the level of CLTC, a protein that was not regulated by APOE (Table S5), remained unchanged (Figure 5F). We obtained similar results when we isolated macrophages from Ldlr−/− mice, suggesting that LDLR is not an important regulator of the MSRN (Figure S2).

These observations provide evidence for coordinate regulation of MSRN proteins. As a further test, we treated peritoneal macrophages isolated from Ldlr−/− mice with control siRNA duplexes or siRNA duplexes specific for CTSL, CYSC, or C3. Then, we used immunoblotting to monitor the expression of ACTB, APOE, CLTC, CTSL, CYSC, and C3 protein in macrophage-conditioned media (Figure S3). siRNA-mediated knockdown of CYSC significantly reduced ACTB (p = 0.02), CLTC (p = 0.006), and C3 (p = 0.04) levels without altering APOE or CTSL levels. On the other hand, downregulation of CTSL lowered C3 expression (p = 0.04) without affecting ACTB, APOE, CLTC, or CYSC levels. Finally, reducing C3 expression elevated APOE levels (p = 0.05) and lowered CTSL expression (p = 0.003) but had no effect on ACTB, C3, CLTC, and CYSC levels. Taken together, these studies provide strong evidence for coordinate regulation of MSRN proteins.

### Statins Normalize the MSRN in Ldlr−/−, but Not Apoe−/−, Macrophages

Our observations indicate that APOE is an important regulator of the MSRN. Moreover, previous studies have demonstrated that simvastatin retards atherosclerosis in Ldlr−/− mice, mice but not in Apoe−/− mice (Wang et al., 2002). Thus, antiatherosclerotic interventions might normalize the network by restoring APOE levels.

To test this idea, we compared the effects of simvastatin therapy (100 mg/kg/day for 2 weeks) on the MSRN in macrophages isolated from Ldlr−/− and Apoe−/− mice fed a western diet. Statin therapy reversed the expression pattern of MSRN proteins in macrophages isolated from Ldlr−/− mice, but not in those of Apoe−/− mice (Figures 6A and 6B). This difference could not be explained by differential cholesterol accumulation because simvastatin therapy reduced macrophage cholesterol levels to a similar extent (~40%) in both genetic backgrounds.
These findings are consistent with the proposal that APOE regulates the MSRN to inhibit atherogenesis.

**Antiatherosclerotic Interventions Restore Lesion APOE Expression**

Taken together, our data suggest that restoring levels of macrophage APOE normalizes the MSRN expression pattern and that this normalization is a critical component of simvastatin’s action. We also found that APOE was one of the most strongly downregulated MSRN proteins in cells harvested from Ldlr−/− mice fed a western diet. To explore the possibility that simvastatin and rosiglitazone inhibit atherosclerosis partly by increasing APOE levels in artery wall macrophages, we used immunohistochemistry to investigate expression of APOE and the macrophage marker MAC2 in aortic lesions of Ldlr−/− mice fed a western diet for 14 weeks.

The lesions contained high levels of APOE. However, the bulk of immunoreactive material localized to their necrotic cores (Figures 6C and S5). Treating the animals for 2 weeks with simvastatin or rosiglitazone markedly increased macrophage APOE protein levels (simvastatin, p = 0.04; rosiglitazone, p = 0.03) without appreciably altering the amount of APOE detected in the necrotic core (Figures 6C, 6D, and S5). Collectively, these observations provide strong evidence that macrophage foam cells in the artery wall, like foam cells isolated from the peritoneal cavity of hypercholesterolemic Ldlr−/− mice, have abnormally low APOE levels that are normalized by antiatherosclerotic interventions.

**DISCUSSION**

In contrast to traditional biochemical studies that focus on individual proteins, our approach seeks to identify protein networks that coherently respond to environmental, pharmacological, and genetic interventions that promote cardiovascular disease. We therefore used tandem mass spectrometry in concert with bioinformatics to obtain a comprehensive view of the shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.

Networks of highly interconnected proteins can develop new, disease-causing properties when strongly perturbed (Lusis et al., 2008; Chen et al., 2008), and two lines of evidence suggest that modulating the expression of key MSRN proteins can influence atherosclerosis. First, relative to the overall shed and secreted proteome of macrophage foam cells generated in vivo. By determining the known physical interactions and biological functions of differentially expressed proteins, we identified the macrophage sterol-responsive network (MSRN), a highly integrated network of proteins that are coordinately regulated by sterol loading.
Ldlr sterol in vivo. We observed a similar reversal in macrophages atherosclerosis without affecting plasma cholesterol levels in this Apoe proposal that pharmacological interventions target the MSRN in tin or rosiglitazone treatment. Thus, our observations support the olemic Ldlr treated animals. These results imply that the APOE levels are readily detectable in both locations in statin- or rosiglitazone- the acellular necrotic core of atherosclerotic lesions but was failed to influence the expression of those genes in atheroscle- of cultured macrophages and foam cells generated in vivo. For have demonstrated that APOE secretion is upregulated by sterol loading with acetyl-LDL (Brown and Goldstein, 1983 ). In contrast, we found that APOE was one of the most downregu- lated proteins in the MSRN of macrophage foam cells harvested from the peritoneum. However, APOE expression increased markedly when we incubated cultured peritoneal macrophages of Ldlr–/– mice with acetyl-LDL (Figure S6). Other investigators have also noted significant differences in the expression patterns of cultured macrophages and foam cells generated in vivo. For example, Li et al. found that PPARα, PPARγ, and PPARγ agonists induced cultured macrophages to express ABCA1 and LXRα (Li et al., 2000). In striking contrast, all three agonists failed to influence the expression of those genes in atheroscle- rotic lesions or in foam cells derived from hypercholesterolemic mice. These observations support the proposal that macro- phages of Ldlr–/– mice loaded with cholesterol ester in vivo provide a relevant model for studying foam cells’ roles in atherosclerosis.

Because APOE is one of the most downregulated MSRN proteins, statins and thiazolidinediones might inhibit atherogen- esis, in part, by increasing APOE expression by artery wall macrophages. Indeed, in immunohistochemical studies of Ldlr–/– mice fed a western diet, APOE was readily visualized in the acellular necrotic core of atherosclerotic lesions but was essentially undetectable in macrophages. In contrast, it was readily detectable in both locations in statin- or rosiglitazone-treated animals. These results imply that the APOE levels are likely to be diminished in artery wall foam cells of hypercholesterolemic Ldlr–/– mice and that this effect is partly reversed by statin- or rosiglitazone treatment. Thus, our observations support the proposal that pharmacological interventions target the MSRN in Ldlr–/– mice and that the ability to regulate the network is one atheroprotective effect of statin and rosiglitazone in this model of hypercholesterolemia.

Unexpectedly, we found that expression of one-third of the MSRN proteins was dysregulated in macrophages isolated from Apoe–/– mice on the low-fat diet. These cells showed little evidence of sterol accumulation, implying that APOE modulates the expression of a subset of MSRN proteins and that the network is dysfunctional in macrophages lacking APOE. Genetic ablation of other highly interconnected proteins within the MSRN might similarly potentiate network dysregulation and atherogen- esis. This hypothesis is strengthened by the demonstration that mice deficient in ADFP (Paul et al., 2008), APOE (Bellotta et al., 1995), LPL (Babaev et al., 2000), LRP1 (Laper et al., 2000), LYZ (Liu et al., 2006), or MFGE8 (Ait-Oufella et al., 2007), all of which map to the network, have macrophage-specific phenotypes. Moreover, we found that attenuating levels of individual proteins in the MSRN with siRNA affected the expression of other network proteins.

Proteins involved in lipid binding, cytoskeletal regulation, and vesicle-mediated transport were overrepresented in the MSRN, and mice lacking proteins implicated in those functions have atherosclerotic phenotypes (Buono et al., 2002; Overton et al., 2007; Ait-Oufella et al., 2007; Feil et al., 2004). The network was also greatly enriched in proteins with known physical interactions, raising the possibility that macromolecular assemblies might partly account for the link between sterol-responsive proteins and functional categories. Remarkably, when macro- phage-conditioned medium was centrifuged at 100,000 × g, more than two-thirds of the MSRN proteins localized to the pellet, indicating that they likely resided in the microvesicle frac- tion. Colocalization in microvesicles, which transfer proteins and RNA between neighboring cells and have been implicated in thrombosis, cytokine release, and inflammation (Cocucci et al., 2009), would readily account for the enrichment of physical and functional interactions that we observed in MSRN proteins.

We therefore propose that the atherogenic actions of choles- terol-loaded macrophages are an emergent property that results when the normal balance of MSRN proteins in microvesicles is perturbed. We further suggest that certain dietary factors or genetic variations can disturb this network, thereby promoting vascular disease. By integrating mouse and human data, we hope to better understand the MSRN’s role in foam cell forma- tion, with the long-term goal of identifying therapeutic interven- tions for targeting networks rather than individual proteins.

**EXPERIMENTAL PROCEDURES**

**Macrophage Foam Cell Formation**

Male Ldlr–/– and Apoe–/– mice on the C57BL/6J genetic background received a chow (4% fat [w/w]; Picolab, Rodent diet 20:50503) or western diet (21% fat, 1.25% cholesterol; Harlan Teklad, #TD96121) for 14 weeks. For antiather- osclerotic interventions, animals were fed the western diet to which simvasta- tin (100 mg/kg/day; Merck & Co Inc.) or rosiglitazone (10 mg/kg/day; GlaxoSmithKline) were added during the last 2 weeks of the diet. Four inde- pendent biological replicates were obtained for each experimental condition. Peritoneal macrophages were harvested from the mice 5 days after thioglyco- late was injected. Cells were washed with phosphate-buffered saline (PBS), seeded into T-75 flasks (20 × 10⁶/flask), incubated at 37°C for 2 hr in serum-free Dulbecco’s minimum essential medium (DMEM), and washed three times with PBS. Macrophages were then cultured for 6 hr in DMEM.

Linton et al., 1995; Buono et al., 2002; Bengtsson et al., 2005; Overton et al., 2007; Liu et al., 2006; Ait-Oufella et al., 2007). In contrast, ADFP levels were enhanced in cholesterol-loaded macrophages, and transgenic expression of ADFP promotes atherosclerosis in mice (Paul et al., 2008). These observations further support our proposal that MSRN proteins are coordinately regulated and that network dysregulation is important for foam cell formation and atherogenesis.

Second, treating Ldlr–/– mice with rosiglitazone, which retards atherosclerosis without affecting plasma cholesterol levels in this genetic background (Li et al., 2000), reversed the protein expression pattern that is seen when macrophages are loaded with sterol in vivo. We observed a similar reversal in macrophages isolated from statin-treated Ldlr–/– mice, but not in macro- phages isolated from statin-treated Apoe–/– mice. Importantly, statins are known to inhibit atherosclerosis in Ldlr–/– mice, but not Apoe–/– mice (Wang et al., 2002). Thus, our results with two different pharmacological interventions in two different genetic backgrounds support a model in which sterol-responsive proteins in macrophages form a highly interconnected network, the MSRN, that promotes atherosclerosis when dysregulated.

APOE, which plays a key role in macrophage cholesterol homeostasis, is a particularly striking example of an MSRN protein. When this protein is missing from macrophages, mice suffer from accelerated atherosclerosis (Bellotta et al., 1995; Linton et al., 1995). Classical studies of cultured macrophages have demonstrated that APOE secretion is upregulated by sterol loading with acetyl-LDL (Brown and Goldstein, 1983). In contrast, we found that APOE was one of the most downregulated proteins in the MSRN of macrophage foam cells harvested from the peritoneum. However, APOE expression increased markedly when we incubated cultured peritoneal macrophages of Ldlr–/– mice with acetyl-LDL (Figure S6). Other investigators have also noted significant differences in the expression patterns of cultured macrophages and foam cells generated in vivo. For example, Li et al. found that PPARα, PPARγ, and PPARγ agonists induced cultured macrophages to express ABCA1 and LXRα (Li et al., 2000). In striking contrast, all three agonists failed to influence the expression of those genes in atheroscle- rotic lesions or in foam cells derived from hypercholesterolemic mice. These observations support the proposal that macro- phages of Ldlr–/– mice loaded with cholesterol ester in vivo provide a relevant model for studying foam cells’ roles in atherosclerosis.
Protein Isolation
Macrophage-conditioned medium (~10 μg protein/ml) was collected, clarified by centrifugation (5 min at 1000 × g), supplemented with 0.02% sodium deoxycholate and 20% trichloroacetic acid, and incubated overnight at 4°C. Proteins were harvested by centrifugation (15,000 × g for 30 min at 4°C). The protein pellet was washed twice with ice-cold acetone, reconstituted in digestion buffer (0.1% Rapigest [Waters Corp.], 50 mM Tris-HCl [pH 8.8]), and then reduced, alkylated, and digested overnight at 37°C with sequencing grade trypsin (1:50, w/w, trypsin:protein; Promega). Tryptic digests were mixed with acetic acid (1:1, v/v) and subjected to solid-phase extraction on a C18 column (HLB, 1 ml; Waters Corp.) according to the manufacturer’s protocol. Fractions containing peptides were dried under vacuum and resuspended in 0.3% acetic acid/5% acetonitrile (1 mg protein/ml) for analysis.

Liquid Chromatography-Electrospray Ionization-Tandem MS
Tryptic digests (2 μg protein) were injected in duplicate into a trap column (Paradigm Platinum Peptide Nanotrap, 0.15 × 50 mm; Michrom Bioresources, Inc.) and desalted for 5 min with 5% acetonitrile, 0.1% formic acid (50 μl/min). Peptides were then eluted onto an analytical reverse-phase column (0.150 × 150 mm, 5 μm beads; Magic C18AQ, Michrom Bioresources, Inc.) and separated at a flow rate of 1 μl/min over 180 min, using a linear gradient of 5% to 35% buffer B (90% acetonitrile, 0.1% formic acid) in buffer A (5% acetonitrile, 0.1% formic acid). Mass spectra were acquired in the positive ion mode, using electrospray ionization and a linear ion trap mass spectrometer (LTQ, Thermo Electron Corp.) with data-dependent acquisition (Vaisar et al., 2007). MS/MS scans were obtained on the eight most abundant peaks in each survey MS scan.

Peptide and Protein Identification
MS/MS spectra were searched against the mouse International Protein Index (IPI) database (version 2006/04/18) (Kersey et al., 2004), using the SEQUEST search engine with the following search parameters: unrestricted enzyme specificity, 2.8 amu precursor ion mass tolerance, 1.0 amu fragment ion mass tolerance, fixed Cys alklylation, and variable Met oxidation. SEQUEST results were further validated with PeptideProphet (Keller et al., 2002) and ProteinProphet (Nesvizhskii et al., 2003), using an adjusted probability of ≥ 0.90 for peptides and ≥ 0.96 for proteins. Proteins considered for analysis had to be identified in every biological replicate of at least one biological condition. When MS/MS spectra could not differentiate between protein isoforms, all isoforms were included in the analysis.

Protein Quantification
Proteins detected by liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS) were quantified by spectral counting (the total number of MS/MS spectra detected for a protein). Two replicate injections for each sample were averaged to obtain spectral counts.

Microvesicles
Microvesicles were isolated from macrophage-conditioned medium, using differential centrifugation (Hegmans et al., 2004). Conditioned medium was preclarified by centrifugation at 1000 × g for 10 min, two spins at 4000 × g for 10 min, and one spin at 10,000 × g for 30 min. Microvesicles were isolated from the supernatant by ultracentrifugation (two 100,000 × g spins for 1 hr). The protein pellet was washed with PBS after each spin. Microvesicles were reconstituted in digestion buffer (0.1% Rapigest [Waters Corp.], 50 mM Tris-HCl [pH 8.8]), digested with trypsin, and analyzed with MS/MS. Results are the average of duplicate analyses of four pooled microvesicle isolations for each condition. Differences in spectral counts between the conditions were assessed with the G-test (Sokal and Rohlf, 1995).

Biochemical Assays
Cholesterol levels were determined using cholesterol oxidase and a fluorescence-based peroxidase assay (Invitrogen). Protein concentration of macrophage-conditioned medium was determined by the Bradford assay, with albumin as the standard.

siRNA
Adhesion-purified peritoneal macrophages from wild-type C57BL/6J mice were transfected with control siRNA (30 nM, Ambion) or two siRNAs specific for APOE (30 nM, Ambion) using the DelivEX Plus siRNA transfection kit (Pamomics). Cells were washed three times with PBS and incubated in serum-free DMEM for 24 hr. Macrophage-conditioned medium was then collected, and levels of APOE, CTSL, CYSC, and CLTC were quantified by immunoblot analysis.

Immunoblot Analysis
Macrophage-conditioned medium was subjected to SDS-PAGE on 4%–12% gradient gels, transferred to PVDF membranes, and probed with antibodies (0.5 μg/ml) raised against murine ACTB (Abcam), ADFP (Abcam), APOE (Abcam), C3 (Abcam), CTSL (R&D Systems), CYSC (Abcam), and LR1P (Abcam). Proteins were quantified by densitometry, using Quantity One software (Biorad).

Analysis of Atherosclerotic Lesions
For immunocytochemical staining, deparaffinized sections from lesions in the aortic sinus were rinsed in PBS, incubated for 10 min in Peroxo-Block (Zymed Laboratories Inc., San Francisco, California, USA), and boiled for 10 min in 0.01 M citrate buffer (pH 6.0). Adjacent sections were incubated overnight at 4°C with rat anti-MAC2 (Cedarlane, 1:2000) or rabbit anti-APOE (Abcam, 1:500) antibody in PBS containing 1% BSA. Antibodies were detected with a peroxidase-chromagen system (Dako NovaRed) in sections counterstained with hematoxylin and eosin. APOE and MAC2 immunoreactivity were quantified in lesions using Image Pro Plus (Media Cybernetics).

Functional Annotation
Functional enrichments in gene ontology annotations in the MSRN, relative to the mouse genome, were identified using the Bingo 2.0 plug-in in Cytoscape (V2.5.2) (Maere et al., 2005). Statistical significance was assessed using the Fisher’s exact test with Benjamini-Hochberg false discovery rate correction (Benjamini and Hochberg, 1995). To identify macrophage proteins associated with an atherosclerotic phenotype in mice, we conducted keyword searches, using PubMatrix (Becker et al., 2003). The search term was “protein name,” and the modifier terms were “atherosclerosis and knockout,” “atherosclerosis and deficiency,” and “atherosclerosis and expression.” All positive results were verified by manual inspection.

Protein Interaction Networks
Protein interaction networks were established from a manually compiled database based on > 200,000 full-text, peer-reviewed articles with Ingenuity Systems (Calvano et al., 2005). Interactions were validated using the BIND, DIP, MINT, IntAct, BioGRID, and MINT databases.

Statistical Analysis
Spectral count differences between foam cells and control cells were assessed using a two-tailed Student’s t test and the G-test (Old et al., 2005). The G-test evaluates the null hypothesis that observed values are due to random sampling from a distribution with a given expected value, using an approximation of the χ² distribution with one degree of freedom. Permutation analysis was used to empirically estimate the false positive rate. The false discovery rate, FDR, was estimated as the ratio of the false positive rate to that of the sum of the false and true positive rates (Benjamini and Hochberg, 1995). Significance cutoff values for the G-statistic and t test were determined by minimizing the empirical FDR and maximizing the number of differentially expressed proteins.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and five tables and can be found with this article online at doi:10.1016/j.cmet.2010.01.003.

ACKNOWLEDGMENTS
We thank Alan Attie (University of Wisconsin) and Stan Fields and Bill Parks at the University of Washington for critical review of the manuscript. This
research was supported by grants from the National Institutes of Health (HL030086, HL018645, HL074223, HL075827, HL092969, HL086798). L.B. and T.V. were supported by a Canadian Institutes of Health Research Fellowship Award (L.B.) and a Pilot and Feasibility Award (T.V.) from the Diabetes and Endocrinology Research Center. Mass spectrometry experiments were supported by the Mass Spectrometry Resource (Department of Medicine), the Proteome Resource (School of Medicine), and the Mass Spectrometry Core (Diabetes and Endocrinology Research Center) at the University of Washington.

Received: August 12, 2009
Revised: December 16, 2009
Accepted: January 14, 2010
Published: February 2, 2010

REFERENCES


