The Impact of Macrophage Insulin Resistance on Advanced Atherosclerotic Plaque Progression

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The Impact of Macrophage Insulin Resistance on Advanced Atherosclerotic Plaque Progression

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Abstract: Atherothrombotic vascular disease is the major cause of death and disability in obese and diabetic subjects with insulin resistance. Although increased systemic risk factors in the setting of insulin resistance contribute to this problem, it is likely exacerbated by direct effects of insulin resistance on the arterial wall cells that participate in atherosclerosis. A critical process in the progression of subclinical atherosclerotic lesions to clinically relevant lesions is necrotic breakdown of plaques. Plaque necrosis, which is particularly prominent in the lesions of diabetics, is caused by the combination of macrophage apoptosis and defective phagocytic clearance, or efferocytosis, of the apoptotic macrophages. One cause of macrophage apoptosis in advanced plaques is activation of a proapoptotic branch of the unfolded protein response, which is an endoplasmic reticulum stress pathway. Macrophages have a functional insulin receptor signaling pathway, and downregulation of this pathway in the setting insulin resistance enhances unfolded protein response–induced apoptosis. Moreover, other aspects of the obesity/insulin-resistance syndrome may adversely affect efferocytosis. These processes may therefore provide an important mechanistic link among insulin resistance, plaque necrosis, and atherothrombotic vascular disease and suggest novel therapeutic approaches to this expanding health problem. (*Circ Res*. 2010;106:58-67.)

Key Words: atherosclerosis ■ insulin resistance ■ diabetes ■ macrophages ■ apoptosis

The incidence of insulin resistance, metabolic syndrome, and type 2 diabetes is rising rapidly because of the epidemic of obesity in the industrialized world.[1] Although a number of disease processes are associated with insulin resistance and type 2 diabetes, the leading cause of morbidity and mortality is cardiovascular disease.[2] An important factor in accelerated heart disease in type 2 diabetes is likely to be insulin resistance and hyperinsulinemia. For example, the risk of cardiovascular disease is increased in metabolic syndrome, which is characterized by insulin resistance without overt hyperglycemia.[3–5] Moreover, rapid weight gain during childhood leads to hyperinsulinemia and increased coronary artery

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disease risk in adult life.[6] Part of the association between insulin resistance and cardiovascular disease is related to associated risk factors, including dyslipidemia (increased very low–density lipoprotein, reduced high-density lipoprotein, and possibly altered low-density lipoprotein [LDL]), hypertension, and a prothrombotic state.[3] However, insulin resistance may have direct proatherogenic effects at the level of the arterial wall, and an emerging concept that will be explored in this review is that insulin resistance in lesional macrophages promotes a series of cellular events critical for advanced plaque progression. After a brief review of atherogenesis, we will focus on new findings related to plaque progression and the role of macrophage insulin resistance that have appeared in the literature since the last review of this topic in this journal in 2007.[7]

Non-standard Abbreviations and Acronyms

Apoe	apolipoprotein E
CaMKII	calcium/calmodulin-dependent protein kinase II
CHOP	CEBP-homologous protein
EPA	eicosapentanoic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
Insr	insulin receptor
IP3	inositol 1,4,5-triphosphate
LDL	low-density lipoprotein
Ldlr	LDL receptor
MEK	MAPK/ERK kinase
Mertk	c-mer tyrosine kinase
NF-κB	nuclear factor κB
PRR	pattern recognition receptor
STAT	signal transducer and activator of transcription
SERCA	sarco-/endoplasmic reticulum calcium-dependent ATPase
UPR	unfolded protein response

Principles of Atherogenesis

Plaque Initiation and Progression

Atherogenesis begins with the retention of atherogenic lipoproteins in the subendothelium of susceptible areas of the arterial tree.[8] In response to these retained lipoproteins, particularly those that undergo atherogenic modifications such as oxidation and aggregation, a series of biological and maladaptive inflammatory responses ensue: (1) monocytes and other inflammatory cells enter the intima; (2) monocytes differentiate into macrophages, which then ingest retained and modified lipoproteins and become cholesteryl ester–loaded foam cells; (3) macrophages and other inflammatory cells contribute to a state of inflammation that fails to properly resolve; and (4) smooth muscle cells populate the intima, leading to collagen synthesis.[9–12] At this stage, the plaques are usually asymptomatic because of outward remodeling of the artery to preserve lumenal blood flow and a fibrous cap that protects the lesion from disruption.[13,14] However, some of these plaques, unrelated to plaque size per se, may undergo necrotic breakdown, thinning of the fibrous cap, a heightened state of inflammation, and an accumulation of unesterified cholesterol.[13–19] Many of the hallmarks of impaired inflammation resolution are evident in these plaques, including continued entry and poor egress of inflammatory cells, defective clearance of apoptotic cells, and a suppressed fibrotic "scarring" response.[12,20] These so-called "vulnerable plaques" are at risk for plaque disruption through fibrous cap rupture or endothelial erosion, which in turn can trigger acute thrombosis. If the thrombosis is extensive and not quickly resolved, acute vascular occlusion and tissue infarction occurs, leading to acute myocardial infarction, unstable angina, sudden cardiac death, or stroke.

The exact mechanisms of plaque disruption are not known. Cap thinning per se may be caused by a combination of protease-mediated digestion of extracellular matrix molecules, particularly by matrix metalloproteinases, and decreased collagen synthesis, perhaps exacerbated by death of the collagen-synthesizing cells in the intima.[13] These processes, as well as coagulation and thrombosis, are likely promoted by inflammatory cytokines, many of which are secreted by lesional macrophages.[13] Lesional necrosis of vulnerable plaques, which is caused by the combination of macrophage death and defective phagocytic clearance, or "efferocytosis," of dead macrophages,[21–23] can promote plaque disruption by a number of mechanisms.[15,22,24–27] For example, although matrix proteases are secreted by living macrophages in lesions, they may also be released by dead and dying macrophages.[28] Moreover, lesional necrosis triggers a heightened state of inflammation, which, as mentioned above, promotes matrix metalloproteinase secretion, coagulation, and thrombosis.[29] Finally, the necrotic core is rich in lipids and poor in cells and extracellular matrix, and the structural properties resulting from this composition are thought to contribute to mechanical stresses in the overlying cap, which may contribute to cap rupture.[30] Thus, macrophage death and defective clearance of the dead cells, leading to lesional necrosis, are important processes in the formation of the vulnerable plaque, and, as described in this review, exacerbations of these processes may help explain accelerated atherothrombotic disease in insulin-resistant states.

Mechanisms and Consequences of Macrophage Death and Defective Efferocytosis in Advanced Atheromata

To understand how insulin resistance may promote advanced plaque progression in general, and plaque necrosis in particular, it is necessary to review our latest understanding of the mechanisms and consequences of macrophage death in advanced atheromata. A number of hypotheses have been conceived to explain advanced lesional macrophage apoptosis, and undoubtedly more than one mechanism is involved. Examples include growth factor deprivation, toxic cytokines, and oxidized lipids or lipoproteins,[31] but there is as yet little proof for these ideas in vivo. Recent mechanistic data in cultured cells and correlative and genetic causation evidence in vivo support a role for endoplasmic reticulum (ER) stress in advanced lesional macrophage apoptosis and its major consequence, plaque necrosis. As had been previously dem-

onstrated in other models of ER stress–induced apoptosis, macrophages subjected to ER stress undergo apoptosis in a manner that is partially dependent on the CEBP-homologous protein (CHOP) (GADD153 [growth arrest and DNA damage]) branch of the ER stress pathway known as the unfolded protein response (UPR).[32,33] CHOP-mediated apoptosis can be modeled in cultured macrophages by either potent inducers of ER stress or by the combination of more subtle ER stressors and a "second hit." An example of an atherosclerosis-relevant inducer of single-hit ER stress apoptosis is 7-ketocholesterol,[33,34] the most abundant oxysterol in advanced atherosclerotic lesions. Examples of the two-hit model include the combination of low-level ER stressors with pattern recognition receptor (PRR) ligands, such as modified lipoproteins.[35,36] Another example of the two-hit model is incubation of macrophages with atherogenic lipoproteins under conditions of genetic or pharmacological inhibition of intracellular cholesterol re-esterification.[37,38] In this model, which is often referred to as the "free cholesterol" model and is designed to mimic free cholesterol–loaded macrophages in advanced atheromata,[39,40] the ER stress hit is provided by excess accumulation of unesterified cholesterol in the ER membrane, and the second hit is activation of PRRs by the lipoproteins themselves. The contribution of the second hit to apoptosis involves both amplification of proapoptotic pathways and suppression of cell-survival pathways that are activated in ER-stressed cells.[36] The tendency of macrophages to undergo apoptosis when subjected to ER stress in combination with PRR activation may have evolved as a host defense mechanism against intracellular organisms that require living macrophages to survive.

Although it has been known that activation of the CHOP pathway of the UPR can cause apoptosis, the molecular mechanisms linking CHOP to death execution pathways is poorly understood. Recent work in our laboratories has provided evidence for a calcium-dependent mechanism in ER stress–induced macrophage apoptosis. ER stress in macrophages leads to the release of calcium from the ER lumen into the cytosol.[32] The cytosolic calcium chelator BAPTA-AM [acetoxymethyl ester of 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] can block ER stress–induced apoptosis in macrophages, and recent work has shown that a key integrator of cytosolic calcium and death execution in these cells is a calcium-responsive kinase called calcium/calmodulin-dependent protein kinase (CaMK)II.[36,41,42] Activation of CaMKII leads to multiple death pathways, including induction of the cell-surface death receptor Fas; stimulation of mitochondrial calcium uptake and release of proapoptotic cytochrome c from the mitochondria; activation of proapoptotic STAT-1 (signal transducer and activator of transcription 1); and accumulation of reactive oxygen species through activation of NADPH oxidase.[42] CHOP amplifies this calcium-death pathway by leading to activation of inositol 1,4,5-triphosphate (IP3) receptors, which are calcium-release channels in the ER membrane.[43] The mechanism involves oxidative activation of IP3 receptor by the downstream CHOP transcriptional target, ER oxidase-1α. Net calcium release can also be promoted through inhibition of sarco-/endoplasmic reticulum calcium-dependent ATPase (SERCA), which pumps calcium back into the ER lumen. SERCA is inhibited by alterations in the ER membrane by certain ER stressors, such as unesterified cholesterol or saturated fatty acids,[44] and SERCA is downregulated in the setting of insulin resistance, as will be summarized below.

Macrophage apoptosis by itself would not be expected to be detrimental, because apoptotic cells are normally cleared rapidly by phagocytosis ("efferocytosis") in a manner that prevents postapoptotic cellular necrosis and that promotes antiinflammatory processes.[27] Indeed, manipulations that accelerate early lesional macrophage apoptosis decrease lesion cellularity and plaque progression, and vice versa,[23,45] suggesting that efferocytosis is very efficient in the early stages of atherogenesis. This principle has been applied recently to a mouse model of type 2 diabetes and early atherosclerosis.[46] However, in the later stages of atherosclerosis, macrophage apoptosis is associated with plaque necrosis,[23] and there is evidence in humans that efferocytosis is defective in advanced plaques.[47] The mechanisms of defective efferocytosis in advanced lesions are not known, but several interesting ideas have been advanced based on in vitro and in vivo observations. For example, oxidized lipids and proteins exist in these plaques, and some of these molecules can competitively inhibit efferocytosis by binding to efferocytosis receptors.[48] Thus, to the extent that these oxidized molecules accumulate as lesions progress,[10] they may reach a high enough level in advanced atheromata to take on this competitive inhibitory role. In another scenario, the efferocytosis receptor Mertk (c-mer tyrosine kinase) has been shown to play a role in efferocytosis and plaque necrosis in mouse lesions,[49,50] and inflammation-induced cleavage of this receptor by membrane sheddases[51] may contribute to defective clearance of apoptotic cells in advanced plaques. The fact that inflammation increases as lesions progress may offer an explanation as to why this antiefferocytic process occurs only in advanced plaques.

The concept that ER stress–induced macrophage apoptosis in combination with defective efferocytosis in advanced lesions promotes plaque necrosis is supported by a number of genetic-causation studies in mice and by correlative studies in humans. In fat-fed apolipoprotein E–null $(Apoe)^{-/-}$ or LDL receptor–null $(Ldlr)^{-/-}$ mice, ER stress markers are induced as lesions progress.[32,52–56] Most importantly, genetic targeting of CHOP and STAT-1, the proapoptotic signaling transducer activated by the CHOP calcium–CaMKII pathway (above), as well as prevention of cholesterol-induced ER damage, inhibit advanced lesional macrophage apoptosis and plaque necrosis.[33,42,52] Moreover, deletion of two "second-hit" PRRs (SRA and CD36) decreases macrophage apoptosis and plaque necrosis in the lesions of fat-fed $Apoe^{-/-}$ mice.[57] In humans, there are close correlations among markers of ER stress, apoptosis, and plaque vulnerability in coronary arteries.[55] In terms of efferocytosis, studies have shown an increase in plaque necrosis that correlates with a worsening of lesional efferocytosis in several mouse models in which efferocytosis effectors have been targeted, including c-mer tyrosine kinase, MFG-E8 (milk fat globule epidermal growth factor 8), transglutaminase-2, and complement factor C1q.[49,58] In summary, in vitro and in vivo evidence support a model in which

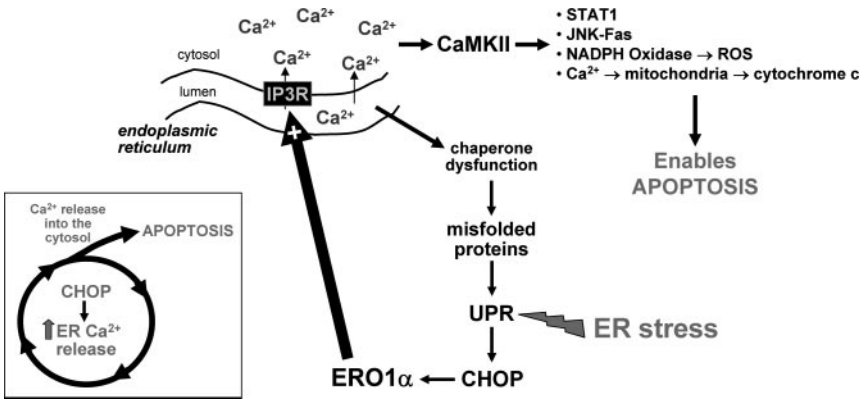

Figure 1. A CHOP–calcium pathway of ER stress–induced apoptosis in macrophages. A diverse array of ER stress–provoking events, many of which exist in advanced atheromata, triggers the UPR and leads to induction of the downstream effector CHOP. CHOP induces ER oxidase-1α (ERO1α), which in turn oxidatively activates IP3 receptor (IP3R) calcium release channels in the ER. IP3 receptor–mediated calcium release begins a proapoptotic cascade involving activation of CaMKII by cytosolic calcium and subsequent downstream apoptotic processes, as listed in the figure and as described in the text. In addition, the resulting low level of calcium in the ER lumen likely causes dysfunction of calcium-dependent protein chaperones, which amplifies UPR activation. The central concept is that proapoptotic CHOP functions, at least in part, by promoting calcium-induced death as part of a positive feedback cycle (see inset).

macrophage apoptosis in advanced lesions, induced in part by a proapoptotic ER stress–calcium pathway, plus defective efferocytosis promote plaque necrosis (Figure 1). Because plaque necrosis is strongly associated with disrupted plaques and acute lumenal thrombosis,[59] and because plaque necrosis is particularly prominent in atherosclerotic lesions from diabetic subjects, as described in the following section, these insights should be useful in our understanding of and therapeutic approaches to accelerated plaque progression in the setting of insulin resistance.

Macrophage Death and Plaque Progression in Insulin Resistance

Plaque Necrosis in Human Diabetic Coronary Artery Lesions

It is now well-established that type 2 diabetes and insulin resistance are major risk factors for atherothrombotic vascular disease.[3–5] Although many theories have arisen to explain this relationship,[60,61] a common end point of plaque progression associated with atherothrombotic vascular disease, as mentioned in the previous section, is plaque necrosis. In this context, a number of independent studies have found that advanced atherosclerotic lesions in diabetic subjects are characterized by particularly large necrotic cores when compared to similarly sized lesions from nondiabetic individuals.[62–67] For example, Burke et al[62] found that necrotic core size in the coronary arteries of subjects who died suddenly was positively correlated with the presence of diabetes independently of other factors. Similar results were found when coronary atherectomy specimens of diabetics and nondiabetics were compared.[63] Nasu et al[64] used virtual histology based on intravascular ultrasound data to assess coronary arterial necrotic cores in nondiabetic and diabetic patients with stable angina and found an approximate 50% increase in the percent area covered by necrotic cores in the diabetic group. Almost identical findings were reported in similar studies conducted by Hong et al[65] in Korea and Pundziute et al[66] in the Netherlands. A prospective study of subjects with coronary artery disease in which radiofrequency data from intravascular ultrasound was used to assess necrotic core size in coronary arteries found that only diabetes and age were associated positively with necrotic core size in logistic regression analysis.[67] These collective data raise the issue as to whether the cellular events described in the previous sections, particularly advanced lesional macrophage apoptosis and/or defective efferocytosis, are enhanced in the setting of diabetes, leading to increased plaque necrosis and, ultimately, accelerated atherothrombotic vascular disease.

The Effect of Insulin Resistance on Macrophage Death Pathways

In view of the role of insulin resistance in diabetic heart disease and the larger necrotic cores in the coronary arteries of diabetic subjects, we and others have examined how insulin resistance at the level of the macrophage affects mechanisms and consequences of macrophage death in vitro and in vivo. Macrophages have insulin receptors, and acute exposure of the cells to insulin in vitro results in phosphorylation of the insulin receptor, insulin receptor substrate-2, and Akt, leading, among other responses, to nuclear exclusion and inactivation of FoxO transcription factors.[7,68] Moreover, pretreatment of macrophages in vitro with high-dose insulin leads to downregulation of their insulin receptors and suppression of insulin receptor signaling, which is also observed in freshly isolated macrophages from insulin-resistant mice, such as the hyperinsulinemic leptin-deficient *ob*/*ob* mouse.[68] Thus, macrophages show the hallmarks of "insulin resistance" at a cellular level in the setting of high insulin concentrations.

Macrophages rendered insulin resistant through preincubation with insulin, genetic deletion of the insulin receptor, or pharmacological inhibition of insulin signaling, and macrophages freshly isolated from hyperinsulinemic mice, show an increase in the levels of the scavenger receptor SRA.[7,68] As mentioned in the previous section, SRA can serve as a "second-hit" PRR in ER stress–induced macrophage apoptosis both in vitro and in advanced lesional macrophage death and plaque necrosis in vivo. In this regard, insulin-resistant macrophages show markedly enhanced apoptosis in vitro when exposed to ER stress conditions plus an SRA-mediated second hit, as is the case with macrophages loaded with lipoprotein-derived unesterified cholesterol.[56,69,70]

ER stress in macrophages triggers compensatory cell-survival pathways, notably those activated by Akt and nuclear factor (NF)-κB, and apoptosis is temporally correlated with a

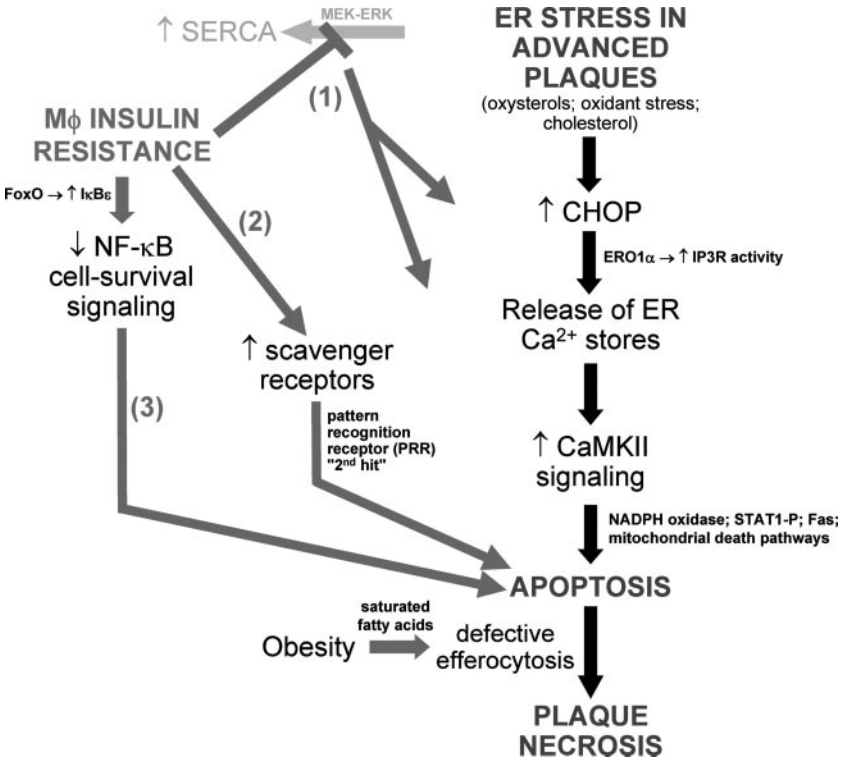

Figure 2. Cellular molecular mechanisms by which macrophage insulin resistance promotes ER stress–induced macrophage apoptosis and advanced plaque progression. At least 3 proapoptotic processes are enhanced in ER stressed macrophages: (1) ER stress normally activates a compensatory MEK-ERK-SERCA pathway to lower cytoplasmic calcium and replenish ER lumenal stores. This pathway is blocked in the setting of insulin resistance, leading to enhanced activation of calcium-mediated apoptotic pathways (increased cytosolic calcium) and further UPR-CHOP activation (decreased ER lumenal calcium); (2) pattern recognition receptors such as scavenger receptors are upregulated in insulin-resistant macrophages and, when activated, are synergistic with ER stress in inducing apoptosis (second hit concept); (3) increased nuclear FoxO in insulin-resistant macrophages induces IκBε, thereby suppressing a compensatory NF-κB cell survival pathway. In addition to these proapoptotic processes, increased levels of saturated fatty acids in the setting obesity compromise the ability of macrophages to engulf apoptotic cells. Apoptotic cells that are not efficiently cleared become secondarily necrotic and, over time, accumulate into necrotic cores in advanced plaques. These necrotic cores, which are particularly large in diabetic atheromata, are thought to contribute to plaque disruption. See text for details.

downregulation of these pathways and can be accelerated by their inhibition.[36,71,72] Moreover, Akt deficiency in *Apoe*$^{-/-}$ mice was shown to enhance lesional macrophage apoptosis and inflammation and plaque progression.[73] In this context, an important observation was that phosphorylation of Akt is suppressed in ER-stressed, insulin-resistant macrophages.[69,71] Consistent with a decrease in Akt phosphorylation, Senokuchi et al[71] found an increase in nuclear FoxO1 in insulin-resistant, ER-stressed macrophages, which normally translocates to the cytoplasm in response to Akt-dependent phosphorylation.[74] Moreover, macrophages genetically lacking FoxO1, 3 and 4, were resistant to ER stress–induced apoptosis.[71] However, FoxO-overexpression experiments indicated that nuclear localization of these transcription factors was not by itself sufficient for macrophage apoptosis but rather led to an enhancement of apoptosis in the setting of ER stress. The apoptosis-enhancing mechanism of FoxO1 is directly related to the role of another compensatory cell-survival factor in ER-stressed macrophages, namely NF-κB.[71,72,75,76] In ER-stressed macrophages, FoxO1 induces the expression of the NF-κB inhibitor IκBε and thereby enhances apoptosis.[71]

Importantly, insulin resistance potentiates the ER stress response itself.[77] ER stress in macrophages leads to activation of the mitogen-activated protein kinase extracellular signal-regulated kinase (ERK),[76] and Liang et al[77] found that this response was blunted in insulin-resistant macrophages. Additional studies revealed that the mitogen-activated protein kinase/ERK kinase (MEK-ERK) pathway induces SERCA,[77] which, as explained above, can abrogate ER stress by replenishing ER lumenal calcium stores and can protect macrophages from ER stress–induced apoptosis by lowering cytosolic calcium levels. Thus, the blunted MEK-ERK-SERCA pathway in insulin-resistant macrophages exacerbates the ER stress response and the calcium-mediated apoptosis pathway described above, and restoration of MEK1 in these cells is protective against both ER stress and apoptosis.[77]

In summary, mechanistic studies using various cell culture models of insulin-sensitive and insulin-resistant macrophages, including primary macrophages freshly harvested from *ob/ob* mice, have revealed an integrated pathway of cell signaling events responsible for the increased apoptotic response to ER stress in the setting of insulin resistance. Key among these events are those related to the compromise of compensatory cell survival pathways and the exacerbation of proapoptotic calcium signaling pathways (Figure 2).

The Effect of Macrophage Insulin Resistance on Murine Atherosclerosis

To test relevance of enhanced ER stress–induced apoptosis in insulin-resistant macrophages in vivo, irradiated *Ldlr*$^{-/-}$ mice were transplanted with bone marrow from insulin receptor (*Insr*)$^{+/+}$ or *Insr*$^{-/-}$ mice.[69] It should be noted that this proof-of-concept model represents the most extreme form of "insulin resistance." After recovery of the graft, the mice were fed a high-fat diet, and lesions were analyzed for overall area and, most importantly, plaque morphology. Consistent with the in vitro data, the advanced lesions of the *Insr*$^{-/-}$→*Ldlr*$^{-/-}$ mice fed the diet for 12 weeks had more apoptotic cells, particularly in macrophage-rich regions of the plaque, and more plaque necrosis than those of the *Insr*$^{+/+}$→*Ldlr*$^{-/-}$ control mice. Overall lesion area, the less important end point for the hypothesis being tested, showed

no change after 8 weeks of diet and only a modest increase after 12 weeks. Baumgartl et al[78] used the cre-lox system to create *Apoe*$^{-/-}$ with macrophage-targeted deficiency of insulin receptors. After 4 months on a high-fat diet, these mice had a modest decrease in lesion area compared with control *Apoe*$^{-/-}$ mice. Apoptotic cells and necrotic areas were not quantified. Immortalized macrophages derived from these mice had a marked reduction in LPS-induced interleukin-6 secretion. The authors also tested the effects of global and bone marrow-derived insulin receptor substrate-2 deficiency in fat-fed *Apoe*$^{-/-}$ mice. In the holo-knockout model, lesion area was modestly increased, and in the bone marrow transplant model, lesion area was modestly decreased. Plaque morphology was not quantified. The authors interpreted these data as showing that myeloid-derived insulin receptors suppress atherosclerosis by blunting the inflammatory response.[78] Senokuchi et al[71] also observed decreased inflammatory responses during ER stress in insulin-resistant macrophages. In that study, reduced NF-κB responses led to both increased apoptosis, as noted in the previous section, and decreased expression of some inflammatory genes. In summary, a careful comparison of Han et al[69] and Baumgartl et al[78] reveal a common finding of relatively modest effects of macrophage insulin resistance on overall lesion size, with subtle differences between the two studies perhaps arising from differences in genetic background (mixed versus inbred C67Bl/6J), diets used (Western-type diet versus the proinflammatory high-cholesterol/bile salt diet), and stage of lesion development. As noted, those specific features of atherosclerotic lesions related to the novel concept that insulin-resistant macrophages are more susceptible to apoptosis, ie, advanced lesional macrophage apoptosis and plaque necrotic area, were assessed in only one of the two studies, and the data supported that concept.[69]

Our laboratories have been working with another model of advanced lesional apoptosis and plaque necrosis in mice that may relate to the findings above. During certain types of ER stress, macrophages respond with activation of a compensatory cell-survival pathway in which the MAP kinase p38α enhances phosphorylation/activation of Akt, a potent survival signal in these cells (see previous section).[79] In essence, this ER stress–activated pathway delays or suppresses apoptosis, but eventually the survival pathway gets overwhelmed, and apoptosis ensues. As predicted by this concept, we found that gene-targeting of macrophage p38α partially impedes Akt activation and promotes ER stress–induced apoptosis both in vitro and in advanced plaques in fat-fed *Ldlr*$^{-/-}$ mice. Because insulin resistance also partially impedes Akt signaling, we reasoned that the two pathways might be additive. Indeed, treatment of macrophages from *Insr*$^{-/-}$;*Ldlr*$^{-/-}$ mice with an ER stressor plus a p38 inhibitor enhanced apoptosis to a very high level, ie, above the high level already seen when these macrophages are subjected to ER stress alone[79] (see previous section). These findings further demonstrate the importance of defective Akt signaling, a critical component of intact insulin receptor signaling, in ER stress–induced macrophage apoptosis and raise caution about the use of p38 inhibitors, currently under development as antiinflammatory agents in a number of diseases including type 2 diabetes,[80] in insulin-resistance subjects.

Two models of global insulin resistance have also shown an effect on plaque necrosis. A recent study examining Western diet-fed *ob/ob*;*Ldlr*$^{-/-}$ mice, which have obesity and insulin resistant secondary to leptin deficiency, showed an increase in necrotic core size compared to similarly fed *Ldlr*$^{-/-}$ mice.[81] As explained below, the mechanism not only involves increased susceptibility to apoptosis but also defective efferocytosis in the macrophages of these mice. Hsueh and colleagues[82] compared 3 months/old and 12 months/old *Ldlr*$^{-/-}$ mice fed a high-fat diet for 3 months. The older mice developed worse insulin resistance and worse atherosclerosis than the younger mice, and the lesions in the older mice appeared to be associated with a marked increase in plaque necrosis. The insulin-resistant older mice had a blunted antioxidant response that might be caused by a defective DJ-1–Nrf2 antioxidant pathway,[83] and a higher lesional expression of the NADPH oxidase subunit, p47. Atherosclerosis and plaque morphology were improved by treating the mice with the NADPH oxidase inhibitor and antioxidant, apocynin. One implication of these findings is that aging, a major risk factor for cardiovascular disease in humans,[84] may interact with insulin resistance to promote plaque necrosis, and in this regard it is interesting to note that aging is associated with both enhanced ER stress and defective efferocytosis.[85,86] Second, a critical downstream proapoptotic effector of ER stress and ER calcium release is activation of NADPH oxidase, and, given the pathways described in Figure 2, this response may be further enhanced in the setting of insulin resistance. Although vitamin E has not been shown to be effective in decreasing cardiovascular risk in humans,[87] more targeted antioxidants, such as NADPH oxidase inhibitors, in the specific setting of insulin resistance and possibly aging, may be more mechanistically justified and have more promise.

How Insulin Resistance Might Affect Efferocytosis

The increase in plaque necrosis in diabetic lesions raises the important issue as to whether efferocytosis is defective in these lesions and, if so, how this is mechanistically linked to insulin resistance. For example, defective phosphatidylinositol 3-kinase signaling in the setting of insulin resistance could, in theory, lead to a defect in efferocytosis in general and a specific defect in c-mer tyrosine kinase–mediated efferocytosis in particular.[88,89] Using an in situ assay that quantifies the percentage of apoptotic cells that have been engulfed by phagocytic macrophages versus not associated with phagocytes,[49] Li et al[81] found that the aortic root lesions of Western diet–fed *ob/ob*;*Ldlr*$^{-/-}$ mice had evidence of defective efferocytosis and, as predicted, increased plaque necrosis compared with lesions of Western diet–fed *Ldlr*$^{-/-}$ mice. In vitro studies showed that primary macrophages isolated from *ob/ob* mice have a defect in efferocytosis that was associated with defective PI3 kinase activity, but those from *Insr*$^{-/-}$ mice do not. Further studies revealed that the key defect in *ob/ob* macrophages was an increase in the saturated fatty acid:unsaturated fatty acid ratio in the macrophage membranes, perhaps through "stiffening" the plasma

membrane to the point where phagocytosis is compromised.[90] The efferocytosis defect on *ob/ob* macrophage could be corrected by treating the cells with the omega-3 polyunsaturated fatty acid eicosapentanoic acid (EPA), and similar results were found when macrophages were harvested from EPA-fed *ob/ob* mice. Most importantly, lesional efferocytosis was improved in *ob/ob*;$Ldlr^{-/-}$ mice by EPA feeding, which interestingly has also been associated with protection from heart disease in humans.[91] The precise mechanism of how saturated fatty acid impair efferocytosis and how EPA improves it is still under investigation, as are other possible links between insulin resistance and clearance of apoptotic cells. Nonetheless, we can begin to imagine an integrated model in which direct effects of insulin resistance on advanced lesional macrophage apoptosis, combined with defective efferocytosis caused by systemic fatty acid defects in the setting of insulin resistance, can at least partially explain the large neurotic cores and accelerated thrombotic vascular disease in diabetics (Figure 2).

Conclusions and Future Directions

This review focused on one key feature of type 2 diabetes, insulin resistance; one type of lesional cell, the macrophage; and one overall context of atherosclerosis, advanced plaque progression. Even within this focused area of research, more work is needed to further define mechanisms whereby insulin resistance affects specific signaling pathways involved in the panoply of atherosclerosis-relevant macrophage activities, including, interaction with lipoproteins and intracellular metabolism of lipoprotein-derived lipids; inflammation and the resolution thereof; stress responses, including oxidative, heat shock, and ER stress; secretion of proteases, procoagulant molecules, and other factors involved in plaque progression; phagocytosis, efferocytosis, and antigen presentation; apoptosis-cell survival balance; and interaction with other cells and extracellular matrix. Moreover, it is likely that insulin resistance affects these processes differently in different subsets of macrophages and in other types of myeloid cells, notably dendritic cells, mast cells, and neutrophils. A limitation of our in vivo studies has been the lack of a mouse model that fully recapitulates features of human plaque disruption and atherothrombosis,[92] and so further developments to improve mouse models of diabetic atherothrombotic vascular disease is an important goal. Nonetheless, it is becoming clear that key morphological features of such plaques are worsened by ER stress[33] and insulin resistance in macrophages.[69]

Beyond the specific areas of plaque macrophages, insulin resistance, and advanced plaque progression, other areas of focus may offer additional clues as to why heart disease is enhanced in type 2 diabetes.[61] For example, decreased insulin signaling in endothelial cells, through impaired Akt signaling, is also likely to have important proatherogenic consequences through decreased endothelial nitric oxide synthase activity and increased expression of inflammatory genes and vascular cell adhesion molecule-1.[73] In the liver, hyperinsulinemia and insulin signaling may increase VLDL secretion while having the opposite effects on LDL receptor expression.[93] The other major feature of type 2 diabetes, hyperglycemia, may promote plaque instability by enhancing the inflammatory response in macrophages through effects on plasma triglyceride-rich lipoproteins and free fatty acids.[94] Hyperglycemia may also cause endothelial cell abnormalities, including oxidative stress and RAGE-induced inflammation, that promote the earlier stages of atherogenesis.[95,96] Interestingly, there are recent data suggesting that hyperglycemia may exert some of its proatherogenic effects in endothelial cells through FoxO1 and also through the induction of ER stress.[97,98] These hyperglycemia–endothelial cell studies, together with the insulin resistance–macrophage studies described in this review, raise the interesting possibility that hyperglycemia may affect mostly the earlier stages of atherogenesis, whereas insulin resistance has its greatest effect on promoting advanced plaque progression. In this context, a recent analysis of the Veterans Affairs Diabetes Trial found that intensive glucose lowering reduced cardiovascular events in diabetics with a coronary artery calcium store <100 (multivariable hazard ratio [HR]$=0.08$, $P=0.03$), but not in those with a calcium score >100 (HR$=0.74$, $P=0.21$).[99] Smooth muscle cells, a key cell type in the generation of the "protective" fibrous cap in advanced lesions, and platelets, the final effector of acute vascular occlusion, may be affected by insulin resistance, hyperglycemia, or fatty acid abnormalities, which provide additional opportunities for investigation.[61] Continued progress in these areas will provide a more complete understanding of how multiple features of diabetes promotes heart disease.

The ultimate goal of these studies is to complement our current efforts at identifying and treating systemic risk factors that promote cardiovascular disease in diabetics. Despite the relative success of this strategy, risk is still very high,[100] and the tremendous scale of this epidemic is such that overall risk will still be high even if compliance is improved and the experimental modalities prove useful. Further understanding of the specific mechanisms of increased vascular disease in diabetics, particularly at the molecular level in arterial wall cells, may be a promising approach for further eradication in the future, and one that should be additive or even synergistic with reduction of lipid and other systemic risk factors. One approach is to increase insulin sensitivity in diabetic macrophages, such has been demonstrated recently using a peroxisome proliferator-activated receptor γ activator in vivo[68] and $1,25(OH)_2$ vitamin D in vitro.[101] Another approach is to develop agents to prevent ER stress or downstream proapoptotic processes in macrophages by pharmacological means, eg, through the use of chemical chaperones[102] or inhibitors of the calcium-mediated proapoptotic pathway.[103,104] Moreover, in view of the importance of defective efferocytosis in the generation of plaque necrosis and the *ob/ob* efferocytosis study described above, experimental therapeutic modalities designed to enhance efferocytosis[58,105] may be particularly useful in diabetics. Delivery of such drugs to plaques might be facilitated by specific vehicles targeted to plaques,[106] whereas clinical assessment in phase II and phase III studies could be assisted by imaging techniques, such as carotid MRI, that have the capacity to measure important plaque features such as necrotic core area and cap thickness.[107] Studies in these areas occurring in parallel with ongoing efforts at systemic risk reduction offer the best chance to curb

the growing epidemic of diabetes-associated atherothrombotic vascular disease.

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