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Is immunity a mechanism contributing to statin-induced diabetes?

Brandyn D Henriksbo and Jonathan D Schertzer*

Department of Biochemistry and Biomedical Sciences; McMaster University; Hamilton, ON Canada

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Abbreviations: HMGCR; HMG-CoA reductase, LDL; Low density lipoprotein, GGPP; geranylgeranyl pyrophosphate, IL; interleukin, CRP; C-reactive protein, TNF; Tumor necrosis factor, SNP; single nucleotide polymorphisms, NLRP3; NOD-like receptor family pyrin domain containing 3, PRR; pattern recognition receptors, BMDM; bone marrow derived macrophages, AMPK; AMP-activated protein kinase, PTEN; phosphatase and tensin homolog, SREBP; sterol response element-binding protein, P2X7; P2X purinoceptor 7, PI3K; Phosphoinositol-3-kinase, PIP_2; phosphatidylinositol 4,5-bisphosphate, PIP_3; phosphatidylinositol 3,4,5-trisphosphate, PDK1; phosphoinositide-dependent kinase-1, MAPK; Mitogen activated protein kinase, JNK; c-Jun N-terminal kinase, ERK; extracellular regulated mitogen-activated protein kinase, mtROS; mitochondrial reactive oxygen species, ATP; adenosine triphosphate, FTase; Farnesyltransferase, GGTase-I and GGTase-II; Geranylgeranyltransferase I and II, IR; insulin receptor.

Statins lower cholesterol and are commonly prescribed for prevention and treatment of cardiovascular disease risk. Statins have pleotropic actions beyond cholesterol lowering, including decreased protein prenylation, which can alter immune function. The general anti-inflammatory effect of statins may be a key pleiotropic effect that improves cardiovascular disease risk. However, a series of findings have shown that statins increase the pro-inflammatory cytokine, IL-1β, via decreased protein prenylation in immune cells. IL-1β can be regulated by the NLRP3 inflammasome containing caspase-1. Statins have been associated with an increased risk of new onset diabetes. Inflammation can promote ineffective insulin action (insulin resistance), which often precedes diabetes. This review highlights the links between statins, insulin resistance and immunity via the NLRP3 inflammasome. We propose that statin-induced changes in immunity should be investigated as a mechanism underlying increased risk of diabetes. It is possible that statin-related insulin resistance occurs through a separate pathway from various mechanisms that confer cardiovascular benefits. Therefore, understanding the potential mechanisms that segregate statin-induced cardiovascular effects from those that cause dysglycemia may lead to improvements in this drugs class.

*Correspondence to: Jonathan D Schertzer; Email: schertze@mcmaster.ca
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Statins, Cholesterol and Inflammation

Statins (such as Lipitor™, Crestor™, Lescol™, Pravachol™, Mevacor™, Zocor™ and Livalo™) are the most widely prescribed drug class in North America and are used for the prevention and treatment of cardiovascular disease risk.[1,2] Statins inhibit 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) a rate limiting step in the conversion of HMG-CoA to mevalonate, a precursor for several cellular processes. One result of HMGCR inhibition is reduced cholesterol biosynthesis and coupled with increased LDL-receptor mediated sequestration of LDL, statins are effective at lowering circulating LDL-cholesterol levels.[3,4] Statins have "pleotropic" actions beyond cholesterol lowering. Importantly, many cholesterol-independent, pleotropic actions of statins also involve inhibition of the mevalonate pathway.[5,6] Mevalonate is a precursor for the generation of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP). These isoprenoids are required for ubiquinone, sterols, dolichol and for prenylation of proteins, an irreversible addition of isoprenyl lipids that occurs on ~0.5% of cellular proteins.

Statins are generally anti-inflammatory. HMGCR inhibition and targeted decrease in protein prenylation predominantly result in skewing immune responses toward anti-inflammatory characteristics, an effect that can occur independently of cholesterol lowering.[5-9] This anti-inflammatory effect is thought to be a significant component of the efficacy of statin therapy and the reduction in C-reactive protein (CRP, an inflammatory marker) has been directly associated with a reduction of myocardial infarction risk.[10,11] This has fostered large scale efforts to understand the cholesterol- and inflammatory-mediated contributions to the efficacy of statins and testing of the "inflammatory hypothesis" to see if other non-statin, anti-inflammatory strategies can improve cardiovascular outcomes.[12] In addition to CRP, statins have been shown to reduce many inflammatory processes, including cell adhesion/migration and skewing cytokine profiles away from

pro-inflammatory (Th1) toward Th2 characteristics.[7] Statins reduce pro-inflammatory cytokines such as Tumor necrosis factor (TNF) and Interleukin (IL)-6.[13,14] However, statins are not simply anti-inflammatory and a series of findings, including our own, have demonstrated the paradoxical statin-induced increase in IL-1β secretion as a result of decreased protein prenylation in immune cells such as macrophages.[15-20]

Statins and Diabetes

Epidemiological evidence has revealed that several statins have been associated with small, but significant increased risk of new onset diabetes.[21,22] It is not yet clear if higher potency statins (often defined in terms of greater cholesterol lowering) equate to increased diabetes risk.[23,24] Also, there is not yet clear consensus if the use of specific statins confer greater risk.[21,25,26] The importance and relevance of statin-induced diabetes is hotly debated. All drugs have side effects and there are many factors to consider in this debate, including the prodigious evidence regarding the benefits of statin therapy for reducing major cardiovascular events and all-cause mortality.[27,28] It is also important to carefully consider the message that should be given to patients regarding statin therapy.[29] Much of the debate pertains to the cost/benefit analysis of statins. It has been argued that the cumulative benefits of statins outweigh the potentially small rise in new onset diabetes or other side effects such as myopathy.[30,31] This is a worthwhile debate for clinical practice; however, it is surprising that a drug class that improves blood lipid profiles and is largely anti-inflammatory does not improve blood glucose control, but can actually worsen it.

Why not try to discover ways to remove the risk of statin-related rise in blood glucose and limit the increased risk of diabetes? Understanding the potential mechanisms that separate the benefits of statins from those that cause dysglycemia may lead to improvements in this drugs class. This is increasingly relevant since statin use in primary prevention is expanding and raising concerns.[32,33] Understanding the cholesterol-dependent and pleotropic effects of statins may allow therapeutic strategies that separate biological pathways that control blood glucose from those that provide cardiovascular benefits. Such strategies may reduce diabetes incidence and actually improve the efficacy of statins since dysglycemia is an independent risk factor for premature death.[34] How do we start to separate the actions of statins on glycaemia versus those on cholesterol and other potentially beneficial pleotropic actions? Genetic analysis of single nucleotide polymorphisms (SNP) in the *HMGCR* gene across several statin trials demonstrated that the actions of statins on HMGCR inhibition partially explain the increased risk of type 2 diabetes.[35] This reinforces the importance of the mevalonate pathway, but does not show that the cholesterol lowering effect of statins are linked to risk of diabetes. Interrogation of other effectors that are dependent on the mevalonate pathway and relevant to blood glucose control is warranted.

The balance between insulin sensitivity and insulin secretion controls blood glucose. If increasing insulin resistance (i.e. ineffective insulin action in target cells) is not matched by increased insulin secretion, blood glucose rises and this is typical of the progression to type 2 diabetes. Inflammation has emerged as a critical factor in promoting insulin resistance and often precedes the development of type 2 diabetes. The inflammatory underpinnings of insulin resistance (and commonalties with cardiovascular disease) have been reviewed.[36-38] Much of this work is focused on the immunometabolism of obesity, a major risk factor of insulin resistance and consequent type 2 diabetes. An important task in this area is to understand how shared elements of nutrient and bacterial/pathogen sensing systems propagate obesity-related insulin resistance. In addition to nutrients, and bacterial factors (i.e., the microbiota), we should consider how drugs may engage the immunometabolism responses relevant to type 2 diabetes. Pattern recognition receptors of the innate immune system, such as Toll-like receptors and Nod-like receptors are a key point of convergence that can link immune responses to various metabolic characteristics of insulin resistance.[39-43]

Statins, Insulin Resistance and NLRP3 Inflammasome

The NOD-like receptor family, pyrin domain containing (NLRP)3 inflammasome has been identified as a key connection between obesity-related immune responses and type 2 diabetes.[44,45] Type 2 diabetes patients have increased NLRP3 activation in immune cells and blocking or deleting specific components of the inflammasome consistently reduces insulin resistance in obese animal models.[46-48] The NLRP3 inflammasome has been described as a metabolic danger sensor and its relevance as a bridge between immunity and insulin resistance may be based on the ability of this inflammasome to detect and respond to a wide range of stimuli (in all of the major tissues) associated with insulin resistance and dysglycemia.[49,50] The NLRP3 inflammasome is a complex of at least NLRP3, ASC, and Caspase-1 that is best known for regulating biologically active IL-1β and IL-18.[51] Generation of downstream effectors by this inflammasome requires adequate priming followed by a signal that promotes assembly/activation. Priming can occur through activation of other pattern recognition receptors (PRR) resulting in NF-κB mediated transcription of inflammasome components such as NLRP3 and effectors such as pro-IL-1β and IL-18.[52] Activation signals for this inflammasome can involve changes in cellular metabolism that produce reactive oxygen species, K^+ efflux, and lysosomal leakage.[51] The links between cellular metabolites and the NLPR3-mediated inflammatory basis of many diseases has been reviewed.[53] Blocking this inflammasome or its key effectors has shown promise for several chronic diseases, including diabetes.[54] In fact, blocking IL-1β is the current strategy for directly testing the inflammatory hypothesis of cardiovascular disease risk, which was derived from work with statins.[55] Conversely, it is logical that drugs could activate the NLRP3 inflammasome, particularly those that alter the cellular metabolic status.

We recently hypothesized that statins could promote insulin resistance by activating the NLRP3 inflammasome.[20] Our first finding corresponded with previous research showing statins increase IL-1β secretion in bone marrow derived macrophages (BMDM's) given adequate priming.[56] Statin-induced

IL-1β secretion was dependent on NLRP3 and reversed with (a high dose of) the known inflammasome inhibitor, glyburide.[57] We also showed that adding back the mevalonate pathway intermediate GGPP prevented IL-1β release from BMDMs demonstrating a role for prenylated proteins. Next, we provided new information showing the importance of this statin-NLRP3 effect in metabolic tissues that help control blood glucose. Feeding a statin for several weeks to obese mice reduced insulin-stimulated glucose uptake into adipose tissue. Statin exposure increased caspase-1 activity in explanted adipose tissue, which was NLRP3-dependent and also was reduced with glyburide. Most importantly, statin treatment impaired insulin signaling in adipose tissue explants, but insulin signaling was normal in adipose tissue from mice that had lacked NLRP3 or when explants were treated with glyburide. Finally, we showed that statins could impair insulin signaling via an adipocyte autonomous cell response, but this occurred despite undetectable IL-1β.

Future Directions: Downstream of the Inflammasome

These findings connected statin treatment to insulin resistance through the NLRP3 inflammasome. However, it is not known; (1) what effector(s) downstream of the inflammatory complex is/are

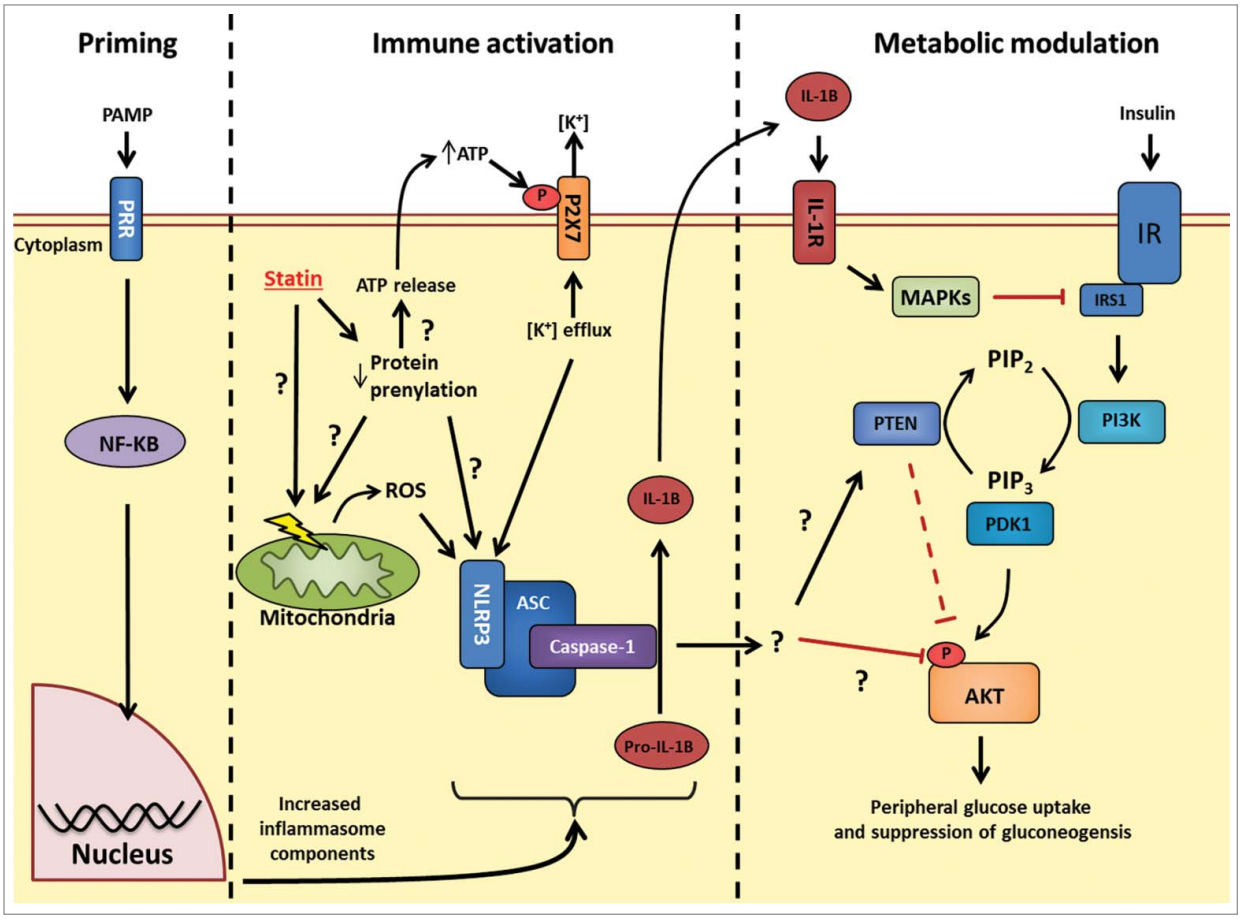

Figure 1. Possible mechanisms linking statin-induced NLRP3 inflammasome activation and insulin resistance. *Priming*: Following PRR stimulation, NF-κB stimulates transcriptional events that increase levels of the inflammasome (such as NLRP3) and inflammasome effectors (such as pro-IL-1β). *Immune activation*: HMGCR inhibition with statins causes pleotropic effects through decreased protein prenylation. Decrease in protein prenylation is a suspected cause for signals to promote increase NLRP3 inflammasome activity, but how these signals conspire to activate this inflammasome is not fully understood. Statins have been shown to cause mitochondrial membrane dysfunction, increase intracellular reactive oxygen species and also promote release of cellular ATP. Extracellular ATP can bind to the P2X7 receptor and promote potassium (K^+) efflux, a key trigger for increased NLRP3 inflammasome activity. The identity of the prenylated protein(s) responsible for statin-induced inflammasome activation is not known. Following inflammasome activation, caspase-1 cleaves pro-IL-1β to biologically active IL-1β. *Metabolic modulation*: The connection between NLRP3 inflammasome activation and insulin signaling may occur through either IL-1β-mediated inflammation and activation MAPKs (JNK, ERK, p38) which inhibit insulin signaling at the level of receptor substrate-1 (IRS1); or through an unknown target of caspase-1 which may alter insulin signaling at the level of phosphatase and tensin homolog (PTEN) or another site such as AKT phosphorylation. PRR, pattern recognition receptor; NLRP, NOD-like receptor family, pyrin domain containing; HMGCR, HMG-CoA reductase; IL-1β, interleukin-1 β; P2 × 7, P2X purinoceptor 7; PI3K, Phosphoinositol-3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositide-dependent kinase-1; MAPK, Mitogen activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular regulated mitogen-activated protein kinase; IR, insulin receptor.

responsible for perturbing insulin signaling and (2) where the defect in insulin signaling cascade originates. IL-1β is a likely culprit given that this pro-inflammatory cytokine has been heavily implicated in insulin resistance and type 2 diabetes. IL-1β signaling can impair insulin signaling through engagement of MAPKs.[58] A direct role for IL-1β has not yet been shown and the role of NLRP3-mediated regulation of biologically active IL-1β in statin-induced insulin resistance should be tested. This is particularly important because our recent paper showed that fluvastatin impaired insulin signaling in 3T3-L1 adipocytes in a cell autonomous manner, which coincided with increased caspase-1 activity, but without a detectable rise in IL-1β. Further, it is important to understand the molecular regulation of IL-1β. In addition to statins and other NLRP3 inflammasome activators that are known to increase levels of (17 kDa) biologically active IL-1β, simvastatin and cerivastatin have recently been shown to processes pro-IL-1β to a 28 kDa intermediate form of IL-1β through a caspase-1-independent mechanism in macrophages.[59] Simvastatin produced this intermediate 28 kDa form of IL-1β independent of HMG-CoA reductase inhibition. This appears to be a different type of pleiotropic action of statins compared to those that arise from inhibition of various arms of the mevalonate pathway. This intermediate 28 kDa form of IL-1β was reported to not properly activate IL-1 receptor signaling and could potentially attenuate the actions of mature IL-1β. For example, this intermediate form of IL-1β can reduce IL-6 induction by mature IL-1β in macrophages by presumably interfering with IL-1 receptor activation. Therefore, if IL-1β turns out to be a critical link between statins and diabetes or if this single cytokine is directly implicated in statin-induced insulin resistance, the caspase-1-dependent and independent molecular regulation of IL-1β should be determined in the context of insulin signaling. In particular, it would be interesting to know the relative importance and net effect of intermediate and mature forms of IL-1β in altering insulin action in the metabolic cells responsible for glucose homeostasis.

IL- 1β it is not the only candidate that could link the NLRP3 inflammasome to insulin action. The NLRP3 inflammasome also regulates biologically active IL-18. IL-18 tracks with obesity and insulin resistance, but IL-18 has recently been found to reduce weight gain and insulin resistance in mice through activation of AMP-activated protein kinase (AMPK).[60,61] Caspase-1 cleaves an array of proteins such as caspase-7, parkin, and enzymes of glycolysis.[62-64] It is not known if caspase-1 cleaves insulin signaling components or how it could facilitate crosstalk from the immune response to insulin signaling (**Fig. 1**). Recent findings from Birnbaum et al. demonstrated that rosuvastatin may relay signals through phosphatase and tensin homolog (PTEN) that inhibit AKT phosphorylation.[65] This builds on a very interesting connection between statin-induced increases in PTEN transcription via a sterol response element-binding protein (SREBP) pathway.[66] PTEN is a component in cell cycle regulation and inhibits transduction of the insulin signal by converting phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) back to PIP_2 (**Fig. 1**), preventing activation of phosphoinositide-dependent kinase-1 (PDK1) and subsequent phosphorylation of AKT.[67] The relevant connections between statin-induced NLRP3 inflammasome activation, SREBPs, PTEN and insulin signaling should be investigated.

Future Directions: Upstream of the Inflammasome

The statin-mediated cellular processes that elicit activation of the NLRP3 inflammasome in different cells are ill-defined. One common theme is the ability of statins to alter mitochondrial metabolism and cellular energy status in both immune and metabolic cells. Increased production of mitochondrial reactive oxygen species (mtROS), altered mitochondrial membrane potential and decreased intracellular ATP levels have been observed with concentrations of statin as low as 1 μM.[68-72] Statins have also been shown to promote a modest increase in cellular ROS and an increase of ATP release in THP-1 monocytes.[56] Statin-induced IL-1β production was also shown to be dependent on P2X purinoceptor 7 (P2X7) activation. Increased extracellular ATP can activate the P2X7 receptor, an ATP-gated ion channel resulting in cellular efflux of K^+ ions. A drop in cellular K^+ due to efflux has been shown to be necessary and sufficient for Caspase-1 activation and has been proposed as the unifying signal for NLRP3 inflammasome activation from various known stimuli.[73] It is still unclear how statin-related mitochondrial stress or changes in cellular nucleotides relate to K^+ efflux and NLRP3 inflammasome activation. Statin-mediated inhibition of HMGCR, decreased mevalonate pathway intermediates and reduced protein prenylation may be a critical link, since addition of GGPP to statin-treated culture has been shown to restore ATP levels and inhibit IL-1β release.[17,20,56,59] However, it is not clear which prenylated protein(s) is/are involved. Three distinct prenyltransferases (Farnesyltransferase, FTase; Geranylgeranyltransferase I and II, GGTase-I and GGTase-II) are responsible for prenylation of an array of proteins including Ras, Rho and Rab family proteins.[74,75] Peripheral blood mononuclear cells isolated from healthy individuals when treated with an inhibitor of GGTase-I in the presence of LPS caused IL-1β secretion, but inhibition of FTase does not increase IL-1β.[17] Interestingly, blocking GGTase-II which prenylates Rab family proteins, prevents GGPP from restoring cellular ATP levels following addition of statin to C2C12 muscle cells.[69] This positions geranylgeranylated proteins, but not farnesylated proteins as key upstream signals mediating statin-induced inflammasome activation. Combining statins with potential NLRP3 inflammasome inhibitors that do not interfere with the ability of statins to reduce LDL-cholesterol appears worthy of testing. However, it is likely that such strategies should not interfere with a global reduction in protein prenylation. This may be critical in maintaining the efficacy of statins since reduced protein prenylation itself improves cardiovascular indices and lifespan -at least in flies.[76] Significant challenges arise in identifying which particular prenylated protein is responsible for linking statins and inflammasome activation as multiple proteins may be important and as one example there are over 60 known Rab proteins.[75]

Priming of the NLRP3 inflammasome also should be investigated, particularly *in vivo* because it may reveal susceptibility to statin-induced NLRP3 activation and possibly statin-related insulin resistance or diabetes. We found no priming effect of statins alone in macrophages.[20] Cell or tissue culture models are typically primed with bacterial components such as LPS or

peptidoglycan before an NLRP3 activating agent is tested. In theory, any stimuli that increases NF-kB-mediated transcription of inflammasome components (and/or pro-IL-1/18) is a potential priming agent for this inflammasome. It is not clear which *in vivo* environment contains sufficient priming signals for statin-induced inflammasome activation. An intriguing possibility would be if specific microenvironments such as expanded adipose tissue contain sufficient priming signals that are poised to be NLRP3 inflammasome activated. We speculate that there are numerous endogenous compounds that could prime the NLRP3 inflammasome including endogenous lipids, metabolic endotoxemia and/or neighboring cell death in specific tissues. All of these factors can regulate NF-κB signaling and have links to obesity and pre-diabetes. One could speculate that priming is associated with increased statin-induced diabetes risk in certain metabolic disease prone populations. Our data suggests this is worthwhile investigating because we have yet to find a protocol for feeding a statin to a healthy chow-fed mouse that alters glucose control or insulin sensitivity (unpublished), but we found that fluvastatin feeding to ob/ob mice promotes insulin resistance in adipose tissue.[20] Ob/ob mice have several features that could equate to increased priming of the NLRP3 inflammasome, including those related to increased adiposity (endogenous lipids, death of adipose resident cells) and substantial metabolic endotoxemia.[77,78] Hence, it is possible that increased priming of the NLRP3 inflammasome in ob/ob mice promotes statin-induced insulin resistance in the adipose tissue microenvironment.

Conclusion

Statins have proven effective in reducing cardiovascular events and all-cause mortality. All drugs have side effects. In addition to cost/benefit analysis, we believe it is important to understand the immunometabolism of statins in order to generate potential strategies to mitigate the side-effects of statins, including the increased risk of diabetes. Given this foundational knowledge, there may be opportunities to target the statin-mediated processes that regulate blood glucose, if they turn out to be separate from statin-mediated processes that provide cardiovascular benefits. Statin-mediated activation of the NLRP3 inflammasome is one possible immunometabolism link to the dysglycemia associated with statins. Whether this inflammasome can be targeted without altering the effectiveness of statin treatment is a key therapeutic question. Identifying the relative importance of the NLRP3 inflammasome in the face of the general anti-inflammatory effect of statins is an important biological question. These two questions are important because it is not clear why statins are effective at reducing cardiovascular disease, but increase diabetes risk. To the best of our knowledge, there has not been a definitive demonstration of an immune response that segregates the actions of statins on cardiovascular disease and insulin resistance. In fact, many elements of inflammation are shared between these 2 interrelated metabolic diseases.[79] One could speculate that divergent effects of statins on CVD and diabetes could be derived from the different cells types that instigate or propagate each disease process. For example, this could involve different effects of statins on endothelial cells that are more relevant to cardiovascular disease vs. the effects of statins on adipocytes, myocytes or hepatocytes that are more relevant to insulin resistance. In addition, the divergent effects of statins on cardiovascular disease versus diabetes may be the relative importance of cholesterol lowering. Statin-induced LDL-cholesterol-lowering may be far more important in preventing cardiovascular disease processes compared to those processes that drive insulin resistance or diabetes. Further, it will be important to position insulin resistance in context with statin-induced changes in insulin secretion, given that NLRP3 inflammasome in the pancreas and resident immune cells have been implicated in type 2 diabetes.[45,80,81] Many mechanistic questions remain about the connection between statins, NLRP3 and insulin action.[82] Understanding upstream and down-stream NLRP3 inflammasome effectors may foster new therapeutic or diagnostic strategies, including combination strategies or biomarkers for statin-induced disease risk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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