Fluvastatin causes NLRP3 inflammasome-mediated adipose insulin resistance

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Abstract

Statins reduce lipid levels and are widely prescribed. Statins have been associated with an increased incidence of type 2 diabetes, but the mechanisms are unclear. Activation of the NOD-like receptor family, pyrin domain containing (NLRP)3/caspase-1 inflammasome promotes insulin resistance, a precursor of type 2 diabetes. We showed that four different statins increased IL-1β secretion from macrophages, characteristic of NLRP3 inflammasome activation. This effect was dose-dependent, absent in NLRP3−/− mice and prevented by caspase-1 inhibition or the diabetes drug, glyburide. Chronic fluvastatin treatment of obese mice impaired insulin stimulated glucose uptake in adipose tissue. Fluvastatin-induced activation of the NLRP3/caspase-1 pathway was required for the development of adipose tissue insulin resistance in adipose tissue explants, an effect also prevented by glyburide. Fluvastatin impaired insulin signaling in LPS primed 3T3-L1 adipocytes, an effect associated with increased caspase-1 activity, but not IL-1β. Our results define an NLRP3/caspase-1-mediated mechanism of statin-induced insulin resistance in adipose tissue and adipocytes, which may be a contributing factor to statin-induced development of type 2 diabetes. These results warrant scrutiny of insulin sensitivity during statin use and suggest combination therapies with glyburide, or other inhibitors of the NLRP3 inflammasome may be effective in preventing the adverse effects of statins.
INTRODUCTION

Statins inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis and reduce low-density lipoprotein (LDL) cholesterol levels. Statins have actions beyond lipid lowering effects, which includes modulation of immune function (1). Statins decrease intermediates in the mevalonate pathway that lie upstream of cholesterol formation; reducing protein prenylation, a post-translational lipid modification that occurs on many proteins, including those involved in immune responses (1). Immune proteins such as pattern recognition receptors (PRRs) have emerged as integrators of nutrient and pathogen sensing systems and the inflammation during obesity (i.e. metaflammation) has been characterized in terms of excess nutrients and energy (2). Drug-mediated changes in inflammation via engagement of PRRs should also be considered, particularly for therapeutics such as statins that are used to treat aspects of metabolic disease.

Statin-mediated decreases in protein prenylation are generally associated with anti-inflammatory responses and can reduce TNF and IL-6 in lipopolysaccharide (LPS)-treated peripheral blood (3). In contrast, statins have been associated with increased secretion of the pro-inflammatory cytokine IL-1β; an effect that requires caspase-1 activity and priming with another immunogenic agent such as LPS (4). These features are indicative of regulation by the inflammasome containing the PRR, NOD-like receptor family, pyrin domain containing 3 (NLRP3/NALP3/Cryopyrin) (5; 6). The NLRP3 inflammasome is causally linked to the development of insulin resistance in rodents (7) and has recently been shown to be activated in macrophages of newly diagnosed insulin resistant type 2 diabetics (8). Statin therapy has been associated with increased incidence of type 2 diabetes; as high as 48% in certain populations (9; 10). Glyburide, a widely prescribed diabetes drug, inhibits the NLRP3 inflammasome independently of cyclohexylurea-mediated insulin secretion (11). We hypothesized that statin-mediated activation of the NLRP3 inflammasome promotes insulin resistance, which could be attenuated by glyburide.
RESEARCH DESIGN AND METHODS

Mice and materials. McMaster University Animal Ethics Review Board approved all procedures. Male WT C57BL/6 (#000664) and leptin-deficient ob/ob (# 000632) mice were from JAX. NLRP3\(^{-/-}\) mice (>10 generations backcrossed to C57BL/6) were from Professor Nicolas Fasel (Université de Lausanne, Switzerland,) and were kindly provided by Dr. Dana Philpott (University of Toronto, Canada). To determine the effect of chronic statin treatment on insulin-stimulated tissue glucose uptake, ob/ob mice were orally administered 40-50 mg/kg fluvastatin or vehicle 5 days a week for 6 weeks, a dose of fluvastatin used in other mouse models (12). 24 h after the last dose, mice were injected with 2 \(\mu\)Ci of \(^3\)H-2-deoxy-D-glucose (2DG) via tail vein, immediately followed by 4U/kg of insulin (i.p.). Blood samples were taken at baseline, 5, 10, 15, 20 min and analyzed for 2DG. Mice were sacrificed by cervical dislocation and tissues were snap frozen in liquid nitrogen. Brown adipose tissue (BAT) and gonadal white adipose tissue (WAT) was analyzed for 2DG radioactivity with and without deproteinization (0.3M BaOH and 0.3M ZnSO4) to calculate rates of tissue specific glucose uptake. Statins were from Cayman Chemical (Ann Arbor, MI). Invivogen (San Diego, CA) supplied ultra-pure LPS (Escherichia coli 0111:B4). Z-WHED-FMK and caspase-1/3 kits were from R&D Systems (Denver, CO). The MTT kit and all other chemicals were from Sigma (St. Louis, MO).

Macrophages. Bone-marrow derived macrophages (BMDMs) were cultured 7-10 d in DMEM containing 10% FBS and 15% L929 conditioned-media. BMDMs were washed in serum-free media and exposed to statin (1\(\mu\)M fluvastatin, unless otherwise stated) for 18 h in serum-free DMEM and LPS (200ng/mL) was added during the final 4 h. GGPP (10\(\mu\)M), Z-WHED-FMK (10\(\mu\)M), and glyburide (200\(\mu\)M), were used during the statin treatment period. IL-1\(\beta\) and IL-6 were quantified by ELISA. Transcript levels were analyzed by quantitative PCR, as described (13; 14).
Adipose explants and adipocytes. Mice were killed by cervical dislocation and PBS-rinsed gonadal adipose tissue was minced into ~5mg pieces in DMEM containing 10% FBS. After 2 h equilibration, explants were placed in serum-free DMEM and exposed to 10µM fluvastatin (18 h) and 2µg/mL of LPS (4 h) and were stimulated with 0.3 nM insulin for 10 minutes. Adipose tissue lysates were used for caspase-1/3 activity (over 4 h), immunoblotting or ELISA determination of cytokines, as described (14). 3T3-L1 pre-adipocytes (ATCC, Rockville, MD, USA) were differentiated (14), and fluvastatin/LPS treated similar to explants. 3T3-L1 media was used for ELISAs and lysates were used to measure caspase-1 enzymatic activity fluorometrically or for immunoblotting after insulin stimulation at 0.3nM or 100 nM for 10 min.

Statistical Analysis. Significance was determined by unpaired, two-tailed T-tests or ANOVA, as appropriate. Bonferroni or Tukey’s post hoc test was used when appropriate (Prism 4-6, GraphPad Software, USA).

RESULTS

Statins activate the NLRP3 inflammasome.

All statins (10µM, 18 h) increased secretion of IL-1β from WT BMDMs compared to LPS alone (Fig 1A). Fluvastatin increased IL-1β secretion in a dose-dependent manner only with LPS priming (Fig 1B), but LPS alone increased IL-6 secretion in BMDMs (Fig 1C). Fluvastatin up to 100 µM did not lower BMDM viability detected using the MTT assay (data not shown). The isoprenyl intermediate GGPP prevented fluvastatin-induced IL-1β secretion in LPS-primed BMDMs (Fig 1D), suggesting that decreased prenylation drives statin-mediated inflammasome activation. Inhibition with z-WHED or glyburide prevented statin-induced IL-1β secretion in LPS-primed BMDMs (Fig 1E). LPS, but not...
Fluvastatin treatment alone, increased transcript levels of NLRP3, IL-1β and IL-6 (Fig 1F, G). Therefore, statins alone did not alter inflammasome priming events such as increased NLRP3 transcript levels (15). The combination of fluvastatin and LPS synergistically increased both IL-1β and IL-6 transcript levels (Fig 1F). Fluvastatin did not increase IL-1β in LPS primed BMDMs from NLRP3<sup>−/−</sup> mice (Fig 1H). LPS increased IL-6 secretion BMDMs from NLRP3<sup>−/−</sup> mice (Fig 1I).

**Fluvastatin impairs adipose tissue insulin signaling via the NLRP3 inflammasome.**

We first established that chronic oral administration of fluvastatin impaired insulin-simulated glucose disposal into adipose tissue using an *in vivo* mouse model of obesity. 2DG uptake was >50% lower in WAT, but not BAT of fluvastatin-treated ob/ob mice (Fig 2A). We then used WAT explants to determine the mechanisms of statin-induced insulin resistance. Fluvastatin increased caspase-1 activity in LPS-primed adipose tissue from WT, but not from NLRP3<sup>−/−</sup> mice (Fig 2B). Glyburide prevented this increased caspase-1 activity (Fig. 2B). Fluvastatin increased caspase-3 activity in LPS-primed adipose tissue from WT and NLRP3<sup>−/−</sup> mice and independently of glyburide treatment (Fig. 2C). Therefore, fluvastatin activated a NLRP3-dependent, glyburide sensitive caspase-1 inflammasome in adipose tissue.

Surprisingly, LPS alone increased IL-1β in adipose explants from both WT and NLRP3<sup>−/−</sup> mice (Fig 2D). Fluvastatin plus LPS further increased IL-1β levels compared to LPS in adipose explants from WT, but not NLRP3<sup>−/−</sup> mice (Fig 2D). LPS alone did not change the ability of insulin to phosphorylate Akt (pAkt) at serine 473 in adipose tissue explants (Fig 2E). Fluvastatin alone impaired insulin-mediated pAkt in adipose tissue from WT, but not NLRP3<sup>−/−</sup> mice (Fig 2E). The combination of LPS and fluvastatin prevented insulin’s ability to increase pAkt in adipose tissue explants from WT, but not NLRP3<sup>−/−</sup> mice (Fig 2E). Glyburide reversed fluvastatin-induced suppression of insulin-mediated pAkt in LPS-primed adipose explants, but glyburide did not increase pAkt on its own (Fig. 2F).
Interestingly, changes in caspase-1 activity, but not IL-1β secretion mirrored statin-induced insulin action in adipose explants. There are many non-adipocyte cell types and potential sources of IL-1β processing in adipose tissue (16), so we next tested the adipocyte cell autonomous response. Fluvastatin plus LPS increased caspase-1 activity in 3T3-L1 adipocytes, but did not increase IL-1β or IL-6 secretion (Fig 3A-D). However, fluvastatin plus LPS significantly lowered insulin-stimulated pAkt in 3T3-L1 adipocytes (Fig 3E).

**DISCUSSION**

Statins lower blood lipids and reduce cardiovascular disease-related events (17). Paradoxically, statins have been associated with increased incidence of diabetes. This has sparked debate over reassessing the benefits and risks of statin use (18). Understanding how statins promote adverse effects such as the progression to diabetes may promote improvements in this drug class. We show that statins activate the NLRP3 inflammasome in various immune and metabolic cells of adipose tissue. Fluvastatin-induced impairments in insulin signaling were dependent upon the NLRP3 inflammasome. The commonly used diabetes drug, glyburide, inhibited statin-induced inflammasome activation and prevented defects in adipose tissue insulin action.

Endogenous and exogenous stimuli activate the NLRP3 inflammasome, which prompted the theory that this PRR is a metabolic danger sensor (19). Production of bioactive IL-1β (or IL-18) by the NLRP3 inflammasome requires priming and stimuli promoting assembly of a caspase-1 protein complex. We confirm that statins increased IL-1β secretion in adequately primed macrophages (5; 6; 20) and we demonstrated the requirement of the NLRP3 inflammasome. Glyburide, an existing diabetes drug, inhibited statin-induced increases in IL-1β in macrophages, which is consistent with its inhibitory effect on other inflammasome activators (11). All statins tested activated NLRP3-mediated
increases in IL-1β, which is similar to the class effect of these HMG-CoA reductase inhibitors increasing the risk of diabetes, independently of potency or lipophilic properties (18). A standard dose of fluvastatin can equate to µM serum levels in humans (21) and other statins can reach serum levels beyond 10 µM (22), which corresponds with the effective dose range our in vitro models. The dose-response of fluvastatin-induced IL-1β secretion that we report is consistent with higher doses of statins increasing the risk of diabetes to a greater extent (18). This is important because of the diminishing returns of lipid lowering as the dose of statins is increased, the high dose of stains required to achieve adequate lowering in many patients, and the incidence of statin-intolerance in clinical practice (23).

Adipose tissue is a key site of inflammation during insulin resistance and the NLRP3/caspase-1 inflammasome regulates adipose tissue inflammation and function (24). We first showed that 6 weeks of oral fluvastatin treatment of ob/ob mice impaired insulin-stimulated glucose uptake in WAT, a depot where insulin normally increases glucose disposal from the blood. Statin feeding in these mice had no effect on glucose uptake in BAT, highlighting the specificity of this statin-mediated effect. We then provided genetic evidence that statins impaired adipose tissue insulin action via the NLRP3 inflammasome, which was also prevented with glyburide ex vivo. The combination of LPS and fluvastatin was most effective in preventing insulin-mediated signals in adipose tissue explants. However, fluvastatin did not require LPS to cause impaired insulin action, suggesting that adipose tissue contains endogenous NLRP3 inflammasome priming signals. This is consistent with the NLRP3 inflammasome mediating obesity-associated insulin resistance in response to saturated lipids (7).

Intriguingly, NLRP3 was not necessarily required for IL-1β secretion from adipose tissue explants. Since the regulation of IL-1β did not mirror changes in insulin action, our results suggest that caspase-1 rather than IL-1β provides the link to adipose tissue insulin resistance. Further, a cell-autonomous program that increases caspase-1 activity can be engaged by fluvastatin in LPS primed clonal adipocytes. This response in 3T3-L1 adipocytes did not increase IL-1β secretion, but impaired insulin
action. Therefore, our results suggest that fluvastatin acts through the NLRP3/caspase-1 inflammasome in multiple cells within adipose tissue and culminates in insulin resistance that does not necessarily require IL-1β. Our results on insulin resistance have focused on fluvastatin, but the type of statin and pleiotropic actions on inflammation (that are often conflicting) in immune cells, liver, muscle, adipose and pancreas should be considered in obese and pre-diabetic mice and patients (25; 26). Little is known about the contributing factors to the statin-diabetes relationship. We propose a role for inflammation and that inflammasome-mediated insulin resistance is positioned as a contributor to the development of diabetes. It is enticing to speculate that metabolic endotoxemia or other priming agents for the inflammasome may play a role (27).

We propose that the inhibition of NLRP3/caspase-1 inflammasome may attenuate statin-induced insulin resistance. This is particularly relevant in mitigating any contribution of statins to insulin resistance leading to diabetes in obese, hyperlipidemic patients who commonly use this class of drugs for lipid lowering, but are often at risk for developing diabetes. The next generation of statins may be driven by combination therapy or statin derivatives that maintain or enhance lipid lower properties, but allay adverse effects by evading the NLRP3 inflammasome.

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discussion and edited the manuscript. JDS researched data, derived the hypothesis, and wrote the
manuscript and is the guarantor.
References

Figure Legends

Figure 1. Statins activate the NLRP3 inflammasome in macrophages. BMDMs from WT mice were treated with various statins (10 µM, 18 h) and/or LPS (200 ng/mL, 4 h) and IL-1β in the media was quantified (A). BMDMs were treated with various doses of fluvastatin and/or LPS and IL-1β (B) and IL-6 (C) in the media was quantified. BMDMs were treated with LPS and 1 µM of fluvastatin combined with vehicle or 10 µM GGPP and IL-1β in the media was quantified (D). BMDMs were treated with LPS and 1 µM of fluvastatin combined with vehicle or 10 µM z-WHED-FMK or 200 µM glyburide and IL-1β in the media was quantified (E). Transcript levels of cytokines (F) and PRRs (G) in BMDMs after 1 µM fluvastatin (18h) and 200 ng/mL LPS (4 h) treatments. BMDMs from NLRP3−/− mice were treated various doses of fluvastatin and/or 200 ng/mL LPS and IL-1β (H) and IL-6 (I) in the media was quantified. *Significantly different from LPS alone or as indicated by connecting bars. †Significantly different from conditions without LPS. #Significantly different from fluvastatin plus LPS. ^Significantly different from control or statin alone. Statin = fluvastatin. Values are means + SEM, n > 3 for all conditions.

Figure 2. Fluvastatin activates the NLRP3 inflammasome and impairs insulin signaling in adipose tissue.

*In vivo* insulin-stimulated 2DG uptake in brown adipose tissue (BAT) and white adipose tissue (WAT) from ob/ob mice orally treated with vehicle (Control) or fluvastatin (Statin) for 6 weeks (A, n ≥ 3 mice per group). Caspase-1 (B) and caspase-3 (C) activity in adipose tissue explants from WT mice and NLRP3−/− mice, where explants were treated with vehicle (control), LPS (2µg/mL) plus 10 µM fluvastatin (L+S) or L+S plus 10 µM glyburide (L+S+Glyb). Quantification of IL-1β in adipose tissue lysates from WT and NLRP3−/− mice after treatment of explants with vehicle (control), LPS, fluvastatin
or LPS plus fluvastatin (D). Representative immunoblots (left) and quantification (right) of basal (Bas; i.e. no insulin) and insulin-mediated phosphorylation of Akt (serine 473) after treatment with fluvastatin and/or LPS in adipose tissue explants from WT and NLRP3-/- mice (E). Representative immunoblots (left) and quantification (right) basal and insulin-mediated phosphorylation of Akt after treatment with fluvastatin and LPS with various doses of glyburide in adipose tissue explants from WT mice. $Significantly different from control or basal; εSignificantly different from L+S. ^Significantly different from LPS alone in WT. #Significantly different from vehicle control (with insulin). Statin = fluvastatin. Values are means ± SEM, n ≥ 8 explants per group.

**Figure 3. Cell-autonomous actions of fluvastatin in adipocytes.**

Time-course (A) and quantification (B) of relative caspase-1 activity in 3T3-L1 adipocytes after treatment with vehicle (Control) or LPS plus fluvastatin (L+S) (n ≥ 7 / group). Quantification of IL-1β (C) and IL-6 (D) secreted in the media after control or L+S (n ≥ 8 / group). Representative immunoblots (left) and quantification (right) of 0.3 nM and 100 nM insulin-stimulated phosphorylation of Akt (serine 473) in 3T3-L1 adipocytes after treatment with control or L+S (E; n = 4 /group). *Significantly different from control (no insulin). #Significantly different from control at the same dose of insulin. Statin = fluvastatin. nd = not detected. Values are means ± SEM.
Fig 1
Diabetes

Fig 2

274x367mm (300 x 300 DPI)
Diabetes

Fig 3

154x120mm (300 x 300 DPI)