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Addenda

# A Two-Carbon Switch to Sterol-Induced Autophagic Death

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**KEY WORDS**

macrophage, cholesterol, sitosterol, autophagy, necroptosis, cell death, atherosclerosis

**ABBREVIATIONS**

| | |
|---|---|
| ACAT | acyl-CoA:cholesterol acyl transferase |
| ER | endoplasmic reticulum |
| FC | free cholesterol |
| MAPK | mitogen-activated protein kinase |
| SERCA | sarcoplasmic-endoplasmic calcium ATPase |
| UPR | unfolded protein response |

Addenda to:

*Sitosterol-Containing Lipoproteins Trigger Free Sterol-Induced Caspase-Independent Death in ACAT-Competent Macrophages: Implications for Sterol Structure-Dependent Mechanisms of Cell Death and for Atherosclerotic Vascular Disease in Sitosterolemia*

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## ABSTRACT

Although both cholesterol and plant sterols are abundant in our diets, our intestinal epithelial cells selectively and efficiently rid the body of plant sterols. However, a rare mutation in plant sterol excretion in humans results in the accumulation of plant sterols, particularly sitosterol, in the plasma and tissues. Sitosterol differs from cholesterol only in an extra ethyl group on the sterol side chain. Significantly, sitosterolemia is associated with accelerated atherothrombotic vascular disease, notably myocardial infarction. An important process that promotes atherothrombosis is advanced lesional macrophage death, leading to plaque necrosis. One of the causes of atherosclerotic macrophage death is sterol-induced cytotoxicity. We therefore compared the effects of excess intracellular sitosterol vs. cholesterol on macrophage death. Whereas excess cholesterol kills macrophages by caspase-dependent apoptosis, sitosterol kills macrophages by a caspase-independent pathway involving necroptosis and autophagy. The finding that an ethyl group on the sterol side chain fundamentally alters the way cells respond to excess sterols adds new insight into the mechanisms of sterol-induced cell death and may provide at least one explanation for the excess atherosclerotic heart disease in patients with sitosterolemia.

## EXCESS INTRACELLULAR STEROL ACCUMULATION AS AN INDUCER OF MACROPHAGE DEATH AND PLAQUE NECROSIS IN ADVANCED ATHEROSCLEROTIC LESIONS

Cholesterol is an essential component of all cells in vertebrates.[1] The key structural features of cholesterol include a planar array of 6- and 5-carbon rings to which is attached a polar hydroxy group at one end and a hydrophobic aliphatic side chain at the other end (Fig. 1).[1] This structure is ideally suited to meet the three major functions of cholesterol in vertebrate cells: regulating the structure of cellular membranes; serving as a precursor to essential molecules like steroids and bile acids; and carrying out regulatory functions such as negative feedback control of sterol biosynthesis.[1] These and other functions of cholesterol are critical for vertebrate cell viability. Excess cholesterol, however, is cytotoxic, because a high cholesterol:phospholipid ratio in cell membranes perturbs membrane structure and function (below).[2] Not surprisingly, excess cellular cholesterol is normally prevented by a number of regulatory mechanisms.[3] Among the most important of these mechanisms is fatty acyl esterification of excess cholesterol by an endoplasmic reticulum (ER) enzyme called acyl-CoA:cholesterol acyltransferase (ACAT).[4] Cholesterol fatty acyl esters are too hydrophobic to intercalate into cell membranes, and thus cytotoxicity is prevented.[2]

A critically important example of these processes occurs during the progression of atherosclerosis, which is the cause of heart attacks, strokes, and other occlusive vascular diseases. The major cell type in atherosclerotic lesions is the macrophage, which ingests large amounts of cholesterol-rich lipoproteins in focal regions of the arterial wall.[5] For the most part, lesional macrophages remain viable, and a key reason is their ability to esterify the excess ingested cholesterol ('foam cells"). As atherosclerotic lesions progress, however, something appears to go awry with the esterification process, because some of the macrophages accumulate excess unesterified, or "free," cholesterol (FC).[2] As expected from the above discussion, excess FC is a potent inducer of macrophage death. Advanced lesional macrophage death induced by FC and similar mechanisms, together with defective phagocytic clearance of dead cells in these lesions, leads to the development of large areas of necrosis.[6] Lesional necrosis is thought to promote plaque disruption and

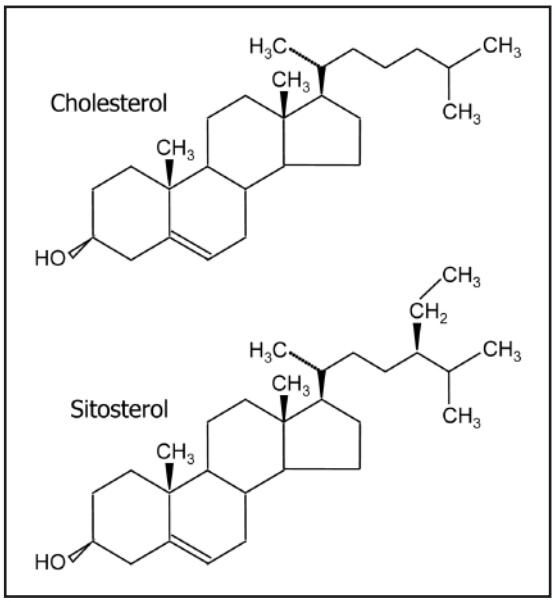


Figure 1. Structures of cholesterol and sitosterol. Compared with cholesterol, sitosterol has an extra ethyl group on C24.

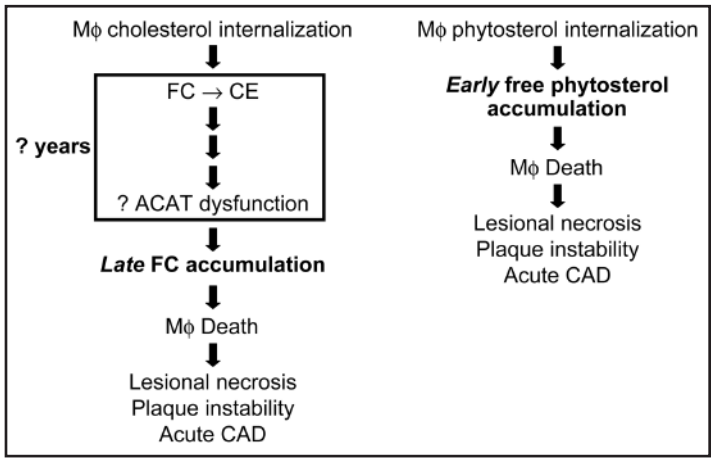


Figure 2. Hypothesis of how excess phytosterols might promote premature advanced atherosclerotic macrophage death and coronary artery disease (CAD). The key point, as explained in the text, is that the long period to ACAT dysfunction that is needed to enable FC accumulation in macrophages would not be required for plant sterols, because they are poor substrates for ACAT. Thus, lesional macrophage death, and the detrimental consequences that follow, would occur at an accelerated rate during lesion progression. In vivo studies are needed to prove this hypothesis.

lumenal thrombosis through the release of inflammatory and thrombotic inducers.[2,6]

The cholesterol that ultimately leads to these devastating events originates from endogenous cholesterol synthesis in the liver and absorption of dietary cholesterol in the intestine. Notably, as much as 50% of dietary sterols come from plant sources ("phytosterols").[7] Intestinal epithelial cells internalize phytosterols, but there is almost complete excretion back into the intestinal lumen through the action of the ABC transporters ABCG5/G8.[7] Thus, the plasma level of phytosterols in most humans is very low.[7] However, there exists a rare mutation in humans that perturbs phytosterol excretion, and as a result these subjects have high circulating levels of phystosterols.[8-10] A major phytosterol that accumulates in these subjects is sitosterol, which differs from cholesterol by an extra ethyl group on carbon 24 of the sterol side chain (Fig. 1). Importantly, patients with this disorder, called sitosterolemia, have premature atherothrombotic vascular disease.[11-15]

The mechanism of accelerated atherosclerosis in sitosterolemic subjects is not known. However, previous work showing that phytosterols are relatively poor substrates for ACAT[16-19] suggested to us a novel hypothesis, namely, accelerated lesional macrophage death in the setting of sitosterolemia (Fig. 2). Recall that FC accumulation and FC-induced apoptosis in lesional macrophages require ACAT dysfunction,[2] which undoubtedly takes a long time to develop throughout the course of atherosclerosis. When lesional macrophages ingest sitosterol-containing lipoproteins, however, there should be a more rapid accumulation of free sterol and free sterol-induced death, because ACAT dysfunction is not required for free sterol accumulation. Indeed, whereas most cell-culture models of FC accumulation must include an ACAT inhibitor or use ACAT-deficient cells, macrophages accumulate excess free sitosterol and undergo sitosterol-induced cell death in the absence of ACAT inhibitors or deficiency.[20]

Although the relevance of these findings to accelerated atherothrombotic disease in sitosterolemia remains to be investigated, further mechanistic work revealed a number of surprises that should prove interesting to members of the autophagy community. Previous work in our laboratory showed that the mechanism of FC-induced death is caspase-dependent apoptosis, which is triggered by two key events: activation of an ER stress pathway called the Unfolded Protein Response (UPR) and activation of the mitogen-activated protein kinase JNK.[21,22] We have evidence to suggest that the UPR is triggered by FC excess in the ER membrane bilayer, which is normally cholesterol poor.[23] For example, FC enrichment of the ER membrane dramatically changes the structure of that membrane and inhibits a key integral ER membrane protein called sarcoplasmic-endoplasmic calcium ATPase (SERCA).[23] SERCA inhibition by FC is not only a marker of ER membrane dysfunction but also may contribute directly to UPR activation via a calcium-dependent mechanism.[23]

## CHOLESTEROL AND SITOSTEROL DIFFER FUNDAMENTALLY IN THE WAY THEY TRIGGER DEATH IN MACROPHAGES (FIG. 3)

Given this background, we assumed that free sitosterol, which differs from cholesterol only by an extra ethyl group in the side chain (Fig. 1), would induce macrophage death by the same mechanism. Indeed, we showed that sitosterol, like cholesterol, requires trafficking to the ER to cause cell death and inhibits SERCA.[20] However, much to our surprise, sitosterol did not activate either the UPR or JNK, and sitosterol-induced macrophage death was caspase-independent.[20] These surprising findings led us to investigate caspase-independent mechanisms of cell death. In that context, we read with great interest a recent study by Yuan and colleagues showing that a form of caspase-independent death induced by TNFα and a caspase inhibitor was characterized by necrotic-like changes culminating in autophagy.[24] A key feature of this type of death, which the authors called "necroptosis," was inhibition by a compound called methylthiohydantoin-DL-tryptophan (necrostatin-1, or Nec-1).[24] Thus, we tested Nec-1 in both FC- and sitosterol-induced macrophage death

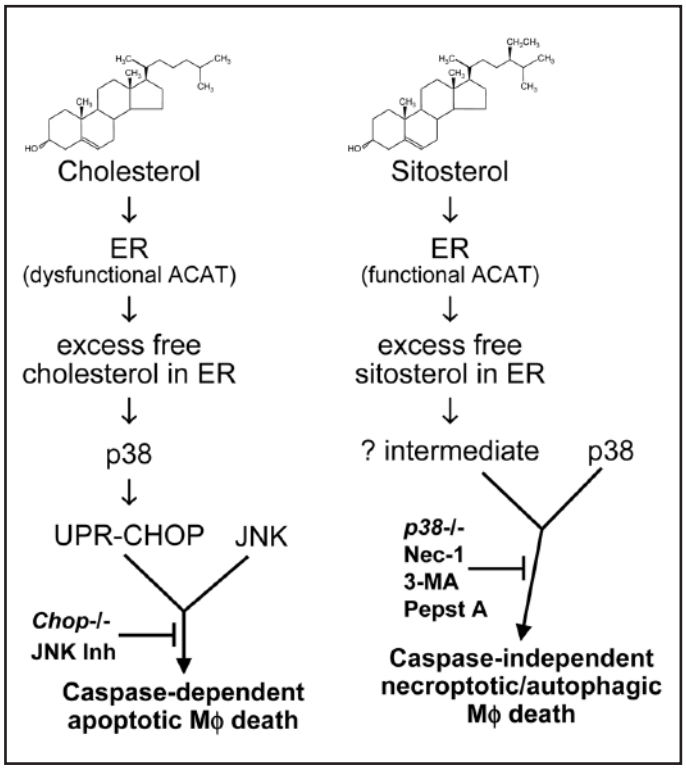


Figure 3. Summary of mechanisms distinguishing cholesterol- vs. sitosterol-induced cell death in macrophages. See text and reference 20 for details. Chop$^{-/-}$ and p38$^{-/-}$ refer to macrophages lacking these proteins; Jnk Inh = JNK inhibitor or JNK-deficient macrophages; Nec-1 = necrostatin-1; 3-MA = 3-methyladenine; and Pepst A = pepstatin A.

and found that it selectively inhibited the latter.[20] Moreover, the macrophages showed biochemical and morphological signs of autophagy, including expression of LC3-II[20] and the appearance of cytoplasmic vacuoles (unpublished data). The study by Yuan and colleagues showed that autophagy in their model was downstream of necroptotic-induced death: inhibition of autophagy with 3-methlyadenine and pepstatin A did not block cell death in their model, whereas inhibition of necroptosis using Nec-1 blocked autophagy.[24] However, sitosterol-induced macrophage death was inhibited by both 3-methyladenine and pepstatin A.[20] Thus, autophagy does appear to play an important role in sitosterol-induced macrophage death.

Autophagy plays critical roles in cellular physiology, particularly in development and as a response to nutrient deprivation, and thus autophagy is often critical for cell survival.[25] Nonetheless, under unusual conditions in which cellular physiology is perturbed, autophagy can trigger a caspase-independent death mechanism.[25] Why sitosterol happens to trigger autophagic cell death is an interesting question, particularly in view of the fact that cholesterol does not do this at all, Despite the structural similarities between cholesterol and sitosterol, previous studies have shown that their effects on membrane structure and function are not identical.[26,27] The fact that sitosterol trafficking to the ER is necessary for autophagic-induced death is intriguing in view of the key involvement of the ER in autophagy. A key question in this regard is whether sitosterol triggers autophagic death by perturbing ER membrane structure or by uniquely interacting with a key autophagy mediator in the ER membrane. Our study also showed that activation of p38 MAPK by sitosterol was necessary for sitosterol-induced macrophage death[20] and so how processes downstream of p38 might contribute to autophagic death in macrophages needs to be investigated.

Atherosclerotic disease, which usually kills subjects only in post-reproductive life, does not drive evolution. Nonetheless, it is tempting to contemplate whether the findings in our study have anything to do with the fact that nature designed the cholesterol side chain without a C-24 ethyl group and that vertebrates evolved an extremely efficient mechanism involving specific ABC transporters to rid the body of a sterol that does have this ethyl group. There is no evidence to date that patients or genetically altered mice with sitosterolemia have a survival disadvantage in early life, i.e., up to reproductive age, let alone evidence of autophagic death in macrophages or other cells. However, this effect may become apparent only under certain stressful conditions, such as conditions in which macrophages participate in host defense against infectious agents. Sitosterolemic humans are too rare to have been studied in this manner, and the mouse models of sitosterolemia have not been investigated under such conditions. The concept of the "two-carbon" switch to sterol-induced autophagic cell death now provides the rationale for such studies.

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