The impact of mitochondrial oxidative stress on bile acid-like molecules in *C. elegans* provides a new perspective on human metabolic diseases

Ju-Ling Liu and Siegfried Hekimi*
Department of Biology; McGill University; Montreal, Québec, Canada

*Correspondence to: Siegfried Hekimi; Email: siegfried.hekimi@mcgill.ca

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**Cholesterol and Bile Acid Metabolism in Mammals**

In mammals, cholesterol is a structural component of the cell plasma membrane that affects permeability and fluidity of the lipid bilayer as well as being a substrate for the biosynthesis of signaling molecules such as steroids hormones, vitamin D and bile acids (BAs). After cholesterol is taken up from food, it is associated with lipoprotein particles and then transported between organs predominantly as cholesterol esters. Cholesterol is mostly excreted in the bile after having been converted into bile acids in the liver. Bile acids are amphipathic molecules comprising the steroid nucleus of cholesterol with a shortened side chain (Fig. 1). They act as biological detergents that help in the intestinal uptake of lipids, hydrophobic vitamins and cholesterol itself. They also act as steroid hormone-like substances that integrate several aspects of metabolism, including lipid, glucose and energy metabolism by regulating gene expression through nuclear hormone receptors such as the farnesoid X receptor (FXR), the pregnane X receptor (PXR) and the vitamin D receptor (VDR) (BA biology is reviewed in detail in refs. 3 and 4). As sterols play multiple essential roles in mammals, alterations of their normal metabolism or transport cause diseases. For example, in mammals, BA excretion and recirculation depend on several membrane transporters such as ATP8B1 and ABCB11. Mutations in ATP8B1 cause progressive familial intrahepatic cholestasis type1 (PFIC1) characterized by a pathological retention of bile. ATP8B1, a type 4 P-type ATPase, is a predicted phospholipid flipase. Studies in mice suggest that ATP8B1 deficiency causes loss of canalicular membrane phospholipid asymmetry and as a result the resistance of the canalicular membrane to hydrophobic BAs is decreased, which causes cholestasis by impairing the activity of ABCB11, the BA export pump.
obtained through the diet, and when cultivated in the laboratory cholesterol has to be supplemented in the culture media (generally at 5μg/ml cholesterol). Therefore, by reducing the level of dietary cholesterol or replacing it with labeled or modified sterols, the worm can be used to investigate the metabolism and transport of sterols in a living organism. Worms grown on plates with a reduction in sterol supplementation produce a complex phenotype that includes abnormal molting and inappropriate dauer formation. A complete lack of sterol supplementation leads to lethality. Sterols appear to be required only in very small amounts for normal physiology in worms, suggesting that sterols are unlikely to be structural components in worm membranes. However, they are clearly used for the synthesis of signaling molecules, as reviewed below.

Worm signaling molecules derived from cholesterol were described in detail only recently when a number of studies showed that dafachronic acids (DAs), which are molecules that have some of the characters of BAs (Fig. 1), act as hormones that support reproductive development under favorable conditions by binding to a nuclear hormone receptor encoded by daf-12. Under unfavorable conditions, such as when worms are starved or overcrowded, DAs are not produced and un-liganded DAF-12 associates with its co-repressor DIN-1, which promotes worms entering into the dauer diapause. Genetic approaches and biochemical studies of the regulation of dauer formation revealed that a crucial step for production of DAs is performed by a cytochrome P450, DAF-9, which hydroxylates cholesterol twice in the C-26 position to produce a carboxyl group. In addition to DAF-9, two other enzymes required for the synthesis of DAF-12 ligands are DAF-36, a Rieske oxygenase that acts as a cholesterol 7-desaturase converting cholesterol to 7-dehydrocholesterol and HSD-1, a 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase. Although no ecdysteroids similar to those in insects have been identified in C. elegans, many observations suggest that molting, the process by which a new cuticle is synthesized and the old cuticle is shed, is also likely regulated by

**Figure 1.** The structures of (A) cholesterol, (B) a mammalian bile acid, chenodeoxycholic acid (CDCA), (C) dafachronic acid.
a steroid hormone. In insects, ecdysones, which are polyhydroxylated sterols, initiate molting through the activation of the ecdysone receptor, a heterodimeric receptor composed of two nuclear receptors including EcR (the ecdysone receptor) and USP (ultraspiracle) (reviewed in ref. 17). Although no homolog of the ecdysone receptor is known in C. elegans, cholesterol deprivation produces a phenotype of incomplete shedding of the old cuticle, which is similar to that observed by disruption of two nuclear hormone receptors, nhr-23 and nhr-25, which are C. elegans homologs of Droso phila orphan nuclear receptors that are induced by ecdysone, DHR3 and BFTZ-F1, respectively.\(^{19,20}\) Furthermore, mutants displaying molting defects are enhanced by cholesterol deprivation such as mutations in let-767, a steroid modifying enzyme that is a homolog of human 17-estradiol dehydrogenase\(^{21}\) and mutations in trp-1, the worm homolog of gp330/megalin, a mammalian protein with homology to the LDL receptor.\(^{22}\) These activities might thus be required for the production and function of a steroid hormone that regulates molting.

**Cholesterol Transport Through Lipoproteins Distinct from Yolk**

In mammals, after BA-facilitated absorption, ingested cholesterol, phospholipids, triglycerides and lipid-soluble vitamins, are transported from the gut to other tissues that need them via lipoproteins such as chylomicrons. Other lipoproteins such as low-density lipoproteins (LDL) serve to re-distribute lipids and cholesterol from the liver to peripheral tissues, and high density lipoproteins (HDL) transport cholesterol from peripheral tissues back to the liver in a process termed reverse cholesterol transport. In humans, defects in cholesterol transport and distribution are associated with several diseases such as atherosclerosis, obesity, type C Niemann-Pick disease and others. The best-known lipoproteins in C. elegans are the yolk particles. The protein moieties of yolk particles are vitellogenins, distant homologs of apolipoprotein B (ApoB), which is the major protein in chylomicrons and LDL.\(^{23}\) In C. elegans there are five genes that code for vitellogenins, including vit-2 to vit-6.\(^{24}\) To provide nutrients for growth and development of the embryo in C. elegans, cholesterol, fatty acids and possibly other nutrients are transported from the gut to developing oocytes through the pseudocoelomic cavity by means of yolk particle, indicating that the transport mechanisms of cholesterol in C. elegans are similar to those in mammals.\(^{25,26}\) Yolk is taken up by oocytes by a conserved pathway of receptor-mediated endocytosis via a yolk receptor, RME-2, which is a member of the lipoprotein receptor superfamily.\(^{25}\)

Several observations suggest that there are other cholesterol transport systems in worms besides yolk.\(^{27}\) For example, hermaphrodites are capable of transporting cholesterol before the vitellogenins are expressed and males do not express vitellogenins but accumulate cholesterol in developing sperm.\(^{28}\) Furthermore, a mutation in dsc-4/mtp, the worm homolog of the microsomal triglyceride transfer protein (MTP),\(^{29}\) whose activity in mammals is required for production and secretion of ApoB-containing lipoproteins, does not affect yolk production as, in contrast to rme-2 mutants, it produces no defect in oocyte maturation or embryo production. However, disruption of dsc-4/mtp by mutation or RNAi has phenotypic effects on several tissues, shortening the development rate of the germline and the length of the defecation cycle, a rhythmic behavior that is driven by the physiology of the gut (Fig. 2).\(^{25}\) This suggests that DSC-4/MTP is required for the secretion of a type of lipoprotein that is distinct from yolk and that serves to transport lipids between tissues, as do mammalian lipoproteins. Possibly, the core apoprotein for this hypothetical lipoprotein is a vitellogenin that is folded and lipidated by DSC-4/MTP into a particle that is distinct from yolk, as suggested by previous studies that show that human MTP is able to act upon Xenopus egg vitellogenins.\(^{30}\) Furthermore, RNAi knockdowns of dsc-4/mtp and of some vitellogenins, in particular vit-5, have the same phenotypic effects on germline development.\(^{28}\) Another possibility is that DSC-4/MTP might be the apoprotein itself as it is evolutionarily related to vitellogenins and ApoB.\(^{25}\) dsc-4/mtp was identified as a mutation that suppresses the slow defecation and the slow germline development of clk-1 mutants.\(^{31}\) CLK-1 is a conserved mitochondrial enzyme that is necessary for the biosynthesis of the antioxidant and redox cofactor ubiquinone (coenzyme Q: CoQ). Several observations suggest that dsc-4/mtp suppresses clk-1 by reducing the level of a type of MTP-dependent, LDL-like lipoprotein. For example, reducing the level of dietary cholesterol, which is a major constituent of lipoproteins whose reduction leads to reduce LDL levels in vertebrates, mimics the effects of dsc-4 on the slow germline development and the slow defecation cycle length of clk-1 mutants,\(^{28,32}\) suggesting that those phenotypes of clk-1 mutants are due to high levels of LDL-like lipoproteins biosynthesis and secretion. It is not clear how elevated lipoprotein biosynthesis and secretion slow down the defecation cycle; however, the MTP-dependent lipid transport system appears to be so well conserved between mammals and C. elegans that drugs that have been developed to lower lipid levels in humans can act as suppressors of the slow defecation phenotype of clk-1 mutants.\(^{33}\) For example, the slow defecation is suppressed by drugs that (1) antagonize high LDL levels by increasing HDL levels (e.g., phenylthiourea inhibitors of the HDL receptor SR-BI\(^{35}\)), that (2) stimulate reverse cholesterol transport by regulating gene expression through nuclear hormone receptors (e.g., gemfibrozil\(^{34}\)) or that (3) inhibit the activity of HMG-CoA reductase to lower cholesterol levels (e.g., flu- vastatin and lovastatin). This suggests that the targets of these drugs are conserved to a sufficient degree in worms for these compounds to be active and that C. elegans provides a pharmacological platform that could be used to develop or identify compounds that affect lipid metabolism in mammals as well. In fact, such a compound screen has been performed and a number of molecules that are active on clk-1 mutants have been found to reduce apoB secretion in cultured human cells and to reduce plasma lipoprotein levels in a mouse model of dyslipidemia.\(^{35}\)
A Conserved Pathway of Bile Acid Biosynthesis and Secretion in C. elegans

In the same genetic screen in which disc-4/mtp was identified, we also found another suppressor of the slow defecation phenotype of clk-1 mutants, disc-3/tat-2 (Fig. 3A). The two mutations appear to function in a common pathway as their effects are not additive.1 DSC-3/TAT-2 (TAT stands for transbilayer amphipath transporters) is a homolog of ATP8B1, which is necessary for normal secretion of BAs in mammals (see above).6,7 Following up this lead we found that feeding clk-1 mutants with cholestyramine, a BA-binding resin that targets BAs and reduces BAs availability and, thus, lowers circulating LDL in mammals,35 suppresses the slow defecation of clk-1 mutants, like the disc-3/tat-2 and the disc-4/mtp mutations (Fig. 3A). Knockdown of the C. elegans homologs of BA-biosynthetic enzymes similarly suppresses the phenotype (see below). This lead us to propose a model in which C. elegans secretes molecules that are similar to BAs in mammals and in which the altered defecation phenotype of clk-1 mutants is due to enhanced synthesis of such molecules.1 In addition, we found that the regulation of defecation in response to BA availability is deregulated in clk-1 mutants as shown by the observation that treatment with mammalian BAs enhances the phenotype of the mutants but is without effect on the wild-type6 (Fig. 3A).

Although the exact molecular structures of the C. elegans BA-like molecules are not yet known, an activity that can be extracted from C. elegans by ether mimics the effect of mammalian BAs in enhancing the slow defecation cycle of clk-1 mutants. Furthermore, as expected from the genetics-based model, this activity is more abundant in extracts from clk-1 mutants1 (Fig. 3B).

The C. elegans BA-like Molecules are Derived from Cholesterol and are Distinct from Dafachronic Acids

In mammals, BAs are produced in the liver as oxidized breakdown products of cholesterol through a series of oxidation reactions and a shortening of the side chain.2 Enzymes that catalyze these multi-step reactions are located in different cellular compartments, including the endoplasmic reticulum, cytosol, mitochondria and peroxisomes. For example, the oxidation of the side-chain takes place in the mitochondria, but side-chain shortening takes place in the peroxisomes by β-oxidation.2 To understand whether BA-like molecules in worms are synthesized through pathways similar to those in mammals, we used RNAi on clk-1 mutants to screen homologs of mammalian BA-biosynthetic enzymes. A number of genes were identified, including 13 P450 oxidases and enzymes of peroxisomal β-oxidation.1 In mammals, peroxisomal β-oxidation is involved in cholesterol side-chain shortening. In addition, the clk-1 phenotype is also suppressed by a mutation in daf-36, which encodes a cholesterol 7-desaturase. These data suggest that C. elegans BA-like molecules are indeed cholesterol derivatives and that they might have a shortened side-chain. This is in contrast to DAs, which have some characteristics of BAs, but whose oxidation is not extensive and whose side-chain is not shortened.10 Moreover, neither knockdown of daf-12, the nuclear receptor of DAs nor depletion of daf-9 and hsd-1, two activities that are known to participate in the
been evidenced but its mechanistic basis has not yet been elucidated. BAs are signaling molecules that regulate steroid, lipid and glucose metabolism. Our findings with worm BAs thus suggest the attractive hypothesis that the mitochondrial oxidative stress that is typical of the aging pattern of a wide variety of animals, including people, could lead to abnormal metabolism of BAs, which could in turn deregulate metabolic pathways and, thus, lead to the classical pattern of age-dependent metabolic diseases that is prominent in our society. Interestingly, the unexplained variety of phenotypes observed in clk-1 mutants might also reflect an

_in both worms and mice, mutations in clk-1 affect mitochondrial function, with increased mitochondrial oxidative stress, elevated mitochondrial ROS production, and increased sensitivity to pro-oxidant drugs. To determine whether the elevated mitochondrial oxidative stress was responsible for the altered BA metabolism, we tested the effects on clk-1 of treatment with an antioxidant, NAC (N-acetyl-cysteine) and of depletion of SOD-2, the main mitochondrial superoxide dismutases whose reduction should elevate mitochondrial oxidative stress. Both treatments were successful in suppressing and enhancing, respectively, the slow defecation cycle. Furthermore, these treatments also consistently altered the level of BA-like activity that could be extracted from treated worms (Fig. 3B). In mammals, a link of oxidative stress with dyslipidemia and with the other cardiovascular risk factors that constitute the metabolic syndrome has repeatedly been evidenced but its mechanistic basis has not yet been elucidated. BAs are signaling molecules that regulate sterol, lipid and glucose metabolism. Our findings with worm BAs thus suggest the attractive hypothesis that the mitochondrial oxidative stress that is typical of the aging pattern of a wide variety of animals, including people, could lead to abnormal metabolism of BAs, which could in turn deregulate metabolic pathways and, thus, lead to the classical pattern of age-dependent metabolic diseases that is prominent in our society. Interestingly, the unexplained variety of phenotypes observed in clk-1 mutants might also reflect an

**Figure 3.** The elevated mitochondrial oxidative stress of clk-1 mutants alters the metabolism of bile acid-like molecules, resulting in the slow defecation mutant phenotype. The length of the defecation cycle (defined as the time between pBocs) for each genotype or condition is schematically represented by blue arrows. The decreased or increased size of arrows means that the defecation rates are shortened or lengthened, respectively. (A) The slow defecation phenotype of clk-1 is suppressed by mutations in dsc-4/mtp or dsc-3/tat-2 and treatment with the BA-binding resin cholestyramine. On the other hand, the slow defecation phenotype is enhanced by treatment with mammalian BAs. (B) Lipid extracts from wild type worms contain an activity that mimics the enhancing effect of mammalian BAs on clk-1. Furthermore, extracts from clk-1 mutants contain more of the activity and thus have a stronger enhancing effect. In addition, suppression of clk-1 mutant by treatment with the antioxidant NAC, which suppresses the slow defecation phenotype, reduces the amount of activity in extracts, while enhancement of the phenotype by increase of mitochondrial oxidative stress through loss of SOD-2 increases the amount of activity in extracts.
effect of abnormal regulation of metabolic processes by BAs.

Perspectives

In this commentary, we have briefly reviewed findings about several processes (such as dauer formation, molting and defecation) that appear to be regulated by sterol metabolites. However, until now, only DAs have been structurally characterized and the corresponding nuclear hormone receptor identified. Likely, the BA-like molecules that regulate defecation will likely be found to target at least some of the nearly 260 potential nuclear hormone receptors (NHRs). Identifying these receptors might prove particularly useful to understand the pleiotropy of dkr-1 and help to provide a more complete model of how the deregulation of BA synthesis by mitochondrial oxidative stress can lead to metabolic disorders.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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