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

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C. elegans is a model used to study cholesterol metabolism and the functions of its metabolites. Several studies have reported that, in worms, cholesterol is not a structural component of the membrane as it is in vertebrates. However, as in other animals, it is used for the synthesis of steroid hormones that regulate physiological processes such as dauer formation, molting and defecation. After cholesterol is taken up by the gut, mechanisms of transport of cholesterol between tissues in *C. elegans* involve lipoproteins, as in mammals. A recent study[1] shows that both cholesterol uptake and lipoprotein metabolism in *C. elegans* are regulated by molecules whose activities, biosynthesis and secretion strongly resemble those of mammalian bile acids, which are metabolites of cholesterol that act on metabolism in a variety of ways. Importantly, it was found that oxidative stress upsets the regulation of the synthesis of these molecules. Given the known function of mammalian bile acids as metabolic regulators of lipid and glucose homeostasis, future investigations of the biology of *C. elegans* bile acid-like molecules could provide information on the etiology of human metabolic disorders that are characterized by elevated oxidative stress.

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).[2] After cholesterol is taken up from food, it is associated with lipoprotein particles and then transported between organs predominantly as cholesteryl 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.[5] ATP8B1, a type 4 P-type ATPase, is a predicted phospholipid flippase.[6] 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.[7]
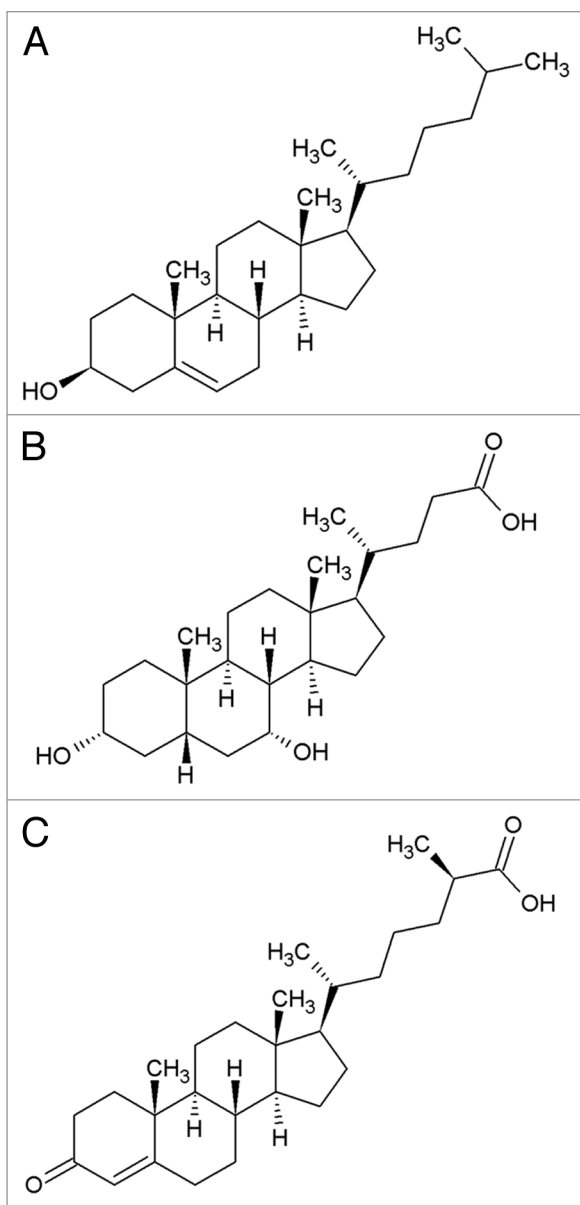
Figure 1. The structures of (**A**) cholesterol, (**B**) a mammalian bile acid, chenodeoxycholic acid (CDCA), (**C**) dafachronic acid.

Cholesterol Metabolism and Functions of Cholesterol Metabolites in *C. elegans*

Data accumulated in recent years indicate that *C. elegans* is starting to become a valuable model for studying sterol metabolism and function. In this commentary, we summarize recent advances in our understanding of *C. elegans* steroid signaling pathways and their regulations of physiological features, in particular dauer formation, molting and defecation. Much about sterols and sterol metabolites in

worms, including biosynthesis, transport and targets such as lipoproteins, appears to be evolutionarily conserved, thus providing a model system that can be used to understand metabolic disorders in mammals.

C. elegans and other nematodes, like many other animals such as *Drosophila* and other insects, are auxotrophic for sterols because they do not possess the enzymes that are required for de novo sterol synthesis but are capable of modifying sterols (reviewed in ref. 8). Although the worm does not synthesize sterols, they are

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,[9] 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*.[10] 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.[11-14] 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[15] and HSD-1, a 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase.[16]

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

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*,[18] 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 *Drosophila* 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 *lrp-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.[26] Furthermore, a mutation in *dsc-4/mtp,* the worm homolog of the microsomal triglyceride transfer protein (MTP),[28] 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**).[29] 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.[23]

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 vertebrate, 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.[32] 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[33]), 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., fluvastatin 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.[32]
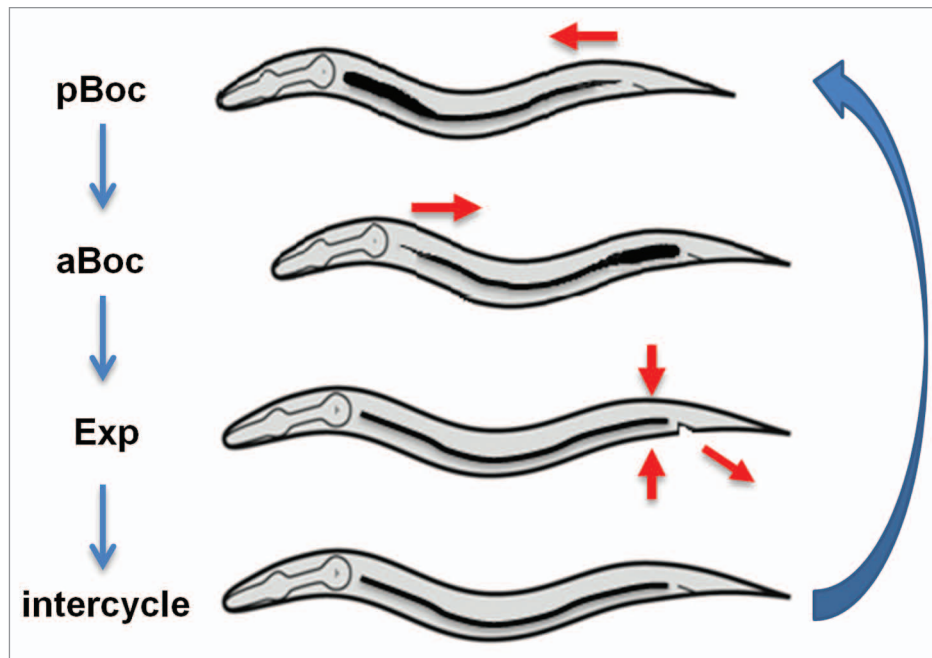
Figure 2. Steps of the defecation motor program (DMP). The first step involves the posterior body muscle contraction (pBoc), which pushes the gut contents anteriorly, followed by the second step, the anterior body muscle contraction (aBoc), which concentrates the gut contents in the pre-anal region. Finally, the enteric muscle contractions result in the expulsion (Exp) of the gut contents. The red arrows indicate the directions in which muscles contract during the various steps of the motor program. The defecation motor program is executed rhythmically, about once a minute.

A Conserved Pathway of Bile Acid Biosynthesis and Secretion in *C. elegans*

In the same genetic screen in which *dsc-4/mtp* was identified, we also found another suppressor of the slow defecation phenotype of *clk-1* mutants, *dsc-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 *dsc-3/tat-2* and the *dsc-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-type[1] (**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* mutants[1] (**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
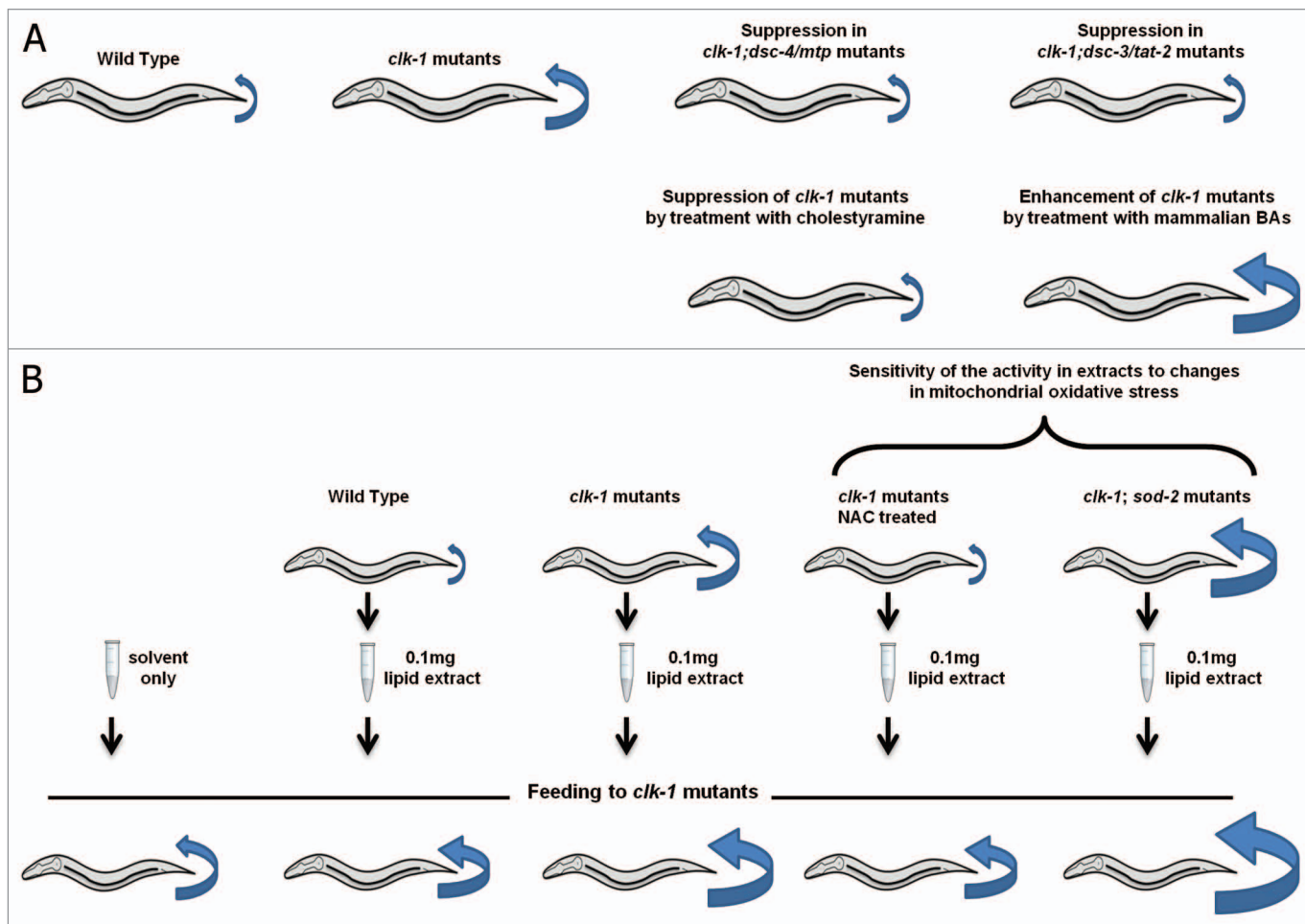
Figure 3. The elevated mitochondrial oxidative stress of *clk-1* mutants alters the metabolism of bile acid-like molecules, resulting in the slow defecation mutant phenotype. The length of the defecation cycle (defined as the time between pBocs) for each genotype or condition is schematically represented 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.

synthesis of DAs, affect the *clk-1* defecation phenotype.[1]

C. elegans as Model for Studying the Effect of Oxidative Stress on Metabolic Disorders

In both worms and mice, mutations in *clk-1* affect mitochondrial function,[36,37] with increased mitochondrial oxidative stress,[38,39] elevated mitochondrial ROS production,[38] elevated oxidative damage[40,41] and increased sensitivity to prooxidant drugs.[42] 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.[1] 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[43-45] 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[46] might also reflect an

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.[10] 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).[47] Identifying these receptors might prove particularly useful to understand the pleiotropy of *clk-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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