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Medical Bioremediation: A Concept Moving Toward Reality

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Abstract

A major driver of aging is catabolic insufficiency, the inability of our bodies to break down certain substances that accumulate slowly throughout the life span. Even though substance buildup is harmless while we are young, by old age the accumulations can reach a toxic threshold and cause disease. This includes some of the most prevalent diseases in old age—atherosclerosis and macular degeneration. Atherosclerosis is associated with the buildup of cholesterol and its oxidized derivatives (particularly 7-ketocholesterol) in the artery wall. Age-related macular degeneration is associated with carotenoid lipofuscin, primarily the pyridinium bisretinoid A2E. Medical bioremediation is the concept of reversing the substance accumulations by using enzymes from foreign species to break down the substances into forms that relieve the disease-related effect. We report on an enzyme discovery project to survey the availability of microorganisms and enzymes with these abilities. We found that such microorganisms and enzymes exist. We identified numerous bacteria having the ability to transform cholesterol and 7-ketocholesterol. Most of these species initiate the breakdown by same reaction mechnism as cholesterol oxidase, and we have used this enzyme directly to reduce the toxicity of 7-ketocholesterol, the major toxic oxysterol, to cultured human cells. We also discovered that soil fungi, plants, and some bacteria possess peroxidase and carotenoid cleavage oxygenase enzymes that effectively destroy with varied degrees of efficiency and selectivity the carotenoid lipofuscin found in macular degeneration.

Introduction

As a side effect of being alive, various waste substances are produced in our bodies. Most of these are routinely broken down by enzymes catalyzing the processes, summarized as human catabolism. However, some molecules are resistant to human catabolism and accumulate in our bodies over the adult life span. This phenomenon has been termed age-related catabolic insufficiency, and it is thought to be a major mechanism driving aging and age-related diseases.[1] For example, atherosclerosis and age-related macular degeneration are thought to be such age-related storage diseases. Atherosclerosis is associated with the accumulation of cholesterol and oxidized cholesterol derivatives in the artery wall. Age-related macular degeneration is associated with the accumulation of carotenoid lipofuscin in retinal pigment epithelial cells. We discuss each of these processes and the accumulating compounds in more detail below.

It has been proposed to treat the whole class of age-related storage disorders by using enzymes from environmental microorganisms that can break the accumulating substances down.[2] Because this method is commonly used in the successful field of environmental bioremediation, the new approach targeting aging diseases has been termed "medical bioremediation."

Atherosclerosis

Atherosclerosis is the leading cause of death in developed countries. The disease progresses slowly and mostly asymptomatically over the life span until rupture of brittle arterial

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plaques causes a heart attack or stroke.[3] Atherosclerosis is attributed to the accumulation of cholesterol and oxidized cholesterol derivates, such as 7-ketocholesterol (7KC), in the artery wall. Endothelial cells and macrophages attempt to clear these aggregates, but are unable to do so. Thus, they become engorged with lipid material and turn into dysfunctional foam cells, which may slowly die. Inflammation and fibrosis follow, and further immune cells are attracted, which are equally unable to resolve the storage problem, but instead add to it.[4]

Foam cell formation can be recapitulated *in vitro*.[5] Oxysterols, such as 7KC, promote foam cell formation in these models,[6] possibly by inhibition of cholesterol efflux from the macrophage.[7] The mechanisms underlying this may involve lysosomal enzyme inhibition and lysosomal membrane labilization by the oxysterols.[8] Another potential mechanism is iron capture by the oxysterols followed by iron-catalyzed lipid peroxidation.[9] In particular, 7KC appears to inhibit the processing and efflux of normal cholesterol by foam cells.[7] However, better tools are needed to assess the relevance for these cell culture models in the *in vivo* situation.

In vitro and *in vivo*, foam cells are characterized by pronounced deposits of cholesterol, cholesteryl esters, 7KC, and 7KC ester in the foam cell lysosomes and in the cytoplasm.[10,11] Even though much of the free oxysterols are located in the cytoplasm, they do traffic through the lysosomes and might be accessible there to a lysosomally delivered enzyme therapy.[12] Foam cell death leads to the formation of a necrotic core in the advanced lesion, and the core contains extracellular oxidized lipids and cell debris.

Current therapies, importantly statin therapy, can slow down the progression of atherosclerosis by virtue of their lipid-lowering, antiinflammatory, antithrombotic, plaque-stabilizing, and cytoprotective properties.[13] A bioremediation treatment directly targeting existing cholesterol and oxysterol deposits would be an important addition to the existing options. In the following, we report novel results from our studies into the degradation of 7KC by soil microbes.

Macular degeneration

Age-related macular degeneration is the most frequent cause of blindness in the elderly.[14] The disease is characterized by progressive apoptosis of retinal pigment epithelial (RPE) cells, inflammation, extracellular matrix changes, and sometimes aberrant vascularization of the tissue.[15] Accumulations of pathogenic materials, such as drusen and intracellular lipofuscin, are associated with the disease. Lipofuscin is a heterogeneous mixture of indigestible molecules that accumulate in postmitotic cells.[1] RPE lipofuscin is different from other lipofuscins in that it is derived mostly from phagocytosed photoreceptor segments. Thus, it contains high levels of vitamin A–derived fluorescent compounds, such as the pyridinium bisretinoid A2E.[16] A2E has multiple pathogenic effects, including disruption of lysosomal membranes due to detergent-like activity,[17] inhibition of the lysosomal adenosine triphosphatase (ATPase),[18] and phototoxicity.[19] Congenitally accelerated A2E accumulation causes Stargardt macular degeneration, a condition closely mimicking age-related macular degeneration in mice[20] and humans.[21] It is presently unclear how A2E may relate to the development of the dry and wet subtypes of the disease and how it is implicated in the associated inflammatory and angiogenic pathways.[22]

A selective means to remove A2E from the affected cells' lysosomes could provide an important test for the hypothesis that A2E is indeed causing the disease, and, if a causal relationship exists, simultaneously provide a bioremediation treatment with the potential to arrest or reverse the disease. In the following, we report for the first time the discovery that certain naturally occurring enzymes destroy A2E in various ways.

Enzyme therapy

An enzyme therapy targeting lysosomal accumulations of oxysterols or lipofuscin would require a safe and effective means to delivery enzyme drugs to the affected cells' lysosomes. Currently marketed "enzyme replacement therapies" (ERT) for heritable lysosome storage diseases provide a roadmap for delivering therapeutic enzymes to human cell lysosomes.[2,23,24] These strategies rely on certain "tags" on the enzymes that bind specific endocytic cell-surface receptors. Binding of the enzyme to the receptor results in endocytosis. According to the classic endocytic route, endosomes fuse with lysosomes, resulting in targeted lysosomal delivery. The successful clinical use of these systems suggests that ERT would provide a means to deliver oxysterol- or lipofuscin-destroying enzymes to the target cell lysosomes.

Two primary receptors are used for ERT delivery: (1) the mannose receptor, which is found on phagocytic cells, and (2) the cation-dependent mannose-6-phosphate (M6P)/insulin-like growth factor-2 (IGF-2) receptor, which is found on most cell types.[25] For example, Gaucher disease is caused by mutations in glucocerebrosidase. As a result, glucocerebroside accumulates selectively in macrophages. Recombinant glucocerebrosidase (Cerezyme, Imiglucerase) is delivered via the mannose receptor to treat this disease.[26] For therapy of other lysosome storage diseases, such as Fabry disease, which affect nonphagocytic cell types, the respective therapeutic enzymes are delivered via the M6P/IGF2R route.[27] Strategies for implementing each kind of tag on a recombinant enzyme have recently been discussed elsewhere, and will be recognized here only by reference.[28–32]

Immunogenicity is a further important concern for therapies using foreign proteins. In ERT, supplying enzymes "foreign" to the patient with a congenital defect can indeed pose problems. For therapies targeting very slowly accumulating substances, such as A2E or 7KC, the enzyme therapy would be needed only once or extremely infrequently. Thus, immunogenicity may be less of a concern. However, we recognize methods of managing immunogenicity if it does occur by reference.[33–36] It is beyond the scope of this review to discuss these methods in detail.

In the following, we report the discovery and characterization of enzymes with the ability to degrade 7KC and A2E and discuss their potential applications as bioremediation therapies for atherosclerosis and macular degeneration.

Materials and Methods

Target compounds

All compounds were ACS grade from Sigma, unless indicated otherwise. We synthesized isotope-labeled 7KC from

cholesterol (Cambridge Isotopes) using a series of published methods. Briefly, we protected the 3′-hydroxyl group as an acetyl ester with I_2 catalysis,[37] oxidized the allylic 7-position with pyridinium chlorochromate,[38] and cleaved the ester by cold K_2CO_3 hydrolysis.[39] We purified 7KC on an Agilent HP 1100 system with a preparative column (25 cm×2.5 cm× 6 μm). The method was isocratic 100% methanol at a flow rate of 20 mL/min. We synthesized A2E using a published method.[40] Briefly, we dissolved all-*trans*-retinal in ethanol, and then added 0.5 equivalents of ethanolamine and 0.5 equivalents of acetic acid. The mxiture was stirred in the dark at room temperature for 3 days. We purified crude A2E by flash chromatography against 6-nm silica using a two-step gradient (5% CH_2Cl_2 in methanol for binding, then 8% CH_2Cl_2 with 0.1% trifluoroacetic acid [TFA] for elution). Subsequently, we purified A2E from the flash product on an Agilent HP 1100 system with a preparative column (25 cm×2.5 cm×6 μm) in the dark. The method was isocratic 92.5% methanol, 7.5% water, 0.1% TFA at a flow rate of 20 mL/min. We synthesized 7KC esters from fatty acid chlorides.[41] Briefly, we incubated 7KC with 1 equivalent of fatty acid chlorides in anhydrous pyridine at 55C for 3 h. We purified the esters by flash chromatography against 6-nm silica in 100% diethyl ether.

Enrichment culturing

We dissolved or suspended by sonication 0.1% of the target compound in M9 mineral medium. We inoculated 50-mL cultures with 100 mg of environmental samples and incubated them with shaking at 160 rpm, at 28°C. We sampled 100-μL aliquots over time and analyzed the concentration of target compounds by high-performance liquid chromatography (HPLC). For hydrophobic targets that formed clumps, we averaged up to five such samples per time point to compensate for the variance due to clumping.

HPLC analysis

We sampled 100 μL of culture or enzyme reaction and extracted the hydrophobic targets (A2E, 7KC) with one volume of chloroform/methanol (1:1 vol/vol). We analyzed all compounds on a Waters Acquity ultra-high-pressure liquid chromatograph (UPLC) equipped with a C18 high-strength silica column (10 cm×2.1 mm×1.7 μm). Methods were: 7KC, isocratic 100% methanol, 1 mL/min; A2E, isocratic 92.5% methanol, 7.5% water, 0.1% TFA, 700 μL/min. Detection was with a Waters photodiode array detector. Preferred wavelengths were: A2E, 440 nm; 7KC, 238 nm.

Liquid chromatography/mass spectrometry analysis of metabolites

We performed liquid chromatography/mass spectrometry (LC/MS) analyses on a Beckmann System Gold Programmable Solvent Module, using a Supelcosil LC-18 column (250 mm×4.6 mm, 5 μm). The solvent system was isocratic isopropanol:acetonitrile (30:70) containing 0.1% formic acid at a flow rate of 0.3 mL/min. We suspended the culture extracts in 500 μL of solvent and injected 50 μL into the system. We performed mass spectrometry with a PE SCIEX API 365 MS/MS turbo ionspray mass spectrometer. Settings were: Scan mode $m/z = 300$–500, positive mode, ionspray voltage − 3500, declustering potential = 40, focusing potential = 200, entrance potential = 9.6, nebulizer gas = 9, and curtain gas = 9.

In vitro *enzyme reactions*

For enzyme expression, we used a standard *Escherichia coli* expression system, pET21a in the strain BL21-Star (Invitrogen), and followed standard procedures.[42] Briefly, we inoculated an overnight culture from a frozen glycerol stock in LB medium using 100 mg/L ampicillin selection. The next day, we transferred 5 mL of the culture to a new 50-mL culture and incubated it at 25°C for ≈4 h, until the optical density at 600 nm reached 0.600. Then, we added isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM to induce the T7-lac promoter system of the BL21-Star strain. We allowed the proteins to be expressed for 4 h and then lysed 1.5 mL of culture volume with 100 μL of the BugBuster/Lysonase system (Novagen). We centrifuged down debris at 16,000×*g* for 10 min and used the supernatant as the enzyme solution. For commercial enzymes, we suspended the enzymes in 50 mM Na_2PO_4 buffer at the pH as indicated in the results section. We prepared the substrate solution by adding 10 μL of 1 mg/mL A2E in methanol to 1 mL of 50 mM Na_2PO_4 buffer containing 0.2% Triton X-100 at the indicated pH. In the case of the carotenoid cleavage oxygenases (CCOs), the buffer also contained antioxidant–iron mixture (final concentrations 10 mM ascorbic acid, 10 mM dithiothreitol, 0.5 mM $FeSO_4$). We initiated the *in vitro* enzyme reactions by adding 1 volume of enzyme solution to 2 volumes of substrate solution.

Mammalian cell toxicity

We cultured human embryonic kidney cells 293T (ATCC #CRL-11268) in Eagle minimal essential medium (EMEM) with 10% fetal bovine serum (FBS) under 5% CO_2. We dissolved sterols in 45% β-hydroxypropyl cyclodextrin and delivered them to 293T cells grown in 96-well plates (100 μL Dulbecco modified Eagle medium [DMEM]/well) to make a final concentration of 18 μM sterols in the medium, and we incubated the plates for 24 h. We used Cell Counting Kit-8 (Dojindo) to determine the viability of cultured cells and a colorimetric assay for cell viability. Briefly, we added 10 μL of [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium] bromide (MTT) solution to cells and incubated them for and additional 4 h before measuring the absorbance at 450 nm.

Results and Discussion

Figure 1 shows the 7KC-biodegradation patterns for six strains of bacteria that were able to grow due to their biodegradation of 7KC. Approximately 1 out of 5 independent environmental samples contained microorganisms able to biodegrade 7KC within several days, and we have isolated over 30 7KC-degrading species. The six strains shown in Fig. 1 are the ones for which we recorded time curves during their biodegradation. We have published more details about these experiments and characterized the degraders in detail elsewhere.[43,44]

Since the earlier publications, we performed isotope-labeling and LC/MS experiments to identify 7KC-derived metabolites in the culture supernatant, which we report here for the first time. The labeling experiments were carried out with *Nocardia nova*, one of our fastest degrading isolates.

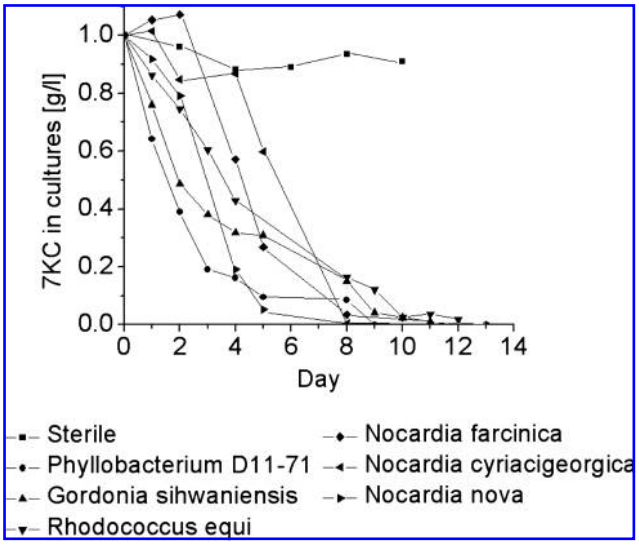

FIG. 1. Biodegradation of (7KC) by environmental microorganisms. We exposed 7-ketocholesterol in M9 medium to 10^6 colony-forming units (CFU) of pure cultures of 7KC degraders previously isolated. We took samples at the indicated time points, extracted 7KC with methanol/chloroform, and quantified it by high-performance liquid chromatography (HPLC). These species efficiently deplete 7KC from the cultures, apparently using it as the sole source of carbon and energy.

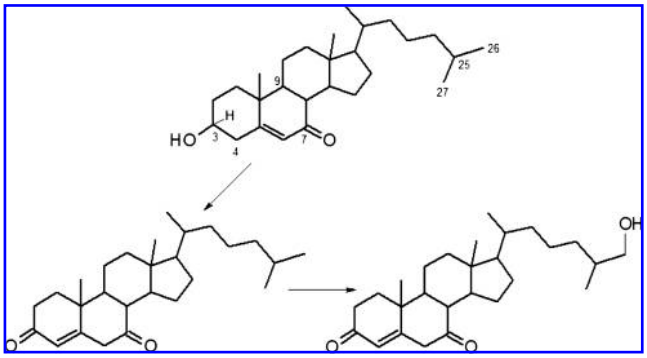

FIG. 2. Based on our isotope-labeling and liquid chromatography/mass spectrometry (LC/MS) experiments, we postulate a 7-ketocholesterol (7KC) degradation pathway in which head oxidation is followed by tail hydroxylation. This is analogous to classical microbial cholesterol degradation pathways.[45]

Under electrospray ionization (ESI), 7KC ($M = 400$) appears as a proton adduct $M + H^+ = 401$ and as a sodium adduct $M + Na^+ = 421$. LC/MS of the culture supernatant revealed 7KC, as well as two minor peaks with $M + H^+ = 399$, $M + Na^+ = 420$ and $M + H^+ = 415$, $M + Na^+ = 437$. These masses are consistent with a two-proton elimination and subsequent hydroxylation of 7KC on a hydrocarbon atom. When grown on 4-^{13}C 7KC, all of the above masses increased by 1, which demonstrates that the products are directly derived from 7KC. Furthermore, 3-D 7KC appeared at an m/z increased by 1, but did not give rise to products with increased m/z, suggesting elimination of the deuterium label in the 3-position. This is consistent with an oxidation of the 3-hydroxyl group to a ketone, forming 3,7-diketocholesterol (37DKC). We could not label the OH-proton itself, because this label was unstable in aqueous solution. 25,26,27-D_6 7KC appeared at an m/z increased by 6. This gave rise to an oxidized product whose mass was increased by 6, but its hydroxylated-product's mass was increased only by 5. This is consistent with hydroxylation somewhere at the "tail" end of 7KC, at either of the 25, 26, or 27 carbon atoms. On the basis of these results, we postulate the 7KC-degradation pathway in Fig. 2. Degradation of 7KC is initiated by head oxidation (a cholesterol oxidase-like reaction), followed by tail hydroxylation. These steps are analogous to known cholesterol degradation pathways by microorganisms, although some microbes use a second dehydrogenation step at the head ring before they hydroxylate the tail.[45]

Thus, most of these species appear to employ a cholesterol-like degradation pathway that is initiated by cholesterol oxidase and continues with tail hydroxylation, suggesting that they use the same enzymes for cholesterol and 7KC breakdown. Potentially, this may limit the usefulness of these enzymes as therapeutics, because cholesterol oxidation may cause the production of a potentially toxic 3-ketone product.[46]

We discovered only one bacterial species, *Staphylococcus haemolyticus*, that slowly degraded 7KC, but had neither the $M + H^+ = 399$ nor the $M + H^+ = 415$ signal. Our mass spectrometric assay cannot rule out the absence of a metabolite based on the absence of a signal. However, it is of note that the genome sequence of *S. haemolyticus*[47] does not appear to contain any homologs of cholesterol oxidase. Thus, *S. haemolyticus* may be an interesting species for further investigation, to discover alternative pathways of 7KC catabolism.

Enzymatic degradation of 7KC

After we had detected a diketone compound in the supernatant of some cultures by LC/MS, it was natural to suspect the involvement of cholesterol oxidase in this reaction. We purchased all forms of cholesterol oxidase available from Sigma (St. Louis, MO) and determined their action on cholesterol and 7KC. As we expected, all forms of cholesterol oxidase oxidized cholesterol. Only one form, from *Brevibacterium sterolicum*, oxidized 7KC and formed the same diketone compound we had observed in the culture extracts. This further corroborates the role of cholesterol oxidase in 7KC degradation by certain microbial species.

Mammalian cell toxicity

To get a preliminary picture of the toxicity of the product of the 3,7-DKC cholesterol oxidase to mammalian cells, we assessed the survival of HEK cells exposed to the sterols using an MTT cell viability assay (Dojindo). Figure 3 shows that cholesterol had minimal toxicity compared to untreated cells, but 7KC caused nearly complete loss of viability staining. The toxicity of 3,7-DKC was about one-half of that of 7KC. It is currently unknown whether the residual toxicity of 3,7-DKC would compromise therapeutic utility of the cholesterol oxidase enzyme. Questions about the therapeutic potential of cholesterol oxidase should be asked in a foam cell model.[5,9] If cholesterol oxidase can protect macrophages from 7KC-mediated inhibition of sterol efflux and foam cell

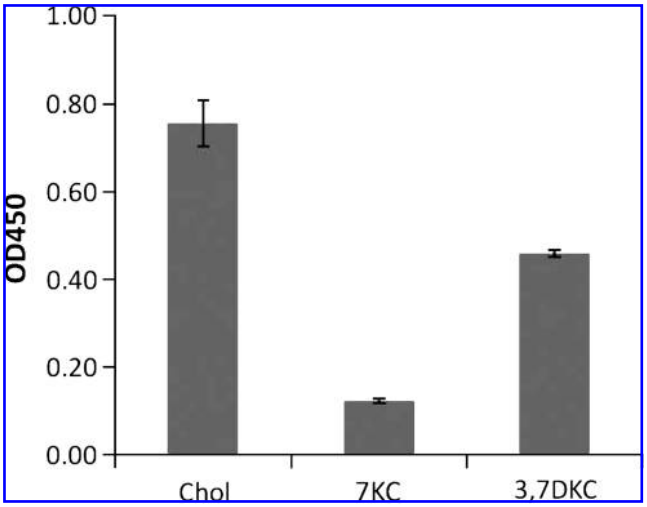

FIG. 3. Toxicity of sterols to 293T cells. After overnight exposure to 18 μM sterols in beta-hydroxypropyl cyclodextrin (BCD), cholesterol was nontoxic, 7-ketocholesterol (7KC) caused near-complete cell death, and the toxicity of 3,7-diketocholesterol (DKC) was intermediate. This suggests that an enzyme transforming 7KC into 3,7-DKC (cholesterol oxidase) may have some usefulness in protecting cells from 7KC toxicity.

formation, then such potential may exist. If it cannot afford such protection, then the search for selective 7KC modifying enzymes should continue.

Hydrolysis of 7KC esters

We tested whether our 7KC-degrading isolates would also degrade 7KC esters of palmitate and linoleate using the same methods as for 7KC. All of our 7KC-degrading isolates also degraded the esters and employed them as the sole source of carbon and energy. Thus, microbial esterases capable of cleaving 7KC-esters appear to be frequent. This is especially significant because no human lysosomal enzyme is known to hydrolyze these compounds. We also purchased *Pseudomonas fluorescens* cholesterol esterase from Sigma. This enzyme effectively cleaved cholesterol palmitate and linoleate, as well as 7KC palmitate and linoleate *in vitro*. Free cholesterol and 7KC were formed in the respective reactions.

The 7KC-degrading species also degraded 7KC linoleate and palmitate, and some microbial cholesterol esterases accept 7KC-esters as substrates. These enzymes may be investigated further for oxysterol ester cleavage inside cells. Cell culture evidence suggests that oxysterol esters are long lived in human macrophage lysosomes, and their accumulation may play a role in atherosclerosis.[10,11] This hypothesis could be investigated further by introducing microbial oxysterol esterases into the foam cell lysosome to effect oxysterol ester cleavage. Ultimately, these enzymes could also be delivered to animal models using ERT and thereby help to shed light on the relevance of the cell culture models of ester storage for the *in vivo* situation.

Another interesting question for future study is whether cholesterol and oxysterol esters oxidized at the fatty acid moiety ("core aldehydes") could be hydrolyzed in a similar manner. These compounds contaminate the lysosomal ester pool, and are likely resistant to lysosomal hydrolysis themselves.[10,11]

Absence of A2E microbial biodegradation

Each of the soils from the 7KC-project (described in detail elsewhere[43,44]) was also tested for A2E biodegradation in parallel. Even though cell viability (assessed by colony-forming unit [CFU] plating) usually remained high, we did not detect A2E biodegradation in any of the samples. We screened more than 250 independent environmental samples from diverse habitats around the planet, but never found A2E biodegraders. In fact, the presence of soils usually seemed to protect A2E from spontaneous decomposition over very long times. Whereas 30% of the A2E decomposed spontaneously after 3 weeks in sterile M9, spontaneous decomposition was reduced to 5–20% over the same time period in the presence of soils, and pure cultures of some bacteria had the same effect (data not shown).

Given the impressive catabolic diversity of the microbial communities in soil,[48] we were surprised that biodegradation of an energy-rich molecule composed of relatively common isoprenoid units was not readily achieved. Even the organisms from which we obtained A2E-degrading enzymes later were unable to utilize A2E as the only carbon and energy source. We speculate that the protection of A2E from spontaneous degradation by soils may be due to strong association of A2E with surfaces, which protect it from attack by hydrolysis or oxidation. Whether the lack biodegradation was caused by a lack of catabolic potential, insufficient enzyme induction and/or low bioaccumulation remains to be determined.

Enzymatic degradation of A2E by carotenoid cleavage oxygenases

Because microbial biodegradation of A2E could not be achieved, we set our sights on enzymes already known to degrade similar compounds (carotenoids). Two families of enzymes known to transform carotenoids are the highly substrate-specific CCOs[49] and nonspecific enzymes from the peroxidase/laccase family.[50]

We obtained CCO genes cloned into pET21a as gifts from Claudia Schmidt-Dannert (University of Minnesota), expressed the proteins in *E. coli*, and tested the activity of the lysate directly against A2E. Of all the CCOs described by the Schmidt-Dannert group in 2006,[45] only one, NSC1 (all1106) bleached A2E visibly. The HPLC analyses in Fig. 4 show that the NSC1-containing lysate had depleted A2E, and a new product had formed.

We reasoned that, because A2E is an unusual carotenoid due to its charged central moiety, it is not accepted by most CCOs as a substrate. To test this idea, we obtained by *de novo* gene synthesis[51] an *E. coli*-optimized gene for crocus sativus zeaxanthin cleavage dioxygenase (CSZCD).[52] CSZCD cleaves its carotenoid substrates at the far outer ends (9,10-bond) of the zeaxanthin molecule. The analogous bond in A2E would be far away from the central charged moiety. We found that lysates expressing CSZCD (as described for NSC1) significantly degraded A2E in comparison to non-degrading control enzymes (data not shown). Thus, natural CCOs may indeed work better on A2E if they bind and cleave far away from the unusual charged moiety.

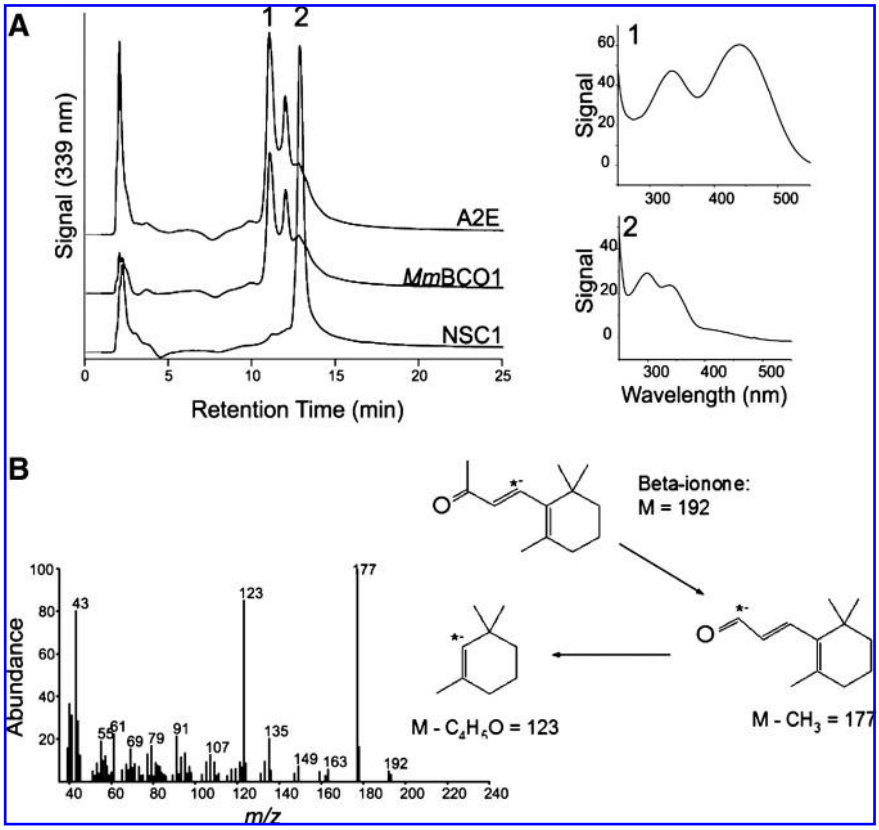

FIG. 4. Cleaveage of A2E by the carotenoid cleavage oxygenase NSC1. We exposed synthetic A2E to a lysate from an *E. coli* culture expressing NSC1. After overnight incubation in the dark, the reaction vessel with NSC1, but not other enzymes appeared bleached. (**A**) High-performance liquid chromatography (HPLC) analysis showed depletion of A2E by NSC1, but not other enzymes (only MmBCO1 shown). (**B**) Gas chromatography/mass spectrometry (GC/MS) analysis of the vessel headspace revealed the formation of gaseous β-ionone in the A2E-degrading reaction, which is also seen when NSC1 acts on other carotenoid substrates.[45]

Enzymatic degradation of A2E by peroxidases

We purchased peroxidases from commercial sources, as indicated in the Fig. 5 legend. We used the absorbance of the *heme* group at 403 nm ($\varepsilon = 102\,\mathrm{mM^{-1}cm^{-1}}$) to adjust the final concentration of each enzyme spectrophotometrically in the reactions to 5.88 μM. Figure 5 shows that the peroxidases differed widely in their ability to degrade A2E. Without peroxide addition, the laccase was the fastest. This is expectable because this enzyme is known to catalyze a four-electron reduction of molecular oxygen in a much more efficient manner than the peroxidases.[53] With 2% hydrogen peroxide addition, the versatile peroxidase was by far the fastest enzyme, depleting A2E virtually instantaneously under these conditions (data not shown due to its fast rate).

We attempted to determine key enzyme-kinetic parameters for these enzymes (K_M/k_{cat}). However, we were hampered by the low solubility of A2E. Dissolving A2E at a concentration approaching the K_M of these enzymes required such high amounts of detergent that most of the enzymes would be inactivated. We were able to determine the parameters only for the laccase: $K_M = 336\,\mu$M and $k_{cat} = 9.7\,\mathrm{min^{-1}}$. The enzymes appeared to work on undissolved A2E clumps in a detergent-free system (similar to those that would be found in lysosomes), but, due to the irregularities

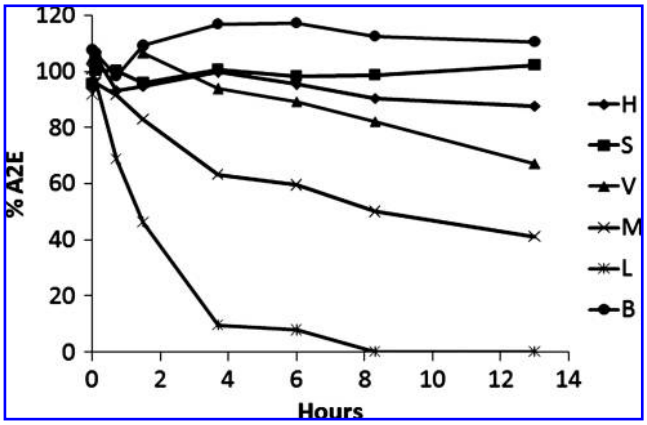

FIG. 5. A2E degradation by peroxidases. We exposed synthetic A2E to peroxidases obtained from commercial sources *in vitro*. We took samples at the indicated time points, extracted A2E with methanol/chloroform, and quantified it by high-performance liquid chromatography (HPLC). These peroxidases destroyed A2E over time with varying degrees of efficiency. H, Horseradish peroxidase type II, Sigma # P8250; S, soybean peroxidase, Bio Research Products; V, versatile peroxidase, Jena Biosciences # EN 203L; M, manganese peroxidase, Jena Biosciences # EN-201L; L, laccase, Jena Biosciences # EN-204L; and B, bovine serum albumin, Sigma # A3059-10G.

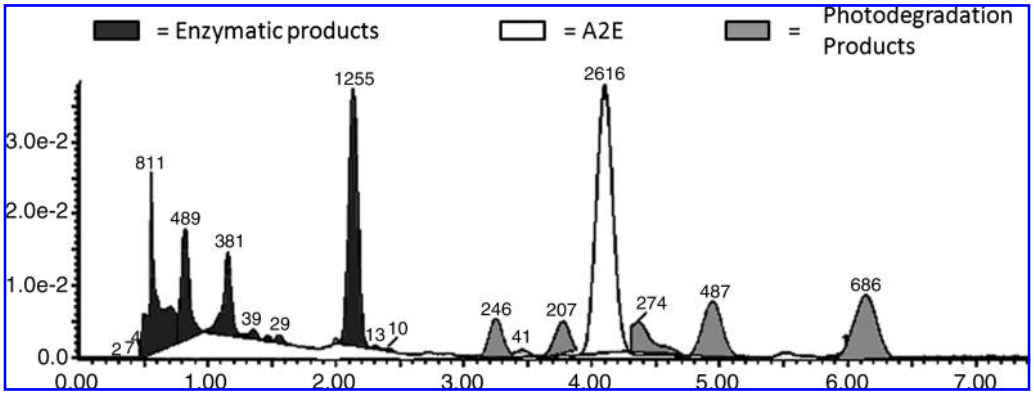

FIG. 6. Reaction products formed during A2E degradation. We exposed A2E (white peak) to horseradish peroxidase *in vitro*, until approximately 60% of the A2E was degraded (dark grey peaks). In a separate reaction, we exposed A2E to room light to achieve the same amount of degradation (light grey peaks). The figure is an overlay of traces from untreated, light treated and enzyme treated samples. The products formed by our enzymes are clearly different from the toxic photodegradation products. The enzymatic products are further degraded by the enzymes, forming increasingly hydrophilic and colorless compounds until they can no longer be detected by our assay.

of the clumping behavior of A2E, quantitative statements could not be made under these conditions. Most of the peroxidases degraded A2E without impairment at lysosomal pH (5.2), except for horseradish peroxidase, which was inhibited by 30%. For comparison the activity of NSC1 also was impaired 30% at this pH, but CSZCD was not tested.

Figure 6 shows HPLC traces of the reaction products formed by horseradish peroxidase. All peroxidases and laccases formed the same set of reaction products, and they successively degraded each reaction product further. The products were different from the toxic photodegradation products and similar to dark autooxidation products.[54] The products were increasingly hydrophilic and less intensely colored. All products stained strongly with 2,4-dinitrophenylhydrazine, suggesting the presence of aldehyde groups.

By surveying A2E-degrading enzymes directly, we discovered that CCO action on A2E is rare. However, we found two CCOs that cleave A2E at defined bonds, NSC1 and CSZCD. Furthermore, fungal and plant peroxidases and laccases efficiently destroy A2E, probably through successive steps of oxidative double-bond cleavage under aldehdye formation. Aldehyde formation does not necessarily lead to toxicity, because many aldehydes are products of normal metabolism (e.g., glyceraldehyde-6-phosphate, glucose). The toxicity of the specific products formed during peroxidase-mediated A2E degradation in retinal pigment epithelium RPE lysosomes remains to be rigorously assessed.

Currently, it is not clear whether the defined cleavage and high substrate selectivity of the CCOs would be more desirable therapeutically than the broad substrate range and complete molecular destruction of A2E caused by the peroxidases. COO-mediated defined cleavage may have the advantage of avoiding side reactions against other beneficial lysosomal molecules. Peroxidase-mediated nonselective complete destruction may have the advantage of destroying other species of the carotenoid lipofuscin that the CCOs might not act on, as well as the further destruction of any toxic reaction products. However, it is worth pointing out that some peroxidases are natural residents of the plant or fungal vacuole, which has properties similar to a lysosome. The fact that no toxicity occurs in the vacuole makes us hopeful that this may also be true in the lysosome. However, further evaluation of these enzymes in preclinical models of A2E toxicity is needed to answer these questions.

Both classes of enzymes were active over a broad pH range. This broad pH activity is likely desirable, because initially lysosomes are acidic, but may become neutralized under heavy A2E loading.[55]

We have begun a collaboration to test A2E-degrading enzymes in cell models of macular degeneration and will publish these results separately (Janet Sparrow and Bruce Rittmann groups, manuscript in preparation). Briefly, an A2E-degrading peroxidase can be delivered to cultured RPE cell lysosomes with a protein transduction reagent, and it can destroy A2E there without acute toxicity to the cells. These preliminary studies further support our view that the aldehydic reaction products may be either nontoxic, or may be further degraded before significant toxicity occurs.

In conclusion, we believe that our work has answered initial questions around the ambitious proposal of medical bioremediation and has raised many more. We now know that oxysterol- and A2E-degrading enzymes can be readily discovered in nature. Future work should aim at characterizing the toxicity of our oxysterol- and A2E-degrading enzymes and discovering additional such enzymes to compare them against ours. Simultaneously, the best ways of achieving enzyme drug delivery for age-related storage diseases should begin in preclinical models.

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Author Disclosure Statement

J. Schloendorn, K. Kemmish, T. Webb, and B. Rittmann have filed for patent protection of the A2E-degrading enzymes and may be eligible for royalty payments under the Arizona Board of Regents Intellectual Property Policy (6-908). No other competing interests exist.

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