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Emerging applications of zebrafish as a model organism to study oxidative mechanisms and their role in inflammation and vascular accumulation of oxidized lipids

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Abstract

With the advent of genetic engineering, zebrafish (*Danio rerio*) were recognized as an attractive model organism to study many biological processes. Remarkably, the small size and optical transparency of zebrafish larvae enable high-resolution imaging of live animals. Zebrafish respond to various environmental and pathological factors with robust oxidative stress. In this article, we provide an overview of molecular mechanisms involved in oxidative stress and antioxidant response in zebrafish. Existing applications of generically-encoded fluorescent sensors allow imaging, in real time, production of H₂O₂ and studying its involvement in inflammatory responses, as well as activation of oxidation-sensitive transcription factors HIF and NRF2. Oxidative stress, combined with hypelipidemia, leads to oxidation of lipoproteins, the process that contributes significantly to development of human atherosclerosis. Recent work found that feeding zebrafish a high-cholesterol diet results in hypercholesterolemia, vascular lipid accumulation and extreme lipoprotein oxidation. Generation of a transgenic zebrafish expressing a GFP-tagged human antibody to malondialdehyde (MDA)-modified LDL makes possible *in vivo* visualization of MDA epitopes in the vascular wall and testing the efficacy of antioxidants and dietary interventions. Thus, using zebrafish as a model organism provides important advantages in studying the role of ROS and lipid oxidation in basic biologic and pathologic processes.

Introduction

Applications of zebrafish models become increasingly popular in various fields of biomedical research and drug development. Traditionally, many toxicological studies have been using zebrafish embryos and larvae for initial characterization of environmental hazards or therapeutic candidates, before advancing to larger animal models. Small size, multiple progeny from a single mating, the straightforward route of chemicals administration, especially for water-soluble compounds, are among the advantages of using zebrafish for the survival/lethality type of experiments. Recent studies have introduced more advanced read-out assays, such as assessing neuronal and liver damage, renal dysfunction, gene expression analysis, DNA damage and fluorescence-based reporter assays [1].

For the reasons that will be discussed in this article, zebrafish respond to many pathological factors with robust oxidative stress. Thus, measuring parameters of oxidative stress is one of

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the most common categories of assays used in zebrafish toxicological studies [2–4]. The oxidative stress assays were also applied to test detrimental effects of administering oxidized low-density lipoprotein (OxLDL) and pro-survival effects of high-density lipoprotein (HDL), including specific mutants of the HDL protein APO-AI that enhance its function [5,6].

In addition to the toxic effects of high doses of ROS, intracellular production of ROS is intimately involved in regulation of normal cellular function and in inflammatory responses [7,8]. Inflammation is now recognized as a major factor in pathogenesis of many chronic diseases, such as atherosclerosis, the metabolic syndrome and diabetes [9–12]. We will discuss recent work demonstrating the advantages of zebrafish as an animal model to elucidate the connection between oxidative mechanisms and inflammatory processes relevant to early stages in development of these chronic conditions. We will describe chemical probes used to measure specific ROS in zebrafish and the latest advances in applications of transgenic zebrafish, which express genetically-encoded sensors for ROS, oxidation-regulated transcription factors and oxidized lipids. We will conclude with the discussion of future directions for mechanistic studies as well as for testing novel therapeutic approaches that would aim to regulate oxidative processes involved in pathogenesis of human disease.

Zebrafish as a model organism

General characteristics

Zebrafish (*Danio rerio*) are small, fresh water, teleost fish. Adult zebrafish are 30–40 mm long and weigh 300–500 mg. They reach fertile age by 3 months and live for up to 2 years under laboratory conditions (water at 28.5°C, 14/10 hours light/dark cycle). Female lay up to 200 eggs per mating and male fertilize the eggs with sperm *ex vivo*. Following fertilization, embryos develop in a chorion and then hatch approximately 48 hours post fertilization (hpf). Embryos can be mechanically dechorionated for experimental work at any time. By the 5–6th day post fertilization (dpf) zebrafish larvae consume the yolk nutrients and begin free feeding. Maintenance of zebrafish is relatively inexpensive and large numbers of animals can be produced for each experiment.

Among the major advantages of using zebrafish is the optical transparency of embryos and larvae for up to 30 dpf. They are small and thin enough to be imaged with a confocal microscope at high resolution (63× objective). Embryos can be immobilized to capture time lapse images for several days and older larvae can be anesthetized and remain immobile for up to 20 minutes, the time sufficient to capture high quality optical z-sections, and the imaging sessions can be repeated several times.

Since efficient methods to manipulate zebrafish gene expression have been developed and because zebrafish larvae are transparent, numerous transgenic animals expressing fluorescent proteins in various cell types and tissues have been generated. Stable transgenic zebrafish lines expressing EGFP, DsRed, mCherry and other fluorescent proteins in endothelial cells, neutrophils, macrophages, skeletal muscle and other cell types can be obtained from investigators who develop them and from Zebrafish International Resource Center (ZIRC; <http://zebrafish.org>). ZIRC also maintains a large collection of zebrafish mutants in which the phenotype has been described and the genetic mutation defined. Zebrafish knockout technology has also been reported [13,14]. There are no yet embryonic stem cells isolated from zebrafish and, thus, current gene knockout techniques used in mice cannot be applied to zebrafish. However, an alternative method using zinc finger nuclease mediated site-specific DNA double strand breaks is now used to produce knockout zebrafish [15,16]. A newly developed method of transcription activator-like effector nucleases

(TALEN)-mediated gene knockout [17,18] has spurred the great interest in the zebrafish research community. The zebrafish genome has been completely sequenced (http://www.sanger.ac.uk/Projects/D_rerio/). Comparison of the genome sequences reveals highly conserved genes involved in oxidation, lipid metabolism and inflammation, and many of the corresponding zebrafish proteins have been demonstrated to function in the same way as their mammalian homologs [19–33].

The characteristics of the zebrafish model listed above and its many other features that we have not mentioned to keep this article focused, make zebrafish an attractive model organism to study oxidative mechanisms and their role in pathogenesis of acute and chronic inflammatory diseases. In particular, using the advantage of *in vivo* high resolution imaging enables direct monitoring of physiologic and pathologic processes in live animals.

Oxygen- and electrophile-responsive transcription and enzymatic antioxidant systems

Many of the oxidation-sensitive transcription factors are conserved in zebrafish. Zebrafish express the critical components of the oxygen-sensing signaling system, including hypoxia inducible factor (HIF), the von Hippel-Lindau tumor suppressor protein (pVHL), and several isoforms of prolyl hydroxylase (PHD). At normal oxygen tension in tissues, PHD specifically hydroxylates HIF α , which triggers pVHL binding to HIF α , followed by HIF α ubiquitination and degradation. However, under hypoxic conditions, HIF α escapes hydroxylation and degradation and translocates to the nucleus where it associates with HIF β and initiates a diverse transcription program aimed to alleviate the detrimental effects of hypoxia [34]. Since the hypoxia inflicted damage in large part is due to overproduction of free radicals, HIF targets include heme oxygenase-1 (HO-1), an enzyme important in cellular defense against oxidative stress [35]. Because systemic hypoxia accompanies human pulmonary dysfunction and local hypoxia is a characteristics of atherosclerotic lesions and rapidly proliferating tumors, elucidating HIF-dependent mechanisms is of particular importance for understanding and treatment of human disease.

Recent studies have demonstrated that *vh1* zebrafish mutants display systemic hypoxic response, characterized by hyperventilation, cardiomegaly and elevated cardiac output, and severe polycythemia [36]. The gene expression profile of *vh1* mutants shows enrichment in genes related to the anaerobic metabolism, oxygen sensing and transport, angiogenesis, and hematopoietic proliferation. The *vh1* zebrafish mutants are also predisposed to carcinogen-induced hepatic and intestinal tumors [37], supporting the role of HIF in regulation of tumorigenesis. Maeda and coworkers have found that zebrafish Cullin-2 (Cul2), the protein involved in pVHL-mediated ubiquitination of HIF α , is required for normal vasculogenesis, the effect at least in part mediated by Cul2 regulation of Hif-mediated expression of Vegf and Flk [38]. Using a pharmacologic inhibitor of PHD enzymes and dominant-negative and dominant-positive variants of zebrafish Hif α , Elks et al. have shown that Phd-dependent hydroxylation of Hif α is critical for timely resolution of neutrophilic inflammation [39]. Stabilization of Hif resulted both in reduced neutrophil apoptosis and in increased retention of neutrophils at the site of a tail fin wound.

Zebrafish have also been used to study nuclear factor E2-related factor 2 (NRF2), a transcription factor that plays an important role in the regulation of antioxidant gene expression via its interaction with antioxidant/electrophile response elements (ARE/EPRE). Similarly to HIF α , under normal redox conditions, NRF2 is associated with a repressor protein, Kelch-like ECH-associated protein (KEAP1), which targets NRF2 for ubiquitination. KEAP1 contains redox-sensitive cysteines, which undergo oxidation if the balance shifts toward electrophiles, leading to release of NRF2 and its translocation to the nucleus and activation of ARE-regulated genes [40].

Zebrafish *nrf2a* and *nrf2b* are the two homologs of human *NRF2*, which have different distribution and expression levels in the zebrafish embryo [41]. On the functional level, zebrafish Nrf2a plays a similar protective function as its mammalian NRF2 ortholog, while Nrf2b negatively regulates expression of antioxidative genes [41]. As in mammals, zebrafish Nrf2a activates expression of enzymes involved in *de novo* glutathione (GSH) biosynthesis, such as 3-glutamylcysteine synthetase (γ Gsh), as well as glutathione-S-transferase (Gst), which recycles oxidized glutathione (GSSG), and a detoxifying enzyme NAD(P)H:quinone oxidoreductase (Nqo1) [30].

The second pathway of regulation of these antioxidant enzymes is via aryl hydrocarbon receptor (AHR), which is activated by polycyclic aromatic hydrocarbons and binds to xenobiotic response elements (XREs). In addition to various *gst* and *nqo* genes, the AHR/XRE axis regulates enzymes of the cytochrome P450 (*cyp1*) family, glutathione peroxidase 1 (*gpx1*), and the glutamate-cysteine ligase catalytic subunit (*gclc*) [33]. The expression of Mn- and Cu, Zn-dependent superoxide dismutases (Sod) and catalase (Cat), the enzymes that reduce superoxide radical and hydrogen peroxide, is well documented in zebrafish [33,42–44]. The expression levels of these enzymes are markedly increased in zebrafish exposed to pollutants and drugs [42,45].

Lipid metabolism

Polyunsaturated fatty acids (PUFA) and PUFA-containing phospholipids (PL) and cholesterol esters (CE) are highly susceptible to oxidation, yielding a variety of lipid free radicals, peroxides, epoxides, endoperoxides, aldehydes, and ketones, many of which in turn modify other lipids, sterols, proteins and nucleic acids. In humans, hyperlipidemia often leads to increased lipid oxidation, and oxidation of LDL is considered a leading pathogenic factor in development of atherosclerosis [46–48].

Lipid metabolism in zebrafish, and in teleost fish in general, is surprisingly similar to that in humans. Moreover, fish preferentially use lipids rather than carbohydrates as an energy source and would be classified, using standards applied to mammals, as hyperlipidemic and hypercholesterolemic [49,50]. Analytical ultracentrifugation identified VLDL, LDL and HDL lipoprotein classes in rainbow trout (*Oncorhynchus mykiss*), with HDL dominating the lipoprotein profile [50]. Native agarose gel electrophoresis of normal zebrafish plasma shows mostly the HDL fraction as well; however, the lipoprotein profile of zebrafish fed a high-cholesterol diet (HCD) is identical to that of human plasma, with LDL and VLDL found at higher proportions than HDL [51]. The nature and the distribution of apolipoproteins in different classes of fish lipoproteins resembles that in mammals, but plasma concentration of apolipoproteins in rainbow trout accounts for 36% of total protein, compared with only 10% in humans [50]. Antibodies against human APOB-100 and APOA-I recognize proteins of a similar molecular mass in zebrafish plasma [51]. During embryonic fish development, yolk syncytial layer actively synthesizes Apoe and other apolipoproteins and forms VLDL from yolk lipids, which then enter the circulatory system and deliver nutrient lipids to the tissues [20,52]. As in humans, zebrafish microsomal triglyceride transfer protein (Mtp) is involved in the VLDL assembly in the yolk and, later, in the intestinal lipoprotein synthesis [26,53]. Lipoprotein lipase, hepatic lipase and lecithin:cholesterol acyltransferase (LCAT) activities have been shown in several fish species, and there is evidence suggesting the presence of cholesterol ester transfer protein (CETP) activity in rainbow trout plasma [50]. Zebrafish express *cetp*, homologous to human *CETP*, and *Cetp* activity in zebrafish plasma increases with hypercholesterolemia, reaching the levels comparable to that in humans [54,55]. In fact, measuring *Cetp* activity is an important advantage of the zebrafish model compared to studies with mice who do not express CETP. CETP is considered an important therapeutic target for human dyslipidemia.

Studies of intestinal lipid metabolism in zebrafish have identified annexin2-caveolin1 and fat-free as important factors in cholesterol absorption and the targets for anti-hyperlipidemic therapies [27,56]. Zebrafish also express *npc111*, an ortholog of the human *NPC1L1* gene encoding intestinal Niemann-Pick C1-like protein 1. NPC1L1 is involved in intestinal absorption and transfer of cholesterol and is a target of ezetimibe, an important cholesterol-lowering drug. The ezetimibe-binding domain in zebrafish Npc111 is highly homologous to the corresponding domain in human NPC1L1, with the preserved phenylalanine and methionine residues required for high-affinity binding of ezetimibe [57]. Larvae treated with ezetimibe shows 25–75% reductions in intestinal fluorescence derived from NBD-cholesterol [57]. These data suggest that the mechanism of inhibition of intestinal cholesterol absorption by ezetimibe in zebrafish is similar to that in humans, but direct evidence of the involvement of Npc111 in zebrafish cholesterol metabolism targeted by ezetimibe has not yet been reported. We found that ezetimibe was very effective in reducing total cholesterol levels in HCD-fed zebrafish larvae. Already at the concentration of 1 μ M, ezetimibe reduced cholesterol to the levels observed in larvae fed control diet. In contrast, simvastatin, the drug targeting HMG-CoA reductase, was less effective in reducing cholesterol levels in zebrafish [58].

The reports summarized in this section demonstrate that redox signaling, antioxidant enzymes and lipid metabolism, the areas of importance to researchers interested in oxidative mechanisms and their role in human disease, are strikingly similar in humans and in zebrafish. Combined with the advantages of using zebrafish as a model organism, these similarities enable *in vivo* studies of specific physiologic and pathologic mechanisms that would be impossible or difficult to conduct with other animal models.

***In vivo* imaging of ROS and oxidation-dependent signaling**

Chemical probes

Because zebrafish larvae are optically transparent, they can be used to monitor ROS *in vivo*. A number of fluorescent probes have been adapted for use in zebrafish experiments. The common fluorogenic dye 2',7'-dihydrodichlorofluorescein diacetate (H₂DCFDA), which is oxidized by ROS to produce green fluorescent dichlorofluorescein (DCF), was used in zebrafish embryos to demonstrate ROS production in response to PMA [59]. The same probe was used in a high-throughput assay in which ROS, produced in response to hydrogen peroxide, arsenic trioxide, and phenethyl isothiocyanate, was quantified in zebrafish larvae. The ROS response was inhibited in the presence of the antioxidant N-acetylcysteine [60]. Although H₂DCFDA has low selectivity for various oxidants, it is a good general sensor of ROS.

During inflammation, hypochlorous acid (HOCl) is produced by myeloperoxidase (MPO)-mediated peroxidation of chloride ions in activated neutrophils and macrophages. HOCl contributes to the destruction of bacteria in living organisms as well as to modification of lipoproteins during atherogenesis. A large number of probes have been synthesized to detect MPO activity and HOCl [61–65]. For example, the water-soluble dihydrofluorescein-ether probe FCN2 was demonstrated to detect micromolar concentrations of HOCl both *in vitro* and *in vivo* in zebrafish [66]. A rhodamine-hydroxamic acid-based fluorescent probe for HOCl was used in similar applications [64]. However, in these experiments, HOCl was added to water rather than produced in zebrafish in response to e.g. a bacterial challenge. Thus, fluorescent HOCl probes are suitable for toxicologic studies, but their use for biologic experiments in zebrafish are yet to be demonstrated.

Genetically-encoded sensors

Genetically-encoded sensors and expression reporters offer, in most cases, a more sensitive and more selective method to detect ROS and related signaling events than chemical probes. Belousov et al. have engineered a highly selective probe for H₂O₂ based on the prokaryotic H₂O₂-sensing protein OxyR [67,68]. The probe, named HyPer, consists of circularly permuted YFP inserted in the regulatory domain of OxyR (Fig. 1A). H₂O₂-mediated oxidation of cysteines in OxyR results in the formation of a disulfide bond, a conformational change in YFP and its excitation spectrum shift, characterized by an increased peak at 500 nm and a decreased peak at 420 nm. Reduction of the OxyR disulfide bond reverses the spectral shift. Niethammer et al. used HyPer to generate a transgenic H₂O₂ reporter zebrafish and studied spatiotemporal gradients of H₂O₂ in wound responses [69]. They have found that the wound-inflicted H₂O₂ gradient is created by dual oxidase (Duox), and that it is required for rapid recruitment of leukocytes to the wound. The HyPer transgenic zebrafish was also used in a different study to demonstrate that a Src family kinase Lyn is a redox sensor that mediates leukocyte recruitment to the wound [70]. Cys466 in Lyn is responsible for direct oxidation-mediated activation of the kinase. This series of studies using transgenic zebrafish revealed that oxidation events occur before myeloid cell recruitment, and provided important spatiotemporal characteristics and mechanistic insights by identifying Duox as a source of H₂O₂ and Lyn as an oxidation sensor.

Oxygen and electrophile stimulated signaling events have been studied in transgenic zebrafish in which fluorescent proteins replaced coding regions of the proteins expressed in response to oxidative stress. Thus, Santhakumar et al. generated a zebrafish with EGFP expression driven by the *phd3* promoter/regulatory elements (Fig. 1B) [37]. Phd3 is a robust target of Hif-mediated transcription and, thus, *phd3* promoter-driven expression of EGFP is an indicator of hypoxia. Using this reporter zebrafish and *vhl* mutants, the authors established the role of pVhl as a genuine tumor suppressor [37]. Suzuki et al. used a similar approach to identify pi-class Gst as a target for Nrf2a in zebrafish [71]. They subsequently used a transgenic zebrafish expressing GFP under control of the *gstp1* promoter to identify exogenous nitro-oleic acid as an activator of Keap1-Nrf2 [72]. The reporter was also sensitive to application of a cyclopentenone prostaglandin 15d-PGJ2, but not of H₂O₂, suggesting that nitro-oleic acid and 15d-PGJ2 share an analogous cysteine code as electrophiles and also have similar anti-inflammatory roles. Kusik et al. generated a transgenic zebrafish expressing a luciferase–GFP fusion protein under the regulation of the electrophile response element (EPRE) [73]. This construct allows visual confirmation of transgene expression in live fish through fluorescence microscopy and quantitative assessment of gene expression by measuring luciferase activity in embryo extracts. This EPRE reporter zebrafish was used to detect mercury in aquatic environments.

The studies referred to in this section, most of them conducted in last 2–3 years, demonstrate the power of transgenic zebrafish models to monitor ROS and assess oxidation-dependent signaling events, as well as to address specific questions of embryonic development, inflammation, cancer and toxicology.

Lipoprotein oxidation in hypercholesterolemic zebrafish

In 2009, we suggested to use zebrafish as a model organism to study selected pathological events relevant to early development of human atherosclerosis [51]. Feeding zebrafish a HCD results in marked hypercholesterolemia and development of vascular lesions, which resemble early atherosclerotic lesions in humans and in hypercholesterolemic mice. Remarkably, hypercholesterolemia in zebrafish is associated with strong lipoprotein oxidation. Oxidative modification of LDL is widely believed to increase LDL atherogenicity and to drive the initial formation of atherosclerotic lesions in both humans and experimental

animals [12,46,74]. Thus, the hypercholesterolemic zebrafish model, which is characterized by extremely high levels of OxLDL and oxidized lipids, offers attractive approaches to studying lipoprotein oxidation and its pro-atherogenic effects and to developing new diagnostic tools and therapies.

Immunoassays and mass spectrometry

Oxidation of LDL renders it immunogenic, and antibodies against oxidation-specific epitopes (OSE) in OxLDL arise naturally in patients with cardiovascular disease and in hypercholesterolemic animals. Witztum and colleagues have cloned an array of natural monoclonal antibodies that recognize OSE and have developed immunoassays to detect OSE in plasma of different animal species and in humans [75–81]. The monoclonal antibody EO6 is used to measure oxidized phospholipids (OxPL) in plasma LDL. This immunoassay has been used in over 25 published papers to assess OxPL/APOB levels in a large number of clinical trials and has proven to have a high prognostic value in predicting risk of cardiovascular disease [12,82–95].

The EO6/APOB levels in HCD-fed zebrafish plasma are as much as 20–30 times higher than in plasma of control animals [51], the values that are rarely observed in human plasma. The EO6 immunoreactivity with APOA-I lipoproteins is as high as with APOB lipoproteins in zebrafish plasma, which is also unusual for human or mouse plasma.

Oxidized lipids in zebrafish larvae, the major focus of our vascular imaging studies [51,96], have been analyzed using liquid chromatography/mass spectrometry. Because it is impractical to draw blood from a tiny zebrafish larva, the whole euthanized larvae were gently homogenized and bodily liquids collected. A 2-week HCD feeding resulted in a 4-fold increase in total cholesterol and triglycerides [58,97]. Non-oxygenated CEs (cholesteryl arachidonate, linoleate and oleate) were increased 4–13-fold in HCD-fed larvae, indicating increases in circulating lipoproteins and/or accumulation of lipid in tissues. Remarkably, oxygenated CEs were increased as much as 10–70-fold, suggesting that the HCD feeding leads to profound lipid oxidation. The OxCE molecules identified in HCD-fed zebrafish are also found in human and mouse atherosclerotic lesions and in plasma of patients with cardiovascular disease [97–99]. Some of these OxCE have been demonstrated to elicit proinflammatory and proatherogenic responses in endothelial cells and in macrophages [100–102].

We have also characterized non-oxidized and oxidized phosphatidylcholines (PC) in hypercholesterolemic zebrafish. While the levels of non-oxidized PC remained unchanged in HCD-fed zebrafish larvae, the levels of many lysoPC and OxPC increased 5–25 fold [97]. The OxPC analyzed include 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-*sn*-glycero-3-phosphocholine (PEIPC), and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC), the OxPL molecules shown to induce numerous proinflammatory responses in endothelial cells, vascular smooth muscle cells and macrophages [48,103–109]. The increased levels of lysoPC suggest activation of phospholipase A₂ (PLA₂). In agreement, an increased PLA₂ activity was detected in the vasculature of HCD-fed zebrafish larvae using a fluorogenic PLA₂ substrate [51]. Homogenates of HCD-fed zebrafish larvae competed for oxidized LDL binding with macrophages and activated ERK, Akt and JNK and induced macrophage spreading, the effects characteristic for macrophages activated with oxidized LDL.

Why are zebrafish so prone to oxidative stress and lipid oxidation?

Papers reviewed in this article suggest that zebrafish possess rather sophisticated antioxidative systems, complete with oxygen- and electrophile-sensing signaling pathways

and the enzymes that detoxify free radicals and replenish small molecule antioxidants. Then, why are zebrafish so sensitive to any insult, ranging from environmental pollutants to high-cholesterol feeding, and respond to these stimuli with extreme oxidative stress? This exacerbated oxidative response, of course, makes zebrafish an attractive model organism to study mechanisms and implications of oxidative stress, but it is also important to understand the causes of this phenomenon. Here, we will briefly consider three factors that may contribute to the susceptibility of zebrafish to oxidative stress – water, temperature and lipids, but as more data become available, the topic merits further detailed discussion.

Levels of dissolved oxygen in water vary significantly depending on the depth, turbulence, sediments and environmental pollutants. Fish deploy complex behavioral, physiologic and molecular strategies to maintain blood oxygenation, but intermittent hypoxia is inevitable. The metabolic response to hypoxia is to increase anaerobic respiration via glycolytic production of ATP. The glycolysis pathway inherently produces more ROS than the aerobic respiration.

Furthermore, zebrafish are an ectothermic organism, which regulates its body temperature largely by exchanging heat with its surroundings. This is in contrast to endothermic mammals who generate heat to maintain body temperature, typically above the ambient temperature. In wild, fish experience thermal fluctuations during day/night and seasonal cycles, which affect hemoglobin affinity to oxygen and are a stressor by itself. For example, exposure to cold increases activity of glycolytic enzymes in the brain of green sunfish (*Lepomis cyanellus*) [110] and expression of glucose transporters and uncoupling proteins in the brain of zebrafish [111]. Thus, exposure to temperature fluctuations has predisposed zebrafish to activation of glycolysis as a defensive mechanisms, with its attendant oxidative stress.

Cooler body temperature in fish (zebrafish are maintained at 28.5°C) than in mammals also affects membrane lipid composition. Relatively high levels of unsaturated fatty acids help maintain membrane fluidity in species exposed to cold [112,113], but also make these membranes more susceptible to oxidation. In addition, as we discussed above, fish preferentially use lipids as an energy source and are hyperlipidemic, using standards applied to mammals [50]. Thus, predisposition to oxidative stress associated with hypoxia and anaerobic respiration, together with abundance of lipids that are easily oxidized, result in elevated lipid oxidation, the condition which is greatly exacerbated upon feeding zebrafish a HCD, as evident from our experiments [51,97].

***In vivo* imaging of lipid oxidation products with oxidation-specific antibodies**

In the reports that we discussed so far, oxidized lipids were measured using immunoassays and mass spectrometry techniques in the materials obtained from euthanized zebrafish (blood draw is a terminal procedure in zebrafish). Although these analytical techniques provide important insights into lipid oxidation processes, a method to visualize oxidized lipids in live transparent zebrafish larvae would have clear advantages. The approach that we used was to image oxidized lipids using fluorescently tagged oxidation-specific antibodies. For these studies, we used the antibody IK17, cloned in the Witztum laboratory from a patient with advanced cardiovascular disease [79]. IK17 binds to malondialdehyde (MDA)-modified LDL and other MDA-modified proteins and stains mouse and human atherosclerotic lesions [79,90,114]. IK17-reactive epitopes are increased in thin cap fibroatheroma and in ruptured human lesions, suggesting their pathophysiological role and the importance in the identification of plaques vulnerable to rupture (Sotirios Tsimikas, personal communication).

Injection of recombinant antibodies

Feeding zebrafish larvae a HCD for as short as 5 days results in lipid accumulation in the vascular wall, which is the initial step in formation of human atherosclerotic lesions. The vascular lipid accumulation in zebrafish is visualized by supplementing the HCD with a fluorescent cholesterol ester (cholesteryl BODIPY 576/589 C11) and imaging live larvae under a confocal microscope. Bright red fluorescence in the vascular wall indicates vascular lipid accumulation. Since the BODIPY 576/589 fluorophore is on the acyl chain of the cholesterol ester, which is likely hydrolyzed in the digestive system, and the fluorescent fatty acid is then re-esterified into cholesterol esters, triglycerides and/or phospholipids, the red fluorescence represents all classes of lipids. To test whether these vascular lipid deposits contain MDA-modified proteins, we injected IK17 into HCD-fed zebrafish larvae. A single chain Fv fragment was cloned from the heavy and light chains of the IK17 Fab fragment and the recombinant IK17 antibody was expressed in *E. coli*, followed by its labeling with Alexa Fluor 488 [96]. The labeled single chain IK17 retained its binding to the MDA epitope. IK17-Alexa488 injection into the posterior cardinal vein resulted in the antibody binding to selected areas of lipid deposits in the vascular wall of HCD-fed zebrafish [96]. The incomplete colocalization of lipid and IK17 signals in larvae suggests that MDA epitopes accumulate only in selected areas of vascular lesions. This selective accumulation of IK17-specific epitopes in zebrafish vasculature agrees with the selective IK17 staining of mouse and human atherosclerotic lesions.

The approach of injecting recombinant labeled antibodies for imaging oxidized lipids in live zebrafish larvae has its own advantages and disadvantages. The procedure is quick and informative and thus suitable for initial characterization of the experimental system. However, it requires a skilled operator to inject intravenously into a tiny larva, the procedure that may be traumatic to the zebrafish. We found that the recombinant antibody must be endotoxin-free; otherwise, endotoxin induces non-specific binding of an antibody to the vasculature. The steps of producing, labeling and purification of the antibody may be time consuming. A different approach, using a transgenic zebrafish with conditional expression of IK17, as described below, gives a researcher the ability to simply and efficiently control transient antibody expression, repeatedly and at specified time points, and the opportunity to study therapeutic effects of diets, antioxidants, and oxidation-specific antibodies themselves, without repetitive injections of recombinant antibody.

Conditional expression of antibodies in transgenic zebrafish

The Tg(hsp70:IK17-EGFP) zebrafish that we have produced (Fig. 1C), transiently express IK17-EGFP only after heat shock [96]. Placing zebrafish larvae, which are normally maintained at 28.5°C, in water at 37°C larvae for 1 hour induces robust expression of the transgene in somites, but not in the vasculature. IK17-EGFP is secreted into the circulation and binds to the vascular lipid deposits and also is detected in other tissues, suggesting the presence of MDA throughout the body of hypercholesterolemic zebrafish. Zebrafish larvae tolerate a 1-hour long heat shock well and the heat shock can be repeated several times. This allows measuring the time course of MDA vascular deposition in the same animal. By using multi-color fluorescent microscopy, a researcher can correlate accumulation of MDA epitopes in the vasculature with the extent of overall lipid deposition (with a fluorescent lipid added to the diet), activation of PLA₂, matrix metalloproteinases or other enzymes for which fluorogenic substrates are available, recruitment of macrophages or neutrophils to the vasculature (using transgenic zebrafish with *mpeg1* or *lyz* promoter driven fluorescent proteins) [51,96] or any other pathologic process of interest. All measurements are performed in live animals, in time-dependent and quantitative manner.

We have used the Tg(hsp70:IK17-EGFP) zebrafish to test several hypotheses. First was that accumulation of oxidized lipids in the vasculature can be reversed by switching to normal diet to which no cholesterol was added. The possibility of such a reversal has been suggested by Tsimikas et al. who demonstrated that switching the *Ldlr*^{-/-} mice in which extensive atherosclerosis was achieved by a 6-month long HCD, to a chow diet for additional 6 months resulted in little change in the atherosclerotic lesion area but in remarkable reductions in binding of a MDA-specific antibody [115]. In our Tg(hsp70:IK17-EGFP) zebrafish experiment, a 5-day feeding with a HCD led to vascular accumulation of IK17-positive MDA epitopes, which was completely reversed following an additional 5-day feeding with control diet.

Therapeutic effects of antioxidants and oxidation-specific antibodies

The second hypothesis tested with Tg(hsp70:IK17-EGFP) zebrafish was that the antioxidant probucol would reduce accumulation of MDA epitopes as well as the overall vascular lipid deposition in HCD-fed zebrafish. Probucol is a potent lipophilic antioxidant that can potentially decrease atherogenesis in rabbits independent of changes in plasma cholesterol levels [116,117]. Probucol added to the zebrafish HCD reduced the areas of vascular lipid deposits. Even more profound was a reduction in the vascular lesions to which IK17-EGFP bound, with many lipid deposits devoid of IK17-reactive epitopes [96]. These studies agree with the results of other work in which other antioxidants were tested in zebrafish, albeit using different read-out measurements. Zebrafish cannot synthesize vitamin C; thus, feeding zebrafish a vitamin C-deficient diet resulted in robust oxidative stress as demonstrated by elevated levels of MDA, measured as thiobarbituric acid reactive substances [118]. Consumption of a vitamin E-deficient diet led to lower levels of long chain PUFA, which may be a result of either increased lipid peroxidation or an impaired ability to synthesize sufficient PUFA, or both [119].

The third hypothesis was that constitutive expression of IK17 in zebrafish would inhibit vascular lipid deposition. This hypothesis is based on the observations that IK17 inhibits MDA-LDL binding to macrophages and that raising the titers of antibodies to MDA-LDL by immunization or by adenovirus-delivered expression of IK17 is atheroprotective in mice and rabbits [90,120,121]. Homogenates of zebrafish expressing IK17-EGFP (i.e. following heat shock but not before that) indeed inhibited MDA-LDL binding to macrophages *in vitro* [96]. These results suggest that *in vivo* IK17 expression may have a therapeutic effect. To test this possibility, we subjected Tg(hsp70:IK17-EGFP) zebrafish to repeated heat shocks to ensure sustained IK17-EGFP expression during the whole HCD feeding period. The resulting vascular lipid accumulation was dramatically reduced. Moreover, when heat shock was applied for the first time only on the 5th day after the start of HCD feeding, the ensuing IK17-EGFP expression reduced the area of lipid deposits measured 5 days later. These studies suggest that oxidation-specific antibodies themselves may have therapeutic applications by binding to oxidized lipids and neutralizing their proinflammatory and proatherogenic effects.

Conclusions

In aggregate, the results of the studies reviewed in this paper strongly support the use of zebrafish as a model organism to study oxidative mechanisms and search for effective therapies to reduce detrimental effects of oxidative damage. Such studies may range from work seeking answers to fundamental question of inflammation, such as with transgenic HyPer zebrafish used for imaging of H₂O₂ gradients (Fig. 1A), to applied research, as with zebrafish expressing an oxidation-specific antibody (Fig. 1C). Several examples reviewed in this article suggest that the zebrafish model can enable rapid initial screening of interventions that can inhibit LDL oxidation and/or uptake of oxidized LDL by

macrophages. Although we have reviewed mostly the work dissecting lipid oxidation processes relevant to atherosclerosis, which is the focus of our own experimental studies, the evidence suggests that oxidative processes relevant to many other human diseases can be effectively studied using zebrafish models as well.

We think that the possibility to generate transgenic zebrafish greatly expands research capabilities to study oxidative mechanisms. Fluorescent protein-based reporter systems, such as those to detect H₂O₂, HIF α activity or oxidized lipids, as illustrated in Fig. 1, offer the greatest sensitivity and selectivity, as well as the possibility of conditional expression and time-resolved studies. Fluorescent microscopy allows multiplexing, and transgenic zebrafish can be crossed to produce complex, multi-color reporter systems or can be used in combination with small-molecule fluorescent assays. Another interesting application would be to over express or conditionally express enzymes involved in antioxidative defense or the enzymes that promote lipid oxidation, such as lipoxygenases. Combined with the reporter systems reviewed, these transgenic animals would provide valuable mechanistic insights into oxidative mechanisms and suggest methods to shift the balance toward reduction of oxidative damage, resolution of inflammation and eventually, the reversal of chronic inflammatory conditions.

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Highlights

- Zebrafish respond with robust oxidative stress to many pathological stimuli
- Genetically-encoded reporters enable monitoring of H₂O₂ and HIF and NRF2 activities
- Feeding zebrafish a high cholesterol diet leads to excessive lipoprotein oxidation
- Transgenic zebrafish with GFP-tagged antibody enables imaging of oxidized lipids
- Fluorescent reporter zebrafish provide methods for in vivo antioxidant screening

Figure 1. Transgenic reporter zebrafish to study ROS and oxidative processes

A, H_2O_2 reporter. The HyPer construct expressed in zebrafish is a ratiometric sensor for H_2O_2 . It uses the selectivity of cysteine oxidation by H_2O_2 in the bacterial protein OxyR and its subsequent conformational change. cpYFP inserted in the OxyR molecule responds to this H_2O_2 -induced conformational change by the shift in its excitation spectrum: reduced emission with excitation at 420 nm and increased emission with excitation at 500 nm [67]. It is illustrated by blue color for a low YFP_{500}/YFP_{420} ratio and red color for the high ratio. In zebrafish, wound inflicted to the tail fin induces a gradient of H_2O_2 , sensed by Lyn kinase and leading to recruitment of neutrophils [69,70].

B, HIF α activity reporter. At normal oxygen tension, HIF α is specifically hydroxylated and binds with pVHL, which mediates HIF α ubiquitination and degradation. However, under hypoxic conditions, HIF α is not hydroxylated, becomes stabilized and translocates to the nucleus, where it associates with HIF β and activates a transcription program. Expression of Phd3 is one of the strongest responses to hypoxia, mediated by HIF. In zebrafish, the HIF β activity reporter expresses GFP under control of the *phd3* promoter [37].

C. Reporter for oxidized lipids. Feeding zebrafish a high-cholesterol diet results in rapid accumulation of lipid deposits in the vasculature and in lipoprotein oxidation [51,97]. One of the lipid oxidation-specific epitopes, LDL modified by malondialdehyde (MDA) is detected in the transgenic zebrafish vasculature by the GFP-tagged antibody IK17. The conditional expression of the secreted IK17-GFP antibody is under control of the *hsp70* promoter and is transiently induced in zebrafish subjected to heat shock [96].