RETINAL PIGMENT EPITHELIAL ACID LIPASE ACTIVITY AND LIPOPROTEIN RECEPTORS: EFFECTS OF DIETARY OMEGA-3 FATTY ACIDS

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ABSTRACT

Purpose: To show that fish oil–derived omega-3 polyunsaturated fatty acids, delivered to the retinal pigment epithelium (RPE) by circulating low-density lipoproteins (LDL), enhance already considerable RPE lysosomal acid lipase activity, providing for more efficient hydrolysis of intralysosomal RPE lipids, an effect that may help prevent development of age-related macular degeneration (ARMD).

Methods: Colorimetric biochemical and histochemical techniques were used to demonstrate RPE acid lipase in situ, in vitro, and after challenge with phagocytic stimuli. Receptor-mediated RPE uptake of fluorescently labeled native, acetoacetylated, and oxidized LDL was studied in vitro and in vivo. LDL effects on RPE lysosomal enzymes were assessed. Lysosomal enzyme activity was compared in RPE cells from monkeys fed diets rich in fish oil to those from control animals and in cultured RPE cells exposed to sera from these monkeys.

Results: RPE acid lipase activity was substantial and comparable to that of mononuclear phagocytes. Acid lipase activity increased significantly following phagocytic challenge with photoreceptor outer segment (POS) membranes. Receptor-mediated RPE uptake of labeled lipoproteins was determined in vitro. Distinctive uptake of labeled lipoproteins occurred in RPE cells and mononuclear phagocytes in vivo. Native LDL enhanced RPE lysosomal enzyme activity. RPE lysosomal enzymes increased significantly in RPE cells from monkeys fed fish oil–rich diets and in cultured RPE cells exposed to their sera.

Conclusions: RPE cells contain substantial acid lipase for efficient metabolism of lipids imbibed by POS phagocytosis and LDL uptake. Diets rich in fish oil–derived omega-3 fatty acids, by enhancing acid lipase, may reduce RPE lipofuscin accumulation, RPE oxidative damage, and the development of ARMD.

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INTRODUCTION

The risk for development of age-related macular degeneration (ARMD) may be reduced by dietary fish oil that is rich in omega-3 polyunsaturated fatty acids.[1-3] This clinical observation leads to the following hypothesis: Dietary omega-3 polyunsaturated fatty acids, delivered to the retinal pigment epithelium (RPE) by circulating low-density lipoproteins (LDL), favorably enhance already considerable RPE lysosomal lipolytic activity in vivo. This mechanism would assist in the efficient degradation of intralysosomal lipid-rich material that otherwise might accumulate in RPE cells and contribute to the indigestible lipid-protein complexes of lipofuscin, which have been implicated in the development of ARMD.

To test this hypothesis, a series of studies was conducted to do the following: (1) apply a simple, chromogenic biochemical assay and a novel histochemical technique for the measurement and detection of RPE lysosomal acid lipase; (2) show that purified RPE acid lipase is responsible for triglyceride and cholesteryl esterase activity and may esterify cholesterol to fatty acids, depending on substrate kinetics; (3) demonstrate that RPE acid lipase activity is comparable to the high levels known to exist in mononuclear phagocytes; (4) show that RPE acid lipase activity is enhanced by incorporation of photoreceptor outer segment (POS) membranes or LDL; (5) demonstrate avid RPE native and modified LDL uptake in vitro and in vivo; (6) show that dietary fish oil enhances RPE lysosomal acid lipase and cholesteryl esterase activities, thereby improving RPE lysosomal efficiency in live monkeys; and (7) demonstrate that the serum from fish oil–fed monkeys enhances these RPE lipolytic activities in vivo.

In so doing, this study presents a new method to study RPE lysosomal lipid metabolism, shows that the RPE monolayer incorporates circulating lipoproteins in

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vivo, and demonstrates that diets may be favorably altered to deliver beneficial lipids via LDL to the RPE in vivo. Caveats for dietary fish oil augmentation are likely to include the need for antioxidant supplements, avoidance of smoking and, possibly, protection from excess blue and UV light to avoid in vivo oxidation of LDL protein and lipid that may, in turn, damage RPE cells and promote RPE aging and the development of ARMD.

BACKGROUND

The RPE consists of a monolayer of cells that is interposed between the fenestrated vasculature of the choriocapillaris and the photoreceptor cells of the neurosensory retina.[4] In this strategic location, the cells of this pigmented monolayer envelop the light-sensitive tips of the photoreceptor cells with their apical processes and lie on Bruch's membrane, which consists of the basement membranes of the RPE and choriocapillaris together with intervening collagen and elastic layers.[4-6] The continuous RPE monolayer forms the outer blood-retina barrier by virtue of the tight junctions joining adjacent RPE cells.[7-10] The RPE is thereby an intermediary between the systemic circulation and the avascular outer one third of the neurosensory retina, which relies on various RPE functions for its survival and homeostasis.[11]

Among the essential physiologic functions of the RPE are the selective diffusion and transport of ions, metabolites, and serum components to the outer retina[12-17]; transport, storage, and processing of vitamin A and its derivatives[18-27]; the absorption of scattered light by RPE melanin granules[6,28,29]; and the synthesis of basement membrane components, including fibronectin, laminin, collagen, and glycosaminoglycans, all of which are important to RPE adhesion to and maintenance of Bruch's membrane.[30,31]

A prominent and cardinal RPE function, first described by Young and Bok,[32] is the phagocytosis and intracellular lysosomal degradation of aged, light-sensitive membranes which are shed from the apices of the POS in a diurnal cycle.[29,33-37] The RPE must phagocytize approximately 10% of the POS material daily insofar as each photoreceptor renews its outer segment every 10 days.[38] Each RPE cell may underlie as many as 200 photoreceptors and may, therefore, ingest an equivalent of 20 entire outer segments per day.[38] The steps in POS phagocytosis and degradation[35,36,38,39] include the recognition, binding, and internalization of the shed membrane material by apical RPE processes leading to fusion of the material with lysosomes. This process is highly dependent on aerobic metabolism[40] and involves microtubules,[41,42] intermediate filaments, and actin.[39,43-46] The daily phagocytosis of the shed, lipid-rich POS membranes, therefore, imposes an enormous metabolic burden on the RPE. After fusion of phagocytic debris with lysosomes, homeostasis requires efficient lysosomal enzymatic degradation of engulfed POS material as well as lysosomal elimination of autophagocytic debris derived from damaged RPE organelles.[47] Fusion of the phagocytic POS debris or autophagocytic and RPE-derived debris with lysosomes forms secondary lysosomes called phagosomes, in which enzymatic degradation of lipids and proteins leads to the recycling of their basic components for RPE and photoreceptor metabolism.[48]

RPE lysosomes contain an impressive array of nearly 40 hydrolytic enzymes that have been identified by a variety of biochemical and histochemical techniques.[6,49,50] In fact, RPE lysosomal fractions are sevenfold more potent than liver lysosomes in degrading POS.[51] Cathepsin D is the major RPE lysosomal protease and, together with cathepsin S, appears to be most important in the degradation of POS protein.[52-54] Opsin, a glycoprotein composing about 75% of POS mass, is completely degraded by cathepsin D and other RPE acid hydrolases.[55-57] To date, studies of key RPE acid hydrolases as a function of aging and in ARMD have yielded mixed results.[50,58] Cathepsin D, ß-glucuronidase, and ß-galactosidase appear to actually increase with age,[59-61] while acid phosphatase activity is unaffected[62] and glycosidases show reduced activities.[63,64]

The remaining 25% of POS mass consists chiefly of polyunsaturated lipids that are degraded by RPE phospholipases[65,66] and acid lipases,[55,67-69] releasing fatty acids that are recycled to photoreceptors for use in POS renewal.[70,71] Studies of RPE acid lipase have employed acid lipase enzyme substrates, which are notoriously insoluble,[67] or have used neutral triglycerides, whose hydrolyzed fatty acids require organic extraction and fractionation for their detection.[68,69] In a lone cytochemical study of acid lipase in humans, Feeney[72] showed no age-dependent reduction in detectable enzyme activity.[73] Other studies have examined acid lipase in the interphotoreceptor matrix[74] or in canine lipofuscinosis.[75] However, a simple, reproducible chromogenic biochemical assay and histochemical technique for acid lipase, though available,[76-78] has not been applied to the study of RPE lysosomal lipid metabolism and its modulation by phagocytosis or exposure to other variables.

The importance of RPE lysosomal lipid metabolism is emphasized by the fact that despite robust RPE lysosomal hydrolytic activity, particularly of the cathepsins, nondigestible material accumulates progressively in tertiary lysosomes called residual bodies.[72] The nondigestible material forms yellow, autofluorescent lipid-protein aggregates known as lipofuscin, which may fill up to one fifth of the RPE cytoplasm by age 80.[79,80] One of the putative factors leading to lipofuscin accumulation in this nonrenewable, pigmented cell monolayer is the highly oxida-

tive retinal environment, which may oxidize proteins and polyunsaturated fatty acids that are abundant in POS and autophagocytic debris, rendering them refractory to lysosomal action.[81-84] In this scenario, the cross-linked, nondigestible residues accumulate in the RPE, which may then discharge lipid-protein complexes, as well as secondary lysosomes,[85] into Bruch's membrane.[86,87] This results in progressive lipid accumulation seen with aging[88-91] and the formation of basal laminar and basal linear deposits and drusen that are present in lesions of ARMD and frequently predate them.[86,92-98]

Another factor that may lead to the accumulation of lipofuscin may be directly related to enzymatic actions within RPE lysosomes. Acid-catalyzed, irreversible crosslinking of two retinaldehyde molecules (A2), derived slowly and incrementally from vitamin A metabolism, and an ethanolamine molecule (E), derived from POS degradation, produces the dominant fluorophore of lipofuscin granules: A2-E.[99] This amphiphilic, quaternary nitrogen compound concentrated in RPE tertiary lysosomes inhibits lysosomal enzyme activity, particularly if photoactivated to produce free radicals.[82,83,100] A2-E may also leak from lysosomes on account of its detergentlike structure or upon photoactivation, leading to RPE plasma membrane dysfunction, the formation of RPE basal deposits, and RPE detachment and apoptosis.[101-103] Whatever its source, lipofuscin is a photoinducible generator of reactive oxygen metabolites that cause lipid peroxidation, impairment of lysosomal enzyme activity, loss of lysosomal integrity, and eventually RPE cell death.[81,104,105]

In addition to the major roles of degrading engulfed, aged POS and autophagocytic debris, the RPE may incorporate other material requiring lysosomal action. Extensive studies of RPE phagocytosis have been performed in vivo and in vitro, the latter utilizing both retinal-choroidal organ cultures and cultured RPE cells. RPE phagocytosis of particulate matter, including isolated POS,[45,106-111] latex spheres,[39,112-115] liposomes,[116] bacteria,[112,115] and lectin- and sugar-coated beads,[117,118] have been examined. In these studies, RPE cell phagocytosis varied with the type of phagocytic stimulus, presumably on account of variable cell surface–particle binding, but receptors mediating their uptake remained unknown.

Subsequently, RPE cell surface receptors mediating the recognition and binding of specific ligands and leading to selective, rapid, and efficient incorporation of particulates into the RPE were identified. These include CD16 for immunoglobulin,[119,120] CD11b/CD18 and CD35 for complement,[119] mannose-6-phosphate receptors,[121-125] and CD14 for endotoxins.[120,126,127] Specific, high-affinity RPE uptake of lipid-laden particulates may be mediated by receptors for native (N-) LDLs and scavenger type I/II receptors for acetylated/acetoacetylated (A-) LDL, for which RPE cells bear specific receptors.[128-130] Like A-LDL, oxidized (O-) LDL may be bound and internalized into lysosomes by scavenger type I/II receptors[131-134] and other scavenger receptors, including CD68 and CD36[85,135-139] that have also been identified on RPE cells.[140-142] CD36 is also a receptor for thrombospondin, which appears to act together with the vitronectin receptor, avb3 integrin, on macrophages to clear senescent erythrocytes and neutrophils[143-146] and on RPE to recognize and internalize aged POS membranes.[105,147] O-LDL internalized by scavenger receptors, including CD36, inhibits RPE POS membrane degradation by interfering with secondary lysosomal function.[148] Nevertheless, receptor-mediated uptake of native or modified LDL has not been shown on primate cells in vitro, and the RPE incorporation of these lipoproteins, which must cross Bruch's membrane, has not been demonstrated in vivo. Furthermore, the effects of these lipoproteins, whose abundance and composition may be altered by diet and other exogenous factors, on RPE lysosomal lipid hydrolytic activity has not been investigated.

The unique relationship between the photoreceptors and the RPE extends to the peculiar composition of the POS membranes and the RPE role in preserving it. The principal polyunsaturated fatty acid of POS membranes is the omega-3 fatty acid, docosahexaenoic acid (DHA), and arachidonic acid (AA).[148-150] Of all mammalian cells, DHA is the most abundant in POS, and dietary intake of DHA, related omega-3 fatty acids, or its precursor fatty acids, principally linoleic acid, is required for normal brain and retinal development.[151] Infant monkeys[152,153] and humans[154-156] deficient in dietary DHA have reduced visual acuity function. The main dietary sources of DHA and related fatty acids are fish oils, nuts, and other seeds. When ingested, hepatic processing places these fatty acids in LDLs that are delivered via the serum to tissues where receptor-mediated LDL uptake delivers the lipids containing omega-3 fatty acids to cells.[157,158] The RPE metabolizes the delivered lipid and transports DHA to the photoreceptors, which incorporate these fatty acids into POS. Inasmuch as the photoreceptors have an absolute requirement for DHA in substantial quantity on a daily basis to renew POS membranes, the RPE has sophisticated pathways to synthesize and conserve DHA cleaved from phagocytized POS membranes and recycle it back to the photoreceptors.[159-162] These pathways include transient RPE storage of cleaved DHA in triglycerides followed by efficient hydrolysis and transport of free DHA via fatty acid binding proteins.[163-166] Thus, the photoreceptors and the RPE have a high stake in the efficient DHA metabolism within POS-laden secondary lysosomes that is necessary for physiologic functioning and homeostasis. Alterations of this process may lead to accumulation of abnormal DHA-containing lipids driving aging and ARMD.[163]

Accordingly, experimental models have shown that oxidized DHA moieties including DHA hydroperoxide[167] and linoleic hydroperoxide are involved in oxidative-mediated retinal and RPE cell injury.[168] Deprivation of linoleic acid, the dietary precursor of omega-3 fatty acids in rats, reduced RPE lysosomal enzyme activity and altered the size and distribution of POS phagosomes in RPE cells and was associated with reduced electroretinographic amplitudes.[169] These studies imply that omega-3 fatty acids or their precursors may modulate RPE lysosomal function, DHA metabolism, and RPE cell integrity. To date, however, no investigations regarding lysosomal lipid metabolism in response to omega-3 fatty acids have been performed, perhaps in part because of difficulty in biochemically assaying lysosomal acid lipase and unavailability of a reliable histochemical stain to assess its activity in situ.

Reported epidemiologic risk factors for ARMD include cigarette smoking, atherosclerosis, high levels of serum cholesterol, low levels of serum antioxidants, and exposure to blue and UV light,[130,170-177] while consumption of dietary fish oils appears to have protective effects.[1-3] All of these factors may potentially modulate RPE lipid metabolism by altering the quantity and character of circulating lipoproteins that are delivered from the blood to the RPE monolayer and by locally oxidizing cellular and extracellular lipids and proteins, including lipoproteins that have emerged from the choriocapillaris to reach the RPE monolayer.

Cigarette smoking is the most unequivocal risk factor increasing risk for ARMD.[175-177] Among its protean effects, smoking causes peroxidation of circulating LDL,[178,179] producing elevated plasma levels of thiobarbituric acid–reactive substances (TBARS),[180] isoprostanes,[181] and other measures of lipid peroxidation.[182-183] Increased levels of circulating O-LDL, a known risk factor for atherosclerosis,[170, 179-182] have been implicated as a possible risk factor for ARMD.[183] Depletion of antioxidant vitamins and antioxidant enzymes[172,173,184,185] and increased exposure to blue or UV light[174] are other factors that have been purported to lead to increased oxidative stress. Oxidized LDL and other locally oxidized lipids, proteins, and extracellular matrix components are thereby altered to forms that may be recognized and engulfed by specific RPE scavenger receptors.[128,129,140-142] Once internalized, O-LDL has been shown to interfere with RPE POS membrane degradation, leading to oxidation of additional lipid and protein in RPE cells.[148]

Dietary fish oil is a newly recognized factor that may reduce the risk of developing ARMD.[1-3] Its main constituent, DHA, also demonstrates a trend for reduced risk.[2] This contrasts with high consumption of vegetable fats containing polyunsaturated fatty acids, most notably linolenic acid, which is positively associated with the development of atherosclerosis and ARMD and mitigates the positive effects of high fish oil intake.[1,186,187] The mechanisms underlying this disparity are complex and involve the types of lipoprotein elaborated by the liver and their promotion of intracellular cholesterol esterification when they are incorporated into target cells.[188-193] These differences underlie the proatherogenic nature of linolenic acid and the antiatherogenic qualities of fish oil.[186,187,194-197] However, both types of unsaturated fatty acid may be readily oxidized when circulating or within tissues, resulting in oxidized forms that may be injurious to tissue, especially those containing cells expressing specific receptors for oxidized or otherwise damaged lipoproteins.[182,188] Therefore, better understanding of the effects of fish oil–rich diets and serum on RPE lysosomal lipid metabolism may assist in illuminating how diet and environmental oxidative factors, such as smoking and light exposure, alter the progression of RPE aging and the development of ARMD.

MATERIALS AND METHODS

ANIMALS, TISSUES, AND CELLS

Normal New Zealand White rabbits and monkeys (*Macaca mulatta*) were fed standard chow diets or diets supplemented with cholesterol, coconut oil, and Menhaden fish oil until sacrifice. All animals were treated and cared for according to National Institutes of Health guidelines for the humane treatment of laboratory animals. All experimental protocols were approved by committees on animal use and care in research at the University of Chicago and the University of Michigan.

Tissues and Cells for Comparative Acid Lipase Studies in New Zealand White Rabbits

For histochemical studies, the albino rabbits were exsanguinated under sodium pentobarbital anesthesia. Whole eyes and tissue blocks of lung, liver, and spleen not exceeding 5 mm thick were fixed at 4°C for 24 hours in formal sucrose and rinsed in gum sucrose for 24 hours.[77] The fixative consisted of 4% formaldehyde prepared from paraformaldehyde by depolymerization, after which the osmolality of the fixative, buffered to pH 5.8 with 0.067 M sodium phosphate buffer, was raised by adding 7.5% wt/vol sucrose. Formal sucrose consisted of a solution of 1% (wt/vol) gum acacia and 30% (wt/vol) sucrose.[198]

For comparative biochemical studies in the rabbits, immediately upon death, the lungs and peritoneal cavity were lavaged with lactated Ringer's solution and cell suspensions were obtained by centrifugation for 15 minutes at 110*g* followed by resuspension of the cells into Eagle's minimum essential medium (MEM) supplemented with 15% fetal calf serum (FCS). Pieces of spleen were finely

minced into MEM with 15% FCS. Blood obtained at sacrifice was collected in glass tubes containing sufficient ethylenediaminetetraacetic acid (EDTA) to obtain a final concentration of 2.0 mM. White blood cells were then obtained following centrifugation and resuspension in MEM with 15% FCS. Aortic tissue samples (0.5 cm^2) were excised, gently minced, and plated to establish smooth-muscle cell cultures that were maintained in MEM supplemented with 15% FCS. Rabbit eyes were removed and placed on ice and kept in darkness. Within 2 hours of death, the anterior ocular segment and vitreous were carefully removed under dim, red light. The posterior segment was immersed in Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution (HBSS) containing 2.0 mM EDTA for 30 to 60 minutes at 4°C in darkness. The neurosensory retina was then carefully peeled from the RPE monolayer, which was subsequently collected by the method of Heller and Jones.[199] RPE cells were harvested by gentle brushing into phosphate-buffered saline (PBS), pelleted at 1,000*g* for 10 minutes, and resuspended in MEM with 15% FCS. RPE, alveolar, peritoneal, splenic, and white blood cell suspensions were overlaid onto glass beads (Microbeads class IV-C, Cataphote Corp, Toledo, Ohio) in plastic culture dishes. After 3 hours, the glass beads were rinsed thoroughly with three changes of MEM containing 15% FCS and once in PBS to remove nonadherent cells.

Bovine RPE Cells for Acid Lipase Purification Studies

Cow eyes were obtained within 1 hour of slaughter from a local abattoir and maintained on ice and in dim light. Within 2 hours, RPE cells were harvested as described for the rabbit eyes and kept on ice in MEM with 15% FCS until acid lipase extractions were performed.

Monkey RPE Cells, Fibroblasts, Smooth-Muscle Cells, Alveolar Macrophages, and POS for In Vitro Studies

At autopsy, monkey RPE cells were obtained immediately after death by exsanguination under thiamylal sodium anesthesia as described for rabbit and bovine eyes. The monkey RPE cells were established and characterized in culture as previously described. POS of monkeys was prepared by a modification of the method of Plantner and Kean.[200,201] Briefly, carefully peeled, fresh neurosensory retinas were gently homogenized in 5mM Tris-acetate buffer, pH 7.4, containing 0.2 mM $MgCl_2$, 65 mM NaCl, and 36.5% sucrose (wt/wt) using a Teflon pestle and then centrifuged at 1,000*g* for 5 minutes. After repeating this procedure, the supernatant containing POS was centrifuged in the Tris-acetate buffer at 12,000*g* for 15 minutes, resuspended, and centrifuged again to obtain POS for RPE in vitro studies. POS were then resuspended in MEM, counted in a hemocytometer, and adjusted to a final concentration of 2×10^6/mL for use in experimental incubations. Alveolar macrophages were obtained by lavage with lactated Ringer's solution, and aortic smooth-muscle cells were harvested, seeded, and established as described for the rabbits. Orbital fibroblasts were obtained by mincing orbital fat into culture dishes. Fibroblasts grew from the tissue pieces and were established as cultures in MEM with 15% FCS.

Tissues and Cells from Monkeys On Fish Oil or Coconut Oil Diets

Twenty-four adult ($2^1/_2$- to 3-year-old) rhesus monkeys were purchased from the National Institutes of Health (Alice, Texas). The monkeys were divided into three groups of eight each and fed the diets as previously described. In brief, low-fat Purina monkey chow was used as the basic ration, to which was added 25% fat and 2% cholesterol. The 25% fat consisted of coconut oil alone, Menhaden fish oil and coconut oil (1:1), or Menhaden fish oil and coconut oil (3:1). The fish oil contained cholesterol (432 mg/dL); therefore, monkeys consumed slightly more cholesterol on the fish oil diets. Fasting serum lipid values were determined at the onset and at monthly intervals throughout the 12-month experimental period.[190,192] Each chow-based diet supplied sufficient essential fatty acids and other nutrients. The animals were autopsied after 12 months by exsanguination under thiamylal sodium anesthesia. For biochemical enzyme assays, the eyes from each monkey were opened in the coronal plane anterior to the equator, and the RPE cells were harvested as described for rabbit and bovine eyes. The RPE cells were harvested by gentle brushing into PBS, pelleted at 1,000*g* for 10 minutes, and homogenized in distilled, deionized water at 0°C in the desired volume. Portions of one of the posterior ocular segments from each monkey were fixed at 4°C for 24 hours in 4% paraformaldehyde (wt/vol) containing 7.5% sucrose (wt/vol), followed by a 24-hour wash at 4°C in 1% gum acacia (wt/vol) containing 30% sucrose (wt/vol). Frozen sections (6 µm) were cut and stained by histochemical localization of nonspecific acid esterase and acid lipase lysosomal enzyme activity.[77] Samples of liver, spleen, and lung were also placed in water (1/10, wt/vol) and homogenized. The homogenates of these organs were then centrifuged at 5,000*g* for 10 minutes, and the supernatant was collected for enzyme activity studies. Tissues for biochemical assays were used immediately or frozen at -20°C for up to 1 month until assayed.

ADAPTATION OF A NOVEL CHROMOGENIC TECHNIQUE FOR ACID LIPASE ACTIVITY

A histochemical and biochemical technique to detect and measure acid lipase activity utilizing detergent micelles containing α-naphthyl palmitate was previous reported.

This technique is adapted for use in analyzing RPE lysosomal acid lipase and nonspecific lysosomal acid esterase activity.

Histochemical Assays for Acid Lipase and Acid Esterase Activity

Histochemical staining for acid lipase activity was performed on fixed, frozen tissue sections 4 to 10 μm thick or cultures fixed for 30 minutes in formal sucrose and rinsed for 15 minutes in gum sucrose prior to histochemical staining. Fixed sections or cultured cells rinsed in chilled 0.1 M sodium acetate buffer, pH 5.2, were transferred within 30 minutes to 40 mL of incubation medium that contained substrate micelles consisting of 1.0 mM α-naphthyl palmitate (15.4 mg; Sigma Chemical Co, St Louis, Missouri), 10 mM Triton X100 (0.65% wt/vol) and 1.0 mL hexazotized pararosaniline (HPR) prepared immediately before use. HPR was obtained by vigorously mixing equal volumes of 4% sodium nitrite and 4% pararosaniline HCl (Sigma) dissolved in 2.5 N HCl for 60 seconds. After addition of the HPR solution to the buffer-containing substrate micelles, the pH was readjusted to 5.2 with 5.0 N NaOH. For acid esterase activity, the incubation medium was prepared by injecting and swirling 10 mg of α-naphthyl acetate, previously dissolved in 1.0 mL acetone, into 40 mL of 0.1 M sodium acetate buffer containing 1.0 mL of freshly hexazotized pararosaniline. Incubation times, at 25°C, ranged from 15 minutes to 24 hours, depending on enzyme activity present in the tissues or cells and the purpose of the experiment. Incubations were terminated by thorough rinsing in distilled water. All sections and tissue cultures were counterstained with 1% purified methyl green in 1.0 M sodium acetate buffer, pH 4.0, and subsequently cleared with graded alcohols and xylenes prior to mounting in synthetic resin.

To inhibit acid lipase activity, fixed sections or cultures were preincubated in diethylparanitrophenylphosphate (DEPP, E600, Mintacol; Bayer Ag, Leverkrusen, Germany) or parahydroxymercuribenzoate (PHMB; Sigma) in 0.1 M sodium phosphate buffer, pH 6.5, for 30 minutes at 25°C. The inhibitors were also included in the incubation media. Control sections were heat-inactivated by incubating unmounted sections in a moist chamber at 56°C for 30 minutes.

Biochemical Assays for Acid Lipase and Acid Esterase Activity

Acid lipase activity was determined with substrate micelles which were prepared using 1.0 mM α-naphthyl palmitate and 10 mM Triton X100 in 0.1 M sodium acetate buffer, pH 4.2, containing 0.1% fatty acid–poor bovine serum albumin. Acid esterase activity was determined using the same buffer containing 1.0 mM α-naphthyl acetate which was first dissolved in 0.5 to 1.5 mL of acetone and then rapidly and forcefully injected into the buffer. Aliquots (50 to 100 μL) of enzyme fractions, tissue homogenates, cell homogenates, or conditioned culture media or aliquots of glass beads with adherent rabbit cells were assayed in 1.0 mL of substrate at 25°C with continuous agitation for 1 to 6 hours. The reactions were stopped by placing the samples in boiling water for 2 minutes. One milliliter of 1.0 M sodium acetate buffer, pH 4.2, containing 10% Tween 20 (wt/vol) and 0.5 mg fast garnet GBC salt was added to each sample. Diazocoupling of liberated α-naphthol was performed at 25°C for 16 hours. Absorbance of samples, heat-inactivated blanks, and α-naphthol standards were then measured at 535 nm.

QUANTITATION OF CELLS AND DNA

For comparative acid lipase rabbit studies, cells were enumerated to normalize assayed enzyme activity. For all other studies, cell and tissue enzyme activity was normalized to DNA. DNA quantitation of cell cultures did not reveal any statistically significant differences after different cell culture treatments with phagocytic stimuli, LDL, or sera.

Enumeration of Cells for Comparative Acid Lipase Studies in New Zealand White Rabbits

Aliquots of glass beads were weighed and subsequently incubated to determine the number of glass-adherent cells per gram of glass beads to determine acid lipase activity present per cell number. Aliquots of third-passage rabbit aortic smooth-muscle cells rinsed and scraped into PBS from tissue culture flasks were also assayed for cell number to determine acid lipase activity per 10^6 cells/hour. Some aliquots of glass beads with adherent cells were agitated for 5 to 10 minutes in 2.0 mM EDTA according to the method of Siakotos and associates[202] to dislodge the glass-adherent cells, which were then smeared or dried onto glass slides. The cells in the slide preparations were either counted to assess the number of monocytes, macrophages, and RPE cells present in the various adherent cell populations or stained histochemically for up to 6 hours to determine the number of acid lipase–positive cells adherent to the glass beads. The numbers of glass-adherent monocytes, macrophages, and RPE cells per gram of glass beads were determined by enumerating cell nuclei using a modification of the method of Levine and associates.[203] Briefly, 0.50- to 1.0-g aliquots of glass beads with adherent cells were incubated at 37°C in 3.0-mL aliquots of 1.0 M citric acid containing 0.1% crystal violet. After 1 hour, the glass beads were agitated to dislodge cell remnants from the beads, and liberated cell nuclei were counted in a hemocytometer. For

smooth-muscle cell cultures, 100-µL aliquots of smooth-muscle cells scraped into PBS were assayed in 1.0 mL of the citric acid–crystal violet solution.

DNA Quantitation of RPE Cells and Tissue Homogenates
The DNA contents of the cultured cell monolayers scraped from each tissue culture flask and of homogenates of freshly obtained RPE cells, liver, and spleen were determined according to the method of LaBarca and Paigen.[204] Briefly, the cells grown in tissue culture flasks were scraped into distilled water in the cold, briefly sonicated, and maintained on ice until DNA assays were performed. Then 25- to 100-µL aliquots of the cell lysates, raised when necessary to a total of 100 µL by adding distilled water, were added to 4.7 mL of a phosphate-saline buffer solution (0.05 M Na_3PO_4, 2.0 M NaCl), followed by addition of a 0.5-µg Hoechst 33258, a fluorescent dye which quantitatively binds to double-stranded DNA, in 200 µL of distilled water. The samples were then mixed vigorously and kept in the dark until fluorescence was measured using a Beckman Fluorocolorimeter with a 370-nm narrow-band interference filter for the excitation light and a Kodak 2A cutoff filter for light emission. Calf thymus DNA standards were used in each assay.

ACID LIPASE PURIFICATION FROM FRESHLY ISOLATED BOVINE RPE CELLS

To demonstrate the specificity of the chromogenic assay for acid lipase and to show that RPE acid lipase may actually esterify free fatty acids to cholesterol, acid lipase activity, corresponding to a single protein band, was purified from bovine RPE and shown to possess the ability to hydrolyze α-naphthyl palmitate and esterify cholesterol to fatty acids hydrolyzed from triolein.

Molecular Sieve Gel and Ion Exchange Chromatography
Bovine RPE cell pellets were resuspended and sonicated in distilled water, 1:10 (wt/vol), and combined with 2.0 mL of 0.02 M Tris-HCl buffer, pH 7.4. The sample was then applied to and run at 4°C on a 90.0 x 2.0-cm Glenco Column of Sephadex G-150, equilibrated with degassed 0.02 M Tris-HCl buffer, pH 7.4. Column flow was 16 mL/hour and 4.0-mL fractions were collected and assayed for acid lipase activity and cholesteryl ester synthesis. Peak fractions were pooled and concentrated by ultrafiltration in 0.05 M sodium acetate buffer, pH 5.5, containing 0.025 M NaCl using an Amicon stirred cell with a UM 10 membrane (10,000 MW cutoff) under 70 psi of nitrogen. The pooled concentrate was applied to a 20.0 × 1.0-cm column of SP Sephadex C-50 equilibrated and eluted with degassed 0.05 M sodium acetate buffer, 0.025 M NaCl, pH 5.5. Two-milliliter fractions were collected and assayed for cholesteryl ester formation and for the hydrolysis of α-naphthyl palmitate, triglycerides, and cholesteryl esters.

Polyacrylamide-SDS Gel Electrophoresis
To test the purity of the isolated protein, electrophoresis in polyacrylamide-SDS slab gels was performed using 0.1% SDS in 0.05 M Tris-glycine buffer, pH 8.3, at an initial voltage of 200 V. The slab gels consisted of a 13% polyacrylamide running gel and a 6% stacking gel made in a 0.5 M Tris-HCl buffer at pH 8.8 and pH 6.8, respectively. Protein samples and molecular weight markers were prepared by heating a mixture containing equal volumes of 4% SDS, 20% glycerol, 10% mercaptoethanol, 0.002% bromophenol blue, and 0.05 M Tris-HCl buffer, pH 6.8, for 5 minutes at 90°C. The resulting gel profiles were stained with either Coomassie blue or silver nitrate, destained, dried, and photographed.[205,206]

Isoelectric Focusing Zymography
The isolated fractions were also examined by isoelectric focusing zymograms. The fractions were dialyzed against distilled water and added to a mixture of degassed 5% (wt/vol) acrylamide, 0.25% (wt/vol) bisacrylamide, 15% (wt/vol) glycerol, and either a broad-range (pH, 3.0-10.0) or narrow-range (pH, 4.0-6.5) ampholyte solution (Pharmacia, Uppsala, Sweden). Polymerization was induced by adding 50 µL/mL of a freshly prepared 0.3% ammonium persulfate solution. Gels were poured into 10.0 × 0.5-cm glass tubes and allowed to solidify for 30 minutes. The gels were run in a Buchler disc electrophoresis cell containing 0.025 N NaOH in the cathode chamber, 0.025 N H_2SO_4 in the anode chamber cooled to 5°C by a circulating cold water jacket. Initially, isoelectric focusing was performed at 50 V for 30 minutes, and subsequently the voltage was increased stepwise for 8 to 18 hours to a terminal voltage of 375 V and a current of 0.5 mA/gel. Some gels were stained for protein with Coomassie blue by the method of Vesterburg,[206] while other gels run under the same conditions were cut in 2-mm gel slices and assayed for enzyme activities.

ASSAY FOR CHOLESTERYL ESTERASE AND TRIGLYCERIDE HYDROLYSIS

Cholesteryl ester and triglyceride hydrolytic activity was determined using a radioactively labeled vesicle substrate system modified from the method of Brecher and associates.[207] Vesicles were prepared by drying 25 mg of egg yolk lecithin with either 2.0 mg cholesteryl oleate and 2.0 µCi cholesteryl-1-[^{14}C]-oleate (66:1 phospholipid/substrate molar ratio) or 4.5 mg trioleate and 2.0 µCi glyceryl tri-1-[^{14}C]-oleate. The mixtures were sonicated in 2.0 mL of glycerol, and the glycerol sonicate was added to 20 mL of 0.1 M sodium acetate buffer, pH 4.2, and resonicated.

Aliquots (200 µL) of media or distilled water containing sonicated cells were incubated for 4 hours in 1 mL of substrate. The reaction was terminated by adding 6.0 mL of benzene:chloroform:methanol (1.0:0.5:1.2). Free oleate was extracted by the method of Pittman and associates.[208] In brief, the mixture was made alkaline with 1.2 mL of 0.3 M NaOH. After phase separation, 0.5 mL of the upper phase was added to 10.0 mL of PCS scintillation cocktail (Amersham Inc, Arlington Heights, Illinois) and counted by liquid scintillation spectrometry. Counting efficiencies were determined from sample channel ratio values and quenching curves derived from quenching standards (Amersham). Appropriate blanks and controls were included in each assay.

ASSAY FOR ACID LIPASE CHOLESTEROL ESTERIFICATION WITH FATTY ACIDS HYDROLIZED FROM TRIOLEIN

Esterification of cholesterol to fatty acids liberated from hydrolyzed triglycerides was determined by incubating aliquots of enzyme isolates and homogenized tissue samples for 16 hours after 25°C in a substrate media containing 0.1 M sodium acetate buffer, pH 4.2, and lecithin-[^{14}C]-cholesterol-triolein vesicles. The vesicles were prepared by drying under a nitrogen stream, 25 mg lecithin, 0.6 mg cholesterol, 9.0 mg triolein, and 2.0 µCi 4-[^{14}C] cholesterol (0.013 mg), sonicating the mixture in 4.0 mL of glycerol, adding 46 mL of buffer, and resonicating. The substrate vesicles were sized with a cross-linked Sephrose 6B column and were found to have an apparent M_r of 2.5×10^6 with a calculated hydrodynamic radius of 100 angstroms, consistent with unilamellar vesicles. Incubations terminated by adding methanol and lipids were extracted by the method of Bligh and Dryer.[209] Extracted lipid was dried under nitrogen to a minimal volume and spotted on silica gel (IB2 Baker-Flex) thin-layer chromatography plates. The plates were developed in petroleum ether:ethyl ether:acetic acid (75:24:1). Lipid profiles were visualized with iodine vapor and cholesterol ester fractions excised and counted by liquid scintillation spectrometry.

ISOLATION, FLUORESCENT LABELING, AND MODIFICATION OF MONKEY LDL

Human and monkey LDL was isolated and labeled as previously described by Havel and associates.[210] Briefly, human or monkey LDL (d=1.02-1.05 g/cm^3) and lipoprotein-deficient serum (LDS) were obtained by sequential density gradient ultracentrifugation from normal plasma. For fluorescent lipoprotein uptake studies, isolated human LDL was then labeled with fluorescent, 3,3′-dioctadecylindocarbocyanine (DiI) according to the method of Pitas and associates.[211] One milligram of LDL was added to 2 mL of LDS, and the serum was sterilized with a 0.45-µm polycarbonate filter. Then 50 µL of DiI (3.0 mg/mL in dimethyl sulfoxide) was added, followed by brief, gentle agitation and incubation at 37°C overnight. To reisolate the DiI-labeled LDL, the density of the incubation mixture was raised to 1.063 g/cm^3 with NaCl, and the DiI-native LDL was isolated at 10°C by 24 hours of centrifugation at 29,000 rpm in a Beckman 30.2 rotor. The DiI-labeled N-LDL was then either dialyzed against 0.9% NaCl containing 0.01% EDTA, pH 7.0, or 0.1 M borate buffer, pH 8.4, in the sample to be acetoacetylated. Acetylation of LDL was performed according to the method of Basu and associates[212] using acetic anhydide and subsequently N,N-dimethyl-1,3-propanediamine and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide. Extensive dialyses for 24 hours at 4°C against 12 L of buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4, were performed between the steps for LDL modification. For oxidation of LDL, LDL (100 µg/mL) was dialyzed for 16 hours at 37°C against 5 µM copper sulfate in PBS followed by dialysis for 24 hours against PBS containing 0.5 mM EDTA to remove Cu^{++} ions according to the method of Quinn and associates.[213] To confirm successful acetylation and oxidation of LDL, electrophoretic mobility was assessed on 1% agarose gels.[214]

MODULATION OF LYSOSOMAL ENZYME ACTIVITY IN CULTURED MONKEY RPE CELLS BY PHAGOCYTIC CHALLENGE OR EXPOSURE TO LIPOPROTEINS

To begin experimental incubations, monkey RPE cells (5×10^5 cells/well) were rinsed twice with PBS to remove serum. They were then overlaid for 24 hours with serum-free medium or media containing 0.93 µm latex spheres (10 mg/mL) or POS (5×10^6 POS/well) or LDL (50 to 100 µg/mL). Media were then collected and centrifuged to remove debris. The RPE cell layers were rinsed and either fixed and stained for acid lipase and acid esterase activity or scraped from the culture flasks and sonicated on ice for biochemical assays. Cell sonicates and media were maintained at -20°C for up to 1 month prior to biochemical assays.

RPE NATIVE AND MODIFIED LIPOPROTEIN UPTAKE

In vitro assays were performed to demonstrate receptor-mediated uptake of lipoproteins that may be specifically inhibited, while in vivo perfusions of labeled lipoproteins were used to demonstrate rapid and avid lipoprotein uptake in a primate model.

Receptor-Mediated Native and Modified LDL RPE Uptake In Vitro

Monkey RPE cells, alveolar macrophages, smooth-muscle cells, and fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 15%

FCS, which was replaced with DMEM containing 10% LDS 1 to 2 days prior to incubation with DiI-labeled lipoproteins. Experimental incubations were begun by changing the medium to DMEM containing 10% LDS and 15 µg/mL of either DiI-labeled, N-, A-, or O-LDL. For competitive inhibition, tenfold excess of the corresponding unlabeled lipoprotein was used. Fucoidin, a competitive inhibitor of A-LDL and a partial inhibitor of O-LDL binding and uptake, was added to cell cultures 15 minutes prior to and during DiI-labeled, AA, and O-LDL incubations at a concentration of 150 µg/mL. Dextran sulfate (30 µg/mL), a polyanionic inhibitor of N- and A-LDL binding and uptake, was also used in some incubations. To confirm the specific uptake of the N-, A-, and O-LDL, assays on cultured macrophages, smooth-muscle cells, and fibroblasts were performed according to the method of Pitas and associates.[211] Experimental assays on cultured RPE cells were performed simultaneously with assays on the other cell types. Following 2-hour incubations with DiI-labeled lipoproteins at 37°C, cells were washed five times with PBS containing 2.0 mg/mL bovine serum albumin, rinsed two times with PBS, and fixed for 20 minutes in HBSS, pH 7.4, containing 4% paraformaldehyde. The cell preparations were mounted in Bacto FA fluid, pH 7.2 (Difco Inc, Detroit, Michigan) and observed using an Olympus BH microscope equipped with a BH-DMG dichroic mirror and a O-590 barrier filter. At least 200 cells were counted in each sample. Photomicrographs were taken using Ektachrome 400 film with exposures varying from 10 to 45 seconds.

Native and Modified LDL RPE Uptake In Vivo

In vivo perfusion studies were performed on monkeys under thiamylal sodium anesthesia and in rabbits under sodium pentobarbital anesthesia by catheterizing the common carotid arteries. Following carotid perfusion with 75 to 100 mL of isotonic, 0.9% saline solution at 100 mm Hg, 100 mL of DiI-labeled, N-, A-, or O-LDL (40 µg/mL) in PBS was perfused over 20 minutes in dim light. The jugular veins were then cut to permit outflow, and normal saline solution was perfused until clear fluid return was obtained. In situ fixation was obtained by subsequent perfusion with 500 mL of 4% buffered formaldehyde at a perfusion pressure of 150 mm Hg. The enucleated eyes and pieces of lung, liver, and spleen were immersed in the same fixative for 1 to 7 days. Six- and 12-µm frozen tissue sections were cut in darkness and viewed and photographed as described for cell cultures. Control tissues were obtained as described above except that in vivo perfusion was performed with 50 mL of PBS lacking lipoproteins.

Statistical Analysis

Statistical significance of differences between control and experimental groups was determined using Student's two-tailed *t* test. Differences between groups were considered to be statistically significant for $P<.05$.

RESULTS

COMPARATIVE STUDIES ON ACID LIPASE IN RPE CELLS AND MONONUCLEAR PHAGOCYTES

A histochemical and biochemical technique to detect and measure acid lipase activity using detergent micelles containing α-naphthyl palmitate was previously reported.[77] This technique was adapted for use in analyzing RPE lysosomal acid lipase activity and nonspecific lysosomal acid esterase activity.

Acid lipase activity was initially assessed in fixed, frozen sections of New Zealand White, albino rabbit eyes to eliminate obfuscation of staining by endogenous RPE pigment. The rabbit RPE monolayers stained selectively and intensely for acid lipase activity in full-thickness sections of the posterior ocular segment histochemical incubations of up to 6 hours (Figure 1A and 1B). The dense, red-brown reaction product was present throughout the cytoplasm of all cells in the RPE monolayer.

Histochemical staining of fixed, frozen sections of liver, spleen, and lung demonstrated selective, intense staining of mononuclear phagocytes in histochemical incubations lasting from 30 minutes to 4 hours, as previously reported in other species.[76,77] In liver, Kupffer cells, resident macrophages within the hepatic sinusoids, stained intensely (Figure 2A). Only faint, diffuse staining of hepatocytes was present. Histochemical staining of the spleen for acid lipase activity (Figure 2B) demonstrated heavy deposition of red-brown reaction to be limited to mononuclear phagocytes principally in interfollicular regions, but also within phagocytes distributed in the lymphoid follicles. In the lung (Figure 2C), staining for acid lipase was limited to alveolar macrophages, which exhibited deep, red-brown staining corresponding to high levels of enzyme activity in these phagocytes. Smears of peripheral monocytes and cultures of third-passage smooth-muscle cells showed no appreciable histochemical staining in overnight incubations.

To compare the acid lipase activity of freshly isolated rabbit RPE cells to that of macrophages isolated from other tissues, biochemical acid lipase assays were performed on aliquots of glass beads bearing adherent RPE cells or macrophages (Table I). The highest lipolytic activity was found in freshly isolated alveolar macrophages, followed by splenic and peritoneal mononuclear phagocytes. All macrophages demonstrated acid lipase activities well above the 36 $\mu M/10^6$ cells/hour threshold of α-naphthyl palmitate hydrolysis required for histochemical staining in fixed, frozen tissue sections despite 80% to 90% loss of

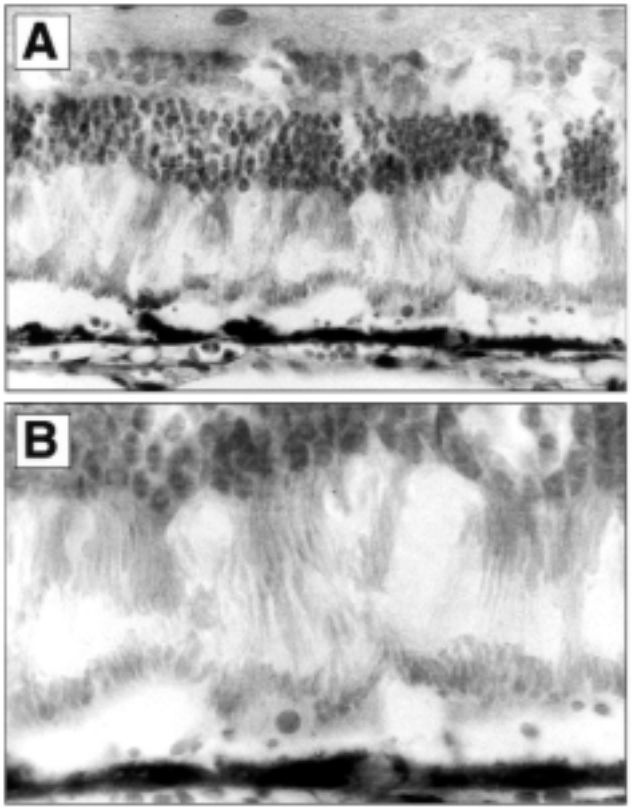

FIGURE 1

Histochemical staining for acid lipase activity in New Zealand White rabbit retinal pigment epithelial (RPE) cells. A, Deposition of red-brown reaction product corresponding to sites of α-naphthyl palmitate hydrolysis is limited to RPE cells in fixed, frozen sections of posterior ocular segment. Variable staining of a few hyalocytes in cortical vitreous is also present (methyl green counterstain, x100). B, Selective staining of RPE cells for acid lipase activity is readily visible at higher power. Lipid vacuoles are also noted in rabbit RPE monolayer (methyl green counterstain, x400).

acid lipase activity associated with formal sucrose fixation.[76,77] Freshly isolated, glass-adherent RPE cells liberated 81 μM of α-naphthol/10^6 cells/hour (Table I), which is also compatible with the positive in situ histochemical staining present in these cells (Figure 1). Subthreshold activities for histochemical staining were present in monocytes and third-passage, cultured smooth-muscle cells, the latter containing the least acid lipase activity of all cell types assayed.

Enumeration of cells adherent to glass bead aliquots was performed by counting cell nuclei extracted from cells with citric acid and stained with crystal violet. Using this technique, cell counts varied less than 10% per sample. To determine the purity of cells adherent to glass beads, adherent cells were dislodged from aliquots of glass beads using EDTA. The cells were dried onto glass slides and counted microscopically. Greater than 90% of adherent cells were macrophages, RPE cells, or monocytes in their respective preparations.

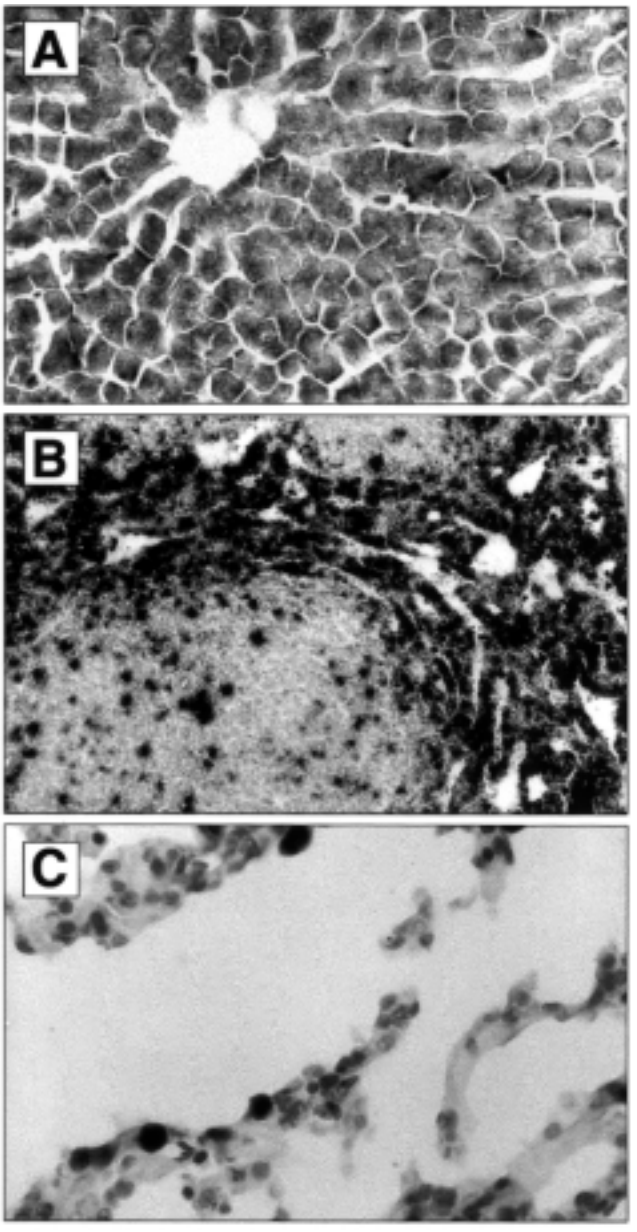

FIGURE 2

Histochemical staining for acid lipase activity in fixed, frozen sections of New Zealand White rabbit lung, liver, and spleen. A, Liver Kupffer cells are deeply and selectively stained with reaction product following incubation with α-naphthyl palmitate substrate. Deposition of red-brown product due to enzyme activity is just visible in centroacinar hepatocytes (methyl green counterstain, x100). B, Splenic acid lipase activity is detected selectively in mononuclear phagocytes of red pulp and germinal centers of spleen by red-brown reaction product (methyl green counterstain, x100). C, Lung alveolar macrophages contain selective, deep red-brown reaction product following incubation with α-naphthyl palmitate–Triton X100 substrate micelles (methyl green counterstain, x100).

Preincubation of isolated cells or the tissue sections with the organophosphate DEPP (10^{-4} M) or the sulfhydryl blocking reagent, PHMB (5×10^{-5} M), but not the carboxylic esterase inhibitor, bis-p-nitrophenylphosphate, virtually eliminated acid lipase activity in the RPE

TABLE I: ACID LIPASE ACTIVITY OF FRESHLY ISOLATED RABBIT RPE CELLS AND MONONUCLEAR PHAGOCYTES

CELL TYPE*	α-NAPHTHYL PALMITATE HYDROLYZED (μM)/10^6 CELLS/HR
Alveolar macrophages (14)	4,312 ± 9,042
Splenic macrophages (24)	805 ± 2,785
Peritoneal macrophages (35)	322 ± 2,212
RPE cells (25)	81 ± 395
Blood monocytes (14)	25 ± 12
Smooth-muscle cells† (6)	10 ± 4.4

RPE, retinal pigment epithelium.
*Number of separate glass-adherent cell samples pooled from experiments.
†Third-passage rabbit aortic smooth-muscle cells.

histochemical and biochemical assays, a feature of acid lipases in other tissue preparations.[77]

ACID LIPASE PURIFIED FROM FRESHLY ISOLATED BOVINE RPE CELLS

To demonstrate the specificity of the chromogenic assay for acid lipase and to show that RPE acid lipase may actually esterify free fatty acids to cholesterol, acid lipase activity, corresponding to a single protein band, was purified from bovine RPE cells and shown to possess the ability to hydrolyze α-naphthyl palmitate and esterify cholesterol to fatty acids hydrolyzed from a triglyceride (ie, triolein). Freshly isolated RPE cells from bovine eyes were used, since adequate numbers of cells for biochemical studies could be obtained from this source.

Sonicated, isolated bovine RPE cells run on a Sephadex G-150 column exhibited similar profiles for α-naphthyl palmitate hydrolysis and cholesteryl ester formation (Figure 3). The activities were associated with an apparent M_r of 66,000 d with this sizing column. Pooled peak fractions were concentrated and subsequently run on an SP-Sephadex C-50 ion exchange column under conditions that would isolate acid lipase and minimize enzyme binding to the column. This was done to obviate the need for subsequent elution with high salt or pH solutions, which decreases enzyme-specific activity. Void volume fractions showed active α-naphthyl palmitate, cholesteryl ester, and triglyceride hydrolysis as well as cholesterol esterification in the same fractions (Figure 4). Polyacrylamide-SDS gel electrophoresis was performed on samples from the SP-Sephadex C-50 purification. Electrophoresis of void volume fractions following SP-Sephadex C-50 chromatography resulted in a single Coomassie blue–stained band with an apparent M_r of 65,000 d when compared to molecular weight markers. Silver staining demonstrated a similar profile with a single stainable band in gels overloaded intentionally to ascertain any other proteins that might be present (Figure 5). These findings indicated the single identity of the protein hydrolyzing α-naphthyl palmitate, cholesteryl esters, and triglycerides. Isolectric focusing of the same void volume fractions from the Sephadex C-50 column resulted in several bands stainable by Coomassie blue at isoelectic points between pH 5.5 and 6.0. Alpha-naphthyl palmitate hydrolytic and cholesterol esterification assays at acid pH were performed on 2-mm gel slices of simultaneously run gels not stained for protein. Both enzyme activities were found in the gel slices from areas that corresponded to the Coomassie blue–stained bands (Figure 6).

INDUCTION OF LYSOSOMAL ENZYME ACTIVITY IN CULTURED MONKEY RPE CELLS BY PHAGOCYTIC CHALLENGE AND LDL

To demonstrate that RPE cells may upregulate lysosomal enzyme activity in response to phagocytic stimuli, particularly to lipid-rich POS, which the RPE naturally ingests, RPE lysosomal activities were measured after phagocytic

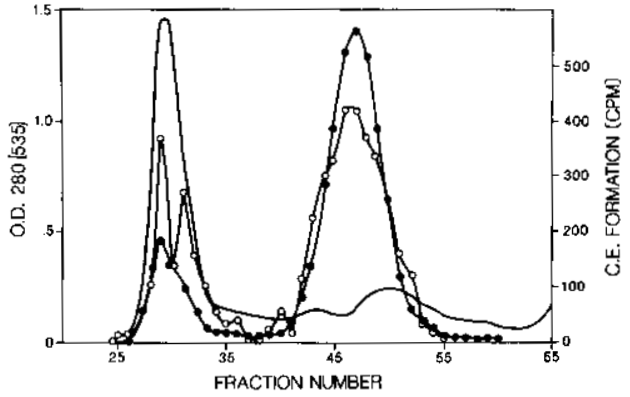

FIGURE 3
Elution profile of acid lipase activity obtained by molecular sieve gel chromatography of bovine retinal pigment epithelial (RPE) cell sonicate. Fractions collected from a Sephadex G-150 column were assayed continuously for protein (solid line) by measuring absorption at 280 nm. α-Naphthyl palmitate hydrolytic (OD, 535 nm •-----•) and cholesterol esterification (CPM o-----o) activities were measured in biochemical assays at acid pH. Both enzyme activities eluted in the same fractions corresponding to an apparent M_r of 66,000. Bovine RPE cell pellets were sonicated in distilled water at a ratio of 1:10 (wt/vol) and combined with 2 mL of 0.02 M Tris-HCl, pH 7.4, and applied to and run on the column at 4°C.

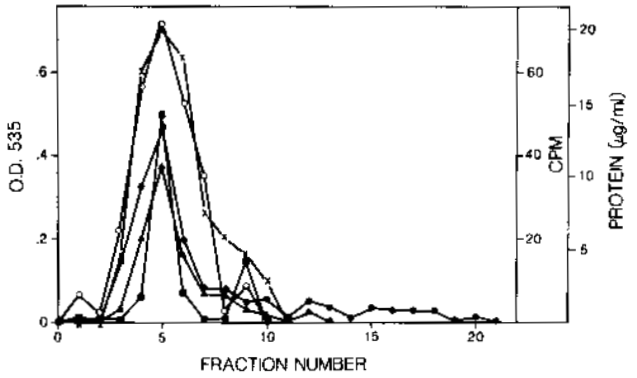

FIGURE 4

Elution profile of pooled, concentrated Sephadex G-150 fractions demonstrating peak bovine retinal pigment epithelial (RPE) acid lipase activity following application to an ion exchange column. Fractions (2 mL) eluted from SP Sephadex C-50 were assayed for protein (•-----•) and for the hydrolysis of α-naphthyl palmitate (OD, 535 nm x-----x), cholesteryl esters (CPM ■-----■), and triglycerides (CPM ▲-----▲) in addition to the formation of cholesteryl esters at acid pH (CPM o-----o). All three enzyme activities are found in the same fractions. Pooled Sephadex G-150 fractions that contained peak acid lipase activity were concentrated by ultrafiltration and applied to the SP Sephadex C-50, which was equilibrated and eluted at 4°C with 0.05 M sodium acetate buffer, pH 5.5, containing 0.025 M NaCl. The low salt concentrations necessary to avoid enzyme inactivation resulted in obligate void volume elution of the enzyme activities.

challenge with POS. Latex beads were used in control preparations to assess the inductive effect of nonspecific phagocytosis on RPE lysosomal activities. Assays for acid lipase, nonspecific acid esterase, and cholesteryl esterase were performed on sonicates of control cultured monkey RPE cells and compared with cultured monkey RPE cells overlaid for 24 hours with either latex beads or POS in serum-free media (Table II). Sonicates of RPE cells that had internalized latex microspheres exhibited statistically significant increases of acid lipase activity ($P<.05$) when compared with control cultured monkey RPE cells. Monkey RPE cells showed more than twofold increases in acid lipase, acid esterase, and cholesteryl esterase (all $P<.001$) after phagocytic challenge with POS compared with control RPE cells (Table II).

The media of the RPE cultures were also assayed for acid lipase, acid esterase, and cholesteryl esterase activity (Table III). Media aliquots from cultures after 24 hours phagocytic challenge with latex beads revealed statistically significant increases in media for all three lysosomal enzyme activities over control media. Interestingly, RPE cell monolayers exposed to POS released substantially less lysosomal activity, reaching statistical significance only for cholesteryl esterase ($P<.05$).

Histochemical studies of latex-fed and POS-fed monkey RPE cells showed readily visible staining for enzyme activity (Figure 7). The red-brown precipitates indicative

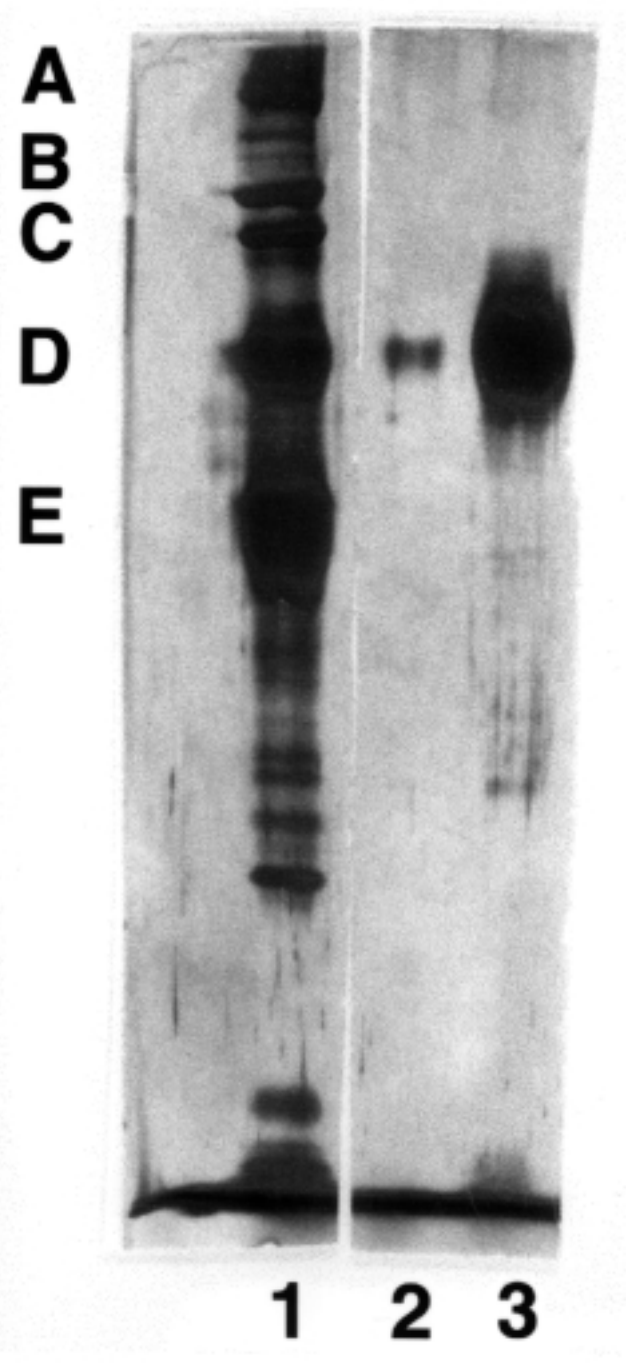

FIGURE 5

Polyacrylamide-SDS electrophoretic patterns of bovine retinal pigment epithelial (RPE) acid lipase following sequential molecular sieve gel and ion exchange chromatography. Silver-stained, polyacrylamide-SDS gel of the SP-Sephadex C-50 void volume fraction, which showed peak α-naphthyl palmitate hydrolysis in biochemical assays, demonstrates a single stainable protein band (lane 2). An overloaded gel run simultaneously (lane 3) demonstrates little contamination with other proteins. Bovine RPE acid lipase (lane 2) has an apparent M_r of 65,000 when compared with molecular weight markers (lane 1): A, myosin; B, ß-galactosidase; C, phosphorylase B; D, bovine serum albumin; E, ovalbumin.

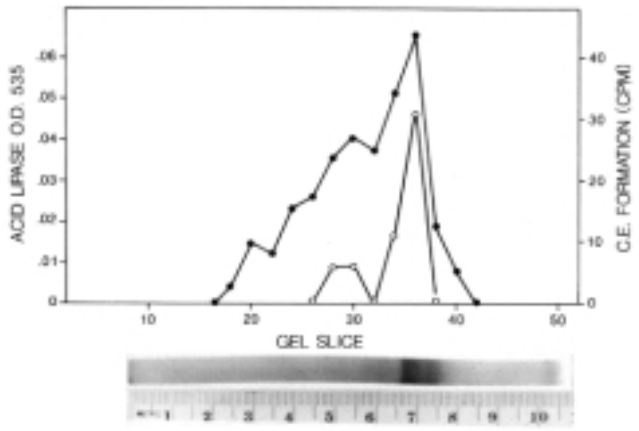

FIGURE 6

Isolectric focusing zymogram of pooled, eluted fractions demonstrating peak bovine retinal pigment epithelial (RPE) acid lipase activity following molecular sieve gel and ion exchange chromatography. α-Naphthyl palmitate hydrolytic (•-----•) and cholesterol esterification (o-----o) activities were assayed in successive 2-mm gel slices at pH 4.2. Peak enzyme activities corresponded to Coomassie blue–stainable protein bands of gels run in parallel. Isoelectric focusing (pH, 4.0-6.5) of the peak SP-Sephadex C-50 void volume fractions was performed in polyacrylamide gels for 8 to 18 hours at 5°C.

of nonspecific acid esterase activity were present in control cultures, but substantially increased in intensity in cells exposed to latex particles or POS. In contrast, control RPE cell layers showed almost no reaction product when stained for acid lipase activity, but distinct reaction product corresponding to lipolytic activity after latex-bead or POS challenge.

RPE cells phagocytize substantial amounts of lipid derived from POS and enhance lysosomal lipolytic activity in response to this physiologic stimulus (Table II, Figure 7). Since circulating LDL is a rich source of lipid for which RPE cells bear specific receptors,[128,129] monkey RPE acid lipase, acid esterase, and cholesteryl esterase activities were assessed after 24 hours challenge with native monkey LDL (Table IV). Exposure of cultured RPE cells to N-LDL significantly enhanced acid lipase ($P<.01$) and cholesteryl esterase ($P<.05$) activities. Lipoprotein-deficient serum, derived from the same samples from which the N-LDL was isolated, served as control for these assays, leading to the conclusion that LDL was responsible for enhanced RPE lysosomal activity observed. These findings paralleled those of RPE degradation assays in which ^{99}I-labeled N-LDL was actively degraded by the monkey RPE cells (results not shown).

RECEPTOR-MEDIATED RPE UPTAKE OF N-LDL, A-LDL, O-LDL BY MONKEY RPE CELLS IN VITRO AND IN VIVO

To demonstrate receptor-specific uptake of native and modified LDL by RPE cells, in vitro assays of fluorescently labeled lipoprotein uptake in the presence or absence of specific inhibitors was performed. In vivo 29-minute perfusions of labeled lipoproteins were then performed to demonstrate actual, rapid, and avid lipoprotein uptake in monkeys.

Specific receptor-mediated uptake of native and modified lipoproteins by monkey RPE cells was initially assessed in vitro. N-LDL labeled with the red, fluorescent dye, DiI, was avidly incorporated into approximately 90% of cultured monkey RPE cells that exhibited diffuse cytoplasmic positivity (Figure 8A). A tenfold excess of unlabeled LDL effectively inhibited RPE uptake of the labeled LDL (Figure 8B), while dextran sulfate (30 µg/mL) completely blocked uptake of the fluorescent, native lipoprotein. Neither A nor O-LDL blocked DiI-N-LDL uptake.

Eighty percent of monkey RPE cells exposed to DiI-A-LDL displayed intense, granular cytoplasmic fluorescence (Figure 8C), presumably due to lysosomal incorporation of the modified lipoprotein.[133,134] Excess unlabeled A-LDL (Figure 8D) and fucoidin, (150 µg/mL), a specific inhibitor of A-LDL binding and uptake,[211,215,216] were effec-

TABLE II: LYSOSOMAL ENZYME ACTIVITY INDUCED IN MONKEY RPE CELLS BY PHAGOCYTIC CHALLENGE

	LYSOSOMAL ENZYME ACTIVITY*		
LYSOSOMAL ENZYME†	CONTROL RPE CELLS‡	LATEX-FED RPE CELLS	POS-FED RPE CELLS
Acid lypase (8)	1.00 ± 0.12 (32)	1.59 ± 0.22§	2.53 ± 0.66 ‖
Acid esterase (8)	1.00 ± 0.30 (84)	1.30 ± 0.34	2.06 ± 0.37 ‖
Cholesteryl esterase (6)	1.00 ± 0.20 (217)	1.14 ± 0.22	2.60 ± 0.37 ‖

POS, photoreceptor outer segment; RPE, retinal pigment epithelium.
*Activities ±SD expressed relative to mean activities of control cell groups with mean activity of each control group expressed as unity ±SD.
†Number of separate cell samples pooled from experiments.
‡Mean activity of pooled control cell cultures expressed as µM substrate hydrolyzed per hour per µg DNA for acid lipase and acid esterase and as counts per minute per hour per µg DNA for cholesteryl esterase.
§$P<.05$, compared to control.
‖ $P<.001$, compared to control.

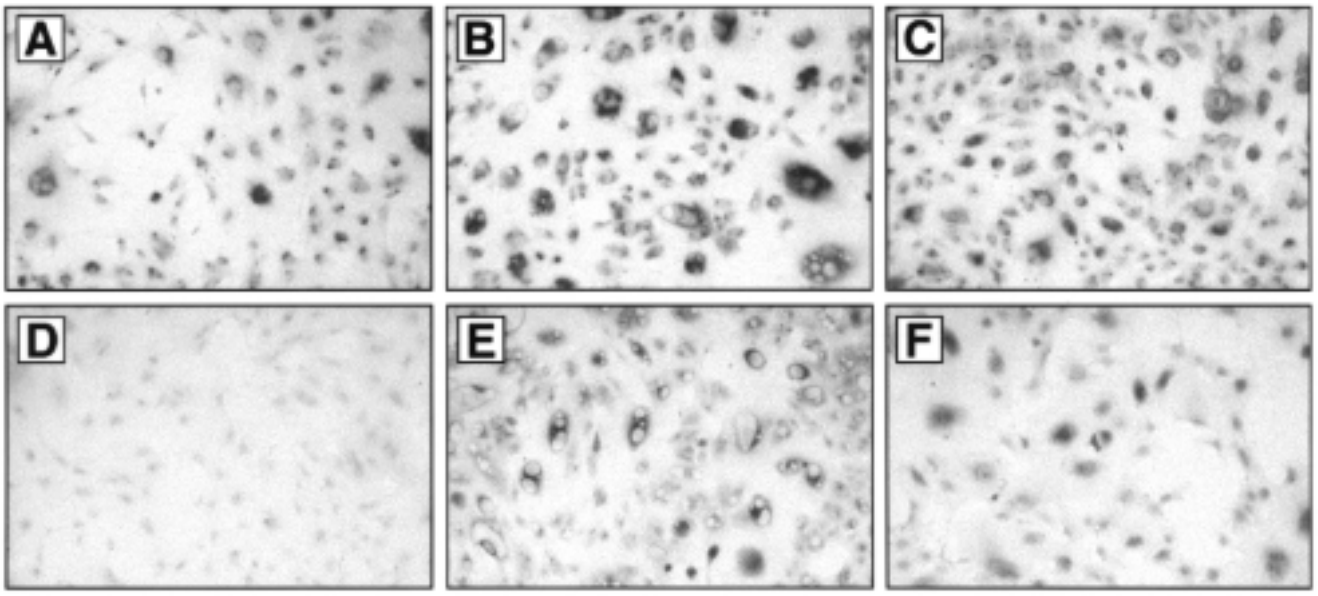

FIGURE 7

Histochemical demonstration of monkey retinal pigment epithelial (RPE) lysosomal enzyme induction following phagocytic challenge. A, Histochemical staining for nonspecific acid esterase using α-naphthyl acetate demonstrates red-brown reaction product in cultured RPE cells (methyl green counterstain, x200). B, RPE cell cultures overlaid with 0.93-µm latex microspheres (40 mg/mL) contain numerous internalized beads and increased enzyme reaction product (methyl green counterstain, x200). C, After overlay of photoreceptor outer segments (40 mg/mL), increased nonspecific esterase activity is also visualized compared with control cultures (methyl green counterstain, x200). D, Staining for acid lipase activity using α-naphthyl palmitate fails to reveal distinct reaction product in control RPE cultures (methyl green counterstain x200). E, Intense, red-brown acid lipase cytoplasmic reaction product is present and surrounds latex microspheres that have been phagocytized (methyl green counterstain, x200). F, RPE cells contain distinct, enhanced visible reaction product, corresponding to acid lipase activity, after overlay of photoreceptor outer segments (methyl green counterstain, x200).

TABLE III: LYSOSOMAL ENZYME ACTIVITY RELEASED BY MONKEY RPE CELLS DURING PHAGOCYTIC CHALLENGE

	LYSOSOMAL ENZYME ACTIVITY*		
LYSOSOMAL ENZYME†	CONTROL RPE CONDITIONED MEDIA‡	LATEX-FED RPE CONDITIONED MEDIA	POS-FED RPE CONDITIONED MEDIA
Acid lypase (6)	1.00 ± 0.29 (6.2)	2.75 ± 0.30§	1.08 ± 0.22 ‖
Acid esterase (6)	1.00 ± 0.09 (8.4)	2.30 ± 0.47	1.18 ± 0.27 ‖
Cholesteryl esterase (6)	1.00 ± 0.10 (53)	1.58 ± 0.34¶	1.34 ± 0.29 ‖

POS, photoreceptor outer segment; RPE, retinal pigment epithelium.
*Activities ±SD expressed relative to mean activities of control cell groups with mean activity of each control group expressed as unity ±SD.
†Number of separate cell samples pooled from experiments.
‡Mean activity of pooled control cell cultures expressed as µM substrate hydrolyzed per hour per µg DNA for acid lipase and acid esterase and as counts per minute per hour per µg DNA for cholesteryl esterase.
§$P<.001$, compared to control.
‖$P<.05$, compared to control.
¶$P<.01$, compared to control.

tive inhibitors of RPE DiI-A-LDL uptake. N-LDL did not block DiI-A-LDL uptake. O-LDL reduced, but did not completely block, the RPE uptake of fluorescent A-LDL.

Seventy percent of monkey RPE cells incubated with DiI-O-LDL exhibited delicate, red granular cytoplasmic fluorescence (Figure 8E)[131,132,148] that was also likely due to lysosomal incorporation. DiI-O-LDL uptake was effectively inhibited by a tenfold excess of unlabeled O-LDL (Figure 8F). Fucoidin also inhibited RPE uptake of the fluorescently labeled oxidized LDL, but it was less effective than excess unlabeled O-LDL and also less effective than when it was used to block RPE DiI-A-LDL uptake (Figure 8D). A-LDL, but not N-LDL, partially blocked DiI-O-LDL RPE uptake, presumably by blocking some, but not all, of the scavenger receptors mediating O-LDL uptake.[131,132,140,142]

In control incubations, about 95% of monkey fibro-

TABLE IV: EFFECTS OF LOW-DENSITY LIPOPROTEINS (LDL) ON LYSOSOMAL ENZYME ACTIVITY IN MONKEY RPE CELLS

	LYSOSOMAL ENZYME ACTIVITY*	
LYSOSOMAL ENZYME†	LIPOPROTEIN-DEFICIENT SERUM‡	NATIVE LDL
Acid lypase (6)	1.00 ± 0.14 (14)	1.28 ± 0.16§
Acid esterase (6)	1.00 ± 0.09 (20)	1.17 ± 0.23
Cholesteryl esterase (6)	1.00 ± 0.27 (170)	1.38 ± 0.31‖

RPE, retinal pigment epithelium.
*Activities ±SD expressed relative to mean activities of control groups with mean activity of each control group expressed as unity ±SD.
†Number of separate cell samples pooled from experiments.
‡Mean activity of pooled control cell cultures expressed as µM substrate hydrolyzed per hour per µg DNA for acid lipase and acid esterase and as counts per minute per hour per µg DNA for cholesteryl esterase.
§$P<.01$, compared to LDS.
‖$P<.05$, compared to lipoprotein-deficient serum.

blasts, which are known to possess N-LDL receptors, became fluorescent when incubated with DiI-N-LDL, but less than 5% of fibroblasts incorporated A- or O-LDL. As expected, fibroblast uptake of DiI-N-LDL was inhibited by dextran sulfate or excess unlabeled LDL. Known to express both A-LDL and O-LDL receptors, 85% and 80% of monkey alveolar macrophages became brightly fluorescent upon exposure to DiI-A-LDL and DiI-O-LDL, respectively. Excess unlabeled A- and O-LDL inhibited macrophage uptake of their respective labeled, modified LDL while fucoidin was much more effective at inhibiting DiI-A-LDL uptake than DiI-O-LDL uptake. Ten percent to 40% of alveolar macrophages incubated with DiI-labeled, native LDL incorporated the lipoprotein but were less fluorescent than those exposed to either DiI-labeled, A- or O-LDL.

In vivo assessment of native and modified LDL uptake by retina, liver, spleen, and lung was performed after carotid perfusion of monkeys with DiI-labeled lipoproteins. Frozen sections of formalin-fixed monkey eyes after DiI-N-LDL perfusion revealed specific and intense diffuse red cytoplasmic fluorescence within the entire RPE monolayer (Figure 9A). Much less uptake was present in the choroid and inner neural retina. Posterior segments of eyes from monkeys perfused with A-DiI-LDL exhibited intense, red granular fluorescence along the entire RPE monolayer. The only other red fluorescence seen was delicate labeling of vascular endothelial cells within choroidal blood vessels (Figure 9C). Selective, delicate, red granular fluorescence was also present throughout the RPE monolayers of eyes from monkeys perfused with DiI-labeled O-LDL (Figure 9E) but was less intense than that observed with DiI-A-LDL. As in the case of A-LDL, no other staining was visible in the posterior ocular segment. The granular fluorescence mimicked that observed in the cultured RPE cells and presumably was due to lysosomal incorporation of the modified lipoproteins.[131-134,148] In all of the monkey eyes, background, endogenous, yellow autofluorescence could be readily distinguished from the distinctive, red fluorescence of the DiI label.

To compare the patterns of DiI-labeled N-, A-, and O-LDL uptake by monkey RPE cells to those of other resident tissue phagocytes in vivo, the livers, spleens, and lungs of the same animals were harvested, fixed, frozen sectioned, and examined by fluorescence microscopy. Tissue sections of liver after DiI-N-LDL perfusion demonstrated diffuse and discrete red granular fluorescence in hepatocytes without evidence of labeled lipoprotein uptake in resident mononuclear phagocytes (Kupffer cells) or sinusoidal endothelial cells (Figure 10A). In contrast, perfusion of DiI-A-LDL resulted in selective, avid incorporation of the modified lipoprotein by Kupffer cells, which became very brightly fluorescent (Figure 10B). Lesser, but distinctive, red granular fluorescent staining of endothelial cells within the sinusoids corresponded to A-LDL uptake by these cells also. DiI-O-LDL perfusions resulted in hepatic uptake that was similar in distribution and intensity to that of DiI-A-LDL (Figure 10C) and reiterated the fluorescent pattern of uptake reported by Esbach and associates.[217]

Frozen sections of monkey spleens after in vivo perfusion of DiI-N-LDL demonstrated only weak paratrabecular incorporation as well as faint staining of a few stellate and fusiform cells, chiefly in interfollicular regions (Figure 10D). The splenic capsule, however, stained brightly, presumably because of the presence of fibroblasts (not shown). Fluorescence microscopy of spleens perfused with DiI-A-LDL was markedly different. The interfollicular regions contained numerous brightly fluorescent mononuclear phagocytes as well as delicate, but uniform, bright red fluorescence of vascular endothelium (Figure 10E). Mononuclear phagocytes distributed in the lymphoid follicles also displayed bright fluorescence.

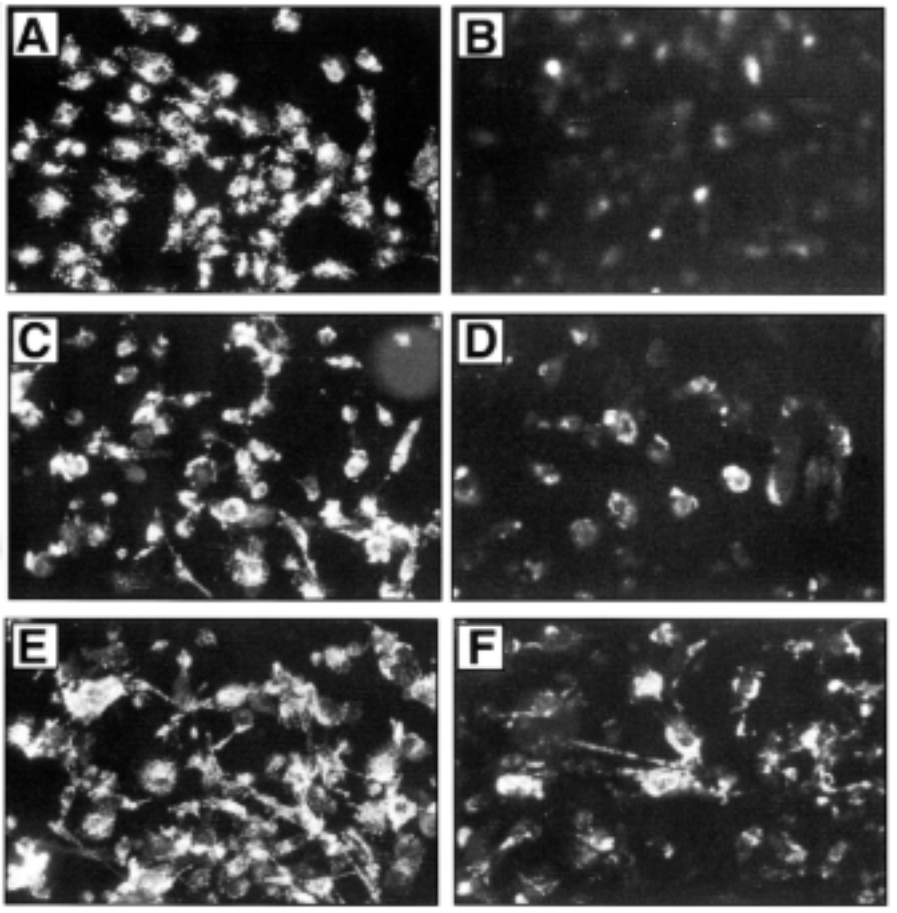

FIGURE 8

Detection of receptors for native (N)-, acetylated (A)-, and oxidized (O)-LDL on cultured monkey RPE cells. A, Photomicrograph of fluorescent, cultured RPE cells exposed to DiI-labeled N-LDL. Intense cellular fluorescence following exposure to N-LDL (15 µg/mL) indicates avid incorporation of the lipoprotein. Cytoplasmic fluorescence contrasts with nuclei, which are hypofluorescent. Uptake of the fluorescent lipoprotein is also observed in the stromal cells of the choroid (x200). B, RPE cells exposed to fluorescent N-LDL in the presence of excess unlabeled LDL (150 µg/mL). RPE cells are significantly less fluorescent owing to competitive inhibition of uptake by unlabeled lipoprotein. Dextran sulfate (30 µg/mL) completely inhibited RPE N-LDL uptake (x200). C, Monkey RPE cells after incubation with DiI-labeled A-LDL (15 µg/mL) display intense, red, granular fluorescence due to intracellular accumulation of modified lipoprotein. Contrasting cell nuclei are hypofluorescent (x200). D, Cultured RPE cells exposed to fluorescent A-LDL in the presence of excess unlabeled A-LDL (150 µg/mL) show substantially less fluorescence, indicating marked competitive inhibition of labeled, modified lipoprotein uptake by RPE cells. Fucoidin (100 µg/mL), a competitive inhibitor of A-LDL receptor binding, abolished DiI–A-LDL binding and uptake (x200). E, Moderately overexposed photomicrograph of RPE cells exposed to DiI-labeled, O-LDL. Granular fluorescence due to uptake of red, fluorescent oxidized lipoprotein within cytoplasm contrasts with hypofluorescent nuclei (x200). F, Competitive inhibition of DiI-labeled O-LDL uptake by excess unlabeled O-LDL (150 µg/mL) renders RPE cells much less fluorescent. Fucoidin also inhibited DiI-labeled O-LDL uptake, but to a lesser extent than excess unlabeled O-LDL (x200).

Perfusion with DiI-labeled, O-LDL also demonstrated fluorescence, which was limited to resident tissue macrophages and vascular endothelial cells (Figure 10F) but was less intense than that found with DiI-A-LDL.

In the lung, uptake of DiI-N-LDL was seen as intense, red fluorescence of bronchial and vascular smooth muscle as well as faint positivity in alveolar septae (Figure 10G). There was no evident uptake by alveolar macrophages or vascular endothelium. In contrast, perfusions with DiI-A-LDL and DiI-O-LDL resulted in bright fluorescence of some of the alveolar macrophages (Figure 10H,10I), but overall fluorescence was less intense than that of the liver or spleen.

New Zealand White rabbit perfusions with DiI-labeled native and modified LDL exhibited uptake patterns in retina, liver, spleen, and lung that were similar, if not identical, to those observed in the monkeys, confirming previous in vivo results.[128] In vitro studies on rabbit cells yielded entirely similar results to those described for monkey cells.[128]

FISH OIL MODULATION OF MONKEY RPE LYSOSOMAL ENZYMES

Since diets containing fish oil have been associated with reduced risk of ARMD and severity of atherosclerosis, presumably because of their effects on circulating lipopro-

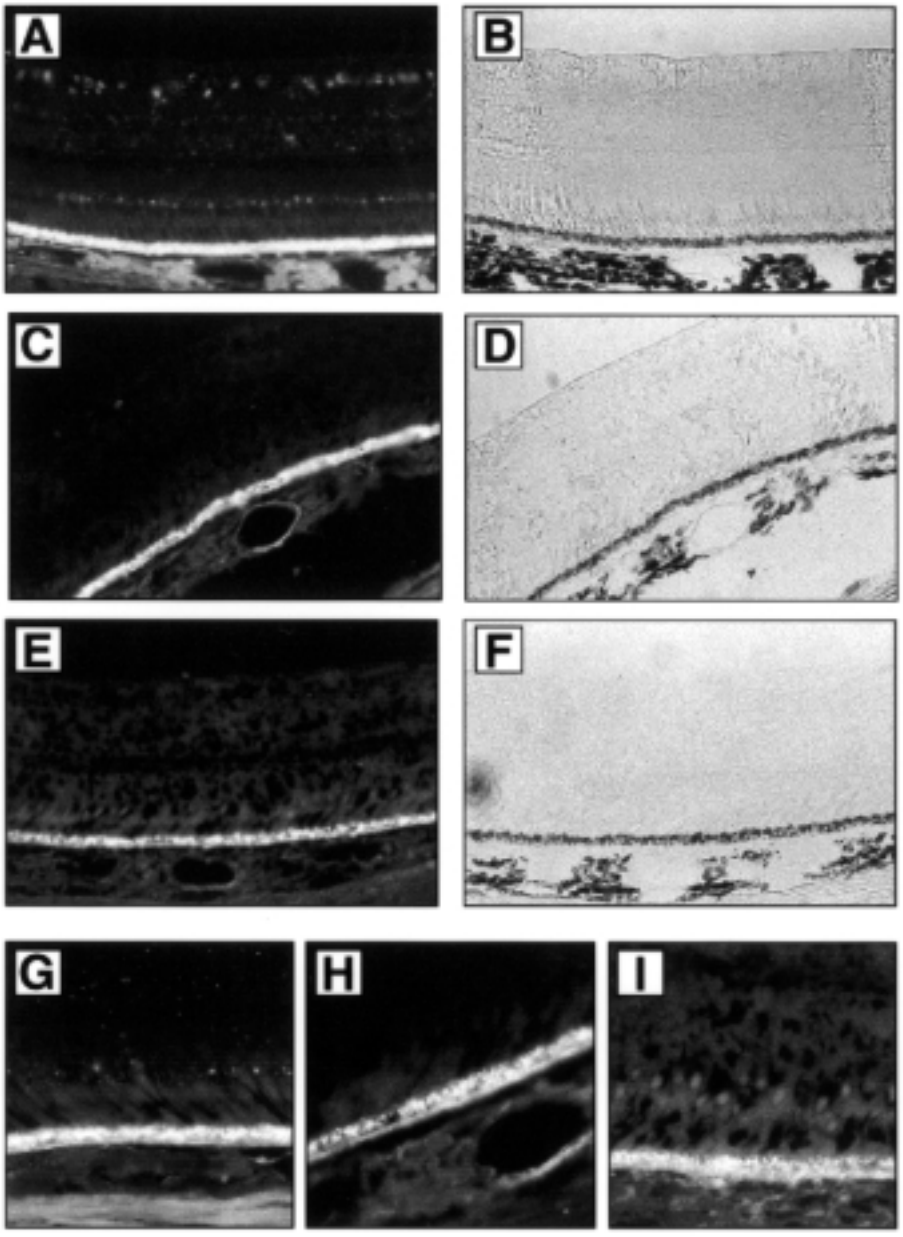

FIGURE 9

Demonstration of native (N)-, acetylated (A)-, and oxidized (O)-LDL uptake by monkey RPE in vivo. A, Histologic frozen section of formalin-fixed monkey eye after in vivo carotid arterial perfusion of DiI-labeled N-LDL (1 µg/mL). Intense, diffuse red fluorescence of retinal pigment epithelial (RPE) monolayer indicates avid N-LDL uptake. Less N-LDL uptake is seen within choroid and inner retinal layers (x200). B, Phase contrast photomicrograph of same field seen in A (x200). C, Frozen section of fixed monkey eye after in vivo perfusion with DiI-labeled A-LDL (1 µg/mL). RPE monolayer is selectively stained with intense granular fluorescence. RPE nuclei are hypofluorescent. Delicate staining of cells lining a choroidal vessel indicates endothelial incorporation of DiI-labeled A-LDL. Neural retina sclera, and other portions of choroid are unstained (x200). D, Phase contrast photomicrograph of same field seen in C (x200). E, Monkey eye following in vivo perfusion with DiI-labeled O-LDL (1 µg/mL). RPE monolayer exhibits selective, delicate, granular fluorescence due to uptake of the modified lipoprotein. RPE nuclei are hypofluorescent. Neural retina, sclera, and choroid are unstained (x200). F, Phase contrast photomicrograph of same field seen in E (x200). G, DiI–N-LDL uptake by RPE seen at higher power. Intense fluorescence is seen throughout cytoplasm. Uptake is also seen in sclera (x400). H, DiI–A-LDL uptake. Intense granular fluorescence, with overall less intensity than that seen due to DiI–N-LDL uptake, is present in RPE cells (x400). I, Moderately overexposed photomicrograph demonstrating DiI–O-LDL uptake as granular fluorescence within RPE cells. Uptake is also seen in vessels within choroid to a greater extent than that observed with DiI–A-LDL (x400).

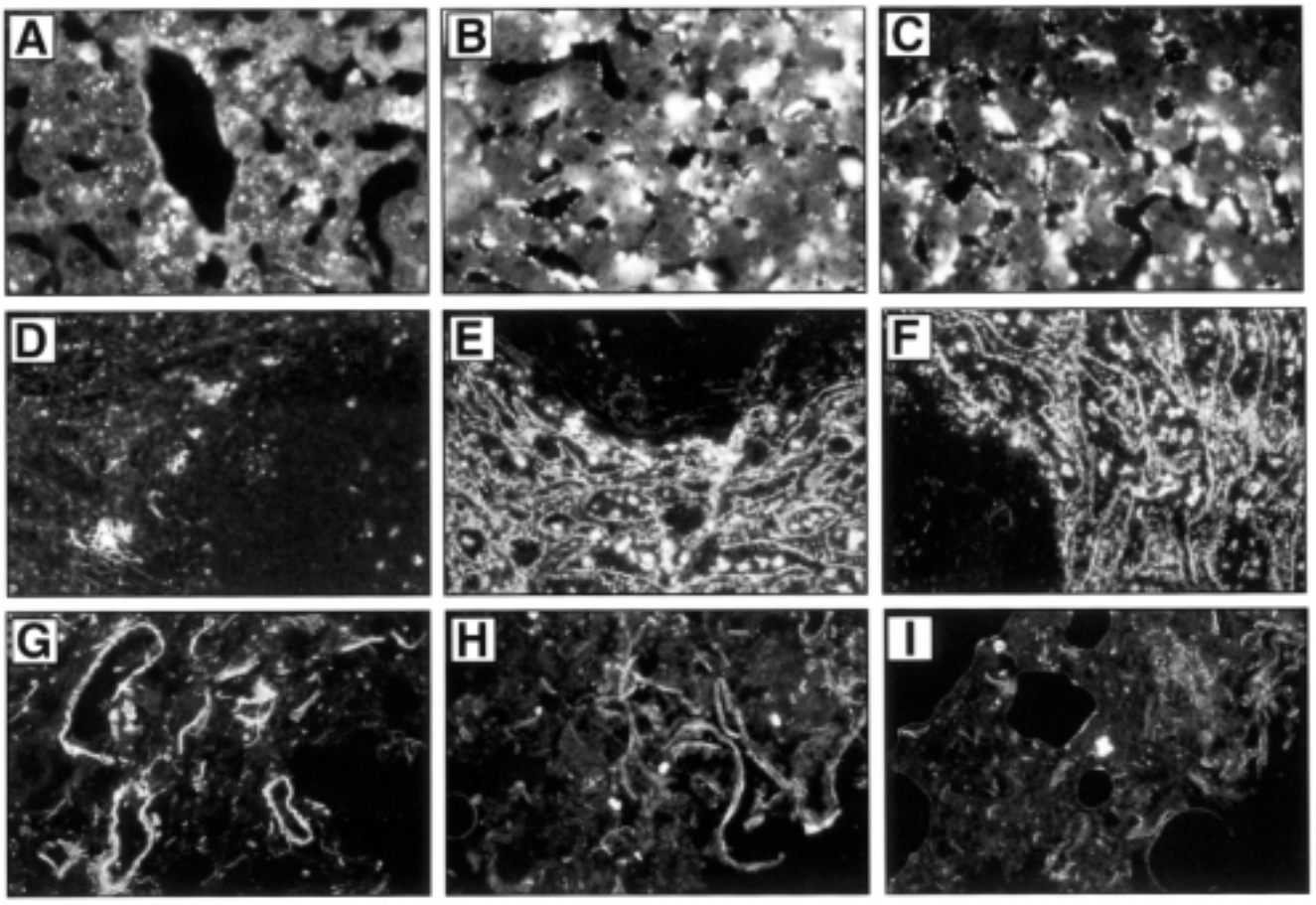

FIGURE 10

Demonstration of native (N)-, acetylated (A)-, and oxidized (O)-LDL uptake by monkey liver, spleen, and lung in vivo. A, Histologic frozen section of fixed monkey liver following in vivo perfusion of DiI-labeled N-LDL. Discrete, granular fluorescence is present in hepatocytes and surrounding central vein, indicating uptake of N-LDL. Kupffer cells and endothelium of sinusoids are not stained (x200). B, Monkey liver following in vivo perfusion of DiI-labeled A-LDL. Kupffer cells are brightly fluorescent owing to active uptake of modified lipoprotein. Sinusoidal and vascular endothelium is also distinctly fluorescent, indicating lipoprotein uptake by this cell type (x200). C, DiI–O-LDL incorporation in monkey liver after in vivo perfusion displays pattern of distribution similar to A-LDL (x200). D, Frozen section of monkey spleen following in vivo perfusion with fluorescently labeled N-LDL. Weak paratrabecular incorporation of DiI-labeled N-LDL is seen. There is faint staining of a few stellate and fusiform cells. Intense staining of splenic capsule, however, was present (not shown) (x200). E, Monkey spleen following in vivo perfusion with DiI–A-LDL. Numerous mononuclear phagocytes in interfollicular region are brightly fluorescent, as are a few mononuclear phagocytes located within lymphoid follicles. Vascular endothelium also demonstrates delicate, bright fluorescence (x200). F, DiI–O-LDL incorporation in perfused monkey spleen displays pattern of distribution similar to A-LDL (x200). G, Monkey lung after in vivo perfusion with DiI-labeled N-LDL. Intense staining of bronchial and vascular smooth muscle due to uptake of native lipoprotein is present. Weak incorporation of DiI-labeled N-LDL is seen in alveolar septae (x200). H, Fixed, frozen section of lung following DiI–A-LDL perfusion. Selective, intense fluorescence of alveolar macrophages due to uptake of labeled, modified lipoprotein is evident (x200). I, DiI–O-LDL incorporation in perfused monkey lung displays selective distribution in alveolar macrophages similar to, but less dramatic than, DiI–A-LDL (x200).

teins for which RPE cells bear receptors mediating their uptake in vivo, the effects of fish oil–rich diets on RPE lysosomal lipolytic activity were investigated. Monkeys were fed high-cholesterol diets supplemented with coconut oil (saturated fatty acids) alone or similar diets containing Menhaden fish oil (highly polyunsaturated omega-3 fatty acids) and coconut oil in 1:1 or 3:1 ratios for 12 months. Freshly isolated RPE cells from eyes removed at autopsy were assayed for lysosomal enzyme activity (Table V). Statistically significant elevations of acid lipase ($P<.05$), acid esterase ($P<.01$), and cholesteryl esterase ($P<.01$) activity were observed when comparing the activity in RPE cells from monkeys fed the 3:1 fish oil and coconut oil diet to those from control monkeys fed coconut oil alone. RPE cells from monkeys whose diets contained equal amounts of fish oil and coconut oil (1:1 diet) also contained increased activity of all three enzymes, but these increases did not reach statistical significance.

Fixed, frozen sections of portions of a posterior ocular segment from each monkey were assayed histochemically for nonspecific acid esterase and acid lipase activity (Figure 11). Red-brown reaction product, identical to

TABLE V: DIETARY FISH OIL MODULATION OF MONKEY RPE LYSOSOMAL ENZYMES

	LYSOSOMAL ENZYME ACTIVITY*		
DIET	**ACID ESTERASE**	**ACID LIPASE**	**CHOLESTERYL ESTERASE**
25% coconut oil/ 2% cholesterol	167 ± 61	145 ± 39	1,105 ± 150
12.5% coconut oil/12.5% fish oil/ 2% cholesterol	212 ± 65	183 ± 42	1,306 ± 327
6.25% coconut oil/ 18.75% fish oil/ 2% cholesterol	261 ± 56†	217 ± 68‡	1,778 ± 619†

RPE, retinal pigment epithelium.
*Mean activities ±SD of RPE cell isolates from eyes of 8 monkeys in each group expressed as µM substrate hydrolyzed per hour per µg DNA for acid lipase and acid esterase and as counts per minute per hour per µg DNA for cholesteryl esterase.
†P<.01, compared to 25% coconut oil/2% cholesterol diet.
‡P<.05, compared to 25% coconut oil/2% cholesterol diet.

TABLE VI: FISH OIL MODULATION OF HISTOCHEMICAL STAINING FOR MONKEY RPE LYSOSOMAL ENZYMES

	INTENSITY OF HISTOCHEMICAL ACTIVITY*	
DIET	**ACID ESTERASE**	**ACID LIPASE**
25% coconut oil/ 2% cholesterol	2.0 ± 1.1	1.4 ± 0.7
6.25% coconut oil/18.75% fish oil/ 2% cholesterol	3.4 ± 4†	3.2 ± 0.7‡

RPE, retinal pigment epithelium.
*Mean grading of staining intensity ±SD of eyes from 8 monkeys in each group on a 0 to 4 scale by 2 masked observers.
†P<.01, compared to 25% coconut oil/2% cholesterol diet.
‡P<.001, compared to 25% coconut oil/2% cholesterol diet.

that observed in the albino rabbit studies (Figure 11C, 11F), could be readily discerned from endogenous RPE pigment. Staining, corresponding to enzyme activity, was greater for acid esterase (Figure 11A) and acid lipase (Figure 11D) in animals fed the 3:1 fish oil and coconut oil diet than in those fed coconut oil alone as shown in Figures 11B and 11E, respectively. The distinctive red-brown reaction product of the enzymatic histochemical assays was distinct from the red fluorescence due to DiI-labeled N-, A-, and O-LDL incorporation in RPE monolayers (Figure 11G, 11H, 11I) and in RPE cells exposed to the fluorescent lipoproteins (Figure 11D, 11K, 11L). Quantitation of the degree of histochemical staining on a scale of 0 (no stain) to 4 (intense stain) by two masked observers confirmed that the increased RPE histochemical staining was statistically significant for nonspecific acid esterase (P<.01) and acid lipase (P<.001) (Table VI).

Fish oil modulation of liver and spleen acid lipase (P<.001) and cholesteryl esterase activity generally corroborated the RPE results (Table VII). Acid lipase and cholesteryl esterase were significantly elevated in the livers of monkeys fed a 3:1 diet of fish oil and coconut oil when compared with those fed coconut oil alone. The same was true for splenic acid lipase (P<.01) and cholesteryl esterase (P<.05). Acid lipase enzyme activity was also significantly elevated in livers of monkeys fed equal amounts of coconut and fish oils (P<.05). These data indicate a systemically beneficial effect in enhancing acid lipolytic efficiency.

To determine whether circulating lipoproteins from the monkeys fed diets supplemented with fish oil directly enhance RPE lysosomal enzyme activity, cultured monkey RPE cells were incubated for 24 hours with 10% sera removed at autopsy prior to RPE biochemical enzyme assays (Table VIII). Statistically significant increases in acid lipase (P<.01), acid esterase (P<.05), and cholesteryl esterase (P<.01) activity were present in RPE cells exposed to serum from monkeys fed a 3:1 diet of fish oil

TABLE VII: FISH OIL MODULATION OF MONKEY LIVER AND SPLEEN LYSOSOMAL ENZYMES*

DIET/ORGAN	ACID LIPASE	CHOLESTERYL ESTERASE
Liver		
25% coconut oil/ 2% cholesterol	61 ± 2	1,474 ± 76
12.5% coconut oil 12.5% fish oil/ 2% cholesterol	106 ± 11†	1,380 ± 125
6.25% coconut oil/ 18.75% fish oil/ 2% cholesterol	223 ± 24‡	2,883 ± 494§
Spleen		
25% coconut oil/ 2% cholesterol	101 ± 10	5,053 ± 309
12.5% coconut oil 12.5% fish oil/ 2% cholesterol	120 ± 12†	4,572 ± 236
6.25% coconut oil/ 18.75% fish oil/ 2% cholesterol	194 ± 35§	5,935 ± 395†

*Mean activities ±SD of homogenates from eyes of 8 monkeys in each group expressed as µM substrate hydrolyzed per hour per µg DNA for acid lipase and acid esterase and as counts per minute per hour per µg DNA for cholesteryl esterase.
†$P<.05$, compared to 25% coconut oil/2% cholesterol diet.
‡$P<.001$, compared to 25% coconut oil/2% cholesterol diet.
§$P<.01$, compared to 25% coconut oil/2% cholesterol diet.

TABLE VIII: EFFECTS OF FISH OIL-FED MONKEY SERUM ON LYSOSOMAL ENZYME ACTIVITY IN CULTURED MONKEY RPE CELLS

	LYSOSOMAL ENZYME ACTIVITY OF RPE CELLS OVERLAID WITH SERUM*			
LYSOSOMAL ENZYME†	LIPOPROTEIN-DEFICIENT SERUM‡	25% COCONUT OIL/ 2% CHOLESTEROL	12.5% COCONUT OIL/ 12.5% FISH OIL/ 2% CHOLESTEROL	6.25% COCONUT OIL/ 18.75% FISH OIL/ 2% CHOLESTEROL
Acid lipase (8)	1.00 ± 0.29 (25)	1.21 ± 0.24	1.36 ± 0.22	1.61 ± 0.26§
Acid esterase (8)	1.00 ± 0.20 (32)	1.15 ± 0.14	1.31 ± 0.22	1.43 ± 0.32 ‖
Cholesteryl esterase (8)	1.00 ± 0.13 (161)	1.22 ± 0.25	1.54 ± 0.50	1.81 ± 0.45§

*Cells overlaid with media containing 10% vol/vol of each type of monkey serum; activities ±SD expressed relative to mean activities of control groups with the mean activity of each control group expreessed as unity ±SD.
†Number of separate cell samples pooled from experiments.
‡Mean activity of pooled control cell cultures expressed as µM substrate hydrolyzed per hour per µg DNA for acid lipase and acid esterase and as counts per minute per hour per µg DNA for cholesteryl esterase.
§$P<.01$, compared to serum from 25% coconut oil/2% cholesterol-fed animal.
‖$P<.05$, compared to serum from 25% coconut oil/2% cholesterol-fed animal.

and coconut oil when compared with the enzyme activity measured in RPE cells exposed to sera from coconut oil–fed monkeys. Elevations in RPE enzyme activity after exposure to sera from monkeys fed 1:1 fish oil and coconut oil were measurably, but not statistically significant, elevated over the levels of enzyme activity in RPE cells exposed to sera from monkeys fed coconut oil alone.

Of note, RPE cells exposed to sera from animals fed only coconut oil did not induce statistically significant increases in RPE lysosomal enzyme activity over that measured in RPE cells exposed to LDS from chow-fed monkeys (Table VIII). This finding contrasts with the significant elevations observed for native LDL from chow-fed animals (Table IV) and may indicate that even diets

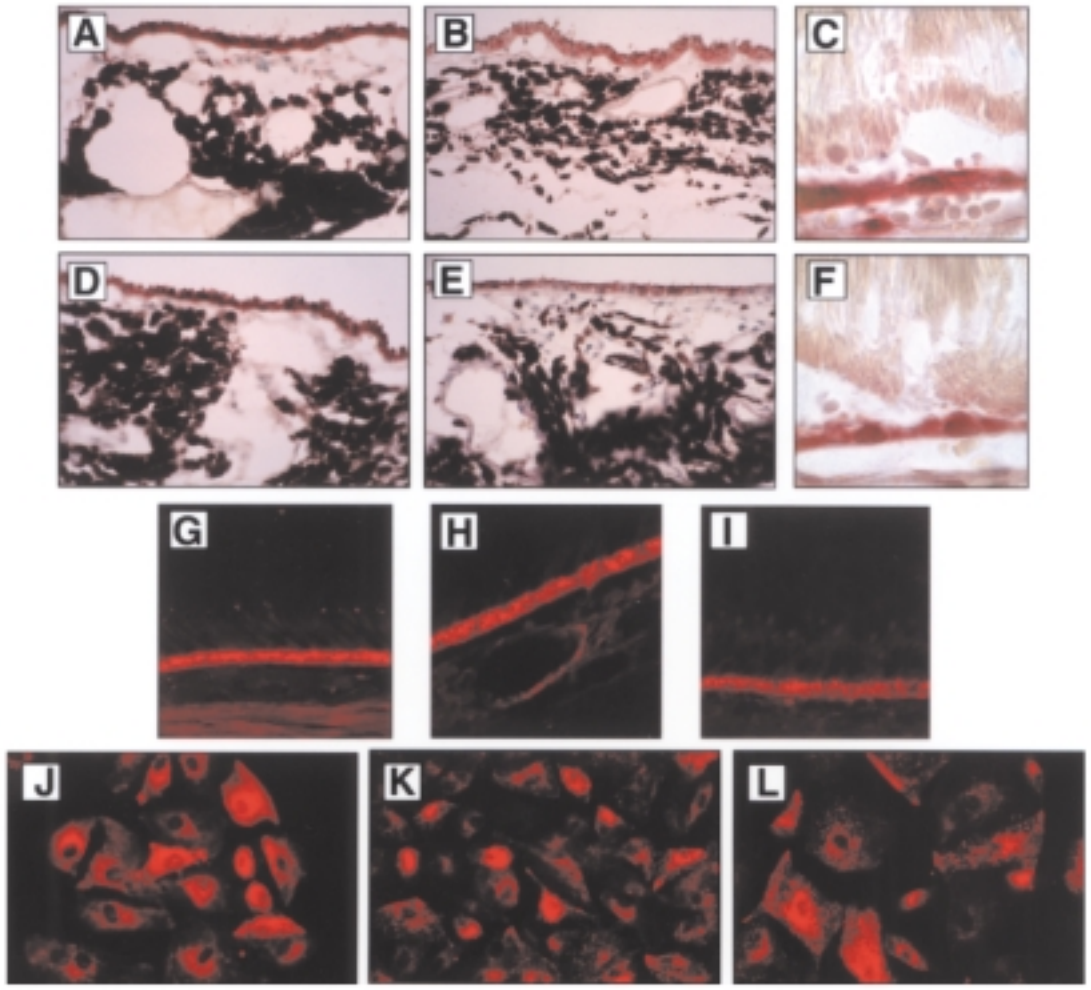

FIGURE 11

Histochemical detection of acid lipase and nonspecific acid esterase activity in fixed, frozen sections of eyes from monkeys fed coconut oil–rich and fish oil–rich diets. Retinal pigment epithelial (RPE) monolayers in monkeys fed fish oil show more intense red-brown staining for acid lipase (A) and acid esterase (B) than RPE monolayers of coconut oil–fed monkeys stained simultaneously for acid lipase (C) and acid esterase (D) activity (all methyl green counterstain, x100). Reference histochemical staining acid esterase (E) and acid lipase (F) in New Zealand White rabbit albino eye lacking autogenous brown pigment (both methyl green counterstain, x400). Comparative color of red, fluorescent DiI-labeled N-LDL (G), A-LDL (H), and O-LDL (I) uptake by RPE monolayer after in vivo perfusion of monkeys (all x400) and if DiI-labeled N-LDL (J), A-LDL (K), and O-LDL (L) uptake by RPE cells in vitro (all x400).

not supplemented with fish oil may affect RPE lysosomal function.

DISCUSSION

STUDIES ON ACID LIPASE IN RPE CELLS

The prominent phagocytic function of the RPE correlates with the high activities of hydrolytic lysosomal enzymes found in RPE cells.[49-51] Studies on RPE lipid metabolism have involved assessments of RPE lipid profiles, particularly of retinyl–fatty acid esters and phospholipids containing various saturated and unsaturated fatty acids, including high levels of DHA and arachindonic acid.[21,148-150,218-221] RPE lipolytic enzymes, especially acid lipase, may be of particular importance in RPE metabolism because of the massive amount of lipid-rich, POS material that the RPE phagocytizes and degrades daily. However, a simple reproducible biochemical assay and a histochemical technique for the in situ or in vitro detection of acid lipase activity have not been applied to the study of RPE lipid metabolism.

In this study, as reported previously,[76,77] consistent and specific staining for acid lipase was obtained in tissues when dispersion of α-naphthyl palmitate with the nonionic detergent Triton X100 was used (Figures 1, 2, and 11). Histochemically, acid lipase activity was readily detected in cells active in the uptake and processing of neutral lipids, namely, the mononuclear phagocytes of the reticuloendothelial system, as well as in RPE cells in the eye. With use of the same artificial substrate, enzyme

activity had not been detectable in a case of human acid lipase deficiency (Wolman's disease).[77] One important advantage of utilizing naphthyl palmitate-Triton X 100 substrate micelles for the detection of acid lipase activity is the associated histochemical staining technique that may be employed for in situ or in vitro studies. Although albino New Zealand White rabbits initially were used in this study to avoid obscuration of the red-brown histochemical reaction product by endogenous RPE melanin (Figures 1 and 2), discrete and selective in situ staining for monkey RPE acid lipase activity was also obtained (Figure 11). The ability to stain for acid lipase activity may be particularly valuable in the study of human retinal lesions, since (1) eyes with retinal diseases are available only in small numbers, (2) RPE cell harvesting necessitates disruption of the specimen, and (3) RPE cells are few in number and difficult to harvest for cell culture studies, particularly when diseased.

Studies were then performed to prove that the chromogenic biochemical assay and histochemical technique were specific for acid lipase and show the lipid metabolic functions subserved by this enzyme. Acid lipase derived from isolated, sonicated bovine RPE cells hydrolyzed triglycerides and cholesteryl esters as well as the artificial substrate α-naphthyl palmitate at acid pH (Figure 4). Biochemical evidence for the exclusive hydrolysis of this artificial fatty acid ester substrate by acid lipase at low pH included sequential purification of bovine RPE acid lipase activity by gel and ion exchange chromatography, followed by electrophoresis, resulting in a single silver-stained band with acid lipolytic activity. Acid lipase activity, detected by α-napthyl palmitate hydrolysis, was competitively inhibited by a natural triglyceride substrate of acid lipase, triolein, as well as by the organophosphate, DEPP, and the organomercurial, PHMB, consistent with previous observations.[77] Bovine RPE acid lipase activity detected by hydrolysis of triolein in triolein-lecithin substrate vesicles could not be separated from that detected by the hydrolysis of α-naphthyl palmitate in α-naphthyl palmitate-Triton X100 micelles at any stage in the purification process (Figures 3 through 6). The very similar, if not identical, migratory behavior of both of these hydrolytic activities purified by sequential protein purification techniques suggests that both the natural substrate, triolein, and the artificial substrate, α-naphthyl palmitate, are hydrolyzed by the same enzyme. The natural substrate, triolein, was a potent competitive inhibitor of α-naphthyl palmitate hydrolysis by bovine RPE cell extracts and monkey RPE cell sonicates. This effect is not due to the mere physical presence of triolein in the α-naphthyl palmitate-Triton X100 micelles, since introduction of equivalent amounts of trioleylglycerylether did not produce a detectable inhibitory effect.[77] Bovine RPE acid lipase had an apparent M_r of 64,000 to 66,000 d, as estimated by polyacrylamide-SDS electrophoresis (Figure 5), comparing favorably with that of rat liver acid lipase reported by Kaplan and Teng.[222]

Bovine RPE lysosomal acid lipase was first demonstrated by Rothman and associates[67] and Hayasaka and associates[68,69] employing substrates that were poorly soluble or required organic extraction and fractionation. Hayasaka and associates[68,69] purified acid lipase from crude RPE extracts. As in this study, they found acid lipase activity to be very labile, with 50% loss of activity during 12-hour dialysis at 0°C or during 48-hour storage at -20°C while purifying the enzyme using ammonium sulfate fractionation, void volume separation with Sepharose 6B gel chromatography, and diethyl-aminoethyl Sephadex ion exchange chromatography (Figure 4).

Rothman and associates[67] used 4-methylumbelliferyl palmitate and Hayasaka and associates[68,69] used [^{3}H]-labeled triglycerides as substrates for acid lipase activity. The latter investigators found bovine RPE acid lipase to enrich in the lysosomal fractions, to have a pH optimum of 4.0 to 5.0, to be heat-sensitive, and to be inactivated by the organomercurial, p-chloromercuribenzoate. All of these findings are consistent with the enzyme activity measured with the α-naphthyl palmitate-Triton X100 micelle chromogenic assay and histochemical technique. The Triton X100 activation of acid lipase found by these investigators was probably due to optimizing surfactant-substrate ratios and micellar surface properties, including charge.[223] The addition of this nonionic detergent thus contributed to maximally efficient substrate hydrolysis by acid lipase, a distinct advantage of the methodology used in this study.

This study also confirms the findings of Hayasaka and associates[68,69] and Teng and Kaplan,[224] who found that purification of lysosomal acid lipase by molecular sieve and/or ion exchange chromatography results in activity elution in trailing or multiple peaks and in poor enrichment of acid lipase activity due to the formation of enzyme aggregates. Pitfalls in the purification of acid lipase include not only the inactivation of even semipurified forms during storage, but also instability in the presence of inorganic salts and high affinity for hydrophobic surfaces.[225] In this study, bovine RPE acid lipase also was eluted from SP-Sephadex C-50 in the void volume due to the low salt concentrations necessary to avoid enzyme inactivation. Significant loss of acid lipase activity also occurred during the purification procedures in this study, presumably on account of a combination of (1) contaminating proteases, (2) denaturation, (3) aggregation, (4) exposure to salts,[225] and (5) extraction of the hydrophobic enzyme from cell membranes or other hydrophobic constituents necessary for maximal enzyme activity.

Copurification of triglyceride lipase, as detected by α-naphthyl palmitate hydrolysis, and cholesteryl esterase activities from bovine RPE cells has been reported in other tissues. Brown and Sgoutas[226] purified rat liver lysosomal cholesteryl esterase with an apparent M_r of 60,000 d, and also found cholesteryl esterase and triglyceride lipase activities to cochromatograph throughout their isolation procedures. Burton and Mueller[227] and Warner and associates[228] demonstrated the hydrolysis of cholesteryl oleate, triolein, and 4-methylumbelliferyl oleate at acid pH by human placental and hepatic acid lipases during sequential purification steps, during which considerable acid lipolytic activity was lost. The authors of these studies also concluded that acid lipase was responsible for cholesteryl ester and triglyceride hydrolysis.

In addition to hydrolyzing α-naphthyl palmitate, triolein, and cholesteryl oleate, the purified RPE enzyme fraction was found to form cholesteryl esters at acid pH (Figures 3 through 6). The substrate used to demonstrate cholesteryl ester formation in the present study contained no cholesteryl ester, but the kinetic conditions were in favor of cholesteryl ester synthesis by the purified enzyme. In the cholesterol esterification assay, the substrate contained only phospholipid, triglyceride, and radioactively labeled and unlabeled cholesterol in vesicle form. Since there was no free source of fatty acid to esterify to cholesterol, only hydrolysis of the lecithin or triglyceride could provide fatty acids for cholesteryl esterification. Burrier and Brecher[229] and Brecher and associates[207] used similar lecithin substrate vesicles to demonstrate cholesteryl esterase and triglyceride hydrolysis. They found that at pH 4.5, only cholesteryl esters and triglycerides were hydrolyzed, and not phospholipids. In this study, purified acid lipase from bovine RPE cells also showed triglyceride hydrolytic activity, indicating that the enzyme may hydrolyze triglycerides and subsequently esterify the liberated fatty acids to cholesterol, probably only after the free fatty acid concentration is sufficient to drive the reaction thermodynamically to form cholesteryl esters. Further support for cholesterol esterification by acid lipase is provided by a previous study of monkey splenic acid lipase, which had similar hydrolytic and esterification activities, as well as an M_r of 64,000 d when purified as described for the bovine RPE cell sonicates.[77]

The hydrolysis of the artificial substrate α-naphthyl palmitate to free α-naphthol and palmitic acid in chromogenic biochemical assays paralleled the activities for triglyceride and cholesteryl ester hydrolysis and cholesterol esterification. This simple chromogenic technique appeared to correlate well with assays for activities associated with acid lipase activity and therefore is an effective means for indirect assay of acid lipase hydrolytic and esterification activities. When applied to retinal tissue, this methodology may demonstrate the significance of acid lipase in RPE accumulation of cholesteryl esters, lipid metabolites, and peroxidized, undigestible lipids, including lipofuscin. Cholesterol and fatty acid concentration–dependent, thermodynamically driven cholesteryl ester synthesis by acid lipase might occur in RPE cells that phagocytize lipid-rich POS membranes,[29,33-37] have active autophagocytic mechanisms,[47] and incorporate exogenous native and modified LDLs in vivo.

The chromogenic biochemical assay confirmed the findings of the histochemical diazocoupling technique. RPE cells were found to contain lysosomal acid lipase activity comparable to that of mononuclear phagocytes (Table I). New Zealand White rabbit RPE acid lipase activity was one fortieth of that found in alveolar macrophages, which demonstrated the highest activity of all mononuclear phagocytes assayed, but one fourth of that found in peritoneal macrophages. Although RPE cells had less activity than all of the tissue histiocytes examined, they demonstrated considerably more activity than monocytes and cultured smooth-muscle cells. The acid lipase activity found in rabbit RPE cells was consistent with the acid lipase activity necessary for positive histochemical staining in fixed, frozen tissue sections, namely, 36 μM of hydrolyzed substrate/10^6 cells/hour.[76] Accordingly, New Zealand White rabbit RPE cells stained selectively in sections of the posterior ocular segment (Figures 1 and 11), a finding which corresponds to the observation of selective mononuclear phagocyte staining for acid lipase activity in fixed, frozen sections of liver (Figure 2A), spleen (Figure 2B), and lung (Figure 2C). The high and histochemically stainable level of acid lipase activity in RPE cells may reflect the consequence of their prominent role in the phagocytosis and turnover of lipid-rich POS and incorporation of LDL from the systemic circulation, inasmuch as POS (Table II) and LDL (Table IV) stimulate RPE acid lipase activity.

Hayasaka and associates[68,69] also revealed high levels of acid lipase activity in RPE cells when compared to liver, kidney, and blood tissue homogenates, but RPE acid lipase activity was not evaluated with respect to isolated cell types. Our studies utilized a modified microcarrier culture technique, which allows comparison of freshly isolated, glass-adherent RPE cell enzyme activities to those of freshly isolated, glass-adherent mononuclear phagocytes (Table I). Assays on these cell preparations yielded direct measurements of enzyme activities in specific cell types. This technique may be adapted to cell culture studies and yield information that cannot be obtained using whole-tissue homogenates.

Deficiencies or defective augmentation of RPE lysosomal acid lipase activity in response to various metabolic, particulate, inflammatory, or immunologic stimuli may be

important to normal RPE aging and to the pathogenesis of various heritable and nonheritable retinal diseases. Of particular interest are those characterized by the accumulation of indigestible lipid residues that are incorporated into RPE cytoplasmic residual bodies as the lipopigment lipofuscin.[72,82] Deposition of lipofuscin has been shown to accumulate during aging[79,116] and reach massive proportions in various RPE degenerations[5,230-243] and to be extruded from RPE basal surfaces,[87,154] contributing to progressive lipid accumulation in Bruch's membrane,[88] and the formation of basal laminar and linear deposits and drusen.[86,87,92-98,230,234] Deficient acid lipase activity may also be important in diseases such as retinitis pigmentosa, where the inefficient elimination of POS debris[37,244-249] may contribute to outer retinal degeneration. A role for acid lipase deficiency in the pathogenesis of outer retinal disease is underscored by observations of tapetoretinal degeneration in patients with biochemically verified acid lipase deficiency and clinically manifest neutral lipid storage disease.[250,251] Alternatively, lipid esterification by acid lipase might occur under appropriate kinetic conditions as shown in this study (Figures 3, 4, and 6), thereby accumulating lipids in the RPE cytoplasm, where they may become subject to lipid peroxidation, leading to lipofuscin formation and RPE dysfunction,[81,82,101-105] including further loss of RPE antioxidant protection.[81] This scenario might then result in RPE deposition[86,87] of lipids, cholesterol, and cholesterol esters that are seen in Bruch's membrane with aging and in drusen.[89-91] The presented methodology may be helpful to assist in vitro investigations of RPE cell enzyme activity and in situ studies of the retina in health and disease that could not be previously performed.

Variations in intracellular and secreted RPE lysosomal enzyme activities in vitro may be ascribed to culture conditions and to the type and duration of inducing stimuli. Basal RPE cell activation and, hence, response to various phagocytic stimuli may be influenced by in vitro conditions, including RPE cell confluency, culture flask surface, culture media, and serum supplementation. Numerous studies have documented variable RPE avidity for and time course of uptake of various particles, including latex microspheres, under different organ culture and tissue culture conditions.[39,112,114,247,252] In this study, monkey RPE cells synthesized and secreted substantial basal levels of lysosomal enzymes in vitro (Tables V and VI). Significant increases over basal RPE intracellular lysosomal enzyme activities of monkey cells (Table V) were induced to a much greater degree by POS than nonspecific, control latex microsphere phagocytic challenge (Table VI). In contrast to latex, the greater POS-induced increases in RPE intracellular lysosomal enzyme activities were not accompanied by significant increases in enzyme secretion over the already substantial basal levels secreted by the unstimulated RPE cell layers (Table VI). The specificity of RPE cellular responses to various phagocytic stimuli that was observed may be related to early-response genes such as *zif-268*, *c-fos*, and *tis-1*, which are rapidly and transiently expressed in cultured rat RPE cells during phagocytosis of bovine POS.[253] These inductions are thought to modulate a gene cascade of intracellular responses to POS phagocytosis, but are not activated in RPE cells during the phagocytosis of nonspecific particles such as latex beads. Moreover, increases in RPE intracellular and secreted lysosomal enzyme activities were not observed in response to the soluble, nonspecific stimulant, phorbolmyristic acid (PMA) (results not shown), further supporting the specificity of RPE cellular responses to phagocytic stimuli.

Native LDL also significantly enhanced RPE acid lipase and cholesteryl esterase activity (Table IV). Although it is likely that other serum constituents may affect RPE lysosomal function, the fact that RPE cells exposed to the lipoprotein-deficient serum from which the native LDL was derived had significantly less activity implies that the LDL per se induced RPE lysosomal lipolytic activity in this study. These increases in acid lipolytic activity suggest that receptor-mediated uptake of lipid-rich particulates may regulate RPE biochemical events after their incorporation. Such regulation may involve receptor-dependent induction of the aforementioned genetic responses[253] that may also assist in perpetuating the efficient uptake and subsequent degradation of the large amounts of lipid-rich material that is continually presented to these cells.

Mechanisms regulating RPE extracellular release of lysosomal enzymes have not yet been elucidated. However, acid hydrolases have been reported in the space beneath the neurosensory retina under physiologic conditions[74,254] and in cases of rhegmatogenous retinal detachment[255] and retinal dystrophies.[75,256,257] Moreover, the accumulation of lysosomal enzyme activities appears to be carefully controlled in a highly selective manner from excessive accumulation by specific cell surface receptors.[258] In this study, RPE phagocytosis of latex beads, and to a much lesser extent of POS, resulted in release of lysosomal enzyme activity into the overlying media (Table VI). This apparent stimulus-dependent release of RPE enzyme activity during phagocytosis may be due to leakage of lysosomal hydrolases when nonphysiologic or large debris are engulfed or persist in RPE cells. This observation raises the possibility that damage to patches of the RPE monolayer may occur because of leakage of RPE lysosomal enzymes when abnormal particulates are engulfed or persist in RPE cells. Such particulates may include abnormal POS in retinal dystrophies, peroxidatively damaged autophagocytic debris, oxidized or other

modified lipoproteins, and indigestible residues, including lipofuscin and A2E, which compromise RPE lysosomal function and integrity.[81-83,101,148] Moreover, extracellular nonlysosomal enzymes, including metalloproteinases, are known to be secreted as inactive forms that require postsecretory enzymatic cleavage for activation.[259,260] RPE lysosomal leakage into the extracellular space may activate these MMP by cleaving them into active forms.[261-264] The principal lysosomal enzymes subserving this function are cathepsins B and D,[261,262] the latter being the principal RPE protease that degrades engulfed POS protein.[56,57] Once hydrolyzed by the activated metalloproteinases, the extracellular lysosomal enzymes are then capable of further degrading fragments generated by metalloproteinase activity.[265,266] Thus, hydrolytic enzymes released into the pericellular environment because of inefficient lysosomal degradation of debris may also result in localized damage to connective tissues as well as neighboring RPE cells and cells of the neurosensory retina. Ultrastructural cytochemical evidence of discharge of secondary lysosomes from aged RPE cells directly into Bruch's membrane provides direct evidence that such a process indeed occurs.[85]

Analogous to RPE cells, intracellular mononuclear phagocyte lysosomal enzyme activity and, to a lesser extent, enzyme release may be enhanced by phagocytosis of particulates, including latex microspheres, zymosan, opsonized erythrocytes, antigen-antibody complexes, and bacteria.[215,264,267-270] Moreover, mononuclear phagocytes that have phagocytized indigestible bacteria or have been activated by thioglycollate synthesize and release substantially more lysosomal enzyme activities following subsequent phagocytic challenge.[264,271,272] These mononuclear phagocyte responses support our findings that show RPE lysosomal enzyme synthesis and secretion to be dependent on the type of phagocytic stimulus.

LIPOPROTEIN UPTAKE BY MONKEY RPE CELLS IN VITRO AND IN VIVO

DiI-labeled, A-LDL is bound and internalized by specific receptors on macrophages and may be used to distinguish bone marrow–derived mononuclear phagocytes from other cells in cell culture and in tissue sections.[211,273] Control incubations using cultured macrophages and smooth-muscle cells confirmed that successful modification of isolated N-LDL for our RPE studies. Accordingly, only macrophages avidly incorporated A-LDL and O-LDL while smooth-muscle cells only internalized N-LDL. Macrophage uptake of A-LDL was abolished by fucoidin, a specific A-LDL that did not block N-LDL binding and uptake and only partially reduced O-LDL uptake, presumably because of O-LDL uptake by other O-LDL receptors not blocked by fucoidin.[85,132,135-137,139]

RPE cells incubated with either DiI-A-LDL or O-LDL, like macrophages, exhibited intense surface and cytoplasmic granular red fluorescence, presumably owing to lysosomal incorporation of the modified lipoproteins (Figures 8 and 11). The incomplete blockage of DiI-O-LDL uptake by fucoidin was probably due to the fact that the RPE possesses other scavenger receptors, such as CD68 and CD36, that may also mediate RPE O-LDL uptake.[140-142] RPE cells also avidly incorporated DiI-N-LDL, indicating that these cells do not demonstrate the selectivity of mononuclear phagocytes or smooth-muscle cells for lipoproteins but, rather, actively bind and internalize native and modified lipoproteins.[128,129] Following in vivo perfusion with the DiI-labeled, modified lipoproteins, the RPE exhibited intense fluorescence (Figures 9 and 11) comparable to that of resident mononuclear phagocytes of liver, spleen, and lung removed from the same animals (Figure 10). In these organs, perfused DiI-A-LDL and DiI-O-LDL were selectively incorporated into mononuclear phagocytes, in sharp contrast to their lack of labeling by perfused N-LDL, which was avidly incorporated into hepatocytes. In all of the tissues examined histologically, discrete uptake of labeled A-LDL and O-LDL was visualized in vascular endothelial cells as previously reported.[274] The lesser degree of DiI-O-LDL fluorescence present in tissues other than the liver may indicate rapid clearance by this organ in spite of the site of perfusion (ie, the carotid arteries). These in vivo observations, together with the selective alveolar macrophage and smooth-muscle cell lipoprotein uptake and the successful use of specific inhibitors, leads to the conclusion that RPE cells possess specific receptors for rapid, high-affinity N-, A-, and O-LDL binding and uptake in vitro[128,129] and in vivo.

High-affinity N-LDL receptors on RPE cells are likely to subserve physiologic functions by efficient culling of native lipoproteins from the blood to provide nourishment for the outer retina (Figures 9 and 11). It is possible that lipids derived from lipoproteins, from phagocytized, lipid-rich photoreceptor membranes, and from autophagocytosis might converge in, and possibly overwhelm, the RPE lysosomal pathway, leading to the intracellular accumulation of lipid in aging and disease. The results of this study suggest that this is unlikely, since POS membranes and N-LDL appear to induce compensatory increases in lysosomal enzyme activity (Tables II and IV).

However, RPE sequestration of modified lipoproteins, particularly oxidized LDL, may have adverse consequences. Intracellular accumulation of lipid, particularly cholesteryl esters, is specifically promoted by lysosomal hydrolysis of lipids internalized via scavenger receptor pathways.[131-134,216,275] Intracellular accumulation of lipid per se does not appear to cause cellular dysfunction and death, but light- and oxygen-induced free radicals and peroxides may cause peroxidation of accumulated lipid,

leading to indigestible lipid metabolites. The RPE contains many antioxidants for neutralizing reactive oxygen intermediates that are generated by the combined actions of high RPE oxidative metabolism and light absorption by RPE pigment.[6,28,40,276,277] High levels of vitamin C,[278,279] vitamin E,[280-283] peroxidase,[284] superoxide dismutase,[285-287] catalase,[286,288,289] glutathione,[185,290] and selenium[291] serve to protect the RPE cells and surrounding tissue by neutralizing reactive oxygen species,[292,293] some of which are generated in response to POS phagocytosis.[294] When these protective mechanisms are overwhelmed, however, extracellular modifications of N-LDL, similar to in vitro acetoacetylation or oxidation,[133,134,216] may result in forms recognized by the scavenger receptors,[85,131,135,136,143] some of which have been identified on RPE cells.[140-142] These modifications include exposure to oxidizing vascular endothelial cell metabolites,[275,295,296] peroxide,[297] and highly reactive, oxidized by-products of arachadonic acid, such as malonaldehyde and malondialdehyde, that are secreted by platelets, macrophages, and vascular endothelial cells in vivo.[298,299]

In the outer retina, RPE cells may participate in this process, since they contain high levels of POS-derived arachidonic acid[218,219,300] that may undergo reactive oxygen intermediate-induced lipid peroxidation to produce reactive fragments that may then oxidize or otherwise modify native LDL arriving from the choroid.[301] The RPE also contains significant levels of phospholipases A_1 and A_2,[51] the latter of which may induce vascular endothelial cells to produce oxygen metabolites[275,295,296] that cause LDL oxidation and modification to forms recognized by scavenger receptors. The same local modifications of circulating LDL that are likely to occur within the choriocapillaris and at the RPE-choriocapillaris interface may also result in local denaturation and oxidation of other extracellular lipids, proteins, and glycoproteins. Such modified molecules might then become subject to uptake by RPE scavenger receptors that mediate A- or O-LDL incorporation.[214,302] Lysosomal dysfunction may thus be further aggravated by high-affinity uptake and lysosomal incorporation of oxidized or denatured extracellular components by means of scavenger receptors.[148]

Local generation of reactive oxygen intermediates may also inhibit POS membrane phagocytosis[303] and degradation,[82,83,148] perpetuating the accumulation of denatured oxidized lipids and proteins, leading to formation of lipofuscin that further embarrasses POS degradation.[81-83] Lipofuscin is normally found in aging RPE cells,[5,47,72] but massive RPE cytoplasmic accumulations and basilar excrescences of this indigestible end product are seen in ARMD and in retinas from animals and humans with dietary antioxidant deficiency.[235,288] Clearly, inducing robust lysosomal lipolytic activity for efficient degradation and recycling of lipid debris may be beneficial by mitigating this pathologic sequence of events. These observations, taken together with the demonstrated ability of LDL to induce acid lipase and to penetrate the RPE monolayer in vivo, leads to the speculation that dietary lipids incorporated into circulating LDL may reach the RPE and modulate its role in the development of ARMD.

EFFECTS OF FISH OIL ON RPE ACID LIPASE ACTIVITY

This study describes RPE lipid metabolic findings in the first and only primate study on the effects of long-term dietary fish oils as a portion of dietary fat in a highly atherogenic diet.[190,192,193] Analysis of these animals showed that fish oil in diet may effectively reduce atherosclerotic progression during severe atherogenic stimuli, even though high-density lipoprotein cholesterol was depressed by the administration of fish oil.[190,192,193] Administration of fish oil depressed the concentrations of all lipoproteins, a remarkable finding, since rhesus monkeys usually show a marked rise in LDL and LDL cholesterol when fed a 2% cholesterol diet no matter what other food fat is included to a 25% level.[304,305] In animals fed a diet of 3:1 fish oil to coconut oil, triglycerides were less prevalent than those found animals fed coconut oil, presumably because of the fact that there were progressive declines in triglyceride secretion by hepatocytes handling fatty acids of increasing length and unsaturation (oleate > linolenate > arachidonate > eicosapentaenoate [EPA] > DHA). The lipoproteins of animals fed the 3:1 fish oil to coconut oil diet promoted significantly less cholesterol esterification in mononuclear phagocytes than lipoproteins of monkeys fed coconut oil alone.[190,192] The reduction in cholesterol esterification was at least partly due to the omega-3 fatty acids contained in the fish oil, since the percentage of these fatty acids in the circulating LDL of monkeys fed the 1:1 and 3:1 fish oil to coconut oil diets correlated with reduced acyl-CoA:cholesterol acyltransferase (ACAT) cholesterol esterification in mononuclear phagocytes.[192] In the animals fed the 3:1 fish oil to coconut oil diet, the concentration of lipoprotein particles in the plasma, their reduced apoprotein E–apoprotein B ratios, their predominant triglyceride and reduced cholesteryl ester cores, and their reduced ability to induce cholesterol esterification contributed to the reduction in atherosclerosis in these monkeys.[190]

RPE cells from the eyes of these monkeys fed the 3:1 fish oil to coconut oil diet also demonstrated marked differences. Enhanced lysosomal lipolytic profiles were demonstrated in histologic sections of the eyes (Table VI, Figure 11) and in biochemical assays for acid lipase, acid esterase, and cholesteryl esterase activities (Table V). The effect of fish oil diets on lysosomal lipolytic activity was not limited to the RPE cells, but appeared to be a sys-

temic effect demonstrated by enhanced activities measures in liver and spleen homogenates from the same animals (Table VII). Thus, the RPE appeared to share in the beneficial systemic effects bestowed by the fish oil diet. The effect of fish oil on RPE lysosomal lipolytic profiles may be due to RPE uptake of omega-3 fatty acid containing lipoproteins, which reduce cholesterol esterification and promote acid lipase and cholesterace activities.[190-192]

Experiments in which cultured RPE cells were exposed to the sera of these animals confirmed that RPE lysosomal induction was likely due to favorable lipoproteins impacting the RPE monolayer in vivo. Because of the fact that the monkey sera were needed for several analyses,[190,192,193] only small amounts of sera could be used for the RPE studies. This precluded LDL isolation and incubation with RPE cells, since much more than the available sera would have been required to perform such assays. Cultured monkey RPE cells exposed to the sera from monkeys fed the 3:1 fish oil to coconut oil diet exhibited increased lysosomal hydrolytic activity (Table VIII). Of particular interest was the observation that sera from monkeys fed coconut oil alone did not significantly increase lysosomal enzyme activity in cultured RPE cells when compared with LDS from chow-fed animals (Table VIII). This finding contrasted with the significant induction of enzyme activity in RPE cells exposed to native LDL from chow-fed animals over that obtained with LDS (Table IV). These data imply that dietary alterations in LDL, even without fish oil supplementation, may affect induction of RPE lysosomal enzyme activities. Taken together, these results show that diet may modulate RPE lysosomal activity in response to alterations in circulating lipoproteins for which the RPE cells express receptors, which mediate rapid LDL uptake by the monolayer. The enhanced RPE lysosomal activities observed in RPE cells exposed to LDL from monkeys fed fish oil–containing diets, but not coconut oil–containing diets, indicates that the type of fatty acids contained in LDL is important in the modulation of RPE lysosomal activity. Such increased activity is likely to be beneficial because it results in the efficient metabolism of fats and prevents intracellular accumulation of lipids that may then be subject to oxidative processes that may embarrass RPE cellular functions.

The beneficial effects of fish oil on RPE lysosomal lipid metabolism may be only one of several mechanisms by which omega-3 polyunsaturated fatty acids may reduce the risk for ARMD. A prominent feature of ARMD lesions is the presence of leukocytes that have infiltrated diseased retinal tissue from the blood to become intimately associated with RPE cells.[306-308,326] Proinflammatory cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF), and interferon (IFN)-γ, derived from infiltrating leukocytes and RPE cells and surface receptors, such as intercellular adhesion molecule (ICAM)-1, are increasingly recognized as key participants in the evolution of ARMD lesions.[306,307,326]

Fish oils appear to have potent immunoregulatory and anti-inflammatory effects,[309] including suppression of (1) arachidonic acid–derived eicosanoids[309,310,327-329]; (2) major histocompatibility type II (MHC II; HLA-DR) antigen expression[311,312]; (3) cell adhesion molecules, including ICAM-1[309,330,331]; (4) responses to endotoxins[310,321,322,327,328,332,333]; and (5) production of and response to proinflammatory cytokines.[309,313,319,323] Cultured cells and cells from animals fed fish oils rich in omega-3 polyunsaturated fatty acids, particularly DHA and EPA, incorporate them into their cell membranes, which become depleted of arachidonic acid.[309] The membranes become more fluid,[329] imparting reduced activity to proinflammatory surface receptors, including IFN-γ and TNF receptors and scavenger receptors for A- and O-LDL.[314-317,330] DHA and EPA also inhibit the elaboration of IL-1[318,328,333] and TNF[318,319,321,328,333] from leukocytes and reduce responses to TNF, IL-1, and IFN-γ.[312,313,332] The reduced inflammatory responses to these cytokines are at least partially mediated by omega-3 polyunsaturated fatty acid inhibition of intracellular signaling cascades and transcription factors controlling gene transcription.[334] In animal models, fish oil reduces the severity of autoimmune disease and indicators of disease activity, including TNF and IL-6.[310,323-325] The myriad of mechanisms by which omega-3 polyunsaturated fatty acids inhibit the production and actions of proinflammatory cytokines, reduce expression of cellular adhesion molecules that bind and stimulate leukocytes, and inhibit intracellular responses mediating inflammatory responses, all of which have been shown to be operative in RPE cells,[4,306-308,326] is likely to ameliorate mechanisms that lead to RPE damage and the development of ARMD.

Another important effect of dietary fish oil is omega-3 polyunsaturated fatty acid–induced reduction of nitric oxide[321-323,332,335] and reactive oxygen intermediates,[325,336] in vitro and in vivo. Reduced nitric acid and superoxide responses to TNF, IL-1, and IFN-γ prevent the formation of peroxynitrite, a strong oxidizer causing cellular damage.[310,319-322] Inhibiting reactive oxygen species may be important to preventing systemic and localized LDL modification in the retina that would subject LDL[317,325,330] reaching the RPE monolayer to lysosomal incorporation by RPE A-LDL and O-LDL receptors.[128,129] Nevertheless, there is strong evidence that the LDL of animals and humans eating fish–oil–rich diets contains high levels of omega-3 polyunsaturated fatty acids that are subject to peroxidation.[336-340] Such oxidative processes utilize polyunsaturated fatty acids as substrates for peroxidation yielding reactive oxygen intermediates, lipoperoxides, and O-LDL, resembling the pathologic retinal process of

lipofuscin formation and toxicity in aged, damaged RPE cells.[81-83,101-105,148] Cells, including RPE cells, incorporating O-LDL that reaches the RPE monolayer from the blood (Figures 9 and 11) may then suffer adverse consequences, including embarrassment of RPE lysosomal, antioxidant, and POS degradative activities.[81,317,325,330] Ikeda and associates[183] provided recent clinical evidence that such processes may indeed contribute to ARMD in humans. These investigators showed that genetic polymorphisms of paraxonase, a glycoprotein that prevents O-LDL formation, represent a risk factor for ARMD. Furthermore, enhanced RPE lysosomal acid hydrolytic activity occurring in response to N-LDL (Table IV) sera from monkeys fed fish oil (Table VIII) may be antagonized by O-LDL,[183] negating the beneficial effects of fish oil–rich diets in reducing the risk for ARMD.[122,123,127] In fact, significantly reduced acid lipase, acid esterase, and cholesteryl esterase (all $P<.05$) activities were observed in cultured RPE cells exposed to O-LDL when compared to LDS from the same plasma (data not shown).

The propensity for omega-3 polyunsaturated fatty acid–rich LDL to be oxidized may underlie apparently conflicting evidence from some reports.[337,339,341] This has led to the recommendation that dietary intake of omega-3 fatty acids should be accompanied by adequate amounts of antioxidants found in fruits and vegetables.[336,338,340,341] The peculiar milieu of the RPE may render adequate dietary antioxidants even more important.[173,174,184,185] Light,[138,174] vitamin C and, particularly, vitamin E deficiency result in outer retinal degeneration with RPE pathologic alterations consistent with lipid peroxidation injury.[280,281] Increased circulating levels of O-LDL have also been demonstrated in non-insulin-dependent diabetic patients,[342-344] prompting more aggressive management of hyperglycemia and hyperlipidemia, both known risk factors for vascular complications in these patients, who may require antioxidant supplementation as part of a dietary regimen high in fish oil. The most unequivocal risk factor for the development of ARMD is cigarette smoking.[175-177] In smokers, fish oil ingestion may actually be detrimental, insofar as they suffer significantly increased peroxidation of LDL, with O-LDL rising up to 50% above baseline levels after 4 weeks of fish oil ingestion.[179] Furthermore, vitamin E does not counteract oxidation of LDL in smokers as it does in smokers in whom vitamin E may idiosyncratically be pro-oxidant.[179,182] These observations imply that dietary fish oil, rich in omega-3 polyunsaturated fatty acids, may enhance O-LDL delivery to the RPE, aggravating RPE dysfunction that leads to ARMD.[1-3] Thus, dietary fish oil supplementation may potentially have negative effects on RPE metabolism in the presence of smoking, excess UV light exposure, inadequate dietary antioxidants, and diabetes with increased O-LDL levels.

The enhanced RPE lysosomal lipid hydrolytic activity due to native LDL may reflect physiologic compensation for increased lipid incorporation and one reason that elevated lipoproteins have not been implicated as a risk factor for ARMD.[345] It is likely that LDL modifications, be they beneficial or pejorative, are likely to affect the RPE by efficient delivery to the RPE monolayer. In the absence of the aforementioned factors, which promote lipid oxidation, most notably smoking, dietary fish oil may be a beneficial agent that favorably modulates RPE lysosomal lipid metabolism as it reduces the risk for ARMD.[1-3]

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