Volume 10 number 11 1991, 1009–1014

Current Eye Research

Chemotactic activity of the peroxidized retinal membrane lipids in experimental autoimmune uveitis

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ABSTRACT

We investigated the mechanism for amplification of intraocular inflammation in rats with experimental autoimmune uveitis by examining the chemotaxis potentials of peroxidized lipids extracted from the retinas. Utilizing thin layer chromatography, we found that the peroxidized products isolated from the inflamed retinas were fatty acid hydroperoxides that corresponded to the autooxidized products from commercial methyl docosahexaenoate, with Rf values ranging from 0.30 to 0.37. These were not demonstrated in similar preparations from normal retinas or in unoxidized docosahexaenoate. Boyden chamber assay revealed that the hydroperoxides isolated from inflamed eyes and the products of oxidized methyl docosahexaenoate possessed significantly higher chemotactic activity than did the retinal lipids isolated from normal eyes ($P < 0.01$). These findings may help to explain the mechanism of inflammatory amplification induced by peroxidized retinal lipids that is seen in this animal model of uveitis.

INTRODUCTION

The inflammatory response is composed of events that can be grouped according to type: vascular, cellular, or humoral. The hallmark of the cellular component of acute inflammation is the neutrophil. Migration and accumulation of neutrophils is governed largely by a group of mediators, called chemotactic agents, that are liberated at the site of the inflammatory initiation. Known chemotactic agents, such as arachidonic acid metabolites, have been implicated in inflammation throughout the body, including uveitis. Temporally, inflammation is thought to consist of an initiation phase, followed by a perpetuation or amplification phase that is believed to be associated with the inflammatory mediators. However, these mediators may not be the only factors involved in amplification of the inflammatory response.

During the inflammatory reaction, a variety of substances, including oxygen metabolites, proteases, and arachidonic acid metabolites, are released by the inflammatory cells in an attempt to destroy the target of the inflammation. Recently, oxygen metabolites, such as superoxide, hydrogen peroxide, hypochlorous acid and hydroxyl radicals, have been shown to cause tissue damage via peroxidation of the membrane lipids, and are responsible also for the amplification of a variety of inflammatory conditions, including those involving the uvea(1,2).

We have investigated the mechanism of the perpetuation and amplification of inflammation by analyzing the chemotactic activity of the peroxidized lipids extracted from retinal tissue. We hypothesize that these lipids, following the peroxidation that accompanies inflammation, contribute to the perpetuation of chemotaxis and to the amplification of inflammation that characterizes experimental autoimmune uveitis.

MATERIALS AND METHODS

Induction of experimental autoimmune uveitis (EAU)

Animal experimentation was performed in accordance with the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23).

Following anesthesia with intramuscular ketamine hydrochloride and xylazine hydrochloride, Lewis rats (weighing approximately 175 g each) were given a single hind foot-pad injection of 50 μg bovine S-antigen in complete Freund's adjuvant containing 0.2 mg heat killed Mycobacterium. Animals were observed for 15 days, at which time they were killed with an overdose of intramuscular ketamine hydrochloride and xylazine hydrochloride.

Received on May 22, 1991; accepted on September 5, 1991

Preparation of lipids

Following enucleation, retinas of rats were dissected from the globes and the membrane lipids were extracted using the method of Folch et al(3). Briefly, the combined retina tissues from 6 eyes were homogenized in 6 ml of chloroform/ methanol (2:1) containing 0.5 mg of butylated hydroxytoluene per 100 ml of solvent. The pooled extracts were then washed with 1.2 ml of water and centrifuged. The solvent was then evaporated from the organic layer and total lipids was obtained as residue. In vitro preparation of peroxidized retinal lipids was also performed by isolating the lipids from normal control rats and incubating these lipids with a radical-generating system consisting of 200 μM of 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) (Polysciences, Inc., Warrington, PA) dissolved in Hanks' balanced salt solution (HBSS) (4).

Commercial methyl docosahexaenoate (Nu-Chek-Prep, Elysian, MN) was used in its pure and oxidized forms. The oxidized methyl docosahexaenoate was prepared by air oxidizing the commercial pure methyl docosahexaenoate (Nu Chek Prep, Inc., Elysian, MN) at room temperature for 5 to 7 days. The extent of autoxidation was monitored by measuring the diene conjugation formed in the sample. For this measurement, an aliquot of the sample was dissolved in 1 ml of ethanol and the absorption range of 200-400 nm was recorded using a Shimadzu spectrophotometer model UV-160. For estimating the quantity of conjugated dienes formed in the sample, absorbance at 233 nm was measured and molar extinction coefficient of 25,200 was used for the calculation (5). In a typical experiment, 50 mg of methyl docosahexaenoate was oxidized and 40 to 50% of the oxidized lipid could be obtained in 7 days.

To confirm the presence of fatty acid hydroperoxides and hydroperoxide-derived hydroxy fatty acids in the inflamed retinal tissue, thin layer chromatography (TLC) was performed. Total lipids extracted from the normal and inflamed retinas were transesterified to obtain fatty acid methyl esters. Transesterification was performed by reacting the lipids with METH-PREP II (0.2 M m-trifluoromethylphenyl-trimethylammonium hydroxide in methanol, Alltech Associates, Deerfield, IL) using the method of van Kuijk et al (6). This procedure has been shown to give nearly quantitative conversion of phospholipids and triglycerides to fatty acid methyl esters (6,7). In a typical experiment, to a solution of crude lipids (8-10 mg obtained from 12 eyes) dissolved in 0.2 ml of methanol, 0.3 ml of METH-PREP II was added. The reaction mixture was then stirred under nitrogen at room temperature for 30 minutes. At the end of this period, 0.8 ml of water was added and the mixture was then extracted twice with 2 ml each of ethyl acetate. The organic layer was separated by centrifugation, pooled and evaporated under nitrogen to obtain usually 4-5 mg of total faty acid methyl esters. TLC was carried out using precoated silica gel 60, Merck plates (0.25 mm thick) and a solvent system consisting of petroleum ether/diethyl ether/acetic acid (70:30:1). The spots on the plates were visualized by dipping in a solution of 3% cupric acetate in 8.5% phosphoric acid and then charring at 140^{o}C. The extent of oxidation of the commercially available methyl docosahexaenoate was also monitored by TLC using the same solvent system.

Chemotaxis assay

Human polymorphonuclear leukocytes (PMNs) were isolated from heparinized peripheral blood utilizing dextran sedimentation and centrifugation of Ficoll-Hypaque gradients, as previously described(8). Chemotactic responses were measured in a modified multiwell Boyden chamber(8). A portion of lipids to be used for chemotactic activity measurement was initially dissolved in 95% ethanol and the solvent was then removed via evaporation under nitrogen. The residue was suspended in 200 μl of HBSS, and this solution was placed in the lower compartment of the chamber. A suspension of PMNs ($2x10^6$/ml) in HBSS with 0.5% bovine serum albumin was placed in the upper compartment. Two chamber wells were divided by a single nitrocellulose filter with a 5.0 μm pore diameter. The chamber was incubated at 37° in 5% CO_2 for 60 minutes. Filters were removed, fixed in alcohol, and stained with hematoxylin and eosin. The chemotactic activity was quantitated as the average number of cells per high power field (x400) migrating completely through the filter in four different locations(8). Cell counts were performed by two observers, masked as to the filter group. Each observer independently examined all of the filters and chose four different locations on each that

they felt were representative of the entire filter. All assays were carried out in triplicate.

RESULTS

Detection of lipid peroxidation

The peroxidized products isolated from inflamed retinas were compared with the autooxidized products obtained from the commercial methyl docosahexaenoate by measuring conjugated dienes and by TLC. The major products formed in both cases were fatty acid hydroperoxides, which were seen as multiple spots with R_f values ranging from 0.30 to 0.37 (Figs. 1 and 2).

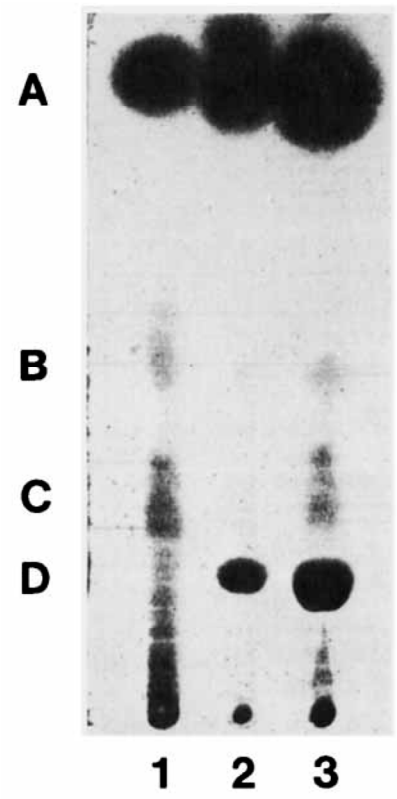

Figure 1. Thin layer chromatogram of retinal lipids isolated from both normal and EAU rats. Channel 1, oxidized soybean phosphatidylcholine standard; channel 2, total fatty acid methyl esters isolated from the retina and choroid of control animals; channel 3, total fatty acid methyl esters isolated from the retina and choroid of EAU animals; A, unchanged fatty acid methyl esters (R_f=0.57-0.65); B, hydroperoxy fatty acid methyl ester (R_f=0.30-0.37); C, hydroxy fatty acid methyl esters (R_f=0.19-0.27); D, cholesterol. Note the presence of hydroperoxides and hydroxy fatty acids in channel 3 and an absence of both hydroperoxides and hydroxyfatty acid in channel 2.

During reduction by glutathione peroxidase in tissue and transesterification by METH-PREP II in processing, most of the hydroperoxy fatty acid methyl esters are converted to hydroxy fatty acid methyl esters. These hydroperoxide spots were not recognizable in fatty acid preparations from the normal retinas or in unoxidized methyl docosahexaenoate.

Chemotactic activity

Boyden chamber assay revealed that the peroxidized retinal lipids possessed much greater chemotactic ability than did retinal lipids from normal rats (Table 1). The lipids isolated from inflamed eyes (10 mg/ml) were more chemotactic than were those isolated from normal eyes ($P < 0.01$). The same extent of increased chemotaxis was observed in the samples of lipids that were oxidized in vitro using the AAPH radical generating system. The

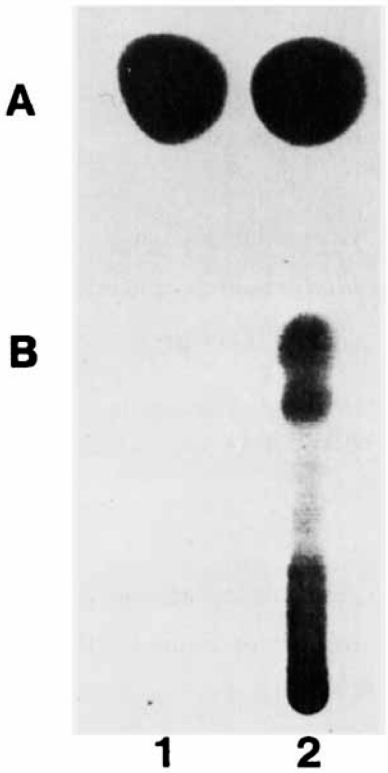

Fig. 2. Thin layer chromatogram of air-oxidized methyl docosahexaenoate Channel 1, commercially pure 22:6; channel 2, air-oxidized 22:6; A, unchanged fatty acid methyl esters (R_f=0.58-0.67); B, hydroperoxy fatty acid methyl esters (R_f=0.29-0.38).

assays using reduced concentrations of the same lipids (1 mg/ml) yielded similar increases in chemotaxis. Using the commercially available docosahexaenoic acid, the chemotactic activity of the air-oxidized fatty acid was found to be 3-fold higher than that of the unoxidized form ($P < 0.01$).

DISCUSSION

Inflammatory processes, including uveitis, appear to occur in two steps: induction, followed by perpetuation or amplification. In the amplification phase of EAU, chemotactic factors are produced that serve to attract PMNs and other inflammatory cells to the site of inflammation. In the case of EAU induced by S-antigen, the infiltration of PMNs and macrophages can be seen in the retina, as well as in the uvea. These leukocytes then release inflammatory mediators, including oxygen metabolites(9).

In inflammation, several biologically active lipids, such as the arachidonic acid metabolites, have been implicated as chemotactic factors. In this study, we have shown that the lipids isolated from the retinas of animals

Table 1. Chemotactic ability as measured by Boyden chamber analysis

Source of chemoattractant	Concentration of lipids (mg/ml)	Chemotaxis (Net PMNs/HPF*)	p value†
Retinal lipid extracted from normal rats (control)	10.0	0.3 ± 3.4	
	1.0	0.3 ± 0.1	
Retinal lipid extracted from EAU rats	10.0	28.2 ± 0.4	< 0.01
	1.0	3.7 ± 1.3	< 0.01
Retinal lipid extracted from normal rats and oxidized via AAPH in vitro	10.0	28.7 ± 6.6	< 0.01
	1.0	2.0 ± 1.0	< 0.01
AAPH alone		.0 ± 3.6	
Unoxidized, pure methyl docosahexaenoate§ (control for oxidized form)	1.04.1 ± 1.9		
	0.5	2.3 ± 1.2	
Oxidized methyl docosahexaenoate§	1.0	13.4 ± 1.2	< 0.05
	0.5	7.2 ± 1.2	< 0.05

*PMNs/HPF = Number of polymorphonuclear neutrophils per high power field. Shown as mean ± standard deviation.
†Compared with control.
§Refers to commercially available methyl docosahexaenoate.

with EAU have chemotactic ability. These chemotactic properties could be due to the peroxidized retinal lipids, since lipids isolated from control animals also showed chemotactic abilities when they were exposed in vitro to an effective radical generating system, AAPH. This system is a well known radical initiator, and the spontaneous decomposition of this azo compound to free radicals is followed by reactions with oxygen molecules, rapidly yielding peroxyl radicals(10). The oxidized polyunsaturated fatty acids have been found to possess chemotactic properties(11).

In the outer segment of photoreceptors, the membrane phospholipids contain more than 50 mole percent of docosahexaenoic acid (22:6), and only a small percentage of arachidonic acid(12). Arachidonic acid is derived from dietary omega-6 series and is the precursor of prostaglandins and thromboxanes. Docosahexaenoic acid, on the other hand, is derived from dietary omega-3 series, and cannot take part in the synthesis of arachidonic acid pathway products. In EAU, the reactive oxygen radicals released by PMNs are capable of oxidizing retinal polyunsaturated fatty acids and it has been shown the 22:6 is oxidized during this process(9). The peroxidized 22:6 was found to be mostly hydroperoxides(9, 13). As one of the detoxification mechanism, tissue glutathione converts hydroperoxides to the corresponding hydroxy fatty acids. However, this protective mechanism might have been destroyed by the initial influx of reactive oxygen species(9). In the peroxidation of 22:6, there are ten possible isomers of hydroperoxides, resulting from the five sets of 1, 4-dienes in the molecule. Using mass spectrometry/gas chromatography, we have positively identified five major isomers of hydroperoxide derived hydroxydocosahexaenoic acids from the inflamed retinas(9).

Lipid peroxidation products are known to lead to cell edema and increased vascular permeability(14). We have found that the most abundant polyunsaturated fatty acid in photoreceptor membranes, 22:6, upon subjecting to PMN-mediated peroxidation could be chemotactic. This, undoubtedly, contributes to further elicitation of PMNs and thus amplifies the inflammatory process. In this study, the contribution from other chemotactic membrane lipids was not excluded, the studies are being conducted to refine the system, thus to evaluate the contribution from other chemotactic factors, in particular oxygenated arachidonic products.

ACKNOWLEDGMENTS

Supported in part by grant EY O5662 from the National Institutes of Health and by an unrestricted grant from Research to Prevent Blindness, Inc., New York, NY.
Ann Dawson, medical editor, reviewed this manuscript.

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