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Significance and Immunoassay of 9- and 13-Hydroxyoctadecadienoic Acids

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Linoleic acid, the predominant polyunsaturated fatty acid in the diet, can be metabolized by cyclooxygenase, lipoxygenase and P450 enzymes. The monohydroxy lipoxygenation products of linoleic acid, 9- and 13-hydroxyoctadecadienoic acids (9(S)- and 13(S)-HODEs), are the most widely distributed of the known linoleic acid metabolites. These compounds exhibit interesting biological activities, including regulation of platelet function, maintenance of vascular thromboresistance and transduction of the cellular responses to certain growth factors. In view of their biological significance, we have produced polyclonal antibodies for the first time to these bioactive lipids to develop an easy, inexpensive, sensitive, specific and rapid enzyme immunoassay method for these bioactive lipids.

Linoleic acid, the predominant polyunsaturated fatty acid in the human diet, is a major component of membrane fatty acids in leukocytes and other tissues (1–3). Linoleic acid is also a major component of triglyceride stores in endothelial cells (4). More recently, oxidized derivatives of linoleic acid itself have been identified in a variety of tissues. These linoleic acid metabolites have been shown to exhibit a number of interesting biological functions (5,6). Recent evidence indicates that these compounds may have important functions in cellular regulation (7,8).

Linoleic acid metabolism. Linoleic acid can be oxidized by several enzymes. Lipoxygenase (LX) enzymes are widely distributed. They were first shown to metabolize arachidonate to a variety of biologically active molecules, including leukotrienes and hydroperoxy/hydroxyeicosatetraenoic acids (HPETE/HETE's). LX's which specifically oxygenate arachidonate at positions 5(5-LX), 12(12-LX) or 15(15-LX) have been identified in mammalian tissue (9). LX enzymes have also been shown to metabolize linoleic acid to HODEs in several tissues. 12-LX has been reported to produce 13-HODE in porcine (1) and bovine leukocytes (10). Significant epoxy and trihydroxy derivatives were also detected in porcine leukocytes, although they may be produced by non-enzymatic mechanisms (11). Linoleic acid was also shown to be a better substrate than arachidonate for the porcine leukocyte 12-LX. The portion of linoleic acid (22%) in the membrane phospholipids of leukocytes is much greater than arachidonate (8%), suggesting that linoleic acid is a more readily available substrate than arachidonate for this LX enzyme (2).

Monohydroxy derivatives of linoleic acid and their regulatory roles. In mammals, monohydroxy derivatives of linoleic acid are produced by various cells of the circulatory system and vascular endothelium (12). Both platelets and endothelial cells produce 9-HODE and 13-HODE. 13-HODE appears to have important roles in the regulation of platelet function and in the regulation of interactions between platelets and the vascular endothelium (19). 13-HODE causes an inhibition of thrombin-induced thromboxane B_2 production and stimulation of 12-HETE production in platelets, and in the stimulation of prostacyclin synthesis by endothelial cells (12).

In addition to the regulatory influences of 13-HODE on vascular interactions, monohydroxy

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Abbreviations: HETE, hydroxyicosatetraenoic acid., oxo-ODE, oxo-octadecadienoic acid., HODE, hydroxyoctadecadienoic acid., ELISA, enzyme linked immunosorbent assay.

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metabolites of linoleic acid may also have roles in the pathology of atherosclerosis (13). As discussed above, endothelial cells metabolize linoleic acid to both 9-HODE and 13-HODE. Cholesterol esters of 9-HODE and 13-HODE are found in atherosclerotic plaques and, the presence of 9-HODE and 13-HODE cholesterol esters are particularly associated with more advanced atherosclerotic lesions (13). These findings suggest a role for monohydroxy linoleic acid derivatives in atherosclerosis, which is in distinct contrast to their proposed role in vascular thromboresistance.

Recent studies (14) suggest that 13-hydroperoxy and 13-hydroxy derivatives of linoleic acid may function through the regulation of cellular responses to growth factors. They have shown that 13-HPODE and 13-HODE stimulate the induction of DNA synthesis in BALB/c 3T3 fibroblasts in response to epidermal growth factor.

Similarly, exogenous application of 13-HODE and 13-HPODE is mitogenic to colonic epithelium (15). Numerous epidemiological and animal studies have demonstrated a strong correlation between high-fat, high linoleate, diets and the development of colon cancer. Therefore, it is possible the mitogenic effects of linoleic acid metabolites play a role in enhancing the development of colon cancer.

The studies highlighted above strongly support important physiologic and pathophysiologic roles for 9-HODE and/or 13-HODE. Further progress in our understanding of the biochemical actions of these compounds, including their involvement in tumor growth, requires the development of a facile method for the detection and quantitation of 9/13-HODE in physiological samples.

MATERIALS AND METHODS

9-HODE and 13-HODE were enzymatically produced from linoleic acid (18:2) using soybean 15-lipoxygenase (Sigma Chemicals, St. Louis, MO) and HPLC-purified. The purity of these compounds was higher than 98%. 9/13-HODEs were coupled to keyhole limpet hemocyanin (KLH) by the dicyclohexylcarbodiimide method as described by Levine et al. (16). Aliquots of the 9-HODE-KLH and 13-HODE-KLH conjugates were administered to goats as follows: one hundred fifty micrograms of conjugate in Complete Freund's Adjuvant was administered at multiple subcutaneous sites for the primary immunization. At subsequent 2 week intervals, 100 μg of conjugate in Incomplete Freund's Adjuvant were administered at multiple subcutaneous sites to boost the titer. Serum (500 mL per bleed) was collected one week following each boost and titers were determined by ELISA using 9/13-HODE conjugated to ovalbumin as detailed below.

Costar high-binding strip well microplates were coated with 9/13-HODE-ovalbumin by the addition of 50 μL/well of a 0.02 μg/mL solution of 9/13-HODE-ovalbumin in Dulbecco's Phosphate-Buffered Saline, pH 7.4 (DPBS). Plates were then covered with parafilm. Plates were initially incubated for 2 hrs at room temperature. However, overnight incubation was determined to improve reproducibility and all subsequent experiments employed overnight plate coating. The coated wells were gently washed five times with tris buffered saline (TBS) pH 7.5, non-specific sites were blocked by the addition of 5% non-fat dried milk in TBS, pH 7.5. plates were incubated for one hour at room temperature and washed with deionized water as above. Serial dilutions of the anti-9/13-HODE sera, 50 μl prepared in TBS, were added to triplicate wells, after

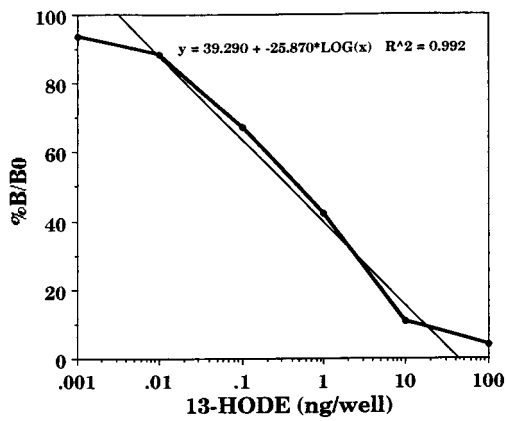

FIG. 1. A typical standard curve showing the reaction of anti-13-hydroxyoctadecadienoic acid with 13-hydroxyoctadecadienoic acid.

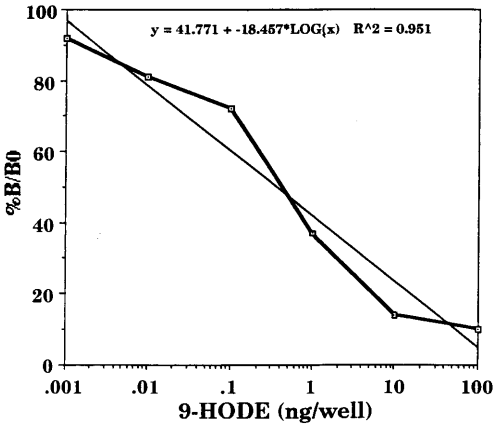

FIG. 2. A typical standard curve showing the reaction of anti-9-hydroxyoctadecadienoic acid with 9-hydroxyoctadecadienoic acid.

which the plates were incubated for one hour at room temperature and washed with deionized water as above. Wells were again blocked with 5% non-fat dried milk in TBS as above. Donkey anti-goat IgG coupled to horseradish peroxidase (2°-HRP) was used as a secondary antibody at 1:2500 dilution with the same binding conditions as for the primary antisera. Following incubation with the 2°-HRP, wells were washed and the color was developed by the addition of 150 μL of 3,3′,5,5′-tetramethylbenzidine reagent (TMB), a substrate for HRP that is converted to a colored product. The plate was then incubated for 15 min, the reaction was stopped by addition of 75 μL of 1N H_2SO_4, and the absorbance at 450 nm was determined using a microtiter plate reader. Non-specific binding to uncoated plates was negligible.

Immunoglobulins were purified from goat antisera by caprylic acid method as described by Reik et al. [17]. The protein concentrations of 9-HODE and 13-HODE IgG were determined which were found to be 23.6 mg/mL and 38 mg/mL respectively. The IgGs were aliquoted and stored at −20°C and used for subsequent experiments. After subtraction of blank, the mean maximum absorbance reading (B_0) in the absence of unconjugated 9/13-HODE was determined.

To develop a solid phase competitive ELISA for 9-HODE and 13-HODE, 9-HODE and 13-HODE were directly coupled to Horseradish peroxidase (Sigma Chemicals, St. Louis, MO) by the carbodiimide method as described for KLH conjugation. To obtain suitable conditions for the development of solid phase competitive ELISAs for 9-HODE and 13-HODE, a wide variety of assay parameters have been evaluated. During these studies it was found that coating of wells with 1.9 μg (13-HODE-IgG) and 23.6 μg (9-HODE-IgG) and working dilutions of 13-HODE-HRP at 1:1000 dilutions and 9-HODE-HRP at 1:5000 diltutions gave optimal conditions to get the standard curves.

RESULTS AND DISCUSSION

In order to be useful for potential clinical diagnostic applications, it is critical that an ELISA be able to specifically measure a given analyte without interference due to other compounds in complex physiologic fluids, and that it be sensitive enough to detect levels of the analyte that are present in normal individuals. The sensitivity of the assay was assessed simply by performing the assay with increasing dilutions of authentic 9-(S)HODE and 13-(S)HODE, and plotting absorbance

TABLE 1
Cross-reactivity of Anti 9-HODE

9(S)-HODE	**100.0**%
9(R)-HODE	100.0%
13(S)-HODE	1.2%
13(R)-HODE	1.2%
15(S)-HETE	0.0%
11(S)-HETE	0.0%
Linoleic acid	0.0%
13-oxo-ODE	2.4%
9-oxo-ODE	1.2%

TABLE 2
Cross-reactivity of Anti 13-HODE

13(S)-HODE	**100.0**%
13(R)-HODE	11.7%
9(S)-HODE	0.6%
9(R)-HODE	0.8%
15(S)-HETE	6.0%
11(S)-HETE	2.7%
Linoleic acid	0.5%
13-oxo-ODE	0.4%
9-oxo-ODE	11.7%

versus the log of the 9-(S)HODE and 13-(S)HODE concentration. Since the sensitivity of the ELISA is dependent on the concentration of certain assay components (e.g. the IgG bound per microtiter well, enzyme-9/13-HODE and/or standard ligand used per well), the effect of each assay parameter was systematically evaluated and optimized for sensitivity and reproducibility. The standard curves for 9-HODE and 13-HODE are typical of several that were performed after optimizing assay parameters (Figures 1 & 2). For these experiments, the r^2 values for the fit of the data to an equation describing an inverse logarithmic relationship of free 9/13-HODE to B/Bo was 0.990. For several independent runs, r^2 values ranged from 0.950-0.990 indicating that this prototype ELISA is highly reproducible.

The specificity of the prototype two separate ELISAs was investigated using authentic 9/13-HODE and a panel of structurally related compounds such as 9 (R)-HODE, 13 (R)-HODE, 11 (S)-HETE, 15 (S)-HETE, 9-oxo-ODE and 13-oxo-ODE. Based on their structure, might be anticipated to compete with 9-HODE and 13-HODE for binding to the polyclonal antibody that was produced and thus interfere with such assays if they are not specific. Results are presented in tables 1 and 2. It should be noted that the specificity of a given polyclonal antibody is determined in part by the nature and purity of the immunogen and the protocols used for immunization, but is also largely dependent on the immune response of a given animal. We found that that the competitive enzyme immunoassay developed in this study was very very sensitive for 9/13-HODE so that as little as 10 pg can be quantitated. The high specificity of the anti-13-HODE antibody is more than adequate for specific determination of 13(S)-HODE in biological fluids by ELISA. Although the antisera thus far produced against 9(S)-HODE reacted equally well with 9(R)-HODE, they did not crossreact with any of the other test compounds and should still prove be suitable for assays of 9-HODE in biological system. The high specificity indicated of this assay (Tables 1 & 2) is more than adequate for specific determination of 9/13-HODE in biological fluids by ELISA. The high specificity indicated of this assay (tables 1 & 2) is more than adequate for specific determination of 9/13-HODE in biological fluids by ELISA.

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