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Toxoplasma gondii Stimulates the Release of 13- and 9-Hydroxyoctadecadienoic Acids by Human Platelets†

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Received October 17, 1991; Revised Manuscript Received March 25, 1992

ABSTRACT: We have recently demonstrated a novel cytotoxic effect of human platelets against *Toxoplasma gondii* and a role for thromboxane (TX) in this process (Yong et al., 1991). We now report on the spectrum of lipid mediators released by human platelets after interaction with *T. gondii*. In addition to TXB_2, human platelets after incubation with *T. gondii* for 90 min released 12-hydroxyheptadecatrienoic acid (12-HHT), 12-hydroxyeicosatetraenoic acid (12-HETE), and an unidentified peak (UV_{max} 234 nm) as determined by reverse-phase high-performance liquid chromatography. Thermospray-liquid chromatography/mass spectrometry analysis and straight-phase HPLC identified the unknown peak as a mixture of 13-hydroxyoctadecadienoic acid (HODE) and 9-HODE. Radiolabeling studies with [^{14}C]linoleic acid indicated that the platelets were the cellular source of the octadecanoids with 13-HODE (87.7%) > 9-HODE (12.3%). Inhibitor studies with indomethacin indicated that 13-HODE was a lipoxygenase product and 9-HODE was a cyclooxygenase product of linoleic acid. Thus, *Toxoplasma*-stimulated platelets release oxygenated products of both arachidonic acid and linoleic acid which may be important in the host response to *T. gondii* infection.

Toxoplasma gondii is an important pathogen in immunocompromised individuals, especially in patients with the acquired immunodeficiency syndrome (AIDS)[1] (Pfefferkorn, 1990; Yong et al., 1991). *T. gondii* can exert a variety of antiinflammatory effects including inhibition of mononuclear phagocyte respiratory burst activity (Wilson et al., 1980), phagolysosomal fusion (Jones & Hirsch, 1972), and 5-lipoxygenase activity (Locksley et al., 1985). For example, LTB_4, LTC_4, and LTD_4 formation in murine peritoneal macrophages is blocked by intracellular parasitism with *Toxoplasma* organisms (Locksley et al., 1985). Macrophages activated by cytokines such as γ-interferon (Murray et al., 1985; Suzuki et al., 1988), monocytes (Wilson & Remington, 1979; Murray et al., 1985), neutrophils (Wilson & Remington, 1979), and recently platelets (Ridel et al., 1988; Auriault et al., 1990; Yong et al., 1991) have been implicated in the host defense against *T. gondii* infection because of their toxoplasmacidal activities.

Platelets may interact with *T. gondii* during the bloodstream dissemination of the parasites throughout the body that occurs

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[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; DEAE, diethylaminoethyl; DEAEC, diethylaminoethyl chloride; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; 12-HETE, 12-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid; 12-HHT, 12-hydroxy-5(*Z*),8(*E*),10(*E*)-heptadecatrienoic acid; HODE, hydroxyoctadecadienoic acid; 9-HODE, 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-HODE, 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid; 12-HPETE, 12-hydroperoxy-5(*Z*),8(*Z*),10(*E*),14-(*Z*)-eicosatetraenoic acid; IFA, indirect fluorescence antibody; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; PBS, phosphate-buffered saline; RIA, radioimmunoassay; TSP-LC/MS, thermospray-liquid chromatography/mass spectrometry; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2.

0006-2960/92/0431-5356$03.00/0

early in the disease process. *T. gondii* parasitemia has been demonstrated in acute infections in humans (Kayhoe et al., 1957; Frenkel et al., 1960) and in immunocompromised patients with reactivation toxoplasmosis (Shepp et al., 1985; Hofflin & Remington, 1985). Ridel et al. (1988) have demonstrated that *Toxoplasma* tachyzoites are killed by rat platelets removed during infection with this parasite. Further, the adoptive transfer of these platelets into Nu/Nu Fischer rats which are highly susceptible to *Toxoplasma* infection conferred significant protection against the organisms. These data indicate that, in immunocompromised rats, platelets are effector cells which limit *T. gondii* dissemination. We have recently demonstrated that human platelets exert cytotoxic activity against tachyzoites of *T. gondii* (Yong et al., 1991); cytotoxicity was associated with the release of the arachidonic acid metabolite TXA_2 by the *Toxoplasma*-stimulated platelets (Yong et al., 1991).

In the present study, we have examined whether other biologically active lipid mediators of inflammation are generated as a consequence of the interaction of human platelets with *T. gondii*. We report that human platelets release the octadecanoids 13-HODE and 9-HODE from endogenous linoleic acid when stimulated by *Toxoplasma*. Further, *Toxoplasma*-activated platelets release the eicosanoids 12-HHT and 12-HETE in addition to thromboxane.

Experimental Procedures

Materials. 12(*S*)-HETE, 9(*S*)-HODE, 13(*S*)-HODE, and 12(*S*)-HHT were obtained from Cayman Chemical Co. (Ann Arbor, MI), 9(*R*)-HODE was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), 13(*R*)-HODE was from Cascade Biochem Limited (Reading, England), Dulbecco's PBS and Ca^{2+}/Mg^{2+}-free PBS were from GIBCO Laboratories (Grand Island, NY), K_2EDTA and indomethacin were from Sigma Chemical Co. (St. Louis, MO), and DEAEC was from Aldrich Chemical Co., Inc. (Milwaukee, WI). Indomethacin was dissolved at a concentration of 10^{-2} M in Tris buffer (0.1 mL), pH 8.1, with further dilutions made in PBS.

T. gondii. The RH strain of *T. gondii* (generously provided by Dr. C. B. Wilson, University of Washington) was maintained by intraperitoneal passage in Balb/c mice (Locksley et al., 1985). After peritoneal harvesting in Ca^{2+}/Mg^{2+}-free PBS and filtration through a 3-μm polycarbonate filter (Nucleopore, Pleasanton, CA) for separation from leukocytes, *Toxoplasma* were centrifuged at 1000*g* for 15 min at 4 °C and washed twice with Ca^{2+}/Mg^{2+}-free PBS before resuspension in PBS. Greater than 95% of these *T. gondii* tachyzoites were viable as assessed by trypan blue dye exclusion.

Human Platelets. Platelets were isolated from normal human volunteers who were negative for *Toxoplasma*-specific IgM and IgG antibodies as determined by an IFA test (Microbiological Research Corp., Bountiful, UT) as previously described (Yong et al., 1991). The absence of prior acute or chronic infection with *T. gondii* was indicated by the respective lack of fluorescence at a 1:8 serum IgM–IFA titer and at a 1:16 IgG–IFA titer. After collection of platelet-rich plasma in K_2EDTA, the platelets were centrifuged at 1000*g* for 15 min at 4 °C, washed twice in Ca^{2+}/Mg^{2+}-free PBS, and resuspended in PBS immediately prior to use. There was less than 0.05% contamination with nucleated cells in the platelet preparations.

Platelet–T. gondii Reaction Mixtures. Human platelets, *T. gondii*, and other components of the reaction mixtures were incubated in PBS, pH 7.2, at the concentrations indicated in the legends to the figures and tables. The reactions (2-mL total volume each) were performed in 12 × 75 mm polystyrene tubes in an oscillating water bath at 37 °C for 90 min. For kinetic studies on the rates of formation of arachidonic acid and linoleic acid metabolites, the reactions were stopped by the addition of 8 mL of ice-cold methanol. The reaction mixtures were centrifuged at 1000*g* for 15 min at 4 °C. The supernatants were collected and stored at −70 °C prior to chromatography or mass spectral analysis.

Reverse-Phase HPLC. Arachidonic acid and linoleic acid metabolites were assayed in each supernatant by reverse-phase HPLC. After the addition of HPLC-grade water to the samples to make a final methanol:water ratio of 20:80 (v/v), the samples underwent solid-phase extraction using octadecyl Baker-10 columns (J. T. Baker Chemical Co., Phillipsburg, NJ). After evaporation to dryness and reconstitution in methanol, the samples were centrifuged at 3000*g* for 5 min at 4 °C. The clear supernatants underwent reverse-phase HPLC on a 5-μm particle size Ultrasphere ODS C_{18} column (4.6 × 250 mm, Beckman Instruments, Inc., Berkeley, CA) using a Hewlett-Packard Model 1090 HPLC system (Hewlett-Packard Co., Palo Alto, CA). The solvent employed was methanol/water/acetic acid (75:25:0.01 v/v/v), pH 4.7, at a flow rate of 1 mL/min (HPLC condition I). Eluting peaks were monitored simultaneously by rapid UV spectral scanning at up to eight different wavelengths (190–600 nm) using a Hewlett-Packard Model 1040 diode array detector. Identified peaks were quantitated using the Hewlett-Packard Model DPU multichannel integrator. Peaks that coeluted with authentic octadecanoid and eicosanoid standards were collected and rechromatographed on a 3-μm particle size Beckman Ultrasphere ODS C_{18} column (4.6 × 75 mm) using the same solvent at a flow rate of 1.6 mL/min (HPLC condition II).

TSP-LC/MS. Analyses were performed on a Vestec Model 201 dedicated TSP-LC/MS instrument (Vestec Corp., Houston, TX), equipped with a directly-heated probe vaporizer, and mass spectra were acquired in the positive ion mode. A Hewlett-Packard 59979C ChemStation data system (Palo Alto, CA) was used to control data acquisition. The mass spectrometer was operated in the full scan mode (200–550 Da). The HPLC system consisted of two LKB Model 2150 HPLC pumps (LKB-Produkter AB, Bromma, Sweden). The separation for TSP-LC/MS analyses was performed on a 5-μm particle size Beckman Ultrasphere ODS C_{18} column (4.6 × 150 mm). For underivatized samples, the mobile phase consisted of acetonitrile/water containing 0.05 M ammonium acetate buffer (60:40 v/v) adjusted to pH 4.0 with acetic acid and was delivered at a flow rate of 1.2 mL/min (HPLC condition III). Under these conditions, the TSP interface temperatures were as follows: T_1 (control) = 110–115 °C, T_2 (tip) = 174–177 °C, T_3 (jet) = 221–225 °C, T_4 (source block) = 275–280 °C, and lens = 125–130 °C.

In some studies, the products of the incubations and reference standards were derivatized using DEAEC to form the DEAE esters as previously described by Voyksner et al. (1987). For the DEAE ester derivatives, HPLC pump A was connected to the HPLC column and delivered a mobile phase consisting of acetonitrile/water (90:10 v/v) containing 0.5 g/L ammonium acetate at a flow rate of 1.2 mL/min. HPLC pump B was used for post-column addition of acetonitrile/0.1 M ammonium acetate buffer (20:80 v/v) through a tee-joint connected at right angles to the column effluent and in-line with the TSP interface. This additive, which was delivered at a flow rate of 0.75 mL/min, served to optimize the sensitivity of the HPLC/MS system by increasing the ammonium acetate and water content of the effluent entering the TSP ion source. Under these conditions (HPLC condition IV), the TSP in-

terface temperatures were optimized as follows: T_1 = 105 °C, T_2 = 190 °C, T_3 = 195 °C, T_4 = 280 °C, and lens = 125–130 °C.

Straight-Phase and Chiral-Phase HPLC. In selected experiments to determine the cellular source and identity of linoleic acid metabolites, either the platelets or *Toxoplasma* were preincubated with [^{14}C]linoleic acid (950 mCi/mmol; NEN, Wilmington, DE) in the absence of unlabeled linoleic acid. [^{14}C]Linoleic acid metabolites (i.e., 9-HODE and 13-HODE) released by platelet–*T. gondii* reaction mixtures were resolved by straight-phase HPLC using a Hewlett-Packard Hypersil–silica column (5-μm particle size; 2.1 × 200 mm); the solvent employed was hexane/2-propanol/acetic acid (85.5:14.5:0.02 v/v/v) at a flow rate of 1 mL/min (HPLC condition V). A radioactive flow detector (FLO-ONE/Beta, Model IC; Radiomatic Instruments & Chemical Co., Inc., Meriden, CT) was employed to detect the radioactivity of radiolabeled eluting peaks using Radiomatic Flo-Scint I at a ratio of 2:1 (scintillant/HPLC solvent) (Martin et al., 1987).

Chiral-phase HPLC of linoleic acid metabolites was performed as previously described by Kühn et al. (1987). Unlabeled platelet–*T. gondii* reaction mixture supernatants first underwent straight-phase HPLC (HPLC condition V) to resolve 13-HODE from 9-HODE. 13-HODE generated by *T. gondii*-stimulated platelets and authentic 13(*S*)-HODE and 13(*R*)-HODE standards (Cayman Chemical Co.) were converted to their methyl esters with excess diazomethane in diethyl ether. These methyl esters then underwent chiral-phase HPLC on a 5-μm particle size Bakerbond DNBPG (covalent) chiral HPLC column (4.6 × 250 mm; J. T. Baker Chemical Co., Phillipsburg, NJ). Isocratic elution of products was performed with hexane/2-propanol (99.6:0.4 v/v) at a flow rate of 1 mL/min (HPLC condition VI).

RIA. TXB_2, the stable hydrolysis product of TXA_2, was assayed by RIA. TXB_2 was assayed by the measurement of competitive inhibition of [^{3}H]TXB_2 to anti-TXB_2 binding. The TXB_2 antiserum was produced in rabbits as previously reported (Geissler & Henderson, 1988). The TXB_2 antiserum at a dilution of 1:100 000 had a sensitivity of 1 pg/0.1-mL sample and the following cross-reactivities at B/B_0: PGD_2, 0.53%; $PGF_{2\alpha}$ <0.2%; 6-keto-PGE_1, PGE_2, $PGF_{1\alpha}$, and 6-keto-$PGF_{1\alpha}$, each <0.02%. [^{3}H]TXB_2 tracer was obtained from New England Nuclear (Boston, MA) and unlabeled TXB_2 standard from Cayman Chemical Co. (Ann Arbor, MI). In preparation for RIA, the methanol in the samples was evaporated using a SpeedVac concentrator (Model SVC-100H; Savant Instruments, Inc., Farmingdale, NY) with a −60 °C refrigerated condensation trap. The samples were reconstituted in 10 mM potassium phosphate buffer, pH 7.3, containing 1 mM Na_2-EDTA and 0.25 mM thimerasol. The assays were performed in duplicate according to standard protocols (Fitzpatrick, 1982).

Statistical Analysis. The data are reported as the mean ± SE of the combined experiments. Differences were analyzed for significance using Student's two-tailed *t*-test for independent means (not significant, $p > 0.05$).

Results

Reverse-Phase HPLC of Platelet–T. gondii Reaction Mixtures. No UV peaks at 235 nm were identified after reverse-phase HPLC of supernatants obtained from human platelets incubated in buffer alone (Figure 1A). In contrast, chromatography of the supernatants resulting from the interaction of platelets with *Toxoplasma* at a ratio of 5:1 (platelets:*Toxoplasma*) for 90 min resolved UV peaks (235 nm) at 10.4, 17.5, and 21.1 min (Figure 1B). Peaks at 10.4 and 21.1 min coeluted with authentic 12-HHT and 12-HETE standards, respectively, on rechromatography. The UV_{max} of the unknown peak X was 234 nm (Figure 1B) in comparison to 237 nm for 12-HETE (not shown).

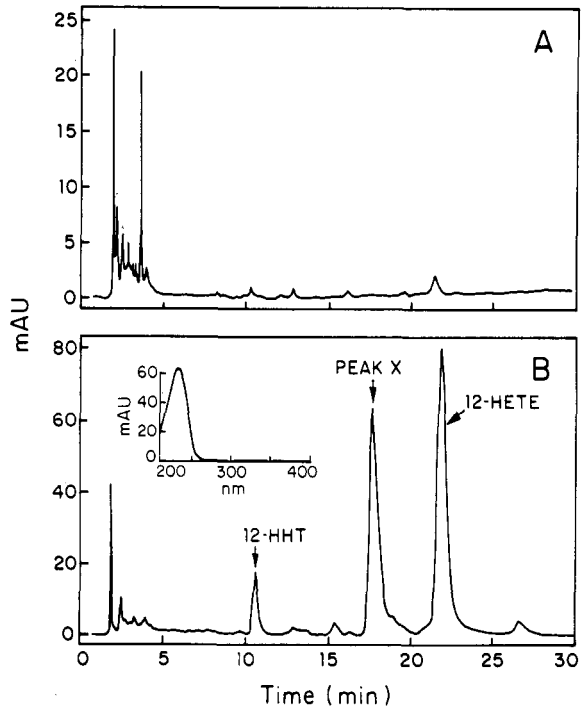

FIGURE 1: Platelet–*T. gondii* interaction. Reverse-phase HPLC chromatograms (HPLC condition I, as described under Experimental Procedures) of supernatants of human platelets (2×10^8) incubated in the absence (A) or presence (B) of *T. gondii* (4×10^7) in 2 mL of PBS for 90 min at 37 °C. UV absorbance [milliabsorbance units (mAU)] was measured at 235 nm. The peaks at 10.4 and 21.1 min in (B) coeluted with 12-HHT and 12-HETE standards, respectively. The unidentified peak X (17.5 min) had an UV_{max} of 234 nm (panel B insert).

Characterization of the Products of Platelet–T. gondii Interactions by TSP-LC/MS. The concentrated supernatant obtained from pooled samples of rechromatographed peak X and 12-HETE (Figure 1B) from the platelet–*T. gondii* reaction mixtures was analyzed by TSP-LC/MS. The chromatogram (not shown) obtained showed two peaks at 8.3 and 10.7 min (HPLC condition III). The latter peak was determined to be 12-HETE both by cochromatography with authentic standard and by giving an identical mass spectrum (Figure 2A) as authentic 12-HETE. The spectrum showed an ammoniated molecular ion at *m/z* 338. Also present were ions at *m/z* 320 and 303 due to loss of H_2O from the ammoniated molecular ion and from the protonated molecular ion, respectively (Voyksner & Bush, 1987). The unknown peak X at 8.3 min gave the spectrum shown in Figure 2B. The spectrum showed three major ions at *m/z* 314, 296, and 279. The mass differences between these three ions were the same as in the case of 12-HETE. This suggests that the ion at *m/z* 314 is an ammoniated ion that loses H_2O to give the ion at *m/z* 296, and the ion at *m/z* 279 is due to loss of H_2O from a protonated molecular ion at *m/z* 297. Therefore, a tentative molecular mass for this (these) compound(s) would be 296 Da in comparison to 320 Da for 12-HETE. The mass difference between peak X and 12-HETE (24 Da) and structural similarity to 12-HETE by TSP-LC/MS suggested that the unknown peak was one or more mono-HODEs such as 9-HODE and/or 13-HODE.

To confirm the molecular mass of the unknown compound, 12-HETE and peak X were derivatized to their DEAE esters

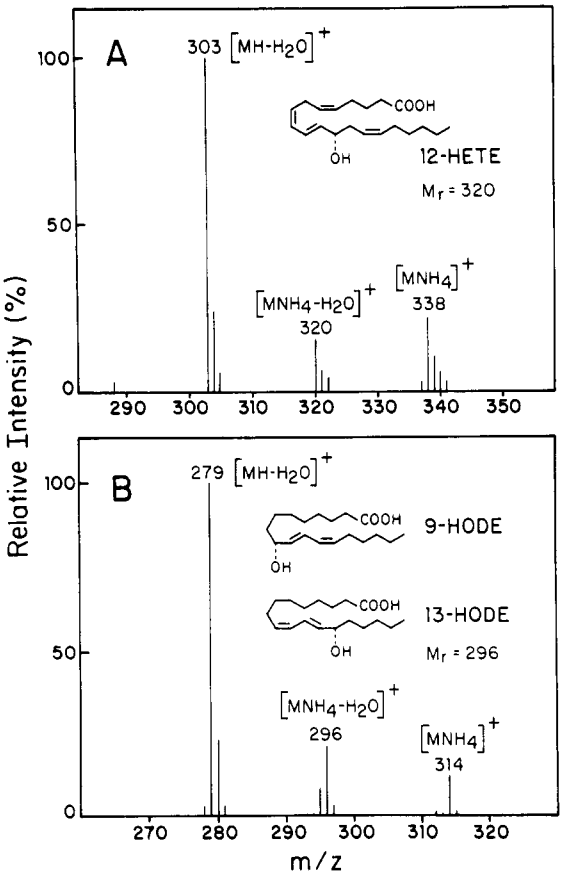

FIGURE 2: TSP-LC/MS spectra of underivatized peak X and 12-HETE from platelet–*T. gondii* reaction mixtures. Using HPLC condition III, the spectrum in (A) is for the peak eluting at 10.7 min and the spectrum in (B) is for the peak eluting at 8.3 min.

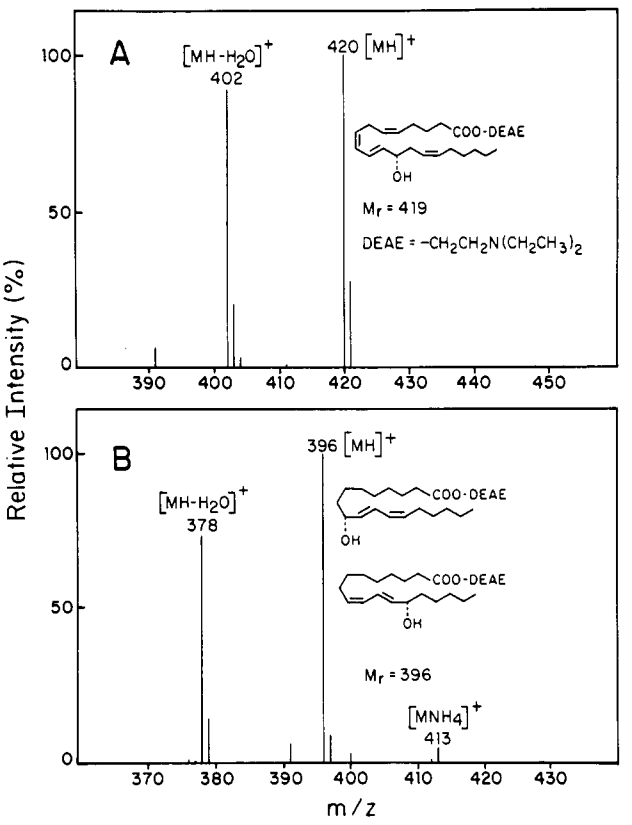

FIGURE 3: TSP-LC/MS spectra of DEAE-derivatized samples of 12-HETE and peak X using HPLC condition IV: (A) spectrum of the DEAE ester of 12-HETE; (B) spectrum of the DEAE ester of peak X.

and analyzed by TSP-LC/MS. The spectrum of the DEAE ester of 12-HETE showed a major ion at m/z 420 $[M + H]^+$ (Figure 3A), which represents the expected 99-Da shift from the $[M + H]^+$ ion for underivatized 12-HETE. The major ion in the spectrum of the peak X DEAE ester was at m/z 396 (Figure 3B), which is also a 99-Da mass shift from the suggested $[M + H]^+$ of the underivatized sample, confirming a moleuclar mass of 296 Da for the unknown compound(s). The spectrum also showed a weak ammoniated adduct at m/z 413 and ions at m/z 378 corresponding to the $[M + H - H_2O]^+$ ion. When authentic 9-HODE and 13-HODE were derivatized to their DEAE esters and analyzed by TSP-LC/MS, they each cochromatographed with and gave the same mass spectra as the DEAE ester of peak X, indicating that the unknown compound was 9- and/or 13-HODE.

The rates of formation of arachidonic acid and linoleic acid metabolites by human platelets stimulated by *T. gondii* were compared. TXB_2, 12-HHT, and 12-HETE were more rapidly released than HODEs by the *Toxoplasma*-stimulated platelets (Figure 4). By 5 min of incubation, the mean percentage of maximal release for the three arachidonic acid products (TXB_2, 12-HHT, and 12-HETE) was 64.2% in comparison to 14% for the linoleic acid products (9-/13-HODE) (Figure 4). To determine the cellular source and identity of the HODEs produced by the reaction mixtures, platelets that had been preincubated with [^{14}C]linoleic acid were washed and incubated with unlabeled *T. gondii* for 60 min at 37 °C; 99.4 ± 0.7% (n = 2) of the total radioactivity released by the *T. gondii*-stimulated platelets comigrated with peak X (i.e., 9-/13-HODE) by reverse-phase HPLC. Since the reverse-phase system does not resolve 9-HODE from 13-HODE, the reaction mixtures underwent straight-phase HPLC, and radiolabeled 13-HODE (87.7 ± 4.7%; n = 3) and 9-HODE (12.3 ± 4.7%; n = 3) were recovered (Figure 5A). In contrast, when unlabeled platelets were incubated with *Toxoplasma* that had been preincubated with [^{14}C]linoleic acid for 30 min and extensively washed prior to use, no radiolabeled products were recovered by straight-phase HPLC (Figure 5B). Chiral-phase HPLC indicated that 13-HODE generated by *Toxoplasma*-stimulated platelets was predominantly of the *S* configuration (Figure 6).

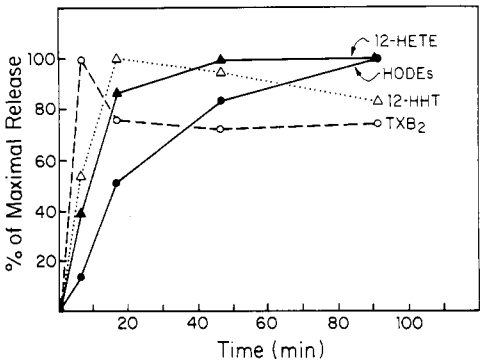

FIGURE 4: Rates of formation of arachidonic acid and linoleic acid products by *T. gondii*-stimulated platelets. Human platelets (2×10^8) were incubated with *T. gondii* (4×10^7) in 2 mL of PBS at 37 °C with the reactions stopped at varying time periods up to 90 min by the addition of 8 mL of ice-cold methanol. The extracted supernatants underwent either RIA for the TXB_2 assay or reverse-phase HPLC (HPLC condition I) for the 12-HHT, 12-HETE, and 9-/13-HODE assays. The percentage of maximal release for TXB_2 (○), 12-HHT (△), 12-HETE (▲), and HODEs (9-/13-HODE; ●) is shown. The data are the mean of two studies.

Effect of Cyclooxygenase Inhibition on 9- and 13-HODE Formation. Oxygenation of linoleic acid to HODEs can be catalyzed by either fatty acid cyclooxygenase or a lipoxygenase.

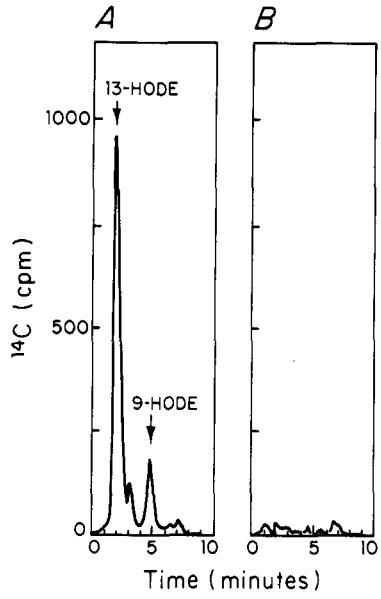

FIGURE 5: Cellular source of 13-HODE and 9-HODE. Human platelets (7.5×10^8) that had been incubated for 30 min with [^{14}C]linoleic acid were washed three times and incubated with *T. gondii* (1.5×10^8) in 2 mL of PBS for 60 min (A). Unlabeled platelets (7.5×10^8) were incubated in 2 mL of PBS for 60 min with *T. gondii* that had been preincubated with [^{14}C]linoleic acid for 30 min and washed three times before use (B). The supernatants underwent straight-phase HPLC using condition V.

Table I: Effect of Indomethacin on 9-HODE, 13-HODE, and 12-HETE Release by *T. gondii*-Stimulated Platelets[a]

indomethacin (M)	% of control		
	9-HODE	13-HODE	12-HETE
10^{-5}	2.2 ± 2.2 (5)[b]	206.7 ± 28.4 (5)	197.8 ± 31.5 (5)
10^{-6}	44.8 ± 23.5 (7)	152.6 ± 20.2 (7)	193.4 ± 25.1 (7)
10^{-7}	62.0 ± 23.3 (6)	125.2 ± 20.3 (6)	149.5 ± 40.2 (6)
10^{-8}	103.0 ± 12.2 (4)	113.5 ± 34.6 (4)	98.3 ± 25.1 (4)

[a] The *T. gondii*–platelet reaction mixture was the same as described in Figure 1B with indomethacin added at the concentrations indicated. The supernatants from the reaction mixtures underwent straight-phase HPLC (HPLC condition V, as described under Experimental Procedures). The amount of 9-HODE, 13-HODE, and 12-HETE released by the *T. gondii*–platelet reaction mixtures in the presence of indomethacin was expressed as percent release compared to control (i.e., *T. gondii*–platelet reaction mixtures in the absence of indomethacin). [b] Mean ± SE of (*n*) experiments.

The effect of indomethacin upon formation of 9-HODE and 13-HODE by the platelet–*T. gondii* reaction mixtures was undertaken to determine whether the compounds were formed by a fatty acid cyclooxygenase-catalyzed oxygenation of linoleic acid. 9-HODE biosynthesis was inhibited in a concentration-dependent manner with 97.8% inhibition by 10^{-5} M indomethacin, indicating that 9-HODE is a cyclooxygenase product of linoleic acid metabolism (Table I). In contrast, 13-HODE release by the platelet–*T. gondii* reaction mixtures was increased approximately 2-fold in the presence of 10^{-5} M indomethacin, suggesting that this octadecanoid may be formed by a lipoxygenase-catalyzed oxygenation of linoleic acid. 12-HETE formation by the reaction mixtures was similarly augmented by cyclooxygenase blockade to further suggest that 13-HODE is a lipoxygenase product.

Discussion

These data indicate that human platelets release oxygenated derivatives of both linoleic acid (13-HODE and 9-HODE) and arachidonic acid (12-HETE, 12-HHT, and TXA_2) after interaction with *T. gondii* tachyzoites. To our knowledge, this is the first study demonstrating that a stimulus (i.e., *T. gondii*) of pathophysiological relevance can induce the release of 13- and 9-HODE from endogenous linoleic acid by human platelets. Previous studies by Daret et al. (1989) have indicated that human platelets can convert exogenous linoleic acid to mono-HODEs; our work identifies a novel stimulus for octadecanoid synthesis by platelets. TSP-LC/MS was employed to identify 13-HODE and 9-HODE in the platelet–*T. gondii* reaction mixtures. Radiolabeling studies with [^{14}C]-linoleic acid indicated that 13-HODE (87.7%) was the predominant octadecanoid released and that platelets were the cellular source of the HODEs. As with human leukocytes which synthesize 13(*S*)-HODE (Reinaud et al., 1989), the platelet-derived 13-HODE in our study was primarily of *S*. chirality. The chiral purity of 13-HODE is consistent with enzymatically-catalyzed oxygenation of linoleic acid by *T. gondii*-stimulated platelets.

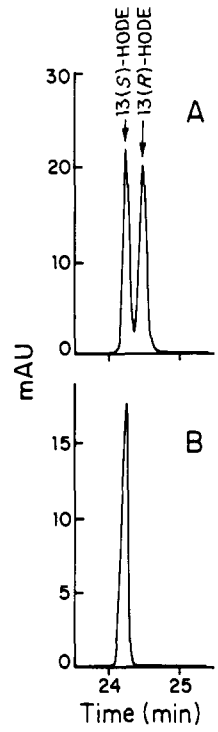

FIGURE 6: Chirality of 13-HODE. After methylation, synthetic 13(*S*)- and 13(*R*)-HODE (panel A) and 13-HODE generated by the *T. gondii*-stimulated platelets (Panel B) underwent chiral-phase HPLC (HPLC condition VI). UV absorbance [milliabsorbance units (mAU)] was measured at 235 nm.

Daret et al. (1989) have demonstrated that human platelets convert exogenous linoleic acid to 13-HODE (85%) and 9-HODE (15%) at a rate similar to which they convert [^{14}C]-arachidonic acid to [^{14}C]-12-HETE. 12-Lipoxygenase of bovine polymorphonuclear leukocytes also transforms linoleic acid to 13-HODE and arachidonic acid to 12-HETE at similar rates (Walstra et al., 1987). In *Toxoplasma*-stimulated human platelets, the rates of formation of arachidonic acid metabolites are more rapid than that of linoleic acid products. Both cyclooxygenase and lipoxygenase systems have been implicated in the formation of HODEs from exogenous linoleic acid in human platelets (Daret et al., 1989). In our study of HODE production, we found that *T. gondii*-stimulated platelet formation of 9-HODE was dependent on prostaglandin endoperoxide synthase (cyclooxygenase) since 9-HODE release was inhibited by indomethacin ($IC_{50} \simeq 5 \times 10^{-7}$ M). 9-HODE formation by guinea pig alveolar macrophages is similarly catalyzed by cyclooxygenase (Engels et al., 1986).

Although 15-lipoxygenase may contribute to 13-HODE formation by endothelial cells (Buchanan et al., 1985), Kaduce et al. (1989) have reported that cyclooxygenase is primarily responsible for both 9-HODE and 13-HODE formation in human endothelial cells. The conversion of linoleic acid to 9-HODE and 13-HODE in rabbit VX_2 carcinoma tissue (Hubbard et al., 1980), rabbit peritoneal tissue (Claeys et al., 1982), and sheep vesicular gland tissue is also mediated by cyclooxygenase (Hamberg & Samuelsson, 1967).

In contrast, we found that generation of 13-HODE like 12-HETE was augmented by indomethacin at concentrations $\geq 10^{-7}$ M, suggesting that this octadecanoid is generated by a lipoxygenase-catalyzed reaction by *T. gondii*-stimulated platelets. Lipoxygenases have previously been demonstrated to catalyze formation of 13-HODE by rat and rabbit aortae (Funk & Powell, 1985), human neutrophils (Soberman et al., 1985), and rat and human epidermis (Nugteren & Kivits, 1987). 15-Lipoxygenase has been implicated in this transformation of linoleic acid by neutrophils (Soberman et al., 1985) and skin (Nugteren & Kivits, 1987).

15-Lipoxygenase most likely accounts for the formation of 13-HODE by *T. gondii*-stimulated human platelets. 15-Lipoxygenase activity has been demonstrated in human platelets (Wong et al., 1985). Linoleic acid is a poor substrate (<5% conversion) for 12-lipoxygenase from either disrupted (Nugteren, 1975) or intact (Walstra et al., 1987) bovine platelets. The bovine platelet 12-lipoxygenase has an ω-9 specificity for cis double bonds (Nugteren, 1975), whereas an ω-6 specificity is required for 13-HODE formation. Our data (i.e., release of 12-HETE, TXB_2, and 13-HODE but not 15-HETE by *Toxoplasma*-stimulated human platelets) suggest that 12-lipoxygenase and PGH synthase/TXA_2 synthase utilize arachidonic acid as substrate, whereas 15-lipoxygenase uses linoleic acid preferentially over arachidonic acid as substrate. Similarly, when human platelets are incubated with exogenous arachidonic acid (1.3×10^{-5} M) in the absence or presence of bradykinin (10^{-7} M), the formation of 12-lipoxygenase products predominates over that of 15-lipoxygenase products (Wong et al., 1985). Human polymorphonuclear leukocyte 15-lipoxygenase acts at the ω-6 position of cis-unsaturated fatty acids to preferentially form 13-H(P)ODE from linoleic acid over 15-H(P)ETE from arachidonic acid (Soberman et al., 1985). Endothelial cells which contain 15-lipoxygenase activity (Hopkins et al., 1984; Mayer et al., 1986) generate 13-HODE from endogenous linoleic acid stores but do not form 15-HETE from endogenous arachidonic acid stores (Buchanan et al., 1985).

The octadecanoids and eicosanoids released by *T. gondii*-stimulated platelets have various biologic activities that may influence host inflammatory reactions against *Toxoplasma* organisms in vivo. For example, 13-HODE stimulates release of both PGI_2 by endothelial cells (Kaduce et al., 1989; Yamaja Setty et al., 1987a) and 12-HETE by platelets (Yamaja Setty et al., 1987b). We have recently found that exogenous 13-(*S*)-HODE at concentrations $\geq 10^{-8}$ M has toxoplasmacidal activity causing disruption of surface membranes and cytoplasmic contents of the organisms as demonstrated by transmission electron microscopy (Henderson & Chi, 1992). In contrast, linoleic acid and 12(*S*)-HETE over the same concentration range do not exert cytotoxic activity against the *Toxoplasma*. 12-HETE and 12-HHT have chemokinetic and chemotactic activity for human leukocytes (Goetzl & Gorman, 1978). 12-HETE also induces degranulation of specific granules in human neutrophils (Stenson & Parker, 1980), and its precursor 12-HPETE activates 5-lipoxygenase in human neutrophils (Maclouf et al., 1982; Kanaji et al., 1986). Such effects could amplify the immune response of phagocytic cells against *Toxoplasma*.

In summary, we have presented novel findings that human platelets synthesize 9- and 13-HODE from endogenous linoleic acid as well as lipoxygenase and cyclooxygenase products of arachidonic acid when activated by the pathogen *T. gondii*. The extracellular release of biologically active linoleic acid and arachidonic acid metabolites by platelets may be important in the host inflammatory response against these organisms.

Acknowledgments

We thank Dr. Thomas A. Baillie and Dr. Walter C. Hubbard for valuable discussions during the course of these studies and for critical review of the manuscript, the Mass Spectrometry Facility of the School of Pharmacy, University of Washington, Seattle, Margot McCready for technical help, and Rachel Norris for typing the manuscript.

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Conformational Equilibrium of an Enzyme Catalytic Site in the Allosteric Transition†

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Received December 9, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: The dynamic equilibrium of a catalytic site between active and inactive conformations, the missing link between the structure and function of allosteric enzymes, was identified using protein engineering and NMR techniques. Kinetic analyses of the wild-type and three mutants of *Thermus* L-lactate dehydrogenase established that the allosteric property of the enzyme is associated with a concerted transition between the high-affinity (R) and low-affinity (T) states. By introducing mutations, we prepared an enzyme in which the R and T states were balanced. The conformation of the enzyme-bound coenzyme, NAD^+, which interacts directly with the substrate, was analyzed using NMR spectroscopy. NAD^+ bound to the mutant enzyme was in a conformational mixture of the active and inactive forms, while NAD^+ took on predominantly one of the two forms when it was bound to the other enzymes we had analyzed. We interpret this to mean that the catalytic site is in equilibrium between the two conformations. The ratio of the coformers of each enzyme agreed with the [T]/[R] ratio as determined by kinetic analyses. Therefore, it is the identified conformational equilibrium of the catalytic site that governs the allosteric regulation of the enzyme activity.

The allosteric regulation of enzyme activity has been attributed to the transition of the protein conformation between two states since the proposal of relevant models in the 60's (Monod et al., 1965; Koshland et al., 1966). Crystallographic studies on several enzymes have led to the successful identification of two different conformations, which explain the difference in activity between the two states (Kantrowitz & Lipscomb, 1988; Barford & Johnson, 1989; Gouaux & Lipscomb, 1990; Schirmer & Evans, 1990; Ke et al., 1991). On the other hand, we know little about the dynamic transition of allosteric enzymes between different conformations, though almost all the structural research on allostericity was based on the assumption of a two-state transition. Even for the global conformation, there have been few demonstrations of the allosteric transition (Hervé et al., 1985; Eisenstein et al., 1990). In particular, we know nothing about the conformational

† This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. S.K. was supported by a fellowship from the Japan Society for the Promotion of Science.

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0006-2960/92/0431-5362$03.00/0