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Anti-Inflammatory Actions of Neuroprotectin D1/Protectin D1 and Its Natural Stereoisomers: Assignments of Dihydroxy-Containing Docosatrienes

Charles N. Serhan, Katherine Gotlinger, Song Hong, Ya-Lu, Jeffrey Siegelman, Tamara Baer, Rong Yang, Sean P. Colgan, and Nicos A. Petasis

Protectin D1, neuroprotectin D1 when generated by neural cells, is a member of a new family of bioactive products generated from docosahexaenoic acid. The complete stereochemistry of protectin D1 (10,17-docosatriene), namely, chirality of the carbon-10 alcohol and geometry of the conjugated triene, required for bioactivity remained to be assigned. To this end, protectin D1/ neuroprotectin D1 (PD1) generated by human neutrophils during murine peritonitis and by neural tissues was separated from natural isomers and subjected to liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry. Comparisons with six 10,17-dihydroxydocosatrienes prepared by total organic and biogenic synthesis showed that PD1 from human cells carrying potent bioactivity is 10R,17-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid. Additional isomers identified included trace amounts of 15-trans-PD1 (isomer III), 10S,17-dihydroxy-docosa-4Z,7Z,11E,13Z,15E,19Z-hexaenoic acid (isomer IV), and a double dioxygenation product 10S,17-dihydroxy-docosa-4Z,7Z,11E,13Z,15E,19Z-hexaenoic acid (isomer I), present in exudates. 18O2 labeling showed that 10S,17-diHDHA (isomer I) carried 18O in the carbon-10 position alcohol, indicating sequential lipooxygenation, whereas PD1 formation proceeded via an epoxide. PD1 at 10 nM attenuated (50%) human neutrophil transmigration, whereas 15-trans-PD1 was essentially inactive. PD1 was a potent regulator of polymorphonuclear leukocyte (PMN) infiltration (~40% at 1 ng/mouse) in peritonitis. The rank order at 1- to 10-ng dose was PD1 ≈ PD1 methyl ester ≫ 15-trans-PD1 > 10S,17-diHDHA (isomer I). 10S,17-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid (isomer VI) proved ≥ PD1 in blocking PMN infiltration, but was not a major product of leukocytes. PD1 also reduced PMN infiltration after initiation (2 h) of inflammation and was additive with resolvin E1. These results indicate that PD1 is a potent stereoselective anti-inflammatory molecule. The Journal of Immunology, 2006, 176: 1848–1859.

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2 Address correspondence and reprint requests to Dr. Charles N. Serhan, Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative, and Pain Medicine, Brigham and Women’s Hospital, 75 Francis Street, Boston, MA 02115. E-mail address: cserhan@zeus.bwh.harvard.edu

3 Abbreviations used in this paper: LT, leukotriene; LX, lipoxin; PMN, polymorphonuclear leukocyte; DHA, C22:6, docosahexaenoic acid; EPA, C20:5, eicosapentaenoic acid; NPD1, neuroprotectin D1; LO, lipooxygenase; PD1, protectin D1/ neuroprotectin D1; pSLLO, 5-LO from potato; LC, liquid chromatography; MS, tandem mass spectrometry; GC, gas chromatography; 17β-Hp(DHA), 17β-hydroperoxy)-DHA; NMR, nuclear magnetic resonance; 10S,17-diHDHA, 10S,17-dihydroxy-docosa-4Z,7Z,11E,13Z,15E,19Z-hexaenoic acid (the dioxygenation product); RvE1, resolvin E1, 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid.
During spontaneous resolution, cell-cell interactions and transcellular biosynthesis lead to the production of these new families of potent bioactive lipid mediators from omega-3 essential fatty acid precursors, and were termed resolvins (resolution phase interaction products derived from docosahexaenoic acid (DHA, C22:6) and eicosapentaenoic acid (EPA, C20:5)) and protectins (docosatrienes derived from DHA; Refs. 1, 3, and 18 and recently reviewed in Ref. 19). These novel di- and tri-hydroxy-containing products from EPA and DHA that are generated by previously unrecognized enzymatic pathways display potent anti-inflammatory and immunoregulatory actions in vitro and in vivo in murine models of acute inflammatory actions (1, 3, 18).

In 1929, the omega-3 polyunsaturated fatty acids were assigned essential roles because their exclusion from the diet gave rise to a new form of deficiency disease (20). Many recent reports document the importance of fish oil (omega-3) fatty acids EPA and DHA in human diseases associated with inflammation. In particular, omega-3 DHA and EPA are protective in inflammatory bowel disease and colitis (21), cardiovascular disease (22–25), and Alzheimer’s disease (26). However, the molecular mechanisms responsible for these well-documented beneficial actions of omega-3 fatty acids remain an important challenge. DHA is enriched in neural tissues, where it appears to play functional as well as structural roles (27, 28). Along these lines, results from earlier studies indicated that DHA was enzymatically converted to products coined docosanoids, which might be linked to retinal protection (29) and neuronal function (30). The structures of the molecules involved, however, were not established.

Human whole blood isolated leukocytes, and glial cells enzymatically convert DHA to 17S-hydroxy-containing docosanoides and 17S-series resolvins (1, 3). The novel 10,17S-docosatriene, first identified in Ref. 3 and its basic structure established, displayed potent anti-inflammatory actions, i.e., reducing PMN numbers in exudates in vivo and down-regulating production of proinflammatory cytokines by glial cells in vitro (1). During the resolution phase of peritonitis, unesterified DHA levels increase within exudates, and 10,17S-docosatriene is generated within the resolving exudates, where it appears to promote catabasis, or the return to homeostasis, by shortening the resolution interval (31). Of special interest, this DHA-derived 10,17S-docosatriene is generated in vivo during strokes in murine tissues and limits the entry of leukocytes into the area of neural damage, reducing the magnitude of tissue injury (32). In collaboration with Bazan and colleagues (2), we found that 10,17S-docosatriene is neuroprotective in retinal pigmented cells and introduced the term neuroprotectin D1 (NPD1) for this potent compound, which accumulates in the ipsilateral hemisphere of the brain following focal ischemia (33).

Recent results indicate that NPD1 is formed from DHA in cornea in a lipoxigenase (LO)-dependent fashion to protect from thermal injury as well as promote wound healing (34). It is noteworthy that NPD1, resolin D1, and resolin D5 are all produced by trout brain cells from endogenous DHA, suggesting that the structures of these DHA-derived mediators are conserved from fish to humans (35). Together, these recent findings underscore the need to establish the complete stereochromy of endogenous biologically active 10,17S-docosatriene, namely its carbon-10 position alcohol chirality and double bond geometry of its conjugated triene system. In recognition of its wide scope of formation and actions, protectin D1/neuroprotectin D1 (PD1) is used to denote the structure of this chemical mediator, and the prefix neuro before protectin D1 is used to note its tissue origin and address. In this study, we report the complete stereochromy of protectin D1 and its related natural isomers (i.e., Δ15-trans-PD1) as well as their anti-inflammatory properties.

### Materials and Methods

#### Materials

Zymosan A, soybean LO (fraction V), and calcium ionophore, A-23187, were purchased from Sigma-Aldrich. DHA and 5-LO from potato (pt5LO) were obtained from Cayman Chemical. Additional materials used in liquid chromatography (LC)-UV-tandem mass spectrometry (MS-MS) analyses were obtained from vendors reported in Refs. 1 and 3. 18O2 isotope was purchased from Cambridge Isotopes.

#### Isolation, LC-MS-MS, and gas chromatography (GC)-MS analyses

Incubations were extracted with deuterium-labeled internal standard (prostaglandin E2) (Cayman Chemicals) for LC-MS-MS analysis using a Finnigan LCQ LC ion trap tandem mass spectrometer equipped with a LUNA C18-2 (150 × 2 mm 5 µm) column and a rapid spectra scanning UV diode array detector using mobile phase (methanol:water:acetate at 65:35:0.01) with a 0.2 ml/min flow rate that monitored UV absorbance ~0.1 min before samples entered the MS-MS. The scan acquisition rates were 11/min for MS-MS and 60/min for UV, which give rise to a lag interval in retention times that was corrected for the results presented for each molecule. All intact cell incubations and in vivo exudates were stopped with 2 vol cold methanol and kept at 20°C for >30 min. Samples were extracted using C18 solid phase extraction and further analyzed using GC-MS, using a Hewlett-Packard 6890 with a HP 5973 mass detector (Table 1), and LC-MS-MS. Detailed procedures for isolation, quantitation, and structural determination of these DHA and related lipid-derived mediators, as reported recently in Refs. 1 and 36, were used in this study for elucidation of new products. Biogenic synthesis of some of the DHA-derived products were performed using isolated enzymes, i.e., 5-LOX from potato and 15-LO were each incubated in tandem sequential reactions (see Refs. 1, 3, 37, and 38) with either DHA, 17S-hydroxy-DHA, or 17S-hydro(peroxy)-DHA (17S-Hp(DHA)) to produce the compounds in quantities suitable for isolation and incubation with cells and tissues as well as confirmation of physical properties and assignment of biological actions. Incubations in an 18O2-enriched atmosphere were performed and analyzed as in Ref. 39.

#### Nuclear magnetic resonance (NMR) for protectin D1

1H NMR (400 MHz, MeOH-d4) were as follows: δ 6.52 (dd, J = 14.1 Hz and 11.8 Hz, 1H), 6.26 (m, 2H), 6.07 (dd, J = 11.1 Hz and 11.1 Hz, 1H), 5.50–5.28 (m, 7H), 4.90 (s, 2H), 5.50–5.60 (m, 2H), 4.55 (m, 1H), 4.14 (m, 1H), 3.65 (s, 3H), 2.82 (m, 2H), 2.40–2.13 (m, 8H), 2.06 (m, 2H), 2.07 (m, 2H), 0.96 (t, J = 7.5 Hz, 3H).

13C NMR (125 Hz, MeOH-d4) were as follows: δ 174.93, 137.59, 134.56, 134.47, 134.35, 131.01, 130.52, 130.17, 129.92, 128.57, 128.52, 126.14, 124.89, 72.60, 68.18, 36.00, 35.97, 34.45, 26.30, 23.43, 21.31, 14.20. The total organic synthesis of PD1 and related isomers will be reported separately (3).

#### Incubations with human PMN and whole mouse brain

Human venous whole blood (~10 ml) was collected into heparin (0.01 U/ml) via venipuncture from healthy volunteers (who declined taking medication for 2 wk before donation; Brigham and Women’s Hospital protocol 88-02642). Human PMN were freshly isolated from whole blood using Ficoll gradient and enumerated as described in Ref. 40. PMN (30 × 106 cells/ml) were exposed to ionophore A23187 (5 µM) with either DHA or 17S-Hp(DHA) (15 µg/ml) Cell suspensions were incubated for 30 min at 37°C in a covered water bath. Lipidomics and lipid mediator profile analyses were conducted for DHA-derived products, resolvins, and docosatrienes, as described in Refs. 1, 3, 18, and 36. Whole mouse brains (Charles River Laboratories) were excised, and then homogenized in PBS (minus Mg2+ and Ca2+) (Cambrex Bioscience). Brain homogenates were washed once (800 rpm, 4°C, 5 min) in PBS, and then suspended in 1 ml of PBS minus divalent cations. Homogenates were placed in the incubator (5 min) with an atmosphere of 5% CO2 at 37°C, and calcium ionophore A23187 (5 µM) or DHA (30 µM) was added. Brain homogenates were then placed in the incubator for 30 min, with an atmosphere of 5% CO2 (37°C). Incubations were stopped with 2 vol of ice-cold MeOH, and, after 30 min at 4°C, the suspensions were pelleted, and the supernatants were extracted using solid phase C18 cartridges (Alltech Associates). Material eluted with methanol was taken to dryness using a stream of nitrogen gas, and resulting material was further analyzed.

#### Human neutrophil transmigration

PMN were freshly isolated from whole blood obtained by venipuncture from healthy human donors (who declined taking medication for 2 wk before
donation; Brigham and Women’s Hospital (Boston, MA), protocol no. 88-02642) and anticoagulated with acid citrate dextrose as described in Refs. 40 and 41. Briefly, plasma and mononuclear cells were removed by aspiration from the buffy coat after centrifugation (400 × g; 20 min) at room temperature. Histopaque (density 1.077) was obtained from Sigma-Aldrich. RBCs were sedimented using 2% gelatin, and residual RBCs were removed by lysis in ice-cold NH4Cl buffer. The cell suspensions were >90% PMN, as determined by light microscopic evaluation. PMN were suspended at 5 × 10⁶ cells/ml in HBSS with 10 mM HEPES (pH 7.4) and without Ca²⁺ or Mg²⁺ (Sigma-Aldrich). PMN were used within 2 h of their isolation.

Human microvascular endothelial cells (HMEC; a gift from Francisco Candal, Centers for Disease Control, Atlanta, GA) were obtained as primary cultures. For preparation of experimental HMEC monolayers, confluent 5-mm-thick cultures were grown on 0.33-cm² ring-supported polycarbonate filters (5-µm pore size; Costar) in the apical-to-basolateral direction. Cells were grown for ~1 wk before transmigration experiments.

Transmigration was conducted essentially as described in Ref. 41. PMN were incubated with either vehicle-containing buffer or compound for 15 min at 37°C. A chemoattractant gradient was established by placing HMEC inserts that had been washed in HBSS with Ca²⁺ and Mg²⁺ (denoted "+/+" in 10⁻⁴ M LTB₄ in the lower chambers. Neutrophils (10⁶ cells) were added to 50 µl of HBSS "+/+" in the upper chambers, and cells were incubated at 37°C for 90 min. Transmigrated PMN were quantified by assessing the PMN azurophilic marker myeloperoxidase and a calibration curve. PMN were lysed by the addition of Triton X-100 to a final concentration of 0.5%. The samples were acidified with citrate buffer (final concentration 100 mM; pH 4.2). An aliquot of each sample was added to an equal volume of ABTS solution (1 mM ABTS, 0.05% H₂O₂, 100 mM sodium citrate buffer pH 4.2) in a 96-well plate. The resulting color was monitored using a plate reader at 405 nm, and a calibration curve was used to determine cell number.

Acute inflammatory exudates: murine peritonitis

Peritonitis was conducted using 6- to 8-wk-old FVB male mice (Charles River Laboratories) that were fed laboratory Rodent Diet 5001 (Purina Mills) that were anesthetized with isoflurane, and compounds to be tested were administered i.p. Zymosan A in 1 ml of saline (1 mg/ml) was injected i.p. 1–1.5 min later in the peritoneum. Each compound tested or vehicle alone was suspended in 1 ml of ethanol and mixed in sterile saline (120 µl). After i.p. injections (either 2 or 4 h of acute inflammation), the mice were sacrificed in accordance with the Harvard Medical Area Standing Committee on Animals (protocol no. 02570), and peritoneal lavages were collected rapidly and placed in an ice bath (4°C) for enumeration, differential counts, and further analysis.

### Results

**Complete stereochemistry of PD1**

The DHA-derived 10,17-dihydroxy conjugated triene-containing product PD1 is generated by several human cell types, murine exudates, skin, and brain tissues (1–3, 32), as well as isolated fish tissues, indicating that it is a conserved structure in evolution (35). PD1 displays potent protective and anti-inflammatory actions (1, 2, 18, 32). To determine the complete stereochemical assignment of PD1 (i.e., the chirality at C10 and the geometry of the triene), we directly compared the physical and biological properties of DHA-derived PD1 and related 10,17 dihydroxy-docosatriene stereoisomers to those prepared by total organic synthesis and biogenic synthesis. These included the following, as illustrated in Figs. 1 and 2: compound I, 10S,17S-dihydroxy-docosa-4Z,7Z,11E,13Z,15Z,19Z-hexaenoic acid; compound II, 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid, coelute in this HPLC system; compound III, 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid, coelute in this HPLC system; compound IV, 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid, coelute in this HPLC system; compound V, 10S,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid, coelute in this HPLC system; compound VI, 10S,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid. The total synthesis of these will be reported elsewhere (N. A. Petasis, R. Yang, and C. N. Serhan, manuscript in preparation). Of interest, compounds I and VI coelute in this system, as did both compounds II and V (Figs. 1 and 2). Bioactive PD1 was generated by isolated human cells and murine brain tissue and during inflammation in vivo. Fig. 1 shows representative chromatographic profiles for PD1 generated by isolated human neutrophils incubated with DHA that was separated into several positional and geometric isomers (Fig. 1A, top panel). The main positional isomer of 10,17S-docosatriene was 7,17-diHDHA (denoted as resolvlin D5), as documented earlier (1, 3), and a double dioxygenation product (37). Also, a representative profile of products is given for those obtained from murine inflammatory exudates (Fig. 1A, middle panel) and neural tissues (data not shown). A direct comparison of these materials is shown in Fig. 1A, along with the profile obtained for synthetic materials (Fig. 1A, bottom panel) and chromatograms recorded by MS-MS (Fig. 1, right side) and UV at 270 nm (Fig. 1A, left side).

The complete stereochemistry of bioactive 10,17-docosatriene, PD1, namely the double bond geometry of the conjugated triene unit and chirality of its carbon-10 position remained to be established (Fig. 2, top middle). To assign the complete stereochemistry of bioactive PD1 and its related natural geometric isomers, it was necessary to carry out total organic synthesis and side-by-side matching experiments with murine and human systems because PD1 is generated in only nanogram quantities commensurate with its potent actions in vivo and in vitro, but preclude direct NMR analysis. The mixture of synthetic isomers used is shown in Fig. 1A, bottom. The human- and murine-generated bioactive PD1 matched the physical and biological properties of synthetic 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13Z,15Z,19Z-hexaenoic acid (compound II). In addition to both LC-MS-MS and GC-MS analyses with these materials (Table I), experiments were conducted with both biological and synthetic isomers prepared with the same overall backbone structure, namely 10,17-dihydroxydocosatriene. A 17R-containing isomer was prepared and included in these experiments (isomer V; Fig. 2), but could be eliminated in these assignments, because 17R-containing products are the major series produced from DHA when aspirin is used (3, 18). Thus, although compounds II and V coelute in this system, V could be eliminated as a major DHA-derived product in these incubations (Figs. 1 and 2).

The chirality of the alcohols and double bond geometry of the triene were systematically addressed. Fig. 1B shows the MS-MS spectrum of PD1 obtained from murine peritonitis (4 h) generated in vivo upon challenge with zymosan A. Fig. 1C shows the mass spectrum recorded using the same instrument settings and conditions with synthetic 10R,17S-diHDHA (compound II; Figs. 1 and 2). To obtain additional evidence for matching, GC-MS analyses were performed. Fig. 1D illustrates a representative mass spectrum and prominent ions obtained with GC-MS for PD1 treated with diazomethane and subsequently converted to its corresponding trimethylsilyl derivative. Hence, chromatographic behavior and prominent ions in two mass spectrometry systems (LC-MS-MS and GC-MS), together with biological activity (Figs. 5 and 6), permitted criteria for assignment of the physical properties of PD1 and related isomers. Because the parent and daughter ions were the same for each isomer, retention time in two chromatographic systems and bioactivity were key requirements for assigning the stereochemistry of the endogenous PD1 (vide infra).

Compounds synthesized for these matching experiments are given in Fig. 2. PD1 isolated and identified earlier carries alcohol groups at carbon-10 and -17 positions flanking the conjugated triene portion of this molecule (1, 3). The stereochemistry of the carbon-17 position alcohol was retained from the precursor predominantly in the S configuration when derived from the LOX product 17S-H(p)DHA precursor (1, 3), eliminating isomer V from the matching panel in Figs. 1 and 2. The double bond geometry...
and stereochemistry of the alcohol group at position 10 were tentatively assigned based on biogenic evidence, i.e., the formation of alcohol trapping products in murine brain and human leukocytes as well as identification of two vicinal diols 16,17-S-diHDHA; hence, the complete stereochemical assignment remained as illustrated in Fig. 2, top. To this end, each of the double bond isomers likely to be biosynthesized was prepared in view of potential biosynthetic routes involved in PD1 formation, namely, the involvement of epoxide-containing intermediates and/or double dioxygenation intermediates (1–3). The R and S configuration of the alcohol group at the carbon-10 position were each prepared by stereocontrolled total organic synthesis. The strategy for the synthesis of these is outlined in Fig. 3. Each of the stereocontrolled steps from defined precursors enabled preparation of geometric isomers of the conjugated triene region that were confirmed by NMR (see Materials and Methods) (N. A. Petasis, R. Yang, and C. N. Serhan, manuscript in preparation). Also, for these experiments, we prepared dihydroxydocosanoids using isolated plant LO(s) to obtain, as in earlier experiments (37), both positional isomers 7,17S-diHDHA and 10,17S-diHDHA (1, 3). The preparation of these isomers using micellar substrate was given in further detail in Ref. 37. These reference compounds were useful in analyses of biosynthetic routes.

Table I shows the prominent ions and chromatographic behaviors for each of the double bond and positional isomers prepared (Fig. 2). As expected, each of these isomers gave characteristic UV λ<sub>max</sub>MeOH for a conjugated triene chromophore with a λ<sub>max</sub>MeOH at ~270 nm, with shoulders at 260 and 282 nm (± 2 nm). Each isomer gave a specific λ<sub>max</sub>MeOH, which appeared to reflect the geometry of the double bond system. For example, the Δ15-trans isomer in the conjugated triene portion of 10,17-diHDHA gave an UV λ<sub>max</sub>MeOH of 269 nm (Table I). Only one of these products (compound II) matched the chromatographic behavior using both LC-MS and GC-MS as well as biological activity. As expected, each of the major prominent ions for these isomers in both LC-MS-MS and GC-MS were essentially identical (i.e., daughter and parent ions were essentially the same for each).

The main materials isolated from human PMN coeluted with compound II and, in some preparations, a trace amount of compound IV. Compound IV differs from compound II in its triene
configuration, which is 11E,13Z,15E. This change in two double bonds was unlikely in view of the earlier identification of alcohol trapping products (1). Also, compound IV was not observed in all PMN incubations, which might reflect some degree of donor variation. A second representative profile from another human donor is illustrated in Fig. 1A, top right panel, at m/z 260.8 to 261.8 of the MS-MS for M-1 at m/z = 359. As noted for this ion plot and donor, compound II is the most abundant, and compound IV is not present. Also, compound I is clearly present along with an unknown material denoted with an asterisk. In Fig. 1A, top right, the retention time of isomer III is plotted in gray for direct comparison. Only trace amounts of compound III, the 15-trans isomer of PD1, were routinely identified. The appearance of this 15-trans isomer with its triene in the all-trans configuration likely reflects workup-induced isomerization at the 15 position, which may account for its varied presence in LC-MS-MS-based analysis. This is in addition to 15-trans-PD1 formation via nonenzymatic hydrolysis of the proposed epoxide intermediate (Fig. 4B).

Compound I was consistently identified in profiles obtained from murine peritonitis (Fig. 1A, middle). However, it was not the major product of human cells nor did it carry potent actions as compound II did (Figs. 5 and 6), also reported earlier (1, 3, 39). Consistent with its biosynthesis (vide infra), the appearance of this double dioxygenation product was time-dependent in vivo and in vitro (data not shown). Although isomer VI coeluted with I in this system, it was excluded on the basis that it was not a major product of human cells and is not likely to be generated from an epoxide intermediate without a specialized enzyme (see Fig. 4 and Discussion). The stereoselective insertion of oxygen from H₂O can be expected to give rise to predominantly a 10R configuration when attaching a carbonium cation intermediate; proposed in Ref. 1. Compound VI differs from PD1 in its C10 position, which is 10S rather than 10R and is not a double dioxygenation product because its double bond geometry in the triene portion of the compound is not consistent with the biosynthesis of the triene in the trans,cis,trans configuration. Because these and other lipid mediators are highly conserved structures found in many species from fish to human (35), a species difference between mouse and human in PD1 structure is not likely. Hence, although compound VI carries bioactivity (Fig. 6), it was excluded on the basis of the above findings and because compound VI was not a major isomer in human profiles, as was compound II. Hence, compound II matched PD1 formation and physical properties as well as potency of action (see Figs. 5–7).

The biosynthesis of 7,17-diHDHA in inflammatory exudates (3, 18) and its formation from DHA or 17-hydroxy-DHA with isolated human neutrophils suggested that the biosynthesis of this compound involved formation via double dioxygenation (18). That is, in addition to using molecular oxygen for insertion at the 17 position, lipoxygenation could also insert molecular oxygen at the 7 position in sequential fashion. The identification of this novel compound from DHA and the sequential lipoxygenation events in its formation (1, 3) appeared to be similar to that of 5S,15S-dihydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid generated from arachidonic acid (42, 43). Hence, it was of particular interest in earlier studies (37) when sequential actions of pt5LO and/or 15-LO with...
The substrates in micellar configuration were noted to produce both 7,17-diHDHA and 10,17-diHDHA isomers as major products as well as multiple geometric isomers as minor products following hydrolysis of enzymatically generated epoxides in vitro (cf 1, cf 3). The formation of the minor isomers was dependent on substrate, pH, and enzyme concentration.

To test the role of sequential LO actions in the proposed mechanism of PD1 formation (compound II; Fig. 2) and its isomer 10\(^{\text{S}}\),17\(^{\text{S}}\)-diHDHA (compound I), incubations were conducted in an atmosphere enriched in isotope \(^{18}\text{O}_2\) with 17\(^{\text{S}}\)-H(p)DHA as substrate and isolated pSLO (see Materials and Methods). Note that compounds I and II differ in both chirality at carbon-10 as well as geometry of their respective triene configurations (Fig. 2). After extraction and isolation, the product profiles, GC-MS and LC-MS-MS results indicated that \(^{18}\text{O}\) was incorporated in the carbon-10 position in 10\(^{\text{S}}\),17\(^{\text{S}}\)-diHDHA (Fig. 4). Chromatographic separation of 10\(^{\text{S}}\),17\(^{\text{S}}\)-diHDHA (Fig. 4) gave prominent ions with MS-MS (Fig. 4A), indicating on average \(/\text{H}1^{10}22\) 75% incorporation of \(^{18}\text{O}\) originating from molecular oxygen in the carbon-10 position with a range of 51.4 to 91.8% increase in diagnostic ions. Because these enzymes use molecular oxygen as a substrate, it is not possible, under these conditions, to completely replace enzyme-associated \(^{16}\text{O}\) for the \(^{18}\text{O}\) isotope as calculated earlier for LXA4 in Refs. 39 and 44. The extent of \(^{18}\text{O}\) present in diagnostic ions was determined for m/z 181/183, 261/263, 289/291, 297/299, 315/317, 323/325, 341/343, and 359/361, and the ratio of \(^{16}\text{O}\):\(^{18}\text{O}\) was calculated from ion intensities and averaged. These results indicate that 10\(^{\text{S}}\),17\(^{\text{S}}\)-diHDHA can be produced via double lipoxygenation.

Results from matching studies indicated that the double bond geometry for the conjugated triene portion of this molecule was in the \(\text{trans,cis,trans}\) configuration (matching compound I; Fig. 2). Hence, double dioxygenation to form 10\(^{\text{S}}\),17\(^{\text{S}}\)-diHDHA was also a mechanism to generate this compound in vivo, because it is a prominent product in murine exudates from peritonitis, and, to some extent, present in suspensions of human leukocytes incubated with DHA (Fig. 1 and Ref. 1) and murine brain (3, 18), as well as trout leukocytes and brain (35). Fig. 4B outlines the proposed scheme and proposed role for double dioxygenation and its products 10\(^{\text{S}}\),17\(^{\text{S}}\)-diHDHA and 7\(^{\text{S}}\),17\(^{\text{S}}\)-diHDHA. The double bond geometry in the conjugated triene portion of the molecule

| Table 1. Physical and chemical properties by LC-MS-MS and GC-MS analyses |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Compound        | LDMS RT         | LCMS Major ions | GCMS Major ions | UV \(\lambda_{\text{max}}\) |
| 10,17S-diHDHA   | 30.2            | 310, 314, 315   | 518, 503, 499   | 290 nm          |
| 10,17S-diHDHA   | 30.2            | 310, 314, 315   | 518, 503, 499   | 290 nm          |
| 10,17S-diHDHA   | 30.2            | 310, 314, 315   | 518, 503, 499   | 290 nm          |
| 10,17S-diHDHA   | 30.2            | 310, 314, 315   | 518, 503, 499   | 290 nm          |
| 10,17S-diHDHA   | 30.2            | 310, 314, 315   | 518, 503, 499   | 290 nm          |
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| 10,17S-diHDHA   | 30.2            | 310, 314, 315   | 518, 503, 499   | 290 nm          |

\(^a\) LC-MS-MS was performed with a Finnigan LCQ liquid ion trap tandem mass-spectrometer equipped with a LUNA C18-150 (2 × 2 mm × 5 \(\mu\)m) column and a UV diode array detector using an isocratic mobile phase (MeOH:H₂O:AcOH at 65:35:0.01) with a 0.2 ml/min flow rate.

\(^*\) GC-MS was performed with a Hewlett-Packard 6890 equipped with a HP 5973 mass-detector. A HP5MS cross-linked 5% ME siloxane column (30 cm × 0.25 mm × 0.25 \(\mu\)m) was employed with a temperature program; the initial temperature was 150°C, followed by 230°C (2 min), and 280°C (10 min) with a helium flow rate of 1.0 ml/min. Trimethylsilyl derivatives were prepared with each compound following treatment with diazomethane.

\(^c\) Spectra were recorded in methanol using a Hewlett-Packard 8453 UV spectrophotometer with ± 2 nm accuracy.
(trans,cis,trans) is consistent with oxygenation, using molecular oxygen with two sequential lipoxigenation steps. Given the biological actions, chromatographic and physical properties of PD1 as well as the results from epoxide trapping experiments with human PMN, and the isolation of two vicinal diol 16,17-dihydroxy-docosatrienes as minor products (1), it is likely that, once a 16,17-epoxide-containing intermediate is generated in situ (as illustrated in Fig. 4), an enzymatic reaction is needed to efficiently produce PD1 carrying the 10R,17S-dihydroxy-trans,trans,cis configuration arising from an epoxide intermediate as depicted in Fig. 4B.

Anti-inflammatory actions of PD1
As indicated above, the complete stereochecmical assignment for synthetic PD1 also relied on determining biological action of the related isomers. Earlier results indicated that PD1’s anti-inflammatory properties were comprised of blocking leukocyte infiltration in murine systems (1, 3, 32, 37). Results in Fig. 5 show that synthetic PD1 reduced PMN transmigration in response to LTB4. Amounts as small as 1.0 nM gave 30% inhibition. The /H9004 15-trans isomer of PD1, where the conjugated triene portion of the molecule was in the trans configuration, did not block PMN transmigration in vitro. Although PD1 is a potent inhibitor in neutrophil transmigration, the degree of inhibition observed with monolayers of HMECs and human neutrophils from /H11022 5 separate donors did not achieve values greater than IC 50 in each experiment.

These experiments with transmigration were conducted in parallel with murine acute inflammation. In these experiments, acute peritonitis was initiated by challenge with the microbial isolate zymosan A, and the actions of five isomers were assessed in vivo. Two compounds (compound V and compound VI) were excluded from matching with PD1 because the physical retention times on LC and GC-MS (Fig. 1 and Table I) and biosynthetic considerations indicated that they were not likely candidates for endogenous human PD1 or isomers produced. It is noteworthy that PD1 (compound II; Fig. 2) at doses as low as 1 ng/mouse gave striking inhibition of PMN infiltration within the exudates. In these experiments, the double dioxygenation product 10S,17S-docosatriene (compound I) was substantially less potent. In this context, the double dioxygenation product was not active at 0.1 ng compared directly to synthetic PD1. At higher doses, 10S,17S-HDHA (compound I) blocked PMN infiltration, but it was less potent than PD1. Compound IV, which is the 10R version of the double dioxygenation products, was essentially equipotent at a 1 ng dose (compound IV ~ compound I), but did not increase potency in a dose-dependent fashion at 10-ng and 100-ng doses (data not shown). The /H9004 15-trans isomer of PD1 was, at equal doses of 1 ng/mouse, substantially less potent. Also, a rogue isomer for this series, compound V (Fig. 2) was not likely to be produced in vivo from the 17S-hydroxy precursor because its 10S,17R-diHDHA was essentially without activity in this dose range. Of interest, compound VI was the most potent of these isomers in vivo; however, only trace amounts were noted in human PMN extracts. Hence, a rank order of potency at the 0.1-ng dose of these 10,17-diHDHA isomers was compound VI >> PD1 > 10S,17S-DT (the double dioxygenation product) > the 15-trans-PD1 >> compound V. We also tested the carboxy methyl ester of PD1 vs the native synthetic PD1. Fig. 6B demonstrates the potent dose response of PD1 as it dramatically reduced the infiltration of PMN into the peritoneum. The carbon-1 position carboxy methyl ester was similar in its ability to block in vivo the hallmark of acute inflammation, namely PMN infiltration. The methyl ester of compound VI also proved to be a potent regulator of PMN infiltration.
cytes while reducing inflammation, a hallmark of resolution.

Differential results indicate that they have a potential additive rather than synergistic anti-inflammatory action when administered together in vivo. RvE1 and PD1 both reduced the infiltration of PMN when administered i.p. following PMN in vivo during zymosan-induced peritonitis (Fig. 7).

We also addressed whether the anti-inflammatory actions of DHA-derived PD1 and EPA-derived resolin E1 (RvE1) were synergistic or additive in vivo. Similar and striking results were obtained with the carboxy methyl ester of PD1, also administered i.p. Hence, once PD1 was given, essentially no further infiltration of PMN into the peritoneum was obtained with essentially >90% blocking of further PMN infiltration to the site. We also addressed whether the anti-inflammatory actions of DHA-derived PD1 and EPA-derived resolin E1 (RvE1) were synergistic or additive in vivo. RvE1 is derived from EPA and is another omega-3-derived counterregulatory anti-inflammatory lipid mediator that we recently isolated and identified (3, 45). When administered together, RvE1 and PD1 both reduced the infiltration of PMN in vivo during zymosan-induced peritonitis (Fig. 7B). These results indicate that they have a potential additive rather than synergistic anti-inflammatory action when administered together in vivo. We also prepared and tested a chemically more stable form of synthetic PD1, i.e., 15,16-dehydro-PD1, that proved to retain activity in vivo, reducing PMN infiltration, albeit was slightly less potent than the native PD1 (Table II). Of interest, differential counts on light microscopy also revealed that both PD1 and its chemical analog 15,16-dehydro-PD1 reduced PMN infiltration and increased the nonphlogistic recruitment of monocytes and lymphocytes (Table II) while reducing inflammation, a hallmark of resolution (31).

Discussion

PD1 (10,17-docosatriene) is a potent bioactive lipid mediator derived from DHA that displays anti-inflammatory actions (1, 3) and is generated during the resolution phase of an acute inflammatory response (31). The basic structure of this novel potent DHA-derived mediator was determined, i.e., 10,17-di hydroxydocosatriene (1, 3, 37); its potent role in neural protection was recently uncovered (2), and thus it is denoted as NPD1 when produced in neural tissues. Given the importance of establishing the molecular basis of endogenous anti-inflammation and natural resolution (17), because knowledge of these pathways and mechanisms in vivo may provide new therapeutic approaches to human disease, we sought evidence for the complete stereochemistry of PD1. On the basis of physical, biosynthetic, and biological properties in matching results with human cells and synthetic materials, the complete stereochemistry of PD1 was assigned 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid (compound II; Figs. 1, 2, and 6A, and Table I).

On identification of 10,17-diHDHA in resolving inflammatory exudates (3, 37) and potent anti-inflammatory actions, it was critical to establish its biosynthesis from DHA. To address this action, we studied isolated human PMN, whole blood, microglial cells, and murine exudates and tissues (1, 3). The isolation and identification of alcohol trapping products indicated the involvement of an epoxide intermediate in the conversion of DHA to 10,17S-dihydroxy-docosatriene, a docosatriene containing a characteristic conjugated triene structure involving three of the six double bonds present in this compound. The role of a 16(17)epoxide intermediate generated from the 17S-H(p)DHA precursor was further supported by the identification of two vicinal diols, i.e., 16,17S-dihydroxydocosatrienes present in these LC-MS-MS profiles also generated from DHA (1). The 16(17)epoxy-DHA intermediate could open via nonenzymatic hydrolysis to a racemic mixture, i.e., 16R/5,17S-diHDHA, or to a single 16,17S-vinyl alcohol by the actions of an appropriate epoxide hydrolase in a reaction similar to that demonstrated earlier in the biosynthesis of LXA4 (39, 44, 46). The biosynthesis of PD1 by human cells (compound II) with this stereochemistry from a 16(17)epoxide intermediate would require an enzymatic reaction to move the double bond configuration to set the triene geometry to 11E,13E,15Z and direct the attack of H2O and insertion of its oxygen into the carbon-10 position of PD1 determined in the present experiments to be in the 10R stereochemical configuration.

In addition to PD1 (compound II) in human cell extracts, which carried potent bioactions, an isomer 10S,17S-diHDHA (compound I) was also identified in murine exudates with lesser amounts in isolated human cells (Fig. 1). Compound I was found to be a double dioxygenation product and was also formed from DHA, but in a reaction that required two sequential lipoxygenation steps and oxygen incorporation that was directed at the carbon-10 position derived from molecular oxygen (i.e., 18O2 in an enriched atmosphere in vitro). This reaction producing 10S,17S-diHDHA is markedly different from the proposed enzymatic hydrolysis of the epoxide intermediate in mammalian tissues to produce PD1. The double dioxygenation product formed in vivo is different from PD1 in four key ways: 1) PD1 carbon-10 position alcohol is predominantly in the 10R configuration, whereas the dioxygenation product is mainly in the 10S configuration; 2) the double bond structure of PD1 conjugated triene is in the 11E,13E,15Z configuration, and the 10S,17S-dioxygenation product conjugated triene system is in the 11E,13Z,15E configuration; 3) most importantly, PD1 is more potent than the dioxygenation product (PD1 (compound II) >> 10S,17S-diHDHA (compound I)); and 4) PD1 is generated by isolated human leukocytes and tissues.

Also in support of the stereospecific basis of these DHA-derived products in human and murine systems is the bioaction of the 15-trans-PD1 isomer (compound III), which can arise via workup-induced isomerization of PD1 and possesses little bioactivity in vitro or in vivo within the dose or concentration range (Fig. 5) observed with biogenic or synthetic PD1 (Figs. 6 and 7). Also,
compound IV, identified in human leukocytes (Fig. 1) and which differed from compound I at the 10R position and carried the same double bond geometry, was essentially equipotent at a 1-ng dose (Fig. 2). Hence, the biosynthesis of PD1 from DHA, from the results of the present experiments, appears to require stereoselective enzymatic steps to evoke bioactions. This requirement for stereoselective enzymatic reactions is widely appreciated in the biosynthesis of eicosanoids (9, 44). The nature of the PD1 epoxide hydrolase in vivo is therefore of interest, particularly in view of the bioactivity results with compound VI, which was the most potent of the isomers (Fig. 6). Compound VI shares the triene geometry of PD1, differing only in the C10 chirality in the S configuration. However, only trace amounts of this isomer appear to be generated by human cells (vide infra). Thus, the fidelity of the enzyme that produces PD1 from the proposed carbonium cation intermediate (1), in its ability to direct insertion of H2O-derived alcohol at carbon-10 exclusively in the 10R with apparently trace amounts of 10S as in compound VI (Figs. 2 and 6), is an intriguing point for further studies.

Earlier results indicated that DHA, which is not a natural substrate for p5LO, is converted to 10-HDHA by this enzyme and the double dioxygenation product 10,20-diHDHA (47). In addition, Whelan et al. (48) demonstrated that this plant LO is very versatile with DHA as a substrate and identified multiple monohydroxy-DHA products at carbon positions 4, 7, 8, 11, 13, 14, 16, and 17 to give positional isomers of HDHA; each was an enzymatic product of this flexible enzyme. This regioselectivity also likely reflects the degree of enzyme purity as well as the geographic source of the potato. As with other LOs, we found that potato 5-LOX and soybean 15-LO gave specific diHDHA profiles of products that were dependent on pH, enzyme, and substrate concentrations used in the incubations (3). When the substrates were presented in micellar configuration with the enzymes, hydroperoxy intermediates were converted to epoxides that, on hydrolysis, gave many of the isomers as relatively minor products but were nonetheless in quantities useful for in vitro and in vivo studies (37). These findings were advantageous in the preparation of intermediates (i.e., 7,17-diHDHA, 17S-HDHA, and 17S-H(p)DHA) used in biosynthesis studies and determining the identity and actions of enzymatic products generated in vivo as well as by isolated human cells from DHA (1, 3).

The DHA potato 5-LOX products 10,20-diDHA and 10-HDHA were originally reported by J. Whelan (Ref. 47; doctoral thesis with C. Reddy as advisor). Recently, classic steric analysis of 10S-HDHA and the formation of 10,20-diHDHA and 17-H(p)DHA were reportedly optimized for the plant LOs (49). In the present studies, the double dioxygenation product prepared, matched, and identified in both suspensions of human PMN (Fig. 1A; m/z 261

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**FIGURE 6.** Dose-dependent inhibition of acute inflammation in vivo with synthetic PD1 and its isomers. Peritonitis was initiated in 6- to 8-wk-old male FVB mice (Charles River Laboratories) by peritoneal injection of 1 mg of zymosan A. Mice received injections with the following: (A) the double dioxygenation product 10S,17S-diHDHA (●) (compound I; Fig. 2), synthetic PD1 (●) (compound II), Δ15-trans-PD1 (△) (compound III), the 10S,17R-diHDHA isomer (○) (compound V), compound VI (●), or vehicle alone. *, p < 0.05; n ≥ 3 for each compound. n ≥ 7 for PD1; and n ≥ 4 for compound I. B, PD1 (●), PD1 methyl ester (○), or the rogue isomer (compound VI) methyl ester (△). Peritoneal lavages were obtained at 2 h and leukocytes enumerated. Results are expressed as percentage inhibition compared with mice receiving injections with zymosan A (1 mg) and vehicle alone. *, p < 0.05; n ≥ 4 for each compound.
profile) and in vivo during peritonitis carries its alcohols as expected in the 10S,17S configuration in this diHDHA (compound I). Hence, this natural isomer of PD1 (compound II) formed in vivo from DHA has its Δ13 position double bond in the cis-configuration (i.e., 13Z) and its Δ11 in the trans configuration within the conjugated triene portion of the molecule (11E,13Z,15E) and possesses some anti-inflammatory activity in vivo, albeit proved to be much less potent than natural or synthetic PD1 (compound I), the remainder in the 10R configuration as in compound IV.

In earlier experiments, when administered i.v., 10,17-docosatriene (PD1, compound II; Fig. 2) was found to be more potent than indomethacin in reducing PMN infiltration in murine peritonitis, i.e., ~40% inhibition at 100 ng/mouse (1). Synthetic PD1 in as small a dose as 1 ng/mouse gave ~40% inhibition of PMN infiltration that was maintained at the 10- and 100-ng doses. Thus, synthetic PD1 (compound II) matched with the natural compound is a potent regulator of PMN infiltration in vivo but does not completely block PMN recruitment, which is consistent with its counterregulatory and autacoid actions and apparently would not

Table II. Leukocyte infiltration in murine peritonitis: actions of PD1 and chemically stable analogs

<table>
<thead>
<tr>
<th>Injection</th>
<th>Neutrophils (X10^6)</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymosan A</td>
<td>9.22 ± 0.47</td>
<td>2.54 ± 0.15</td>
<td>0.15 ± 0.11</td>
</tr>
<tr>
<td>Zymosan A + PD1</td>
<td>5.93 ± 0.62±δ (35.7% ↓)</td>
<td>4.43 ± 0.62±δ (74.4% ↑)</td>
<td>0.33 ± 0.13 (120% ↑)</td>
</tr>
<tr>
<td>Zymosan A + 15,16-dehydro-PD1</td>
<td>6.18 ± 0.80±δ (33.0% ↓)</td>
<td>3.79 ± 0.53 (49.3% ↑)</td>
<td>0.42 ± 0.11 (180% ↑)</td>
</tr>
</tbody>
</table>

*Peritonitis was carried out as in experimental procedures but extended to 4 h. Mice received injections with 10 ng/mouse of either PD1 (n = 3) or 15,16-dehydro-PD1 (n = 3) followed by 1 mg of zymosan A. Leukocyte infiltration was determined 4 h after injection. Results are expressed as mean ± SEM, and percentage inhibition of neutrophils and stimulation of monocytes as compared to mice receiving injections with zymosan A (1 mg) alone. Statistically different from ±zymosan A-injected mice (p < 0.05) but not between δ PD1 and 15,16-dehydro-PD1-injected mice (p > 0.05).
compromise host defense via immune suppression of effector cell function. This was also the case with human PMN transmigration, which required 10–100 nM PD1 to reduce PMN transmigration by 35–45% in vitro. Thus, although PD1 stereoselectively reduces PMN transmigration in vitro, given its potent actions in vivo, PD1 (compound II) likely targets additional cell types in vivo to evoke its potent anti-inflammatory actions in vivo (Fig. 7 and Table II). In murine peritonitis, PD1 also regulated both monocyte and lymphocyte traffic to the exudates. Alternatively, these potencies in vivo vs isolated human cells might also reflect species differences. As an inducer of peritonitis, zymosan stimulates the initial formation of many endogenous chemottractants for PMN, i.e., LTβR, the complement component C5a, chemokinies, and cytokines (37). Because PD1 stops PMN recruitment in vivo, it counteracts these several different sets of PMN chemotactants that regulate traffic of these cells in vivo (Table II and Ref. 37). Given the inherent chemical liabilities of PD1, we also prepared and tested a more chemically stable form denoted 15,16-dehydro-PD1 (Table II). Although less potent, chemical stabilization of the conjugated double bonds with an acetylenic form proved useful because the molecule retained activity in vivo (Table II). These results are consistent with the approximately 40% inhibition obtained with a 4,5-acetylenic analog of PD1 (37).

In addition to this LO-initiated route of biosynthesis for PD1, an aspirin-triggered route with a 17R epimer of PD1 (17R series) is generated via acetylated cyclooxygenase and subsequent reactions (3, 32); the complete stereochemistry of this bioactive epimer is in progress. It is of interest to note that compound V, 10,17,18 (3, 32); the complete stereochemistry of this bioactive epimer is in progress. It is of interest to note that compound V, 10,17,18 S-trienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis.9

**Disclosures**

In association with Brigham and Women’s Hospital, Boston, MA, C.N.S. is the inventor of the patent “Resolvins: biotemplates for novel therapeutic interventions.” C.N.S. and S.H. are among the inventors of the patent “Neuroprotectin D1 protects against cellular apoptosis, neuronal stroke damage, neurodegenerative diseases, and retinal degeneration” with joint assignees Louisiana State University and Brigham and Women’s Hospital.

**References**


CORRECTIONS


Figure 8 is incorrect. The corrected figure is shown below.

![Corrected Figure](image)


The second author’s middle initial was omitted. The correct name is Robert L. Ferris.


The eighth author’s last name was misspelled. The correct name is Leda Q. Vieira.

The ninth author’s last name was misspelled. The correct name is Hideaki Nakajima.


In Figure 2A, the three left hand dot plot panels from Ly9+/+ cells were mistakenly duplicated in the three right hand dot plot panels of Ly9−/− cells. The numbers in each of the quadrants are correct and the error does not change any interpretation in the article. The corrected figure is shown below.


One of the first author’s affiliations was omitted. The corrected list of authors and affiliations is shown below.

Meng-Tsung Tien,2*†‡ Stephen E. Girardin,2* Béatrice Regnault,7 Lionel Le Bourhis,8 Marie-Agnès Dillies,7 Jean-Yves Coppée,7 Raphaëlle Bourdet-Sicard,9 Philippe J. Sansonetti,3* and Thierry Pévron*8

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In Figure 1B, the WT Ca flux data line is missing from the Ca flux graph. The corrected figure is shown below.

In Discussion, the last reference in the paper is incorrect. The corrected sentence and reference are shown below.

It is known that the cytoplasmic domains of several components of the TCR complex tend to homo-oligomerize at high concentrations (41); perhaps ligand-induced clustering of the TCR drives the cytoplasmic domains of proximal receptors to rearrange, exposing the Nck binding epitope and propelling other signaling cascade processes.


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In Discussion, in the second sentence of paragraph six, 10S-HDNA should have been 10S-HDHA. The corrected sentence is shown below.

Recently, classic steric analysis of 10S-HDHA and the formation of 10,20-diHDHA and 17-H(p)DHA were reportedly optimized for the plant LOs (49).

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The title of the article is incorrect. The corrected title is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.

Thymocyte Negative Selection Is Mediated by Protein Kinase C- and Ca\(^{2+}\)-Dependent Transcriptional Induction of Bim