Introduction

The metabolism of dietary fatty acids is of particular interest in prostate cancer, the most frequently diagnosed cancer and a leading cause of death in American males. Epidemiological studies suggest that intake of the n-3 marine polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) reduces prostate cancer risk [1,2]. Moreover, tissue levels of n-3 PUFA were inversely associated with prostate cancer progression [3,4,5]. In addition, cell culture and animal models have shown that n-3 PUFA protect against prostate cancer through inhibition of tumor cell proliferation [6,7,8]. PUFA are incorporated into cell membrane phospholipids and are substrates for oxygenase (cyclooxygenase and lipoxygenase) enzymes to be metabolized to bioactive lipids. One proposed mechanism for the tumor-inhibitory activity of n-3 PUFA is competitive inhibition of the oxygenases used by n-6 PUFA to form tumor-promoting metabolites (reviewed in [9,10]). Our studies [11,12] and those of others [6,13] have shown that DHA is a strong inhibitor of prostate cancer cell growth, a property that is regulated by a 15-lipoxygenase (15-LOX) (unpublished studies).

Previous studies have indicated that two isoforms of 15-LOX identified in humans may play opposing roles in the development and progression of prostate cancer through metabolism of n-6 PUFA. 15-LOX-1 is more highly expressed in malignant than in normal human prostate tissue and its levels correlate positively with the disease’s severity [14,15,16]. It prefers linoleic acid (LA) to arachidonic acid (AA) and in consequence makes mainly the AA metabolite, 13-hydroxy-octadecaenoic acid (13-HODE) [14,17,18]. Prostate cancer has higher levels of 13-HODE and converts LA to 13-HODE to a greater extent than normal prostate tissue [14,15,16]. 15-LOX-2, in contrast, prefers AA over LA, makes mainly the AA metabolite, 15-hydroxy-eicosatetraenoic acid (15-HETE), is under-expressed or absent in prostate cancer, and its levels correlate negatively with disease severity [14,17,18,19,20]. Human prostate cancer has relatively little ability to convert AA to 15-HETE [15]. Experimental studies have supported and expanded these clinical observations.

Mice made to express in their prostate glands human 15-LOX-1 develop prostate intraepithelial neoplasia (PIN) [21]; when similarly engineered to express human 15-LOX-2, they develop prostates enlarged with senescent cells [22]. Correlating with these results, the forced expression of human 15-LOX-1 speeds and 15-LOX-1 knockdown slows the proliferation of cultured and explanted human prostate cancer cells [23]. Forced expression of human 15-LOX-2 causes these cells to stop proliferating and become senescent [24,25]. The effect of 15-LOX-1 appears due to its production of 13-HODE, which enhances the ability of growth factors to stimulate prostate cancer cell proliferation [23,26,27]. The effect of 15-LOX-2 is attributed in part to its production of 15-HETE, which inhibits prostate cancer cell proliferation.
Prostate cancer cells can readily convert shorter chain n-3 metabolites to their corresponding DHA, which is known to be irrelevant to DHA's activity since men as well as cultured prostate cancer cell lines do not use DHA [31,32,33]. Moreover, the anti-proliferative action of 15-LOX metabolites of DHA. These metabolites are of the proliferation of prostate cancer cells [35,36]. The existing data thus warrant searches for other 15-LOX/PUFA metabolite models.

We here examine the activity, potency, and mechanism of action of 15-LOX metabolites of DHA. These metabolites are of particular interest because 1) DHA is a member of the n-3 PUFA family suggested to suppress prostate cancer in epidemiological studies [31,32,33]; 2) DHA is a key contributor to the anti-proliferative effect of n-3 PUFA in prostate cancer cells [11,12] and 3) the activity of shorter chain n-3 PUFA, including EPA, is also less likely mediators of 15-LOX metabolites because both 15-hydroxy-eicosatetraenoic acid and 15-hydroxy-eicosapentaenoic acid require ≥1 μM to slow the proliferation of prostate cancer cells [35,36].

Materials and Methods

DHA and AA (NuChek Prep); soybean 15-LOX type 1a (sLOX), sodium borate, and sodium borohydride (Sigma); HPLC columns (Waters); HPLC or optima grade organic solvents and diethyl ether (Fisher); anti-SDC-1 (H-174) antibody (Santa Cruz Biotechnology, Inc); anti-HRP-conjugated secondary antibody against rabbit antibody (Cell Signaling Technology); and CellTiter 96® Aqueous One Solution Cell Proliferation Assay and Caspase-Glo® 3/7 assay (Promega) were purchased. PC3, DU145, and LNCaP human prostate cancer cell lines (American Type Culture Collection, Manassas, VA) were grown in advanced Dulbecco’s Modified Eagle’s Medium (Invitrogen) containing 1% fetal bovine serum (FBS). Due to their instability [40,41], 15-HpDHA and 17-HpDHA were stored in methanol under an argon atmosphere at −80°C; freedom of methanol by a stream of nitrogen; taken up in culture media; and added to cell cultures. Due to their 17-HpDHA and 17-HpDHA were used within 5 weeks of preparation.

Metabolite Preparations

We prepared 15S-hydroperoxy-eicosatetra-5Z,8Z,11Z,13E-enonic (15-HpETE), 15S-hydroxy-eicosatetra-5Z,8Z,11Z,13E-enonic (15,17-diHETE), 5S,15,18-trihydroxy-eicosatetra-6E,8Z,11Z,13E-enonic (5,15,17-diHETE), and 8S,15S-dihydroxy-eicosatetra-5Z,9E,11Z,13E-enonic (5,15-diHETE) acids by reacting arachidonic acid with sLOX and used this same method to prepare DHA metabolites. Briefly, DHA (10 μM) was reacted with 0.8 μg of sLOX in 50 ml of aerated sodium borate buffer (50 mM; pH 9; 4°C, 30 min). Reactions were extracted with diethyl ether; the 1S,15S-hydroperoxy-docosa-hexa-4Z,7Z,10Z,13Z,15E,19Z-enoate (4Z,7Z,10Z,13Z,15E,19Z-enoate) was separated and purified by silicic acid column chromatography followed by HPLC (3.5×150 mm; hexane:isopropanol:glacial acetic acid, 950:50:1, v/v; 3 ml/min; eluting at ~34 min), and isotopic μ-Porasil HPLC (1.5×300 mm; hexane:isopropanol:glacial acetic acid, 950:50:1, v/v; 3 ml/min; eluting at ~6 min). Elution UV spectra were monitored with a G1315A diode array spectrophotometer run with ChemStation 51 software (Agilent Technologies). 17-HpDHA was reacted with sodium borohydride in methanol and re-purified by Bondapak HPLC to obtain 17S-hydroxy-docosa-hexa-4Z,7Z,10Z,13Z,15E,19Z-enoate (17-HDHA). For dihydroxy products, DHA (10 μM) in 500 ml of heptane was reacted with 10 μg of sLOX added at 0, 45, 90, 150, and 240 min. After 300 min, the reaction was processed like 17-HpDHA though the Bondapak HPLC step; the peak eluting in this system at ~10 min with a triene absorbance spectra (maxima: 280, 270, and 261 nm) dominating its left side and 5,15-diHETE-like absorbance spectra (maximum: 243; adiabatic hump: ~223 nm) dominating its right side was collected; reduced with sodium borohydride; and, following Butovich et al. [40,41], resolved by isocratic 5SW HPLC (3×250 mm; hexane: isopropyl alcohol:glacial acetic acid, 974:26:1, v/v; 1 ml/min) into peaks at ~17 and 22 min with respective UV spectra for 10S,17-diHETE (maxima: 280, 270, and 261 nm) and 7S,17S-dihydroxy-docosa-hexa-4Z,8E,10Z,13Z,15E,19Z-enoate (7,17-diHDHA [or protectin D5]); maximum: 222 nm; adiabatic hump: 242 nm) [40,41,43]. In addition to their HPLC elution times and UV spectra, the structures of the AA metabolites were confirmed by MS [39] and of the DHA metabolites by MS and nuclear magnetic resonance (NMR). 17-HDHA and 10,17-diHETE gave electrospray spectra (Quattro II MS, MassLynx 3.5 software, negative ion mode) similar to those published [40,41,43,44,45,46]. The molecular ion for 17-HpDHA was 16 AMU greater than that for 17-HDHA. NMR spectra (1D and 2D double-quantum-filtered COSY in d4-methanol; 25°C; Bruker 699 MHz Avance NMR spectrometer) for 17-HDHA and 10,17-diHETE had chemical shifts and coupling patterns matching published reports [40,41,44]; the deduced conjugated double bond geometries were for 17-HDHA, 13Z,15E; for 10,17-diHETE, 11E,13Z,15E; and for 7,17-diHDHA, 8E,10Z and 13Z,15E. The four DHA metabolites lacked resonances at 6.1–6.2 ppm indicating the absence of a trans-trans conjugated double bond. The DHA and metabolites were stored in methanol under an argon atmosphere at −80°C; freedom of methanol by a stream of nitrogen; taken up in culture media; and added to cell cultures. Due to their instability [40,41], 15-HpETE and 17-HpDHA were used within 5 weeks of preparation.

Proliferation and Caspase Assays

Proliferation was assayed with Cell Titer96 Aqueous One Solution Cell Proliferation Assays (Promega) as described [47]. To measure apoptotic activity, cells were seeded in 96-well plates at a density of 1000 cells/well for 24 h, then treated with the compounds for 48 h prior to measurement of caspase activity using the Caspase-Glo® 3/7 assay (Promega) according to the manufacturer’s directions.

PPARγ Activation Assay

2×10³ PC3 cells were seeded on 35 mm dishes in 1 ml of advanced DMEM with 1% FBS, for 24 h and transfected with 1 μg of lacZ and 1 μg of PPRE DNA (PPAR response element-luciferase reporter) [34,38] using FuGENE 6 Transfection Reagent (Roche). In some studies, cells were co-transfected with a vector (1 μg) encoding dominant negative (d/n)-PPARγ (L465/ E471) [34] or empty vector pcDNA3 (Invitrogen, Carlsbad, CA) for 24 h or were treated for 30 min with the PPARγ antagonist, GW9662. The cells were then challenged with a DHA

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metabolite or a PPARγ agonist, troglitazone, for 24 h. Cells were scraped into Reporter Lysis Buffer (Promega). Samples were frozen for 18 h and centrifuged (200 g, 4 min, 20°C). Supernatant fluids were assayed for luciferase and β-galactosidase (Promega Luciferase and β-Galactosidase Enzyme Assay Systems). Luciferase was corrected for transfection efficiency based on β-galactosidase as in [34,38].

SDC-1 Assay

To detect SDC-1 message, total PC3 cell RNA was prepared and amplified in triplicate using the Applied Biosystems 7500 Real-Time PCR System. Primers for human SDC-1 were 5′-ggagcaggtcaccttgc (forward) and 5′-ctccagaccccttcctc (reverse). Data were normalized to the housekeeping control peptidylprolyl isomerase B and are presented as relative to control. To detect SDC-1 protein, PC3 cells were homogenized and lysed in ice-cold buffer (25 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 0.1 mg/ml phenyl-methanesulfonyl fluoride, 1× proteinase, and 1× phosphatase inhibitors [Roche Applied Science]), dialyzed against 100 mM Tris and 30 mM sodium acetate, pH 8.0, for 24 h at 4°C, and digested by chondroitinase ABC (Seikagaku, Ijamsville, MD) and heparinase III (Sigma-Aldrich) at 37°C overnight. Protein extracts were prepared for Western blot analysis as described using the indicated antibody [12]. Band densities on photographic films were analyzed using Image J 1.37v (National Institutes of Health, Bethesda, MD). To silence SDC-1, 1×10⁵ PC3 cells per well were plated in 96-well plates, transfected with a siRNA or not transfected (Fig. 3D). Although 10,17-diHDHA and 7–17-HDHA were weak activators of PPARγ in this study inducing respectively a 69% and 68% increase in activity over control that trended toward but did not achieve statistical significance (P<0.1) (Fig. 2B). We have previously shown that both 15-HETE and 5,15-dihETE (1–200 μM) failed to activate this reporter [34]. Finally, the DHA metabolites stimulated PC3 cells to express SDC-1 mRNA (Fig. 2C) that resulted in increased SDC-1 protein (Fig. 2D); these effects also matched those of troglitazone and DHA [12,38,48,49]. The relative potencies of the metabolites in producing these responses approximated their relative potencies in slowing PC3 cell proliferation. The discrepancy between weak activation of PPARγ by 10,17-diHDHA and 7–17-HDHA (Fig. 2B) and their more robust effect on the accumulation of SDC-1 protein over 72 h (Fig. 2D) may suggest a slower uptake and/or metabolism of these two more polar products by the cells.

Results

17-HpDHA, 17-HDHA, 10,17-diHDHA, and 7,17-HDHA slowed the proliferation of PC3 cells by 25% at about 0.1, 1, 8, and 10 μM, respectively (Fig. 1A). Under the same conditions, DHA required >60 μM to achieve this effect [12] and the analogous AA-derived 15-LOX metabolites, 15-HpETE, 15-HETE, and 8–15-dihETE, had far less or no activity while 5,15-dihETE, slightly stimulated proliferation (Fig. 1B). The comparable 15-hydroxy metabolites of EPA and GLA reportedly require ≥5 μM to slow proliferation by 25% [35,36] while the 15-LOX-dependent metabolites of LA, 13-HODE, and 9-HODE, lacked anti-proliferative activity at <100 μM and actually stimulated proliferation at ≥0.1 and 1 nM, respectively (unpublished observations). 17-HpDHA and 17-HDHA also proved more potent than 15-HpETE or 15-HETE in slowing the proliferation of LNCaP and DU145 prostate cancer cells (Fig. 1C and 1D). Similar results occurred in the three cell lines when incubated with the metabolites for 48 or 96 h (results not shown). Under identical conditions, DHA required ≥30 μM to inhibit the proliferation of these cells [12].
metabolites inhibit proliferation [15,19,20,22,24,25,26]. Based on their in vitro activity and dominance as 15-LOX-2 metabolites, 15-HETE [24,25,26,34], 15-hydroxy-eicostrienoic acid, and 15-hydroxy-eicosapentaenoic acid [35,36] are candidate mediators of 15-LOX-2's anti-proliferative effect. However, the low potency of these metabolites allows that products derived from other PUFA might be more potent and therefore more important in mediating the 15-LOX-2 effect. We find that members of the 17-series of DHA metabolites, 17-HpDHA, 17-HDHA, 7,17-diHDHA, and 10,17-diHDHA, inhibit the proliferation of androgen-independent (PC3 and DU145) and androgen-dependent (LNCaP) prostate cancer cells. The most potent of these, 17-HpDHA and 17-HDHA, significantly slowed proliferation at concentrations of 1 and 100 nM, respectively, and therefore are >1,000-fold more

Figure 1. The proliferation responses of PC3, LNCaP, and DU145 prostate cancer cells to selected DHA and AA metabolites. The indicated cell types were incubated for 3 days with the indicated metabolite and their proliferation presented as the mean ± SEM (n=3 independent experiments) fractions of that found in cells treated with the vehicle (culture media) for the metabolites.

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Figure 2. Stimulatory effects of DHA metabolites on caspase, PPARγ activity, and SDC-1 expression in PC3 cells. A. Cells were incubated with the indicated concentration of 17-HDHA or 17-HpDHA for 24 h and caspase-3 activity was measured by Caspase-Glo® 3/7 assay. Results are presented as mean ± SD (N = 3) relative to control cells treated with the medium for the metabolites. Responses to all doses at and above $10^{-8}$ M for 17-HpDHA and at or above $10^{-7}$ M for 17-HDHA were significantly greater than that of control cells (two way ANOVA, $P<0.05$). B. Cells transfected with luciferase PPARγ reporter gene were stimulated for 24 h with 10 μM of the indicated metabolite (the lowest dose where all had a clear effect on cell growth) or 5 μM of troglitazone and assayed for luciferase. Values represent the mean ± SD (N = 3). Bars labeled with the same letters are not significantly different from each other; bars labeled with different letters are significantly different from each other (one-way ANOVA, $P<0.05$). C. Cells were treated with medium (control) or 10 μM of the indicated metabolite for 8 or 24 h and their SDC-1 mRNA was measured. Values represent the mean ± SD (N = 3). Within each time group, bars labeled with the same letters are not significantly different from each other; bars labeled with different letters are significantly different from each other (one-way ANOVA, $P<0.05$). D. Cells were treated with medium (control) or 10 μM of the indicated metabolite for 72 h and their lysates were analyzed for SDC-1. The Western blot is representative of 3 independent experiments. Values in graphs represent the mean ± SEM (N = 3 independent experiments). Bars labeled with different letters are significantly different from each other (one-way ANOVA, $P<0.05$).

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potent than the corresponding metabolites of AA; they also appear far more potent than the 15-LOX metabolites of EPA and GLA as reported in [35,36]. The DHA metabolites clearly acted in a structural specific manner as evidenced by their decidedly different individual potencies and by their greater potencies than their counterparts in the 15-series of AA metabolites. We note that the activities of the 17-series of DHA metabolites found here do not exclude possibilities that their effects involve their further cellular...
metabolism to even more potent anti-proliferative products. We are just beginning to examine this issue.

Studies have shown that DHA suppresses the proliferation of prostate cancer cells including PC3 cells by a pathway that involves the activation of PPARγ, the binding of PPARγ to the SDC-1 promoter, the induction of SDC-1, and SDC-1-induced apoptosis [12,38]:

DHA → PPARγ → SDC-1 → Apoptosis

The DHA metabolites studied here stimulated PC3 cells to activate a PPARγ reporter, express SDC-1, and activate caspase-3, thus suggesting an additional important step in this pathway i.e. the metabolism of DHA to more potent intermediates. Moreover, the PPARγ antagonist, GW6992, d/nPPARγ, and SDC-1 silencing blocked the DHA metabolites’ anti-proliferative action; d/nPPARγ also blocked their induction of SDC-1. The metabolites thus used the same signaling pathway as DHA to slow the proliferation of PC3 cells. This set of findings opens the possibility that the anti-proliferative effect of DHA is mediated at least in part through its metabolism by 15-LOX-2 to the 17-series of metabolites, particularly 17-HpDHA and 17-HDHA. There are, however, several problems with this scheme.

Studies disagree on the ability of PC3 cells to metabolize PUFA with some finding the cells make no [26] or very little [23,24] 13-HODE and 15-HETE and others finding they make appreciable amounts of 13-HODE [15,16] but little 15-HETE [15] even after exposure to high concentrations of LA or AA. Since 15-LOX-1 prefers LA to AA while 15-LOX-2 prefers AA to LA [14,17,18,19,20], these results indicate that PC3 cells have no or little 15-LOX-2 metabolizing activity, a result fully compatible with findings that these cells have 15-LOX-1 but little or no 15-LOX-2 message and protein [15,24,54]. In addition, the relative ability and specificity of the two human enzymes to use DHA as a substrate have not been defined although a 15-LOX-1 knockdown study in retinal pigment epithelial cells suggests that 15-LOX-1 but not 15-LOX-2 is responsible for metabolizing DHA to the 17-series of metabolites [55]. It is clear that the 17-series of DHA metabolites are made by various cell types in vitro and numerous tissue types in vivo [45,55,56,57,58]. However, the ability of malignant as well as normal prostate cells and tissues to make these metabolites and the contribution of 15-LOX-1 versus 15-LOX-2 to this is not known. We found that PC3 cells challenged with an anti-proliferative concentration (i.e. 100 μM) of DHA for 0.5–96 h converted only very small quantities (<0.003%) of it to 17-HDHA, 7,17-diHDHA, plus 10,17-diHDHA, as detected by selective ion-monitoring-MS (unpublished studies). These quantities seemed insufficient to slow proliferation. Faced with these findings, it might be profitable to consider other avenues by which prostate cancer could be subjected to these metabolites. Human prostate cancer juxtaposes with normal tissue. This normal tissue could provide the 17-series of DHA metabolites through the activity of 15-LOX-1, 15-LOX-2, or cytochrome P450 [59,60]. 17-series DHA metabolites also form through auto-oxidation [61]; cultured neuroblastoma cells, for example, metabolize DHA to 17-HDHA and other cytotoxic DHA derivatives through auto-oxidation as well as 15-LOX-dependent pathways [56]. One or more of these alternative paths may be the means by which dietary n-3 PUFA ultimately act to reduce the mortality of prostate cancer [31].

In conclusion, we find that a series of 15-LOX-derived metabolites of DHA, particularly 17-HpDHA and 17-HDHA, are far more potent than their parent molecule or 15-LOX metabolites of other PUFA in inhibiting the proliferation of androgen positive and androgen negative human prostate cancer cell lines. Similar to their parent molecule, the DHA metabolites’ mechanism of action involves the PPARγ/SDC-1 apoptosis-signaling pathway. We propose that the prostate cancer-suppressing effect of dietary DHA is mediated in part by the conversion of DHA to one or more of these metabolites.

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Author Contributions

Conceived and designed the experiments: JTO IJE. Performed the experiments: YH REW HS MPS. Analyzed the data: JTO YH IJE. Wrote the paper: JTO IJE.

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