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Omega-3 fatty acids, EPA and DHA induce apoptosis and enhance drug sensitivity in multiple myeloma cells but not in normal peripheral mononuclear cells

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Abstract

The n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to enhance the effect of chemotherapeutic drugs in clinical studies in cancer patients and to induce apoptotic tumor cell death *in vitro*. Until now, EPA and DHA have never been investigated in multiple myeloma (MM). Human myeloma cells (L363, OPM-1, OPM-2 and U266) and normal peripheral blood mononuclear cells were exposed to EPA and DHA, and effects on mitochondrial function and apoptosis, caspase-3 activation, gene expression and drug toxicity were measured. Exposure to EPA and DHA induced apoptosis and increased sensitivity to bortezomib in MM cells. Importantly, they did not affect viability of normal human peripheral mononuclear cells. Messenger RNA expression arrays showed that EPA and DHA modulated genes involved in multiple signaling pathways including nuclear factor (NF) κB, Notch, Hedgehog, oxidative stress and Wnt. EPA and DHA inhibited NFκB activity and induced apoptosis through mitochondrial perturbation and caspase-3 activation. Our study suggests that EPA and DHA induce selective cytotoxic effects in MM and increase sensitivity to bortezomib and calls for further exploration into a potential application of these n-3 polyunsaturated fatty acids in the therapy of MM.

Keywords: EPA; DHA; Multiple myeloma; Apoptosis; Oxidative stress; NFκB; Mitochondria

1. Introduction

Multiple myeloma (MM) is a hematologic neoplasm of terminally differentiated B cells (plasma cells). Malignant plasma cells infiltrate the bone marrow, and taking advantage of bone marrow microenvironment protection, they become resistant to most drugs and apoptotic signals. Even with new and effective drugs, most MM patients develop intrinsic drug resistance or become chemoresistant in the course of treatment. For instance, bortezomib, the first-in-class antimyeloma drug, is a potent proteasome inhibitor that imposes its effects mostly through inhibiting the nuclear factor (NF) κB pathway [1]. However, several studies indicate NFκB resistance to bortezomib in MM primary cells or human myeloma cell lines (HMCLs) [2,3]. To potentiate the effect of bortezomib and overcome this resistance, the use of combined drug or drug/nutrition regimens can be considered in MM. Currently, the n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to enhance the effect of chemotherapeutic drugs in clinical studies in cancer patients and induce apoptotic tumor cell death *in vitro* through several mechanisms (reviewed in Ref. [4]). Nutritional intervention with medical food enriched with fish oil showed that EPA and DHA rapidly increase the percentage EPA and DHA in white blood cell phospholipids [5,6]. EPA and DHA have been shown to inhibit the growth of pancreatic tumor cells by hampering the Wnt/β-catenin signaling pathway [7] and to induce cell death through the production of reactive oxygen species (ROS), through caspase-8 activation or through autophagy [8]. Moreover, these fatty acids have been described to prevent the expansion of colorectal cancer cells by down-regulating cancer stem-cell-like genes [9] and to increase cell death and chemosensitivity of B-cell chronic lymphocytic leukemia cells by induction of ROS generation [10]. The different functional features of n-3 PUFAs make them potentially beneficial in combination with chemotherapeutic drugs. Additionally, the anti-inflammatory effects of n-3 PUFAs are reflected by a decrease in production of inflammatory cytokines, modulation of inflammatory genes and inhibition of NFκB activity [4]. Indeed, studies support the involvement of an inflammatory process in the pathogenesis or progression of some cancers [11–13] with possibly NFκB functioning as the key linking element [14–16]. In this respect, MM is not an exception. A history of chronic inflammatory diseases has been reported in some MM patients [17]. Furthermore, up-regulation of some inflammatory cytokines (e.g., interleukin-6 which is also the main growth factor for MM cells) [18], Toll-like receptors [19–21] and constitutive activation of NFκB [4,22,23] in a large fraction of MM cells and HMCLs suggests that an inflammatory process could be in effect in the BM which

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probably render tumor cells viable and drug resistant. The latter features of n-3 PUFAs might be another reason why they could be beneficial in combination with antineoplastic drugs.

In this study, we explored how omega-3 fatty acids, EPA and DHA, influence the viability and drug sensitivity of MM cells.

2. Materials and methods

2.1. Reagents and antibodies

EPA, DHA and arachidonic acid (AA) were obtained from Sigma (St. Louis, MO, USA) and dissolved in pure ethanol to make 100 mM stocks. The maximum concentration of ethanol in all experiments was kept at 0.05%. Controls with only ethanol were also run with each experiment. Fluorescein isothiocyanate (FITC)-conjugated Annexin-V and propidium iodide were from eBioscience (San Diego, CA, USA). JC-1 mitochondrial membrane potential assay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). The following antibodies were used in Western blotting: Rabbit monoclonal antihuman cleaved caspase-3 (clone D3E9) and NFκB-p65 (clone C22B4) (Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal antihuman pro-caspase-3 (clone E83-103; Epitomics, Burlingame, CA, USA), Horse radish peroxidase (HRP)-conjugated antirabbit immunoglobulins (DAKO). Bortezomib was supplied by LC Laboratories (Woburn, MA, USA) and dissolved in dimethyl sulfoxide (DMSO) to make a 100 mM stock solution. 7-Amino-4-methylcoumarin (AMC) powder and caspase-3 substrate acetyl–Asp–Glu–Val–Asp–AMC (Ac–DEVD–AMC) were obtained from Sigma and Bachem (Bubendorf, Switzerland), respectively, and also dissolved in DMSO. In relevant experimental conditions, DMSO final concentration was maintained below 0.01%. 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and phenazine methosulfate (PMS) were also obtained from Sigma.

2.2. Cell lines and cell culture

The HMCLs, L363, OPM-1, OPM-2 and U266 were obtained from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in RPMI medium containing 2 mM L-glutamine, supplemented with 5% fetal bovine serum (FBS) and intermittently with 100 U/ml penicillin and 100 μg/ml streptomycin at a humidified 37°C incubator providing 5% CO_2. Human peripheral blood mononuclear cells (PBMCs) were isolated from normal buffy coats (Sanquin Blood Bank, Amsterdam, the Netherlands), suspended in above-mentioned medium (but with 10% FBS) and treated as described below.

2.3. Drug cytotoxicity assay

The assay for drug cytotoxicity has been described earlier [23]. Briefly, HMCLs were first incubated with 30 μM EPA or DHA in RPMI medium supplemented with 5% FBS for 72 h. The cells were then washed and treated with different concentrations of bortezomib for 1 h (*acute exposure*) in round-bottomed 96-well plates, washed and resuspended in fresh drug-free medium and further incubated for 48 h. At the last 4 h, 50 μl of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) reagent premixed with PMS was added to each well, and after 4 h, the optical density of each well was determined with a plate reader. Some wells were also specified as blank (medium+ethanol), solvent control (cells+medium+ethanol) and growth control (cells+medium). Percent survival (% of viability) was calculated using nonlinear regression.

2.4. Annexin-V apoptosis assay

HMCLs were first incubated with 5–100 μM of EPA/DHA/AA in RPMI medium with 5% FBS or treated in parallel with ethanol for 24, 48 and 72 h. Cells were then washed with ice-cold FACS buffer (PBS+1% bovine serum albumin (BSA)+0.01% sodium azide), pelleted and suspended in Annexin-V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) containing 5 μl FITC–Annexin-V and left at room temperature in the dark for 10–15 min. After washing and suspending in binding buffer, 5 μl propidium iodide was added, and samples were applied to FACS analysis in a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FACS Diva software (BD Biosciences). To determine the effect of n-3 PUFAs on drug sensitivity of MM cells, in separate experiments, cells were first incubated with 30 μM of EPA or DHA for 72 h, washed and exposed to 5 nM of bortezomib for 48 h. After this time, samples were washed and applied to apoptosis FACS analysis as mentioned above.

2.5. Gene expression array (real-time polymerase chain reaction)

Gene expression following EPA and DHA treatment was analyzed using the Human Signal Transduction Pathway Finder RT^2 Profiler PCR Array (PAHS-014Z; SABiosciences). Working on a real-time polymerase chain reaction (PCR) basis, this array profiles the expression of 84 genes involved in various signaling pathways, and it also

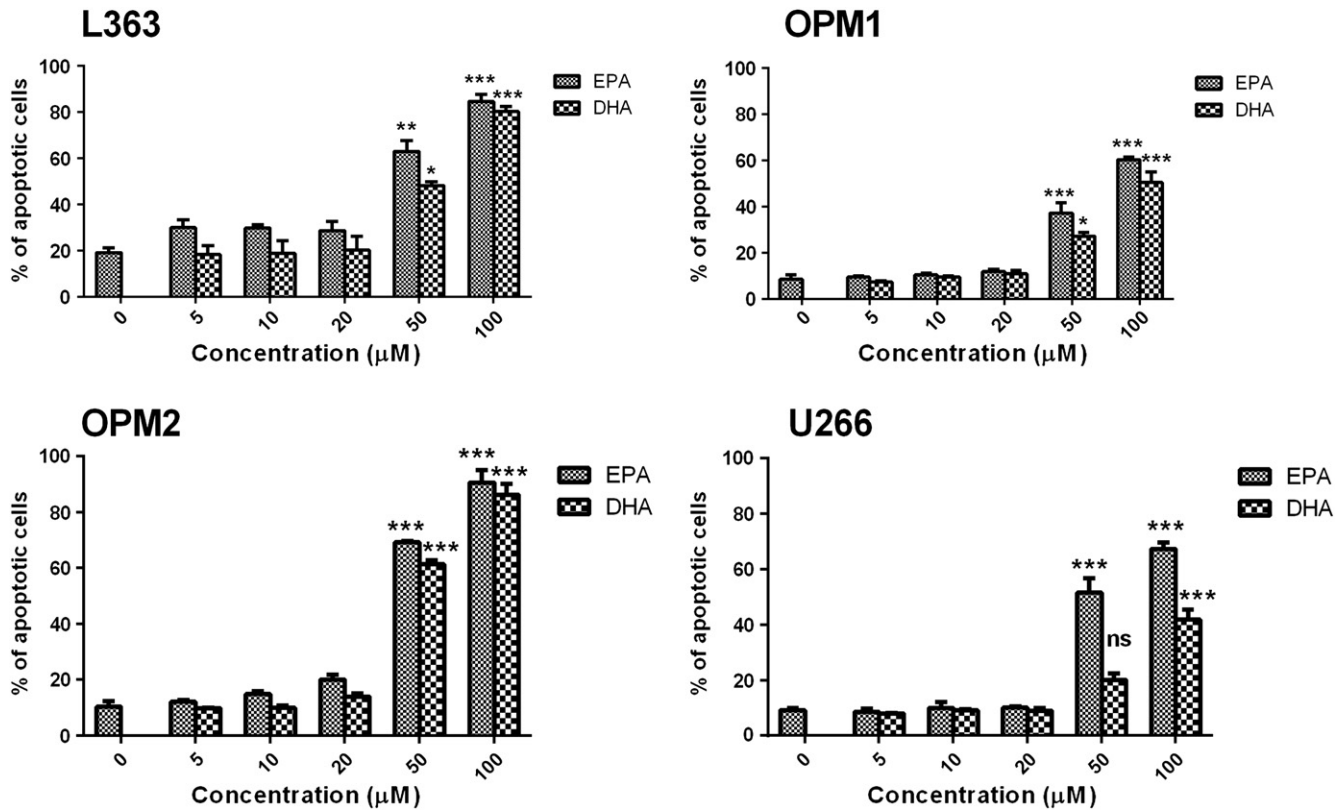

Fig. 1. n-3 PUFAs induce apoptosis in HMCLs in a dose-dependent manner; however, this effect is highly significant at or above 50 μM compared to the untreated (zero concentration) conditions. Data are mean±S.E.M. of two independent experiments. *P<.05, **P<.01, ***P<.001.

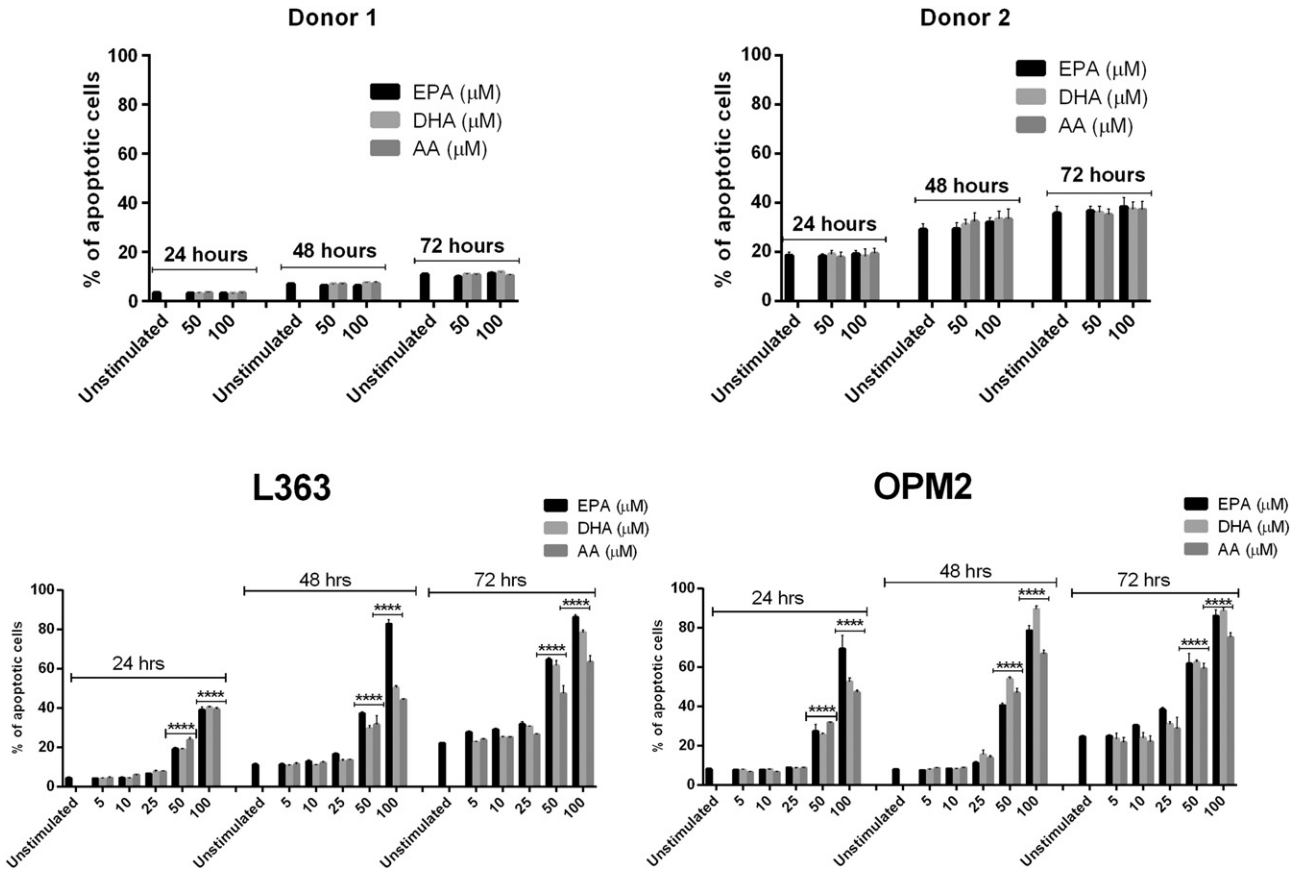

Fig. 2. Time and dose-dependent induction of apoptosis in HMCLs and PBMCs by EPA, DHA and AA. L363 and OPM2 cell lines were treated with 5–100 μM and PBMCs with 50 and 100 μM of above PUFAs for 24, 48 and 72 h, and applied to Annexin-V FACS analysis. Data are mean±S.E.M. of two independent experiments. *P<.05, **P<.01, ***P<.001.

includes five different housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, *HGDC*) and highly sensitive controls for detecting genomic DNA contamination and quality control of the assay. Briefly, L363 and OPM2 cell lines were incubated with EPA and DHA (50 μM) (test) or treated with ethanol (control) for 24 h, washed and pelleted. Using RNeasy mini kit (Qiagen), messenger RNA (mRNA) was isolated, and 1 μg of total mRNA was reverse-transcribed using RT^2 First Strand Kit (SABiosciences). The prepared cDNA was added to array plates which were then applied to real-time PCR using protocols and thermal profiles recommended by SABiosciences. Threshold cycle values (Ct values) were extracted, and using the $2^{-\Delta\Delta Ct}$ algorithm, fold regulation (up or down) of genes in EPA/DHA-treated compared to ethanol-treated conditions was determined.

2.6. Cleaved caspase-3 enzymatic activity

Cleaved caspase-3 enzymatic activity was measured as described previously with slight modifications [23]. The assay is based on the release of the fluorescent AMC moiety following hydrolysis of the peptide substrate Ac–DEVD–AMC by the activated enzyme. Briefly, cells were lysed in a buffer consisting of 10 mM HEPES (pH 7.5), 1% IGEPAL, 10% sucrose, 50 mM NaCl, 40 mM β-glycerophosphate, 2 mM $MgCl_2$, 5 mM EDTA and supplemented with a cocktail of protease inhibitors (Complete Mini; Roche) and left on ice for 30 min. After spinning, the samples at 10,000×*g* for 15 min at 4°C, supernatants were collected and incubated with 5 μM of caspase-3 substrate Ac–DEVD–AMC in each well of a 96-well plate. The plate was placed in a fluorescent plate reader with a built-in 37°C incubator (Fluoroskan *A*scent FL; Labsystems) for 1 h. During this time, substrate was cleaved (AMC release) by active caspase-3 and the fluorescent signals were recorded (excitation 340 nm, emission 460 nm). The activity of caspase-3 was determined as nM AMC/min/ml of cell lysate. A calibration curve was also created using free AMC.

2.7. Mitochondrial membrane potential measurement

JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was used to measure changes in mitochondrial membrane potential (ΔΨm) following apoptosis induction by EPA/DHA/AA. JC-1 is a cytofluorometric and lipophilic cationic dye that selectively enters the mitochondria and changes color from green (monomers) to red (aggregates) when mitochondrial membrane potential increases. During apoptosis in which mitochondrial membrane becomes permeable, the ΔΨm decreases leading to reduction in the ratio of aggregates to monomers. Briefly, HMCLs (5.0×10^5 cells/ml) were stimulated with different concentrations of EPA/DHA/AA for 24 h. Without washing the cells, 5 μl of the JC-1 solution was added to 100 μl of cell suspension in wells of a round-bottom 96-well plate and the plate was kept at 37°C for 30 min. Cells were then washed with special assay buffer, resuspended in assay buffer and applied to FACS (FACSCanto; BD Biosciences) analysis or fluorometry in a fluorescent plate reader (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany). Using FACS, the mean fluorescent intensity (MFI) of apoptotic cells in FL2 channel (aggregates) was measured and divided by MFI in FL1 channel (monomers). In fluorometric experiments, readings for J-aggregates (excitation: 565 nm, emission: 595 nm) were divided by those for monomers (excitation: 485 nm, emission: 535 nm) A reduction in above ratios was considered as a decrease in ΔΨm.

2.8. Preparation of nuclear fractions

HMCLs from different experiments were washed in cold PBS, pelleted and suspended in buffer A [10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% IGEPAL (Sigma), pH 7.9] containing a cocktail of protease inhibitors (Complete Mini; Roche), and left on ice for 10 min. After spinning at 3000 rpm 4°C, supernatants were collected and pellets were lysed in buffer B [5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 30% glycerol (vol/vol), pH 7.9] for 30 min on ice. Finally, supernatants were collected after spinning the lysates at 20,000×*g* 4°C, for 15 min. Protein concentration was determined using a BCA kit (Pierce). A fixed amount of protein was subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis/blotting as mentioned below.

2.9. Western blotting

For caspase-3 protein analysis in blotting, HMCLs were harvested from EPA/DHA-treated or ethanol-treated conditions, washed in cold PBS and pelleted. Cell pellets were then lysed in cold RIPA buffer (150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) or another buffer (150 mM NaCl, 1% IGEPAL, 50 mM Tris, pH 8.0, 10% glycerol), both containing a cocktail of protease inhibitors (Complete Mini; Roche), and left on ice for 30 min. After spinning at 10,000×*g* 4°C for 15 min, the supernatants were removed and the protein

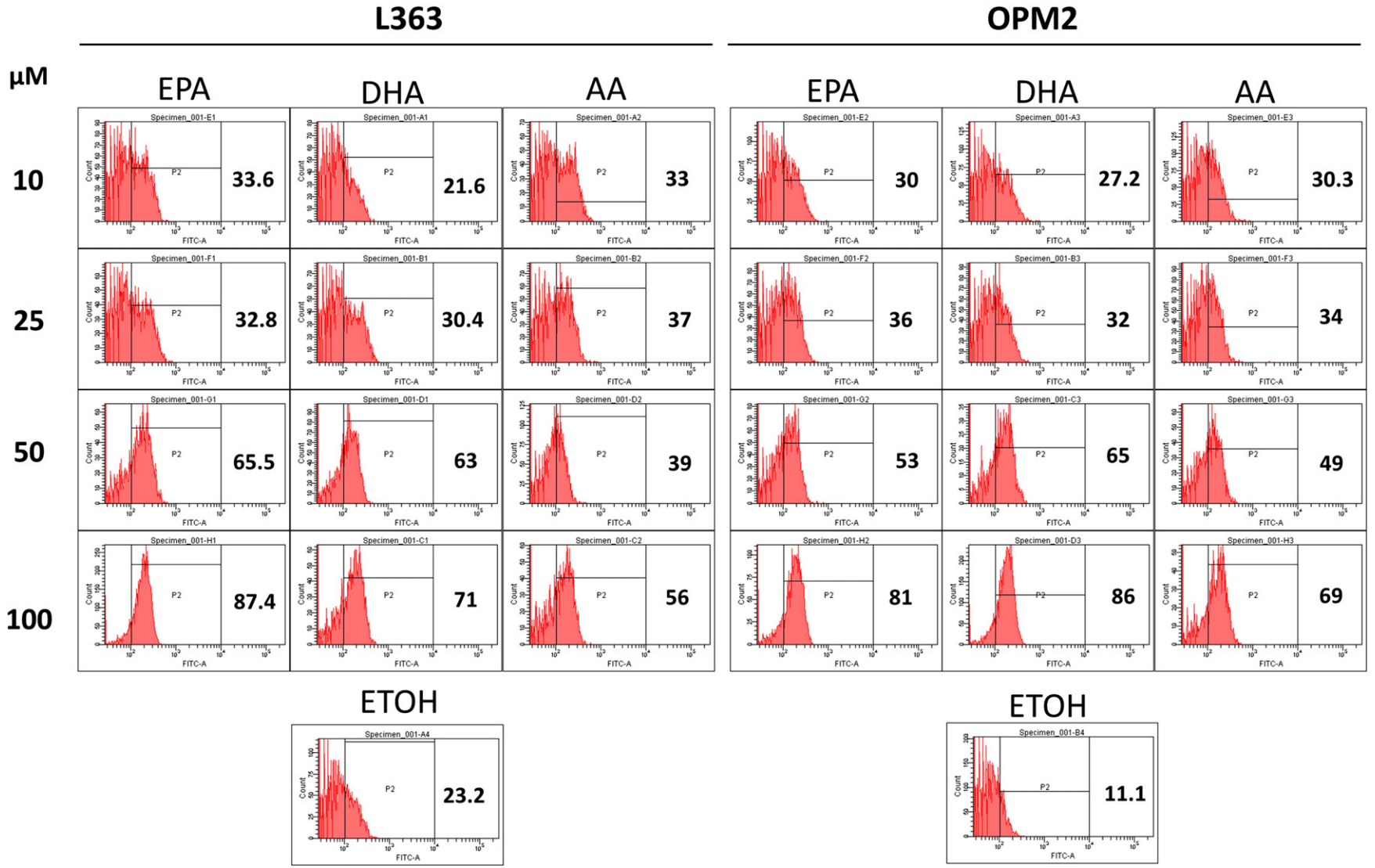

Fig. 3. Typical examples of flow cytometry histograms showing EPA-, DHA- and AA-induced apoptosis in L363 and OPM2 MM cells as demonstrated by binding of FITC-conjugated Annexin-V. Ethanol (EtOH) was used as solvent control.

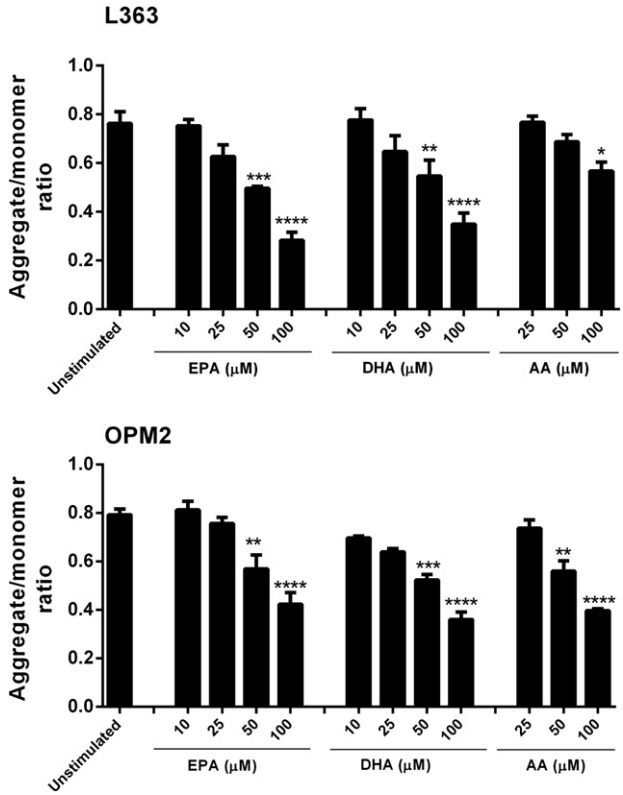

Fig. 4. PUFAs induce apoptosis in HMCLs through mitochondrial perturbation. HMCLs were incubated for 24 h with 10–100 μM of EPA/DHA/AA and applied to JC-1 staining for analysis of mitochondrial membrane potential change (ΔΨm) using FACS as explained in Materials and Methods. All three PUFAs decreased membrane potential dose dependently indicating mitochondrial membrane disruption due to apoptosis. Data represent mean±S.E.M. of four independent experiments. *P<.05, **P<.01, ***P<.001.

concentration was measured with a BCA kit (Pierce). Twenty-Thirty micrograms of total protein was applied to a 12% SDS gel which was then electroblotted onto a polyvinylidene fluoride (PVDF) membrane. After incubation in blocking buffer, the membranes were probed with rabbit antihuman primary antibodies (NFκB-p65, cleaved and pro-caspase-3, beta-actin or GAPDH) followed by specific secondary antibodies. Finally, the signals were detected using ECL prime reagent (Amersham) or SuperSignal West Femto Chemiluminescent Substrate (Pierce) if required.

2.10. Statistical analysis

We used one- or two-way analysis of variance in GraphPad prism 6 software for data analysis in all experiments, and P<.05 was considered significant.

3. Results

3.1. EPA, DHA and AA induce apoptosis in myeloma cells dose dependently, but have no effect on normal human PBMCs

Since it is currently unknown at which time point different cell types incorporate PUFAs into their membranes, L363, OPM-1, OPM-2 and U266 HMCLs were incubated with 5–100 μM EPA or DHA for 72 h, and afterward, the percentage of apoptotic cells was determined by flow cytometry. As depicted in Fig. 1, EPA and DHA induced a dose-dependent cell death and very significant cell death occurred at 50 μM and higher. No effects of the solvent ethanol (ETOH) were observed. The HMCLs U266 and OPM-1 were found significantly less sensitive to EPA/DHA than L363 and OPM-2. At the next steps, to explore whether the effects really reflect the occurrence of apoptosis (not necrosis), L363 and OPM-2 cell lines were selected to perform additional analyses. FACS experiments were performed at three different time periods (24, 48, 72 h) in parallel with two more controls: AA, to investigate whether the effects were associated with specific structural features of PUFAs, and normal human PBMCs, to investigate whether these PUFAs were toxic to primary healthy cells, as well. EPA/DHA/AA induced apoptosis in MM cells after 24 h, and these effects increased over time reaching the highest level at 72 h (Fig. 2, a histogram sample at Fig. 3). Moreover, the highest effect was observed with EPA, especially in L363 cell line, but AA showed significantly less strength in L363 cells. Importantly, EPA/DHA/AA was not found to have any effect on the viability of normal human PBMCs at the same concentrations and incubation times (Fig. 2).

3.2. EPA/DHA/AA-induced apoptosis in HMCLs is accompanied by mitochondrial perturbation

Disruption of active mitochondria is a hallmark of early apoptosis leading to changes in mitochondrial membrane potential (ΔΨm) due to formation of pores in mitochondrial membrane and passage of ions, which leads in turn to the respiratory chain being decoupled and cytochrome C being released. L363 and OPM2 were stimulated with different concentrations of EPA, DHA and AA for 24 h and analyzed for changes in ΔΨm using JC-1 staining. As depicted in Fig. 4, the results suggest that all three PUFAs decreased ΔΨm dose dependently indicated by a decrease in the ratio of aggregates to monomers. However, the significant effect for EPA and DHA in both cell lines appeared at 50 μM and higher, but that for AA started at 100 μM in L363 and at 50 μM in OPM-2 suggesting that AA was generally less potent than EPA and DHA in L363 cell line. We also observed exactly the same trend in fluorometry (plate reader) as in FACS, but only the results in FACS are shown here. These observations imply that EPA/DHA-induced apoptosis was also accompanied by early changes in the mitochondrial pathway.

3.3. Induction of apoptosis in HMCLs by EPA, DHA and AA is caspase dependent

We next investigated if EPA/DHA/AA-induced apoptosis in MM cells occurred in a caspase-dependent manner. Lysates from treated and untreated cells were incubated with caspase-3 substrate (Ac–DEVD–AMC), and enzymatic activity was determined based on AMC release over time. EPA, DHA and AA triggered caspase-3 activity dose dependently, with EPA showing the most pronounced effects. By contrast, caspase-3 was not activated following treatment of normal PBMCs with any of the three PUFAs (Fig. 5). Furthermore, at protein level, caspase-3 was significantly up-regulated dose dependently following EPA/DHA/AA treatment of L363 and OPM2 cell lines (Fig. 6). The above findings indicate that EPA/DHA/AA triggers cell death in MM cells through a caspase-dependent mechanism.

3.4. EPA and DHA sensitize HMCLs to cytotoxic effects of bortezomib and enhance bortezomib-induced apoptosis

In the next step, the effects of EPA and DHA on the cytotoxic effects of bortezomib were determined. HMCLs were prestimulated with EPA and DHA (30 μM) and showed a significantly lower viability following exposure to increasing doses of bortezomib (0.1–5.0 μM) (Fig. 7). Considering the IC50 of the drug in EPA/DHA-treated or untreated conditions, the level of sensitivity differed for both PUFAs. In L363 and OPM-2, EPA was more potent in sensitizing the cells than DHA (IC50 0.70 for EPA vs. 1.450 for DHA in L363, and IC50 0.35 for EPA vs. 1.03 for DHA in OPM-2). Generally, L363 and OPM-2 cell lines were much more sensitive than U266. Furthermore, the increase in susceptibility to bortezomib after EPA and DHA treatment was also accompanied by an increase in percentage of apoptotic cells (Fig. 7). Notably, EPA was

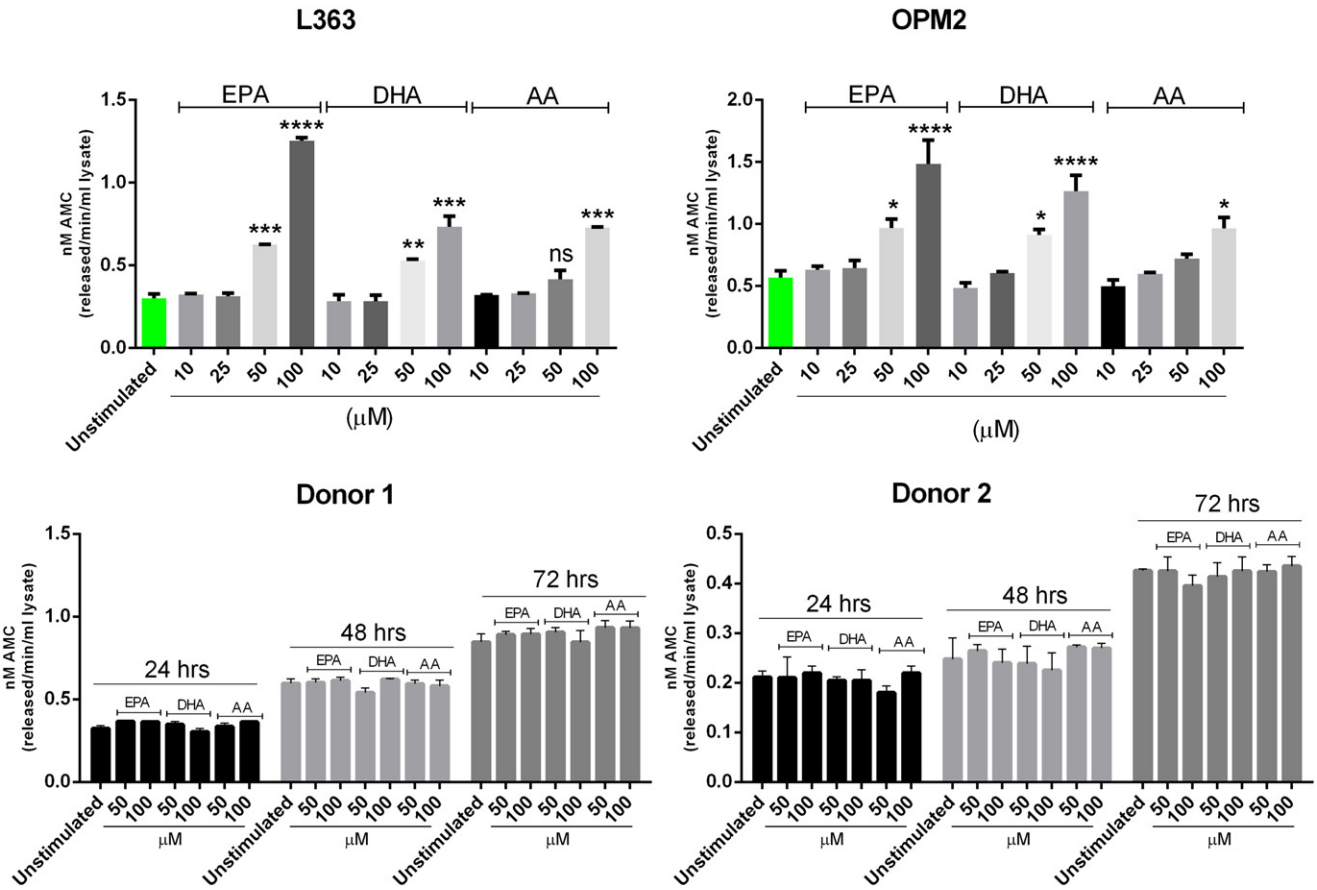

Fig. 5. The effect of EPA/DHA/AA on caspase-3 activity in HMCLs and normal PBMCs incubated with different concentrations of PUFAs. HMCLs were treated with 10–100 μM of PUFAs for 24 h, lysed and assessed for enzymatic activity of caspase-3 as explained in Materials and Methods. Data represent mean±S.E.M. of three separate experiments in HMCLs and two measurements on each PBMC donor. *P<.05, **P<.01, ***P<.001. For PBMCs, lysates were prepared from the samples in Fig. 2 and assessed for casapse-3 activity as explained in Materials and Methods. During 24-, 48-and 72-h incubation times, no changes were observed in enzymatic activity of caspase-3. Data represent mean±S.E.M. of two independent measurements for each donor.

more potent than DHA in L363 and OPM-2 cell lines in enhancing the apoptotic effect of bortezomib.

3.5. EPA and DHA have inhibitory effect on NFκB baseline activity in HMCLs

Considering the constitutive activation of NFκB pathway in most HMCLs [4,23–25], we next investigated the effect of EPA and DHA on NFκB pathway in HMCLs at steady state. HMCLs were cultured for 72 h with 50 μM EPA or DHA, and the expression of NFκB-p65 protein was analyzed in nuclear fractions with Western blotting (Fig. 8). EPA or DHA down-regulated NFκB-p65 protein in OPM-2 and L363 cell lines, suggesting they probably inhibited nuclear translocation of NFκB, hence NFκB deactivation. However, this effect was more pronounced in OPM2 cells.

3.6. EPA and DHA modulate a variety of genes involved in various signaling pathways in HMCLs

Little is known about the modulatory effects of EPA and DHA at gene levels in cancer cells. Therefore, L363 and OPM2 cells were incubated with 50 μM EPA/DHA and applied to qPCR array. EPA and DHA modulated heterogeneously a wide range of genes which were clustered in various signaling pathways including PPAR, NFκB, p53, oxidative stress, hypoxia, notch, hedgehog, JAK/STAT, TGFβ and Wnt. The pattern of change in gene expression by EPA and DHA seemed to be different between the two cell lines, but also between the compounds (Fig. 9, and Supplementary Tables 1 and 2). In L363 (EPA) and OPM2 (EPA and DHA), several genes (*GCLC*, *GCLM*, *NQO1*, *FTH1*, *TXNRD1*, *GSR*, *HMOX1*, *SQSTM1*) involved in oxidative stress pathway were up-regulated (2.0- to 3.5-fold). In the hypoxia pathway, the *EPO* gene was down-regulated (2- to 5-fold) in both cell lines by both PUFAs, and *SERPINE1* was down-regulated only by DHA in both cell lines (19-fold L363, 6.6-fold OPM2). These findings suggest a possible role of hypoxia modulation in EPA/DHA-induced apoptosis. The p53 pathway was apparently not affected in L363, whereas in OPM2, the genes *BTG2* (5.2-fold), *BBC3* (4-fold) and *BAX* (2.3-fold) were down-regulated by EPA and up-regulated by DHA (*BTG2* 2.7-fold, *BBC3* 3.8-fold). PPAR and NFκB pathways were differentially modulated in the two cell lines. For example, *OLR1* gene in PPAR pathway was up-regulated in L363 by EPA/DHA (2.0-fold), while it was down-regulated in OPM2 by both PUFAs (5.3-fold EPA, 13.5-fold DHA). In the NFκB pathway, only two genes, *TNF* (EPA and DHA) and *BCL2A1* (EPA), were down-regulated by the two PUFAs in L363. On the contrary in OPM2 cells, the *TNF* gene was up-regulated by EPA/DHA and several other genes (*BCL2L1*, *ICAM1*, *BIRC3* and *IFNG*) were down-regulated implying some level of NFκB pathway inhibition by these PUFAs in HMCLs. Several genes involved in Hedgehog, Notch and Wnt signaling pathways were differentially modulated in OPM2 cell line (but much less in L363). Intriguingly, EPA mostly down-regulated and DHA mostly up-regulated the genes involved in latter pathways.

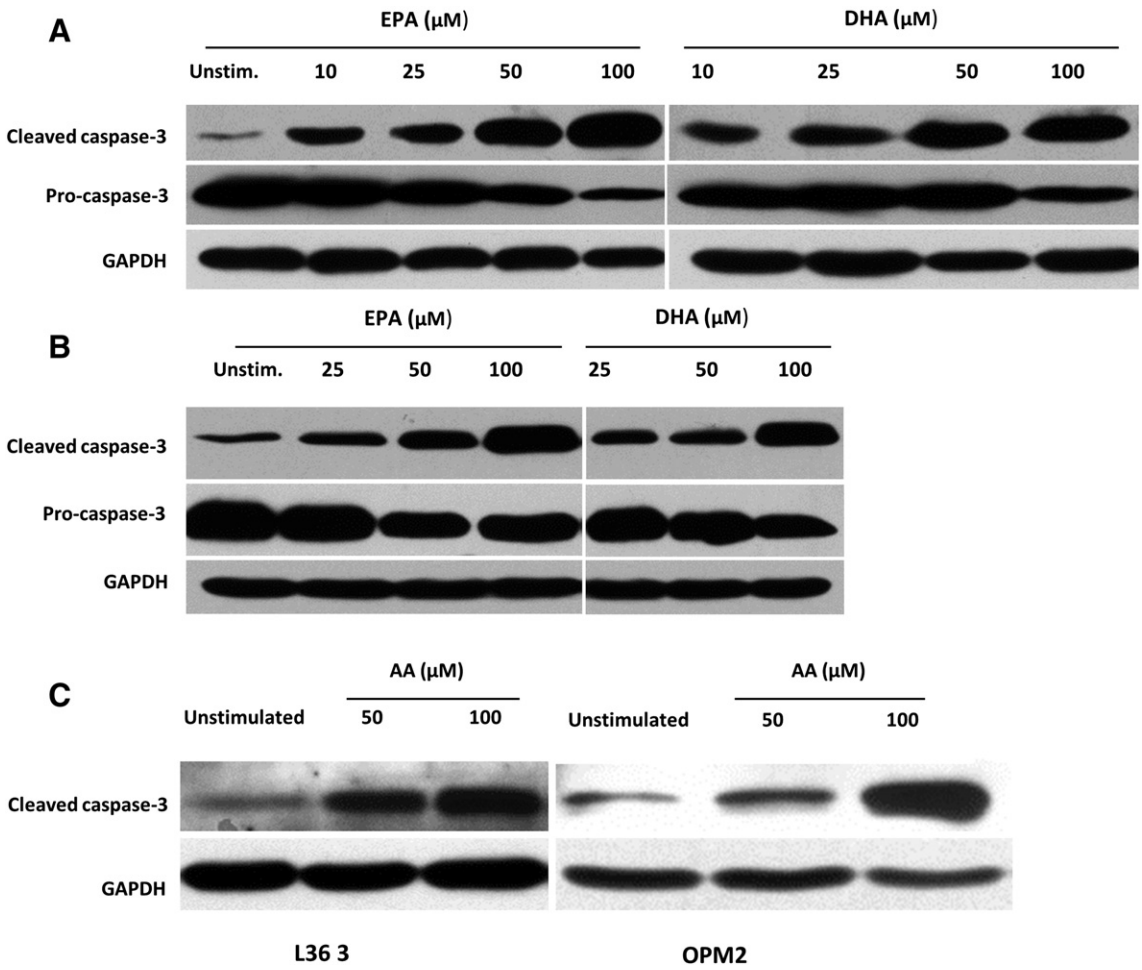

Fig. 6. The effect of PUFAs on caspase-3 protein levels in HMCLs. L363 and OPM2 cell lines were incubated with 10–100 μM of EPA/DHA for 24 h, and lysates were probed for the expression of caspase-3 protein (cleaved and pro forms). Both PUFAs up-regulated caspase-3 dose dependently and in parallel decreased the proenzyme form indicating cleavage of the precursor protein.

4. Discussion

This is the first study to describe the proapoptotic effects of EPA and DHA in myeloma cells and to show that EPA and DHA sensitize myeloma cells to bortezomib and increase bortezomib-induced apoptosis. n-3 PUFAs are well known to influence cell physiology through multiple mechanisms [26] and have been found to induce apoptosis in other cancer types [8,27–29]. Thus far, multiple mechanisms have been suggested to mediate their apoptotic effects in cancer cells [10,30]. Here, we found that EPA and DHA induced apoptosis in HMCLs in a caspase-3- and mitochondrial-dependent mechanism implying an intrinsic pathway of apoptosis. Consistent with our findings, another study demonstrated that EPA induced apoptosis in human pancreatic cell lines and *in vivo* models through a caspase-dependent pathway associated with ROS accumulation [8]. It is important to note that increasing evidence support the role of ROS production and oxidative stress pathway in EPA and DHA-mediated cell death in various cancer cells [4,8,10]. Transcription analysis of different signaling pathways in OPM-2 and L363 showed that EPA and DHA have multiple effects on various members in these pathways (Fig. 9). We found several genes from the oxidative stress pathway up-regulated. Some of these genes including *TXNRD1*, *HMOX1*, *GCLC* and *GCLM* were also reported as being up-regulated in EPA/DHA-induced cell death in human colon cancer cells [30]. EPA and DHA modulated these genes differentially in OPM2 cells, with EPA down-regulated and DHA up-regulated most genes suggesting that multiple mechanisms may lead to the induction of cell death. Presently, little is known about the effect of up- or down-regulation of these genes on apoptosis in HMCLs. Our findings provide further evidence on the role of oxidative stress pathway in EPA/DHA-induced apoptosis in HMCLs.

Inhibition of NFκB pathway is reported to be one of the mechanisms for omega-3 fatty acids to inhibit inflammatory reactions [31,32]. In EPA- and DHA-treated HMCLs, the NFκB-p65 protein was down-regulated. Moreover, mRNA expression array also yielded a pattern of NFκB-pathway-related genes alteration which suggests a possible inhibitory effect on this pathway by, e.g., down-regulation of *TNF* gene in L363, or down-regulation of *BCL2L1*, *ICAM1* and *BIRC3* in OPM2. The incubation of L363, OPM-2 and U266 with 50 μM EPA or DHA for 72 h decreased NFκB-p65 protein in L363 and OPM-2 but not in U266 cells. This suggests that a resistant NFκB phenotype in U266 may explain its low drug sensitivity or apoptosis in EPA/DHA-treated conditions. It remains to be determined in future experiments to what extent increased drug sensitivity and apoptosis in HMCLs following EPA/DHA treatment is due to NFκB inhibition.

Several genes related to Notch, Hedgehog and Wnt signaling pathways were also differentially modulated. These pathways are widely known as key regulators of stem cell development and biology, and their role in MM pathogenesis has been described earlier [25,33,34]. n-3 PUFAs trigger apoptosis in pancreatic [7] and hepatocellular carcinoma [35] cells through inhibition of Wnt

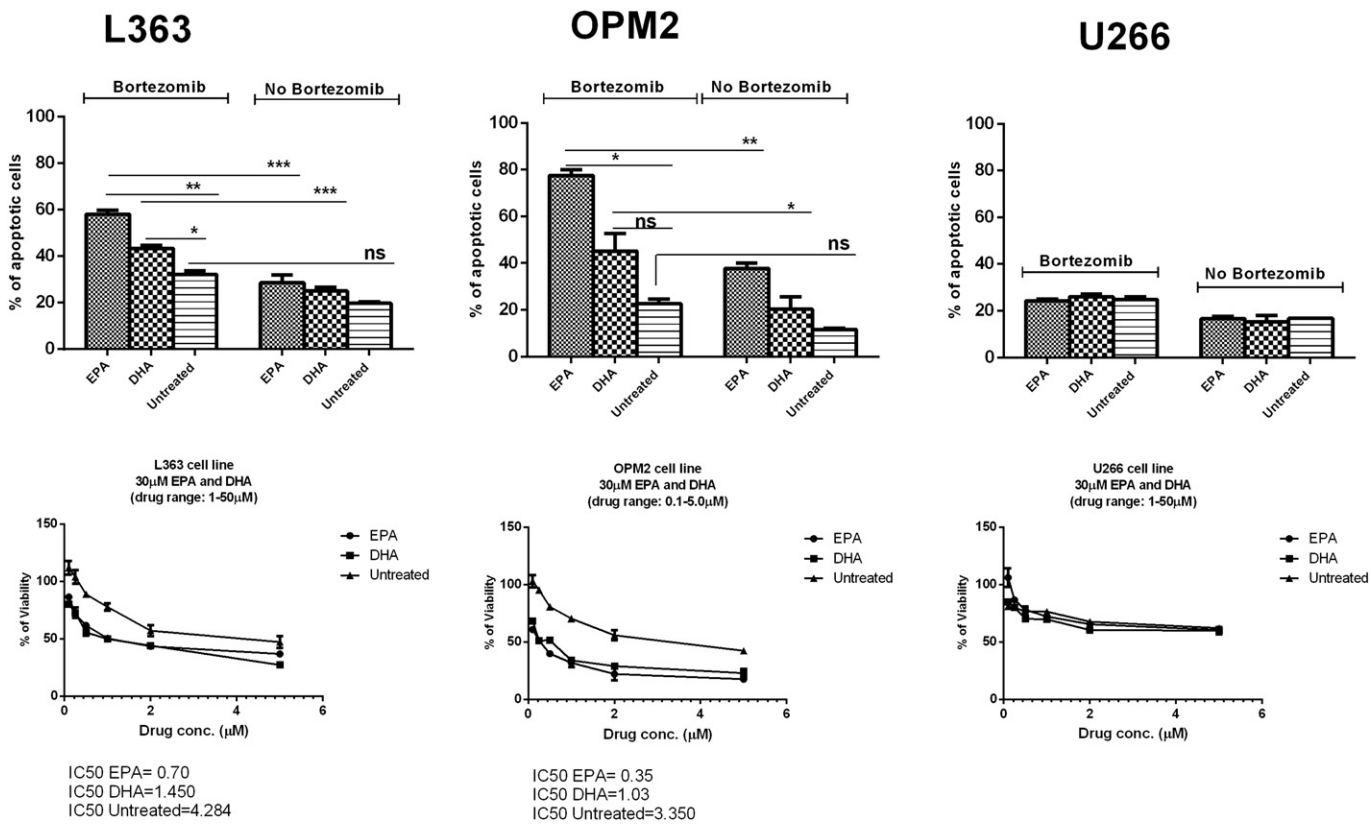

Fig. 7. The effect of EPA and DHA on bortezomib-induced apoptosis (A) and bortezomib sensitivity (B) in HMCLs. For drug-induced apoptosis, HMCLs were incubated for 72 h with 30 μM EPA or DHA, washed and exposed to bortezomib (5 nM) for 48 h and analyzed for Annexin-V apoptosis in FACS. For drug cytotoxicity, HMCLs were pretreated with 30 μM EPA or DHA for 72 h, washed and exposed to different concentrations of bortezomib (0.1–5.0 μM) for 1 h (acute exposure), and then washed again and the incubation was extended to 48 h in bortezomib-free medium. Data are mean±S.E.M. of two independent experiments. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$.

pathway. DAB2 which is reported to be a tumor suppressor gene was up-regulated only in OPM2 by EPA (1.5-fold) and DHA (3.0-fold) in Wnt pathway. Other pathways including TGFβ, p53, PPAR, JAK/STAT, and hypoxia also showed varying levels of modulation following EPA/DHA treatment. In the hypoxia pathway, the *EPO* gene is down-regulated in all conditions, suggesting that these PUFAs may inhibit hypoxia pathway in HMCLs to trigger cell death. Furthermore, it is possible that n-3 PUFAs influence cytokine signaling to induce cell death. For example in L363, *SOCS3* gene (encoding a protein which inhibits cytokine signaling) from JAK/STAT pathway is up-regulated by EPA, and *EMP1* gene from TGFβ pathway is down-regulated by EPA and DHA. Taken together, the analyses at gene level indicate that n-3 PUFAs modulate a wide range of genes involved in various signaling pathways in HMCLs. Further research is needed to show which pathways are crucial to control the induction of apoptotic cell death.

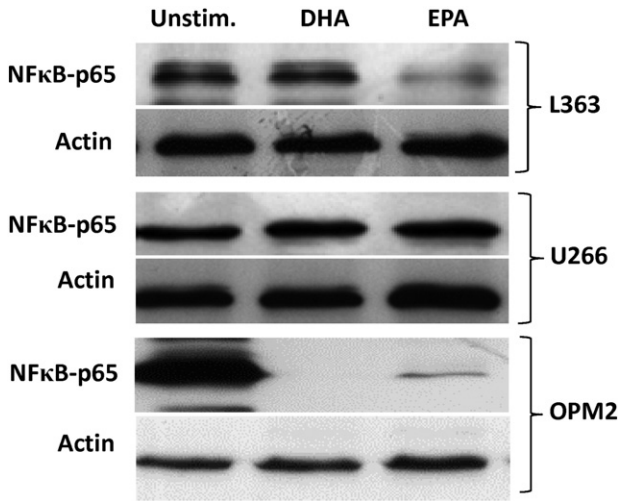

Fig. 8. The effect of EPA and DHA on NFκB-p65 protein levels in nuclear fractions. HMCLs were treated with 50 μM EPA or DHA for 72 h. Nuclear fractions were isolated, electrophoresed and immunoblotted with an NFκB-p65 specific Ab as described in Materials and Methods.

In our study, AA an n-6 PUFA also triggered apoptosis in HMCLs. The latter finding may indicate that apoptotic effects of PUFAs are not dependent on intrinsic structural features (position of double bonds). In support of this, AA-derived prostaglandin, 15d-PGJ2, has been reported to trigger apoptosis in leukemia stem cells [29]. Interestingly, in L363 we observed a lower potency for AA than EPA and DHA.

Additionally, the combination of bortezomib and the n-3 PUFAs EPA or DHA increased the antimyeloma activity of bortezomib with respect to apoptosis and drug sensitivity. Supplementation or combination therapy with n-3 PUFAs in MM chemotherapy may help reduce the resistance to chemotherapeutic drugs such as bortezomib which has recently been shown to end in resistance in some MM patients [2,3,36,37]. In conclusion, our study indicates that the n-3 PUFAs EPA and DHA stimulate apoptosis in MM cells, which is associated with modulation of the expression of genes involved in various signaling pathways. The sensitivity for n-3 PUFAs seems unique for MM cells, because normal PBMCs are not affected by EPA and DHA. Further research is warranted to investigate

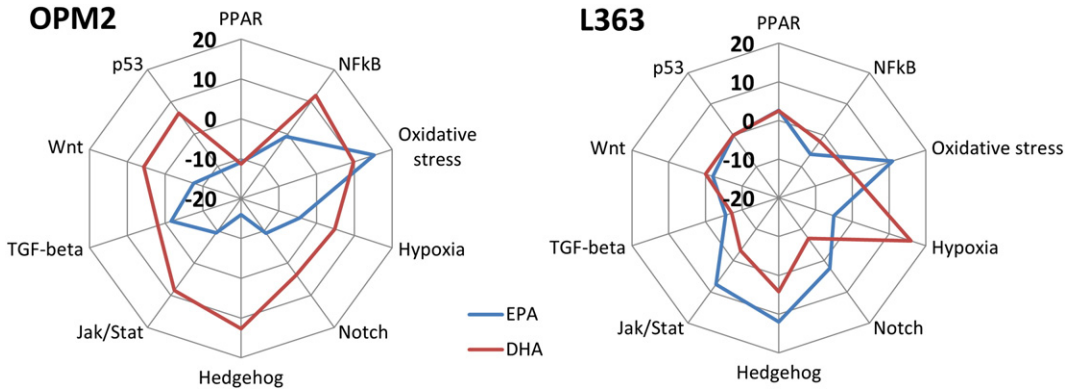

Fig. 9. Graphical view of the overall effects of EPA and DHA on gene transcription within different signaling pathways in HMCLs OPM-2 (left panel) and L363 (right panel). Transcriptional changes (up-regulation of down-regulation) for different members within a signaling pathway induced by EPA or DHA were added up and are depicted in a Web graph. Individual gene changes are listed in Supplementary Tables 1 and 2.

if supplementation with omega-3 fatty acids in MM therapy could increase drug sensitivity of the MM tumor and potentially reduce chemoresistance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2014.06.013.

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