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PUFA and Oxidative Stress. Differential Modulation of the Cell Response by DHA

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Abstract:

Although an increased dietary intake of long-chain n-3 PUFA is considered an effective preventive strategy, a theoretical concern related to the possible increase of lipid peroxidation induced by a PUFA-rich diet still remains. In this study, the effects of different PUFA (linoleic, α-linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acid) on cytotoxicity, lipid oxidation, and modulation of antioxidant defenses were evaluated in HepG2 cells submitted to an oxidative stress (H_2O_2). Results clearly evidenced that all supplemented PUFA but DHA enhanced cell susceptibility to H_2O_2. Overall, our results underline that PUFA cannot be considered as a single category but as individual compounds, and research on mechanisms of action and preventive effects should deal with the individual fatty acids, particularly in the case of DHA.

Keywords: HepG2 cells; hydrogen peroxide; cytotoxicity; antioxidant enzymes

1. Introduction

There are converging opinions among experts, organizations and health professionals that a recommendation for a daily individual consumption of 500 mg of eicosapentaenoic (EPA)/ docosahexaenoic (DHA) acids would provide health benefits (Salem & Eggersdorfer, 2015). An increased dietary intake of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), EPA and DHA, is considered an effective strategy in the prevention and modulation of cardiovascular (Ohnishi & Saito, 2013; Cao et al., 2015) and neurological diseases (Patrick & Ames, 2015), for the optimization of cognitive performance (Muldoon et al., 2014) and brain development (Brenna & Carlson, 2014).

Since DHA and other PUFA are highly oxidizable molecules (Miyashita, 2014), a theoretical concern remains on their use at high concentration in the prevention of chronic diseases having the oxidative stress as one of the underlying mechanisms.

Besides being prone to oxidation, PUFA have been reported to activate the stress-signaling pathway controlling the genetic program used by cells to induce the synthesis of proteins responsible for detoxification and counteraction of the oxidative stress (Ma, 2013). In this way, PUFA can modulate the activity of antioxidant enzymes (Wang et al., 2004) to ensure survival and reduced status.

Supplementing cultured cells with individual PUFA we previously observed diverse effects on oxidation and modulation of antioxidant defenses. The different effects were not simply related to the length of the carbon chain, or to the number and position of double bonds in the fatty acid (Di Nunzio et al., 2011). In particular, DHA supplementation did not cause any adverse effect, suggesting that its use as preventive dietary strategy does not induce a higher sensitivity to oxidation. It is conceivable that the overall effects of the different PUFA are related to their individual ability to increase the intracellular concentration of pro- or antioxidants.

In this light, the concomitant oxidative status of the cell could have a role in its response to PUFA supplementation, and the differential effect of DHA than other PUFA needs to be confirmed in cells submitted to an oxidative insult. To evaluate it, in the present work, we supplemented HepG2 cells with linoleic acid (18:2n-6, LA), α-linolenic acid (18:3n-3, ALA), arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA), then a mild oxidative stress reflecting physiological condition was induced (Halliwell et al., 2000; González-Flecha et al., 1993; Khassaf et al., 2001). HepG2 cells were used as model system since liver is a major organ attacked by reactive oxygen species (ROS) (Sànchez-Valle et al., 2012), and parenchymal cells are primary cells subjected to oxidative stress induced injury in the liver. Cells were supplemented with physiological concentration (60 µM) of n-6 and n-3 PUFA, and cell survival, lipid oxidation, and antioxidant defenses were evaluated after exposure to hydrogen peroxide.

2. Experimental Section

2.1. Chemical

Dulbecco's modified Eagle's medium (DMEM), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Lonza (Milan, Italy). All chemicals and solvents were of the highest analytical grade and were purchased from Sigma-Aldrich (Milan, Italy).

2.2. HepG2 cells tissue culture

HepG2 human hepatoma cells were maintained at 37°C, 95% air, 5% CO_2 in DMEM supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Di Nunzio et al., 2010). Once a week cells were split 1:20 into a new 75cm^2 flask, and medium was refreshed after 72 h.

2.3. Fatty acid supplementation and oxidative stress

Cells were seeded in 6-well plates, and after 24 hours, at 75-80% confluence, they were incubated for 21 h with the different fatty acids at 60 µM concentration. Free fatty acids were dissolved in 100%

isopropanol, and complexed to bovine serum albumin (BSA). Fatty acid-BSA complexes were prepared fresh each time, and BSA final concentration was 0.5% in serum free DMEM. Unsupplemented (US) cells received corresponding amounts of BSA and isopropanol, which final concentration in the media was kept below 1% (v/v). LA, ALA, ARA, EPA, and DHA were used for cell supplementation. After 21 h incubation cells were washed twice with warm DPBS, and then exposed for 20 or 60 min to 0.2 mM H_2O_2 in Earle's Balanced Salt Solution (EBSS) (116 mM NaCl, 5.4 mM KCl, 0.8 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2.38 mM $CaCl_2$, 0.39 mM $MgSO_4$). Some unsupplemented cells received EBSS only (control cells). Then EBSS medium was collected, centrifuged at 400 g for 3 min, and used to evaluate LDH release and TBARS concentration. Cells were washed twice with warm DPBS, and processed for further analysis.

2.4. Lactate dehydrogenase (LDH) release

LDH activity in the EBSS was assessed following spectrophotometrically for 1 min the rate of NADH oxidation at 340 nm (Di Nunzio et al., 2013), and enzyme activity was expressed as mUnits/ml.

2.5. Thiobarbituric acid reactive substances (TBARS) concentration

TBARS, the end products of lipid peroxidation, were assayed in EBSS as previously reported by Valli *et al.* (Valli et al., 2012), and their concentration was expressed as relative fluorescence units (RFU) normalized for mg protein in the corresponding well.

2.6. Cell viability

Cell viability was evaluated by the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal by metabolic active cells (Danesi et al., 2011). Results were expressed as percent of control cell viability, assigned as 100%.

2.7. Conjugated diene (CD) level

Cellular level of CD containing lipids was assayed as reported by Di Nunzio *et al.* (Di Nunzio et al., 2011), and expressed as percent of the value detected in control cells, assigned to 100%.

2.8. Cytoplasmic total antioxidant activity (TAA)

Cytoplasmic TAA was measured as reported by Di Nunzio *et al.* (Di Nunzio et al., 2013), based on the ability of the antioxidant molecules in the sample to reduce the radical cation of ABTS. Values were normalized for protein content in the sample, and expressed as micromoles of trolox equivalent (TE)/mg protein.

2.9. Reduced glutathione (GSH) content

Cytoplasmic GSH content was determined as previously described (Valli et al., 2012), normalized for protein content in the sample, and expressed as nmoles of GSH/mg protein.

2.10. Antioxidant enzymes activity

Cytoplasmic superoxide dismutase (SOD) activity was assayed using a commercial kit (Cayman Chemical, Michigan, USA) following the manufacture instructions. SOD activity (Units) was then normalized for mg of protein in the sample. Cytoplasmic catalase (CAT) activity was assayed spectrophotometrically at 240 nm following the extinction of H_2O_2 (Danesi et al., 2006), normalized for protein content in the sample, and expressed as Units/mg protein. Cytoplasmic glutathione-S-transferase (GST) activity was assayed as previously reported by Di Nunzio *et al.* (Di Nunzio et al., 2011). GST activity (nmol/min) was then normalized for protein content in the sample. Cytoplasmic glutathione peroxidase (GPx) activity was assayed using a commercial kit (Cayman Chemical, Michigan, USA) following the manufacture instructions. GPx activity (nmol/min) was then normalized for protein content in the sample.

2.11. Protein content

Protein content was determined according to Bradford (Bradford, 1976), using BSA as standard.

2.12. Statistical analysis

Data are reported as mean ± SD of at least six samples derived from three independent cell cultures. Statistical analysis was by the one way ANOVA with Dunnett as post-test.

3. Results

To evidence the detrimental effect of H_2O_2 addition, control cells were compared to stressed cells, both US and fatty acid supplemented. Then a statistical comparison was performed among stressed cells, to verify the differential effect of the single fatty acids, and to evidence whether the diverse supplementation could protect or exacerbate the outcomes of the oxidative stress.

3.1. H_2O_2-induced cell oxidative damage

The H_2O_2-induced cell damage was evaluated by measuring cell viability, LDH release, TBARS level and CD content (figure 1). As shown in figure 1A, in all cells H_2O_2 exposure caused a significant reduction of viability with respect to controls. Among stressed cells, viability decreased in all supplemented cells, but DHA.

The oxidative stress caused a significant increase in LDH activity and TBARS concentration in the medium of all cells except the US and DHA supplemented ones (figure 1B and 1C, respectively). Notwithstanding the increase of TBARS in almost all stressed cells, the level of CD, the first products of lipid peroxidation, increased in LA and ARA supplemented cells only (figure 1D).

3.2. Modulation of antioxidant defenses

To evaluate the effect of PUFA supplementation on the cell response to an oxidative stress, cytosolic TAA and GSH content (figure 2), and the activity of SOD, CAT, GST and GPx enzymes (figure 3) were determined.

Compared to controls, H_2O_2 exposure caused a significant decrease of cytosolic TAA in US and LA supplemented cells only (figure 2A), and no modification in GSH content apart from a significant increase in DHA supplemented cells (figure 2B). Comparing stressed cells, TAA appeared higher in ARA and DHA supplemented hepatocytes, the latter also showing a higher GSH concentration.

Compared to controls, upon H_2O_2 exposure a significant increase of SOD activity was detected in LA and n-3 PUFA supplemented cells (figure 3A), and CAT activity increased in all cells (figure 3B). GST activity was not modified in US cells, while it significantly increased in all supplemented cells but DHA ones (figure 3C). Furthermore, a significant increase in GPx activity was detected in LA supplemented cells only (figure 3D). Comparing stressed cells, a differential influence of the supplemented fatty acids on enzyme activities was evidenced. SOD activity being higher in EPA and DHA supplemented, CAT in ARA and EPA supplemented, GST in ARA supplemented, and GPx in LA supplemented cells than in US ones.

Since cellular oxidative status may be influenced by nonlethal oxidative stress exposure in a time depending manner (Brunk et al., 1995), SOD, CAT, GST and GPx activities were also evaluated after 20 min H_2O_2 exposure (figure 4A, 4B, 4C and 4D respectively).

Compared to controls, upon the shorter H_2O_2 exposure time SOD activity increased in ARA and EPA supplemented cells only, and no significant modification in CAT and GPx activity was detected. GST activity was reduced in all supplemented cells except DHA and US ones. Comparing stressed cells a differential effect of the supplementation on SOD, CAT and GST activities was still detected, while no differences were evidenced in GPx activity.

4. Discussion

n-6 and n-3 PUFA are well known for their physiological functions and preventive actions (Liu et al., 2015; Pelliccia et al., 2013) that are ascribable to different mechanisms including regulation of membrane functionality, cell signaling, and gene expression (Robichaud & Surette, 2015; Righi et al., 2011; Bordoni et al., 2007). In contrast to their health effects, PUFAs are known to be prone to rapid peroxidation, which has been shown to impair membrane functions and inactivate proteins and enzymes, eventually leading to various disorders and diseases (Csala et al., 2015). To explain this apparent oxymoron, it has been speculated that the lipid peroxidation occurring in PUFA-rich cells causes a low level, sub-toxic stress that could induce a protective adaptations over time (i.e. hormesis), contributing to PUFA health effects. Actually, n-3 PUFA have been shown to up-regulate the antioxidant systems in various cells (Giordano & Visioli, 2014), so counteracting the oxidative stress (Zanatta et al., 2014; Tourtas et al., 2012). Although different PUFA share common chemical characteristics, in a previous work we evidenced that they differently modulate the activity of antioxidant enzymes, and have a different impact on cell oxidative status (Di Nunzio et al., 2011). A concomitant oxidative stress could have an impact on the effects of PUFA, this representing an important issue since a higher intake of PUFA, particularly n-3 PUFA, is suggested for the prevention/modulation of a wide range of diseases associated with an increased production of ROS

(Fischer & Maier, 2015; Reuter et al., 2010; Pashkow, 2011; Salomone et al., 2016). To further elucidate it, in the present work we have supplemented different PUFA to HepG2 cells, which were then submitted to a mild oxidative stress.

Results herein reported clearly evidence that the cell response to a subsequent oxidative stress can be modulated by a preceding PUFA supplementation.

All supplemented fatty acids except DHA enhance cell susceptibility to H_2O_2. This could be due to an increased lipid peroxidation inducing disturbance of fine structures, alteration of integrity, fluidity, and permeability, and functional loss of biomembranes, and generates potentially toxic products (Ayala et al., 2014), as indicated by the increase in TBARS concentration in the medium of all supplemented cells but DHA supplemented ones. Since CD containing lipids, the first products of lipid peroxidation, are usually quickly cleaved to their corresponding breakdown products (Yin et al., 2011), the higher CD concentration in n-6 PUFA than n-3 PUFA supplemented hepatocytes could indicate a longer time lasting lipoperoxidation.

Although we already observed a PUFA sensitizing effect in basal condition (Di Nunzio et al., 2011), results herein reported cannot be simply considered as its consequence but as an additive effect due to the concomitant presence of PUFA and H_2O_2. It can be clearly evidenced comparing present and previous data; as example, in basal condition LDH release was significantly higher in ARA supplemented cells only (Di Nunzio et al., 2011), while it is significantly increased in all supplemented cells except DHA ones after H_2O_2 addition, the increase being higher in ARA supplemented cells. It is worth noting that DHA supplementation has no adverse effect either in basal condition (Di Nunzio et al., 2011) or after the oxidative insult.

This differential modulation of the cell response to the oxidative stress is in part ascribable to the modulation of the antioxidant enzymes activities. The effect of PUFA on the antioxidant enzymes status have been extensively studied leading to controversial results. To the Authors knowledge, previous studies have been performed using fish oil (Avramovic et al., 2012; Romieu et al., 2008) or other oils (Umesha & Naidu, 2015; Taranu at al., 2014; Haggag et al., 2014) containing different combinations of PUFA, this not allowing to extrapolate the effect of the single fatty acid. Results herein reported clearly evidence that each fatty acid has specific modulatory effects on antioxidant enzymes, and promotes a specific profile of antioxidant enzyme activities that cannot be predicted using oils containing different proportion of PUFA. The importance of the proportion of PUFA in a mixture has been also shown by Molinar-Toribio *et al.* (Molinar-Toribio et al., 2015) who evidenced in rat supplemented with EPA plus DHA mixtures that the activity of antioxidant enzymes increases with magnitudes depending on the EPA:DHA ratio. Notably, the highest DHA concentration reduced the oxidative stress.

Our study confirms that exposure time to H_2O_2 modulates anti-oxidant enzymes activity, as previously reported by Shull *et al.* (Shull et al., 1991) and Franco *et al.* (Franco et al., 1999). In addition, our results evidence that PUFA supplementation exacerbates this time-depending modulation. Assuming that the observed increased activity of antioxidant enzymes is based on an increased transcription of the corresponding encoding genes and an increased synthesis of the enzymatic proteins, a lag time between the insult and the cell response is conceivable. This can explain the less evident changes in antioxidant enzymes activities after 20 than 60 min H_2O_2 exposure. In addition, the weaker cell response after shorter time of exposure could be due to the lower intracellular

concentration of H_2O_2. In fact, H_2O_2 diffusion across the plasma membrane is low during the first 10 minutes of exposure (Alexandre et al., 2006).

Oxidative stress underlies a complex array of metabolic alterations. Under this condition, derangement of cell biochemistry and function is known to involve misregulation of key components responsible for cell signaling, such as membrane receptors, kinases, phosphatases, and transcription factors (Leonarduzzi et al., 2011; Brigelius-Flohé et al., 2011). In this view, the overall differential effect of supplemented DHA could rely on different mechanisms. Among them, a different extent of incorporation/metabolism of the supplemented fatty acid inside the cell. Although all supplemented PUFA are readily incorporated by HepG2 cells at the expense of monounsaturated fatty acids, in a previous work we observed different conversion rate (Di Nunzio et al., 2010). LA and ALA are further metabolized upon cellular uptake, and EPA is partially desaturated/elongated to DHA. On the contrary, no DHA retro-conversion is detected. Therefore the effects of all PUFA except DHA could be ascribable to both the supplemented PUFA and its derivatives. The differential metabolism of supplemented PUFA could also impact on the modification of membrane composition, and consequently functionality, since the majority of PUFA is incorporated in the cell membrane phospholipids (Abbott et al., 2012). The oxidative stress may selectively impair cell PUFA concentration, which in turn may modulate the effect of the oxidative stress (Zaloga et al., 2006). Oxidative condition modifies fatty acid composition in neonatal rat cardiomyocytes (Bordoni et al., 2005), and a slight increase in the saturated and a decrease in monounsaturated and PUFA content has been detected in the liver of pro-oxidant pesticide-treated rats (Nakbi et al., 2010). At membrane level, exposure to an oxidative stress leads not only to fatty acid oxidation but also to phospholipid breakdown due to activation of phospholipase A_2 (Nalbone et al., 1990). DHA released from phospholipids is the substrate for the formation of resolvins and protectins, that act through distinct receptors to unfold biological effects (Calder, 2015) including oxidation preventing effects (Liu et al., 2014; Tian et al., 2015).

A higher DHA cellular content may play an important role in the protection from oxidative damage because 4-hydroxyhexenal, an aldehyde derived from DHA peroxidation, up-regulates Nrf2-HO-1 antioxidant signaling pathway, which induces antioxidant effects (Gao et al., 2007).

Many studies indicate that PUFA, particularly EPA and DHA, control gene expression also by directly governing the transcription/activity of peroxisome proliferator-activated receptor-alpha (PPARα) (Di Nunzio et al., 2009; Georgiadi et al., 2012), whose activation may result in enhanced expression/activity of antioxidant enzymes such as CAT and SOD (Toyama et al., 2004; Inoue et al., 2001). PPARs exert their effect on gene transcription by dimerization with the 9-cis retinoic acid receptors (RXRs) (Feige et al., 2005). RXR is also able to bind PUFA, mainly DHA (Crawford et al., 2003). Other PUFAs such as EPA or ARA can activate RXR but with lower efficiency (Egea et al., 2002). Thus, DHA can be considered a more efficient activator of PPARα (Bordoni et al., 2006).

In conclusion, based on reported results each PUFA seems to possess specific pro- and antioxidative characteristics, not predictable on chemical basis such as the length of the carbon chain or the unsaturation degree. According to many *in vivo* (Mas et al., 2010; Patten et al., 2013) and *in vitro* (González-Périz et al., 2006; Mukherjee et al., 2004) studies, DHA appears the only fatty acid not increasing cell susceptibility to H_2O_2, although its effect is not ascribable to a stronger induction of antioxidant enzymes activity.

Overall, results herein reported clearly evidence that fatty acid supplementation can modify the cell response to an oxidative stress, and the response is dependent on the supplemented fatty acid. We are beginning to understand the physiology and molecular basis of the effects of n-3 and n-6 fatty acids, and thereby gradually encompass the full biological potential of these compounds. Our results underline that PUFA cannot be considered as a single category, but as individual compounds. It appears particularly evident for DHA, and although observed results must be confirmed *in vivo*, this apparent "DHA paradox" represents an important step ahead in the use of DHA supplementation as preventive strategy in the clinical practice.

Disclosure statement

The authors declare no conflict of interest.

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Figure 1. Cell viability A), LDH activity B), TBARS concentration C) and CD level D) in basal condition and after 1h H_2O_2 exposure.

*Data are means ± SD of at least six samples in each group, coming from three independent cell cultures. Statistical analysis was by the one-way ANOVA p<0.001) using Dunnett's post-test to compare: 1) stressed cells both US and supplemented) to controls *p<0.05; **p<0.01; ***p<0.001); and 2) supplemented stressed cells to US stressed cells °p<0.05; °°°p<0.001).*

Figure 2. TAA A) and GSH level B) in basal condition and after 1h H_2O_2 exposure.

*Data are means ± SD of at least six samples in each group, coming from three independent cell cultures. Statistical analysis was performed by the one-way ANOVA p<0.01) using Dunnett's as post-test to compare: 1) stressed cells both US and supplemented) to controls *p<0.05); and 2) supplemented stressed cells to US stressed cells °p<0.05; °°p<0.01).*

Figure 3. SOD A), CAT B), GST C) and GPx D) activity in basal condition and after 1h H_2O_2 exposure.

*Data are means ± SD of at least six samples in each group, coming from three independent cell cultures. Statistical analysis was performed by the one-way ANOVA A, B and C p<0.001; D p<0.01) using Dunnett's as post-test to compare: 1) stressed cells both US and supplemented) to controls *p<0.05; **p<0.01; ***p<0.001); and 2) supplemented stressed cells to US stressed cells °p<0.05; °°p<0.01; °°°p<0.001).*

Figure 4 SOD A), CAT B), GST C) and GPx D) activity in basal condition and after 20 min H_2O_2 exposure.

Data are means ± SD of at least six samples in each group, coming from three independent cell cultures. Statistical analysis was performed by the one-way ANOVA A and B p<0.01; C p<0.001) using Dunnett's as post-test to compare: 1) stressed cells both US and supplemented) to controls

**p<0.05; **p<0.01; ***p<0.001); and 2) supplemented stressed cells to US stressed cells °p<0.05; °°p<0.01; °°°p<0.001).*