

Fish Oil Supplementation Decreases Oxidative Stress but Does Not Affect Platelet-Activating Factor Bioactivity in Lungs of Asthmatic Rats

A. L. Zanatta · D. T. S. Z. Miranda · B. C. L. Dias ·
R. M. Campos · M. C. Massaro · P. V. Michelotto Jr ·
A. L. West · E. A. Miles · P. C. Calder · A. Nishiyama

Received: 18 September 2013 / Accepted: 10 May 2014 / Published online: 25 May 2014
© AOCs 2014

Abstract Dietary fish oil supplementation increases the content of n-3 polyunsaturated fatty acids (PUFA) in cellular membranes. The highly unsaturated nature of n-3 PUFA could result in an enhanced lipid peroxidation in the oxidative environment characteristic of asthma. The oxidative reaction cascade culminates in an increased production of components associated to oxidative stress and of an important proinflammatory mediator platelet-activating factor (PAF)-like lipid. We evaluated the effect of fish oil supplementation in asthmatic rats upon the PAF bioactivity and parameters related to oxidative stress in the lung. Fish

oil supplementation of asthmatic rats resulted in lower concentrations of nitrite (1.719 ± 0.137 vs. 2.454 ± 0.163 nmol/mL) and lipid hydroperoxide (72.190 ± 7.327 vs. 120.200 ± 11.270 nmol/mg protein). In asthmatic animals, fish oil increased the activities of superoxide dismutase (EC 1.15.1.1) (33.910 ± 2.325 vs. 24.110 ± 0.618 U/mg protein) and glutathione peroxidase (EC 1.11.1.9) (164.100 ± 31.250 vs. 12.590 ± 5.234 U/mg protein). However, fish oil did not affect PAF bioactivity in lung tissue of asthmatic rats (0.545 ± 0.098 340/380 vs. 0.669 ± 0.101 340/380 nm ratio). Considering the two-step process—oxidative stress and PAF bioactivity—fish oil exhibited a divergent action on these aspects of asthmatic inflammation, since the supplement lowered oxidative stress in the lungs of asthmatic rats, presenting an antioxidant effect, but did not affect PAF bioactivity. This suggests a dual effect of fish oil on oxidative stress and inflammation in asthma.

A. L. Zanatta (✉) · D. T. S. Z. Miranda · B. C. L. Dias ·
R. M. Campos · M. C. Massaro · A. Nishiyama
Departamento de Fisiologia, Centro Politécnico, Universidade
Federal do Paraná, Bairro Jardim das Américas,
Curitiba CEP 81531-990, Brazil
e-mail: analucia_zanatta@yahoo.com.br;
analuciazanatta@ufpr.br

P. V. Michelotto Jr
School of Agricultural Sciences and Veterinary Medicine,
Pontifícia Universidade Católica do Paraná, Rodovia BR 376,
Km 14, Bairro Costeira, São José dos Pinhais CEP 83010-500,
Brazil

A. L. West · E. A. Miles · P. C. Calder
Human Development and Health Academic Unit, Faculty of
Medicine, MP887 Southampton General Hospital, University of
Southampton, Southampton SO16 6YD, UK

P. C. Calder
NIHR Southampton Biomedical Research Centre, University
Hospital Southampton NHS Foundation Trust, University of
Southampton, Southampton SO16 6YD, UK

P. C. Calder
Department of Biological Sciences, Faculty of Science, King
Abdulaziz University, Jeddah, Saudi Arabia

Keywords Fish oil · Asthma · Oxidative stress ·
PAF/PAF-like

Abbreviations

A	Asthmatic group
AS	Asthmatic group supplemented with fish oil
ASM	Airway smooth muscle
BALF	Bronchoalveolar lavage fluid
BHT	Butylated hydroxytoluene
C	Control group
CAT	Catalase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GPx	Glutathione peroxidase
OVA	Ovalbumin
PAF	Platelet activating factor

PBS	Phosphate-buffered saline
PUFA	Polyunsaturated fatty acid(s)
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S	Supplemented with fish oil group
SOD	Superoxide dismutase
TPP	Triphenylphosphine

Introduction

Asthma is a disease characterized by airway hyperresponsiveness accompanied by inflammation and oxidative stress, which are important pathophysiological components of this disease [1–5]. The oxidative stress is caused by an imbalance between the antioxidant defense mechanisms, especially the activity of antioxidant enzymes superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (GPx; EC 1.11.1.9) and catalase (CAT; EC 1.11.1.6), and the generation of oxidants, such as reactive oxygen and nitrogen species (ROS and RNS) [2, 4, 5]. The excessive production of ROS during the inflammatory response seen in asthma [2, 5] promotes damage to DNA, proteins and membrane lipids [6]. The oxidizing agents generated are related to the disease severity [5, 7], since as a consequence of the increased lipid peroxidation, airway hyper-responsiveness, production of chemotactic agents and vascular permeability are all increased [8]. These effects observed in asthma are also caused by platelet activating factor (PAF) [9–12], that has a central role in the promoting asthmatic inflammation [12].

The oxidative stress observed in asthma fosters an environment conducive to oxidative reactions involving polyunsaturated fatty acids (PUFA) of both n-6 and n-3 families resulting in the production of inflammatory mediators [13]. Dietary fish oil supplementation increases the content of n-3 PUFA in cellular membranes [14–16]. Although this is related to the anti-inflammatory effects of these fatty acids, their highly unsaturated nature makes them susceptible to lipid peroxidation, so enhancing the production of lipid hydroperoxides and the important pro-inflammatory lipid mediator, the PAF-like lipid. PAF-like molecules are produced by a non-enzymatic lipid oxidation pathway and they possess biological activity similar to PAF [12]. Although less potent than enzymatically produced PAF, the PAF-like lipids can play an important role in asthmatic inflammation because of their uncontrolled generation [17]. The current study addresses whether fish oil, a source of the n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), modifies the PAF bioactivity as a consequence of changes in oxidative stress in

an animal model of asthma. We evaluated the effect of fish oil supplementation in asthmatic animals on PAF bioactivity and on parameters related to oxidative stress (concentrations of nitrite and lipid hydroperoxides and activities of the antioxidant enzymes SOD, GPx and CAT) in lung fluid.

Materials and Methods

Reagents

Fish oil capsules were donated by Herbarium Foundation (Colombo, Paraná, Brazil). GPx and SOD assay kits were purchased from Bioassay Systems (Hayward, California, USA). The PAF receptor antagonist BN52021 (ginkgolide B) and PAF (β -acetyl- γ -O-hexadecyl-L- α -phosphatidylcholine) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Fura-2 AM was purchased from AnaSpec (San Jose, California, USA). Ovalbumin (OVA) (Grade II), aluminum hydroxide ($\text{Al}(\text{OH})_3$), triphenylphosphine, hydrogen peroxide and all other reagents were purchased from Sigma (St. Louis, Missouri, USA).

Animals and Dietary Supplementation

Adult male Wistar rats weighing 220 ± 30 g were obtained from the Biotery of the Biological Sciences Sector, Federal University of Paraná (Curitiba, Brazil). The animals had free access to water and to food (52 % by weight of carbohydrates, 21 % by weight of proteins and 4 % by weight of total lipids; Nuvilab CR1, Nuvital Nutrientes Ltda, Curitiba, Paraná, Brazil) and were maintained on a reversed 12 h light/dark cycle. The animals were randomly divided into 4 groups: control (C), supplemented with fish oil (S), asthmatic (A), asthmatic supplemented with fish oil (AS). Fish oil was administered into the mouth using a micropipette at a daily dose of 1 g/kg body weight for 21 days. This supplementation significantly increased the content of n-3 PUFA (both EPA and DHA) in lung tissue (data not shown). Groups A and C received water at the same dose. The body weight of the animals was recorded every 2 days throughout this period.

The fatty acid composition of the standard diet was: 12:0 1.3 %; 16:0 13.7 %; 18:0 2.4 %; 18:1n-9 20.0 %; 18:2n-6 56.0 %; 18:3n-3 6.0 %; 20:4n-6 0.3 %; 20:5n-3 0.2 %. The fish oil capsules had the following fatty acid composition: 12:0 7.0 ± 0.1 %; 16:0 14.2 ± 1.9 %; 18:0 1.4 ± 0.4 %; 18:1n-9 10.6 ± 1.1 %; 18:2n-6 3.3 ± 0.3 %; 20:4n-6 13.7 ± 0.8 %; 20:5n-3 29.5 ± 0.7 %; 22:6n-3 23.6 ± 0.2 % [18]. Thus, a rat weighing 200 g would receive approximately 60 mg EPA and approximately 48 mg DHA each day.

All protocols were approved by the Ethics Committee on Animal Experimentation of the Federal University of Paraná, protocol 468, according to the Brazilian College on Animal Experimentation (COBEA) guidelines.

Induction of Asthma

On the 5th and 12th day of the dietary supplementation, allergic sensitization was induced in the A and AS groups by a subcutaneous injection of 1 mL of phosphate buffer saline (PBS) containing 1 mg of OVA and 200 mg of $\text{Al}(\text{OH})_3$. The animals in the C and S groups received only PBS containing $\text{Al}(\text{OH})_3$. On days 18, 19 and 20 of supplementation, the rats were submitted to antigenic challenge by exposure for 30 min to an aerosol of 5 g/100 mL OVA in PBS. The C and S groups were exposed to an aerosol of PBS only. 24 h after the last antigenic challenge the animals received a lethal dose of urethane (3 mg/g) intraperitoneally.

As demonstrated in a previous publication of ours [19], the animals were considered asthmatic based upon the increased leukocyte infiltration, eosinophilia, vascular permeability, expressed by the obliteration of the bronchioles by mucus, and alterations of lung compliance and the force of contraction of tracheal smooth muscle of animals. Here, we present a histological analysis of the lungs of animals showing leukocyte infiltration in asthmatic animals.

BALF and Lung Collection

The bronchoalveolar lavage fluid (BALF) was collected for the determination of nitrite concentration and of the activities of SOD, GPx and CAT. The BALF collection procedure was performed through a tracheostomy at the upper part of the trachea. The lung was washed ten times with 5 mL of PBS and 90–95 % of the total volume was recovered. The collected BALF was kept on ice.

After BALF collection, the animals underwent thoracotomy and the lungs were removed and washed in PBS. The right lower lobe was collected for immediate lipid hydroperoxide determination. The intermediate lobe and the lower pulmonary left lobe of the lungs were collected, immediately frozen in liquid nitrogen and stored at -80°C for subsequent determination of PAF bioactivity and analysis of fatty acid composition, respectively.

Histological Analysis of the Lung

Lungs were collected and immediately fixed in Karnovsky. Serial sections (3 μm sections taken every 100 μm) of paraffin-embedded lungs were prepared and stained with hematoxylin and eosin. Each section was analysed by a

light microscope (Axio-Imager Z2 Zeiss) coupled to a computer.

Nitrite Concentration in BALF

BALF was centrifuged for 8 min at 290g and 4°C . In a 96-well microplate, 100 μL of Griess reagent (1:1 mixture of 1 g/100 mL sulfanilamide in 5 % (v/v) orthophosphoric acid and 0.1 g/100 mL *N*-[1-naphthyl]ethylenediamine) was added to 100 μL cell-free BALF. After five minutes the absorbance was measured at 550 nm using a microplate spectrophotometer (TECAN Infinite M200). Nitrite concentration was determined by interpolating absorbance values in a standard curve generated by known concentrations of sodium nitrite. Results are expressed as nmol/mL of BALF.

Superoxide Dismutase and Glutathione Peroxidase Activities in BALF

BALF was centrifuged for 8 min at 290g and 4°C . The colorimetric determination of SOD and GPx activities in BALF was performed according to the kit manufacturer's protocols (bioassay systems).

Catalase Activity in BALF

BALF was centrifuged for 20 min at 10,600g and 4°C . The supernatant was discarded and the cells were resuspended in 100 μL of PBS and homogenized. An aliquot of 10 μL of cell extract was added to 990 μL of the reactive medium (20 mM H_2O_2 , 0.05 M tris[hydroxymethyl] aminomethane, 0.25 mM ethylenediaminetetraacetic acid, pH 7.4, aqueous solution). Starting immediately, the absorbance was monitored for 2 min, with an interval of 5 s between each reading, at 240 nm using a spectrophotometer (TECAN Infinite M200) [20]. Enzyme activity is determined by the H_2O_2 consumption from the medium, expressed as absorbance variation at 240 nm. CAT activity was corrected for the sample protein concentration and the results are expressed as consumption of H_2O_2 per min per mg of protein (mmol/min mg protein).

Lipid Hydroperoxide Concentration in Lung

A methanol extract of lung tissue samples was done by mixing 400 mg of the right lower lung lobe in 1 mL of methanol, using an electric homogenizer (GGS 27, Bosch). The homogenate was centrifuged for 5 min at 5,000g and 4°C . For each sample, aliquots of 90 μL of the supernatant were disposed in six centrifuge vials (1.5 mL). To three of these tubes were added 10 μL of methanolic triphenylphosphine (TPP) 10 mM. To the other three tubes were

added 10 μL of methanol. All the six tubes were vortexed and then incubated for 30 min at room temperature. After that 900 μL FOX2 reagent (100 μM xylenol orange, 4 mM butylated hydroxytoluene (BHT), 25 mM sulfuric acid and 250 μM ammonium ferrous sulfate, 90 % (v/v) methanol, 10 % (v/v) ultrapure water) was added to all tubes. All the six tubes were vortexed and then incubated for another 30 min at room temperature. After the incubation time, absorbance was measured at 560 nm using a spectrophotometer (Ultrospec 2,000, Pharmacia Biotech). Lipid hydroperoxide concentration was determined by interpolating absorbance values in a standard curve generated by known concentrations of H_2O_2 [21]. Results were corrected for the sample protein concentration and are expressed as nmol/mg protein.

Lipid Extraction From Lungs for Use in PAF Bioactivity Assay

Extraction of lipid from the lungs was performed as described by Folch et al. [22]. A 100-mg portion of lung intermediate lobe from each rat was homogenized in 1.33 mL of methanol/chloroform (1:2 v/v) with an electric homogenizer (GGS 27, Bosch) and 0.480 mL of methanol was added. The samples were then homogenized and centrifuged for 10 min at 5,000g and 4 °C. The supernatant was transferred to a glass tube (5 mL) and 0.96 mL of chloroform was added. The extract was washed once with ultrapure water and three times with washing solution (1.8 mM CaCl_2 , 1.8 mM MgCl_2 , 49.7 mM NaCl, 3.0 % (v/v) chloroform, 49 % (v/v) methanol, 48 % (v/v) ultrapure water). After that, the extract was dried with a nitrogen stream at 37 °C and stored at -80 °C under nitrogen. To analyze the PAF bioactivity the lipid extract was solubilized with 100 μL of DMSO and then diluted with PBS (1:500 v/v).

PAF Bioactivity in Lung

The determination of the PAF bioactivity was performed as described by Muehlmann et al. [12], by detection of calcium influx into neutrophils by a fluorescent indicator. Horse blood was collected into sodium citrate as anticoagulant, diluted in PBS (1:2 v/v) and 40 mL of diluted blood was layered over 6 mL of Ficoll Paque Plus and centrifuged for 40 min at 400g and 4 °C; the upper layer was discarded. The lower layer was divided into two centrifuge tubes of 50 mL. Each tube was completed with hemolytic solution (0.77 % NH_4Cl , 0.21 % tris[hydroxymethyl]aminomethane, pH 7.4, aqueous solution). After incubation for 20 min at 37 °C, polymorphonuclear cells were isolated by centrifugation for 6 min at 400g and 4 °C. The supernatant was discarded and polymorphonuclear

cells were washed with PBS and centrifuged for 6 min at 400g and 4 °C. After removing the supernatant, cell pellet was resuspended in 5 mL of PBS containing 5 mM glucose. Cell density was determined by counting cells in a Neubauer chamber and then adjusted to 1.0×10^7 viable cells/mL.

FURA-2 AM (1 μM) was added to the polymorphonuclear cells suspension and then incubated for 45 min at 37 °C in the dark. After incubation, cells were washed twice with Ringer solution (110 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10 mM tris, 1 mM glucose, pH 7.4, aqueous solution) and resuspended in Ringer solution for the final density of 2.25×10^6 viable cells/mL. The fluorescence was measured at 37 °C with dual excitation at 340 and 380 nm with the emission recorded at 510 nm by a microcomputer-coupled spectrofluorometer (RF5301PC, Shimadzu) controlled by the software Super Ion Probe (Shimadzu). For analysis, 2 mL of polymorphonuclear cells suspension were placed in a cuvette and incubated in the dark for 4 min at 37 °C with 10 μL of PBS or 10 μL of a PAF antagonist, BN52021 (150 μM). After a 50 s basal reading, 10 μL of the lung lipid extract was added to the polymorphonuclear cells suspension and the fluorescence resulting from calcium influx was monitored for 100 s. The difference of the response obtained in the absence and in the presence of BN52021 is a semi-quantitative indicator of the PAF bioactivity in the samples; activity of both PAF and PAF-like lipid is measured by this assay. The polymorphonuclear cells suspension was also tested with 1, 10 and 100 μM PAF, with and without BN52021. The results are expressed as 340/380 nm ratio.

Fatty Acid Analysis

Fatty acids were analyzed in the total lipid fraction of lung homogenates. Total lipid was extracted into chloroform:methanol (2:1, vol:vol) from homogenized lung; BHT (50 mg/L) was added as an antioxidant. The lipid extract was dried under nitrogen and redissolved in toluene. Fatty acid methyl esters (FAME) were formed by incubation with methanol that contained 2 % (vol:vol) H_2SO_4 at 50 °C for 2 h. After allowing the tubes to cool, samples were neutralized with a solution of 0.25 M KHCO_3 and 0.5 M K_2CO_3 . FAME were extracted into hexane, dried down, redissolved in a small volume of hexane, and separated by using gas chromatography. Gas chromatography was performed on a Hewlett-Packard 6,890 gas chromatograph fitted with a BPX-70 column (30 m $\times$ 0.22 mm $\times$ 0.25 μm). The inlet temperature was 300 °C. The oven temperature was initially 115 °C and was maintained for 2 min after injection. The oven temperature was programmed to increase to 200 °C at the rate of 10 °C/min to hold at 200 °C for 16 min and increase to 240 °C at the

rate of 60 °C/min to hold at 240 °C for 2 min. The total run time was just longer than 29 min. Helium was used as the carrier gas. FAME were detected by using a flame ionization detector held at a temperature of 300 °C. The instrument was controlled by, and data collected, with HPChemStation software (Hewlett-Packard). FAME were identified by comparison of retention times with those of authentic standards run previously. Within-run coefficient of variation for analysis of EPA and DHA as methyl esters were 3 and 2 %, respectively. Between-run coefficient of variation for analysis of EPA and DHA as methyl esters were 5 and 2.5 %, respectively.

Statistical Analysis

All values are expressed as means $\pm$ standard error of the mean (SEM) for at least three replicates. Data were analyzed by one-way ANOVA followed by Tukey's post test. Differences were considered significant for $P < 0.05$.

Results

Body Weight

The weight of the animals in the different groups was not modified by fish oil supplementation, although there was a trend for unsupplemented asthmatic animals to weigh less than the other groups (Fig. 1).

Histological Analysis of the Lung

The observational histological analysis showed that leukocytes were increased in lung tissue of asthmatic rats with no effect of fish oil (Fig. 2).

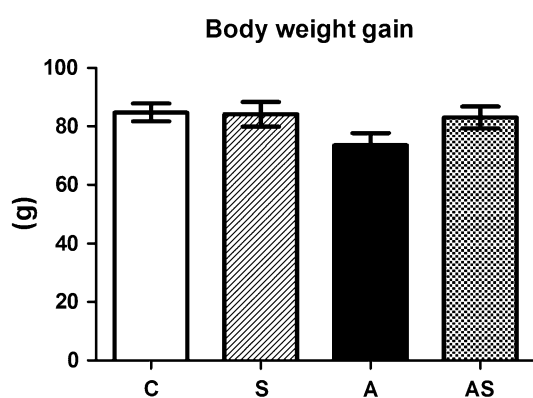


Fig. 1 Body weight of the rats for the following groups: control (C, $n = 39$), supplemented with fish oil (S, $n = 34$), asthmatic (A, $n = 35$), asthmatic supplemented with fish oil (AS, $n = 36$)

Nitrite Concentration in BALF

Nitrite concentration was measured in BALF as an indicator of nitric oxide production. As shown in Fig. 3, asthma caused a significant increase in nitrite concentration but the supplementation of asthmatic animals with fish oil partly prevented this effect. Nitrite concentration in asthmatic animals supplemented with fish oil was an average of 30 % lower than in the asthmatic control group ($P < 0.005$).

Superoxide Dismutase Activity in BALF

BALF SOD activity was higher in non-asthmatic rats supplemented with fish oil than in control non-asthmatic rats (Fig. 4). Asthma lowered BALF SOD activity but this effect was prevented by fish oil supplementation. Activity in fish oil supplemented asthmatic rats was an average of 40 % higher than in the control asthmatic rats ($P < 0.05$) (Fig. 4).

Glutathione Peroxidase Activity in BALF

GPx activity in BALF was lower in the asthmatic group compared with non-asthmatic controls (Fig. 5). This effect of asthma was prevented by fish oil supplementation. GPx activity in fish oil supplemented asthmatic rats was an average of 13-times higher than in the control asthmatic group ($P < 0.05$) (Fig. 5).

Catalase Activity in BALF

CAT activity in BALF was significantly lower for asthmatic rats compared to non-asthmatic controls (Fig. 6). CAT activity in the asthmatic rats supplemented with fish oil was also significantly lower when compared to controls and was not different from that in the asthmatic group. Fish oil resulted in lower CAT activity in non-asthmatic rats ($P < 0.001$) (Fig. 6).

Lipid Hydroperoxide Concentration in Lung

Figure 7 shows the lipid hydroperoxide concentration in lung. Asthma significantly increased lung lipid hydroperoxide concentrations compared with non-asthmatic controls ($P < 0.05$). Fish oil supplementation prevented this effect of asthma: fish oil resulted in an average 40 % lower lipid hydroperoxide concentration than in the asthmatic group ($P < 0.001$) and this was not different from that seen in the non-asthmatic control group (Fig. 7).

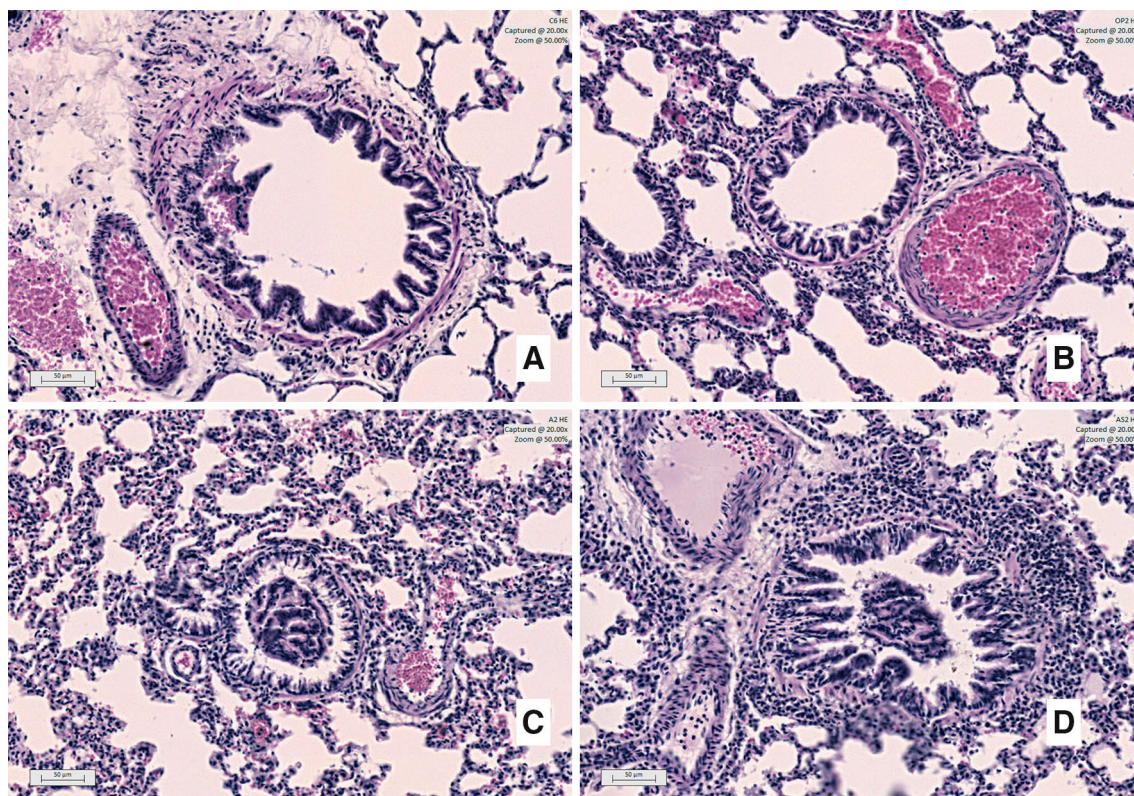


Fig. 2 Histological analysis of the lung for the following groups: control (C), supplemented with fish oil (S), asthmatic (A, photo C), asthmatic supplemented with fish oil (AS, photo D). Peribronchiolar

leukocytic infiltrates are visible, especially in images of groups A and AS. Representative images for 4 samples. Scale white bars correspond to the length of 50 μ m

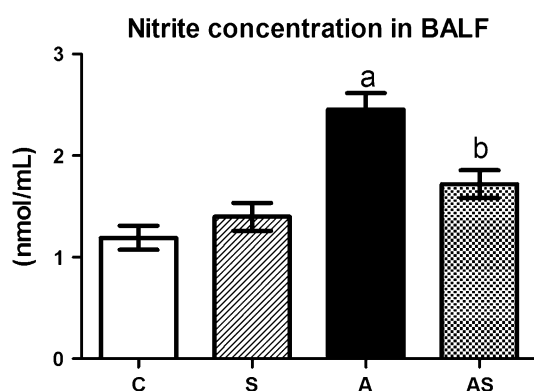


Fig. 3 Nitrite concentration in BALF for the following groups: control (C, $n = 30$), supplemented with fish oil (S, $n = 34$), asthmatic (A, $n = 31$), asthmatic supplemented with fish oil (AS, $n = 36$). Data are presented as means $\pm$ SEM. *a* $P < 0.0001$ versus group C and S; *b* $P < 0.05$ versus group C and A

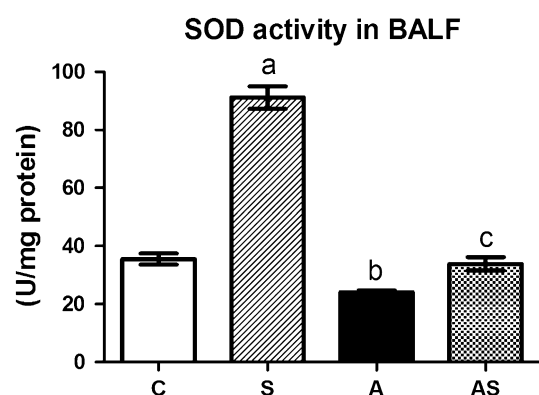


Fig. 4 Superoxide dismutase activity in BALF for the following groups: control (C, $n = 9$), supplemented with fish oil (S, $n = 8$), asthmatic (A, $n = 8$), asthmatic supplemented with fish oil (AS, $n = 8$). Data are presented as means $\pm$ SEM. *a* $P < 0.0001$ versus group C, A and AS; *b* $P < 0.05$ versus group C; *c* $P < 0.05$ versus A

PAF Bioactivity in Lung

The PAF bioactivity in the lung increased with the asthmatic inflammation compared with controls ($P < 0.0001$) (Fig. 8). Fish oil had no effect on PAF bioactivity (Fig. 8).

Lung Fatty Acid Levels

Lung fatty acid composition is shown in Table 1. Asthma itself had a limited effect on lung fatty acids, only significantly altering 18:1n-9 content (lower by about 10 % in A versus C). In contrast, fish oil supplementation had a major

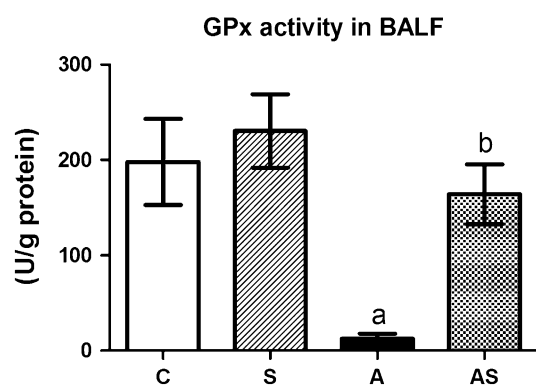


Fig. 5 Glutathione peroxidase activity in BALF for the following groups: control (C, $n = 4$), supplemented with fish oil (S, $n = 6$), asthmatic (A, $n = 5$), asthmatic supplemented with fish oil (AS, $n = 5$). Data are presented as means $\pm$ SEM. *a* $P < 0.001$ versus group C and $P < 0.0001$ versus group S; *b* $P < 0.05$ versus group A

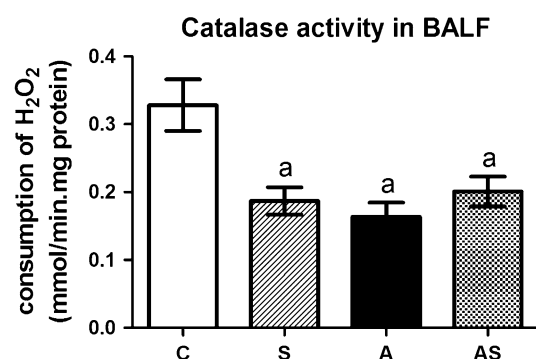


Fig. 6 Catalase activity in BALF for the following groups: control (C, $n = 32$), supplemented with fish oil (S, $n = 32$), asthmatic (A, $n = 26$), asthmatic supplemented with fish oil (AS, $n = 29$). Data are presented as means $\pm$ SEM. *a* $P < 0.001$ versus group C

impact on lung fatty acids, with very similar effects being seen in both non-asthmatic and asthmatic rats. Fish oil resulted in higher lung concentrations of EPA, docosapentaenoic acid (DPA) and DHA and a lower concentration of arachidonic acid (ARA) than seen in animals not receiving fish oil. The increase in lung EPA was 2.7 times and 5.2 times in S versus C and in AS versus A, respectively, while for DPA the increases were 2.7 and 2.6 times, respectively and for DHA 2.1 and 2 times, respectively. The result of these changes was a higher ratio of n-3 to n-6 fatty acids in the fish oil supplemented rats (2.8 times higher in AS than in A and 2.9 times higher in S than in C). The ratio of saturated to unsaturated fatty acids was not altered by fish oil supplementation in asthmatic or non-asthmatic rats.

Discussion

In the present study asthma increased markers of lung oxidative stress (lipid hydroperoxides and nitrite), lowered

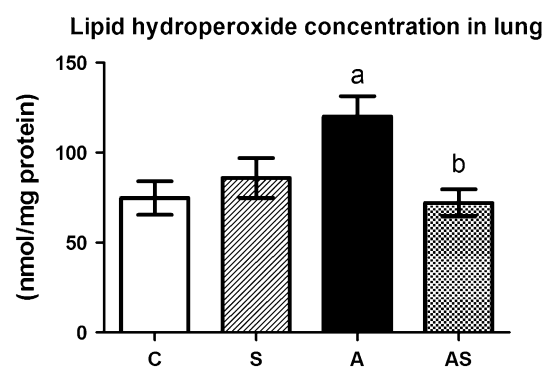


Fig. 7 Lipid hydroperoxide concentration in lung for the following groups: control (C, $n = 25$), supplemented with fish oil (S, $n = 28$), asthmatic (A, $n = 30$), asthmatic supplemented with fish oil (AS, $n = 26$). Data are presented as means $\pm$ SEM. *a* $P < 0.05$ versus group C; *b* $P < 0.001$ versus group A

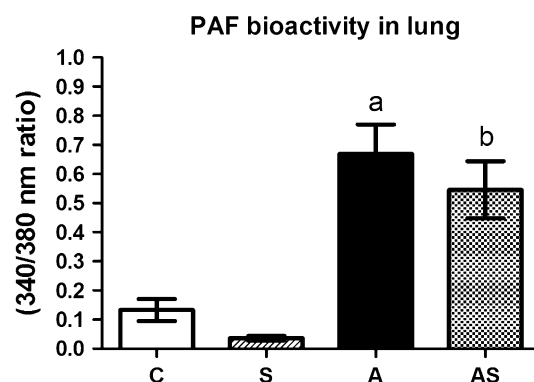


Fig. 8 PAF bioactivity in lung for the following groups: control (C, $n = 9$), supplemented with fish oil (S, $n = 8$), asthmatic (A, $n = 10$), asthmatic supplemented with fish oil (AS, $n = 8$). Data are presented as means $\pm$ SEM. *a* $P < 0.0001$ versus group C and S; *b* $P < 0.001$ versus group C and $P < 0.0001$ versus group S

the activities of the antioxidant protective enzymes in the lung and increased PAF bioactivity, an indicator of inflammation. The dietary supplementation of asthmatic rats with fish oil was able to counter all of these effects of asthma apart from that on PAF bioactivity. Fish oil supplementation resulted in a higher content of EPA and DHA in lung tissue. The fatty acids present in large quantities in fish oil can be incorporated into cell membranes at the expense of ARA [23]. Indeed a change in the fatty acid composition of the lung was clearly demonstrated. Twenty-one days of fish oil supplementation was sufficient to decrease the ARA content and to increase the EPA and DHA contents in the lungs of both asthmatic and non-asthmatic rats (Table 1). EPA and DHA are widely accepted as the key n-3 PUFA eliciting anti-inflammatory effects [23, 24]. The current study showed that, as for EPA and DHA, DPA was increased in the lung tissue with fish oil supplementation. DPA can be formed via the rapid

Table 1 Percentage of fatty acids from lung total lipid extract from asthmatic and non-asthmatic rats supplemented or not with fish oil

Fatty acids	C	S	A	AS
14:0	1.52 ± 0.06	1.63 ± 0.06	1.56 ± 0.04	1.66 ± 0.07
15:0	0.58 ± 0.02	0.60 ± 0.02	0.63 ± 0.02	0.60 ± 0.03
16:0	34.61 ± 0.58	33.77 ± 0.38 ^a	36.97 ± 0.47 ^c	36.13 ± 0.80 ^c
17:0	0.90 ± 0.10	1.18 ± 0.17	0.79 ± 0.07	0.70 ± 0.07 ^c
18:0	12.57 ± 0.33	12.98 ± 0.24	12.78 ± 0.18	12.29 ± 0.30
20:0	0.26 ± 0.01	0.28 ± 0.01	0.24 ± 0.01	0.25 ± 0.02
22:0	0.32 ± 0.01	0.40 ± 0.02 ^a	0.31 ± 0.02 ^c	0.35 ± 0.02
24:0	0.37 ± 0.03	0.34 ± 0.03	0.38 ± 0.02	0.30 ± 0.01
18:3n-3	0.21 ± 0.01	0.26 ± 0.02	0.21 ± 0.01	0.20 ± 0.01 ^c
20:4n-3	0.21 ± 0.01	0.27 ± 0.02	0.21 ± 0.01	0.21 ± 0.02
20:5n-3	0.33 ± 0.09	0.89 ± 0.07 ^{ab}	0.17 ± 0.01	0.89 ± 0.05 ^{ab}
22:5n-3	1.11 ± 0.08	3.05 ± 0.03 ^{ab}	1.15 ± 0.05	3.00 ± 0.16 ^{ab}
22:6n-3	1.19 ± 0.04	2.52 ± 0.13 ^{ab}	1.32 ± 0.06	2.72 ± 0.13 ^{ab}
18:2n-6	10.91 ± 0.19	10.98 ± 0.24	10.44 ± 0.13	10.58 ± 0.14
18:3n-6	0.27 ± 0.01	0.22 ± 0.01	0.30 ± 0.01 ^c	0.27 ± 0.02
20:3n-6	0.75 ± 0.03	0.88 ± 0.03 ^{ab}	0.77 ± 0.02	0.87 ± 0.02 ^{ab}
20:4n-6	15.42 ± 0.47	13.04 ± 0.18 ^{ab}	16.54 ± 0.23	14.22 ± 0.26 ^{ab}
16:1n-7	1.79 ± 0.08	1.79 ± 0.03 ^b	2.00 ± 0.05	1.76 ± 0.05 ^b
18:1n-9	11.48 ± 0.49	11.09 ± 0.10	10.00 ± 0.11 ^a	9.69 ± 0.28 ^{ac}
18:1n-7	1.97 ± 0.03	1.94 ± 0.03	1.93 ± 0.03	2.00 ± 0.04
20:1n-9	0.33 ± 0.01	0.36 ± 0.01	0.34 ± 0.02	0.30 ± 0.02 ^c
20:2n-6	0.74 ± 0.02	0.73 ± 0.02	0.81 ± 0.01	0.71 ± 0.03 ^b
24:1n-9	0.18 ± 0.01	0.24 ± 0.01	0.21 ± 0.01	0.23 ± 0.02
	0.696 ± 0.012	0.722 ± 0.011	0.654 ± 0.010 ^c	0.696 ± 0.014
n-3/n-6	0.099 ± 0.003	0.289 ± 0.008 ^{ab}	0.106 ± 0.002	0.276 ± 0.008 ^{ab}

Data are reported as means ± SEM from C (*n* = 16), S (*n* = 16), A (*n* = 16) and AS (*n* = 16) groups

^a *P* < 0.05 versus C

^b *P* < 0.05 versus A

^c *P* < 0.05 versus S

elongation of EPA in inflammatory cells and can lead to COX inhibition and modification of eicosanoid production [25].

As can be seen in our study, fish oil had a differential effect on oxidative stress and on PAF bioactivity. Oxidative stress was lower with fish oil, based on the determination of the activities of antioxidant enzymes and nitrogen reactive species and lipid hydroperoxide concentration. SOD plays an important role in cellular protection against oxidative stress [1] by catalyzing the conversion of superoxide anion to hydrogen peroxide [1, 2, 5]. GPx is essential for removing toxic products of lipid peroxidation and hydrogen peroxide [1, 5, 8, 26], which is continuously generated as a result of the infiltration of inflammatory leukocytes into the lung [8]. The findings of the current study suggest a role for n-3 PUFA from fish oil in controlling lung oxidative stress associated with asthma: the higher activity of antioxidant enzymes may explain the lower concentrations of oxidative stress markers. The lower enzyme activity observed in asthmatic rats is associated with increased ROS and RNS, which lead to modifications of proteins and changes in their functions that are biologically relevant for the initiation and maintenance of inflammation [1]. Such alterations include the reduced antioxidant capacity of SOD, GPx and CAT

[1, 2, 5, 7, 27] that strongly contributes to oxidative stress in asthmatics. In agreement with the literature [1, 4, 8, 28], in this study we detected that the activity of SOD, GPx and CAT are decreased in the lungs of asthmatic animals. Oxidative stress plays a role directly in several aspects of asthmatic inflammation and can influence the severity of the pathology. Thus, the addition of dietary antioxidants may be a means of protection against noxious oxidizing agents and may inhibit subsequent steps of inflammatory response amplification. Considering that the oxidative stress strongly affects the severity of asthma, the use of antioxidant therapies associated with conventional treatment could improve the quality of life of patients with asthma [9]. Although CAT activity was not altered by fish oil supplementation, the reversal of the SOD and GPx activities demonstrates an antioxidant action of fish oil in asthma and may indicate an important role of fish oil supplementation in the asthmatic condition, helping in the control of oxidative stress. The antioxidant action of fish oil supplementation has also been shown in previous studies in ulcerative colitis, atherosclerosis and renal disease [29–32]. However, such effects in asthma have not been reported previously. The mechanism of action by which the fish oil exerts its effect in the antioxidant enzymes in asthma may be by a direct action on the

enzymes regardless of inflammatory mediators, since in the absence of inflammation the supplement had an effect on the activity of SOD and CAT.

The antioxidant action of fish oil in asthma was also observed in other oxidative stress-related parameters, such as BALF nitrite concentration. Fish oil, when administered to asthmatic animals, decreased nitrite concentration. Nitrite concentration is an indication of production of nitric oxide, which is a precursor of reactive species involved in lipid peroxidation [33–36]. It is likely therefore that the fish oil suppressed the production of extremely toxic free radicals present in asthmatic inflammation, such as the hydroxyl radical, which is formed from nitric oxide. The nitric oxide reacts with superoxide anion generating peroxynitrite [28]. Among various possibilities, from peroxynitrite the hydroxyl radical can be formed [37] and this is a major reactive species responsible for lipid peroxidation [38]. Within this context, the antioxidant action of fish oil on nitrite production is also important for the control of oxidative stress in asthma. It is not clear how fish oil might decrease nitric oxide production but this could be through an effect on nitric oxide synthase expression. This was not investigated here.

The proposal that fish oil suppressed free radical generation was reinforced by the fact that fish oil supplementation also reduced the concentration of lipid hydroperoxides, a known marker of oxidative stress and lipid peroxidation resulting from asthmatic inflammation [39]. In tissues where oxidative stress occurs, such as in the lung of asthmatics, large amounts of lipid hydroperoxides are formed. In asthmatic animals, in accordance with findings in the literature [12], lung lipid peroxidation was high. In asthma, lipid peroxidation may have detrimental consequences such as airway hyper responsiveness, plasma overflow, smooth muscle constriction and consequent airway obstruction [12]. Considering the damage caused by lipid peroxidation in asthma, the reduction of nitrite and lipid hydroperoxide production reinforces the antioxidant role of the supplement in this pathology.

Through lipid peroxidation caused by ROS and RNS, the PAF-like lipid can be formed. In the present study, dietary supplementation of asthmatic animals with fish oil had no effect on PAF bioactivity. As fish oil contains a large amount of PUFA that could be substrates for lipid peroxidation, PAF-like lipids could be formed and thereby increase the PAF bioactivity. On the other hand, based on the results presented here, oxidative stress may be controlled by supplementation of asthmatic rats with fish oil, and so the PAF bioactivity would decrease with the supplement. In fact, no effect of fish oil on lung PAF bioactivity was seen in asthmatic rats. Thus, it is likely that the observed PAF bioactivity is due to enzymatically formed PAF and not as a result of oxidative stress. In asthma, PAF

is involved in bronchial hyperresponsiveness and the consequent bronchoconstriction. In addition, it is associated with an increased vascular permeability and chemotaxis and activation of leukocytes [11, 40, 41]. When PAF is produced it stimulates the airways cells to secrete leukotriene B₄ [42], a potent proinflammatory mediator that is directly related to airway responsiveness [43]. Since anti-leukotriene drugs are very effective in reversing the hyper responsiveness observed in asthma, PAF action on bronchial responsiveness may be mediated by leukotrienes. Moreover, in a previous study we found that dietary supplementation of asthmatic animals with fish oil did not affect the force of contraction of airway smooth muscle, which is the main feature that promotes asthmatic exacerbation, an immediate reaction of bronchoconstriction [19]. Considering that PAF is related to bronchoconstriction, the ineffective action of fish oil on parameters associated to airway smooth muscle function reported in previous studies may be related to its lack of effect on the PAF bioactivity.

Although the findings of this work are interpreted in the context of a therapeutic effect on asthma, because we provided the fish oil through the period of sensitization to OVA we cannot rule out an effect on n-3 PUFA on the sensitization process itself. Moreover, some differences between asthmatic and control animals may be due to the instillation of protein into the lungs for 3 days prior to sampling. However since some of the measurements made were not different between asthmatic rats and asthmatic rats given fish oil (as seen in histology of lungs and body weight of animals) it would seem likely that the sensitization process was similarly effective in both these groups of rats.

In summary, dietary supplementation with fish oil had an antioxidant effect in asthmatic animals, since the supplement was able to increase the activity of SOD and GPx and to reduce the concentrations of nitrite and lipid hydroperoxides. However, fish oil had no effect on the PAF bioactivity, a proinflammatory molecule with a central role in asthmatic inflammation and that is related to oxidative stress. Considering the two-step process—oxidative stress and PAF bioactivity—fish oil exhibited a divergent action in these two aspects of asthmatic inflammation. In conclusion, fish oil supplementation has a dual effect in asthmatic rats, reducing oxidative stress, but having no effect on the PAF bioactivity.

Acknowledgments We thank the Fundação Herbarium for donating of fish oil capsules, Marlon Alves Mota and Fabrício Azevedo for help with horse blood collection and the biochemistry department of UFPR for the loan of the spectrofluorometer for our analyses.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Comhair SA, Erzurum SC (2010) Redox control of asthma: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal* 12(1):93–124
- Owayed A, Dhaunsi GS, Al-Mukhaizeem F (2008) Nitric oxide-mediated activation of NADPH oxidase by salbutamol during acute asthma in children. *Cell Biochem Funct* 26(5):603–608
- Carlo T, Levy BD (2008) Chemical mediators and the resolution of airway inflammation. *Allergol Int* 57(4):299–305
- Kaleli S, Akkaya A, Akdogan M, Gültekin F (2006) The effects of different treatments on prolidase and antioxidant enzyme activities in patients with bronchial asthma. *Environ Toxicol Pharmacol* 22(1):35–39
- Reynaert NL, Aesif SW, McGovern T, Brown A, Wouters EFM, Irvin CG, Janssen-Heininger YMW (2007) Catalase overexpression fails to attenuate allergic airways disease in the mouse. *J Immunol* 178(6):3814–3821
- Zimmerman GA, Prescott SM, McIntyre TM (1995) Oxidatively fragmented phospholipids as inflammatory mediators: the dark side of polyunsaturated lipids. *J Nutr* 125(6 Suppl):1661s–1665s
- Mak JCW, Leung HCM, Ho SP, Law BKW, Lam WK, Tsang KWT, Ip MSM, Chan-Yeung M (2004) Systemic oxidative and antioxidative status in Chinese patients with asthma. *J Allergy Clin Immunol* 114(2):260–264
- Nadeem A, Chhabra SK, Massod A, Raj HG (2003) Increased oxidative stress and altered levels of antioxidants in asthma. *J Allergy Clin Immunol* 111(1):72–78
- Zanatta AL, Michelotto PV Jr, Nishiyama A (2012) The prevention of oxidative stress improves asthmatic inflammation. *Adv Biosci Biotechnol* 3:1087–1090
- Ishii S, Nagase T, Shindou H, Takizawa H, Ouchi Y, Shimizu T (2004) Platelet-activating factor receptor develops airway hyperresponsiveness independently of airway inflammation in a murine asthma model. *J Immunol* 172(11):7095–7102
- Uhlig S, Goggel R, Engel S (2005) Mechanisms of platelet-activating factor (PAF)-mediated responses in the lung. *Pharmacol Rep* 57(Suppl):206–221
- Muehlmann LA, Zanatta AL, Farias CLA, Bieberbach EW, Mazzonetto AC, Michelotto PV Jr, Fernandes LC, Nishiyama A (2010) Dietary supplementation with soybean lecithin increases pulmonary PAF bioactivity in asthmatic rats. *J Nutr Biochem* 21(6):532–537
- Marathe GK, Harrison KA, Murphy RC, Prescott SM, Zimmerman GA, McIntyre TM (2000) Bioactive phospholipid oxidation products. *Free Radic Biol Med* 28(12):1762–1770
- Calder PC (2006) n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr* 83:1505S–1519S
- Calder PC (2009) Polyunsaturated fatty acids and inflammatory processes: new twists in an old tale. *Biochimie* 91:791–795
- Calder PC (2011) Fatty acids and inflammation: the cutting edge between food and pharma. *Eur J Pharmacol* 668:S50–S58
- Marathe GK, Davies SS, Harrison KA, Silva AR, Murphy RC, Castro-Faria-Neto H, Prescott SM, Zimmerman GA, McIntyre TM (1999) Inflammatory platelet-activating factor-like phospholipids in oxidized low density lipoproteins are fragmented alkyl phosphatidylcholine. *J Biol Chem* 274(40):28395–28404
- Rodacki L, Rodacki AL, Pereira G, Naliwaiko K, Coelho I, Pequeto D, Fernandes LC (2012) Fish oil supplementation enhances the effects of strength training in elderly women. *Am J Clin Nutr* 95(2):428–436
- Miranda DT, Zanatta AL, Dias BC, Fogaça RT, Maurer JB, Donatti L, Calder PC, Nishiyama A (2013) The effectiveness of fish oil supplementation in asthmatic rats is limited by an inefficient action on ASM function. *Lipids* 48(9):889–897
- Aebi H (1984) Catalase in vitro. *Methods Enzymol* 105:121–126
- Nourooz-Zadeh J, Tajaddini-Sarmadi J, Wolff SP (1994) Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylene orange assay in conjunction with triphenylphosphine. *Anal Biochem* 220(2):403–409
- Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226(1):497–509
- Miles EA, Calder PC (2012) Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. *Br J Nutr* 107(2):S171–S184
- Weylandt KH, Chiu C-Y, Gomolka B, Waechter SF, Wiedemann B (2012) Omega-3 fatty acids and their lipid mediators: towards an understanding of resolvin and protectin formation Omega-3 fatty acids and their resolvin/protectin mediators. *Prostaglandins Other Lipid Mediat* 97(3–4):73–82
- Norris PC, Dennis EA (2012) Omega-3 fatty acids cause dramatic changes in TLR4 and purinergic eicosanoid signaling. *Proc Natl Acad Sci USA* 109(22):8517–8522
- Rhee SG, Yang K, Kang SW, Woo HA, Chang T (2005) Controlled elimination of intracellular H₂O₂: regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. *Antioxid Redox Signal* 7(5–6):619–626
- Perišić T, Srećković M, Matic G (2007) An imbalance in antioxidant enzymes and stress proteins in childhood asthma. *Clin Biochem* 40(15):1168–1171
- Rahman I, Biswas SK, Kode A (2006) Oxidant and antioxidant balance in the airways and airway diseases. *Eur J Pharmacol* 533(1–3):222–239
- Barbosa S, Cecchini R, Kadri MZ, Rodríguez MAM, Burini RC, Dichi I (2003) Decreased oxidative stress in patients with ulcerative colitis supplemented with fish oil ω -3 fatty acids. *Nutrition* 19:837–842
- Kim YJ, Yokozawa T, Chung HY (2005) Effects of energy restriction and fish oil supplementation on renal guanidino levels and antioxidant defences in aged lupus-prone B/W mice. *Br J Nutr* 93(6):835–844
- Kim YJ, Yokozawa T, Chung HY (2005) Suppression of oxidative stress in aging NZB/NZW mice: effect of fish oil feeding on hepatic antioxidant status and guanidino compounds. *Free Radic Res* 39(10):1101–1110
- Wang H, Hung T, Wei J, Chiang A (2004) Fish oil increases antioxidant enzyme activities in macrophages and reduces atherosclerotic lesions in apoE-knockout mice. *Cardiovasc Res* 61(1):169–176
- Berry M, Eisenberg S, Haratz D, Friedlander Y, Norman Y, Kaufmann NA, Stein Y (1991) Effects of diets rich in monounsaturated fatty-acids on plasma-lipoproteins: the Jerusalem nutrition study: high MUFAs versus high PUFAs. *Am J Clin Nutr* 53:899–907
- Stamler S, Hausladen A (1998) Oxidative modifications in nitrosative stress. *Nat Struct Biol* 5(4):247–249
- Jourd'heuil D, Mills L, Miles AM, Grisham MB (1998) Effect of nitric oxide on hemoprotein-catalyzed oxidative reactions. *Nitric Oxide* 2(1):37–44
- Matés M, Perez-Gomez C, Blanca M (2000) Chemical and biological activity of free radical 'scavengers' in allergic diseases. *Clin Chim Acta* 296(1–2):1–15
- Halliwell B (1991) Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *Am J Med* 91(3C):14S–22S
- Spitteler G (2001) Lipid peroxidation in aging and age-dependent diseases. *Exp Gerontol* 36(9):1425–1457

39. Wood G, Gibson PG, Garg ML (2003) Biomarkers of lipid peroxidation, airway inflammation and asthma. *Eur Respir J* 21(1):177–186
40. Zimmerman GA, McIntyre TM, Prescott SM, Stafforini DM (2002) The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. *Crit Care Med* 30(5 Suppl):S294–S301
41. Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM (2000) TM platelet-activating factor and related lipid mediators. *Annu Rev Biochem* 69:419–445
42. Gabrijelcic J, Acuña A, Profita M, Paternò A, Chung KF, Vignola AM, Rodríguez-Roisin R (2003) Neutrophil airway influx by platelet-activating factor in asthma: role of adhesion molecules and LTB4 expression. *Eur Respir J* 22(2):290–297
43. Foster HR, Fuerst E, Lee TH, Cousins DJ, Woszczek G (2013) Characterisation of P2Y12 receptor responsiveness to cysteinyl leukotrienes. *PLoS One* 8(3):e58305