

## Anti-inflammatory effects of long-chain *n*-3 PUFA in rhinovirus-infected cultured airway epithelial cells

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Long-chain *n*-3 PUFA (LCn-3PUFA) including DHA and EPA, are known to decrease inflammation by inhibiting arachidonic acid (AA) metabolism to eicosanoids, decreasing the production of pro-inflammatory cytokines and reducing immune cell function. The aim of this study was to determine if EPA and DHA reduced the release of inflammatory mediators from airway epithelial cells infected with rhinovirus (RV). Airway epithelial cells (Calu-3) were incubated with EPA, DHA and AA for 24 h, followed by rhinovirus infection for 48 h. IL-6, IL-8 and interferon- $\gamma$ -induced protein-10 (IP-10) released by cells were measured using ELISA. Viral replication was measured by serial titration assays. The fatty acid content of cells was analysed using GC. Cellular viability was determined by visual inspection of cells and lactate dehydrogenase release. DHA (400  $\mu$ M) resulted in a significant 16% reduction in IL-6 release after RV-43 infection, 29% reduction in IL-6 release after RV-1B infection, 28% reduction in IP-10 release after RV-43 infection and 23% reduction in IP-10 release after RV-1B infection. Cellular DHA content negatively correlated with IL-6 and IP-10 release. None of the fatty acids significantly modified rhinovirus replication. DHA supplementation resulted in increased cellular content of DHA at the cost of AA, which may explain the decreased inflammatory response of cells. EPA and AA did not change the release of inflammatory biomarkers significantly. It is concluded that DHA has a potential role in suppressing RV-induced airway inflammation.

### EPA: DHA: Inflammation: Rhinovirus: Acute asthma

A low prevalence of chronic inflammatory diseases, such as CHD and asthma, was first reported in 1979 among people of Greenland who had a high intake of long-chain *n*-3 PUFA (LCn-3PUFA)<sup>(1)</sup>. Later this was attributed to the anti-inflammatory effect of LCn-3PUFA<sup>(2)</sup>. LCn-3PUFA include EPA and DHA. LCn-3PUFA have been shown to decrease inflammation via (1) inhibiting the inflammatory pathway of arachidonic acid (AA), (2) decreasing production of pro-inflammatory cytokines and (3) reducing immune cell function<sup>(3–8)</sup>.

Long-chain fatty acids are the substrate for production of eicosanoids, which are inflammatory regulators in the human body<sup>(9,10)</sup>. Eicosanoids produced from AA are more inflammatory than those produced by LCn-3PUFA<sup>(11–13)</sup>. For example, leukotriene B<sub>4</sub> (produced from AA) is 10–30 times more potent as a chemo-attractant than leukotriene B<sub>5</sub> (produced from EPA)<sup>(4,12,13)</sup>. Leukotriene B<sub>4</sub> increases neutrophil influx, which further potentiates inflammation<sup>(14–16)</sup>. It has

been shown that supplementation with EPA and DHA decreases leukotriene B<sub>4</sub> production and neutrophil chemotaxis<sup>(10)</sup>. It has also been demonstrated that EPA and DHA reduce synthesis of AA, compete with AA for incorporation into *sn*-2 position of membrane phospholipids, competing at the cyclo-oxygenase and lipoxygenase enzymes, thereby resulting in a reduction of potent eicosanoid production<sup>(4,5)</sup>.

There is evidence suggesting that increased levels of LCn-3PUFA in cellular membranes decreases cytokine production<sup>(7,8)</sup>. *In vivo*<sup>(17,18)</sup> and *in vitro*<sup>(19,20)</sup> studies show a decrease in inflammatory biomarkers such as IL-6, IL-8 and C-reactive protein following LCn-3PUFA supplementation. Animal studies have also shown a decrease in circulating levels of IL-6, IL-10, TNF- $\alpha$ <sup>(21–23)</sup>, IL-12, IL-1 $\beta$  and interferon- $\gamma$ <sup>(22)</sup> following LCn-3PUFA supplementation. These anti-inflammatory effects of LCn-3PUFA are attributed to their potent effect on suppression of NF- $\kappa$ B<sup>(24–28)</sup> most probably via inactivation of signalling through toll-like

**Abbreviations:** AA, arachidonic acid; 10% FCS/MEM, minimum essential medium containing 10% fetal calf serum; IP-10, interferon- $\gamma$ -induced protein-10; LCn-3PUFA, long-chain *n*-3 PUFA; RV, rhinovirus; TCID<sub>50</sub>, tissue culture infectious dose at 50%; TLR, toll-like receptor.

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receptor-2 (TLR-2)<sup>(29,30)</sup>. TLR-2 activation leads to the induction of a signalling cascade that results in the activation of NF- $\kappa$ B<sup>(31)</sup>. NF- $\kappa$ B has been shown to stimulate production of various pro-inflammatory cytokines (reviewed in Blackwell & Christman<sup>(32)</sup>) including IL-6 and IL-8 production in rhinovirus infection<sup>(32–34)</sup>. Recently, it has been found that DHA is more potent than EPA in suppression of NF- $\kappa$ B<sup>(26)</sup>.

It has also been reported that supplementation with high levels of EPA and DHA *in vivo* decreases (1) lymphocyte proliferation, (2) natural killer cell and monocyte activation and (3) neutrophil and monocyte chemotaxis<sup>(7,8,12,19,35,36)</sup>.

Rhinovirus (RV) is the most common cause of the common cold<sup>(37)</sup> and the major cause of asthma exacerbation in adults<sup>(38)</sup> and children<sup>(38,39)</sup>. RV infection can also worsen airway obstruction in asthmatics<sup>(40)</sup>, though the mechanism is not completely understood<sup>(41)</sup>. RV target epithelial cells, in which they replicate<sup>(42)</sup> and initiate innate immune responses via activation of TLR-3<sup>(43,44)</sup>. As a result, epithelial cells produce various inflammatory mediators that contribute to the host defence and result in increased airway inflammation<sup>(41)</sup>. These include IL-6<sup>(8,14,15)</sup>, IL-8<sup>(5,7,8,15,16,45)</sup> and interferon- $\gamma$ -induced protein-10 (IP-10)<sup>(41)</sup>.

Evidence regarding the ability of LCn-3PUFA to improve inflammation in airway epithelial cells infected with RV is lacking. However, some *in vitro* studies have shown that EPA and DHA can reduce the production of inflammatory biomarkers<sup>(18)</sup> and decrease inflammation induced by the innate immune stimulus lipopolysaccharide, which acts via TLR-4<sup>(24,26,27)</sup>. These studies found that EPA and DHA suppress NF- $\kappa$ B activation<sup>(24,26)</sup>. It is hypothesised that LCn-3PUFA may also decrease inflammation in airway epithelial cells infected with a virus. The aim of the present study was to determine if EPA and DHA reduced the release of inflammatory mediators from airway epithelial cells infected with RV.

## Materials and methods

### Airway epithelial cell culture

Airway epithelial cells (Calu-3, Passage 40–43, from ATCC, USA) were cultured in minimum essential medium containing 10% fetal calf serum (10% FCS/MEM), containing 2% penicillin–streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine and 2.2 g/l NaHCO<sub>3</sub> (all from Invitrogen Corporation, Carlsbad, CA, USA) at 37°C in the presence of 5% CO<sub>2</sub>. All experiments were carried out in cells with >80% confluence.

### Long-chain n-3 PUFA enrichment of cultured epithelial airway cells

In order to prepare the medium containing DHA, EPA and AA, the method of Nair *et al.*<sup>(46)</sup> was employed. Final concentrations of 0, 10, 200 and 400  $\mu$ M of each fatty acid (all purchased from Sigma) were dissolved in 0.04% ethanol (400  $\mu$ l ethanol/l medium) and added to 10% FCS/MEM which contains 2.2 g/l NaHCO<sub>3</sub>, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 1 mM-sodium pyruvate, 0.1  $\mu$ M-non-essential amino acids, 2 mM-L-glutamine and 10% fetal calf serum (all purchased from Invitrogen Corporation). Calu-3 cells were separately incubated with different concentrations

(0, 10, 200, 400  $\mu$ M) of EPA, DHA and AA for 24 h at 37°C in the presence of 5% CO<sub>2</sub>.

### Rhinovirus infection of Calu-3 cells

After removing from the fatty acid-containing media, cells were infected with RV-43 (multiplicity of infection: 7.2) and RV-1B (multiplicity of infection: 7.2) in fresh 1% FCS/MEM. Plates were incubated for 48 h at 37°C in the presence of 5% CO<sub>2</sub>.

### Visual inspection of Calu-3 cells

Cellular viability of cultured Calu-3 cells was checked after each step of supplementation and infection (Olympus microscope, TL4).

### Cytokine analysis

IL-6, IL-8 and IP-10 concentrations of medium were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

### Lactate dehydrogenase assay

Lactate dehydrogenase concentration in the media was measured by the enzymatic method on the Dade Behring RXL Dimension platform (Dade Behring Inc., USA). The assay has a CV of 4.9% at 350 U/l.

### Cellular content of fatty acids

The Calu-3 cellular content of fatty acids (including EPA, DHA and AA) was analysed using GC. The cell pellet was suspended in 2 ml of a methanol–toluene mixture (4:1, v/v), containing C19:0 (0.02 mg/ml) and butylated hydroxytoluene (0.12 g/l) and vortexed vigorously. The samples were methylated by adding 200  $\mu$ l acetyl chloride drop-wise while vortexing, followed by heating to 100°C for 1 h. After cooling, the reaction was stopped by adding 5 ml 6% K<sub>2</sub>CO<sub>3</sub> followed by vigorous mixing by vortex. The sample was centrifuged at 3000 g at 4°C for 10 min to facilitate separation of layers. The upper toluene layer containing the fatty acid methyl esters was transferred to a 2 ml glass vial and crimp sealed with a teflon-lined cap for analysis by GC. GC analysis was conducted using a 30 m  $\times$  0.25 mm (DB-225) fused carbon-silica column, coated with cyanopropyl-phenyl (J & W Scientific, Folsom, CA, USA). Both injector and detector port temperatures were set at 250°C. The oven temperature was 170°C for 2 min, increased 10°C/min to 190°C, held for 1 min, then increased 3°C/min up to 220°C and was maintained to give a total run time of 30 min. A split ratio of 10:1 and an injection volume of 3  $\mu$ l were used. The chromatograph was equipped with a flame ionization detector, autosampler and autodetector. Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of a standard mixture of fatty acid methyl esters and quantified using a Hewlett Packard 6890 Series Gas Chromatograph with Chemstations version A.04.02.

### Viral titration assay

Viral titration was performed using confluent RD-ICAM-1 cells seeded in ninety-six-well tissue culture plates (NUNC, Roskilde, Denmark). Cells were infected by either media alone or virus containing media at varying dilutions. Serial 10-fold dilutions of the samples were prepared and four individual wells were infected with each dilution. For titration of samples six dilutions were prepared. Additionally, for every dilution two controls wells were prepared with media alone. After 4 d the plates were read and the tissue culture infectious dose at 50% (TCID<sub>50</sub>) was calculated. Infected wells were scored based on the cytopathic effect seen, >50% cytopathic effect (wells where more than 50% of their cells are dead) demonstrated by light microscopy was considered a positive result. Viral titres of the samples were determined by cell titration assay using RD-ICAM-1 cells and the viral titre was calculated and expressed as a log value (TCID<sub>50</sub> log<sub>10</sub>)<sup>(47)</sup>, using the Karber formula for TCID<sub>50</sub><sup>(48)</sup>.

### Statistics

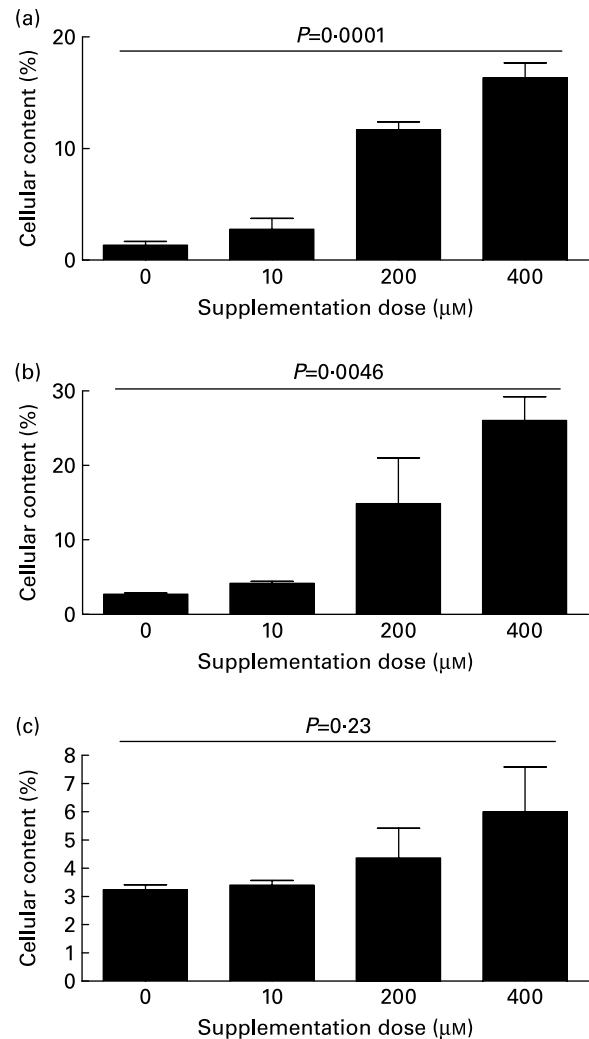
Paired *t* tests, ANOVA and correlations were performed by GraphPad Prism 4 software (GraphPad Prism, San Diego, CA, USA). *P* values less than 0.05 were considered as statistically significant.

### Results

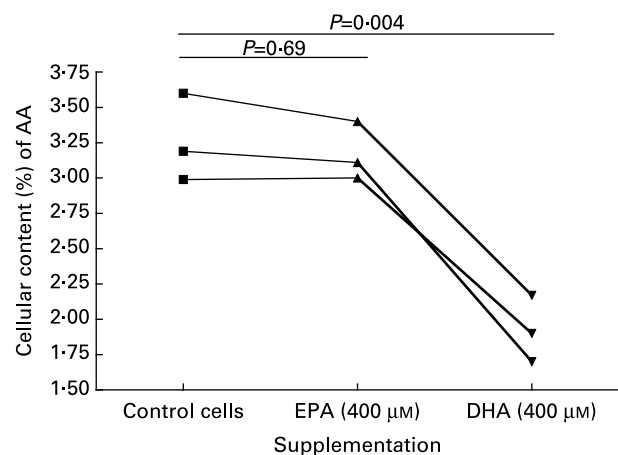
Fatty acids were incorporated into the cultured airway epithelial cells in a dose-dependent manner (Fig. 1). EPA and DHA content of cells increased significantly as the concentration of the fatty acids was increased in the medium. The results show that the highest concentration (400 μM) of EPA, DHA and AA resulted in 16, 26 and 6% incorporation into Calu-3 cells, respectively. Pretreatment with 400 μM-DHA led to a significant decrease in AA content of Calu-3 cells (Fig. 2, from three independent experiments). It was also found that supplementation of DHA (400 μM) resulted in lower cellular levels of AA compared to EPA.

The concentration of lactate dehydrogenase released by Calu-3 cells was similar across all groups supplemented with various concentrations (0, 10, 200, 400 μM) of EPA, DHA and AA. Furthermore, visual inspection of the cultured Calu-3 cells after each step confirmed that the cells were viable under all conditions and supplementation regimens.

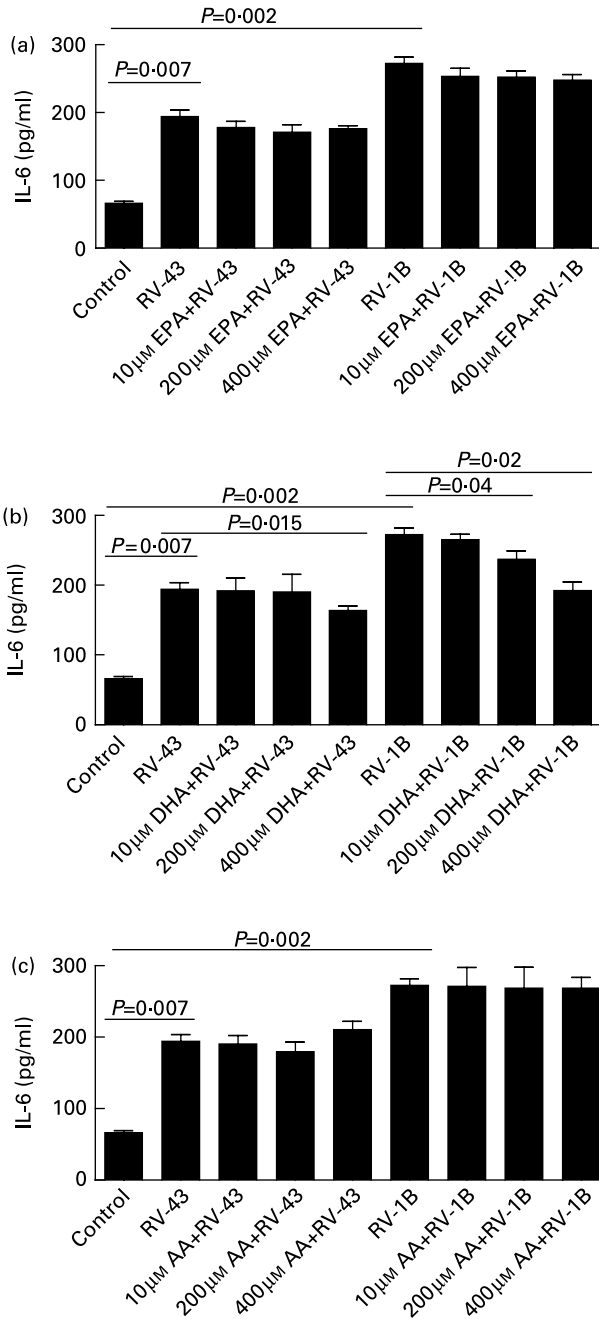
Infection with either RV-43 or RV-1B resulted in increased release of IL-6, IL-8 and IP-10 (Figs. 3, 4 and 5). DHA (400 μM) resulted in a significant 16% reduction in IL-6 after RV-43 infection (Fig. 3), 29% reduction in IL-6 after RV-1B infection (Fig. 3), 28% reduction in IP-10 after RV-43 infection (Fig. 5) and 23% reduction in IP-10 after RV-1B infection (Fig. 5). DHA suppression of IL-6 release by cells infected with RV-1B occurred in a dose-dependent manner (Fig. 3 (b)). DHA content of the cells had a negative correlation with IL-6 (Spearman  $\rho$  -0.775,  $P=0.003$ ) and IP-10 (Spearman  $\rho$  -0.69,  $P=0.012$ ) levels (Fig. 6). EPA and AA had no effect on the release of cytokines by cultured cells. Pretreatment of cells with the fatty acids had no significant effect on RV replication (data not shown).



**Fig. 1.** Cellular content (weight % of total fatty acids) of EPA (a), DHA (b) and arachidonic acid (AA; c) in Calu-3 cells before and after treatment with different concentrations of EPA, DHA and AA. Values are means with their standard deviations depicted by vertical bars (from triplicate experiments).



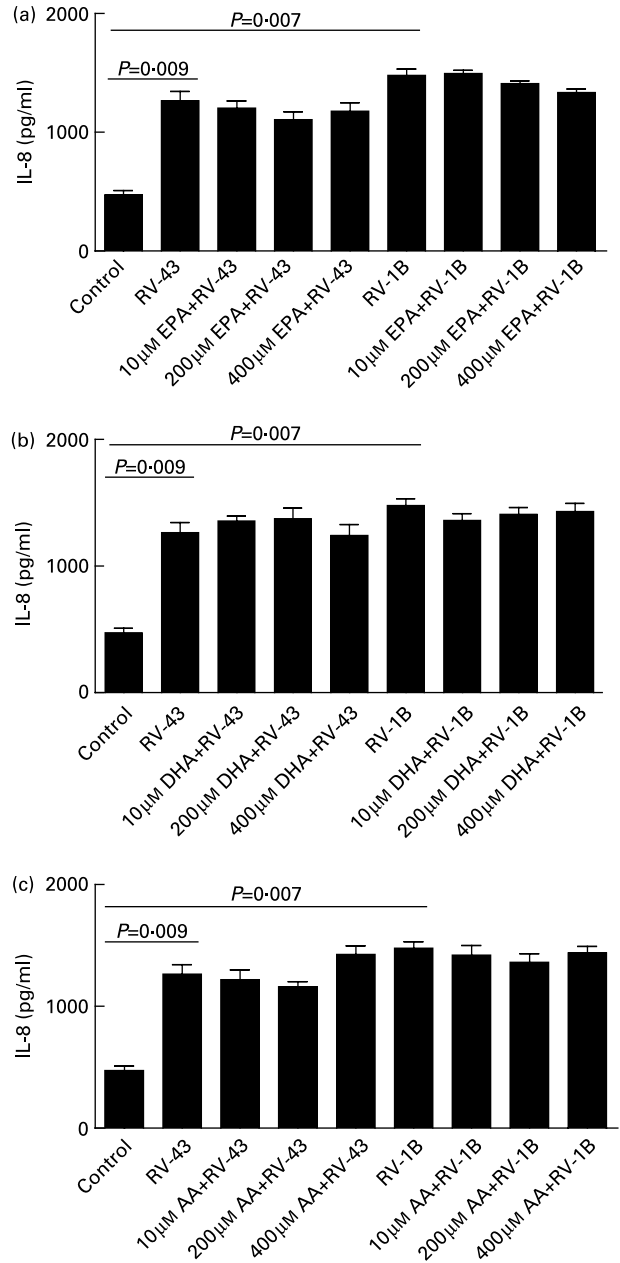
**Fig. 2.** Arachidonic acid (AA) content (weight % of total fatty acids) of control Calu-3 cells compared to the cells supplemented with EPA and DHA. Values are means (from three independent experiments).



**Fig. 3.** IL-6 release following rhinovirus (RV) infection by Calu-3 cells enriched with different concentrations of EPA (a), DHA (b) and arachidonic acid (c). Values are means with their standard deviations depicted by vertical bars (from triplicate experiments).

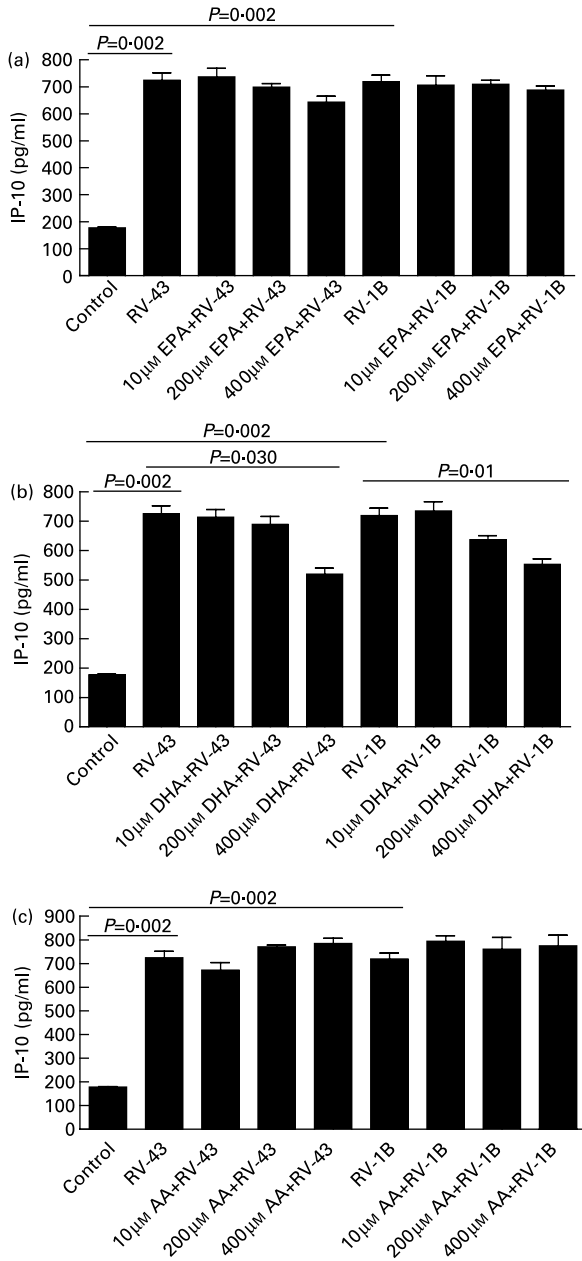
**Discussion**

This is the first study investigating the anti-inflammatory effect of EPA and DHA on RV-infected airway epithelial cells (Calu-3 cells). We showed that DHA supplementation increased DHA and decreased AA content of the cells and decreased the release of IL-6 and IP-10 by cells infected with RV-43 and RV-1B. We also showed that EPA and AA have no effect on the release of inflammatory biomarkers. Furthermore, DHA, EPA and AA have no effect on the replication of RV-43 and RV-1B.



**Fig. 4.** IL-8 release following rhinovirus infection by Calu-3 cells enriched with different concentrations of EPA (a), DHA (b) and arachidonic acid (c). Values are means with their standard deviations depicted by vertical bars (from triplicate experiments).

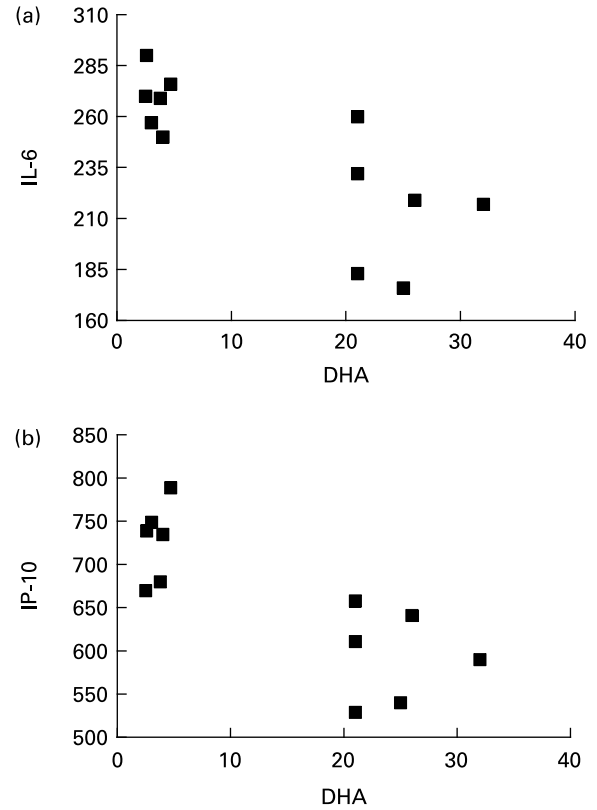
The present results showed that EPA, DHA and AA uptake into the cultured airway epithelial cells occurred in a dose-dependent manner (Fig. 1). The highest concentration of supplemented fatty acids (400 µM) in the present study has been used successfully on cultured porcine cardiomyocytes cells by Nair *et al.* (46). At the highest concentration (400 µM), DHA was most efficiently incorporated, being taken up by 26%, which was nearly twice EPA (16.3%) and nearly four times the uptake of AA (6%). Other studies have reported dose-dependent uptake of LCn-3PUFA into human breast cancer cells after 24 h (49). It has been shown that higher uptake of DHA compared to EPA into cellular membrane is



**Fig. 5.** Interferon- $\gamma$ -induced protein-10 (IP-10) release following rhinovirus infection by Calu-3 cells enriched with different concentrations of EPA (a), DHA (b) and arachidonic acid (c). Values are means with their standard deviations depicted by vertical bars (from triplicate experiments).

attributed to more efficient incorporation of DHA than EPA into phospholipids of the membrane<sup>(50)</sup>.

The results show supplementation with DHA caused a reduction in AA levels compared to control cells (Fig. 2). This is in agreement with other studies that show that LCN-3PUFA decrease the cellular content of AA in airway epithelial cells<sup>(6)</sup>. We have also shown that DHA is more effective than EPA in decreasing cellular content of AA. The substitution of DHA/EPA for AA is important, as the anti-inflammatory effect of LCN-3PUFA is dependent not only on increasing the level of LCN-3PUFA, but reducing the level of AA, thus ensuring a reduction in production of pro-inflammatory eicosanoids.



**Fig. 6.** Correlations between DHA content (weight % of total fatty acids) of Calu-3 cells v. IL-6 (a) and interferon- $\gamma$ -induced protein-10 (IP-10; b) levels after rhinovirus-1B infection (from triplicate experiments). (a), Spearman  $\rho$  - 0.775,  $P$ =0.003; (b), Spearman  $\rho$  - 0.697,  $P$ =0.012.

Supplementation of different concentrations (10, 200, 400  $\mu$ M) of EPA, DHA and AA did not cause any cytotoxic effect on the cultured cells. Cellular viability was confirmed by visual inspection of the cells as well as lactate dehydrogenase released by the cells after supplementation. Lactate dehydrogenase is commonly used as a cell death biomarker<sup>(51)</sup>. *In vitro* studies have shown that incorporation of high levels of DHA and EPA (more than 500  $\mu$ M) increases cell cytotoxicity and apoptosis in different cell types<sup>(52-54)</sup>. However, in the low concentrations used in the present experiment, no cell toxicity has been observed<sup>(24,46)</sup>.

The present results showed that the concentration of IL-6, IL-8 and IP-10 increased significantly after infection with RV-43 and RV-1B. This is in agreement with the other studies reporting that RV infection increases the production of IL-6, IL-8<sup>(33,34,55)</sup> and IP-10<sup>(41)</sup> in different types of cultured cells. The present results also showed that pre-supplementation with DHA (400  $\mu$ M) significantly decreased the release of IL-6 and IP-10 by Calu-3 cells infected with RV-43 and RV-1B. DHA at a lower concentration (200  $\mu$ M) also decreased IL-6 released by cells infected with RV-1B. DHA content of the cells has a significant negative correlation with pro-inflammatory cytokine levels (Fig. 6). The present data agree with other *in vitro*<sup>(18,19)</sup>, human<sup>(17)</sup> and animal studies<sup>(21)</sup> that have also reported a decrease in IL-6 and IL-8 release after supplementation with LCN-3PUFA. Weldon *et al.*<sup>(26)</sup> reported that DHA



is more potent than EPA in decreasing the production of inflammatory biomarkers. In their study, cellular uptake of DHA and EPA was not compared. Therefore, the present study shows that increased cellular uptake of DHA compared to EPA is probably the explanation for the higher anti-inflammatory effect of DHA.

The present data also showed that DHA decreased IP-10 production of Calu-3 cells infected with RV. There are no previous reports regarding the effect of LCn-3PUFA on the release of IP-10 from airway epithelial cells or other types of cells. It has been reported that response elements of NF- $\kappa$ B in the promoter region of the IP-10 gene are involved in transcriptional activation of IP-10<sup>(56–58)</sup>. Therefore, decreased NF- $\kappa$ B activation may affect the release of IP-10. It has been suggested that IP-10 is involved in viral replication in cells<sup>(41)</sup>. However, the present results show that viral replication of RV-43 and RV-1B was not changed by supplementation with fatty acids. There are no previous studies examining the effect of EPA, DHA and AA on the viral replication of RV in the literature. Although it appears that the decreased concentration of IP-10 that we observed due to DHA must be independent of viral replication. The viral replication-independent release of IP-10 has been reported previously<sup>(59)</sup>.

The increased cellular content of DHA and decreased content of AA is a probable explanation for the significant anti-inflammatory effect of DHA compared to EPA, as the substitution of DHA for AA is known to reduce production of inflammatory markers<sup>(4,17,19,21–23,60,61)</sup>. The potential effect of LCn-3PUFA on decreasing cytokine production via suppression of NF- $\kappa$ B has been well described<sup>(24–27)</sup>. NF- $\kappa$ B has a proven effect on production of a vast variety of pro-inflammatory cytokines (reviewed in Blackwell & Christman<sup>(32)</sup>) including IL-6 and IL-8<sup>(33,34)</sup>. It has been reported that LCn-3PUFA suppress NF- $\kappa$ B via (1) inactivation of TLR<sup>(27,29,30)</sup>, (2) blocking I- $\kappa$ B (NF- $\kappa$ B inhibitor) degradation and also (3) blocking mitogen-activated protein kinase<sup>(24)</sup>. LCn-3PUFA also inhibit the AA inflammatory pathway via competing with AA to produce less potent inflammatory eicosanoids<sup>(4,5,60)</sup>, decreasing the release of AA from the phospholipids of cellular membrane via decreasing the enzymatic activity of phospholipase A<sub>2</sub><sup>(3)</sup>, decreasing AA content of cells<sup>(6)</sup> and suppressing the activation of cyclo-oxygenase-2 which converts AA to PG<sub>2</sub> and thromboxane 2<sup>(25,29)</sup>. The probable mechanism for this suppression is also related to inactivation of TLR<sup>(29,30)</sup>. Therefore the most likely mechanisms by which DHA decreased the production of inflammatory biomarkers in the present study is its inhibitory effect on (1) NF- $\kappa$ B activation and (2) AA inflammatory pathway, both via decreasing TLR activity.

In summary, we found that DHA decreased inflammation in airway epithelial cells infected with RV-43 and RV-1B via decreasing IL-6 and IP-10 release. It was found that because of the higher cellular uptake of DHA, after supplementing with equivalent amounts of EPA and DHA, DHA content of airway epithelial cells was higher than EPA. DHA also decreased cellular content of AA. It is likely that these two findings explain the anti-inflammatory effect of DHA. Therefore, DHA supplementation may be useful in decreasing the inflammatory response of airway epithelial cells to RV infection.

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