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Dehydroascorbic acid as pre-conditioner: Protection from lipopolysaccharide induced mitochondrial damage

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Abstract

Oxidative stress-induced mitochondrial dysfunction is a common consequence of severe sepsis. However, oxidative stress also activates signalling cascades which enable protection of cells against subsequent oxidative damage. This study hypothesized that cellular uptake of vitamin C as dehydroascorbic acid rather than ascorbic acid would up-regulate antioxidant enzyme systems and impart a protective effect to mitochondria in cells subsequently exposed to lipopolysaccharide (LPS) in an iron free environment. Treatment of monocytes with dehydroascorbic acid, but not ascorbic acid, caused oxidative stress ($p < 0.001$). Dehydroascorbic acid exposure also resulted in increased manganese superoxide dismutase ($p = 0.018$) and catalase ($p = 0.003$) expression. Pre-treatment of monocytes with dehydroascorbic acid followed by LPS resulted in higher mitochondrial membrane potentials than cells without pre-treatment ($p < 0.0001$). Lower cytochrome *c* in cytosol ($p < 0.05$) and higher mitochondrial expression of the anti-apoptotic Bcl-2 protein ($p = 0.029$) was also found in monocytes pre-treated before subsequent LPS exposure, compared to cells without pre-treatment. In conclusion, acute exposure of monocytes to dehydroascorbic acid in an iron free environment induces cytoprotective antioxidant enzymes and protected mitochondria from the harmful effects of oxidative stress prior to a septic insult, which was abrogated when cells were pre-incubated with the DHA uptake inhibitor cytochalasin B.

Keywords: *Vitamin C, reactive oxygen species, monocyte, endotoxin, mitochondria, pre-conditioning*

Introduction

Sepsis and septic shock are the most common causes of mortality on the intensive care unit. The standard feature of sepsis is a deregulated host response to microbial components of bacterial, fungal or viral origin and often results in multiple organ failure and death [1,2]. There is growing evidence that mitochondrial oxidative damage inhibits mitochondrial ATP production and plays a part by which cell death and organ failure occur during sepsis [3,4]. We and others have shown that patients with sepsis have low circulating antioxidant levels, including vitamin C [5], low total plasma antioxidant capacity [6,7], increased concentrations of lipid peroxidation products [5,8,9] and altered bioenergetic status [10]. Mitochondrial damage was found in livers from patients who died of severe sepsis [11] and we have also shown deranged mitochondrial redox state in patients with sepsis [12]. If oxidative damage to mitochondria is central to the pathology of sepsis then antioxidants could be potential therapies. *N*-acetylcysteine was recently shown to be of benefit in a rat model of sepsis, with reduced oxidative damage and less mitochondrial dysfunction [13]. However, no studies have yet provided conclusive evidence of the beneficial effect of antioxidant supplementation in critically ill patients.

Vitamin C is bio-available equally as either dehydro-L-ascorbic acid (DHA) or L-ascorbic acid (AA) and indeed DHA is regarded as an important dietary source of vitamin C [14]. Cells can take up ascorbate through Na^{+}−dependent ascorbate co-transporters (SVCT1 and SVCT2). However, not all nucleated cells possess SVCTs and incubation of these cells with DHA *in vitro* increases intracellular AA levels via

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the facilitative glucose transporters (GLUT1, GLUT3 and GLUT4) [15–17]. Once inside the cell, DHA is immediately converted to AA by NADPH-dependent thioredoxin reductase or glutathione-dependent DHA reductase [18]. Reduction of DHA to AA 'snatches electrons' from intracellular components, generating a mild oxidative stress, such that DHA has been regarded as an intracellular 'pro-oxidant' [19,20].

Many studies have shown that brief exposures to ischemia or to sub-lethal concentrations of exogenous oxidants have amplified oxidant protection enzyme mechanisms and provided subsequent protection against cell death [21,22]. Generation of reactive oxygen species (ROS) is now accepted as the basis of the initiation of this protection (conditioning) and is believed to involve the modulation of a number of molecules and signalling pathways and influence mitochondrial function [23,24]. Peripheral blood monocytes contain membrane transporters for uptake of both AA (SVCT2) and DHA (GLUT1 and GLUT3) [25,26]. Therefore, we proposed that pre-exposure of monocytes to DHA may induce protection against subsequent oxidative stress induced by endotoxin (lipopolysaccharide, LPS) when compared to pre-treatment with AA. We hypothesized that cellular uptake of DHA may up-regulate antioxidant enzyme systems and impart a protective effect to mitochondria in cells subsequently exposed to LPS.

Materials and methods

Monocyte isolation and treatment

Following Local Research Ethics Committee approval and informed consent, mononuclear leucocytes were isolated from heparinized blood from 10 healthy Caucasian volunteers using single density gradient separation through Ficoll-paque™ PLUS (GE Healthcare UK Ltd., Bucks, UK) (density = 1.077 g/ml). A highly enriched monocyte fraction was then obtained using Percoll™ (GE healthcare UK Ltd) density gradient separation of the purified mononuclear leucocytes (density = 1.064 g/ml). Following centrifugation, monocytes (> 97% pure, assessed by flow cytometry after staining a sample of the cells with an anti-CD14 antibody) were washed using Isocove's modified Eagles medium (Invitrogen, Paisley, UK) devoid of bicarbonate ions and containing 5 mM glucose, 10% autologous plasma, 25 mM HEPES, 500 μg/ml gentamicin and 1 mM sodium pyruvate (Sigma, Dorset, UK). Cell viability was assessed using Trypan blue and cells with viability > 98% were plated into 6-well culture dishes. Monocytes were allowed to attach to the culture dish for 1 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. Following monocyte attachment, any remaining contaminating lymphocytes were washed from the culture dishes before being washed gently with the above medium containing 10% autologous plasma, 500 μg/ml gentamicin, 1 mM sodium pyruvate and 0.4 mM desferrioxamine mesylate (DFO, Sigma, Dorset, UK) to remove free iron. Adherent monocytes were then cultured for 24 h in the same medium containing DFO plus 100 μM AA or DHA (Sigma) to allow for the conditioning effect to develop, 2 μg/ml LPS derived from *E. coli* strain O111:134 (Sigma) or phosphate buffered saline (PBS), AA or DHA. In separate cells were exposed to AA or DHA plus 10 μM cytocholasin B or dihydrocytocholasin B as controls.

In some experiments conditioned medium was then removed and the monocytes were washed gently with culture media devoid of AA or DHA and replaced with fresh culture medium containing DFO and either 2 μg/ml LPS derived from *E. coli* strain O111:134 (Sigma) or PBS as control and incubated for a further 18 h. Instead of LPS, in some experiments cells were exposed to 50 μM hydrogen peroxide or 0.2 mM xanthine plus 1 unit/ml xanthine oxidase as positive controls.

Ascorbic acid accumulation

Indirect measurement of intracellular AA accumulation was achieved by determining the rate of extracellular ferricyanide oxidation to ferrocyanide. Attached monocytes were washed twice in the above culture media. Monocytes were then incubated with AA or DHA and cytocholasin B or dihydrocytocholasin B in culture media for 15 min. Cells were then washed three times in Krebs-Ringer Hepes (KRH) buffer that consisted of 128 mM NaCl, 10 mM HEPES pH 7.4, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$, 1.4 mM $CaCl_2$, 5 mM glucose and contained 1 mM potassium ferricyanide. Following 30 min incubation the supernatant was removed. Ferrocyanide production was determined in diluted supernatant by incubating with 300 mM sodium acetate, 20 mM citric acid, 165 μM $FeCl_3$ dissolved in 100 mM acetic acid and 3 mM 1,10-phenanthroline for 5 min and absorbance was read at 510 nm.

Radical formation

For measurement of the relative oxidative stress generated in response to AA, DHA or LPS, monocytes were pre-loaded with 10 μM of the oxidation-sensitive 5,6-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCHF) or 10 μM of the oxidation-insensitive 5,6-carboxy-2,7-dichlorofluorescein diacetate (DCF, negative control) dissolved in dimethylformamide (Molecular Probes, Invitrogen, Paisley, UK) in 96-well plates for 1 h at 37°C. Cells were then cultured as described above in medium containing DFO plus either AA, DHA or LPS and the rate of increase in intracellular fluorescence measured over 24 h with a FLUOstar Optima 96 well fluorescence plate reader (BMG Lab Tech, Aylesbury, UK) at 37°C (excitation 488 nm, emission 535 nm). The mean fluorescence

intensity was corrected for auto-fluorescence from unlabelled cells. Cells pre-loaded with DCF showed no increase in fluorescence.

GSH determination

Oxidative stress from intracellular DHA reduction to AA was determined by measuring GSH levels. Monocytes were incubated with AA or DHA and cytocholasin B or dihydrochytocholasin B in the above culture media for 15 min. Cells were then washed twice with PBS and incubated at 37°C with 20 μM monobromobimane in PBS for 15 min. Cells were then washed twice with PBS and fluorescence was measured using a FLUOstar Optima fluorescence plate reader (excitation 355 nm and emission 520 nm).

Monocyte survival

The protective effect of pre-treatment with AA or DHA prior to hydrogen peroxide exposure on monocyte survival was determined by measuring acid phosphatase activity. Monocytes were cultured in 96-well plates in medium containing DFO plus AA, DHA, DHA plus cytocholasin B or dihydrocytocholasin B for 24 h. Cells were then washed and the medium replaced with that containing 400 μM hydrogen peroxide or PBS as control and further incubated for 18 h. After further washing with PBS, 0.1 M sodium acetate buffer pH 5.0 containing 0.1% triton X-100 and 5 mM *p*-nitrophenyl phosphate was added to each well and cells were incubated at 37°C for 1 h. The reaction was stopped by the addition of sodium hydroxide and the absorbance was measured at 405 nm.

Cell fractionation

Adherent monocytes pre-treated with AA or DHA with and without subsequent LPS exposure as described above were lysed in a solution containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1%v/v Triton X-100, 2 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4 and protease inhibitor cocktail (Roche, Hertfordshire, UK) for 30 min on ice. The lysates were then centrifuged at 300 × g to remove cell debris and nuclei. Cytosolic and mitochondrial fractions were obtained by centrifugation of the cleared lysate for 10 min at 13 000 × g. Protein concentrations of the cytosolic and mitochondrial fractions were determined by the Bio-Rad protein assay procedure (Bio-Rad, Hertfordshire, UK).

Protein expression

Antioxidant and anti-apoptotic protein expression in cytosolic fractions and isolated mitochondria was measured by western blotting; briefly, 50 μg protein was loaded onto each well of a 12% SDS gel. Separated proteins were transferred to Immobilon-P™ transfer membrane (Millipore, Bedford, MA) using standard techniques. Following transfer, the membranes was stained using Ponceau to check for transfer efficiency using densitometry. Blocked membranes were probed with primary polyclonal antibodies using sheep anti-human manganese superoxide dismutase (MnSOD) and B-cell leukaemia/lymphoma 2 (Bcl-2) (mitochondrial fractions) and mouse anti-human catalase and anti-human glutathione peroxidase (GPx) with appropriate horseradish peroxidase-linked secondary antibodies (cytosolic fractions) (Abcam, Cambridge, UK). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as loading control. Bands were visualized by enhanced chemiluminescence (ECL+ plus) according to the manufacturer's protocol (GH Healthcare UK, Ltd, Bucks, UK) and quantitated using GeneTools™ (SynGene, Synoptics Ltd, Cambridge, UK).

Cytochrome c release

Cytochrome *c* in cytosolic fractions devoid of mitochondria was measured using enzyme immunoassay (R&D systems, Abingdon, UK).

Mitochondrial membrane potential

Mitochondrial membrane potential was analysed in intact cells using the fluorescent probe 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Briefly, following cell treatments, monocytes were washed twice with PBS and then incubated for 30 min with 10 μg/ml JC-1 diluted in PBS at 37°C in the dark. Following incubation, cells were washed twice with PBS and the red/green fluorescence was immediately measured using a FLUOstar Optima at 37°C (excitation 490 nm and emission 590/520 nm).

Statistical analysis

Data obtained from ferricyanide reduction, GSH, western blots, JC-1 fluorescence and cytochrome *c* measurements were not normally distributed and were compared using Kruskal Wallis with *post hoc* testing using the Mann Whitney U-test. DCF fluorescence and acid phosphatase data were normally distributed and are reported as mean and standard deviation (SD) and statistically analysed using Student's paired *t*-est. Statistical analysis was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA) and *p*-values of < 0.05 were considered significant.

Results

Ascorbic acid accumulation

A sensitive but indirect method of determining intracellular AA accumulation within monocytes was used

to determine intracellular AA-dependent extracellular ferricyanide reduction. Ferricyanide reduction was 2-fold higher in monocytes incubated with DHA compared to control cells. However, when monocytes were pre-incubated with cytocholasin B and exposed to DHA, ferricyanide reduction was substantially reduced. Ferricyanide reduction by monocytes pre-incubated with dihydrocytocholasin B was similar to DHA treated cells ($p < 0.01$, Figure 1).

Extracellular ferricyanide reduction was slightly higher than control cells incubated with AA, but was substantially lower than DHA treated cells and no difference in ferricyanide reduction from monocytes pre-incubated with either cytocholasin B or dihydrocytocholasin B and incubated with AA was seen ($p < 0.01$, Figure 1).

Oxidative stress

To assess whether AA, DHA or LPS induced intracellular oxidative stress, we incubated cells with AA, DHA or 2 μg/ml LPS after pre-loading cells with DCF. The rate of increase in DCF fluorescence when monocytes were cultured in the presence of AA was 42 ± 3.0 ΔF/units/min which was similar to control cells (39 ± 1.8 ΔF units/min, $p = 0.2$, Figure 2). The rate of change in fluorescence was higher when monocytes were cultured with DHA or LPS indicating oxidative stress (50 ± 2.1 ΔF units/min and 58 ± 1.8 ΔF units/min, respectively, both $p < 0.001$, Figure 2). There was no significant difference between DHA and LPS treated cells ($p = 0.8$). No free iron was detected in the culture media and no oxidation of AA in the media was seen over the time course of the experiment (data not shown).

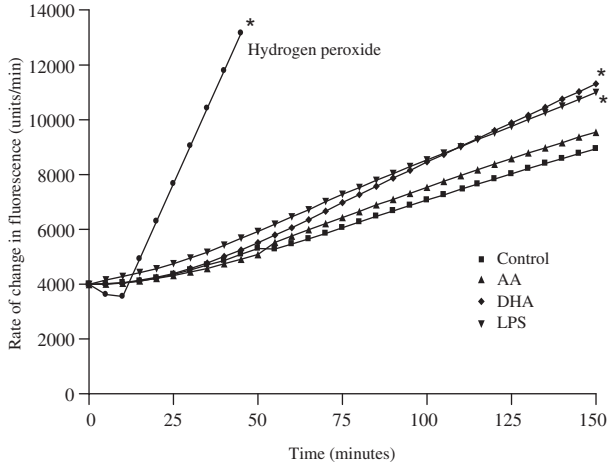

Figure 2. Oxidative stress (total radical production) as measured by the rate of change in fluorescence in monocytes cultured in medium containing 0.4 mM deferroximine (DFO) plus either phosphate buffered saline (control), 100 μM ascorbic acid (AA), 100 μM dehydroascorbic acid (DHA), 2 μg/ml lipopolysaccharide (LPS) or 50 μM hydrogen peroxide as a positive control. Values are mean rate of change ($n = 10$). p-value shown is ANOVA. *Significantly higher than in control or AA treated cells (T-test, $p < 0.0001$).

GSH determination

Substantiation of intracellular oxidative stress due to intracellular DHA reduction to AA was determined by

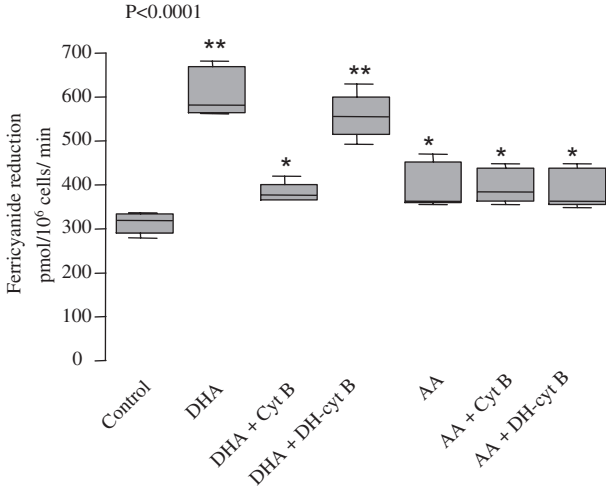

Figure 1. Reduction of extracellular ferricyanide by AA and DHA loaded monocytes. Monocytes were pre-treated in media containing 0.4 mM deferroximine (DFO), 100 μM ascorbic acid (AA) or 100 μM dehydroascorbic acid (DHA) plus 10 μM cytocholasin B (cyt B) or 10 μM dihydrocytocholasin B (DH-cyt B) for 15 min, washed and then incubated in the presence of 1 mM ferricyanide. Box and whisker plots show median, interquartile and full range ($n = 10$). p-value shown is Kruskal Wallis. *Significantly higher than in control cells (Mann Whitney $p = 0.01$); **significantly higher than in control cells or AA treated cells (Mann Whitney $p < 0.001$).

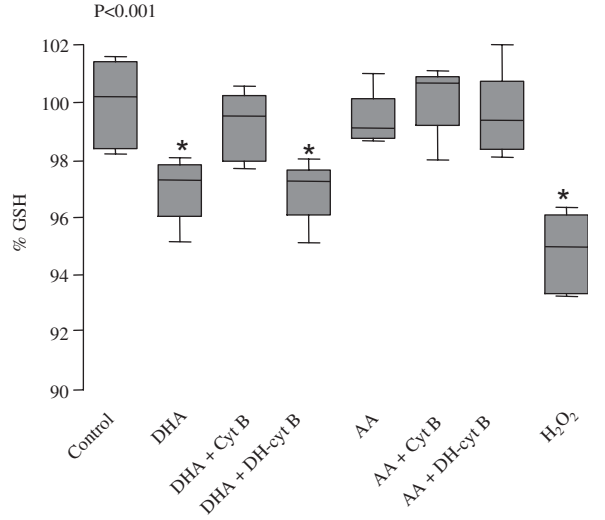

Figure 3. Effect of AA and DHA loading on GSH oxidation. Monocytes were pre-treated in media containing 0.4 mM deferroximine (DFO), 100 μM ascorbic acid (AA) or 100 μM dehydroascorbic acid (DHA) plus 10 μM cytocholasin B (cyt B) or 10 μM dihydrocytocholasin B (DH-cyt B) and hydrogen peroxide (H_2O_2) as positive control for 15 min prior to measuring GSH content. Results are shown as the percentage of GSH content of control cells. Box and whisker plots show median, interquartile and full range ($n = 10$). p-value shown is Kruskal Wallis. *Significantly lower than in control cells (Mann Whitney $p < 0.01$).

measuring intracellular GSH levels. Monocytes loaded with DHA showed a small but significant fall in intracellular GSH content when compared to untreated control cells. No reduction in GSH content was observed in cells incubated with cytocholasin B, but GSH oxidation was similar in monocytes incubated with dihydrocytocholasin B ($p < 0.001$, Figure 3).

Antioxidant enzyme protein expression

To ascertain the effect of AA and DHA on induction of cellular protection against oxidative stress, we measured the expression of MnSOD, catalase and GPx protein. MnSOD protein expression was higher in mitochondria from monocytes cultured for 24 h with DHA when compared to control cells, AA treated and control cells ($p < 0.01$, Figure 4). MnSOD protein expression was the same as control cells when DHA treated monocytes were pre-incubated with cytocholasin B, but expression was similar to DHA treated cells when DHA treated monocytes were pre-incubated with dihydrocytocholasin B ($p < 0.01$, Figure 4). MnSOD protein expression in cells pre-treated with either AA or DHA before LPS was similar to those without pre-treatment ($p < 0.01$, Figure 4).

Catalase expression was also higher in DHA treated cells compared to control, AA or control cells ($p < 0.05$, Figure 5) and the highest expression of catalase was seen following exposure to LPS (Figure 5). Cytocholasin B/DHA treatment inhibited the increase

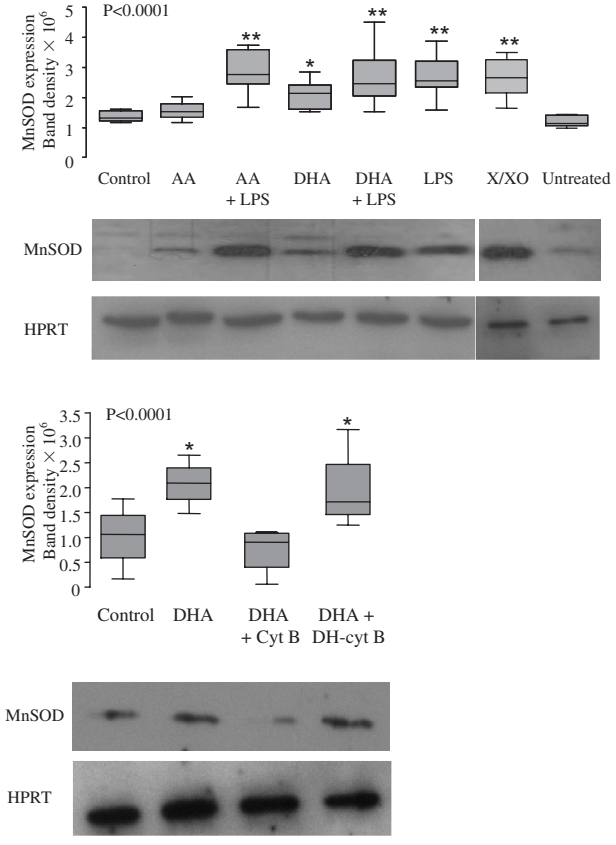

Figure 4. Densitometry analysis and representative western blot of protein expression of MnSOD (extracted from mitochondria). Protein was extracted from monocytes cultured in media containing 0.4 mM deferroximine (DFO) plus either phosphate buffered saline (control), 100 μM ascorbic acid (AA) or 100 μM dehydroascorbic acid (DHA) plus 10 μM cytocholasin B (cyt B) or 10 μM dihydrocytocholasin B (DH-cyt B) for 24 h. In some cases cells were subsequently exposed to 2 μg/ml LPS for 18 h. Untreated monocytes were cultured in media devoid of DFO. Xanthine, 0.2 mM and 1 unit/ml xanthine oxidase (X/XO) was used as a positive control. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a loading control. Box and whisker plots show median, interquartile and full range ($n = 10$). p-value shown is Kruskal Wallis. *Significantly higher than in control cells, AA treated cells or untreated cells (Mann Whitney $p < 0.05$). **Significantly higher than DHA treated cells (Mann Whitney $p < 0.05$).

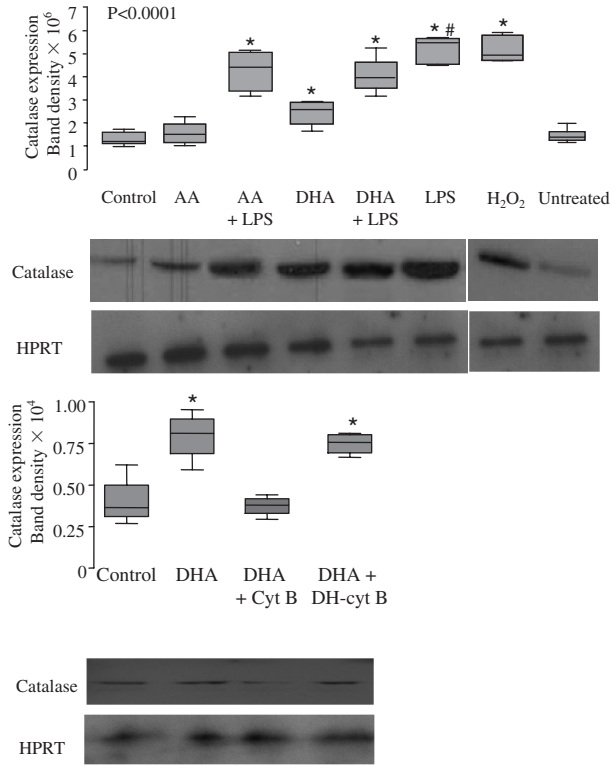

Figure 5. Densitometry analysis and representative western blot of protein expression of catalase (from cytosol). Protein was extracted from monocytes cultured in media containing 0.4 mM deferroximine (DFO) plus either phosphate buffered saline (control), 100 μM ascorbic acid (AA) or 100 μM dehydroascorbic acid (DHA) plus 10 μM cytocholasin B (cyt B) or 10 μM dihydrocytocholasin B (DH-cyt B) for 24 h. In some cases cells were subsequently exposed to LPS for 18 h. Untreated monocytes were cultured in media devoid of DFO. Hydrogen peroxide, 50 μM was used as a positive control. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a loading control. Box and whisker plots show median, interquartile and full range ($n = 10$). p-value shown is Kruskal Wallis. *Significantly higher than in control cells, AA treated cells or untreated cells (Mann Whitney $p < 0.05$); **significantly higher than DHA treated cells (Mann Whitney $p < 0.05$); #significantly higher than in cells pre-treated with AA/DHA before exposure to LPS (Mann Whitney $p < 0.05$).

expression; this was not seen with dihydrocytocholasin B/DHA treated cells ($p < 0.001$, Figure 5). When cells were pre-incubated with either AA or DHA and then stimulated with LPS, catalase expression was lower than that seen without pre-treatment ($p = 0.03$ and $p = 0.008$, respectively, Figure 5).

There was no difference in mitochondrial GPx protein expression between any test treatments apart from the positive control ($p = 0.574$, data not shown).

Monocyte cell survival

To determine if pre-treatment with AA or DHA could enhance monocyte survival to a subsequent more severe oxidative insult, monocytes pre-treated with AA or DHA with/without cytocholasin B/dihydrocytocholasin B were exposed to 400 μM hydrogen peroxide. Acid phosphatase activity in either hydrogen peroxide treated control cells or those pre-incubated with AA or DHA and cytocholasin B was significantly lower upon exposure to hydrogen peroxide, indicating a lower number of viable cells (all $p < 0.006$, Figure 6). However, acid phosphatase activity in monocytes which were pre-treated with DHA or DHA plus dihydrocytocholasin B before hydrogen peroxide exposure was higher than without pre-treatment, demonstrating a higher number of viable cells ($p < 0.001$, Figure 6).

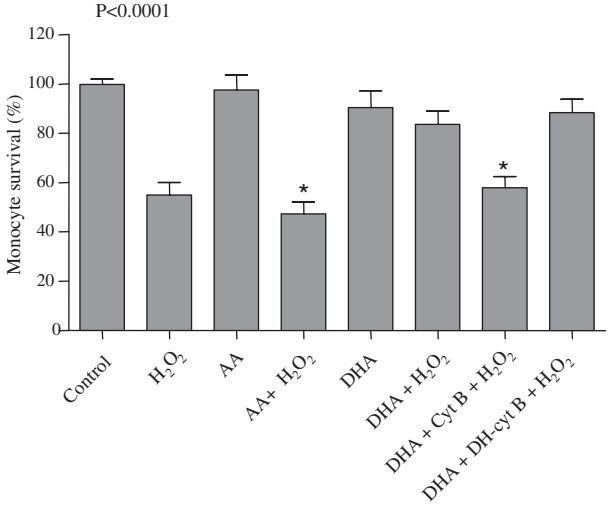

Figure 6. Monocyte survival measured by acid phosphatase assay. Monocytes were pre-treated in media containing 0.4 mM deferroximine (DFO) and either phosphate buffered saline (control), 100 μM ascorbic acid (AA) or 100 μM dehydroascorbic acid (DHA) plus 10 μM cytocholasin B (cyt B) or 10 μM dihydrocytocholasin B (DH-cyt B) for 24 h prior to exposure to 400 μM hydrogen peroxide for 18 h. Mean and SD ($n = 10$) is shown. *p*-value shown is ANOVA. *Significantly lower than without hydrogen peroxide (*T*-test, $p < 0.05$).

Mitochondrial membrane potential

As a measure of the effect of vitamin C on mitochondrial function, we assessed mitochondrial membrane potential as the ratio of red/green JC-1 fluorescence. Higher red/green fluorescence reflects enhanced mitochondrial membrane potential. Monocytes treated with AA, DHA or LPS alone had similar red/green ratios to control cells (Figure 7). However there was a significantly higher red/green fluorescence ratio from monocytes that were pre-treated with DHA or DHA plus dihydrochytocholasin B prior to LPS exposure compared to without pre-treatment ($p < 0.0001$, Figure 7). Pre-treatment with AA did not have this effect.

Cytochrome c release and mitochondrial Bcl-2 protein expression

We also examined the effects of AA and DHA on cytosolic cytochrome *c* concentration and mitochondrial Bcl-2 protein expression. Cytochrome *c* was similar in AA, DHA and control cells (Figure 5) but was higher in cells treated LPS alone ($p = 0.001$, Figure 8). However, pre-treatment with either AA or DHA before LPS exposure resulted in significantly lower cytochrome *c* in cytosol from monocytes, compared without pre-treatment ($p < 0.05$, Figure 8).

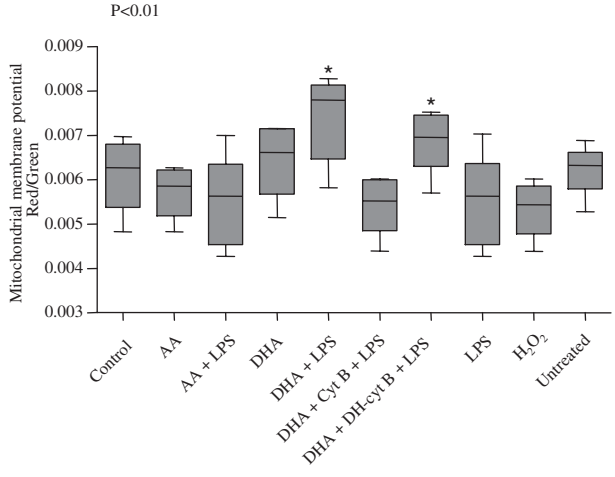

Figure 7. Mitochondrial membrane potential measured using red (mitochondrial)/green (cytosol) JC-1 fluorescence. Monocytes were cultured in media containing 0.4 mM desferrioxamine (DFO) and either phosphate buffered saline (control), 100 μM ascorbic acid (AA) or 100 μM dehydroascorbic acid (DHA) plus 10 μM cytocholasin B (cyt B) or 10 μM dihydrocytocholasin B (DH-cyt B) for 24 h. In some cases cells were subsequently exposed to lipopolysaccharide (LPS) for 18 h. Untreated monocytes were cultured in media devoid of DFO and hydrogen peroxide, 50 μM was used as a positive control. Box and whisker plots show median, interquartile and full range ($n = 10$). *p*-value shown is Kruskal Wallis. *Significantly higher than in LPS or DHA treated cells (Mann Whitney $p < 0.05$).

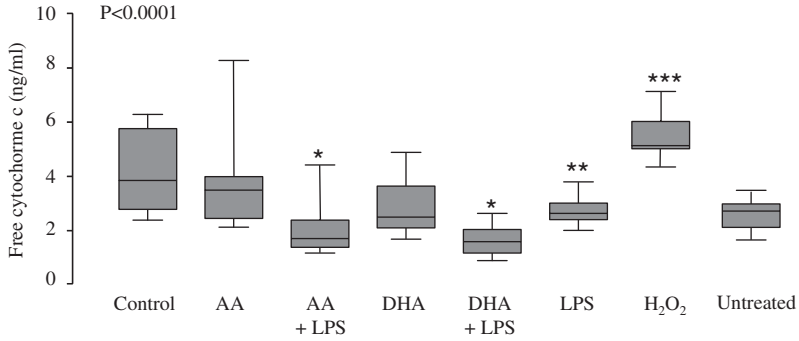

Figure 8. Cytochrome *c* in cytosol. Monocytes were cultured in media containing 0.4 mM desferrioxamine (DFO) plus either phosphate buffered saline (control), 100 μM ascorbic acid (AA) or 100 μM dehydroascorbic acid (DHA) for 24 h. In some cases cells were subsequently exposed to lipopolysaccharide (LPS) for 18 h. Untreated monocytes were cultured in media devoid of DFO. Cytosolic cytochrome *c* was determined by enzyme immunoassay in cell lysates devoid of mitochondria. Box and whisker plots show median, interquartile and full range ($n = 10$). *p*-value shown is Kruskall Wallis. *Significantly lower than in LPS or DHA alone treated cells (Mann Whitney $p < 0.05$); **significantly lower than in control (PBS) treated cells (Mann Whitney $p = 0.01$); ***significantly higher than in control (PBS) treated cells (Mann Whitney $p = 0.05$).

Bcl-2 protein expression in mitochondria was higher with all cell treatments compared to PBS treated control monocytes ($p = 0.029$, Figure 9).

Discussion

We have shown that DHA treatment of monocytes induced intracellular oxidative stress, up-regulated MnSOD, inhibited cytochrome *c* release and augmented mitochondrial membrane potential in cells subsequently exposed to LPS, in addition to protecting cells against subsequent exposure to cytotoxic concentrations of hydrogen peroxide. AA did not provide such protection, suggesting that protection was enhanced due to the pre-conditioning pro-oxidative effects of DHA uptake and subsequent reduction to AA, which was blocked by the GLUT (DHA uptake) inhibitor cytocholasin B. In contrast an analogue of the GLUT inhibitor dihydrochytocholasin B did not block the effects of DHA uptake.

Endotoxaemia, sepsis and septic shock are associated with oxidative stress, consumption of endogenous antioxidants and mitochondrial damage [3–12]. Therefore, various strategies need to be explored to prevent oxidative stress induced damage and to protect mitochondrial membrane integrity in critically ill patients.

Peripheral blood monocytes contain membrane transporters for uptake of both AA (SVCT2) and DHA (GLUT1 and GLUT3) [25,26] and accumulate intracellular AA to levels of up to 3 mM [27]. Human monocytes and U937 cells have been shown

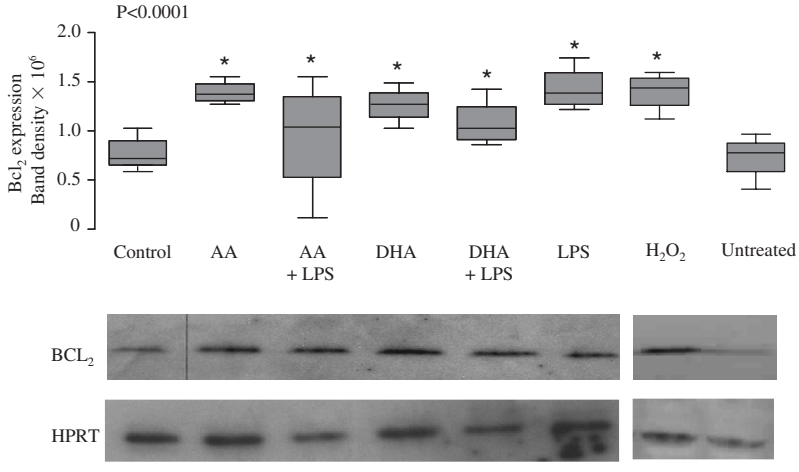

Figure 9. Densitometry analysis and representative western blot of protein expression of Bcl2. Mitochondrial protein was extracted from monocytes cultured in media containing 0.4 mM deferroximine (DFO) plus either phosphate buffered saline (control), 100 μM ascorbic acid (AA) or 100 μM dehydroascorbic acid (DHA) for 24 h. In some cases cells were subsequently exposed to LPS for 18 h. Untreated monocytes were cultured in media devoid of DFO and hydrogen peroxide, 50 μM was used as a positive control. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a loading control. Box and whisker plots show median, interquartile and full range ($n = 10$). *p*-value shown is Kruskal Wallis. *Significantly higher than in control cells (Mann Whitney $p < 0.001$).

to take up DHA rapidly and reach intracellular steady state levels of AA within 60 min, which is not affected by normal glucose concentrations. However, it has been shown that AA uptake by monocytes is approximately two orders of magnitude slower than DHA [28,29]. We also showed that DHA uptake and reduction to intracellular AA enhanced the ability of monocytes to reduce extracellular ferricyanide, which was blocked by cytocholasin B. Incubation of monocytes with AA resulted in lower ferricyanide reduction, indicating that AA uptake was much slower. To model the impact of intracellular oxidative stress from acute supplementation with AA or DHA in cell culture, we used the iron chelator DFO and bicarbonate free media, which slow AA and DHA degradation respectively, and inhibit the effect of Fenton chemistry under culture conditions for AA. DFO can chelate extracellular iron in a 1:1 ratio [30,31]. We found no detectable free iron in the culture media following DFO treatment. DFO has been suggested to be capable of inducing pre-conditioning [32] although we found no effect of DFO treatment alone on any of any parameters measured in this study. DHA is rapidly converted to 2,3-diketogulonate (2,3-DKG) in media. To determine if the conditioning effect could be due to DHA uptake and reduction to AA and not the effects of extracellular DHA degradation to 2,3-DKG, monocytes were incubated with cytocholasin B to block GLUT dependent DHA uptake. Diydrocytocholasin B does not inhibit DHA uptake and was used to control for the pleiotropic effects of cytochalasin B. We show that inhibiting DHA uptake and reduction to AA in monocytes abrogated the conditioning effect.

Oxidative stress was not increased in cells treated with AA. However, pre-incubation of monocytes with DHA resulted in oxidative stress which is in agreement with previous studies in oocytes and liver cells [17,33]. However, this was not seen in other cell types such as Jurkat and H9 human lymphocytes [18,34] and is probably due to the energy requirements of the particular cells used. The oxidative stress also resulted in a small reduction of intracellular GSH which was not shown in studies using U937 cells at the concentration of DHA used in the present study [35]. Oxidative stress caused by DHA uptake and intracellular reduction back to AA has been shown to induce apoptosis in PC12 cells [19]. Several groups have also shown that DHA increases glutathione levels in Jurkat cells [18] and primary astrocytes [36] by stimulating the pentose phosphate pathway [19] and can also induce cellular protection in experimental stroke models *in vitro* [37]. Nevertheless, intracellular oxidative stress is now emerging as a common mechanism of cytoprotection of organs to subsequent larger oxidative insults by hypoxia (ischaemia-reperfusion) [21] and chemicals such as ozone [38]. Sharma and Singh [39] demonstrated that pre-conditioning with oxidative stress provided cardioprotection similar to ischaemic pre-conditioning.

Increases in antioxidant enzyme expression are presumed to be a secondary event following oxidative stress, with the aim of re-establishing redox homeostasis. Mild oxidative stress is known to immediately activate *c-fos* and *c-jun* and to elevate a variety of transcription factors, resulting in the up-regulation of antioxidant protective enzymes and survival signals [40]. To ascertain the potential protective effects of treatment with AA and DHA we measured the protein expression of MnSOD, catalase and GPx. DHA treatment increased MnSOD and catalase protein expression. Intracellular oxidative stress due to DHA reduction is presumed to be the cause of the increased expression of these proteins as inhibition of DHA uptake by cytocholasin B prevented this up-regulation. However, neither AA nor DHA pre-treatment had any effect on MnSOD expression following subsequent LPS exposure, but DHA pre-treatment did reduce subsequent LPS-induced catalase expression. This may be due to a number of reasons; DHA pre-treatment may cause either a smaller increase in cellular hydrogen peroxide production upon subsequent LPS exposure or LPS exposure increased hydrogen peroxide production and its subsequent elimination by catalase, resulting in down-regulation of the enzyme. This effect has been shown in LPS and tumour necrosis factor α stimulated rat liver cells [41,42] and is thought to be due to either changes in gene expression or translation rates being decreased in DHA pre-exposed LPS stimulated cells. In addition, no effect of AA, DHA or LPS treatment on glutathione peroxidase protein expression was seen. Other studies have shown that induction of MnSOD following ischaemic, hyperthermic or chemical pre-conditioning and catalase induction with ceramide are important mechanisms in protection against subsequent reperfusion injury [43,44]. We also found that monocytes pre-incubated with DHA were protected against further oxidative stress caused by cytotoxic concentrations of hydrogen peroxide. Therefore, it may be concluded that a mild intracellular oxidative stress due to reduction of DHA to AA is capable of inducing antioxidant enzyme protection that ultimately can prime the cell to a subsequent larger oxidative stress. Likewise, pre-incubating glutathione deficient HL-60 cells with DHA protected them from subsequent oxidative stress [45] and in another study DHA protected HL-60 cells against hydrogen peroxide and x-ray induced toxicity [46].

Oxidative stress can modulate mitochondrial membrane potential and increase cytochrome *c* release from the inner mitochondrial membrane, leading ultimately to cell death [47,48]. However, we have shown that when monocytes were pre-exposed to DHA for 24 h prior to LPS exposure, they had a significantly higher mitochondrial membrane potential when compared to non-pre-treated LPS exposed cells or monocytes

pre-incubated with cytocholasin B and exposed to DHA and LPS. Endotoxin is known to induce an anti-apoptotic phenotype in monocytes, which may impact on mitochondrial membrane potential. However, intracellular vitamin C is important for the maintenance of mitochondrial membrane potential [49]. Li et al. [50] showed intracellular AA recycling (DHA back to AA) during LPS stimulation, which can lead to mitochondrial AA accumulation (mitochondria can only take up DHA). However, our data suggest that the mild intracellular oxidative stress as a result of DHA reduction to AA is also required to maintain mitochondrial membrane potential upon subsequent exposure to LPS, which may be due to effects of additional NADPH requirements of the cell. Loss of mitochondrial membrane potential is a key step in the apoptotic cascade, but does not necessarily parallel the release of cytochrome *c* into the cytoplasm [51]. None of the treatments in our study induced apoptosis within the time frame of the experiments as measured by caspase 3 activation (data not shown). Cytochrome *c* release into cytosol was significantly lower in cells treated with LPS alone (anti-apoptotic effect), but was even lower in cells pre-treated with AA and DHA prior to LPS exposure. It is interesting that release of cytochrome *c* in cells pre-treated with AA before LPS exposure was also lower than in LPS treated cells alone. This may be because acute AA supplementation has effects on global gene expression by modulating the nuclear binding of redox sensitive transcription factors such as nuclear factor kappa B and activator protein-1. Furthermore, all treatments resulted in similar increases in expression of Bcl-2 protein, which is known to protect mitochondrial membrane integrity, inhibit cytochrome *c* release, modulate antioxidant protection pathways and protect cells from apoptosis [52].

Many studies have also shown that increases in myocardial mitochondrial protection resulting from ischaemic or chemical pre-conditioning involve various protein kinase pathways [53] and have indicated a role of the inner membrane mitochondrial K_{ATP} channel and mitochondrial ROS production [53,54]. However, Liu et al. [55] showed that oxidative stress signalling upstream of kinase activation can protect mitochondria in a pre-conditioning model, although much work is still needed to determine the exact signalling mechanisms involved in mitochondrial protection by oxidative stress.

In summary, we have shown that DHA is able to protect against oxidative damage *in vitro*. Although mitochondria are a source of oxidative stress during sepsis there are other potential sources. However, our study has shown that the use of DHA to increase antioxidant defences and enhance mitochondrial membrane potential may be protective. DHA has been shown to provide antioxidant protection against cerebral stroke *in vivo* in animals, but it is not known if DHA could be beneficial in critically ill patients with sepsis.

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