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DHA Protects Against Zinc Mediated Alterations in Neuronal Cellular Bioenergetics

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Key Words

Zinc • Docosahexaenoic acid • DHA • Human neuronal cells • Mitochondrial function • Bioenergetics

Abstract

Zinc accumulation may impair cellular bioenergetics, which is associated with neuronal apoptosis. We simultaneously assessed anaerobic and aerobic metabolism in live cells to characterise this effect and hypothesised that the omega 3 fatty acid docosahexaenoic acid (DHA) would protect against any zinc mediated alterations in bioenergetics. In this study we observed a decrease in cellular oxygen consumption, but not glycolytic rate, following chronic zinc exposure, which was specific for neuronal cells. This was due to impaired ATP turnover, without any other effects on mitochondrial function, and was restored by DHA. DHA had no further effects on bioenergetics. These data suggest that zinc disrupts bioenergetics at a point distal to the respiratory chain, which is restored by DHA.

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Introduction

Zinc is an important metal cofactor for normal neuronal cell function [1]. However, impaired zinc homeostasis has a profound effect on neuronal cell viability, with zinc accumulation being a potent mediator of neuronal cell injury that is implicated in neurodegenerative diseases such as Alzheimer's disease (AD; [2]). The mechanisms by which zinc impairs neuronal viability are thought to include impaired bioenergetics [3]. Indeed, zinc is thought to inhibit a number of metabolic pathways, including glycolysis [4], the tricarboxylic (TCA) cycle [5] and the mitochondrial respiratory chain [6]. However, much of this data has been gathered from *in vitro* reactions with purified metabolic enzymes and studies of isolated mitochondria. Therefore, the role of zinc on metabolism in intact human neuronal cell lines is not entirely clear. Furthermore, many of these studies have used acute supraphysiological levels of zinc to induce bioenergetic dysfunction, such that the role of chronic zinc exposure at moderate pathophysiological levels on bioenergetics is also unclear.

We have recently found that the omega 3 fatty acid docosahexaenoic acid (DHA) protects against neuronal

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cell apoptosis associated with altered zinc homeostasis [7, 8]. DHA represents a large portion of the poly unsaturated fatty acids found within the brain [9] and functions by influencing membrane fluidity, ion channels and signal transduction pathways [10]. Increased DHA intake and incorporation into the brain are associated with protection from neurodegenerative diseases like AD [11, 12], highlighting the importance of this omega 3 fatty acid on normal neuronal function. However, the effect of DHA on zinc mediated alterations in cellular bioenergetics and mitochondrial function have not been examined. Therefore, the aims of the present study were to; 1. determine whether zinc impairs bioenergetics in live M17 neuroblastoma cells; 2. establish whether DHA can protect against any alterations in cellular bioenergetics; and 3. determine whether the effects of zinc and DHA on bioenergetics are specific to neuronal cell lines.

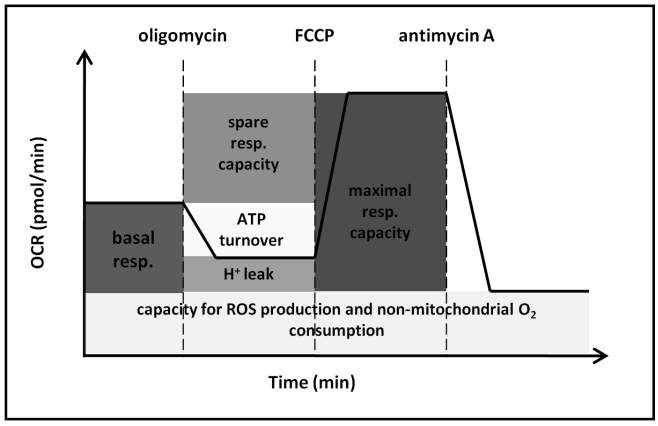

Fig. 1. Schematic of the mitochondrial function test. Multiple oxygen consumption measurements are made basally and after the injection of the ATP synthase inhibitor oligomycin, the proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) and the complex III inhibitor antimycin A. From these analyses, basal respiration, proton leak, ATP turnover, spare respiratory capacity and maximal respiratory capacity can be calculated.

Materials and Methods

Cell culture

Human neuroblastoma M17 cells were maintained in Opti-MEM media supplemented with 2.5% heat inactivated fetal bovine serum (FBS). Human HaCaT keratinocytes were maintained in DMEM media supplemented with 10% FBS. All cultures were growth at 37°C in humidified air containing 5% CO_2.

Bioenergetics analysis and mitochondrial function tests

All bioenergetic and mitochondrial function analyses were performed using the Seahorse XF24 Extracellular Flux Analyser (Seahorse Bioscience, Billerica, USA). M17 neuroblastoma and HaCaT cells were seeded into 24 well Seahorse V7 plates at $2.5x10^4$ cells/well. The following day, cells were treated with either vehicle, zinc (5 µM), DHA (10 µg/mL) or zinc and DHA in combination for 48 hours. Prior to assay, cells were washed twice with assay running media (unbuffered DMEM, 25mM glucose, 1mM glutamine, 1mM sodium pyruvate), before being resuspended in 675µl of running media. Cells were equilibrated in a non-CO_2 incubator for 60 min prior to assay. The assay protocol consisted of repeated cycles of 2 min mix, 2 min wait and 4 min measurement periods, with oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured simultaneously through each measurement period by excitation of fluorophores for O_2 and H^+. This gives measurement of oxidative and non-oxidative metabolism, respectively. Basal energetics were established after three of these cycles, followed by sequential exposure of the ATP synthase inhibitor oligomycin, the proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) and the complex III inhibitor antimycin A, all to a final concentration of 1µM. These compounds were introduced to the cell media by the Seahorse injection system. Three mix, wait and measurement cycles separated each compound injection. Using these compounds as modulators of mitochondrial function, it is possible to determine a number of bioenergetic and mitochondrial parameters, including basal respiration, ATP turnover rate, proton leak, maximal and spare respiratory capacity (Fig. 1). All treatment conditions were analysed as ten replicates, over at least two independent experiments and data was pooled to give average values for each treatment. At the completion of each assay, the assay plate was frozen at -80°C, prior to determination of cell number in each well using the CyQuant kit (Invitrogen, Carlsbad, USA) according to manufacturer's instructions.

Statistics

All values are reported as means ± standard error of the mean (SEM) and were evaluated for statistically significant differences using analysis of variance (ANOVA) and Tukey *post-hoc* testing where appropriate. Differences between groups were considered statistically significant where $p<0.05$.

Results

Zinc impairs basal cellular bioenergetics in M17 neuroblastoma, which is restored by DHA

OCR for M17 neuroblastoma throughout the bioenergetics analysis are shown in Fig. 2A. Basal OCR and ECAR are shown in Fig. 2B. Zinc significantly reduced basal OCR when compared with control cells (Fig. 2A and B), without altering basal ECAR (Fig. 2B), which is indicative of mitochondrial dysfunction without non-oxidative compensation. Basal OCR was not different

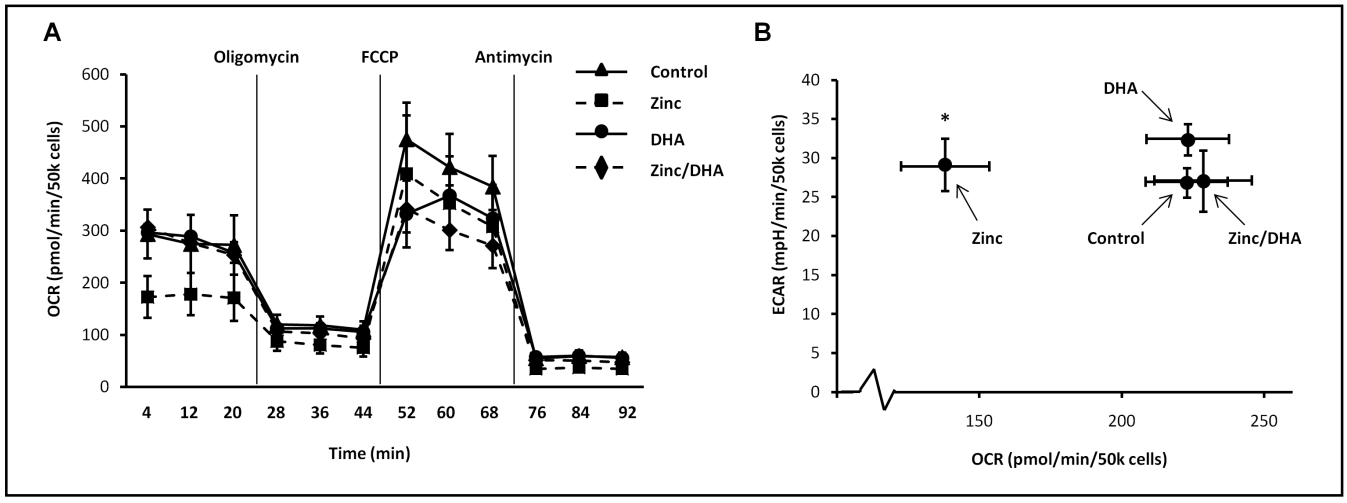

Fig. 2. Mitochondrial function and basal bioenergetics in M17 neuroblastoma. (A) Oxygen consumption rate (OCR) throughout mitochondrial function testing, and (B) basal OCR and extracellular acidification rate (ECAR) of M17 neuroblastoma exposed for 48 hr to either vehicle (Control), zinc, docosahexaenoic acid (DHA) and zinc and DHA together (zinc/DHA). All values are reported as means ± SEM (n=12-15 per group). *denotes significantly different OCR from all other groups ($p<0.05$).

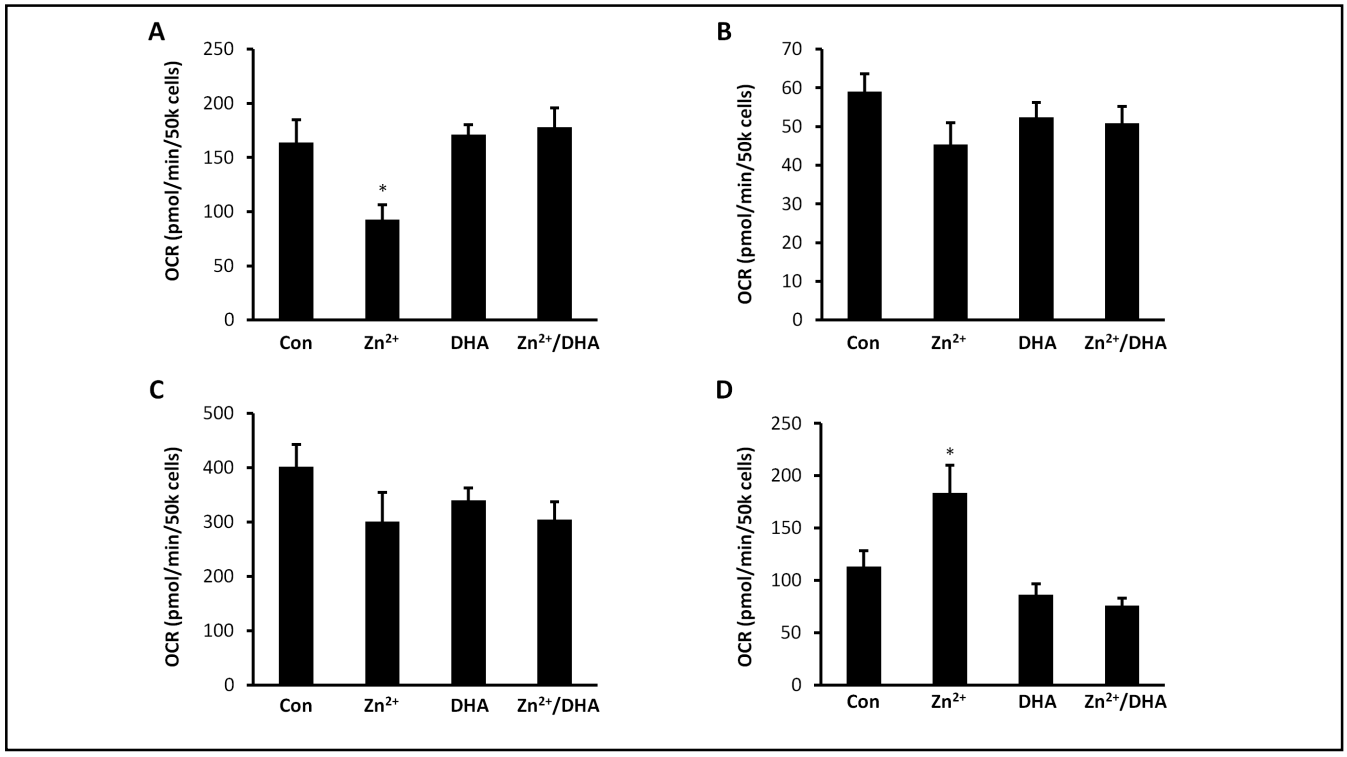

Fig. 3. Mitochondrial function parameters in M17 neuroblastoma. (A) ATP turnover rate, (B) proton leak, (C) maximal respiratory capacity and (D) spare respiratory capacity were calculated from oxygen consumption rates of M17 neuroblastomas exposed for 48 hr to either vehicle (Con), zinc (Zn^{2+}), docosahexaenoic acid (DHA) and zinc and DHA together (Zn^{2+}/DHA). All values are reported as means ± SEM (n=10 per group). *denotes significantly different from all other groups ($p<0.05$).

in cells exposed to both zinc and DHA, suggesting that DHA can protect against zinc mediated alterations in basal OCR (Fig. 2B). There was no effect of DHA exposure alone on cellular bioenergetics (Fig. 2B).

Zinc reduces oxidative ATP turnover, which is restored by DHA

To further examine why basal oxidative respiration was impaired following zinc exposure in M17

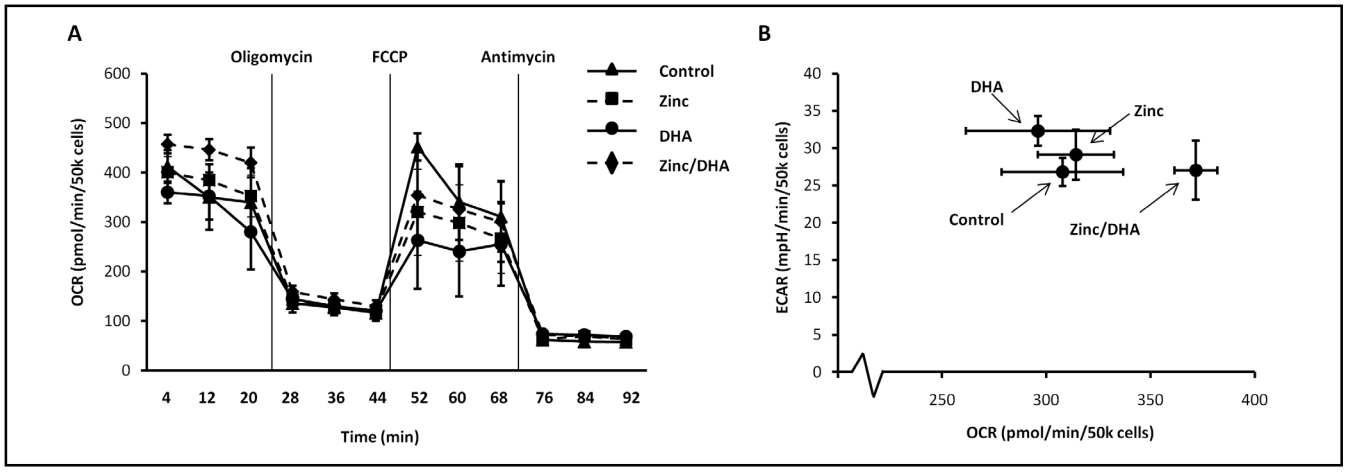

Fig. 4. Mitochondrial function and basal bioenergetics in HaCaT keratinocytes. (A) Oxygen consumption rate (OCR) throughout mitochondrial function testing, and (B) basal OCR and extracellular acidification rate (ECAR) of HaCaT keratinocytes exposed for 48 hr to either vehicle (Control), zinc, docosahexaenoic acid (DHA) and zinc and DHA together (zinc/DHA). All values are reported as means ± SEM (n=12-15 per group).

neuroblastomas, we assessed mitochondrial function in these cells. Zinc decreased oxidative ATP turnover, which was restored with co-exposure of DHA (Fig. 3A). Both zinc and DHA, either alone or in combination, did not significantly affect proton leak (Fig. 3B) and maximal respiratory capacity (Fig. 3C). However, as zinc decreased basal respiration without any significant effect on maximal respiratory capacity, zinc increased spare respiratory capacity when compared with all other treatments (Fig. 3D).

Zinc and DHA have no effect on cellular bioenergetics in HaCaT keratinocytes

It has been proposed that due to their relatively high oxidative ATP demand and low spare respiratory capacity, neuronal cell bioenergetics, and in turn viability, are particularly sensitive to factors that induce mitochondrial dysfunction [13]. To determine whether the effects of zinc and DHA on bioenergetics are unique to neuronal cell lines, we examined HaCaT keratinocyte bioenergetics following chronic zinc exposure. OCR for HaCaT keratinocytes throughout the bioenergetics analysis are shown in Fig. 4A and basal OCR and ECAR are shown in Fig. 4B. This cell line was used as they too have a relatively high oxidative ATP demand and low spare respiratory capacity, which was confirmed in our analyses (Fig. 4A). Zinc and DHA had no effect on basal keratinocyte bioenergetics (Fig. 4B), suggesting that neuronal cell lines are more sensitive to the effects of zinc and DHA. No differences in other mitochondrial function parameters were observed (data not shown).

Discussion

Here we show that chronic exposure of M17 neuroblastomas to moderate pathophysiological levels of zinc impairs cellular bioenergetics, through inhibition of basal respiration. Our data showed that ATP turnover was the primary parameter of mitochondrial function that was impaired and that there was no compensatory response through anaerobic energy sources. This fits with previously published data showing that chronic zinc exposure can induce neuronal apoptosis through a decline in cellular ATP stores [14], albeit at higher zinc concentrations. However, using indirect measures of metabolism, this study found that the primary defect in bioenergetics was glycolysis [14]. We saw no alterations in glycolytic rate, as measured by cellular proton production, using a bioanalyser that simultaneously measures both anaerobic and aerobic flux. While these differences could be due to the different analytical methods employed, the concentrations of zinc were also different. The present study used moderate pathophysiological zinc concentrations over a 48 hr period. Importantly, it should be noted that cells were not exposed to zinc in the 60 min prior to, and throughout the assays. This suggests that chronic exposure to moderate zinc concentrations induces alterations in oxidative metabolism that are not readily reversible following withdrawal of zinc.

Our analysis of mitochondrial function localised the defect in basal respiration to a reduction in mitochondrial ATP turnover. This means that chronic exposure to moderate pathophysiological zinc concentrations induces

a defect in mitochondrial metabolism that is distal to the respiratory chain. We are unaware of any studies showing that zinc inhibits ATP synthase directly, or the availability of ADP for conversion to ATP by ATP synthase. Indeed, studies using isolated mitochondria with a wide range of zinc concentrations and various substrates for metabolism have found inhibition of the respiratory chain, most likely at the transfer of electrons between complexes II and III [15, 16]. However, zinc has also been found to dissipate the mitochondrial membrane potential through regulation of the mitochondrial transition pore [17]. As ATP synthase requires the proton gradient of the mitochondrial membrane potential to generate ATP [18], this mechanism could account for the impairment of ATP turnover induced by zinc. This mechanism has also been associated with increased production of mitochondrial reactive oxygen species, release of cytochrome c and initiation of apoptosis [3]. These data suggest that inhibition of ATP turnover is the primary bioenergetic parameter modulated by chronic exposure to moderate pathophysiological concentrations of zinc and highlight the complex role that this transition metal has on both normal cell function, such as synaptic transmission, and pathological processes, such as initiation of apoptosis.

Data from the present study also shows that DHA is able to protect against reductions in oxidative ATP turnover. As dissipation of the mitochondrial membrane potential appears to be the mechanism by which zinc impairs ATP turnover, we can speculate on how DHA might protect mitochondrial metabolism. One of the major functions of DHA is its incorporation into membranes [10], where they can assist with membrane fluidity and function. It is possible that DHA might protect the transition pore in response to zinc through its role in membranes. Indeed, it has recently been shown that DHA can prevent mitochondrial permeability transition in the heart [19, 20]. However, our previous work has showed that DHA may impair cellular zinc uptake [7], which could contribute to its protective effects. Nonetheless, these data from the present study are the first to show that DHA normalises cellular bioenergetics in response to chronic pathophysiological zinc exposure. As we have showed that DHA can protect against neuronal apoptosis, part of this protective effect could be ascribed to protection against alterations in bioenergetics secondary to altered zinc transport.

A final aim of the present study was to examine whether the effects of zinc and DHA on cellular bioenergetics were specific to neuronal cells. To address this aim, we examined anaerobic and aerobic metabolism in HaCaT keratinocytes. Indeed, it has been proposed that neurons are particularly susceptible to apoptosis by insults that perturb bioenergetics due to their high ATP demand and relative low spare respiratory capacity [13]. HaCaT keratinocytes were used in the present study because of their similar properties (Fig. 4A). However, we found that these cells were not sensitive to zinc mediated alterations in metabolism. A potential explanation for this finding could be altered zinc uptake in these cells when compared with neuronal cells. However, isolated liver mitochondria are also insensitive to the effects of zinc on the transition pore [21], which could suggest that intrinsic differences in neuronal mitochondria make them more sensitive to this effect of zinc. As there was no alteration in metabolism following chronic zinc exposure in these cells, DHA had no effect on bioenergetics. These data are consistent with the fact that DHA had no effect on metabolism independent of zinc in M17 neuroblastoma. Furthermore, these data could highlight that neuronal reliance on zinc for functions such as synaptic transmission could in turn make this cell type particularly susceptible to dysregulation of zinc homeostasis.

In conclusion, this study shows that chronic exposure of M17 neuroblastoma to moderate pathological levels of zinc impairs bioenergetics by inhibiting mitochondrial ATP turnover. We also showed that co-exposure of zinc with DHA protected against this effect and that this was specific for neuronal cell lines. These data show that the protective effect of DHA on neurons in response to pathological stimuli also includes modulation of metabolic processes.

Abbreviations

AD (Alzheimer's disease); ATP (Adenosine triphosphate); DHA (Docosahexaenoic acid); ECAR (Extracellular acidification rate); FCCP (Carbonylcyanide p-trifluoromethoxyphenylhydrazone); OCR (Oxygen consumption rate); TCA (Tricarboxylic acid).

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