Saturated very long chain fatty acid-induced neuronal dysfunctions playing essential roles in Alzheimer's disease: impairment of mitochondrial activity, induction of oxidative stress and cytoskeleton disorganization

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Introduction: Inherited peroxisomal disorders mostly develop a neuronal phenotype, suggesting important functions of this organelle in nervous tissue. In Alzheimer's disease (AD), lipid alterations are present early during disease progression. Some of these alterations point towards peroxisomal dysfunctions. Indeed, lipid analyses of cortical regions of patients with AD revealed accumulation of very long chain fatty acids (VLCFA: C22:0, C24:0 and C26:0), all substrates for peroxisomal β-oxidation, in patients with stages V-VI pathology (Kou J et al., Acta Neuropathol 2011; 122(3): 271-283; Lizard G et al., J Alzheimers Dis, 2012; 29(2): 241-254). On human neuronal cells, which are strongly implicated in AD, it was therefore of interest to determine the effects of these VLCFA on mitochondrial activity and oxidative stress playing crucial roles in AD (Verri M et al., Int J Immunopathol Pharmacol 2012; 25(2): 345-353), as well as on cytoskeleton organization, which could influence peroxisomal metabolism (Schrader M et al., Microsc Res Tech 2003; 61: 171–178; Nguyen T et al., J Cell Science, 2006; 119: 636-645).

Methods: Human neuronal cells (SK-N-BE) were treated with VLCFAs (C22:0, C24:0 or C26:0 at 0.1; 1; 5; 10 and/or 20 µM; 48 h). Cells were seeded at 200,000 – 400,000 cells/mL (per well of 12 well plates). The effects of VLCFAs on mitochondria were evaluated by various methods: MTT test; flow cytometric analyses with $DiOC_6(3)$ and MitoSOX to measure the transmembrane mitochondrial potential (Δψm) and the mitochondrial production of superoxide anion ($O_2^{\cdot -}$), respectively; fluorescence microscopy (mitochondrial staining with MitoTracker Red) associated with flow cytometry and transmission electron microscopy to evaluate mitochondrial morphology, topography and density. Oxidative stress was estimated by flow cytometric analyses with DHE, DHR123, and DAF to quantify $O_2^{\cdot -}$, hydrogen peroxide (H_2O_2), and nitric oxide (NO), respectively; fluorescence microscopy after staining with monochlorobimane to identify the cellular level of reduced glutathione (GSH)), and gas chromatography coupled with mass spectrometry to measure cholesterol autoxidation products (cholesterol oxide derivatives (oxysterols) oxidized at C7). Cytoskeleton organization (actin, tubulin and neurofilament network) was evaluated by fluorescence microscopy with Rhodamine-phalloidin (reacting with filamentous actin (actin-F)) and specific antibodies (mouse monoclonal anti-160 kDa neurofilament (Abcam, ab7794); rabbit polyclonal anti-tubulin antibody recognizing α/β tubulin subunits (Abcam, ab59680).

Results: On human neuronal cells SK-N-BE, under treatment with VLCFAs (C22:0, C24:0 or C26:0), some mitochondrial changes were observed: reduced succinate dehydrogenase activity, and loss of Δψm (**Figure 1**); increased of the mitochondrial mass (**Figure 2**), identification of elongated mitochondria (**Figure 3**), and overproduction of mitochondrial superoxide anions (**Figure 4**). An overproduction of superoxide anions was also revealed on whole cells (Figure 4), no or slight overproduction of H_2O_2 and NO was found, and a decrease of GSH associated with an increased lipid peroxidation (increased level of cholesterol oxide products oxidized at C7) was detected (**Table 1**). A more or less pronounced disorganization of the cytosqueleton was observed with VLCFAs at 5 and 10 µM (**Figure 5**). **(This work is in part published: Zarrouk A *et al.*, Oxidative Medicine and Cellular Longevity, 2012; Vol 2012, ID 623257).**

Effects of VLCFAs on mitochondrial activity

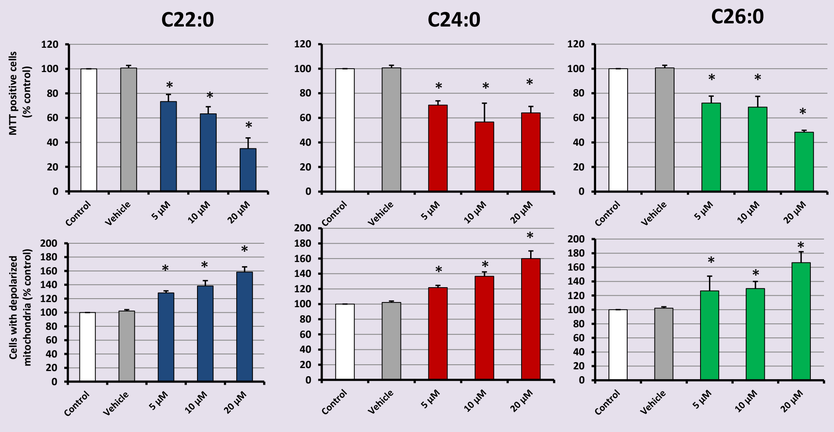
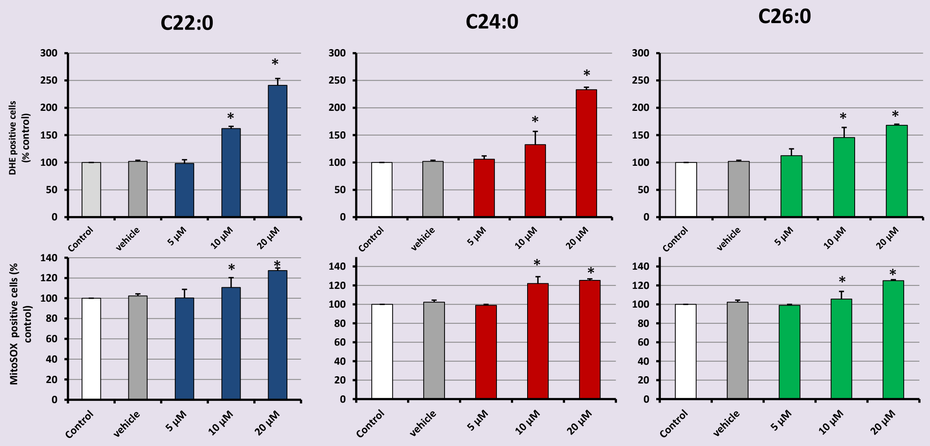


Figure 1: Evaluation of the effects of VLCFAs on mitochondrial activity with MTT and $DiOC_6(3)$. Data are mean ± SD. Significance of the difference between vehicle (α-cyclodextrin)- and fatty acids-treated cells (Mann Whitney test; * $P < 0.05$ or less). No significant difference was observed between control and vehicle-treated cells.

Effects of VLCFAs on oxidative stress



Effects of VLCFAs on mitochondrial topography and mass

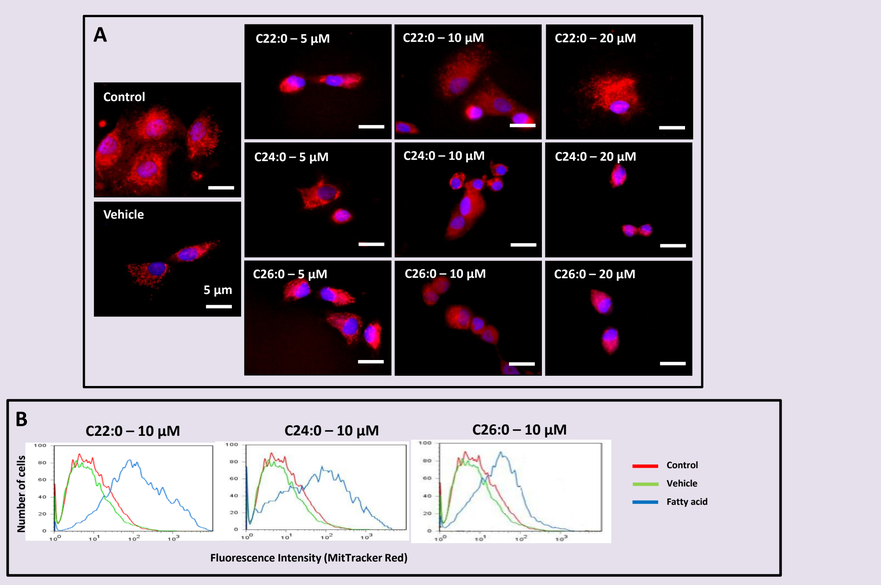


Figure 2: Analysis by fluorescence microscopy and flow cytometry of the effects of VLCFAs on mitochondrial topography and mitochondrial mass with MitoTracker Red. **A**: evaluation of mitochondrial topography by fluorescence microscopy; the punctuated aspect of fluorescence observed in untreated (control) and α-cyclodextrin (vehicle)-treated cells is not observed in the presence of fatty acids whereas a more diffuse and irregular staining pattern is detected. **B**: quantification of mitochondrial mass by flow cytometry.

Figure 4: Flow cytometric evaluation of VLCFAs on superoxide anion production on whole cells and at the mitochondrial level. The production of superoxide anions on whole cells (HE positive cells) (**A**) and at the mitochondrial level (MitoSOX positive cells) (**B**) was determined after staining with DHE and MitoSOX, respectively. Significance of the difference between vehicle (α-cyclodextrin) - and fatty acids-treated cells (Mann Whitney test; * $P < 0.05$ or less). No significant difference was observed between control and vehicle-treated cells.

- With DHR123 allowing H_2O_2 quantification on whole cells, an overproduction of H_2O_2 was mainly observed under treatment with C24:0 and C26:0, especially at 10 and 20 µM.
- With DAF, no or slight overproduction of NO was observed in the presence of VLCFAs.
- The overproduction of $O_2^{\cdot -}$ detected on whole cells with DHE and at the mitochondrial level with MitoSOX, as well as the overproduction of H_2O_2 , were associated with a decrease of the intracellular level of reduced glutathione (GSH) and with lipid peroxidation revealed by higher levels of the ratio [(total cholesterol oxidized at C7 in VLCFAs-treated cells)/(total cholesterol oxidized at C7 in untreated cells)] (**Table 1**).

	SK-NB-E cells - Treatments (48 h)										
Parameters measured			C22:0			C24:0			C26:0		
	Control	Vehicle	5 µM	10 µM	20 µM	5 µM	10 µM	20µM	5 µM	10 µM	20 µM
% GSH positive cells	88 ± 5	90 ± 10	82 ± 10	62 ± 8 *	40 ± 10 *	55 ± 9 *	50 ± 7 *	30 ± 10 *	65 ± 10 *	70 ± 5 *	45 ± 8 *
Lipid peroxidation (x 1000)	3.34 ± 0.32	3.26 ± 0.14	ND	3.81 ± 0.04 *	ND	ND	4.05 ± 0.15 *	ND	ND	4.52 ± 0.85 *	ND

Table 1: VLCFAs-induced disruption of the RedOx equilibrium. GSH was detected on whole cells by flow cytometry after staining with monochlorobimane. Lipid peroxidation was evaluated on whole cellular lipid extract by GC/MS by the ratio [(total cholesterol oxidized at C7 in VLCFAs-treated cells)/(total cholesterol oxidized at C7 in untreated cells)]. ND: not determined. Significance of the difference between vehicle (α-cyclodextrin) - and fatty acids-treated cells (Mann Whitney test; * $P < 0.05$ or less). No significant difference was observed between control and vehicle-treated cells.

Effects of VLCFAs on ultrastructural features of mitochondria

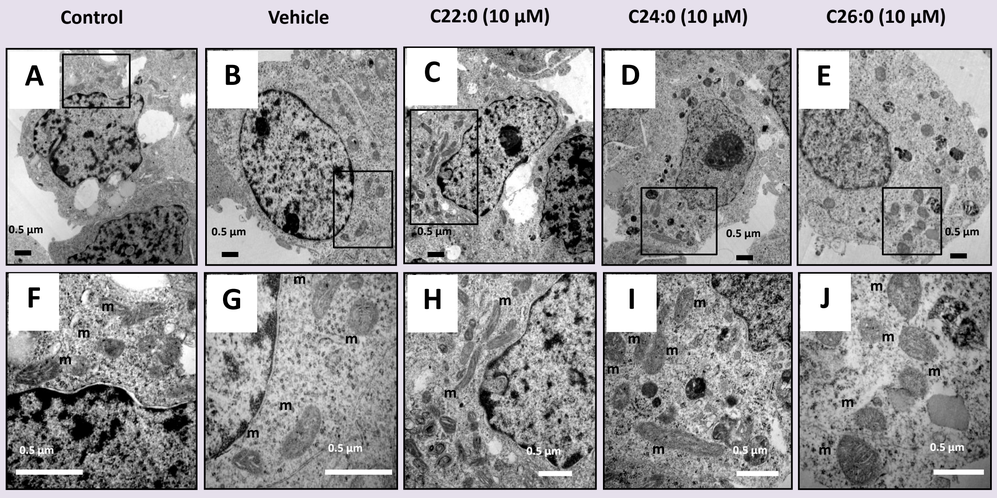


Figure 3: Evaluation by transmission electron microscopy of the ultrastructural characteristics of mitochondria in Human Neuronal Cells (SK-N-BE) treated by C22:0, C24:0 and C26:0. Transmission electron microscopy of SK-N-BE cells cultured for 48 h in the absence (control cells) (**A, F)**, or presence of α-cyclodextrin (1 mg/mL) (vehicle) (**B, G**), C22:0 (10 µM) (**C, H**), C24:0 (10 µM) (**D, I**), or C26:0 (20 µM) (**E, J**). The insets in Figures A, B, C, D and E, correspond to Figures F, G, H, I, and J, respectively.

Effects of VLCFAs on cytoskeleton organization

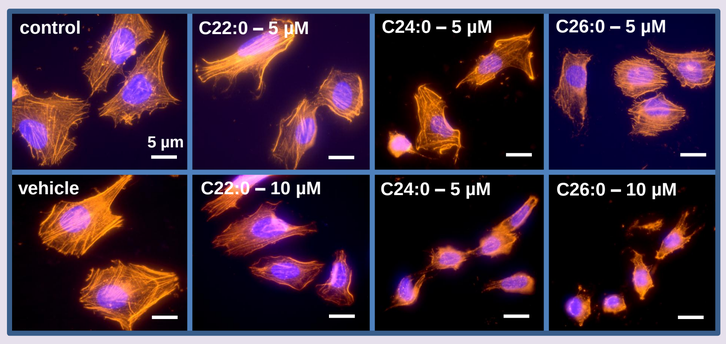


Figure 4: Microscopical evaluation of VLCFAs effects on cytoskeleton organization. In order to evaluate the effects of VLCFAs (48 h of treatment) on the cytoskeleton organization, actin-F was stained with Rhodamin-Phalloidin and the cells were observed by fluorescence microscopy. In VLCFAs-treated cells, comparatively to untreated cells and vehicle, the area of the cytoplasm was decreased, and the actin-F filaments were more compact. Similar data were obtained with antibodies raised against 160 kDa neurofilament and α/β tubulin subunits.

Conclusion

✓ **VLCFA (C22:0 ; C24:0 ; C26:0) are able to induce neuronal damages: mitochondrial dysfunctions, induction of oxidative stress and disorganization of the cytoskeleton.**

✓ **Thus, increased levels of VLCFA (C22:0; C24:0 ; C26:0) in cortical lesions of patients with Alzheimer's disease might contribute to the development of this disease.**