Article

Neuronal metabolism and neuroprotection. Neuroprotective effect of fingolimod on menadione induced mitochondrial damage.

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Abstract:

Imbalance in the oxidative status in neurons, along with mitochondrial damage, are common characteristics in some neurodegenerative diseases. The maintenance in energy production is crucial to face and recover from the oxidative damage and the coexistence of different sources of energy production, such as mitochondrial and glycolytic ATP, allows faster adaptative mechanisms to situations of high energy demand and may help in the maintenance of neuronal function in stress situations. Fingolimod phosphate is a drug with neuroprotective and antioxidant actions, used in the treatment of Multiple Sclerosis. This work has been performed in a model of oxidative damage on neuronal cell cultures exposed to menadione, in presence or absence of fingolimod phosphate. We have studied the mitochondrial function and several pathways related with glucose metabolism, including oxidative, glycolytic and pentose phosphate in neuronal cells cultures. Our results showed a beneficial effect on neuronal survival probably based in the recovery of all, oxidative balance, glycolysis and pentose phosphate, promoted by fingolimod phosphate. These effects are mediated, at least in part by the interaction with its specific receptor. These actions would make this drug a potential tool to the treatment of neurodegenerative processes, either to slow progression or alleviate symptoms.

Keywords: Sphingosine-1-phosphate receptor analogue; fingolimod phosphate; neuroprotection; mitochondrial damage; glycolytic pathway; pentose phosphate pathway; REDOX homeostasis.

1. Introduction

Energy is a key factor in maintaining brain function, especially for the generation of action potentials, axonal transport, synthesis and release of neurotransmitters and synaptic function. In brain, the main sources of energy are based in the uptake and metabolism of glucose and oxygen [1]; and the choice between one of those may have important consequences for brain function in both health and disease [2]. Several studies have demonstrated that many neurodegenerative diseases are triggered or maintained by metabolic alterations [3,4]. During aging, a certain decrease in glucose and oxygen metabolism can be seen [3], but a more dramatical decrease is found in disorders such as Alzheimer's (AD), amyotrophic lateral sclerosis (ALS), Parkinson's (PD), and Huntington's (HD)

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diseases [5]. The need of energy to maintain its function and integrity is much more manifest in neurons susceptible to degeneration, therefore the failure in energy metabolism, along with the enormous energy demand can eventually result in higher cellular stress in these neurons [5].

Some neurodegenerative diseases, such as PD, Multiple Sclerosis (MS), ALS or AD, are characterized by an imbalance in the oxidative status of the cells, leading to neuronal damage and death, contributing to the disease pathogenesis [6]. Several external and/or internal noxious stimuli are able to produce neuronal damage that in most occasions trigger an imbalance in the neuronal oxidative homeostasis, resulting in most cases in accumulation of oxygen free radicals damaging proteins, nucleic acids and lipid membranes, leading to neuronal death [7]. These pathologies have in common a mitochondrial damage that in turn increase, even more, the oxidative damage on the neurons, in such way that they could be grouped as mitochondriopathies [8]. In these, and probably in most neurodegenerative diseases, the importance of glycolysis on synapse, axonal conduction and plasticity among other neuronal functions has been previously stablished [9]. The coexistence of different sources of ATP production, such as mitochondrial and glycolytic ATP, allows faster adaptative mechanisms to situations of high energy demand and may help in the maintenance of neuronal function in stress situations [10]. A clearer knowledge of the influence of the oxidative damage on these pathways could be crucial in the development of potential therapeutic strategies targeting these diseases [7].

Several natural and endogenous products, such as polyphenols, N-acetylcysteine (NAC) and IGF-I/IGF-II have shown antioxidant properties in different oxidative damage models. Polyphenols and NAC can promote suppression of reactive oxygen species (ROS) formation by either, inhibition of enzymes involved in their production, scavenging of ROS, or upregulation or protection of antioxidant defences [11,12], whereas hormones such as IGF-I/IGF-II mediate its antioxidant properties through specific receptors, modulating the expression and/or activity of antioxidant molecules and preventing mitochondria to become a new source of oxidative radicals [13,14]. Fingolimod is one of the few drugs available orally for treatment of MS; after phosphorylation is converted in fingolimod phosphate (FP), the active part of the compound. Recent works have indicated a neuroprotective effect of this drug that could promote an improvement in cognitive function in ischemic processes [15] and neurodegenerative disorders like HD [16] or AD [17]. In the present study, we resort to the analysis of glucose metabolism pathways (Glycolysis and pentose phosphate (PPP)), along with mitochondrial respiration, to investigate the molecular events associated with the protective effect of fingolimod against menadione (VitK3)-induced oxidative cell damage in SN4741 neuronal cells. The results of this work will give an insight into the underlying mechanisms in oxidative damage and neuroprotection.

2. Materials and Methods

2.1. Cell culture and treatments

The SN4741 (RRID:CVCL_S466) dopaminergic cell line derived from mouse substantia nigra [18] were cultured in D-MEM high glucose supplemented with 10% FCS penicillin-streptomycin, and L-glutamine (Fisher Scientific SL, Madrid, Spain) were incubated at 37 °C in 5% CO_2 up to about 70–80% confluence. Cells were seeded in 100 mm^2 dish ($5x10^6$ cells) or glass bottom 35 mm^2 dish and 6-well plates ($2x10^5$ cells each) and treated with 5 μM of VitK3 (menadione, CAS nº 58-27-5. Sigma, Madrid, Spain), in the absence or presence of 50 nM FP. In order to obtain a tighter control on the concentration of drug in the culture media, in this work we have used in all the incubations the active metabolite FP kindly provided by Novartis Pharmaceutical, instead of the prodrug. The sphingosine-1-phosphate (S1P) receptor antagonists, W123 10 μM (CAS nº 1345982-24-2. Cayman Chemicals, Michigan, USA), was also co-incubated with Vitk3 and FP. The treatments were carried out in Locke's solution modified (137 mM NaCl, 5 mM $CaCl_2$, 10 mM KCl, 25 mM glucose, 10 mM Hepes, pH: 7.4) supplemented with penicillin-streptomycin and L-glutamine during 2 hours at 37 °C, in some experiments, such as extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), the incubation time was extended.

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In ECAR experiments, dishes, plates and coverslips were pre-coated with poly-D-lysine. In some experiments, performed to assess the effect of FP in the recovery of VitK3 damage, after incubation of 2 h with Vitk3, the buffer was changed by only buffer (in control cells) or buffer with 50 nM FP (treated cells) and the same but in presence of 10 μM W123 to clarify the contribution of the S1P receptor on this recovery; measurements were obtained over 4 additional hours.

2.4. Antioxidant enzyme activity

Antioxidant enzyme activity of superoxide dismutase (SOD; E.C.1.15.1.1), glutathione peroxidase (E.C.1.11.1.9) (GPX) and catalase (E.C.1.11.1.6) (CAT), was measured spectrophotometrically using a Cobas Mira autoanalyzer (ABX Diagnostics, Montpellier, France) and the different commercial kits described previously [19].

2.5. Protein nitrosylation

Protein nitrosylation was measured in a homogenate of neuronal cultures using the commercial kit OxiSelect™ Nitrotyrosine ELISA Kit; (Cell Biolabs, Inc., San Diego, CA, USA) following the supplier instructions.

2.6. Determination of Aldolase

Aldolase activity (EC 4.1.2.13) in cell homogenates was assessed using an Aldolase assay kit (Spinreact SP, Gerona, Spain) adapted to a Cobas Mira Autoanalyzer. This assay is based on observation of the decrease in absorbance at 340 nm caused by the conversion of NADH to NAD^+ [20].

2.7. Glucose-6-Phosphate-Dehydrogenase activity

Glucose-6-Phosphate-Dehydrogenase activity (EC 1.1.1.49) (G-6-PDH) in cell homogenates was assessed using a G-6-PDH assay kit (Spinreact SP, Gerona, Spain) adapted to a Cobas Mira Autoanalyzer. This assay is based on observation of the decrease in absorbance at 340 nm caused by the conversion of NADPH to $NADP^+$ [20].

2.8. Extracellular acidification rate (ECAR) and mitochondrial oxygen consumption rate (OCR)

Extracellular acidification rate and OCR were measured using a Seahorse Bioscience XF24 analyser (Agilent Technologies. Agilent, CA, USA) [21,22]. Cells were seeded at $2x10^4$ cells per well during 18 h prior to the analysis and each experimental condition was performed on 8 replicates. Before each measurement, the cells were washed with PBS, and 590 μL of Agilent Seahorse XF Base Medium (without phenol red and bicarbonate) supplemented with 1 mM pyruvate and 25 mM glucose was added to each well. Measurements were normalized according to protein concentration in each well. For OCR experiments, we used the commercial kit "Seahorse XF cell Mito Stress test kit" (Agilent Technologies. Agilent, CA, USA) following the manufacturer instructions. In all the experiments, data was normalized with protein content in each well as described previously [23].

2.9. Immunocytochemical staining

Immunocytochemical staining was performed as described previously [24], primary antibody was Anti-S1P Receptor EDG1 (1:100 v/v) (CAS nº AB_10745373. Sigma, Madrid, Spain) in PBS/3% BSA/0.02% sodium azide, incubated at 4 °C over-night. Secondary antibody was Alexafluor™ 488 (Fisher Scientific SL, Madrid, Spain), in PBS/BSA, incubated for 30 min at room temperature in the dark. Images were acquired using an Olympus BX51 epifluorescence microscope at 40X magnification and processed using ImageJ software (US National Institute of Health; http://rsbweb.nih.gov/ij/).

2.10. Statistical analysis

Statistical differences were determined using one-way ANOVA. Pairwise comparisons were performed using a post hoc Newman-Keuls multiple comparison test. Statistical significance was considered to be $p < 0.05$. For data in which the measured units were arbitrary, the respective values represent the percentage relative to the control value unless specified.

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3. Results and discussion

In our work, we have resorted to the analysis of glycolytic pathway and PPP, along with mitochondrial function, to study the molecular events responsible of the protective effect of FP against VitK3-induced oxidative damage. VitK3 is a exogenic toxic that produce a mitochondrial damage linked to an increase in ROS [24] distorting cellular structures such as endoplasmic reticulum, but also influencing important metabolic pathways such as glycolysis or pentose shunt [25]. VitK3 interfere in quinone redox-cycle, uncoupling the mitochondrial respiratory chain, triggering the release of $O2^{-\bullet}$ that in presence of Fe from the respiratory complex, produce an increase in free radicals [24]; with increase in respiration without increase in ATP production and decrease of spare respiratory capacity (SRC) and maximal respiratory capacity. Furthermore, oxidative stress could also activate/inactivate other metabolic pathways that may contribute to VitK3 damage [26]. This study has been performed on neuronal cell cultures and so, one of the limitations of the work is to fail in knowing the protective effect of FP in the whole animal. In any case, a clearer knowledge of these ways could help to understand the mechanisms involved in oxidative damage, common in several neurodegenerative diseases, and hence, to ameliorate symptoms and/or develop new therapeutic strategies.

Based in the importance of glycolysis to maintain neuronal metabolism when mitochondrial disfunction appears, along with previous work by our group and others, showing the influence of ROS in energy production by neurons [24,27,28], we decided to go dipper on the study not only of mitochondrial function but also on the effect of FP on glucose metabolism.

In order to determine the influence of oxidative radicals on mitochondrial respiration chain function, we studied the OCR in neuronal cultures after oxidative damage induced by VitK3, in presence or absence of FP; figure 1 shows the bioenergetic assessment. The OCR data obtained in these experiments enable determination of oxygen consumption due to a) ATP synthesis, b) mitochondrial oxygen reserve capacity, c) related to proton leak, d) spare respiratory capacity and e) maximal respiration.

In basal situations (without addition of oligomycin, FCCP and rotenone/antimycin), the incubation of neurones with VitK3 showed an increase in OCR of 17% compared to control, agreeing with our previous results and others [24,26]; whereas neurones incubated with VitK3 in presence of FP maintained the same levels than control cells (Figure 1a).

Mitochondrial ATP-dependent OCR is the OCR required for the synthesis of ATP at complex V, and can be easily studied as it is sensitive to Oligomycin. Regarding oxygen consumption by mitochondrial ATP synthesis, this decreases by 25% after incubation with VitK3, agreeing with the results of Lakhter et al [29] who also found a similar decrease after incubation with this toxic. In our experiments, FP partially rescue the VitK3-induced loss of ATP linked to OCR, agreeing with Bai et al [30], who found that increased ATP production by FP could be the reason for its neuroprotective effect; in our case, FP restores OCR linked to ATP production to levels close to control neurons (Figure 1b).

We did not find differences in proton leak (Figure 1c), although we could see a clear difference in SRC, the difference between OCR at basal and at maximal respiratory activity, after oligomycin and FCCP addition. In our experiments, incubation of neurons with VitK3 produce a great decrease in SRC (40%) compared to control, which is reverted to values close to control in presence of FP (Figure 1d).

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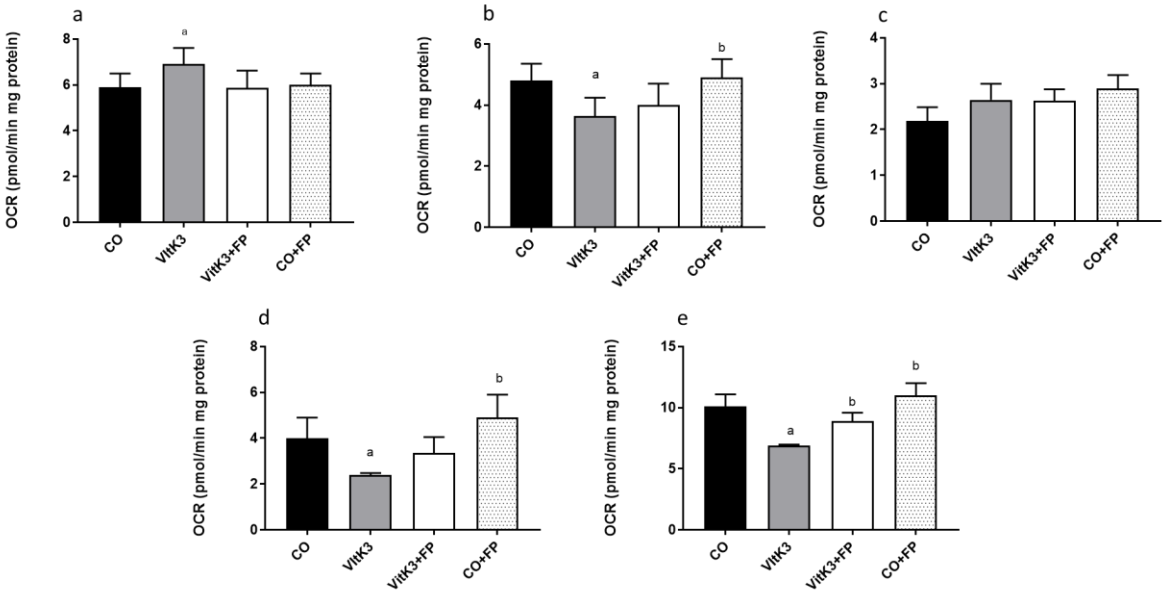

Figure 1. Study of neuronal mitochondrial function after treatment of cell with VitK3 in absence or presence of FP. The oxygen consumption rate was assessed in a) basal after 2 hours of incubation with VitK3; b) ATP production, measured after addition of oligomycin; c) proton leak, considered as the residual oxygen consumption after oligomycin addition; d) SRC, as the difference between maximal respiration and basal respiration and e) maximal respiration, obtained as the oxygen consumption after subsequent addition of oligomycin and FCCP. The sequence of mitochondrial toxics added was: oligomycin 1µM, FCCP 0.5 µM, Rotenone/antimycin A (0.5/0.5) µM. The inclusion of Rotenone/antimycin A serves to measure respiration by non-mitochondrial processes as these compounds abolishes mitochondrial respiration, and was subtracted in all OCR values obtained. Values represent mean and SD of at least five experiments per situation performed in triplicated. (a: $p<0.05$ versus control, b: $p<0.05$ versus VitK3).

SRC is essential to maintain neuronal homeostasis against oxidative and/or other type of cellular stress [31]. The use of mitochondrial SRC by neurons is very variable, ranging from approximately 6-7% in resting situation to up to 80% in firing neurons; and so, deterioration in mitochondrial SRC can be fatal to neurons [31]. The ability to increase the SRC by FP, will allow mitochondria to produce more ATP and overcome an increase in oxidative stress imbalance. FP cold be then considered a long-term regulator of mitochondrial respiration through the changes promoted in SRC; this modulation could be related with an increase in the expression of neuronal nitric oxide synthase (NOS) and a consequent increase in nitric oxide (NO) levels [32], agreeing with our results on protein nitrosylation commented in table 1. These long-term regulators produce permanent changes in mitochondrial respiration which seem to be tissue specific [33]. This would make FP a valuable therapeutic tool in neurodegenerative diseases, such as PD, HD or MS in which imbalance in oxidative stress would produce a dramatical mitochondrial affectation, in such a way that they have been grouped as mitochondriopathies [8,34,35].

We also found similar result in maximal respiratory activity, which is the maximum rate of respiration achieved by a neurone after addition of uncoupling FCCP and oligomycin and it is an indicator of potential mitochondrial disfunction [31]. In these experiments, incubation of neurones with VitK3 produce a clear decrease (32%) in maximal respiratory activity. This decrease was counteracted by coincubation in presence of FP, being able to recover 20% of maximal respiratory activity (Figure 1e). The effect of FP on mitochondrial function could be related with the modulation of mitochondrial translocator protein expression (TSPO), as seen in MS, where FP decreases the expression of TSPO [36]. In all the experiment in figure 1, control cells were incubated in presence of FP without any significative change in the values obtained compared to control cells.

In the study of enzymes related with $O2^{-\bullet}$ metabolism, we found an increase (77.3%) of mitochondrial SOD2 activity in VitK3 treated neurones compared to control (table 1); in our

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experiments, that increase was not compensated by variations in CAT or GPX activities (see ratio in table 1).

Table 1. Intracellular redox measurements. The main enzymes related to superoxide radical metabolism were measured; also, nitrosylated proteins were measured as marker of oxidative damage. Each experiment was performed in triplicate; samples were a homogenate from a pool of neuronal cells. The results are expressed as mean ± SD. (a: $p<0.05$ versus control, b: $p<0.05$ versus VitK3, c: $p<0.05$ versus VitK3+FP, d: $p<0.05$ versus VitK3+FP+W123).

	SOD2 U/10^6 cells	GPX U/10^6 cells	CAT KU/10^6 cells	Nit-Prot pmol/10^6 cells	SOD2/GPX	SOD2/CAT
CO	43.1±3.0	21.9±4.6	27.9±3.1	0.76±0.17	2	0.15
VitK3	76.4±4.0 (a)	4.5±0.8 (a)	27.1±6.3	4.5±0.35 (a)	17 (a)	0.28
VitK3+FP	40.3±3.0 (b)	10.6±0.9 (a)(b)	27.8±4.2	0.45±0.15 (b)	5 (a)(b)	0.14
VitK3+FP+W	79.9±6.0 (a)	27.6±4.8 (b)(c)	14.4±2.5 (a)(b)(c)	3.75±0.20 (a)(c)	3 (b)(c)	0.55 (a)(b)(c)
FP	50.1±3.0 (b)(c)(d)	23.9±4.0 (b)(c)	44.1±8.5 (a)(b)(c)(d)	1.87±0.5 (a)(b)(c)(d)	2 (b)(c)	0.11 (b)(d)

Increased SOD2 activity, would in turn produce an increase in H_2O_2 that, in order to maintain homeostasis, should be followed by increases in CAT and/or GPX to remove H_2O_2 excess and avoid oxidative damage. This would suggests that mitochondria are involved in the generation of oxidative stress triggered by VitK3, agreeing with our previous results [24]. SOD2 is an inducible mitochondrial enzyme that promotes the formation of H_2O_2, being one of the factors that induce its expression the increase in $O2^{-\bullet}$ [37]. Interestingly, in the experiments in presence of FP, the SOD2 activity was normalised to control levels indicating that FP, as shown above, exerts a protective effect on mitochondrial function; it also reduces $O2^{-\bullet}$ production, as demonstrated previously [24] and both in turn, could contribute to the reduction in the induction of SOD2.

GPX is an enzyme family with peroxidase activity that protects cells from oxidative damage, based in its ability to reduce free H_2O_2 to water at low H_2O_2 concentration. In our experiments we found a decrease (79.4%) in GPX activity in neurones treated with VitK3 compared to control, probably as a consequence of the increase in $O2^{-\bullet}$ and decrease in total thiols, as demonstrated previously [24]. In these experiments, the inclusion of FP in the incubation media partially recovers (50%) the activity of this enzyme, agreeing with the finding of other authors [38]; this effect could be mediated by the Nrf2 translocation to the nucleus as demonstrated previously [39].

CAT is an enzyme that efficiently remove H_2O_2 at high concentrations of the oxidant. In our experiments, we did not see any modification after incubation with VitK3 alone or in presence of FP; only in control cells produced a clear increase (57%) in CAT activity; this could be related with ability of FP to promote an increase in Nrf2 expression and translocation demonstrated in a previous work [24].

To assess the putative damage produced by oxidative stress induced by VitK3 treatment we resorted to the study of protein nitrosylation. The nitrosylation of proteins triggered by NO is greatly enhanced in presence of $O2^{-\bullet}$ with the formation of peroxinitrites [40], and it is an important regulator of mitochondrial activity involved in energy-transducing systems [41]. NO is an important molecule in neuronal cell signalling, but its overproduction can be toxic to neurones causing protein nitrosylation and mitochondrial damage [42]. In our experiments we found a great increase (almost 600%) in protein

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nitrosylation in neurones treated with VitK3; again coincubation in presence of FP restores nitrosylation to levels close to control neurones, this could be explained by the ability of FP to inhibit the inducible NO demonstrated previously [43] that in turn would decrease protein nitrosylation. We also could see an increase in control neurones treated with FP (246%), which could be due to the stimulation of neuronal NO [32]; also FP has been involved in an increase in neuronal plasticity mediated by NO [44,45].

Expression of genes associated with plasticity and synaptic remodelling processes are associated in adult brain with aerobic glycolysis [10]. Active synapses obtain its energy by glucose breakdown through glycolysis, being this the main source of cellular energy, and the preferred method for certain neuronal functions, such as fast axonal transport [46].

In presence of VitK3, we found a great decrease (46% compared to control) in glycolysis, measured as ECAR after 2 hours of incubation; coincubation in presence of FP recovered values to control levels (Figure 2a,b). This effect is similar to that found with some sphingosine-1-phospate mimetics that increase the expression of genes related with glycolysis, ECAR and glycolytic capacity [47]. In the study of glycolytic capacity which measures the maximum rate of conversion of glucose to pyruvate or lactate, we found that VitK3 produces a decrease by 47% compared to control, being this decrease totally reverted by coincubation in presence of FP (Figure 2a,c). When we studied the glycolytic reserve; which is the ability of cells to adapt to extra energetic demands when the glycolytic function is working at maximum, neurones incubated in presence of VitK3 showed also a reduction in glycolytic reserve by 48% compared to control; again, coincubation in presence of FP reverted this value to control levels (Figure 2a,d). Our results partially agree with those from Lee et al [47] who found an increase of glycolytic capacity with S1P mimetics in mesenchymal cells, although they did not find differences in glycolytic reserve, may be due to the different type of cells. This recovery in glycolytic capacity would supply ATP in situations where it is needed rapidly and the mitochondrial metabolism, although with high capacity to produce ATP, is not totally functional as demonstrated before.

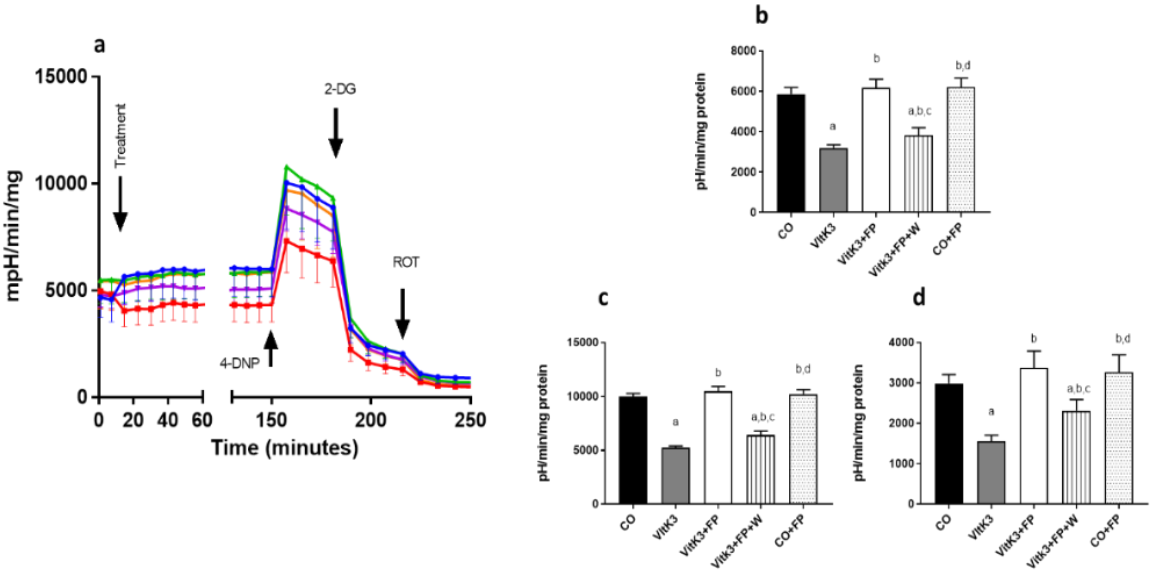

Figure 2. FP recovers the glycolytic damage induced by VitK3 on neuronal SN4741 cells. a) Time course of ECAR and glycolytic function after incubation with VitK3 in presence or absence of FP (Blue line: Control; Red line: Vitk3; Green line: Vitk3+FP; Purple line: VitK3+FP+W123). To uncouple mitochondrial respiration from ATP synthesis we used 2,4-DNP 100 µM; 2-DG 100 mM was used to inhibit glycolysis and rotenone 1µM was used to inhibit NADH hydrogenase/complex I; b) Basal glycolysis after two hours of treatment with VitK3 in presence or absence of FP; c) Glycolytic capacity obtained after incubation with 2,4-DNP and rotenone; d) Glycolytic reserve obtained as the difference between the maximal glycolytic capacity and basal. In all situations the non-glycolytic ECAR, obtained after addition of rotenone, was subtracted. Values represent mean and SD of at least five experiments

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per situation performed in triplicated. (a: p<0.05 versus control, b: p<0.05 versus VitK3, c: p<0.05 versus VitK3+FP, d: p<0.05 versus VitK3+FP+W123).

As commented above, we have found an increase in the expression of metabolic pathways related to glycolysis. This has also been described before in situations of energy stress, where increase in glycolytic metabolic enzymes have been seen in presynaptic buttons [48]. The decrease in glycolytic enzymes activity as consequence of oxidative damage, seen in early stages of some neurodegenerative diseases, can lead to a decrease in ATP synthesis which in turns would lead to a less reduced environment and an increase in free radical production [49]. In some neurodegenerative diseases, especially in those where axonal conduction is damaged, neurones tend to restore that conduction by increasing energy consumption to restore axonal function [50]. In AD, low glucose metabolism is associated with cognitive fails [51]; also Rone et al, highlights the role of glycolysis to obtain energy in cells under metabolic stress [52]. One of the key enzymes involved in glycolysis is aldolase; in this work we have found a decrease (23%) in aldolase activity after treatment of neurones with VitK3; again, FP restores values to those found in control neurones (Figure 3), agreeing with Lee et al, who also found an increase in aldolase activity with S1P mimetics [47]; also Geffin et al found an upregulation of genes involved in glycolysis, including aldolase, in cells exposed to HIV and treated with FP [53]. Based on these findings, FP could be considered as a new therapeutic approach to the treatment of neurodegenerative diseases showing a decrease in glycolysis, such as PD, AD or MS [27].

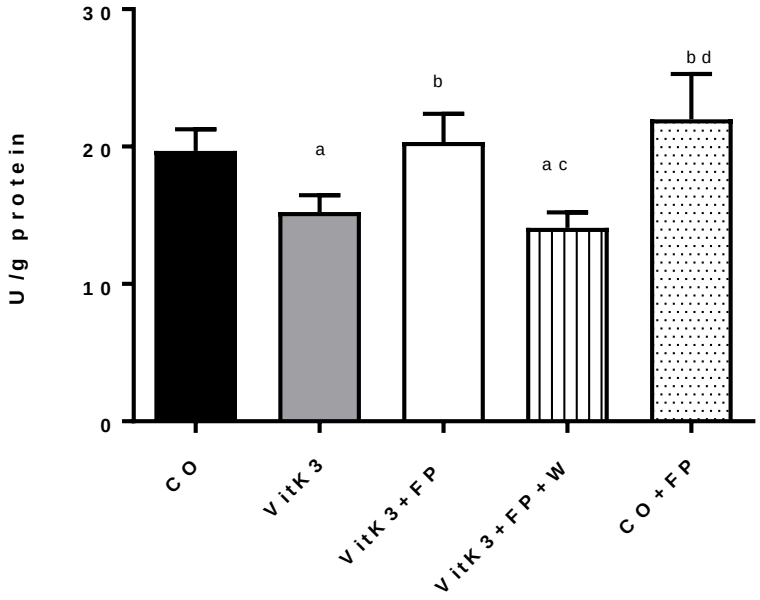

Figure 3. FP recovers the enzyme aldolase from the inhibition induced by VitK3. Aldolase was measured in a homogenate of neurones after two hours of incubation with VitK3 in presence or absence of FP. Values represent mean and SD of at least five experiments per situation performed in triplicated. (a: p<0.05 versus control, b: p<0.05 versus VitK3, c: p<0.05 versus VitK3+FP, d: p<0.05 versus VitK3+FP+W123).

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Although our model is based in a mitochondrial oxidative damage, we see a decrease in glycolytic function, recovered in presence of FP. Some authors have demonstrated that withdrawal of FP after a period of treatment, could trigger relapses in MS patients [54], so we decided to maintain FP in one of the groups; and extend the study time, to determine the need of FP in the medium to maintain its beneficial effects. In these experiments, we found a decrease in ECAR compared to control neurones; FP almost totally reverted the damage produced by VitK3, so maintaining glycolytic activity to levels close to control up to the maximum time studied, whereas in those neurones not in presence of FP the damage was maintained over time (Figure 4). This could be of great interest as many neurodegenerative diseases have in common a decrease in energy metabolism and hence in ATP levels [55], in these situations, enhancement of glycolytic processes may represent a good approach to slow progression and/or alleviate symptomatology [55,56].

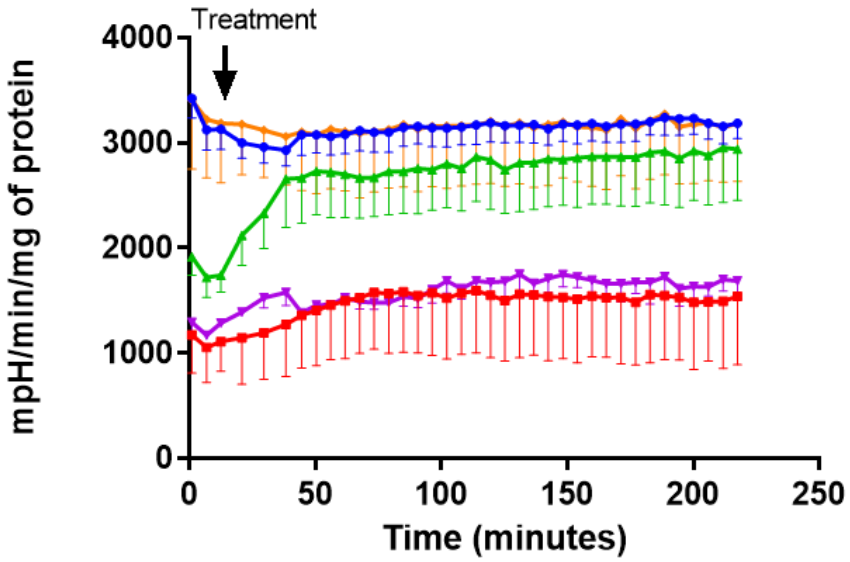

Figure 4. FP is necessary in the medium to maintain its actions. Time course of ECAR after 2 h of incubation with Vitk3 in presence or absence of FP. After 2 h of incubation, Vitk3 was removed from the media and cells were incubated for four additional hours in the following conditions: two groups were incubated with media (Vitk3 group (red line) and control group (blue line)); one with FP (VitK3+FP group (green line)); one with FP+W123 (VitK3+FP+W group (purple line)) and one with FP (FP group (orange line). Values represent mean and SD of at least four experiments per situation performed in triplicated.

In neuronal cells, glucose is metabolized via glycolysis, the PPP, the tricarboxylic acid cycle and oxidative phosphorylation to produce ATP. In the processing of glucose, the balance between glycolysis and PPP seem to be important, as the PPP processing of glucose serve as a source of NADPH that in turn will regenerate oxidized antioxidants such as glutathione and thioredoxin, and so, the exclusive processing of glucose by the glycolytic pathway would produce a decrease in NADPH availability, increase oxidative stress and may produce cell death [57]. NADPH serve also as a cofactor of NQO1 detoxifier activity, synthesis of fatty acids and myelin, neurotransmitter turnover, and to maintain REDOX homeostasis [3,24].

G-6-PDH is one of the key enzymes in the PPP and it has been related with increases in NADPH levels related to the improvement in REDOX homeostasis [58]; also a decrease of this enzyme has been found in neurodegenerative diseases such as PD [59]. Taking in to account the relevance of this enzyme, we decided to study the level of G-6-PHD in neuronal cultures after oxidative damage induced by menadione in presence or absence of FP. The results in Figure 5 show a decrease in the activity of G-6-PHD after incubation with VitK3. This decrease was abolished when FP was included in the incubation media, producing a great increase in G-6-PHD activity (three-fold the control value). This marked

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increase could be due to the stimulation in synthesis and translocation of Nrf2, an inductor of PPP key enzymes synthesis, triggered by FP after the damage induced with VitK3, as demonstrated previously by our group [24]. Also other authors have previously established the relation between Nrf2 synthesis and translocation, and the expression of PPP key enzymes [60,61].

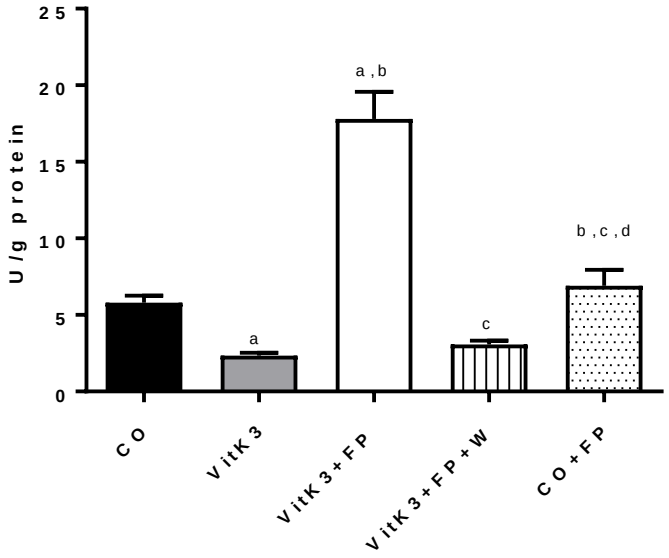

Figure 5. FP increases the enzyme G-6-PDH reduced by VitK3. G-6-PHD was measured in a homogenate of neurones after two hours of incubation with VitK3 in presence or absence of FP. Values represent mean and SD of at least five experiments per situation performed in triplicated. (a: $p<0.05$ versus control, b: $p<0.05$ versus VitK3, c: $p<0.05$ versus VitK3+FP, d: $p<0.05$ versus VitK3+FP+W123).

Classically FP is thought to develop its effects through the interaction whit S1P receptors. We have demonstrated in this study the presence of S1P receptors in soma and axons of SN4741 neuronal cells (Figure 6).

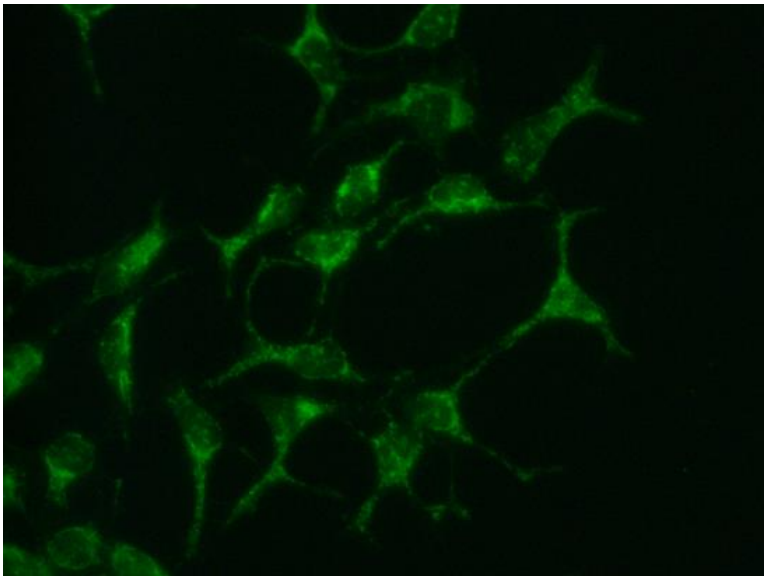

Figure 6. Immunocytochemistry of S1P receptors in SN4741 neuronal cells. Image were acquired using an Olympus BX51 epifluorescence microscope at 40X magnification.

 doi:10.20944/preprints202011.0709.v1

In order to determine the contribution of these receptor in the protective effect of FP, we have performed some experiments in presence of the S1P antagonist W123. In the study of the influence of S1P receptors on the enzymes involved in redox balance we found intriguing results. Whereas the effect on SOD can be attributable to the interaction of FP with S1P receptors; in such a way that we see an increase in SOD by the increase in free radicals triggered by VitK3, that is totally reverted in presence of FP, when the S1P receptor is blocked with the antagonist, the beneficial FP effects disappear, free radicals increase again [24], and SOD values return to those found in VitK3 treated neurones (table 1). The influence of FP on GPX is not so clear, in these experiments FP has demonstrated to exert its action not only by its interaction with S1P receptors, but also by other mechanisms. When blocking the S1P receptor with the antagonist, we abolish that receptor-interaction effects but we still would have other non-receptor mechanisms. These would include an increase in expression of Nrf2, OH1 and thioredoxin, and also an increase in Nrf2 translocation to the nucleus [24], being these factors, especially Nrf2 and OH1 important to increase antioxidant enzymes such as GPX. Something similar occurs with CAT where no clear results on the influence of S1P were obtained, in these experiments, we did not see effects of VitK3 incubation on CAT activity, nor with the addition of FP. Interestingly when the S1P receptor was blocked, we found a decrease in the activity of this enzyme without a clear explanation; although we could argue that the decrease could be related to the increase in SOD and GPX previously found, that would reduce the H_2O_2 and in turns would decrease CAT [62]. In the study of protein nitrosylation, we found a similar behaviour than in SOD. Neurones incubated with VitK3 showed an increase in protein nitrosylation that was reverted in presence of FP, the blockade of S1P receptor abolishes FP beneficial effects, increasing nitrosylation again to levels close to those found in VitK3 treated neurones, agreeing with other authors [63]. All the effects of FP on glycolysis were mediated through its interaction with S1P receptors, as all of these effects disappear when the S1P antagonist is present, being affected the basal glycolysis, glycolytic capacity, glycolytic reserve and the capacity to maintain the aldolase activity; this finding is common with other authors who also found the need of interaction of FP whit its receptor to develop its glycolytic protective actions [47,53]. In the study of G-6-PHD activity, the effect of FP was also mediated by the interaction with its specific S1P receptor, as the increase in G-6-PHD activity was totally abolished when the S1P antagonist was present in the incubation media.

4. Conclusions

In global, our data support that in extreme neuronal situations such as in neurodegenerative diseases, where damaged mitochondria fail to cover the ATP needs and oxidative environment turns aggressive, other sources of ATP and antioxidants need to be used. Our results show, in one hand a beneficial effect of FP increasing glycolytic and pentose phosphate metabolism and hence ATP production and redox buffering capacity, which are important factors against neurodegeneration. On the other hand, FP also exerts a beneficial effect on mitochondrial oxidative damage, which would improve neuronal condition by improving mitochondrial function and restoring oxidative balance in neurones. Both actions would make this drug a potential therapeutic tool for the treatment of neurodegenerative processes, either to slow progression or alleviate symptoms.

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