Efavirenz alters mitochondrial respiratory function in cultured neuron and glial cell lines

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Background: The NNRTI efavirenz is among the most widely employed antiretroviral drugs. Although it is considered safe, efavirenz has been linked with several adverse effects including neurological manifestations, which appear in the majority of the patients on efavirenz-containing regimens. The molecular mechanisms responsible for these manifestations are not understood, but mounting evidence points to altered brain bioenergetics.

Methods: We evaluated the effect of short-term efavirenz treatment on the mitochondrial respiratory function of cultured glioblastoma and differentiated neuroblastoma cell lines using a Seahorse Extracellular Flux Analyzer.

Results: Incubation with efavirenz provoked a significant and concentration-dependent decrease in basal respiration and specifically in ATP production-coupled O₂ consumption in both SH-SY5Y and U-251MG cells, with the effect being more pronounced in the latter. In contrast, efavirenz did not alter mitochondrial proton leakage in either of the cell types. Efavirenz led to a decrease in the respiratory control ratio as well as to a reduction in the maximal respiration rate and spare respiratory capacity in both U-251MG and SH-SY5Y cells, the former cells being more susceptible.

Conclusions: These findings reveal that efavirenz specifically alters mitochondrial respiration, which is of relevance for a better understanding of the molecular mechanisms responsible for the efavirenz-associated neurological effects that have been recorded in clinical situations.

Keywords: mitochondria, neurotoxicity, HIV, respiration, side effects

Introduction

Efavirenz is an NNRTI that is widely employed as part of the combined treatment of HIV infection. Although it is considered safe, efavirenz has been linked to several adverse effects, with CNS reactions being the most frequent (occurring in more than half of patients and often requiring discontinuation of therapy). The cellular mechanisms responsible for the appearance and progression of these effects are still not known, but a growing body of evidence, both in vitro and in vivo, has pinpointed the ability of efavirenz to interfere with mitochondrial function. We recently reported that efavirenz undermines mitochondrial respiration in cultured neurons and glial cells, paralleled by a decreased mitochondrial membrane potential and an increased generation of mitochondrial reactive oxygen species (ROS). Of note, the bioenergetic response of this action is differential: glial cells, but not neurons, up-regulate glycolysis. Detailed measurements of mitochondrial respiration in intact cells can help to define metabolism and its dysregulation and have been proven useful in toxicological analysis. The most informative assays involve the equivalent measurement of cell respiratory control reporting the ATP production-linked rate, proton leakage rate, coupling efficiency, maximum respiratory capacity (MRC), respiratory control ratio (RCR) and spare respiratory capacity (SRC). In the present work, we dissect the effect of efavirenz on mitochondrial O₂ consumption by providing a detailed analysis of these parameters through a comparison between cultured cancer neuron and glial cell lines.

Materials and methods

Cell culture, reagents and treatments

The chemical reagents were acquired from Sigma-Aldrich (Steinheim, Germany). The cell lines employed were human glioma U-251MG (CLS 300385) and neuroblastoma SH-SY5Y (ATCC CRL-2266) cells, which were subject to differentiation prior to efavirenz treatment (details provided as Supplementary data, available at JAC Online).

Analysis of mitochondrial respiratory function using an extracellular flux analyser

Extracellular flux analysis has become a reference method for analysing mitochondrial function in intact cells. It provides real-time quantification...
of the O2 consumption rate (OCR) (Figure 1) that can be ascribed to mitochondrial respiration as well as the change in pH that can be related to glycolysis. In this study, detection was performed at 37°C using the Seahorse XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Copenhagen, Denmark), following the manufacturer’s instructions. To allow a comparison between different experiments, the data obtained for each condition were normalized to the cell number per well and expressed as the OCR in pmol/min (details provided as Supplementary data). The short-term treatment with efavirenz (1 h) did not have any impact on the cell viability of either the U-251MG cells or the SH-SY5Y cells. The experiment was repeated three times (n=3) and the SH-SY5Y cells were differentiated independently for each separate measurement. No significant inter-assay differences were detected between the three repetitions regarding the appearance and viability of cells or the values of O2 consumption that were obtained.

Data analysis

Data were analysed using GraphPad Prism v5 software with the Student’s t-test and values were expressed as the mean ± SEM (the arithmetic mean of three independent experiments; n=3). Statistically significant differences versus vehicle were designated: *P<0.05, **P<0.01 and ***P<0.001.

Results

Efavirenz provoked a significant and concentration-dependent decrease in the basal respiration of the SH-SY5Y and U-251MG cells (Figure 2a). The effect was more pronounced in the glioblastoma cells, where 25 μM efavirenz induced a 57.1% reduction compared with the 39.3% seen for differentiated neuroblastoma cells. Basal respiration is strongly controlled by ATP turnover and partly by substrate oxidation and proton leak; therefore basal OCR measurements are sensitive to ATP demand, but rather insensitive to small changes in MRC or in proton leakiness. In order to assess the effect of efavirenz on these specific parameters, the OCR was recorded after the addition of several mitochondrial inhibitors. ATP production-coupled O2 consumption was diminished in both cell types and again the U251-MG cells were more susceptible (SH-SY5Y cells displayed reductions of 14.2% and 51.5% whereas the corresponding values in the glioblastoma cells were 65.9% and 73.8% upon exposure to 10 and 25 μM efavirenz, respectively). Of note, efavirenz did not alter mitochondrial proton leakage in either of the cell types. The SRC is the difference between the MRC, obtained after the addition of an uncoupler, and the basal OCR. Importantly, both the MRC and the SRC were diminished in cells treated with efavirenz. This was recorded only with the higher concentration of efavirenz in the differentiated neuroblastoma cells whereas the effect was greater in the U-251MG cells and was observed with both concentrations of efavirenz (Figure 2a). In addition, the OCR that corresponds to the non-mitochondrial O2 consumption was affected by efavirenz in a concentration-dependent manner in both cell types.

Furthermore, we assessed the parameters in comparison with the basal respiration (expressed as the percentage of basal respiration; Figure 2b). The aim was to determine whether efavirenz had the ability to alter the relative proportions of the distinct participants of the basal OCR. In this sense, efavirenz not only undermined basal respiration in both cell types, but also significantly reduced the relative participation of respiration that was linked to ATP generation. In vehicle-treated SH-SY5Y, 66% of the basal respiration was used for ATP synthesis, but this value dropped to 52.7% with 25 μM efavirenz, an effect that was even more pronounced in U-251MG cells (with values of 49.6% and 30.3%, respectively). In addition, the relative participation of the proton leakage rate to the basal respiration increased significantly in both cell types, being recorded only with the higher concentration of efavirenz in SH-SY5Y cells. In the differentiated neuroblastoma cells, ~80% of the basal respiration rate was mitochondrial and this figure did not change when efavirenz was present. In U-251MG cells, 61.9% of the basal OCR was of mitochondrial origin and 10 μM efavirenz modified this proportion, diminishing it by ~10%. Interestingly, the higher efavirenz concentration did not alter the relative proportions of mitochondrial and non-mitochondrial respiration owing to the fact that it produced similar major decreases in both sources of O2 consumption. The reserve capacity and the maximal respiration have also been expressed as percentage of change with regard to mitochondrial O2 consumption (Figure 2c). We also evaluated the RCR, calculated as the ratio between the maximal uncoupled OCR and the respiration that occurred in the presence of oligomycin. The RCR is a useful and versatile indicator of mitochondrial dysfunction as it is strongly influenced by almost every functional aspect of mitochondrial function.
oxidative phosphorylation (OXPHOS). Notably, efavirenz provoked a major decrease in RCR, which was again more prominent in U-251MG cells (Figure 2d).

**Discussion**

We have described here the effect of clinically relevant plasma concentrations of efavirenz on the mitochondrial respiratory function of cultured neuroblastoma and glioma cell lines. Importantly, although the absolute values of efavirenz in CSF differ from those in plasma, the protein-free drugs levels have been found to be similar. In both cell types, efavirenz provoked a decrease in basal mitochondrial O₂ consumption. This inhibitory action of efavirenz is pinpointed by the fact that mitochondria are considered to possess a high reserve capacity of respiratory complexes and their activities typically need to be reduced by >60% before major changes in basal O₂ consumption can be detected. Glial cells were more susceptible to efavirenz exposure than neurons and displayed a reduction of roughly 50% in their basal OCR with 25 µM efavirenz. These results are in agreement with previously published data in the same cellular models where intact cell respiration was detected with a Clark-type O₂ electrode immediately after the addition of efavirenz to the respiration chamber or the activity of the individual electron transport chain (ETC).

![Figure 2. Effect of efavirenz treatment on mitochondrial respiratory function. The OCR was measured in SH-SY5Y and U-251MG cells exposed to vehicle, 10 µM efavirenz or 25 µM efavirenz for 1 h. (a) Left: Graphical representation of the OCR measurement over time; sequential additions are indicated as I, II, III and IV: 2 µM oligomycin (I), 0.6 µM FCCP (II), 0.4 µM FCCP (III) and 1 µM rotenone plus 1 µM antimycin A (IV). Right: Quantification of the mean OCR in SH-SY5Y and U-251MG cell lines exposed to efavirenz is shown for respiration under basal conditions (basal) and after the addition of oligomycin (proton leak) and FCCP (maximal respiration). The basal respiration rate minus the respiration rate recorded with oligomycin provides a measure of OCR due to ATP turnover whereas the respiration rate obtained with FCCP minus the basal O₂ consumption values represents the reserve respiratory capacity. Non-mitochondrial respiration (rotenone plus antimycin A) was subtracted from each condition. (b and c) Relative parameters of mitochondrial respiratory function. The histograms show several parameters where data are expressed as the percentage of basal respiration (100% = mitochondrial respiration + non-mitochondrial respiration). (d) Representation of the mean RCR. The RCR was calculated as the ratio between the maximal uncoupled mitochondrial respiration and the respiration rate detected in the presence of oligomycin. Non-mitochondrial respiration (recorded upon the addition of rotenone plus antimycin A) was subtracted from each condition. Data (mean ± SEM; n = 3) were compared with those for the vehicle and analysed by Student’s t-test; significance versus vehicle: *P < 0.05, **P < 0.01 and ***P < 0.001. Veh, vehicle; Efav 10, 10 µM efavirenz; Efav 25, 25 µM efavirenz; Mito. res., mitochondrial respiration; Non-mito., non-mitochondrial respiration.]
complexes was assessed spectrophotometrically after short-term treatments. The present findings expand these results by revealing further details of this respiration defect through an in-depth respirometric profiling performed using the Seahorse Extracellular Flux Analyzer. This accurate, automated, multi-well plate-based technology examines intact, adherent cells and offers a comprehensive assessment of both the present mitochondrial respiration efficiency and that potentially occurring under different energetic and metabolic conditions. The detection of a decrease in OCR induced by a short-term exposure to efavirenz in the present study is in line with recently reported data from galactose-conditioned HepG2 cells using the same extracellular flux assay. Importantly, we did not detect alterations in the absolute values of OCR linked to proton leakage in either differentiated neuroblastoma cells or glioma cells; however, relative to the basal O2 consumption, efavirenz increased the rate of proton leakage, an effect that was more pronounced in U-251MG cells. In addition to a decrease in the basal OCR, we also observed a major reduction in the MRC and consequently in the SRC. This crucial parameter of bioenergetics (i.e. SRC) reflects the difference between the ATP produced by OXPHOS at a basal level and at maximal ETC activity. It expresses the capability of a cell to operate in energetically demanding conditions and is mainly the result of the bioenergetic capacity of mitochondria, determined by many factors including efficient substrate delivery to mitochondria and the

Proton leakage dissipates about 20% of the energy derived from mitochondrial function in animal cells and its physiological function is believed to include heat production and the prevention of oxidative stress though a decrease in ETC-generated ROS. Importantly, we did not detect alterations in the absolute values of OCR linked to proton leakage in either differentiated neuroblastoma cells or glioma cells; however, relative to the basal O2 consumption, efavirenz increased the rate of proton leakage, an effect that was more pronounced in U-251MG cells. In addition to a decrease in the basal OCR, we also observed a major reduction in the MRC and consequently in the SRC. This crucial parameter of bioenergetics (i.e. SRC) reflects the difference between the ATP produced by OXPHOS at a basal level and at maximal ETC activity. It expresses the capability of a cell to operate in energetically demanding conditions and is mainly the result of the bioenergetic capacity of mitochondria, determined by many factors including efficient substrate delivery to mitochondria and the
functional capacity of the ETC complexes. It has been proposed that SRC maintenance is a major factor defining the vitality and/or survival of cells and its decrease has been correlated with a variety of pathologies including heart disease, neurodegenerative disorders and ageing. Importantly, OXPHOS, and thereby respiratory capacity, is critical for neuronal susceptibility to cellular stress caused by hypoxia, nutrient depletion or excitatory stimuli. The dependency of the human brain on O$_2$ for OXPHOS is emphasized by the fact that although the brain accounts for only 2% of the entire body mass, it consumes ~ 16% of the body’s O$_2$. Therefore, neuronal SRC exhaustion can have fatal consequences, as while resting neurons utilize ~ 6% of their MRC, firing neurons utilize up to 80%. The decreased MRC and SRC values we report reveal that mitochondria in cells subject to efavirenz treatment operate closer to their bioenergetic limit.

We also detected a decrease in RCR, another crucial parameter of bioenergetics that reflects the capacity of mitochondria to idle at a low rate while being able to generate ATP at a high rate. The decreased RCR implies either a drop in the ability of mitochondria to undertake substrate oxidation and ATP turnover and/or the presence of increased proton leak.

Finally, mitochondria consume >95% of the O$_2$ of aerobic higher organisms; however, in vitro measurements can reveal higher rates of non-mitochondrial respiration, which result from substrate oxidation and peroxisomal and cell surface O$_2$ consumption. There is a great variability in the extent to which mitochondrial respiration accounts for total cellular O$_2$ consumption among cell types. Here, we report an extramitochondrial O$_2$ consumption of 25% and 40% of the basal OCR in cancer cell lines of neurons and glial cells, respectively, which is in a similar range to that reported by other studies using the same detection system, for example 30% in podocytes.

We are aware of the limitations of our study. The measurements were performed in cancer cell lines whose cellular bioenergetics is not fully comparable to that of primary cells. Moreover, these data may not reflect the bioenergetic profile of neurons or glial cells in vivo, where these cells are surrounded by a complex extracellular matrix with a lower O$_2$ tension than is typically seen in cell cultures.

In summary, we have analysed the direct inhibitory effect of efavirenz on the mitochondrial respiratory function of cultured glioblastoma and differentiated neuroblastoma cell lines. These findings may be of relevance for a better understanding of the molecular mechanisms responsible for the CNS effects associated with efavirenz use in clinical situations.

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