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Effect of hypoxia and KCl depolarization in autofluorescence and ROS changes at the hippocampal CA3 area

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Abstract. The increasing incidence of neurodegenerative and other diseases is considered to involve an excessive production of reactive oxygen species (ROS). Water supplies are often characterized by excessive organic waste that is decomposed by bacteria, using dissolved oxygen, leading to oxygen depletion. The potassium content of these waters may also affect negatively the mitochondrial metabolism and cellular ROS formation. This work focused on characterizing mitochondrial autofluorescence changes, with flavoprotein origin, and fluorescence ROS signals measured using the 2',7'-dichlorodihydrofluorescein diacetate indicator H_2DCFDA. All signals were evoked by hypoxia or by the depolarizing agent KCl (20 mM), at the hippocampal mossy fiber synapses of CA3 area. It was observed that both hypoxia and KCl-induced depolarization elicited a small rise in the autofluorescence and ROS changes. The hypoxia-induced signals were maintained upon normal reoxygenation, but of those evoked by KCl, the autofluorescence signals recovered during washout, while the ROS changes were irreversible.

Key words: Reactive oxygen species (ROS) — Flavoprotein — Flavin adenine dinucleotide (FAD) — 2',7'-dichlorodihydrofluorescein diacetate indicator (H_2DCFDA) — Rat hippocampus — Mossy fiber synapses

Introduction

The presence of large amounts of both organic and inorganic loads in wastewaters, from domestical, industrial and agricultural activities may have public health consequences (Liotta et al. 2009; Mosse et al 2011). For this reason, wastewaters are normally treated by a variety of processes before being delivered to the natural environment (Rashid et al. 2021). In wastewaters, excessive organic material is decomposed by bacteria, using oxygen, which leads to low oxygen levels in the waters. Hypoxia and higher amounts of inorganic content, like potassium, may cause various mitochondrial and synaptic dysfunctions, including higher formation of mitochondrial reactive oxygen species (ROS)

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(Chan 2001; Bancila et al. 2004). A very important signal of the mitochondrial metabolism is autofluorescence, the intrinsic fluorescence emitted by biological structures when excited by UV/Vis radiation. Some of these structures are associated with mitochondria including proteins that contain aromatic amino acids, such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) (Reinert et al. 2004; Shuttleworth 2010). When excited, with radiation of the appropriate wavelength, these proteins get in an excited state and decay to the ground state emitting fluorescence due to this energy loss (Monici 2005). The autofluorescence signals that are associated with the oxidation/reduction properties of the intrinsic proteins NADH and FAD (Reinert et al. 2004), have different spectral properties, with opposite polarities. In particular, FAD, which is more fluorescent than its reduced form, $FADH_2$ has a peak of excitation in the visible region, at 450 nm and an emission peak around 530 nm (Kosterin et al. 2005; Bartolomé and Abramov 2015). In mitochondria, the formation of ATP requires the donation, by the electron carriers NADH and $FADH_2$, of the tricarboxylic acid cycle (TCA), to complexes I and II (Nsiah-Sefaa and Mckenzie 2016). In this work the autofluorescence signals obtained had flavoprotein (FAD) origin.

Another major mitochondrial signal is formed by ROS, which serve as signaling molecules to regulate biological and physiological processes (Chan 2001). They are derived from oxygen and can oxidize other molecules so that they are considered as toxic byproducts of aerobic metabolism and can be used to describe many different molecules and free radicals derived from molecular oxygen (Turrens 2003; Sena and Chandel 2012; Schieber and Chandel 2014). The most abundant ROS in a living cell exist in the form of radicals, such as the superoxide anion (O_2^{-}), and hydroxyl radicals (•OH) and as non-radicals like hydrogen peroxide (H_2O_2) and singlet oxygen $^1O_2$ (Murphy 2009; Sena and Chandel 2012; Schieber and Chandel 2014; Zorov et al. 2014). Mitochondria are one of the most important sources of ROS within eukaryotic aerobic organisms. Its metabolic work cannot be performed without oxygen, which is involved in oxidative reactions in order to satisfy energy requirements (Duchen 1999). However, as oxygen can behave as a toxic agent, in excess, ROS is often associated with the occurrence of oxidative stress, which can affect mitochondrial metabolism therefore inducing cellular damage and physiological dysfunction. These changes, which can take place in lipids, proteins and nucleic acids, may give rise to neurodegenerative diseases, diabetes, cancer, and premature aging (Murphy 2009; Sena and Chandel 2012; Venditti et al. 2013; Schieber and Chandel 2014; Zorov et al. 2014; Fuhrmann and Brüne 2017; Ryu et al. 2018). When O_2 accepts an electron, the superoxide anion formed is considered a radical with one unpaired electron requiring a strong reducing agent in the electron transport chain. When receiving an electron, superoxide is reduced to hydrogen peroxide, which is more stable and can reach concentrations 100 times higher in the mitochondria, and is decomposed by catalase into oxygen and water (Marks et al. 1996; Buettner et al. 2006). Thanks to the high reactivity, ROS can also act as a cellular signaling agent, causing DNA damage, and altering the cellular antioxidant system (Comporti 1989; Liochev and Fridovich 1999). However, some antioxidant enzymes, like dismutase superoxide (SOD) are able to eliminate the excess of produced ROS.

Studying the oxidative activity can be a hard task, due to the presence of several ROS in one same living cell (Kalyanaraman et al. 2012). In this work, fluorescence changes were obtained from slices incubated with the fluorescent ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (ThermoFisher Scientific 2010). This dye diffuses into the cell and is deacetylated by cellular esterases forming a non-fluorescent compound which is oxidized by ROS, forming DCF, 2',7'-dichlorofluorescein. This compound, which is highly fluorescent, is usually detected using excitation and emission wavelenghts of approximately 495 nm and 529 nm, respectively (ThermoFisher Scientific 2010; Kalyanaraman et al. 2012). The ROS changes were obtained after subtracting, point by point, the FAD autofluorescence signals detected from non-incubated slices that were measured from H_2DCFDA containing slices.

These studies were performed in hippocampal slices of Wistar rats, which are members of various substrains originated from a common lineage during the last decades (McCormick 2017). Since the early times of the electrophysiology, the hippocampus has been the most studied brain region, because its structure is formed by a few parallel layers, in which the main synaptic systems are kept intact. The measurements were made undo severe hypoxic conditions (95% N_2, 5% CO_2), applying extracellular KCl (20 mM), to evoke membrane depolarization. It has been shown that severe hypoxia and oxygen-glucose deprivation increases neuronal activity after a short adaptation period. This enhancement is explained by the fall of the production of ATP and subsequent decrease of ATP-dependent potassium transporters (K^+ ATP-ases), leading to an increase in extracellular potassium concentration (Müller and Somjen 2000; Dzhala et al. 2001). Thus, in both types of stimulation, the rise of external potassium content depolarizes the cell and activates glutamate release, calcium entry and, in general, neuronal activity.

As mentioned in the Methods, the hippocampal slices were obtained from the brain of pregnant female rats, with normal pregnancies, whose fetuses were used by other research groups in order to share the animals. Many research works have addressed the characteristics of ROS formation during pregnancy in various organs, both in humans and

animals (Agarwal et al. 2005; Hussain et al. 2021). It has been found that a variety of physiological processes occurring at the placenta, which has important nutritional and oxygen requirements during pregnancy, are mediated by physiological amounts of ROS formation. Excessive ROS production may lead to pregnancy-associated diseases or reproductive failure (Agarwal et al. 2005; Hussain et al. 2021). During pregnancy, the rate of formation of ROS is proportional to the rate of consumption of oxygen, suggesting that enzymatic antioxidant activity increases with elevated ROS (Mover-Lev and Ar 1997). For these reasons, the same authors measured the activity of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and SOD, in various organs of pregnant Wistar rats. They have found that, in normoxia, there was a small effect of pregnancy on the brain enzymatic antioxidant activity, while that from the lungs was enhanced (Mover-Lev and Ar 1997).

Materials and Methods

All studies were carried out in accordance with the Directive 2010/63/EU of the European Parliament and Council to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

For the experiments, 8 to 13 weeks old pregnant Wistar-Hannover [CRL: WI (Han)] rats, with 16 to 18 days of gestation, were used. The study was carried out in brain slices while the fetuses were used in other experiments, by other groups, in order to reduce the number of animals used in research. The animals had a normal pregnancy and were fed *ad libitum* with free access to water, at a constant temperature (20–24°C) and relative humidity (50–75%) conditions, under 12 hours light/dark cycle. Before the experiments, the animals went to a one-week acclimatization period. The animals were anesthetized with isoflurane and then sacrificed by cervical dislocation followed by decapitation. The brain was immediately immersed in a previously oxygenated (95% O_2 and 5% CO_2) ice cold (5–8°C) ACSF solution. Then, 400 μm thick slices were obtained and kept in oxygenated (95% O_2 and 5% CO_2) ACSF, at room temperature. They remained there at least 1 hour before being used in an experiment. The ACSF medium had the following composition (in mM): NaCl 124; KCl 3.5; $NaHCO_3$ 24; NaH_2PO_4 1.25; $MgCl_2$ 2; $CaCl_2$ 2 and D-glucose 10; pH 7.4. The slices were subsequently transferred to the experimental chamber where they were perfused with ACSF, at a rate of 1.5 to 2 ml/min, at temperatures in the range 30–32°C. The KCl solution consisted of ACSF but with a 20 mM higher concentration of KCl. During the hypoxia periods, the normal oxygenation was replaced by an oxygen-free gas mixture (95% N_2, 5% CO_2).

The optical arrangement consisted of the fluorescence microscope Zeiss Axioskop, with an halogen light source and an excitation filter (480 ± 10 nm). The emitted light was collected through a water immersion lens (40×, N.A., 0.75) and a 500 nm long pass filter. Fluorescence signals were detected by a silicon photodiode connected to current-to-voltage converter with a feedback resistance of 1 GΩ. These signals were processed by means of a 16-bit A/D converter, at a frequency of 0.017 Hz, and were analyzed using the Signal Express ^{TM} software from National Instruments.

The fluorescent dye used to detect the formation of ROS, was H_2DCFDA, at a concentration of 20 μM. In the experiments designed to study autofluorescence changes, which were detected using 480 nm incident light and, thus, had FAD origin, the slices were not incubated.

The measurements are presented as mean ± SEM. Significance of statistical analysis was performed using a one-way ANOVA with *post-hoc* Tukey test. The results were considered significant for $p < 0.05$ and compare the baseline values with the ones obtained in the other two conditions.

Results

The production and the elimination of ROS is an important indicator of mitochondrial oxidative stress. In this work, autofluorescence and ROS activity was studied in non-incubated or in H_2DCFDA containing slices, making this indicator fluorescent complexes with certain ROS species.

In order to evaluate the effect of hypoxia in both autofluorescence and ROS production, the data shown in Figure 1 was obtained from non-incubated and incubated slices during periods of both normoxia (95% O_2, 5% CO_2) and hypoxia (95% N_2, 5% CO_2). All slices were initially perfused with ACSF for 30 min in normoxic conditions, being the baseline formed by the data collected from the last 10 min of this step. Figure 1A shows the total fluorescence trace obtained from slices incubated with the ROS indicator H_2DCFDA, with the same hypoxia protocol, which represents the sum of two components: autofluorescence and ROS fluorescence. It can be observed that the total fluorescence changes increased during hypoxia, being their amplitude 11 ± 2% at 65–70 min ($n = 5$) of control. When normal oxygenation was reintroduced, the signal returned to the baseline. Figure1B presents an autofluorescence signal that increased during the 60 min period of hypoxia, reaching an amplitude of 8 ± 1% (mean ± SEM, $n = 5$) at 65–70 min, with respect to baseline. Upon returning to normal oxygenation, the amplitude of the signal decreased, having amplitude of 4 ± 2% at 125–130 min. The fluorescence corrected by the autofluorescence component, which represents the true ROS changes, is shown in Figure 1C. The hypoxic conditions caused a small increase in the ROS signal, which reached a steady level, with an amplitude of 5 ± 2% at 65–70 min ($n = 5$).

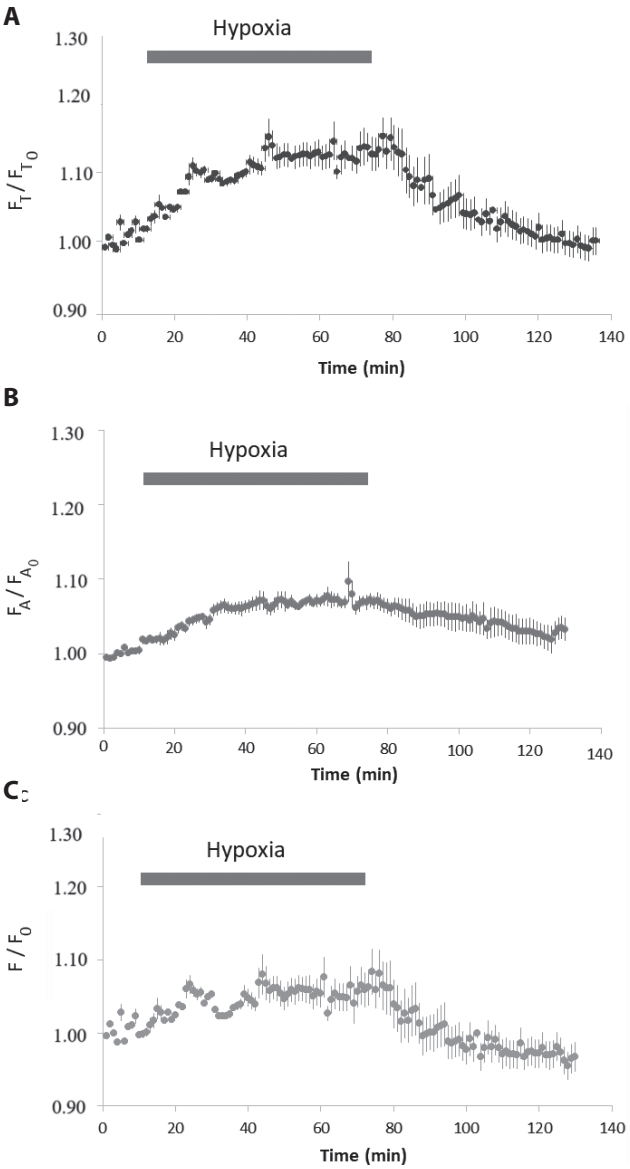

Figure 1. Effect of 60 min of hypoxia (95% N_2, 5% CO_2) on total fluorescence (F_T), autofluorescence (F_A) and ROS (F) signals. The ROS profile (**C**) was calculated as the point-by-point difference between traces (**A**) and (**B**). In all graphs, n = 5. All values were normalized by the average of the first 10 data points and represent the mean ± SEM. F_{A0}, basal autofluorescence; F_{T0}, basal total fluorescence; F_0, basal ROS fluorescence.

The next group of experiments addressed another possible source of neuronal autofluorescence and ROS potentiation: the depolarization evoked by extracellular application of KCl (20 mM). After the initial perfusion with ACSF, the KCl (20 mM) solution was applied during 30 min, followed by the reperfusion of the ACSF medium, during the same period of time. The total fluorescence signal shown in Figure 2A, had an amplitude 15 ± 1% (n = 5) higher than that of the baseline. In these experiments, upon ACSF reperfusion, the signals increased more before starting to decrease, exhibiting a small recovery. It can be observed in Figure 2B that in the presence of KCl, the amplitude of the autofluorescence rose by 11 ± 3%, at 35–40 min (n = 5), with respect to the basal values. After the removal of KCl, the signal declined without reaching the baseline, thus showing only a partial recovery. Subsequently, the fluorescence of slices incubated

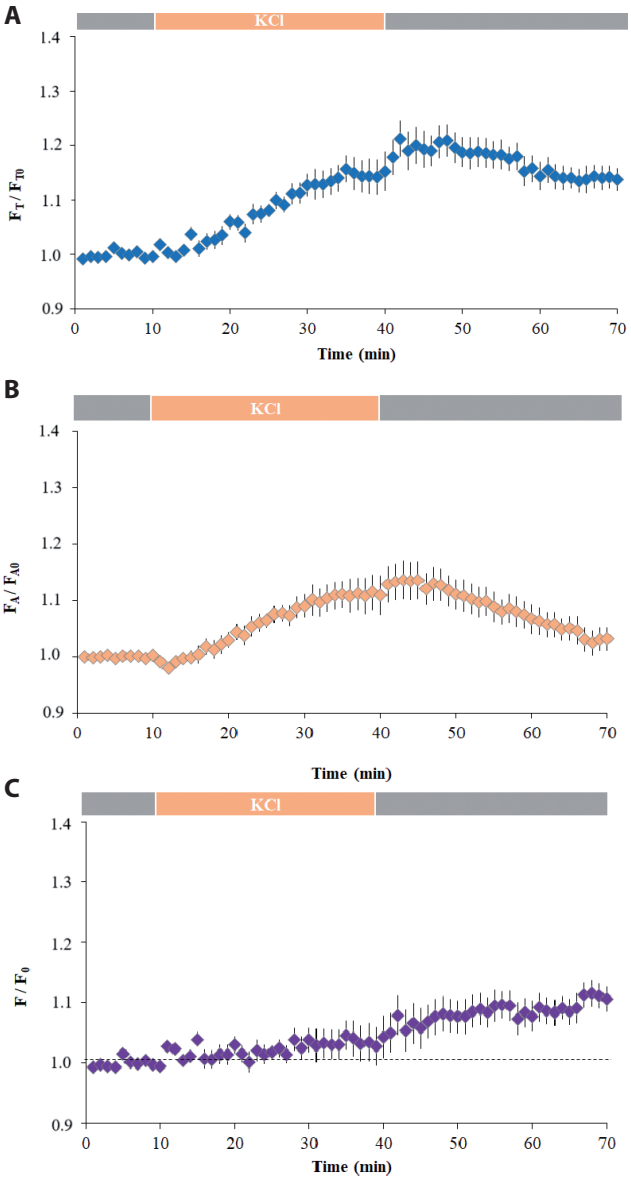

Figure 2. Total fluorescence (F_T; **A**), autofluorescence (F_A; **B**) and ROS (F; **C**) signals evoked by the application of 20 mM KCl for 30 min. F_T and F_A signals (n = 5) were obtained from H_2DCFDA incubated slices and F signals were calculated as the difference between the data from traces (A) and (B). All values were normalized by the average of the first 10 responses and represent the mean ± SEM. For more abbreviations, see Figure 1.

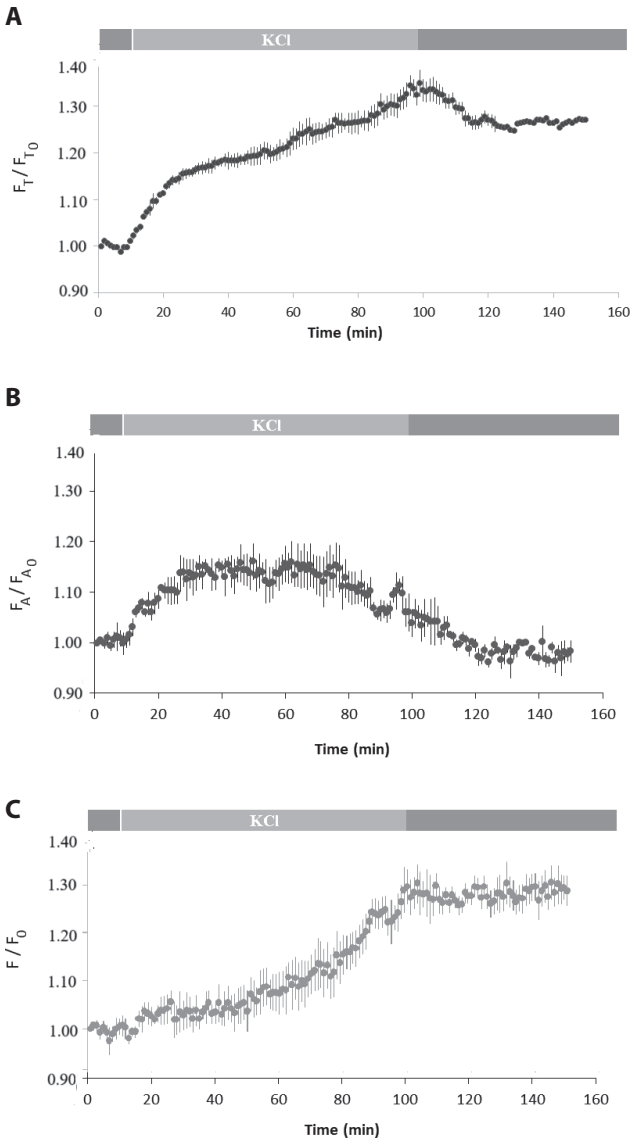

Figure 3. Intrinsic and ROS associated changes induced by a longer (90 min) application of KCl (20 mM). **A.** Total fluorescence (F_T) signals from H_2DCFDA incubated slices, $n = 5$. **B.** Autofluorescence (F_A) signals, $n = 5$. **C.** ROS (F) signals obtained as the difference between the data from panels (A) and (B). All values were normalized by the average of the first 10 responses and represent the mean ± SEM. For more abbreviations, see Figure 1.

with the ROS indicator H_2DCFDA was measured in order to determine the total fluorescence, which includes, again, the intrinsic fluorescence and the fluorescence associated with the production of ROS. The experimental procedures were similar to those applied in the non-incubated slices. Figure 2C represents the true ROS fluorescence signal, obtained as the difference between the data of Figure 2A and B, which was enhanced by 4 ± 3%, at 35–40 min, but its effect increased afterwards (Fig. 2C). Thus, adding KCl (20 mM) to the extracellular medium of hippocampal slices lead to both FAD and ROS potentiation that remained after KCl washout.

In order to determine the effect of a longer application of the same KCl solution, the second group of experiments involved the detection of the same types of signals, i.e. autofluorescence and total fluorescence changes, but evoked by KCl (20 mM) during a 90 min period. In H_2DCFDA containing slices there was a higher increase in the total fluorescence signal, which reached 34 ± 3%, at 95–100 min, as shown in Figure 3A and recovered only partially in the absence of KCl. In Figure 3B, containing data from indicator-free slices, it can be observed that in the presence of KCl (20 mM) the signal rose, having an amplitude of 9 ± 3%, at 95–100 min ($n = 5$). This signal recovered completely upon washout, as can be seen in the same panel. Panel 3C represents, as in the previous figure, the ROS fluorescence signal. This trace reached 25 ± 4%, at the same period of time, 95–100 min. A comparison with the first KCl results presented in this work suggests that, both the shorter (30 min) and the longer (90 min) expositions to KCl, cause a higher and reversible enhancement of the autofluorescence signal than that of the ROS signal that appears to be irreversible.

The bar graphs in Figure 4 summarize and compare the FAD autofluorescence, total fluorescence and ROS results, considered in the previous figures. It can be observed that the application of hypoxia evoked significant changes in both FAD-linked autofluorescence ($F = 56.51$; $df = 29$; $p < 0.05$) and ROS production ($F = 22.40$; $df = 29$; $p < 0.05$). The statistical analysis obtained for the application of KCl revealed that this agent affected significantly both FAD-linked autofluorescence ($F = 68.27$; $df = 33$; $p < 0.05$) and ROS production ($F = 14.47$; $df = 33$; $p < 0.05$).

Discussion

The preparation used in our work consisted of hippocampal slices from female rats that had normal pregnancies and thus were not subjected to externally induced oxidative stress. It is considered the existence of proportionality relationships between the rate of ROS production, the rate of tissue oxygen consumption and the activity of antioxidants enzymes (Mover-Lev and Ar 1997). The mentioned study reported that, during normoxia, pregnancy did not alter much brain enzymatic antioxidant activity, but increased the same type of activity in the lungs (Mover-Lev and Ar 1997). In our study, hypoxic conditions were only applied during the *in vitro* brain slice experiments and, thus, it is considered that the major conclusions of this work were not affected by pregnancy.

The main observations in these optical studies were that both autofluorescence and ROS enhancements were evoked by severe hypoxia and by KCl (20 mM) depolarization. These

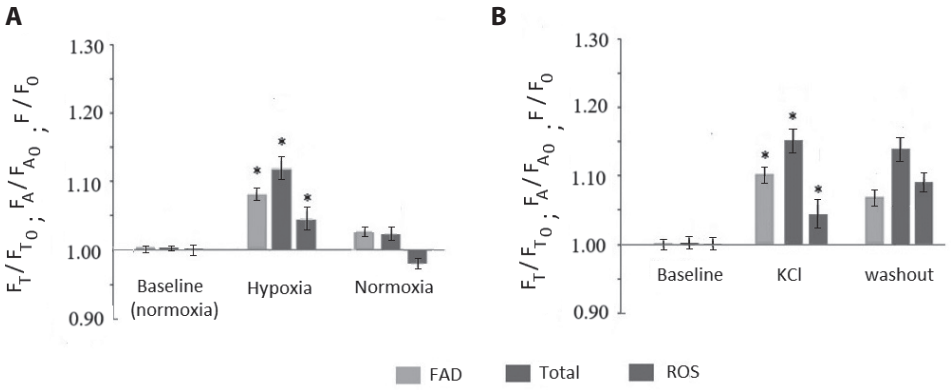

Figure 4. Comparison of the amplitude of FAD autofluorescence (F_A), total fluorescence (FT) and ROS (F) signals, from the hypoxia and KCl experiments. **A.** 60 min of hypoxia. **B.** 30 min of application of 20 mM KCl. The height of the bars represents the mean ± SEM. The statistical significance of the data was assessed by using a one-way ANOVA test with Tukey *post-hoc*, comparing the baseline with the last 5 min of each condition. Left side: for FAD fluorescence, the statistical values obtained were F = 56.51; df = 29 and the result was significant (* $p < 0.05$). In ROS production, the values were F = 22.40; df = 29 and the result was significant (* $p < 0.05$). Right side: both FAD-linked autofluorescence (F = 68.27; df = 33; * $p < 0.05$) and ROS production (F = 14.47; df = 33; * $p < 0.05$) were affected by KCl. The averaged values were obtained during the last 5 min of each perfusion medium, from the traces in Figures 1 and 2. For more abbreviations, see Figure 1.

results call the attention for the need to investigate the impact that wastewaters with high organic and inorganic contents may have in health and, in particular, in brain activity. The mentioned intrinsic and ROS enhancements may be due to various cellular processes that are represented in Figures 5 and 6. The observed increase of the FAD-linked autofluorescence is in the agreement with the idea that hypoxia may initiate an adaptive response that may be mediated by the hypoxia inducible factor (HIF), as indicated in Figure 5. This

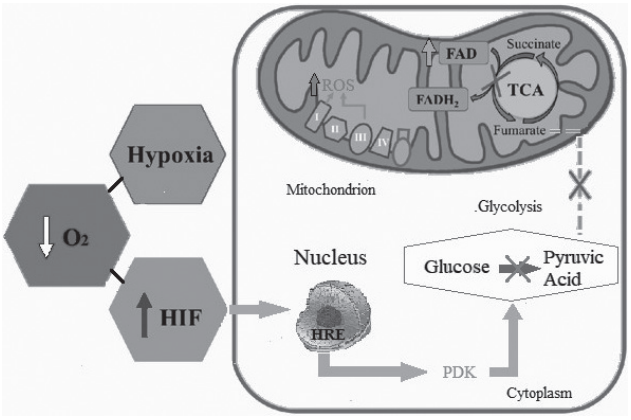

Figure 5. Cellular mechanisms of ROS production during hypoxic conditions. Hypoxia inducible factor (HIF) production is enhanced by hypoxia. This factor acts in the DNA nuclear chains, namely binding with hypoxia-responsive elements (HRE) and subsequentially activating the enzyme pyruvate dehydrogenase kinase (PDK). This enzyme blocks glycolysis, leading to a disruption in the tricarboxylic acid (TCA) cycle in the mitochondrial complex II. Consequently, there is a less efficient reduction of flavin adenine dinucleotide (FAD) to $FADH_2$.

factor binds to DNA fragments and activates the transcription of a large amount of genes allowing the cells to adapt to the hypoxic environment (Schumacker 2002; Solaini et al. 2010). HIF has the ability to bind to the hypoxia response element (HRE) and modify the expression of many genes, affecting the formation of pyruvate dehydrogenase kinase (PDK). This enzyme may inhibit glycolysis and thus the conversion of glucose to pyruvic acid. Without this transformation, mitochondrial activity is affected, namely TCA cycle, which occurs in the mitochondrial complex II. The inhibition of this cycle leads to a decrease in the reduction of FAD to $FADH_2$, thus enhancing FAD fluorescence under hypoxia (Semenza et al. 1991; Papandreou et al. 2006; Brahimi-Horn and Pouysségur 2007; Fuhrmann and Brüne 2017), as represented in the mitochondrion of Figure 5.

One possible explanation for the fact that the ROS signals had a less significant increase than autofluorescence may be that most of the oxygen arriving in mitochondria (nearly 90%) is used in the metabolic activity of the mitochondrial complex IV, and, thus, never gets reduced to ROS (Schumacker 2002). This suggests that during severe hypoxic conditions (95% N_2 and 5% CO_2), the absence of O_2 limits the production of mitochondrial ROS due to the lack of O_2 as a substrate for electrons (Schumacker 2002). On the other hand, it is known that complex III assumes an important role in ROS generation. An increasing ROS production may be explained by the ability of the complex III Q_0 center to not be affected by antioxidant defenses. This way, complex III releases ROS in the intermembrane space, far from the matrix with antioxidant defenses, favoring the release of ROS from mitochondria (Chen et al. 2003; Bell et al. 2007). An overproduction of ROS may also result from acquired and genetic defects in the respiratory chain of mitochondria near a binding site to antimycin A, an effective ROS inducer

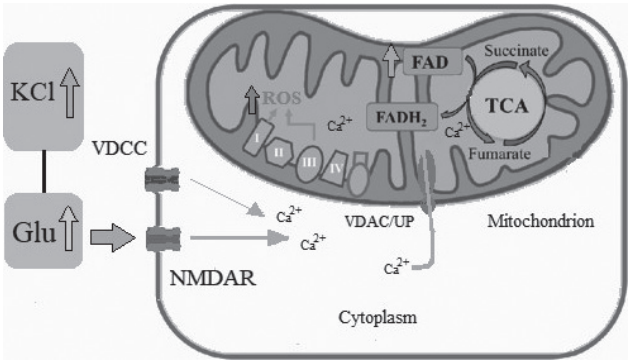

Figure 6. Neuronal mechanisms of FAD autofluorescence and ROS potentiation evoked by the application of KCl. The plasma membrane depolarization induced by KCl causes a rise in glutamate (Glu) release and in the subsequent calcium (Ca^{2+}) influxes through postsynaptic NMDA receptors (NMDARs) and voltage-dependent calcium channels (VDCCs). Therefore, cytosolic calcium increases and may enter mitochondria *via* voltage-dependent anion channels/calcium uniporter (VDAC/UP), stimulating the TCA cycle.

(Turrens et al. 2003; Brahimi-Horn and Pouysségur 2007; Zorov et al. 2014). In addition, under hypoxic conditions, antioxidant defense systems, such as SOD and GPx, significantly decrease in the brain. The consequent ROS overload is a major concern, since these species are highly reactive and capable of oxidizing lipids, proteins and DNA, leading to structural and functional cellular changes that can cause oxidative damage, apoptosis and necrosis in neurons (Lages et al. 2015; Coimbra-Costa et al. 2017).

The application of 20 mM KCl also caused an increase in autofluorescence and ROS production, involving a variety of cellular mechanisms that are shown in Figure 6. The increase of extracellular potassium caused a shift of the plasma membrane potential, from about −70 mV to −54 mV (Bancila et al. 2004). This depolarization leads to higher presynaptic calcium entry through voltage-dependent calcium channels (VDCCs) and to the subsequent potentiation of glutamate release. This, in turn, leads to an increase in the calcium influxes through postsynaptic N-methyl-D-aspartate (NMDA) receptors and VDCCs. The increase of the cytosolic calcium concentration in the postsynaptic cell evokes calcium entry into the mitochondria through voltage-dependent anion channels (VDACs) or the calcium uniporter (UP). Therefore, high levels of calcium stimulate the mitochondrial respiratory chain activity, potentiating the FAD-linked autofluorescence and also the formation of ROS. Furthermore, calcium stimulates enzymatic activity in the TCA cycle and promotes ATP synthesis and oxidative phosphorylation in mitochondria resulting in an increase of ROS levels (Görlach et al. 2015).

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