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CICLO XXI

H_2O_2 RELEASE BY ISOLATED MITOCHONDRIA AND A NEW APPROACH FOR THE EVALUATION OF H_2O_2 SOURCES IN INTACT CELLS

Direttore della Scuola: Ch.mo Prof. Giuseppe Zanotti

Supervisore: Ch.mo Prof. Adolfo Alexandre

Correlatore: Dr. Lucia Cavallini

Dottorando: Silvia Bortolami

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RIASSUNTO

L' H_2O_2 è un metabolita accertato nelle cellule viventi. A lungo considerata una forma attivata dell'ossigeno particolarmente dannosa, recentemente è stata rivalutata come mediatore fondamentale della funzionalità cellulare per i suoi molteplici ruoli in situazioni sia fisiologiche che patologiche. Oltre a riconoscerne un ruolo importante nei meccanismi di difesa contro patogeni viene ampiamente suggerita come molecola messaggero in una serie di comunicazioni intra e intercellulari grazie alla sua caratteristiche di stabilità e diffusibilità uniche fra i derivati reattivi dell'ossigeno (ROS). Può essere prodotta direttamente da alcune ossidasi o in seguito a dismutazione del superossido prodotto principalmente dalle NADPH ossidasi di membrana e dalla catena respiratoria mitocondriale.

Nella catena respiratoria mitocondriale il superossido ($O_2^{\bullet -}$), dismutato ad H_2O_2 dalla superossido dismutasi Mn-dipendente, viene prodotto soprattutto a livello del complesso I e risente del potenziale e dello stato redox di vari componenti la catena respiratoria. Tale produzione è bassa in presenza di substrati NAD dipendenti (nel nostro caso glutammato più malato), e alta, e rotenone sensibile, in presenza di succinato. Nella prima parte della tesi si evidenzia come la produzione di $O_2^{\cdot -}$ in mitocondri isolati possa avvenire a concentrazioni submillimolari di succinato, anche in presenza di substrati NAD dipendenti, condizioni ritenute simili a quelle fisiologiche. Inoltre la presenza di acil-CoA a lunga catena inibisce la produzione di H_2O_2 dipendente da succinato, in un modo indipendente dalla loro ossidazione. I mitocondri appaiono quindi una delle più probabili fonti di produzione fisiologica modulabile di H_2O_2 intra-cellulare.

La critica più pesante a studi fatti su preparazioni subcellulari è la loro effettiva congruità al modello cellulare o tissutale. Obiettivo del lavoro era quindi estendere i dati ottenuti in mitocondri isolati a un modello più complesso: la cellula intera. Il grosso limite allo studio è determinato dalla particolare esiguità della produzione di H_2O_2 in condizioni fisiologiche e dalla scarsa affidabilità dei metodi di misura intracellulari. Infatti, le principali sonde fluorescenti, come la diidrorodamina (DHR) o la diclorofluoresceina (DCFH) utilizzate per la detection dell'H_2O_2 in cellula intera non sono affidabili, come ampiamente discusso in

letteratura. Il basso segnale e la mancanza di specificità di queste sonde, a nostro avviso, è dovuta soprattutto alla carenza di uno catalizzatore per la reazione sonda/H_2O_2. Abbiamo pensato quindi di esprimere all'interno delle cellule l'enzima Horseradish Peroxidase (HRP), ampiamente utilizzato per la misura dell' H_2O_2 in esperimenti in vitro accoppiato a sonde spettroscopiche.

L'espressione dell'HRP all'interno delle cellule aumenta visibilmente la sensibilità delle sonde e la specificità della reazione con l'H_2O_2. Infatti è stato possibile misurare, per la prima volta in cellula intera, la produzione di H_2O_2 derivata dall'attività Mono-Ammino Ossidasica (MAO) come incremento di velocità della ossidazione della DHR o $DCFH_2$ indotta da Tiramina, substrato della MAO. L'integrità cellulare e la risposta metabolica non vengono modificate significativamente dalla trasfezione con HRP. Inoltre la specificità della misura (verificata con l'aggiunta esogena di H_2O_2 e per competizione con i sistemi di rimozione endogeni) conferma che le cellule trasfettate con HRP sono un buon modello per lo studio del coinvolgimento dell'H_2O_2 nella fisiologia cellulare. Nell'ultima parte della ricerca vengono presentati risultati preliminari che indicano come il metodo sia sufficientemente sensibile alla misura della quota di H_2O_2 di origine mitocondriale. Infatti si evidenzia una maggiore velocità di ossidazione della DHR in presenza di rotenone, simile all'incremento ottenuto nei mitocondri isolati in presenza di Glutammato/Malato. Inoltre si evidenzia un aumento dell'ossidazione della DHR in presenza del dietil-succinato, una forma permeabile di succinato. L'aumentata ossidazione viene inibita da difenilene iodonio, che si dimostra essere anche un potente inibitore dell'ossidazione e quindi della produzione di H_2O_2 sia dei substrati NAD dipendenti che del succinato in mitocondri isolati. Inoltre, come già ipotizzato in letteratura, mostriamo che la produzione di H_2O_2 dipendente dalla MAO è largamente superiore a quella mitocondriale indotta da alte concentrazioni di dietil-succinato in cellula.

Complessivamente il modello di cellule trasfettate con HRP sembra essere sufficientemente sensibile per misurare il contributo alla produzione di H_2O_2 di varie fonti intracellulari e quindi tale da poter essere utilizzato in varie condizioni fisiologiche o patologiche.

ABSTRACT

Reactive Oxygen Species (ROS), considered for many years as the unwanted toxic by-products of living systems in an aerobic environment, are now recognised as mediators of physiological functions. For H_2O_2, the more stable among ROS, a role as second messenger is suggested also for its permeability across membranes. H_2O_2 can be produced directly by several oxidases or after dismutation of superoxide ($O_2^{\cdot -}$) originating from the membrane NAD(P)H oxidases and from mitochondrial electron transport chain.

In the first part of the thesis are summarised some evidences obtained in brain and heart mitochondrial suspensions, of how Complex I can be responsible of most of the mitochondrial $O_2^{\cdot -}/H_2O_2$ release. The measure of H_2O_2 release was performed with the sensitive fluorescent method Amplex red/HRP. H_2O_2 release is low during the oxidation of the NAD linked substrates but high during succinate oxidation, a condition considered not physiological in the scientific literature being attributed to the high succinate concentration used. In this work the succinate dependent H_2O_2 production was measured also at submillimolar succinate concentrations, more consistent with physiological conditions. Moreover long chain fatty acyl-CoAs, but not fatty acids, act as strong inhibitors of the succinate dependent H_2O_2 release, showing that it can be modulated by the presence of these important metabolites. It was also shown that the inhibitory action of acyl-CoAs is independent of their oxidation, being relieved by carnitine and unaffected or potentiated by malonyl-CoA.

These data pose the question of how and in which conditions the mitochondrial ETC can give rise to a sustained H_2O_2 production in intact cells. In fact the majority of the evidences of the mitochondrial involvement comes from subcellular preparations. The aim of my work was then to extend the work done in mitochondria to intact viable cells

We soon realized that the use of “specific” fluorescent probes, often used for the H_2O_2 measurements, were unreliable as also largely debated in literature. We tried to overcome the general problem of the inadequacy of the H_2O_2 measurements in intact cells by transiently expressing HRP in two mammalian

cell lines, C_2C_{12} and SHSY-5Y. HRP is a well known specific H_2O_2 reagent when coupled to fluorescent probe and this detection system is widely accepted for H_2O_2 measurements in subcellular preparations.

HRP expression largely enhance the sensitivity and specificity of the detection of H_2O_2 by the cell ROS sensitive fluorescent probes $DCFH_2$ and DHR. In HRP expressing cells we are able to measure with fluorescence microscopy, but also directly at a fluorescent plate reader, the H_2O_2 production derived from a constitutive MAO activity for the first time in intact cells.

The viability and metabolic response confirmed that HRP transfected cells behave as the corresponding wild type. The specificity of the measurements (obtained with exogenous H_2O_2 delivery and for competition with endogenous H_2O_2 removal systems), and the limits and reliability of this procedure largely confirm that HRP transfected cells are suitable for studies involving H_2O_2 in cell physiology.

In the last part of the research we also obtained preliminary results, confirmed in isolated mitochondria in parallel, that the method allows measurements of the mitochondrial H_2O_2 release. We give evidence that the dynamic H_2O_2 measurements performed following the rate of oxidation of DHR is increased by addition of rotenone. The rise in cells is expected since the prevailing NAD dependent substrates should induce an higher H_2O_2 release in the presence of the Complex I inhibitor Rotenone as largely reported in mitochondria. The oxidation rate is also increased, again as in mitochondria, when a permeant form of succinate is exogenously added, and the increase is inhibited by DPI, which inhibits the oxidation of both NAD dependent substrates and succinate. Comparative analysis of the maximal rate of H_2O_2 production by succinate and by the MAO activity in HRP transfected cells shows that the ETC contribution to H_2O_2 production in the cells is far lower than the maximal production following the addition of the MAO substrate tyramine. Our data in cells are in agreements with our data in isolated mitochondria and as also suggested by several authors.

On the whole HRP transfected cells appear a suitable model to study different experimental conditions mimicking physiological or pathological conditions where ROS are thought to be involved.

1.0 INTRODUCTION

The presence of free radicals in biological materials was discovered less than 50 years ago [1]. Soon thereafter, Denham Harman hypothesized that oxygen radicals may be formed as by-products of enzymatic reactions in vivo. In 1956, he described free radicals as a Pandora's box of evils that may account for gross cellular damage, mutagenesis, cancer, and, last but not least, the degenerative process of biological aging [2]. The science of free radicals in living organisms entered a second era after McCord and Fridovich discovered the enzyme superoxide dismutase (SOD) and, finally, convinced most colleagues that free radicals are important in biology. A third era began with the first reports describing advantageous biological effects of free radicals. Mittal and Murard provided suggestive evidence that the superoxide anion ($O_2^{\bullet -}$), through its derivative, the hydroxyl radical, stimulates the activation of guanylate cyclase and formation of the "second messenger" cGMP [3]. At the beginning of the 21st century, there is now a large body of evidence showing that living organisms have not only adapted to an unfriendly coexistence with free radicals but have, in fact, developed mechanisms for the advantageous use of free radicals. Important physiological functions that involve free radicals or their derivatives include: regulation of vascular tone, sensing of oxygen tension and regulation of functions that are controlled by oxygen concentration, enhancement of signal transduction from various membrane receptors including the antigen receptor of lymphocytes, and oxidative stress responses that ensure the maintenance of redox homeostasis. The field of redox regulation is also receiving growing attention from clinical colleagues in view of the role that oxidative stress has been found to play in numerous disease conditions. These pathological conditions demonstrate the biological relevance of redox regulation. The delicate balance between the advantageous and the detrimental effects of free radicals is clearly an important aspect of life. The science of biological "redox regulation" is a rapidly growing field of research that has impact on diverse disciplines including physiology, cell biology, and clinical medicine.

Among reactive oxygen species the more stable H_2O_2 could be the link in the so called “redox regulation”. In fact in many instances these physiological phenomena have been first demonstrated using the exogenous addition of H_2O_2 and only later, and sometimes not, demonstrated with the endogenous generation of H_2O_2. In the light of the recent discover a sensitive method to detect endogenous H_2O_2 production needs to be developed.

1. 1 REACTIVE OXYGEN SPECIES

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. This unpaired electron(s) usually gives a considerable increase of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems. Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet -}$). Superoxide anion, arising either through metabolic processes or following oxygen “activation” by physical irradiation, is considered the “primary” ROS, and can further interact with other molecules to generate “secondary” ROS, either directly or prevalently through enzyme- or metal-catalysed processes. With estimates of 1–2% of the total daily oxygen consumption going to mitochondrial $O_2^{\bullet -}$ generation, a 60 kg woman would produce 160–320 mmol of $O_2^{\bullet -}$ per day, and correspondingly an 80 kg man 215–430 (based on an O_2 consumption of 6.4 l/kg/day) [4].

In biological systems, $O_2^{\bullet -}$is short-lived owing to its rapid dismutation to O_2 and H_2O_2 by SODs (superoxide dismutases). The charge on the $O_2^{\bullet -}$makes it unable to cross cellular membranes, except possibly through ion channels. In contrast, H_2O_2 has a longer biological lifespan than $O_2^{\bullet -}$, is relatively stable, and is easily diffusible within and between cells [5]. Under physiological conditions, the production of hydrogen peroxide is estimated to account for about 2% of the total oxygen uptake by the organism.

The hydroxyl radical, $OH^{\bullet}$, is the neutral form of the hydroxide ion is a strong oxidant specie. The $OH^{\bullet}$ has a high reactivity, making it a very dangerous radical with a very short in vivo half-life of approx. 10^{-9} s. Thus when produced in vivo $OH^{\bullet}$ reacts close to its site of formation. The redox state of the cell is largely linked to an iron (or copper) redox couple and is maintained within strict physiological limits. It has been suggested that iron regulation warrants that iron is essentially not free; however, in vivo, under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules. The released Fe^{2+} can participate in the Fenton reaction, generating highly reactive $OH^{\bullet}$:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

The superoxide radical participates in the Haber–Weiss reaction:

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{-}$$

keeping iron in Fe^{2+} state by the reduction of Fe^{3+} by superoxide:

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$

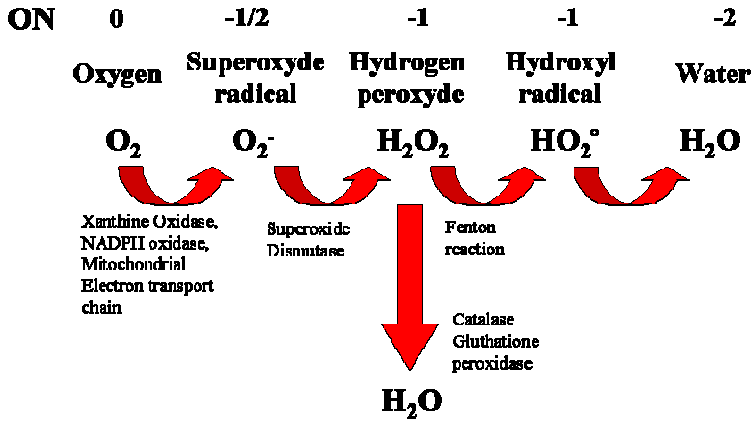


Figure 1: Consecutive reduction of O_2 yields ROS. Step 1, the conversion of dioxygen to superoxide is endothermic. The following steps are exothermic. Under the arrow are indicated enzymes for the convertion of O_2 into superoxide radical and further conversion. The reduction via the $OH^{\bullet}$ to water occurs non-enzymatically.

Additional reactive radicals derived from oxygen that can be formed in living systems are peroxyl radicals ($ROO^{\bullet}$). The simplest peroxyl radical is $HOO^{\bullet}$, which is the protonated form (conjugate acid; pKa 4.8) of superoxide and is usually termed either hydroperoxyl radical or perhydroxyl radical.

1.2 SOURCES OF H_2O_2 IN CELLS

H_2O_2 can be produced inside the cells in two ways: directly or after $O_2^{\bullet-}$ dismutation. Among the source of H_2O_2, direct and indirect, inside the cells the most important are:

- Mitochondria: in the 1973 Boveris and his colleagues estimate the total rate of the H_2O_2 production in the order of 90 nmol/min per g wet of liver; and about 13% of that production (i.e. 12 nmol/min per g wet of liver) was attributed to the mitochondria. Now, a measurable ROS production by at least nine of the mammalian mitochondrial enzymes has been reported. The nine enzymes are ubiquitously present in mammalian mitochondria but their capacity in producing ROS, as well as their expression, varies greatly among tissues and species.

- Amine oxidases (AOs): are widely distributed among all living organisms and catalyze the oxidative deamination of biogenic amines accompanied by release of H_2O_2. This is a quantitatively large source of H_2O_2 that contributes to an increase in the steady state concentrations of reactive species.

- NADPH oxidase: is a membrane-bound enzyme complex. It can be found in the plasma membrane as well as in the membrane of phagosome. The complex is normally latent in neutrophils and is assembled and activated in the membranes during respiratory burst. It generates superoxide by transferring electrons from NADPH inside the cell across the membrane to molecular oxygen to produce the superoxide. After the discovering from Lambeth and his co-workers that this

enzyme is present also in non-phagocytic cells [6] the hypothesis that H_2O_2 could be a second messenger became more strong.

- Hypoxantine/xanthine oxidase: dehydrogenases involved in conversion of hypoxanthine to xanthine and xanthine to uric acid, as the final catabolism of purines. Mammalian native xanthine dehydrogenase can be by-oxidatively converted to xanthine oxidase which produces both superoxide anion and hydrogen peroxide [7]. It has been implicated as a key oxidative enzyme in diseases as hypertension and ischemia.

- In addition, microsomes and peroxisomes are sources of ROS. Microsomes are responsible for the 80% H_2O_2 produced in vivo concentrated at peroxisomal sites. Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet -}$, under physiologic conditions. Although the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these H_2O_2-generating mechanisms. Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H_2O_2 production as a result of prolonged starvation [8].

1.2.1 Mitochondrial membrane

ROS generation within mitochondria is closely associated with the primary function of these organelles, namely oxidative metabolism and ATP synthesis. The machinery of mitochondrial metabolism ultimately leads to ATP synthesis by the F1Fo-ATPase (also termed Complex V) in the inner membrane of mitochondria. This process involves oxidation of metabolites in the tricarboxylic acid cycle, resulting in the formation of NADH and $FADH_2$. These reducing equivalents supply electrons derived from the metabolism of substrates to the respiratory chain, which comprises four enzyme complexes, ubiquinone and cytochrome c. Electron transfer is driven by the redox potential of the individual components of the respiratory chain, and protons are translocated across the inner membrane by Complexes I, III and IV functioning as proton pumps. The drop in

redox potential during the transfer of electrons through the respiratory chain generates the protonmotive force (pmf or Δp, expressed in mV), which drives the synthesis of ATP by the F1Fo-ATPase. The Δp has two components: the mitochondrial membrane potential (Δψ) and the pH gradient across the mitochondrial inner membrane (ΔpH). However, under most conditions, Δp is dominated by Δψ. This potential, estimated to be 150–180 mV (negative on the matrix side), influences virtually all mitochondrial functions and is a key parameter that indicates the bioenergetic competence of mitochondria. During oxidative phosphorylation, electrons are eventually transferred to molecular oxygen, and H_2O is produced by Complex IV via a sequential four-electron transfer. However, a proportion of oxygen in mitochondria is reduced only partially, and this one-electron reduction results in the generation of superoxide.

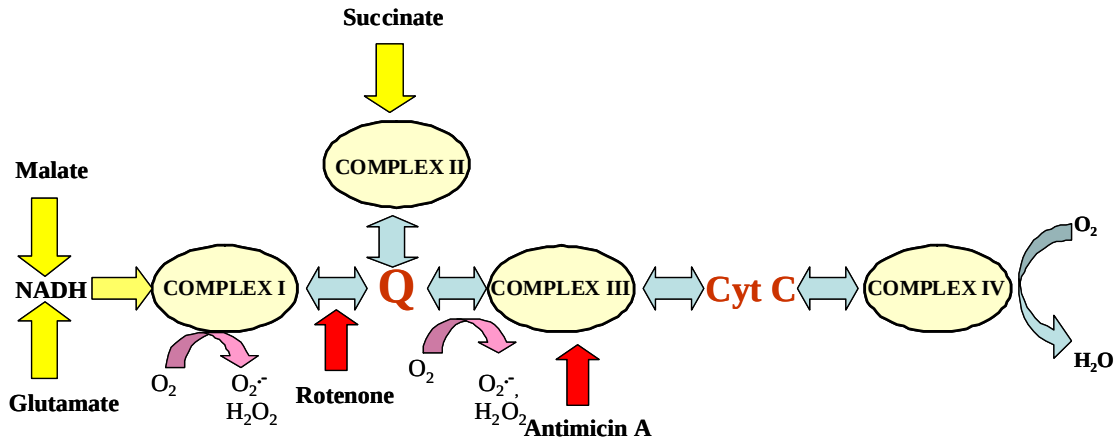


Figure 2: General view of ROS generation in mitochondrial electron transfer chain

In mammalian mitochondria most of the superoxide originates from Complex I (NADH:ubiquinone oxidoreductase) and is released into the mitochondrial matrix [9-12]. Forward electron transport into Complex I from NAD-linked substrates produces little superoxide. When Complex I inhibitors such as rotenone are added, this low rate increases, but not usually to the rate seen with reverse electron transport. Instead when mitochondria oxidize the Complex II substrate succinate in the absence of electron transport chain inhibitors, the rate of superoxide production is high. However, this rate is almost abolished when the Complex I inhibitor rotenone is added [13]. High superoxide production from

Complex I is driven by the high proton motive force generated from proton pumping by Complexes III and IV. This superoxide production is primarily on the matrix side of the inner membrane [12, 14]. This asymmetry of superoxide production between forward and reverse electron transport has been investigated leading to the conclusion that high rates of superoxide production from Complex I in vitro require an adequate supply of electrons, a high pH gradient across the membrane[14], and a particular configuration achieved by reverse electron transport or by inhibition of Complex I with high concentrations of myxothiazol (more usually used as a potent Complex III inhibitor). Moreover superoxide production rate is linked to the magnitude of the membrane potential, and the high superoxide production from Complex I during reverse electron transport is particularly sensitive to membrane potential and to mild uncoupling [11].

The physiological rate of the mitochondrial production of $O_2^{\bullet-}$ and H_2O_2 associated with the electron-transfer chain is dependent on the mitochondrial metabolic state: the resting mitochondrial state 4 is characterized by a relatively slow rate of respiration and no availability of ADP and is associated with a relatively high rate of $O_2^{\bullet-}$ and H_2O_2 production, probably as a consequence of the high reduction state of the components of the respiratory chain. Conversely, the active mitochondrial respiratory state 3, with a high rate of oxygen uptake and ample availability of ADP, shows a relatively slow rate of $O_2^{\bullet-}$ and H_2O_2 production due to the highly oxidized state of the components of the respiratory chain. Finally, in the anoxic state 5, with a limitation in O_2 supply and a lack of respiration, no partial reduction of O_2 to $O_2^{\bullet-}$ or H_2O_2 occurs. In addition to metabolic state, pO_2, and ADP availability, mitochondrial production of active oxygen species is also modulated by nitric oxide through mechanisms involving reversible binding to cytochrome oxidase, inhibition of electron transfer at the bc1 segment, and oxidation of mitochondrial ubiquinol to ubisemiquinone with subsequent autoxidation and generation of superoxide [15].

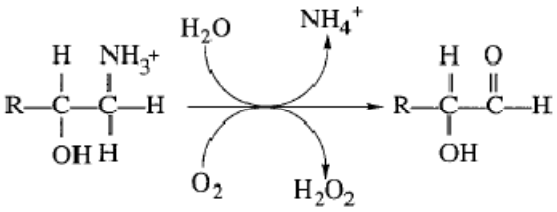
Complex III (ubiquinol: cytochrome *c* oxidoreductase) also has a large capacity to produce superoxide, at least in the presence of antimycin, a specific inhibitor of center ‘‘i’’ of Complex III. Myxothiazol (an inhibitor of center ‘‘o’’ of Complex III) decreases superoxide production, suggesting that center o is

responsible for its production within Complex III. Structurally, center o faces the cytosolic side of the membrane, but it seems that the superoxide produced at this site appears at both sides of the inner membrane [12, 16]. Although robust ROS generation by Complex III has been repeatedly demonstrated, this appears of limited physiological importance because it is produced by Complex III only under artificial conditions.

In addition to the respiratory chain, a significant amount of ROS can be produced by the isoenzyme α-glycerophosphate dehydrogenase (α-GPDH). The α-GPDH is localized on the outer surface of the mitochondrial inner membrane donating electrons to the respiratory chain via co-enzyme Q. α-GPDH is part of the glycerophosphate shuttle, with a function of transporting reducing equivalents from the cytosol to the mitochondria. Mitochondrial oxidation of α-glycerophosphate (α-GP) is able to generate a membrane potential and has been reported to result in ROS generation in mitochondria deriving from different tissues including the brain. Results concerning the mechanism of α-GP-supported ROS generation in brain mitochondria are controversial, but as reported by Adam-Vizi [17] it seems that ROS formation in the metabolism of α-GP involves reverse electron flow via Complex I, similarly to the succinate-supported formation. α-GP-mediated ROS production presents also a component independent of reverse electron transfer associated with the autoxidation of the flavoenzyme α-GPDH.

1.2.2 Amine oxidases

Amine oxidases (AOs) are widely distributed among all living organisms and catalyze the oxidative deamination of biogenic amines accompanied by release of H_2O_2, aldehyde and ammonia (primary amines) or a substitutes amines (secondary amines).



This class of enzymes is divided into two subclasses: FAD-containing amine oxidases (FAD-AOs) and Cu-containing amine oxidases (Cu-AOs).

1.2.2.1 Monoamine oxidase

Monoamine Oxidase (MAO; EC 1.4.3.4) are flaovoenzymes located in the outer mithocondrial membrane. They exists in two iso-forms, MAO-A and MAO-B, distinguished by their substrate specificity and inhibitor affinity [18-20]. MAO-A preferentially deaminates 5-hydroxytryptamine (serotonin), adrenaline and noradrenaline, and is irreversibly inhibited by clorgyline at low micromolar concentrations [21]. MAO-B preferentially deaminates β-phenylethylamine and benzylamine and is inhibited by deprenyl and pargyline [22, 23]. Dopamine, tyramine, and tryptamine are common substrates for both forms of MAO. Classification by means of specific inhibitors has been more convenient for the identification of different types of amine oxidase, however the substrates and inhibitors which are generally used to distinguish MAO-A and MAO-B, are not entirely specific for the respective MAO form [24].

The two iso-forms are encoded by separate, highly homologous genes located in the X-chromosome and are independently regulated [25].

While both MAO-A and MAO-B can be detected in most human tissues examined, they are however distributed differently in certain cells and tissues. For example, human placenta expresses predominantly MAO-A; human platelets and lymphocytes contain only MAO-B [26, 27]. The neural distributions of MAO-A and B enzyme activities are different. In human brain, high levels of MAO-B are expressed in astrocytes and serotonergic neurons, while high levels of MAO-A are expressed in catecholaminergic neurons. A similar distribution has been observed in rats and in monkeys. Furthermore, MAO-A and B expression is also different during development. MAO-A activity appears before MAO-B activity in fetal brain, whereas MAO B activity is higher than MAO-A in adult human brain. The results of Northern analysis of MAO-A and B transcripts are consistent with the distribution of their catalytic activities [28].

It is commonly accepted that MAO may play a critical role in the regulation of central nervous system activity and contribute to the pathogenesis of human neurodegenerative and depressive disorders. Fifty years ago the first generation of

MAO inhibitors was developed and applied in therapy as anti-depressive compounds. However, for many years MAO inhibitors were considered useless in therapy due to the serious side effects induced by these drugs. Recently, MAO and its inhibitors are again in the centre of scientific and pharmacological interest, providing new drugs for the therapy of Parkinson's disease, Alzheimer's disease, and various types of depression.

1.2.2.2 Semicarbazide-sensitive amine oxidase

Semicarbazide-sensitive amine oxidase (SSAO) is a group of enzymes containing copper and topaquinone and sensitive to semicarbazide. Topaquinone cofactor derives from a tyrosine that is post-translationally modified to a quinone in an autocatalytic reaction. The enzyme is present either as membrane or soluble forms located in the vascular system and adipocytes [29]. SSAO essentially metabolizes amine compounds similar to MAO-A and MAO-B. However, the enzyme activity is not inhibited by clorgyline and deprenyl, whereas it is almost completely inhibited by semicarbazide. In contrast, semicarbazide has a weak inhibitory effect on MAO at a concentration of 0.1-1mM. Clorgyline and deprenyl have been regarded as virtually inactive against the tissue-bound SSAO, nevertheless they showed moderate reversible competitive inhibition against bovine and human plasma SSAO at a concentration of 0.1-1mM. Aminoguanidine has been used for many years as an inhibitor of Cu-AO, but it is a relatively weak and nonselective inhibitor of SSAO [24].

It has been shown that this enzyme may be involved in detoxifying xenobiotic amines, regulating glucose uptake, affecting cell adhesion, modulating leukocyte trafficking and may be involved in angiogenesis. Increased serum SSAO activities were found in patients with diabetic complications, vascular disorders and heart disease [30].

Interestingly, the SSAO is now recognised also as VAP-1 The discovery was completely independent from SSAO research. The sequence of the protein called VAP-1 has been found to be identical to SSAO [31]. Indeed, VAP-1 has been shown to be capable of deaminating amines. Its distribution is also very similar to that of SSAO. VAP-1, which contains polysialic acid, induces cell adhesion and regulates lymphocyte trafficking. VAP-1 is involved in granulocyte extravasation

and its level has been shown to be up-regulated during inflammation. It is intriguing that a single protein possesses such completely different functions. It remains to be established whether or not the two functions of this protein may act in a concerted fashion. It is perhaps interesting to note that formaldehyde, the deaminated product of methylamine, can induce inflammation and has been used as an agent to elicit inflammation (i.e. arthritis).

1.2.3 NADPH oxidases

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (also called NOX enzymes) are enzymes that catalyze the production of superoxide ($O_2^{\cdot -}$) from oxygen and NADPH, according to the following reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet -}$$

These enzymes, which makes very large amounts of superoxide, are found in professional phagocytes (neutrophils, eosinophils, monocytes and macrophages) at certain stages of their development. The function of NADPH oxidase in professional phagocytes, however, is to provide agents that kill organisms that are in contact with the phagocytes. These organisms can be ingested, in the case of neutrophils, monocytes and macrophages, and activate eosinophils, which kill metazoans such as worms. The oxidizing agents generated by NADPH oxidase include H_2O_2, which is produced by the dismutation of superoxide. Other oxidizing agents generated by NADPH oxidase include HOCl, which is generated by the H_2O_2-mediated oxidation of Cl^-, a reaction catalyzed by myeloperoxidase, $^1O_2$ which is derived by the reaction of HOCl and H_2O_2, ozone whose origin is at present mysterious, and $OH^{\bullet}$, which is postulated to arise from the oxidation of reduced metals (Fe^{2+} or Cu^+) by H_2O_2.

The structure of NADPH oxidase is quite complex, consisting of two membrane-bound elements (gp91PHOX and p22PHOX), three cytosolic components (p67PHOX, p47PHOX and p40PHOX), and a low-molecular-weight G protein (either rac 2 or rac 1). The racs are kept inactive by binding to a guanine nucleotide dissociation inhibitor, which prevents the exchange of guanine

nucleotides from the rac proteins. Activation of NADPH oxidase is associated with, and probably caused by, the migration of the cytosolic components to the cell membrane so that the complete oxidase can be assembled [32].

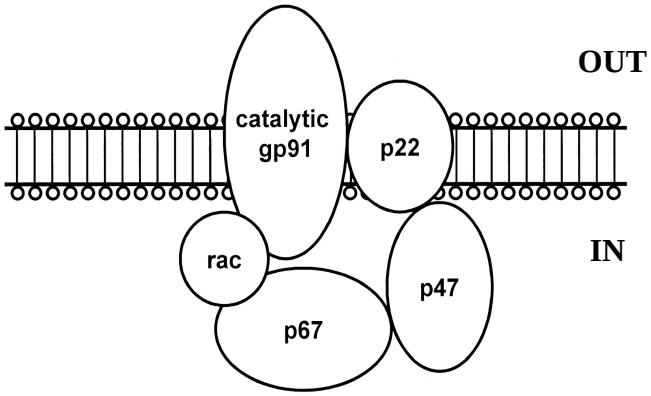


Figure 3: NADPH oxidase structure

The discovery of a group of NADPH oxidases (the Nox family) [6] with unique tissue localization in non-immune cells and whose activity or level is regulated by growth factors has reinforced the idea that ROS play a biologically important role. Evidence is rapidly accumulating that low level of ROS can be produced by NADPH oxidase homologs in non-phagocytic cells. To date, six human homologs (Nox-1, Nox-3, Nox-4, Nox-5, Duox-1 and Duox-2) have been recently identified in a variety of non-phagocytic cells. The identification of Nox-1 was quickly followed by the cloning of Nox-3, Nox-4, and Nox-5. In parallel, two very large members of the Nox family were discovered, namely Duox-1 and Duox-2, initially also referred to as thyroid oxidases. The physiological functions of Nox-dependent ROS generation are in progress and still require detailed characterization.

1.3 OXIDATIVE DAMAGE TO MOLECULES

1.3.1 Nuclear and mitochondrial DNA damage

Reactive oxygen species are formed through a variety of events and pathways. It has been estimated that one human cell is exposed to approximately 1.5×10^5 oxidative hits/day from $OH^{\bullet}$ and other reactive species [33]. The $OH^{\bullet}$ is known to

react with all components of the DNA molecule: damaging both the purine and pyrimidine bases and also the deoxyribose backbone [34]. Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis and ageing. In fact, as it is well established, in various cancer tissues free radical-mediated DNA damage has occurred. To date, more than 100 products (like 8-hydroxyguanine, 8-OH-G) have been identified from the oxidation of DNA. ROS-induced DNA damage involves single or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis.

1.3.2 Lipid peroxidation

It is known that metal-induced generation of ROS results in an attack not only on DNA, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation [35]. Once formed $ROO^{\bullet}$ can be rearranged via a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) [36]. The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE). These unsaturated highly reactive aldehydes can modify protein structure and function reacting with protein amino groups. MDA is found mutagenic in bacterial and mammalian cells and carcinogenic in rats. HNE is weakly mutagenic but appears to be the major toxic product of lipid peroxidation.

1.3.3 Amino acids

Exposure of proteins to radical attack in the presence of O_2 results in multiple changes in the target molecule. These can include the oxidation of side-chain groups, backbone fragmentation, crosslinking, unfolding, changes in hydrophobicity and conformation, altered susceptibility to proteolytic enzymes, and the formation of new reactive groups (e.g., carbonyls, hydroperoxides, and 3,4-dihydroxyphenylalanine). Ultimately, these processes can result in the loss of

structural or enzymatic activity of the protein and, hence, biological perturbations. Backbone fragmentation is believed to occur predominantly via the formation of carbon-centered radicals, and subsequently peroxyl species, at the α-carbon position. The peroxyl radicals are believed to give rise to fragmentation via two major pathways: one involving elimination of HO_2^- and hydrolysis of the subsequent imine to form an amide and α-diketo species, and a second involving β-scission of an α-carbon alkoxyl radical, formed from the $ROO^{\bullet}$ (either via dimerization and decomposition of a tetroxide or via hydroperoxide formation and subsequent decomposition) [37]. The oxidation of other amino acids residues bring to formation by many different mechanisms of carbonyl groups The concentration of carbonyl groups so generated is then a measure of ROS-mediated protein oxidation. A number of highly sensitive methods have been developed for the assay of protein carbonyl groups [38].

Also the side chains cysteine and methionine residues are susceptible to oxidation. Numerous classes of proteins contain free cysteine residues that are highly conserved across species, suggesting regulatory possibilities beyond structural roles and metal ion coordination. In addition, cysteine residues can be modified through alternative redox-based modifications (SNO, SOH, SSG, S-S) that may enable differential effects on protein function. In other words, distinct reversible modifications of cysteines may lead to unique functional outcomes as well as providing a mechanism through which differential responsivity can be achieved. For example, SOH modification (hydroxylation) of a single allosteric cysteine has been shown to elicit effects on structure and function that are distinct from those elicited by mixed disulfide (glutathionylation). Cysteines are thus believed to serve as molecular switches, capable of processing different redox-based signals into distinct functional responses [39].

1.4 TRANSPORT OF H_2O_2

The multifunctionality of H_2O_2 is due to its chemistry. The oxygens of H_2O_2 have an intermediate oxidation number of "−1", explaining both reducing and

oxidizing properties. For thermodynamic reasons one would expect hydrogen peroxide to exist primarily in the linear or trans-planar conformation (fig. 4A,B). In this way, the two hydrogen atoms would stay as far as possible from each other resulting in a nonpolar arrangement due to a symmetrical distribution of charge. The determination of the dipole moment, however, revealed a permanent dipole moment even higher than that of water suggesting a skewed cis conformation (Fig. 4C) with the hydrogen atoms pointing in the same direction relative to the O\O bond and the lone electron pairs oriented in the opposite direction to the hydrogen atoms. The lone electron pairs do not allow free rotation around the O—O bond, resulting in a permanent dipole. With its two pKa values of about 11 and 16 it is uncharged and protonated at physiological pH. Although chemically H_2O_2 is a ROS it shares surprisingly many physical features with water. The permanent dipole is likely to be important for cellular function and transport of H_2O_2.

The concentration of H_2O_2 in a cell is defined by influx and intracellular formation as well as by scavenging and efflux.

$[H_2O_2]$= Influx + Production - Efflux - Scavenging

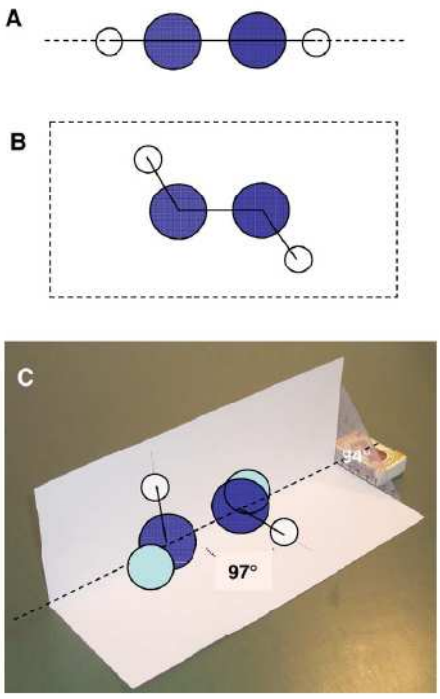


Figure 4: Three possible conformations of H_2O_2. Oxygen atoms are in blue and hydrogen atoms are in white. (A) Linear conformation resulting in a non-polar arrangement. (B) Trans-planar conformation resulting in a non-polar arrangement. (C) Skewed cis conformation resulting in a polar arrangement with both hydrogen atoms oriented to the same side relative to the oxygen–oxygen bond [43].

H_2O_2 has often been believed to freely cross membranes [40], which adequately explains various physiological facts. Contrary to this, recent studies [41, 42] point out that some membranes are rather poorly permeable to H_2O_2. This implies that transport of H_2O_2 may be

regulated and that this regulation constitutes a major factor in the determination of cellular H_2O_2 concentration. These differences in permeability could either be explained by changes in membrane lipid compositions or by diffusion-facilitating channel proteins or a combination of both.

Membrane composition indeed affects transmembrane H_2O_2 diffusion. Also, mechanical changes imposed by osmotic stretching of lipid bilayers change H_2O_2 diffusion [43]. Consequently, cells are potentially able to control transmembrane H_2O_2 diffusion by changing their osmotic pressure.

Another possible way to transport hydrogen peroxide are aquaporins. Aquaporins are known as diffusion facilitators for a growing number of non-charged and partially polar solutes such as glycerol, urea, CO_2, polyols, purines, pyrimidines, NH_3 and trivalent inorganic forms of arsenic and antimony. Water, for example, can cross membranes. Nevertheless, water also passes through aquaporins with much higher capacity, a diffusion facilitation that is believed to be physiologically highly relevant. The size and the electro-chemical properties of solutes are the main factors determining the diffusion through aquaporins.

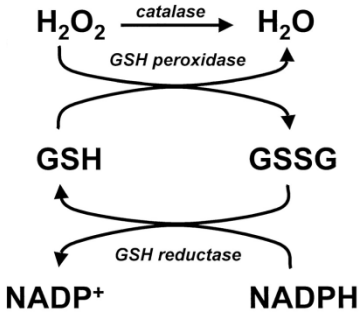
Exactly these features qualify H_2O_2 as a possible aquaporin substrate. H_2O_2 possesses almost the same dipole moment, dielectric properties and capacity to form hydrogen bonds as does water. The slightly larger dipole moment of H_2O_2 makes simple diffusion through the hydrophobic lipid bilayer even less likely than for water. The mean diameter of about 0.25–0.28 nm of H_2O_2 compared to the pore size of human AQP1, a typical aquaporin, of 0.30 nm is also compatible with passage through aquaporins. When water channels were inhibited with the blocker mercuric chloride ($HgCl_2$), the permeabilities of both water and H_2O_2 were substantially reduced. In fact, for the latter, it was not measurable. It is suggested that some of the water channels in *Chara corallina* (and, perhaps, in other species) serve as ‘peroxoporins’ rather than as ‘aquaporins’ [44].

1.5 DEFENCE MECHANISMS

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms [45]. Defence mechanisms against free radical-induced oxidative stress involve: preventive removal systems, repair mechanisms, physical defences, and antioxidant defences. Halliwell and Gutteridge [46] have defined antioxidants as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates. This definition includes non-enzymatic antioxidants represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione, carotenoids, flavonoids and the enzymatic antioxidant defences [47].

1.5.1 Enzymatic defence

- **Glutathione peroxidase:** is the general name for a family of multiple isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor:



$$H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2\ H_2O$$

In mammalian tissues, there are four major selenium dependent GPx isozymes: (a) classical GPx (GPx1), which is found in red cells, liver, lung and kidney;

(b) gastrointestinal GPx (GPx2);

(c) plasma GPx (GPx3), which is present in different organs such as kidney, lung, epididymus, vas deferens, placenta, seminal vesicle, heart and muscle;

(d) phospholipid GPx (PHGPx4 or GPx4), which is also broadly distributed in different tissues.

GPxs also have distinct subcellular locations: GPx1 was identified in the cytosol, nucleus and mitochondria; GPx2 accumulates in the cytosol and nucleus;

GPx3 is a secreted protein also found in the cytosol, whereas GPx4 is present in the nucleus, cytosol, mitochondria and bound to membranes. Two other isozymes, GPx5 and GPx6, identified in mammals, are both closely related to GPx3. However, GPx5 lacks the selenocysteine at the active site and is secreted in epididymus. GPx-6 was identified in humans and pigs and is a selenium-dependent GPx found in the olfactory epithelium [48].

- **Catalase:** the reaction catalyzed by catalases is a dismutation (a redox reaction involving two molecule of the same specie one acting as reductant and the other as oxidant) of two molecules of hydrogen peroxide to water and oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

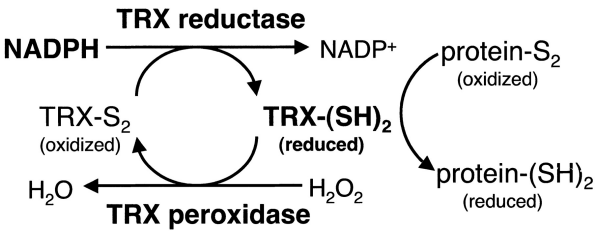
It is a tetrameric enzyme consisting of four identical, tetrahedrally arranged subunits of 60 kDa, each containing in its active center a heme group and NADPH. Catalase has two enzymatic activities depending on the concentration of H_2O_2. If concentration of H_2O_2 is high, catalase acts catalytically, i.e. removes H_2O_2 by forming H_2O and O_2 (catalatic reaction). However, at low concentration of H_2O_2 and in the presence of a suitable hydrogen donor, e.g. ethanol, methanol, phenol, and others, catalase acts peroxidically, reducing H_2O_2 to H_2O but oxidizing its substrate (peroxidatic reaction).

- **Superoxide dismutase (SOD)** catalyses the dismutation of $O_2^{\bullet-}$ to dioxygen (O_2) and H_2O_2:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

H_2O_2 is then eliminated by glutathione peroxidase or catalase. The biochemistry and molecular structure of three SOD isoforms found in different body compartments have been characterized in humans. Cu/Zn-SOD or SOD1 is found in the cytoplasm, whereas EC-SOD or SOD3 is extracellular. Both isoforms use copper and zinc as cofactors. Iron and Manganese are the cofactor for SOD2, which is found in mitochondria.

- **Thioredoxin (TRX) system** (made of TRX, TRX reductase, and NADPH) reduces oxidized cysteine groups on protein through an interaction with the redox-active center of TRX (Cys-Gly-Pro-Cys) to form a disulfide bond, which in turn can be reduced by TRX reductase and NADPH. TRX seems to exert most of its antioxidant properties through TRX peroxidase, which uses SH groups as reducing equivalents. TRX reduces the oxidized form of TRX peroxidase, and the reduced TRX peroxidase scavenges ROS, such as H_2O_2. A second TRX (TRX-2) with conserved TRX catalytic site and a consensus signal sequence for mitochondrial translocation was identified in mitochondria but little is known regarding its function [49].



1.5.2 Non-enzymatic antioxidants

- **Vitamin C:** (ascorbic acid) is a very important, and powerful, antioxidant that works in aqueous environments of the body, such as are present in the lungs and in the lens of the eye. Its primary antioxidant partners are Vitamin E and the carotenoids, as well as working along with the antioxidant enzymes. Vitamin C co-operates with Vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins. Some in vitro and animal supplementation studies explored the pro-oxidant properties of ascorbate. The pro-oxidant effect of ascorbate was attributed to the release of metal ions from damaged cells. In addition, it has been reported that Vitamin C induces lipid hydroperoxide decomposition to the DNA-reactive bifunctional electrophiles 4-oxo-2-nonenal, 4,5-epoxy-2(E)-decenal and 4-hydroxy-2-non-enal. The compound 4,5-epoxy-2(E)-decenal is a precursor of etheno-2′-deoxyadenosine a highly mutagenic lesion found in human DNA. Recent in vitro and ex vivo studies revealed that Vitamin C in plasma increases dose-dependently resistance to-lipid peroxidation, even in the presence of redox-active iron or copper and H_2O_2. Overall, in vitro studies have shown that Vitamin C either has no effect or inhibits transition metal (Fe, Cu)-ion dependent lipid peroxidation in plasma and other

biological fluids. In contrast, Vitamin C may be able to promote metal ion-dependent hydroxyl radical formation in biological fluids, but only under unphysiological conditions .

- **Vitamin E:** Vitamin E is a fat-soluble vitamin that exists in eight different forms. α-Tocopherol is the most active form of vitamin E in humans and is a powerful biological antioxidant which is considered to be the major membrane-bound antioxidant employed by the cell. Its main antioxidant function is protection against lipid peroxidation. Recent evidence suggests that α-tocopherol and ascorbic acid function together in a cyclic-type of process. During the antioxidant reaction, α-tocopherol is converted to an α-tocopherol radical by the donation of a labile hydrogen to a lipid or lipid $ROO^{\bullet}$. The α-tocopherol radical can then be reduced to the original α-tocopherol form by ascorbic acid.

- **Glutathione:** The major thiol antioxidant is the tripeptide, glutathione. Glutathione (GSH) is a multifunctional intracellular non-enzymatic antioxidant. It is considered to be the major thiol-disulphide redox buffer of the cell. GSH is highly abundant in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) and is the major soluble antioxidant in these cell compartments. The reduced form of glutathione is GSH, and the oxidised form is GSSG, glutathione disulphide. GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. An oxidative environment leads to rapid modification of protein sulphydryls (protein-SH): two-electron oxidation yields sulphenic acids (protein-SOH) and one-electron oxidation yields thiyl radicals (protein-$S^{\bullet}$). These partially oxidised products react with GSH and form S-glutathiolated protein (protein-SSG), which is reduced further by the glutathione cycle through glutathione reductase and small proteins such as glutaredoxin and thioredoxin, to restore protein sulphydryls (protein-SH). However, if the process of oxidation of protein sulphydryls is not trapped by GSH, further oxidation leads to the formation of irreversibly oxidised forms such as sulphinic (protein-SO_2H) and sulphonic (protein-SO_3H) acids. Generally, the antioxidant capacity of thiol compounds is due to the sulphur atom which can easily accommodate the loss of a single electron. In addition the lifetime of sulphur radical species thus generated, i.e. a thiyl radical ($GS^{\bullet}$), may be

significantly longer than many other radicals generated during the stress. The reaction of GSH with the radical R can be described:

$$GSH + R \rightarrow GS^{\bullet} + RH$$

Thiyl radicals generated may dimerise to form the non-radical product, oxidised glutathione (GSSG):

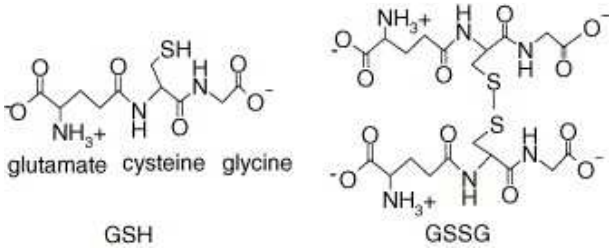
$$GS^{\bullet} + GS^{\bullet} \rightarrow GSSG$$

Oxidised glutathione GSSG is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organisms. Too high a concentration of oxidised glutathione GSSG may damage many enzymes oxidatively. GSSG can react with protein sulphydryl groups to produce protein–glutathione-mixed disulphides

$$GSSG + \text{protein-SH} \leftrightarrow \text{protein-SSG} + GSH$$

The mixed disulphides (protein-SSG) have a longer half-life than GSSG, most probably due to protein folding.

The main protective roles of GSH against oxidative stress are: that (i) GSH is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione transferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv) glutathione is able to regenerate the most important antioxidants, vitamins C and E back to their active forms; GSH can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semidehydroascorbate to ascorbate.



- **Lipoic acid:** α-Lipoic acid (ALA), a disulphide derivative of octanoic acid, is a natural compound and has the full chemical name 1,2-dithiolane-3-pentanoic acid. α-Lipoic acid is both water and fat-soluble and therefore is widely distributed in both cellular membranes and the cytosol. α-Lipoic acid is readily absorbed from the diet and is converted rapidly in many tissues to its reduced dithiol form, dihydrolipoic acid (DHLA). Both ALA and DHLA are powerful antioxidants. Their antioxidant functions involve: (i) quenching of reactive oxygen species; (ii) regeneration of endogenous and exogenous antioxidants involving vitamins C and E and GSH; (iii) chelation of redox metals including Cu(II) and Fe(II); (iv) repair of oxidised proteins. ALA is a possible chelator for Cd(II), but much less effectively than DHLA.

The positive effect of ALA has been associated with being beneficial to cardiovascular ailments, HIV infections and several different neurodegenerative diseases. Lipoic acid has a long history in Germany as being safe to use in the treatment of complications arising from diabetes mellitus. ALA prevents β-cell destruction, stimulates glucose uptake, protects against atherosclerosis and cataract, and decreases symptoms from diabetic neuropathy. In ischemia-reperfusion injury, ALA has also been shown to prevent, or ameliorate, the damage that occurs from ROS, produced when the ischemic tissue is re-oxygenated. ALA also possesses radio-protective properties and furthermore minimises the pathological consequences of cigarette smoke.

- **Carotenoids:** Carotenoids are pigments that are found in plants and microorganisms. There are over 600 carotenoids occurring in nature. Various studies have indicated that carotenoids may prevent or inhibit certain types of cancer, artherosclerosis, age-related muscular degeneration, and other diseases. The antioxidant activity of carotenoids arises primarily as a consequence of the

ability of the conjugated double-bonded structure to delocalise unpaired electrons. This is primarily responsible for the excellent ability of β-carotene to physically quench singlet oxygen without degradation, and for the chemical reactivity of β-carotene with free radicals such as the $ROO^{\bullet}$,$OH^{\bullet}$, and $O_2^{\bullet-}$. At sufficiently high concentrations, carotenoids can protect lipids from peroxidative damage.

Generally three mechanisms are proposed for the reaction of free radicals ($ROO^{\bullet}$, $R^{\bullet}$) with carotenoids: (i) radical addition, (ii) hydrogen abstraction from the carotenoid and (iii) electron-transfer reaction.

- **Flavonoids:** Polyphenolic compounds constitute one of the most commonly occurring and ubiquitous groups of plant metabolites and represent an integral part of human diet. Flavonoids constitute the most important single group of polyphenols; with more than 4000 compounds described, and which can be subdivided into 13 classes. Their common structural feature is the diphenylpropane moiety, which consists of two aromatic rings linked through three carbon atoms that together usually form an oxygenated heterocycle. Recent interest in phenolic compounds in general, and flavonoids in particular, has increased greatly owing to their antioxidant capacity and their possible beneficial implications in human health. These include the treatment and prevention of cancer, cardiovascular disease and other pathological disorders.

Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalysing lipid peroxidation. Phenolic antioxidants (PhOH) interfere with the oxidation of lipids and other molecules by the rapid donation of hydrogen atom to radicals

$ROO^{\bullet} + PhOH \rightarrow ROOH + PhO.$

The phenoxy radical intermediates are relatively stable so they do not initiate (propagate) further radical reactions. They even act as terminators of the reaction chain by interacting with other free radicals. However, under certain conditions, e.g. a high concentration of phenolic antioxidants, the presence of redox-active metals (copper, iron) and a high pH, they may behave as pro-oxidants.

The key factors affecting the biological activity of flavonoids (both in vivo and in vitro) are the extent, nature, and position of the substituents and the number of hydroxyl groups. All these factors influence whether a flavonoid will act as an antioxidant or as a modulator of enzyme activity, or whether it possesses antimutagenic or cytotoxic properties.

One of the most actively studied properties of flavonoids is their protection against oxidative stress. For example, flavonoids are ideal scavengers of peroxyl radicals due to their favourable reduction potentials relative to alkyl peroxyl radicals and thus, in principle, they are effective inhibitors of lipid peroxidation. Of particular importance is the hydrogen (electron) donating ability of a flavonoid molecule which acts to scavenge a reactive radical species, and is primarily associated with the presence of a B-ring catechol group (dihydroxylated B-ring). The nutritional benefit of flavonoids is generally linked to their healing potential. A regular intake of flavonoids, such as polyphenols and quercetin, is linked to lower rates of stomach, pancreatic, lung and possibly breast cancer. Some studies indicate that a diet high in flavonoids, particularly quercetin, may help prevent blood clots and blocked arteries, significantly reducing the chance of death from stroke or heart.

- **Selenium:** Selenium appears to function as an antimutagenic agent, preventing the malignant transformation of normal cells. These protective effects of Se seem to be primarily associated with its presence in the glutathione peroxidases (GSH-Pxs) and thioredoxin reductase, which are known to protect DNA and other cellular components from oxidative damage. Generally, seleno-enzymes are known to play roles in the control of cell division, oxygen metabolism, detoxification process, induction of apoptosis in cancer cells and the functioning of the immune system. Other modes of actions involve inactivation of oncogenes.

1.6 ROS AND DISEASE

There is a growing awareness that oxidative stress plays a role in various clinical conditions. Malignant diseases, diabetes, atherosclerosis, chronic

inflammation, human immunodeficiency virus (HIV) infection, ischemia-reperfusion injury, and sleep apnea are important examples. These diseases fall into two major categories. In the first category, diabetes mellitus and cancer show commonly a pro-oxidative shift in the systemic thiol/disulfide redox state and impaired glucose clearance, suggesting that skeletal muscle mitochondria may be the major site of elevated ROS production. These conditions may be referred to as "mitochondrial oxidative stress". Without therapeutic intervention these conditions lead to massive skeletal muscle wasting, reminiscent of aging-related wasting. The second category may be referred to as "inflammatory oxidative conditions" because it is typically associated with an excessive stimulation of NAD(P)H oxidase activity by cytokines or other agents. In this case increased ROS levels or changes in intracellular GSH levels are often associated with pathological changes indicative of a dysregulation of signal cascades and/or gene expression, exemplified by altered expression of cell adhesion molecules.

1.6.1 Malignant diseases

Direct oxidative damage to DNA represents a common explanation offered for ROS-induced cancer. It has been proposed that $OH^{\bullet}$ generated close to DNA by iron-mediated processes are mainly responsible for this damage. Indeed, hydroxyl radicals generate multiple mutagenic products after oxidation of DNA, including purine, pyrimidine, and deoxyribose oxidation products. Other reactive oxidants like peroxynitrite ($ONOO^{-}$) and hypochlorous acid (HOCl), when produced at appreciable amounts, may also induce oxidative DNA modifications that may be relevant to cancer development [50].

Extensive experimental support has accumulated, indicating that ROS, apart from inducing oxidations on DNA, can also exert a wide range of potential effects on proliferating cells, through modulation of signalling pathways that influence transformation of the cells. The elucidation of the exact molecular mechanisms underlying these effects remains poorly understood. It is likely that the steady-state levels of different ROS in combination with the particular cell-type represent important determinants for the ultimate fate of cells. So, while a certain intracellular concentration can promote cancer development by increasing

proliferation, angiogenesis, and by suppressing apoptosis, slightly higher levels can easily act as anti-cancer agents, inducing cell-cycle arrest, senescence, and cell death either by apoptosis or necrosis. The above ideas are experimentally supported by genetic manipulation experiments. Thus, genetic elimination of CuZn-SOD or decreased Mn-SODs activity in mice led to increased rates of cancer development in these animals [51, 52]

A placebo-controlled clinical study of patients with previous adenomatous colonic polyps, i.e., a group with an increased risk for colon cancer and increased proliferative index of colonic crypts, revealed a significant decrease in the proliferative index after treatment with N-acetylcysteine. Even normal cells often show increased proliferation and expression of growth-related genes if exposed to hydrogen peroxide or superoxide. In addition, certain types of cancer cells produce substantial amounts of ROS [53]. Anti-tumor activity of conventional chemotherapeutic agents is enhanced by antioxidants treatment [54].

Also during angiogenesis, the process of new blood vessel formation from the pre-existing vessels that plays an important role in tumor growth and metastasis, ROS function as signalling molecules in many aspects of growth factor-mediated responses. Vascular endothelial growth factor is a key angiogenic growth factor and stimulates proliferation, migration, and tube formation of endothelial cells.

On the other side overproduction of these highly reactive oxygen metabolites can initiate lethal chain reactions, which involve oxidation and damage to structures that are crucial for cellular integrity and survival. In fact, many antitumor agents, such as vinblastine, cisplatin, mitomycin C, doxorubicin, camptothecin, inostamycin, neocarzinostatin and many others exhibit antitumor activity via ROS-dependent activation of apoptotic cell death, suggesting potential use of ROS as an antitumor principle. Thus, a unique anticancer strategy named "oxidation therapy" has been developed by inducing cytotoxic oxystress for cancer treatment. This goal could be achieved mainly by two methods, namely, (i) inducing the generation of ROS directly to solid tumors and (ii) inhibiting the antioxidative enzyme system of tumor cells. Since 1950s, many strategies have been employed based on the first method, namely, administration of ROS per se (e.g. H_2O_2) or ROS generating enzyme to tumor bearing animals. However no

successful and practical results were obtained probably because of the lack of tumor selective ROS delivery and hence resulting in subsequent induction of severe side effects [55].

1.6.2 Diabetes Mellitus

Elevated ROS levels have also been implicated in diabetes mellitus.

Hyperglycemia is a hallmark of both non-insulin-dependent (type 2) and insulin-dependent diabetes mellitus (type 1). Chronic exposure to elevated glucose and fatty acid concentrations can cause damage in different types of cells by a variety of mechanisms ('glucolipotoxicity'), but oxidative stress may be a common link in cell dysfunction. Fatty acids were first shown to be oxidized by isolated cardiac and skeletal muscles, so inhibiting glucose utilization. Increased oxidation of fatty acids resulted in an increase in the intramitochondrial $NADH/NAD^+$ ratio, so reducing pyruvate dehydrogenase activity and thus glucose oxidation. Moreover FFAs increase the rate of ROS generation in the forward mode of electron transport because of slowing down the rate of electron flow through Complexes I and III of the respiratory chain due to interaction within the complex subunit structure, and between Complexes III and IV due to release of cytochrome c from the inner membrane [56]. It has been reported that glucose or free fatty acids initiate the formation of ROS in muscle, adipocytes, pancreatic β-cells and other cells [57].

Interestingly, compared to many other cell types, the β-cell may be at high risk for oxidative damage with an increased sensitivity for apoptosis. In fact expression levels of antioxidant enzymes such as catalase, and glutathione peroxidase are very low in β-cells compared to other tissues, so β-cells are thought of as targets for oxidative stress-mediated tissue damage. Thus it is likely that production of ROS and subsequent oxidative stress is involved in β-cell deterioration in diabetes [58].

Moreover insulin resistance at the molecular level may be mediated by inhibition of signal transduction at the apex of the signalling pathway, which involves the insulin receptor (IR) β-subunit, which contains an intrinsic tyrosine kinase activity. Exposure of different cell lines to micromolar concentrations of

hydrogen peroxide leads to the activation of stress kinases such as c-Jun N-terminal kinase, p38, IκB kinase, and extracellular receptor kinase 1/2. This activation is accompanied by a down-regulation of the cellular response to insulin (insulin resistance), leading to a reduced ability of insulin to promote glucose uptake, and glycogen, lipid and protein synthesis [59].

1.6.3 Atherosclerosis

Atherosclerosis is a multifactorial disease characterized by hardening and thickening of the arterial wall. The vascular areas affected by this disease contain mononuclear cells, proliferating smooth muscle cells, and extracellular matrix components. Atherosclerosis is commonly viewed as a chronic inflammatory disease and is associated with certain risk factors such as hyperlipidemia, diabetes, and hypertension. Excessive ROS production has been implicated in the pathogenesis of atherosclerosis and hypertension. Lipid peroxidation and atherogenesis may be ameliorated by vitamin E. A study of atherosclerosis-susceptible APO-lipoprotein E knock-out mice revealed that induction of vitamin E deficiency by disruption of the α-tocopherol transfer protein gene increased the severity of atherosclerotic lesions in the proximal aorta.

1.6.4 Neurodegenerative Diseases

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive decline in cognitive function and extensive neuronal loss. The brains of affected patients show numerous amyloid plaques and neurofibrillary tangles. The production of ROS in the brains of AD patients and its implication in AD pathogenesis are implicated by the significant amount of lipid peroxidation detected in the brain as well as by the increased levels of 4-hydroxynonenal found in postmortem cerebrospinal fluid of AD patients. Furthermore, ROS were found to mediate amyloid -protein damage .

Parkinson's disease (PD) involves a selective loss of neurons in an area of the midbrain called the substantia nigra. The cells of the substantia nigra use dopamine (a neurotransmitter-chemical messenger between brain and nerve cells) to communicate with the cells in another region of the brain called the stratium.

Thus, a reduction in nigral dopamine levels results in a decrease in stratial dopamine that is believed to cause PD symptoms. A majority of studies explored the effect of oxidative stress that contributes to the cascade of events leading to dopamine cell degeneration in PD. The occurrence of oxidative stress in PD is supported by both post-mortem studies and by studies demonstrating the capacity of oxidative stress to induce nigral cell degeneration. There is evidence that there are high levels of basal oxidative stress in the substantia nigra pars compacta (SNc) in the normal brain, but that this increases in PD patients. However, other factors involving inflammation, excitotoxic mechanisms, toxic action of nitric oxide, and mitochondrial dysfunction play roles in the aetiology of PD.

Amyotrophic lateral sclerosis is a neurodegenerative disease that affects primarily motor neurons in the spinal cord and brain stem. Approximately 10% of the cases are inherited in an autosomal dominant manner. One-fifth of these familial amyotrophic lateral sclerosis patients carry mutations in the Cu/Zn-SOD gene, suggesting the involvement of ROS in this neurodegenerative disease. Several lines of transgenic mice carrying mutant SOD transgenes have been shown to develop a pathology and clinical phenotype similar to that of familial amyotrophic lateral sclerosis patients. The mutation in the Cu/Zn-SOD gene causes neuronal death by apoptosis through the sequential activation of caspase-1 and caspase-3.

Down's syndrome or trisomy 21 is the most frequent genetic cause of mental retardation and is commonly associated with the development of Alzheimer's disease in adult life. Cultured cortical neurons from foetal Down's syndrome cases exhibit a three to-fourfold higher intracellular ROS level than age-matched normal brain cells. Treatment with free radical scavengers or catalase prevents the degeneration of Down's syndrome neurons in culture [60].

1.6.5 Ischemia and Reperfusion Injury

Ischemia and reperfusion can lead to tissue injury and are serious complications in organ transplantation, myocardial infarction, and stroke. Massive ROS production was identified as an important causative factor. Xanthine dehydrogenase, which normally utilizes NAD^+ as electron acceptor, is converted

under the conditions of ischemia/reperfusion into xanthine oxidase which uses oxygen as substrate. During the ischemic period, excessive ATP consumption leads to the accumulation of the purine catabolites hypoxanthine and xanthine, which upon subsequent reperfusion and influx of oxygen are metabolized by xanthine oxidase to yield massive amounts of superoxide and hydrogen peroxide. Neutrophils are the principal effector cells of reperfusion injury, and the inhibition of neutrophil adhesion to the endothelium attenuates the process. Antioxidant treatment ameliorates both leukocyte adhesion and leukocyte-mediated heart injury in the postischemic period. Also, treatment with a synthetic SOD mimetic was shown to ameliorate tissue damage in a rat model of ischemia/reperfusion injury.

1.6.6 The Free Radical Theory of Aging

Multicellular organisms generally undergo qualitative changes with time (aging) that are associated with progressive degeneration of biological functions, increased susceptibility to diseases, and increased probability of death within a given time period. The widely popular free radical theory of aging states that the age-related degenerative process is to a large extent the consequence of free radical damage. Genetic evidence linking oxidative stress to life span has been obtained for different animal species.

1.7 H_2O_2 CAN ACT AS SECOND MESSENGER

As discussed previously, oxidative stress has long been considered an “accident” of aerobic metabolism; a stochastic process of free radical production and nonspecific tissue damage which is fundamentally unregulated aside from the normal phalanx of antioxidant defence mechanisms. In recent years, a paradigm shift has been occurring wherein certain ROS and RNS have become appreciated as signalling molecules whose production may be regulated as a part of routine cellular signal transduction.

The seminal work by Baeurle and colleagues first showed that certain transcription factors of the NFκB/rel family can be activated not only by receptor-

targeted ligands but also by direct application of oxidizing agents (particularly H_2O_2) or ionizing radiation [61].

Second messengers are generated at the time of receptor activation, are short-lived, and act specifically on effectors to transiently alter their activity. Indeed, ROS and RNS can be generated at the time of receptor activation and are short-lived, as are other second messengers, but the specificity of their action has been difficult to assess, except for that of ·NO, which binds specifically to the heme of the regulatory domain of soluble guanylate cyclase, resulting in its activation. Clearly, a reactive species such as ·OH cannot have any specificity, as it reacts at nearly the rate of diffusion with almost any molecule [62, 63]. Instead H_2O_2, under physiological conditions, is relatively stable and less reactive compared to other ROS species but is able to perform a number of rather specific chemical reactions. A recent mass-spectrometry analysis revealed that protein modifications by ROS are not a random process but appear specific to certain target proteins. Protein modifications in mammalian neuronal cell lines were dependent on the particular ROS used. Treatment with hydrogen peroxide either promoted or inhibited disulfide bonding of select proteins in a concentration dependent manner. Interestingly, many of these targets are involved in translation and energy production. This clearly demonstrates that oxidation by ROS is not simply destructive but rather a specific modification used for signalling. Glyceraldehyde-3-phosphate dehydrogenase isoenzymes, aconitase, pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, fatty acid synthase, fructose bisphosphatase and Cu–Zn superoxide dismutase are only a few examples of very important metabolic enzymes, which are directly or indirectly modified by H_2O_2. H_2O_2 can contribute to the carbonylation of proteins and oxidation of methionine residues or thiol groups of cysteines.

Oxidation of cysteines is the best-studied modification. Oxidation of the sulfhydryl group (–SH) of one cysteine molecule can lead to a sulfenic (–SOH), sulfinic ($–SO_2H$) or sulfonic ($–SO_3H$) derivate. This can change the enzyme activity of a protein or the binding capacity of a transcription factor. One investigated example is the regulation of the activity of the protein-tyrosine phosphatase protein family (PTP), which encompasses 103 members in humans

alone. It has been shown that the conserved catalytic cysteine residues sensitive to oxidation are essential for the catalysis and that the degree of oxidation determines PTP enzyme activity. Protein structure and consequently protein function can be altered by the intra- and intermolecular oxidation of two cysteine residues causing formation of disulfide linkages. While intramolecular disulfides lead to alterations of enzyme activity by conformational changes, intermolecular disulfides cause di- or oligomerization. Such conformational changes can expose or shape the reaction centres of enzymes, shape DNA binding motifs or uncover localization signals like in the case of transcription factors.

The role of ROS has been demonstrated for nerve growth factor (NGF) signalling in neuronal cells [64], for epidermal growth factor (EGF) signalling in human epidermoid carcinoma cells [65], and for platelet derived grow factor (PDGF). Stimulation by any of these growth factors results in a transient increase in intracellular ROS through the signalling protein Rac1. Elimination of hydrogen peroxide by catalase was shown to inhibit EGF and NGF-induced tyrosine phosphorylation of various cellular proteins, including phosphorylation of the growth factor receptor itself.

As previous mentioned activation of NFκB is one of the best studied models of redox regulation in mammalian cells. NFκB is involved in the induced expression of the interleukin-2 gene and in a wide variety of biological responses. In particular, it is implicated in inflammatory reactions, growth control, and apoptosis and is the first eukaryotic transcription factor shown to respond directly to oxidative stress in certain types of cells. ROS or changes in the thiol/disulfide redox state are not strictly required for NFκB activation but induce or amplify NFκB activation in various cell types under various conditions. At least two different mechanisms contribute to the enhancement of NFκB activation. One of these mechanisms is based on the enhanced proteolytic degradation of the NFκB inhibitor IB after exposure to ROS. The second mechanism involves the increase in IB kinase- activity after exposure to hydrogen peroxide or to pro-oxidative changes in the intracellular glutathione redox state.

The redox control of the heme oxygenase-1 (HO-1) gene is another well known model of redox regulation. HO-1 is a 32-kDa a ubiquitous enzyme that

degrades heme to biliverdin, free iron, and carbon monoxide. Expression of HO-1 responds to chemical and physical agents that directly or indirectly generate ROS. Depletion of cellular reduced GSH may act as a signal for HO-1 transcriptional activation. Furthermore, antioxidants and metal-chelating compounds can modulate HO-1 expression. Several signalling molecules (e.g., mitogen-activated protein kinases), transcriptional regulators (activator protein-1, NF-E2-related factor-2, hypoxia-inducible factor-1, Bach-1), as well as two enhancer regions in the ho-1 5' regulatory region, participate in the regulation of the ho-1 gene. HO-1 protein expression can occur in the lung in response to oxidative stress associated with infection, altered oxygen tension, and inflammatory diseases [66, 67]. Activation of the HO-1 pathway is part of a complex homoeostatic adaptation of cells to the redox imbalance inflicted by stressful stimuli, and it is becoming evident that increased CO production reflects a dynamic and active involvement of this by-product in the cytoprotective response. Indeed, CO is an important signalling mediator possessing vasodilatory properties, which are achieved by activation of the guanylate cyclase–cGMP pathway as well as large-conductance potassium channels. Moreover, comprehensive studies published in the last decade corroborate the anti-ischaemic, antioxidant and anti-inflammatory properties of the endogenous HO-1/CO system in a number of experimental models. [66, 67]. The sustained induction of HO-1 mRNA and its inducibility in many tissues and various mammalian species has rendered HO-1 mRNA a useful marker for cellular oxidative stress at the mRNA level [68].

1.8 ROS DETECTION

Recent elucidation of the physiological and pathologic roles played by ROS makes its detection and quantitation a very important task. This task is, however, far from simple due to their low steady-state concentrations and short half-life under physiological conditions. Moreover there is a large variety of antioxidants that are capable of capturing these reactive species. The most common approach is an indirect detection of ROS via quantitation of products of their reactions. These reactions may involve endogenous cell compounds, extracellular matrix, or

exogenous probes. A spectroscopic probe is a substance (usually exogenous) that changes its spectroscopic properties (light absorption or emission, or magnetic characteristics) upon reaction with ROS. An ideal spectroscopic probe should be highly specific for one ROS form and react with it efficiently so that it can be used at low concentration without perturbing the system studied. Electron spin resonance and paramagnetic resonance (ESR or EPR) are unique techniques that specifically and directly ‘‘see’’ unpaired spins of reactive species. Because the ESR technique requires a specialized and expensive ESR spectrometer, alternative methods have been developed for ROS detection with more readily available equipment. These alternative methods are based on the detection of ROS reaction products with a variety of probe molecules. Although spectrophotometric probes [69]are perhaps the simplest, their sensitivity is usually much lower when compared to other probes. Because of this limitation, the much more sensitive fluorescent probes are more widely used in practice. Luminometry is used less frequently, however, this highly sensitive technique provides direct visualization of singlet oxygen formation as well as excited states of other molecules. Apart from direct luminometry, luminescent probes can be employed and permit ROS detection with less sensitive equipment. Alternatively, the detection and quantitation of ROS reaction products can also be accomplished with detector molecules using high performance liquid chromatography (HPLC), mass spectrometry and immunochemistry. Other methods of detecting ROS reactions include monitoring lipid peroxidation and oxidative damage to protein as well as DNA. Currently, fluorescence is most frequently used for ROS detection in cell culture and tissue. This technique has the advantages of good sensitivity and versatility [69-71]. Fluorescence may be measured or observed with a fluorimeter, microplate reader, microscope or cytometer. Confocal microscopes offer the possibility of additionally observing cellular topography of ROS production and can provide some degree of specificity through use of various fluorescent probes. It should be noted, however, that the techniques of fluorescence, spectophotometry and luminometry are less direct and less specific for the detection of ROS than ESR. As can be appreciated, an alternative to any single-dimensional approach is to combine methodologies in collaboration with other

investigators. This strategy typically provides more in-depth and comprehensive analysis. Although the use of spectroscopic probes appears to be a simple and easy means for the detection and quantification of ROS production in cellular systems, there are inherent limitations of this methodology and many sources of artefacts. These include: low stability of some probes and/or products formed that may be additionally metabolized in cellular systems; lack of probe specificity with oxidants (reductants); undesired probe reactions in the system studied; problems with probes accessing cellular sites of ROS production; unwanted extracellular export of probes and/or products of their reactions; production of ROS by the probes themselves; and perturbation of the systems studied by the probes. Dangers or artefacts associated with spectroscopic probes are also considered. Another question concerns the feasibility of quantifying the rate of ROS production based on measurements of spectroscopic probes.

1.8.1 Spectrophotometric probes

The best and simplest ROS detectors are substances with optical properties that change in reaction with ROS and show some specificity for different ROS species [69-71]. Their principal disadvantage, however, is low sensitivity in comparison with other probes. Superoxide has both oxidizing and reducing properties. Its ability to reduce cytochrome c and various tetrazolium salts, specifically Nitro Blue Tetrazolium (NBT), has been most frequently utilized to detect superoxide formation. One classic way to detect and quantify superoxide production is via reduction of cytochrome c leading to increased light absorption at 550 nm:

$$\text{Cyt c-Fe}^{3+} + O_2^{\cdot -} \rightarrow \text{Cyt c -Fe}^{2+} + O_2$$

NBT has also been widely used for the detection of superoxide radical. The product of univalent reduction of NBT is tetrazoinyl radical in which its dismutation generates a stable formazan. The formazan is insoluble. This property is advantageous in studies to locate superoxide production, but a drawback for quantitation.

$NBT^{2+} + O_2^{\cdot -} \leftrightarrow NBT^{\cdot +} + O_2$

Moreover NBT and the other tetrazolium salts can be also reduced by cellular reductases in a superoxide independent manner.

Phenol Red is oxidized by hydrogen peroxide in a reaction catalyzed by horseradish peroxidase (HRP) to a product of different spectral properties, including increased absorbance at 610 nm. This unique property provides a simple mechanism for measurement of hydrogen peroxide in micromolar concentration range (1–60 nmol/mL).

1.8.2 Luminescence probes

Luminol-derived chemiluminescence in the presence of a peroxidase, such as HRP, has been used to detect cellular H_2O_2 production under various experimental conditions. One-electron oxidation of luminol by the H_2O_2/peroxidase system leads to the formation of a luminol radical. The luminol radical can then reduce O_2 to superoxide that subsequently reacts with an additional luminol radical to yield an unstable endoperoxide. Decomposition of the luminol endoperoxide produces N_2 and aminophthalate. The aminophthalate is in an electronically excited state, which emits a photon upon relaxation to the ground state. Luminol-derived chemiluminescence elicited by the H_2O_2/peroxidase is inhibited by catalase and SOD indicating the dependence of chemiluminescence on both superoxide and H_2O_2. Among the most widely used compounds with higher specificity in their light emission with superoxide is bis-Nmethylacridinium nitrate (i.e., lucigenin). Other compounds used are cypridina luciferin analogues. However, the chemiluminescence intensity of the probe is relatively low [69, 71].

1.8.3 Fluorescence probes

Fluorescent probes permit detection of ROS with much higher sensitivity versus spectroscopic probes.

We can differentiate the fluorescence probes into two big family: the positive and the negative fluorescent probes. The “negative” probes is naturally fluorescent compound that in presence of ROS originate non fluorescent compound. Most of

the fluorescent probes used for detection of ROS are ‘‘positive’’ probes. These compounds are non-fluorescent (or weakly fluorescent), but yield fluorescent products upon reaction with ROS. These called fluorogenic probes offer a larger field of application. Advantages of assays based on increased fluorescence are: higher sensitivity; linear response to a wide range of ROS concentrations; and low background fluorescence [69-71].

1.8.3.1 Scopoletin

Scopoletin (7-hydroxy-6-methoxy-coumarin) is an examples of ‘‘negative’’ fluorescence probes used for the detection of hydrogen peroxide. H_2O_2, in presence of HRP, or catalase, forms a complex, which oxidizes scopoletin ($\lambda_{excitation}$=360 nm; $\lambda_{emission}$=460 nm), originating a non-fluorescent product. Although being widely used as an H_2O_2 monitoring probe, either in isolated mitochondria, or in stimulated neutrophils and eosinophils, scopoletin has some disadvantages: i) has low extinction coefficient; ii) presents short wavelength spectra of excitation and emission which makes it susceptible to interference from autofluorescence when biological samples are used; iii) due to its low fluorescent power scopoletin requires a significant signal amplification to quantify its fluorescence changes, which results in an increase of the background fluorescence therefore affecting the method’s sensibility; iv) presents low stability in biological media. With time its fluorescence decreases spontaneously and is highly dependent on pH and temperature; v) might suffer interference from reductive compounds such as NADPH, NADH, ascorbic acid and glutathione (GSH).

1.8.3.2 2,7-Dichlorodihydrofluorescein (DCFH)

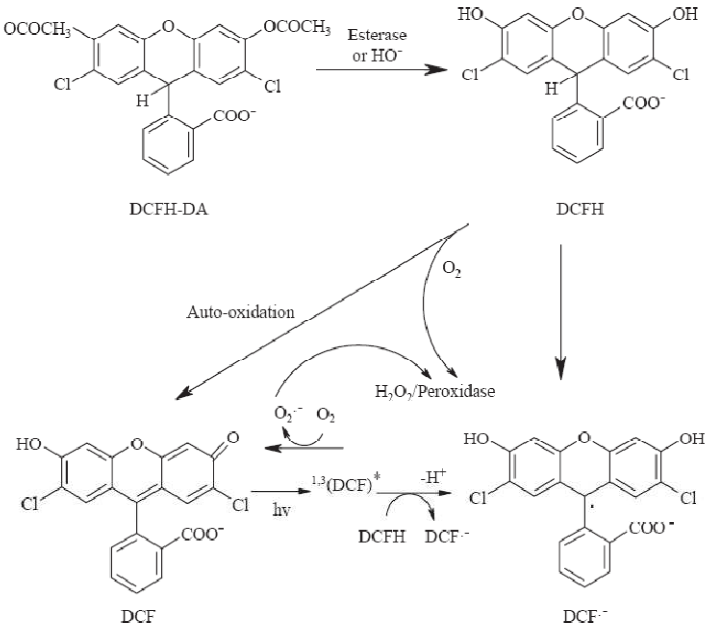
The oxidation of 2,7-dichlorodihydrofluorescein (DCFH) originates 2,7-dichlorofluorescein (DCF), a fluorescent compound ($\lambda_{excitation}$=498 nm; $\lambda_{emission}$=522 nm) initially thought to be useful as a specific indicator for H_2O_2 [37]. However, it was already demonstrated that DCFH is oxidized by other ROS, such as $HO^{\bullet}$ and $ROO^{\bullet}$ and also by RNS like $^{\cdot}NO^{\bullet}$ and $ONOO^{\bullet-}$.

The DCFH diacetate form (DCFH-DA) can be applied in cell studies due to its ability to diffuse through the cellular membrane, being then enzymatically hydrolysed by intracellular esterases to DCFH.

The presence of cellular peroxidases is important for the oxidation of DCFH to DCF by H_2O_2. But Horseradish peroxidase (HRP) is also capable of oxidizing DCFH even in the absence of H_2O_2. These observations for HRP led to the hypothesis that oxidation of DCFH could also be directly performed by other oxidases or peroxidases. Moreover haematin and cytochrome c are substances that highly increase the formation of DCF. Noteworthy, Burkitt et al. [72] demonstrated that cytochrome c is a powerful catalyst of DCFH oxidation, and so use of DCFH-DA to probe oxidative stress during apoptosis should be approached with caution, since a rise in cytosolic cytochrome c levels could result in a higher fluorescence without any change in cellular peroxide levels.

DCFH oxidation also occurs by action of H_2O_2 in presence of Fe(II), being, nevertheless, highly probable that, in this case, $HO^{\cdot}$ is the species responsible for the oxidation. These probe is considered also sensible for the determination of $ONOO^{-}$.

DCF can suffer photoreduction in presence of visible light, or by action of UVA radiation [72]. This reduction's mechanism is supposed to involve the generation of semiquinone radical from DCF which, by reaction with O_2, originates $O_2^{\cdot -}$. In its turn, the dismutation of $O_2^{\cdot -}$ generates H_2O_2, which leads to an artificial increase of DCFH oxidation and consequently to an amplification of DCF fluorescence.

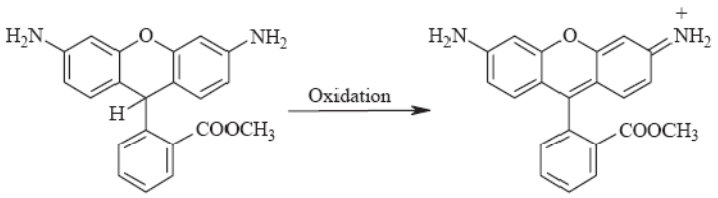


Due to the existence of several substances that interfere with the formation of DCF, this probe, when used in cellular systems, has better use as a marker of the cellular oxidative stress than as indicator of the formation of H_2O_2 or other ROS and RNS.

Intracellular oxidation of DCFH tends to be accompanied by leakage of the product, 2',7'-dichlorofluorescein, which makes quantitation or detection of the slow oxidation difficult. To enhance retention of the fluorescent product, new improved versions of DCFH are now commercially available: the carboxylated DCFH-DA analog (carboxy-DCFH-DA), which has two negative charges at physiological pH, and its di-acetoxymethyl ester which should more easily pass through membranes during cell loading and the 5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-DCFH-DA), a chloromethyl derivative of DCFH-DA that should exhibit much better retention in live cells (CM-DCFH-DA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular GSH and other thiols, subsequent oxidation yields a fluorescent adduct that is trapped inside the cell, thus facilitating long-term studies). These modifications however do not solve the specificity problems.

1.8.3.3 Dihydrorhodamine 123 (DHR)

Dihydrorhodamine 123 (DHR) is a non-fluorescent molecule that, by oxidation, yields rhodamine 123, a fluorescent cationic and lipophilic probe ($\lambda_{excitation}$ =505 nm; $\lambda_{emission}$ =529 nm).



Dihydrorhodamine 123 (Non fluorescent) — Rhodamine 123 (Fluorescent)

The lipophilicity of DHR facilitates its diffusion across cell membranes. Upon oxidation of DHR to the fluorescent rhodamine 123, one of the two equivalent

amino groups tautomerizes into an imino, effectively trapping rhodamine 123 within cells. H_2O_2 oxidizes DHR in the presence of peroxidases, and also this probe has low specificity for this ROS since it can also be oxidized by other reactive oxidants, namely $ONOO^-$, Fe(II), Fe(III)/ascorbate, Fe(III)/EDTA, cytochrome c, or HOCl. On the other hand, DHR is not directly oxidizable by H_2O_2 alone (Fig. 5A), by $O_2^{\cdot -}$, and by xanthine/ xanthine oxidase. As shown in fig. 5B [73] DHR is more efficiently oxidized by HRP and H_2O_2 than DCFH and is less efficiently oxidized by HRP alone.

DHR is oxidized 8 fold more efficiently than DCFH by hypochlorous acid (HOCl), that is produced by the enzyme myeloperoxidase, which is found almost exclusively in neutrophils. In these cells systems where myeloperoxidase is present, DHR titrate mainly the HOCl [73].

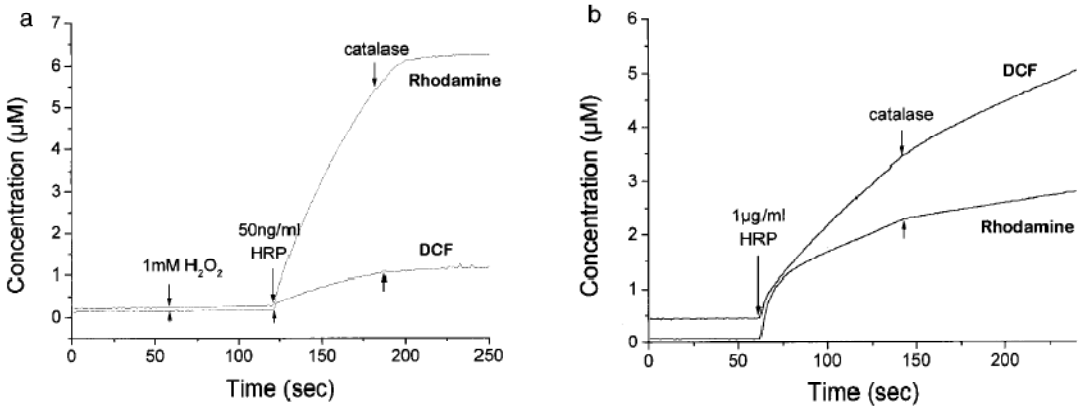
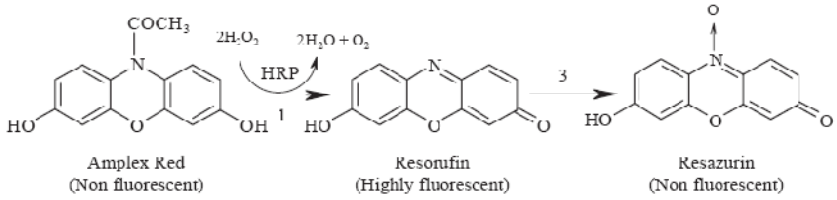


Figure 5: (A) Effects of H_2O_2 and HRP on $DCFH_2$ and DHR (B) Effects of HRP. HRP alone was added to solutions of $DCFH_2$ or DHR [2] [73].

1.8.3.4 N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red)

N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red) is a non-fluorescent molecule that when oxidized by H_2O_2 in presence of HRP originates resorufin, a highly fluorescent product ($\lambda_{excitation}$ =563 nm; $\lambda_{emission}$ =585 nm). Amplex Red consumes stoichiometric amounts of H_2O_2. This probe can also be used for measurement of $O_2^{\cdot -}$, by using SOD in the reaction mixture to convert this radical into H_2O_2. Main advantages of Amplex Red are its low background fluorescence (in the reduced form) as well as the stability and high fluorescence of its oxidation product. Furthermore, this probe's excitation and emission maximum wavelengths

are in a spectral zone that has little susceptibility to interference from autofluorescence in assays where biological samples are used. Altogether this results in a sensibility increase in H_2O_2 detection. However, resorufin per se is also a substrate of HRP. Further oxidation of resorufin, to nonfluorescent resazurin and other oxidation products, may result in significant loss of fluorescence intensity.



Mohanty et al. [74] carried out tests where catalase was used in order to confirm that the observed fluorescence resulted indeed from action of H_2O_2. In these tests they verified that the fluorescence decreased significantly when catalase was added to the reaction mixture and completely prevented fluorescence developing when added before Amplex Red. This probe appears far more specific for H_2O_2 since does not react with $O_2^{\cdot -}$, generated by the hypoxanthine/xanthine oxidase system, and reaction with HOCl caused an increase on the probe's oxidation at very high concentrations (>1 mM).

Thus, Amplex Red is a probe sensible and specific for the detection of H_2O_2, and can be used not only in activated phagocytic cells but also in other types of cells or even in non-cellular systems. The sensitivity of the Amplex Red when detecting H_2O_2 is at least 10 times higher than scopoletin's under the same conditions . Zhou et al. [75] referred a 50 nM detection limit for H_2O_2 in a plate reader methodology in which Amplex Red was used as probe.

Nevertheless, it must be taken into account that in biological systems, NADH and GSH may interfere with HRP/Amplex red assay system. Indeed, auto-oxidation and HRP-mediated oxidation of NADH and GSH may produce H_2O_2 at levels found in biological systems. Also, this methodology, like every other HRP dependent methodologies, is susceptible to interference from substances that oxidize this enzyme. Another aspect to be considered in this methodology is that Amplex Red seems to be a substrate for endogenous peroxidases present in eosinophils and in neutrophils, which can also cause interference.

1.8.3.5 Homovanillic acid

Homovanillic acid (4-hydroxy-3-methoxy-phenylacetic acid; HVA) represents an important metabolite of dopamine in the brain. It is a non-fluorescent molecule that by reaction with H_2O_2, in presence of HRP, originates a fluorescent dimer ($\lambda_{excitation}$ =312 nm; $\lambda_{emission}$ =420 nm). Unfortunately, the dimer spectrum overlaps with the absorption of common cellular components such as NAD(P)H.

1.8.3.6 Other reduced fluorogenic probes for ROS

Other fluorogenic reduced probes show reacting activity on species other than H_2O_2 so are considered specific detector of some other ROS and can be used to discriminate which other ROS species are formed in the biological systems.

Dihydroethidium (Hydroethidine) commonly used to analyze respiratory burst in phagocytes. Recent reports suggest that oxidation of dihydroethidium by superoxide produces a fluorescent product that is distinctly different from ethidium and that exhibits shorter-wavelength fluorescence upon binding to DNA or in organic solvent. Thus it is considered as a superoxide specific probe.

2-[6-(4V-hydroxy)phenoxy-3Hxanthen- 3-on-9-yl]benzoic acid (HPF) and 2-[6-(4V-amino)- phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) have been proposed for detection of highly reactive ROS such as the $OH^{\bullet}$, peroxynitrite or Compound I of peroxidases and hypochlorite. Moreover APF is also highly reactive with hypochlorite in contrast to HPF. These probes were reported to be superior to DCFH in having lower auto-oxidation, and present much higher specificity to the $OH^{\bullet}$, and much lower reactivity with H_2O_2, nitric oxide, peroxyl radicals and singlet oxygen than DCFH.

Mito tracker orange-CM-H_2 TM ROS and Mito Tracker Red-CM-H_2 X Ros are chemically reduced rosamine that enter active respiring cells and become fluorescent after oxidation by ROS and then sequestered in the mitochondria.

2.0 PURPOSE OF THE RESEARCH

The described scenario depicts H_2O_2 as one of the most important ROS, involved both in physiological and pathological events.

The growing importance that mitochondria are gaining as source of ROS, was the starting point of my PhD work. In particular I studied the mechanisms underlying the mitochondrial release of H_2O_2, and the conditions that appear to modulate its release. The physiological role of mitochondrial H_2O_2 production is, in fact, still under debate. Other sources are more prone to release H_2O_2 responding to external stimuli like the NADPH oxidase at least in some particular cells/tissues. The discussion, besides the modest level of mitochondrial H_2O_2 production compared to other sources, must take into account that a constitutive mitochondrial H_2O_2 production (less than 2% of daily O_2 consumption is suggested to undergo reactive oxygen species formation) should respond to modulation in order to have the requirements for being considered a cell signal event. My work, in particular, was addressed to the involvement of succinate and other mitochondrial substrates (as the NAD-dependent substrates and fatty acids) mimicking physiological conditions. The study was conducted in mitochondrial preparations from brain and heart.

The question is however how to correlate the results largely obtained in mitochondrial suspensions to a realistic *in vivo* condition. We asked ourselves if the particular conditions, found by us and largely reported by other studies in isolated mitochondria, are really representative of physiological conditions.

To answer this question the first problem is how to measure H_2O_2 in intact cells since the level of peroxide released by cells is too low to be detected outside. The current available methods based on the use of fluorescent probes are not reliable, as largely discussed in the introduction, since do not measure the H_2O_2 oscillation, but, at the best, are detectors of the oxidative state of the cells. We then addressed our study on how to improve the measure of H_2O_2 production in a more complex system like intact cells in order to obtain indication on the contribution of different sources and in particular on the role of mitochondria.

To obtain this measure we projected to set up an innovative procedure based on the expression of a protein like the HRP, required to induce an H_2O_2 dependent oxidation of some reduced probes inside the cells. We coupled the test to the more common probes used for detection of oxidative reactive species, since the fluorimetric approach constitutes one of the most simple direct and sensitive detection systems. The second part of my PhD deals with the set up of this new procedure based on the transient expression of the HRP in some cell lines. The model of HRP transfected cells will be validated, analysed for its peculiar features and for its possible applications.

The procedure will be verified and then applied firstly on the measure of MAO activity, used as tool to induce an active H_2O_2 production in viable cells, and then will be used to obtain at least some preliminary data on the study of the involvement of mitochondria in cell H_2O_2 production.

3.0 EXPERIMENTAL PROCEDURES

3.1 REAGENTS

The fluorescent probes Amplex Red (*N*-acetyl-3,7-dihydroxyphenoxazine), DHR (dihydro-rhodamine 123),DCFH2-DA (2',7'-dichlorodihydro fluorescein diacetate), Hoechst 33342, DAPI (4,6-diamidino-2-phenylindole) were purchased by Molecular Probe, Inc. (Eugene, OR) were dissolved in analytically pure DMSO and stored at -20°C.

Fatty acid free Bovine Serum Albumin (BSA), D-MEM phenol red free, Horseradish Peroxidase (E.C. 1.11.1.7), glucose oxidase from Aspergillus Niger (E.C. 1.1.3.4) resazurin, monobromobimane, Propidium Iodide, MTT (3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl Tetrazolium Bromid), CDNB (1-chlor-2 ,4-dinitrobenzene), and BSO (buthionine sulfoximine or S-(n-buty1)homocysteine sulfoximine), α-ketoglutarate (α-KG) dehydrogenase from pig heart (EC 1.2.4.2) and β-hydroxybutyrate dehydrogenase (from Pseudomonas Lemoignei EC 1.1.1.30), long and short chain acyl CoAs were from Sigma.

LipofectamineTM 2000, trypsine, D-MEM (41966-029), Opti-mem and FCS was all obtained from Invitrogen.

The BCA protein assay (23227) was from Pierce and the Cytotoxicity detection kit (LDH) (11 644 793 001) was from Roche.

All other chemicals were of analytical grade from commercial sources.

3.1 MOST USED MEDIA AND SOLUTIONS

3.1.1 Mitochondria media and solutions

Heart Isolation Medium

0,25 M sucrose, 10 mM HEPES, 0,2 mM EGTA, BSA 0,5 mg/ml pH 7.3

Brain Isolation Medium I

320 mM sucrose, 5 mM HEPES, 0.5 mM EDTA, 0.05 mM EGTA, pH 7.3

Brain Isolation Medium II

250 mM sucrose, 10 mM K-HEPES, pH 7.2

Mannitol Medium

0,25 M mannitol, 10 mM HEPES, 0,2 mM EGTA pH 7.3 and after protein determination 10 mg/ml BSA

Standard Incubation Medium

125 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 100 μM EGTA, 20 mM HEPES, 1mg/ml BSA pH 7.2

Gornall Solution

1 liter solution contain: 1,5 g $CuSO_4$, 6 g of Sodium and Potassium Tartrate, 30 g NaOH

3.2.2 Cells culture mediums and solutions

KRB medium

118,5 mM NaCl, 4,74 mM KCl, 2,5 mM $CaCl_2$, 1,18 mM KH_2PO_4, 1,18 mM $MgSO_4$.

Complete KRB

KRB medium additioned with BSA 5mg/ml, HEPES 20 mM, Glucose 25 mM, penicillin and streptomycin

Complete D-MEM

Medium with high glucose (4500 mg/l) supplemented with 10% (v/v) fetal calf serum

3.3 PREPARATION OF MITOCHONDRIA

3.3.1 Rat heart mitochondria

Rat heart mitochondria were isolated from 6-7 weeks old rats with the Polytron procedure essentially as described in [76]. Rat heart was first homogenized with Polytron in Heart Extraction Medium and then centrifuge at 1500 X g for 10 minutes at 4° C. The surnatant was centrifuged at 10500 x *g* for 10 min, and the resulting pellet was resuspended in 20 ml of Heart Extraction Medium and centrifuged at 10500 X g for 10 minutes at 4° C. The pellet was then resuspended in Mannitol Medium and the protein was quantified with Gornall reagent at 540 nm.

3.3.2 Brain mitochondria

The cerebral cortices of two 6–7-week-old rats were rapidly removed into 20 ml if ice-cold Brain Isolation Medium and homogenized. The homogenate was centrifuged at 900 x *g* for 5 min at 4 °C. The supernatant was centrifuged at 8500 x *g* for 10 min, and the resulting pellet was resuspended in 1 ml of Brain Isolation Medium. This was layered on a discontinuous gradient consisting of 4 ml of 6% Ficoll, 1.5 ml of 9% Ficoll, and 4 ml of 12% Ficoll (all prepared in isolation medium) and centrifuged at 75,000 x *g* for 30 min. The myelin, synaptosomal, and free mitochondrial fractions formed were respectively above the 6% layer, as a doublet within the 9% layer and as a pellet. The pellet was resuspended in Brain Isolation Medium II, and centrifuged at 8500 x *g* for 15 min before being resuspended in Mannitol Medium and the protein was quantified with Gornall reagent at 540 nm.

3.3.3 Hydrogen Peroxide Measurements

Mitochondrial H_2O_2 release were assessed by the oxidation of Amplex Red (40 μM) by horseradish peroxidase (6 μg/ml, 1 unit) induced by H_2O_2 [77, 78]. Amplex Red fluorescence was monitored at excitation and emission wavelengths of 544 and 590 nm, respectively, on a Fluoroskan Ascent FL plate reader [74] in 24 wells plates thermostatted at 30 °C with the indicated concentration of substrates. The reaction was started with addition of the Amplex red/ HRP mixture. H_2O_2 was detected by the formation of the fluorescent product resorufin. Internal standard of 1 nmol H_2O_2 was added at the end of each assay. The wells were read every minute and mean reading of every well was considered. The first 10 minutes rates were calculated. Values are reported as pmoles /min/mg protein.

3.3.4 Assay of α-Ketoglutarate

The α-Ketoglutarate assay was performed as in [79]. Mitochondria (4 mg/ml) were incubated in the standard incubation medium under agitation for 10 minutes with the indicated amount of substrates. During incubation substrate concentrations of glutamate, malate and succinate were maintened constant by addition of 0,3 mM glutamate and malate and 0,5 mM succinate at 5 minutes.

Reactions were stopped by addition of cold PCA (12 %) and samples left in ice 20 minutes before centrifugation (15000 X g, 10 min).

The method depends on the oxidative decarboxylation α- Ketoglutarate to succinyl- CoA:

$$\alpha\text{- Ketoglutarate} + \text{CoASH} + NAD^+ \rightarrow \text{succinyl CoA} + CO_2 + \text{NADH} + H^+$$

Enzymatic fluorimetric titration of NADH formed by α- Ketoglutarate oxidation was carried out in 100 µl aliquots of the supernatant neutralized with KOH in 200 µl (final volume) of medium containing 100 mM Na-phosphate (pH 7.3), $CaCl_2$ 5 mM, $MgCl_2$ 1 mM, EGTA 5 mM, NAD^+ 1 mM, and 0.2 mg/ml (160 mU) of α- Ketoglutarate dehydrogenase in the plate reader Fluoroskan at 355/460 nm ex/em. The reaction was started by addition of 100 µM CoASH. α- Ketoglutarate concentrations were calculated from the titration curve of the rates obtained with known comparable amounts of standard α- Ketoglutarate. Values are reported as nmoles/min/mg protein. Similar results were obtained by spinning down mitochondria at the end of incubation and measuring α- Ketoglutarate in the PCA-treated supernatant.

3.3.5 Assay of β-hydroxybutyrate

The β-hydroxybutyrate assay was performed as in [80]. The incubation was for 10 min at 2 mg/ml mitochondrial suspension. During incubation the substrate concentrations were: 5 mM acetoacetate, 1 mM succinate, 10 and 20 µM palmitoleyl-CoA, 1µg/ml rotenone and succinate concentration was maintened constant by addition of 0,3 mM succinate at 5th minute. Reactions were stopped by addition of cold perchloric-acid (12 %) and samples left on ice 20 minutes before centrifugation (15000 X g, 10 min).

The method depends on the oxidation of β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase:

$$\beta\text{-hydroxybutyrate} + NAD^+ \leftrightarrow \text{Acetoacetate} + \text{NADH} + H^+$$

Enzymatic fluorimetric titration of NADH formed by β-hydroxybutyrate oxidation was carried out in 100 µl aliquots of the supernatant neutralized with KOH at pH 8,5 in 200 µl (final volume) of 0,1M Tris-HCl (pH 8,5) containing 1,5 mM EDTA 0,5 mM NAD+, 100 mM hydrazine-HCl. At pH 8.5 approximately 40% of β-hydroxybutyrate is oxidized to acetoacetate. In the presence of hydrazine to trap the acetoacetate formed as hydrazone the reactions proceeds from left to right. The reaction was started by additions of 20 mU of β-hydroxybutyrate dehydrogenase in the plate reader Fluoroskan (ex/em 355/460 nm). β-hydroxybutyrate concentrations (reported as µM) were calculated from the titration curve of the rates obtained with known comparable amounts of standard β-hydroxybutyrate.

3.3.6 Measurement of Mitochondrial Membrane potential

Membrane potential ($\Delta\psi$) was measured using fluorescence quenching of the cationic dye safranine (3 µM) at ex/em 495/586 nm in 0,3 mg/ml of mitochondrial suspension in Incubation Medium in a Shimatzu spectrofluorimeter as in [81]

3.3.7 Measurement of Mitochondrial NAD(P)H redox state

Fluorescence of NAD(P)H redox state was followed fluorimetrically at ex/em 340/460 nm in Shimatzu Spectrofluorimeter in a 3 ml quartz cuvette under stirring in Incubation Medium.

3.3.8 Measurement of Mitochondrial Respiration

Oxygen consumption was monitored with a Clark-type oxygen electrode in a 1.6 ml closed chamber with continuous stirring in Incubation Medium.

3.4 CELL CULTURE

C_2C_{12} (ATCC Nr: CRL-11268) and SHSY-5Y (ECACC Nr. 85051005) adherent cells were grown at 37°C and 5% CO_2 in complete D-MEM unless otherwise specified. Cultures were grown to 70–80% confluence.

3.4.1 Transfection

For transfection both with peYFP (Clonotech) and ssHRP-KDEL pRK5 cells were grown in complete D-MEM. Cells were transfected using TransFectin Lipid Reagent (BioRad) or Gene Jammer (Stratagene) or Lipofectamine^{TM} 2000 (Invitrogen).

3.4.1.1 Classic transfection

Transfectant was added in solution to 70-80% confluent cells. Basically DNA and lipocation were diluted in Opti-Mem by following the suitable manufacture's protocols. The lipid was then added to the DNA and mixed by gentle pipetting. Following a 15 min incubation at room temperature, the lipoplex formed was added directly into the wells with complete D-MEM. After 6, 12 and 24 hours the medium was removed and replaced with fresh complete D-MEM.

3.4.1.2 Inverted phase transfection

The transfection performed with LipofectamineTM 2000 was optimized to increase its efficiency with an inverted procedure based on [82] and synthetically described. Essentially C_2C_{12} and SHSY-5Y were transfected with LipofectamineTM 2000 and plasmid in 25 cm^2 polystyrene flasks. The DNA (125 μg /ml) and the Lipofectamine (250-350 μg/ml) were each pre-diluted in Opti-MEM. The 1:1 (v/v) DNA/Lipofectamine mixture prepared was used to pre-treat polystyrene flasks (DNA was omitted in vehicle-treated cells) before seeding cells suspended in complete D-MEM medium after trypsinization. 18 hours after, the medium was removed and replaced with fresh complete medium D-MEM. At 24 hours cells were trypsinized, then seeded at the desired concentrations on polistyrene multiwell plates for the subsequent determinations or on coverslips for the HRP immunodetection. Vehicle treated cells or not transfected WT cells were used as negative controls.

Expression of YFP was observed by a inverted fluorescence microscope (Zeiss) and image was analyzed with Image J software. At least 5 field/samples at low resolution were considered.

Expression of HRP was monitored with the anti-HRP antibody (Jackson ImmunoResearch laboratories) on glass fixed cells (at 48 hours). Cells were

transferred on glass cover slips after tripsinization at 24 hours. At least 5 field/samples at low resolution were considered.

3.4.2 HRP protein detection

3.4.2.1 HRP protein immuno-detection

Cells fixed in methanol/acetone/ H_2O (2/2/1) for 20 minutes at -20°C were saturated with PBS containing 1% BSA overnight at 4 °C. The saturated cells was incubated with goat antibody anti-HRP Tetramethyl Rhodamine conjugated (TRITC) (Jackson Immuno Research laboratories) 1:100 diluted in saturating buffer for 1 hour and then incubated for 10 minutes with 10 μM Hoechst 3310 to stain nuclei. After three washing in PBS and one in H_20 coverslips were treated with a drop of ProLong® Gold antifade reagent (Molecular Probes) mounted on slide and analyzed with fluorescent microscope.

3.4.2.2 Functionality of the expressed HRP protein:

24 h after transfection cells were trypsinized and aliquots of 8000 were seed in 96 wells plates (Falcon) in complete D-MEM. After 24 hour the D-MEM was collected replaced with complete KRB. The functionality of the expressed protein and the time course of its expression was followed by titration of the HRP activity in total cell with Amplex red 40 μM, H_2O_2 20 μM after solubilization with 0,1 % Triton X100. The rate before and after additions of H_2O_2 were measured and reported as mean net increase of fluorescence /min at 544/590 nm.

3.4.3 Viability test

3.4.3.1 Propodium Iodide staining

After 4 hours in incubation medium at 37° cells were treated for 20 more minutes with 20 μM Propidium Iodide and 10 μM Hoechst. The permeability to the PI nuclear stain measured on the fluorescent microscopy images was used as index of cell death and reported as Mean ± SD percentage of PI stained nuclei/ total (Hoechst stained) nuclei [83]. Data were calculated from the corresponding images acquired in a Zeiss fluorescent inverted microscope by the Image J

software. At least 3 fields/sample in triplicate conditions at 80X magnification were considered

3.4.3.2 Lactate dehydrogenase released:

The amount of lactate dehydrogenase released from cells, after 4 hours in incubation medium as death index [84], was performed with the Cytotoxicity Detection Kit (Roche) on the supernatant and in the total cell lysates. The percentage of LDH released is expressed as Mean ± SD of triplicate analysis. The total amount of LDH was corrected for the protein content.

3.4.3.3 Metabolic activity

Cell viability was assessed with MTT dye [85]. The MTT assay is based on the reduction of the methyl tetrazolium salt to the purple formazan by the mitochondrial enzymes of viable cells. The MTT test was performed after 30 minutes of incubation in complete D-MEM with 1 mg/ml of MTT followed by the colorimetric determination of the formazan salt formed as net absorbance (540-620 nm) of DMSO solubilised samples. The values of the MTT test normalized to the protein content (in parallel wells) and reported as % of the corresponding WT cells (Mean ± SD of triplicate analysis).

3.4.4 Protein Assay

The micro-method of BCA protein assay was used on aliquots of Triton X100 cell lysates essentially as described by manufactures protocol (Pierce) and measured at 540 nm at the plate reader (Multiscan XE Thermo Labsystems).

3.4.5 Cell GSH depletion

The GSH content was lowered by coniugation with CDNB (1-chlor-2 ,4-dinitrobenzene), through the glutathione-S-transferase action [86], or by inhibition of " de novo " biosinthesis with buthionine sulfoximine [87]. In both cases preincubations (one hour with 30 μM CDNB and 14-16 hours with 1 mM BSO) were performed in complete D-MEM and reagents removed before the experiments.

3.4.6 MAO activity assay

3.4.6.1 Radiochemical assay

The oxidase activity, based on the radiochemical method described [88], was measured with $^{[14]}C$ 5-hydroxytriptamine (serotonin) in viable C2C12 cells. Briefly near confluent C_2C_{12} cells in 6 wells plates (106 cells/well) were incubated in humidified termostated chamber at 37°C in the KRB containing BSA buffer for 12 hours with the labeled MAO A substrate 5 hydroxytriptamine (0.3 mM, 0. 5 μCi/ mM) after a 30-min preincubation without (total oxidation) or with the MAO A inhibitor clorgyline (300 nM). Supernatants and 0,1% cell lysates were separately collected and reaction stopped with 0,5 N HCl. Reaction products were extracted by addition of 3 volume of solvent (toluene/ ethyl acetate, v/v). The radioactivity present in the organic phase was counted in a β-scintillation counter. The sum (supernatant + lysates) of recovered labeled serotonin metabolites found in viable C_2C_{12} cells was about 1 nmol of oxidised serotonin 106 cells/60 min. MAO A activity accounted for 95% as calculated the difference between 5 hydroxytriptamine oxidation observed in the absence and in the presence of clorgyline.

3.4.6.2 Fluorescent assay in permeabilyzed cells

Hydrogen peroxide production from Amine Oxidase activity was measured with Amplex Red/HRP [75] in a plate reader [74]. The amount of H_2O_2 production is followed as increase of the fluorescent (at 544/590 nm) resorufin formed by the oxidation of the non fluorescent Amplex red (40 μM) in the presence of HRP (50 mU/ml). Cells were treated with tyramine (1mM), clorgyline (300 nM) and deprenyl (1 μM) in the presence of 0,3 mM NaN_3. Clorgyline and deprenyl were also pre-incubated for at least 30 minutes before tyramine additions. The Amplex Red oxidation rate was recorded before and after permeabilization with 0,1 % Triton X100. At the end of every experiments H_2O_2 was added as internal standard.

3.4.6.3 Fluorescent assay in intact cells

96 wells plates with HRP transfected or WT cells ($8x10^3$/well) of both cell lines were used at the fluorescent plate reader or at the inverted fluorescent microscopy at 80X magnification. At 0 time the complete D-MEM was replaced with the complete KRB containing the fluorescent probe 10 μM DCFH2-DA or 10 μM DHR. Tyramine (1mM) was added to start H2O2 production. When present, the MAO inhibitors (clorgyline 300 nM, deprenyl 1mM) were pre-incubated before tyramine and fluorescent probes additions.

Direct readings (10 minutes cycles) were performed in triplicate on Fluoroskan Ascent FL plate reader at 485-527 nm, the oxidation rates were calculated and data reported as arbitrary units of fluorescence increase (AUF /min).

Fluorescence microscopy evaluation was performed after three hours of incubation. One shot (at fixed exposition time of 2-3 seconds) images were acquired at the Zeiss inverted fluorescence microscope in the different experimental conditions soon after washing away reactants and replacing medium with fresh incubation medium, at the proper excitation and emission filters. Images acquired with the Metamorph software were analyzed by the Image J software for the fluorescence signals. Data reported represent the total cell intensity as means ± SD of at least 200-300 cells/field for every condition.

3.5 STATISTICAL ANALYSIS

All data are presented as mean ± standard deviation (SD) in at least triplicate samples. Where appropriate, Student's *t* tests were performed, to give significance values.

4.0 RESULTS

4.1 GENERAL PROPERTIES OF H_2O_2 RELEASE AT COMPLEX I

Mitochondria are considered one of the main sites of ROS production in mammalian cells, and $O_2^{\bullet -}$ appears to be the primary ROS produced as the result of mono-electronic reduction of O_2.

Mitochondrial Complex I is the most complicated and the least understood electron carrier in the respiratory chain. Its primary function is to pump protons across the inner membrane. Another property associated to Complex I activity is a highly modulated $O_2^{\bullet -}$ production.

At the Complex I $O_2^{\bullet -}$ is generated on the matrix side of the inner membrane [12] and is transformed into H_2O_2 by mitochondrial Mn-SOD (manganese superoxide dismutase). The highly permeable H_2O_2 can be measured in the extra-mitochondrial space in the presence of an excess of HRP, coupled to the oxidation of a fluorescent probe. We used Amplex red that, as mentioned above, is the most sensitive fluorogenic probe.

In the present study, we have considered the most probable condition where a physiological production of superoxide/H_2O_2 can occur, i.e. where both NAD-linked substrates (in our experiments we use glutamate plus malate) are present together with a low concentration of succinate, both in brain and heart mitochondria. All experiments were also performed in the absence of ADP that inducing the oxidation of the redox electron transfer chain and the decrease of membrane potential components inhibits the $O_2^{\bullet -}$/H_2O_2 production as previously reported [15]. Moreover as summarized in the introduction, H_2O_2 is generated during coupled respiration of NAD-linked substrates and the release is increased by the electron transfer inhibitor rotenone; it is also generated, at a much higher rate, during coupled succinate respiration and this production is strongly inhibited by rotenone and by decreasing membrane potential (fig. 6). These properties are taken as evidence that succinate-dependent H_2O_2 generation occurs in Complex I

upstream of the rotenone inhibition site (i.e. via energy-dependent reverse electron transfer from Complex II to Complex I).

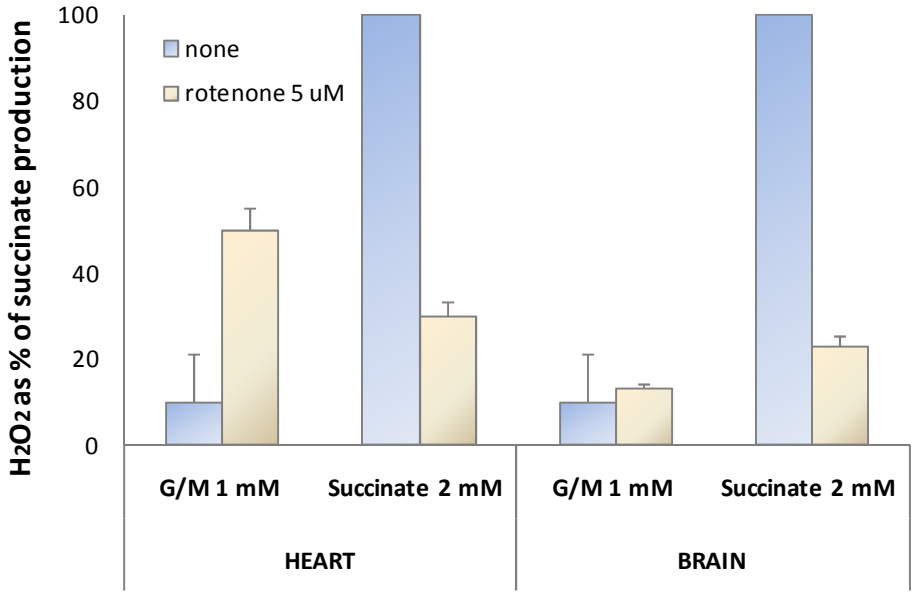


Figure 6: Rotenone effect on H_2O_2 release by Complex I in heart and brain mitochondria

So the generally accepted view is that succinate promotes H_2O_2 release by pushing electrons back into Complex I in a reverse mode of electron transfer and the production starts from submillimolar succinate (fig. 7), a concentration more physiological than the high succinate concentration (5-10 mM) used elsewhere. In fact below 0,5 mM a succinate dependent H_2O_2 production is already revealed, reaching the maximum at 2-3 mM succinate both in heart and brain mitochondria. With NAD-linked substrates alone the H_2O_2 release is slow and accounts for 10% of the maximal production with succinate. Moreover low succinate promotes H_2O_2 release also in the presence of high NAD-linked substrates. The succinate dose dependent increase of H_2O_2 release is shown also when G/M (1 mM each) are co-incubated. The curve of H_2O_2 release is obtained at slight higher succinate concentrations, showing an inhibition at low succinate concentration by the possible flow of electrons coming from the NADH dependent oxidation but still reaches approximately the same maximum at 2-3 mM. Only a limited competition is shown at the lower succinate concentrations between electrons coming from NADH and succinate.

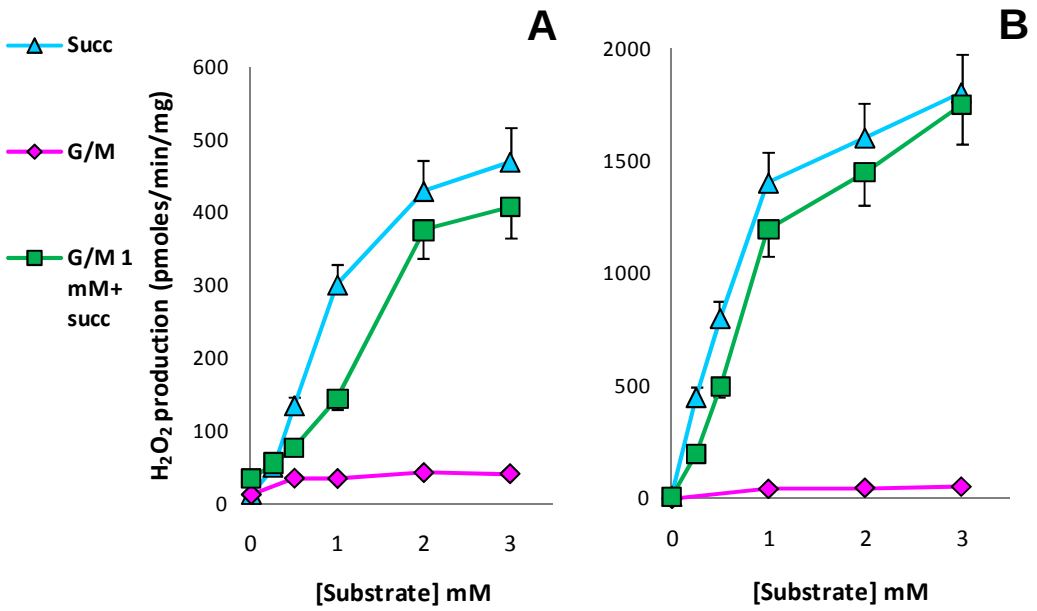


Figure 7: H_2O_2 release in heart (A) and brain (B) mitochondria in the presence of different concentrations of NAD-dependent substrates (G/M), succinate and both. Where G/M and succinate were present together G/M were 1 mM each

4.1.1 Succinate oxidation does not completely inhibit NAD-dependent substrates oxidation

It can be questioned whether succinate in these conditions operates a 'reverse' mode of electron flux, i.e. whether the oxidation of G/M is prevented. To solve this problem, first we analysed the rates of O_2 consumption with G/M (1 mM each), succinate and a mixture.

O_2 consumption in state 4 (ngA Ox min $^{-1}$ x mg^{-1})	***RHM***	***RBM***
Succinate 1 mM	50 ± 5	31 ±3
G/M 1 mM	20 ± 3	10,5 ± 2.5
G/M 1 mM + succinate 1 mM	35 ± 4	25 ± 2

table 1

Results are reported in Table 1, which shows the experiment with 1 mM succinate (a concentration that promotes a large H_2O_2 release). The oxidation rate is higher with succinate than with G/M, reflecting the lower number of protons pumped by succinate, however, the O_2 consumption rate with the mixture is intermediate between that of the two constituents alone, a possible indication that both succinate and G/M are contributing to O_2 consumption when they are present together.

Although these results provide evidence in favour of the hypothesis that electrons actually flow downwards in Complex I also during the succinate-promoted H_2O_2 release in the presence of G/M, they are not a direct demonstration that this really occurs. As a second approach to prove this point, we measured the generation of α-ketoglutarate with G/M in the absence and presence of succinate.

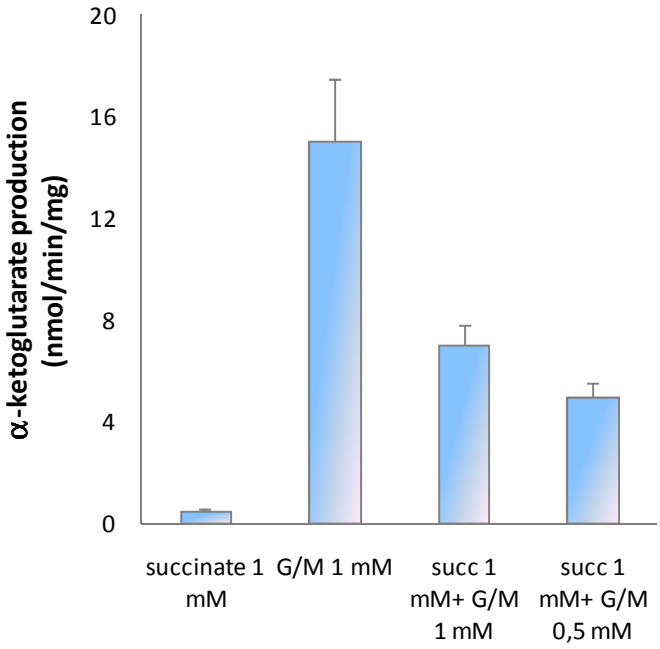


Figure 8: α-ketoglutarate is produced during coupled oxidation of G/M and succinate

The accumulation of α-ketoglutarate is connected directly to the oxidation of G/M, via glutamate dehydrogenase, or via glutamate/OAA (oxaloacetate) transaminase, where OAA originates from malate oxidation. As shown in fig. 8, α-ketoglutarate accumulated during the oxidation of G/M, but not during succinate oxidation. With G/M, the further presence of succinate progressively

decreased, but did not abolish, α-ketoglutarate production. At 1 mM succinate, α-ketoglutarate production was still 30% that of the control without succinate. These results are in agreement with the data on respiration presented above.

These experiments demonstrate conclusively that electrons keep flowing down from Complex I in the presence of succinate and during the process of succinate-supported H_2O_2 production.

4.1.2 H_2O_2 release at Complex I is controlled by ΔpH

The Complex I H_2O_2 release is dependent on Δp across the inner mitochondrial membrane. Δp is the sum of two components, ΔΨ and ΔpH. It has been shown previously that ΔpH is a major controller of succinate-dependent H_2O_2 production [14]. In fact, the inclusion of the K^+/H^+ ionophore nigericin (in a high K^+ medium) allows K^+ equilibration and hence H^+ equilibration (ΔpH=0); this results in a corresponding equivalent increase of ΔΨ to maintain Δp constant [14]. Under these conditions, the succinate promoted H_2O_2 release is lower. On the contrary, increasing ΔpH by omitting phosphate (whose influx via the Pi–H^+ symporter decreases ΔpH) promotes a higher succinate-dependent H_2O_2 release. ΔpH controls also the G/M dependent H_2O_2 production. Unlike with succinate, low ΔpH (with nigericin) slightly stimulates the G/M induced H_2O_2 release and high ΔpH (obtained omitting Pi) inhibits the H_2O_2 release (fig.9).

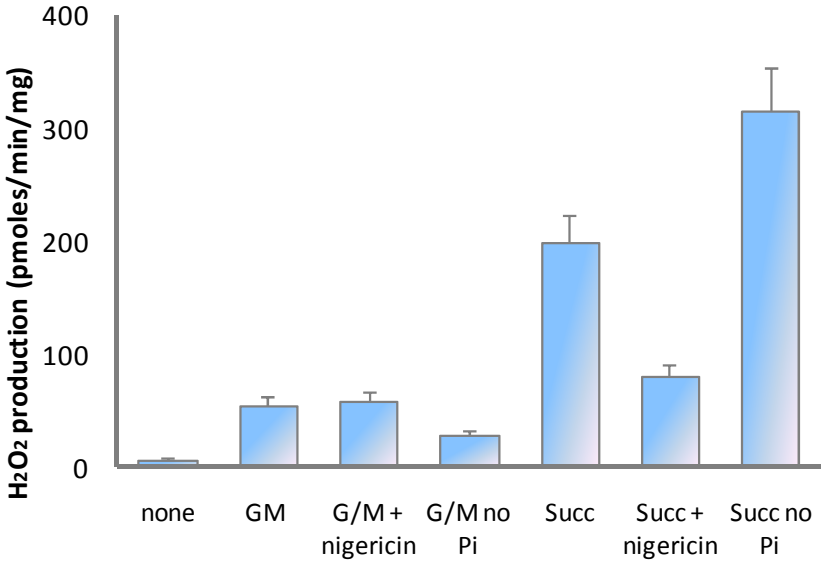


Figure 9: H_2O_2 production rate is dependent on ΔpH

So ΔpH appears to control the superoxide/H_2O_2 release in opposite way. High ΔpH decreases monoelectronic O_2 reduction when electrons flow down from NADH and increases it when electrons derive from the FAD dependent succinate oxidation. High ΔpH behaves like the inhibitor rotenone.

4.2 SUCCINATE INDUCED H_2O_2 GENERATION IS INHIBITED BY LONG CHAIN ACYL-COAS

In an attempt to investigate the property of the other major FAD-dependent process in heart mitochondria, i.e. fatty acid degradation, we studied the effect of different free fatty acids on H_2O_2 production. Free fatty acids *per se* did not produce nor modify the succinate dependent H_2O_2 production, independently of their oxidation (fig. 10).

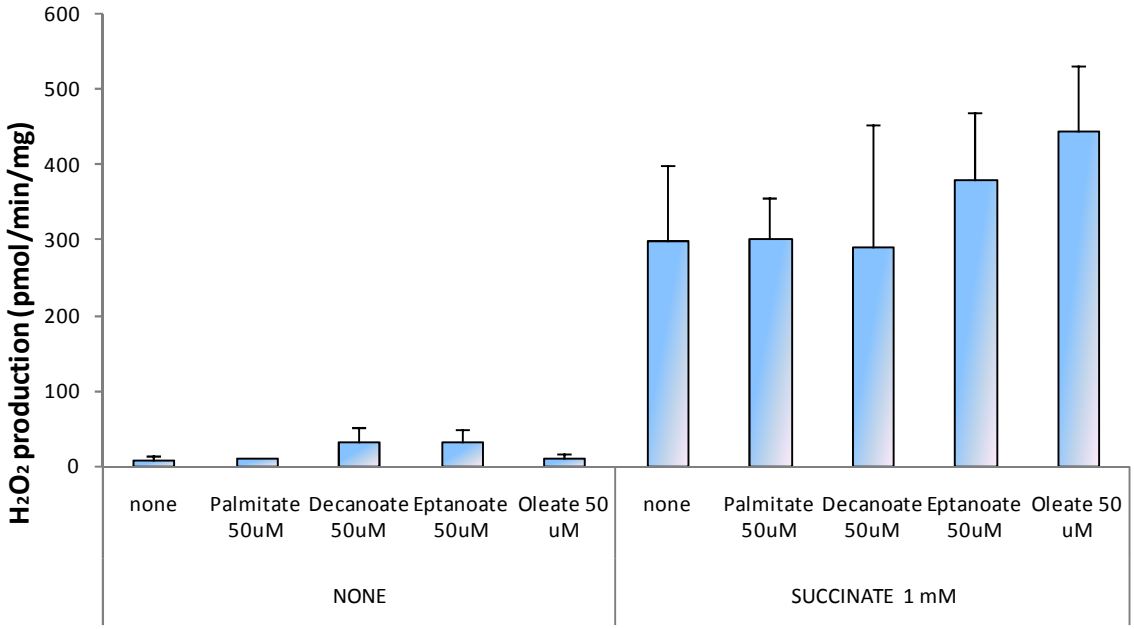


Figure 10: Effect of free fatty acids on mitochondrial H_2O_2 production with or without succinate

Medium chain fatty acids oxidation occurs without the help of carnitine and ATP. Indeed when added to mitochondria they generate a high Δψ (fig. 11 trace

a). Long chain fatty acids instead require activation to CoA thioesters (via the acyl- CoA synhetase present in intact heart mitochondria [89] in the presence of ATP and CoASH) and in the presence of carnitine are then transformed into fatty acyl-carnitine (by means of carnitine palmityl transferase I) to be transported into the matrix and oxidized. This is reported in the same fig. 11 trace b showing that oleate, in the presence of ATP, CoASH and oligomycin (to prevent the direct ATP-dependent mitochondrial energization), is unable to increase membrane potential significantly, until carnitine is also added. This demonstrates that energization by oleyl-CoA depends on its transport inside mitochondria, while external oleyl-CoA *per se* is not metabolized.

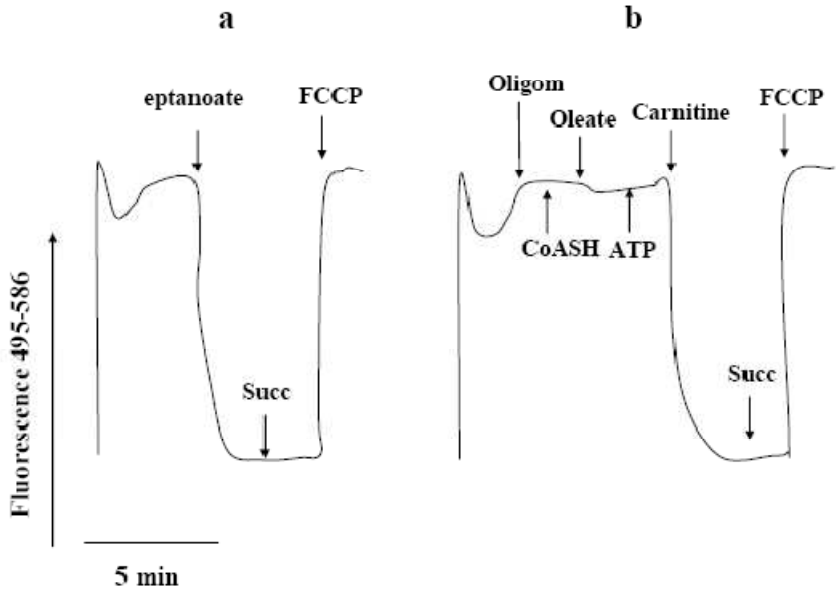


Figure 11: Effect of long or short fatty acid on safranine measured Δψ When indicated eptanoate or oleate (40 µM), oligomycin (1 µM), CoASH (100 µM), ATP (2 mM), carnitine (2 mM) and succinate (2 mM) were added.

While oleate does not modify succinate dependent H_2O_2 production (fig. 10 and 12), its CoA ester, indeed inhibits peroxide formation. In fig.12 it is shown that in the presence of CoASH and ATP where oleyl-CoA is generated, oleate acts as an inhibitor of succinate dependent H_2O_2 production. Carnitine addition removes instead the inhibition. These results strongly suggest acyl-CoAs as potential inhibitors of the succinate-dependent H_2O_2 production.

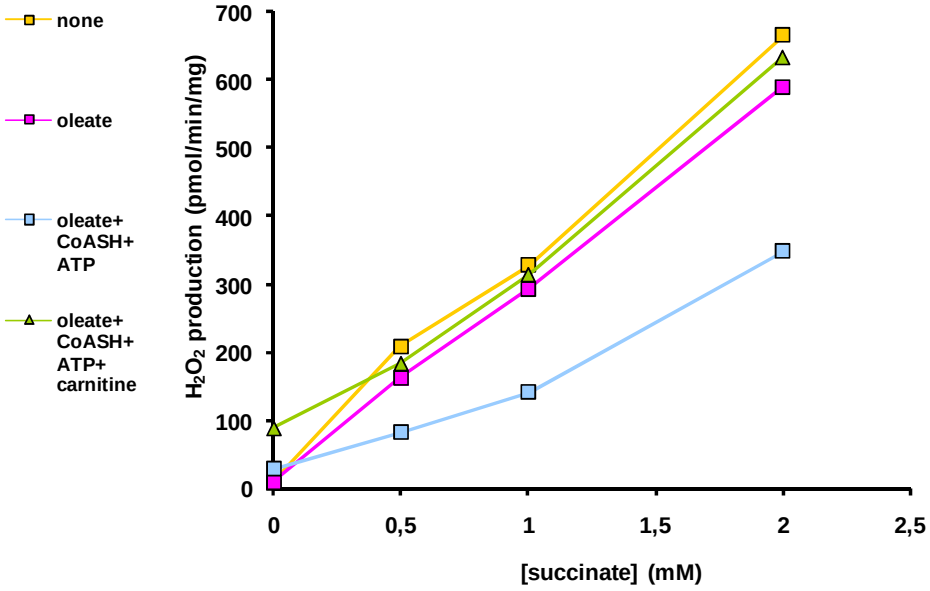


Figure 12: Effect of oleate-derived activated forms on succinate H_2O_2 production

Direct addition of fatty acyl-CoA esters has been then used to confirm their inhibition on the succinate dependent H_2O_2 release avoiding interferences of ATP, oligomycin and CoASH.

We performed titrations of the inhibition exerted by both the saturated long chain acyl-CoA, palmitoyl-CoA, and the unsaturated long chain acyl-CoA, palmitoleoyl-CoA (fig. 13A). Both acts as powerful inhibitors. An apparent IC_{50} value of 5 µM was found with palmitoyl-CoA. A similar curve, obtained with palmitoleoyl-CoA, gives an higher IC_{50} (12 µM) showing a lower inhibitory efficiency of the corresponding mono-unsaturated fatty acyl-thioester, always in the low micromolar range. Data strongly indicate that the fatty acyl-CoAs exhibit "*per se*" a powerful inhibition on the succinate driven H_2O_2 production. To definitely prove their action as acyl-CoA we next added carnitine. The inhibition by acyl-CoA is progressively removed by addition of carnitine which induces the decrease of palmitoyl-CoA versus the production of the non inhibitory palmitoyl-carnitine (fig. 13B). Maximal effect was obtained at 2 mM carnitine but it is largely evident at 0,5 mM that carnitine reduces acyl-CoA inhibition. To prove that the inhibitory effect depends on the CoA esters we have also shown that in the presence of 0,5 mM carnitine, when the acyl-CoA inhibitory effect is largely

prevented, the inhibition is dose-dependently restored by malonyl-CoA, the physiological inhibitor of carnitine palmitoyl transferase I; in this condition palmitoyl-carnitine formation is prevented and the H_2O_2 release is again inhibited by acyl-CoA. All data so far obtained clearly indicate a direct inhibition of succinate dependent H_2O_2 release by long chain acyl-CoAs.

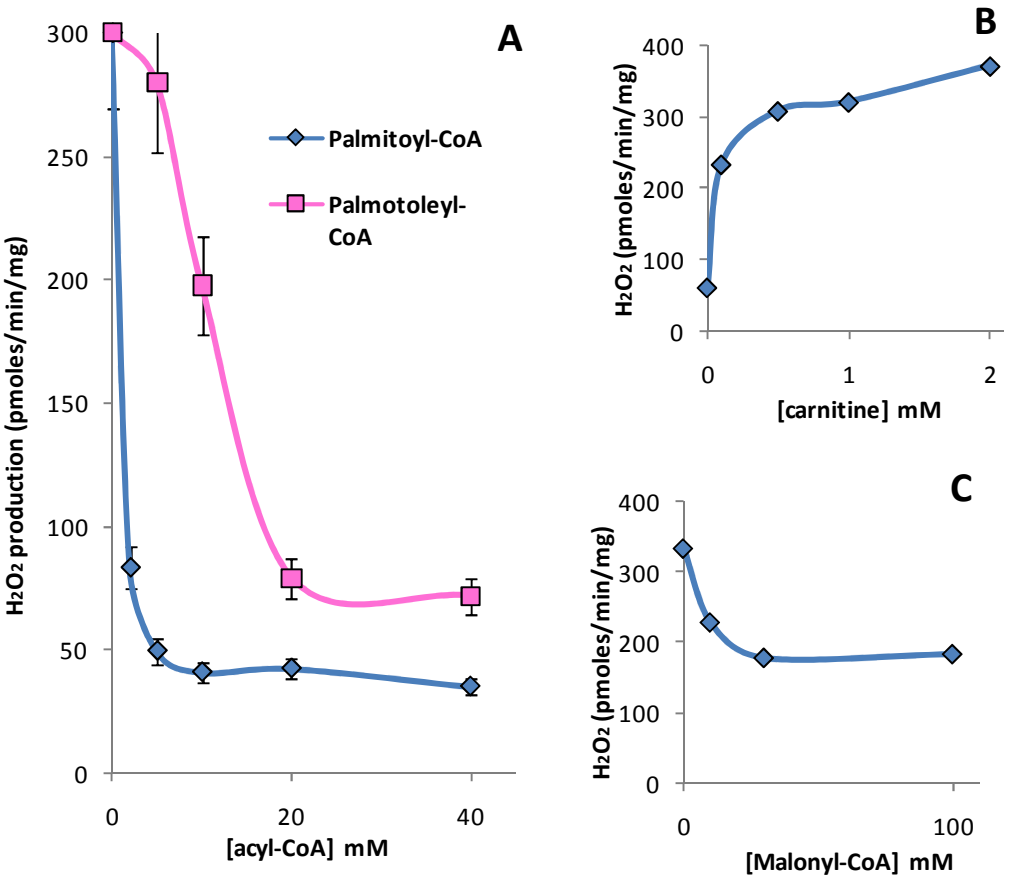


Figure 13: (A) Inhibition of succinate dependent H_2O_2 release by palmitoyl-CoA and Palmitoleyl-CoA is dose-dependent; (B) carnitine restore succinate dependent H_2O_2 release in the presence of palmitoyl-CoA 10 µM; (C) malonyl-CoA restore palmitoyl-CoA inhibition in the presence of 500 µM carnitine

To exclude any other effect of acyl-CoA on the succinate transport and/or oxidation, succinate respiration was measured in the presence of palmitoyl-CoA. In fact an inhibition of dicarboxylate transporter by long chain acyl-CoAs as reported by [90] has been excluded since in the presence of BSA (our experimental conditions and also as reported by [91]), palmitoyl-CoA slightly

affects succinate oxidation only at concentrations higher than 20 μM (18 ± 2 % inhibition by 20 μM palmitoyl-CoA). The respiration is essentially unaffected at the concentration used (0 ± 5 % inhibition by 10 μM). The presence of BSA besides being required for detection of H_2O_2 in mitochondrial suspension, mimics a more physiological status of the fatty acid presence in a cell. In fact in the cells acyl-CoAs are not free but are linked to the family of the acyl-CoA binding proteins (ACBP). It has to be recalled that the binding activity of BSA and ACBP are superimposable as reported in liposomal models [92, 93]. To address the structural requirements of the acyl-CoA also short chain acyl-CoAs were tested. Short chain acyl-CoAs instead were without activity on succinate-induced peroxide as shown in fig. 14, where short medium and long chain acyl-CoAs are compared. Only long chain fatty acyl-CoAs appeared inhibitors, and the inhibition depended strictly on the chain length of the CoA thioesters which seem to act at the outer face of the inner membrane since their inhibition is prevented by the presence of carnitine.

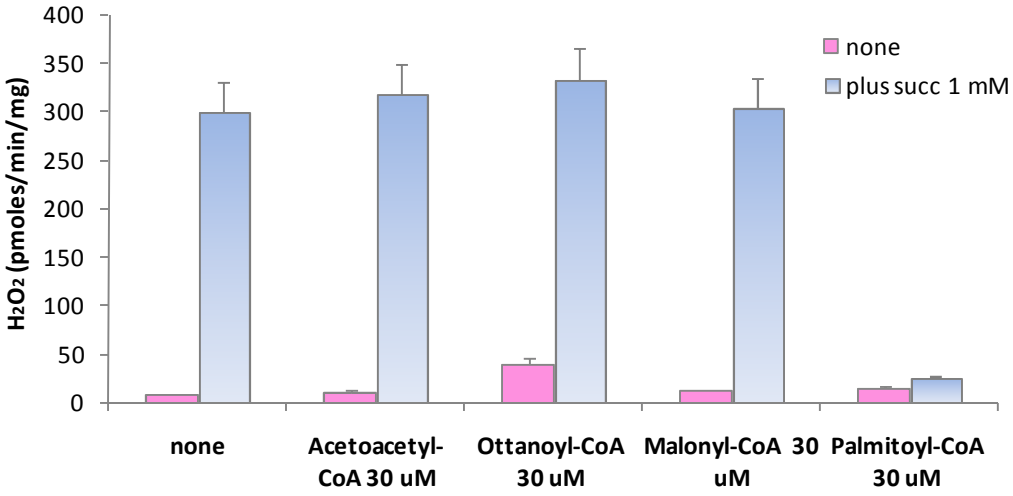


Figure 14: H_2O_2 release by different length acyl-CoA thioesters in the absence or in the presence of 1 mM succinate

To better characterize the possible mechanism underlying the acyl-CoA inhibition we measured their possible involvement in modifying the ΔΨ and ΔpH. Palmitoyl-CoA does not decrease the succinate-generated membrane potential, ruling out the possibility that its effect depended on a partial uncoupling (fig. 15).

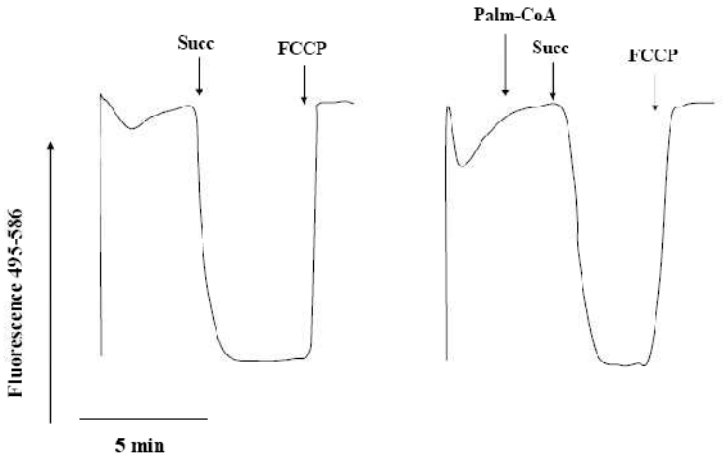


Figure 15: Effect of palmitoyl-CoA on succinate dependent Δψ. Δψ was measured by safranine distribution. When indicated 1 mM succinate, 10 µM palmitoyl-CoA and 1 µM FCCP were added

The inhibition is also independent of ΔpH. In fact 10 µM palmitoyl-CoA inhibits H_2O_2 generation both in the absence of phosphate (maximal ΔpH) and in the presence of phosphate (intermediate ΔpH) to an extent similar to that obtained with the H^+/K^+ exchanger nigericin that abolishes the ΔpH. A limited effect was shown if added together with nigericin (fig. 16).

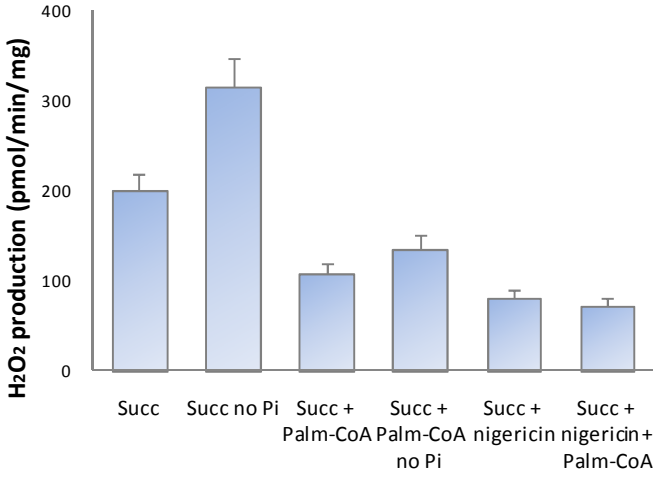


Figure 16: Inhibition of succinate dependent H_2O_2 release by palmitoyl-CoA is independent of ΔpH

Palmitoyl-CoA appears to act similarly to nigericin and to rotenone. In fact, as shown for both agents, while inhibiting the succinate dependent H_2O_2 production,

it slightly stimulates the low G/M dependent H_2O_2 release, both at high and low ΔpH.

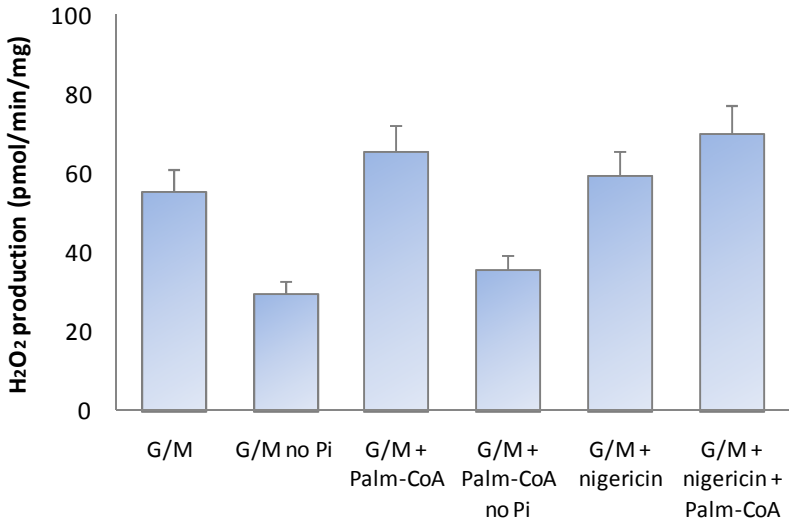


Figure 17: Palmitoyl-CoA stimulate the low G/M dependent H_2O_2 release both at high and low ΔpH

We can conclude that external long chain fatty acyl-CoAs appear as the most probable physiological modulators of the Complex I H_2O_2 production. The prevailing effect is to inhibit the large succinate stimulated H_2O_2 release, also if the low G/M dependent H_2O_2 production appears stimulated. In that they act similarly to rotenone. Acyl-CoAs can physiologically mimic the effect of the unphysiological rotenone.

Other effects can also be ascribed to long chain acyl-CoAs since they are reported also as inhibitors of the energy dependent transhydrogenase and of ADN translocator in mitochondria.

4.2.1 Long chain acyl-CoAs inhibit NAD(P) reduction and reverse electron flow at Complex I

Long chain acyl-CoAs are known to be powerful inhibitors of mitochondrial energy dependent transhydrogenase [94, 95]. This enzyme is the principal responsible for NADP reduction in mitochondria. In turn, H_2O_2 removal depends largely on NADPH (via glutathione or thioredoxin peroxidase) [40, 96, 97]. Acyl-CoAs should therefore enhance H_2O_2 release. Inhibition of the NADPH dependent

removal of H_2O_2 could be however masked by the activity of catalase that is present heart mitochondria [98, 99].

We decided to investigate the effect of palmitoyl-CoA on mitochondrial NAD(P) reduction. Fig. 18 shows that during coupled succinate oxidation a large NAD(P) reduction is observed (trace a), which is completely removed upon uncoupling. The subsequent addition of rotenone promotes a partial reduction (originating from the oxidation of succinate-derived malate). Due to the lack of energy-dependent transhydrogenase activity in this condition the reduced specie is mostly NADH. In the presence of palmitoyl-CoA the succinate-dependent pyridine nucleotide reduction is strongly decreased (fig. 18 trace c), to a level close to that of NADH determined in the previous trace (fig. 18 trace a) and in trace b where malonyl-CoA is added before succinate. In these experiments malonyl-CoA was also included to prevent slow palmitoyl-CoA oxidation. The strong inhibition by palmitoyl-CoA of NAD(P) reduction tentatively reflects the inhibition of transhydrogenase but may involve also the inhibition of reverse electron flow from succinate.

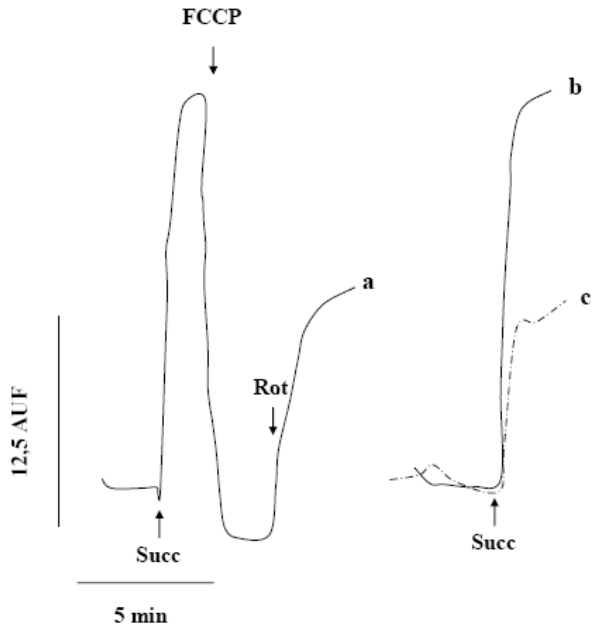


Figure 18: Effect of FCCP, rotenone and palmitoyl-CoA on NAD(P) reduction by succinate. Trace a: total NAD(P) was reduced by succinate. 50 µM malonyl-CoA (trace b) or malonyl-CoA plus palmitoyl-CoA 10 µM (trace c) were added before succinate.

To confirm the last effect a direct measurement of the rate of NADH formation from succinate in coupled mitochondria was performed by measuring succinate supported acetoacetate reduction (by means of the NADH dependent mitochondrial β-hydroxybutyrate dehydrogenase) in the presence and absence of palmitoyl-CoA. The amount of β-hydroxybutyrate formed was slightly inhibited by 10 μM palmitoyl-CoA and more strongly by 20 μM palmitoyl-CoA an indication that a lower level of NADH is produced in the presence of palmitoyl-CoA. The level reached in the presence of 20 μM palmitoyl-CoA was similar to that obtained with rotenone, that is a more specific inhibitor of the reverse electron transport, indicating a residual NAD^+ sustained reduction by succinate, probably dependent on the activity of malate dehydrogenase (fig. 19).

These results support a possible interference in the Complex I by palmitoyl-CoA that can partially inhibit the reverse electron transfer from succinate in heart mitochondria. Thus the acyl-CoA effects appear to represent a metabolic control of the mitochondrial respiratory chain-dependent H_2O_2 release.

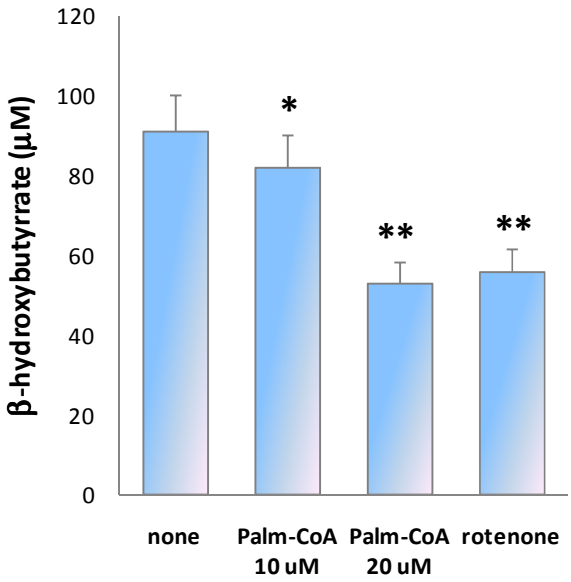


Figure 19: Succinate dependent acetoacetate reduction, effect of palmitoyl-CoA and rotenone. β-hydroxybutirate formation (μM) is reported in the indicated experimental conditions. Values are means ± SD of at least three different preparation in duplicates. Significantly different from control *P<0.05; ** P<0.01(Student's t test)

To extend the physiological meaning of acyl-CoAs we also compared the inhibition in non fatty acids oxidizing tissues. Fatty acid oxidation is reported to be essentially absent in brain mitochondria, due to the low activity of 3-ketoacyl-CoA thiolase [100]. We confirmed that 10 μM palmitoyl-CoA inhibited succinate-dependent H_2O_2 production as in heart mitochondria.

On the whole we suggest for acyl-CoAs another possible relevant physiological effect as modulators of the mitochondrial H_2O_2 production among the most physiological effects attributed to these intracellular metabolites [101].

The significance of the mitochondrial production of H_2O_2, due to the large number of conditions affecting its production and its effective role in cell metabolism is however still under debate.

We next tried to extend the measurements made in mitochondrial preparations to intact cells.

The inadequacy of the usual methods for detection of H_2O_2 were soon evident and will be stressed in the following section.

4.3 DETECTION OF H_2O_2 IN INTACT CELL SYSTEM

The general problem of measuring H_2O_2 in intact cells is the inadequacy of most detection methods. Among the several approaches to measure intracellular ROS production in living cells the use of “specific” fluorescent probes is the most common tool as tested by many scientific data in the literature. Many of the ROS sensitive probes are substrates for peroxidases and thus their oxidation has been referred to as specific for detection of intracellular H_2O_2. The most popular are 2',7'-dichlorodihydrofluorescein ($DCFH_2$) with its permeant derivatives or Dihydrorhodamine 123 (DHR). Their use as detectors of H_2O_2 or others ROS in intact cells is now amply criticised suggesting that data obtained are to be taken with high caution especially for the lack of specificity of these probes and their low stability to photo-irradiation. We confirmed with our experiment the difficulties in obtaining relevant results with the use of the most popular fluorescent probes. First of all we also considered the possible sources that could be easily handled in intact cell systems. Aware that the mitochondrial contribution

is perhaps the most difficult to be studied for the impermeability of the cell to majority of mitochondrial substrates we addressed our study to the monoamine oxidase activity that is reported as one of the main direct H_2O_2 producer.

4.3.1 H_2O_2 produced in cells by MAO is not measurable outside

Monoamine Oxidases activity has been chosen to induce and measure the H_2O_2 production in intact cell systems. The Monoamine Oxidases are outer mitochondrial membrane enzymes largely expressed in mammalian tissues involved in the oxidative deamination of endogenous and exogenous amines coupled to a primary production of H_2O_2. The amount of MAO derived H_2O_2 production is indeed suggested to exceed the levels generated by mitochondrial respiration [42], and can be manipulated by permeant substrates and inhibitors. We first tried to measure this activity in human neuroblastoma SHSY-5Y and mouse myoblast C_2C_{12} cells with the reported method based on the detection of H_2O_2 by means of the Amplex Red and HRP. This methods is generally applied to the subcellular preparations or in tissue lysated or purified MAO enriched fractions.

In agreement with previous reports where MAO-A was detected either immunologically or by means of the mRNA [102, 103], we confirmed in the SHSY-5Y cells a prevalent MAO-A activity (fig. 20A). The same activity was also found in C_2C_{12} cells with the same procedure (fig. 20A e B). No data were found in literature for this cell line so MAO activity was also confirmed in C_2C_{12} cells using the classical method based on the measure of the radiolabeled product formation from oxidation of the MAO-A substrate [^{14}C] 5–hydroxytriptamine. Determination was performed in intact C_2C_{12} cells and the activity found was 1 nmol/10^6 cells/60 min. The activity was completely inhibited by 0,3 μM clorgyline a specific inhibitor of MAO-A.

What is to be noticed is that HRP and the most sensitive probe Amplex red added to the culture medium fail to detect hydrogen peroxide unless cells exposed to MAO substrates are permeabilized (fig. 20A), clearly indicating that H_2O_2 produced within the cells by MAO activities does not reach appreciably the extracellular compartment.

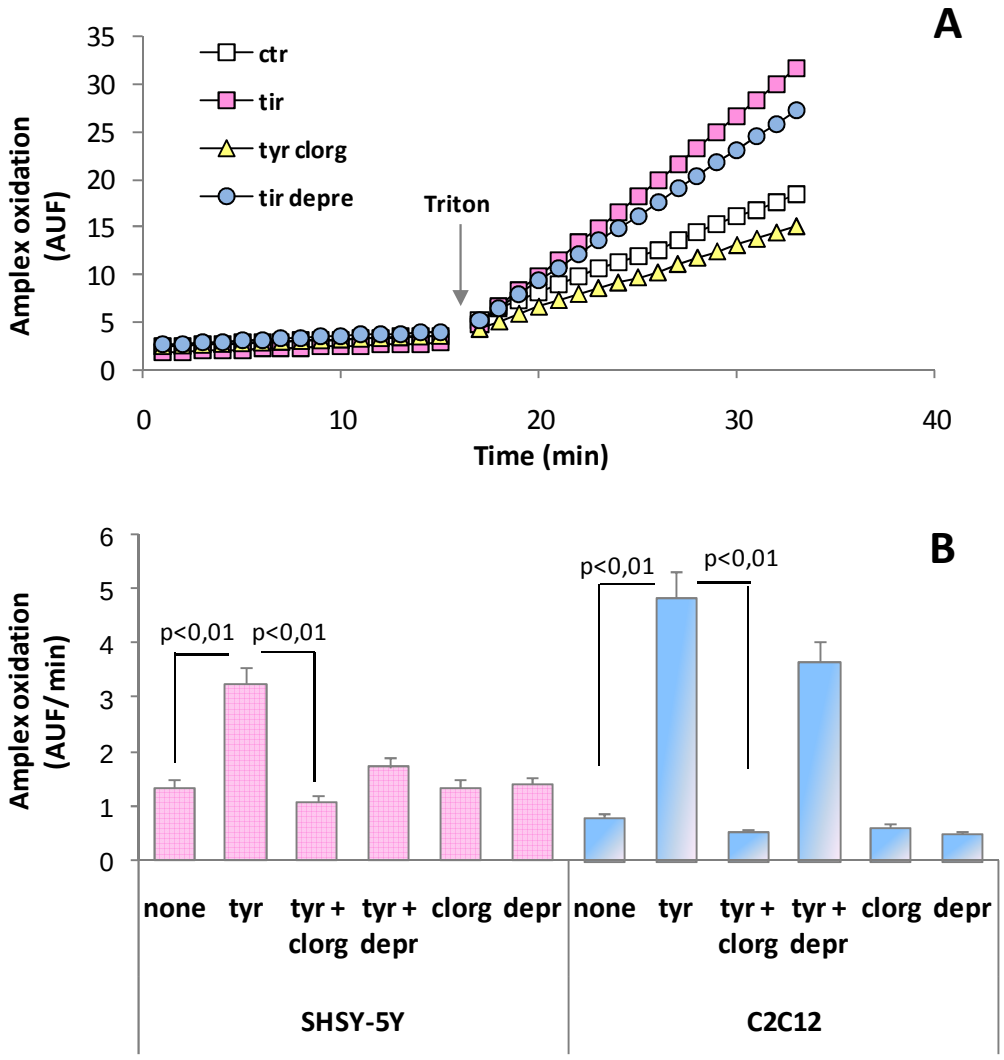


Figure 20: Hydrogen peroxide production by MAO activity. A) Rate of Amplex Red oxidation in the presence of HRP. At 15 min 0.1% Triton X100 was added to C_2C_{12} cells. The rate was measured in control, untreated cells; in the presence of Tyramine, to which were added the MAO inhibitors clorgyline and deprenyl B) Oxidation rates of Amplex Red in C_2C_{12} and SHSY-5Y permeabilized cells in the presence of substrate and inhibitors of MAO activity. When indicated tyramine was 1 mM, clorgyline 300 nM and deprenyl 1 μM. Relevant statistically significant differences are indicated.

The larger inhibitory effect of clorgyline over deprenyl on H_2O_2 production indicates that in both cell lines MAO-A prevails over MAO-B.

The detected background oxidation of the probe in the absence of MAO substrates insensitive to inhibitors, is reasonably accounted for other intracellular sources of H_2O_2 .

Then lack of evidentiation of the MAO-dependent H_2O_2 production in intact viable cells should depend on its efficient removal by endogenous systems, a confirmation that cytosolic H_2O_2 production by MAO activation is essentially undetectable outside cells. So we compared the response of four classic fluorescent probes (DHR, $DCFH_2$-DA, its chlor-methyl derivative CM-$DCFH_2$ and Mitotraker orange) that, as mentioned in the introduction, are largely used as measure of the intracellular reactive oxygen species for the detection of MAO-dependent H_2O_2 production with fluorescence microscopy. The experiments were performed for different times and in different ways paying attention to avoid the possible drawbacks of fluorescence probes. Every image was acquired with one shot at fixed exposition time. The best data in C_2C_{12} was obtained after 3 hours with 1 mM tyramine treatment and are reported in fig. 21.

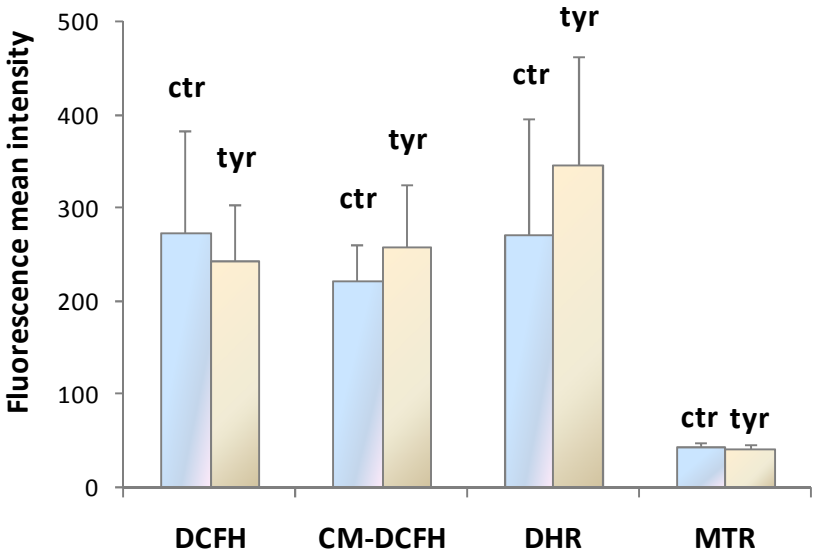


Figure 21: ROS sensitive probes fluorescent products determined at the fluorescence microscopy after 3 hours of 1 mM tyramine treatment in C_2C_{12}.

As it appears, non significant increases of oxidation products were evidenced upon tyramine treatment, sometimes actually the contrary could be observed.

Other approaches as the direct fluorescence detection in cells with a fluorescence plate reader, following different protocols found in literature (detection in adhering cells with or without washing of the excess probe, or after solubilisation of the cells) were however similarly not clearly indicative of a real measure of the H_2O_2 arising from the MAO activity and are not reported. This confirmed that measurements of intracellular H_2O_2 (or other ROS) are tricky also in a model where the MAO-dependent H_2O_2 production reasonably occurs. We thought that expression of HRP inside the cells could overcome large part of the criticisms in the use of the ROS sensitive probes by increasing the reliability of the intracellular measure. In fact the expression of the HRP inside the cells could increase the extent of the reaction occurring between the fluorescent probes and H_2O_2. Moreover large transfection yield could allow direct determinations of the oxidation rates in fluorescence plate readers avoiding cell selection and photo-irradiation side effects. We then address our attention to the finding of an HRP containing plasmid to be used for the transfection of the cells and to set up an efficient transfection procedure for our cell lines. Prof D Cutler kindly furnished us the ssHRP-KDEL pRK5 an HRP encoding plasmid.

4.4 METHOD FOR HIGH EFFICIENCY TRANSFECTION

Traditionally, nonviral vectors are formed by complexation of plasmid DNA with cationic lipids which reduces the negative surface charge of DNA, protects against degradation, and facilitates cellular internalization and intracellular trafficking of the plasmid. In vitro studies typically employ addition of these complexes to the media, resulting in internalization of approximately 20–50% of plasmid [104]. Preliminarily we tested the efficacy of three popular transfectant agents following the suppliers suggested protocols. SHSY-5Y were transfected with peYFP, and the expression of YFP can be directly followed by fluorescent microscopy. In table 2 is shown that the higher efficiency of transfection (made in triplicate) was obtained with Lipofectamine™ 2000 in SHSY-5Y, a value higher than that obtained with TransFectin Lipid Reagent or Gene Jammer. None of the cationic lipids however reached a reasonably high transfection yield

although Lipofectamine appeared the more suitable. Changing the ratio of tranfectant/plasmid, the ratio of the mixture/number of cells and the time of exposition only minor improvements of the transfection efficiency were obtained.

Agent trasfectant	*% transfection maximum*
Jene Jammer	3,9
TransFectin	2
Lipofectamine	8

table 2

Alternative delivery strategies have shown that increasing the concentration of DNA in the cellular microenvironment can increase transfection [105]. Immobilizing DNA to the cell culture substrate prior to cell seeding has been proposed as a mechanism to increase the concentration of DNA in the cellular microenvironment. Reverse transfection [106] is being developed to enhance gene transfer and to create transfected cell arrays [106, 107]. We simplified and improved the reverse transfection method [108]. Essentially after drying of the lipocationic DNA complexes onto the polystyrene flasks, mammalian cells were passaged onto the flasks using standard tissue culture techniques, and then cultured. Under ideal conditions, cells plated developed intracellular YFP fluorescence over the time course of 12–72 h, with maximal fluorescence occurring between 24 and 48 h after cell plating. The amounts of complexes must be optimized for the number of cells adhering to the spotted complex. The transfection efficiency (the percentage of cells expressing the transfected reporter protein) indeed varies with different ratios of complexes/cells that must be calibrated for any cell line. This procedure increases the transfection efficiency of SHSY-5Y cells. Forty-eight hours after transfection the maximal efficiency is reached (about 50% of the cells). This is a good level if compared to the 6–10% of efficiency reported previously for SHSY-5Y [109] and the procedure was applied to the other cell line. The efficiency of transfection increased significantly also in

C_2C_{12} (about 45% instead of 2-3% previously reported with lipocationic transfection [110]). The results are summarized in table 3 where different ratios of lipocationic/plasmid ratio for both cell lines was tested. A proper ratio is to be adjusted for every cell line to avoid excess agents that could also be differently toxic for the cells.

Lipofectamine : DNA	*% transfection C_2C_{12}*	*% transfection SHSY-5Y*
1:1	10 ± 4	32 ± 3
2:1	49 ± 8	51 ± 5
3:1	45 ± 5	26 ± 4

table 3

The equivalent procedure repeated with the other two adjuvants resulted in a much lower efficiency of that obtained with the Lipofectamine™ 2000 (in this condition the maximal efficiency reached with Gene Jammer was 0,18 % for SHSY-5Y and of 0,05 % for C_2C_{12}).

YFP expression was followed during four days upon transfection by microscopy in order to evaluate the time for the optimal experimental conditions and the percentage of YFP positive cells is maximal between 29-48 hours, when it starts to decrease following cellular duplication.

4.4.1 HRP transfection and activity

The optimal ratio of Lipofectamine/DNA was then used to transfect both cell lines with ssHRP-KDEL pRK5. In this case HRP expression was measured with a specific anti-HRP antibody on fixed cells. Cells were tripsinizated 24 hours after transfection, seeded on glass slide, fixed after 5, 24 and 48 hours and immuno-detected with the fluorescent TRITC conjugated anti-HRP antibody. We confirmed an even higher efficiency of transfection at 48 hours after transfection (24 hours after trypsinization), similarly to the YFP expression. The resulting

transfection efficiency with the HRP plasmid was 51±5 % in SHSY-5Y and 49±11% in C_2C_{12}.

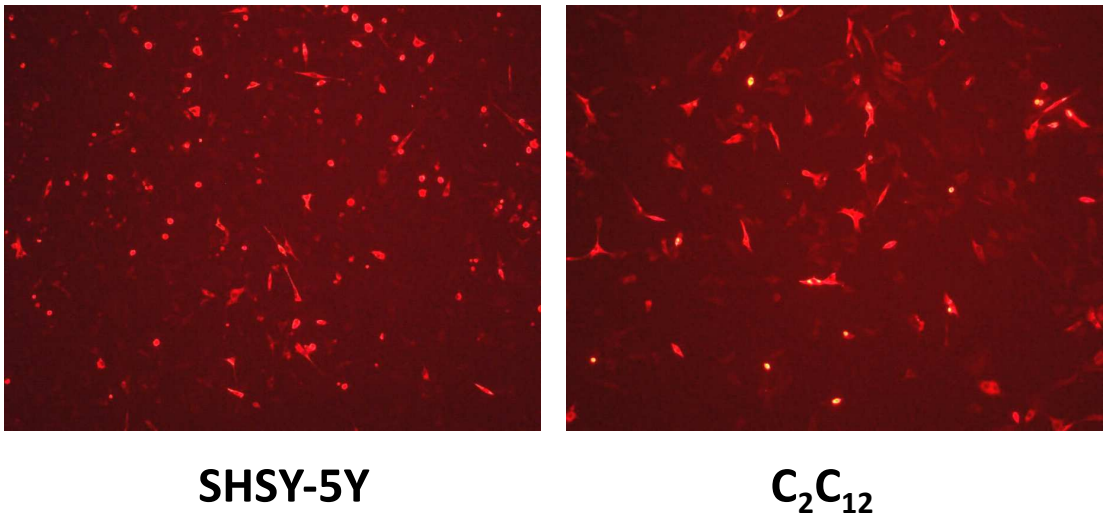


Figure 22: HRP trasfected cells after immuno-detection

The percentage of HRP positive cells is maximal at 29-48 hours, when it starts to decrease following cellular duplication. Anyway, the actual number of HRP positive cells/field remains constant for at least the next two days. Most importantly however the protein should be expressed in active form. In parallel the peroxidase activity was measured in cell lysates with added Amplex red and H_2O_2 as described in the method. Data are comparatively reported in the same figure for both cell lines. The HRP activity increases up to 72 hours (fig.23) indicating that the protein accumulates in the active form in both kind of cells. Remarkably, the activity of HRP at 48 hours strictly correlates with the number of transfected cells and this permits calibration of the model and the extrapolation of data to 100% transfected cells. A value of 10 mU/1x10^6 cells was calculated, where 1 U corresponds to 1 μmole of H_2O_2 consumed/min, in both SHSY-5Y and C_2C_{12} independently measured by Amplex red. Calculation are based on the 1:1 Amplex red stoichiometry with added H_2O_2 in the presence of HRP [75].

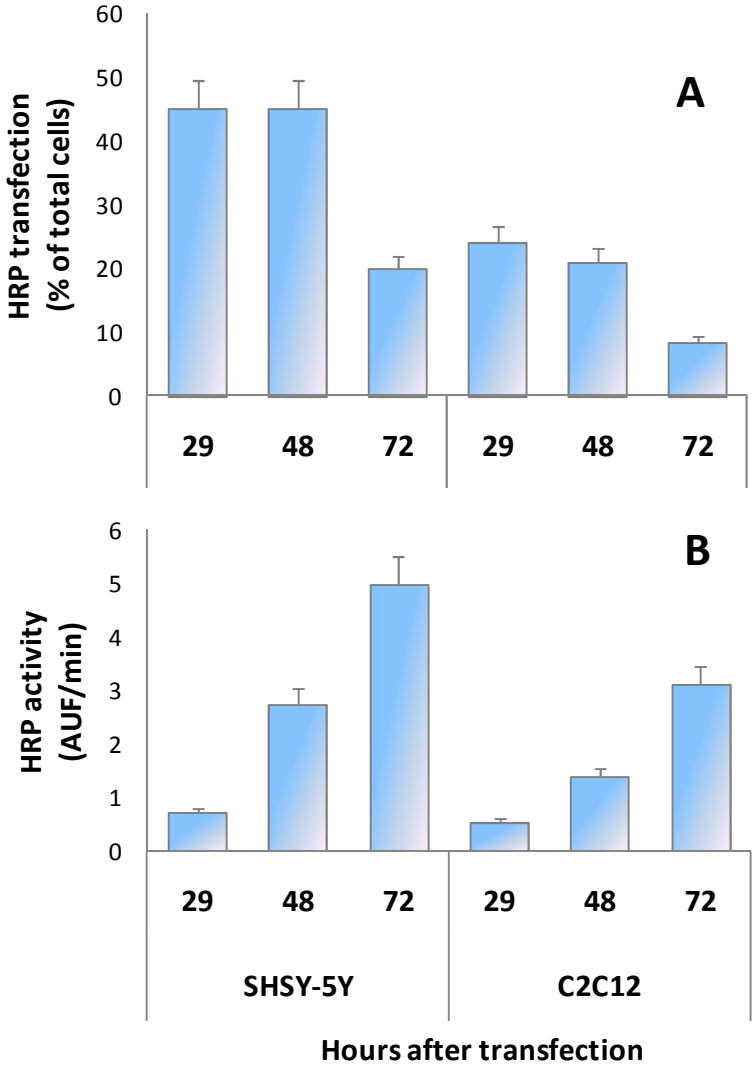


Figure 23: Efficiency of transfection and expression of HRP in C_2C_{12} and SHSY-5Y cells. In (A) is reported the percentage of immune-detected cells containing HRP and in (B) is reported the specific activity of protein.

4.4.2 Cell viability and metabolism in HRP transfected cell

To validate cell model we next assessed if HRP transfection perturbed cell viability and metabolic response. For the measurement of cell viability we used propidium iodide (PI) as indicator the percentage of dead cells. PI in fact is only permeant to dead cells. Similarly to PI also the release of lactate dehydrogenase (LDH) into the medium is a measure of the loss of membrane integrity as occurring in damaged dead cells. Both PI and LDH test are not affected by HRP

transfection (fig. 24A). The same occurs for the redox metabolic activity of cells, that we measured as MTT reduction (fig. 24B). The reduction of MTT operated by intracellular dehydrogenases is largely used also as toxicity test. For both, the death index and the redox metabolic activity, minimal and not relevant negative effects were found with Lipofectamine not worsened by HRP expression.

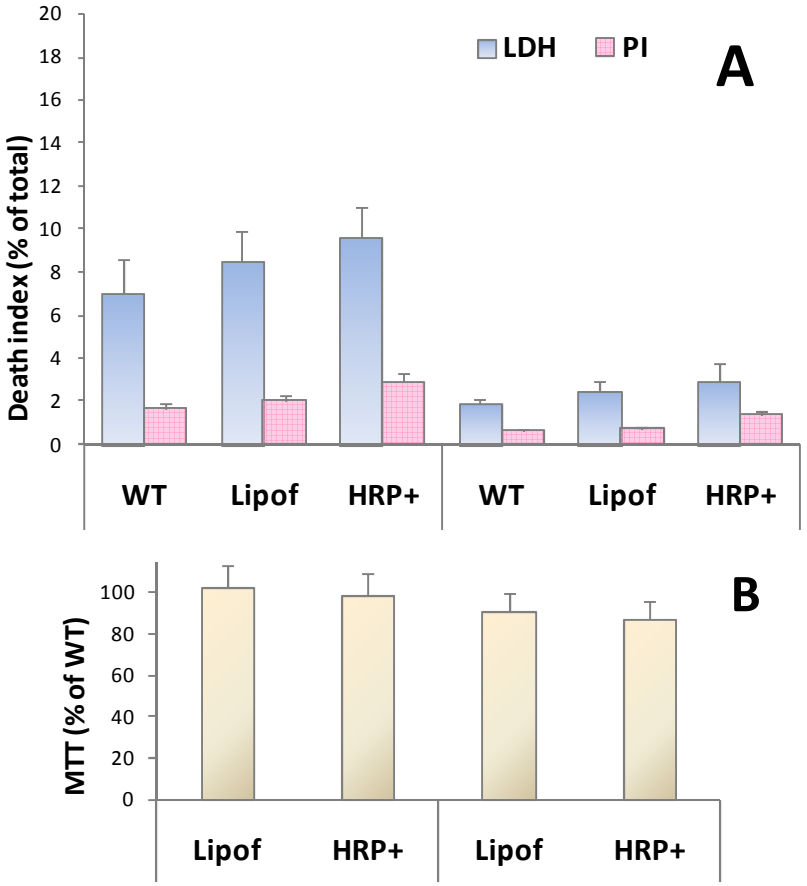


Figure 24: Transfection with HRP does not affect cell viability measured as LDH release and PI permeability (A) or MTT reduction (B)

4.4.3 Intracellular MAO activity can be monitored as enhanced oxidation in HRP transfected cells.

HRP transfected cell were then used to measure H_2O_2 arising from the MAO activity. HRP transfected C_2C_{12} cells were observed by fluorescence microscopy, and a net accumulation of the oxidized fluorescent rhodamine was clearly observed following the addition of the MAO substrate tyramine (fig. 25). No increase was detectable in the corresponding WT cells similarly treated. Data

obtained in both cell lines also in the presence of the MAO specific inhibitor clorgyline and of deprenyl are summarized in the lower part of fig. 25. Moreover data are in line with the total activity measured in fig. 20.

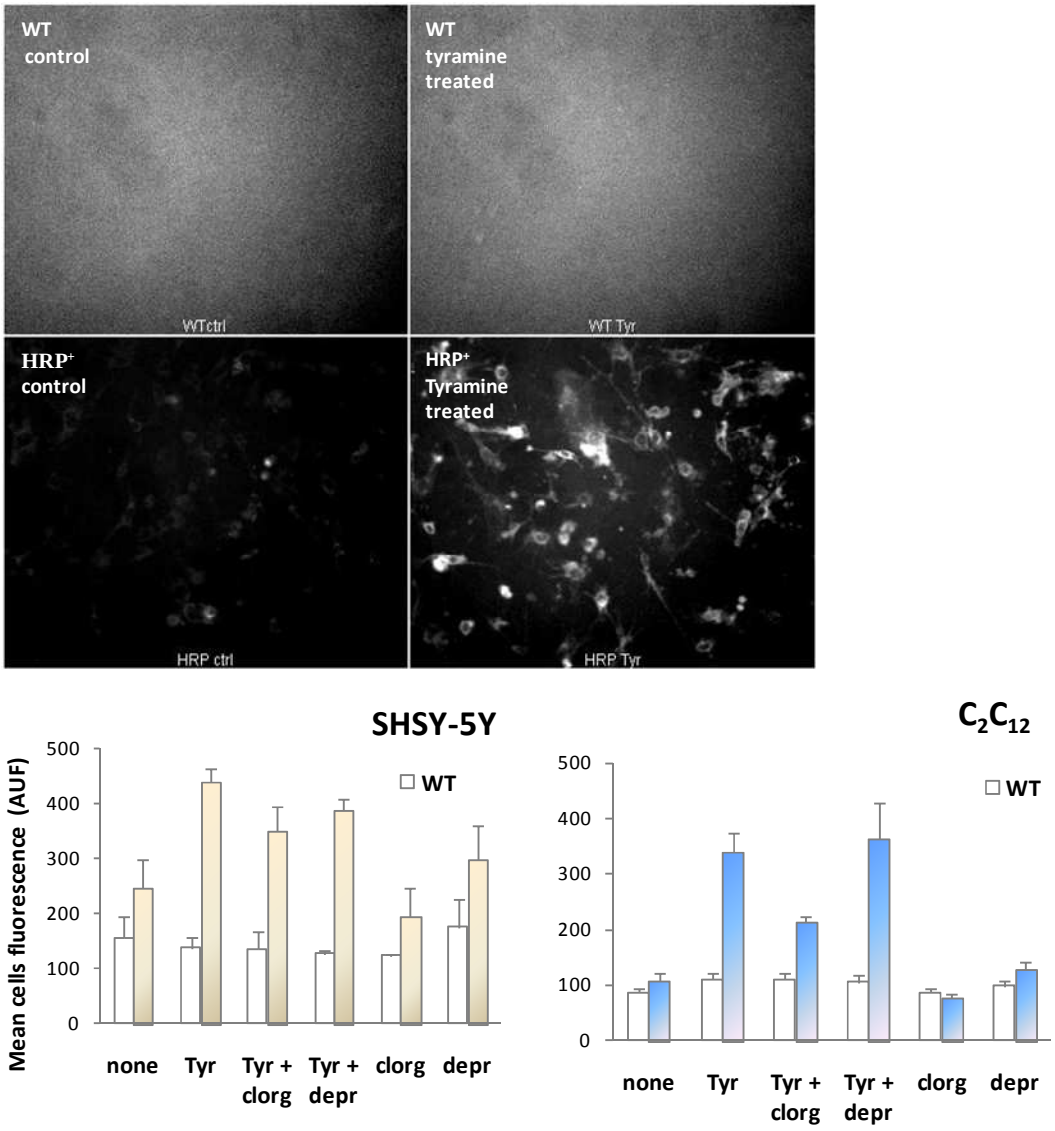


Figure 25: Detection by fluorescence microscopy of DHR oxidation. In the upper part of the figure an example of images acquired in SHSY-5Y transfected cells (HRP^+) or not (WT) is reported. In the lower part total fluorescence emission was measured on SHSY-5Y or C_2C_{12} in the presence of MAO substrate and inhibitors. Only in HRP transfected cells tyramine induces a significant increase of DHR oxidation ($p<0,01$) in both cells line, that is selectively inhibited by clorgyline ($p<0,05$ in SHSY-5Y and $p<0,01$ in C_2C_{12})

The inhibition of DHR oxidation in the presence of clorgyline supports the specificity for MAO-A of the observed production of the oxidant H_2O_2. This is the first evidence of a measure of H_2O_2 production depending on the MAO activity in an intact monolayer of cells.

Since fluorescent microscopy is not suitable for multiple analyses and carries on practical disadvantages such as long time of acquisition and likely photoreaction artefacts, we adopted the strategy of performing a direct fluorimetric detection in a multiwell plate reader. Different probes can also be tested to confirm data in parallel avoiding possible side effects of any particular probe. Moreover this can be obtained from any single transfection experiment where the transfected preparation in batch (about $1x10^6$ cells) can be tripsinized at 28 hours and aliquoted in a 96 wells plate with less than 10 % variability of the amount of transfected cells/well. To HRP transfected cells, uniformly seeded in 96 wells plates in parallel with the corresponding WT, the fluorescent probes DHR and $DCFH_2$ were added and their oxidation recorded in a fluorescence plate reader, following addition of substrates and inhibitors of MAO.

A faster oxidation rate of both DHR (fig. 25A) and $DCFH_2$ (fig. 25B) was indeed detected upon tyramine addition in HRP transfected C_2C_{12} cells. As expected, clorgyline decreased the rate of oxidation, while deprenyl induced a minor inhibition. The discrepancy with the results obtained on cell lysates where deprenyl was not inhibitory could depend by some cross inhibition of deprenyl in intact cells also on MAO-A as already reported [24]. Similar data were obtained in SHSY-5Y.

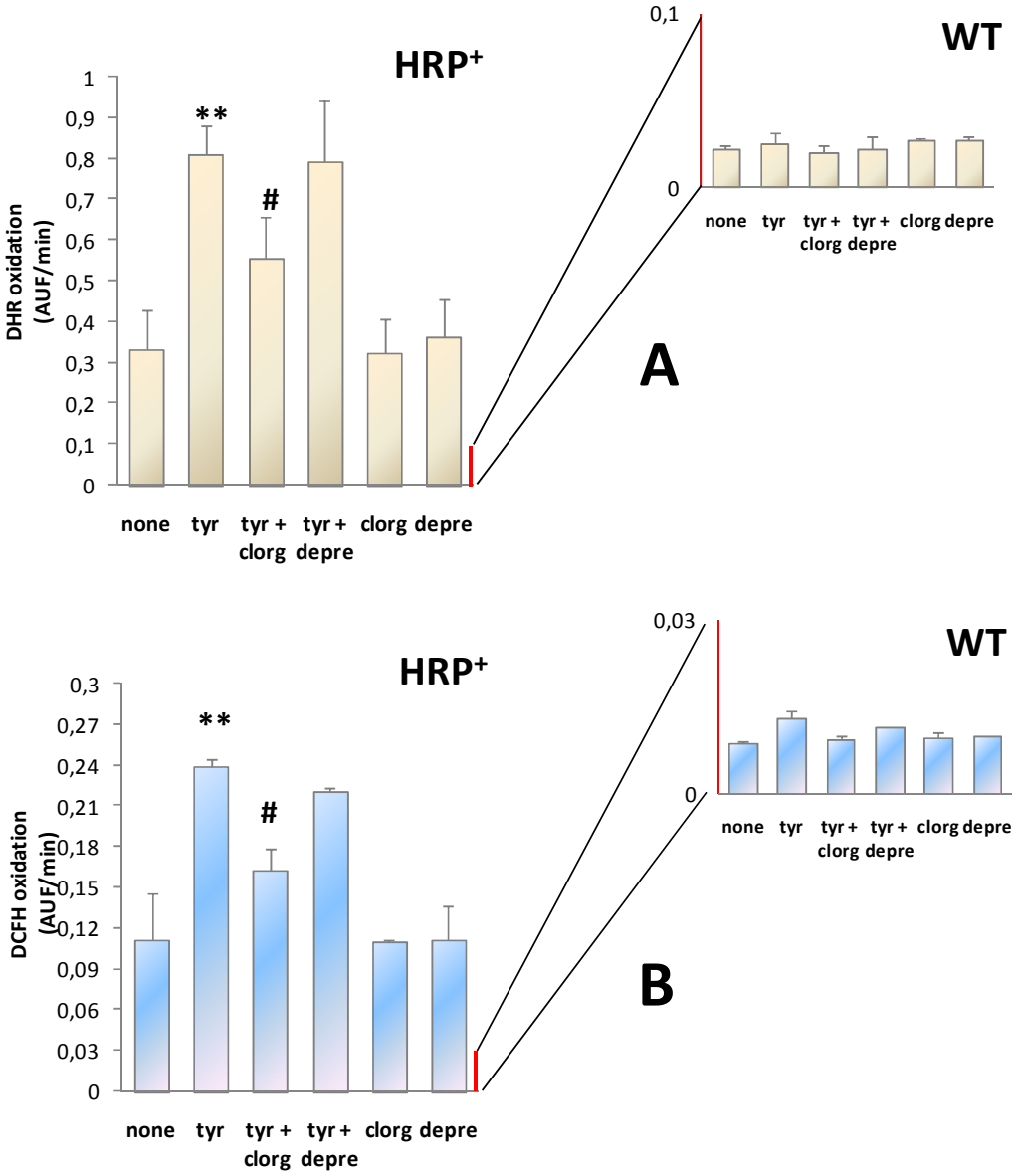


Figure 26: Direct determination of MAO induced DHR and $DCFH_2$ oxidation rate using a fluorescent plate reader. Significantly different from control: **P<0.01; significantly different from tyramine # P<0.05 (Student's t test)

4.4.4 Specificity of the measure for the H_2O_2

Specificity of the detection of H_2O_2 in the HRP expressing cells was then stressed confirming its sensitivity to other H_2O_2 delivery systems or by acting on modulation of intracellular endogenous ox exogenous H_2O_2 removal systems.

The use of cells transfected with HRP for measuring intracellular H_2O_2 was further validated by titrating the diffusion to the cytoplasm of H_2O_2 generated in

the culture medium. As shown in fig. 27 A-B in HRP transfected SHSY-5Y cells the addition of glucose oxidase to the glucose containing medium produces a known amount of H_2O_2 (determined in parallel experiments with Amplex red/HRP) that dose dependently increases the rate of oxidation of the intracellular probe. Maximal DHR or $DCFH_2$ oxidation rate was reached at 200 nmoles/min of H_2O_2. In these experiments the metal ions chelator DTPA (1 mM) was present in the external medium to minimize the artifactual oxidation of DHR or $DCFH_2$ (same results was obtained in C_2C_{12}, not shown).

The specificity for H_2O_2 of the oxidation of intracellular probes was confirmed using pyruvate as competitor of HRP [111]. Pyruvate, indeed, easily enters in cells, probably by means of the monocarboxylate transporters, where it interacts with H_2O_2 [112]. The nucleophilic attack of the peroxide species at the C-2 carbonyl group carbon centre brings to decarboxylation to acetate. This reaction is reported to occur stoichiometrically with H_2O_2 at physiological pH, and pyruvate is reported as the most efficient scavenger of H_2O_2 among several physiological α cheto-acids [111-115]. As expected, in the presence of 10 mM pyruvate, the rates of $DCFH_2$ and DHR oxidation at basal level and following tyramine addition are deeply lowered (fig. 27C-D) .

An opposite effect is expected for agents that decrease the efficiency of endogenous H_2O_2 removal systems. To test this, we decreased the efficiency of the GSH dependent endogenous removal system. We used 1-chlor-2,4-dinitrobenzene (CDNB), that act as GSH depleting agent being a substrate of glutathione-S-transferase [86], and buthionine sulfoximine (BSO) an inhibitor of GSH biosynthesis [87]. Both compounds, by limiting the efficiency of the removal of hydroperoxides, increased the rate of oxidation of intracellular probes (fig. 27E-F). This clearly indicates that, in transfected cells, HRP competes with endogenous peroxidases for the reduction of H_2O_2 produced by MAO and provides further specificity for the assay. Unfortunately, the relevance in this respect of catalase could not be tested since aminotriazole, the most efficient inhibitor of catalase, inhibits also HRP.

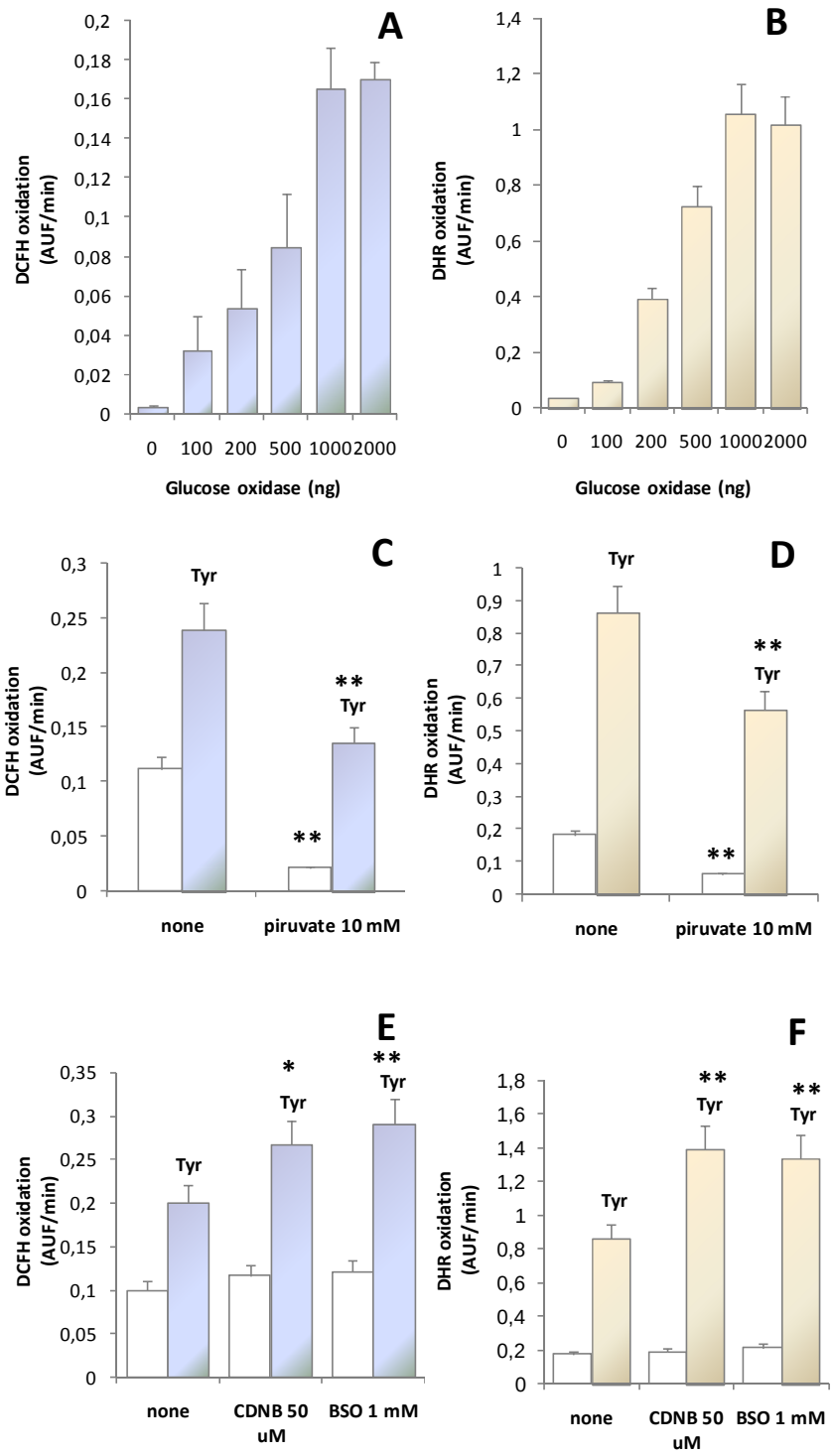


Figure 27: Positive evidences that the oxidation rate of redox probes in HRP transfected cells depends on H_2O_2. The oxidation rate in SHSY-5Y cells of $DCFH_2$ (A,C,E) and DHR (B,D,F) in the presence of glucose oxidase to generate extracellular H_2O_2 (A,B) ; Pyruvate to scavenge H_2O_2 (C,D); CDNB or BSO to lower GSH concentration (E,F). * P<0,05, ** P < 0,01 of treated cells (pyruvate, CDNB or BSO) versus the respective conditions in controls.

4.4.5 The detection of intracellular H_2O_2 production requires a minimal number of transfected cells.

The last point to the validation of the method is the range of transfection needed for its useful application in a plate reader. To find out the minimal amount of transfected cells required to run reliable H_2O_2 measurement experiments, we prepared mixed cultures diluting transfected with WT cells. The percentage of HRP positive cells for each mixture was tested in parallel by immuno-detection. For both cell lines the oxidation rate of both probes was a linear function of the percentage of transfected cells (fig. 28).

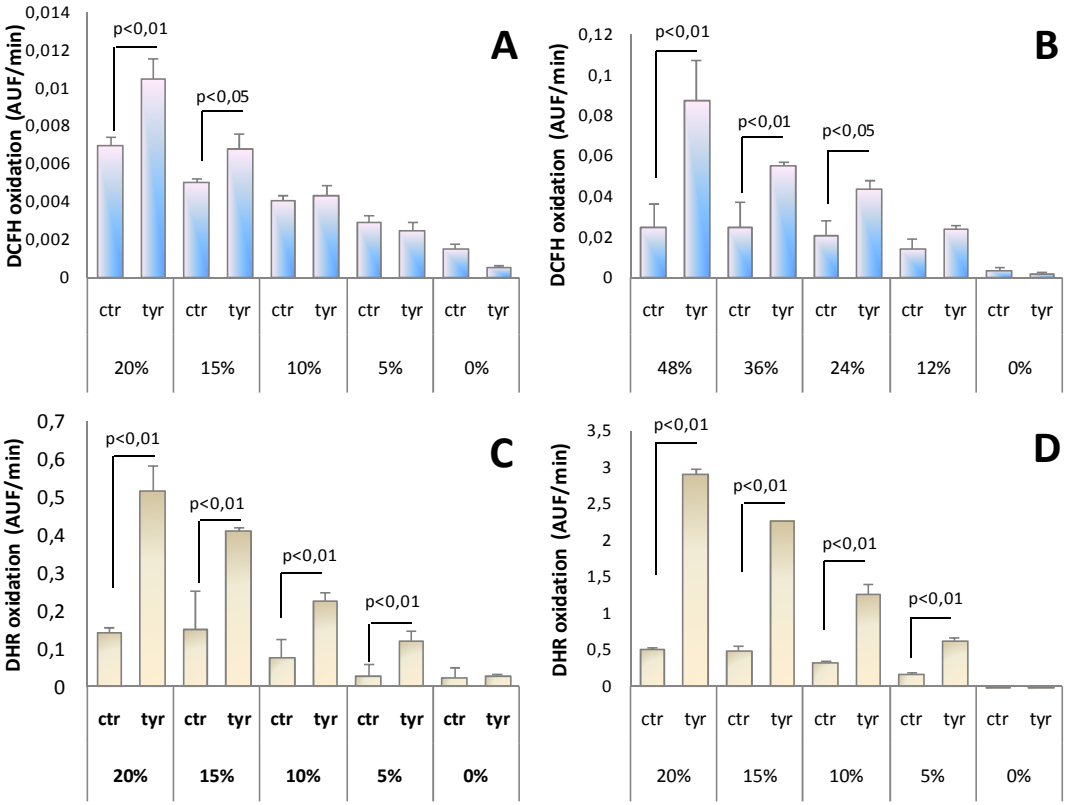


Figure 28: Detection of intracellular H_2O_2 formation requires a minimal percentage of transfected cells. Fluorescence readings of $DCFH_2$ and DHR oxidation rates in C_2C_{12} cells (A, B) and SHSY-5Y cells (C,D) mixed with WT cells at the reported percentage. Tyramine was 1 mM. Data in triplicate are representative of three independent preparations. Relevant statistically different data are indicated.

These results indicate a limit of sensitivity of the procedure of approximately 500-1000 transfected cells per well, corresponding to a limit of 5-10% of

transfection efficiency depending on the probe used. Do to the higher quantic efficiency, DHR is more sensitive than $DCFH_2$ and can be used for the detection of lower H_2O_2 amounts, or with a lower transfection efficiency (less than 5 % with DHR).

4.4.6 Application to other intracellular sources: mitochondrial contribution to H_2O_2 production

The method can be applied to other sources and, as previous demonstrated with the decrease of basal DHR oxidation rate by pyruvate, appears sensitive enough to allow the measure of mitochondrial H_2O_2 production. This production is estimated to be lower than MAO-dependent production [4], but nobody measured it before in intact cells. In fig. 29 the rate of DHR oxidation rate following tyramine addition is compared to the oxidation rate obtained in the basal condition in the presence or in the absence of the Complex I inhibitor rotenone.

As shown in fig. 29A rotenone induces an increase of the DHR oxidation rate, and this is consistent with the prevalent presence of NAD-dependent substrates inside the cells, whose capability to release H_2O_2 outside mitochondria is stimulated by rotenone, as largely reported. The amount of this stimulation is however very low if compared with the tyramine dependent production. This confirms the suggested low production by the mitochondrial electron transfer chain. Addition of a permeable form of succinate (diethyl succinate) however can increase succinate intracellular concentration when hydrolysed by intracellular esterases and as shown in fig. 29B it induces a dose dependent increase of the oxidation rate of both DHR and $DCFH_2$ only in HRP transfected cells. To exclude any possible interference of ethanol arising from succinate diethyl ester hydrolysis, we verified that a dose dependent increase of ethanol up to 12 mM does not modify oxidation of DHR. The maximal succinate dependent H_2O_2 production is relevant and measurable in our HRP transfected cells but still remains largely lower that that derived from MAO as shown in the same figure where the MAO activation with tyramine was induced in the same cells.

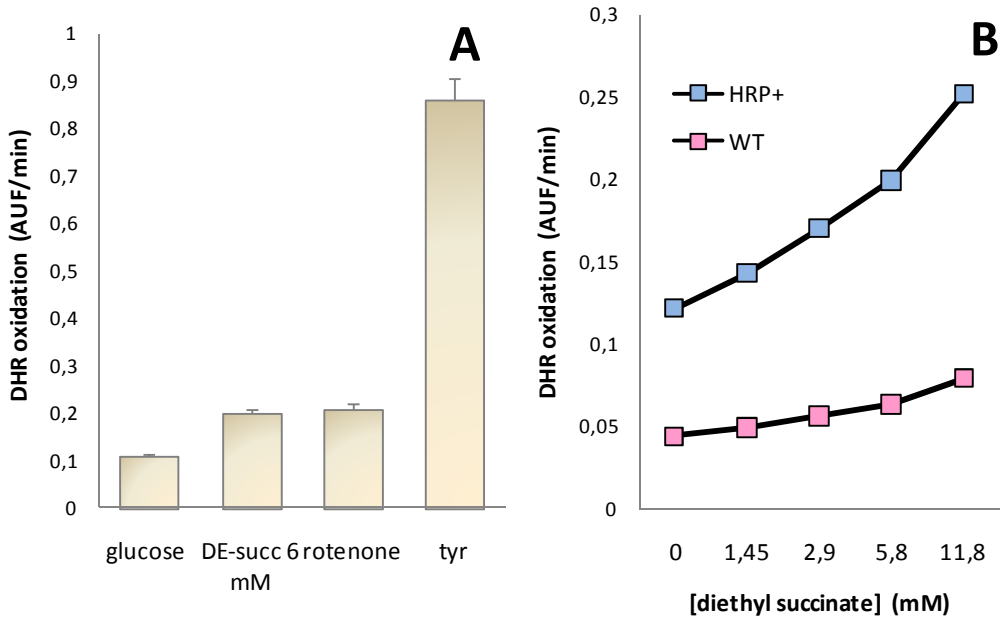


Figure 29: H_2O_2 production in HRP^+ SHSY-5Y by MAO and mitochondrial activity (A) and dose response production induced by diethyl succinate in HRP^+ and WT SHSY-5Y (B)

A direct measurement (with Amplex red/HRP) of MAO and succinate H_2O_2 contribution in a mitochondrial preparation was performed and shown in fig. 30. The H_2O_2 production induced by maximal activation of the MAO with 1 mM tyramine is about four times higher than that obtained with maximal succinate (4 mM) confirming the same ratio shown in cells.

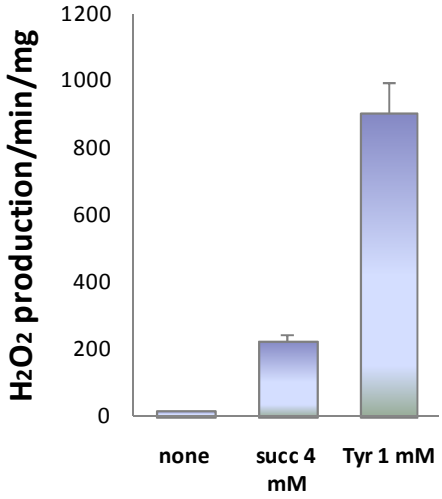


Figure 30: Comparison of maximal succinate and MAO dependent H_2O_2 production in reat heart mitochondria

In the presence of 5 mM pyruvate the same pattern is observed but the oxidation rates both with and without succinate are decreased by the competition (already observed in fig. 27) of pyruvate on the H_2O_2 removal. Also in these conditions a clear stimulation of DHR is observed with the diethyl succinate. Moreover in order to keep the intracellular pyruvate concentration low we used lactate (permeable through the same monocarboxylate transporter) that through lactate dehydrogenase gives rise to a steady state formation of the mitochondrial substrate pyruvate. In this condition we are able to evidence an even higher DHR oxidation rate when diethyl succinate is added.

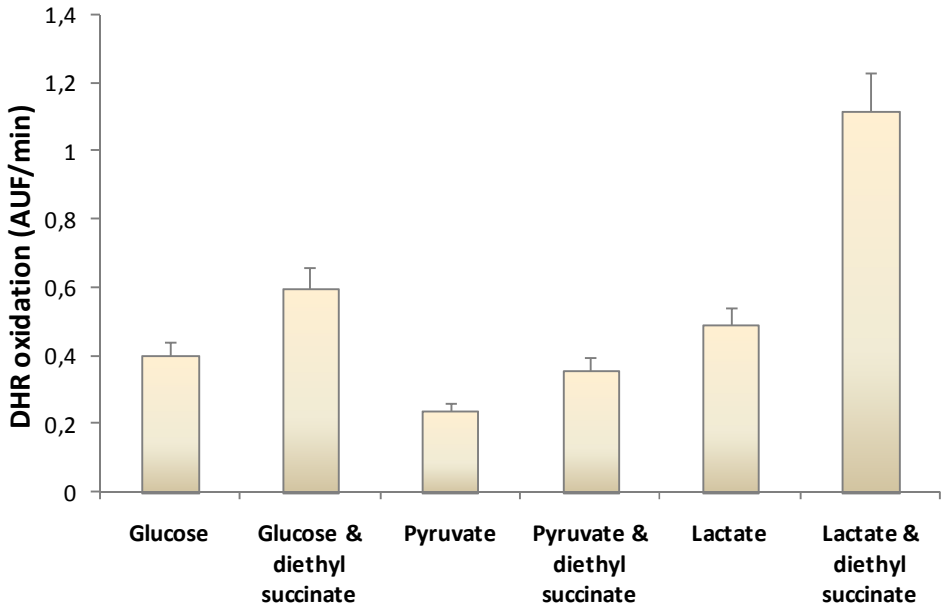


Figure 31: Diethyl succinate dependent H_2O_2 production in HRP^+ SHSY-5Y incubated with different substrates

On the whole we are able to show a mitochondrial succinate dependent contribution to H_2O_2 production in intact cells, a production that depends on the mitochondrial NAD substrates availability presumably higher when NAD dependent substrates are low (when lactate is the only exogenous substrate) than in the presence of high glucose (where glycolitic pyruvate production is conceivably more efficient). When instead 5 mM pyruvate is added to the cells the competitive removal of H_2O_2 contributes also to a decrease the HRP-dependent detection system efficiency shown both in the presence or absence of succinate.

The succinate induced increase is the first indication that also in intact cell systems (i.e. with physiological ADP/ATP ratio, ionic strength and mitochondrial potential and pH) succinate can dose dependently induce an increased rate of H_2O_2 production similarly to the increase evidenced in mitochondrial suspensions of several origins but amply discussed for its physiological relevance. We can confirm, also, that a stimulated MAO-dependent H_2O_2 production can be higher than mitochondrial H_2O_2 release in the same cell.

4.4.7 Diphenyleneiodonium inhibition in whole cells can be partly attributed to mitochondrial H_2O_2 production

Main cell sources of superoxide/H_2O_2 are the NADPH oxidase in phagocytic cells but their involvement in other cells has been suggested and studied by means of the use of the inhibitors apocinyn and diphenyleneiodonium (DPI). We performed some preliminary experiments with both inhibitors to set up a measure of the NADPH contribution in our transfected cells.

Apocinyn induces a large stimulation of the DHR oxidation, an effect exhibited in the absence of any typical NADPH oxidase stimulator. This effects confirms what is reported by [116] where the large apocinyn interferences due to ROS formation makes its inhibitory effects evident only in the oxidative burst of professional cells. On the contrary a significant inhibition was shown in our HRP transfected cells by DPI again in basal unstimulated cells (fig. 32A). DPI is reported to be a broad-spectrum flavoprotein inhibitor, via slow formation of a covalent flavin-DPI adduct [117]. However when DPI is incubated with isolated Complex I, it also slowly inhibits both the oxidation of NADH and the reduction of the iron-sulphur centres, suggesting that it inhibits at a site upstream of these centres, i.e. flavin in Complex I [119]. DPI inhibits superoxide formation during reverse electron transfer ([118] and our data). Although Lambert [118] found no inhibition by DPI on the low superoxide production by forward electron transport measured with pyruvate plus malate in state 4 (not phosphorylating condition) in rat skeletal mitochondria, we found instead that DPI as an efficient inhibitor of superoxide release in rat heart mitochondria measured with either G/M or

Succinate (fig. 32B). The inhibition shown in state 4 is evident also in the lower superoxide production of state 3. Thus DPI behaves a powerful inhibitor of the H_2O_2 production in isolated mitochondrial suspensions, inhibiting also their oxidation. We have measured in parallel the O_2 consumption of both G/M and succinate. Both are inhibited by increasing amounts of DPI added to the RHM in the presence of ADP. Inhibition reached the 80% at 20 μM DPI and was lower (50%) at the concentration used (10 μM).

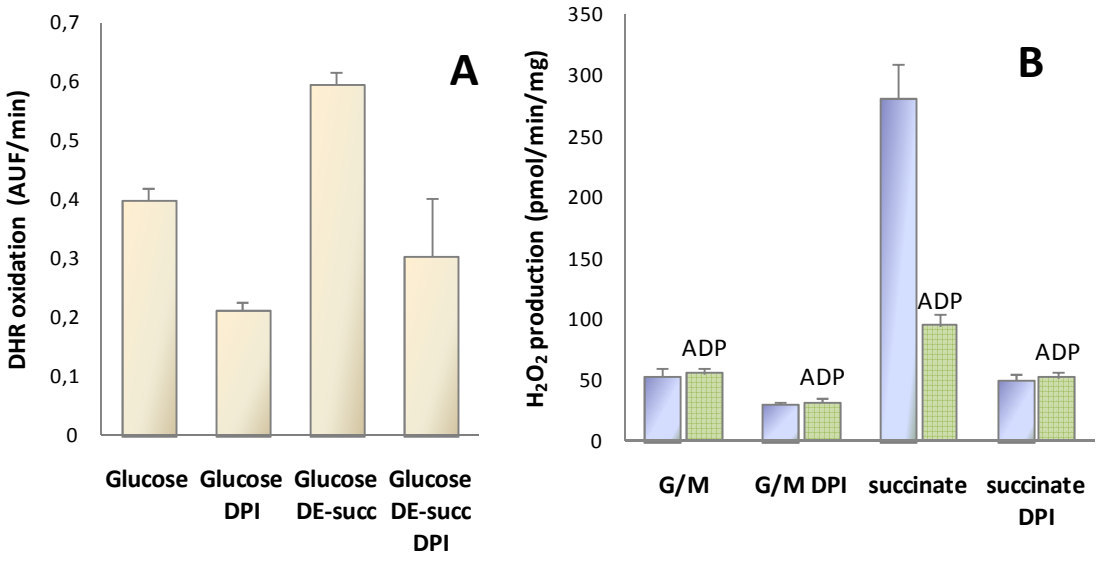


Figure 32: Effect of DPI on H_2O_2 production in HRP^+ SHSY-5Y (A) and in isolated RHM (B) state 3 (ADP) or state 4.

We next studied the effect of DPI on succinate stimulation in HRP transfected C_2C_{12} (fig. 32A). DPI inhibits both the basal rate of H_2O_2 production and almost completely abrogates the increase by diethyl succinate. These effects in intact cells can be explained by a net inhibition of the mitochondrial contribution to cell H_2O_2 production.

Recent studies show that DPI exerts several effects on cell metabolism: such as inhibition of the redox metabolism, loss of GSH and induction of apoptosis in addition to its expected effects as NADPH oxidases inhibitor [120-122], and how many of these effects could depend on the inhibition of mitochondrial H_2O_2 production is unknown. The inhibitory effects of DPI however indicate again a

mitochondrial contribution to the overall cell H_2O_2 production and allows to answer if the Complex I is involved on this production. In fact while the rotenone effect is not detectable in the cell because of the contemporary inhibition of the succinate and stimulation of the NAD dependent H_2O_2 production, this Complex I inhibitor lets us suggest that mitochondria can be considered a modulated source of H_2O_2 in cells.

5.0 CONCLUSION

Reactive oxygen species (ROS) influence many physiological processes including host defense, and cellular signalling [62]. Increased ROS production (termed "oxidative stress") has been implicated in various pathologies, including hypertension, atherosclerosis, diabetes, and chronic kidney disease. Among ROS, H_2O_2 is the most important for his stability and his permeability across membrane. This reactive oxygen species is generated in the cells by specific enzymes (like several aerobic dehydrogenases, amine-oxidase and some NADPH oxidases called Duox) or from dismutation of superoxide released by mitochondrial electron transfer chain and NADPH oxidases [123].

Mitochondria are considered to be one of major sources of $O^{\bullet -}$, that is immediately dismutated to O_2 and H_2O_2 by the actions of Mn-SOD dismutase. There is a consensus that succinate-driven ROS generation is largely due to reverse electron transport and that this is quantitatively more significant than ROS formation in the presence of NADH-linked substrates [15]. During normal metabolism in cells, however, the major reducing equivalent that supports the respiratory chain is NADH generated by the tricarboxylic acid cycle. Furthermore, the physiological concentration of succinate in tissues is valued at least one order of magnitude smaller than is commonly used (5–10 mM) in experiments with isolated mitochondria. We have demonstrated that the succinate stimulation of mitochondrial H_2O_2 release occurs at low and more physiologically succinate concentrations (0,5 mM), and that the presence of G/M modifies only marginally the succinate promotion of H_2O_2 release. Furthermore, G/M oxidation is decreased, but not prevented, by succinate, since α-ketoglutarate formation is not halted in its presence [124]. Moreover we also found that long chain acyl-CoAs, while not inhibiting succinate oxidation, powerful inhibit the succinate-supported H_2O_2 production. This supports the possibility that acyl-CoAs are physiological negative modulators of succinate-dependent H_2O_2 release and this role may be exerted also in tissues incapable of fatty acid oxidation [125].

While detection and measurements of H_2O_2 in subcellular preparations are reliably performed with the coupled reaction HRP/Amplex red, as used for our

experiments, there are no methods that give a reliable measure of H_2O_2 generation in intact cells. Nonetheless to understand the physiological role of H_2O_2 in vivo reliable measure of this reactive oxygen specie is necessary. The most used approach is based on the detection of the oxidation of intracellular probes such as $DCFH_2$ and DHR. Their oxidation can occur with other most oxidative species but does not occur with H_2O_2. Reaction with the peroxide requires a catalyst that is first oxidized by H_2O_2, forming a transient species able to oxidize the probe changing its spectroscopic features. The limiting factors for the application of this analytical approach are the concentration in the cells of a hemoprotein competent for the catalysis of the oxidation, and the long time of irradiation required for the acquisition under the microscope [69-71].

We addressed the issue of increasing the sensitivity and the specificity of the detection of intracellular H_2O_2 by introducing the use of HRP transfected cells. Since much larger part of H_2O_2 is moved to HRP, sensitivity and specificity increase. Also the possible pitfall of the specificity limited stoichiometry between DHR and $DCFH_2$ oxidation and H_2O_2, partly due to autoxidations or reaction with HRP also in the absence of H_2O_2 [126], if not fully overcome, is definitely less relevant. In the presence of HRP, indeed, the specific reaction rate largely increases and prevails over the background of unspecific oxidative chain reactions. The high sensitivity achieved, eventually, permits the substitution of a fluorescence microscope with the much more convenient fluorescence micro plate readers, suitable for multiple analyses. Using the Lipofectamine procedure, as modified in our protocol, the transfection efficiency is particularly high. The excellent correlation between the number of transfected cells and signal recorded permits a calibration and data can be extrapolated to 100% of transfection efficiency.

The procedure was tested by measuring H_2O_2 diffusing to the cytosol from the extracellular compartment (formed by the Glucose oxidase/glucose) and was applied to the direct determination of the MAO-dependent intracellular H_2O_2 production [127] in intact viable cells as induced by tyramine addition and to selective inhibition by MAO inhibitors thus leading to the measure of the MAO activity. Notably, this activity has never been directly measured before in intact cells and was not detectable in non HRP transfected cells. This constitutes the first

application of a procedure that has been validated for its low influence on the cell metabolism and for its high sensitivity and specificity.

No apparent toxicity or heavy viability modifications have been observed after the transfection procedure and HRP expression, thus the procedure can be referred as safe.

The specificity for H_2O_2 was confirmed by the competition of pyruvate that chemically scavenges H_2O_2 and thus decreases the HRP dependent oxidation rate of the probes. An increased HRP dependent response is instead found when the endogenous peroxidase activities are compromised by GSH depletion. The HRP dependent probe oxidation is faster when the GSH dependent removal of H_2O_2 is prevented, although the possibility that GSH also competes with the probe for the oxidized form of HRP cannot be positively ruled out.

Using fluorescent plate readers kinetic measurements of hydrogen peroxide can be performed in less than 1000 HRP transfected cells/well and reaction can be followed by repeated short reading cycles. This is crucial for long lasting experiments, when limiting the stress of cells maintained in a thermostat chambers is appropriate.

The inhibition of the basal level of oxidation in the presence of the competing pyruvate indicates that the measurements could be ideally applied to very low intracellular H_2O_2 sources. In fact preliminary data indicate that the procedure allow the measure of mitochondrial H_2O_2 production, that appears stimulated by the presence of rotenone. This is expected from the prevalence of NAD dependent substrates in whole cell, whose capability to release H_2O_2 is stimulated by the Complex I inhibitor as shown in isolated mitochondria. We have shown that also the maximal succinate dependent H_2O_2 release in isolated RHM is far lower than that arising from the MAO activity as also estimated by [4]. The same results is indeed obtained in our cell models: in the presence of increasing concentration of diethyl-succinate (a cell permeable form of succinate) we can measure a corresponding increase on the oxidation rate of DHR only in the HRP transfected cells, an indication that also in intact cell systems (i.e. with physiological ADP/ATP ratio, ionic strength and mitochondrial potential and pH) succinate can dose dependently induce an increased rate of H_2O_2 production as already

evidenced in mitochondrial suspensions. This data suggest that intracellular increase of succinate can act as physiological inducer of a mitochondrial increased production of H_2O_2. Consistently with data in mitochondrial suspensions the succinate dependent production is lower than that measured in the same cells upon MAO activation by tyramine.

In an attempt to measure the contribution of the NADPH oxidase (NOX) we have found that Diphenyleneiodonium, a broad-spectrum flavoprotein inhibitor and one of the most used inhibitors to prove activation of the NADPH oxidase [117], may act also as inhibitor of the mitochondrial H_2O_2 production [119]. We measured an inhibition of both NAD and succinate-dependent H_2O_2 production which correlated with the inhibition of both G/M and succinate dependent O_2 consumption in isolated mitochondria. Thus Diphenyleneiodonium in isolated mitochondria inhibits the prevailing succinate dependent H_2O_2 production without increasing but rather inhibiting the quote arising from NAD dependent substrates. In HRP transfected cells DPI correspondently inhibits both basal (cells in high glucose medium have prevalent NAD substrate) and diethyl succinate stimulated production. Even if these data are only preliminary and must be confirmed they strongly support our procedure as a good tool to measure intracellular levels of H_2O_2. The dynamic H_2O_2 measurements can give informations on the cell thiol redox state and on the efficiency of the endogenous scavenger systems in different experimental conditions mimicking physiological or pathological conditions where ROS are thought to be involved.

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7.0 ABBREVIATIONS

α-KG: α-ketoglutarate

8-OH-G: 8-hydroxyguanine

AD: Alzheimer's disease

Amplex Red: 10-acetyl-3,7-dihydroxyphenoxazine

AO: Amine oxidases

BSA: Bovine Serum Albumin

BSO: buthionine sulfoximine

CDNB: 1-chlor-2 ,4-dinitrobenzene

Clorg: clorgiline

DCFH: 2,7-Dichlorodihydrofluorescein

Depre: deprenyl

DHR: Dihydrorhodamine 123

DPI: diphenyleneiodonium

DTPA: di-ethylentriamine pentacetic acid

FCS: Fetal Calf Serum, FCS

G/M: glutamate plus malate

GPx: Glutathione peroxidase

GSH: glutathione

H_2O_2 : hydrogen peroxide

HNE: 4-hydroxy-2-nonenal

HO-1: heme oxigenase 1

HOCl: hypochlorous acid

HRP: Horseradish Peroxidase

HRP^+: HRP positive cells

KRB: Krebs Ringer buffer

LDH: lactate dehydrogenase

MAO: Monoamine Oxidase

Mn-SOD: manganese superoxide dismutase.

MTT: 3-(4,5- dimethyldiazol-2-yl)-2,5 diphenyl Tetrazolium Bromid)

NBT: Nitro Blue Tetrazolium

NDA: malondialdehyde

$O_2^{\bullet -}$: superoxyde

$OH^{\bullet}$: hydroxyl radical

$ONOO^-$: peroxynitrite

Palm CoA: palmitoyl- CoA

PD: Parkinson's disease

PI: Propidium Iodide

RBM: rat brain mitochondria

RHM: rat heart mitochondria

RNS: reactive nitrogen species

$ROO^{\bullet}$: peroxyl radicals

ROS: reactive oxygen species

SOD: superoxyde dismutase

SSAO: Semicarbazide-sensitive amine oxidase

Succ: succinate

TRITC: Tetramethyl Rhodamine conjugated

TRX: Thioredoxin

Tyr: tyramine

VEGF: Vascular endothelial growth factor

WT: Wild Tipe

YFP: yellow fluorescent protein

α-GP: α-glycerophosphate

β–OH: β-hydroxybutyrate

8.0 APPENDIX

8.1 PUBLICATIONS

8.1.1 Biochem J. 2007 Aug 15; 406:125

Succinate modulation of H_2O_2 release at NADH:ubiquinone oxidoreductase (Complex I) in brain mitochondria.

Zoccarato F, Cavallini L, Bortolami S, Alexandre A.

Complex I (NADH:ubiquinone oxidoreductase) is responsible for most of the mitochondrial H_2O_2 release, both during the oxidation of NAD-linked substrates and during succinate oxidation. The much faster succinate-dependent H_2O_2 production is ascribed to Complex I, being rotenone-sensitive. In the present paper, we report high-affinity succinate-supported H_2O_2 generation in the absence as well as in the presence of GM (glutamate/malate) (1 or 2 mM of each). In brain mitochondria, their only effect was to increase from 0.35 to 0.5 or to 0.65 mM the succinate concentration evoking the semi-maximal H_2O_2 release. GM are still oxidized in the presence of succinate, as indicated by the oxygen-consumption rates, which are intermediate between those of GM and of succinate alone when all substrates are present together. This effect is removed by rotenone, showing that it is not due to inhibition of succinate influx. Moreover, alpha-oxoglutarate production from GM, a measure of the activity of Complex I, is decreased, but not stopped, by succinate. It is concluded that succinate-induced H_2O_2 production occurs under conditions of regular downward electron flow in Complex I. Succinate concentration appears to modulate the rate of H_2O_2 release, probably by controlling the hydroquinone/quinone ratio.

8.1.2 J Bioenerg Biomembr. 2008;40:9-18.

Long chain fatty acyl-CoA modulation of H_2O_2 release at mitochondrial complex I.

Bortolami S, Comelato E, Zoccarato F, Alexandre A, Cavallini L.

Complex I is responsible for most of the mitochondrial H_2O_2 release, low during the oxidation of the NAD linked substrates and high during succinate oxidation, via reverse electron flow. This H_2O_2 production appear physiological since it occurs at submillimolar concentrations of succinate also in the presence of NAD substrates in heart (present work) and rat brain mitochondria (Zoccarato et al., Biochem J, 406:125-129, 2007). Long chain fatty acyl-CoAs, but not fatty acids, act as strong inhibitors of succinate dependent H_2O_2 release. The inhibitory effect of acyl-CoAs is independent of their oxidation, being relieved by carnitine and unaffected or potentiated by malonyl-CoA. The inhibition appears to depend on the unbound form since the acyl-CoA effect decreases at BSA concentrations higher than 2 mg/ml; it is not dependent on DeltapH or Deltap and could depend on the inhibition of reverse electron transfer at complex I, since palmitoyl-CoA inhibits the succinate dependent NAD(P) or acetoacetate reduction.

8.1.3 S.G. Pandalai, Biogenic Amines: biochemical, Physiological and Clinical Aspects, May 14-18 2008, Trento, In press.

Evaluation of the measurement of MAO activity as source of H_2O_2 and ROS in intact biological systems and cells

Bortolami S.; Cavallini L.

In the field of reactive oxygen species (ROS) as signalling molecules of both pathological or physiological phenomena the knowledge of the intracellular sources specifically involved in different tissues is receiving much attention. Among the primary sources of H_2O_2 monoamine oxidases are frequently regarded as main inducers of oxidative stress. Their involvement is indirect or largely deduced from ROS sensitive probes now amply criticised as detectors of H_2O_2,or even others ROS, in intact cells. We compared two methods of detection of the H_2O_2 arising from MAO activity in intact and permeabilized cell lines. The first was the sensitive fuorimetric detection of extracellular H_2O_2 by Amplex red/HRP and the second was the fluorescent microscopy detection of the oxidation product originating from reaction with H_2O_2/ ROS of DCFH, CM-DCFH, DHR and Mitotracker, four different ROS sensitive probes, in intact cells. The first was unable to detect any activity in intact cells but it detected a prevalent MAO-A activity after permebilization in both SHSY-5Y and in $C_2C_{12\ cells}$. The second did not show any reliable indication of an increased H_2O_2/ROS production dependent on MAO activity in the same cell lines considering the mean intensity of the totality of cells. We present some preliminary data indicating that expression of the HRP in cells increased the specificity of the reaction with endogenous H_2O_2 and amplified the signal bringing to the detection of the MAO-A activity for the first time in intact viable cell (Bortolami & Cavallini submitted). We speculate about the possible application of the HRP expression as a mean to measure H_2O_2 in viable cell systems.

8.2 PATENT

Cavallini L., Bortolami S.

Metodo per la misurazione della produzione di H_2O_2 in cellule eucariotiche mediante test accoppiato all'espressione di HRP

Ref. PD2009A000016

Università di Padova.

Italia