Use of H$_2$O$_2$ to Cause Oxidative Stress, the Catalase Issue

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Abstract: Addition of hydrogen peroxide (H$_2$O$_2$) is a method commonly used to trigger cellular oxidative stress. However, the doses used (often hundreds of micromolar) are disproportionately high with regard to physiological oxygen concentration (low micromolar). In this study using polarographic measurement of oxygen concentration in cellular suspensions we show that H$_2$O$_2$ addition results in O$_2$ release as expected from catalase reaction. This reaction is fast enough to, within seconds, decrease drastically H$_2$O$_2$ concentration and to annihilate it within a few minutes. Firstly, this is likely to explain why recording of oxidative damage requires the high concentrations found in the literature. Secondly, it illustrates the potency of intracellular antioxidant (H$_2$O$_2$) defense. Thirdly, it complicates the interpretation of experiments as subsequent observations might result from high/transient H$_2$O$_2$ exposure and/or from the diverse possible consequences of the O$_2$ release.

Keywords: reactive oxygen species; oxidative damage; DNA strand break; cellular respiration; aconitase; fumarase

1. Introduction

Exposure to hydrogen peroxide (H$_2$O$_2$) is a widely used procedure to cause oxidative damage/stress in cellular models. The Fenton’s reaction between H$_2$O$_2$ and Fe$^{2+}$ ions generates the highly reactive OH radical and is thought to be the main mechanism for oxidative damage [1]. Then the question arises as to whether the conditions used are relevant to the mechanism underlying endogenous oxidative damage. Oxidative damage to cells is supposed to result primarily from oxygen using reactions within cells: cellular respiration accounts for most of the cellular oxygen consumption hence mitochondria are supposed to be oxidative stress generators. A quick survey of the literature reveals that the concentrations of H$_2$O$_2$ used to trigger oxidative damage appear disproportionately high to anyone used to dealing with cellular respiration for the following reasons: Dissociation of oxygen from hemoglobin starts below 50 µM and interstitial concentration is in the 20 µM range [2] hence even lower within cells. The affinity of mitochondria for oxygen is extremely high [3,4] and consequently mitochondria can exhaust all incoming oxygen with minimal decrease in their respiratory rate. This allows a sustained oxygen consumption flux while oxygen concentration is close to zero [5]. This points to the distinction to be made between concentrations and fluxes. Several questions arise with regard to this H$_2$O$_2$ model of oxidative damage: (1) Why are these high concentrations of H$_2$O$_2$ necessary to observe oxidative damage? (2) Are there other consequences than direct oxidative damage by H$_2$O$_2$? (3) Are the induced modifications relevant to physiology and to the redox balance associated with aerobic life?

The aim of the present report is to attract attention to the fact that direct application of H$_2$O$_2$ to cells results in the generation of oxygen (O$_2$) because of the presence of catalase [6–8]. This is not unexpected but seems generally overlooked and is likely relevant to the above first two questions.
With regard to the third question, which lies well beyond the scope of the present article, two comments arise: (1) High concentration of \( \text{H}_2\text{O}_2 \) may be justified with the belief that they would increase the frequency of oxidative damages but would not alter their nature. (2) In addition to normal aerobic metabolism, local concentrations might be closer to the experimental values mentioned before in the inflammatory state where enzymatic reactions generate oxygen radicals with the aim to kill unwanted organisms/cells.

Actually, this report originates from our attempts to generate DNA strand breaks in mitochondrial DNA with hydrogen sulfide [9,10]. In our hands hydrogen sulfide could neither cause nuclear [11] nor mitochondrial DNA damage, while \( \text{H}_2\text{O}_2 \) as a positive control required the high micromolar concentrations found in the literature (Appendix A).

Lipids and proteins are other targets for oxidative stress. The enzyme aconitase contains an iron atom (\( \text{Fe}^{2+} \)) sensitive to oxidative stress [12]. Its mitochondrial isoform is part of the tricarboxylic acid cycle (Krebs cycle). In contrast, fumarase, whose mitochondrial isoform is also part of the Krebs cycle, is resistant to oxidative stress. Similar experimental procedures detect aconitase and fumarase activities allowing sequential monitoring of these two reactions in the same reaction medium. Therefore, the aconitase to fumarase ratio is a convenient index of the extent of oxidative damages/stress [12].

Examination of this ratio in cells exposed to \( \text{H}_2\text{O}_2 \) could not detect indication of intracellular oxidative damage to aconitase unless \( \text{H}_2\text{O}_2 \) approached the millimolar concentration.

2. Results

2.1. Values for \( \text{H}_2\text{O}_2 \) Concentration in the Literature

The PubMed database was searched with the keywords «Oxidative damage and Hydrogen Peroxide» and only publications in open access were considered. This request yielded 5147 publications. They were sorted according to best match. The first 201 were scrutinized for \( \text{H}_2\text{O}_2 \) treatment with concentration values, yielding 112 publications, whose PMID identifiers are listed in Appendix B. Different experimental profiles had to be considered: 46 publications used a single \( \text{H}_2\text{O}_2 \) concentration value, 36 publications presented a dose response curve leading to the choice of a single concentration used throughout the rest of the study (this concentration was considered here), and 30 publications used a set of increasing concentrations with no privileged concentration leading us to consider the mean value between minimal and maximal concentrations. Then the repartition of the \( \text{H}_2\text{O}_2 \) concentrations used was as follow: <10 \( \mu\text{M} \) 4 publications, 10–99 \( \mu\text{M} \) 14 publications, 100–500 \( \mu\text{M} \) 55 publications, 501–1000 \( \mu\text{M} \) 13 publications, and >1000 \( \mu\text{M} \) 26 publications. The concentrations of \( \text{H}_2\text{O}_2 \) used to generate oxidative damage in cellular models are therefore in the high micromolar range with 84% above 100 \( \mu\text{M} \). Notably the 100–500 \( \mu\text{M} \) range was used in half of the publications.

2.2. A Proportionate Increase in Dioxygen Follows High Micromolar \( \text{H}_2\text{O}_2 \) Addition

Our initial aim was to evaluate mitochondrial DNA strand break in conditions of induction of mitochondrial sulfide oxidation in wild type chinese hamster ovary (CHO) cells or in cells with overexpressed human sulfide quinone reductase [13]. We used the O2k oxygraph, which allows simultaneous measurement in two identical chambers (Figure 1). It showed that addition of high (hundreds) micromolar \( \text{H}_2\text{O}_2 \) resulted in an immediate release of molecular oxygen apparently proportionate to the \( \text{H}_2\text{O}_2 \) input, and 500 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) led to oxygen concentrations above 250 \( \mu\text{M} \), hence higher than that resulting from air saturation of the medium (approx. 200 \( \mu\text{M} \) at 37 °C). However, with 25 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) the oxygen release was hardly detected.

We hypothesized that catalase enzymatic activity (\( \text{H}_2\text{O}_2 + \frac{1}{2}\text{O}_2 \)) underlay the observed phenomenon. The reaction equation predicts that the oxygen concentration increase would equal half the concentration of \( \text{H}_2\text{O}_2 \) added, hence 12.5, 50, and 250 \( \mu\text{M} \) in the examples shown in Figure 1. Figure 2 shows the percentage of this theoretical oxygen increase that was actually measured in the experiments shown in Figure 1. More than 80% of predicted oxygen was recovered with 500 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \),
roughly 50% with 100 µM, and this percentage dropped below 10% with 25 µM H2O2, at which the slow linear increase in the long term (120 s and more) is rather explained by small experimental differences exaggerated by the conversion in % of absolute values differing by two orders of magnitude. When the difference between two simultaneous experiments shown in Figure 1 was considered (empty circles in Figure 3), the percentage of O2 recovery was higher and reached 100% when the difference between 500 µM and 100 µM traces (hence 400 µM H2O2) was considered.

Figure 1. O2 release upon H2O2 addition. Chinese hamster ovary (CHO) cells suspension at approx. 2 × 10^6 cells/mL; X axis = time in seconds; left Y axis = recorded oxygen concentration expressed in µM (lines); right Y axis = oxygen flux rate in pmol/(s.mL) calculated over a two second interval (dots); and thick upward black arrow = H2O2 addition performed at time 180 s. The stable negative value of oxygen rate before H2O2 addition is the oxygen consumption due to cellular respiration. Different H2O2 additions are represented: (a) 25 µM (black symbols) or 100 µM (grey symbols) H2O2 in the cellular suspension, (b) 500 µM (black symbols) or 100 µM (grey symbols) H2O2 in the cellular suspension.

Figure 2. Increase in oxygen content in the medium compared to a full catalase reaction: X axis = time in seconds with addition of H2O2 considered as zero time; Y axis = oxygen release expressed as percentage of the amount expected from a 100% yield for the catalase reaction (O2 = 1/2 H2O2). The two experiments presented in Figure 2a are in black symbols (+100 and +500 µM H2O2) while those presented in Figure 2b are in grey (+25 µM and +100 µM). Empty symbols represent results obtained when subtracting the oxygen release in the two concomitant chambers, i.e., either 100 − 25 = 75 µM H2O2 or 500 − 100 = 400 µM, with one data point out of ten shown for the sake of clarity.
Recapitulation of experimental data led to a model for the reaction between H$_2$O$_2$ and CHO cells. CHO cells suspension ranged from 1.5 to 2.2 × 10$^6$/mL. (a) X axis = final H$_2$O$_2$ concentration in the medium, Y axis = maximal observed increase in O$_2$ (see Figure 2 for an example); dotted black line = linear fitting according to the equation shown in grey (with values in µM) in the panel upper part; grey lines = released oxygen given by the predicted equation of the model. (b) Expression of these O$_2$ increases as percentage of the value expected from full engagement into catalase reaction, same X axis and symbols as in panel a. (c) Resulting model: upon H$_2$O$_2$ addition, 40 nanomoles H$_2$O$_2$ are immediately engaged in reactions not leading to O$_2$ release; the rest of H$_2$O$_2$ is subjected to catalase action.

Putting together the results of all the experiments made with H$_2$O$_2$ additions in the 25–500 µM range and with CHO cells suspensions close to 2 × 10$^6$ cells/mL suggested a simple model (Figure 3).

In that model two reactions took place: an immediate titration of a part of H$_2$O$_2$ and catalase reaction on the rest of H$_2$O$_2$. With the lowest H$_2$O$_2$ concentration (25 µM) the immediate titration checked almost 100% of the H$_2$O$_2$ and little was made available to catalase (Figures 1 and 2).

Analysis of a range of cell concentrations strengthened the proposed role of cellular catalase (Figure 4). With increasing cell concentration, the lag time between H$_2$O$_2$ addition and maximal O$_2$ release decreased (Figure 4a). A single experiment with medium alone led to a low rate of O$_2$ release that reached a peak of 27 µM, hence 11% of the value explained by a catalase like reaction, after 1518 s (not shown on Figure 5). Altogether, this meant that the more cells, the faster the catalase reaction.

However, when the yield of the catalase reaction was considered it declined when lower amounts of cells were used. Time needed for completion of catalase reaction was then likely to be the determinant factor. To a significant extent experimental limitation could explain this because the determination of time and extent of the maximal O$_2$ release became increasingly inaccurate when it took a long time. Another proposal would be that in addition to fast titration (see above) other slower reactions, which could involve components of the culture medium or renewal of H$_2$O$_2$ quenchers, contributed to H$_2$O$_2$ elimination.
Within three minutes, $2 \times 10^6$ CHO cells neutralized 500 µM H$_2$O$_2$ (Figure 4a). This corresponds to an average rate while the initial rate was much faster (Figure 1). We estimated this initial rate and Figure 5a represents its dependence on H$_2$O$_2$ concentration with CHO cells. This initial rate of oxygen release remained proportionate to H$_2$O$_2$ concentration in the range 0–1000 µM with no
saturation. The oxygen release rate could largely outperform the cellular oxygen consumption rate (cellular respiration) with rates that are more than one order of magnitude higher in the opposite direction (Figure 5a). This fits with the very high turnover rate of the catalase catalytic cycle [6] as a consequence while minutes were needed to reach the maximal increase in O$_2$ (Figure 4a), hence to exhaust all H$_2$O$_2$ in the external medium the H$_2$O$_2$ concentration decreased sharply within seconds after being in contact with cells.

We observed comparable rates for this catalase reaction in eight cell lines. Interestingly, the O$_2$ release rate (catalase reaction) apparently correlated with the endogenous respiration rate, i.e., the cellular oxidative metabolism (Figure 5b).

### 2.4. Cellular Respiration and Aconitase Activity Resistant to H$_2$O$_2$ in the High Micromolar Range

We evaluated intracellular oxidative stress using the aconitase to fumarase ratio, which is considered a sensitive index [12]. Furthermore, we discriminated the cytosolic enzymes, revealed after mild digitonin treatment of the cells, from the mitochondrial enzymes, requiring drastic disruption of the mitochondrial inner membrane using Triton X100 (Figure 6). Increasing concentrations of H$_2$O$_2$ up to 500 µM did not alter the aconitase to fumarase ratio, either in cytosol or in mitochondria (Figure 6a). One experiment with H$_2$O$_2$ concentration raised up to 1mM induced a detectable decrease in cytosolic aconitase activity that remained partial for the mitochondrial enzyme (Figure 6b).

![Figure 6](image_url)

**Figure 6.** Evaluation of cellular oxidative stress using the aconitase to fumarase ratio in CHO cells exposed to H$_2$O$_2$. Y axis = aconitase to fumarase ratio; X axis = H$_2$O$_2$ concentration. (a) Empty bars = values observed in cells treated with digitonin (cytosolic activities); filled bars = values observed in cells treated with Triton X100 (both cytosolic and mitochondrial activities); values expressed as mean ± SD; the number of independent experiments is indicated at the bottom of the histogram bars. (b) Single experiment with the concentration of H$_2$O$_2$ raised to 1 mM; values expressed as mean and SD of the wells observed in that experiment.

Evaluation of a potential impact of H$_2$O$_2$ impact on cellular oxygen consuming reactions was impossible in the short term after H$_2$O$_2$ addition because of the O$_2$ release. However, with the rapid decline of H$_2$O$_2$ concentration, cellular oxygen consumption rate became detectable, gradually converging towards a new stable value. Comparison of that value with the value observed just before H$_2$O$_2$ addition, used as reference, did not show any change with H$_2$O$_2$ ranging from 0 to 750 µM (Figures 7 and 8). This was true within the period considered, limited to 30–40 min after H$_2$O$_2$ addition. Respiration showed a mild increase (>110%) with 1mM H$_2$O$_2$, likely explained by the huge increase in O$_2$ concentration up to three times the level for saturation with air (not shown). With the reservation that we could not distinguish between mitochondrial respiration and other oxygen consuming processes, this suggested that intense exposure to H$_2$O$_2$ and subsequently to oxygen did not result in fast and irreversible damage to any critical component necessary for normal cellular respiration. This was fully consistent with the resistance of mitochondrial aconitase to the exogenous H$_2$O$_2$. 

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**Figure 4a** shows the effect of H$_2$O$_2$ on cellular respiration in CHO cells. **Figure 4b** demonstrates the effect of H$_2$O$_2$ on aconitase activity in the cytosol of CHO cells. **Figure 5a** illustrates the relationship between catalase activity and endogenous respiration. **Figure 6a** and **Figure 6b** depict the impact of H$_2$O$_2$ concentration on aconitase activity and catalase reaction, respectively.
with generation of superoxide from H\textsubscript{2}O\textsubscript{2} within a few minutes. This makes the H\textsubscript{2}O\textsubscript{2} (largest) part from the hydroxyl radical liberated by Fenton’s reaction. However, a surge in superoxide of a well and surrounded by a still medium, O\textsubscript{2} the cellular suspension necessary for the measurement of cellular oxygen consumption. Consequently, intracellular O\textsubscript{2} damages. The conversion of hundreds of micromolar H\textsubscript{2}O\textsubscript{2}.

3. Discussion

The results presented here indicate that cells subjected to H\textsubscript{2}O\textsubscript{2} exposure convert H\textsubscript{2}O\textsubscript{2} into O\textsubscript{2} within a few minutes. This makes the H\textsubscript{2}O\textsubscript{2} exposure considerably shorter and less intense than expected. It may well explain why such high H\textsubscript{2}O\textsubscript{2} concentrations were required to record detectable damages. The conversion of hundreds of micromolar H\textsubscript{2}O\textsubscript{2} into oxygen has the consequence of exposing cells to O\textsubscript{2} concentrations higher than that resulting from equilibration of the aqueous medium with air, a concentration already way above physiological oxygen concentrations [2].

The oxidative damages taking place after H\textsubscript{2}O\textsubscript{2} exposure are thought to result for a significant (largest) part from the hydroxyl radical liberated by Fenton’s reaction. However, a surge in superoxide has been associated with exposure to H\textsubscript{2}O\textsubscript{2} [14]. Superoxide results from O\textsubscript{2} reduction with a single electron, and is converted into H\textsubscript{2}O\textsubscript{2} by superoxide dismutase. Occurrence of the reverse reaction, with generation of superoxide from H\textsubscript{2}O\textsubscript{2}, is not immediately obvious and is expected to be an indirect consequence of H\textsubscript{2}O\textsubscript{2} exposure [15]. Our experiments indicate that a large increase in oxygen concentration is expected to take place at the site of catalase action hence inside the cell. This rise in intracellular O\textsubscript{2} would greatly increase the probability of its reduction by leakage of a single electron from cellular metabolism. It may therefore provide a direct explanation for the observed increase in cellular superoxide production.

In our experiments, the equilibration of concentrations was immediate because of the stirring of the cellular suspension necessary for the measurement of cellular oxygen consumption. Consequently, cellular catalase could access added H\textsubscript{2}O\textsubscript{2} within a short time. However, in cells attached at the bottom of a well and surrounded by a still medium, O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} may have restricted diffusion leading to

![Figure 7](image_url) Absence of impact of H\textsubscript{2}O\textsubscript{2} on CHO cells respiration. Y axis = cellular oxygen consumption after H\textsubscript{2}O\textsubscript{2} had been exhausted and expressed as percent of the initial rate (rate recorded just before H\textsubscript{2}O\textsubscript{2} addition). (a) X axis = lag time between H\textsubscript{2}O\textsubscript{2} addition and measurement oxygen consumption rate; black triangles refer to concomitant control experiments (without H\textsubscript{2}O\textsubscript{2}). (b) X axis = amount of H\textsubscript{2}O\textsubscript{2} injected in the experiments represented in panel a.

![Figure 8](image_url) Absence of impact of H\textsubscript{2}O\textsubscript{2} on the respiration is common to different cells. Oxygen consumption rate after H\textsubscript{2}O\textsubscript{2} had been exhausted is expressed in % of the reference rate for cells exposed to 500 µM H\textsubscript{2}O\textsubscript{2}; values expressed as mean ± SD; CHO-K1: 21 preparations of CHO-K1 with cell density ranging from 0.35 to 2.08 × 10\textsuperscript{6} cells per mL. Others: 7 preparations of the cell lines used in Figure 5b (one value per cell line cell and density ranging from 0.86 to 3 × 10\textsuperscript{6} cells per mL).
delayed access to catalase. H$_2$O$_2$ may then represent an additional oxygen reserve. Interestingly, the dose response curve showed a rather positive effect of H$_2$O$_2$ at low concentrations, before the deleterious effect took place/dominated [16]. Reactive oxygen species (ROS) signaling might explain this observation but the improvement of cellular bioenergetics by H$_2$O$_2$/catalase mediated O$_2$ supply to mitochondria may also deserve consideration.

At low concentrations of H$_2$O$_2$, a significant proportion escaped from catalase action (Figures 2 and 3). One could easily envision that H$_2$O$_2$ would first titrate all the molecules able to react quickly with it. They would represent a “sink for H$_2$O$_2$”, which includes components of the medium as well as cellular antioxidant defenses. This sink shows highest affinity but limited size (40 nanomoles in our experimental settings). In contrast, catalase shows relatively low affinity but a large activity able to neutralize within a few minutes surrounding H$_2$O$_2$ concentrations approaching the millimolar range, hence a micromole of H$_2$O$_2$ in our settings.

Altogether these observations render the interpretation of H$_2$O$_2$ experiments far more complex than considering a sudden increase in the probability for Fenton’s reaction to occur (Figure 9).

![Scenario for H$_2$O$_2$ mediated oxidative damage](image)

**Figure 9.** Scenario for H$_2$O$_2$ mediated oxidative damage. **Top:** normal cellular physiology, endogenous oxygen metabolism is essentially devoted to mitochondrial ATP generation (1). This and other oxygen using enzymes generate oxygen radicals (2) and a vast majority is eliminated by antioxidant defenses. Some oxidative damages (Damages) occur and cellular bioenergetics contributes to their reparation resulting in a steady state with no accumulation of oxidative lesions. **Bottom:** addition of H$_2$O$_2$ (high μM) has three immediate consequences: it increases the probability of Fenton’s reaction (3), H$_2$O$_2$ reacts within seconds with existing reactive molecules and annihilates the existing antioxidant defenses (4), and H$_2$O$_2$ concentration drops abruptly through action of cellular catalase. This increases greatly the intracellular O$_2$ concentration (5) and hence endogenous oxygen radicals production (2). On one side oxygen radical generation is increased (2 and 3) and on the other side antioxidant defenses are invalidated (4). This is expected to generate oxidative damages at rates exceeding repair: they accumulate and become detectable and may result in cell death.

ROS release by mitochondria often leads to considering them as permanent cell threatening ROS generators, checked by antioxidant defenses. Decline of the latter would explain increase in oxidative damage. Mitochondrial superoxide dismutase (MnSOD) quickly converts mitochondrial superoxide, the essential mitochondrial ROS, into H$_2$O$_2$. Then, if the largest part of cellular oxidative damage has a mitochondrial origin, the mitochondrial exposure to H$_2$O$_2$ would be maximal as well as the possible damage. Indeed, coincidence between mitochondrial aconitase inactivation and cell death has
been observed [12]. However, when considering exposure to exogenous H$_2$O$_2$, our study indicated protection from H$_2$O$_2$ action of the mitochondrial aconitase. This highlights that the topology is inversely related to mitochondrial ROS mediated damage and H$_2$O$_2$ treatment. Possible explanation of the relative immunity of the mitochondrial matrix with regard to exogenous H$_2$O$_2$ could be diffusion limitations [17] or large excess in antioxidant defense, the latter expected to be also efficient against mitochondrial H$_2$O$_2$.

In conclusion, one may consider that mitochondria protect themselves and other cellular components from oxygen damage by three different means: avidly consuming oxygen, antioxidant defenses, and contribution to cellular bioenergetics to feed the renewal of damaged components. Mitochondrial impairment would in the first instance downgrade the above-mentioned protective roles. Further degradation would render mitochondria unable to check for their endogenous ROS and, worse, could increase their generation rate. Therefore, mitochondrial ROS release would not cause but rather highlight the decline in cellular bioenergetics and both would contribute cell death.

4. Materials and Methods

All cells were grown at 37 °C with 5% CO$_2$ in media supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. CHO-K1 cells (ATCC CCL-61) were grown in Ham’s F-12 medium containing 10mM glucose, 1mM glutamax, and 1mM sodium pyruvate (Gibco 31765-027). Human lymphoblasts (HPB-All, Jurkat, DND, Mol4) were obtained from Françoise Pflumio (CEA, Fontenay aux Roses France) and were grown in RPMI medium (Gibco 61870-010). Human immortalized fibroblast cell lines were established by one of the co-authors (AL) and grown in DMEM with 1mM Glutamax 5mM glucose. For oxygen flux measurements the cells were resuspended in their culture medium after action of trypsin 0.05 g/L in phosphate-buffered saline (PBS) containing 1 g/L EDTA.

Oxygen concentration at 37 °C was monitored with an Oroboros “O2k” (http://www.oroboros.at/index.php?home). Oxygen consumption/production rate was calculated from oxygen concentration variation either with the DatLab software, which averaged rates over 80 s, or by calculation of individual values between two successive measurements (2 s) when fast rates had to be considered (early phase of H$_2$O$_2$ consumption and O$_2$ release). H$_2$O$_2$ solution was prepared each day of experiment from stock solution (Sigma ref. H1009). The concentration was checked by UV absorbance using the extinction coefficient at 240 nm of 46.3 L × mol$^{-1}$ × cm$^{-1}$.

To evaluate the impact of H$_2$O$_2$ on the aconitase to fumarase ratio H$_2$O$_2$ was added to CHO cells (approximately 2 × 10e6 cells per mL) incubated in the O2k 37 °C with stirring, and cell sampling was done 30 min after H$_2$O$_2$ addition. The aconitase and fumarase activities were measured in 96 well plates allowing measurement at 240 nm (Greiner Bio-One 675801) with a microplate reader equipped with two injectors (TECAN Infinite M200). Preparation of the plate was as follows: first 20 μL of detergent solution in PBS was introduced in the wells, followed by 20 μL of the cellular suspension in PBS, and, finally, by 50 μL of Tris Buffer 0.5 M pH 7.4. In our study 36 wells were used simultaneously (12 samples in triplicates), and optimization led to 100,000 CHO cells per well with final concentrations of 0.01% digitonin and 0.05% Triton X100 to probe for cytosolic enzymes or cytosolic and mitochondrial enzymes, respectively. The plate was introduced in the plate reader to start the reading protocol for measurement of OD240 nm: (1) Shaking (15 s, 2 mm linear); (2) Four readings (each 60 s) to evaluate the background and stability; (3) aconitase reaction with injection of isocitrate solution 120 mM (25 μL at 200 μL/s), shaking as before, twelve readings (each 40 s); (4) fumarase reaction with injection of malate 250 mM, rest of the procedure as before. Calculation of the aconitase to fumarase ratio was made with the assumption that the OD240 increase summed the rates of aconitase and fumarase reactions, which furthermore shared the same proportionality between OD increase and enzymatic reaction rate.

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### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| CHO          | Chinese Hamster Ovary |
| ROS          | Reactive Oxygen Species |
| MnSOD        | Manganese SuperOxide Dismutase (mitochondrial form of SOD) |
| EDTA         | Ethylene Diamine Tetra-acetic Acid |
| OD           | Optical Density |

### Appendix A

**Figure A1.** Quantitation of mitochondrial DNA damage: DNA was prepared from CHO cells exposed to Na$_2$S (H$_2$S) or H$_2$O$_2$. qPCR of samples compared with that of a known amount of target sequence allowed absolute quantitation. This quantitative measurement was made either directly with CHO DNA preparation or after eight cycles of a long PCR amplifying a 8Kb fragment of mitochondrial DNA (2976–11031) including the qPCR target sequence (6744–6918). Therefore, after the long PCR the theoretical amplification factor of the mtDNA target sequence is 64 times. The graph represents the experimental amplification factor (Y axis) when cells were exposed to increasing concentrations (X axis) of H$_2$S (empty symbols) or H$_2$O$_2$ (filled symbols). The number of experiments (independent treatment with H$_2$O$_2$ or H$_2$S) is indicated. A log scale is used for both axes with following modifications: H$_2$S values are represented with X values 10% higher than reality, this allows distinction of error bars, the 10$^6$ value (1M) Na$_2$S is represented as if 10$^4$.

### Appendix B

**Figure A2.** The histogram shows the percentage (Y axis) and the number of publications according to H$_2$O$_2$ concentrations.
Table A1. The list hereafter indicates the PMID identifiers of the articles considered.

| $\text{H}_2\text{O}_2 \leq 10 \mu\text{M}$          |                  |
|-------------------------------------------------|-----------------|
|                                               | 20572791        |
|                                               | 29550333        |
|                                               | 23044977        |
|                                               | 9188618         |

| $\text{H}_2\text{O}_2$ range 10–99 $\mu\text{M}$ |                  |
|-------------------------------------------------|-----------------|
|                                               | 17925005        |
|                                               | 19840190        |
|                                               | 14981287        |
|                                               | 12673362        |
|                                               | 12832847        |
|                                               | 15830107        |
|                                               | 17451824        |
|                                               | 29470430        |
|                                               | 29208995        |
|                                               | 10082315        |
|                                               | 16331539        |
|                                               | 18463796        |
|                                               | 21293477        |
|                                               | 26310930        |

| $\text{H}_2\text{O}_2$ range 100–500 $\mu\text{M}$ |                  |
|-------------------------------------------------|-----------------|
|                                               | 21284033        |
|                                               | 12195838        |
|                                               | 25383141        |
|                                               | 31465957        |
|                                               | 20072135        |
|                                               | 28188272        |
|                                               | 22885031        |
|                                               | 25279550        |
|                                               | 19151733        |
|                                               | 12067712        |
|                                               | 23484085        |
|                                               | 14578137        |
|                                               | 26861269        |
|                                               | 27105496        |
|                                               | 31562333        |
|                                               | 26764141        |
|                                               | 9012815         |
|                                               | 22766155        |
|                                               | 28204825        |
|                                               | 29983082        |
|                                               | 18501193        |
Table A1. Cont.

|        |        |        |        |        |        |        |        |        |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 23326372 | 27582624 | 31353362 | 31683554 | 17879239 | 26225431 | 29121706 | 29386875 | 18497977 |
| 20847286 | 23519197 | 9551742 | 22914987 | 26991551 | 29165341 | 28977633 | 20559505 | 11905987 |
| 18520066 | 10409635 | 14627202 | 28122344 | 26898812 | 29431851 | 27479053 | 18003976 | 31112588 |
| 24695490 | 11558568 | 25381633 | 28067784 | 22796327 | 15060142 | 29386875 |        |        |

H$_2$O$_2$ range 501–1000 µM

|        |        |        |        |        |        |        |        |
|--------|--------|--------|--------|--------|--------|--------|--------|
| 23349856 | 25236744 | 24642881 | 9453543 | 24634295 | 20224152 | 6292103 |        |
### Table A1. Cont.

|                |                |                |                |
|----------------|----------------|----------------|----------------|
| 30662338       | 30662323       | 15138265       | 22580126       |
| 16150148       | 18954526       | 10846797       | 22302021       |
| 8176261        | 31783479       | 30513827       | 30366994       |
| 16101127       | 22542292       | 23357941       | 9422344        |
| 8628669        | 9735602        | 26555819       | 25528699       |
| 21429293       | 17085975       | 20824890       | 29351805       |
| 22208537       | 29661935       | 14688260       | 20566850       |
| 16194237       | 16936020       | 21176282       | 30634966       |

|                |                |                |                |
|----------------|----------------|----------------|----------------|
| H₂O₂ range > 1000 μM |                |                |                |
| 10846797       | 22302021       | 8176261        | 31783479       |
| 30513827       | 30366994       | 16101127       | 22542292       |
| 23357941       | 9422344        | 26555819       | 25528699       |
| 21429293       | 17085975       | 20824890       | 29351805       |
| 22208537       | 29661935       | 14688260       | 20566850       |
| 16194237       | 16936020       | 21176282       | 30634966       |

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