Effects of chronic Hydrogen Peroxide Exposure on mitochondrial Oxidative Stress genes, ROS production and lipid peroxidation in HL60 Cells

Montserrat-Mesquida, M[1,2,3]; Ferrer, MD[1]; Pons A[1,2,3]; Sureda, A[1,2,3,*]; Capó, X[1,4]

1- Research Group in Community Nutrition and Oxidative Stress, University of the Balearic Islands-IUNICS, 07122 Palma, Spain.
2- Health Research Institute of Balearic Islands (IdISBa), 07120 Palma, Spain.
3- CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III (ISCIII), 28029 Madrid, Spain.
4- Translational Research in Aging and Longevity (TRIAL) Group, Health Research Institute of the Balearic Islands (IdISBa), 07120 Palma, Spain.

Correspondence:
antoni.sureda@uib.es; Tel.: +34-971172820
Research Group on Nutrition and Oxidative Stress,
Universitat de les Illes Balears,
Crtra. Valldemossa, km 7,5
E-07122 - Palma de Mallorca, Balears, Spain.
e-mail: antoni.sureda@uib.es
Phone: +34-971-172820

Abstract

Hydrogen peroxide (H_2O_2) is a reactive species that is also involved in the redox regulation of cells because of it is relative stability. In numerous pathological situations, a chronic increase in the production of reactive species is observed, which is related to oxidative stress and cellular damage. This study aimed to evaluate the effects of long-term exposure to different H_2O_2 concentrations on oxidative stress biomarkers and mitochondrial dynamics in HL60 cells. HL60 cells were treated with a sustained production (0.1, 1.0 and 10.0 nM/s) of H_2O_2 for one hour. H_2O_2 production and malondialdehyde (MDA) levels, as a lipid peroxidation marker, increased progressively in HL60 cells in accordance with higher H_2O_2 exposure, with significant differences between the 10 nM/s H_2O_2 group and the control and 0.1 nM/s groups. Similarly, progressive increased expression in genes related to the mitochondrial antioxidant defences and mitochondrial dynamics were also observed. Significantly increased gene expression in the 10 nM/s H_2O_2 with respect to the control group was observed for manganese superoxide dismutase (MnSOD), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG1α), nuclear respiratory factor 2 (Nrf2), mitochondrial transcription factor A (Tfam), mitofusins 1 and 2 (Mfn1 and Mfn2) and uncoupling protein 3 (UCP3), whereas no significant changes were observed in the cytochrome c oxidase subunit IV (COXIV) gene expression. In conclusion, exposure to different sustained production of H_2O_2 is related to a progressive increase in the gene expression of mitochondrial dynamics and redox processes in HL60 cells, but also to oxidative damage at higher H_2O_2 production levels.

Keywords: reactive oxygen species, antioxidants, mitochondria, gene expression

1. Introduction

Molecular oxygen is essential for the survival of all aerobic organisms, especially for its role as a final electron acceptor in oxidative phosphorylation. However, during chemical reactions, especially those related to aerobic energy metabolism, oxygen can be partially reduced and generate highly reactive metabolites known as reactive oxygen species (ROS) [1]. Hydrogen peroxide (H_2O_2) is an important metabolite that is also involved in the redox regulation of cells because of it is relative stability and high half-life [2]. In this sense, H_2O_2 can act as a signalling molecule by activating redox-sensitive signalling pathways such as nuclear factor kappa B (NF-κB) and nuclear factor, erythroid 2 like 2 (Nrf2), mainly through the reversible oxidation of specific cysteine residues [2,3]. The main mechanism involved in redox regulation takes place via the thiol peroxidases which involves the reversible oxidation of protein cysteines to the sulfenylated form [4–6]. ROS overproduction can initiate lipid peroxidation processes, damaging both membrane structure and function, and may be responsible for the oxidation of key proteins for cellular metabolism and function, finally causing the oxidation of nucleic acids [7,8]. In fact, the excessive production of reactive species appears in numerous chronic inflammatory diseases, such as autoimmune disorders (rheumatoid arthritis, celiac disease and psoriasis) or and neurodegenerative diseases (Alzheimer's disease, Parkinson's disease), among others [9]. Moreover, all these actions can trigger apoptotic processes by affecting the mechanisms involved in the regulation of the cell life cycle [10,11].

Mitochondria play an extensive diversity of roles in cell physiology and are the principal producers of cell energy and ROS production [12]. ROS overproduction induces mitochondrial dysfunction and changes in the metabolism and dynamics of cells. Specifically, an increased mitochondrial oxidative stress is related to a decreased

efficiency of the electron transport systems, further elevating ROS levels, and reducing ATP production [13]. Mitochondrial biogenesis requires the synthesis and assembly of lipids, proteins and mitochondrial DNA expanding from the pre-existing mitochondrion instead of a new generation [14]. This biogenesis needs fine-tuned coordination between nuclear transcription and the mitochondrial genes, mainly regulated by the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG1α) [15]. PCG1α, is a transcription coactivator which is a crucial component to regulate and limit the effects of oxidative stress and inflammation, due to its ability to increase the expression of antioxidant proteins and the downregulation of NF-kB [16,17]. PCG1α also acts in coordination with Nrf1 and Nrf2 to increase the nuclear transcription of most mitochondrial genes. Nrf2 is one of the most important intracellular antioxidant factors to regulate oxidative stress [18]. Nrf2 activation protects cells from inflammation and apoptosis across oxidative stress regulation [19]. In addition, this interaction increases the transcription of the mitochondrial transcription factor A (Tfam), which is essential for mitochondrial transcription [20]. Moreover, mitochondrial fusion involves the joining of the inner mitochondrial membrane and outer mitochondrial membrane of two different mitochondria or different regions of the mitochondrial reticulum, resulting in a mixture of membranes, as well as of the contents of the mitochondrial intermembrane space and the matrix. Furthermore, this fusion depends on the transmembrane GTPases, mitofusins 1 and 2 (Mfn1 and Mfn2) and optic atrophy protein 1 (OPA-1) [21].

HL60 cell line is a human promyelocytic leukaemia cell line, commonly used in research, due to several characteristics such as genetic stability, immortality and capability to differentiate into different cell types [22]. Moreover, the HL-60 cells, as phagocytic cells, are a relevant component in the acute inflammatory response and can

be used to provide significant information about the cell response to potential stressful situations [23,24]. In this sense, H_2O_2 production in these cells can be influenced by several factors such as oxygen levels, redox state or any pro-inflammatory stimulus [24–26].

The experimental design using glucose/glucose oxidase to produce hydrogen peroxide in a sustained manner allows to reproduce the effects of chronic production of ROS *in vitro* to distinguish those situations in which H_2O_2 generates oxidative imbalance or promotes beneficial responses in HL60 cells [24]. The aim of this study was to evaluate the effects of the exposure to different H_2O_2 concentrations on the expression of mitochondrial dynamics genes, ROS production and lipid peroxidation in HL60 cells.

2. Materials and Methods

2.1 Cell culture

HL60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 units/ml penicillin, 0.1 ng/ml streptomycin and 2 mM L-glutamine, in a humidified atmosphere with 5% CO_2 at 37 ºC in an incubator (Inco-153, Memmert, Germany). The cells were cultured into 250 ml tissue culture flasks at an initial concentration of 2 x 10^5 cells/ml.

2.2 Cell treatments and experimental design

Cell treatments were performed in 6-well plates containing 6 x 10^5 cells/ml. Cells were incubated for one hour with glucose and glucose oxidase (GOX) system with controlled GOX activity in order to produce a sustained rate of H_2O_2 production at 0.1, 1 and 10 nM H_2O_2/s [27]. Briefly, glucose oxidase oxidizes glucose to glucono-δ-lactone using molecular oxygen as an electron acceptor with concurrent production of H_2O_2. Glucose

oxidase type X-S from *Aspergillus niger* (~75% protein, 151,000 U/g solid, Sigma-Aldrich) was added at concentrations of 0.01, 0.1 and 1 μg solid/ml to obtain the sustained productions of 0.1, 1 and 10 nM H_2O_2/s, respectively. The concentration of H_2O_2 in the culture medium was controlled using horseradish peroxidase and tetramethylbenzidine (TMB) monitoring the absorbance at 405 nm [28]. H_2O_2 levels were quantified using a standard curve of known concentration.

2.3. Cell viability

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method [29]. Briefly, cells were treated in triplicate with the different glucose/GOX treatments for 1 hour. Then, cells were washed with PBS to remove the remaining H_2O_2 or glucose oxidase and MTT (0.5 mg/ml) was added to each well. The cells were incubated for 4 h at 37 ºC, and subsequently the plates were centrifuged (2 min, 1500 rpm) and the supernatants were discarded. Tetrazolium crystals were resuspended in DMSO and the absorbance was measured at 570/620 nm.

2.4. Malondialdehyde levels

Malondialdehyde (MDA), a marker of lipid peroxidation, was determined using the specific colorimetric assay kit (Sigma-Aldrich Merck®, St. Louis, MO, USA). In this method, MDA reacted with a chromogenic reagent generating a stable chromophore. Cells were set in glass tubs containing n-methyl-2-phenylindole in acetonitrile:methanol (3:1), followed by adding HCl (12 N) and incubating the cells for one hour at 45 ºC. Finally, the absorbance was measured using a spectrophotometer Microplate Reader (Epoch Microplate Spectrophotometer, Bio-Tek, Agilent Technologies) at 586 nm. A

149 standard curve of known concentration was used to calculate the MDA concentration.
150 Total protein content was used to normalize MDA levels (Biorad® Protein Assay).
151
152 ***2.5. H_2O_2 production***
153 H_2O_2 production was measured in 50 μL of cell suspension with PBS (containing about
154 6 x 10^5 cells), which were added to a 96-well microplate. Then 2,7-dichloroflourescein-
155 diacetate (DCFH-DA) in ethanol was diluted in Hanks' Balanced Salts Medium at 30
156 μg/mL) and 50 μL of this solution was added to each well. Finally, fluorescence (Ex,
157 480 nm; Em, 530 nm) was determined in a FLx800 Microplate Fluorescence Reader
158 (Bio-tek Instruments, Germany) at 37 ºC for 60 minutes by punctual ultraviolet light
159 exposures, and emission readings were recorded every minute with 60 total readings.
160 ROS concentration was calculated by measuring the fluorescence of a standard curve of
161 known ROS concentration after its reaction with DCFH-DA in the same conditions as
162 the samples and normalizing the results using the protein content.
163
164 ***2.6. RNA Extraction and Real-Time PCR***
165 The mRNA expression of Nrf2, manganese superoxide dismutase (MnSOD),
166 uncoupling protein 3 (UCP3), Mtf1, Mtf2, cytochrome c oxidase subunit IV (COXIV),
167 PGC1α and TFAM were determined by Real-Time PCR based on the incorporation of a
168 fluorescent reporter dye. For this purpose, mRNA was isolated by extraction with
169 Tripure Isolation Reagent (Roche). cDNA was synthesized from 1 μg total RNA using
170 reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the
171 LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. For all
172 PCRs there was one cycle of 95 ºC for 10 min, followed by 40 cycles at the conditions
173 shown in Table 1. The relative quantification was performed by standard calculations

considering $2^{(-\Delta\Delta Ct)}$. Basal mRNA levels of control samples were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to ribosomal 18S.

Table 1. Primer sequence and conditions used in Real-Time PCRs

Gene	Primer	Conditions	
18S	Fw: 5'-GACTCAACACGGGAAACCCTCAC-3' Rv: 5'-GACTCAACACGGGAAACCCTCAC-3'	95ºC 60ºC 72ºC	10s 10s 15s
MnSOD	Fw: 5'-GAGAAGGTACCAGGAGGCGTTG-3' Rv: 5'-CAAGCCAACCCCAACCTGAGC-3'	95ºC 64ºC 72ºC	10s 10s 15s
COXIV	Fw: 5'- AGAAGCACTATGTGTACGGCCC-3' Rv: 5'- GGTTCACCTTCATGTCCAGCAT-3'	95ºC 60ºC 72ºC	10s 10s 15s
PGC1α	Fw: 5'- TCAGTCCTCACTGGTGGACA-3' Rv: 5'-TGCTTCGTCGTCAAAAACAG-3'	95ªC 60ºC 72ºC	10s 10s 15s
Nrf2	Fw: 5'-GCGACGGAAAGAGTATGAGC-3' Rv: 5'-GTTGGCAGATCCACTGGTTT-3'	95ºC 60ºC 72ºC	10s 10s 15s
TFAM	Fw: 5'-CAAGACAGATGAAACCACCTC-3' Rv: 5'-AGATTGGGGTCGGGTCACT-3'	95ºC 60ºC 72ºC	10s 10s 15s
Mtf1	Fw: 5'-TGTTTTGGTCGCAAACTCTG-3' Rv: 5'-CTGTCTGCGTACGTCTTCCA-3'	95ºC 60ºC 72ºC	10s 10s 15s
Mtf2	Fw: 5'-ATGCATCCCCACTTAAGCAC-3' Rv: 5'-ATGCATCCCCACTTAAGCAC-3'	95ºC 60ºC 72ºC	10s 10s 15s
UCP3	Fw: 5'-CGTGGTGATGTTCATAACCTATG-3' Rv: 5'-CGGTGATTCCCGTAACATCTG-3'	95ºC 60ºC 72ºC	10s 7s 15s

Fw: Forward; Rv: Reverse; manganese superoxide dismutase (MnSOD); cytochrome c oxidase subunit IV (COXIV); peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α); nuclear factor erythroid 2-related factor 2 (Nrf2), mitochondrial transcription factor A (TFAM), mitofusin 1 (Mtf1); mitofusin 2 (Mtf2), uncoupling protein 3 (UCP3).

2.7. Statistical analysis

Statistical analyses were carried out using a statistical package for social sciences (SPSS v.28 for Windows, IBM Software Group, Chicago, IL, USA). Results are expressed as mean ± S.E.M and $p < 0.05$ was considered statistically significant. The normality of data was evaluated using the Shapiro-Wilk test. The statistical significance of the data

was assessed using a one-way ANOVA. A Bonferroni post hoc test was performed when significant differences were found between groups. The relationships between variables were studied using Spearman correlations.

3. Results

3.1 H_2O_2 production and cell viability

Figure 1 shows H_2O_2 production by glucose/GOX system. All treatments maintained a constant H_2O_2 production for the 60 minutes of the treatment. Three sustained productions of H_2O_2 were used: 0.1, 1 and 10 nM/s.

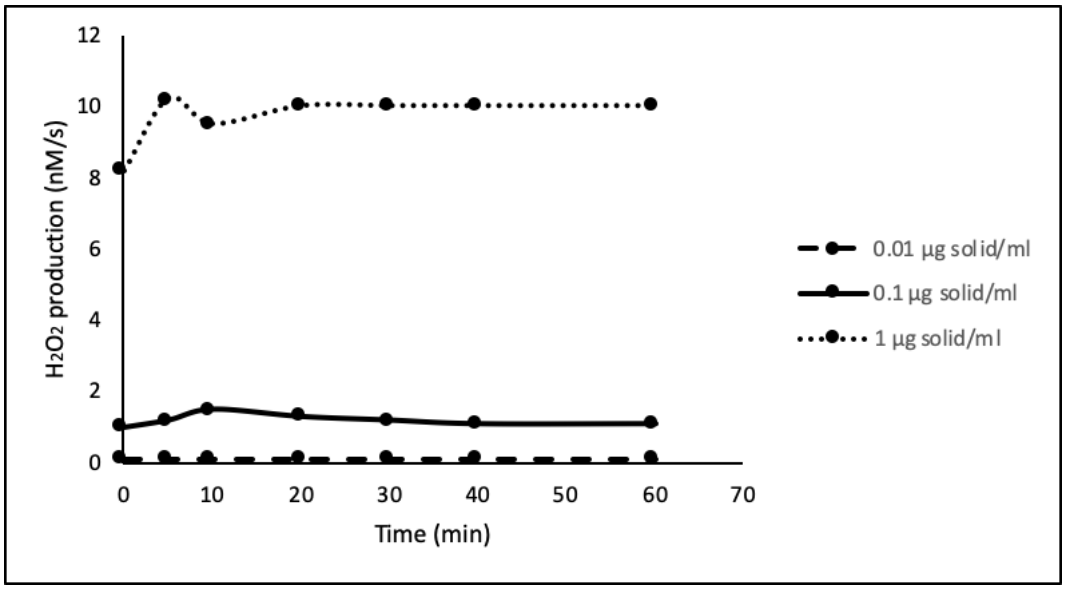

Figure 1. H_2O_2 production with the glucose/glucose oxidase system. H_2O_2 concentration was measured in RPMI medium at 0, 5, 10, 20, 30, 40 and 60 min after the addition of glucose oxidase and glucose.

The treatments with different H_2O_2 production rates did not significantly affect cell viability (Figure 2).

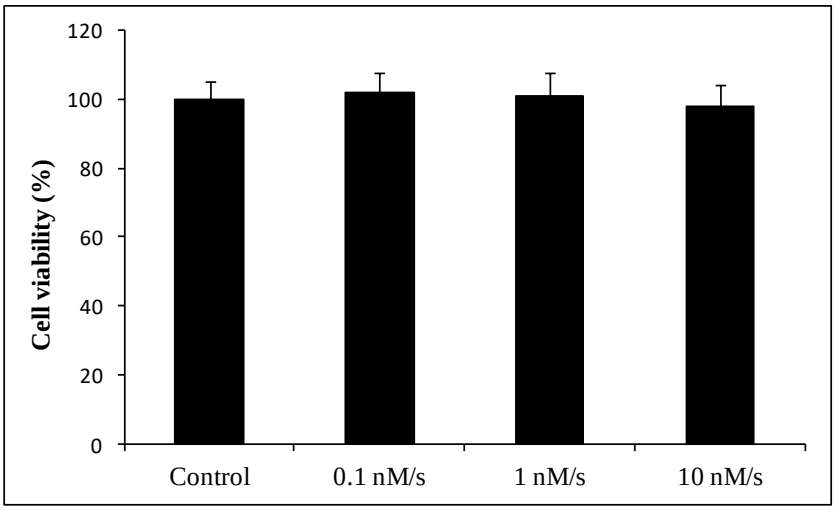

Figure 2. Cell viability of HL60 untreated or treated with increasing sustained levels of H_2O_2 generated by a glucose/glucose oxidase system. Statistical analysis: One-way ANOVA. No significant differences were reported.

3.2. ROS production and MDA levels

Figure 3 shows ROS production (Fig. 3A) and MDA levels (Fig. 3B) in HL60 cells untreated or treated with increasing sustained levels of H_2O_2 production by the glucose/GOX system. Cells treated with 10 nM/s H_2O_2 produced significantly higher levels of ROS and presented higher MDA levels with respect to the control and 0.1 nM/s groups.

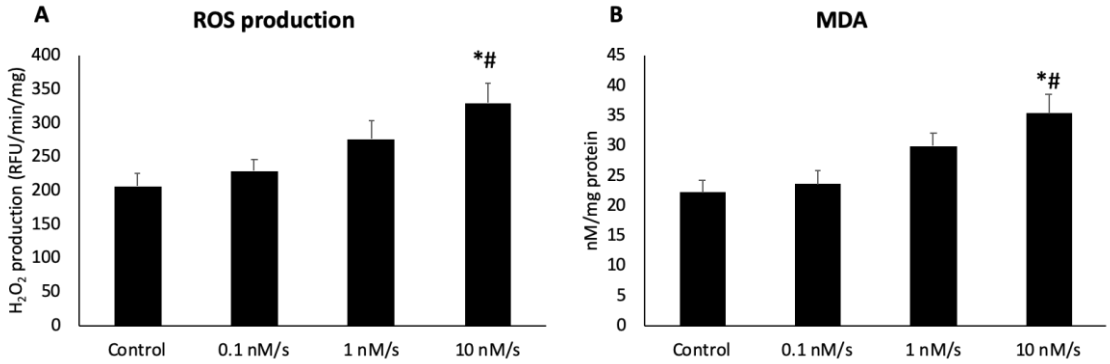

Figure 3. Reactive oxygen species (ROS) production and malondialdehyde (MDA) levels in HL60 cells untreated or treated with increasing sustained levels of H_2O_2 generated by a glucose/glucose oxidase system. (A) ROS production, (B) MDA levels. Statistical analysis: One-way ANOVA with Bonferroni post hoc test. (*) Significant differences vs Control. (#) significant differences vs 0.1 nM/s, $p<0.05$ (n=6)

3.3. Enzymatic gene expression

Figure 4 represents the gene expression of enzymes MnSOD (Fig. 4A) and COIXV (Fig. 4B) in HL60 cells untreated or treated with increasing sustained levels of H_2O_2. A significant increase in MnSOD gene expression was observed in HL60 cells treated with 10 nM/s H_2O_2 when compared to the control group. No significant changes were observed in the COXIV gene expression levels.

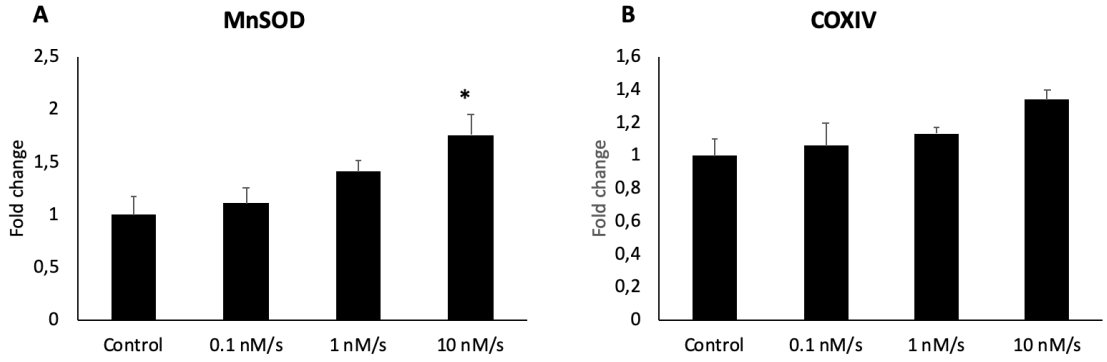

Figure 4. Enzymatic gene expression in HL60 cells untreated or treated with increasing sustained levels of H_2O_2 generated by a glucose/glucose oxidase system. (A) Manganese superoxide dismutase (Mn-SOD), (B) cytochrome c oxidase subunit IV (COXIV). Statistical analysis: One-way ANOVA with Bonferroni post hoc test. (*) Significant differences vs Control, $p<0.05$ (n=6)

3.4. Mitochondrial dynamics gene expression

Figure 5 shows the changes in gene expressions of PGC1α, Nrf2, TFAM, Mtf1, Mtf2 and UCP3. The mRNA levels of PGC1α, Nrf2, TFAM, Mtf1, Mtf2 and UCP3 were significantly increased in HL60 cells treated with 10 nM/s H_2O_2. Moreover, PGC1α, TFAM and Mtf2 were also significantly higher in HL60 cells treated with 1 nM/s H_2O_2 with respect to controls.

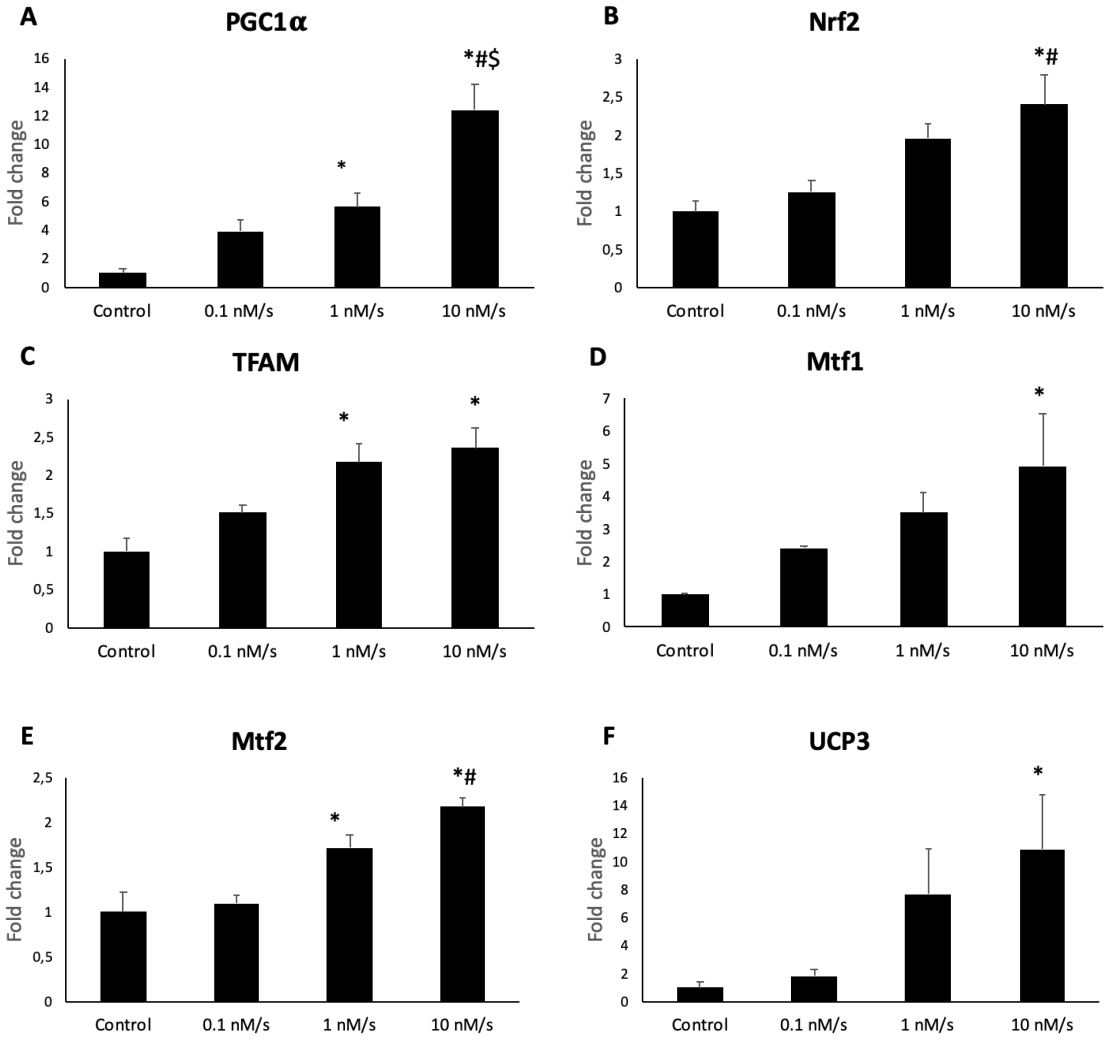

Figure 5. Mitochondrial dynamic of the gene expression in HL60 untreated or treated with increasing sustained levels of H_2O_2 generated by a glucose/glucose oxidase system. (A) peroxisome proliferator-activated receptor gamma coactivator 1-aplha (PGC1α), (B) nuclear factor erythroid 2-related factor 2 (Nrf2), (C) mitochondrial transcription factor A (TFAM), (D) mitofusin 1 (Mtf1), (E) mitofusin 2 (Mtf2), (F) uncoupling protein 3 (UCP3). Statistical analysis: One-way ANOVA with Bonferroni post hoc test. (*) Significant differences vs Control. (#) Significant differences vs 0.1 nM/s. ($) Significant differences vs 1 nM/s, $p<0.05$ (n=6)

3.5. Correlation analysis

Table 2 shows the correlations between the different genes analysed. All genes were directly correlated between them, so all genes were activated as H_2O_2 exposure increased.

Table 2. Correlation between genes expression of HL60 cells.

		COXIV	Mtf2	TFAM	PGC1α	Mtf1	Nrf2	UCP3	Mn-SOD
COXIV	***r***	1.000	0.771**	0.902**	0.578**	0.487*	0.736**	0.581**	0.508*
	p		0.000	0.000	0.003	0.016	0.000	0.003	0.011
Mtf2	***r***		1.000	0.882**	0.825**	0.517**	0.839**	0.776**	0.559**
	p			0.000	0.000	0.010	0.000	0.000	0.004
TFAM	***r***			1.000	0.727**	0.755**	0.982**	0.664**	0.718**
	p				0.000	0.000	0.000	0.001	0.000
PGC1α	***r***				1.000	0.671**	0.678**	0.860**	0.462*
	p					1.000	0.000	0.000	0.023
Mtf1	***r***						0.741**	0.695**	0.469*
	p						1.000	0.000	0.021
Nrf2	***r***							0.517**	0.706**
	p							0.010	0.000
UCP3	***r***							1.000	0.329
	p								0.117
MnSOD	***r***								1.000

Abbreviations: COXIV, cytochrome c oxidase subunit IV; TFAM, mitochondrial transcription factor A; Nrf2, nuclear factor erythroid 2-related factor 2; UCP3, uncoupling protein 3; Mtf1, metal regulatory transcription factor 1; PGC1α, peroxisome proliferator-activated receptor gamma; coactivator 1-aplha; MnSOD, Manganese superoxide dismutase. Bivariate Correlation: (*) Indicates a correlation at $p<0.05$. (**) Indicates a correlation at $p<0.01$.

4. Discussion

The main findings of the current study are evaluate that chronic exposure of HL60 cells to different H_2O_2 concentrations induced a progressive response in the cells in parallel with the H_2O_2 production rate. This exposure for one hour resulted in an increase in the expression of antioxidant enzymes and proteins related to mitochondrial dynamics, but also with an increase in oxidative damage at the highest treatment of 10 nM H_2O_2/s. Previous studies have suggested that H_2O_2, which is defined as a reactive species produced endogenously in the breakdown of superoxide, may mediate the stimulation of diverse redox-sensitive pathways but also induce oxidative damage and apoptosis if overproduced [30,31]. The exposure of H_2O_2 in HL60 cells has been commonly described to induce the appearance of oxidative damage and oxidative stress, although

283 in practically all of the studies, a one-time or repeated H_2O_2 bolus was used [32]. In fact,
284 H_2O_2 bolus treatments generally rely on applying H_2O_2 concentrations in the upper
285 micromolar or even millimolar range to elicit a cellular response due to the rapid
286 degradation of this ROS by cellular catalase and peroxidases [33]. This contrasts with
287 the much lower concentrations of H_2O_2 under physiological conditions, where
288 exogenous concentrations rarely exceed the low micromolar range (up to 10 µM) [34].
289 Therefore, the present study was designed to mimic the physiological condition where
290 cells are continuously exposed to oxidants such as those which occur in pathological or
291 general stressful situations. The maximum H_2O_2 production of 10 nM/s is comparable
292 to values observed in neutrophil suspensions [34]. Furthermore, none of the treatments
293 caused changes in cell viability, indicating that the treatments used were not toxic to the
294 cells.
295 In general, exposure to pro-oxidant agents is related to a burst of ROS that subsequently
296 initiates a stress response that leads to mitochondrial dysfunction, posing a risk to cell
297 survival [35]. In HL60 cells, acute treatment with H_2O_2 (100 µmol/L) has been shown
298 to suppress cell growth and induce an increase in the production of ROS, MDA and
299 DNA damage [36]. In the present study, ROS production and MDA levels progressively
300 increased after continuous H_2O_2 exposure for 1h, becoming significantly higher in cells
301 treated with 10 nM H_2O_2/s, indicating the establishment of a state of oxidative stress.
302 Similarly, the exposure of HL-60 to immune stimulus such as phorbol myristate acetate
303 (PMA) or with the pro-oxidant peroxynitrite induces the oxidative burst in these cells
304 leading to a significant increase in the production of ROS [37,38]. In fact,
305 undifferentiated HL-60 cells contain a NADPH oxidase enzyme that can generate of
306 superoxide anion which is subsequently dismutated into hydrogen peroxide [39]. In this
307 sense, it was previously described that the formation of ROS could induce apoptosis in

HL60 cells, but also activate pro-survival pathways depending on the ability of the cell to control intracellular ROS levels [37,40].

The present study revealed that HL60 cells, treated with 10 nM/s of H_2O_2 generated by a glucose/GOX system, showed an increase in PGC1α expression. These results were similar to those reported in previous studies, where PGC1α activation has been observed after exposure to acute high concentrations of H_2O_2 [41,42]. PGC1α is a transcriptional coactivator reported as a regulator of mitochondrial biogenesis and function, including ROS detoxification and oxidative phosphorylation. In addition, PGC1α dysregulation modifies redox homeostasis in cells and aggravates inflammatory responses [43]. It is suggested that H_2O_2 can influence PGC1α activity through various mechanisms, including AMPK activation and the modulation of PGC1α turnover. However, it is important to note that the specific details and mechanisms of PGC1α activation by H_2O_2 may vary depending on the cell type and experimental conditions [41,42,44]. Several studies have described that PGC1α activation could induce specific dockings with particular transcription factors, which might offer theoretical possibilities for metabolism control [45]. Particularly, PGC1α is able to increase mitochondrial respiratory capacity ratio, enhance ATP production through the oxidation of fatty acids and induce mitochondrial biogenesis, resulting in an overall increased mitochondrial mass [46,47]. Moreover, PGC1α can induce the activation of several transcription factors related to mitochondrial dynamics and antioxidant protection, such as Nrf2 and Tfam [48,49]. In this sense, the present results show an increase in the expression of both Nrf2 and Tfam transcription factors after chronic H_2O_2 exposure and also the activation of downstream genes such as UCP3 and Mn-SOD.

Nrf2 is a nuclear factor that plays a crucial role in cellular resistance to oxidative stress. This factor is involved in the regulation of genes that encode antioxidant enzymes and

other cytoprotective proteins [50]. When cells are subjected to oxidative stress, Nrf2 is activated and translocates to the nucleus, where it binds to antioxidant response elements (AREs) in the DNA and initiates the transcription of target genes [50,51]. In this sense, it has been reported that preconditioning of mesenchymal stem cells with H_2O_2 increases their survival under conditions of oxidative stress (induced by H_2O_2 at 0.25 mM or 0.5 mM) by stimulating their antioxidant defences [52]. Our results are in accordance with previous studies that observed an increase in Nrf2 expression under oxidative stress conditions [53–55]. It has been described that among the target genes of Nrf2 are genes required for mitochondrial respiration and biogenesis and genes related to cell protection [56,57]. Considering this, increased Nrf2 expression could be related to the increased expression of the mitochondrial antioxidant proteins Mn-SOD and UCP3 to help maintaining the integrity of the mitochondria in the face of a potentially damaging situation.

Mn-SOD is an endogenous mitochondrial antioxidant enzyme essential for the maintenance of mitochondrial bioenergetics and function in virtually all aerobic organisms [58]. In this sense, and although no significant differences were observed, the expression of COXIV, as an indicator of mitochondrial function, also increased progressively with the increase in the rate of H_2O_2 production. Mn-SOD detoxifies the free radical superoxide, the major by-product of mitochondrial respiration [59]. These results are consistent with previous evidence in hepatocytes, which reported an increase in Mn-SOD mRNA expression after H_2O_2 exposure [60]. Similarly, UCP3 expression followed a similar pattern to Mn-SOD. UCP3 is a member of the mitochondrial uncoupling protein family, mainly detected in skeletal muscle, that has been reported to act as an antioxidant by reducing ROS production [61]. It has been shown, in both in vivo and in vitro studies, that hyperoxia increases UCP3 mRNA expression, which

might regulate ROS production in response to an oxidative stress situation [62]. Moreover, in accordance to our study, it has also been observed that the acute treatment with H_2O_2 increases UCP3 expression in mouse cardiomyocytes [63].

Our results showed a significant increase in Mtf1 and Mtf2 as consequence of H_2O_2 exposure in HL60 cells. This fact puts in evidence the capacity of H_2O_2 to induce the mitochondrial remodeling since both Mtf1 and Mtf2 are related to mitochondrial biogenesis. This increased expression of Mtf1/2 could be also considered as an antioxidant mechanism to avoid oxidative stress, as well as a mitochondrial protective quality-control process [64–66]. Specifically, some studies evidenced that Mtf1 can induce the expression of metallothioneins and other proteins such as SOD in order to reduce or avoid oxidative damage [67]. Moreover, it has been evidenced that the primary physiologic role of Mtf1 and Mtf2 is coupled to the preservation of mitochondrial DNA (mtDNA) content by regulating the expression of the crucial mitochondrial transcription factor Tfam [68]. Tfam is a nuclear-encoded protein that plays a vital role in maintaining the integrity and function of mitochondria. While its primary function is related to mtDNA replication and transcription, Tfam is also linked to the cellular response to oxidative stress [69,70]. The present results reported an increase in Tfam expressions after H_2O_2 stimulation. In addition to Mtf1/2, the nuclear expression of Tfam is also regulated by the activation of transcription factors such as Nrf2 and ERRα induced by PGC1α [71]. Upregulation of Tfam drives an increase in the replication and expression of mtDNA, modulating mitochondrial biogenesis. In this sense, previous studies reported that Tfam can induce an increase in the mtDNA copy number, which is a strategy to protect mitochondria integrity in an oxidative stress situation [72,73].

5. Conclusions

These results show the double action of ROS as not only cellular messengers, but also as inducers of oxidative damage, especially as their concentration increases. Overall, this study revealed that HL60 exposure to extracellular sustained physiological levels of H_2O_2 activates the expression of transcription factors that regulate the expression of antioxidant proteins such as Mn-SOD and UCP3 and proteins that modulate mitochondrial structure.

6. Declaration of interest

The authors declare no conflict of interest.

7. Funding

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