Free Radical Research, April 2009; 43(4): 340–347

informa
healthcare

Effect of acute vs chronic H_2O_2-induced oxidative stress on antioxidant enzyme activities

FERNANDA MIGUEL, AMANDA C. AUGUSTO, & SONIA A. GURGUEIRA

San Francisco University, Bragança Paulista, SP, Brazil

(Received 24 September 2008; revised 20 December 2008)

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

Abstract

H_2O_2 can freely crosses membranes and in the presence of Fe^{2+} (or Cu^{+}) it is prone to participate in Fenton reaction. This study evaluated the concentration and time-dependent effects of H_2O_2-induced oxidative stress on MnSOD, Se:GPx and catalase and on aconitase. Acute and chronic H_2O_2 treatments were able to induce oxidative stress in HeLa cells as they significantly decreased aconitase activity and also caused a very significant decrease on antioxidant enzyme activities. The inhibition of enzyme activities was time- and concentration-dependent. Chronic treatment with 5 μM H_2O_2/h after 24 h was able to decrease all enzyme activities almost at the same level as the acute treatment. Acute and chronic treatments on antioxidant enzyme activities were prevented by cell treatment with ascorbic acid or N-acetylcysteine. These results indicate that antioxidant enzymes can also be affected by the same ROS they produce or neutralize if the time of exposure is long enough.

Keywords: *Acute oxidative stress, catalase, chronic oxidative stress, hydrogen peroxide, glutathione peroxidase, superoxide dismutase*

Abbreviations: *CAT, catalase; GO, glucose oxidase; G/GO. glucose/glucose oxidase; GSH, gluthatione; NAC, N-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle*

Introduction

Reactive oxygen species (ROS), which include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and singlet oxygen ($^1O_2$), are physiologically generated in biological systems [1], but they are also involved in many pathological processes [2,3] and in ageing [4,5]. The generation of ROS is a constant process; their levels can fluctuate even under normal conditions and the accumulation of $O_2^{\bullet -}$ and H_2O_2 is prevented by specific detoxification systems as the antioxidant enzymes superoxide dismutases (SODs), and catalase (CAT) and glutathione peroxidase (GPx), respectively [6–8]. Non-enzymatic antioxidants, such as ascorbic acid [4,9] and N-acetylcysteine [10,11], contribute to ROS scavenging and prevention or minimization of oxidative stress. Since oxidative stress is classically defined as a redox unbalance with an excess of oxidants or a deficiency in antioxidants [12], ROS enzymatic defense systems must also change in order to adapt to a particular oxidative status and may be affected by a variation in non-enzymatic antioxidants.

Hydrogen peroxide is a normal metabolite produced by SODs during the dismutation of superoxide anion [1]. It is also produced by TNF-α and cytokines to increase their own activities [13]. This peculiar ROS can freely cross membranes and in the presence of Fe^{2+} (or Cu^{+}) is prone to participate in Fenton reaction to generate hydroxyl radical ($^{\bullet}OH$), the most reactive free radical [1]. H_2O_2 has also been

Correspondence: Dr Sonia A. Gurgueira, Universidade São Francisco, Avenida São Francisco de Assis # 218, Bragança Paulista, SP, Brazil, CEP: 12916-900. Tel: 55-11-4034-8393. Fax: 55-11-40341825. Email: sonia.gurgueira@saofrancisco.edu.br

ISSN 1071-5762 print/ISSN 1029-2470 online © 2009 Informa UK Ltd.
DOI: 10.1080/10715760902751894

RIGHTSLINK

reported to be involved in apoptosis [14] and necrosis as well as in cell proliferation [15]. Besides, H_2O_2 generated by glucose oxidase system (GO) can damage nuclear and mitochondrial DNAs [16].

Based on these facts, we decided to evaluate the effect of concentration and incubation time on H_2O_2-induced oxidative stress in some enzyme activities. The activity of the antioxidant enzymes MnSOD, Se:GPx (Selenium-dependent GPx) and catalase (CAT) was determined after exposure of HeLa cells to H_2O_2-induced oxidative stress. In order to better characterize the differences between acute or chronic oxidative stress both conditions were used in the characterization of time-course and concentration-dependent effects of H_2O_2 exposure.

To confirm the occurrence of oxidative stress induced by H_2O_2 we evaluated the activity of aconitase, a tricarboxylic acid cycle (TCA) enzyme which is a physiological intracellular target sensitive to modification by superoxide anion [17,18]. $O_2^{\bullet -}$ converts the active $[4Fe\text{-}4S]^{2+}$-containing form of aconitase to the inactive $[3Fe\text{-}4S]^{1+}$-containing form, allowing a change in its activity to be used as a marker of $O_2^{\bullet -}$ production and also a marker of oxidative stress [17,19].

We also evaluated the protective effect of ordinary antioxidants as ascorbic acid [9] and N-acetylcysteine [10,11] on the activities of the mentioned enzymes in order to compare their ability to avoid both acute and chronic H_2O_2-oxidative stress in our experimental conditions.

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

Materials and methods

Materials

Dulbecco's modified Eagle's medium; penicillin; streptomycin and foetal bovine serum were from Cultilab (Campinas, Brazil). The other reagents were purchased from Sigma Chemical Co (St. Louis, MO). HeLa cells were purchased from ATCC (Rockville, MD).

Cell culture and H_2O_2 treatments

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, under a humidified 5% CO_2 atmosphere at 37°C.

We studied the dose-dependence and time course of induction of oxidative stress in two different *in vitro* models. Acute and chronic stresses were simulated by treating the cells with boluses of H_2O_2 or with an H_2O_2-generating system, respectively. For the acute treatment, cells received H_2O_2 at initial concentrations ranging from 1–2 mM during 3 or 6 h. For the chronic treatment, glucose oxidase (GO) was added to the culture medium at concentrations that delivered controlled fluxes of H_2O_2 from 5×10^{-2} to 1 mM/h during the incubation times specified on the figure legends. The glucose concentration in the culture medium did not substantially change during incubations [20]. H_2O_2 fluxes delivered by the glucose/glucose oxidase (G/GO) mixtures were calculated from the values of oxygen consumption measured using a Clark-type oxygen electrode [20].

At the indicated times, adherent cells were harvested, homogenized and aliquots from the supernatant were assayed for enzyme activities as described below.

Homogenization of HeLa cells

HeLa cells (7×10^5 cells/cm^2) were homogenized with a Dounce homogenizer, in standard medium (120 mM KCl, 30 mM phosphate buffer, pH 7.4) and protease inhibitors cocktail (Calbiochem). The homogenates were centrifuged at 1500 x *g*, for 10 min at 0–4°C, the pellets were discarded and the supernatants were aliquoted and kept at −80°C until the determination of enzymes activity.

Antioxidant enzyme activities

Total superoxide dismutase (SOD) activity was determined through the rate of inhibition of oxidation of ferricytochrome c at 550 nm in a standard reaction medium [21]. The MnSOD activity was measured after inhibition of the Cu/Zn isoenzyme by addition of 1 mM KCN [22]. Catalase (CAT) activity was determined by measuring the decrease in absorption of H_2O_2 at 240 nm [23]. Se:dependent glutatione peroxidase (Se:GPx) activity was determined measuring the rate of oxidized glutathione (GSH) that was evaluated by NADPH oxidation rate in the presence of GSH, GSH reductase and H_2O_2 [24].

Oxidative stress marker enzyme activities

Aconitase activity was measured in cell homogenates spectrophotometrically by monitoring the formation of cis-aconitate at 240 nm in 50 mM Tris-HCl (pH 7.4) containing 0.6 mM $MnCl_2$ and 20 mM isocitrate at 25°C [19].

Fumarase activity was measured spectrophotometrically by following the increase in absorbance at 240 nm at 25°C in a standard reaction mixture containing 30 mM phosphate (pH 7.4), 0.1 mM EDTA and 5 mM L-malate [25].

Protein concentration

The protein concentration was determined by the method of Lowry et al. [26] using bovine serum albumin as a standard.

RIGHTSLINK

Statistical analysis

Values in the figures are mean ± SEM. Data were statistically analysed by factorial analysis of variance followed by Fisher's test.

Results

Effect of acute and chronic oxidative stress in oxidative stress marker enzyme activities

As mentioned before, aconitase is a tricarboxylic acid cycle (TCA) enzyme and its activity has been used as oxidative stress marker since it can be inactivated by $O_2^{\bullet -}$ [17,18]. To confirm that the effect on aconitase activity was selective for aconitase and not due to a global effect on enzymes in the TCA cycle, we also measured the activity of fumarase, another TCA enzyme which is known to be insensitive to $O_2^{\bullet -}$ [19,27,28].

The effect of acute or chronic oxidative stress on aconitase and fumarase activities is shown in Figure 1. Figure 1A shows both enzyme activities after acute oxidative stress induced by incubation of HeLa cells with 1 or 2 mM of H_2O_2 during 3 or 6 h. Both treatments lead to a significant time- and concentration-dependent decrease ($p < 0.001$) on aconitase activity (closed symbols, Figure 1A) when compared to control without H_2O_2 addition. The treatment with 2 mM H_2O_2 after 6 h achieved 87% of inhibition and the treatment with 1 mM H_2O_2 after 3 h achieved 47% of inhibition. Fumarase activity was not affected by H_2O_2 concentration or incubation time (open symbols, Figure 1A), indicating that the decrease in aconitase activity was selective [19,28]. Figure 1B shows also aconitase and fumarase activities but, 3 h after chronic oxidative stress, where H_2O_2 was enzymatic generated by the glucose/glucose oxidase (G/GO) system. As observed for the acute treatment, there was a significant decrease ($p < 0.001$) on aconitase activity for all H_2O_2 concentrations tested (closed symbols, Figure 1B), while there was no modification on fumarase activity (open symbols, Figure 1B). We also observed that a lower H_2O_2 concentration (50 μM/h) produced constantly in the medium, during 3 h, in the chronic treatment (Figure 1B), was able to decrease aconitase activity to the same level (45% of inhibition; 0.6 mU/mg protein) as the acute treatment with 1 mM H_2O_2, during the same incubation time (Figure 1A).

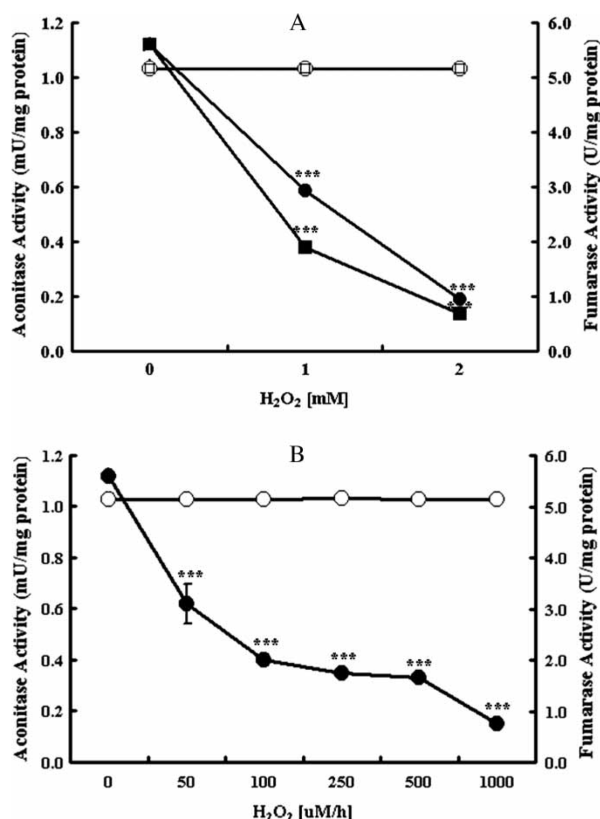

Figure 1. Effect of acute H_2O_2 oxidative stress on aconitase and fumarase activities. (A) HeLa cells were treated with 1 or 2 mM of H_2O_2 during 3 h (circles) or 6 h (squares) or (B) treated with increasing H_2O_2 concentrations, generated by the G/GO system during 3 h (circles). The aconitase (closed symbols) and fumarase (open symbols) activities were quantified in the supernatant from homogenized cells as described in Materials and methods. The results represent the average ± SD ($n = 3$), ***$p < 0.001$, compared to controls without H_2O_2 addition.

Effect of acute and chronic oxidative stress on antioxidant enzymes activity

In order to detect if H_2O_2 oxidative stress was affecting the enzymatic antioxidant activities in HeLa cells, MnSOD, Se:GPx and CAT activities were also determined after acute and chronic H_2O_2 oxidative stress treatments.

Figure 2 shows the effect of acute (Figure 2A) or chronic (Figure 2B) H_2O_2 oxidative stress on MnSOD activity. The acute treatment with 1 or 2 mM H_2O_2, during 3 or 6 h, lead to a significant decrease ($p < 0.001$) on MnSOD activity, at a maximum of 53%. In the chronic treatment (Figure 2B) even a low concentration (100 μM/h) of H_2O_2 in the medium resulted in a significant decrease ($p < 0.001$) in this antioxidant enzyme activity (25%). Compared to the acute treatment, the proportional inhibition of MnSOD in the chronic treatment was achieved with 500 μM/h H_2O_2 ($p < 0.001$) (48%) while 1 mM H_2O_2 resulted in 66% of MnSOD inhibition.

Selenium-dependent GPx (Se:GPx) is an isoenzyme of glutathione peroxidase, an antioxidant enzyme that uses H_2O_2 as substrate [24]. Figure 3 shows Se:GPx activity after acute (Figure 3A) or chronic (Figure 3B) oxidative stress. As shown for aconitase (Figure 1A) and MnSOD (Figure 2A) activities, the acute treatment with 1 or 2 mM of H_2O_2 during 3 or 6 h also resulted in a significant decrease ($p < 0.001$) in this enzyme activity; 1 mM

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

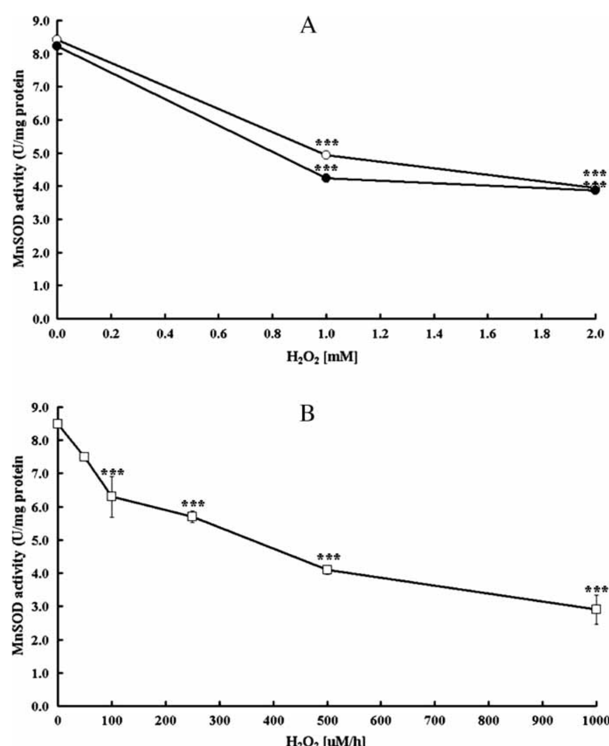

Figure 2. Effect of acute and chronic H_2O_2 oxidative stress on MnSOD activity. (A) HeLa cells were treated with 1 or 2 mM of H_2O_2 during 3 h (open circles) or 6 h (closed circles) or (B) treated with increasing H_2O_2 concentrations, generated by G/GO system during 3 h (closed circles). The MnSOD activity was quantified in the supernatant from homogenized cells as described in Materials and methods. The results represent the average ± SD ($n = 3$), *** $p < 0.001$, compared to controls without H_2O_2 addition.

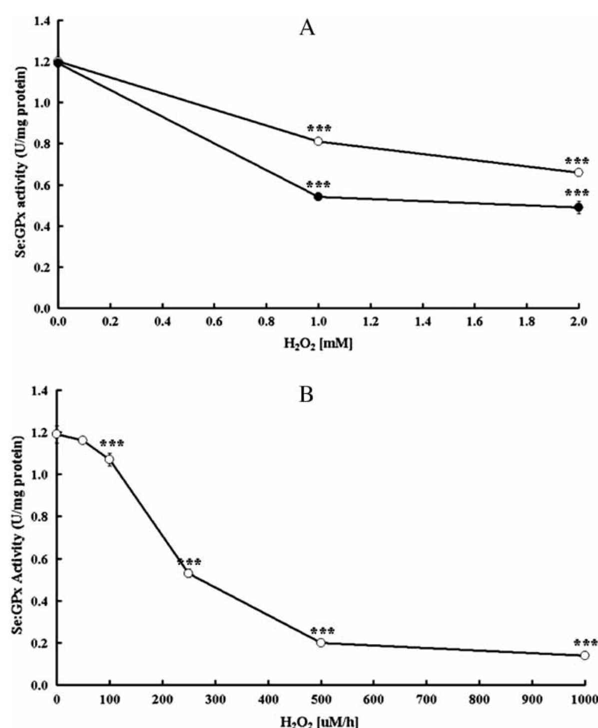

Figure 3. Effect of acute and chronic H_2O_2 oxidative stress on Se:GPx activity. (A) HeLa cells were treated or not (control) with 1 or 2 mM of H_2O_2 during 3 h (open circles) or 6 h (closed circles) or (B) treated with increasing H_2O_2 concentrations, generated by G/GO system during 3 h (open circles). The Se:GPx activity was quantified in the supernatant from homogenized cells as described in Materials and methods. The results represent the average ± SD ($n = 3$), *** $p < 0.001$, compared to controls without H_2O_2 addition.

H_2O_2, after 3 h of incubation resulted in 32.5% of inhibition while the same H_2O_2 concentration after 6 h resulted in 45% of inhibition; 2 mM H_2O_2 after 3 h of incubation decreased MnSOD activity in 55% while the same H_2O_2 concentration after 6 h decreased this activity in 59%. Chronic incubation resulted in a significant decrease on Se:GPx activity from 100 μM/h H_2O_2 (10%) ($p < 0.01$) to higher rates of H_2O_2 production ($p < 0.001$), as shown for other enzyme activities. In this condition the inhibition was significantly higher than in acute treatment and the maximum was achieved with both 500 μM (83%) and with 1 mM H_2O_2 (88%).

Finally, catalase (CAT) activity, an essential cytosolic antioxidant enzyme [29], was also determined after acute (Figure 4A) or chronic H_2O_2 treatment (Figure 4B). Different from other enzyme activities shown here, the acute treatment with 1 mM H_2O_2 lead to a significant decrease in CAT activity only after 6 h of incubation ($p < 0.05$) (33% of inhibition) (Figure 4A). Incubation with 2 mM H_2O_2 during 3 h (19%; $p < 0.05$) or 6 h (39%; $p < 0.001$) resulted in a significant decrease in CAT activity (Figure 4A). In the chronic treatment, we detected a significant decrease ($p < 0.001$) in CAT activity, with H_2O_2 production rates higher than 100 μM/h (Figure 4B).

Time-dependent H_2O_2 chronic oxidative stress

Since chronic oxidative stress requires lower H_2O_2 concentration to inactivate the enzymes assayed here, we also decided to investigate antioxidant enzymes activity after a longer incubation time using a much lower H_2O_2 concentration. Figure 5 shows the 24 h-time plot for aconitase and fumarase activity after incubation with 5 μM and 10 μM/h H_2O_2. Even H_2O_2 production rates as low as 10 μM/h reached 54% of aconitase activity inhibition at longer incubation times (24 h) (closed triangles, Figure 5). At this low H_2O_2 production rates, a significant inhibition in aconitase activity ($p < 0.001$) was observed at all times tested. Even after 3 h of incubation with 5 μM H_2O_2 (closed squares, Figure 5) there was a significant inhibition on aconitase activity ($p < 0.05$). Similarly to what was observed before (Figure 1), no fumarase activity inhibition was detected (open symbols, Figure 5).

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

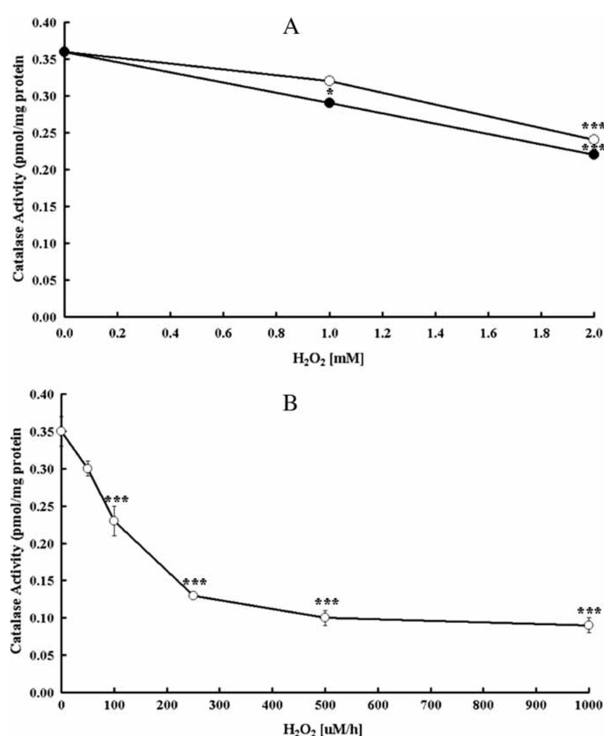

Figure 4. Effect of acute and chronic H_2O_2 oxidative stress on catalase activity. (A) HeLa cells were treated or not (control) with 1 or 2 mM of H_2O_2 during 3 h (open circles) or 6 h (closed circles) or (B) treated with increasing H_2O_2 concentrations, generated by G/GO system during 3 h (open circles). The catalase (CAT) activity was quantified in the supernatant from homogenized cells as described in Materials and methods. The results represent the average ± SD ($n = 3$), $^{***}p < 0.001$, compared to controls without H_2O_2 addition.

Antioxidant enzyme activities were also quantified in these same incubation conditions. MnSOD activity (Figure 6A), Se:GPx (Figure 6B), and CAT (Figure 6C) were also inhibited by these low H_2O_2 concentrations at longer incubation times. MnSOD and Se:GPx

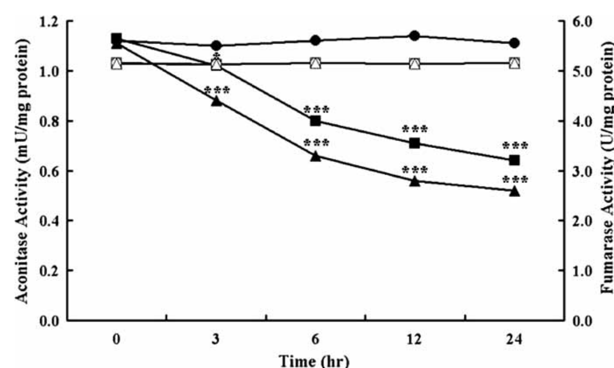

Figure 5. Effect of chronic low level H_2O_2-induced oxidative stress on aconitase and fumarase activities. HeLa cells were treated or not (circles) with 5 μM (squares) or 10 μM (triangles) of H_2O_2 generated by G/GO system during the times indicated in the figure. The aconitase (closed symbols) and fumarase (open symbols) activities were quantified in the supernatant from homogenized cells as described in Materials and methods. The results represent the average ± SD ($n = 3$), $^{***}p < 0.001$, compared to controls without H_2O_2 addition.

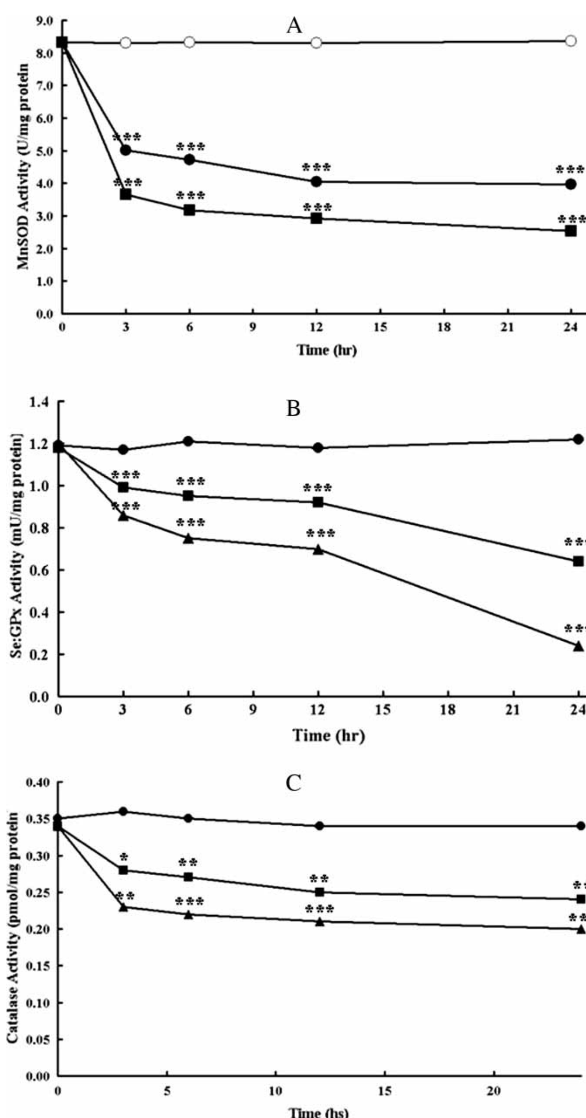

Figure 6. Effect of chronic low level H_2O_2-induced oxidative stress on MnSOD, Se:GPx and aconitase activities. HeLa cells were treated or not (circles) with 5 μM (squares) or 10 μM (triangles) of H_2O_2 generated by G/GO system during the times indicated in the figure. The aconitase (closed symbols) and fumarase (open symbols) activities were quantified in the supernatant from homogenized cells as described in Materials and methods. The results represent the average ± SD ($n = 3$), $^{***}p < 0.001$, compared to controls without H_2O_2 addition.

activities were significantly inhibited even with 5 μM H_2O_2 during 3 h (40% and 17%, respectively), a similar inhibition level as shown before using shorter incubation times and higher H_2O_2 concentration (Figures 2 and 3). CAT activity seems to be a little more resistant to H_2O_2 time-dependent effect (Figure 6C), as detected before in acute treatment (Figure 4A). With 5 μM H_2O_2 there was also a significant decrease in this antioxidant enzyme activity, but the p-value was lower ($p < 0.05$ for 3 h and $p < 0.01$ for other incubation times); 10 μM H_2O_2 inhibited CAT activity more efficiently ($p < 0.01$ for 3 h and $p < 0.001$ for other incubation times). All these results

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

suggest that exposure to low H_2O_2 doses for longer times became as toxic as higher doses, leading to antioxidant enzymes inhibition and making the cells more prone to oxidative damage.

Protective effect of antioxidants on acute and chronic oxidative stress

In order to confirm the role of ROS in antioxidant enzyme inactivation, we tested the same acute (Figure 1) and chronic (Figure 6) oxidative stress conditions in the presence of N-acetylcysteine (NAC) [11,30] or ascorbic acid (AA) [9,31]. NAC [11,30] as well as AA [4,9,31] have been used to prevent oxidative stress in other experimental models. Table I shows the effect of 10 mM NAC on aconitase activity and antioxidant enzymes after acute or chronic H_2O_2 induced-oxidative stress. Oxidative stress was induced by 1 mM H_2O_2 for 3 h in the acute treatment or 10 μM/h H_2O_2 for 24 h in the chronic treatment. These conditions were chosen because they resulted in ~50% aconitase activity inhibition. When cells were incubated with H_2O_2, either in acute or in chronic treatment, in the presence of NAC (10 mM) or AA (1 mM), aconitase and antioxidant enzymes activities were almost completely recovered, indicating a protective effect of these two antioxidants (Table I). NAC and AA treatments alone did not show any effects on basal enzyme activities (not shown), indicating that reduced activities measured in the presence of H_2O_2 and antioxidants are really due to antioxidant protection against H_2O_2-induced oxidative stress (Table I).

Discussion

As mentioned before, H_2O_2 can freely cross membranes and, in the presence of Fe^{2+} (or Cu^{+}) it is prone to participate in Fenton reaction [1] and induce oxidative stress. As reported here, in our experimental conditions, either acute or chronic treatment with H_2O_2, can affect cell function, since both treatments significantly inhibited all three antioxidant enzyme activities as well as aconitase. Besides, oxidative stress produced by lower H_2O_2 concentrations in the chronic treatment seems to be as toxic as the acute treatment, since we detected similar levels of inhibition for MnSOD, Se:GPx, CAT and aconitase in both treatments. One of the relevant findings in the present report is that the time of exposure, more than the concentration, plays a relevant role in decreasing the antioxidant enzyme and aconitase activities. In fact, even H_2O_2 concentrations as low as 5 μM/h, produced constantly in the medium, in the chronic treatment (Figures 5 and 6), can reach the same level of enzyme activity inhibition, after 24 h, compared to acute treatments using higher H_2O_2 concentration but much shorter incubation times (Figures 1 and 2). Inactivation of antioxidant enzymes by oxidative stress has been shown before, but at much higher ROS concentration and shorter incubation times [32].

Since, in our experimental conditions, H_2O_2 was produced or directly added outside the cells and, since it freely crosses membranes, CAT activity was expected to be as affected by this kind of oxidative stress as other antioxidant enzymes. However, our results showed that CAT activity was less affected than other enzymes. We showed before that CAT expression seems not to be affected in gastric diseases while there is a significant decrease on MnSOD and GPx expression only in gastritis. In that case, CAT expression was preserved almost as well as the constitutive GAPDH expression [33]. In general, mitochondria do not exhibit CAT activity and, it is supposed that GPx activity is more important to prevent mitochondrial H_2O_2-oxidative stress [29]. In our case, MnSOD, GPx and aconitase were most seriously affected by H_2O_2 exposition than CAT activity while fumarase, a TCA cycle enzyme, was not affected by these oxidative stress conditions (Figures 1 and 5). We supposed that these differential inhibitions shown here could be the consequence of one of two reasons or both. Some of these enzymes have structural differences that make their activity different in oxidative stress conditions. In this way,

Table I. Effects of N-acetylcystein and ascorbic acid on aconitase and antioxidant enzyme activities after acute or chronic H_2O_2 oxidative stress.

		Aconitase (mU/mg protein)	MnSOD (U/mg protein)	Se:GPX (U/mg protein)	CAT (pmoles/mg protein)
Acute (3 h)	Control	1.12±0.02	8.30±0.04	1.2±0.03	0.35±0.01
	2 mM H_2O_2	0.19±0.02	3.87±0.03	0.16±0.04	0.25±0.02
	2 mM H_2O_2+1 mM AA	1.35±0.02	8.25±0.02	1.21±0.07	0.34±0.05
	2 mM H_2O_2 +10 mM NAC	1.17±0.01	8.20±0.04	1.19±0.02	0.36±0.04
Chronic (24 h)	Control	1.21±0.01	8.42±0.04	1.19±0.02	0.34±0.02
	10 μM/h H_2O_2	0.52±0.02	3.53±0.03	0.12±0.04	0.10±0.03
	10 μM/h H_2O_2+1 mM AA	1.25±0.01	8.37±0.02	1.21±0.03	0.34±0.08
	10 μM/h H_2O_2+10 mM NAC	1.27±0.02	8.35±0.01	1.20±0.02	0.35±0.02

AA, ascorbic acid; NAC, N-acetylcystein.

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

aconitase, as mentioned in Introduction and Results, has been used as an oxidative stress marker because $O_2^{\bullet -}$ oxidizes the active $[4Fe\text{-}4S]^{2+}$ cluster to $[3Fe\text{-}4S]^{1+}$ cluster causing-inactivation and release of Fe^{2+} which may then reduce H_2O_2 to OH^- and $^{\bullet}OH$ [17,19,34]. On the other hand, human fumarase shares sequence identity to FumC from *E. coli*, which are iron-independent and, thus, insensitive to $O_2^{\bullet -}$ [19,27,28]. The second reason could be the kind of oxidative stress used here, because it has been reported before that CAT is inactivated by hydroxyl radicals while GPx and SOD are considerably less affected by these radicals [32]. CAT and SOD are inactivated with similar efficiencies by ozone and/or the radicals derived from their decomposition [35] and these two antioxidant enzymes also have different inhibition kinetics response to oxidative stress induced by singlet oxygen or peroxyl radicals [36].

We have also shown that ordinary non-enzymatic antioxidants can protect cells from the effect of H_2O_2. The ordinary antioxidants NAC and AA seem to protect cells in both H_2O_2-induced oxidative stress models. NAC is known for its role as a precursor for glutathione (GSH) synthesis and essential for GPx to work properly [6,10]. Besides this, NAC is also an antioxidant *per se*, acting as a ROS scavenger [10]. AA is highly efficient in neutralizing free radicals [4,9,31]. In the study, aconitase and antioxidant enzyme activities were protected from the toxic effects of H_2O_2 when NAC or AA were supplemented to the medium.

It has been reported that acute treatment of mammalian cells with micromolar concentrations of H_2O_2 (100 μM) induce oxidative DNA damage more efficiently than millimolar concentrations [37]. H_2O_2 generated by the glucose oxidase system can also preferentially damage mitochondrial DNA [16]. As mentioned before, oxidative stress induced by H_2O_2 was implicated in apoptosis induction [14] and cellular proliferation [15]. Flavonoids as resveratrol [38] and fisetin can induce apoptosis, but only fisetin was also reported to decrease aconitase and antioxidant enzyme activities [39]. Considering all these facts, we may hypothesize that the different responses to H_2O_2 doses and treatment times can also be caused by its chronic effect on antioxidant enzymes, mainly in mitochondrial enzymes. The present report reinforces this idea since even very low H_2O_2 concentrations can significantly decrease the antioxidant enzymatic protection if the time of exposure is long enough and this could ultimately contribute to apoptosis.

The models shown in this report may also be useful to evaluate antioxidant therapies since, if NAC and AA can prevent H_2O_2-induced oxidative stress by protecting antioxidant enzymes, they may also be able to inhibit apoptosis.

Acknowledgements

We would like to thank to Dr Helotonio Carvalho (Federal University of Sao Paulo, Diadema, Brazil) for the valuable discussions and suggestions. We would also like to thank FAPESP (Sao Paulo State Foundation of Support to Research) for supporting this research.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Halliwell B, Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1984;219:1–14.
[2] Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993;90:7915–7922.
[3] Wick G, Jansen-Durr P, Berger P, Blasko I, Grubeck-Loebenstein B. Diseases of aging. Vaccine 2000;18:1567–1583.
[4] Junqueira VB, Barros SB, Chan SS, Rodrigues L, Giavarotti L, Abud RL, Deucher GP. Aging and oxidative stress. Mol Aspects Med 2004;25:5–16.
[5] Ames BN, Shigenaga MK. Oxidants are a major contributor to aging. Ann NY Acad Sci 1992;663:85–96.
[6] Fridovich I. Oxygen toxicity: a radical explanation. J Exp Biol 1998;201:1203–1209.
[7] Fridovich I. Superoxide anion radical ($O_2^{\bullet -}$), superoxide dismutases, and related matters. J Biol Chem 1997;272: 18515–18517.
[8] Meneghini R. Genotoxicity of active oxygen species in mammalian cells. Mutat Res 1998;195:215–230.
[9] Frei B, Stocker R, England L, Ames BN. Ascorbate: the most effective antioxidant in human blood plasma. Adv Exp Med Biol 1990;264:155–163.
[10] Kelly GS. Clinical applications of N-acetylcysteine. Altern Med Rev 1998;3:114–127.
[11] Rhoden CR, Lawrence J, Godleski JJ, Gonzalez-Flecha B. N-acetylcysteine prevents lung inflammation after short-term inhalation exposure to concentrated ambient particles. Toxicol Sci 2004;79:296–303.
[12] Sies H. Oxidative stress. New York: Academic Press; 1985. p 1–7.
[13] Fiers W. Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. FEBS Lett 1991;285: 199–212.
[14] Carvalho H, Evelson P, Sigaud S, Gonzalez-Flecha B. Mitogen-activated protein kinases modulate H(2)O(2)-induced apoptosis in primary rat alveolar epithelial cells. J Cell Biochem 2004;92:502–513.
[15] Sigaud S, Evelson P, Gonzalez-Flecha B. H2O2-induced proliferation of primary alveolar epithelial cells is mediated by MAP kinases. Antioxid Redox Signal 2005;7:6–13.
[16] Salazar JJ, Van Houten B. Preferential mitochondrial DNA injury caused by glucose oxidase as a steady generator of hydrogen peroxide in human fibroblasts. Mutat Res 1997;385:139–149.
[17] Gardner PR, Nguyen DD, White CW. Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. Proc Natl Acad Sci USA 1994;91:12248–12252.
[18] Morton RL, Ikle D, White CW. Loss of lung mitochondrial aconitase activity due to hyperoxia in bronchopulmonary dysplasia in primates. Am J Physiol 1998;274:L127–L133.

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

[19] Evelson P, Gonzalez-Flecha B. Time course and quantitative analysis of the adaptive responses to 85% oxygen in the rat lung and heart. Biochim Biophys Acta 2000;1523:209–216.

[20] Estabrook RW. Mitochondrial respiratory control and polarographic measurements of ADP:O ratios. Methods Enzymol 1967;10:40–47.

[21] McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049–6055.

[22] Beauchamp CO, Fridovich I. Isozymes of superoxide dismutase from wheat germ. Biochim Biophys Acta 1973;317:50–64.

[23] Nelson DP, Kiesow LA. Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C (with molar extinction coefficients of H_2O_2 solutions in the UV). Anal Biochem 1972;49:474–478.

[24] Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun 1976;71:952–958.

[25] Racker E. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim Biophys Acta 1950;4:211–214.

[26] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–275.

[27] Gurgueira SA, Lawrence J, Coull B, Murthy GG, Gonzalez-Flecha B. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. Environ Health Perspect 2002;110:749–755.

[28] Woods SA, Miles JS, Roberts RE, Guest JR. Structural and functional relationships between fumarase and aspartase. Nucleotide sequences of the fumarase (fumC) and aspartase (aspA) genes of Escherichia coli K12. Biochem J 1986;237:547–557.

[29] Li S, Yan T, Yang JQ, Oberley TD, Oberley LW. The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. Cancer Res 2000;60:3927–3939.

[30] Jimenez LA, Zanella C, Fung H, Janssen YM, Vacek P, Charland C, Goldberg J, Mossman BT. Role of extracellular signal-regulated protein kinases in apoptosis by asbestos and H2O2. Am J Physiol 1997;273:L1029–L1035.

[31] Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. Arch Biochem Biophys 1990;280:1–8.

[32] Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, Zachary MD, Remacle J. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. Mech Ageing Dev 1990;51:283–297.

[33] Augusto AC, Miguel F, Mendonca S, Pedrazzoli J Jr, Gurgueira SA. Oxidative stress expression status associated to Helicobacter pylori virulence in gastric diseases. Clin Biochem 2007;40:615–622.

[34] Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem 1995;64:97–112.

[35] Whiteside C, Hassan HM. Induction and inactivation of catalase and superoxide dismutase of Escherichia coli by ozone. Arch Biochem Biophys 1987;257:464–471.

[36] Escobar JA, Rubio MA, Lissi EA. Sod and catalase inactivation by singlet oxygen and peroxyl radicals. Free Radic Biol Med 1996;20:285–290.

[37] Nakamura J, Purvis ER, Swenberg JA. Micromolar concentrations of hydrogen peroxide induce oxidative DNA lesions more efficiently than millimolar concentrations in mammalian cells. Nucleic Acids Res 2003;31:1790–1795.

[38] Yoon SH, Kim YS, Ghim SY, Song BH, Bae YS. Inhibition of protein kinase CKII activity by resveratrol, a natural compound in red wine and grapes. Life Sci 2002;71:2145–2152.

[39] de Sousa RR, Queiroz KC, Souza AC, Gurgueira SA, Augusto AC, Miranda MA, Peppelenbosch MP, Ferreira CV, Aoyama H. Phosphoprotein levels, MAPK activities and NFkappaB expression are affected by fisetin. J Enzyme Inhib Med Chem 2007;22:439–444.

This paper was first published online on iFirst on 11 February 2009.

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK