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Effect of Auranofin on the mitochondrial generation of hydrogen peroxide. Role of thioredoxin reductase

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Abstract

The mitochondrial production of hydrogen peroxide, in the presence of different respiratory substrates (succinate, glutamate, malate and isocitrate), is stimulated by submicromolar concentrations of auranofin, a highly specific inhibitor of thioredoxin reductase. This effect is particularly evident in the presence of antimycin. Auranofin was also able to unmask the production of hydrogen peroxide occurring in the presence of rotenone. However, at variance with whole mitochondria, auranofin does not stimulate hydrogen peroxide production in submitochondrial particles indicating that it does not alter the formation of hydrogen peroxide by the respiratory chain but prevents its removal. As the mitochondrial metabolism of hydrogen peroxide proceeds through the peroxidases linked to glutathione or thioredoxin, the relative efficiency of the two systems and the effects of auranofin were tested. In conclusion, the inhibition of thioredoxin reductase determines an increase of the basal flow of hydrogen peroxide leading to a more oxidized condition that alters the mitochondrial functions.

Keywords: *Auranofin, hydrogen peroxide, mitochondria, selenium, thioredoxin reductase*

Abbreviations: *Auranofin, S-triethylphosphinegold(I)-2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside; NADPH, nicotinamide adenine dinucleotide 3-phosphate; GSH, reduced glutathione; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; H_2O_2, hydrogen peroxide; Trx, Thioredoxin*

Introduction

Mitochondria are considered the major cellular producers of H_2O_2 both in normal and pathological conditions [1–3]. Formation of H_2O_2 occurs both at the inner and outer membranes [2]. At the inner mitochondrial membrane, the autoxidation of specific sites at the level of complex I and III brings to the formation of superoxide anion that, either directly or through manganese superoxide dismutase, is converted to H_2O_2 [3]. In the outer membrane, monoamine oxidase forms H_2O_2 independently of the respiration and in a two-electron process [4].

Since its discovery, the formation of hydrogen peroxide has been deemed as unavoidable consequence of the mitochondrial electron transport and the systems devoted to its removal were considered to simply prevent the potentially toxic consequences of the oxidizing species formed. However, more recently, reactive oxygen species and particularly H_2O_2 are also considered as metabolic regulators, suggesting a potential physiologic role played by the production of H_2O_2 that,

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in response to cytokines and growth factors stimulation, can act as redox signal [5,6]. Consequently, oxygen radicals can be critical in the decision by mammalian cells to undergo proliferation, apoptosis, necrosis or senescence [7,8]. The regulation of the cellular functions by H_2O_2 or, in general, by oxidizing species, can be exerted by different mechanisms such as the reversible oxidation or glutathionylation of specific thiol groups [7–9] or complex formation–dissociation between redox sensible proteins such as thioredoxin and glutaredoxin and specific proteins [10,11].

Hydrogen peroxide produced by mitochondria can either regulate events occurring within mitochondria themselves or can be released outside, therefore influencing cytosolic processes or neighbouring mitochondria. On the whole, the reactive oxygen species alter the mitochondrial thiol redox homeostasis that, in turn, is involved in the modulation of several mitochondrial functions including permeability condition and cell apoptosis.

In mitochondria, two systems able to reduce H_2O_2 and organic hydroperoxides, are present. The first depends on glutathione and the enzymes glutathione reductase and glutathione peroxidase [12]. To the glutathione system is also associated the dithiol-containing protein glutaredoxin able to catalyze the reduction of S-glutathionylated substrates or to act as a disulfide reductase [13]. The second peroxide removing system depends on thioredoxin [14] and the enzymes thioredoxin reductase [15,16] and peroxiredoxin [17,18]. Mitochondrial thioredoxin reductase, similarly to its cytosolic counterpart, is a selenium enzyme containing a couple cysteine-selenocysteine at the C-terminal moiety [16,19]. We have previously observed that the inhibition of mitochondrial thioredoxin reductase by nanomolar concentrations of auranofin or other gold(I) compounds determines an extensive mitochondrial swelling, a decrease of membrane potential and the release of cytochrome c [20,21]. Auranofin (S-triethylphosphinegold(I)-2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside) belongs to the gold(I)-based drug class utilized in the treatment of rheumatoid arthritis [22] and experimentally tested as anticancer compound [23]. In the antitumor action of gold(I) complexes an important role is played by mitochondria [24]. A correlation was therefore established between the action on the thioredoxin system and the alterations of the mitochondrial membranes.

Since H_2O_2 is the terminal substrate of the thioredoxin system we have explored the correlation between the inhibition of thioredoxin reductase and the production of mitochondrial H_2O_2. In the present paper, we report that mitochondria, in the presence of auranofin, are able to elicit a large formation of hydrogen peroxide that depends essentially on a decrease of its removal. Therefore, it is apparent that a balance between production and removal of H_2O_2 occurs and has important consequences for the functioning of mitochondria and the whole cell.

Materials and methods

Preparation of mitochondria, submitochondrial particles and mitochondrial supernatant fraction

Rat liver mitochondria were prepared by differential centrifugation according to Myers and Slater [25] using a medium containing 220 mM mannitol, 70 mM sucrose, 1 mM EDTA and 5 mM Hepes at pH 7.0. EDTA was omitted in the washing and in the final suspension of mitochondria. The functionality of mitochondrial preparations was established by the estimation of the respiratory control ratio by following oxygen uptake measured polarographically utilizing a Clark-type oxygen electrode [26] inserted in a water-jacketed chamber (25°C) with constant stirring. Mitochondria with respiratory control ratio of 4 or greater were utilized. Submitochondrial particles were prepared from the mitochondrial suspension (60 mg protein ml^{-1}) diluted (1:5) with 50 mM Tris-HCl (pH 8.0) by sonicating twice for 30 s each. The obtained suspension was first centrifuged at 10,000*g* for 10 min to get rid of the unbroken mitochondria. The pellet was discarded and the supernatant centrifuged at 105,000*g* for 30 min. The obtained pellet was washed and suspended in a small volume of the same medium used for mitochondria. Mitochondrial supernatant fraction (including matrix and the intermembrane space content) was prepared from mitochondrial suspensions by freezing and thawing followed by sonication and centrifugation at 105,000*g* for 60 min. Pellet was discarded and the supernatant extensively dialyzed and concentrated in a pressure dialysis system. Proteins were estimated with the biuret procedure using bovine serum albumin as standard [27].

Estimation of hydrogen peroxide and superoxide production

Hydrogen peroxide formed by mitochondria and submitochondrial particles was determined by following the loss of fluorescence of scopoletin (7-hydroxy-6-methoxy coumarin) in the presence of horseradish peroxidase [1]. Fluorescence was estimated at 30°C in a microplate reader (Fluoroskan Ascent FL, Labsystems) at 366 nm (excitation wavelength) and 460 nm (emission wavelength). Hydrogen peroxide formed was quantitated by a standard curve obtained by adding known amounts of hydrogen peroxide to the medium in the presence of the detection system (scopoletin and horseradish peroxidase). Superoxide radical anion formed by submitochondrial particles was determined by following the conversion of adrenaline to adrenochrome. The absorbance of adrenochrome was monitored at 30°C in a microplate reader (Multiskan EX, Labsystems) at 492–540 nm.

Estimation of glutathione- and thioredoxin-dependent peroxidase activities in the mitochondrial supernatant fraction

The activities of the thioredoxin and glutathione systems were estimated in the mitochondrial fractions by following spectrophotometrically the decrease of absorbance of NADPH at 340 nm in the presence of H_2O_2. Incubation conditions are indicated in the relevant figure.

Purification and estimation of thioredoxin reductase activity

Cytosolic thioredoxin reductase was prepared from rat liver according to Luthman and Holmgren [28] and from rat liver mitochondria according to Rigobello et al. [15]. Thioredoxin was prepared from mitochondria according to Luthman and Holmgren [28]. Thioredoxin reductase activity was estimated with the NADPH-dependent reduction of DTNB (5,5′-dithiobis (2-nitrobenzoic acid) [28].

Statistical analysis

All the values are the means ± S.D. of not less than five measurements. Multiple comparisons were made by one-way analysis of variance followed by the Tukey-Kramer multiple comparison test.

Results

The production of hydrogen peroxide by rat liver mitochondria is reported in Figure 1. The experiments were performed using succinate as substrate and in state 4 conditions according to Chance and Williams [29]. A small production of H_2O_2 is apparent in the presence of succinate alone (0.29 nmol min^{-1} mg^{-1} protein) and is slightly stimulated by the further addition of auranofin (0.38 nmol min^{-1} mg^{-1} protein). As expected, the addition of antimycin markedly increases the production of H_2O_2 with respect to succinate alone (0.52 nmol min^{-1} mg^{-1} protein). However, the presence of auranofin together with antimycin determines a dramatic increase in the production of H_2O_2 (1.08 nmol min^{-1} mg^{-1} protein) that, after 15 min of incubation, is more than twice as that obtained with antimycin and succinate and about 4 times with respect to the production observed with succinate alone. This large production of H_2O_2 is completely removed by the addition of catalase, therefore confirming the specificity of H_2O_2 estimation.

In Figure 1(B) the dose-response relationship between auranofin action and H_2O_2 formation is reported. Without auranofin, the values of basal and antimycin-stimulated production of H_2O_2 are apparent on the ordinate axis and, after 15 min, are about 1.8 and 6.0 nmol mg^{-1} protein, respectively. Increasing concentrations of auranofin in the nanomolar range stimulate H_2O_2 production that reaches its half-maximal value at concentrations of about 100 nM while at concentrations higher than 200 nM a very slow increase with the time occurs. The rate and extent of H_2O_2 production is only slightly reduced by EGTA or cyclosporin. Both agents act as inhibitors of the mitochondrial membranes permeability transition and, therefore, the lack of effect indicates that the measured production of hydrogen peroxide is not due to membrane swelling.

The strong stimulation of H_2O_2 production in the presence of auranofin is also observed with endogenous substrates (Figure 2) or other substrates added either alone (not shown) or in combination such as succinate/isocitrate and glutamate/malate (Figure 2). The auranofin-induced hydrogen peroxide formation is lower in the presence of rotenone than with antimycin. In fact, in contrast to antimycin, rotenone alone does not increase the production of hydrogen peroxide with respect to the control (Figure 2). Moreover, the effect of auranofin in the presence of rotenone is particularly interesting since it is able to unmask the production of H_2O_2 that, otherwise, appears very low and close to the value of the control.

The production of H_2O_2 was examined in submitochondrial particles treated in different conditions and in the presence of succinate as substrate (Figure 3). A large formation of H_2O_2 is observable when antimycin is present. However, at variance with the result obtained with whole mitochondria, the addition of auranofin does not change the production of H_2O_2 by submitochondrial particles, indicating that this compound is ineffective in directly altering the formation of reactive oxygen species by the respiratory chain. Submitochondrial particle preparations are devoid of the hydrogen peroxide removing systems and, therefore the net production of hydrogen peroxide by the respiratory chain can be observed. In fact, most of the activity of the H_2O_2 removing systems is apparently localized at the level of the soluble fraction of mitochondria comprising the matrix and the intermembrane space. The presence of superoxide dismutase does not increase the production of hydrogen peroxide by submitochondrial particles (Figure 3) indicating that all the measured hydrogen peroxide derives from the spontaneous dismutation of the superoxide anion. To further validate this point the direct production of the superoxide anion was also followed (inset to Figure 3). It can be observed that hydrogen peroxide and superoxide production are strictly correlated. The formation of the latter is almost completely inhibited by superoxide dismutase indicating the specificity of the measurement.

The activities of the two major and well-recognized systems, respectively linked to glutathione and thioredoxin, were comparatively estimated by following the rate of absorbance decrease of NADPH in the presence of hydrogen peroxide as donor of oxidizing equivalents (Figure 4). The dialyzed soluble fraction, devoid of glutathione, gives rise to a slight rate of

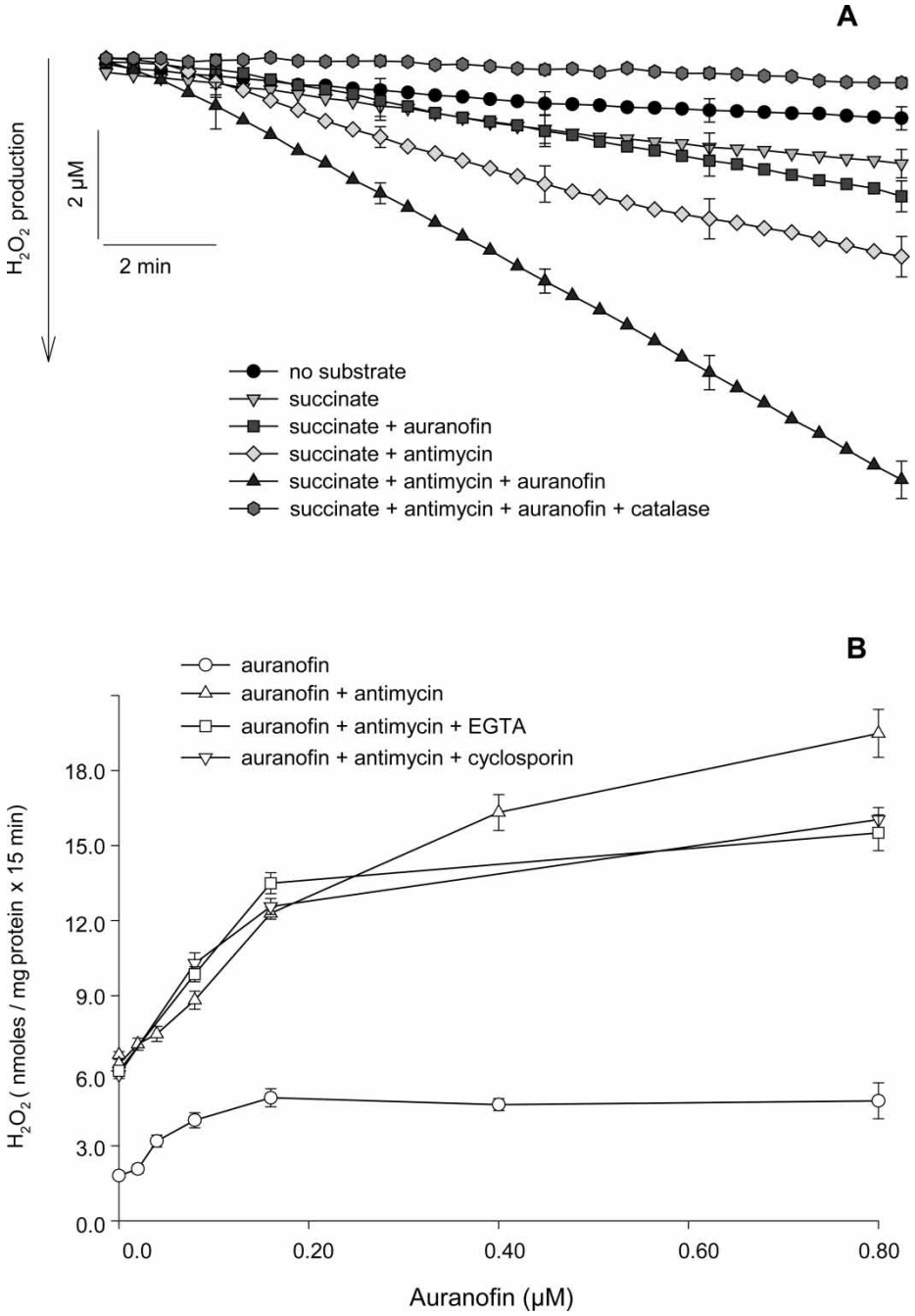

Figure 1. Effect of auranofin on hydrogen peroxide production by rat liver mitochondria. In (A) rat liver mitochondria (0.5 mg protein ml^{-1}) were incubated at 30°C in 100 mM sucrose, 50 mM KCl, 0.5 mM K-phosphate, 20 mM Hepes/Tris (pH 7.4) and, when present, 5 mM succinate, 1 μM antimycin, 0.8 μM auranofin, 1 μM catalase. The hydrogen peroxide production was followed as decrease of fluorescence of scopoletin as indicated under "Materials and methods" section. Reactions were initiated by the addition of horseradish peroxidase. In (B) the effects of increasing concentrations of auranofin on the production of hydrogen peroxide in the presence of 5 mM succinate are reported. Experimental conditions are as in (A). When indicated, 1 μM antimycin, 1 μM cyclosporin and 1 mM EGTA were also present.

NADPH oxidation that probably corresponds to the activity of the endogenous thioredoxin system. However, thioredoxin may be limiting and, in fact, the addition of exogenous thioredoxin increases the rate of NADPH oxidation (Figure 4, trace a) and gives an indication of the contribution of the thioredoxin system to the removal of peroxides. The addition of glutathione, instead of thioredoxin, to the mitochondrial soluble fraction, markedly stimulates the oxidation of NADPH (Figure 4, trace c) showing the activation of the glutathione system that cooperates with the thioredoxin system to the removal of hydrogen peroxide. The presence of auranofin almost completely prevents the stimulation elicited by thioredoxin (Figure 4, trace b), but it is ineffective in inhibiting the GSH-dependent stimulation of NADPH oxidation (Figure 4, trace d). Both the mitochondrial and cytosolic purified isoforms of

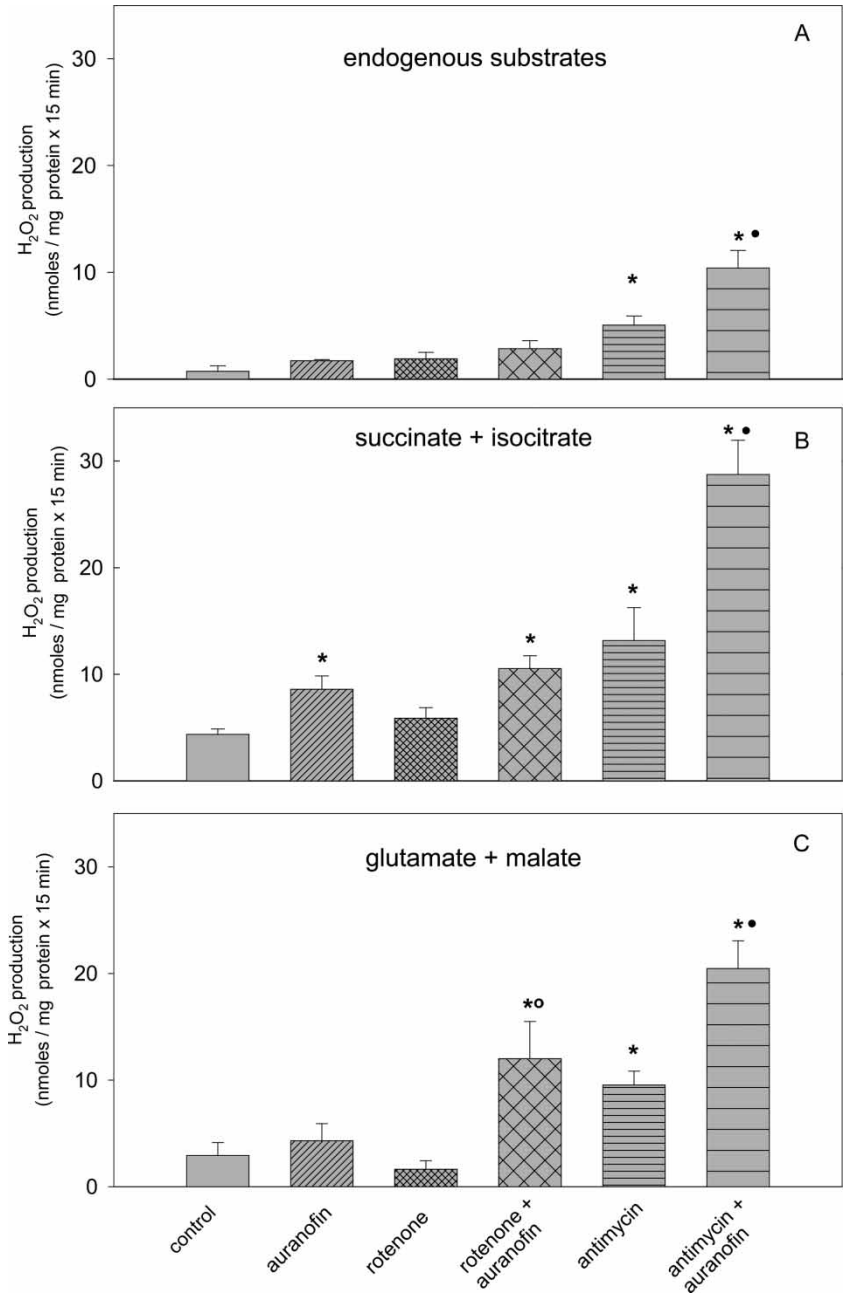

Figure 2. Effect of different respiratory substrates on auranofin-stimulated production of hydrogen peroxide. Rat liver mitochondria (0.5 mg protein ml^{-1}) were incubated at 30°C in 100 mM sucrose, 50 mM KCl, 0.5 mM K-phosphate, 20 mM Hepes/Tris (pH 7.4) in the absence of added substrates (panel A) or in the presence of 5 mM succinate/5 mM isocitrate (panel B) or 5 mM glutamate/5 mM malate (panel C). Auranofin was 0.8 μM in panel A and C and 0.1 μM in panel B. Other additions were: 1 μM rotenone and 1 μM antimycin. Hydrogen peroxide was detected as indicated under "Materials and methods" section and reactions were initiated by the addition of horseradish peroxidase. $^{\star}p < 0.001$ (vs. control), $^{\bullet}p < 0.001$ (vs. antimycin), $^{\circ}p < 0.001$(vs. rotenone).

thioredoxin reductase are inhibited by auranofin at about the same extent (inset to Figure 4).

Discussion

The reported results indicate that auranofin strongly stimulates the mitochondrial hydrogen peroxide production in the presence of antimycin and, to a lower extent, of rotenone. A slight stimulation of hydrogen peroxide production is also apparent in the absence of inhibitors and is particularly significant when succinate + isocitrate are present as substrates (Figure 2). On the contrary, in submitochondrial particles, auranofin is no longer able to stimulate the production of hydrogen peroxide (Figure 3). This result clearly indicates that auranofin does not stimulate nor inhibit the formation of hydrogen peroxide by the respiratory chain but the large stimulation observed with whole mitochondria can be referred to the inhibition of the hydrogen peroxide

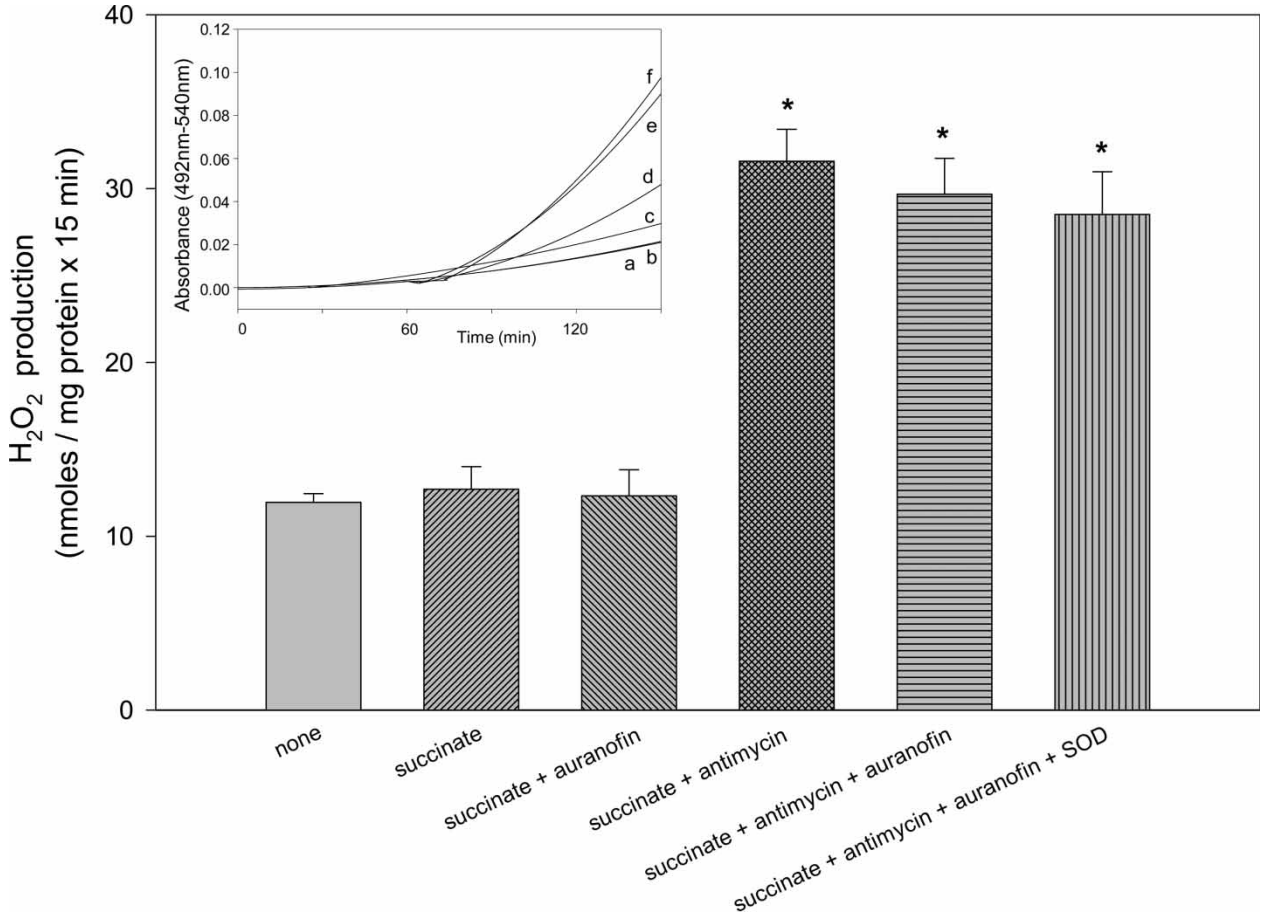

Figure 3. Effect of auranofin on hydrogen peroxide and superoxide production by rat liver submitochondrial particles. Rat liver submitochondrial particles (0.5 mg protein ml^{-1}) were incubated at 30°C in 100 mM sucrose, 50 mM KCl, 0.5 mM K-phosphate, 20 mM Hepes/Tris (pH 7.4) and, when indicated, 5 mM succinate, 1 μM antimycin, 1 μM auranofin and 24 μg/ml superoxide dismutase (SOD). Hydrogen peroxide was detected as indicated under "Materials and methods" section and reactions were initiated by the addition of horseradish peroxidase. $*p < 0.001$ (vs. control). The inset reports the estimation of superoxide production in the presence of 1 mM adrenaline and in the same experimental conditions utilized for hydrogen peroxide measurements. None (a); succinate + auranofin (b); succinate (d); succinate + antimycin (f); succinate + antimycin + auranofin (e); succinate + antimycin + auranofin + SOD (c).

removing systems and, more specifically, to the inhibition of the thioredoxin system. The effect of auranofin is particularly interesting when the respiratory chain is inhibited by rotenone since only in the presence of the latter a net production of H_2O_2 can be observed while, with rotenone alone, there is not apparent stimulation of H_2O_2 formation. According to Chen et al. [30], in the presence of rotenone, H_2O_2 is produced at complex I and is directed towards the matrix of mitochondria where it is completely removed by the various scavenging systems. However, the inhibition of the thioredoxin system by auranofin allows part of H_2O_2 to escape from the mitochondrion and to be detected outside by the scopoletin/horseradish peroxidase system. With antimycin, hydrogen peroxide is produced both at complex I and III and does not appear to be completely removed by the antioxidant systems as it can be estimated in the outside medium. In this case, the extent of stimulation of hydrogen peroxide production by auranofin is very large.

The alterations of the mitochondrial selenium-dependent enzymes elicit an increase of H_2O_2 production. In fact, mitochondria obtained from selenium-deficient rats and incubated with succinate exhibit an increase in H_2O_2 production [31] indicating that the mitochondrial selenoenzymes are important for H_2O_2 removal. Although glutathione peroxidase is considered to have a relevant role in this function, also the mitochondrial isoform of thioredoxin reductase should be taken into account (Figure 4). Furthermore, liver mitochondria isolated from mice deficient in mitochondrial glutathione peroxidase release H_2O_2 at about four times the rate exhibited by mitochondria of control mice [32] again indicating that glutathione peroxidase plays a major role in controlling the level of H_2O_2 in liver mitochondria where this enzyme essentially exerts a detoxifying action. However, according to the same authors [32] liver mitochondria obtained from mice lacking glutathione peroxidase, although forming higher levels of H_2O_2, do not show a tendency greater than that of the control to undergo

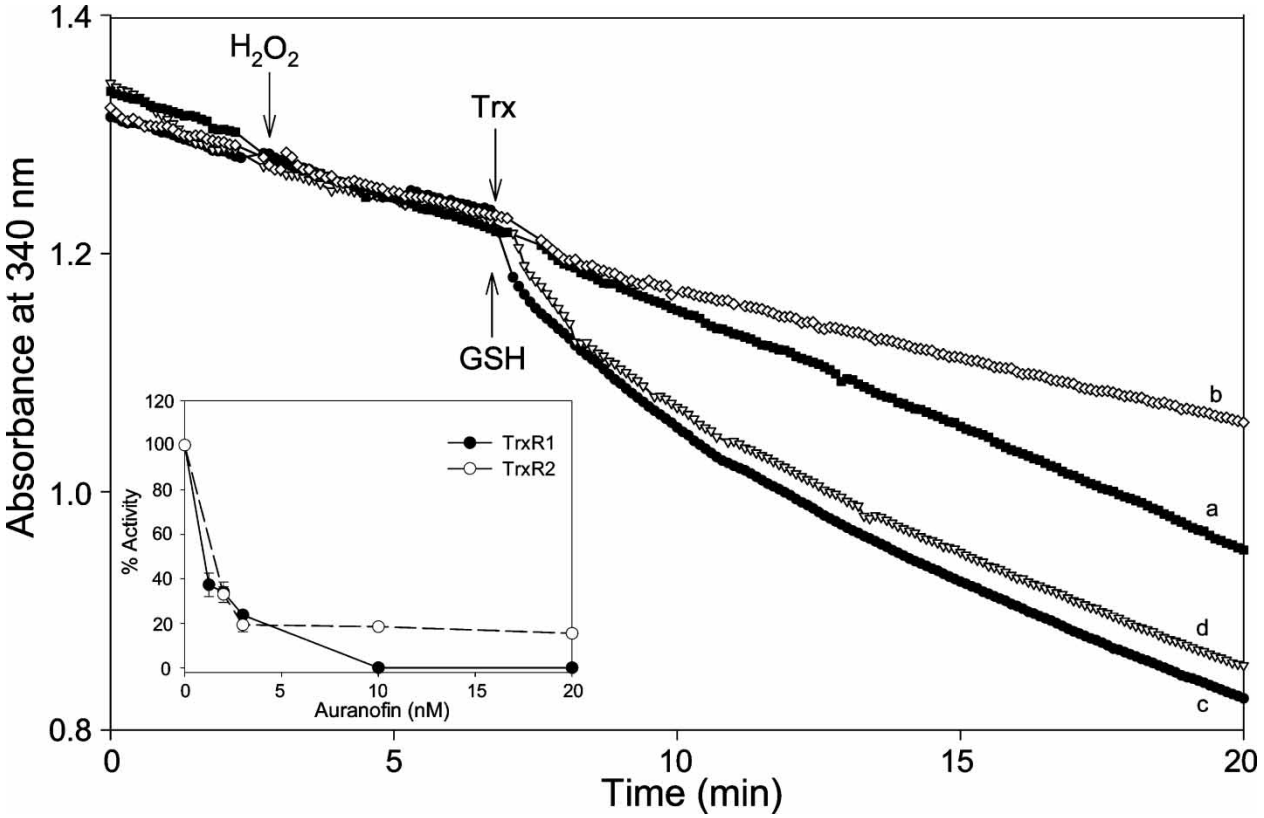

Figure 4. Removal of hydrogen peroxide by the mitochondrial glutathione and thioredoxin systems and effect of auranofin. The dialyzed soluble mitochondrial fraction (0.5 mg protein ml^{-1}) was incubated at 37°C in 50 mM Hepes buffer (pH 7.0) in the presence of 0.25 mM NADPH and 0.5 mM H_2O_2. The activity of the thioredoxin system was sparked by the addition of 40 μM thioredoxin (a and b), while the activity of the glutathione system was initiated by the addition of 8 mM GSH (c and d). In (b) and (d) 1 μM auranofin was also present. The specific activities of the thioredoxin and glutathione systems were 1.54 ± 0.3 nmol min^{-1} mg^{-1} protein and 5.17 ± 0.6 nmol min^{-1} mg^{-1} protein, respectively. The inset reports the effect of increasing concentrations of auranofin on the purified cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductase. The specific activities of cytosolic and mitochondrial thioredoxin reductases were 2.01 ± 0.2 μmol min^{-1} mg^{-1} protein and 1.73 μmol min^{-1} mg^{-1} protein, respectively.

permeability transition indicating that the elevated H_2O_2 levels are not strictly necessary to activate "pore" opening. In addition, the inhibition of glutathione reductase by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), which leads to the oxidation of glutathione in the presence of *tert*-butylhydroperoxide, is not associated with an increased efflux of accumulated calcium [33]. Therefore, when considering the redox control of the permeability properties of mitochondrial membranes, the level of hydrogen peroxide is apparently less important than the type, redox state and regeneration kinetics of the specific sensor targeted by hydrogen peroxide. Auranofin does not alter the glutathione system as none of its components is inhibited (Figure 4 and [20]) and, therefore, the observed effect should be essentially attributed to the inhibition of the thioredoxin system.

As reported in the "Introduction" section, there is increasing evidence indicating that H_2O_2 is a component of membrane receptor signalling [18]. In mammalian cell, several extracellular stimuli, such as interleukin-1, tumor necrosis factor-alpha [34], platelet-derived growth factor [35], and epidermal growth factor [36], induce an intracellular transient increase of hydrogen peroxide that acts as a second messenger. Intracellular reactive oxygen species formed after tumor necrosis factor stimulation are mostly removed by the mitochondrial thiol dependent redox systems causing an imbalance in the thiol status [37]. According to Gitler et al. [38] in the presence of calcium ions an apparent inhibition of thioredoxin reductase occurs. This condition, coupled to the burst of H_2O_2 formation that rapidly reacts with the active site of peroxidases, brings to the formation of peroxiredoxin disulfides. Consequently, a transient redox change in cellular thiol proteins involved in signal transduction occurs. In mitochondria, a similar condition is created by auranofin that strongly prevents the activity of thioredoxin reductase. In fact, in mitochondria, hydrogen peroxide is continuously formed and, in condition where thioredoxin reductase is inhibited, mitochondrial peroxiredoxin oxidizes thioredoxin that cannot be restored again to its reduced state (Figure 5). In turn, oxidized thioredoxin can act on several different targets, leading to the opening of the pore or increasing the permeability of

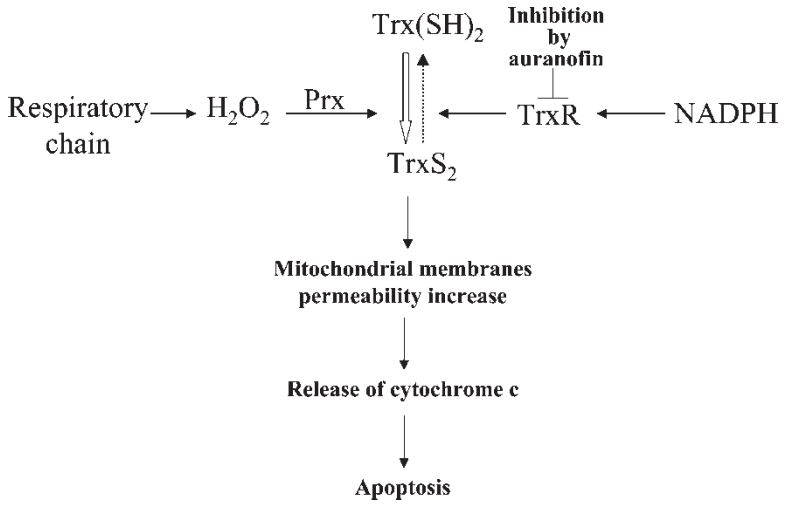

Figure 5. Scheme depicting the mechanism of auranofin alteration of the thiol redox balance in mitochondria. Prx, peroxiredoxin (thioredoxin peroxidase); TrxR, thioredoxin reductase; $Trx(SH)_2$, reduced thioredoxin; $Trx(S)_2$, oxidized thioredoxin.

the outer membrane and, therefore, causing the release of apoptogenic factors (Figure 5). Thioredoxin in its oxidized form can interact with vicinal dithiols of proteins forming intraprotein disulfides. Alternatively, according to its redox conditions thioredoxin can interact with protein targets similarly to the regulation to which ASK-1 (apoptosis signalling kinase) protein is subjected[10].

The thioredoxin/thioredoxin reductase system seems to be involved in the pathogenesis of rheumatoid arthritis since increased levels of thioredoxin were found in the synovial fluid and tissue of patients suffering from rheumatoid arthritis [39]. In the synovial tissue of these patients an increased expression of thioredoxin reductase was also observed [39]. Therefore, thioredoxin reductase can be considered a target for the treatment of rheumatoid arthritis [40] although it is likely that drugs such as auranofin can act on multiple cellular sites [41]. In mitochondria auranofin induces membrane permeability transition and release of cytochrome c [20,21] and, therefore, can be included among the compounds endowed with proapoptotic properties. These actions of auranofin might partially explain at the molecular level its antinflammatory and immunosuppressive properties.

The thioredoxin system plays a critical role in the thiol-mediated cellular redox regulation and in controlling cellular apoptosis or proliferation [40]. It was recently shown that activated p53, which induces apoptosis in a human cell line, is also able to repress the transcription and expression of thioredoxin reductase [42]. Along the same line, it was shown that mitochondrial thioredoxin-deficient cells undergo apoptosis [43] and that the inhibition of thioredoxin reductase with auranofin stimulates mitochondrial permeability transition and release of cytochrome c [20,21]. On the contrary, thioredoxin elicits a protective effect in cells subjected to oxidative stress-induced apoptosis by inhibiting cytochrome c release and procaspase-9 activation [44]. Furthermore, cells overexpressing thioredoxin show an increased resistance to etoposide-induced cytotoxicity [45] and oxidant-mediated apoptosis [46]. Finally, it has been shown that overexpression of mitochondrial thioredoxin reductase is associated with the development of hepatocellular carcinomas [47]. All these results indicate a striking correlation between the thioredoxin system overexpression and the growth of tumors, therefore making this system an attractive target to the development of antitumor drugs [40].

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