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EFFECT OF THE ANTIRETROVIRAL AGENT HYPERICIN ON RAT LIVER MITOCHONDRIA

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Abstract—The photosensitizing effect of hypericin (HY), an antiretroviral agent, on the functions of isolated rat liver mitochondria has been investigated. The respiratory control ratio (RCR), ADP/O and membrane potential of mitochondria were decreased by HY in a light-dependent manner. Uncoupled respiration of mitochondria in the presence of succinate was also inhibited by HY in a light-dependent manner. The ID_{50} of hypericin for these inhibitions was approximately 0.5 μM. These inhibitory effects of HY were not observed when photosensitization was conducted under anaerobic conditions and were not affected by desferrioxamine (DSF) or superoxide dismutase (SOD). Upon photosensitization of HY, mitochondria consumed oxygen in the absence of respiratory substrate with concomitant formation of thiobarbituric acid reactive substance (TBARS). The amount of oxygen consumed was 100-times greater than that of TBARS formed. The oxygen uptake was partially inhibited by NaN_3, and formation of TBARS was inhibited by DSF. Upon photosensitization of HY in the presence of mitochondrial membranes, the electron spin resonance (ESR) signal of 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl (DMPO/·OH) was increased by a mechanism which was suppressed by DSF. An ESR signal for singlet oxygen bound to 2,5-dimethylfuran, 2,2,6,6-tetramethyl-4-piperidone (TEMP) was also detected under light in the presence of mitochondria. This signal of the TEMP-*N*-oxyl radical (TEMPO) was decreased by azide, which physically quenches singlet oxygen, but was increased by DSF. These results indicate that HY might inhibit mitochondrial functions by a type II photodynamic mechanism but that lipid peroxidation of biological membranes through an active oxygen-mediated photodynamic mechanism is not involved.

Key words: free radicals; hypericin; lipid peroxidation; mitochondrial functions; photosensitization; spin trapping

HY**, a natural product of a plant genus *Hypericum* has photodynamic activity both *in vivo* and *in vitro* [1, 2]. Several lines of evidence have suggested that PQP including HY are type II photosensitizers that generate predominantly $^1O_2$, although a supplemental role for O_2^-, ·OH and H_2O_2 should also be considered [3–6]. HY-induced $^1O_2$ generation underlies the mechanism of lipid peroxidation of erythrocyte ghosts [7]. Upon photosensitization, an HY–albumin complex inactivated lysozyme by a $^1O_2$-dependent mechanism [7]. Photosensitization by HY might result in oxidation of lipids, amino acids, and proteins, thereby inhibiting membrane functions. Thus, an oxygen-dependent Type II mechanism that generates $^1O_2$ and O_2^- has been postulated to underlie these effects [2, 3]. However, recent studies by DeWitte *et al.* [8] showed that irradiation of HY also revealed similar effects on EGF-receptor kinase, irrespective of the presence of oxygen. These results suggest that the biological effect of hypericin might depend on type I (involving radical formation) and type II (involving various active oxygens such as singlet oxygen and O_2^-) photosensitization mechanisms.

Clinical studies revealed that low concentrations of HY inhibited the infection and replication cycles of retroviruses *in vivo* and *in vitro* without showing toxic effects in mice and humans [9–11]. Hence, treatment with HY might be expected to be a useful therapy for AIDS. Inhibition of HIV-1

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** Abbreviations: AIDS, acquired immune deficiency syndrome; DETAPAC, diethylenetriaminepentaacetic acid; DNP, 2,4-dinitrophenol; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO/·OH, 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl; DMPO/·OOH, 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl; EGF, epidermal growth factor; DSF, desferrioxamine; ESR, electron spin resonance; HIV, human immunodeficiency virus; HY, hypericin; PKC, Ca^{2+}-phospholipid dependent kinase; KRP, Krebs–Ringer–phosphate buffer; $O^{\cdot-}_2$, superoxide anion; $^1O_2$, singlet oxygen; ·OH, hydroxyl radical; PMA, phorbol myristate acetate; PQP, perylenequinonoid pigments; RCR, respiratory control ratio; SOD, CuZn-superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TEMP, 2,2,6,6-tetramethyl-4-piperidone; TEMPO, 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl radical.

reversetranscriptase [10, 12], succinoxidase [3], and PKC [4, 13, 14] has been postulated to underlie the mechanism for HY-induced antiretroviral activity. In fact, photosensitization of HY inhibited not only Friend leukaemia virus-induced splenomegaly, but also O_2^- generation by PMA-stimulated neutrophils with ID_{50} values similar to that for PKC inhibition [14]. Moreover, Pisanie *et al.* [15] showed that the inhibitory effect of HY on mitochondrial respiration showed positive correlation with its antineoplastic activity [15]. However, the specificity and mechanism of enzyme inhibition by HY remain obscure. The present work describes the photosensitizing effect of HY on the functions of isolated rat liver mitochondria in relation to the generation of active oxygen species and membrane lipid peroxidation.

MATERIALS AND METHODS

Chemicals. ADP and SOD were from Sigma Co. (St. Louis, MO, U.S.A.). The cyanine dye, diS-C_3-(5) was kindly donated by Dr S. Yasui (Research Institute of Kankoshikiso, Okayama, Japan). DMPO and TEMP were from Daiichi Chemical Co. Ltd (Tokyo, Japan) and used without further purification. DSF was from Ciba-Geigy Ltd (Tokyo, Japan). All other reagents were of analytical grade and were from Nacalai Tesque Co. (Kyoto, Japan).

Isolation of mitochondria. Wistar rats (200–250 g) were fasted overnight, and liver mitochondria were isolated by the method of Hogeboom [16]. Briefly, liver mitochondria were isolated in a medium containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA. EDTA was omitted in the final wash and the final mitochondrial pellets were resuspended in 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.4) at 10–30 mg protein/mL at 4°. Mitochondrial protein was determined by Bradford's method using BSA as standard [17].

Measurement of mitochondrial oxidative phosphorylation. Respiration and oxidative phosphorylation were measured polarographically using a Clark type oxygen electrode fitted to a 2 mL water-jacketed closed chamber at 25° [18]. Isolated mitochondria (1 mg protein/mL) were suspended in a medium consisting of 0.2 M sucrose, 10 mM KCl, 3 mM $MgCl_2$, 2 mM sodium phosphate, 5 mM Tris-HCl (pH 7.4) and 5 mM succinate at 25°. State 3 respiration was initiated by adding 250 μM ADP. The respiratory control ratio (State 3 respiration/State 4 respiration, RCR) and the ADP/O ratio were determined as described by Estabrook [19].

Measurement of mitochondrial membrane potential. The membrane potential of the mitochondria was monitored by a cyanine dye method as described previously [20]. Changes in the fluorescence intensity were measured with a fluorospectrophotometer (Hitachi 650–10LC) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer. The wavelength for excitation was set at 622 nm and that for emission at 670 nm.

Measurement of lipid peroxidation. Lipid peroxidation of mitochondria *in vitro* was assayed by the TBA reaction as described previously and expressed in terms of TBARS [21, 22].

Spin trapping and ESR spectrometry for hydroxyl radical and $^1O_2$. Various concentrations of mitochondria (0.2–1.5 mg protein/mL) were incubated in phosphate buffer containing 1–4 μM hypericin and 94 mM DMPO or 10 mM TEMP. The reaction mixture was transferred to a flat quartz ESR cuvette (0.3 mm thick) which was placed into the cavity of an ESR spectrometer (JEOL JES FE-1X with 100-kHz field modulation, X-band). The microwave power used was 8 mW, and the magnetic field was 334.7 ± 5.0 mT. Sweep time was 30 sec or 4 min. The EPR spectra of DMPO/·OOH and DMPO/·OH adducts were identified from hyperfine parameters [23, 24]. The spin trapping of $^1O_2$ by TEMP was used as a method to detect the formation of $^1O_2$ by hypericin according to a modification of the method of Lion *et al.* [25]. Typically, the reaction solution consisted of 0.2–1 mg mitochondrial protein, 15 μM hypericin and 10 mM TEMP as described in [25, 26].

Measurement of protein concentration and incident light. Protein concentration was determined by the method of Lowry *et al.* [27] and illumination was carried out using a high intensity regulated cold light system (NPC Co, Tokyo, Type PICL-NEX) [14].

Statistical treatment of results. At least three independent experiments were performed except where indicated. Results are presented as the mean value ± SD.

RESULTS AND DISCUSSION

Effect of hypericin on the oxidative phosphorylation of rat liver mitochondria

In the dark, low concentrations of HY had no appreciable effect on the oxidation of succinate, RCR and ADP/O of rat liver mitochondria (data not shown). However, upon illumination, HY enhanced state 4 respiration and suppressed state 3 respiration (Fig. 1a and b). Under constant illumination (tungsten light of 29×10^{-3} W/cm^2 for 5 min), the ID_{50} was 0.5 μM, similar to that for PKC [11, 12]. At 1 μM of HY and 5 min exposure, the ID_{50} of light intensity was 15×10^{-3} W/cm^2 (Figs 2a and b). These results suggested that photosensitization of HY might increase the permeability of the mitochondrial membrane for H^+. No such effects were observed when photosensitization was conducted under anaerobic conditions (data not shown), indicating the occurrence of an oxygen-dependent type II reaction. Furthermore, the inhibitory action of HY was not affected by DSF (data not shown). Hence, a superoxide-mediated lipid peroxidation reaction may not underlie the mechanism for HY-induced changes in mitochondrial function.

Effect of hypericin on the mitochondrial membrane potential generated by succinate oxidation

A membrane potential of mitochondria was generated by oxidation of respiratory substrates, which act as energy sources for ATP formation and ion transport [28]. The change in membrane potential was monitored by a cyanine dye, diS-C_3-(5) [20]. A membrane potential generated by succinate oxidation was decreased by Ca^{2+} uptake (Fig. 3). The membrane potential was also decreased by photo-

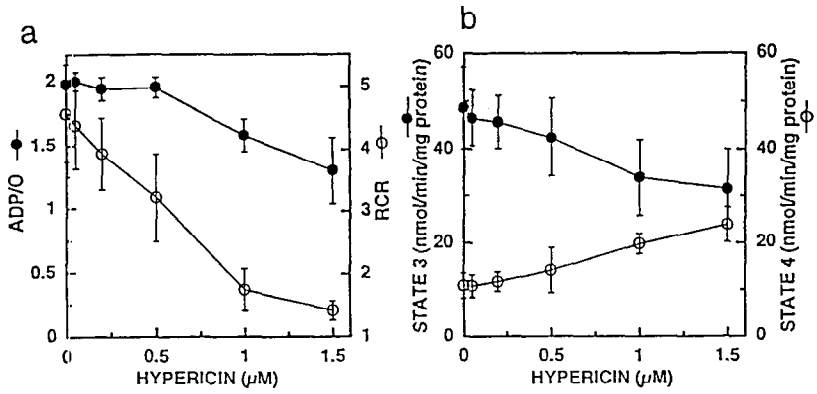

Fig. 1. Effect of HY and illumination on the oxidative phosphorylation of rat liver mitochondria. Rat liver mitochondria (1 mg protein/mL) were incubated in a medium of 0.2 M sucrose, 10 mM KCl, 3 mM $MgCl_2$, 2 mM sodium phosphate, 5 mM Tris-HCl (pH 7.4) with various concentrations of HY and 5 mM succinate at 25° in constant light (29×10^{-3} W/cm^2) for 5 min. Oxidative phosphorylation was measured polarographically and HY added before the addition of ADP. (a) ADP/O (●——●) and RCR (○——○); (b) state 3 (●——●) and state 4 (○——○) respiration. Results are expressed as the mean ± SD from three different experiments.

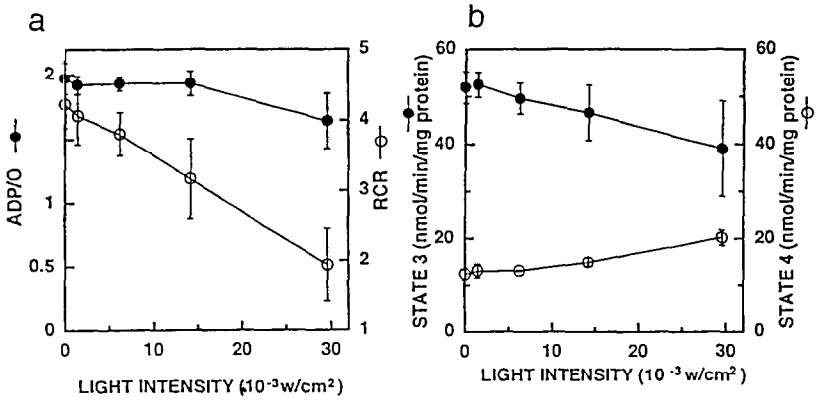

Fig. 2. Effect of light intensity on the inhibition of mitochondrial oxidative phosphorylation by HY. Experimental conditions are as described in Fig. 1 except that the light intensity was variable. (a) ADP/O (●——●) and RCR (○——○); (b) state 3 (●——●) and state 4 (○——○) respiration. Results are expressed as the mean ± SD from three individual experiments.

sensitization with HY and by coupling with energy-requiring reactions, such as Ca^{2+} uptake. In this experiment, we used a low concentration of mitochondria (30 μg protein/mL) to obtain suitably sensitive changes in fluorescence of the cyanine dye. Thus, half-maximum inhibition of membrane potential under constant light (29×10^{-3}W/cm^2) occurred at very low concentrations such as 20 nM of HY.

Effect of hypericin on the succinoxidase activity of mitochondria

Succinoxidase activity in uncoupled mitochondria was inhibited by HY in a light- and concentration-dependent manner [3]. To study the effect of HY on succinoxidase, mitochondrial respiration was uncoupled by repeated cycles of freezing and thawing or by treatment with 10 μM DNP. The ID_{50} of HY for the inhibition of succinoxidase was 0.1 μM (Fig. 4). However, the ID_{50} obtained in this experiment was significantly lower than the previously reported value (2.4 μM) [3]. This difference may be due to different sources of hypericin (Lot. No. of HY used in our experiment was 102H1031, Sigma Chem. Co., St. Louis, MO, U.S.A.) and/or to different conditions of the two experimental systems. To inhibit succinoxidase, oxygen was also required at the time of illumination (Fig. 4). This inhibition was not affected by 1 mM DSF or 100U/mL SOD (data not shown). It is generally accepted that the effects of PQP are mediated predominantly by type II (mediated by $^1O_2$ or other active oxygen species) pathways, and partly by a type I (mediated by PQP semiquinone and/or substrate radicals) reaction [4].

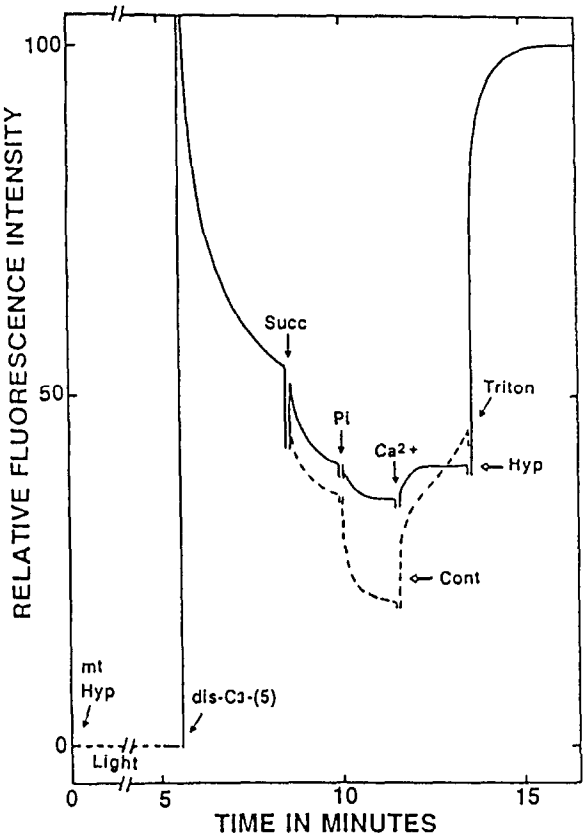

Fig. 3. Effect of HY and constant illumination on the respiration-dependent membrane potential in mitochondria. Mitochondria were incubated in a medium consisting of 0.2 M sucrose, 10 mM KCl, 3 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.4) at 25°. After adding HY, the mitochondrial suspension was illuminated with constant light (29×10^{-3}W/cm^2) for 5 min at 25°. The membrane potential of the mitochondria, generated by succinate oxidation, was monitored by changes in fluorescence intensity of a cyanine dye, 100 μM diS-C_3-(5). The traces show relative fluorescence intensity. Downward and upward deflections indicate polarization and depolarization, respectively. The dotted line shows the control without HY. mt, 30 μg/mL mitochondria; Hyp, 20 nM hypericin; Light, 29×10^{-3}W/cm^2, diS-C_3-(5), 100 μM diS-C_3-(5); Succ, 5 mM sodium succinate; Pi, 2 mM sodium phosphate buffer (pH 7.4); Ca^{2+}, 5 μM $CaCl_2$; Triton, 0.1% Triton X 100; Cont, Control without HY.

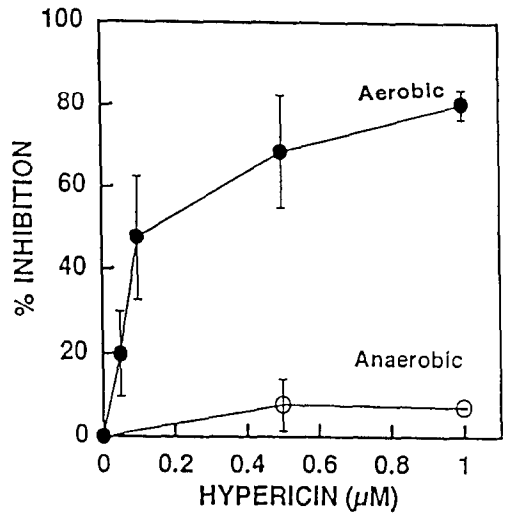

Fig. 4. Inhibition of succinate oxidation of rat liver mitochondria by HY and its dependency on light. Experimental conditions were the same as described in Fig. 1. Mitochondria were uncoupled with 10 μM DNP after illumination for 5 min with constant light (29×10^{-3}W/cm^2) in the presence or absence of various concentrations of HY. Aerobic, in the presence of oxygen; anaerobic, in the absence of oxygen. Results are expressed as the mean ± SD from three individual experiments.

However, several lines of evidence indicate that various active oxygen species play a key role in the photosensitization of PQP [3–5, 29, 30]. The results obtained in this experiment indicate that the inhibition of succinoxidase by HY occurred via a type II $^1O_2$-dependent mechanism and not via a superoxide-mediated photodynamic mechanism.

Effect of HY on the oxygen consumption of mitochondria

To obtain further insight into the mechanism of the photosensitization of HY, the effect of HY on mitochondrial oxygen consumption was studied in the absence of respiratory substrate. Using endogenous respiratory substrates, rat liver mitochondria consume a small amount of oxygen. However, upon illumination, a marked oxygen uptake was induced in the intact and uncoupled mitochondria in the presence of 1 μM HY (Figs 5a and b). Under constant light of 29×10^{-3}W/cm^2, the HY concentration required for half-maximum stimulation was 0.1 μM. This concentration is identical to those for the inhibition of PKC and O_2^- generation in neutrophils [14]. In the presence of 1 μM HY, the light intensity required for half maximum stimulation was 10×10^{-3}W/cm^2.

Effect of HY on lipid peroxidation of the mitochondrial membranes

To elucidate the mechanism of HY-induced oxygen uptake by mitochondria, the relationship between oxygen consumption and lipid peroxidation of uncoupled mitochondria was studied. Both oxygen consumption and TBARS formation [22] by mitochondria linearly increased during the illumination of HY (Fig. 6a). Neither TBARS formation nor oxygen uptake was observed in the dark even in the presence of 10 μM HY. Similar results were also obtained with intact mitochondria (data not shown). However, the amount of oxygen consumed was more than 100 times higher than that of TBARS formed. Furthermore, HY-induced formation of TBARS was suppressed by DSF without oxygen consumption being inhibited (Fig. 6b). The oxygen consumption and formation of TBARS was suppressed by high concentrations of azide which physically quenches singlet oxygen. The results

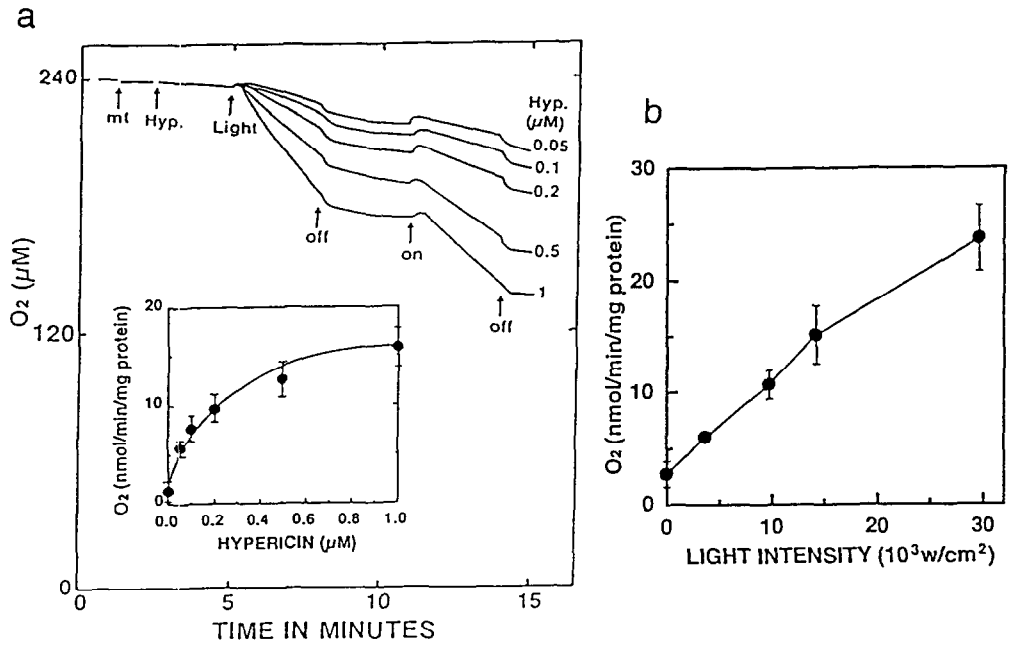

Fig. 5. Stimulation of mitochondrial oxygen consumption by light in the presence of HY. Rat liver mitochondria were subjected to repeated cycles of freezing and thawing and 1 mg of mitochondrial protein was incubated in the medium described in Fig. 1 at 25° under constant light (29×10^{-3}W/cm^2). (a) Shows the dose-dependent stimulation of oxygen consumption by HY, and (b) shows the light dependency of oxygen uptake. Results are expressed as the mean ± SD from three individual experiments.

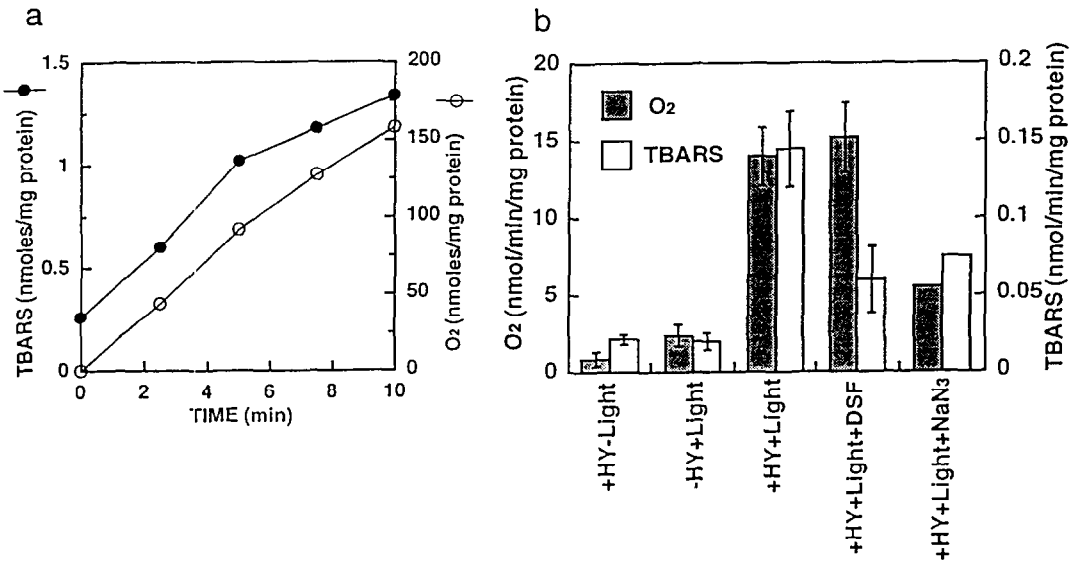

Fig. 6. Lipid peroxidation and oxygen uptake of rat liver mitochondria induced by photosensitization of HY and their inhibition by DSF and azide. Mitochondria (uncoupled by repeated cycles of freezing and thawing) were incubated in a medium consisting of 10 mM Tris-HCl, 0.15 M KCl and 1 μM HY at 25° under constant light (29×10^{-3}W/cm^2). Lipid peroxidation was measured by the TBA reaction and expressed as TBARS [22]. (a) Time course of lipid peroxidation and oxygen uptake of mitochondria induced by photosensitization of HY. (b) Effect of 5 mM desferrioxamine and 10 mM NaN_3 on oxygen uptake and formation of TBARS induced by photosensitization of HY. Results are expressed as the mean ± SD from three individual experiments.

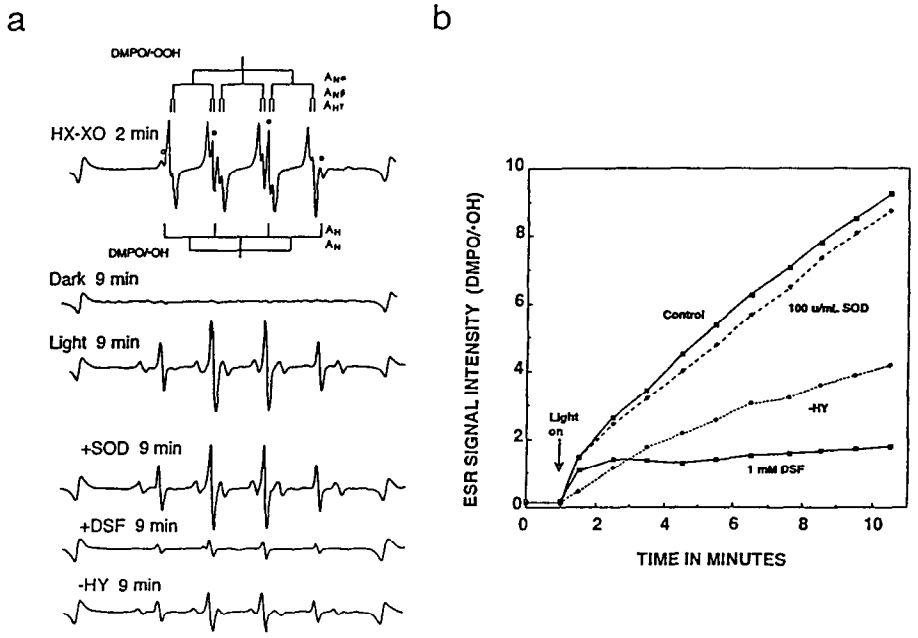

Fig. 7. ESR spectra of DMPO/·OH in mitochondria when illuminated in the presence or absence of HY. Rat liver mitochondria, subjected to repeated cycles of freezing and thawing (2.3 mg protein/mL), were incubated in a medium consisting of 35 mM phosphate buffer (pH 7.4), 1 mM diethylenetriaminepentaacetic acid (DETAPAC), 95 mM DMPO. The spectra were recorded at 0–9 min after exposure to constant light (29×10^{-3}W/cm^2). (a) ESR spectra of DMPO/·OOH and DMPO/·OH in mitochondria and xanthine oxidase preparations (1 mM hypoxanthine (HX)–60 mU xanthine oxidase (XO)) which yielded an ESR spectrum comprised of both DMPO/·OOH ($A\alpha/N$ = 14.3 G, $A\beta/H$ = 11.7 G, and $A\gamma/H$ = 1.25 G) and DMPO/·OH ($A_N = A_H$ = 14.8 G). Open circles indicate DMPO/·OH signal. (b) Time course of the DMPO/·OH signal of mitochondria induced by illumination in the presence of 10 μM HY. On, illumination with constant light (29×10^{-3}W/cm^2); DSF, 5 mM desferrioxamine; SOD, 100 U SOD/mL.

suggest that TBARS formation occurs as a consequence of the reaction of lipid with iron and singlet oxygen. The enhanced oxygen consumption induced by HY may be attributable to the oxidation of lipids and other components of mitochondrial membranes, but cannot be explained on the basis of oxygen consumption by lipid peroxidation which is too small.

Effect of HY on the EPR signals of DMPO/·OH and TEMPO

To clarify the mechanism for photosensitization of HY, EPR signals of DMPO and TEMP were monitored. Figure 7a shows EPR signals of DMPO generated by uncoupled mitochondria after photosensitization of HY. Some unknown signals were observed, but there was an increase of the DMPO/·OH signal during photosensitization of HY. In contrast to the reports of Diwe and Lown [5] and of Weiner and Mazur [6], we could not detect a DMPO/·OOH signal under the present experimental conditions. The DMPO/·OH signal was suppressed by DSF but not by SOD (Fig. 7b). A similar result was also obtained with intact mitochondria (data not shown). To explain these results, much more work is needed but the results do suggest the generation of ·OH via the Fenton reaction. The occurrence of the DMPO/·OH signal was also observed by illuminating mitochondria in the absence of HY. This increase might depend on the presence of mitochondrial components such as pigments and nonheme iron.

Figure 8a shows the change in the ESR spectrum during the illumination of mitochondria with constant light (29×10^{-3}W/cm^2) in the presence of TEMP and HY. The g values and hyperfine splitting constants of the signals were identical to that of authentic TEMPO [4, 5, 31]. In addition to these signals an unknown signal was also observed between the second and third lines. Clearly, more experiments need to be carried out but the center of this species is shifted upfield some 3–4 gauss from TEMPO. This implies a g value of 2.004, typical of semiquinones. The intensity of the TEMPO signal was reduced by NaN_3 [31], and increased by DSF (Figs 8b and c). The increase in TEMPO signal may be due to the inhibition of $O_2^{\cdot-}$ utilization by chelating ferric ions with DSF. These results might suggest that HY generated mostly $^1O_2$ via the type II mechanism and that the $^1O_2$ formed underlies the pathogenesis of mitochondrial dysfunction.

It has been shown that HY inhibits succinoxidase activity by some active oxygen-mediated photosensitizing reaction [3]. The present experiments

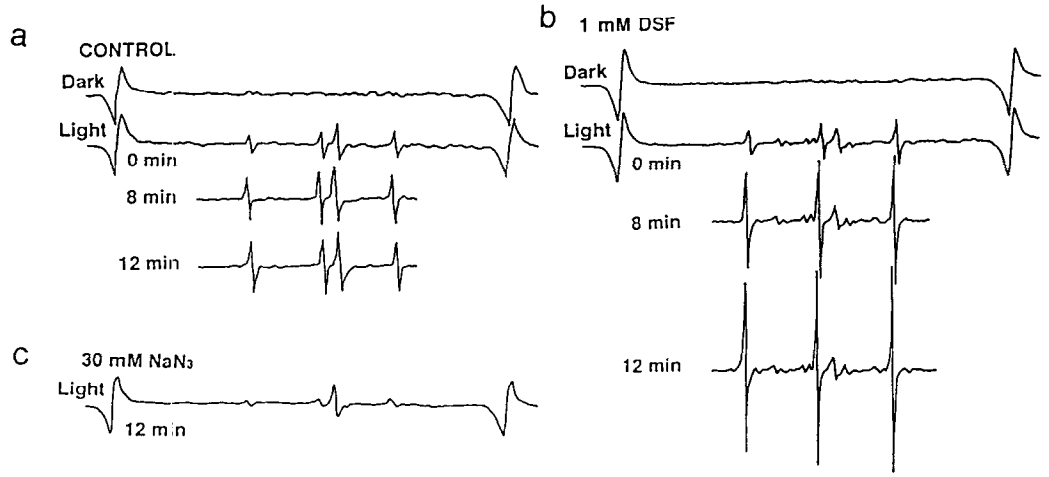

Fig. 8. ESR spectra of TEMPO generated in mitochondria by HY in the presence or absence of DSF. One mg protein of uncoupled rat liver mitochondria was incubated in a medium of 100 mM phosphate buffer (pH 7.8), 10 mM TEMP, 15 μM HY at room temperature. Spectra were recorded 0–12 min after exposure to constant light (29×10^{-3}W/cm^2). The spectrometer settings were: microwave power, 15 db; modulation amplitude, 0.1 G; receiver gain 1.6×10^5. (a) In dark for 12 min or in light for 0–12 min in the presence of HY; (b) in light for 12 min in the presence of 5 mM DSF and HY; (c) in dark for 12 min or light for 0–12 min in the presence of 10 mM NaN_3 and HY.

demonstrated that in addition to succinoxidase, HY also inhibited respiratory control, ADP/O ratio, and the change in respiratory chain coupled membrane potential. Furthermore, HY induced lipid peroxidation with concomitant consumption of oxygen. EPR studies revealed that the increased signals for DMPO/·OH were sensitive to DSF, and that for TEMPO to NaN_3. The details of the mechanism for enzyme-inactivation are not known at present.

Since both succinoxidase and PKC are membrane-bound enzymes [32], perturbation of the membrane by HY may decrease their activity [3]. It is also postulated that modification of membrane lipids by photosensitizing HY may occur by way of oxygen radicals detected by ESR signals from a DMPO adduct, which is an alkoxyl radical (LO·) [26]. However, in contrast to the inhibition of membrane lipid peroxidation, dysfunction of mitochondria induced by photosensitization with HY was not inhibited by DSF. Thus, the mechanism of inactivation of enzymes might not occur via lipid peroxidation of biological membranes but by other photosensitization reactions such as the oxidation of mercapto groups of enzymes through a type II mechanism [4].

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