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Enzymatic and nonenzymatic formation of reactive oxygen species from 6-anilino-5,8-quinolinequinone

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Abstract

The nonenzymatic and enzymatic formation of reactive oxygen species (ROS) from LY83583 (6-anilino-5,8-quinolinequinone) was investigated by electron paramagnetic resonance (EPR) spectroscopy. In the presence of thiol compounds such as glutathione and L-cysteine, LY83583 underwent a one-electron reduction due to low redox potential (-0.3 ± 0.01 V vs. SCE), followed by formation of LY83583 semiquinone anion radical. This species was characterized by EPR spectroscopy under an argon atmosphere at neutral pH. Under an aerobic condition, this species interacts with molecular oxygen to form a superoxide anion radical. GSH-conjugated LY83583 was also identified by NMR and FAB-MS. When LY83583 was applied to PC12 cells, ROS formation was completely inhibited by both the flavoenzyme inhibitor DPI and the DT-diaphorase inhibitor dicumarol. On the other hand, ROS generation occurred independent of intracellular GSH level. These results indicate that LY83583 can generate ROS both enzymatically and nonenzymatically, although the enzymatic formation is dominant over the nonenzymatic system in PC12 cells.

Keywords: Free radical; LY83583; Electron paramagnetic resonance; PC12 cell; Glutathione; Oxidative stress

1. Introduction

The naphthoquinolinedione compound LY83583 (6-anilino-5,8-quinolinequinone, Fig. 1) has been used as a competitive inhibitor of soluble guanylate cyclase [1], which modifies the vasorelaxtion due to nitric oxide [2] through a change of the cyclic GMP levels [3–5], followed by generation of reactive oxygen species (ROS) intermediates [6–8]. In addition, LY83583 acts as an inhibitor of leukotriene release [9].

In general, it has been reported that quinone (Q) derivatives such as benzoquinone [9], adriamycin, daunorbicin, mitomycin C [10], 1,4-benzoquinone [11] and 1,4-naphthoquinone [12–14] can be reduced in intact cells and thereafter generate ROS by the following reactions (reactions (1)–(7)) [10,11,14].

$$Q + e^- \rightarrow Q^{\cdot -} \quad (1)$$

$$Q + 2e^- \rightarrow Q^{-} \quad (2)$$

$$Q^- + Q \rightarrow 2Q^{\cdot -} \quad (3)$$

$$Q^{\cdot -} + O_2 \rightarrow Q + O_2^{\cdot -} \quad (4)$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

$$Q^{\cdot -} + M^n \rightarrow Q + M^{n-1} \ (\mathrm{M}: \text{ transition metal}) \quad (6)$$

$$M^{n-1} + H_2O_2 \rightarrow OH^- + {}^{\cdot}OH + M^n \quad (7)$$

Some flavoenzymes may be candidates for electron donor (reaction (1)). One-electron reduction is catalyzed

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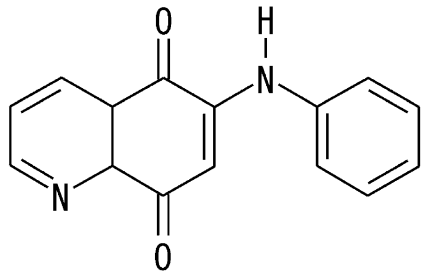


Fig. 1. Structure of LY83583.

by NADPH-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase to form the corresponding semiquinone radical [15,16], which rapidly interacts with molecular oxygen to form the superoxide anion radical ($O_2^{\cdot -}$) and original quinones [17] (reaction (4)). Two-electron reduction of quinones to corresponding quinols (reaction (2)) is catalysed by DT-diaphorase [18], and the quinols interact with parent quinone compounds, which leads to the formation of corresponding semiquinones [19] (reaction (3)). Biological thiol compounds also may be candidates for the electron donor. Menadione, adriamycin, and mitomycin C can be reduced by biological concentrations of glutathione, followed by the generation of ROS (reactions (1) and (4)). However, there are few reports on whether the naphthoquinolidione compound LY83583 is capable of being reduced by thiols and forming ROS and LY83583-thiol adducts.

Therefore, the objective of the present study was to investigate the interactions between LY83583 and thiol compounds. Electron paramagnetic resonance (EPR) spin-trapping was used to detect the LY83583 radical intermediate and the oxygen-centered radicals using the spin-trapping agent in the presence of LY83583 and thiol compounds. The formation of LY83583–GSH adduct was studied by HPLC, and identified by NMR and FAB-MS.

2. Materials and methods

2.1. Materials

LY83583 was purchased from Calbiochem (Darmstadt, Germany). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Labotech (Tokyo, Japan). Glutathione and glutathione disulfide (GSH and GSSG), ethylenediamine pentaacetic acid (EDTA), *N*-ethylmaleimide (NEM), *o*-phthalaldehyde (*o*-PA), superoxide dismutase (SOD; from bovine erythrocyte), deferoxamine mesylate, and L-cysteine were obtained from Wako Pure Chemical Co., Ltd. (Tokyo, Japan). Deuterium oxide and methanol-d4 were obtained from Aldrich. Other chemicals were of analytical grade.

2.2. Method

2.2.1. Growth of PC12 cells and treatment with reagents

The PC12 cells were subcultured in 75-cm^2 polylysine-treated flasks (Iwaki, Tokyo, Japan) in RPMI 1640 culture media supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, streptomycin (50 μg/ml), and penicillin (50 units/ml). After 2 days in culture (passage 50), cells were washed with oxygenated, prewarmed (37 °C) Krebs-Ringer HEPES buffer (KRH buffer), pH 7.4, containing 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES. The cells were then harvested by gentle pipetting, centrifuged to collect the cell pellet, and resuspended at a concentration of 4×10^5 cells/ml with KRH buffer for EPR study, and 5×10^5 cells/ml for GSH and GSSG assay. Cell viability was >95% as determined by exclusion of 0.2% Trypan blue.

2.2.2. Concentrations of cellular GSH and GSSG

The amounts of intracellular GSH and GSSG were measured using the method of Senft et al. [20] with slight modifications as described below. An aliquot of PC12 cell suspension (1 ml, 5×10^5 cells/ml) was introduced in triplicate into polypropylene tubes. Cells were incubated with or without LY83583 for 2–20 min. At the end of the incubation, the supernatant was removed by centrifugation at $1000 \times g$ for 5 min at 4 °C, and then the pellet was washed twice with ice-cold KRH buffer. The cell pellets were resuspended with 100 μl of 0.1 M PBS buffer (pH 6.8) containing 5 mM DTPA, and 10 μl of the suspension was kept for protein assay with a protein assay kit (Pierce, Rockford, IL, USA). The remaining cell suspension was mixed with 100 μl of a cell lysing solution consisting of 40 mM HCl, 10 mM DTPA, 20 mM ascorbate, and 10% trichloroacetic acid. After 5 min of vigorous mixing, the acid-soluble fractions were collected by centrifugation at $14{,}000 \times g$ for 10 min at 4 °C, and these supernatants were stored at −80 °C for GSH and GSSG assay.

Before the GSH and GSSG measurements, we prepared a solution containing 20 mM HCl, 5 mM DTPA, and 10 mM ascorbic acid (medium A). For GSH assay, 4 μl of sample solutions was mixed with 146 μl of 5% TCA in medium A, 20 μl of medium A, 250 μl of 1 M phosphate buffer (pH 7.0), 1000 μl of 0.1M phosphate buffer (pH 6.9), and 150 μl of 5 mg/ml *o*-PA in methanol. To measure the non-glutathione-dependent fluorescence, 20 μl of 7.5 mM NEM in medium A was added instead of 20 μl of medium A. For GSSG assay, 4 μl of sample solutions was mixed with 146 μl of 5% TCA in medium A, 20 μl of NEM in medium A, 250 μl of 1 M phosphate buffer (pH 7.0), 1000 μl of 0.1 M phosphate buffer (pH 6.9), and 150 μl of 5 mg/ml *o*-PA in methanol. To determine the background fluorescence, 30 μl of 0.1 M sodium hydrosulfite was added to the corresponding samples, then the fluorescence was measured. *O*-PA derived fluorescence was measured at 365-

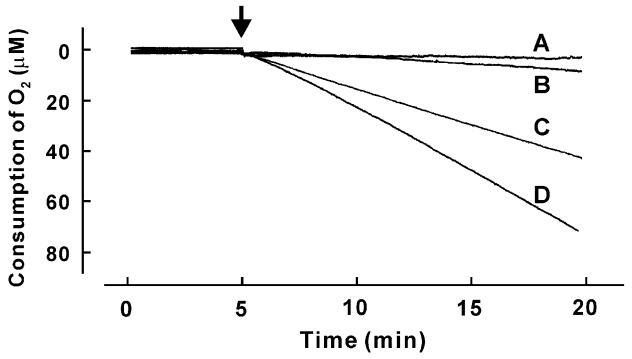


Fig. 2. Oxygen consumption by LY83583 in the presence of thiol compounds. Trace A, 10 mM GSH in HEPES buffer (0.1 M, pH 7.4); trace B, 10 mM L-cysteine in buffer; trace C, 10 mM GSH and 0.25 mM LY83583 in buffer; trace D, 10 mM L-cysteine and 0.25 mM LY83583 in buffer. LY83583 was initially added to the buffer solution (traces C and D). All data were obtained at room temperature (25 °C), and thiol compounds were introduced at the position of the arrow (↓).

nm excitation and 430-nm emission with a Hitachi F-3010 fluorescence spectrophotometer (Tokyo, Japan).

2.2.3. Free-radical analysis by EPR spectroscopy

The free radical metabolites of LY83583 by GSH were examined with a Bruker EMX EPR spectrometer (Bruker Co., Billerica, MA, USA) equipped with a TM_{110} cavity and a 17-mm aqueous quartz flat cell to collect the LY83583-derived radical intermediate. Nonenzymatic and PC12 cell-dependent ROS formation was studied by DMPO spin-trapping using the method of Souchard et al. [21] with slight modifications. A JEOL EPR spectrometer (JES-TE300, JEOL Co., Ltd., Tokyo, Japan) with an ES-UCX2 cavity and 60-μl glass capillary was employed to collect DMPO spin adducts. Spectra were stored on an IBM personal computer for analysis. Hyperfine coupling constants were obtained with the computer program Winsim [22].

2.2.4. Oxygen consumption

Changes of oxygen concentration were monitored on the addition of 18 μl of 10 mM thiol compounds to the chamber of a Clark-type oxygen electrode containing 1.8 ml of HEPES buffer (0.1 M, pH 7.4). Incubations were performed in the presence or absence of 0.25 mM LY83583. In order to estimate the changes of oxygen concentration due to the addition of thiol compounds, the initial oxygen concentration in the incubation was assumed to be 254 μM at room temperature [23].

2.2.5. HPLC assay

HPLC was performed using a JASCO 880-PU pump (JEOL Co., Ltd.) and a manual injector equipped with a 20-μl loop. The separation was carried out on a Chemopack finesil $_5C_{18}$ (4.6 × 250 mm) column. The mobile phase consisted of a mixture of methanol (60%, v/v) and 50 mM phosphate buffer (pH 7.4), filtered through a 0.45-μm filter (Millipore, Bedford, MA, USA). HPLC analysis was performed at room temperature under isocratic conditions, with a flow rate of 1.5 ml/min, then monitored by UV spectrometric detector (Shimadzu, SPD-6A, Tokyo, Japan) at 254 nm with full-scale absorbance set at 0.32 AUFS. The elutant corresponding to each peak was collected, and evaporated for NMR and MS study.

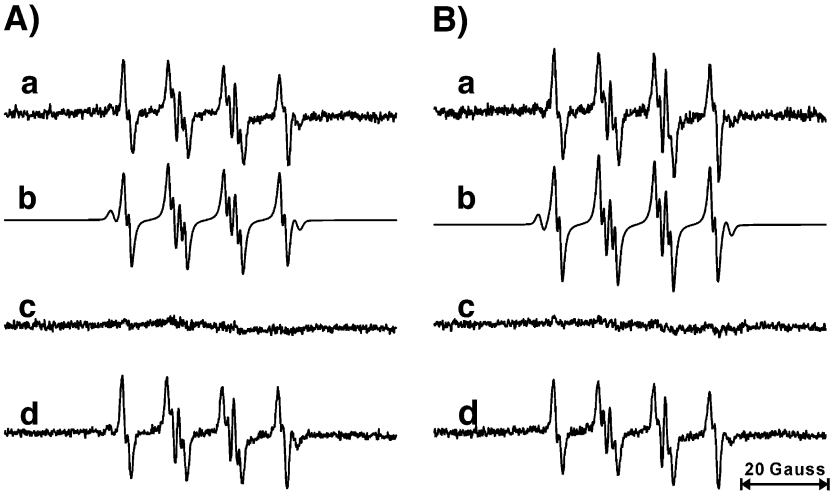


Fig. 3. Spin trapping of LY83583 and thiol compounds with DMPO. A-a: 0.8 mM LY83583, 40 mM GSH, and 0.1 M DMPO in 0.1 M HEPES buffer (pH 7.4) containing 1 mM deferoxiamine. A-b: same as A-a, but with 1000 U/ml SOD. A-c: same as A-a, but with 1000 U/ml catalase. A-d: computer simulation using the hyperfine coupling constants (Table 1) derived from A-a (DMPO/$O_2^{\cdot-}$:DMPO/$^{\cdot}$OH:DMPO/$^{\cdot}$SG = 7:2:1). B-a: 0.8 mM LY83583, 40 mM L-cysteine, and 0.1 M DMPO in 0.1 M HEPES buffer (pH 7.4) containing 1 mM deferoxiamine. B-b: same as B-a, but with 1000 U/ml SOD. B-c: same as B-a, but with 1000 U/ml catalase. B-d: computer simulation using the hyperfine coupling constants (Table 1) derived from B-a (DMPO/$O_2^{\cdot-}$:DMPO/$^{\cdot}$OH = 7:3). EPR spectrometer settings were microwave power, 8 mW; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; sweep time, 2 min; time constant, 0.03 s; receiver gain, 320.

Table 1
Hyperfine coupling constants

Radical	Coupling constants in Gauss (this work)	Literature values	Reference
DMPO/$O_2^{\cdot -}$	$a^N = 14.2$	$a^N = 14.2$	[43]
	$a_\beta^H = 11.4$	$a_\beta^H = 11.3$	
	$a_\gamma^H = 1.3$	$a\gamma^H = 1.3$	
DMPO/$^{\cdot}$OH	$a^N = 15.0$	$a^N = 15.0$	[43]
	$a_\beta^H = 15.0$	$a_\beta^H = 15.0$	
DMPO/$^{\cdot}$SG	$a^N = 15.1$	$a^N = 15.1$	[44]
	$a_\beta^H = 15.8$	$a_\beta^H = 16.0$	

2.2.6. NMR and MS study

NMR spectra were recorded on a Varian UNITY 600 or a JEOL JNM-ECP400 spectrometer in methanol-d4 solution using tetramethylsilane (TMS) as an internal standard. The FAB-MS (Xe gun, 10 kV, *m*-nitrobenzylalcohol as the matrix) were measured on a JEOL JMS-HX-100 mass spectrometer.

2.2.7. Electrochemical measurement

Electrochemical measurements were carried out on a Yanako P-1100 polarographic analyzer (Kyoto, Japan) with a three-electrode system consisting of a platinum wire for working and auxiliary electrodes and a saturated calomel electrode (SCE). One millimolar of LY83583 was prepared with 0.1 M HEPES buffer (pH 6.9 and 7.4) containing 5% EtOH.

2.2.8. Statistics

Statistical analyses were carried out using one-way ANOVA followed by the Student–Newman–Keuls test for pairwise comparisons. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Oxygen-consumption studies

The extent of the oxygen uptake recorded during the addition of reductants is shown in Fig. 2. When 10 mM thiol compounds were introduced into the oxygen electrode containing 1.8-ml HEPES buffer (0.1M, pH 7.4) with LY83583 (0.25 mM), continuous oxygen consumption was observed with L-cysteine (Fig. 2, trace D) and with GSH (Fig. 2, trace C), whereas little oxygen was consumed in the absence of LY83583 (L-cysteine (Fig. 2, trace B) and GSH (Fig. 2, trace A).

3.2. DMPO spin adduct formed from LY83583 and biological thiol compounds

DMPO spin adducts resulting from the interaction between LY83583 and thiol compounds in 0.1 M HEPES buffer (pH 7.4) under aerobic condition are shown in Fig. 3. To prevent formation of artificial spin adducts due to trace amounts of iron, we added 1 mM deferoxamine to the reaction mixture. When 0.8 mM LY83583 and 40 mM thiol compounds were mixed, apparent EPR spectra were observed (Fig. 3, trace A-a; GSH, trace B-a; L-cysteine). The hyperfine splitting constants of these spectra were calculated, and the EPR signals observed here were assigned as a mixture of DMPO/$O_2^{\cdot -}$, DMPO/$^{\cdot}$OH, and DMPO/$^{\cdot}$SG adducts with a ratio of 0.84:0.06:0.10 (Table 1). The addition of SOD (1000 U/ml) completely extinguished (Fig. 3, trace A-c, GSH; trace B-b, L-cysteine) the EPR signal intensity. Catalase (1000 U/ml) did not affect the DMPO/$O_2^{\cdot -}$ adduct, whereas it diminished the DMPO/$^{\cdot}$OH adduct (Fig. 3, trace d). When LY83583 or thiol compounds were absent, no EPR signal was observed (data not shown).

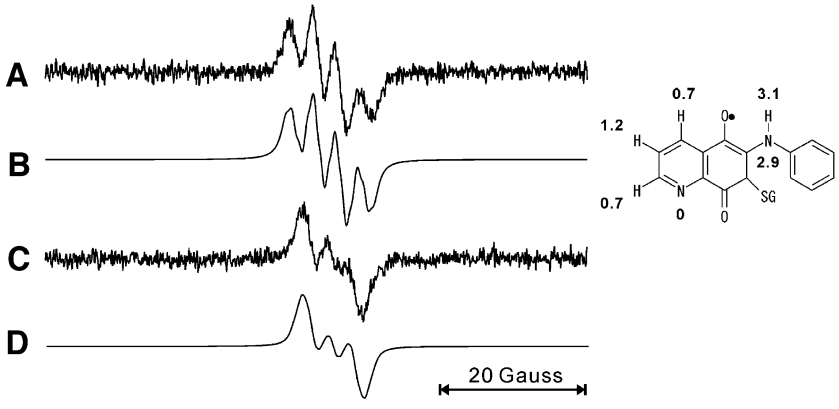


Fig. 4. EPR spectrum of the LY83583 radical intermediate. (A) 250 mM GSH and 20 mM LY83583 in HEPES buffer (0.1 M, pH 7.4 in 50% methanol) under anaerobic conditions. (C) same as A, but GSH and LY83583 were prepared with a D_2O solution of HEPES (0.1 M, pD 6.8 in 50% CD_3OD). (B and D) Simulated spectrum of A and C, respectively. Coupling constants for simulation B are indicated in the figure, and simulation D was obtained using the same coupling constants of B except $a_{ND}^D = 0.6$ G. EPR spectrometer settings were microwave power, 20 mW; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; sweep time, 22.4 min; time constant, 0.33 s; receiver gain, 4×10^6.

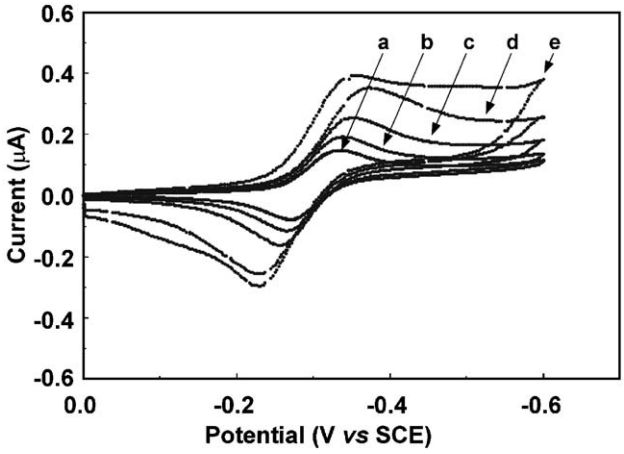


Fig. 5. Cyclic voltammograms of LY83583. 1 mM of LY83583 was dissolved with HEPES buffer (0.1 M, pH 7.4(a–d) and pH 6.9 (e)) containing 5% ethanol, and introduced into the cyclic voltammogram chamber immediately, then the relationship between potential and current was measured at various scan rates (a and e, 5; b, 10; c, 20; d, 50 mV/s). All data were collected at 15 °C.

The EPR simulation spectrum obtained using the hyperfine coupling constants (Table 1) was in good agreement with the original signal (Fig. 3, trace A-b, simulation of A-a; trace B-b, simulation of B-a).

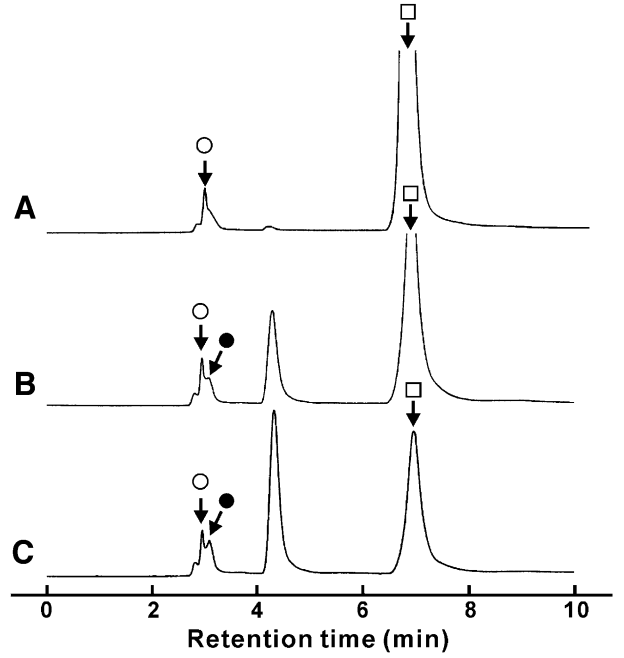


Fig. 6. HPLC chromatograms of the mixture of GSH with LY83583 at various incubation periods. 4 mM LY83583 and 40 mM GSH were dissolved with phosphate buffer (50 mM, pH 7.4) containing 60% methanol, and introduced into the HPLC column immediately (A), 60 min (B), and 120 min (C) after mixing. Open circles (○), closed circles (●), and open squares (□) represent the position of GSH, GSSG, and LY83583, respectively. The chromatogram conditions were described in Materials and methods.

3.3. LY83583 radical intermediate formed by GSH

When 250 mM GSH was incubated with 20 mM LY83583 in HEPES buffer (0.1 M, pH 7.4) containing 50% methanol under an aerobic condition, no EPR signal was observed (data not shown). However, under an anaerobic condition, a four-line EPR signal was detected (Fig. 4A), and these lines could be attributed to interactions of the unpaired electron with hydrogen atoms and a nitrogen ($a^N = 2.9$ Gauss) atom. In addition, when H_2O and CH_3OH were replaced with D_2O and CD_3OD, the hydrogen-derived hyperfine splitting disappeared, and a three-line EPR signal was observed (Fig. 4C). Fig. 4B and D represents a computer simulation of Fig. 4A and C using the hyperfine splitting constants specified in the figure.

3.4. Electrochemical measurements

As shown in Fig. 5, the redox potential of LY83583 obtained by cyclic voltammetry was -0.30 ± 0.01 V (SCE) at 15 °C in 0.1 M HEPES buffer (pH 7.4) containing 5% EtOH, and this redox potential was constant at any scan rate discussed here (Fig. 5a–d). The redox potential was unchanged even if the pH value was shifted to 6.9 (Fig. 5a and e).

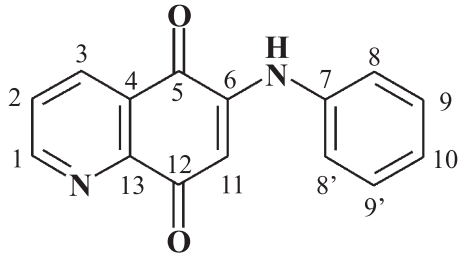
From the result of ^{1}H-NMR, it appeared that a hydrogen atom of the amine group was replaced under the deuterium environment (data not shown).

3.5. HPLC study

Next, we applied the reaction mixture containing LY83583 and GSH to HPLC in order to investigate the formation of LY83583–GSH conjugate. As shown in Fig.

Table 2
^{1}H NMR (600 MHz, J in Hz) and ^{13}C NMR (150 MHz) spectral data of LY83583 in CD_3OD

Position	δ_H	δ_C	Position	δ_H	δ_C
1	8.93	155.5	8, 8′	7.38	125.1
2	7.76	128.4	9, 9′	7.47	130.7
3	8.50	136.0	10	7.28	127.4
4		129.2	11	6.33	103.5
5		182.5	12		183.7
6		148.4	13		149.9
7		139.0			



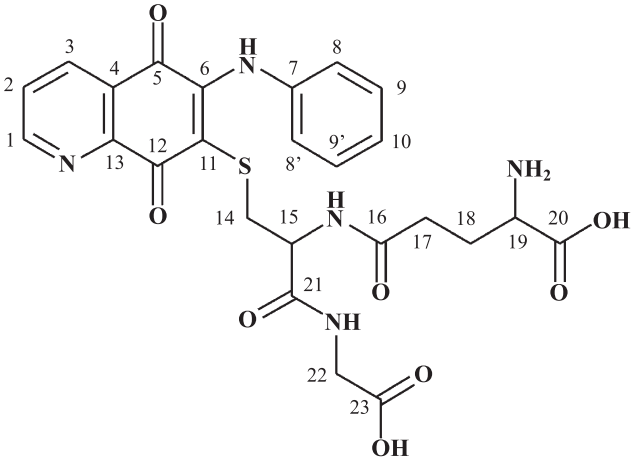
6, when 4 mM LY83583 and 40 mM GSH were mixed under the aerobic condition, a decrease of LY83583 and an increase of a new peak ($t = 4.2$ min) was observed; this peak was augmented with time. To identify this new peak that appeared at $t = 4.2$, we collected the elutant and then evaporated the mobile phase under low pressure to obtain the sample for NMR and FAB-MS measurements.

3.6. NMR and MS of the elutant

When the elutant was introduced to the FAB-MS under the negative and positive conditions, it gave a molecular ion at m/z 554 $[M-1]^-$, m/z 578 $[M+Na]^+$, and m/z 594 $[M+K]^+$, respectively. The ^{1}H-NMR and ^{13}C-NMR spectral data of LY83583 and the elutant are listed in Tables 2 and 3, respectively.

Table 3
^{1}H NMR (600 MHz, J in Hz) and ^{13}C NMR (150 MHz) spectral data of elutant in CD_3OD

Position	δ_H (mult., J, Hz)	δ_C	Position	δ_H (mult., J, Hz)	δ_C
1	8.93 (1H, dd, 4.7, 1.6)	155.3, d	13		150.0, s
2	7.76 (1H, dd, 8.0, 4.7)	128.6, d	14	2.72(β_1, 1H, dd, 14.2, 9.3), 3.03(β_2, 1H, dd, 14.2, 4.8)	36.5, t
3	8.48 (1H, dd, 8.0, 1.6)	136.2, d	15	4.32 (1H, dd, 9.3, 4.8)	53.9, d
4		129.3, s	16		175.0, s
5		181.8, s	17	2.47 (2H, t, 6.9)	32.9, t
6		149.2, s	18	2.09 (2H, dd, 6.9, 6.2)	27.6, t
7		140.1, s	19	3.64 (1H, t, 6.2)	55.4, d
8, 8′	7.17 (2H, dd, 8.0, 1.1)	125.0, d	20		173.9, s
9, 9′	7.34 (2H, t, 8.0)	129.5, d	21		172.5, s
10	7.17 (1H, dt, 8.0, 1.1)	126.4, d	22	3.72 (2H, s)	42.6, t
11		114.3, s	23		173.6, s
12		181.2, s			



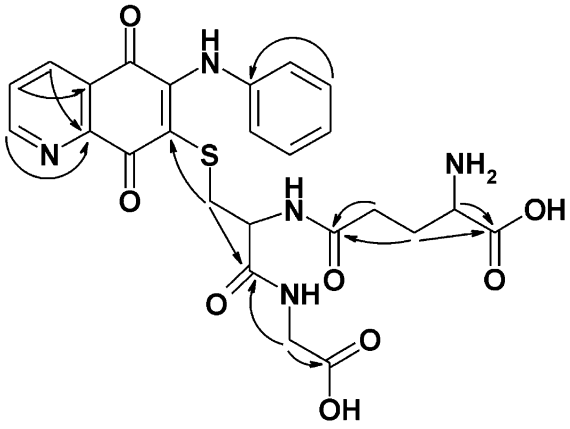


Fig. 7. HMBC correlations of GSH–LY83583 conjugate.

From the NMR and MS data, the structure of the elutant was determined to be as shown in Fig. 7.

3.7. EPR signals detected from PC12 using DMPO as a spin-trap agent

When PC12 cells (4×10^5 cells/ml) were incubated with 0.1 M DMPO alone, no EPR signal was observed (Fig. 8a). However, after 2-min incubation of PC12 with 0.1 M DMPO and LY83583 (1×10^{-4}M), the EPR signal typical of DMPO/·OH was observed (Fig. 8b). When SOD (100 U/ml) was co-incubated with PC12 cells and stimulated by LY83583, the EPR signal disappeared (Fig. 8c).

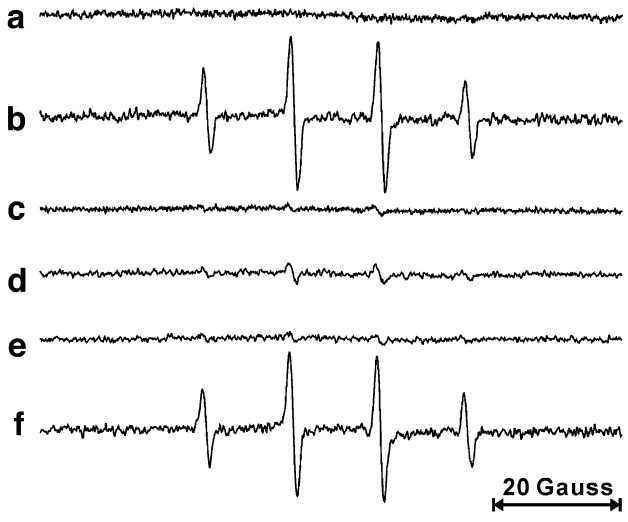


Fig. 8. Effect of LY83583 on the EPR spectra obtained from PC12 cells. The cells (4×10^5 cells/ml) were incubated in the presence of the spin-trap agent DMPO (100 mM) and stimulated with (b) or without (a) 1×10^{-4} M LY83583 for two minutes. In parallel experiments, cells were co-incubated with (c) LY83583 and 100 U/ml SOD, with (d) LY83583 and 1×10^{-3} M DPI, or with (e) LY83583 and 5×10^{-5} M dicumarol. BSO pretreated PC12 cells (1×10^{-4} M BSO, for eight hours) were stimulated with 1×10^{-4} M LY83583 (f). EPR spectrometer settings were the same as in Fig. 3.

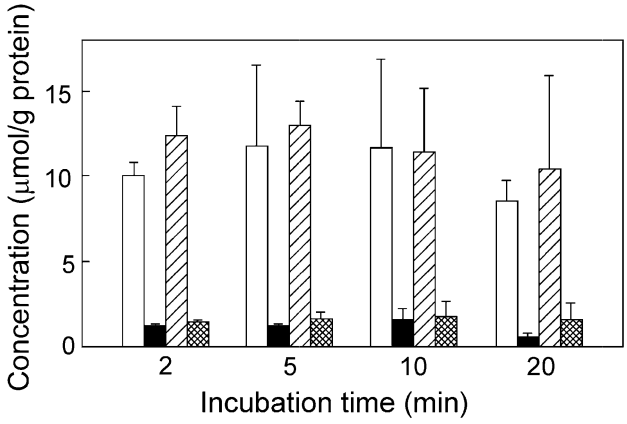


Fig. 9. Effect of LY83583 treatment on the intracellular concentration of glutathione and glutathione disulfide. The PC12 cells were incubated for 2–20 min in the presence of 1×10^{-4} M LY83583 and then collected for fluorescence determination of the intracellular concentrations of the glutathione and glutathione disulfide, as described in Materials and methods. Open column, GSH of untreated PC12 cells; closed column, GSSG of untreated PC12 cells; hatched column, GSH of LY83583-treated PC12 cells; and crosshatched column, GSSG of LY83583-treated PC12 cells. GSH and GSSG concentrations of untreated PC12 cells were 12.0 ± 2.1 and 0.8 ± 0.2 μmol/g protein in three experiments. Each value represents the mean (± S.D.) of three different experiments.

It has been reported that some flavoenzymes and DT-diaphorase are responsible for ROS generation due to the one- or two-electron reduction of LY83583 in vitro [24]. Therefore, in order to study the contributions of LY83583 to enzymatic ROS generation from PC12 cells, the flavoenzyme inhibitor DPI [25] or the DT-diaphorase inhibitor dicumarol [26] was added to the incubation mixture. When PC12 cells were treated with either DPI (1×10^{-4} M) or dicumarol (5×10^{-5} M), the EPR signal of the DMPO/·OH adduct was suppressed (Fig. 8d and e, respectively). To understand the participation of GSH in ROS generation by LY83583 in PC12 cells, BSO, a selective inhibitor of γ-glutamyl cysteine synthetase (γ-GCS) [27], was applied. When PC12 cells were incubated for 8 h in the presence of BSO (1×10^{-4} M), no significant reduction of cell viability was observed (data not shown), as reported previously [28], whereas the GSH concentration dropped to 54% (13 μmol/g protein, control; 6 μmol/g protein, BSO-treated). When 1×10^{-4} M LY83583 was introduced into BSO-treated PC12 cells (4×10^{5} cells/ml), the DMPO/·OH adduct (Fig. 8f) did not change compared to that of the control (Fig. 8b).

3.8. GSH and GSSG concentration after exposure of PC12 cells to LY83583

To examine whether the LY83583 compound modulates the intracellular GSH/GSSG concentrations, we exposed cultured PC12 cells to LY83583 for 2–20 min. As shown in Fig. 9, no significant reduction of the GSH concentration or increment of GSSG was observed.

4. Discussion

Quinone compounds are reduced not only by enzymes but also by biological thiols [29]. Enzymatic reduction is achieved by both one-electron reduction (microsomal NADPH-cytochrome P450 reductase, microsomal NADH-cytochrome b_5 reductase, and mitochondrial NADH-ubiquinone oxidoreductase) and two-electron reduction (DT-diaphorase), which results in the formation of semiquinone radicals and hydroquinones, respectively. Nonenzymatic reduction would be accomplished by cellular nucleophiles, such as GSH, L-cysteine and NADPH, followed by the formation of semiquinone radicals, thiol-semiquinone conjugates, and ROS under aerobic conditions [19].

LY83583, which is a naphthoquinolinedione compound, has some pharmacological effects. It acts as an inhibitor of cellular Ca^{2+} influx and reloading of the Ca^{2+} pool [30], an inhibitor of glutathione reductase [31], a competitive inhibitor of soluble guanylate cyclase [1], and an inhibitor of leukotriene release [9]. LY83583 has a quinone moiety in its molecule, which suggested the possibility for enzymatic and nonenzymatic reduction with subsequent reoxygenation by molecular oxygen and concomitant production of $O_2^{\cdot -}$, as other quinines do. In 1998, Kumagai et al. [24] reported that LY83583 could be an electron acceptor from purified nNOS, p-450 reductase, and DT-diaphorase, which is responsible for the ROS generation by one-electron transfer to molecular oxygen. However, few studies have proposed that LY83583 could be a candidate for an electron acceptor from biological thiol compounds. Therefore, we tried to clarify the interactions between LY83583 and biological thiol compounds.

When LY83583 was mixed with GSH under an aerobic condition, a new HPLC peak appeared (Fig. 6). In addition, when the fraction was applied to NMR, it was found that the chemical shift of the hydrogen at δ 6.33 of LY83583 had disappeared (position 11 in Tables 2 and 3). From the result of the HMBC method, it was found that the carbon at δ 37 (position 14 in Table 3) correlated with the carbon at δ 114 (position 11 in Table 3) and the carbon at δ 173 (position 21 in Table 3) (Fig. 7). Further, FAB-MS gave a molecular ion at *m/z* 554 $[M-1]^-$, which suggested the combination of LY83583 (molecular weight 250) and GSH (molecular weight 307). These results indicated that the addition of GSH to LY83583 had occurred under these conditions.

From the results of the DMPO spin-trapping study, a mixture of DMPO/$O_2^{\cdot}$, DMPO/·OH, and DMPO/·SG adducts was observed when the LY83583 was mixed with GSH under an aerobic condition at neutral pH. These adducts were suppressed by the addition of SOD, which indicated that $O_2^{\cdot -}$ had been produced in this condition, followed by the formation of a thiyl radical (·SG) by following the reaction [32].

$$GS^- + O_2^{\cdot -} + 2H^+ \rightarrow {}^{\cdot}SG + H_2O_2 \quad (8)$$

Because the rate of $O_2^{\cdot -}$ dismutation by SOD (2×10^9 M^{-1} s^{-1}) is faster than that of spontaneous decomposition of $O_2^{\cdot -}$ (5×10^5 M^{-1} s^{-1}) [33], the inhibitory effect of SOD on the formation of the DMPO/$^{\cdot}$SG adduct (Fig. 3A-c) would support reaction (8).

When we used another biological reducing agent, L-cysteine, the EPR intensity of both DMPO/$O_2^{\cdot -}$ and DMPO/$^{\cdot}$OH signals was higher than those of GSH because L-cysteine is a stronger reducing agent than GSH [34].

It has been known that a one-electron reduction of quinone compounds results in the formation of semiquinone radical species, followed by formation of ROS under aerobic conditions [19] (reactions (1) and (4)). Therefore, we tried to detect a semiquinone radical of LY83583 by EPR spectroscopy. Before performing this experiment, we examined whether the LY83583 could be an electron acceptor or not. When 20 mM LY83583 was mixed with 20 mM sodium dithionite in HEPES buffer (0.1 M, pH 7.4) containing 50% methanol, a broadened EPR signal (ΔG(peak to peak) = 4.7 gauss) with a slight shoulder on both sides of the peak appeared, and the shoulders disappeared rapidly (data not shown). We were unable to calculate hyperfine splitting constants from this result due to instability, and we could not compare them with those of the LY83583–GSH system. In any case, it seemed that LY83583 could be an electron acceptor when it was mixed with an electron donor. Direct detection of the LY83583 radical by EPR spectroscopy (Fig. 4) and the results of cyclic voltammetry (Fig. 5) indicated that (1) LY83583 is prone to reduction due to its low redox potential (-0.30 ± 0.01 V vs. SCE), (2) the redox potential was independent of physiological pH value (6.9 and 7.4), which may imply that a hydrogen atom of LY83583 did not participate in the redox process under at these conditions, (3) a one-electron transfer process between quinone and semiquinone radicals was expected from the independence of the scanning rate from the redox potentials, (4) LY83583 has one replaceable hydrogen atom in its molecule.

Next, we studied whether the intracellular GSH contributes to the ROS generation stimulated by LY83583 in PC12 cells. We adopted the EPR spin-trapping method with DMPO as a spin-trap agent. Because DMPO in the spin-trapping method is at least 20 times more sensitive than the reduction of cytochrome *c* for the measurement of $O_2^{\cdot -}$ [35], it provides high reliability compared with lucigenin luminescence [36]. When PC12 cells were incubated with 1×10^{-4} M LY83583 for 2 min under air, formation of DMPO/$^{\cdot}$OH adduct was observed. This EPR signal was completely abolished by the addition of SOD, which indicated that $O_2^{\cdot -}$ was initially produced from PC12 cells in the culture medium by LY83583 stimulation. We could not detect the DMPO/$O_2^{\cdot -}$ adduct from PC12 cells because it decomposes rapidly to the DMPO/$^{\cdot}$OH adduct in cell systems [37]. To determine the contribution of enzymatic processes for $O_2^{\cdot -}$ generation from PC12 cells, DPI was added simultaneously with LY83583 because several authors reported that quinone compounds could accept electrons from flavoenzymes [24,38,39], and that PC12 cells have a DPI-sensitive oxidase system similar to the NADPH oxidase in phagocytes [40]. As shown in Fig. 8, DPI effectively attenuated the formation of DMPO/$^{\cdot}$OH adduct by LY83583, and this result indicated that flavoproteins contribute to ROS generation due to LY83583 stimulation in PC12 cells. However, no significant change of GSH and GSSG concentrations was observed when the PC12 cells were incubated with LY83583 for 20 min (Fig. 9). Although BSO treatment prior to the LY83583 stimulation reduced the GSH level (13 μmol/g protein, control; 6 μmol/g protein, BSO treatment), there was no difference of the intensity of the DMPO/$^{\cdot}$OH adduct (Fig. 8f).

We should not neglect the possibility that LY83583 interacts with intracellular GSH to form a GSH-conjugate, which results in ROS generation in vivo. However, our present data indicated that flavoproteins contributed to ROS generation in PC12 cells mainly by LY83583 stimulation, and the intracellular GSH participated less in ROS generation on short-term (2 min $\geq t$) incubation with LY83583. Recently, the cell toxicity of LY83583 has been reported in several papers. LY83583 causes induction of p21 in both human cell lines and PC12 cells, which results in apoptosis [41] and the activation of NF-κB [42], respectively. Judging from our present results, it is expected that this reported cell toxicity is responsible for the ROS generating activity of LY83583.

In conclusion, we found that LY83583 can be reduced not only by flavoenzymes but also by physiological thiol compounds such as GSH and cysteine, which result in the formation of ROS. Further study will be needed to clarify the pharmacological actions of LY83583 in terms of free radical chemistry.

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