Induction of superoxide in glioma cell line U87 stimulated with lipopolysaccharide and interferon-γ: ESR using a new flow-type quartz cell

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Abstract The production of superoxide and nitric oxide induced in U87 glioma treated with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) was examined by electron spin resonance (ESR) spectroscopy using a newly designed flow-type quartz cuvette without detaching cells from the culture plate. ESR spectra of 2,2,6,6-tetramethyl-4-hydroxy-1-piperidinyloxy (TEMPOL) with U87 cells on a quartz culture plate were measured at 15 min intervals. The signal intensity of TEMPOL decreased in the presence of U87 cells at the pseudo-first order rate. The signal decay was accelerated in the U87 cells treated with LPS/IFN-γ for 24 h, and was suppressed in the presence of superoxide dismutase and catalase. By the spin-trapping method, nitric oxide from U87 cells pretreated with LPS/IFN-γ for 24 h was measured by the ESR, but only a weak signal of nitric oxide adducts was detected. Further, the nitrite and nitrate levels in the medium did not increase for 24 h. By the ESR measurement of cells on culture plates without detachment stress, it was found that the production of superoxide was induced by LPS/IFN-γ, but that of nitric oxide was not, in U87 glioma cells.

Key words: Nitroxide spin label; Spin-trapping; Superoxide; Nitroxide; Glioma cell; Dithiocarboxysarcosine; Endotoxin

1. Introduction

Superoxide and nitric oxide are endogenous radical species, which are considered to play important roles not only in inflammation as protective factors, but also in signal transduction [1–4]. Recently, they have been reported to be involved in neurodegenerative diseases [5–7]. Just like immune cells such as macrophages in peripheral tissues, glial cells in the brain are considered to play a role in protecting against infection and oxidative stress. The production of nitric oxide and superoxide is suggested to be induced by treatment with cytokines or endotoxic reagents in several glioma cell lines. However, there have been only a few attempts to measure the induction of superoxide and nitric oxide [8–10]; by either a colorimetric method or an electrode-sensing method. The former method does not have good sensitivity, and for the latter, a special technique to make the electrodes is needed. Electron spin resonance (ESR) measurement is a useful way to detect radicals in biological systems. However, there have been no reports on the detection of superoxide and nitric oxide in glioma cells using ESR spectrometry. In this study, we investigated the production of superoxide and nitric oxide in a glioma cell line, U87, by measuring the superoxide-dependent spin decay of a nitroxide spin probe, and by spin-trapping of nitric oxide, respectively. The cells were measured by ESR without their detachment from culture glass plates, which does not enforce the stress caused by trypsinization or mechanical detachment, using a new cuvette and culture glass plates designed for adhesive cells.

2. Materials and methods

2.1. Chemicals

Superoxide dismutase (SOD), catalase (Cat), interferon-γ (IFN-γ) and minimum essential medium were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum, a mixture of antibiotics (penicillin–streptomycin–neomycin) and trypsin were from Gibco BRL (Rockville, MD, USA). Lipopolysaccharide (LPS) was from Difco Laboratories (Detroit, MI, USA). *N*-Dithiocarboxysarcosine (DTCS) was from Dojindo Laboratories (Kumamoto, Japan) and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) was from Aldrich (Milwaukee, WI, USA). *N*-Methyl-D-glucamine dithiocarbamate (MGD) was synthesized by following the method of Shinobu et al. [11]. *N*-Methyl-D-glucamine, carbon disulfide, *N*-monomethyl-L-arginine (L-NMMA) and $FeSO_4 \cdot 7H_2O$ were from Wako Pure Chemical Industries (Osaka, Japan). All the chemicals and reagents were of analytical or biochemical grade.

2.2. Cell culture

U87 glioma cells were maintained in minimum essential medium with 10% fetal bovine serum and a mixture of antibiotics at 37°C inside a humidified incubator with 5% CO_2/95% air. Experimental cultures were grown on quartz slide glasses (82 mm × 8.7 mm × 0.2 mm). After confirming U87 cells were grown subconfluently, the culture medium was changed to minimum essential medium with 500 ng/ml LPS and 150 U/ml IFN-γ without fetal bovine serum. After treatment with LPS and IFN-γ for 24 h, U87 cells on slide glasses were washed with Hank's balanced buffer solution in a flow-type cuvette (sample space thickness = 0.5 mm), and subjected to ESR measurements. To detect nitrite and nitrate, U87 cells were seeded into 48 well cell culture plates at 7.3×10^4 cells per well, and incubated at 37°C with 5% CO_2 for 24 h.

2.3. ESR measurement of U87 cells on slide glass

A slide glass with U87 cells was directly inserted into the new flow-type quartz cuvette (Fig. 1) designed for measurement of adhesive cells (Radical Research, Tokyo, Japan). The cuvette was connected

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Abbreviations: ESR, electron spin resonance; LPS, lipopolysaccharide; IFN-γ, interferon-γ; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; Fe-DTCS, iron complex of *N*-dithiocarboxysarcosine; L-NMMA, *N*-monomethyl-L-arginine; SOD, superoxide dismutase; Cat, catalase

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to the flow system as shown in Fig. 1, in which the upstream of the cuvette was connected to a reservoir tank warmed at 37°C, and the downstream to a peristaltic pump connecting to a drain. The cuvette was set in the cavity of a ESR spectrometer (FR-30, JEOL, Tokyo, Japan). The cells in the cuvette were washed with Hank's balanced buffer solution at the rate of 0.5 ml/min for 2 min. The cells were treated with 7 μM TEMPOL (Fig. 2, **1**), 10 mM DTCS-iron complex (Fe-DTCS) (DTCS, Fig. 2, **3**) or 10 mM MGD-iron complex (Fe-MGD) (MGD, Fig. 2, **4**) by introducing 2 ml solutions of these reagents into the cuvette at the rate of 0.5 ml/min, and then ESR spectra were measured. Because the total volume of the cuvette and tubes is 1.2 ml, the buffer solution inside the cuvette is discarded by adding 2 ml of the reagent solution.

2.4. TEMPOL signal decay with U87 cells

U87 cells on culture slide glasses were inserted into the flow-type cuvettes, and washed with Hank's balanced buffer solution. After a 2 ml solution of 7 μM TEMPOL in Hank's balanced buffer solution had been introduced into the cuvettes, ESR spectra were measured at 15 min intervals. ESR measurement conditions were as follows: field, 330–340 mT; modulation frequency, 100 kHz; modulation width, 0.2 mT; time constant, 1 s; sweep time, 8 min/10 mT; microwave frequency, 9.4 GHz; microwave power, 16 mW. The relative signal intensities of the lowest field signals of TEMPOL for MnO as an external standard were plotted as time lapsed. ESR spectra of 7 μM TEMPOL with LPS and IFN-γ-pretreated U87 cells were also measured in the presence of 100 U/ml SOD and 10 U/ml Cat. After the measurement, the cells on the slide glasses were trypsinized and enumerated.

2.5. Spin-trapping of nitric oxide with Fe-DTCS

Culture slide glasses with U87 cells were inserted into cuvettes and washed with Hank's balanced buffer solution. A 2 ml solution of 10 mM Fe-DTCS with or without SOD (1000 U/ml) or 10 mM Fe-MGD in 100 mM Tris–HCl buffer containing 0.25 M glucose was then introduced into each cuvette, and ESR spectra were measured at 8 min intervals. The measurement conditions were as for the TEMPOL measurements except that the field was 324–334 mT and time constant was 3 s.

2.6. Detection of nitrite/nitrate

U87 cells were treated with LPS/IFN-γ or L-NMMA for 24 h in minimum essential medium without fetal bovine serum. The concentration of nitrite and nitrate in the medium was measured with a NO_2/NO_3 detection kit-F (Dojindo, Kumamoto, Japan). Briefly, an aliquot of the medium was collected, and centrifuged at $1000\times g$ for 15 min at room temperature. According to the instructions, 80 μl of the supernatant was treated with nitrate reductase and co-enzyme for 1 h at 37°C, and then reacted with 2,3-diaminonaphtalene under acidic conditions at room temperature for 15 min. After neutralization with sodium hydroxide, the fluorescence from naphtalenetriazole at 460 nm (excited at 355 nm) was measured with a fluorescence plate reader (Fluoroskan Acent, Labsystems, Helsinki, Finland).

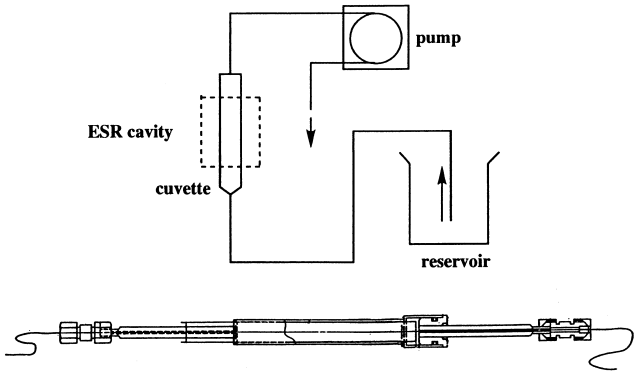

Fig. 1. The structure of the new flow-type quartz cell and a diagram of the ESR measurement system.

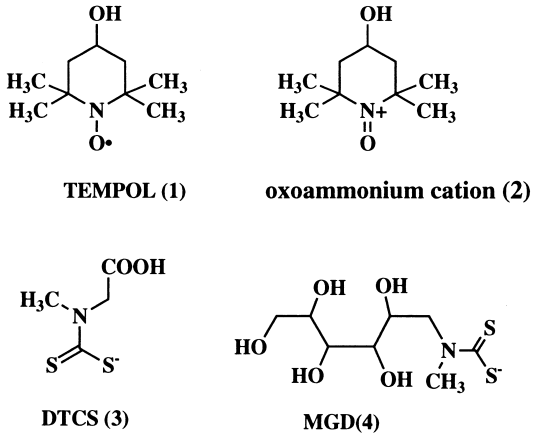

Fig. 2. Structure of TEMPOL (**1**), its oxoammonium cation (**2**), DTCS (**3**) and MGD (**4**).

3. Results

The effect of LPS with IFN-γ on the production of superoxide by U87 glioma cells was examined with a 9.5 GHz ESR spectrometer without detachment of the cells using an order-made flow-type cuvette designed for adhesive cells. The production of superoxide was evaluated by measuring the superoxide-dependent signal decay of TEMPOL. After a 2 ml solution of 7 μM TEMPOL in Hank's balanced buffer solution had been introduced into the cuvette, the ESR spectra of 7 μM TEMPOL were measured at 15 min intervals in the presence of U87 cells pretreated with or without 500 ng/ml LPS and 150 U/ml IFN-γ for 24 h. The relative intensities of the lowest field ESR signals of TEMPOL were measured and plotted as time lapsed. The decrease in the signal intensity of TEMPOL with U87 cells occurred at the pseudo-first order rate (Fig. 3).

Without U87 cells, the signal did not decay. The observed rate constant of the signal decay was increased in the case of U87 cells pretreated with LPS and IFN-γ (Figs. 3 and 4). The observed pseudo-first rate constants were calculated to be 3.05×10^{-3}/min/10^6 cells and 6.14×10^{-3}/min/10^6 cells in the case of non-treated cells and LPS/IFN-γ-pretreated cells, respectively. This increase of the rate constant by LPS/IFN-γ-pretreated U87 cells was inhibited in the presence of SOD and Cat (Fig. 4). The observed pseudo-first rate constant was calculated to be 3.39×10^{-3}/min/10^6 cells in the case of LPS/IFN-γ-pretreated cells with SOD and Cat. From the difference of the observed rate constants in the presence and absence of both SOD and Cat, the signal decay of TEMPOL with superoxide was calculated to be 2.75×10^{-3}/min/10^6 cells. Since the reaction rate constant of TEMPOL with superoxide has been calculated to be 3.90×10^7 M^{-1} min^{-1} [12], the concentration of superoxide in LPS/IFN-γ-treated U87 cells was estimated to be 70.5 pM/10^6 cells.

The effect of LPS with IFN-γ on the production of nitric oxide by U87 glioma cells was also examined by ESR using the flow-type cuvette without detachment of the cells. U87 cells on a culture slide glass were treated with 10 mM Fe-DTCS or 10 mM Fe-MGD for spin-trapping of nitric oxide from the cells [13,14]. ESR spectra were measured at 8 min intervals. In the case of U87 cells pretreated with LPS/IFN-γ, a trace amount of the ESR signal of the nitric oxide adduct of

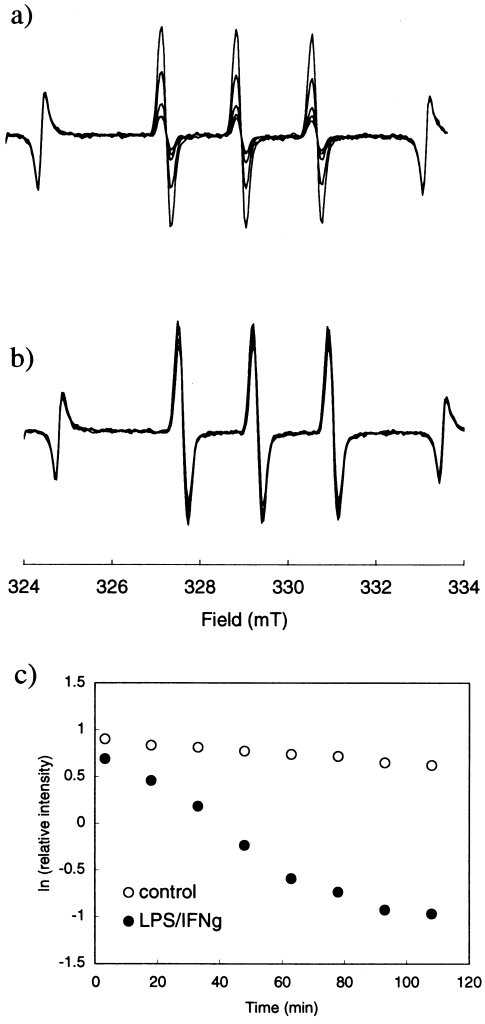

Fig. 3. ESR signal decay of TEMPOL with U87 glioma cells ESR spectra of the TEMPOL solution (the initial concentration is 7 μM) with U87 glioma cells cultivated on a quartz glass plate were obtained at 30 min intervals, (a) with LPS/IFN-γ-treated U87 cells, and (b) with control U87 cells. The time course of the relative signal intensity measured at 15 min intervals is presented in (c).

Fe-DTCS (NO-Fe-DTCS) was detected (Fig. 5) during the first scan (0–8 min after Fe-DTCS treatment), but no nitric oxide adducts were detected thereafter. The same result was obtained in the presence of SOD. Using MGD-ion complex, an ESR signal for nitric oxide adduct was not detected.

To evaluate the production of nitric oxide by U87 cells, the concentration of nitrite/nitrate accumulated for 24 h in the medium was measured with NO_2/NO_3 detection kit-F. It was not affected by treatment of LPS/IFN-γ, and decreased in the presence of L-NMMA (Fig. 6).

4. Discussion

The production of superoxide and nitric oxide by U87 glioma cells was measured by ESR without detachment of the cells using a flow-type cuvette designed for adhesive cells. The cuvette and the flow system made it possible to subject adhesive cells to ESR without the stress of detachment involving trypsinization and mechanical stress.

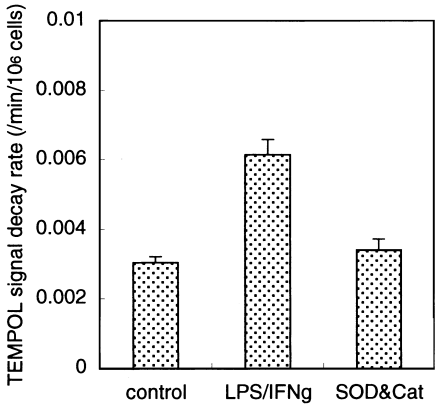

Fig. 4. Signal decay rate of TEMPOL by U87 glioma cells ESR spectra of the TEMPOL solution (the initial concentration is 7 μM) with U87 glioma cells on a quartz glass plate were obtained at 15 min intervals. The signal decay rate of TEMPOL with U87 glioma cells was calculated by measuring the decrease of ESR signal intensities of TEMPOL with U87 glioma cells treated with LPS/IFN-γ in the presence or absence of SOD and Cat. Values are presented as the mean ± S.E.M. of 3–5 experiments. One-way ANOVA indicated significant differences between groups ($P < 0.05$).

For evaluating the production of superoxide, the decay rate of TEMPOL signal was measured by ESR in the presence or absence of both SOD and Cat. The decay rate was increased by treatment with LPS/IFN-γ, but this increase was inhibited in the presence of both SOD and Cat to the control level (Fig. 4). This result suggested that superoxide production by U87 cells was induced by treatment with LPS/IFN-γ for 24 h. Nitroxide spin probes have been reported to act as a superoxide scavenger or a SOD-mimic [12,15–18]. TEMPOL was

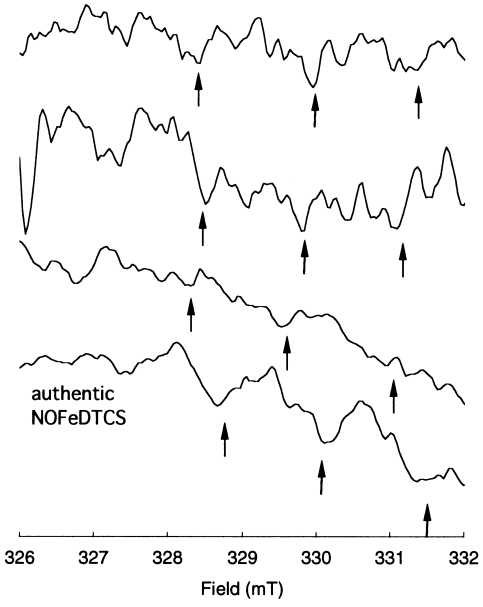

Fig. 5. ESR spectra of LPS and IFN-γ-treated U87 cells with Fe-DTCS U87 cells pretreated with LPS/IFN-γ for 24 h were treated with 10 mM of Fe-DTCS complex as a spin-trapping reagent for nitric oxide. ESR spectra were obtained from the U87 cells from 0 to 8 min after loading of Fe-DTCS. Each spectrum shows ESR signals obtained from independent samples except the bottom one, which is the signal from the authentic nitric oxide adduct of Fe-DTCS. In each spectrum, the weak signals from the nitric oxide adduct are indicated by arrows.

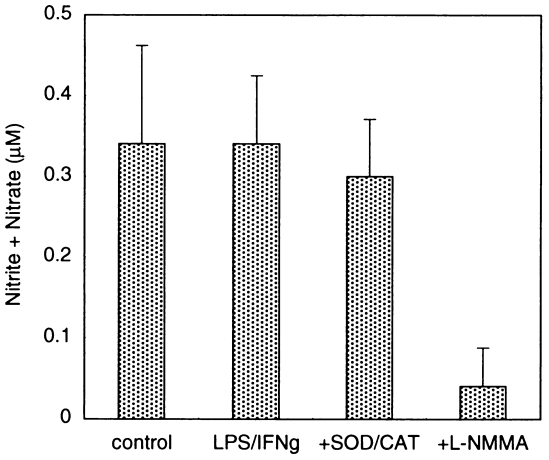

Fig. 6. Concentration of nitrite and nitrate in the culture media of U87 cells treated with LPS and IFN-γ. U87 cells at 7.3×10^4 cells per well were treated with LPS/IFN-γ for 24 h in culture media without serum and phenol red. After a reduction of nitrate to nitrite with nitrate reductase and a reaction with 2,3-diaminonaphtharene under acidic conditions, the concentration of nitrite in 80 μl of the media was evaluated by measuring the fluorescence intensity at 460 nm (excitation at 355 nm). Values are presented as the mean ± S.E.M. of 10 samples.

considered to act a SOD-mimic by the redox mechanism [11]. TEMPOL is readily oxidized by protonated superoxide to oxoammonium cation (Fig. 2, **2**) which in turn oxidizes another superoxide to molecular oxygen [19]. According to these reports, TEMPOL catalytically reacts with superoxide, so that the signal intensity of TEMPOL might not decrease depending on the production of superoxide. However, the decay rate of signal intensity of TEMPOL was considered to reflect the production of superoxide in this study, because the concentration of TEMPOL was much higher than that of superoxide produced in the cells which was practically spent only in the reaction with TEMPOL. The reaction of the oxoammonium cation with superoxide is considered to be negligible as long as the concentration of TEMPOL is high enough in the system.

Although the decay rate of TEMPOL with LPS/IFN-γ-treated U87 cells decreased at the same level as that of the control in the presence of SOD and Cat, the signal still decayed. This slow decay is assumed to be due to biological reductants which have been reported to reduce nitroxide spin probes including TEMPOL [20,21].

The production of nitric oxide by LPS/IFN-γ-treated U87 glioma cells was also examined by ESR without detachment of the cells. However, only a weak ESR signal due to the nitric oxide adduct of Fe-DTCS (NO-Fe-DTCS) was detected (Fig. 5) from U87 cells pretreated with LPS/IFN-γ. From this result, it is not clear whether nitric oxide is induced by LPS/IFN-γ in U87 cells. From the concentration of the nitrite/nitrate that had accumulated over 24 h in the medium, it was apparent that no nitric oxide was produced on treatment with LPS/IFN-γ. Because the concentration of nitrite/nitrate was decreased in the presence of L-NMMA (Fig. 6), a small amount of nitric oxide may be produced by the control cells. The mechanism of induction of nitric oxide for endotoxin and/or for cytokines is considered to be different. Recently, it has been reported that nitric oxide was not produced in some malignant glioma cells, but was involved in the malignant progression of cancers [10]. The loss of the nitric oxide production induced in U87 cells by LPS/IFN-γ is consistent with the tumorigenic nature of U87 glioma cells.

In conclusion, it was found that treatment with LPS/IFN-γ for 24 h induced production of superoxide, but not nitric oxide in a glioma cell line, U87, by ESR for cells using a superoxide-sensitive spin probe, TEMPOL, and spin-trapping reagents for nitric oxide, Fe-DTCS and Fe-MGD. The use of new cuvettes designed for adhesive cells and quartz culture plates is an effective way to measure glioma cells by ESR without their detachment from the plates.

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