Nitric Oxide Release from Porcine Aorta Endothelial Cells Measured by Electron Paramagnetic Resonance Spectroscopy

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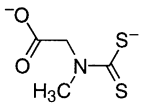
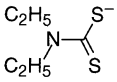
Nitric oxide (NO) generation from porcine aorta endothelial cells has been detected by electron paramagnetic resonance spectroscopy in combination with the spin trapping technique at 77 K or -160 °C. Water-soluble iron(III) complex with *N*-(dithiocarboxy)sarcosine has been used as an NO trapping agent.

Since endothelium-derived relaxing factor has been identified as nitric oxide (nitrogen monoxide; NO) or a closely related compounds derived from L-arginine,[1-3] a large number of studies concerning endogenous NO have been done. As a result, NO synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline and NO, have been found in various organs, tissues, and cells. Three distinct mammalian NOS isozymes are purified and characterized.[4] Among these isozymes, endothelial NOS (eNOS) generates the smallest amount of NO;[2] accordingly it is difficult to detect NO directly from endothelial cells.

Many methods have been developed to detect endogenous NO directly or indirectly. Only two methods have thus far succeeded in direct measurement of endothelium-derived NO. One is a microelectrode method[5,6] and the other is an electron paramagnetic resonance (EPR) spectral method with a spin trapping technique.[7] The advantage of the former one is a high sensitivity. Malinski and Taha could measure the NO released from a single endothelial cell.[5] It is, however, hard to prepare the high performance electrodes. Relative to the electrode method, it is easy to apply the spin trapping technique using iron complexes with dithiocarbamate derivatives (DTC) as spin trapping agents. Mülsch *et al.* used diethyldithiocarbamate (DETC), which yields water-insoluble $Fe(DETC)_2$ complex as an NO trapping agent, and detected the EPR signal of the resultant nitrosyl complex at 77 K by a usual EPR measurement protocol.[7]



DTCS



DETC

It has been reported that the lipophilic Fe-DETC complex can detect the endogenous NO from the lowest level, eNOS[7] to the highest level, macrophage's inducible NOS.[8] Recently we have developed an *N*-(Dithiocarboxy)sarcosine (DTCS) as a new NO trapping agent.[9-12] Unlike DETC, DTCS forms a water soluble iron complex and nitrosyl iron complex. By using the Fe-DTCS complex, we succeeded in detecting the *in vivo* EPR spectrum at 700 MHz and obtaining the *in vivo* EPR image of the endogenous NO in the abdominal region of lipopolysaccharide-treated mice.[10] Considering the sensitivity of the low frequency EPR, high solubility in water (~100 mM) of the trap is required to obtain strong signal intensity.[10] Furthermore, because of its water solubility, the preparation of the DTCS trap is easier than that of the DETC one.[12] However, the hydrophilic nature of the trap may be a disadvantage for the detection of intracellular NO like endothelial cellular one because eNOS localizes in membranes and NO will have tendency to dissolve in the membrane and lipid phases of cells.[4] In this study, therefore, we applied the Fe-DTCS trap to the measurement of the NO generation from endothelial cells to examine whether the hydrophilic trap can detect the lowest level of NO or not.

Porcine aorta endothelial cells (17 -18 passage, 1 x 10^7) were cultured on Petri dishes and grown to confluence. Being washed twice with phosphate buffered saline (PBS), the cells were detached by cell lifter or TE solution (trypsin 0.025%; ethylenediaminetetraacetic acid 0.02%; PBS). The suspended cells were centrifuged and resuspended in modified Hanks' medium (Hepes, 10 mM; L-arginine, 1 mM; pH 7.4). The cells were incubated (20 min, 37 °C) with the Fe-DTCS complex[12] (final 6 mM; [DTCS·2Na]/[$FeCl_3$] = 3) and stimulants in a normal quartz EPR cell (o.d. = 5 mm). Total volume of the sample solution was 0.5 ml. In this study, adenosine 5'-triphosphate (ATP) or calcium ionophore A23187 (CI-A) was used as the stimulant. After incubation, the head space of EPR cell was purged with nitrogen gas and then the cells were frozen in liquid nitrogen for EPR measurements. EPR spectra were recorded at 77 K or -160 °C and at X-band frequency. Here it should be noted that we used not the DTCS diammonium salt but the DTCS disodium salt. The toxicity of the diammonium salt is higher than that of the disodium salt (a gift from Dojindo Laboratories), the LD50 being 765 mg/kg and 1942 mg/kg in mice, respectively.

Figure 1 shows the EPR spectrum of aqueous solution of chemically synthesized $[NO\text{-}Fe(DTCS)_2]^{2-}$ complex at 77 K. Compared with the spectrum at room temperature, which shows isotropic triplet signal at $g = 2.040$,[12] the spectrum at 77 K is an axially symmetric one ($g_\perp = 2.037$ and $g_{//} = 2.015$).[13] Endothelial

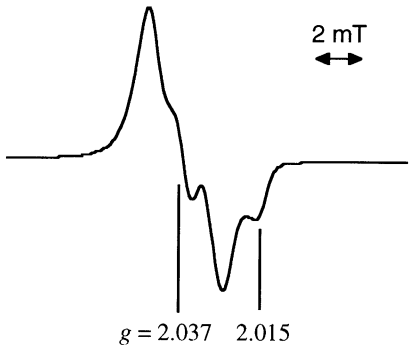


Figure 1. X-band EPR spectrum of $[NO\text{-}Fe(DTCS)_2]^{2-}$ solution at 77 K.

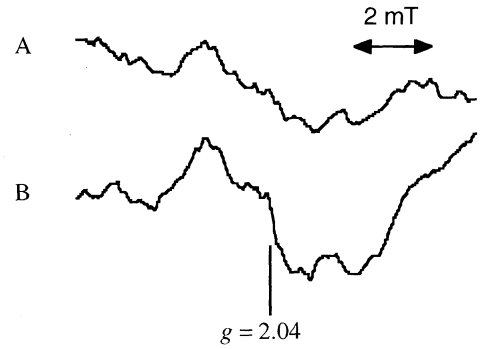


Figure 2. EPR spectra of endothelial cells stimulated by ATP (0.1 mM) at -160 °C. (A) 1 x 10^7 cells. (B) 3 x 10^7 cells. Instrument settings: microwave power 10 mW, modulation width 0.79 mT, and amplitude 2 x 1000.

cells (1 x 10^7) stimulated by ATP (0.1 mM) at – 160 °C exhibited a small EPR signal at g = 2.04 (Figure 2A). Control cells without stimulus did not exhibit such a signal (data not shown); hence, this EPR signal is likely to stem from the $[NO\text{-}Fe(DTCS)_2]^{2-}$ complex (Figure 1). When the number of the cells increased to 3 x 10^7, the signal intensity increases and the distinctive $g_\perp$ component could be observed (Figure 2B). These results indicate that the Fe-DTCS complex can trap the NO derived from eNOS and the resultant NO complex is produced.

More distinct signal could be observed when CI-A (0.1 μM) is used as a stimulant (Figure 3). The CI-A increases the intracellular Ca^{2+} level, so that eNOS is directly activated by Ca^{2+} - calmodulin.[1-4] The fact that the rise in the intracellular Ca^{2+} level increased the signal intensity of the NO adduct is supported by the results of following freeze-thaw experiments. Changes in the spectra are shown in Figure 4. The cells (1 x 10^7) stimulated by ATP (0.1 mM) were frozen and thawed repeatedly. As the number of times increased, the signal became larger. It is widely known that the freeze-thaw process causes a severe damage to the membranes. The damage allows the influx of extracellular Ca^{2+}. As a result, the freeze-thaw process should induce a similar effect to CI-A's.

In conclusion, we have demonstrated that the NO production from endothelial cells can be detected by the water-soluble Fe-DTCS complex. The Fe-DTCS trap, being different from the Fe-DETC trap, scarcely permeate the membrane; consequently, most

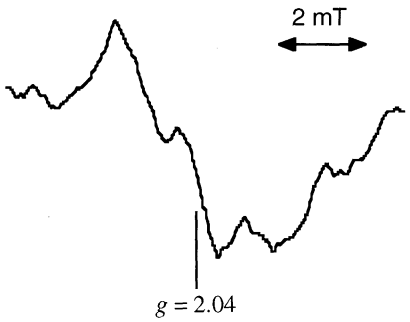


Figure 3. EPR spectrum of endothelial cells (1 x 10^7) stimulated by calcium ionophore A23187 (0.1 μM) at 77 K. At – 160 °C, this signal is larger than the one shown in Figure 2A. Other instrument settings as in the legend of Figure 2.

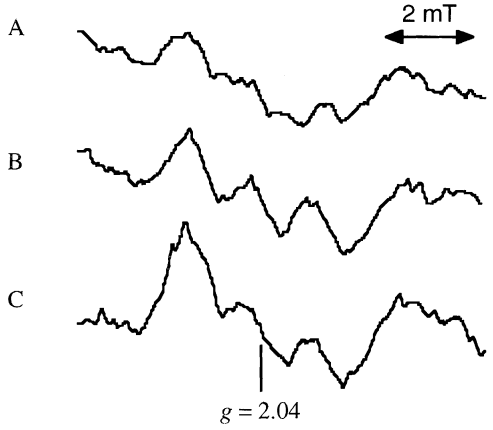


Figure 4. EPR spectra of endothelial cells (1 x10^7) stimulated by ATP (0.1 mM) at – 160 °C. After incubation (20 min, 37 °C), the cells were frozen (A); then the cells were thawed and frozen again (B). (C) is the spectrum of the cells frozen three times. Other instrument settings as in the legend of Figure 2.

of the detected NO are probably the ones which diffuse out from endothelial cells. NO synthesized by endothelial cells diffuses into adjacent vascular smooth muscle cells where it activates guanylyl cyclase, and ultimately leads to vasorelaxation.[1-4] It is, therefore, meaningful to detect and analyze the intercellular NO diffused out from endothelial cells. In addition, since the number of the used cells (~ 10^7) is a suitable level for testing their response to drugs and the preparation of the Fe-DTCS trap is easy, this NO detection method is useful for examining the effects of drugs on the NO production not only from endothelial cells but from other cells, tissues, or organs containing NOS.

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