Indirect Detection of Reactive Oxygen Species by ^{1}H NMR: Monitoring of Reduced Form of Nitroxyl Spin Probes

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Introduction

Nitroxyl spin probes are used for the studies of biological redox systems and oxidative stress in living systems. They are readily reduced to the corresponding N-hydroxylamines by oxidation-reduction enzymes (e.g. cytochrome P-450, mitochondria electron transport), and reactive oxygen species (ROS, e.g. hydroxyl radical (•OH), superoxide (O_2•-)).

Fig. 1. Nitroxyl spin probes are reduced to the corresponding N-hydroxylamines.

Their bioreduction is measured as the decay of nitroxyl ESR signals by time-course *in vitro* and *in vivo* ESR. In previous work from our laboratory, the mechanisms of diseases related to ROS generation were examined by *in vivo* ESR/nitroxyl spin probes technique in various animal models, such as carbon tetrachloride-induced liver injury [1], streptozotocin-induced diabeties [2], and iron-induced diseases [3]. *In vivo* ESR experiments were performed in the low resonance frequency (1.1 GHz for mice, 300 MHz for rats) because of the penetration of electromagnetic waves.

The reduced form of nitroxyl spin probes, N-hydroxylamines should become detectable by NMR because of their diamagnetism. Nitroxyl spin probes have bulky methyl groups to increase their stability. The monitoring of its methyl protons by ^{1}H MRS should make possible that the studies of ROS-related diseases are investigated by the combination of MRS and MRI technique.

The purpose of this study is to establish the indirect detection of ROS by detecting N-hydroxylamines reduced from nitroxyl spin probes by ^{1}H NMR and to evaluate the applicability of this study on the living animal by ^{1}H MRS.

Methods

^{1}H NMR measurements were performed at 400 MHz (9.4 T) or 600 MHz (14.1 T) (UNITY-INOVA, Varian). The nitroxyl spin probe used was 4-Oxo-2,2,6,6-tetramethylpiperidine-1-oxyl (4-oxo-TEMPO, Fig.1). All solutions were prepared in 10 mM phosphate buffer (pH 7.4) with 10% v/v D_2O. •OH was generated by the Fenton reaction (1 mM H_2O_2, 1 mM $FeSO_4$, 20 mM DTPA). For the quantitative studies, N-hydroxylamine was prepared by reducing with ascorbate, and 3-Trimethylsilyl-1-propane sulfonic acid (TSP, 1 mM in a sample) was added as an internal standard for quantitation. ^{1}H NMR spectra were measured with water suppression. T_1 and T_2 were determined with inversion recovery and CPMG method, respectively.

The human hepatoma HepG2 cells were used as an *in vitro* model in this study. Cells (1 x 10^6) were inoculated on polystyrene-coated 100-mm tissue culture dishes in minimum essential medium supplemented with 10 % fetal bovine serum, and cultured at 37^{0}C in humidified atmosphere of 5% CO_2. After preculture for 72 h, 4-oxo-TEMPO (1 mM in medium) was added to the dishes, and then 0.5 ml of the medium were measured by ^{1}H NMR (10% D_2O, 1 mM TSP).

Results and Discussion

The two peaks at 1.2 and 2.6 ppm were detected by ^{1}H NMR after reaction of 4-oxo-TEMPO with the Fenton reagents (Fig. 2). The chemical shift of these two peaks agreed with the authentic sample of the corresponding N-hydroxylamine. The N-hydroxylamine of 4-oxo-TEMPO gave singlet peaks of four CH_3 (i.e. twenty protons), which has large advantage for high sensitive ^{1}H MRS measurement and low dose administration, and two CH_2. Although the chemical shift of the CH_3 group is close to that of the lactate methyl group, these peaks woule be distinguished using the J-modulation method.

The peak height of CH_3 and CH_2 in the N-hydroxylamine increased linearly with the concentration of ascorbate in the 0.1-0.4 mM range (Fig. 3a). Relaxation times of these peaks were not significantly decreased in the presence of the parent radical (Fig. 3b), which should be suitable for the quantitative analysis by ^{1}H MRS.

The reduction of 4-oxo-TEMPO to N-hydroxylamine was gradually occurred in the incubation with cultured cells (Fig. 4), but not without cells. 4-oxo-TEMPO was reduced by not only constitutive ROS but also several oxidoreductases in cells. These observation indicates that this method should be applied to *in vivo* ^{1}H MRS and be a great help to the study using ROS-related disease models or transgenic mice.

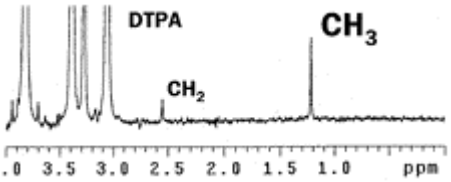

Fig. 2. ^{1}H NMR spectrum of the N-hydroxylamine of 1 mM 4-oxo-TEMPO reduced by hydroxyl radicals generated by the Fenton reaction in phosphate buffer (pH 7.4) at 600 MHz.

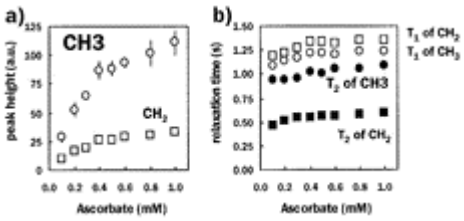

Fig. 3. ^{1}H NMR peak height (a), T_1 (open) and T_2 (filled) (b) of N-hydroxylamine of 1 mM 4-oxo-TEMPO as a function of the concentration of ascorbate (0.1-1.0 mM) in pH 7.4 at 400 MHz, 37^{0}C (n=5) .

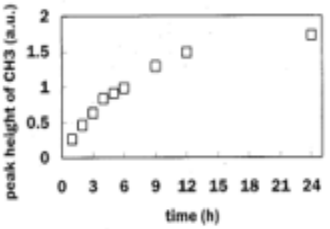

Fig. 4. Time course of CH_3 peak height of N-hydroxylamine of 4-oxo-TEMPO after incubation with HepG2 cell cultures (n=3). SD was within 6% of the mean.

References

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