Sensitive ESR Determination of Intracellular Oxidative Stress by Using Acyl-Protected Hydroxylamines as New Spin Reagents

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Several acyl-protected hydroxylamines were synthesized as new spin reagents for ESR measurements of intracellular oxidative stress. These compounds were stable non-radical compounds, but were easily deprotected with esterase to yield hydroxylamines, which were oxidized by oxidants to yield ESR- detectable nitroxide radical. Using an acyl-protected hydroxylamine, a highly sensitive ESR determination procedure was successfully conducted to analyze oxidative stress in human leukocytes.

It has been believed that oxidative stress caused by active oxygen species plays a role in certain aspects of various biological states. For observing such oxidative stress in biological systems, ESR measurements of spin reagents, including spin trapping reagents and spin probes, have been performed.[1-3] Spin trapping reagents such as nitrones or nitroso compounds can be used for selective detection of active oxygen species such as superoxide or hydroxyl radical; but their signal intensities and stabilities are inadequate in biological systems.[1] Spin probes such as nitroxide radicals are stable radicals. By using them, one can evaluate the reducing capacity of a biological system on the basis of their reduction.[2,3] However, with this technique one is not able to obtain direct information on oxidative stress. It has been reported that hydroxylamines are easily oxidized by oxidative stimulations (such as the production of active oxygen species) and the oxidized products are stable nitroxide radicals.[4-6] We believe that hydroxylamines can function as spin reagents and be used to evaluate oxidative stress. But hydroxylamines are very unstable in aqueous media because of their high susceptibility to oxidants. To overcome this, we treated hydroxylamines with acyl-protectors. In cells, acyl-protected hydroxylamines can be easily deprotected with esterase to yield hydroxylamines, which will be oxidized by oxidants to yield ESR-detectable nitroxide radical. In this study, some acyl-protected hydroxylamines were synthesized as new spin reagents. By employing them, ESR measurements of intracellular oxidative stress were made.

A nitroxide radical, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL, Figure 1a), was reduced by using hydrazine monohydrate to yield a hydroxylamine, 1-hydroxy-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine (Figure 1b).[7] The hydroxylamine was acylated by using acetic, propionic, butyric, or benzoic anhydride to yield 1-acetoxy-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine (ACP, Figure 1c), 1-propionyloxy-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine (PCP, Figure 1d), 1-butyryloxy-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine (BuCP, Figure 1e), or 1-benzoyloxy-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine (BeCP, Figure 1f), respectively.[7,8] Each compound was purified by recrystallization [final yields, 86% (ACP), 85% (PCP), 88% (BuCP), and 95% (BeCP)]. The end products were stable non-radical compounds that were not affected by oxidation. The compounds with larger acyl-sites showed smaller water solubility. BeCP could not be dissolved without an organic solvent such as dimethyl sulfoxide (DMSO). The polarity of the compounds is changed from hydrophobic-ester to hydrophilic-hydroxyl by deprotection. This characteristic is applicable to cell or in vivo studies. All acyl-protectors were deprotected with esterase to yield hydroxylamine; then the latter was easily oxidized by sodium periodate to give nitroxide (carbamoyl-PROXYL), which can be confirmed with an X-band ESR spectrometer.[9] There was a linear relationship between concentrations of esterase and ESR signal amplitudes. The reaction rates of PCP and BuCP were about 10 times greater than those of ACP and BeCP. The differences in reactivity are related to the magnitude of the acyl-sites.

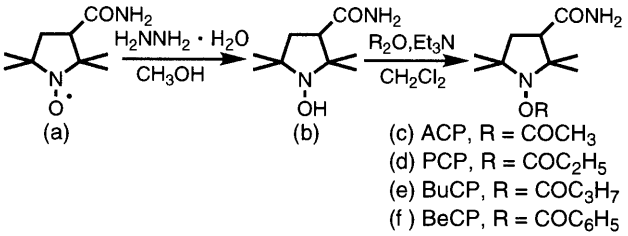

Figure 1. Syntheses of acyl-protected hydroxylamines.

It is well-known that human oral polymorphonuclear leukocytes (OPMNs) release active oxygen species such as superoxide without any stimuli.[10-12] The superoxide release is prompted by neutrophil stimulants such as phorbol 12-myristate 13-acetate (PMA).[10-12] OPMNs were prepared from human saliva.[13] ACP was used as the acyl-protected hydroxylamine because of its high water solubility. An investigation was conducted on the reaction of ACP with OPMNs, enhancement by PMA, and suppression by superoxide dismutase (SOD), a specific enzyme for superoxide disproportionation. ACP (10 mmol dm^{-3}), PMA (16 nmol dm^{-3}), and SOD (100 unit cm^{-3}) were prepared in a phosphate buffer solution (PBS). Four kinds of mixtures were prepared to yield final volumes of 180 mm3: 1) OPMNs and ACP; 2) OPMNs, ACP, and PMA; 3) OPMNs, ACP, PMA, and SOD; 4) OPMNs, ACP, and SOD. These mixtures were subjected to ESR measurements. After 150 min, they were oxidized with sodium periodate, and were again subjected to ESR to evaluate the total amount of hydroxylamine that were formed in these reactions.

Figure 2 shows the time course of the ESR signal intensities. Under all conditions, the signal intensities increased with time. The addition of PMA enhanced the formation of a nitroxide radical. The addition of SOD inhibited the increase independent of the addition of PMA. Thus it is thought that these results reflect appreciable oxidation with intracellular superox-

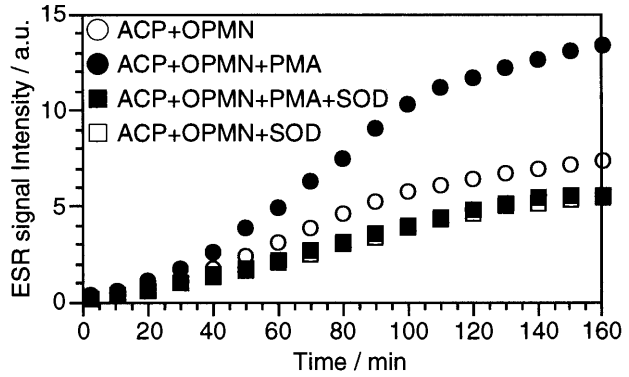

Figure 2. Time course of the ESR signal intensity in the mixture including ACP and OPMN after the mixture.

ide and/or secondary produced hydroxyl radical. Because the ESR signal intensity derived from acyl-protected hydroxylamine reflects nonspecific oxidation (i.e., oxidative stress in a broad sense) in the cell, such an experiment is needed to assign the oxidant. Figure 3 shows the amounts of hydroxylamine and nitroxide: the former indicates the total amount of ACP that was deprotected by intracellular esterase; and the latter indicates the amount of hydroxylamine that was oxidized by the intracellular oxidant. Under all conditions, ACP of almost the same quantity has been deprotected to yield hydroxylamine. However a difference becomes eminent relative to nitroxide formation, indicating the difference in the amount of oxidants.

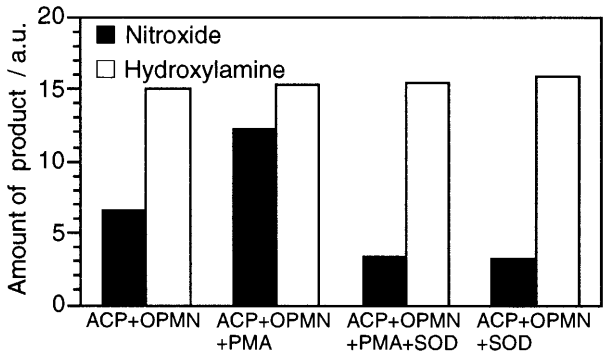

Figure 3. Formation of nitroxide and hydroxylamine in the mixture including ACP and OPMN 150 min after the mixture.

For comparison, a conventional spin trapping reagent, 10 or 100 mmol dm^{-3} of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), was used in the same manner for the ESR measurement instead of ACP. The ESR signal was detected with DMPO in the concentration of 100 mmol dm^{-3} (10 times higher than that for ACP) but not with 10 mmol dm^{-3} of DMPO. The signal obtained is due to a hydroxyl spin-trapped adduct (DMPO-OH) but not a superoxide spin-trapped adduct (DMPO-OOH). However SOD completely inhibited the generation of this spin-trapped adduct. Under this condition, DMPO-OH is the predominant spin-trapped adduct resulting from neutrophil superoxide generation, perhaps due to intracellular reduction of DMPO-OOH to DMPO-OH.[14-17]

These results show high sensitivity in determining oxidative stress by employing acyl-protected hydroxylamines. Because DMPO is not sufficiently stable after spin-adduct formation, the ESR signal intensity decreases. On the other hand, the signal strength derived from acyl-protected hydroxylamines is intensified by oxidizing because nitroxide is more stable. We believe that by using these new spin reagents ESR measurements can be applied to in vivo studies because of the high sensitivity of this technique.

References and Notes

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7 The molecular structures of the synthesized compounds were confirmed by NMR or mass spectrometer. The results were as follows. In 1-hydroxy-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine, ^{1}H-NMR (in DMSO): δ 0.88 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.52 (dd, J_1 = 12.4 Hz, J_2 = 8.1 Hz, 1H, CH_2), 1.96 (t, J = 11.8 Hz, 1H, CH_2), 2.48 (dd, J_1 = 11.8 Hz, J_2 = 8.7 Hz, 1H, CH), 6.81 (s, 1H, $CONH_2$), 7.12 (s, 1H, OH), 7.15 (s, 1H, $CONH_2$). In ACP, ^{1}H-NMR (in DMSO): δ 0.97 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 1.15 (s, 3H, CH_3), 1.64 (dd, J_1 = 12.4 Hz, J_2 = 7.4 Hz, 1H, CH_2), 2.06 (s, 3H, CH_3), 2.07(br, 1H, CH_2), 2.62 (br, 1H, CH), 6.94 (s, 1H, $CONH_2$), 7.27 (s, 1H, $CONH_2$). Mass spectrum (EI^+): *m/z* 228.3 (M^+). In PCP, ^{1}H-NMR (in DMSO): δ 0.97 (s, 3H, CH_3), 1.06 (t, J = 7.4 Hz, 3H, CH_3), 1.09 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.64 (dd, J_1 = 12.4 Hz, J_2 = 7.4 Hz, 1H, CH_2), 2.12 (br, 1H, CH_2), 2.36 (q, J = 7.4 Hz, 2H, CH_2), 2.62 (br, 1H, CH), 6.94 (s, 1H, $CONH_2$), 7.27 (s, 1H, $CONH_2$). Mass spectrum (EI^+): *m/z* 242.3 (M^+). In BuCP, ^{1}H-NMR (in DMSO): δ 0.91 (t, J = 7.4 Hz, 3H, CH_3); 0.97 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.58 (sext., J = 7.4 Hz, 2H, CH_2), 1.64 (dd, J_1 = 12.4 Hz, J_2 = 7.4 Hz, 1H, CH_2), 2.12 (br, 1H, CH_2), 2.32 (t, J = 7.4 Hz, 2H, CH_2), 2.62 (br, 1H, CH), 6.94 (s, 1H, $CONH_2$), 7.27 (s, 1H, $CONH_2$). Mass spectrum (EI^+): *m/z* 256.3 (M^+). In BeCP, ^{1}H-NMR (in DMSO): δ 1.10 (s, 3H, CH_3), 1.20 (br.s, 12H, $3CH_3$), 1.72 (dd, J_1 = 12.4 Hz, J_2 = 7.4 Hz, 1H, CH_2), 2.22 (br, 1H, CH_2), 2.72 (br, 1H, CH), 6.99 (s, 1H, $CONH_2$), 7.32 (s, 1H, $CONH_2$), 7.56 (t, J = 8.1 Hz, 2H, ArH), 7.68 (t, J = 7.4 Hz, 1H, ArH), 7.97 (dd, J_1 = 8.1 Hz, J_2 = 1.2 Hz, 2H, ArH). Mass spectrum (EI^+): *m/z* 290.4 (M^+).
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