Reactive oxygen species generation through NADH oxidation by pterin derivatives

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ABSTRACT

Pterin is an electron transfer compound in biological systems. Among the analogs, 6-formylpterin (6FP) has been demonstrated to have many marked physiological and pharmacological activities. In previous study, we have elucidated that 6FP derivatives in which the 3-position is modified possess reactive oxygen species (ROS), which are involved in the modulation of a variety of cell functions, generation activities through the oxidation of NADH to NAD$^+$ in the dark at neutral pH. In the present study, we have demonstrated that the ROS generation activity by 6FP derivative is enhanced in the presence of 3-methyl-1-phenyl-2-pyrazolin-5-one. In this reaction, 3-methyl-1-phenyl-2-pyrazolin-5-one is reacted with the formyl group on the 6-position of 6FP derivative to give the activated product. The present results would be helpful for designing pharmaceutical ROS generation system in vivo.

INTRODUCTION

Pterin is an electron transfer compound present in biological systems. Some endogenous pterins are known to play important roles in immune and cytokine systems and also work as cofactors for nitric oxide synthase and enzymatic hydroxylation of aromatic amino acids. 6-formylpterin (6FP, Scheme 1), a pterin derivative is produced from folic acid by photodestruction and produced in vivo from folic acid in some pathological conditions, such as carcinoma. In previous studies, we have shown that reactive oxygen species (ROS) such as H$_2$O$_2$ generated by 6FP, induced apoptosis in HL-60 cells, inhibited Fas-mediated apoptosis in Jurkat cells, and suppressed activation of NF-$\kappa$B, cytokine production, and cell proliferation in PanC-1 and human blood T cells. We have also demonstrated that 6FP derivatives such as 2-(N,N-dimethylaminomethyleneamino)-6-formyl-3-pivaloylpteridin-4-one (DFP, Scheme 1), 2-(N,N-dimethylaminomethyleneamino)-6-formyl-3-methylpyrazolin-4-one, and 2-Amino-6-formyl-3-methylpteridin-4-one with modified 3-position can generate H$_2$O$_2$ through NADH to NAD$^+$ oxidation in the dark in phosphate-buffered saline (PBS) at pH 7.4 without the aid of any biological systems. In addition, DFP showed suppressed proliferation of PanC-1 cells. We presume that the above-mentioned chemical activity, which is intracellular ROS generation through oxidation of biomolecules such as NADH, is involved in the mechanism of physiological activities of DFP in Panc-1 cells. As reported previously, it is now widely known that ROS is not only involved in cell death but is also involved in the modulation of a variety of cell functions. Therefore, it is important to understand the relationship between ROS activity and biological events. ROS generation system by pterin derivative which can generate controllable amount of ROS without any biological systems would be useful in pharmaceutical investigations.

RESULTS AND DISCUSSION

PBS solutions containing (a) 300 $\mu$M DFP and 300 $\mu$M NADH (b) 300 $\mu$M DFP, 300 $\mu$M 3-methyl-1-phenyl-2-pyrazolin-5-one (Scheme 1), and 300 $\mu$M NADH were stirred in the dark in open system and the time-dependent concentration change of each component in the sample solutions was analyzed. In the absence of DFP, the concentration of NADH remained constant over 100 h. Although the oxidation of NADH to NAD$^+$ was observed in both systems, when 3-methyl-1-phenyl-2-pyrazolin-5-one was added to the reaction mixture containing DFP and
NADH, the oxidation rate of NADH to NAD$^+$ was dramatically enhanced (Figure 1). In the system (b), a compound named DFP-E was produced as a product from the reaction of DFP with 3-methyl-1-phenyl-2-pyrazolin-5-one. From NMR analysis, 3-methyl-1-phenyl-2-pyrazolin-5-one was reacted with the 6-formyl group on DFP to give DFP-E. When PBS (pH 7.4) suspending solutions of DFP-E containing NADH were stirred in the dark in open system, decrease of NADH and generation of NAD$^+$ was observed (data not shown). From the results, it was found that DFP-E has chemical activity to oxidize NADH to NAD$^+$ in the dark. The reason why the system (b) in Figure 1 showed high NADH oxidation activity is that compared to DFP, DFP-E has stronger chemical activity of the NADH oxidation.

To investigate the O$_2$-related species generated in this NADH oxidation reaction, the EPR spin trapping technique using DMPO as a spin trap was employed. PBS solutions (pH 10) containing 1 mM DFP-E (or 200 mM DFP-E, at pH 7.4) and 2 mM NADH were incubated for 48 h and diethylenetriaminepentaacetic acid (500 μM), FeSO$_4$ (250 μM), and DMPO (100 mM) were added to the reaction mixture, and EPR analysis was performed. Note that in the presence of ferrous ion (Fe$^{2+}$), hydroxyl radical (OH) is generated from H$_2$O$_2$ via the iron-catalyzed Fenton reaction. The solutions containing either DFP-E or NADH did not show EPR signals over 48 h (data not shown). The solution containing DFP-E and NADH showed a quartet with the hyperfine splitting constant (hfsc) of $a$(N) = 1.49 mT and $a$(BH) = 1.49 mT (data not shown). The analogous quartet with the same hfsc as the above quartet was also produced when PBS suspending solution (pH 7.4) containing 200 μM DFP-E and 2 mM NADH was analyzed after 48 h incubation. These peaks were quenched by adding catalase (10000 units/mL) to the reaction crude composed of DFP-E and NADH. Based on these observations, it was found that DFP-E generates ROS such as H$_2$O$_2$ from O$_2$ in the presence of reducing compound such as NADH in the dark. Consequently, it is suggested that NADH was oxidized by DFP-E to NAD$^+$, and then the reduced form of DFP-E generated in the process of NADH to NAD$^+$ conversion reduced O$_2$ to H$_2$O$_2$.

**CONCLUSION**

We developed novel ROS generation system by coexistence of pterin derivative and 3-methyl-1-phenyl-2-pyrazolin-5-one without any biological systems. In the reaction, DFP was activated to DFP-E by the reaction with 3-methyl-1-phenyl-2-pyrazolin-5-one and the ROS generation activity was enhanced. The present results would be helpful for designing pharmaceutical ROS generation system in vivo.

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**REFERENCES**


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