Pteridines
Vol. 10, 1999, pp. 32 – 34

Pterin-6-aldehyde, Xanthine Oxidase Inhibitor and Superoxide Scavenger, Directly React with Peroxynitrite

Hiroko Mori[1], Toshiyuki Arai[§1], Hisanari Ishii[1], Nobuyuki Endo[2], Toshinori Suzuki[2] and Kazuhiko Fukuda[1]

[1]Department of Anesthesia, Kyoto University Hospital, Kyoto, Japan
[2]Institute of Advanced Energy, Kyoto University, Uji, Japan

(Received September 1, 1998)

Summary

The effect of pterin-6-aldehyde (P6A), xanthine oxidase inhibitor and superoxide scavenger, on the production of nitrotyrosine as a footprint of tyrosine nitration by peroxynitrite, was compared with that of uric acid, a peroxynitrite scavenger. The amounts of tyrosine, P6A and nitrotyrosine were quantified using reversed-phase high-performance liquid chromato-graphy (RP-HPLC). P6A suppressed nitrotyrosine formation less effectively than uric acid, that is, 0.25 mM P6A reduced nitrotyrosine formation to 67.9±10.8%, while 0.025 mM uric acid reduced it to 34.2±1.6%.

In living systems, peroxynitrite is generated by the reaction of super-oxide with nitric oxide and has a variety of toxic effects. Our results show that P6A is not necessarily a strong scavenger of peroxynitrite. However, since P6A is a potent scavenger of superoxide, P6A is thought to totally suppress peroxynitrite generation. Compounds that scavenge both superoxide and peroxynitrite may be useful in tissue damage in which reactive oxygen species are involved.

Key words : Pterin-6-aldehyde, Peroxynitrite, Nitrotyrosine

Introduction

Recently, we found that pterin-6-aldehyde (P 6A), a xanthine oxidase (XOD) inhibitor, has a superoxide (O_2^-) scavenging activity in the neutrophil/phorbol myristate acetate (PMA) reaction system (1) and that P6A attenuates the neuronal damage in global brain ischemia in gerbils (2). We hypothesized that the neuro-protective effects of P6A are exerted through O_2^- scavenging activity and XOD inhibition. More recently, however, we found that another XOD inhibitor, oxypurinol, also has a potent O_2^- scavenging activity but does not attenuate ischemic neuronal damage in gerbils (3). This finding raised doubts regarding the mechanism of neuroprotection by P6A and indicated the effects of P6A on other reactive oxygen intermediates (ROI) responsible for ischemic injury.

Peroxynitrite is a strong oxidant generated by nitric oxide (NO) and O_2^- and plays an impor-

§Author to whom correspondence should be addressed.

*Parts of the work were presented at the 5th Meeting of Cytokine and Neopterin held on July 18, 1998, in Nagoya, Japan. Toshiyuki Arai, Department of Anesthesia, Kyoto University Hospital, 54 Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.

tant role in cerebral injury (4). Since it is known that P6A has an O_2^- scavenging activity, P6A may suppress peroxynitrite generation. However, it is not known whether P6A directly reacts with peroxynitrite.

To elucidate the reaction of P6A with peroxynitrite, we examined the effect of P6A on the production of nitrotyrosine as a footprint of tyrosine nitration by peroxynitrite.

Materials and Methods

Reagents

P6A was synthesized and its purity was determined as previously described ADDIN ENRef (1). Tyrosine, nitrotyrosine and uric acid were purchased from Nacalai Tesque (Kyoto, Japan) and dissolved in phosphate-buffered saline (PBS, pH 7.4).

Preparation of Peroxynitrite

Peroxynitrite was prepared as described previously with some modifications ADDIN ENRef (5). 0.6 M $NaNO_2$ and 0.6 M H_2O_2 in 0.7 M HCl were pumped at 7 ml/min into a T-junction and mixed. The peroxynitrous acid that formed, was quenched by pumping 1.5 M NaOH at 14 ml/min. Excess H_2O_2 was destroyed by granular MnO_2. The solution was frozen at -80°C. The yellow solution that contains the high concentration of peroxynitrite was obtained by melting the frozen solution slightly. It was removed and used for further study. The concentration of peroxynitrite was determined by absorbance at 302 nm ($\varepsilon_{302\ nm}$=1670 M^{-1}/cm^{-1}).

Production-analysis

The RP-HPLC system consisted of an LC-6A pumping system, an SCL-6B column oven and a CTO-6A system controller (Shimadzu, Kyoto, Japan). On line UV spectra were obtained with a SPD M6A UV-VIS photodiode-array detector (Shimadzu). For RP-HPLC, a Cosmosil 5C18-MS octadesylsilane column (4.6×150 mm and particle size 5 μm, Nacalai Tesque, Japan) was used. The eluent was 100 mM triethylammonium acetate buffer (pH 7.0). All measurements were performed at the flow rate of 1.0 ml/min at a column temperature of 40°C. The CH_3CN concentration increased in the linear gradient mode (0 min: 0%, 20 min: 20%).

Freshly prepared peroxynitrite was added to PBS containing 0.25 mM tyrosine and appropriate concentrations of P6A. The final concentration of peroxynitrite was adjusted to 0.25 mM and 200 μl of the reaction mixture was injected into the HPLC system. The retention time of nitro-tyrosine was confirmed by the injection of an authentic sample and the concentrations of the generated nitrotyrosine were estimated from the areas of the HPLC chromatogram and the molar extinction coefficients at 260 nm ($\varepsilon_{260\ nm}$). The RP-HPLC peak area of nitrityrosine in the absence of P6A was used as a standard (100%). The peroxynitrite scavenging activity of P 6A was compared with that of uric acid, a peroxynitrite scavenger.

Results and Discussion

In the production-analysis, P6A suppressed nitrotyrosine formation but its efficacy was far less than that of uric acid. For example, 0.25 mM P6A reduced nitrotyrosine formation to 67.9± 10.8%, while 0.025 mM uric acid reduced it to 34.2±1.6% (Fig. 1).

Nitric oxide and O_2^- rapidly react to peroxynitrite and its decomposition is thought to generate a strong oxidant, hydroxyl radical (6). Although it is controversial whether peroxynitrite actually

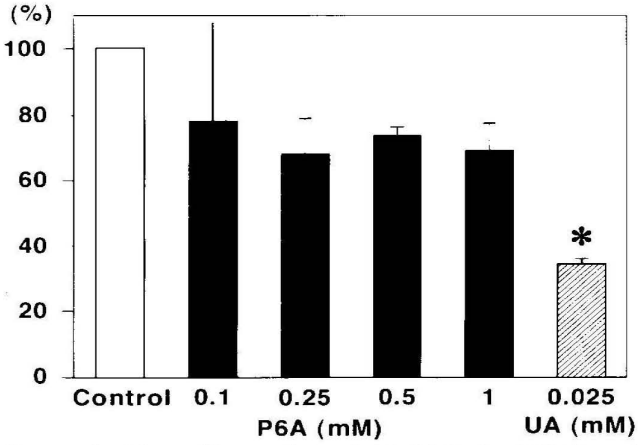

Figure 1. The effects of pterin-6-aldehyde (P6A) and uric acid (UA) on the formation of nitrotyrosine. Peroxynitrite was added to PBS containing 0.25 mM tyrosine and 0, 0.1, 0.25, 0.5 and 1 mM P6A, or 0.025mM UA. The final concentration of peroxynitrite was adjusted to 0.25 mM. The vertical axis shows the percentage of the nitrotyrosine production when that in the absence of P 6A was assigned to 100%. Data are shown as means±SD (n=3). Statistical comparison of results were done by Students t-test. p-values <0.05 were considered to indicate statistical significance ($*p<0.05$).

generates hydroxyl radicals (7), peroxynitrite is toxic and reacts with tyrosine to form nitrotyrosine (4). Since direct detection of peroxynitrite is difficult, we measured nitrotyrosine as a foot print of tyrosine nitration by peroxynitrite, using RP-HPLC.

Scavenging of peroxynitrite is thought to be useful for prevention of tissue damage. It has been reported that uric acid, a peroxynitrite scavenger, can delay the onset of clinical signs of experimental allergic encephalomyelitis in mice (8). Also it has been reported that 7,8-dihydroneopterin completely prevents nitration by peroxynitrite (9). In the present study, P6A inhibited nitrotyrosine formation less effectively than uric acid, which may mean P6A is not an effective peroxynitrite scavenger. However, since P6A has O_2^- scavenging activity, it should reduce the peroxynitrite formation itself in the presence of NO. Direct reaction of P6A with NO is now been investigated.

P6A, a XOD inhibitor and an O_2^- scavenger, has been shown to have a weak but positive scavenging activity of peroxynitrite. Compounds that act both as iNOS inhibitors and as a peroxynitrite scavenger are useful for the prevention of peroxynitrite-induced oxidative damage (10). Therefore, both peroxynitrite and superoxide scavenging activity may provide an account of the neuroprotective effect of P6A.

References

1. Watanabe K, Arai T, Mori H *et al.* Pterin-6-aldehyde, an inhibitor of xanthine oxidase, has superoxide anion radical scavenging activity, Biochem. Biophys. Res. Commun. 1997; 233: 447-450.
2. Mori H, Arai T, Ishii H *et al.* Neuroprotective effects of pterine-6-aldehyde in gerbil global brain ischemia: comparison with those of α-phenyl-*N*-*tert*-butyl nitrone, Neuro. Lett. 1998; 241: 99-102.
3. Arai T, Mori H, Ishii H *et al.* Oxypurinol, a xanthine oxidase inhibitor and a superoxide scavenger, did not attenuate ischemic neuronal damage in gerbils, Life Sci. 1998; 63: 107-112.
4. Beckman JS. Peroxynitrite versus hydroxyl radical: The role of nitric oxide in superoxide-dependent cerebral injury, Ann. N. Y. Acad. Sci. 1994; 738: 69-75.
5. Beckman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite, Meth. Enzymol. 1994; 233: 229-240.
6. Beckman JS, Beckman TW, Chen J, Marshall PM, Freeman BA. Apparent hydroxyl radical production from peroxynitrite: Implications for endothelial injury by nitric oxide and superoxide., Proc. Natl. Acad. Sci. U.S.A. 1990; 87: 1620-1624.
7. Pou S, Nguyen SY, Gladwell T, Rosen GM. Does peroxynitrite generate hydroxyl radical?, Biochem. Biophys. Acta 1995; 1244: 62-68.
8. Hooper DC, Bagasra O, Marini JC, Zborek A, Ohnishi TS, Kean R. Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: Implications for the treatment of multiple sclerosis, Proc. Natl. Acad. Sci. U.S.A. 1997; 94: 2528-2533.
9. Widner B, Baier-Bitterlich G, Wede I, Wirleitner B, Fuchs D. Neopterin derivatives modulate the nitration of tyrosine by peroxynitrite, Biochem. Biophys. Res. Commun. 1998; 248: 341-346.
10. Szabo C, Ferrer-Sueta G, Zingarelli B, Southan GJ, Salzman AL, Radi R. Mercaptoethylguanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite-induced oxidative damage, J. Biol. Chem. 1997; 272: 9030-9036.