Pteridines
Vol. 10, 1999, pp. 202–206

# Modulation of LPS-induced Cell Death by 5,6,7,8-Tetrahydrobiopterin

Hiroyuki Iizuka, Hirofumi Sagara and Shuji Kojima§

Research Institute for Biological Sciences, Science University of Tokyo, Chiba 278-0022, Japan

(Received November 1, 1999)

## Summary

It has been previously reported that 5,6,7,8-tetrahydrobiopterin ($BH_4$) modulates HL-60 cell death induced by a nitric oxide (NO) donor, S-nitroso-N-acetyl-D, L-penicillamine (SNAP). In this study, the role of endogenous BH4 was investigated in lipopolysaccharide (LPS)-induced apoptotic cell death. LPS induced an increase of DNA fragmentation and of nitrite and nitrate content (NOx content) in the macrophage-like RAW 264.7 cell line. 2,4-Diamino-6-hydroxypyrimidine (DAHP), an inhibitor of $BH_4$ synthesis, suppressed both of them. The NOx content of cells treated with LPS and interferon-γ was much higher than that of cells treated with LPS alone. However, the degree of apoptotic cell death induced by LPS and interferon-γ did not differ significantly from that induced by LPS alone. Further investigation revealed that LPS-induced cell death of RAW264.7 cells was mainly mediated by reactive oxygen species, such as hydrogen peroxide ($H_2O_2$). From these data, it was speculated that BH4 might be a modulator in NO-induced apoptosis, in which BH4 involves LPS-induced cell death by its function as a cofactor of inducible NO synthase and it suppresses the cell death mediated by NO and/or $H_2O_2$ via an antioxidative activity.

**Key words:** 5,6,7,8-Tetrahydrobiopterin ($BH_4$), Apoptosis, Nitric oxide (NO), Lipo-polysaccharide (LPS), Hydrogen peroxide

## Introduction

5,6,7,8-tetrahydrobiopterin ($BH_4$) acts as a cofactor of inducible nitric oxide synthase (iNOS) (1). Nitric oxide (NO) generated by iNOS shows feature of radicals, and it induces apoptotic cell death (2). On the other hand, we reported that $BH_4$ has antioxidative activity (3) and shows modulatory effect on S-nitroso-N-acetyl-D,L-penicillamine (SNAP), an NO-donor, -induced apoptosis of HL-60 cell (4). Accordingly, it was speculated that $BH_4$ modulates NO-induced apoptosis by means of its antioxidant activity, in addition to the function as a cofactor of iNOS.

In this study, we evaluated effects of endogenous $BH_4$ in LPS-induced apoptotic cell death.

## Materials and Methods

### *Chemicals*

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui medical Co., Ltd, (Tokyo, Japan). 2,4-Diamino-6-hydroxypyrimidine (DAHP), superoxide dismutase (SOD), catalase (CAT), and

§Correspondence: Dr. S. Kojima, Research Institute for Biological Sciences, Science University of Tokyo, Chiba 278-0022, Japan. Tel/Fax:+81-471-23-9755, E-mail: kjma@rs.noda.sut.ac.jp

*Parts of the work were presented at the 6th Meeting of Cytokine and Neopterin held on July 24, 1999, in Tokyo, Japan

ribonuclease A were obtained from Sigma Chemical Company, (St. Louis, MO, USA). λHindIII, agarose, proteinase K, ethylendiaminetetracetic acid 2Na (EDTA 2Na), boric acid, ethidium bromide, bromophenol blue, glycerol, interferon-g, and lipopolysaccharide (LPS) were from Wako Pure Chemicals Co., Ltd., (Osaka, Japan).

*Culture of cells*

The mouse monocyte/macrophage cell line, RAW 264.7 was purchased from Riken Cell Bank (Tsukuba, Japan). Cells were maitained in DMEM containing 10% fetal bovine serum. Cells ($4\times10^5$ cells/ml) were seeded at a density of $4\times10^5$ cells/ml in multiplates (Corning Co., NY, USA). They were then incubated at 37°C, under $5\%O_2+95\%CO_2$, for 24 hrs. The medium was replaced by fresh medium. One or a combination of the following substances, was added to the culture: 1 μg/ml LPS, 100 U/ml Interferon-γ, and 1 mM DAHP. The cells were incubated for 24 hrs under the same conditions.

*Quantitation of apoptotic cell death*

Following the 24-hr incubation, the supernatant of each of the cell cultures was collected, and applied to the nitrite and nitrate content (NOx content) assay. The NOx content of supernatant is a marker of NO generation. DNA samples for agarose gel electrophoresis were extracted as previously described (4). Each sample was subject to 1.8% agarose gel electrophoresis. The electrophoresis was carried out in TBE buffer (pH 7.4), which contained 89 mM Tris, 89 mM borate, 2 mM EDTA, and ethidium bromide as running buffer, at 100 V for 90 min; Hind III was used as the marker. The DNA fragmentation pattern was examined in photographs taken under UV illumination. The degree of apoptotic cell death, that is the degree of DNA fragmentation, was determined by densitometry. DNA fragments of molecular size below 7 kb, were considered to have undergone apoptotic cell death. The percentage of apoptotic nuclei was expressed as a percentage of total nuclei density.

*Measurement of NOx content*

The NOx content of cell cultures, was measured with the ENO-5000 NOx Analysis System (EICOM, Tokyo, Japan). Briefly, nitrite and nitrate were separated by high-performance liquid chromatography (HPLC), and passed through a Cd column. The concentrations of nitrite and nitrate, were determined using a Griess reagent, which consisted of 1% sulfanilamide, 0.1% naphthyl-ethylenediamine dihydrochloride, and 5% $H_3PO_4$. The absorbance at 540 nm was measured. Nitrite and nitrate were each quantified using $NaNO_2$ and $NaNO_3$, respectively, as standard.

## Results

LPS, a substance which induces iNOS as well as BH4 synthesis, induced apoptotic cell death, as well as DNA fragmentation. DAHP suppressed the LPS-

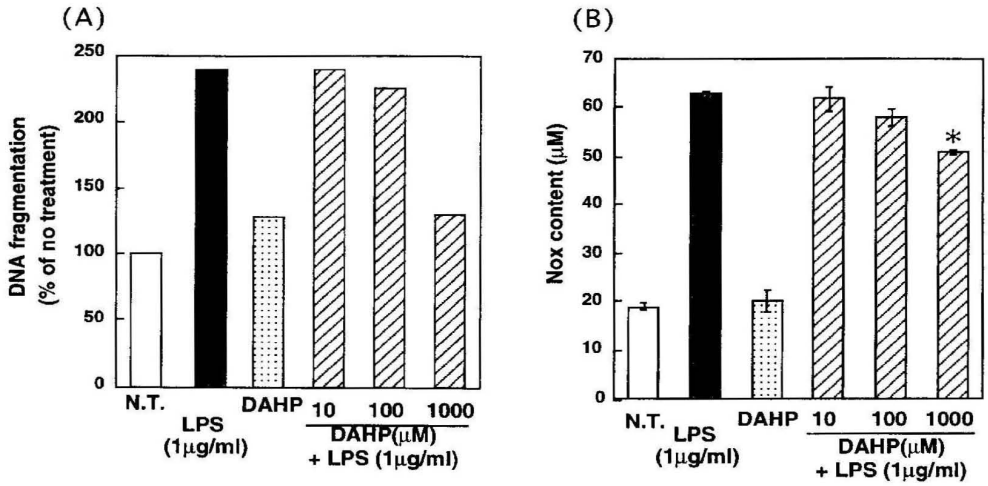


Figure 1. Effect of DAHP on LPS-induced apoptotic cell death of RAW264.7 cells. (A) Degree of DNA fragmentation and B) NOx content in cultured medium. Cells ($4\times10^5$ cells/well) were incubated with LPS (1 μg/ml), DAHP (1000 μM), and LPS (1 μg/ml) plus DAHP (10-1000 μM) for 24hr. (A, B). Each column in Figure 1 (B) represents the mean±S.E.M. of quadruplicate assays. The statistical significance of differences compared with LPS alone group $^*p<0.05$) was determined by means of Student's *t*-test. N.T., no treatment.

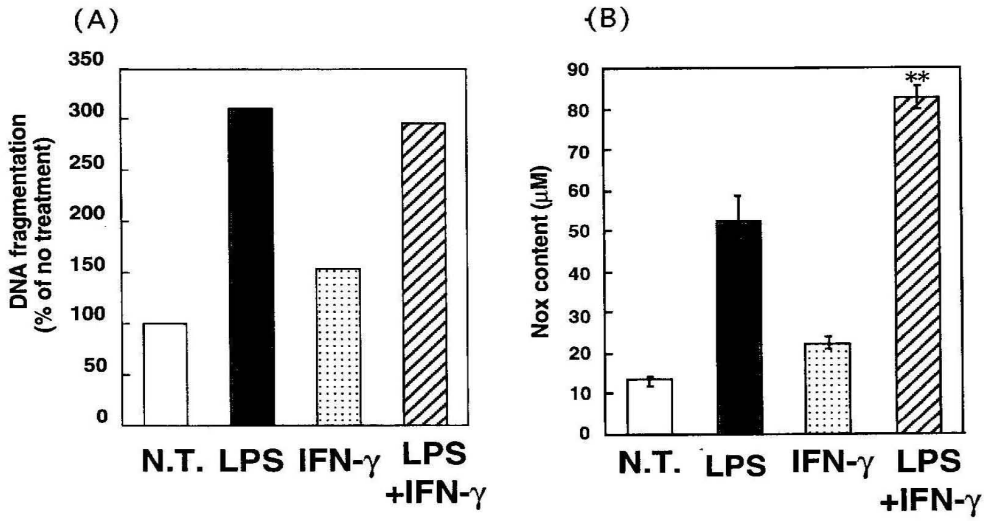


Figure 2. Effect of interferon-γ on LPS-induced apoptotic cell death of RAW264.7 cells. (A) Degree of DNA fragmentation and (B) NOx content in cultured medium. Each column in Figure 2 (B) represents the mean±B S.E.M. of quadruplicate assays. The statistical significance of differences compared with LPS alone group (**$p<0.01$) was determined by means of Student's t-test. N.T., no treatment; LPS, lipopolysaccharide (1 μg/ml); IFN-γ, interferon-γ (100 U/ml); LPS+IFN-γ, lipopolysaccharide (1 μg/ml) plus interferon-γ (100 U/ml).

induced enhancement of DNA fragmentation, in a dose-dependent fashion (Fig. 1-A). DAHP also inhibited the LPS-induced enhancement of NOx generation (Fig. 1-B).

Interferon-γ is a substance that induces the synthesis of pteridines, including $BH_4$. LPS induced an increase in the degree of DNA fragmentation (Fig. 2-A).

Combination of LPS with interferon-γ, however, did not increase much higher degree of DNA fragmentation than LPS alone, though cells treated with LPS and interferon-γ displayed a much higher NOx content than with LPS alone (Fig. 2-B).

Effect of antioxidants on the cell death induced by LPS was examined in order to ascertain an involvement of reactive oxygen species (ROS) in this event. SOD and CAT both suppressed the elevation of lactate dehydrogenase (LDH) activity, a maker of cell damage, induced by LPS, in a dose-dependent manner (Figs. 3). The efficacy of CAT was much stronger than that of SOD, suggesting an involvement of $H_2O_2$ in LPS-induced RAW 264.7 cell death.

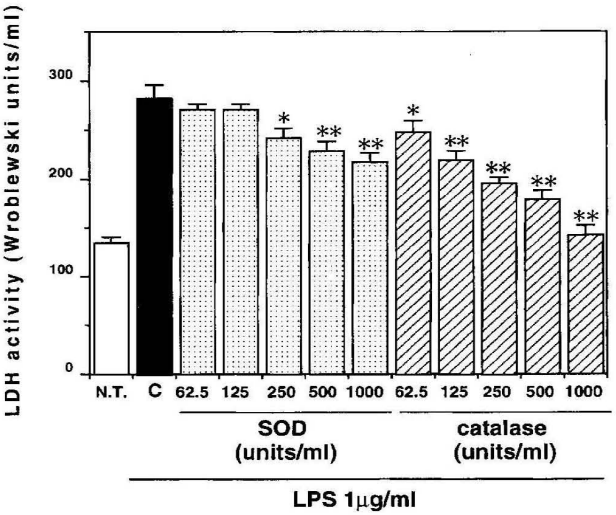


Figure 3. Effects of superoxide dismutase (SOD) and catalase (CAT) on LPS-induced apoptotic cell death of RAW264.7 cells. Lactate dehydrogenase (LDH) activity in the supernatant of cultured medium was assayed as a marker of cell damage. The statistical significances of differences compared with LPS alone (C) group (*$p<0.05$ and **$<0.01$) were determined by means of Student's *t*-test. N.T., no treatment; C, 1 μg/ml of LPS alone. Appropriate concentrations of SOD or CAT were added to the medium in the presence of 1 μg/ml of LPS. Each column represents the mean±S.E.M. of quadruplicate assays.

## Discussion

Role of endogenous $BH_4$ in apoptotic cell death was examined in mouse monocyte/macrophage cell line, RAW264.7 cell. It has been reported that BH4 acts as a limiting factor in LPS-induced cell death, while it also is cofactor of iNOS (5-7). Indeed, we obtained similar results in that DAHP inhibited apoptotic cell death induced by LPS. DAHP inhibited apoptotic cell death, and it inhibited the increase in NOx content to nearly the same content (Fig. 1). Interferon-γ induces the synthesis of BH4, and also induces NOx generation (8-11). In this study, the amount of NOx generated in RAW264.7 cells

treated with LPS and interferon-γ, was apparently higher than that of the cells treated with LPS alone (Fig. 2-B). However, the degree of apoptosis induced by treatment with LPS and interferon-γ, was similar to that induced by treatment with LPS alone (Fig. 2-A). The reason why interferon-γ affected NOx content and degree of apoptotic cell death to a different degree, is obscure. As interferon-γ induces the biosynthesis of $BH_4$, in regard to apoptotic cell death it is assumed that $BH_4$ acts as a cofactor of iNOS and that $BH_4$ also has a suppressive effect on apoptosis. Brune, *et al.* (12) reported that the expression of p53 gene is an important factor in NO-induced apoptotic cell death of RAW264.7 cells. In their study, S-nitrosoglutathione (GSNO)-induced apoptotic cell death was inhibited by treatment with LPS plus interferon-γ, in which the combination of LPS and interferon-γ inhibits p53 gene expression in the presence of NG-monomethyl-L-arginine (L-NMMA), a substance which inhibits iNOS expression. Accordingly, in our experiments, $BH_4$ may have been generated by direct stimulation of interferon-γ and not through the pathway mediated by NO, $BH_4$ might be involved in inhibiting apoptotic cell death through its effect on p53 gene expression.

It has been reported that the concentration of $BH_4$ directly affects the release of neurogenic modulators, such as catecholamines in smooth muscle cells (13, 14). Also, it has previously been reported that neopterin, another pteridine derivative and precursor of $BH_4$, increases the release of cytokines, such as interleukin-6 and GM-CSF, in cultured bone marrow cells (15). Accordingly, it is suspected that $BH_4$ may inhibit apoptotic cell death by affecting p53 gene expression directly, or through a cytokine-mediated mechanism.

As for apoptosis, $H_2O_2$, as well as NO, induce apoptosis of HL-60 cells. In this study, effects of antioxidants, such as SOD and CAT, on the cell death induced by LPS, were also examined. The cell death was clearly suppressed by these antioxidants, in particular CAT, suggesting that $H_2O_2$ is involved in the cell death induced by LPS (Fig. 3). When the concentration of $BH_4$ in the brain is low, NOS in the brain generates $H_2O_2$ instead of NO (16). Furthermore, $BH_4$ shows scavenging activity on reactive oxygen species such as $H_2O_2$ (3). Thus, $BH_4$ may affect the level of $H_2O_2$ by acting as a cofactor, or through direct scavenging activity. This is further evidence that $BH_4$ is involved in suppressing apoptosis.

Furthermore, it was shown that endogenous $BH_4$ induced by treatment with sepiapterin directly increased the degree of apoptotic cell death. However, no significant increase of NOx content was observed (Data not shown). We previously reported that exogenously-added $BH_4$ to HL-60 cells, inhibited apoptotic cell death in the early incubation period, and enhanced apoptotic cell death after a long incubation period (4). Thus, it is supposed that $BH_4$ shows inducible factors for apoptosis, as well as the suppressive factors, under various conditions in which NO is not involved. Volk, *et al.* suggested that NO and/or $H_2O_2$ act as a lethal factor when unregulated production of either species occurs (for instance, after exposure to cytokines), but that NO and $H_2O_2$ regulate normal cellular functions (17).

Accordingly, a high level of cytokines, such as interferon-γ, may reflect a disregulated and/or a disease condition, and that $H_2O_2$, NO, and BH4 are involved in apoptosis through complex pathways. They may each have a direct effect on apoptosis; on the other hand, the relative levels of cytokines may be important.

In any case, it is supposed that $BH_4$ is closely involved in the modulation of apoptotic cell death, and that it also modulates apoptosis in a NO-independent pathway. More detailed investigations are now under way in order to find the exact mechanisms underling the modulation of $BH_4$ on cell death.

## Acknowledgment

This study was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

## References

1. Kwon NS, Nathan CF, Stuehr D J. Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. J Biol Chem 1989; 264: 20496-20501.
2. Albina JE, Cui S, Mateo RB, Reichner JS. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J Immunol 1993; 150: 5080-5085.
3. Kojima S, One S, Iizuka H, Arai T, Mori H, Kubota K. Antioxidative activity of 5,6,7,8-tetrahydrobiopterin and its inhibitory effect on paraquat-induced cell toxicity in cultured rat hepatocytes. Free Rad Res 1995; 23: 419-430.
4. Kojima S, Nimura K, Komatsu H, Taguchi T, Iizuka H. Modulation of S-nitroso-N-acetyl-D,L-penicillamine (SNAP) induced HL-60 cell death by tetrahydrobiopterin. Anticancer Research 1997; 17: 929-938.
5. Giovanelli J, Campos KL, Kaufman S. Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation

of arginine. Proc Natl Acad Sci 1991; 88: 7091-7095.
6. Klatt P, Heinzel B, Mayer B, Ambach E, Wemer-Fermayer G, Wachter H. Werner ER. Stimulation of human nitric oxide synthase by tetrahydrobiopterin and selective binding of the cofactor. FEBS Lett 1992; 305:160-162.
7. Wemer-Fermayer G, Werner E.R, Fuchs D, Hausen A, Reibnegger G, Schmidt K, Weiss G, Wachter, H. Pteridine biosynthesis in human endothelial cells. Impact on nitric oxide-mediated formation of cyclic GMP. J Biol Chem 1993; 268: 1842-1846.
8. Wermer-Felmayer G, Werner ER, Hausen A, Reibnegger G, Wachter H. Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. J Exp Med 1990; 172: 1599-1607.
9. Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Wachter H. Tumor necrosis factor-alpha and lipopolysaccharide enhance interferon-induced tryptophan degradation and pteridine synthesis in human endothelial cells. J Biol Chem 1989; 370: 1063-1069.
10. Werner ER, Werner-Fermayer G, Fuchs D, Hausen A, Reibnegger G, Wachter H. Impact of tumor necrosis factor-alpha and interferon-gamma on tetrahydrobiopterin syntesis in murine fibrobalsts and macrophages. Biochem J 1991; 280: 709-714.
11. Hevel JM, Marletta MA. Macrophage nitric oxide synthase: relationship between enzyme-bound tetrahydrobiopterin and sythase activity. Biochemistry 1992; 31: 7160-7165.
12. Brune B, Golkel, Knethen A V. Cytokine and low-level nitric oxide prestimulation block p53 accumulation and apoptosis of RAW 264.7 macrophages. Biochem Biophys Res Commun 1996; 229: 396-401.
13. Miwa S, Watanabe Y, Hayashi O. 6R-L-erythro-5,6,7,8-tetrahydrobiopterin as a regulator of dopamine and serotonin biosynthesis in the rat brain. Arch Biochem Biophys 1985; 239: 234-241.
14. Koshimura K, Miwa S, Lee K, Fujiwara M, Watanabe, Y. Enhancement of dopamine release in vivo from the rat striatum by dialytic perfusion of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin. Neurochem. 1990; 54: 1391-1397.
15. Aizawa S, Hiramoto M, Araki S, Negishi S, Kimura Y, Hoshi H, Kojima S, Wakasugi K. Stimulatory effects of neopterin on hematopoiesis *in vitro* are mediated by activation of stromal cell function. Hematol Oncol 1998; 16: 57-67.
16. Heinzel B, John M, Klatt P, Bohme E, Mayer B. $Ca^{2+}$ calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. Biochem J 1992; 281: 627-630.
17. Volk T, Ioannidis I, Hensel M, deGroot ,Kox WJ. Endothelial damage induced by nitric oxide: Synergism with reactive oxygen species. Biochem Biophys Res Commun 1995; 213: 196-203.