Effect of 5,6,7,8-Tetrahydrobiopterin (BH₄) on γ -Ray-induced Micronucleus Frequency in RAW264.7 Cells

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Abstract

The effect of 5,6,7,8-tetrahydrobiopterin (BH₄), an endogenous antioxidant, on γ -ray-induced micronucleus frequency was examined in RAW264.7 cells. Exogenously added BH₄ reduced the γ -ray-induced micronucleus frequency. Treatment with 1 mM sepiapterin, a biosynthetic precursor of BH₄, also significantly reduced the micronucleus frequency induced by 3-10 Gy γ -rays, by 20-35% at 18 h post-treatment. The reducing effect of SP on γ -ray-induced micronucleus frequency was blocked by N-acetyl-5-hydroxytryptamine and methotrexate, which are inhibitors of BH₄ biosynthesis. These results suggest that intracellular BH₄ biosynthesized from sepiapterin is involved in the reducing effect of SP on γ -ray-induced micronucleus frequency. To examine possible mechanisms underlying this action, changes of intracellular BH₄ concentration after sepiapterin treatment were examined. Intracellular BH₄ concentration peaked at 6 h and was maintained at a high level until 24 h after sepiapterin treatment. However, the γ -ray-induced micronucleus frequency at 6 h post-treatment with sepiapterin showed no change in comparison with that after irradiation alone. These results indicate that the reducing effect of BH₄ on γ -ray-induced micronucleus frequency is not necessarily due to antioxidant activity, but may involve other mechanisms.

Key words: 5,6,7,8-tetrahydrobiopterin, γ -ray, micronucleus

Introduction

5,6,7,8-Tetrahydrobiopterin (BH₄) is a pteridine derivative that is biosynthesized from guanosine triphosphate (GTP). Its chemical structure and biosynthesis via the salvage pathway are shown in Fig. 1. BH₄ is an essential cofactor for aromatic amino acid hydroxylases, i.e., tyrosine hydroxylase, tryptophan hydroxylase and phenylalanine hydroxylase (1-3), and for nitric oxide synthases (NOS) (4-7). BH₄ is also an endogenous antioxidant, with scavenging activity against reactive oxygen species (ROS) and protective activity against paraquat-induced cell toxicity (8, 9) and nitric oxide (NO)-induced apoptotic cell damage (10).

Ionizing radiation leads to many types of DNA damage, such as base modifications, single-strand breaks (SSBs) and double-strand breaks (DSBs). DSBs are the most critical lesions, because unrepaired or misrepaired DSBs can cause chromosome breaks, be

lethal for cells and lead to mutagenesis and carcinogenesis. Chromosome breaks lacking centromeres in dividing cells are unable to move to the spindle poles during mitosis and are detected in the cytoplasm besides the cell nucleus as small nucleus-like particles called micronuclei (MN) (11, 12). Hence, the cytokinesis-block micronucleus (CBMN) assay is used widely for evaluating chromosome breaks induced by ionizing radiation and chemical mutagens *in vitro*. DNA damage induced by low-linear energy transfer (LET) ionizing radiation such as γ -rays and X-rays is mainly caused via indirect action, and hydroxyl radicals produced by the radiolysis of water are believed to be the primary active species responsible (13-15). Consequently, various antioxidants, such as ascorbic acid, α -tocopherol, β -carotene (16), flavonoids (17), and ethanol (18), reduce the DNA damage induced by low-LET ionizing radiation.

In the present study, the effect of BH₄ on γ -ray-

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(A)

5,6,7,8-Tetrahydrobiopterin (BH₄)

(B)

Figure 1. Chemical structure of 5,6,7,8-tetrahydrobiopterin (BH₄), upper: biosynthesis of BH₄ via the salvage pathway from sepiapterin (SP), lower

induced MN frequency in RAW264.7 cells was examined.

Materials and Methods

Materials

5,6,7,8-Tetrahydrobiopterin was provided by

Daiichi Asubio Pharma Co., Ltd. (Tokyo, Japan). Sepiapterin was obtained from Alexis Co., (Lausen, Switzerland). Cytochalasin B was purchased from MP Biomedicals, Inc. (Irvine, CA, U.S.A.). Thiazolyl blue tetrazolium bromide (MTT), methotrexate (MTX) and bovine serum albumin (BSA) were from Sigma Chemical Co., (St. Louis, MO, U.S.A.). N-Acetyl-5-hydroxytryptamine (NAS), potassium iodide (KI),

iodine (I₂), ascorbic acid and methanol (HPLC grade) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). All other reagents were of analytical grade.

Cell culture

Mouse macrophage-like RAW264.7 cells were obtained from American Type Culture Collection (Virginia, U.S.A.). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) plus 100 mg/ml streptomycin and 100 U/ml penicillin at 37°C in a 5% CO₂ / 95% air atmosphere. The cells were tested and found to be free of mycoplasma. Logarithmic-phase cells were used for all experiments.

BH₄ and sepiapterin treatment

Freshly harvested RAW264.7 cells were seeded into 6-well plates (Corning Co., NY, U.S.A.) at 1.1×10^6 cells per well and allowed to proliferate for 15 h. Various concentrations of BH₄ or sepiapterin were then added to the medium and incubation was continued for an appropriate time.

γ -Ray irradiation

Irradiation was done with γ -rays from a ¹³⁷Cs source (GAMMACELL40, Nordin International Inc., Canada) at a dose of 0-15 Gy (0.93 Gy/min).

Micronucleus determination

The frequency of radiation-induced MN was assessed by using the CBMN assay as described by Fenech and Morley (19) with a slight modification. Briefly, cytochalasin B dissolved in 95% ethanol was added to the culture medium at a final concentration of 3 μ g/ml immediately after the irradiation. After 24 h of culture, cells were harvested and fixed in methanol : acetic acid (3:1) at 20°C. The fixed cell suspension was dropped on glass slides and air-dried. The slides were stained with 8% Giemsa. The number of MN per 500 binucleated (BN) cells was scored for each sample under a light microscope.

Assay of BH₄ concentration

Quantification of intracellular BH₄ concentration was performed by reversed-phase high-performance liquid chromatography (HPLC) with fluorometric detection as described previously by Fukushima and Nixon with a slight modification (20, 21). Cells in 6-well plates were directly treated with 0.3% KI/0.2% I₂ in 0.1 M HCl and 0.3% KI/0.2% I₂ in 0.2 M NaOH. The plates were left for 15 min on ice, then the cells were collected in fresh tubes and kept for 1 h at room temperature under low light. Then, excess I₂ was removed by addition of 1.5% ascorbic acid solution

until the I₂ was just reduced. The solution was centrifuged at 15,000 rpm for 15 min and 200 μ l of the supernatant was analyzed by HPLC. The HPLC system was composed of an LC-6A solvent system (Shimadzu, Kyoto, Japan), a TSK-GEL ODS-80Ts QA column (4.6 mm $\times$ 150 mm) (Tosoh Co., Tokyo, Japan), 821-FP fluorescence detector (JASCO, Kyoto, Japan) and C-R6A Chromatopak (Shimadzu, Kyoto, Japan). Excitation and emission wavelengths were 350 and 450 nm, respectively. The mobile phase was 8% methanol and the flow rate was 1.0 μ l/min. The amount of BH₄ was calculated from the difference in the bipterin concentrations measured after oxidation in acid (total bipterins) and base (bipterin + 7,8-dihydrobipterin (BH₂)) and was calculated as mmol/mg protein. The protein concentration was determined by the method of Lowry *et al.* (22), using BSA as a standard.

Statistical analysis

The statistical significance of differences between groups was determined by using Student's t test for comparison between two groups or two-way repeated-measures analysis of variance (ANOVA) and Dunnett's tests for multiple comparisons, where appropriate. P values of less than 0.05 were considered significant.

Results

Effect of BH₄ on 5 Gy γ -ray-induced MN frequency

To assess the effect of BH₄ on γ -ray-induced MN frequency, 10, 100 and 500 μ M BH₄ was exogenously added to cells for 3 to 24 h before the irradiation. Treatment with 100 μ M BH₄ for 6 h and 10, or 100 μ M BH₄ for 9 h, significantly reduced the MN frequency induced by 5 Gy γ -rays. The relative MN frequencies were 82, 76 and 79 % versus the radiation alone-treated group, respectively (Fig. 2).

Effect of sepiapterin on 5 Gy γ -ray-induced MN frequency

To evaluate the effect of sepiapterin, a precursor of BH₄ biosynthesis (23), cells were treated with 0.1, 1 and 10 μ M sepiapterin for 3 to 24 h before the irradiation. MN frequency induced by the irradiation was significantly reduced when 1 mM sepiapterin was given at 18 and 24 h, or 10 μ M sepiapterin was given at 12 and 24 h before the irradiation (Fig. 3). The relative MN frequencies were about 66, 71, 82 and 51% versus the radiation alone-treated group, respectively.

Changes in MN frequencies induced by different doses of γ -rays at a constant concentration of SP (1 ?M)

Figure 2. Micronucleus (MN) frequency induced by 5 Gy γ -rays at different times after treatment with various concentrations of 5,6,7,8-tetrahydrobiopterin (BH₄) in RAW264.7 cells. RAW264.7 cells were incubated with BH₄ for 0 to 24 h before irradiation with γ -rays at a dose of 5 Gy. After exposure to γ -rays, the cells were kept for 24 h under normal growth conditions before the quantification of micronuclei. Each point indicates the mean $\pm$ SE (n=500). The relative MN frequency was calculated with respect to the MN frequency of the group irradiated with 5 Gy γ -rays alone taken as 100% at each time. * and **, significantly different from the radiation-alone group at $p < 0.05$ and $p < 0.01$, respectively.

The cells were incubated in the presence or absence of 1 μ M SP for 18 h and then irradiated with various doses of γ -rays. MN frequencies were increased by γ -rays in a dose-dependent manner between 0 and 10 Gy, reaching a plateau at more than 10 Gy. Pretreatment with SP significantly reduced the MN frequency induced by γ -rays at doses of between 5 and 15 Gy (Fig. 4).

Effect of inhibitors of BH₄ biosynthesis on the MN-reducing effect of SP

Whether the reductive effect of SP on γ -ray-induced MN frequency was due to the action of SP itself, or BH₄ synthesized via the salvage pathway from SP, was investigated by using inhibitors of BH₄ biosynthesis. NAS is a potent inhibitor of sepiapterin reductase, and MTX is an inhibitor of dihydrofolate reductase (23, 24). As shown in (Fig. 5), NAS pretreatment blocked the reducing effect of SP on γ -ray-induced MN frequency in a dose-dependent manner. MTX treatment dose-dependently blocked the effect of SP as well (Fig. 6).

Figure 3. Micronucleus (MN) frequency induced by 5 Gy γ -rays at different times after treatment with various concentrations of sepiapterin (SP) in RAW264.7 cells. RAW264.7 cells were incubated with sepiapterin for 0 to 24 h before irradiation with γ -rays at a dose of 5 Gy. After exposure to γ -rays, the cells were kept for 24 h under growth conditions before the quantification of micronuclei. Each point indicates the mean $\pm$ SE (n=500). The relative MN frequency was calculated with respect to the MN frequency of the group irradiated with 5 Gy γ -rays alone, taken as 100% at each time. *, ** and ***, significantly different from the radiation-alone group at $p < 0.05$, $p < 0.01$ and $p < 0.005$, respectively.

Changes of intracellular BH₄ concentration after sepiapterin treatment

Intracellular BH₄ concentrations after 1 μ M sepiapterin treatment were assayed. The concentration peaked at 6 h, declined slowly, and remained at a high level until 24 h after sepiapterin treatment (Fig. 7).

Discussion

Ionizing radiation is used for diagnostic as well as therapeutic purposes in modern clinical practice. Exposure of the living body to ionizing radiation or environmental mutagens and carcinogens can sometimes lead to genomic instability. Scoring of MN frequency allows a direct assessment of the genotoxicity of environmental toxicants, such as radiation. The use of compounds such as amifostine (25) and garlic extract (26) may help to reduce or inhibit the genotoxicity, and in turn may inhibit mutagenesis or carcinogenesis (27, 28).

In the present study, the effect of BH₄ on γ -ray-

Figure 4. Micronucleus (MN) frequency induced by various doses of γ -rays at 18 h after treatment with 1 mM sepiapterin (SP) in RAW264.7 cells. RAW264.7 cells were incubated with 1 mM sepiapterin before irradiation with various doses of γ -rays. After exposure to γ -rays, the cells were kept for 24 h under growth conditions before the quantification of micronuclei. In each experiment, 500 binucleated cells were scored for micronuclei. Each point indicates the mean $\pm$ SE. * and ***, significantly different from the radiation-alone group at $p < 0.05$ and $p < 0.005$, respectively.

induced MN frequency was examined. BH₄ has important biological functions as a cofactor and an endogenous antioxidant. It protects cells from damage induced by various kinds of oxidative stresses, including paraquat (8), NO (10), hydrogen peroxide (29) and peroxynitrite (30), and supplementation of BH₄ is efficacious to counteract biochemical pathways which are important in the pathogenesis Parkinson's disease and atherosclerosis, in part via antioxidant activity (31,32). The *in vitro* scavenging activity of BH₄ was previously evaluated by spin-trapping ESR spectrometry. BH₄ scavenged both superoxide anion radical and hydroxyl radical (8). We further examined the effect of BH₄ on the auto-oxidation of brain homogenate, which is considered to be induced by hydroxyl radicals and/or lipid peroxides. BH₄ protected the brain homogenate from oxidation as potently as ascorbic acid and more potently than cysteamine. Several investigators have demonstrated the ability of antioxidants such as free radical-scavenging compounds to protect cellular DNA against low-LET ionizing radiation (16-18). Consequently, it was expected that BH₄ would have a

Figure 5. Influence of N-acetyl-5-hydroxytryptamine (NAS) on the reducing effect of sepiapterin on the micronucleus (MN) frequency induced by 5 Gy γ -rays in RAW264.7 cells. RAW264.7 cells were incubated with 1 mM SP for 18 h in the presence or the absence of NAS and then irradiated. After irradiation, the cells were kept for 24 h under normal growth conditions before the quantification of micronuclei. Each point indicates the mean $\pm$ SE ($n = 500$). The relative MN frequency was calculated with respect to the MN frequency of the group irradiated with 5 Gy γ -rays alone, taken as 100% at each time. ***, Significantly different from the radiation-alone control at $p < 0.005$, # and ###, significantly different from the SP-treated group at $p < 0.05$ and $p < 0.005$, respectively.

radioprotective effect against DNA damage induced by γ -rays.

BH₄ itself slightly reduced the MN frequency by 20-25%, compared with that in the radiation-alone group (Fig. 2). It was reported that only a slight increase in intracellular BH₄ concentration was observed after adding BH₄ exogenously, because BH₄ taken up from the extracellular fluid was immediately oxidized to dihydrobiopterin (BH₂) and released without mixing into the endogenous BH₄ pool (33, 34). On the other hand, administration of SP, a precursor of BH₄ biosynthesis, significantly elevated the intracellular level of BH₄. SP was readily incorporated into the cells and converted into BH₄ via the salvage pathway shown in Fig. 1 (B) (23, 24). Thus, the dose-dependent effect of SP on γ -ray-induced MN frequency was examined. Treatment with SP at doses of 1 mM and 10 mM for more than 12 h significantly reduced the MN frequency induced by 5 Gy γ -rays (Fig. 3). Moreover,

Figure 6. Influence of methotrexate (MTX) on the reductive effect of sepiapterin (SP) on the micronucleus (MN) frequency induced by 5 Gy γ -rays in RAW264.7 cells. RAW264.7 cells were incubated with 1 mM sepiapterin for 18 h in the presence or absence of MTX and then irradiated. After irradiation, the cells were kept for 24 h under normal growth conditions before the quantification of micronuclei. Each point indicates the mean $\pm$ SE ($n=500$). The relative MN frequency was calculated with respect to the MN frequency of the group irradiated with 5 Gy γ -rays alone, taken as 100% at each time. * and ***, significantly different from the radiation-alone control at $p<0.05$ and $p<0.005$, respectively. ###, significantly different from the SP-treated group at $p<0.005$.

the MN frequencies induced by different doses of γ -rays from 5 to 15 Gy were significantly reduced by SP at the fixed concentration of 1 mM (Fig. 4). To examine whether the reducing effect of SP on radiation-induced MN frequency was due to not SP itself, but BH₄ biosynthesized from SP, the influence of two inhibitors of BH₄ biosynthesis on the MN-reducing effect of SP was examined. Treatment with NAS or MTX blocked the reducing effect of SP on γ -ray-induced MN frequency (Figs. 5 and 6). These results suggest that intracellular BH₄ biosynthesized from SP is involved in the MN-reducing effect of SP.

In order to examine the relationship between intracellular BH₄ and the MN-reducing efficacy, changes in intracellular BH₄ concentration after 1 mM SP treatment were examined. The concentration of BH₄ was increased 3 h post-treatment with SP, peaked at 6 h and remained at a high level until 24 h (Fig. 7). The time-course of increase in BH₄ was not coincident with that

Figure 7. Changes of intracellular 5,6,7,8-tetrahydrobiopterin (BH₄) concentrations after treatment with 1 mM sepiapterin (SP) in RAW264.7 cells. Intracellular BH₄ concentrations in RAW264.7 cells were assayed at 0 to 24 h after 1 mM SP treatment. Each point indicates the mean $\pm$ SD of three independent assays. ** and ***, significantly different from the non-treated control group at $p<0.01$ and $p<0.005$, respectively.

of appearance of the MN-reducing efficacy of SP, suggesting the involvement of other factors induced by BH₄.

BH₄ may induce biofactors and enzymatic pathways that prevent radiation-induced damage and/or promote DNA repair (35). It has been reported that induction of antioxidant systems is involved in the radioprotective efficacy of some compounds (36, 37). However, we found no significant alterations in the activities of catalase, superoxide dismutase and glutathione peroxidase, or in the level of glutathione after SP treatment (data not shown).

Radioprotectors such as WR-2721 and nicotinamide have been shown to enhance DNA repair (38), but there is still no report concerning the influence of BH₄ on DNA repair systems. It will be necessary to examine the involvement of BH₄ in promoting DNA repair.

BH₄ is a cofactor of NOS (4-7), leading to NO production in various cells (39, 40). Our previous study showed that NO reduced MN frequency induced by γ -rays via elevation of glutathione and up-regulation of DNA-PK activity for repairing DSBs (41). Thus, the involvement of NO in the effect of BH₄ should also be investigated.

Another possible mechanism is alteration of the cell cycle induced by irradiation. It is well established that

cells in the mitotic (M) and late G₂ phases of the cell cycle are generally more sensitive to radiation than cells in the early S and G₁/G₀ phases (42). BH₄ was found to stimulate DNA synthesis and to induce proliferation in some cell lines (43, 44). Therefore, there is a possibility that BH₄-treated cells were in a more resistant phase than untreated cells, and this would have influenced DNA damage after the irradiation. Detailed analysis of the cell cycle will be required to examine this possibility.

In conclusion, BH₄ protects cells from radiation-induced DNA damage. This action appears to involve not only the antioxidant activity of BH₄, but also other factors that may be induced by BH₄.

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