

## Stimulation of In Vitro Angiogenesis by Tetrahydrobiopterin in Bovine Aortic Endothelial Cells

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**ABSTRACT**—This study examined whether tetrahydrobiopterin (BH4) stimulates angiogenesis by measuring the formation of tube-like structures in vascular endothelial cells. Bovine aortic endothelial cells that were cultured between two layers of collagen type I gel formed tube-like networks. Addition of BH4 or sepiapterin, a precursor of BH4 synthesis, stimulated the formation of tube-like structures. The sepiapterin-stimulated tube formation was completely inhibited by co-treatment with *N*-acetylserotonin, an inhibitor of sepiapterin reductase. These findings show that BH4 stimulates in vitro angiogenesis in vascular endothelial cells.

**Keywords:** Tetrahydrobiopterin, Angiogenesis, Proliferation

Angiogenesis is the formation of new capillaries from existing vessels. The process is tightly restricted and only occurs under a few conditions which include, for example, wound healing and menstruation in the healthy adult. However, angiogenesis also occurs under ischemic and/or hypoxic conditions in various tissues including the heart and brain (1, 2). The induction of angiogenesis may be an important mechanism to protect tissues against ischemic and/or hypoxic stress since neovascular structures can carry oxygen and nutrients to the ischemic and/or hypoxic sites.

We recently reported that tetrahydrobiopterin (BH4), a cofactor of nitric oxide (NO) synthase, has a protective effect against vascular endothelial cell injury induced by H<sub>2</sub>O<sub>2</sub>, NO and a combination of superoxide and NO, which are implicated in the development of reperfusion injury following ischemia (3–5). Kojima et al. (6) have also shown that reactive oxygen species-induced rat hepatocyte injury is reduced by BH4. The protection mechanisms of BH4 are likely to involve the scavenging activity of reactive oxygen species and antioxidative activity (3, 6). Interestingly, BH4 has been shown to stimulate cell proliferation in various cells including PC12, SV40-transfected human fibroblasts and rat glioma cells (7). If BH4 stimulates endothelial cell proliferation, it is possible that BH4 may enhance angiogenesis since enhancement of cell proliferation may affect angiogenesis. Moreover, if BH4 functions as a stimulator of

angiogenesis, it may be effective for treating ischemic and/or hypoxic diseases because it also has a protective effect against reactive oxygen species- and/or NO-induced cell injury. This study investigated whether BH4 stimulates angiogenesis.

Vascular endothelial cells were isolated from bovine thoracic aortae as previously described (8). The endothelial cell layer was removed by scraping with a scalpel. The obtained cells were cultured in minimum essential medium (MEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS), 15 µg/ml gentamicin, 2 µg/ml amphotericin B, 1 µg/ml minocycline and 50 µg/ml ampicillin. When the cells reached confluence, they were trypsinized and then maintained in MEM supplemented with 10% FBS, 100 µg/ml penicillin and 100 units/ml streptomycin. Cells at 4 to 8 passages were used for experiments.

The proliferation of endothelial cells was determined by a trypan blue exclusion assay (9). Cells (3 × 10<sup>4</sup> cells/well) were seeded on 6-well culture plates and incubated with MEM supplemented with 10% FBS for 24 hr and allowed to attach to the wells. The cells were then treated with BH4 or sepiapterin and/or *N*-acetylserotonin (NAS) in MEM supplemented with 2% FBS for 48 hr. After the treatment, the cells were rinsed once with phosphate-buffered saline (PBS), trypsinized and stained with 0.2% trypan blue solution. The number of viable cells were counted using an optical microscope at 100-fold

magnification. The data were expressed as the percentage of the control.

Tube formation was measured in 24-well culture plates using the three-dimensional culture method as previously described (10). Collagen gel solution (0.5 ml) consisting of a mixture of 8 volumes of type I collagen gel solution (Koken Co., Ltd., Tokyo), 1 volume of ten-fold concentrated MEM, 1 volume of 0.05 N NaOH, 200 mM HEPES and 260 mM  $\text{NaHCO}_3$ , was poured into each well of the culture plates and incubated for 60 min at  $37^\circ\text{C}$ . Endothelial cell suspension ( $5 \times 10^4$  cells) in 1 ml of MEM containing 10% FBS was added to the well. After overnight incubation, the medium was removed, and 0.5 ml of collagen gel solution was overlaid. One milliliter of the culture medium containing 2% FBS and various drugs (BH4, sepiapterin and/or NAS) was added to the well and incubated for 3 days at  $37^\circ\text{C}$ . The cultures were washed three times with PBS and fixed with 2.5% glutaraldehyde in PBS. Subsequently, randomly selected fields measuring  $0.86 \times 1.3$  mm were photographed in each well under phase-contrast microscopy. Tube formation was quantified from three randomly selected fields per experiment by measuring the total cumulative length of all cellular structures including all branches, using a computer-assisted image analyzer (MCID; Imaging Research Inc., St. Catharines, Canada).

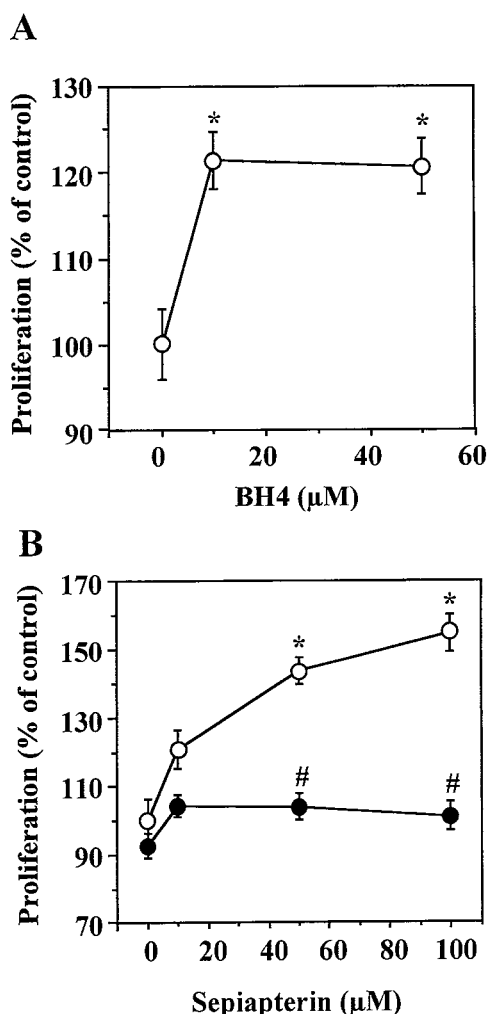
Results were expressed as the means  $\pm$  S.E.M. of  $n$  observations for each experiment. Statistical analysis was performed with analysis of variance followed by Bonferroni's method. Differences among means were considered significant at  $P < 0.05$ .

Addition of BH4 (10 and  $50 \mu\text{M}$ ) enhanced cell proliferation (Fig. 1A). Sepiapterin (10– $100 \mu\text{M}$ ), a precursor of BH4 synthesis, also enhanced cell proliferation in a concentration-dependent manner (Fig. 1B). The sepiapterin-stimulated cell proliferation was strongly reduced by co-treatment with NAS ( $100 \mu\text{M}$ ), an inhibitor of sepiapterin reductase (Fig. 1B). Anastasiadis et al. (7) reported that BH4 stimulates cell proliferation in various cells including PC12, SV40-transfected human fibroblasts and rat glioma cells. Our results show that BH4 also enhances vascular endothelial cell proliferation.

Although vascular endothelial cells grown on uncoated dishes were polygonal at confluence, when the cells were cultured between two layers of collagen type I gel, they formed tube-like networks with multiple branches as shown in our previous report (10). To determine whether BH4 stimulates the formation of tube-like networks in this system, endothelial cells were exposed to various concentrations of BH4 (10– $100 \mu\text{M}$ ). Treatment with BH4 concentration-dependently increased the tube formation, and the maximal stimulation was obtained when cells were exposed to  $50 \mu\text{M}$  BH4 (Fig. 2A). Sepiapterin

also enhanced tube formation (Fig. 2B). The sepiapterin-stimulated tube formation was completely inhibited by co-treatment with  $100 \mu\text{M}$  NAS (Fig. 2C). These findings show that BH4 stimulates *in vitro* angiogenesis.

In the present study, we showed that BH4 stimulates cell proliferation and angiogenesis in vascular endothelial cells. The activity of cell proliferation in vascular endothelial cells is likely to affect angiogenesis, since endothelial cell proliferation is an important process of angiogenesis (11). Although the mechanisms by which BH4 stimulates angiogenesis has not yet been determined, the stimulation of cell proliferation may be at least one of the factors. The mechanisms by which BH4 stimulates angio-



**Fig. 1.** Effects of BH4 and sepiapterin on proliferation in vascular endothelial cells. A: Cells were incubated with or without BH4 (10 or  $50 \mu\text{M}$ ) for 48 hr. B: Cells were incubated with 10– $100 \mu\text{M}$  sepiapterin (○) or sepiapterin plus  $100 \mu\text{M}$  NAS (●) for 48 hr. Results are expressed as the means  $\pm$  S.E.M. of 3 experiments with duplicate assays. \* $P < 0.05$ , compared with the vehicle control group. # $P < 0.05$ , compared with the corresponding value in the sepiapterin alone group.

genesis need to be examined in further detail in the future.

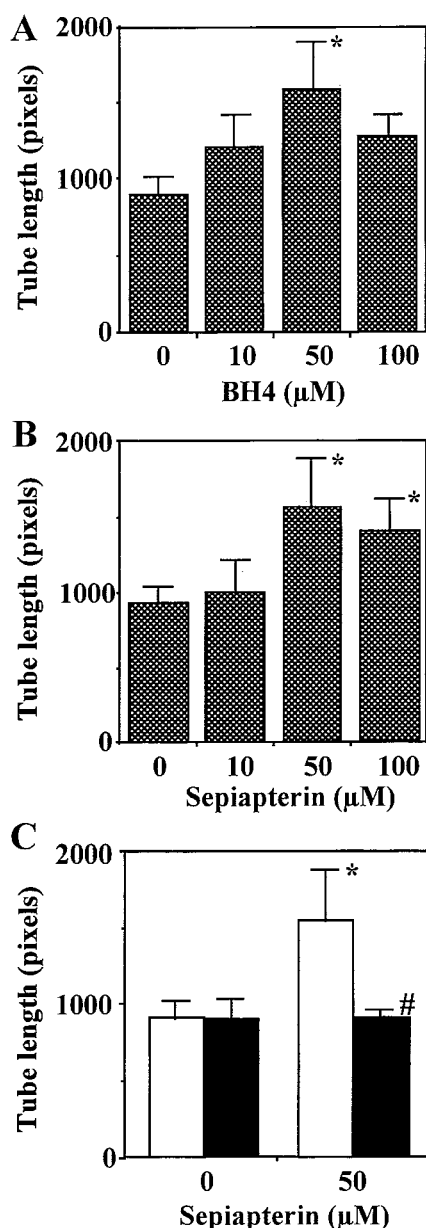
Angiogenesis is known to be observed under ischemic and/or hypoxic conditions in various tissues including the heart and brain (1, 2). The induction of angiogenesis may be an important mechanism for protecting these tissues against ischemic and/or hypoxic stress since neovascular

structures can carry oxygen to the ischemic and/or hypoxic sites. In fact, vascular endothelial growth factor has been shown to stimulate angiogenesis, and it improves myocardial perfusion in pigs (12) and patients (13) with chronic myocardial ischemia. It is possible that BH4 also improves myocardial perfusion by stimulating angiogenesis.

BH4 also has a protective effect against injury in vascular endothelial cells induced by  $H_2O_2$ , NO and a combination of superoxide and NO, which have been implicated in the development of reperfusion injury following ischemia (3–5). BH4 has been shown to scavenge reactive oxygen species and to have an antioxidative activity (3, 6). Therefore, this scavenging of reactive oxygen species and antioxidative activity of BH4 may be one of the mechanisms involved in the protective effect of BH4. Interestingly, NO synthase in endothelial cells releases superoxide instead of NO when the BH4 concentration in endothelial cells is decreased (14, 15). The BH4 concentration in endothelial cells under reperfusion may be lowered since BH4 is easily oxidized by reactive oxygen species. Superoxide release from NO synthase is blocked by the addition of BH4. BH4 may also improve the function of endothelial NO synthase under pathologic conditions. Thus, BH4 not only has a protective effect against NO and/or reactive oxygen species and a regulatory function on endothelial NO synthase, which may be implicated in reperfusion injury following ischemia, but also stimulates angiogenesis. Therefore, BH4 may be useful for treating ischemic diseases.

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**Fig. 2.** Effects of BH4 and sepiapterin on tube formation by vascular endothelial cells. A: Cells were incubated with various concentrations of BH4 (10–100  $\mu$ M) for 72 hr. B: Cells were incubated with various concentrations of sepiapterin (10–100  $\mu$ M) for 72 hr. C: Cells were incubated with or without sepiapterin (50  $\mu$ M) in the presence (■) or absence (□) of 100  $\mu$ M NAs for 72 hr. Results are expressed as the means  $\pm$  S.E.M. of 4–8 experiments with triplicate assays. \* $P < 0.05$ , compared with the vehicle control group. # $P < 0.05$ , compared with sepiapterin (50  $\mu$ M) alone group.

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