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Tetrahydrobiopterin impairs the action of endothelial nitric oxide *via* superoxide derived from platelets

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1 The mechanism by which exogenous tetrahydrobiopterin (BH_4) impairs the action of endothelial nitric oxide (NO) in the presence of platelets was investigated.

2 The endothelial NO generated by shear stress was determined by the anti-aggregating activity of indomethacin-treated endothelial cells and the cyclic GMP concentration in platelets.

3 The inhibitory effect of exogenous BH_4 was suppressed by superoxide dismutase (SOD), or diclofenac sodium at concentrations inhibiting O_2^- generation, but not by allopurinol, a xanthine oxidase inhibitor.

4 BH_4 similarly inhibited the anti-aggregatory effect of sodium nitroprusside (SNP), a NO donor. The inhibitory effect was suppressed by diphenyleneiodonium, a specific inhibitor of NADPH oxidase.

5 Six(S)-BH_4, an inactive diastereoisomer of 6(R)-BH_4, and the 5,6,7,8-tetrahydropterin compounds inhibited the endothelial NO action, whereas sepiapterin and 7,8-dihydrobiopterin (BH_2), 5,6-double bond pterins, were inactive.

6 These tetrahydropterins, but not sepiapterin and BH_2, scavenged superoxide (O_2^-) generated by the hypoxanthine-xanthine oxidase reaction, possibly due to electron transfer during oxidation to its quinonoid-form.

7 BH_4 markedly stimulated the O_2^- generation from platelets, in the presence of NADH, rather than that of NADPH.

8 These findings suggest that BH_4 stimulates platelet NAD(P)H oxidase to generate O_2^-, and inhibits the anti-aggregating effect of NO. SOD activity in the local environment may modify the effect of BH_4 on the endothelial NO activity.
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Keywords: Tetrahydrobiopterin; nitric oxide; endothelial cells; platelets; anti-aggregation; superoxide; NAD(P)H oxidase; electron transfer

Abbreviations: BH_2, 7,8-dihydrobiopterin; BH_4, 5,6,7,8-tetrahydrobiopterin; 6,7-$DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; DPI, diphenyleneiodonium; FAH_4, 5,6,7,8-tetrahydrofolic acid; 6-MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide; SNP, sodium nitroprusside; SOD, superoxide dismutase

Introduction

Tetrahydrobiopterin (BH_4), an essential cofactor of nitric oxide synthase (NOS), plays an important role in the generation of nitric oxide (NO) (Nathan, 1992). The NO synthesis in endothelial cells is triggered by the increase in intracellular concentration of Ca^{2+}, but not by that of BH_4. However, exogenous BH_4 dose-dependently enhanced NO generation in cultured endothelial cells. The intracellular concentration of BH_4 in freshly isolated endothelial cells significantly declined during prolonged culture (Rosenkranz Weiss *et al*., 1994). Therefore, the supplement of BH_4 to cultured endothelial cells may prevent a decrease in NO generation. Recently, it has been suggested that the impairment of endothelial NO-dependent relaxation in smokers (Higman *et al*., 1996) or patients with hypertension (Cosentino *et al*., 1998) or familial hypercholesterolemia (Stroes *et al*., 1997a) may result from a disorder of BH_4 availability. The decrease in BH_4 availability *in vivo* may also impair NO generation in endothelial cells. Indeed, the administration of BH_4 (Stroes *et al*., 1997b) or its precursor (Verhaar *et al*., 1998) could restore NO activity in familial hypercholesterolemia. The supplement of BH_4 may be useful to improve the symptoms of various diseases due to decreased NO synthesis.

In contrast to the beneficial aspect of BH_4 administration, the present study indicates that BH_4 induces the opposite effect in the presence of platelets. We investigate here the mechanism that exogenous BH_4 markedly impaired the effect of endothelial NO in coculture with platelets.

The present study demonstrates that BH_4 stimulates the platelets to produce O_2^-, and inactivates NO.

Methods

Preparation of endothelial cells

Human endothelial cells were prepared from the umbilical vein by digestion with the 500 units ml^{-1} dispase for 25 min at 37°C. The cells were grown in medium 199 supplemented with 10% FBS, heparin sodium (100 u ml^{-1}), penicillin (100 u ml^{-1}), streptomycin (100 μg ml^{-1}) and endothelial cell growth supplement (ECGS, 50 μg ml^{-1}). The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

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Preparation of platelet suspensions

Rabbit blood was treated with sodium citrate (0.38%) and platelet rich plasma (PRP) was harvested by centrifugation at $200 \times g$ for 10 min. EDTA (6 mM final concentration) was added and PRP was centrifuged at $1500 \times g$ for 10 min. Platelets were washed with 20 ml of ice-cold HEPES-Tyrode buffer I (containing in mM.: KCl 2.7, NaCl 137, dextrose 5.6, HEPES 3.8, NaH_2PO_4 0.4, EDTA 1, 0.25% gelatin) and centrifuged at $1500 \times g$ for 10 min. The platelets (6×10^9) were finally resuspended in 20 ml of ice-cold HEPES-Tyrode buffer II (1 mM EDTA in HEPES-Tyrode buffer I was replaced by 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$) at 3×10^8 platelets ml^{-1}.

Assay of endothelial NO

The shear stress-induced NO from endothelial cells was measured by an original method of platelet aggregation. The cuvette was coated with fibronectin (25 μg ml^{-1} PBS, 400 μl) by incubating at 37°C overnight, and was gently rinsed with PBS prior to the addition of endothelial cells. The primary culture of endothelial cells was isolated from three umbilical cords and the cell density was adjusted to 3×10^5 cells ml^{-1} in culture medium containing ECGS. The cell suspension (400 μl) was added to each fibronectin-coated cuvette, and the cuvette was placed horizontally to adhere the cells on its inner wall. Every 10 min, these cuvettes were turned at an angle of 90° to ensure the homogeneous adhesion of cells on inner walls. The endothelial cell-attached cuvettes were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3–4 days. For the experiment, endothelial cells in a cuvette were treated with 10 μM indomethacin for 1 h to inhibit prostacyclin synthesis, and were then washed three times with Hanks' balanced salt solution (HBSS). Immediately, the platelet suspension and a stirring magnet were added into the cuvette coated with indomethacin-treated endothelial cells. After the pre-warming (2 min, 37°C) of cuvettes, the stirring platelet suspension in the cuvette was stimulated by platelet activating factor (PAF, 5 nM). In this model (illustrated in Figure 1), endothelial cells are given the shear-stress by stirring the platelet suspension in a cuvette. NO derived from endothelial cells by shear stress can act on platelets. It is known that shear stress induces the increase of intracellular Ca^{2+} levels (Ando *et al*., 1991) in endothelial cells and triggers NO synthesis (Cooke *et al*., 1990; Buga *et al*., 1991). The NO activity was estimated by the platelet aggregation (Radomski *et al*., 1987a,b) and intracellular cyclic GMP concentration described below.

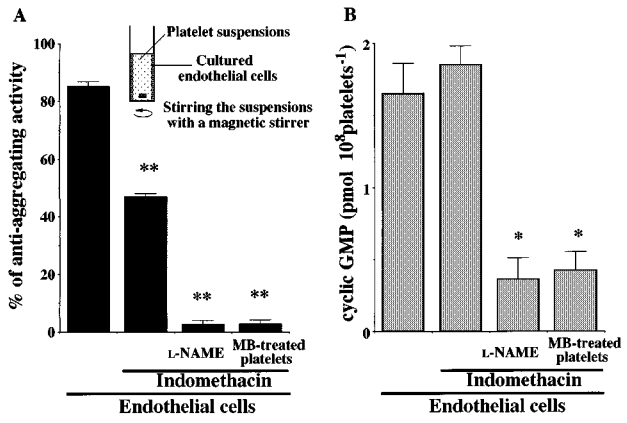

Figure 1 The anti-platelet action of endothelial nitric oxide by the shear stress. Endothelial cells, which were coated on the inner wall of cuvettes, were pretreated with indomethacin (10 μM) and L-NAME (300 μM) for 1 h, and then washed three times with HBSS. The platelet suspensions or methylene blue (MB, 10 μM for 30 min)-treated platelets were added to cell-coated cuvettes, and were stirred at 37°C (insert in A). (A) The platelet aggregation was induced by PAF, and the anti-aggregating activity of endothelial cells was determined. (B) The cyclic GMP levels in platelets was measured before the stimulation of PAF. Values are expressed as the mean ± s.e.mean of 4–8 experiments. $*P<0.01$, $**P<0.001$ compared with control.

Measurement of platelet aggregation

Platelet aggregation was measured in an aggregometer (AA-100, Sysmex) according to the method of Born (1962). Test compounds were added to the platelet suspension 2 min before the addition of PAF (5 nM). Per cent of maximum aggregation (M.A.) during 5 min was measured. The anti-aggregating activity (%) of endothelial cells was calculated using the following equation:

The anti-aggregating activity (%) =

$$(1 - [+\text{ECs}]\text{M.A.}\%/[-\text{ECs}]\text{M.A.}\%) \times 100$$

where [+ECs]: endothelial cell-coated cuvette, [−ECs]: endothelial cell-free cuvette.

Assay of cyclic GMP

Platelet suspensions were stirred at 37°C for 2 min in a cuvette coated with indomethacin-treated endothelial cells. Then 250 μl of platelet suspensions were taken and mixed with ice-cold trichloroacetic acid (TCA, 6%). After centrifugation at $1500 \times g$ for 15 min at 4°C, the supernatant was collected and washed twice with water-saturated ether to remove TCA. The lyophilized samples were dissolved in distilled water for the determination of cyclic GMP by enzyme immunoassay (Biotrak, Amersham). The platelet cyclic GMP was determined before aggregation and without isobutylmethylxanthine.

Assay of O_2^- scavenging activity and assay of O_2^- generation

The scavenging activity of pterin-related compounds against O_2^- was determined in 96 well-plates, according to the modified nitrite-kit method (Oyanagui, 1984). Reaction mixtures (1 mM hydroxylamine, 0.1 μg ml^{-1} hydroxylamine *O*-sulphonic acid, 0.02 u ml^{-1} xanthine oxidase, 100 μM hypoxanthine, 13 mM KH_2PO_4, 7 mM $Na_2B_4O_7$, 0.1 mM EDTA, pterin-related compounds) (pH 8.2) were incubated at 37°C for 30 min. Diazo dye-forming reagent (20 μM *N*-1-naphtylethylenediamine, 2 mM sulphanilic acid, 16.7% acetic acid) was added and the absorption was measured using the multiwell plate reader through a 540 nm filter after 30 min. The generated O_2^- immediately reacted with hydroxylamine to produce NO_2^-, which then initiated the diazo-coupling reaction with naphtylethylenediamine and sulphanilic acid. The O_2^- production was estimated from the different optical densities (OD) determined in the presence or absence of 500 units ml^{-1} superoxide dismutase (SOD).

The BH_4-induced O_2^- generation from platelets was measured using the same buffer without hypoxanthine and xanthine oxidase, as described above.

Statistical analysis

Experimental data are expressed as the mean ± s.e.mean of 4–8 experiments. Student's *t*-test was used to assess the statistical

significance of the differences. A value of $P<0.05$ was considered significant.

Materials

M-199 medium and HBSS were obtained from GIBCO–BRL (NY, U.S.A.). Dispase was obtained from Godo Shusei (Tokyo, Japan). Fibronectin, indomethacin, N^G-nitro-L-arginine methyl ester, L-arginine, alloprinol, diclofenac, SOD, sodium nitroprusside, hypoxathine, xanthine oxidase were obtained from Sigma (St. Louis, MO, U.S.A.). Diphenyleneiodonium was purchased from Dojindo Laboratories (Kumamoto, Japan). Sepiapterin, 6R-BH_4, 6S-BH_4, neopterin, 7,8-dihydrobiopterin(BH_2), 6,7-dimethyl-5,6,7,8-tetrahydropterin, 6-methyl-5,6,7,8-tetrahydropterin, 5,6,7,8-tetrahydrofolic acid were obtained from Alexis (CA, U.S.A.). FBS was obtained from JRH Biosciences (KS, U.S.A.). Endothelial cell growth supplement was obtained from Collaborative Biomedical Products (Bedford, U.S.A.). NADH and NADPH were purchased from Wako (Osaka, Japan). PAF was obtained from Avanti Polar Lipids (AL, U.S.A.).

Results

Anti-aggregatory effect of indomethacin-treated endothelial cells coincides with NO activity

Platelet aggregation was strongly inhibited in the presence of endothelial cells. This inhibition was partially reduced by the treatment of endothelial cells with indomethacin (10 μM). When endothelial cells were treated with both indomethacin and N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), the anti-aggregating activity of endothelial cells practically disappeared. The pretreatment of platelets with methylene blue (10 μM), a soluble guanylate cylcase inhibitor, also cancelled the activity of indomethacin-treated endothelial cells (Figure 1A). At this time, the cyclic GMP levels of platelets in cuvettes completely paralleled with anti-aggregating activity of indomethacin-treated endothelial cells (Figure 1B). These indicate that the anti-aggregating activity of indomethacin-treated endothelial cells coincides with the activity of endothelial NO.

BH_4 impairs endothelial NO via O_2^- production

BH_4 was added to the platelet suspension in a cuvette which was coated with indomethacin-treated endothelial cells on its inner wall. The platelets were first treated with BH_4 and then stimulated by PAF. BH_4 dose-dependently inhibited the anti-aggregating activity of the endothelial cells (Figure 2A), and reduced the intracellular cyclic GMP concentration of platelets before the stimulation of PAF (Figure 2B). This suggests that BH_4 inhibits the action of endothelial NO. The action of BH_4, however, was reversed in the presence of SOD (Figure 2A, B). This suggests that the action of BH_4 might depend on O_2^- production. However, when indomethacin-treated-endothelial cells were pretreated with BH_4 and then washed with HBSS, the anti-aggregating activity was rather enhanced (data not shown). These findings suggest that BH_4 directly interacts with platelets, and inhibits the action of endothelial NO *via* O_2^- production.

When the platelet suspension was pretreated with diclofenac sodium, which has the capacity to inhibit NADPH oxidase (Yuda *et al.*, 1991), but not allopurinol (a xanthine oxidase inhibitor), the inhibitory effect of BH_4 on the anti-aggregating activity of endothelial NO completely disappeared (Figure 3).

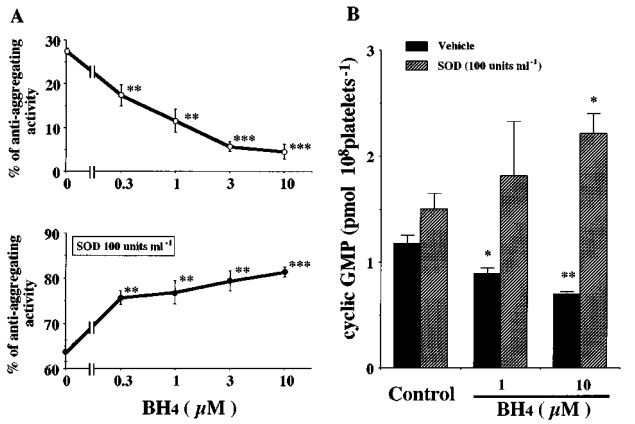

Figure 2 The effects of tetrahydrobiopterin on the anti-platelet action of endothelial nitric oxide in the absence or presence of SOD. The platelet suspensions and tetrahydrobiopterin were added to cell-coated cuvettes, and were stirred at 37°C. (A) The platelet aggregation was induced by PAF, and the anti-aggregating activity of endothelial cells was determined in the presence of the indicated concentrations of BH_4 with or without SOD. (B) The cyclic GMP levels in platelets was measured before the stimulation of PAF. Values are expressed as the mean±s.e.mean of 4–8 experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with control.

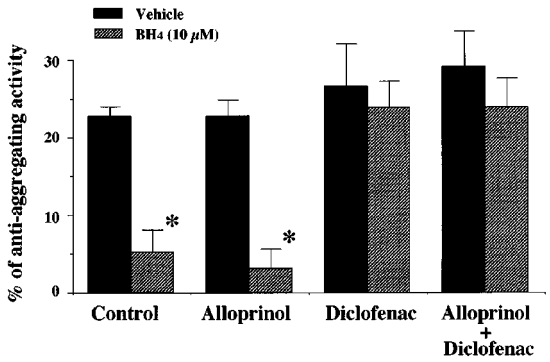

Figure 3 The effect of allopurinol and diclofenac sodium on the BH_4-induced inhibition of endothelial NO. Allopurinol (300 μM) and/or diclofenac sodium (300 μM) were added to platelet suspension 1 min before treatment with BH_4. Values are expressed as the mean±s.e.mean of 4–8 experiments. $*P<0.01$ compared with the vehicle group.

It is possible that BH_4 stimulates the O_2^- production by activation of NADPH oxidase.

BH_4 inhibits exogenous NO by O_2^- production from platelets

SNP, a NO donor, dose-dependently inhibited the platelet aggregation, accompanied by the elevation of cyclic GMP levels in platelets (data not shown). BH_4 also suppressed the anti-aggregation induced by the NO donor used instead of endothelial cells (Figure 4). This inhibitory effect of BH_4 was abrogated by the pretreatment of platelets with either diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, diclofenac or SOD (Figure 5). These findings further support the suggestion that BH_4 stimulates O_2^- generation by platelets, *via* the activation of NADPH oxidase.

Tetrahydropterins impair NO

The pretreatment of indomethacin-treated endothelial cells with BH_4 (6R-BH_4) prior to addition of platelets enhanced the release

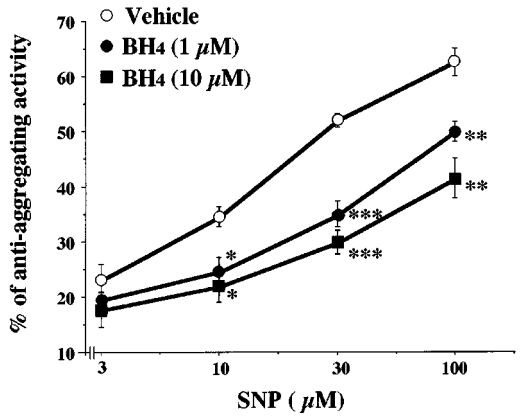

Figure 4 The effect of BH_4 on the anti-aggregating activity of sodium nitroprusside. BH_4 was added to platelet suspension 1 min before treatment with the indicated concentrations of sodium nitroprusside (SNP). Values are expressed as the mean ± s.e.mean of 4–8 experiments. $*P<0.02$, $**P<0.01$, $***P<0.001$ compared with the vehicle group.

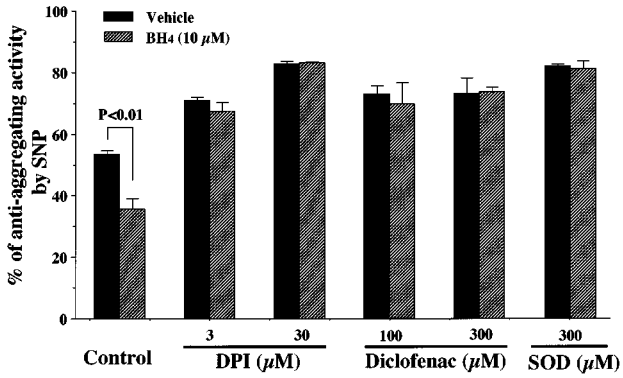

Figure 5 The effect of NADPH oxidase inhibitors on the inhibitory action of BH_4 against the anti-aggregating activity of sodium nitroprusside. Diphenyleneiodonium (DPI), diclofenac sodium and SOD were added to platelet suspension 1 min before the treatment with BH_4. Values are expressed as the mean ± s.e.mean of 4–8 experiments.

of endothelial NO, whereas 6S-BH_4 , an inactive NOS cofactor, was inactive. However, both 6R-BH_4 and 6S-BH_4 inhibited the action of endothelial NO in the presence of platelets (Figure 6). The inactivation of endothelial NO by BH_4 was observed in both diastereoisomers, in contrast to the NOS cofactor. Sepiapterin, a precursor of BH_4 in the salvage pathway, and neopterin did not inhibit the activity of endothelial NO even in the presence of platelets, possibly due to the 5, 6-double bond in the pterin-structure. This suggests the importance of 5,6,7,8-tetrahydropterins for the inhibition of endothelial NO.

This was further supported by our finding that all 5,6,7,8-tetrahydropterins impaired the action of endothelial NO, whereas 7,8-dihydrobiopterin (BH_2) was inactive (Figure 7). When BH_4 is chemically converted from its tetrahydro-form to quinonoid-form, the electron is transferred to the acceptor and stimulates NOS to generate NO.

We therefore investigated whether 5,6,7,8-tetrahydropterins act as electron donors by measuring their activity to scavenge O_2^- generated by hypoxanthine and xanthine oxidase reaction. All 5,6,7,8-tetrahydropterins scavenged O_2^- in a dose-dependent manner, whereas sepiapterin or BH_2, which have 5,6-double bond, were inactive (Figure 8). This O_2^- scavenging activity appears to be due to the electron transfer from 5,6,7,8-tetrahydropterins.

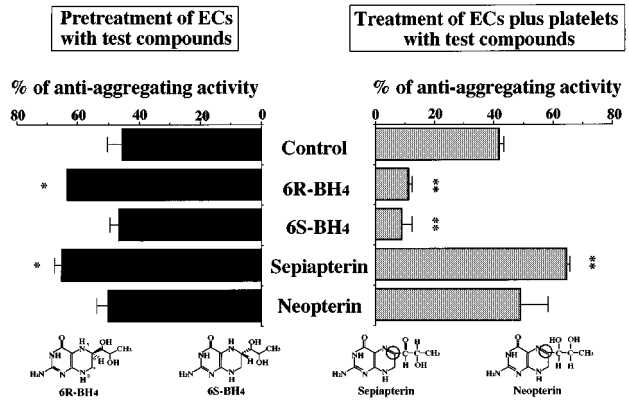

Figure 6 The effects of 6R- and 6S-BH_4 on the generation and degradation of endothelial NO. The endothelial cells (ECs) were incubated with 10 μM pterin compounds for 10 min at 37°C, then these cells were washed three times with HBSS to determine the NO generated by the endothelial cells (left histogram). The endothelial cells with platelets were simultaneously treated with pterins for 5 min at 37°C to determine the NO generated by endothelial cells in the presence of platelets (right histogram). $*P<0.01$, $**P<0.001$ compared with the control group.

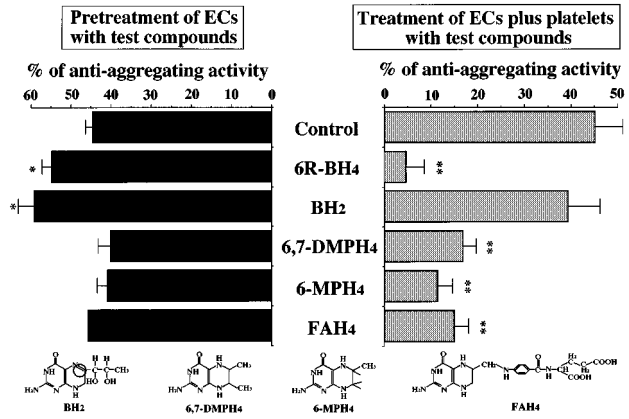

Figure 7 The role of 5,6,7,8-tetrahydropterins for the degradation of endothelial NO. Endothelial cells (ECs) without (left) or with (right) platelets were treated with 10 μM test compounds as described in Figure 6. The left histogram indicates the NO generation of endothelial cells. The right histogram indicates the degradation of endothelial NO in the presence of platelets. All 5,6,7,8-tetrahydropterins impaired the activity of endothelial NO. BH_2, which does not have the 5,6,7,8-tetrahydrostructure, failed to decrease the activity of endothelial nitric oxide. $*P<0.02$, $**P<0.01$ compared with the control group.

BH_4 activates NAD(P)H oxidase of platelets

There was a possibility that BH_4 stimulates O_2^- generation by platelets *via* the activation of NAD(P)H oxidase. To investigate this possibility, the NAD(P)H activity of platelets was measured using the modified nitrite-kit method. In the presence of NADH (100 μM) or NADPH (100 μM), BH_4 significantly enhanced the O_2^- generation by platelets. The stimulation effect of NADH exceeded that of NADPH (Figure 9). These findings support this possibility.

SOD modifies the effect of BH_4

We next investigated the combination effect of SOD and BH_4 on endothelial NO. BH_4 significantly enhanced the NO activity in the presence of higher concentrations of SOD (>10 units ml^{-1}). However, BH_4 inhibited the NO activity in the presence

of lower concentrations of SOD (<0.3 units ml^{-1}) (Figure 10). The action of BH_4 might be significantly affected by the surrounding SOD.

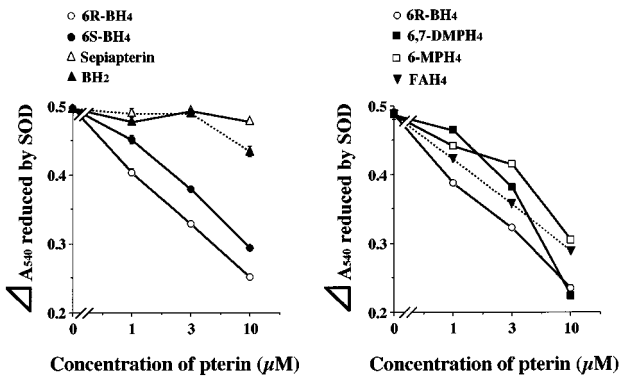

Figure 8 The O_2^- scavenging activity of 5,6,7,8-tetrahydropterins. O_2^- was produced by hypoxanthine (100 μM) plus xanthine oxidase (10^{-3} u ml^{-1}) under the cell-free conditions. Each pterin compound was added to the reaction mixture before the treatment of hypoxanthine plus xanthine oxidase. O_2^- was measured using the modified nitrite-kit method. O_2^- was represented as the reduction of absorbance at 540 nm by SOD. All 5,6,7,8-tetrahydropterin compounds scavenged O_2^- in a dose-dependent manner, whereas sepiapterin and BH_2, which do not have the 5,6,7,8-tetrahydro-structure, were inactive.

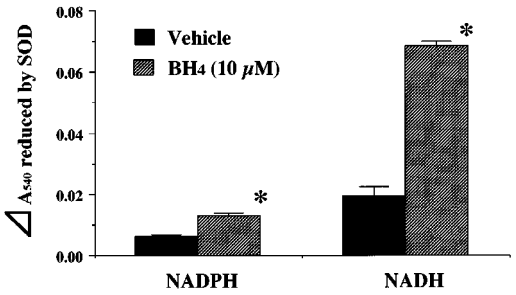

Figure 9 BH_4 enhances O_2^- production by platelets in the presence of NADH or NADPH. The platelet suspension was added NADH (100 μM) or NADPH (100 μM) before the treatment of BH_4 (10 μM). O_2^- produced from platelets was measured using the modified nitrite-kit method. $^*P<0.001$ compared with the vehicle group.

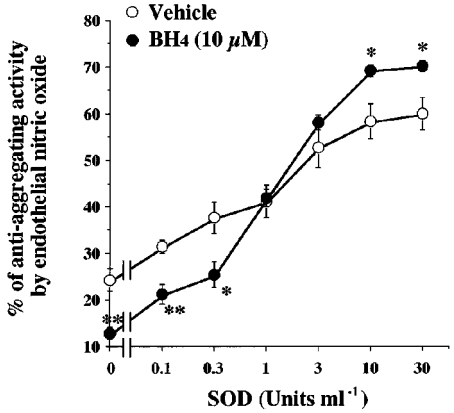

Figure 10 Effect of SOD on the BH_4-inhibition of endothelial NO. The activity of endothelial NO was evaluated as the anti-aggregating activity. Platelet suspensions were added at various concentrations of SOD 1 min before the treatment with BH_4 (10 μM). $^*P<0.05$, $^{**}P<0.01$ compared with the vehicle group.

Discussion

The present study demonstrates that exogenous BH_4, an essential cofactor of NOS, increased the NO activity of cultured endothelial cells in the absence of platelets, whereas BH_4 markedly reduced the NO activity in the presence of platelets. This inhibitory effect of BH_4 depends on the O_2^- generated by the activated NAD(P)H oxidase of platelets. The tetrahydropterins appear to play an important role in the activation of platelet NAD(P)H oxidase.

The NO synthesis in endothelial cells is triggered by the increase in the intracellular Ca^{2+} concentration (Hirata *et al*., 1993), but cultured endothelial cells with lower BH_4 concentration (Rosenkranz Weiss *et al*., 1994) can generate NO by the addition of BH_4 (Wever *et al*., 1997). The pretreatment of indomethacin-treated endothelial cells with BH_4 increased the anti-aggregating activity (NO activity) of endothelial cells.

However, exogenous BH_4 markedly impaired the effect of endothelial NO in the presence of platelets. SOD reversed the inhibitory effect of BH_4, suggesting the O_2^- generation by BH_4. It has been previously shown that auto-oxidation of BH_4 produces O_2^- (Davis & Kaufman, 1993; Mayer *et al*., 1995) and induces endothelium-dependent contractions of cerebral arteries without endothelium-dependent relaxation (Kinoshita & Katusic, 1996), which is consistent with the previous finding that O_2^- induces vascular contractions (Rubanyi & Vanhoutte, 1986a,b).

The present finding, however, cannot be explained by the auto-oxidation of BH_4. We showed that BH_4 produced O_2^- in the presence of platelets, and the inhibition of platelet NAD(P)H oxidase abrogated the inhibitory action of O_2^- against NO. The present experimental model might be applicable for the investigation of the interaction between O_2^- (generated by platelets) and endothelial NO (induced by shear stress). It is known that platelets produce reactive oxygen species (Iuliano *et al*., 1994; Leoncini *et al*., 1991; Marcus *et al*., 1977). The inhibitory effect of BH_4 was suppressed by diphenyleneiodonium (a specific inhibitor of NADPH oxidase) (Cross & Jones, 1986), diclofenac sodium and SOD, but not by allopurinol (a xanthine oxidase inhibitor). It has been shown that diclofenac sodium inhibits O_2^- generation (Oyanagui, 1976; Kaever *et al*., 1992), but does not scavenge O_2^- (Oyanagui, 1976), suggesting that it is based on the inhibition of NADPH oxidase activity (Yuda *et al*., 1991). The present findings indicate that BH_4 induced O_2^- generation *via* the activation of NAD(P)H oxidase, but not *via* that of xanthine oxidase. Recent reports suggest that NAD(P)H oxidase in vascular smooth muscle cells plays an important role in O_2^- production (Griendling *et al*., 1994; Rajagopalan *et al*., 1996). However, it is not fully understood whether the NAD(P)H oxidase of smooth muscle cells has similar characteristics with that of neutrophils. The NAD(P)H oxidase of platelets has not been well characterized. The present findings demonstrate that NADH enhanced the BH_4-induced O_2^- generation by platelets more effectively than NADPH. In this regard, the O_2^- production from platelets may be similar to that of smooth muscle cells.

It has been reported that endothelial NOS can generate O_2^- under L-arginine deficiency (Xia *et al*., 1996). BH_4 regulates the balance of NO and O_2^- generated from endothelial NOS (Cosentino *et al*., 1998; Stroes *et al*., 1998; Vasquez Vivar *et al*., 1998; Wever *et al*., 1997; Xia *et al*., 1998). The presence of NOS in human platelets has also been reported (Radomski *et al*., 1990a,b), although, it is unclear regarding the NO/O_2^- balance in human platelets. However, the rabbit platelets used

in the present study may have very low NO activity, judging from the effect of the NOS inhibitors. In the present experiment, BH_4 did not change the NO activity regardless of the absence or presence of L-arginine. Therefore, it appears unlikely that BH_4-induced O_2^- is generated from the NOS of platelets.

NOS requires 6R-BH_4 but not 6S-BH_4 for NO generation (Tayeh & Marletta, 1989). The 6S-diastereoisomer of BH_4 has little cofactor activity for NOS. The present findings of the anti-aggregating activity of endothelial NO supports this stereospecificity of BH_4. However, the platelet-dependent NO inhibition by BH_4 did not depend on stereospecificity of BH_4. This difference in the requirement of the BH_4-isomer suggests that the situation of the binding site of BH_4 for NO inhibition may be different from that for NO generation. The generation of NO from L-arginine by NOS requires the coupling reaction of oxidation of BH_4 to quinonoid dihydrobiopterin (qBH_2) (Kwon *et al*., 1989; Nathan, 1992). BH_4 acts as a cofactor to provide electrons for oxidation.

NOS is composed of the oxygenase domain, the reductase domain and calmodulin, which is tightly bound to both oxygenase and reductase domains. The oxygenase domain contains NADPH-, FMN- and FDA-binding sites. The reductase domain contains the haeme-, BH_4- and arginine-binding sites. It has been proposed that the electron in NOS flows to NADPH, FDA, FMN, haeme and O_2. Finally, the electron which is transfered to haeme will reduce O_2, and thereby the active oxygen is coupled to the oxidative reaction of L-arginine and N^G-hydroxy-L-arginine. This final reaction is similar to the activation of NAD(P)H oxidase. It is known that NAD(P)H oxidase in the cell surface membrane of neutrophils contains haeme in the active centre (Cross *et al*., 1995). Recently, it has been reported that the 5-methyl analogue of BH_4, which does not react with O_2, is a functionally active pterin cofactor of neuronal NOS (Riethmüller *et al*., 1999). This suggests that the pterin cofactor may play a role besides reductive oxygen activation. The essential role of BH_4 in NOS activation is not fully understood.

The 5,6,7,8-tetrahydropterins appear to be converted to the quinonoid-form, and thereby may cause the electron transfer from these compounds. The present findings indicate that all 5,6,7,8-tetrahydropterins can reduce O_2^-. This reaction may be explained by that the electron transfer from these tetrahydropterins stimulates the reduction of O_2^- to hydrogen peroxide. The electron donation from tetrahydropterins may similarly activate the NAD(P)H oxidase, because the haeme in its active centre may convert O_2 to O_2^-. It is suggested that the NAD(P)H oxidase of platelets can accept electrons from tetrahydropterins because the haeme in NAD(P)H oxidase may be located near the outer cell surface membrane of platelets.

In the present study, the addition of NADH rather than NADPH markedly enhanced BH_4-induced O_2^- generation by platelets. This indicates that BH_4-induced O_2^- generation may be mainly due to the activation of NADH oxidase in platelets. If NOS of platelets generates O_2^-, NADPH should be required as a cofactor for the activation of NOS.

Recent reports indicate that BH_4 plays a role in cytoprotection against oxidative stress (Ishii *et al*., 1997; Shimizu *et al*., 1998). This may be explained that the electrons transfered from BH_4 facilitate the reduction of O_2^-. This effect might alleviate the oxidation-induced damage. However, the electron transfer under non-oxidative stress induces O_2^- generation by the activation of NAD(P)H oxidase in the presence of platelets, resulting in the inactivation of NO. The treatment of BH_4, in contrast, may lead to impair the protective function. Finally, SOD activity surrounding the cells determines whether the treatment of BH_4 displays protective or injurious effects for the endothelial function. The present findings indicate that the enhancement of endothelial NO activity by BH_4 required more than 10 units ml^{-1} of SOD. The SOD activity in human plasma, determined using the highly sensitive nitrite method, was reported to be 22.54 units ml^{-1} (DiSilvestro *et al*., 1990). If the decrease of SOD activity is caused by diseases and aging, the treatment of BH_4 may not effectively enhance the endothelial NO. In such cases, the supplement of sepiapterin, which does not produce O_2^-, may be useful instead of BH_4.

In conclusion, exogenous BH_4 increases both the activity of endothelial NO and enhances the O_2^- production by platelets. The O_2^- immediately inactivates endothelial NO. Consequently, in the presence of platelets, BH_4 will impair the effect of endothelial NO. It is highly possible that the tetrahydrostructure of BH_4 produces O_2^- *via* the activation of platelet NAD(P)H oxidase. Finally, SOD activity in the local environment modifies the effect of BH_4 on the endothelial NO activity.

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