Tetrahydrobiopterin Supplementation: Elevation of Tissue Biopterin Levels Accompanied by a Relative Increase in Dihydrobiopterin in the Blood and the Role of Probenecid-Sensitive Uptake in Scavenging Dihydrobiopterin in the Liver and Kidney of Rats

Akiko Ohashi[1]*, Yusuke Saeki[2], Tomonori Harada[3], Masako Naito[1], Tomihisa Takahashi[1], Shin Aizawa[3], Hiroyuki Hasegawa[1,3]

1 Department of Anatomy, Nihon University School of Dentistry, Chiyoda, Tokyo, Japan, 2 Department of Biosciences, Teikyo University of Science and TechnologyUenohara, Yamanashi, Japan, 3 Division of Anatomical Science, Department of Functional Morphology, Nihon University School of Medicine, Itabashi, Tokyo, Japan

* oohashi.akiko@nihon-u.ac.jp

Abstract

Tetrahydrobiopterin (BH_4) is an essential cofactor of nitric oxide synthase (NOS) and aromatic amino acid hydroxylases. BH_4 and 7,8-dihydrobiopterin (BH_2) are metabolically interchangeable at the expense of NADPH. Exogenously administered BH_4 can be metabolized by the body, similar to vitamins. At present, synthetic BH_4 is used as an orphan drug for patients with inherited diseases requiring BH_4 supplementation. BH_4 supplementation has also drawn attention as a means of treating certain cardiovascular symptoms, however, its application in human patients remains limited. Here, we tracked biopterin (BP) distribution in blood, bile, urine, liver, kidney and brain after BH_4 administration (5 mg/kg rat, i.v.) with or without prior treatment with probenecid, a potent inhibitor of uptake transporters particularly including organic anion transporter families such as OTA1 and OAT3. The rapid excretion of BP in urine was driven by elevated blood concentrations and its elimination reached about 90% within 120 min. In the very early period, BP was taken up by the liver and kidney and gradually released back to the blood. BH_4 administration caused a considerable decrease in the BH_4% in blood BP as an inevitable compensatory process. Probenecid treatment slowed down the decrease in blood BP and simultaneously inhibited its initial rapid excretion in the kidney. At the same time, the BH_4% was further lowered, suggesting that the probenecid-sensitive BP uptake played a crucial role in BH_2 scavenging *in vivo*. This suggested that the overproduced BH_2 was taken up by organs by means of the probenecid-sensitive process, and was then scavenged by counter-conversion to BH_4 via the

BH_4 salvage pathway. Taken together, BH_4 administration was effective at raising BP levels in organs over the course of hours but with extremely low efficiency. Since a high BH_2 relative to BH_4 causes NOS dysfunction, the lowering of the BH_4% must be avoided in practice, otherwise the desired effect of the supplementation in ameliorating NOS dysfunction would be spoiled.

Introduction

Tetrahydrobiopterin (BH_4) is an essential cofactor for a group of aromatic amino acid hydroxylases [1–4]. Inherited BH_4 deficiencies are characterized by hyperphenylalaninemia and defective biosynthesis of classic monoamines such as dopamine, noradrenaline, adrenaline, as well as serotonin. BH_4 therapy using (6R)-L-*erythro*-5,6,7,8-tetrahydrobiopterin dihydrochloride ($6RBH_4$) has been very successful in replacing peripheral BH_4, but not BH_4 in the brain. A guide for the therapeutic use of BH_4 is available [5]. This compound ameliorates hyperphenylalaninemia in cases of inherited BH_4 deficiency [6, 7] and benefits patients with BH_4-responsive phenylketonuria [8]. The most common use of $6RBH_4$ to date is as an orphan drug for these inherited diseases [9–11]. Once $6RBH_4$ is administered, it replaces innate BH_4 and is integrated through endogenous metabolic pathways, similar to the intake of vitamins. However, like most drugs or supplements, large amounts of exogenous BH_4 bypass endogenous pathways and are eliminated in urine [12, 13] and feces [14], therefore it is desirable to minimize this useless exclusion.

We previously observed that $6RBH_4$ administration raised tissue BH_4 levels, however, with a concomitant rise in 7,8-dihydrobiopterin (BH_2) [12, 13]. Urinary excretion was suggested to be the process most responsible for the rapid loss of exogenous BH_4. Furthermore, we demonstrated that urinary excretion of administered $6RBH_4$ at a pharmacological dose predominantly involved secretion across renal tubular epithelium, distinct from glomerular filtration. We observed that the tubular secretion was almost completely suppressed by cyclosporin A (CsA), an inhibitor of transporters with broad specificity in excretion of xenobiotics and metabolic wastes.

In order to obtain greater insight into the renal exclusion of BH_4 and the relative increase in dihydrobiopterin (BH_2), in this study, we analyzed the systemic distribution of biopterin (BP), the sum total of BH_4 and BH_2 (BP = BH_2 + BH_4), in the presence of probenecid (Pbc), a potent inhibitor of uptake transporters with a broad specificity [15]. We confirmed the active role played by the renal secretion of BP in the heavy loss of exogenous BH_4. Furthermore, we revised the role of the salvage pathway of BH_4 biosynthesis in scavenging BH_2 in terms of its passage through the liver and kidney under the conditions of $6RBH_4$ administration.

Materials and Methods

Chemicals

$6RBH_4$ was donated by Suntory (Asubio Pharma, Kobe, Japan). $6RBH_4$ (BH_4•2HCl) was dissolved at 10 mg/mL in 0.9% NaCl before the start of the BH_4 administration experiment. The BH_4 solution was neutralized by 0.1 M Na_2HPO_4 and saline to prepare a 5 mg/mL concentration immediately before injection. Probenecid (Pbc: *p*-(dipropylsulfamoyl)benzoic acid) and cyclosporin A were obtained from Sigma-Aldrich (St. Louis, MO). Pbc was dissolved by dropwise addition of 2M NaOH, followed by the addition of N-2-hydroxyethylpiperazine-N'-2'-

ethanesulfonic acid (HEPES) to neutralize the solution and adjusted the concentration to 40 mg/mL. CsA was dissolved in ethanol, then diluted in 9 vol of olive oil (final concentration, 5 mg/mL). "Gall Powder®" was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Ethics statement

The animal experiments were conducted in accordance with the ethical guidelines of the Teikyo University of Science and Technology Animal Experimentation Committee, and guidelines of the Japanese Pharmacological Society. The protocol was approved by the Teikyo University of Science and Technology Animal Experimentation Committee (Permit Number: B-08005). All efforts were made to minimize suffering. Rats (SD: Sprague Dawley) were obtained from Japan SLC (Hamamatsu, Japan) and bred in our SPF-grade laboratory. The animals were maintained on a constant 12-h light-dark cycle at 21–24°C at 40–60% humidity and were provided with ordinary laboratory chow and sterile tap water ad libitum. For deep but a short period of anesthesia, rats were administered sodium pentobarbital (30 mg/kg for light anesthesia, 50 mg/kg for deep, both i.p.). For experiments taking a longer time (4 ~ 6 hours), rats were anesthetized with an i.p.-administered cocktail of pentobarbital (45 mg/kg), atropine (0.05 mg/kg), dimorpholamine (Theraptique, 1.2 mg/kg), and xylazine (9.5 mg/kg) as described previously [12, 13]. The anesthetized rats were kept on a warm gel pad (40°C) and given a 1/3 to 1/2 dose every hour to maintain them under anesthesia.

Experimental procedure

All rats (8–10 weeks old, male and female random mix) were loaded with $6RBH_4$ (5 mg/mL) at a dose of 5 mg/kg (i.v.) under anesthesia. Administration of Pbc (200 mg/kg, i.p.) or CsA (10 mg/kg, i.p.) was performed 30 min prior to $6RBH_4$ administration. Experiments were carried out by means of two different protocols; (A) by sequentially collecting blood, bile and urine from individual rats under long-lasting anesthesia on a warm gel pad, and (B) by removing the liver, kidney and brain after sacrificing the respective rats under the deep anesthesia at specified times after $6RBH_4$ administration.

(A) Rats were intraperitoneally administered Pbc ("BH_4 + Pbc", n = 5) or saline as a control ("BH_4 alone", n = 7) and were then anesthetized. All rats were given $6RBH_4$, then subjected to sampling at specified times as previously described [12, 13], except that bile was also taken and the animal was given saline containing 2% bovine bile powder (w/v), at a rate of 0.9 mL/hr, which was administered into the intestinal duct. The bovine bile powder did not contain any measurable BH_2 or BH_4. In the following sample acquisition, special care to ensure minimal oxidation of BH_4 was taken throughout the following procedure. In brief, the abdomen was opened and cannulae were set in place for collecting bile and urine. The blood samples were drawn from the tail vein into a heparin-coated capillary containing a solution of 0.1 M ascorbic acid (less than 5% by volume), and they were then subjected to differential iodine oxidation for BP determination. The urine was collected at intervals from the bladder through an indwelling needle connected to an airtight syringe in which the dead space had been filled with saline containing 50 mM each of ascorbic acid and EDTA (pH 6.8). The urine was immediately mixed with 9 volumes of 10 mM HCl containing 5 mM antioxidant. The aliquots were then subjected to quantitation of BP and creatinine. BP excretion was expressed as BP/creatinine (μmol/mg creatinine) based on the BP concentration (nmol/mL) divided by the time-matched creatinine level (μg/mL). In calculating the cumulative total, the amounts of BP in each fraction were added. Calculations were based on the BP concentration (nmol/mL) multiplied by the respective urine volume (mL) produced between each sampling time point regardless of the

creatinine concentration. The bile was also collected from individual rats through a cannula in the bile duct and was immediately subjected to BP determination. The cumulative BP amount was calculated by adding up all samples measured.

(B) Rats were treated with 6RBH$_4$ and drugs (Pbc and CsA), or saline as a vehicle control, and allowed to move freely after recovery from the light anesthesia. In the Pbc experiment, animal used numbered n = 4 each for the respective administration of "BH$_4$ + Pbc", and the vehicle control "BH$_4$ alone". In the CsA experiment, the numbers of rats for groups of "BH$_4$ + CsA" and "BH$_4$ alone" were n = 5, respectively. At 0, 30 and 120 min after 6RBH$_4$ administration, blood samples were taken from the abdominal vein into a syringe in which the dead space was replaced with saline containing 50 mM each of ascorbic acid and EDTA (pH 6.8). Each blood sample was divided into three portions; one served as a "whole blood" specimen, the second was for the hematocrit determination, and the third was centrifuged (10,000 rpm, 4°C, 5 min) to separate red blood cells (RBCs) from the plasma. The whole blood, plasma, and RBCs were separately subjected to differential iodine oxidation for the BP determination described below. The results were expressed as the amount of BP per unit volume of the original blood sample (plasma + RBC, mL) based on the hematocrit value. The kidneys, liver, and whole brain were removed without prior perfusion, rinsed with saline, blotted, weighed, frozen in liquid nitrogen, and then stored at -80°C until BP determination. BP carryover with the blood was ignored in the liver and kidney. However, for the brain, the BP carryover was estimated using an average blood inclusion of 1.2% (v/w), which was determined by a comparison of the heme content of the blood and brain homogenates based on light absorbance at 415 nm [16].

Determinations

Biopterin was determined essentially according to Fukushima and Nixon [17] and modified as described previously [18, 19]. Animal tissues contain at least 4 chemical species of the reduced biopterin family; BH$_4$-4a-carbinolamine (or 4a-hydroxy-tetrahydrobiopterin), quinonoid dihydrobiopterin and 7,8-dihydrobiopterin as well as BH$_4$. The Fukushima and Nixon method includes tissue homogenization and extraction of pterin compounds followed by measurement of amount of fully oxidized biopterin after differential iodine oxidation under acid and alkaline conditions. With their method, the biopterin quantity remaining after the acid-oxidation was taken as the total biopterin (BP = "BH$_4$" + "BH$_2$" + "oxidized biopterin") and that which remained after the alkaline-oxidation, as 7,8-dihydrobiopterin (BH$_2$) plus oxidized biopterin. The amount of oxidized biopterin in extracts without the iodine oxidation was disregarded in this study since the observed amount was too small for the purpose of our work. Accordingly, in this study, the biopterin amount measured after the acid iodine oxidation was taken as the sum of "BH$_4$" and "BH$_2$" ("BP" = "BH$_2$" + "BH$_4$"), and that after the alkaline oxidation, as the amount of "BH$_2$". The difference was therefore taken as the "BH$_4$" amount. The BH$_4$ fraction (BH$_4$%; the percentage of BH$_4$ in BP) was calculated for each individual sample. The amounts of BH$_4$ and BH$_2$ were calculated based on molecular weights of 241 g/mol and 239 g/mol, respectively. The molecular mass of the reagent 6RBH$_4$•2(HCl) was 315g.

The urinary creatinine concentration was measured using a creatininase-HMMPS method according to the manufacturer's instructions (L-type Creatinine M; Wako Pure Chemical Industries, Osaka, Japan).

Data presentation

Data were expressed as means ±S.E. The statistical significance of two groups with non-repeated determination was analyzed by Student's t-test. The significance of difference between

determinations at different times with individual animal groups was analyzed by the Paired t-test. Significance of difference between three groups was analyzed by Tukey's test. All the statistical analyses were performed with Pharmaco Basic Ver.15.0.1 (Scientist Co. Ltd., Tokyo). Using results of pterin kinetics, exponential least square approximation was performed with Excel software using Microsoft Office for Mac. For the best fit by a first-order equation, $BP_t = BP_0 \cdot e^{-kt}$, data were plotted on a semi-log scale of y-axis vs. time (min) on the linear x-axis, forming straight lines as Ln (BP_t) = Ln $(BP_0) - kt$, where BP_0 represents the extrapolated initial concentration of BP and BP_t at time t (in nmol/mL of bile or μmol/mg creatinine of urine). The slope of the plot, k, yields the fractional rate constant (min^{-1}), and gives the corresponding decay half-time, $T_{1/2} = Ln(2)/k$ (min).

Results

Rat biopterin levels in the blood, urine and bile after 6RBH$_4$ administration

We tracked BP contents and BH$_4$ fractions (BH$_4$%) in blood, urine and bile after rats were administered 6RBH$_4$ (5 mg/kg, i.v.) with or without the Pbc treatment (200 mg/kg, i.p.), denoted hereafter as "BH$_4$ + Pbc" or "BH$_4$ alone", respectively. Experimental procedure (A) in the Methods section was used.

Blood and urine. Prior to treatment, the combined BP level in the blood, plasma and red blood cells was 0.57 ± 0.08 nmol/mL and the BH$_4$% was 90% to 95%. The BP level increased after receiving 6RBH$_4$ "BH$_4$ alone", and gradually decreased to ca. 3.3-fold ($P<0.005$) of the initial level at 300 min (Fig 1A, lower panel). The blood BP content of the "BH$_4$ + Pbc" group was much larger than that of the "BH$_4$ alone" group. Reflecting this, the AUC$_{0\text{-}120}$ of the "BH$_4$ + Pbc" group was about 10-fold more than that of the group that received "BH$_4$ alone"; 7,580 vs. 736 (nmol/mL)•min.

In the "BH$_4$ alone" rats, the blood BH$_4$% dropped significantly within 30 min and continued to drop over the experimental period (Fig 1A, upper panel). In the "BH$_4$ + Pbc" rats, the BH$_4$% was substantially decreased but the decrease proceeded gradually; its lowest value of 79% was reached at 180 min and remained low thereafter. The difference in the BH$_4$% between the "BH$_4$ alone" and "BH$_4$ + Pbc" groups was most evident at 180 min (88% vs. 79%) (P = 0.001).

Urinary excretion of BP (relative to creatinine, μmol/mg Cr) after 6RBH$_4$ administration in the presence or absence of Pbc is depicted in Fig 1B. A large amount of BP appeared soon after the 6RBH$_4$ mono-loading of "BH$_4$ alone" rats, with the peak appearing at around 30 min. On the other hand, the early excretion of BP in the "BH$_4$ + Pbc" group at 30 min was strongly suppressed to 11.7% of the amount in the "BH$_4$ alone" group ($P<0.001$) while the blood BP level of "BH$_4$ + Pbc" rats was 8.5-fold higher than of the "BH$_4$ alone" rats (P = 0.03) at the same time point. The peak was delayed and appeared between 60 and 90 min. Since glomerular filtration might not be inhibited by Pbc, the observed decrease in BP flow at 30 min was thought to be due to a transporter-mediated process across the tubular epithelial cell layer, consistent with our previous results using CsA [12, 13]. Despite the effective inhibition of the early outflow by Pbc, the outflow appeared to have been delayed, although the gross amount was not reduced. The creatinine-based AUC$_{0\text{–}270}$ of the "BH$_4$ alone" rats was 731 (μmol/mg Cr)•min, and that of the "BH$_4$ + Pbc" rats was 608 (μmol/mg Cr)•min. The gross outflow was substantially completed by 270 min. The cumulative excretion calculated from the urine-volume-based BP reached 3,150 ±360 and 3,170 ±500 nmol in the "BH$_4$ alone" and "BH$_4$ + Pbc" groups, respectively, representing about 61% of the dose used (Fig 1C). We noted that at as early as 30 min, the BP outflow amounted to 1840 ± 250 nmol in the "BH$_4$ alone" group; about 58% of the gross outflow obtained by 270 min. Similarly, it reached 87% of the gross urinary
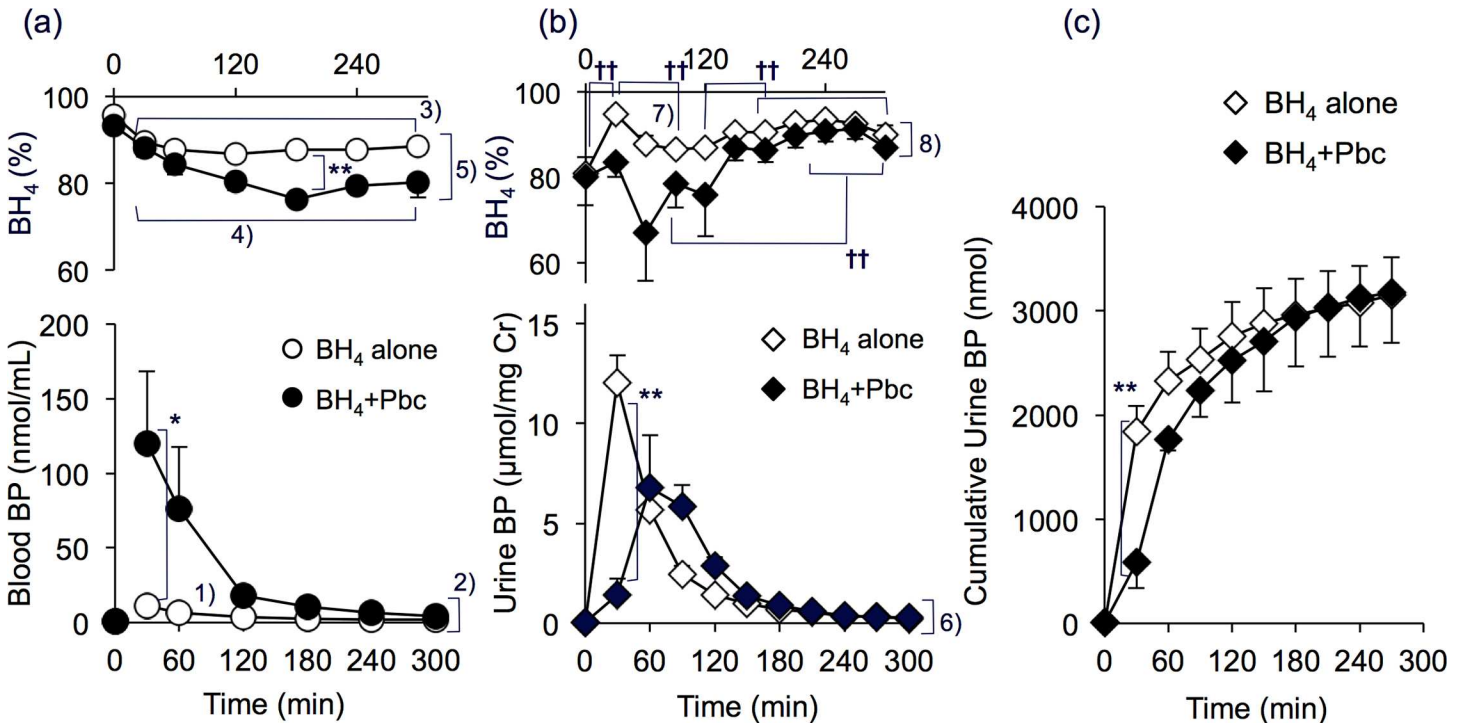
Fig 1. Effect of probenecid on biopterin levels in the blood and urine after 6RBH4 administration. Rats were given 6RBH4 (5 mg/kg, i.v.) with probenecid (200 mg/kg, i.p., "BH4 + Pbc") or without ("BH4 alone"). The blood and urine were collected as in experimental procedure (A) of Materials and Methods. (a) Blood BP (lower panel) and the BH4% (upper panel), "BH4 alone" (open circles) and "BH4 + Pbc" (closed circles). (b) Urinary BP relative to creatinine (μmol/mg creatinine, lower panel) and BH4% (upper panel), "BH4 alone" (open diamonds) and "BH4 + Pbc" (closed diamonds). (c) Cumulative BP content in the urine as a function of time after 6RBH4 administration, "BH4 alone" (open diamonds) and "BH4 + Pbc" (closed diamonds). All data are means ±S.E. of n = 5–7 for "BH4 alone" and n = 3–5 for "BH4 + Pbc". Statistical significance: Student's t-test (non-numbered) $*P<0.05$, $**P<0.01$; Paired t-test (non-numbered) $^{\dagger\dagger}P<0.01$. Numbered significance: (a) 1) "BH4 alone", increased (Paired t-test), 0-time vs. each data point from 30–300 min, $P<0.005$; in "BH4 + Pbc", all greater than at 0-time, $P<0.05$. 2) "BH4 alone" vs. "BH4 + Pbc" (Student's t-test), $P = 0.03$ at 30 min; $P = 0.006$ at 120 min; $P = 0.02$ at 180 min. 3) "BH4 alone", decreased (Paired t-test), 0-time vs. each data point from 60–300 min, $P<0.05$. 4) "BH4 + Pbc", decreased (Paired t-test), 0-time vs. each data point from 30–300 min, $P<0.003$. 5) "BH4 alone" vs. "BH4 + Pbc" (Student's t-test): $P = 0.05$ at 60 min, $P< 0.004$ at each data point from 120–300 min. (b) 6) "BH4 alone" vs. "BH4 + Pbc" (Student's t-test), $P<0.001$ at 30 min; $P<0.006$ at 90 and 120 min; $P = 0.011$ at 150 min. 7) "BH4 alone", increased (Paired t-test), 0-time vs. 30 min, $P = 0.005$; 0-time vs. each data point from 150–270 min, $P<0.04$. 8) "BH4 alone" vs. "BH4 + Pbc" (Student's t-test), $P = 0.002$ at 30 min; $P = 0.04$ at 30 min. (c) "BH4 alone" vs. "BH4 + Pbc" (Student's t-test), $P = 0.008$ at 30 min.

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excretion by 120 min. Most of the remainder may have been broken down to pterin or xanthopterin. However, we have no information about the rest of the BP except for an observation with mice about BP movement in to the rumen of the GI tract in our previous study [14]. We observed that a considerable amount of administered 6RBH4 appeared in the rumen of mouse small intestine and moved to the caecum, where BP seemed not to be retrieved but essentially metabolized to pterin, a bare pterin-ring compound, by the cecal microflora.

The urinary BH4% before receiving 6RBH4 was 81 ± 4% (Fig 1B, upper panel). On BH4 mono-loading, the percentage had risen to 95 ± 1% at 30 min (Paired t-test, $P = 0.005$), presumably representing the amount that reached the kidney bypassing uptake by various organs. Subsequently, the BH4% was decreased to 87 ± 2% at 90 min ($P = 0.01$), followed by a gradual increase up to ca. 94% at 240 min (120 min vs. each data point from 150–240 min, $P<0.005$). Prior to administration of 6RBH4, but 30 min after receiving Pbc, the BH4% was 80 ± 7%. It remained relatively low for 120 min and then rose gradually (90 min vs. each data point from 210–270 min, $P<0.005$).

Bile. Excretion of BP in the bile was increased after administration of 6RBH4 either with or without Pbc. Levels of the bile BP were consistently higher than BP levels of time-matched
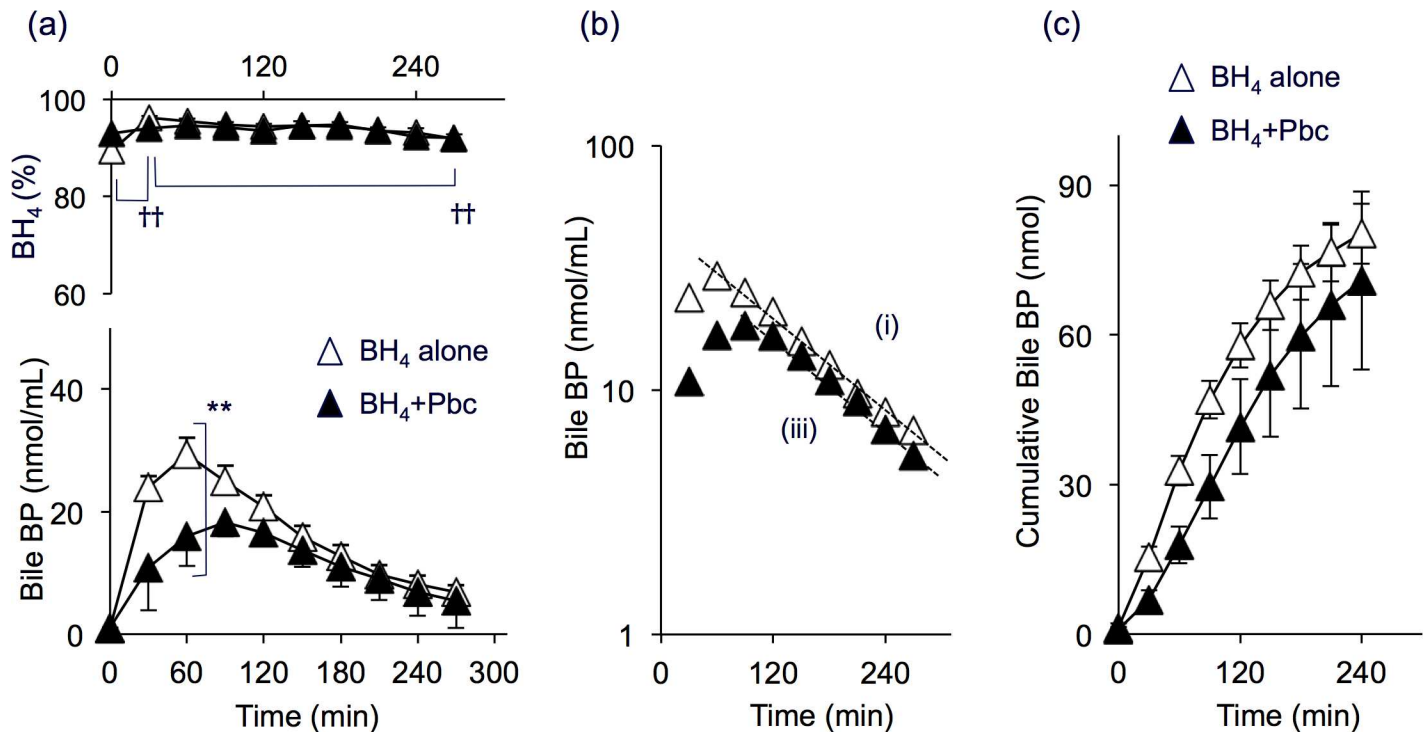
Fig 2. Biliary biopterin excretion after loading rats with 6RBH₄ with and without prior administration of probenecid. Samples were collected from the same rats as those in Fig 1. The bile was collected from the bile duct, and BP levels and BH₄ fractions (BH₄%) were determined following experimental procedure (A) of Materials and Methods. (a) Biliary BP concentration (lower panel) and the BH₄% (upper panel). One group of rats received "BH₄ alone" (open triangles), and the other, "BH₄ + Pbc" (closed triangles). (b) Exponential least-square fitting of biliary BP outflow: "BH₄ alone"; $BP(t) = 47.2 \cdot e^{-0.007t}$, correlation coefficient $|R| = 0.99$. "BH₄ + Pbc"; $BP(t) = 37.2 \cdot e^{-0.007t}$, $|R| = 0.99$. (c) Cumulative BP excretion in the bile. Data in (a) are means ±S.E. of $n = 4–6$ for "BH₄ alone" and $n = 5–7$ for "BH₄ + Pbc". Some error bars are hidden behind symbols. Data in (b) are mean values in (a). Paired t-test, ††$P<0.01$; Student's t-test, **$P<0.01$.

blood samples taken from "BH₄ alone" rats (compare Fig 2A with Fig 1A). At 30 min after 6RBH₄ administration, for example, the BP level in the bile was 2.2-fold higher than that of time-matched blood ($P = 0.001$). The elevation of the BP content in the "BH₄ + Pbc" group was 46% less than that of the "BH₄ alone" group at 60 min ($P = 0.006$). On administration of 6RBH₄, the biliary BH₄% was maintained as high as 95% and was not affected by Pbc treatment over the course of the experiment (30–270 min). The bile BP profile appeared to have a limited correlation with that of the blood BP (compare upper panels in Fig 1A and Fig 2A).

Fig 2B presents kinetics based on the same data (average) of Fig 2A. The BP concentration in both groups fitted two nearly parallel lines:

"BH₄ alone": $BP(t) = 47.2 \cdot e^{-0.007t}$ ($|R| = 0.99$) (i)

"BH₄ + Pbc": $BP(t) = 37.2 \cdot e^{-0.007t}$ ($|R| = 0.99$) (ii)

The common rate constant of 0.007 min⁻¹ denoted a half-decay period of 99 min. This indicated that Pbc caused the BP pool size in bile to become smaller but did not change the rate constant of BP secretion, indicating that Pbc had little effect on the process. The BP pool might represent BP of the liver, the source of this fluid, suggesting that the liver supplied a smaller amount of BP in the presence of Pbc.

As shown in Fig 2C, the cumulative BP amount after administration of "BH₄ alone" for 240 min was 80.3 nmoles per rat, representing 1.6% of the 6RBH₄ dose. In addition, the cumulative BP excretion in the presence of Pbc, i.e., that of the "BH₄ + Pbc" group, was not very different;

70.8 nmoles per rat for 240 min, which was 1.4% of the 6RBH$_4$ dose. In either case, the amount of BP excreted into the bile was much less than that into the urine. We previously described excretion of BP in the bile as a route for its entero-hepatic circulation in mice, and a considerable amount of BP secreted in the small intestine was brought to the caecum [14]. In order to prevent bile BP to return to the circulation, we removed all the bile in the procedure (A). If BP was also excreted by the other route such as *via* the intestinal juice under the present experimental conditions, a certain amount of BP was also secreted to rumen of the small intestine and could have moved to the caecum. Possible loss of administered BP by this route remains elusive.

Organ distribution of biopterin after 6RBH$_4$ administration to rats

Organ distribution of BP was examined after 6RBH$_4$ administration (5 mg/kg, i.v.) with Pbc (200 mg/kg, i.p.) or CsA (10 mg/kg, i.p.) or without the inhibitor. In this experiment, rats were anesthetized for handling and allowed to move freely after recovery as described in experimental procedure (B).

Blood. The initial BP level in the plasma was 0.13 ± 0.02 nmol/mL. After rats were administered 6RBH$_4$, the plasma BP of all groups, "BH$_4$ alone", "BH$_4$ + Pbc" and "BH$_4$ + CsA", was 30- to 50-fold over the initial value (Fig 3A). The elevated BP level in "BH$_4$ + Pbc" rats at 30 min was 1.8-fold higher than that in "BH$_4$ alone" ($P<0.05$). The difference between BP levels in "BH$_4$ + CsA" and "BH$_4$ alone" was not clear. Plasma BP levels in both groups of rats returned to around 1 nmol/mL at 120 min after 6RBH$_4$ administration but they were about 10-fold greater than the initial value. The initial BP level in RBCs was higher than in plasma (0.43 ± 0.06 nmol/mL) and roughly 77% of the blood BP was localized in these cells. The amount of BP in RBCs changed more slowly than that in plasma (Fig 3A and 3B).
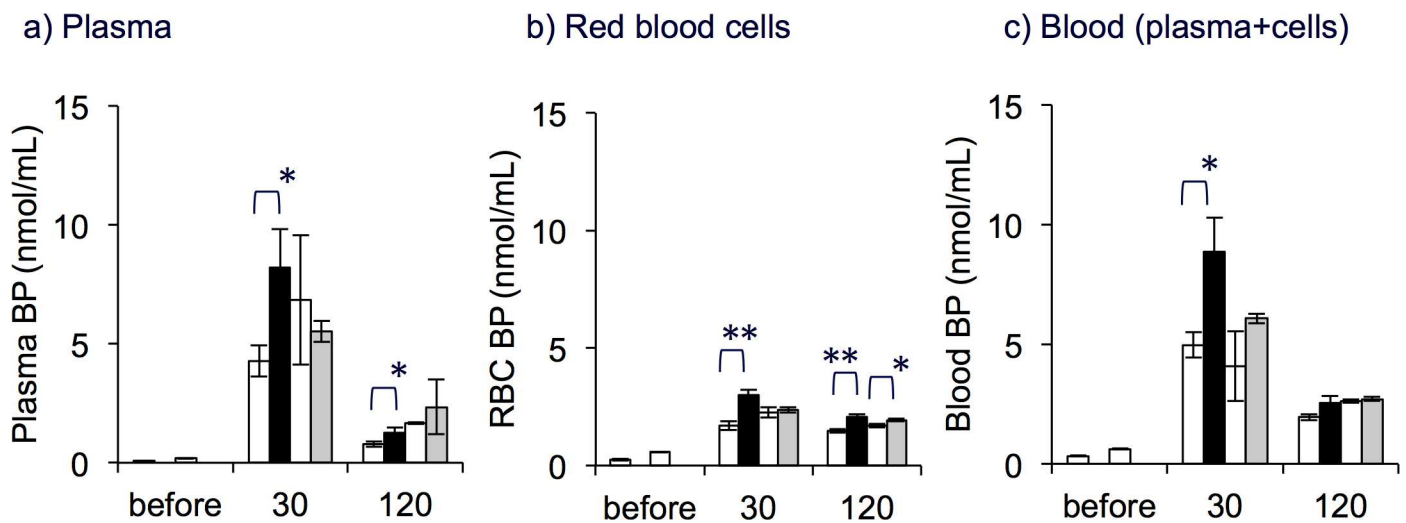

Fig 3. Effect of probenecid and cyclosporin A on the biopterin distribution in blood after 6RBH$_4$ administration. Panels show BP levels (nmol/mL) in plasma (a), red blood cells (b), and whole blood (c). The blood was collected as described in experimental procedure (B) of Materials and Methods. The amount of 6RBH$_4$ administered was 5 mg/kg, i.v. ("BH$_4$ alone", open bars), 6RBH$_4$ plus probenecid, 200 mg/kg, i.p. ("BH$_4$ + Pbc", closed bars), or 6RBH$_4$ plus cyclosporin A, 10 mg/kg, i.p. ("BH$_4$ + CsA", grey bars). The open bars on the left represent the results of "BH$_4$ alone" in experiments performed at the same time with "BH$_4$ + Pbc", and the open bars on the right represent "BH$_4$ + CsA" at the respective time points. In all panels, the rat group "before" received no treatment in all respective drug-administered groups. "BP" denotes BH$_2$ + BH$_4$. The calculation of the amount of BP per volume of original blood (mL) was based on the hematocrit value. Data are means ± S.E. (n = 4 in "BH$_4$ + Pbc" and n = 5 in "BH$_4$ + CsA"). Student's *t*-test, "BH$_4$ alone" *vs.* "BH$_4$ + Pbc", or "BH$_4$ alone" *vs.* "BH$_4$ + CsA", *$P<0.05$, **$P<0.01$.

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Increase in BP contents in the liver, kidney and brain

The BP contents increased in the liver, kidney and brain after $6RBH_4$ administration as shown in Fig 4. The initial BP levels in the organs were as follows: liver, 5.86 ± 0.47; kidney, 0.68 ± 0.04; and brain, 0.41 ± 0.01 nmol/g. The protocol used was experimental procedure (B) in the Materials and Methods section.

Liver. The liver took up a large amount of BP within the short term; the BP content increased to 53.3 nmol/g (9.1-fold over the initial value) in the "BH_4 alone" rats within the first 30 min (Fig 4A). With Pbc and CsA, the effect was different; the increase in the "BH_4 + Pbc" group (5.3-fold) was considerably less than in the "BH_4 alone" group ($P<0.05$ at 30 min), while that of "BH_4 + CsA" was rather increased ($P<0.05$ at 120 min). Two interesting findings emerged: 1) the net uptake by the liver was suppressed considerably, 9.1-fold down to 5.3-fold, even though the elevation of the plasma BP doubled in the presence of Pbc (Fig 3A), suggesting that the rapid uptake was strongly inhibited to nearly one quarter by the drug. This was in sharp contrast to "BH_4 + CsA" rats in which the BP accumulation was elevated to a level corresponding to that in the blood, suggesting that CsA did not inhibit BP uptake in this organ. 2) the release process was not greatly inhibited by the drugs either Pbc or CsA; the decrease in liver BP levels in the three groups seemed proportional over the 30–120 min period. If the BP uptake was mediated by a transporter, the fact that Pbc inhibited BP uptake by the liver suggested that the putative transporter was Pbc-sensitive and was somehow inward-directional for BP transport from the blood. BP uptake by the liver in comparison to uptake by the other organs is summarized in Table 1.

Kidney. Although the endogenous BP in the kidney was only 3% of that in the liver, this organ had a relatively high capacity to uptake exogenous BP. The amount of tissue BP was greatly increased (58-fold over the initial value) in the "BH_4 alone" rats at 30 min (Fig 4B). The BP rise in the "BH_4 + Pbc" rats was even greater (150-fold), as was the rise in "BH_4 + CsA" rats (about 80-fold), both corresponding to the respective rises in the plasma BP (compare Fig 4B
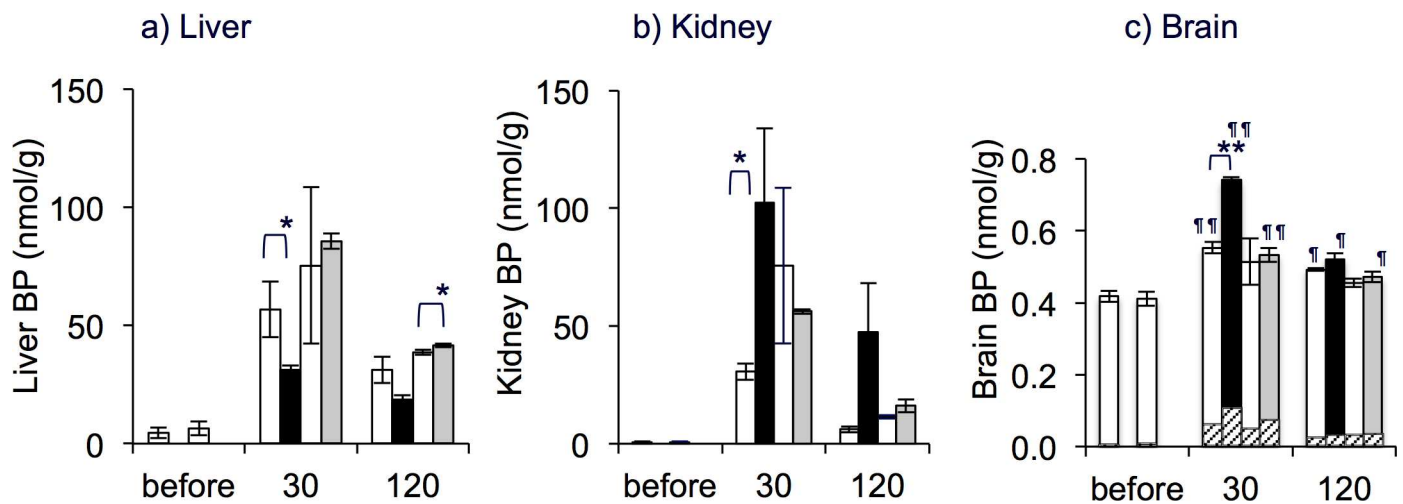

Fig 4. Organ distribution of biopterin after $6RBH_4$ administration and the effect of probenecid and cyclosporin A. BP levels in the liver (a), kidney (b), and brain (c) were determined. Rats were given $6RBH_4$ alone (5 mg/kg, i.v., "BH_4 alone", open bars) or $6RBH_4$ with prior administration 30 min earlier of probenecid ("BH_4 + Pbc", 200 mg/kg, i.p., closed bars) or cyclosporin A ("BH_4 + CsA", 10 mg/kg, i.p., grey bars). The open bars on the left represent the results of "BH_4 alone" in experiments performed at the same time as with "BH_4 + Pbc", and the open bars on the right were those with "BH_4 + CsA" at respective time points. The hatched bars in (c) indicate blood carryover of biopterin based on estimated blood inclusion (1.2%) in the brain. After the indicated periods, rats were sacrificed, and the organs were dissected as described in experimental procedure (B) of Materials and Methods. Samples were taken from the same rats as those in Fig 3. Student's t-test ("BH_4 alone" vs. BH_4 + drug at respective time points), * $P<0.05$; Tukey's test ("before" vs. 30 or 120 min), ¶$P<0.05$, ¶¶$P<0.01$; data are means ± S.E., (n = 4 for Pbc series, n = 5 for CsA series).

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Table 1. Biopterin distribution in organs after 6RBH4 administration.

	organ weight (g)	relative[1] to body	0-time		30 min		120 min	
			BP (nmol)	relative[2] to liver	BP (nmol)	relative[3] to dose	BP (nmol)	relative[3] to dose
Liver	6.9	2.8%	40.4	(100%)	367	7.1%	268	5.2%
Blood	17	6.8%	8.32	21%	76.8	1.5%	39.5	0.76%
Kidneys	1.8	0.72%	1.22	3.0%	70.6	1.4%	17.3	0.3%
Brain	1.5	0.60%	0.62	1.5%	0.8	0.15%	0.71	0.14%
Sub-total	27	11%	50.6	n.a.	515	9.9%	325	6.3%

Biopterin distribution in liver compared with the other organs. Data apply to Figs 3 and 4. BP contents (nmol/organ) are expressed as the mean values and are multiplied by the respective organ weights (g).

1) Relative to a 250 g body weight, adapted from "SD-Rat Control Data 2009" of Charles River Laboratories Japan, Inc.

2) Relative to the amount of BP in whole liver (nmol)

3) Relative to dose: (increase in organ BP over 0-time) / (5180 nmol: 6RBH4 dose per 250 g).

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with Fig 3A). The decrease in BP in all three groups during the period between 30 and 120 min was quite rapid, similar to the change noted in plasma. Taking into account that at 30 min, the peak amount of BP in the kidney was only 4–5% of the urinary BP outflow, in which renal tubular secretion was dominant as mentioned earlier (cf. Fig 1C), it is reasonable to assume that the BP in kidney tissue transited from the plasma to the urine across the tubular epithelium. This is in remarkable contrast to the liver BP which was stored in a large quantity then released gradually into the plasma.

Brain. The brain BP was significantly increased in all three groups, although much less than that of the other organs (Fig 4C). The BP contents of "BH4 alone" rats were about 43% higher after 30 min than those of untreated rats at ($P<0.01$). At this time point, the increase in "BH4 + Pbc" rats was significantly larger, as much as 65% ($P<0.01$), compared to that of "BH4 alone" rats ($P<0.05$). Since the blood content included in the brain samples was about 1.2% (v/w), it was uncertain whether the increase was caused by a blood carryover which contained increased the amount of exogenous BP (cf. Fig 3C). The BP carryover was calculated and compared with the respective BP contents of each group using mean values of BP in time-matched blood (Fig 4C). When the observed increases in BP were adjusted by subtracting the carryover, the net increase in the "BH4 alone" rats for the first 30 min was roughly 15%. Similarly, in "BH4 + Pbc" and in "BH4 + CsA" rats, BP levels were elevated by 41% and 13%, respectively. Therefore, a greater increase in BP content was verified in "BH4 + Pbc" rats than in "BH4 alone" rats ($P<0.05$).

The increase in the brain BP observed in this study might not be sufficient for stimulation of monoamine biosynthesis as reported [20, 21]. In fact, Miwa et al [22] reported that a 50- to 200-fold increase in BH4 was needed, when the preparation was injected directly into the brain ventricle for significant stimulation of monoamine biosynthesis. Although the elevation observed in this study seems to be insufficient for stimulating the biosynthesis of monoamines, it could be beneficial for neural stimulation of their release [23].

The BH4 fraction (BH4%) in organ biopterin and the effect of probenecid

Alterations in the BH4% along with the BP distribution profiles in respective organs (Figs. 3 and 4) are depicted in Fig 5.

The BH4% in untreated whole blood, on which the plasma and RBCs were not separated, was 96.2 ±0.2%, and it decreased significantly along with time (Fig 5A, $P<0.05$). Although the BH4% in the plasma showed a significant but slight increase at 30 and 120 min, the lowering of

a) Blood (unseparated)   b) Plasma   c) Red blood cells
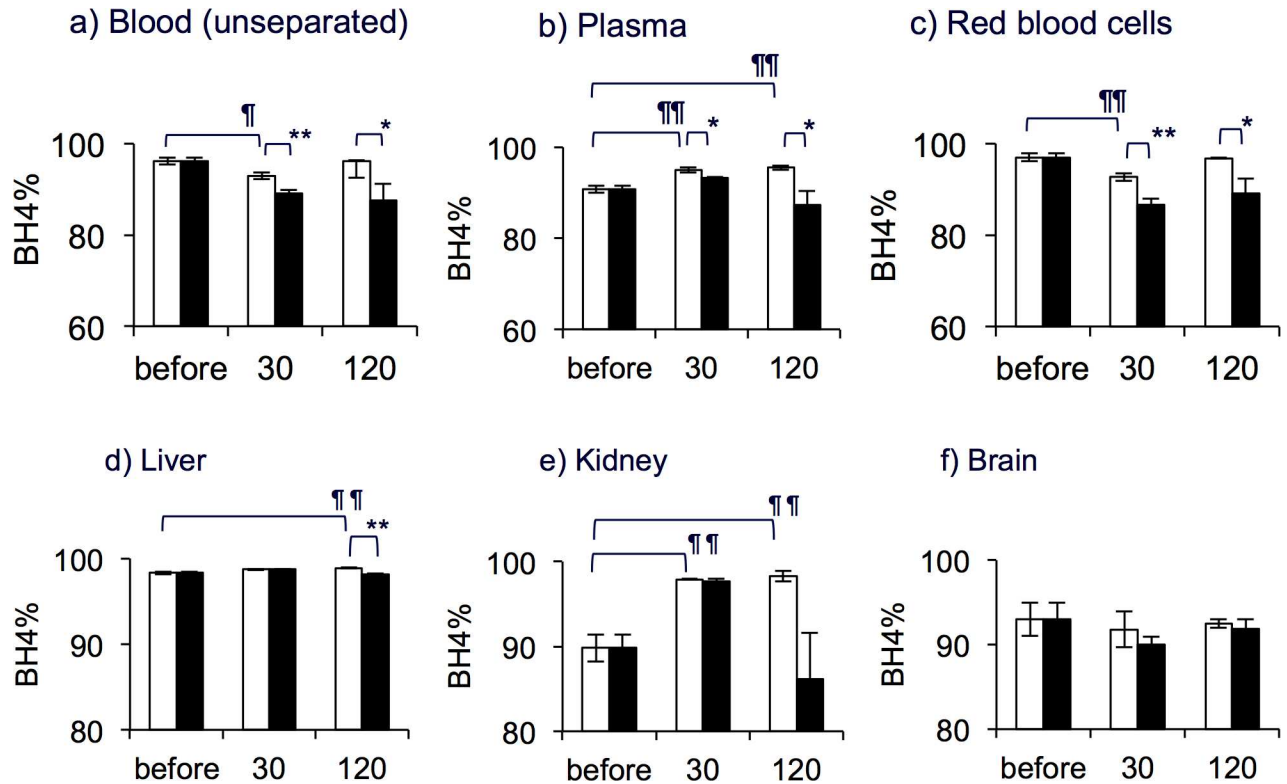
d) Liver   e) Kidney   f) Brain

Fig 5. Effect of probenecid on the BH₄ fraction (BH₄%) in blood biopterin. Panels show levels in whole blood in which the plasma and RBCs were not separated (a), plasma (b) and RBCs (c), liver (d), kidney (e) and brain (f). Rats were given 6RBH₄ alone (5 mg/kg, i.v., "BH₄ alone", open bars) or 6RBH₄ with prior administration 30 min earlier of probenecid ("BH₄ + Pbc", 200 mg/kg, i.p., closed bars). In all panels, the "before" rat group did not receive either 6RBH₄ or Pbc. The experimental procedure was the same as described in Figs 3 and 4. The BH₄% was determined as described in Materials and Methods. Data are means ± S.E. (n = 4). Tukey's test for multiple comparison, "before" *vs*. 30 min and 120 min, ¶$P<0.05$, ¶¶$P<0.01$. Student's *t*-test for two groups, "BH₄ alone" *vs*. "BH₄ + Pbc", *$P<0.05$, **$P<0.01$.

the BH₄% in RBCs was relatively large (Fig 5C, $P<0.01$) and pulled the blood BH₄% down. All samples, plasma, RBCs as well as the unseparated blood, taken from "BH₄ + Pbc" rats showed a significantly lower BH₄% than those of "BH₄ alone" rats.

The BH₄% in the liver, kidney and brain are shown in Fig 5D–5F. The initial BH₄% in these organs were: liver, 98.3 ±0.1%; kidney, 89.8 ±1.6%; and brain, 93.0±2.0%. In the liver, the BH₄% was quite stable at levels higher than 98% in all groups. In the presence of Pbc, however, namely in "BH₄ + Pbc" rats, the BH₄% was lowered slightly but significantly at 120 min (Fig 5D, "BH₄ alone" *vs*. "BH₄ + Pbc", $P<0.01$). The kidney BP showed a considerable increase in its BH₄% after the 6RBH₄ administration (Fig 5E, "before" as the reference, $P<0.01$). It was noted that profiles of the BP content in the blood and kidney looked quite similar (compare Fig 3A and Fig 4B) in the period after the 6RBH₄ administration. The brain took up a small amount of blood BP as seen in Fig 4C, and no significant change in the BH₄% was observed in this study (Fig 5F). The significance of correlation between the BH₄% of blood, liver and kidney is listed in Table 2. In this Table, the BH₄% of all organs after 6RBH₄ administration with or without Pbc or CsA are listed and compared with respect to the statistical significance of differences between the organs. The BH₄% in the liver and kidney was persistently higher than that of blood BP, while the data showed no statistically significant difference between those of liver and kidney.

Table 2. The BH$_4$% in the blood, liver and kidney after 6RBH$_4$ administration.

Time	Rat group	Blood	Liver [1]		Kidney [1], [2]	
		%	%		%	
30 min	BH$_4$ alone	93.9 ±0.6	98.7 ±0.1	**	98.0 ±0.2	**, n.s.
	BH$_4$ + Pbc	89.2 ±0.7	98.7±0.1	**	97.7 ±0.3	**, n.s.
	BH$_4$ + CsA	94.4 ±0.1	98.6 ±0.1	**	98.1 ±0.4	**, n.s.
120 min	BH$_4$ alone	96.2 ±0.1	98.8 ±0.1	**	98.6 ±0.3	**, n.s.
	BH$_4$ + Pbc	87.6 ±3.7	98.1 ±0.1	n.s.	89.2 ±5.5	n.s., n.s.
	BH$_4$ + CsA	95.3 ±0.1	98.5 ±0.1	**	98.4 ±0.1	**, n.s.

The BH$_4$% in organs 30 min and 120 min after administration of 6RBH$_4$ with or without prior treatment with Pbc or CsA. For each rat group, differences in the organ BH$_4$% between blood, liver and kidney were analyzed by Tukey's multiple testing method. BH$_4$% values are means ±S.E. and apply to Fig 5.

[1] Difference between the BH$_4$% of time-matched blood *vs*. liver or kidney. **P<0.01 significance, suggesting the BH$_4$% is higher than that of blood; "n.s.", not significant.

[2] Difference between the BH$_4$% of time-matched liver *vs*. kidney.

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Discussion

Urinary excretion was the major route of BP loss while bile was a minor route

Renal exclusion of BH$_4$ immediately after 6RBH$_4$ administration was remarkably rapid. The major portion of the exclusion, up to ca. 87% of the apparent elimination observed within 270 min, took place in the first 120 min in healthy rats. Kidney exclusion of xenobiotics and metabolic waste generally takes two paths from plasma to urine; glomerular filtration and tubular secretion [24]. The tubular secretion of exogenously administered BH$_4$ was effectively suppressed by prior administration of Pbc, as shown in this report, providing confirmative evidence supporting a previous observation using CsA [12, 13]. The previous report demonstrated that the urinary BP flow involved at least two processes, glomerular filtration with a rate constant of k = 0.013 min^{-1}, and tubular secretion with a 2.3-fold more rapid rate constant of k = 0.030 min^{-1}, which represented the difference between the observed gross rate constant of k = 0.043 min^{-1} minus the glomerular rate constant. The present data on "BH$_4$ + Pbc" rats after 90 min (Fig 1B, lower panel) also fitted the equation $BP(t) = 9.76 \bullet e^{-0.012t}$ (|R| = 0.92), suggesting that the urinary flow was essentially from glomerular filtration at 90 min or later after 6RBH$_4$ administration. The treatment with Pbc greatly increased the blood BP by inhibiting BP uptake by various organs including the kidney. Despite the substantial suppression, BP exclusion within 270 min after 6RBH$_4$ administration was almost the same in "BH$_4$ alone" and "BH$_4$ + Pbc" rats. This was explained by the fact that the concomitantly high concentration of plasma BP caused by the Pbc treatment speeded up the glomerular filtration. It should be noted that the tubular secretion was enhanced when mediated by a drug-sensitive transporter(s) presumably with large capacity but low affinity. This infers that the putative transporter only functions when the plasma BP concentration is high. Actually, it was demonstrated that the dominancy of epithelial secretion over glomerular filtration occurred only when the plasma BP concentration was 10 times higher than ordinary levels [12, 13]. In this context, adjusting the formula or recipe for 6RBH$_4$ administration so as to avoid the tubular secretion by maintaining the plasma BP level around the critical concentration (~ 10-fold) may result in a much higher efficiency in bodily replacement of BH$_4$.

We previously described excretion of BP in the bile as a route for its entero-hepatic circulation [14]. In the present experiment following experimental procedure (A) in the Materials and

Methods section, we removed all the bile and did not let it return to the liver. After $6RBH_4$ administration, the bile BP increased along with the liver BP levels in the presence or absence of Pbc. Further, the $BH_4\%$ of the bile BP was persistently high, similar to the high $BH_4\%$ in the liver, either in the presence or absence of Pbc. Pbc might have inhibited BP uptake by the liver cells but might not have affected its release into the bile. We also noted that the cumulative excretion of BP in the bile was only about 3% that in the urine. Although it was reported that biliary excretion was a major route for the elimination of various drugs or their metabolites [24, 25], our findings revealed that it was rather a minor route with regard to the bodily loss of administered BH_4. However, the fact that the liver excels in secreting BP with a high $BH_4\%$ suggests that it has some role in BH_4 secretion into the gut, however, the physiological function of the bile BH_4 remains elusive.

Distribution and retention of administered $6RBH_4$ in the organs

Hoshiga *et al.* [26] described the body-wide distribution of exogenous BH_4 which they detected by means of whole body autoradiography after i.v. injection of a tracer amount of $(6R)$-$[U-^{14}C]$ BH_4. They found the densest deposition of radioactivity to be in the kidney at 2 hours. Among large organs, the liver had the next densest deposition, while deposition in the brain and striated muscles was unremarkable. Hayashi *et al.* (in a Japanese journal [27]) described BP distribution after administration of $6RBH_4$. According to their report, the BP contents of various organs including the liver were all highest at 30 min (the earliest sampling) and the BP amount in each organ decreased gradually over the course of the 24-hour experiment, suggesting that the uptake phase of BP, in terms of the net balance of uptake minus release, had been virtually completed in organs within the first 30 min. In our present research, we treated rats with Pbc or CsA prior to administration of $6RBH_4$ with the purpose of examining the effect of these inhibitors on BH_4 uptake by various organs and the respective differences in the $BH_4\%$. We started sampling at 30 min, assuming that the net uptake had already been completed by the time that BP was circulating throughout the body based on the data reported by Hayashi *et al.* above. The retention of the BP accumulated in the liver was longer than that of the kidney and brain, and that of RBCs in the blood. Taking its large mass into account, the liver seemed to function as a large reservoir of the administered BH_4 for gradual supply to peripheral tissues including the vasculature. This meant that, over the long period, the liver released BP gradually to the bile and presumably to the blood circulation.

Homeostasis of BH_2/BH_4 after administration of $6RBH_4$

We observed that the $BH_4\%$ in the blood and urine was considerably decreased while the amount of tissue BP was prominently increased in the liver and kidney after $6RBH_4$ administration. Further, Pbc, an uptake inhibitor, enhanced the $BH_4\%$ decrease in the blood and urine. These results raised two questions: first, why did the $BH_4\%$ decrease after $6RBH_4$ administration and secondly, why did the uptake inhibitor promote a further decrease.

As to the first question, we consider that the answer lies in the mechanisms by which cells take up extracellular BH_4. BH_4 is barely able to cross the cell membrane in its tetrahydro-form because as an active coenzyme, it must remain inside the cell. Generally, the cell membrane works as a strong barrier against the in and out movement of such a hydrophilically active coenzyme as BH_4. For the uptake of extracellular BH_4, a complex series of events must occur [28]. First, BH_4 is oxidized, the resultant BH_2 is taken up by the cell, an action presumably mediated by transporters, and then BH_2 is reduced back to BH_4 through the salvage pathway. Although the location and manner of the rapid oxidation of the administered $6RBH_4$ was ambiguous, the systemic oxidation of BH_4 to BH_2 *in vivo* was clearly demonstrated by the

appearance of a BH_2 serge in the blood immediately after $6RBH_4$ administration in the mouse [19]. In the present study using rats under anesthesia, we did not observe such a prominent BH_2 surge after $6RBH_4$ administration, but noted instead a significant decrease in the BH_4%, in other words, an increase in BH_2 relative to BH_4, in the blood and urine. The moderate but long-lasting decrease in the BH_4% in rats seemed to result from essentially the same process responsible for the rapid appearance of the BH_2 serge in mice. We consider that in both rats and mice, these outcomes reflect an inevitable compensatory response of the animal body toward exogenous BH_4.

As for the second question, the answer is contained in the response to the first. The reducing action of BH_2 is an intracellular event but the BH_2 has to be brought into the cell from the plasma. Therefore, BH_2 uptake by the tissues is a prerequisite for scavenging blood BH_2. Although the uptake transporters have not all been identified, at least we know that two equilibrative nucleoside transporters called ENT1 (SLC29A1) and ENT2 (SLC29A2) are able to mediate BH_2 relocation favoring the other dihydropterin-compound, sepiapterin, but not BH_4 [29]. Some portion of the BP uptake observed in this study was obviously mediated by another transporter(s) because ENT1 and ENT2 are sensitive to nitrobenzylthioinosine (NBMPR) but not to Pbc to our knowledge. The fact that the blockade of BP uptake by Pbc enhanced a further decrease in the blood BH_4% suggests that BH_2 was effectively or even preferentially taken up by the putative transporter(s) which was sensitive to Pbc. Since the salvage pathway is thermodynamically favored to proceed, the internalized BH_2 is readily scavenged as in the push-pull accumulation of BH_4 [18, 30]. As for extracellular BH_4, however, we have not uncovered any form of active transport that would allow it to directly accumulate in the cell against a concentration gradient in a tetrahydro-form. A passive transport could not contribute to BH_4 accumulation against the concentration gradient even if a transporter(s) could potentially move it across the plasma membrane.

The endogenous content and the capacity to uptake BH_4 was prominent in the liver and kidney (Table 1). This suggested to us that most BH_2 scavenging might have occurred in these organs. It was noted that the BH_4% in these organs did not decrease when the BH_4% was lowered in the blood and urine after $6RBH_4$ administration. BP uptake by the liver was considerably inhibited by prior treatment with Pbc and it was accompanied by an even greater decline in the BH_4% of the blood and urine. This observation was explained by inhibition of the uptake process by the drug and the consequent inability of the liver to convert BH_2 to BH_4. Taking the liver's large capacity for BP uptake into account, this organ might have played the greatest role in removing the scavenged BH_2.

Considering that the kidney excreted urine with a consistently higher BH_4% than that of the plasma, the strong ability of the kidney to scavenge BH_2 was also noted. The pronounced decrease in the BH_4% in the urine in the presence of Pbc suggested reduced BH_2 scavenging in the kidney. This Pbc sensitivity indicates that the BH_2 scavenging occurred during the transit across the tubular epithelium for secretion. In this manner, the kidney was able to scavenge BH_2 from the plasma. Additionally, the kidney might have released a large amount of BH_2-scavenged "clean" BP back to the plasma resulting in a rise in the plasma BH_4%, but our experiment was not designed to show this explicitly. The details of tubular secretion with regard to the relevant transporter for BH_2 scavenging remain elusive.

When our focus was limited to classic enzyme pathways, the salvage pathway of BH_4 was seen as a mechanism for maintaining BH_4 levels against a loss of BH_4 cofactor activity. The salvage pathway, coupled with uptake of the extracellular precursor, should now be considered as an innate mechanism for scavenging BH_2, a mechanism which covers a wide range of interactions between cells and their environment throughout the body. The liver and kidney are the

major sites of BH_2 scavenging under conditions of $6RBH_4$ administration owing to their large-scale uptake of blood BP.

Remarks concerning NOS dysfunction

In clinical practice, $6RBH_4$ has been prescribed to patients with an inherited BH_4 deficiency as well as to many with BH_4-responsive phenylketonuria. On a search of the literature, we did not encounter any mention of a serious adverse effect of long-term administration in these patients [31]. In another area of medicine, BH_4 supplementation has also drawn increased attention with respect to whether it ameliorates NOS dysfunction in the cardiovascular system as a high BH_2/BH_4 ratio, representing a type of oxidative stress, is a great risk factor. Studies in this area are concerned with the homeostasis of the BH_2/BH_4 ratio, an inverse expression of the $BH_4\%$.

The administration of $6RBH_4$ is followed by a lowering of the blood $BH_4\%$ as an inevitable compensatory reaction, as discussed above. In the case of the lowering of the $BH_4\%$ in the blood, endothelial cells are the ultimate interface in all organs and are active sites of nitric oxide (NO) production regulating vascular contraction. In fact, we previously demonstrated that ENT1 and ENT2 were able to mediate cell membrane permeation of sepiapterin preferentially, permeation of BH_2 moderately and that of BH_4 only slightly [29]. Furthermore, we localized ENT2 on the vascular lumen side of endothelial cells [32], suggesting that these cells are ready to uptake increased amounts of BH_2. Owing to two characteristic properties of the enzyme NOS, once in the cell, BH_2 must be converted to BH_4 promptly. 1) The affinity of NOS for BH_2 is not particularly low compared to its affinity for BH_4; in the case of an nNOS, an isoform of eNOS, the affinity to BH_2 and to $6RBH_4$ was as close as 1: 10 [33]. 2) BH_2-bound NOS is unable to maintain an active dimer and uncouples O_2 reduction to produce superoxide [34–36]. In this context, dihydrofolate reductase is the key enzyme for maintaining a BH_2/BH_4 ratio sufficiently low for sound NO production. However, endothelial cell-derived culture cells, especially of human origin, are poorly furnished with dihydrofolate reductase [37–39], suggesting that the original endothelial cells in the human vasculature have the same vulnerability. Consequently, a high BH_2/BH_4 ratio in the plasma causes eNOS to uncouple locally in endothelial cells and leads to a negative spiral favoring BH_4 oxidation [40]. In this study, we noticed that after $6RBH_4$ administration, the blood $BH_4\%$ persistently dropped below 90%, that is, the BH_2 to BH_4 ratio became less than 1:10 even in the "BH_4 alone" group, while it was maintained at ca. 95% in untreated rats. We also noted that Pbc, a widely prescribed anti-gout drug, further increased the blood BH_2 when used with $6RBH_4$. It is interesting that many experimental studies support the use of $6RBH_4$ administration as a means of ameliorating cardiovascular symptoms, however, translation of these studies to human patients remains limited [41]. The stated vulnerability of endothelial cells suffering from a lowering of the blood $BH_4\%$ might represent a great risk factor for possible exacerbation of cardiovascular dysfunction in cases in which $6RBH_4$ is regularly administered repeatedly at a dose of 5–20 mg/kg/day.

We conclude that supplementation with BH_4 by means of administration of $6RBH_4$ has remarkably low efficacy due to its rapid loss through the urine, nonetheless it is still effective in raising BH_4 levels in peripheral organs. However, considering the general vulnerability of the cardiovascular system to an increasing BH_2/BH_4 ratio, regular administration of $6RBH_4$ may spoil the desired effect of countering NOS dysfunction by lowering the $BH_4\%$. The compensatory lowering of the $BH_4\%$ must be avoided in future improvements to the therapeutic composition. Take together improving $6RBH_4$ preparations to allow for sustained distribution while avoiding a steep elevation in plasma might help to realize the potential benefit of BH_4 supplementation.

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