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# Membrane transport of sepiapterin and dihydrobiopterin by equilibrative nucleoside transporters: A plausible gateway for the salvage pathway of Tetrahydrobiopterin biosynthesis

Akiko Ohashi [a], Yuko Sugawara [b], Kaori Mamada [a], Yoshinori Harada [b], Tomomi Sumi [b], Naohiko Anzai [c], Shin Aizawa [a], Hiroyuki Hasegawa [a,*]

[a] Department of Functional Morphology, Nihon University School of Medicine, Itabashi, Tokyo, 173-8610, Japan
[b] Department of Biosciences, Teikyo University of Science and Technology, Uenohara, Yamanashi 401-0193, Japan
[c] Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, 181-8611, Japan

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ABSTRACT

Tetrahydrobiopterin ($BH_4$) is synthesized *de novo* in particular cells, but in the case of a systemic or local $BH_4$ deficiency, $BH_4$ supplementation therapy is applied. $BH_4$-responsive PKU has also been effectively treated with $BH_4$ supplementation. However, the rapid clearance of the supplemented $BH_4$ has prevented the therapy from being widely accepted. Deposition of $BH_4$ after supplementation involves oxidation of $BH_4$ to dihydrobiopterin ($BH_2$) and subsequent conversion to $BH_4$ by the salvage pathway. This pathway is known to be almost ubiquitous in the body. However, the mechanism for the redistribution and exclusion of $BH_4$ across the plasma membrane remains unclear. The aim of this work was to search for the key transporter of the uptake precursor of the salvage pathway. Based on the observed sensitivity of pterin transport to nitrobenzylthioinosine (NBMPR), we examined the ability of ENT1 and ENT2, representative equilibrative nucleoside transporters, to transport sepiapterin (SP), $BH_2$ or $BH_4$ using HeLa cell and *Xenopus* oocyte expression systems. hENT2 was capable of transporting the pterins with an efficiency of SP>$BH_2$>$BH_4$. hENT1 could also transport the pterins but less efficiently. Non-transfected HeLa cells and rat aortic endothelial cells were able to incorporate the pterins and accumulate $BH_4$ via uptake that is likely mediated by ENT2 (SP>$BH_2$>$BH_4$ ). When exogenous $BH_2$ was given to mice, it was efficiently converted to $BH_4$ and its tissue deposition was similar to that of sepiapterin as reported (Sawabe et al., 2004). $BH_4$ deposition after $BH_2$ administration was influenced by prior treatment with NBMPR, suggesting that the distribution of the administered $BH_2$ was largely mediated by ENT2, although urinary excretion appeared to be managed by other mechanisms. The molecular basis of the transport of SP, $BH_2$, and $BH_4$ across the plasma membrane has now been described for the first time: ENT2 is a transporter of these pterins and is a plausible gateway to the salvage pathway of $BH_4$ biosynthesis, at least under conditions of exogenous pterin supplementation. The significance of the gateway was discussed in terms of $BH_2$ uptake for $BH_4$ accumulation and the release for modifying the intracellular $BH_2$/$BH_4$ ratio.



## 1. Introduction

(6*R*)-L-*erythro*-Tetrahydrobiopterin (6R$BH_4$ or simply $BH_4$) is an enzymically active form of biopterin. The primary role of $BH_4$ is that of an electron donor for a group of monooxygenase reactions such as involving phenylalanine hydroxylase [1], tyrosine hydroxylase [2], and tryptophan hydroxylase [3]. The enzyme complex nitric oxide synthase (NOS) requires $BH_4$ both for the enzyme catalysis [4] and for functional dimerization [5]. In mammals, $BH_4$ is synthesized *de novo* from guanosine triphosphate (GTP), and the pathway involves at least three enzymes, GTP-cyclohydrolase I, pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase (reviews [6–8]). $BH_4$ behaves as a coenzyme. That is, quinonoid dihydrobiopterin ($qBH_2$ ), an oxidation product of $BH_4$ in $O_2$-reducing reactions, is replenished by *in situ* reduction by dihydropteridine reductase (DHPR: EC 1.6.99.7) and converted back to $BH_4$ accompanied by consumption of NADH or NADPH [9]. The coenzyme is converted to inactive 7,8-dihydrobiopterin (7,8$BH_2$ or simply $BH_2$ ) through spontaneous isomerization of $qBH_2$. It has long been known that $BH_4$ is produced from sepiapterin (SP; 6-lactyl-7,8-dihydropterin) by two distinct enzymes, sepiapterin reductase (EC 1.1.1.153, Km to SP, 21 μM [10]) and dihydrofolate reductase (DHFR, EC 1.5.1.3, Km to 7,8$BH_2$, 5–15 μM [11,12]), the latter

* Corresponding author. Department of Functional Morphology, Nihon University School of Medicine, 30-1, Oyaguchi Kamimachi, Itabashi, Tokyo, 173-8610, Japan. Fax: +81 3 3972 0027.
*E-mail address:* hsgw-BH4@asahi-net.email.ne.jp (H. Hasegawa).

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of which converts $7,8BH_2$ to active $BH_4$ [11,13–15]. The route of $BH_4$ retrieval from SP or $BH_2$ was termed the "salvage pathway" as opposed to the *de novo* pathway of $BH_4$ biosynthesis [16]. $BH_4$ is localized almost ubiquitously throughout most major organs as well as within the vascular system and is also detectable in cerebrospinal fluid and in urine. $BH_4$ and its salvage pathway precursors exist in a state of dynamic redistribution between organs via the circulatory system.

In our previous search for effective forms of $BH_4$ supplementation, SP was found to be a good therapeutic choice [17]. Using various culture cells including RBL2H3 and HeLa, it was demonstrated that the transport of SP across the plasma membrane is equilibrative and bidirectional [18]. Although the process of cell entry is equilibrative, the cellular uptake of SP proceeds as an active transport owing to the following: (a) the salvage pathway strongly favors $BH_4$ production, and (b) $BH_4$ confined within the cell is hardly able to leak out. Since SP and $7,8BH_2$ as well as $BH_4$ are inter-convertible, they are members of the "BP group" from a metabolic point of view. Hence, either SP or $7,8BH_2$ may be called a "$BH_4$ precursor" of the salvage pathway. We were interested in the similarity between the role of the putative SP transporter(s) and that of the equilibrative nucleoside transporters (ENTs) as a precursor gateway for their respective salvage pathways. ENTs mediate the trans-membrane relocation of nucleosides and/or nucleobases in a bidirectional and equilibrative manner (reviews [19,20]).

In this study, we examined whether ENT1 and ENT2 participate in the transport of $BH_4$ precursors across the cell membrane in the salvage pathway of $BH_4$ biosynthesis in mammalian cells. We then examined hENT1 or hENT2 in terms of their ability to transport SP and $BH_2$ in a *Xenopus*-oocyte-expressing system. Because these ENTs were observed to transport SP and $7,8BH_2$, their role as the gateway to the $BH_4$-salvage pathway was once again examined with non-transfected HeLa cells as well as for the first time with primary culture cells of rat aortic endothelium (ET). Furthermore, we surveyed the systemic effects of nitrobenzylthioinosine (NBMPR), a potential inhibitor of ENTs, in terms of the distribution and deposition of $BH_4$ caused by exogenous administration of $BH_2$ to individual mice.

## 2. Materials and methods

(6*R*)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin ($6RBH_4$) was donated by Diichi-Asubio Pharma (Tokyo, Japan), and 6-lactyl-7,8-dihydropterin (SP, sepiapterin) and 7,8-dihydrobiopterin ($7,8BH_2$) were purchased from Schircks Laboratories (Jona, Switzerland). N-Acetylserotonin (NAS), methotrexate (MTX), and collagenase (for *Xenopus* oocyte defolliculation) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Nitrobenzylthioinosine (NBMPR, nitrobenzylmercaptopurineriboside), probenecid (4-(dipropylsulfamoyl)benzoic acid), *o*-coumaric acid, *p*-aminohippuric acid, penicillin G, tetraethylammonium sulfate, and cimetidine were obtained from Sigma-Aldrich (St. Louis, MO). Usually, 100× working solutions (100-fold concentration over the final concentration) were prepared with solutions of DMSO, 0.1 M HCl or saline, and the pH of the medium was adjusted if needed.

### 2.1. Cell lines

HeLa cells were maintained as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM, GIBCO® Invitrogen) containing 10% fetal calf serum at 37 °C in 5% $CO_2$/95% air.

### 2.2. Endothelial cells

Endothelium was obtained as capillary outgrowth from rat aorta under 3D-culture on a collagen gel bed (Cellmatrix®, Nitta Gelatin, Osaka) essentially according to the manufacturer's instructions. In brief, the abdominal aorta was isolated from a young adult rat (Sprague–Dawley) under anesthesia, cut to a 5-mm length, placed on a collagen bed supplemented with an "ET-cell medium" containing VEGF, and kept at 37 °C in 5% $CO_2$/95% air until the tissue had extended into a capillary outgrowth with dense branches (about 7 days). The "ET-cell medium" consisted of Nutrient Mixture F-12 Ham (N4888, Sigma-Aldrich) enriched with 20% fetal calf serum, MEM non-essential amino acids (Gibco), 1% glutamine, and 5 ng/mL rat recombinant VEGF (VEGF164®, R&D Systems Minneapolis, MN). The outgrowth was removed and subjected to digestion with collagenase (Collagenase S-1®, Nitta Gelatin). The dispersed cells were collected by centrifugation, washed thoroughly, plated on collagen-coated dishes, and then cultured in the "ET-cell medium". The cells reached confluence at about $2.5\times10^6$ cells on a 10-cm dish 5 days after seeding at $2\times10^5$ cells. They were stocked at the 3 rd passage in liquid nitrogen. Cells restored from the liquid nitrogen were allowed to proliferate until numbers were sufficient for analyses at the 5th to 9th passages.

### 2.3. Xenopus oocytes

African clawed frogs, *Xenopus laevis*, were purchased from Hamamatsu Seibutsu Kyozai (Hamamatsu, Japan). Each gonad was dissected under ice anesthesia and subjected to collagenase treatment (1 mg/mL, 1 h). Mature oocytes were then subjected to manual defolliculation, essentially according to Bianchi and Driscoll [21].

### 2.4. Mice

C57BL/6 J mice were obtained from Japan SLC (Hamamatsu, Japan). The animals were maintained on a constant 12-h light-dark cycle at 21–24 °C and 40–60% humidity with ordinary laboratory chow and tap water supplied *ad libitum*.

### 2.5. Cloning cDNA

hENT1 and hENT2 were cloned from HeLa cells. The total RNA was extracted from the cell mass of HeLa cells using Isogen (Wako Chemical Industries, Osaka, Japan) as the protein denaturant. Transcripts were obtained by a reverse transcriptase reaction (PrimeScript®, TaKaRa, Shiga, Japan) using oligo-dT (17 bases) as the primer. The coding regions of hENT1 (ACCESSION NM_004955) and hENT2 (ACCESSION NM_001532) were separately amplified by PCR in the presence of a DNA polymerase (KOD-plus®, TOYOBO, Osaka, Japan) and appropriate primers with a restriction adaptor:

hENT1 1–25 sense: TCCCCGCGGTTCGAAACCATGACAACCAGTCA CCAGCCTCAGG
hENT1 1346–1371 antisense: GCTCTAGATCACACAATTGCCCGG AACAGGAAGG
hENT2 1–21 sense: ACGCGTCGACTTCGAAACCATGGCGCGA GGAGACGC
hENT2 1342–1371 antisense: CGCCTCGAGTCTAGATCAGAG CAGCGCCTGAAGA

The cDNAs obtained by the following procedure were detected on agar electrophoresis and they were identified by sequencing with the following oligonucleotides as their primer:

hENT1 386–405 sense: TGGTGAAGGTGCAGCTGGAT
hENT2 396–415 sense: CTCCGTATGATTCATCAACT

The PCR products were both cut to provide sticky 5′- and 3′-ends using Csp45I and *Xba*I, respectively. They were inserted into pENTER 11 (Gateway System® Life Technologies). Their mammalian expression vectors, pcDNA3.2/v5/hENT1 and pcDNA3.2/v5/hENT2, were then constructed using pcDNA3.2/v5/DEST and the LR-Clonase reaction according to the manufacturer's instructions.