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Steroid hormones are novel nucleoside transport inhibitors by competition with nucleosides for their transporters

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ABSTRACT

Nucleoside transport is important for nucleic acid synthesis in cells that cannot synthesize nucleosides *de novo*, and for entry of many cytotoxic nucleoside analog drugs used in chemotherapy. This study demonstrates that various steroid hormones induce inhibition of nucleoside transport in mammalian cells. We analyzed the inhibitory effects of estradiol (E2) on nucleoside transport using SH-SY5Y human neuroblastoma cells. We observed inhibitory effects after acute treatment with E2, which lasted in the presence of E2. However, when E2 was removed, the effect immediately disappeared, suggesting that E2 effects are not mediated through the canonical regulatory pathway of steroid hormones, such as transcriptional regulation. We also discovered that E2 could competitively inhibit thymidine uptake and binding of the labeled nucleoside transporter inhibitor, S-[4-nitrobenzyl]-6-thioinosine (NBTI), indicating that E2 binds to endogenous nucleoside transporters, leading to inhibition of nucleoside transport. We then tested the effects of various steroids on nucleoside uptake in NBTI-sensitive cells, SH-SY5Y and NBTI-insensitive cells H9c2 rat cardiomyoblasts. We found E2 and progesterone clearly inhibited both NBTI-sensitive and insensitive uptake at micromolar concentrations. Taken together, we concluded that steroid hormones function as novel nucleoside transport inhibitors by competition with nucleosides for their transporters.

1. Introduction

Steroid hormones are synthesized from cholesterol in various endocrine tissues and have pivotal roles in the regulation of various physiological events. Estradiol (E2), for example, is classically synthesized in the ovary and regulates gonadal development or gametogenesis mainly through transcriptional activities of its receptor. But recent findings show that estradiol is also produced locally and plays important roles in cardiovascular, musculoskeletal, neuronal, and immunological events [1–3]. Although each steroid hormone binds to specific receptors resulting in function, recent findings suggest that there are some non-genomic actions or receptor independent actions of steroid hormones [4].

Homo et al. reported that a group of steroids disturbed uridine transport in thymocytes [5,6], possibly via a non-receptor-mediated process. However, the mechanisms underlying steroid effects on nucleoside transport are unclear. Nucleoside transport across the plasma membrane is largely dependent on nucleoside transporters (NTs). NTs are divided into two groups, the concentrative (SLC28) and equilibrative (SLC29) families on the basis of their mechanism of transport (cation-dependent or passive). NTs can be further distinguished on the basis of their sensitivity to NBTI, with equilibrative nucleoside transporter (ENT) 1 being NBTI-sensitive and ENT2 and the CNTs being NBTI-insensitive. Both ENTs and CNTs contribute to and are involved in various physiological events through regulation of nucleoside recycling and redistribution or augmentation of intrinsic adenosine signals [7].

In brain or heart, NTs modulate extracellular levels of adenosine, and thereby affect neurotransmission and activate cardioprotective responses [8]. In tumors or virus-infected cells, anti-metabolites, such as gemcitabine, 5-fluorouracil, or cytarabine, or anti-viral drugs, such as ribavirin, are delivered via NTs, and expression and activity of NTs are involved in chemoresistance [9–11]. Thus,

Abbreviations: E2, estradiol; NT, nucleoside transporter; NBTI, S-[4-nitrobenzyl]-6-thioinosine; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; P4, progesterone; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered serine; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

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regulatory mechanisms of NTs are extensively studied to understand physiological roles of NTs or to discover novel therapeutic targets.

In this study, we demonstrate that steroid hormones, including E2 and progesterone (P4), inhibit nucleoside uptake in various cell types. We determined that this inhibition was competitive between E2 and nucleoside or NBTI using SH-SY5Y human neuroblastoma cell line. The inhibitory effects of a panel of steroid hormones on nucleoside uptake in NBTI-sensitive or NBTI-insensitive cells were tested.

2. Materials and methods

2.1. Materials

Chemicals and reagents were purchased from either Kanto Chemical Co., Inc. (Tokyo, Japan) or Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) unless noted otherwise. E2, NBTI, P4, testosterone, cortisol, dexamethasone, cholesterol, chenodeoxycholic acid, deoxycholic acid, and cholic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). [*Methyl*-^{3}H] thymidine (1 mCi/ml) was purchased from G.E. healthcare (Waukesha, WI). [^{3}H] NBTI (1 mCi/ml) was obtained from Moravek Biochemicals (Brea, CA). The SH-SY5Y neuroblastoma cell (CRL-2266) was purchased from ATCC (Manassas, VA). The HEK293 human kidney embryonic cell line and the H9c2 cardiomyoblast cell line were kindly provided from Dr. Tomoichiro Asano (Department of Medical Science, Graduate School of Medicine, University of Hiroshima, Hiroshima, Japan).

2.2. Cell culture

SH-SY5Y cells were grown in 1:1 mixture of Eagle's minimum essential medium and Ham's F-12 medium with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), penicillin (50 U/L), kanamycin (100 mg/L), and streptomycin (50 mg/L). HEK 293 cells and H9c2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and antibiotics (as described above). All cells were cultured in 100 mm dishes with 5% CO_2 at 37 °C in humidified incubator.

2.3. Nucleoside uptake assay

Two hundred thousand cells were plated in each well in 24 well cell culture plates, and medium was replaced with serum-free DMEM with 0.1% BSA. After 24 h serum starvation, cells were pre-incubated for 15 min with drug or vehicle alone unless indicated. The [*methyl*-^{3}H] thymidine was immediately added after the pre-incubation period, and the cells were washed once with warmed Hanks' buffered salt solution containing 2 mM of non-radioactive thymidine and solubilized in 0.2 M NaOH/1% SDS at indicated time points after [*methyl*-^{3}H] thymidine addition for radioactivity counting. Non-specific uptake was defined as the amount of uptake in the presence of 2 mM non-radioactive thymidine, which was added 2 min before [*methyl*-^{3}H] thymidine addition, and non-specific uptake was subtracted in each data point.

2.4. [^{3}H] NBTI binding assay

The SH-SY5Y cells were kept in serum-free DMEM with 0.1% BSA for 24 h. Then, 5×10^6 cells were harvested with 0.1% EDTA/PBS, pelleted and re-suspended in 200 μl serum-free DMEM with 0.1% BSA. Cells were pre-incubated for 30 min with either various concentrations of E2 or vehicle on ice. Binding assays were started after addition of various concentration of [^{3}H] NBTI. After 30 min incubation on ice, cells were washed 3 times with ice-cold PBS, and radioactivity was measured. Non-specific binding was defined as the amount of binding in the presence of 10 μM non-radioactive NBTI, and it was subtracted from each data point.

2.5. Statistical analysis

All data were analyzed by using Microsoft Excel and MEPHAS programs (http://www.gen-info.osaka-u.ac.jp/MEPHAS/, provided by research institute for Microbial Diseases, Osaka University). The IC_{50} values of each steroid hormone were calculated using Graded50 (http://chiryo.phar.nagoya-cu.ac.jp/javastat/Graded50-j.htm, provided by Laboratory of CNS Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University). The K_i value was determined by Dixon plot [12], and the B_{max} and K_d value were determined from Scatchard plot [13].

3. Results

3.1. E2 inhibits [methyl-^{3}H] thymidine transport

First, we analyzed the effects of E2 on nucleoside transport in SH-SY5Y cells using standard uptake assays. The cells were pre-incubated in 10 μM E2 for either 0, 15, or 60 min, and [*methyl*-^{3}H] thymidine uptake into cells was measured at 5 min after [*methyl*-^{3}H] thymidine addition. As shown in Fig. 1A, E2 acutely inhibited [*methyl*-^{3}H] thymidine uptake. Time course study indicated that inhibitory effects of E2 lasted from 2 min to at least 3 h in the presence of E2 (Fig. 1B). In addition, when E2 was washed out from the medium, the inhibitory effects disappeared immediately (Fig. 1C). The acute and reversible inhibitory activities of E2 suggest that inhibition of [*methyl*-^{3}H] thymidine transport is not mediated by the canonical receptor-dependent action of E2.

3.2. E2 competitively inhibits [methyl-^{3}H] thymidine transport

To explore the mode of action of E2 on inhibition of thymidine transport, the Dixon plot analysis was conducted (Fig. 2). The results support the conclusion that the E2 affects thymidine transporters in a competitive manner, because a lower slope was obtained in higher substrate concentrations and regression lines intersect each other in the second quadrant (slope value: 0.589 ± 0.39 in 10 nM substrate, $n = 24$; 2.08 ± 0.53 in 2.5 nM substrate, $n = 24$, $P < 0.05$, Student's *t*-test). K_i value was derived from the point of intersection (8.32 ± 3.0 μM, $n = 24$).

3.3. E2 competitively inhibits [^{3}H] NBTI binding to nucleoside transporter

SH-SY5Y cells treated with NBTI prior to thymidine transport showed significant inhibition of thymidine uptake, indicating that the nucleoside transport in this cell line is largely due to NBTI-sensitive nucleoside transporters (Fig. 3A). Since NBTI binds to NBTI-sensitive NTs, leading to inhibition of nucleoside transport, we tested whether E2 affected NBTI binding to NTs. As shown in Fig. 3B, E2 inhibited [^{3}H] NBTI binding by 44 ± 7.8% in a concentration-dependent manner. We performed displacement assays of NBTI in the presence or absence of E2 and a Scatchard plot analysis based on the data is shown in Fig. 3C. The Scatchard plot analysis also showed that E2 decreased NBTI affinity to cells ($K_d^{control}$ = 35.0 ± 3.5 fM, $n = 5$; $K_d^{50\mu ME2}$ = 61.4 ± 0.37 fM, $n = 4$; $P < 0.05$, Student's *t*-test) without affecting the number of NBTI-accessible sites of NT molecules ($B_{max}^{control}$ = 0.127 ± 0.0053 fmol bound/5×10^6 cells; $B_{max}^{50\mu ME2}$ = 0.130 ± 0.0042 fmol bound/5×10^6 cells;

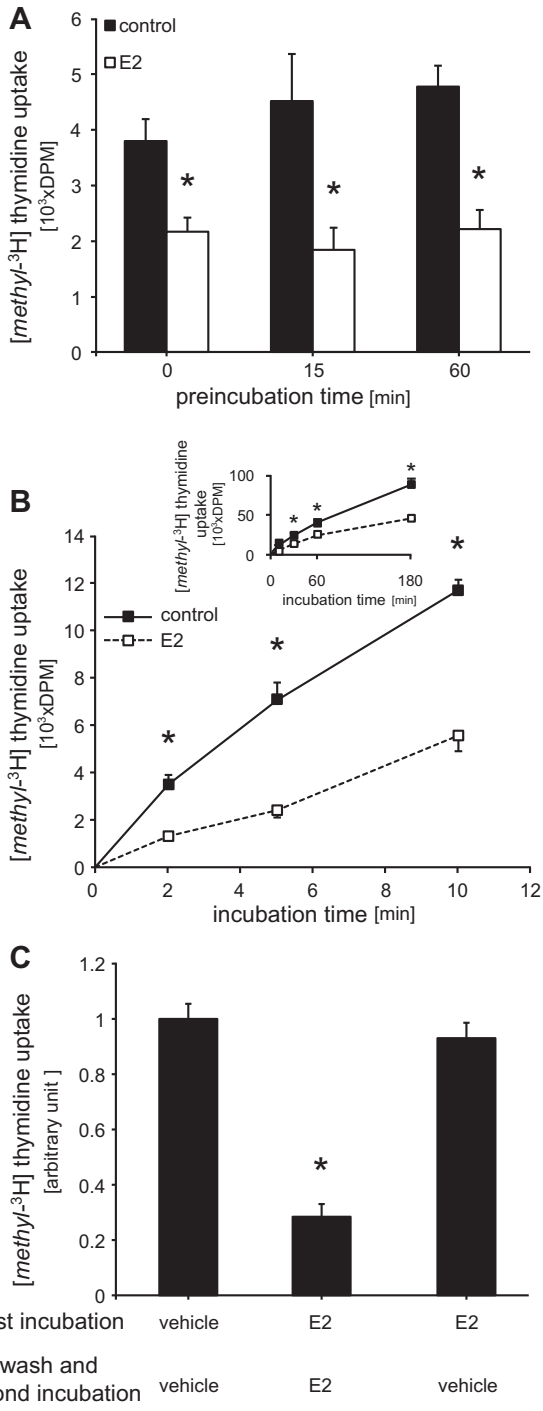

Fig. 1. E2 inhibits [*methyl*-^{3}H] thymidine uptake in SH-SY5Y cells. (A) Effect of pre-incubation time with E2 on thymidine uptake. Starved SH-SY5Y cells were pre-incubated for indicated periods with either 10 μM E2 or vehicle (control). Then, [*methyl*-^{3}H] thymidine uptake for 5 min was measured. (B) Time-course of thymidine uptake with or without E2. Starved SH-SY5Y cells were pre-incubated for 15 min with either 10 μM E2 or vehicle (control). Then, [*methyl*-^{3}H] thymidine uptake for 2, 5, 10, 30, 60, 180 min were measured (inset). The result of 2–10 min is shown in the large graph. (C) E2-mediated inhibition of thymidine uptake can be reversed. Starved SH-SY5Y cells were pre-incubated for 15 min with either 10 μM E2 or vehicle (first incubation). Cells were then washed three times with 10 μM E2-containing media or E2-free media. Soon after washing, [*methyl*-^{3}H] thymidine was added, cells were incubated with either 10 μM E2 or vehicle for 5 min (second incubation), and radioactivity inside cells was determined. The values expressed as a fold of control (first and second incubation with vehicle). Each experimental value represents the mean of three replicate wells (bars represent SEM, $n = 3$), $^*P < 0.05$ vs. control, Student's *t*-test for each condition (A and B), or one-way ANOVA and Tukey's method (C). Data are representative of 3 separate experiments.

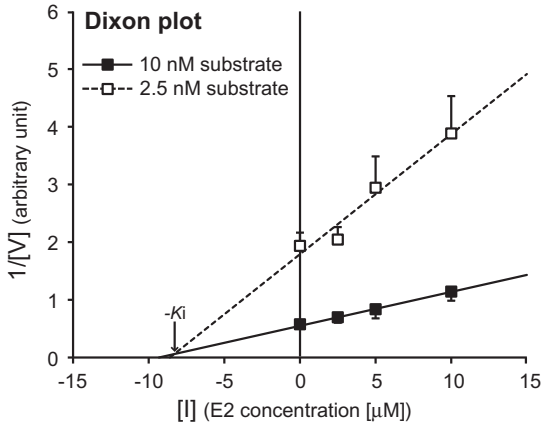

Fig. 2. E2 competitively inhibits [*methyl*-^{3}H] thymidine transport in SH-SY5Y cells. Starved SH-SY5Y cells were pre-incubated for 15 min with various concentrations of E2 or vehicle. Then, [*methyl*-^{3}H] thymidine uptake for 2, 5, 10 min were measured. Transport velocity ([V]) was calculated from the slope of the regression line determined for each concentration of E2. The plot lines were drawn from the results of 6 independent experiments in 4 different E2 concentrations (bar represents SEM, $n = 6$ in each [*methyl*-^{3}H] thymidine concentration).

$P = 0.63$, Student's *t*-test), indicating that E2 competitively inhibits NBTI binding to NTs.

3.4. Steroid hormones inhibit both NBTI-sensitive and insensitive [methyl-^{3}H] thymidine transport

We investigated inhibitory activity of steroidal compounds on thymidine transport in both NBTI-sensitive and insensitive cell lines. [*Methyl*-^{3}H] thymidine uptake assays were conducted with or without 10 μM E2, 10 μM P4, 10 nM or 10 μM NBTI, or vehicle in SH-SY5Y cells, HEK293 cells, and H9c2 cells (Fig. 4A). Thymidine transport in SH-SY5Y and HEK293 cells was sensitive to NBTI as it was largely inhibited by NBTI in the nanomolar range. In contrast, thymidine transport in H9c2 cells was NBTI-insensitive since it was not completely inhibited by NBTI even in the micromolar range. However, E2 and P4 significantly decreased [*methyl*-^{3}H] thymidine uptake in SH-SY5Y cells, HEK293 cells, and H9c2 cells at the same concentration, suggesting that E2 and P4 inhibit both NBTI-sensitive and insensitive nucleoside transport.

Finally, we examined the effects of different concentrations of various steroids on [*methyl*-^{3}H] thymidine transport in NBTI-sensitive cells (SH-SY5Y cells) and NBTI-insensitive cells (H9c2 cells) (Fig. 4B). IC_{50} values were calculated and are shown in Table 1. The IC_{50} value of NBTI was approximately 1000 fold greater in the H9c2 cells than in the SH-SY5Y, and sensitivity of all steroids to inhibition of thymidine uptake in SH-SY5Y cells was generally greater than in H9c2 cells. The inhibitory effects of E2 or P4 on inhibition of thymidine uptake were more obvious compared to testosterone, dexamethasone and cortisol. Additionally, we tested whether bile acids, another group of cholesterol structure-containing molecules, have similar inhibitory effects under the same conditions (Supplementary Data). The results clearly showed that all three bile acids examined, chenodeoxycholic acid, deoxycholic acid, and cholic acid had little or no inhibitory effect on nucleoside uptake. We concluded that some steroid hormones including E2 and P4, but not all cholesterol derivatives, can inhibit both NBTI-sensitive and insensitive nucleoside transport.

4. Discussion

Prior to initiating these studies, we evaluated thymidine incorporation into DNA as a measure of DNA replication activity in

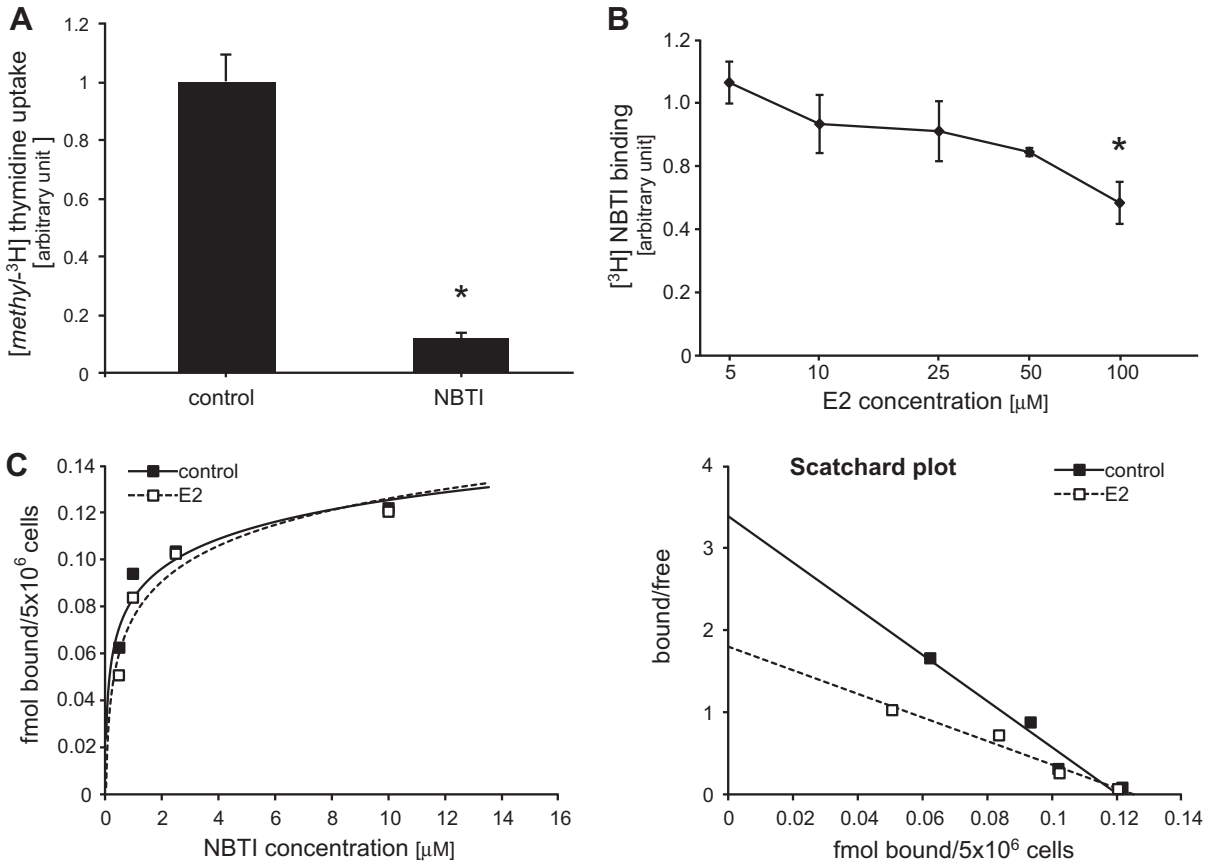

Fig. 3. E2 reduces [^{3}H] NBTI binding to nucleoside transporter in SH-SY5Y cells. (A) Effect of NBTI on thymidine uptake in SH-SY5Y cells. Starved SH-SY5Y cells were pre-incubated for 15 min with either 10 M NBTI or vehicle (control). Then, [*methyl*-^{3}H] thymidine uptake for 5 min was measured. (B) Dose-dependent competition of E2 with NBTI binding sites. Harvested SH-SY5Y cells were pre-incubated for 30 min with various concentrations of E2 or vehicle (control), and 1 nM [^{3}H] NBTI was added. After 30 min incubation on ice, cells were washed, and radioactivity was measured. (C) Competition kinetics of E2 and NBTI. Harvested SH-SY5Y cells were pre-incubated for 30 min with 50 μM E2 or vehicle, and 0.5, 1, 2.5, or 10 nM [^{3}H] NBTI was added. After 30 min incubation on ice, cells were washed, and radioactivity was determined. Competition assay with various NBTI concentrations was shown on left, and Scatchard plot analysis was shown on right (B_{max} and K_d values are described in Section 3). Each values in A and B is expressed as a fold of control and represents the mean of three replicate wells (bars represent SEM, $n = 3$), $^*P < 0.05$ vs. control, Student t test (A) or one-way ANOVA and Tukey's method (B). Data are representative of at least 3 separate experiments.

SH-SY5Y cells treated with high concentrations of E2 (over 10^{-6} M), and the results showed a suppressive effect of the steroid. Subsequent analyses, however, revealed that E2 had little effect on the distribution of S-phase cells in flow cytometric analyses and cell number in cell counting assays (data not shown) leading us to speculate that E2 might inhibit thymidine transport. After finding inhibitory effects of E2 on thymidine transport (as shown in this study), we concluded that the decrease in thymidine incorporation into DNA was a consequence of a decrease in thymidine transport. This is important information for studies on the effects of steroid hormone on DNA synthesis or RNA synthesis, because steroid hormones may affect nucleoside transport.

Here, we show that E2 decreased both binding of thymidine to NTs and binding affinity of NBTI to NTs in a competitive manner. These results indicated that E2 directly inhibited NTs without changing the number of nucleoside-accessible NTs and maximum transport activity of NTs, suggesting that E2 inhibits binding between nucleoside and NTs. How does E2 inhibit this interaction? One possibility is that E2 partially shares the binding site of nucleoside to NTs and directly interferes binding of nucleoside to NTs. However, we could not rule out the possibility that an E2 containing complex interacts with NTs, and thereby indirectly inhibits nucleoside transport. Investigation of these possibilities is in progress in our laboratory. Because inhibition by E2 was observed acutely and the inhibitory effect disappeared after removal of E2, we concluded that these E2 effects represent non-genomic action and the classical receptor-mediated intracellular signaling pathways are not involved.

We found that two steroid hormones, E2 and P4 inhibit nucleoside transport in NBTI-sensitive cell lines and an NBTI-insensitive cell line in low micromolar concentrations. These data indicate that steroid hormones can inhibit nucleoside transport of a number of transporters and the specific steroid receptors, such as estrogen receptors, are not involved in this process. Cholesterol and the three bile acids we tested did not show this inhibitory effect, suggesting that membrane toxic potentials of cholesterol structure are not the cause of this inhibitory effect. Homo et al. reported that diethylstilbestrol, a synthetic estrogen, decreases nucleoside transport in thymocytes [5,6], and our data confirm that inhibition of nucleoside transport occurs in thymocytes and also in various other cell types. E2 and P4 have close IC_{50} values both in NBTI-sensitive and insensitive cells lines. We confirmed mRNA expression of ENT1 in the SH-SY5Y cells (data not shown) and therefore steroid hormones may inhibit nucleoside transport at least through ENT1. Four NBTI-insensitive NTs (ENT2, CNT1, CNT2, and CNT3) have been cloned and well characterized in human. Since ENT2 is the major transporter in H9c2 cells [14] and mRNA expression of CNT1 was confirmed in our laboratory (data not shown), we propose that E2 and P4 can also inhibit nucleoside transport through ENT2 and possibly CNT1. These results showed the inhibition of nucleoside transport by steroid hormones are not specific to particular isoform of NTs.

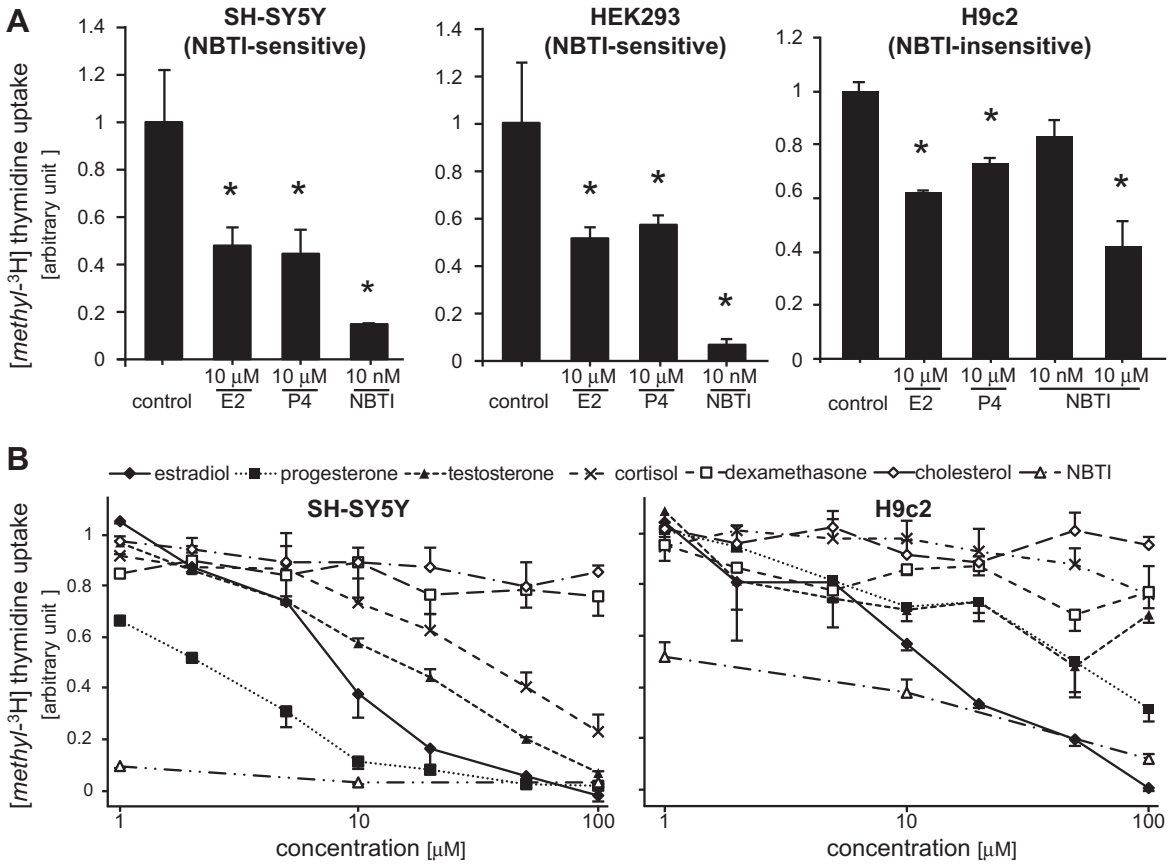

Fig. 4. E2 and P4 inhibit NBTI-sensitive and insensitive [*methyl*-^{3}H] thymidine uptake. (A) E2, P4, and NBTI effect on [*methyl*-^{3}H] thymidine uptake for 5 min in SH-SY5Y cells, HEK293 cells, and H9c2 cells. (B) Dose-dependent effect of steroid hormones, cholesterol, or NBTI on [*methyl*-^{3}H] thymidine uptake for 5 min in the SH-SY5Y cells and the H9c2 cells. Used concentrations of steroid hormones or cholesterol were 1, 2, 5, 10, 20, 50, 100 μM, and those of NBTI were 1, 10, 100 μM. Results are expressed as a fold of control (vehicle), and each experimental point represents the mean of three replicate wells (bars represent SEM, $n = 3$), $^*P < 0.05$ vs. control, one-way ANOVA and Tukey's method. Data are representative of 3 separate experiments.

Table 1
IC_{50} values of steroids and NBTI in NBTI-sensitive and insensitive cell lines. IC_{50} values were calculated from 3 independent experiments including the graphs shown in Fig. 4B (mean value ± SEM, $n = 3$).

Compound	Thymidine uptake IC_{50}	
	SH-SY5Y	H9c2
Estradiol	8.4 ± 2.5 μM	15 ± 2.5 μM
Progesterone	7.8 ± 7.2 μM	30 ± 9.0 μM
Testosterone	10 ± 3.2 μM	88 ± 6.7 μM
Cortisol	34 ± 24.0 μM	>100 μM
Dexamethasone	>100 μM	>100 μM
Cholesterol	>100 μM	>100 μM
NBTI	0.94 ± 2.7 nM	5.5 ± 2.2 μM

Levels of steroid hormones can increase to near micromolar level in plasma or body fluids [15]. For example, over 10 mg/kg of steroidal drug (10 mg in 600 ml body fluid) is sometimes administrated intravenously for steroid pulse therapy [16]. It is possible that such a supraphysiological situation and/or steroid treatment could result in altered nucleoside salvage/reuptake or increased nucleoside signaling in various tissues. Furthermore, steroid-dependent inhibition of nucleoside transport may affect experimental or clinical results in other studies. Several non-specific steroidal effects, which are not fully understood, such as immunosuppressible effect of overdose steroids [17], anti-tumor effect of high-dose estrogen to breast cancer [18] or colon cancer [19], may be mediated by steroid hormone-induced inhibition of nucleoside transport. Apart from pharmacological action of steroids, specific tissues, such as testis, ovary, adrenal gland, and brain are capable of the *de novo* synthesis of steroid hormones, and extremely high concentration of these hormones, which can be achieved in the local area can affect local availability of nucleoside [20–22]. In the placenta of pregnant women, for example, P4 concentration inside the placenta tissue is over 50 μM (25 mg/g placenta tissue), and thus the function of NTs, which are thought to be important in vascularization of placenta, may be inhibited by P4 in such situation [23,24].

Although physiological significance is unknown, few endogenous and many artificial NT inhibitors are now available [8,25–28]. Almost all compounds currently available, however, are selective to NBTI-sensitive transporters, and there are few inhibitors that can block nucleoside transport through both NBTI-sensitive and insensitive NTs at the same concentration [7,29]. ENT1 and NBTI-sensitive NTs are important in thrombosis and coronary circulation and are current pharmacological targets, but the clinical and physiological importance of NBTI-insensitive NTs such as ENT2 are also increasingly recognized [30–32]. Therefore, E2 and P4 may represent new lead compounds for NTs inhibitors.

In conclusion, the steroid hormones E2 and P4 inhibit nucleoside transport at high concentrations possibly by competing binding of nucleosides to nucleoside transporters. Our study suggests that this steroid effect would shed light on understanding of novel steroid action.

Disclosure

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.132.

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