Adenosine transport in peripheral blood lymphocytes from Lesch–Nyhan patients

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We postulated that adenosine function could be related to some of the neurological features of Lesch–Nyhan syndrome and therefore characterized adenosine transport in PBLs (peripheral blood lymphocytes) obtained from Lesch–Nyhan patients (PBL_{LN}) and from controls (PBL_C). Adenosine transport was significantly lower in PBL_{LN} when compared with that in PBL_C and a significantly lower number of high affinity sites for [^{3}H]nitrobenzylthioinosine binding were quantified per cell (B_{max}) in PBL_{LN} when compared with that in PBL_C. After incubation with 25 μM hypoxanthine, adenosine transport was significantly decreased in PBL_{LN} with respect to PBL_C. Hypoxanthine incubation lowers [^{3}H]nitrobenzylthioinosine binding in PBL_C, with respect to basal conditions, but does not affect it in PBL_{LN}. This indicates that hypoxanthine affects adenosine transport in control and hypoxanthine–guanine phosphoribosyltransferase-deficient cells by different mechanisms.

Key words: adenosine, hypoxanthine, hypoxanthine–guanine phosphoribosyltransferase (HPRT), Lesch–Nyhan, transporter.

INTRODUCTION

HPRT (hypoxanthine–guanine phosphoribosyltransferase; EC 2.4.2.8) catalyses the salvage synthesis of IMP and GMP from the purine bases hypoxanthine and guanine respectively utilizing the 5′-phosphoribosyl-1-pyrophosphate as co-substrate. Lesch–Nyhan syndrome [1,2], characterized by hyperuricemia, dystonia, choreoathetosis, mental retardation and self-injurious behaviour that may damage tissue, is associated with deficient HPRT activity. The connection between the aberrant purine metabolism and the neurological and behavioural features of this disease has not been elucidated.

In addition to their well-known intracellular functions related to DNA and RNA synthesis, purine nucleotides are released into the extracellular space where they act as intercellular-signalling molecules. In the nervous system they mediate both immediate effects such as neurotransmission, and trophic effects that induce changes in cell metabolism, structure and function. [3]. Adenosine has been related to motor and behavioural changes through its actions on specific receptors in the neuron cell surface [4]. Clonidine-induced auto-lesioning in mice has been related to adenosine receptor activity [5,6]. Chronic methylxanthine treatment, e.g. with caffeine or theophylline, is known to cause self-injurious behaviour in rats [7,8], and the behavioural effects of caffeine have been related to adenosine receptors, specifically to the A_{2a} subtype, which is abundant in basal ganglia [9]. Adenosine has also been related to motor co-ordination. In one type of dystonia, paroxysmal non-kinesiogenic dystonic choreoathetosis, attacks of generalized dystonic and choreathetotic movements can be provoked by stress or caffeine or theophylline consumption [10]. Moreover, CGS-21680 [2-(*p*-[2-carboxyethyl] phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine; an A_{2a} receptor agonist] has been shown to exert antidystonic effects in a dystonic animal model [11]. Thus we hypothesized that adenosine function could be somehow related to some of the neurological features of Lesch–Nyhan syndrome (dystonia, choreoathetosis and self-injurious biting) and it could be altered in cells with deficient HPRT activity.

In vivo, the extracellular concentration of adenosine depends on a balance between the release of this purine from cells, metabolism of the purine in the extracellular compartment by ecto-nucleotidases, and its re-uptake by a bi-directional adenosine transport process [12]. Adenosine transport seems to be an important regulator of adenosine action, and so, the aim of the present study is to examine adenosine transport in HPRT-deficient cells.

HPRT-deficient neurons cannot be obtained from living patients, and animal models are not totally satisfactory since animals do not reproduce the neurological manifestations of the syndrome [13]. This makes it necessary to find other experimental models. PBLs (peripheral blood lymphocytes) obtained from Lesch–Nyhan patients (PBL_{LN}) show the metabolic characteristics of HPRT-deficient cells: accumulation of 5-phosphoribosyl-1-pyrophosphate, enhanced *de novo* purine synthesis, excessive production and excretion of hypoxanthine, and enhanced turnover rate of adenine nucleotides [14]. Therefore we examined adenosine transport in PBL from HPRT-deficient patients as a model for HPRT-deficient cells.

HPRT deficiency provokes the excretion of increased amounts of hypoxanthine to the extracellular medium. This increase is particularly relevant in the brain, where HPRT enzymic activity is highest [15], probably owing to the fact that the '*de novo*' pathway of purine synthesis functions at a low level [16,17], and the final products of purine nucleotide catabolism are hypoxanthine (from adenine nucleotides) and xanthine (from guanine nucleotides), as there is a high level of guanine deaminase [18] and a very low level of xanthine oxidase in that organ [19,20]. Normal neuronal cells consume very effectively via HPRT, almost all the hypoxanthine formed by nucleotide catabolism and, in culture, neurons excrete

Abbreviations used: ADA, adenosine deaminase; CGS-21680, 2-(*p*-[2-carboxyethyl]phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine; CNS, central nervous system; FBS, foetal bovine serum; HBSS, Hanks balanced salt solution; HPRT, hypoxanthine–guanine phosphoribosyltransferase; NBTI, nitrobenzylthioinosine; PBL, peripheral blood lymphocyte.

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a very small amount or no hypoxanthine into the culture medium [21]. However, under physiological conditions, guanine salvage does not occur in normal neurons, and guanine is transformed to xanthine and excreted to the medium by neuronal cultures [18]. Experimental models of HPRT-deficient neurons, such as the HPRT-deficient rat neuroma cell line B103, show normal excretion of xanthine but a 15-fold increase in the excretion of hypoxanthine to the culture medium [21]. Other HPRT-deficient brain cells, such as primary astroglia cultures obtained from HPRT-deficient transgenic mice, have shown the same metabolic defect with a marked increase in the excretion of hypoxanthine into the culture media, whereas xanthine excretion remains normal [22]. Thus HPRT-deficient cells perform adenosine transport at a higher extracellular hypoxanthine concentration than do normal cells. We tested to ascertain whether this observation influences adenosine transport and function in HPRT-deficient PBL.

MATERIALS AND METHODS

Reagents

Adenosine, hypoxanthine, ADA (adenosine deaminase), NBTI (nitrobenzylthioinosine), CGS-21680, streptomycin, amphotericin B, dipyridamole, RPMI 1640 and HBSS (Hanks balanced salt solution) culture media and Histopaque®-1077 were purchased from Sigma Diagnostic. Quantification of cAMP levels was determined by a commercially available enzyme immunoassay from Amersham Biosciences (Amersham Iberica, Spain). [2-^{3}H]Adenosine (25 Ci/mmol) was obtained from Amersham Biosciences and [^{3}H]NBTI (26.1 Ci/mmol) from Pacisa-Giralt (Madrid, Spain). FBS (foetal bovine serum) was from Gibco (Barcelona, Spain) and Ro-20-1724 from Calbiochem (Nottingham, U.K.). The scintillation cocktail Packard-Ultima-Gold 6013329 was from Packard.

Materials

To study adenosine transport and [^{3}H]NBTI-binding assays, 96-well Filter Plates Multiscreen™ System (Millipore, Bedford, MA, U.S.A.) with 1.0 μm glass fibre Type B was used.

PBL culture

Heparinized blood samples were collected from nine HPRT-deficient patients (PBL_{LN}) [23] and from healthy donors of similar ages (PBL_C). Informed consent was obtained and the method was approved by the Ethics Committee of La Paz Hospital. Isolation of mononuclear cells from peripheral blood was performed by the Ficoll gradient method as described by Boyum [24], by employing a solution of polysucrose and sodium diatrizoate, which was adjusted to a density of 1.077 ± 0.001 g/ml (Histopaque®-1077). PBLs were isolated in a medium containing RPMI 1640 and 10 % (v/v) FBS. Then cells were transferred to a medium containing RPMI 1640, 42 % FBS and 8 % (v/v) DMSO and maintained in liquid N_2 until assay. The cells were carefully thawed 24 h before assay, transferred to a medium containing RPMI 1640, 10 % FBS, streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml), and incubated at 37 °C in a humid atmosphere of 5 % CO_2.

Adenosine transport in basal conditions

Adenosine transport was determined following the methods of Miras-Portugal et al. [25] and Torres et al. [26]. Transport was determined in parallel in PBL_{LN} and PBL_C under the following conditions: cells were placed in 96-well Filter Plates Multiscreen™ at a density of 2.5×10^4 lymphocytes/well in serum-free RPMI 1640 medium. Then 0.5 μCi/well of [2-^{3}H]adenosine (25 Ci/mmol) and unlabelled adenosine up to the required final concentration ranging between 1 and 10 μM was added to a 200 μl volume in serum-free RPMI 1640 medium. Transport was determined during the linear period, which corresponded to the first 1 min from the start. After 1 min, transport was stopped by rapid aspiration with the Multiscreen™ Filtration System and plates were washed twice with 200 μl RPMI 1640 medium containing 10 μM dipyridamole (to inhibit adenosine transport fully) [27]. Glass fibre filters were cut and introduced into vials with LSC cocktail. Radioactivity was determined in a β-counter (Liquid Scintillation Counter LKB-Wallac).

NBTI-binding assays under basal conditions

[^{3}H]NBTI-binding assays were performed in parallel in PBL_{LN} and PBL_C, following the method of Torres et al. [28], under the following conditions. Cells were incubated at 37 °C in a humid atmosphere of 5 % CO_2 in serum-free RPMI 1640 medium, with 2 I.U. (international unit)/ml ADA for 30 min to eliminate any adenosine present in the biological samples. Then PBLs were placed in a 96-well Filter Plate Multiscreen™ at a density of 2.5×10^4 lymphocytes/well in 200 μl serum-free RPMI 1640 medium and incubated in the presence or absence of 10 μM NBTI to determine the non-specific binding, with graded concentrations of [^{3}H]NBTI ranging between 1 and 15 nM. After 30 min, incubation was stopped by rapid filtration with the Multiscreen™ Filtration System. Glass fibre filters were cut and introduced into vials with scintillation cocktail. Radioactivity was determined in a β-counter (Liquid Scintillation Counter LKB-Wallac).

Effect of hypoxanthine on adenosine transport

Cells were incubated for 24 h in RPMI 1640, 10 % FBS, streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml) medium at hypoxanthine concentrations ranging between 0 and 50 μM, at 37 °C in a humid atmosphere of 5 % CO_2. Then cells were transferred to serum-free RPMI 1640 medium, placed in a 96-wells Filter Plate Multiscreen™ at a density of 2.5×10^4 lymphocytes/well, and 0.5 μCi/well of [2-^{3}H]adenosine (25 Ci/mmol) and unlabelled adenosine was added to give a final concentration of 1 μM. After 1 min, transport was stopped by rapid aspiration with the Multiscreen™ Filtration System and plates were washed twice with 200 μl of RPMI 1640 medium containing 10 μM dipyridamole. Glass fibre filters were cut and introduced into vials with LSC cocktail; radioactivity was determined in the β-counter.

Effect of hypoxanthine on cAMP levels

The activation of A_2 adenosine receptors is characterized by the stimulation of adenylate cyclase and can be measured by determining cAMP levels. Control and HPRT-deficient lymphocytes were employed at a density of 0.5×10^6 cells/well. Before assay, cells were incubated for 10 min at 37 °C with 30 μM Ro-20-1724 (a cAMP-specific phosphodiesterase inhibitor) in HBSS medium at pH 7.2. Then cells were incubated with 5 or 25 μM CGS-21680, with or without 5 or 25 μM hypoxanthine for 60 min at 37 °C.

At the end of the incubation period, 500 μl of ice-cold ethanol was used to stop the reaction. Samples were centrifuged at 2000 ***g*** for 20 min at 4 °C and the supernatant was freeze-dried.

Quantification of cAMP levels was determined using a commercially available enzyme-immunoassay kit (cAMP Biotrack; Amersham Biosciences) and supernatants were diluted appropriately in the assay buffer.

Adenosine transport in HPRT-deficient conditions (after 25 μM hypoxanthine incubation)

Cells were incubated for 24 h in RPMI 1640, 10 % FBS, streptomycin (50 μg/ml) and amphoterycin B (2.5 μg/ml) medium with or without 25 μM hypoxanthine at 37 °C in a humid atmosphere of 5 % CO_2. After 24 h incubation, cells were transferred to serum-free RPMI 1640 medium, placed in a 96-well Filter Plate Multiscreen™ at a density of 2.5×10^4 lymphocytes/well, and 0.5 μCi/well of [2-^{3}H]adenosine (25 Ci/mmol) and unlabelled adenosine was added to give the final required concentration in the range of 1–10 μM. After 1 min, transport was stopped by rapid aspiration with the Multiscreen™ Filtration System and plates were washed twice with 200 μl of RPMI 1640 medium containing 10 μM dipyridamole. Glass fibre filters were cut and introduced into vials with LSC cocktail and then radioactivity was determined.

NBTI-binding assays in HPRT-deficient conditions (after 25 μM hypoxanthine incubation)

Cells were incubated for 24 h in RPMI 1640, 10 % FBS, streptomycin (50 μg/ml) and amphoterycin B (2.5 μg/ml) medium with or without 25 μM hypoxanthine at 37 °C in a humid atmosphere of 5 % CO_2. After 24 h incubation, cells were transferred to serum-free RPMI 1640 medium and incubated at 37 °C, 5 % CO_2, with ADA (2 I.U./ml) for 30 min. Then PBLs were placed in a 96-well Filter Plate Multiscreen™ at a density of 2.5×10^4 lymphocytes/well in 200 μl serum-free RPMI 1640 medium and incubated for 30 min in the presence or absence of 10 μM NBTI, with graded concentrations of [^{3}H]NBTI ranging between 1 and 15 nM. Incubation was stopped by rapid filtration with the Multiscreen™ Filtration System. Glass fibre filters were cut and introduced into vials with scintillation cocktail, and radioactivity was determined.

Statistical analysis

Values are the means $\pm$ S.D. from n experiments. P values were obtained according to Student's t test. $P < 0.05$ was considered significant. The amount of non-specific binding was calculated by extrapolation of the displacement curve.

RESULTS AND DISCUSSION

Adenosine transport in HPRT-deficient PBL under basal conditions

In PBLs adenosine transport is a time-linear function during the first minute. System saturation is obtained at 50 s (results not shown). Michaelis–Menten and Lineweaver–Burk representations of the adenosine transport in control PBL cultures give a K_m value of 0.38 ± 0.6 μM and V_{max} value of 19 ± 0.5 pmol of adenosine $\cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$. These results suggest that adenosine transport in PBL cultures is a highly regulated and specific process with a high affinity. These values are of the same order of magnitude as those found in cultured chromaffin cells [25,26] in which nucleoside transport has been thoroughly studied and adenosine transport plays a crucial role in the neurosecretory process. Figure 1(A) shows adenosine transport in cultured PBL from HPRT-deficient patients and from controls, during the linear

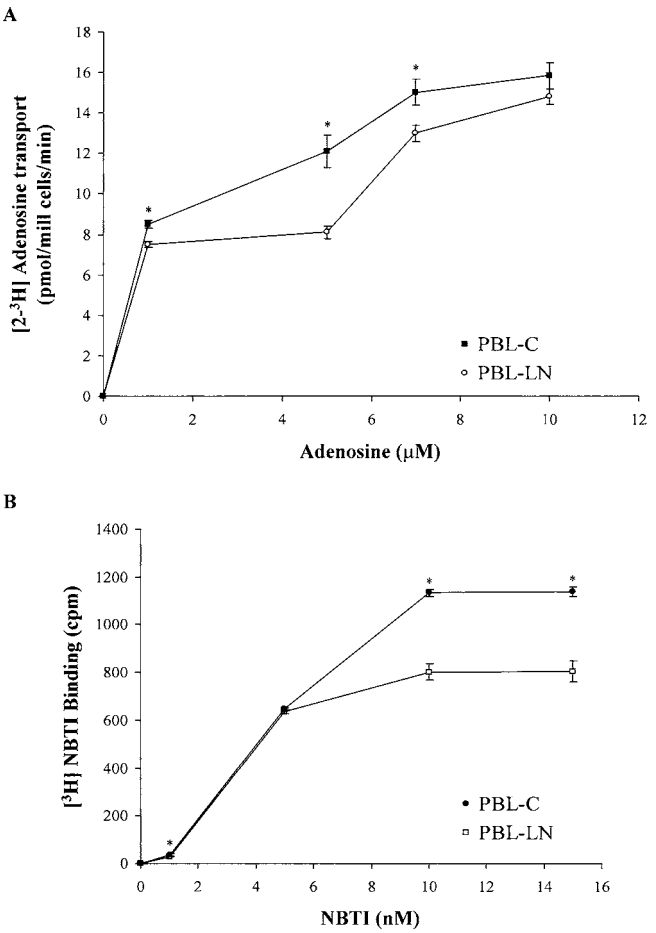

Figure 1 Adenosine transport in PBL_{LN} and PBL_C

(**A**) Means $\pm$ S.D. of adenosine transport in PBL_{LN} and PBL_C ($n = 3$) during the linear period (1 min) at adenosine concentrations ranging from 1 to 10 μM. Cells were incubated at 37 °C, 5 % CO_2, with 0.5 μCi/well of [2-^{3}H]adenosine (25 Ci/mmol) and unlabelled adenosine was added to reach the required final concentration. *Significant difference between PBL_{LN} and PBL_C. (**B**) Means $\pm$ S.D. of [^{3}H]NBTI binding ($n = 3$), expressed as c.p.m./250 000 cells, in PBL_{LN} and PBL_C. Cells were incubated at 37 °C, 5 % CO_2, with [^{3}H]NBTI concentrations ranging from 1 to 15 nM. *Significant PBL_{LN} versus PBL_C.

period, corresponding to the first 1 min after beginning the assay, at different adenosine concentrations ranging from 1 to 10 μM. Adenosine transport displays a sigmoid curve in PBL_{LN} rather than the normal hyperbolic curve observed in PBL_C (Figure 1A). Adenosine transport was significantly decreased in PBL_{LN} with respect to PBL_C at adenosine concentrations of 1 μM [7.5 ± 0.16 versus 8.5 ± 0.18 pmol $\cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$; $P < 0.005$], 5 μM [8.1 ± 0.31 versus 12.1 ± 0.80 pmol $\cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$; $P < 0.005$] and 7 μM [12.99 ± 0.41 versus 15.03 ± 0.66 pmol $\cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$; $P < 0.05$]. No significant difference was observed between PBL_{LN} and PBL_C adenosine transport at a concentration of 10 μM adenosine [14.81 ± 0.38 versus 15.84 ± 0.62 pmol $\cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$; NS (not significant)].

The mechanism whereby HPRT deficiency can affect adenosine transport is unknown. Adenosine transport is performed by different transporters, which differ in their sodium dependence and NBTI sensitivity. Depending on the cell type, different adenosine transporters may be co-expressed [29]. The reported transport activities can be classified as equilibrative and concentrative transportation with the latter being sodium-dependent. At least two types of equilibrative transport and five types of concentrative

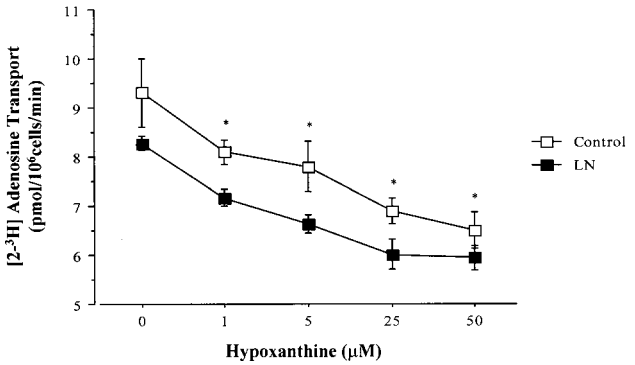

Figure 2 Effects of hypoxanthine on adenosine transport in PBL_{LN} and PBL_C

Means ± S.D. of adenosine transport in PBL_{LN} and PBL_C during the linear period (1 min). PBL were incubated in serum-free RPMI 1640 medium with different hypoxanthine concentrations (0–50 μM), at 37 °C, 5 % CO_2 for 24 h. After 24 h incubation, cells were transferred to a new serum-free RPMI 1640 medium and were incubated at 37 °C, 5 % CO_2, with 0.5 μCi/well of [2-^{3}H]adenosine, (25 Ci/mmol) and unlabelled adenosine was added to give a final concentration of 1 μM adenosine. *Significant versus 0 μM hypoxanthine.

transport have been described [30]. In human lymphocyte line cells, obtained from lymphoma parental lines, at least three nucleoside transport systems were present. The predominant system is the equilibrative system (es). The other two, N1 and N5, are primarily concentrative. The es and N5 transport systems are both NBTI-sensitive [31]. We have found that in the normal PBLs, 75 % of the adenosine transport is inhibited by nM concentrations of NBTI (results not shown). Thus we analysed [^{3}H]NBTI binding in HPRT-deficient lymphocytes to assay the NBTI-sensitive component of that transport activity. We found that [^{3}H]NBTI-binding quantified 6782 ± 395 high-affinity sites per cell (B_{max}) in PBL_{LN} versus 9500 ± 90 in PBL_C; $P < 0.05$ (Figure 1B). The cause for the decrease in NBTI-sensitive transporters in HPRT-deficient lymphocytes is unknown, but it could explain the sigmoid kinetics of adenosine transport observed in PBL_{LN}.

Our study shows that adenosine transport is decreased in HPRT-deficient lymphocytes at non-saturating adenosine concentrations, and this decrease is associated with a decrease in the number of NBTI-binding sites per cell.

Effect of hypoxanthine on adenosine transport in both PBL_C and PBL_{LN}

Figure 2 shows [2-^{3}H]adenosine transport in PBL transferred to serum-free RPMI 1640 medium after 24 h incubation with or without hypoxanthine, during the linear period, at a concentration of 1 μM adenosine. A previous 24 h incubation with hypoxanthine at concentrations of 1, 5, 25 and 50 μM significantly decreased [2-^{3}H]adenosine transport versus basal conditions (0 μM hypoxanthine), in both PBL_C and PBL_{LN} (Figure 2). Therefore we conclude that extracellular hypoxanthine concentration can influence adenosine transport in PBL.

Table 1 shows the results of [2-^{3}H]adenosine transport during the linear period, corresponding to the first 1 min after the start at a concentration of 1 μM adenosine in PBL_C and PBL_{LN} transferred to serum-free RPMI 1640 medium after a 24 h incubation with different hypoxanthine concentrations. If we compare HPRT-deficient and control PBL, we found that following incubation with hypoxanthine at concentrations of 1, 5 and 25 μM, [2-^{3}H]adenosine transport was significantly lower in PBL_{LN} when compared with that in PBL_C. There was no significant difference at 50 μM hypoxanthine.

Table 1 Effects of hypoxanthine on adenosine transport in PBL_{LN} versus PBL_C

Adenosine transport is expressed as means ± S.D.

[Hypoxanthine] (μM)	PBL_C (pmol · $min^{-1} \cdot 10^6$ $cells^{-1}$)	PBL_{LN} (pmol · $min^{-1} \cdot 10^6$ $cells^{-1}$)	PBL_C versus PBL_{LN} (P)
1	8.1 ± 0.25	7.2 ± 0.17	0.0057
5	7.8 ± 0.52	6.6 ± 0.18	0.0212
25	6.9 ± 0.27	6.0 ± 0.30	0.0188
50	6.5 ± 0.36	5.9 ± 0.25	0.0913

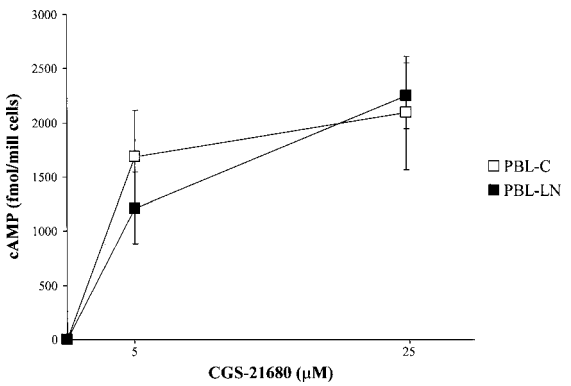

Figure 3 cAMP levels in PBL_{LN} and PBL_C

Means ± S.D. of at least four different experiments. PBLs were employed at a density of 0.5×10^6 cells/well. Before assay, cells were incubated for 10 min at 37 °C with 30 μM Ro-20-1724 in HBSS medium at pH 7.2. Then cells were incubated for 60 min at 37 °C with 5 or 25 μM CGS-21680.

Effect of hypoxanthine on cAMP levels in PBL cultures

Adenosine action is mediated through different receptor subtypes (A_1, A_{2a}, A_{2b} and A_3) that may be activated by different ranges of endogenous adenosine concentrations [12]. In lymphocytes, adenosine leads to increase in cAMP levels through the stimulation of the A_2 receptor subtype.

We found that cAMP levels increased in a dose-dependent manner with the addition of the A_2 agonist CGS-21680 in both PBL_C and PBL_{LN} (Figure 3), and A_2 adenosine receptor responses are not altered in HPRT deficiency.

In PBL_C stimulated with 5 μM CGS-21680, cAMP levels are higher with 25 μM hypoxanthine (705 ± 10 fmol/10^6 cells) when compared with 5 μM hypoxanthine addition (601 ± 11 fmol/10^6 cells; $P < 0.001$). In PBL_C stimulated with 25 μM CGS-21680, 25 μM hypoxanthine (979 ± 27 fmol/10^6 cells) increased cAMP levels with respect to 5 μM hypoxanthine addition (669 ± 32 fmol/10^6 cells; $P < 0.001$). Therefore the extracellular hypoxanthine concentration can influence cAMP levels related to A_2 adenosine receptor stimulation in PBL. No significant difference was found in 25 μM CGS-21680-stimulated cAMP levels between PBL_C and PBL_{LN} or under basal conditions (2091 ± 522 versus 2252 ± 304 fmol/10^6 cells; NS). Similarly, no significant change was observed with respect to the effect of 25 μM hypoxanthine on 25 μM CGS-21680 stimulated-cAMP levels (2930 ± 868 versus 2618 ± 768 fmol/10^6 cells; NS). The response of A_2 adenosine receptor and the effect of excess hypoxanthine on it are not altered by HPRT deficiency.

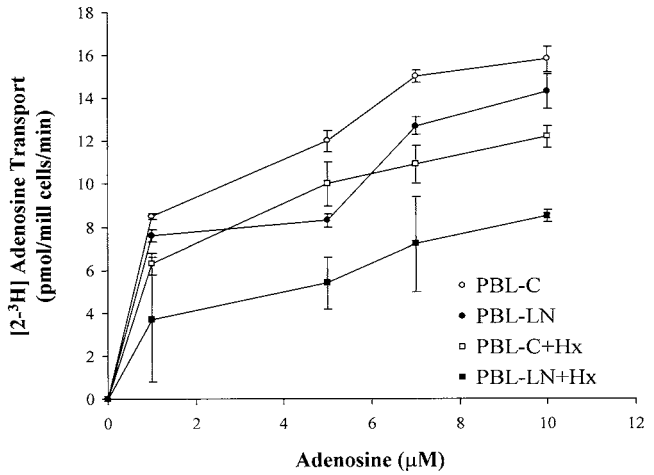

Figure 4 Adenosine transport in PBL_{LN} and PBL_C after hypoxanthine incubation

Means ± S.D. of adenosine transport in PBL_{LN} and PBL_C after 24 h incubation with or without 25 μM hypoxanthine (Hx). After 24 h incubation, cells were transferred to serum-free RPMI 1640 medium, placed in 96-well Filter Plates Multiscreen™ at a density of 2.5×10^4 lymphocytes/well, and 0.5 μCi/well of [2-^{3}H]adenosine (25 Ci/mmol) with the addition of unlabelled adenosine as needed to reach the required final concentration ranging between 1–10 μM.

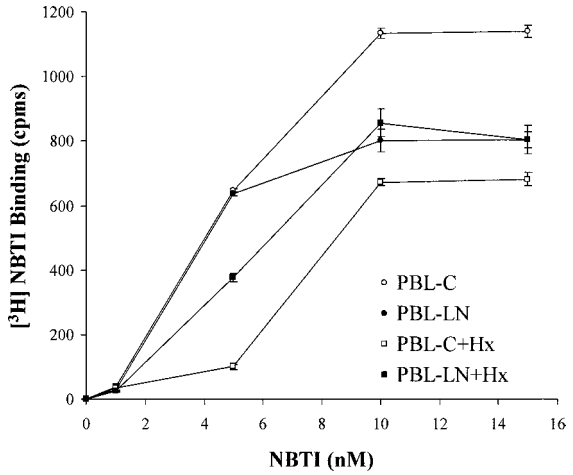

Figure 5 [^{3}H]NBTI binding in PBL_{LN} and PBL_C after hypoxanthine incubation

Means ± S.D. of [^{3}H]NBTI binding, expressed as c.p.m./250 000 cells, in PBL_{LN} and PBL_C, with or without a 24 h incubation in 25 μM hypoxanthine (Hx). After 24 h incubation, cells were transferred to serum-free RPMI 1640 medium and were incubated at 37 °C, in 5 % CO_2, with [^{3}H]NBTI concentrations ranging from 1 to 15 nM for 30 min.

Adenosine transport in HPRT-deficient conditions (after 25 μM hypoxanthine incubation)

Figure 4 shows the kinetics of [2-^{3}H]adenosine transport during the linear period, corresponding to the first 1 min of assay, in PBL_C and PBL_{LN} transferred to serum-free RPMI 1640 medium after 24 h incubation with or without 25 μM hypoxanthine. We found that incubation with hypoxanthine significantly decreases [2-^{3}H]adenosine transport at a final 10 μM adenosine concentration in PBL_C with respect to incubation without hypoxanthine [12.2 ± 0.5 versus 15.8 ± 0.6 pmol · $(10^6$ cells$)^{-1}$ · min^{-1}; $P < 0.005$] and also that the [2-^{3}H]adenosine transport at 10 μM adenosine final concentration, was significantly lower in hypoxanthine incubated PBL_{LN} when compared with those incubated without hypoxanthine [8.51 ± 0.34 versus 14.35 ± 0.81 pmol · $(10^6$ cells$)^{-1}$ · min^{-1}; $P < 0.001$]. Hypoxanthine incubation decreases adenosine transport in both normal and HPRT-deficient PBL.

If we compared HPRT-deficient and PBL_C (Figure 4) under physiological conditions, we found that [2-^{3}H]adenosine transport (during the linear period, which corresponded to the first 1 min from the start at a final 10 μM adenosine concentration) is significantly lower in PBL_{LN} after hypoxanthine incubation [8.51 ± 0.34 pmol · $(10^6$ cells$)^{-1}$ · min^{-1}] when compared with that in PBL_C after incubation without hypoxanthine [15.8 versus 0.6 pmol · $(10^6$ cells$)^{-1}$ · min^{-1}; $P < 0.001$]. If one accepts that HPRT-deficient cells survive in a higher hypoxanthine extracellular medium when compared with normal cells, one can conclude that, under the physiopathological conditions of HPRT-deficient PBL, the latter transport 53.8 % less adenosine than PBL_C at their physiologically normal conditions.

NBTI-binding assays in HPRT-deficient conditions (after 25 μM hypoxanthine incubation)

As Figure 5 shows, [^{3}H]NBTI binding in PBL_C is lower after hypoxanthine incubation when compared with the binding after incubation without hypoxanthine (2728 ± 80 c.p.m./10^6 cells versus 4560 ± 84 c.p.m./10^6 cells; $P < 0.001$). Both adenosine transport and NBTI binding are decreased in PBL_C after hypoxanthine incubation. However, there was no significant difference in [^{3}H]NBTI binding in PBL_{LN} after hypoxanthine incubation in spite of the previously mentioned decrease in adenosine transport (3212 ± 100 versus 3219 ± 178 c.p.m./10^6 cells; NS).

Hypoxanthine incubation decreased adenosine transport in both normal and HPRT-deficient PBL, but a different mechanism is implicated in each cell type. In control cells, hypoxanthine is metabolized to nucleotides via HPRT. After 24 h incubation with hypoxanthine, NBTI-sensitive adenosine transporters decreased and this decrease resulted in lower adenosine transport. However, in HPRT-deficient cells, hypoxanthine cannot be metabolized via HPRT and hypoxanthine incubation does not modify NBTI-binding sites although it seems to decrease NBTI-insensitive adenosine transport. The reason for this difference is unknown and further research is necessary.

In HPRT-deficient cells, HPRT deficiency and hypoxanthine excess both decrease adenosine transport, although by different mechanisms. Irrespective of the mechanism, both situations contribute to the fact that PBL_{LN} in an extracellular medium with high hypoxanthine levels transport less adenosine than do normal cells in a normal medium. Further studies are also necessary to characterize better the adenosine transport in HPRT-deficient cells.

Conclusions

We hypothesized that if adenosine function was somehow related to some of the neurological features of the Lesch–Nyhan syndrome, it would be altered in HPRT-deficient cells characteristic of these patients. As other authors have demonstrated [15,21,22], a consequence of HPRT deficiency is the excretion of increased amounts of hypoxanthine to the extracellular medium and we also postulated that high hypoxanthine levels could influence adenosine function. This paper has demonstrated that decreased HPRT activity and excess hypoxanthine both decrease adenosine transport in PBL, although by different mechanisms. A_2 receptor-mediated cAMP production is not altered in HPRT-deficient PBL. Similarly, excess hypoxanthine increases A_2

receptor-mediated cAMP production in normal and HPRT-deficient PBL. Therefore a lack of HPRT and excess hypoxanthine can both modify adenosine function.

After incubation with 25 μM hypoxanthine, HPRT-deficient lymphocytes can only transport approx. half (53.8 %) of the adenosine that a normal cell transports to the inside of the cell. It is known that several nucleoside transport processes regulate extracellular adenosine levels. Although nucleoside transporters function bi-directionally, it has been reported that inhibition of adenosine transport, *in vivo* and *in vitro*, increases extracellular cerebral adenosine levels [32–34], suggesting that the inhibited transport may predominantly affect adenosine uptake and not adenosine efflux [35]. Thus the inhibition of adenosine transport found in HPRT-deficient cells at increased extracellular hypoxanthine concentrations, probably increases extracellular adenosine levels. If this also occurs in HPRT-deficient CNS (central nervous system) cells, then extracellular adenosine levels would be increased in the CNS of Lesch–Nyhan patients as a result of decreased adenosine transport caused by the sum of effects of the HPRT deficiency and excess hypoxanthine. An increased extracellular adenosine concentration in striatum, obtained after systemic administration of *N*-methyl-D-aspartate, has been correlated with the behavioural effect of motor depression, providing evidence that modifications in extracellular adenosine concentrations may modify neuronal functions [36].

Adenosine effects on the CNS have been implicated in several motor and behavioural changes, including self-injures, similar to one of the manifestations observed in Lesch–Nyhan patients [5–11]. Thus our findings suggest that the decreased adenosine transport found in HPRT-deficient cells, and aggravated with the excess hypoxanthine, could be responsible for some of the neurological features of Lesch–Nyhan syndrome (dystonia, choreoathetosis and self-injurious biting).

It has been postulated that the direct cause of the neurological impairment in Lesch–Nyhan syndrome is defective arborization of dopaminergic neurons [37]. Neuropathological studies of autopsy specimens have revealed no neuroanatomical abnormalities in Lesch–Nyhan patients, but neurochemical studies have demonstrated 60–90 % decreases in the dopamine content of the basal ganglia [38,39]. Recent Positron Emission Tomography studies have documented significant decreases in dopamine transporters and [^{18}F]fluorodopa uptake in the basal ganglia of Lesch–Nyhan patients [40,41]. The results of the present study offer a pathophysiological explanation for these findings. Adenosine and dopamine receptors are coupled in the basal ganglia; thus adenosine agonists and antagonists could influence dopamine neurotransmission [42]. Consequently, a higher extracellular adenosine level could be responsible for the dopaminergic alterations found in HPRT-deficient animal models [43] and in Lesch–Nyhan patients. Further studies are needed to determine whether the cascade originated by deficient HPRT activity, excess hypoxanthine, low adenosine transport, excess extracellular adenosine and dopaminergic alteration can explain the neuropathological symptoms of Lesch–Nyhan syndrome.

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