e-ISSN:2320-1215
p-ISSN:2322-0112

Research and Reviews: Journal of Pharmacy and Pharmaceutical Sciences

Enzyme Kinetics Studies of Nucleoside Diphosphate Kinase in Human Erythrocytes and Frequency Distribution in Healthy Subjects and Transplant Recipients in Chinese Han Population

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Research Article

Received date: 06/11/2015
Accepted date: 17/11/2015
Published date: 27/11/2015

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Keywords: Nucleoside diphosphate kinase, Ion-pair, High-performance liquid chromatogram, Enzyme kinetics, Kidney transplantation, Liver transplantation, Chinese Han population.

ABSTRACT

Nucleoside diphosphate kinase (NDPK), as a house-keeping protein, involves in various molecular processes including signal transduction, energy and drug metabolism. The main objective was to investigate NDPK kinetics in human erythrocytes and to monitor the frequency distribution of NDPK activity levels in Chinese healthy subjects and transplant recipients.

METHODS: NDPK activity in erythrocytes was detected by a validated ion-pair high-performance liquid chromatogram method. NDPK kinetics studies were carried out systematically. NDPK activity levels were determined in 500 healthy subjects, 250 kidney and 250 liver transplant recipients in Chinese Han population.

RESULTS: Thermal and pH stability studies indicated NDPK was relatively stable at temperature 30-45ºC and pH 6.0-9.0. In substrate dependency study, the apparent Michaelis-Menten constant (K_m) and maximum velocity of enzymatic reaction (V_{max}) increased with concentration of substrates. Meanwhile, in product inhibition study, with the increasing concentration of dATP, the V_{max} of dADP decreased with constant K_m and K_m of dGTP increased with constant V_{max}. NDPK activity levels revealed a 7-fold variability and were not normally distributed in all groups. NDPK activity levels were significantly ($P<0.05$) higher in transplant group than those in health group. Additionally, much higher NDPK activity levels had been shown ($P<0.001$) in liver transplant recipients when compared to kidney transplant cases.

CONCLUSIONS: NDPK kinetics studies indicated substrate dependency of NDPK and a “ping-pong” mechanism for production inhibition. Skewness distributions of NDPK activity levels were shown in the study population. The transplant recipients showed higher NDPK activity levels when compared to healthy subjects.

INTRODUCTION

Nucleoside diphosphate kinase (NDPK), initially identified as putative metastasis suppressors and an enzyme required to maintain intracellular nucleoside pools [1,2], has several biochemical functions: (1) binding to DNA to activate or silence transcription [3].

(2) Acting as a phosphotransferase such as phosphorylating guanosine diphosphate to guanosine triphosphate to active small G proteins and histidine protein kinase to regulate mitochondrial function, ion channels, and isoprenoid metabolism [4,5].

(3) Playing a role of 3'-5'-exonuclease during DNA repair and replication to maintain genomic integrity [6,7].

(4) Possessing guanosine triphosphatase activation and subsequently activating specific G-proteins and regulating chromosome stability during cytokinesis [8];

(5) Working as a granzyme A-activated deoxyribonuclease during cytotoxic T lymphocytes-mediated apoptosis [9]. Furthermore, it could be used as a prognostic factor in aggressive non-Hodgkin lymphoma and classical Hodgkin's lymphoma [10,11] and could promote the growth and survival of acute myelogenous leukemia cells [12].

Additionally, it was found that NDPK could participate in thiopurines metabolism and had been correlated with treatment efficacy in childhood leukemia, the incidence of rejection episodes in kidney transplantation as well as in patients with inflammatory bowel disease (IBD) [13,14]. Thiopurines, such as azathioprine and 6-mercaptopurine, were pro-drugs and metabolized step-wise into 6-thioguanosine 5' –phosphate (6-TGTP) by various intracellular enzymes [15], in which NDPK participated in the transfer of γ-phosphate. They were commonly used as immunosuppressant agents in treatment of autoimmune diseases, chronic inflammatory disease, and post-transplant immunosuppression [15,16]. However, various kinds of adverse effects, such as myelotoxicity, hepatotoxicity, and malignant complications, limited their application, and the polymorphism of thiopurine S-methyl transferase, inosine triphosphate pyrophosphatase, hypoxanthine phosphoribosyltransferase could just explain a little [15,16]. According to the previous research [13], it was known that NDPK activity could directly influence the concentration of 6-TGTP that was correlated with treatment efficacy. Therefore, in the transplant recipients the frequency distribution of NDPK activity levels is becoming an increasingly important concern. However, no data are available on the characteristics of NDPK activity levels in Chinese population, especially in the transplant recipients.

There are various methods for NDPK assay such as enzyme-linked immunosorbent assay (ELISA) [10], immunohistochemical staining method [17], Western blotting [18], continuous spectrophotometric assay [19,20], thin layer chromatography (TLC) assay [21], enzyme-coupled spectrophotometric assay [22], and blue native polyacrylamine gel electrophoresis (PAGE) method [23]. ELISA can measure the antigen level of NDPK in serum or tissue [10]. Immunohistochemical staining methods were used to measure local NDPK level in tissue and locate secretory tissue [17]. Western blotting can determine the NDPK protein expression levels in tissues and cells; however, it was time-consuming and semi-quantitative while requiring a high specificity of antibody [18]. Importantly, these above methods can only measure the expression levels but not the activity. Continuous spectrophotometric assay progressed a three-step reaction with five reactants to calculate the reaction rate of enzyme activity except for the complexity of the procedures [19,20]. TLC assay with [γ-^{32}P] adenosine triphosphate as substrate and deoxyguanosine triphosphate (dGTP) as product, but the crude extracts must be adjusted to the same protein concentration and it had radioactivity [21]. Enzyme-coupled spectrophotometric assays were applied to analyze kinetics behavior and to quantify NDPK from a wide variety of animal, microbial, and plant sources, but NDPK needs to be purified and requires a large amount of sample and auxiliary enzymes [22]. Blue native PAGE method can monitor NDPK expressing level and activity but its procedures were complex [23]. Thus, the development and validation of a lower cost and simpler procedure with high sensitivity is crucial for measuring NDPK activity.

Since the importance of NDPK has been revealed, the enzyme kinetics of NDPK had been studied previously [24,25]. On account of that the A and B isoforms of NDPK were the most abundant members in many human tissues including erythrocyte, different kinetic parameters of NDPK-A or NDPK-B with various nucleotides substrate were summarized in review [25]. Additionally, a detailed transient kinetic analysis of the human NDPK-A and NDPK-B had been also conducted to study the half-reaction of NDPK-catalyzed phosphoryl transfer [26]. Because the appropriate temperature, pH and ionic strength were not easily chosen, most of the previous kinetic studies were conducted on purified enzymes. However, NDPK-A and NDPK-B had been demonstrated unequivocally with NDPK activity [27], and the enzyme kinetics of purified or recombinant NDPK may not reflect the actual status and properties. Therefore, it is urgently necessary to investigate the properties and enzyme kinetics of NDPK in internal environment.

In the present paper, an ion-pair gradient high-performance liquid chromatogram (HPLC) method was validated for analyzing the activity of NDPK enzymes (mostly NDPK-A and NDPK-B) in erythrocytes, by measuring the generated deoxyguanosine diphosphate (dGDP) in a γ-phosphate transfer reaction. Furthermore, the properties and enzyme kinetics of NDPK were systematically investigated. Finally, for the first time the frequency distribution of NDPK activity levels was investigated in erythrocytes of healthy subjects, kidney and liver transplant recipients in Chinese Han population.

METHODS

Chemicals and Reagents

The standard compounds of deoxyadenosine diphosphate (dADP), deoxyadenosine triphosphate (dATP), dGDP, dGTP, inosine (internal standard, IS), 1,4-Dithio-D/L-threitol (DTT), and tetrabutylammonium hydrogen sulfate (TBAHS) were purchased from Sigma-Aldrich (St. Louis, USA). Methanol and acetonitrile were provided from Merck KGaA (Darmstadt, Germany, HPLC-grade). KH_2PO_4, K_2HPO_4, $MgCl_2$, and KOH were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Solutions of DTT

(30 mg/mL) were prepared freshly before any experiment. Pure water was obtained from an Ultrapure Water Polishing System (Chengdu, China).

Study Population

Healthy subjects (health group) and patients receiving immunosuppressant (cyclosporine and tacrolimus) after solid organ transplant surgery (kidney and liver) were recruited among March to June in 2012 from the Physical Examination Center of Union Hospital and Department of Organ Transplantation Center of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, P.R. China). All organ donors (kidney and liver) were retrieved from the Accident and Emergency Department and several intensive care units. Donor eligibility screening and testing did not reveal any contraindications to transplantation. None of the transplant donors were from a vulnerable population and all donors or next of kin provided written informed consent that was freely given. All subjects enrolled in this study met the following criteria:

(1) Their body mass index (BMI) was between 18.5 to 24.9 kg/m^2

(2) Their age ranged from 20 to 60 years

(3)They were in good healthy as determined by complete physical examination, 12-lead electrocardiograms (ECGs), chest x-ray, and routine laboratory tests, including routine hematology, blood chemistry, urinalysis, and negative pregnancy test. In transplant recipients the clinical data regarding the dose and duration of immunosuppressant's administration, adverse drug reactions, and laboratory data were assessed by screening of patients' medical records. The study protocol was approved by the independent ethics committee, Huazhong University of Science and Technology (Permit Number: 2012-S019, Wuhan, P.R. China). All subjects were informed of the investigational nature of this study and signed an informed consent prior to any screening procedure.

Preparation of nucleotides standards and internal standard

Stock solutions of the nucleotides (dADP, dATP, dGDP, and dGTP) were prepared in ultrapure water at a final concentration of 10 mM (dADP and dGTP) and 1 mM (dATP and dGDP) and stored at –20ºC. They were diluted in ultrapure water to generate working solutions of dADP (6 mM), dATP (0.26 mM), dGDP (0.25 mM), and dGTP (3 mM). IS was prepared in ultrapure water at a final concentration of 1 mM and stored at 4ºC.

Preparation of erythrocyte lysate

Blood samples were collected in EDTA tubes and prepared within 6 hours after blood sampling by centrifugation at 3000 rotate per min (rpm) for 10 min and washing the remaining erythrocytes with an equal volume of 0.9% sodium chloride twice. Supernatant was again discarded and the packed erythrocytes were immediately frozen at –80ºC until analysis. For determination of NDPK activity, packed erythrocytes were diluted with 19 volumes of potassium phosphate buffer (0.02 M, pH 7.4).

Sample preparation

The activity of NDPK activity was measured by determining the amount of a specific nucleotide triphosphate (dGDP) produced in the presence of phosphate donor (dGTP)-acceptor (dADP) coupled system. To validate the method, the incubation conditions previously described were modified slightly [13]. Briefly, the reaction mixture contained 0.25 mM $MgCl_2$, 0.1 mM DTT, 270 mM $NH_4H_2PO_4$ buffer (pH 7.0), 0.1 mM dGTP, 0.2 mM dADP, and 10 µL of diluted erythrocytes. After incubation at 37ºC for 5 min with gentle shaking, the reaction was stopped by heating at 100ºC for 10 min. After cooling on ice, addition of 10 µL of 1 mM IS, centrifugation (10,000 rpm for 10 min) at 4ºC in Thermo Scientific Sorvall Legend Micro 21R centrifuge (Osterode, Germany), and subsequent dilution of the supernatant with ultrapure water (1:1, v/v), 10-µL aliquots were used for HPLC analysis with a detection wavelength of 260 nm. The NDPK activity was calculated as nmol of dGDP generated per min per milligram of Hb (nmol/min/mgHb).

Chromatography

The separation conditions were prepared according to the method described previously [13]. Determination of dGDP and IS was complemented on the Shimadzu LC-20AT Prominence HPLC system (Shimadzu Corporation, Tokyo, Japan), consisted of a binary pump (LC-20AT), auto sampler (SIL-20A), temperature-controlled column compartment (LGC-1025M), and PDA detector (SPD-M20A). The samples were separated by gradient elution at 25ºC on a Symmetry Shield™ RP_{18} column (3.9 mm×150 mm, 5 µm, Waters, USA) with a pre-column (4.0 mm×10 mm, 5 µm, Shimadzu, Japan). Operation of HPLC equipment and data collection was controlled by LC-solution software version 1.24 (Shimadzu, Japan). The mobile phases consisted of solvent A (20 mM potassium phosphate with 5 mM TBAHS) and solvent B (acetonitrile). The following gradient was used: 0 to 2.0 min, 6% to 13% B, 0.50 mL/min; 2.0 to 7.0 min, 13% B, 0.50 mL/min; 7.0 to 30.0 min, 13% B, 0.80 mL/min, followed by a 10-min post-run interval at 6% B with flow rate of 0.50 mL/min until the next sample was injected.

Method validation

Calibration curve samples with final concentrations of 1.04, 2.08, 4.17, 8.33, 16.67, 25.00, 50.00 µM dGDP per sample were prepared in 300 µL of reaction mixture without adding dADP and dGTP. The sequent procedures were performed as described

above. Calibration curves were constructed by plotting the peak area ratio of dGDP to IS against the dGDP concentrations used with least-squares linear regression analysis. Accuracy and precision were evaluated by assessing QC samples at the following concentrations (n=6): low (2.92 μM), medium (12.50 μM), and high (41.67 μM) for dGDP per sample, respectively. The lower limit of quantification (LLOQ) was determined as the lowest concentration on the calibration curve which should be reproducible with a precision of 20% and accuracy of 80 to 120%. Extraction efficacy of dGDP was measured by comparing the peak areas from spiked diluted erythrocytes samples to those from samples prepared without erythrocytes.

The stability of stock solution of standards storing at -20ºC and IS at 4ºC for 1 week were assessed in sextuplicate. The stability of the dGDP at room temperature and 4ºC within the mixture was determined by preparing six replicates of QC samples, which were injected immediately and after 24 and 72 hours into the HPLC system. In addition, the stability of NDPK activity in erythrocytes after three freeze–thaw cycles was investigated as well as the stability after placing on ice for 6 hours. The stability of NDPK activity in erythrocytes at -80ºC was investigated by analysis of the study samples before and after storing at -80ºC for 3, 6, and 12 months.

Enzyme kinetics study of NDPK in erythrocytes

Incubation experiment

To choose appropriate incubation condition, the relationship were investigated between the generated dGDP and NDPK amounts ranging from 0.05 to 0.88 mgHb or incubation time ranging from 1 to 30 min or incubation temperature ranging from 20 to 85ºC. All experiments were performed in triplicate.

Thermal and pH stability analysis

To assess the thermal and pH stability of NDPK in erythrocytes, the fresh diluted erythrocytes were allowed to place for 2 hours at various temperatures ranging from 20 to 85ºC in water baths, or the reaction mixtures were prepared with different final pH values ranging from 4.0 to 9.0. All experiments were performed in triplicate.

Substrate dependency study

To determine the substrate dependency, 10 μL aliquots of diluted erythrocytes were added to the reaction mixture with varying concentrations of dADP (0.05-0.80 mM) at different fixed concentrations of dGTP (0.025-0.40 mM), as well as with dGTP (0.025-0.40 mM) as the variable substrate and dADP (0.05-0.80 mM) as the changing-fixed substrate, to a final volume of 300 μL under conditions identical to those described above. All the initial velocities were assayed under the same conditions according to the method described previously. All experiments were performed in triplicate. Initial velocities were determined at time points at which no more than 5% of the substrates had been consumed. The kinetics parameters, including apparent Michaelis-Menten constant (K_m) and the maximum velocity of enzymatic reaction (V_{max}), were calculated based on Michaelis-Menten equation by using a least squares regression. The Lineweaver-Burk plots, also described as double-reciprocal plots, were derived from initial rates.

Product inhibition study

For product inhibition studies, different fixed concentrations of dATP were prepared, ranging from 0.0 to 2.0 mM, as the product inhibitor in the presence of various concentrations of dADP ranging from 0.1 to 0.8 mM or various concentrations of dGTP ranging from 0.05 to 0.4 mM. Initial velocities were measured as described above. All of the assays were performed in triplicate.

Determination of NDPK activity in human erythrocytes in Chinese Han population

Based on the validated ion-pair gradient HPLC method, NDPK activity in erythrocytes was determined in 500 solid organ transplant recipients (transplant group), including 250 cases of kidney transplant (kidney group) and 250 of transplantation of liver transplant (liver group), and 500 ethnically and geographically matched healthy subjects (health group). To analyze the influences of gender and age on NDPK activity, we divided the healthy persons into two groups according to gender and four groups for age. Similarly, to analyze the influence of immune suppressants and diseases on NDPK activity, different groups were compared to each other. All samples were analyzed in duplicate.

Statistical analysis

Values are presented as mean ± SD or mean values (range) as specified in results. Data distribution was tested by the method of Kolmogorov-Smirnov (K-S) test. For comparisons between group data that did not display normal distributions, non-parametric analysis was performed by Mann–Whitney U test. Data were fitted and analyzed using Graph Pad Prism version 5.0 software (San Diego, CA, USA). The statistical significance was defined as $P<0.05$.

RESULTS

Chromatography

To achieve optimized chromatographic resolution, peak sharpness, and signal intensity, a variety of mobile phase with different pH, column temperature and several gradient elution procedures were evaluated. The optimized LC condition was a

e-ISSN:2347-7857
p-ISSN:2347-7849

mobile phase consisting of 20 mM phosphate buffer with 5 mM TBAHS in water with gradient elution. Under these conditions, the retention times were approximately 4.0, 13.5, 16.0, 19.0, and 23.5 min for IS, dGDP, dADP, dGTP, and dATP, respectively. The peaks of all the compounds of interest were free of interference from any other peaks presented in the erythrocytes.

Assay validation

The enzymatic reaction was specifically catalyzed by NDPK, and depended on the existence of both substrates (dGTP and dADP). During the method validation analysis, the calibration curves were linear over the concentration range of 1.04 to 50.00 μM of dGDP with r^2=0.9998 and the LLOQ was 1.04 μM (CV<20%). The CVs of the within- and between-day were less than 5% at QC concentrations **(Table 1).**

Table 1. Precision and accuracy for the determination of 2'-deoxyguanosine-5'-diphosphate (dGDP) in human erythrocytes.

Concentration added per sample μM	Day	Intra-day (n=6)			Inter-day (n=18)		
		Mean ± S.D μM	Accuracy %	CV %	Mean ± S.D μM	Accuracy %	CV %
2.92	1	3.10 ± 0.03	106.14	1.04	2.93 ± 0.15	100.36	5.00
	2	2.81 ± 0.12	96.43	4.25			
	3	2.87 ± 0.06	98.51	2.18			
12.5	1	12.23 ± 0.16	97.86	1.28	12.26 ± 0.53	98.04	4.37
	2	12.53 ± 0.48	100.26	3.82			
	3	12.00 ± 0.74	96.01	6.18			
41.67	1	39.58 ± 1.34	95.00	3.38	40.78 ± 1.85	97.86	4.41
	2	41.84 ± 2.44	100.41	5.84			
	3	40.91 ± 0.44	98.17	1.00			

The mean accuracy (%) of dGDP at QC concentrations were 106.14, 97.86, and 95.00, respectively (n=6) and 100.36, 98.04, and 97.86, respectively (n=18). The mean extraction recoveries (%) of dGDP at QC concentrations were 100.49, 99.68, and 113.45, respectively (n=6), with CV less than 5%. The extraction recovery (%) of the IS at the concentration of 16.7 μM was 97.65 with CV less than 5% (n=18). Stock standard solutions of dADP, dGDP, dGTP, and dATP remained stable for at least 1 week at -20°C. The IS solution remained stable for at least 1week at 4°C. The QC samples were stable for 24 hours at room temperature and for 72 hours at 4°C. The NDPK activity levels were stable after three freeze–thaw cycles, placing on ice for 6 hours, storing at -80°C for at least 1 year.

Enzyme kinetics of NDPK in erythrocytes

Incubation experiment

The relationship of generated dGDP and Hb amount revealed a linear increase of dGDP generation up to 0.88 mgHb (r >0.99) **(Figure 1A).** In order to save the amount of erythrocytes and to ensure reaction efficiency, 10 μL of diluted erythrocytes (equivalent to about 0.18 mgHb) were chosen to measure NDPK activity.

The relationship of generated dGDP and incubation time revealed a linear increase of dGDP generation up to 20 min with the correlation coefficient (r)>0.99, and then increased slightly **(Figure 1B)** To save time and ensure optimum reaction rate, a 5-min incubation time was chosen.

As is shown in **(Figure 1C)**, the generated dGDP straightly increased with incubation temperature from 20 to 40°C, slightly increased from 40 to 45°C, and then declined slightly with temperature from 55 to 80°C. To ensure the high activity and be close to the human body environment, incubation temperature 37°C was chosen. In summary, the optimal incubation conditions were chosen as 10 μL of 1:20 diluted erythrocytes incubated at 37°C for 5 min.

Thermal stability

The thermal stability of NDPK activity is presented in **Figure 2A.** Activity with holding at the temperature of 30°C for 2 hours was considered as the reference (100%). A plateau occurred when heat-preservation temperatures ranged from 30 to 45°C with residual activity rate more than 90%. The residual activity rate decreased to about 50% when the temperature increased to 55°C. Furthermore, nearly no activity was detected when the temperature increased to 65-80°C.

pH stability

The pH stability of NDPK activity is presented in **Figure 2B**. The enzyme activity of NDPK apparently increased when pH ranged from 3.0 to 6.0 while the enzyme activity declined slightly with pH increasing from 6.0 to 9.0. Therefore a relatively sharp peak with maximal activity at pH 6.0 formed. Meanwhile, the enzyme appeared to be unstable at lower pH, and nearly no activity was detected when pH was adjusted to 3.0. Therefore, we could draw the conclusion that NDPK was more stable in alkaline environment.

e-ISSN:2347-7857
p-ISSN:2347-7849

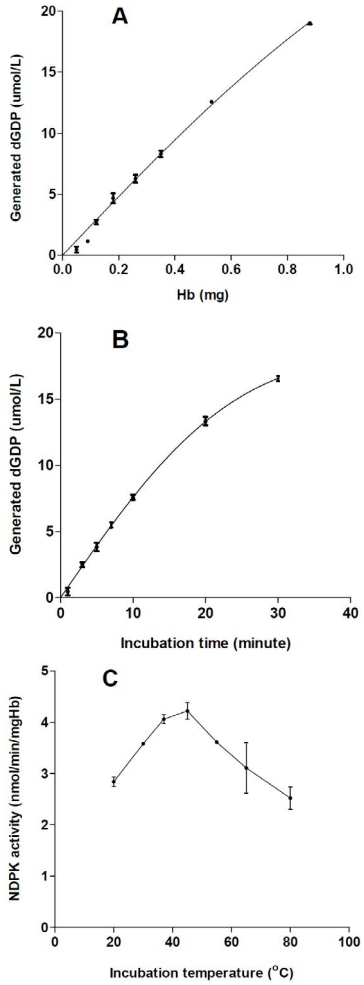

Figure 1. The incubation situations of NDPK in erythrocytes (n=3). (A) Representative plots of the concentration of generated dGDP against the amounts of Hb ranging from 0.05 to 0.88 mg. (B) Representative plots of the concentration of generated dGDP against the incubation time ranging from 1 to 30 min. (C) Representative plots of the concentration of generated dGDP against the incubation temperature ranging from 20 to 85°C.

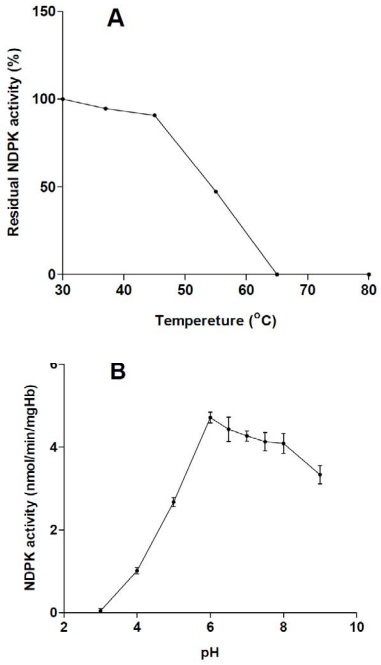

Figure 2. The thermal stability and pH stability of NDPK in erythrocytes (n=3). (A) Representative plots of the residual activity of NDPK after placing at various temperatures ranging from 20 to 85°C in water baths for 2 hours. (B) Representative plots the activity of NDPK incubating in various pH ranging from 3.0 to 9.0.

e-ISSN:2347-7857
p-ISSN:2347-7849

Substrate dependency

Substrate dependency of NDPK activity is presented in **Figure 3**, in which the concentrations of dADP and dGTP varied independently. A series of parallel lines were presented when plotting the reciprocal of initial velocities against the reciprocal of concentrations of dADP at different concentrations of dGTP **(Figure 3A).** Analyzed by GraphPad Prism software, their slopes were not significantly different (P>0.05). Essentially similar findings were obtained when plotting reciprocal of initial velocity against reciprocal of dGTP concentrations at different concentrations of dADP **(Figure 3C).**

Furthermore, the replot of the intercepts of those lines from **Figure 3A** against the reciprocal of dGTP concentrations showed a linear relationship (r>0.99) **Figure 3B.** Similar results were presented in the replot of the 1/velocity (1/v) intercepts of the parallel lines from **Figure 3C** against reciprocal of dADP concentrations **(Figure 3D).** The x and y intercepts in **Figure 3B** and **Figure 3D** represented the K_m and V_{max}. Therefore the K_m and V_{max} of dGTP were determined to be 1.94 mM and 111.35 nmol/min/mgHb, respectively, and the K_m and V_{max} of dADP were determined to be 1.26 mM and 111.41 nmol/min/mgHb, respectively.

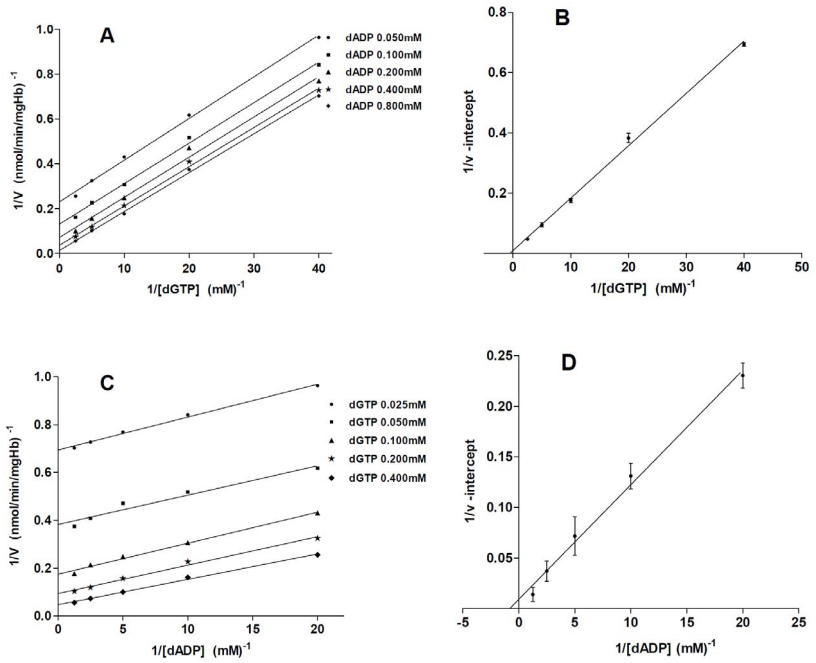

Figure 3. Substrate dependency of NDPK activity (n=3). (A) The reciprocal of initial velocities against the reciprocal of concentrations of dADP at different concentrations of dGTP. (B) The intercepts of those lines from Figure 3A against the reciprocal of dGTP concentrations. (C) The reciprocal of initial velocity against reciprocal of dGTP concentrations at different concentrations of dADP. (D) The intercepts of those lines from Figure 3C against the reciprocal of dADP concentrations.

Additionally, a global fit of the initial rates to velocity equation for double displacement mechanism and velocity equation for sequential mechanism was conducted, suggesting that the data fit best to a Ping-Pong mechanism [28]. These findings were consistent with the concept that the erythrocyte NDPK reaction followed a Ping-Pong mechanism. A tendency was observed towards higher values of the apparent V_{max} and K_m with the increasing concentrations of one of the substrates at the concentrations investigated (0.025 mM to 0.800 mM).

Product inhibition

For product inhibition studies, a series of converging lines were presented when plotting the reciprocal of initial velocities against the reciprocal of concentrations of dADP at different concentrations of dATP as shown in **Figure 4A**, indicating that a typical pattern of non-competitive inhibition was obtained. Meanwhile, competitive inhibition was obtained for the product dATP to the reactant dGTP suggesting that dATP and dGTP were mutually competitive for the same form or binding site of the enzyme **Figure 4B.** With the increasing concentration of dATP (0.0 to 2.0 mM), the V_{max} of dADP decreased with constant K_m and K_m of dGTP increased with constant V_{max}.

Frequency distribution of NDPK activity levels in Chinese Han population

The general characteristics of the study population are evaluated. As listed in **Table 2.**

The three groups had a similar average age (40.38 ± 9.47 years for health group, 39.98 ± 10.37 years for kidney group and 42.96 ± 9.31 years for liver group), respectively, as well as similar gender proportion of males (70.2% for health group, 70.8% for kidney group, and 70.4% for liver group, respectively) with no significant difference (P>0.05). Additionally, with Kolmogorov-Smirnov test analysis, ages of subjects in all groups were normally distributed (P=0.20).

e-ISSN:2347-7857
p-ISSN:2347-7849

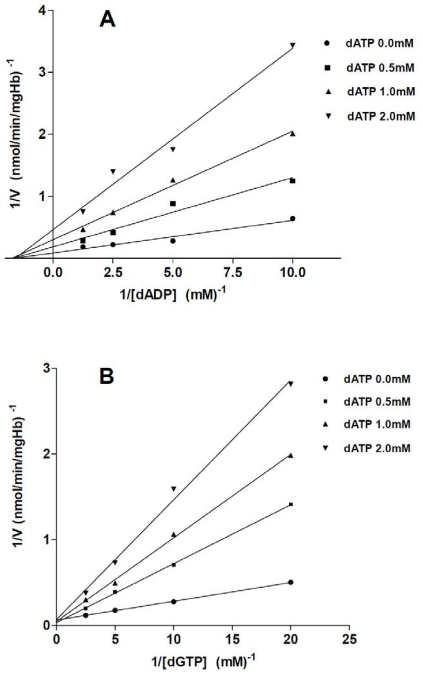

Figure 4. The inhibition of the product dATP on NDPK activity (n=3). (A) The reciprocal of initial velocities against the reciprocal of concentrations of dADP at different concentrations of dATP, indicating that a typical pattern of non-competitive inhibition of dADP by dATP. (B) The reciprocal of initial velocities against the reciprocal of concentrations of dGTP at different concentrations of dATP, suggesting that dATP and dGTP were mutually competitive for the enzyme.

Table 2. Characteristics of Chinese healthy subjects and transplant recipients.

Characteristics	Healthy subjects	Kidney transplant recipients	Liver transplant recipients
Number of subjects (n)	500	250	250
Sex (m/f)	351/149	177/73	176/74
Age (years)	40.38 ± 9.47	39.98 ± 10.37	42.96 ± 9.31
Smoking (%)	30.5	32.1	33.8
Drinking (%)	25.8	25.7	24.1
Years after transplantation		5.0 ± 2.3	4.7 ± 2.5
Cyclosporine (n)		201	195
Tacrolimus (n)		49	55
Prednisone (n)		225	237

The NDPK activity levels in erythrocytes of the every group were determined using the validated ion-pair gradient HPLC method. The NDPK activity levels ranged from 1.50 to 7.87 (mean ± SD: 3.16 ± 0.80; median: 3.01) nmol/min/mgHb in healthy group and 1.13 to 7.71 (3.19 ± 0.67; median: 3.14) nmol/min/mgHb in transplant group, respectively. Additionally, NDPK activity levels ranged from 1.14 to 7.71 (3.10 ± 0.72; median: 3.10) nmol/min/mgHb in kidney group, and ranged from 1.13 to 5.06 (3.28 ± 0.61; median: 3.17) nmol/min/mgHb in liver group, respectively. Furthermore, the NDPK activity levels in human erythrocytes of all groups we're not normally distributed ($P<0.05$, **(Figure 5)**) with non-parametric (Mann-Whitney) tests analysis, the NDPK activity between the health group and transplant group was significantly different ($P<0.05$). Importantly, there was significant difference ($P<0.001$) in the NDPK activity between the kidney group and liver group.

To assess whether NDPK activity levels were related to the gender or age, females and males were divided into four groups according to age, that is, age1 (20-29 years), age2 (30-39 years), age3 (40-49 years) and age4 (50-60 years). However, with non-parametric tests analysis, there was no significant difference among ages and no significant difference between females and males in four groups ($P>0.05$).

DISCUSSION

Our assay presented a viable method to determine NDPK activity in human erythrocytes by measuring the generated dGDP in a γ-phosphate transfer reaction with dGTP and dADP as substrates. It was noteworthy that the method was a little different from previous method [13], in which the NDPK activity was calculated by measuring the resulting product dATP amount. For the first time, in the present study another product dGDP was successfully used to calculate the NDPK activity in human erythrocytes. Through the validation analysis, this ion-pair gradient HPLC method had good linearity, selectivity, specificity, precision, accuracy, recovery, and stability, which could be used in routine clinical practice for the patients to evaluate the therapy efficacy and adverse effects.

e-ISSN:2347-7857
p-ISSN:2347-7849

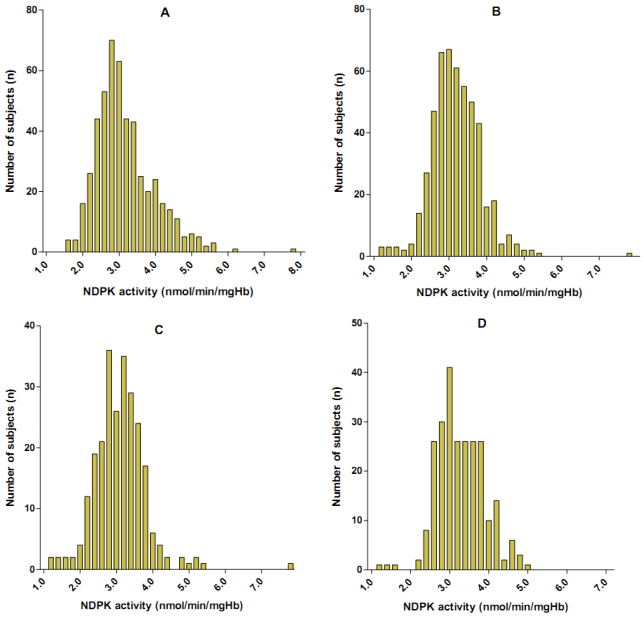

Figure 5. Frequency distribution of NDPK activity in human erythrocytes of 500 healthy subjects (A) and 500 transplant recipients (B) [kidney (C) and liver (D)] in Chinese Han population. NDPK activity levels were not normally distributed in human erythrocytes in the Chinese Han population of each subgroup (A: K-S Dist.=0.0912; P<0.0001; B: K-S Dist.=0.0437; P<0.05; C: K-S Dist.=0.0713; P<0.01; D: K-S Dist.=0.0819; P<0.001).

As a convenient tissue, erythrocytes were indentified with high concentrations of soluble NDPK particularly NDPK-A and NDPK-B, and more completely releasing and less destruction in the extraction process [29]. However, cellular nucleotide homeostasis in erythrocytes may differ substantially from other cells in the vasculature. Therefore, any shifts in erythrocytic NDPK may not be necessarily accompanied by similar enzymatic changes, say in vascular endothelium or hematopoietic cells, and vice versa.

Unlike the previously mentioned methods, the NDPK activity measured in this method significantly represented the mixture of NDPK-A and NDPK-B in erythrocytes rather than a purified monosome for many reasons. Firstly, our main purpose was to determinate the NDPK activity *in vivo* for further study of thiopurines pharmacology and adverse effects. Secondly, four isozymes were shown to present similar kinetics parameters [27]. Finally, it measured the effective activity rather than expression level which could better reflect the participation in thiopurines metabolism. It is noteworthy along with predominant intracellular localization, certain portion of catalytically active NDPK can also be expressed on the cell surface [30,31] and also freely circulate in the bloodstream as soluble enzyme [32].

NDPK was identified in high concentrations in human erythrocytes. Our studies indicated that human erythrocytes had sufficient NDPK to catalyze the synthesis of dATP. These findings are consistent with observations from other laboratories that mammalian erythrocytes have a marked capacity for synthesizing nucleotides [29]. It should be noteworthy that, in the present study, the NDPK activity determined in human erythrocytes should be viewed as approximate values because the erythrocytes contain significant levels of ATPase activity which decrease the reliability [29]. In addition, there may be other enzymes that are capable of phosphorylating nucleoside diphosphate e.g. pyruvate kinase, creatine kinase, phosphoglycerate kinase, succinyl-CoA synthetase, phosphoenolpyruvate carboxykinase and adenylosuccinate synthetase [33,34]. However, it is apparent that NDPK activity in the erythrocytes is up to 10-fold stronger than the activity of other enzymes on the pathway of polyphosphate nucleotide and nucleic acid synthesis [29].

In the present study, according to the results of thermal stability, a plateau occurred when heat-preservation temperatures ranged from 30 to 45ºC, which revealed the stability of the mixture of NDPKs in this temperature range. However, nearly no activity was detected when the temperature increased to 65-80ºC. According to the previous reports, NDPK-A and NDPK-B were remarkably unstable to heat treatment with 60 to 65ºC, 85ºC for NDPK-C, and NDPK-D was highly labile toward heat with 50% inactivated upon pre-incubation at 40ºC [35]. The conclusion could be drawn that our method measured mainly the activity of NDPK-A and NDPK-B, consistent with previous report [35].

On the basis of pH stability results, NDPK enzymes were stable when pH ranged from 6.0 to 9.0 and unstable from 5.0 to 3.0. It dropped to no activity when the pH dropped to 3.0. This phenomenon indicated that the NDPK enzymes were stable in alkaline environment which was the firstly reported, in consistence with human internal environment.

Bisubstrate enzyme-catalyzed reaction mechanisms can be divided into three types, the sequential ordered mechanism, the sequential random mechanism, and the Ping-Pong mechanism. The initial rate equations for sequential ordered and sequential random reaction mechanisms are very similar. However, as no term containing the reciprocal concentrations of both substrates, the initial rate equations of the ping-pong reaction mechanism is much different. Graphically, a double reciprocal plot for a bisubstrate sequential ordered or sequential random mechanism is a family of straight lines that intersect the vertical axis at different points.

However, ping–pong mechanism results in a double reciprocal plot of a family of parallel lines rather than intersecting lines [36]. In the present study, the reciprocal plots of data obtained in initial velocity studies, with dADP as a variable substrate and dGTP as the changing fixed substrate, yielded a family of parallel lines. Essentially similar findings were obtained when dADP replaced dGTP as the changing-fixed substrate. Therefore, these findings are consistent with a ping-pong reaction mechanism.

Consistently, in the Lineweaver–Burk plots, the competitive inhibition lines intersect at a single point on the y-axis, illustrating that such inhibitors do not affect V_{max}. Similarly, the non-competitive inhibition lines intersect at a single point on the x-axis, showing these inhibitors do not affect K_m. In the present study, enzyme-substrate kinetics analysis indicated the substrate dependency of NDPK enzyme, and the product dATP inhibition studies demonstrated that a typical pattern of non-competitive inhibition for dADP and competitive inhibition for dGTP, which revealed a "Ping-Pong" mechanism of NDPK in consistence with Mourad's studies. Mourad [29] used the coupled pyruvic kinase-lactic dehydrogenase assay and the coupled hexokinase-glucose-6-P dehydrogenase assay to study the NDPK kinetics including substrate specificity, initial velocity and nucleoside 5'-monophosphates inhibition.

In the present study, using the validated ion-pair gradient HPLC method, we reported, for the first time, the frequency distribution of NDPK activity levels in 1,000 Chinese Han population, including 500 solid organ transplant recipients and 500 ethnically and geographically matched healthy subjects. In this study population, NDPK activity levels possessed dispersive over the range from 1.13 to 7.87 nmol/min/mgHb and showed skewed distribution in the population and each subgroup. In addition, as the cases and controls groups had similar demographic characteristics (sex, age, smoking, and drinking), the NDPK activity levels between health group and transplant group showed significant difference which suggested the disease or immunosuppressant's might change the NDPK activity. Meanwhile, the activity levels between kidney group and liver group showed significant difference, implying that different disease courses might also change the NDPK activity. In the further study, we will obtain detailed information on the underlying primary renal or hepatic diseases in patients with transplants, which illuminates association analyses of NDPK activity with respect to renal or hepatic diseases factors, such as inflammation and autoimmune disorders. Additionally, it is very important and necessary to conclude the exact contribution of immunosuppressant's on the levels of NDPK activity. Further gene functional studies are urgently needed to confirm and clarify these preliminary data. Importantly, the present study will provide a foundation for further functional studies to reveal the biological and molecular functions of NDPK activity in solid organ transplantation.

CONCLUSION

In conclusion, NDPK enzyme kinetics studies indicated substrate dependency of NDPK and a "Ping-Pong" mechanism for production inhibition. To the best of our knowledge, this is the first report that the NDPK activity levels were simultaneously evaluated in healthy subjects and solid organ transplant recipients in Chinese Han population. Importantly, NDPK activity levels revealed a 7-fold variability and we're not normally distributed in human erythrocytes in the study population. The higher NDPK activity was found in transplant recipients when compared to healthy subjects, which might be affected by immunosuppressant's administration or disease. However, further investigations are needed to clarify this issue.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (grant no. 81273591). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest

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