Enantioselective *N*-Acetylation of *N*-Desisopropylpropranolol by Rat Liver Acetyltransferase

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The enantioselective *N*-acetylation of *N*-desisopropylpropranolol (NDP), one of the main metabolites of propranolol (PL), by rat liver acetyltransferase (AT), was investigated. *R*(+)-NDP or *S*(−)-NDP was used as a substrate at concentrations ranging from 10 to 200 μM. The cytosol fraction of a rat liver containing 3.93 mg protein/ml served as the source of AT. For 1-amino-3-(1-naphthyloxy)-2-propanol (AcNDP) formation from *R*(+)-NDP or *S*(−)-NDP in the presence of infinite AcCoA (250 μM), the K_m value was calculated to be 67.5 or 62.4 μM, and the V_{max} value was 0.462 or 0.205 nmol/min/mg protein. Based on these findings, the enantioselective *N*-acetylation of NDP was proved, *i.e.*, AcNDP formation from *R*(+)-NDP was found to take place more easily than that from *S*(−)-NDP. Furthermore, AcNDP formation from NDP was competitively inhibited by the exogenous arylamine, *p*-aminobenzoic acid (PABA), which is well-known to be a typical substrate of AT. The presence of enantioselective inhibition for AcNDP formation was thus confirmed based on the K_i values, 440 μM in the case of *R*(+)-NDP and 250 μM in the case of *S*(−)-NDP, respectively, *i.e.* two-fold enantioselective inhibition was demonstrated based on the Ki values in *S*(−)-enantiomer in comparison with *R*(+)-enantiomer.

Key words *N*-desisopropylpropranolol (NDP); *N*-acetylation; acetyltransfarase (AT); enantioselectivity; *p*-aminobenzoic acid (PABA)

Racemic propranolol (PL), which is commonly used as a non-selective β-adrenoceptor antagonistic agent, is easily subject to oxidation by cytochrome P4501A2, converting it to *N*-desisopropylpropranolol (NDP).[1] Subsequent *N*-acetylation of NDP to 1-amino-3-(1-naphthyloxy)-2-propanol (AcNDP) was first found by us from the extract of isolated rat hepatocytes after incubation using PL or NDP as a substrate, and also from the extract of human urine after the oral administration of PL.[2] In addition, β-adrenoceptor antagonistic actions and the mutagenicities of *R*(+)- and *S*(−)-enantiomers of NDP and AcNDP were also examined. Weak β_1- and β_2-adrenoceptor antagonistic actions were detected only in *S*(−)-NDP and weak mutagenicity was also observed in *S*(−)-AcNDP when the S-9 mixture was added to the incubation mixture.[3] By further study, AcNDP, which is produced from NDP, was also demonstrated to be catalyzed by the acetyltransferase (AT) in rat liver cytosol (Chart 1). Based on the amount of AcNDP produced from *R*(+)- or *S*(−)-NDP, the presence of enantioselective *N*-acetylation in NDP was suggested. Furthermore, the inhibition of AcNDP formation by *p*-aminobenzoic acid (PABA) was also indicated in a previous paper using racemic NDP as a substrate.[4]

In this study, the *N*-acetylation of *R*(+)- or *S*(−)-NDP by AT was examined in detail. The kinetic parameters of K_m and V_{max} values for AcNDP formation from NDP and the K_i value for the inhibition of AcNDP formation by PABA were calculated in order to prove the enantioselective *N*-acetylation of NDP and the enantioselective inhibition for AcNDP formation from NDP by PABA using *R*(+)- and *S*(−)-NDP in rat liver cytosol, respectively.

MATERIALS AND METHODS

Chemicals NDP, AcNDP and 1,2,3-triazolo[1,5-a]-quinoline (Tri-Q) were synthesized in our laboratory.[2,5] AcCoA, PABA and all other chemicals were purchased from the Sigma Chemical Co., St Louis, MO, U.S.A. The Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Separation of *R*(+)- and *S*(−)-Enantiomers of NDP By HPLC as a device, *R*(+)- and *S*(−)-NDP were separated from racemic NDP synthesized in our laboratory.[1] The purity of each enantiomer of NDP or AcNDP was checked by $[\alpha]_D^{26}$ in methanol; *R*(+)-NDP, +3.59°; *S*(−)-NDP, −3.59°.

Preparation of Rat Liver Cytosol Livers were obtained from male Wistar rats (Japan SLC, Inc., Shizuoka, Japan; 8 weeks of age weighing from 230—260 g). Rats were fasted for 24 h, and water was given *ad libitum* until 1 h before the experiment. A mixture of one part of liver and 9 parts of ice-cooled 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA was homogenized for 2 min using a Potter Elvehjem glass homogenizer with a teflon pestle. The liver homogenate was centrifuged twice at 9000×***g*** for 20 min, and thereafter the sediment was discarded. The supernatant was then centrifuged twice at 105000×***g*** for 60 min, and the resulting supernatant (cytosol) then served as the enzyme source for all subsequent studies. The protein concen-

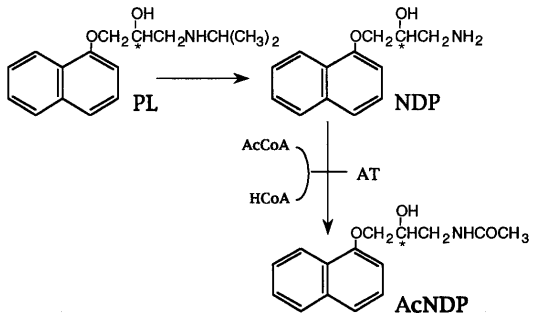

Chart 1. Pathway of *N*-Acetylation of NDP Formed from PL

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tration in the cytosol was determined to be 3.93 mg protein/ml using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard.

Incubation of *R*(+)-NDP or *S*(−)-NDP in Rat Liver Cytosol Coexisted with or without PABA The susceptibility of *N*-acetylation of NDP was measured by determining the rate of AcNDP formation. The experimental conditions were determined by preliminary tests in all cases. Each solution of *R*(+)-NDP, *S*(−)-NDP and PABA was prepared using 50 mM Tris–HCl buffer (pH 7.4), respectively. 0.1 ml of *R*(+)-NDP or *S*(−)-NDP (final concentration 10 to 200 μM) and 0.2 ml of 50 mM Tris–HCl buffer solution were added to a tube containing 0.1 ml of the cytosol fraction (3.93 mg protein/ml) under ice-cooled conditions. On the other hand, in the enzyme inhibitory experiment, 0.1 ml of *R*(+)- or *S*(−)-NDP (final concentration 20, 50 and 100 μM), 0.1 ml of PABA (final concentration 40 to 400 μM) and 0.1 ml of 50 mM Tris–HCl buffer solution was added to a tube containing 0.1 ml of the cytosol fraction (3.93 mg protein/ml) under ice-cooled conditions.

After preincubation of the mixed solution at 37 °C for 3 min, the reaction was initiated by the addition of 0.1 ml of infinite AcCoA (final concentration 250 μM). After incubation for 15 min, sampling was performed. The time of 15 min was determined by one point from the linear part on the time-course curve of AcNDP formation from NDP by preliminary examination. The reaction was terminated by the addition of 2 ml of chloroform. After the addition of Tri-Q (0.7 μg, internal standard (I.S.)), the reaction mixture was shaken for 15 min and centrifuged at 800×***g*** for 10 min. The chloroform layer was separated and evaporated by nitrogen gas. 50 μl of methanol was added to the residue and the solution was finally employed as an HPLC sample.

Regarding the inhibitory study by PABA, a reaction mixture containing 0.1 ml of 20, 50 and 100 μM *R*(+)- or *S*(−)-NDP, 0.1 ml of 40 to 400 μM PABA and 0.1 ml of 50 mM Tris–HCl buffer solution was incubated in a rat liver cytosol fraction (3.93 mg protein/ml) after the addition of 0.1 ml of 250 μM of AcCoA, respectively. The K_i value for AcNDP formation from NDP by PABA was calculated from the data obtained 15 min after incubation.

Determination of AcNDP by HPLC A Shimadzu LC-10AD high performance liquid chromatograph equipped with a spectrofluorometer (Shimadzu RF-550) and a reversed phase column (TSK gel ODS-80TM, 25 cm×4.6 mm i.d., Tosoh Co., Japan) was employed. The column temperature was 37 °C and the flow rate was 0.8 ml/min. A mixture of acetonitrile–methanol–water–acetic acid (35 : 20 : 45 : 0.5) was employed for the mobile phase. The eluate was monitored at 310 nm (excitation) and 380 nm (emission). The retention time of Tri-Q and AcNDP was 6.6 min and 9.5 min, respectively (cf. NDP; 12.6 min).

Data Analysis The kinetic parameters for the *N*-acetylation of *R*(+)- or *S*(−)-NDP by rat liver AT were calculated using a computer program (Kaleida Graph, Synergy Software, Reading PA) designed for nonlinear regression analysis according to the hyperbolic Michaelis-Menten equation.

$$V = V_{max} \cdot [S]/(K_m + [S])$$

An estimation of the K_i values was made by a graphic method (Dixon plots). Statistical data of kinetic parameters between *R*(+)-NDP or *S*(−)-NDP shown in Table 1 were analyzed using a *t*-test for paired data.

RESULTS AND DISCUSSION

Regarding ATs, Weber *et al.*[6] and King *et al.*[7] have studied them for more than three decades, and have published many related papers. Regarding rat liver AT, Land *et al.*[8] and Debiec-Rychter *et al.*[9] demonstrated that a single 32 kD protein in rat liver cytosol carries out all of the acetylation reactions, which are *N*-acetylation of the endogenous arylalkylamine and the exogenous arylamine, *O*-acetylation of arylhydroxylamines, and *N*,*O*-acyltransfer of arylhydroxamic acids. Since 1975, we have studied the metabolism of aminopyrine,[10] isoniazid[11] and hydralazine,[12] including the *N*-acetyl conjugates as their metabolites. We are thus especially interested in the enzyme AT. Fortunately, while studying the metabolism of the chiral drug PL, we succeeded in identifying an *N*-acetylconjugate AcNDP *via* NDP derived from PL. We thus synthesized racemic NDP using a previously reported method[1] following the separation of *R*(+)- and *S*(−)-enantiomers of NDP and AcNDP by HPLC, respectively.[3] This time, each enantiomer of NDP was employed as a substrate and the kinetic parameters for AcNDP formation from NDP were estimated to be the same as those of the *N*-acetylation of NDP. Rat liver cytosol was employed as an enzyme source of AT in coexistence with infinite AcCoA as a cofactor. The present paper describes two important observations on enantioselective *N*-acetylation as follows:

A Confirmation of the Enantioselective *N*-Acetylation of NDP by K_m and V_{max} Values In this paper, the degree of *N*-Acetylation of NDP was expressed in terms of the concentration-dependent changes of AcNDP formation from NDP. As shown in Fig. 1A, the curves due to a simple Michaelis-Menten type for AcNDP formation from *R*(+)- or *S*(−)-NDP were obtained. The K_m and V_{max} values for AcNDP formation were calculated using a nonlinear regression analysis as described in the experimental procedure. The Lineweaver-Burk plots for AcNDP formation from each enantiomer of NDP are shown in Fig. 1B. Chiral conversion was insignificant during incubation using *R*(+)- or *S*(−)-NDP as a substrate (the data were not shown).

For AcNDP formation from *R*(+)- or *S*(−)-NDP in the presence of infinite AcCoA (250 μM), the K_m value was calculated to be 67.5 or 62.4 μM, and the V_{max} value was 0.462 or 0.205 nmol/min/mg protein, respectively. Based on these findings, the intrinsic hepatic clearance (V_{max}/K_m) was calculated. As shown in Table 1, the value for *R*(+)-enantiomer (6.82 μl/min/mg protein) was two times greater than that for *S*(−)-enantiomer (3.36 μl/min/mg protein). The enantioselectivity was thus observed in AcNDP formation from NDP, *i.e.* *R*(+)-NDP was more easily acetylated than *S*(−)-NDP.

A Confirmation of the Enantioselective Inhibition of AT by PABA Using K_i Value in *N*-Acetylation of NDP In a previous paper,[3] we already investigated the *N*-acetylation of racemic NDP. When NDP was incubated with PABA or sulfamethazine (SMZ) in the isolated rat hepatocyte system, PABA indicated a more marked inhibition than SMZ in AcNDP formation. Since PABA is well-known to be a typical substrate of AT, it might participate as a competitive inhibitor in the *N*-acetylation of NDP. We therefore performed

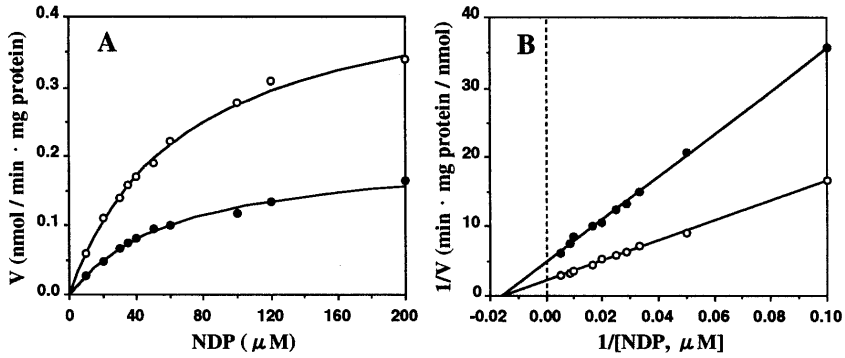

Fig. 1. AcNDP Formation from NDP in Rat Liver Cytosol

A, Michaelis-Menten curves for AcNDP formation; B, Lineweaver-Burk plots for AcNDP formation. Incubation mixtures contained, in final concentrations, 0.1 ml of 10 to 200 μM of either *R*(+)-NDP (○) or *S*(−)-NDP (●), 0.1 ml of the cytosol fraction (3.93 mg protein/ml), 0.1 ml of 250 μM AcCoA and 0.2 ml of 50 mM Tris–HCl buffer solution. Each solution was incubated for 15 min at 37 °C. The reaction was terminated by the addition of 2 ml of chloroform. After the addition of Tri-Q (0.7 μg, I.S.), the residue of the chloroform layer was dissolved in methanol and employed for HPLC. The activity of *N*-acetylation of NDP was measured by determining the rate of AcNDP formation.

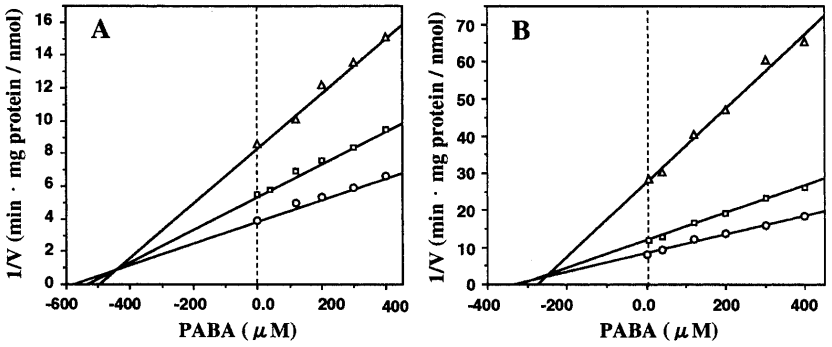

Fig. 2. Dixon-Plots for *N*-Acetylation of *R*(+)-NDP (A) and *S*(−)-NDP (B) Using PABA as A Inhibitor

Incubation mixtures contained, in final concentrations, 0.1 ml of 20 (△), 50 (□) and 100 μM (○) NDP, 0.1 ml of 40 to 400 μM PABA, 0.1 ml of the cytosol fraction (3.93 mg protein/ml) and 0.1 ml of 250 μM AcCoA and 0.1 ml of 50 mM Tris–HCl buffer solution. The incubation was performed under the same conditions indicated in Fig. 1.

Table 1. Kinetic Parameters for AcNDP Formation from NDP and the Influence of PABA on the *N*-Acetylation

Substrates	K_m	V_{max}	V_{max}/K_m	K_i of PABA
	μM	nmol/min·mg protein	μl/min·mg protein	μM
R(+)-NDP	67.5±1.0[a]	0.462±0.031[b]	6.82±0.009[b]	440±10[a]
S(−)-NDP	62.4±0.5	0.205±0.014	3.36±0.04	250±30

The values of K_m and V_{max} were estimated using a computer program fitted to the Michaelis-Menten equation. The values of K_i were calculated from the Dixon plots. Each value represents the mean±S.D. (n=3). Significant different from *S*(−)-enantiomer, *a*) $p<0.01$ or *b*) $p<0.001$.

the reaction using *R*(+)-NDP and *S*(−)-NDP in a rat liver cytosol suspension in order to obtain the K_i value.

Expectedly, the *N*-acetylation was inhibited competitively by PABA and SMZ. The K_i value of PABA , which indicated stronger inhibition than SMZ (data are not shown), was estimated by Dixon-plots from a common intersection of the three lines to be 440 μM for *R*(+)-NDP (Fig. 2A) and 250 μM for *S*(−)-NDP (Fig. 2B), respectively, which shows that a two-fold inhibition was observed in the *S*(−)-enantiomer in comparison with the *R*(+)-enantiomer. The presence of enantioselectivity in the inhibition by PABA in *N*-acetylation of NDP was shown by the K_i value.

This is the first observation to demonstrate the presence of enantioselectivity in the *N*-acetylation of a primary metabolite NDP, which was produced from a chiral compound PL and catalyzed by AT. Further studies are thus necessary to demonstrate the universality of the results obtained in this study of the *N*-acetylation of amino compounds which include a chiral center (drugs, food additives, carcinogens, *etc.*) by AT.

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