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Influence of Ammonium Chloride on the Tissue Distribution of Anticholinergic Drugs in Rats

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Abstract

Ammonium chloride (NH_4Cl) increases lysosomal pH and thereby abolishes intralysosomal accumulation of drugs. Its effect on the tissue distribution of biperiden and trihexyphenidyl in rats has been investigated.

The tissue–plasma concentration ratios (K_p) of these drugs in various tissues were determined by infusion studies at steady-state in the presence or absence of NH_4Cl. Treatment with NH_4Cl reduced the K_p values for both drugs, causing the largest reduction in K_p in the lung (52·1 to 11·8 for biperiden and 59·5 to 18·9 for trihexyphenidyl; ratios of decrease 0·77 and 0·68, respectively), followed by the heart and kidneys, with relatively small reductions in the brain, gut, muscle and fat. Subcellular fractionation studies in the lung indicated that the subcellular fraction–plasma concentration ratio of each drug at the steady state ($K_{p,sf}$) was reduced by treatment with NH_4Cl, with the largest decrease in the post-nuclear fraction (ratio of decrease 0·82 for biperiden and 0·74 for trihexyphenidyl), followed by the nucleus, microsomes and supernatant, in that order. A strong correlation was found between the ratio of decrease in $K_{p,sf}$ after NH_4Cl treatment and the specific activity of acid phosphatases, a marker of lysosomes, in each fraction (biperiden, $r = 0{\cdot}948$; trihexyphenidyl, $r = 0{\cdot}945$).

These results suggest that acidic organelles contribute significantly to the distribution kinetics of anticholinergic drugs.

We have previously reported that the distribution kinetics of the anticholinergic drugs biperiden and trihexyphenidyl are characterized by a large volume of distribution and different tissue–plasma concentration ratios (K_p) in various tissues (Yokogawa et al 1990a, b). Although changes in adipose and muscle tissue volume can cause large inter-individual differences in distribution volume (Nakashima et al 1987), in-vitro studies have shown that basic drugs accumulate in acidic vesicles (e.g. lysosomes) (Poole & Ohkuma 1981). The subcellular distribution of basic drugs in the rat liver in-vivo is concentrated in the lysosome (Ishizaki et al 1996). Basic drugs are distributed in subcellular fractions of the brain and heart in a manner depending on the amount of protein, but their distribution in the lung is not dependent on the amount of protein in the post-nuclear fraction (P_2), which contains lysosomes (Ishizaki et al 1998).

Several investigations have studied the relationship between toxicity and the accumulation of basic drugs in lysosomes (Stoffel et al 1987). Honegger et al (1993) reported a correlation of the side effects of amiodarone, a potent antiarrhythmic drug, with intralysosomal accumulation of both amiodarone and its main metabolite. Studies have also found that NH_4Cl and some basic drugs increase lysosomal pH and thereby abolish drug accumulation (Ishizaki et al 1996). Therefore, lysosomal accumulation of drugs might be involved in drug interactions and toxicity. Although several studies have been performed on pharmacokinetic drug interactions as related to adverse reactions, little is known about the relationship between lysosomal accumulation and drug interactions, except in-vitro (Daniel & Wójcikowski 1997).

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The purpose of this study was to examine the influence of lysosomal inhibition with NH_4Cl on the distribution of basic drugs in the rat in-vivo.

Materials and Methods

Materials

Biperiden (Dainippon Pharmaceutical, Osaka, Japan) and trihexyphenidyl (Nippon Lederle, Japan) were used as supplied. Other chemicals were of reagent grade and were used without purification.

Animal experiments

Male Wistar rats, 262 ± 12 g, mean ± s.d. (Sankyo Laboratory Animal, Toyama, Japan) were treated as described elsewhere (Nakashima et al 1987). Briefly, the femoral vein was cannulated with polyethylene tubing under light anaesthesia. NH_4Cl (3 mg min^{-1} kg^{-1}) or saline was infused via the femoral vein and after 2 h biperiden or trihexyphenidyl solution (3·2 mg kg^{-1}; 0·24 mL) was administered simultaneously by intravenous bolus injection. Rats were killed 1 h later.

Infusion studies were performed to determine the K_p values. NH_4Cl (3 mg min^{-1} kg^{-1}) or saline was infused via the femoral vein, then after 2 h, biperiden or trihexyphenidyl was infused simultaneously at the rate of 16·2 μg min^{-1} kg^{-1} after intravenous bolus injection of a priming dose of 3·2 mg kg^{-1}. The rats were killed for tissue sampling 6 h after the start of infusion of the basic drug. The procedure for preparing tissue homogenates has been described previously (Nakashima et al 1987). Tissue homogenates and plasma samples were stored at −30°C until assayed.

Subcellular fractionation

Rat lungs were fractionated according to the procedures of De Robertis et al (1962). Briefly, excised rat lungs were homogenized with 10 vols ice-cold 0·32 M sucrose. All subsequent steps were performed at 4°C. To obtain nuclear fractions (P_1), homogenates were centrifuged at 1000 *g* for 10 min. To obtain post-nuclear fractions (P_2), the supernatant was centrifuged again at 12 300 *g* for 20 min. To obtain microsome fractions (P_3), the supernatant was centrifuged again at 100 000 *g* for 60 min. The final supernatant was used as cytosol fraction (S_1).

Determination of serum-protein binding

The extent of binding of biperiden and trihexyphenidyl to rat serum protein was measured by equilibrium dialysis, as described previously (Nakashima et al 1987). The concentration of biperiden or trihexyphenidyl ranged from 25 to 1000 ng mL^{-1} in the presence or absence of NH_4Cl (50 mM).

Assay of biperiden and trihexyphenidyl

The concentrations of biperiden and trihexyphenidyl in plasma, tissues and subcellular fractions were determined by gas chromatography, as described previously (Yokogawa et al 1985). Briefly, homogenized tissue or plasma was mixed with an equal volume of 1 M Na_2CO_3 buffer (pH 10), an internal standard was added, and the samples were extracted with diethyl ether. The samples were centrifuged for 5 min at 2000 rev min^{-1} and the organic phase was transferred into another series of test tubes, each containing HCl (1 M; 2 mL). The drug was re-extracted into the HCl and the ether phase was discarded. NaOH (3 M; 1 mL) and diethyl ether (5 mL) were then added to the aqueous phase. The final ether extract was transferred to a glass centrifuge tube, and evaporated. The dried residue was dissolved in diethyl ether (20 μL) and most of the solution was injected into the chromatograph. Analysis was performed with a Shimadzu (Kyoto, Japan) GC-7A gas chromatograph equipped with a Shimadzu FTD-8 nitrogen-phosphorus detector, NPD, and a 25 m × 0·24 mm i.d. flexible fused silica capillary column previously silanized and coated with a 0·2 μm film of OV-101 (Ulbon R HR-52; Sinwa Kako, Kyoto, Japan).

Measurement of protein, blood ammonia concentration and specific activity of acid phosphatases

Protein concentrations were determined colorimetrically by use of a commercial kit (BioRad, Eastern Regional Office, NY). Blood–ammonia concentrations were determined by the nitroprusside-phenol method, by use of a commercial kit (Ammonia-test; Wako, Tokyo, Japan). The specific activity of acid phosphatases was determined by the *p*-nitrophenyl phosphate method, by use of a commercial kit (Acid phospha B-test; Wako, Tokyo, Japan).

Data analysis

Student's *t*-test was used to compare paired or unpaired means of two data sets. A value of $P < 0.05$ was considered to be indicative of significance.

Results

The concentrations of biperiden and trihexyphenidyl in plasma and various tissues 1 h after an

Table 1. The concentrations of biperiden and trihexyphenidyl (ng mL^{-1} or ng (g wet tissue)$^{-1}$) in plasma and tissues 1 h after a 3·2 mg kg^{-1} intravenous bolus injection with or without infusion of ammonium chloride in rats.

Tissue	Biperiden		Trihexyphenidyl	
	Control[a]	With ammonium chloride[b]	Control[a]	With ammonium chloride[b]
Plasma	142±26	174±40	140±13	148±44
Lung	5504±728	3118±655**	7554±934	4444±1116*
Heart	980±423	733±122	1378±248	827±323
Kidney	1907±319	986±431*	2024±831	1796±416
Brain	1803±320	992±210*	1912±348	1654±211
Gut	2251±441	2053±335	2932±220	2740±754
Muscle	606±110	642±93	686±233	974±181
Liver	282±58	337±57	1261±550	1180±123
Fat	4134±641	4436±635	7086±1232	6315±1100

Each value is the mean±s.d. of results from three rats. [a]Concentration in plasma and tissues after an intravenous bolus injection of biperiden or trihexyphenidyl alone. [b]Concentration in plasma and tissues after an intravenous bolus injection of biperiden or trihexyphenidyl with ammonium chloride (3 mg min^{-1} kg^{-1}) infusion. *$P < 0.05$, **$P < 0.01$, significantly different from control.

intravenous bolus dose are summarized in Table 1. The plasma concentration of biperiden was increased from 142±26 to 174±40 ng mL^{-1} by treatment with NH_4Cl, whereas that of trihexyphenidyl changed only slightly (not significant) from 140±13 to 148±44 ng mL^{-1} (mean±s.d.). The tissue distribution pattern of trihexyphenidyl was similar to that of biperiden. Their concentrations were high in lung and fat and low in muscle and heart. In the presence of NH_4Cl the concentration of biperiden decreased in all tissues except in muscle and fat. The decreases in the lung, brain and kidney were statistically significant and the ratios of these decreases relative to the control were approximately 0·4–0·5. Similarly, the ratio of decrease of trihexyphenidyl in the lung was approximately 0·4.

Table 2 summarizes the K_p values of biperiden and trihexyphenidyl in the presence or absence of NH_4Cl and the ratio of the decrease of K_p relative to that of the control in various tissues after treatment with NH_4Cl. The K_p values of biperiden and trihexyphenidyl in each tissue correlated well with each other in the control studies ($r = 0.992$, $P < 0.001$), although the K_p values of trihexyphenidyl obtained from the regression equation were approximately 1·3 times higher than those of biperiden. The K_p values of these drugs in various tissues decreased significantly after treatment with NH_4Cl. The influence of NH_4Cl was most remarkable in the lung, with a ratio of decrease of approximately 0·7–0·8. The ratios of decrease were approximately 0·5 for the heart and kidney, and approximately 0·2–0·4 for the brain, gut, muscle and fat. A strong correlation was obtained between the K_p values of biperiden and trihexyphenidyl after treatment with NH_4Cl ($r = 0.990$, $P < 0.001$). Blood–ammonia concentrations for the control and NH_4Cl-infused (8 h) rats were 81±15 (n=5) and 742±235 μg dL^{-1} (n=7) (mean±s.d.), respectively ($P < 0.001$). The total clearance (CL_{tot}) of biperiden was 67·2±5·5 and 70·4±12·1 mL

Table 2. Effect of ammonium chloride on the tissue–plasma concentration ratio (K_p) of biperiden and trihexyphenidyl.

Tissue	Biperiden			Trihexyphenidyl		
	Control[a]	With ammonium chloride[b]	Ratio of decrease[c]	Control[a]	With ammonium chloride[b]	Ratio of decrease[c]
Lung	52·1±9·7	11·8±2·2**	0·773	59·5±2·6	18·9±7·7**	0·682
Heart	6·9±2·3	3·2±0·1*	0·534	7·3±1·0	3·4±0·7**	0·543
Kidney	8·7±1·5	4·1±0·2**	0·526	13·5±1·1	7·0±1·1**	0·484
Brain	6·6±1·5	3·8±0·1*	0·432	6·8±0·7	4·5±0·7*	0·332
Gut	9·8±2·2	7·0±0·5	0·286	15·0±3·3	8·6±0·7*	0·425
Muscle	3·4±0·7	2·6±0·1	0·232	3·2±0·5	2·3±0·6	0·277
Fat	51·0±6·6	41·5±2·9	0·186	67·8±6·5	47·2±15·0	0·303

Each value is the mean±s.d. of results from three rats. [a]Tissue–plasma concentration ratio after infusion of biperiden or trihexyphenidyl alone. [b]Tissue–plasma concentration ratio after infusion of biperiden or trihexyphenidyl with ammonium chloride. [c]These values are the ratio of decrease of tissue–plasma concentration ratio as a result of the action of ammonium chloride. *$P < 0.05$, **$P < 0.01$, significantly different from control.

Table 3. Effect of ammonium chloride on the lung subcellular fraction–plasma concentration ratio ($K_{p,sf}$) of biperiden and trihexyphenidyl.

Fraction	Biperiden			Trihexyphenidyl		
	Control[a]	With ammonium chloride[b]	Ratio of decrease[c]	Control[a]	With ammonium chloride[b]	Ratio of decrease[c]
Nuclear fractions	5·2 ± 1·2	3·0 ± 0·5*	0·423	4·9 ± 1·2	3·0 ± 1·1*	0·388
Post-nuclear fractions	22·8 ± 9·9	4·2 ± 1·3*	0·816	18·8 ± 1·3	4·9 ± 2·4**	0·739
Microsome fractions	2·3 ± 0·7	0·84 ± 0·17*	0·635	3·1 ± 0·7	1·4 ± 0·52	0·548
Cytosol fractions	12·7 ± 1·8	10·2 ± 2·0	0·197	14·4 ± 1·9	12·1 ± 1·5	0·160

Each value is the mean ± s.d. of results from three rats. [a]Lung subcellular fraction–plasma concentration ratio after infusion of biperiden or trihexyphenidyl alone. [b]Lung subcellular fraction–plasma concentration ratio after infusion of biperiden or trihexyphenidyl with ammonium chloride. [c]These values are the ratio of decrease of lung subcellular fraction–plasma concentration ratio as a result of the action of ammonium chloride. *$P < 0.05$, **$P < 0.01$, significantly different from control.

min^{-1} kg^{-1} (mean ± s.d., n = 3) in control and NH_4Cl-infused rats, respectively. The corresponding values for trihexyphenidyl were 72·2 ± 14·5 and 68·1 ± 17·4 mL min^{-1} kg^{-1} (mean ± s.d., n = 3). The unbound fraction of biperiden in the serum of control and NH_4Cl-infused rats was 0·195 ± 0·019 and 0·212 ± 0·039 (mean ± s.d., n = 5), respectively. The corresponding values for trihexyphenidyl were 0·220 ± 0·03 and 0·216 ± 0·016 (mean ± s.d., n = 5).

The influence of NH_4Cl treatment on the subcellular distribution of the drugs was examined in the lungs. Table 3 summarizes the fraction–plasma concentration ratio ($K_{p,sf}$) of biperiden and trihexyphenidyl in the lungs in the presence or absence of treatment with NH_4Cl and the ratio of decrease of $K_{p,sf}$ relative to the control after NH_4Cl treatment. The $K_{p,sf}$ values of fractions decreased in the order $P_2 > S_1 > P_1 > P_3$. The ratios of decrease of $K_{p,sf}$ value in each fraction after NH_4Cl treatment decreased in the order $P_2 > P_3 > P_1 > S_1$.

Figure 1 illustrates the relationship between the specific activity of acid phosphatases in the control study and the ratio of decrease of $K_{p,sf}$ for biperiden and trihexyphenidyl in lung subcellular fractions after treatment with NH_4Cl. The correlation coefficients were 0·948 and 0·945 for biperiden and trihexyphenidyl, respectively. The specific activities of the acid phosphatases of each fraction after treatment with NH_4Cl were almost the same as for the control.

Discussion

We have demonstrated that treatment with NH_4Cl alters the in-vivo distribution kinetics of the basic drugs biperiden and trihexyphenidyl. The influence of NH_4Cl on tissue distribution was examined by two

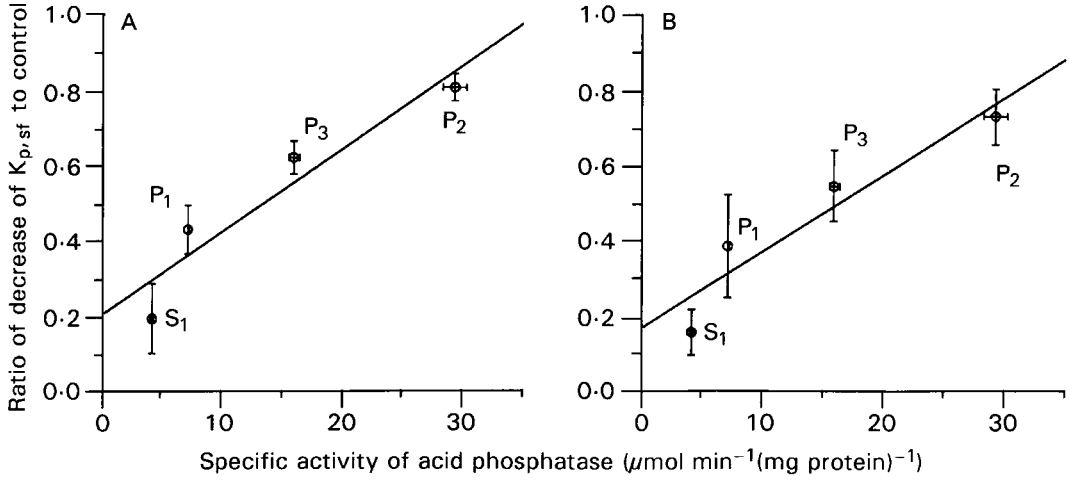

Figure 1. The relationships between the specific activity of acid phosphatases in the control study and the reduction in the ratio of the subcellular fraction–plasma concentration ($K_{p,sf}$) of biperiden (A) and trihexyphenidyl (B) in rat lung after treatment with NH_4Cl. The solid lines are simple linear regression lines. Each point represents the mean ± s.e. of results from three rats. P_1, nuclear fractions; P_2, post-nuclear fractions (e.g. lysosomes, mitochondria); P_3, microsome fractions; S_1, cytosol fractions.

treatments—single dose and infusion. Because these basic drugs equilibrate across well-perfused tissues within 1 h (Yokogawa et al 1990a; Ishizaki et al 1998), drug concentrations were measured 1 h after an intravenous bolus dose. When the drugs were administered as intravenous bolus doses there were no differences between drug plasma concentrations in the presence or absence of NH_4Cl. However, the concentrations of the drugs in the lung, brain, heart and kidney were significantly reduced by treatment with NH_4Cl (Table 1). Thus, we performed infusion studies to examine the influence of NH_4Cl on the distribution kinetics for multiple doses of these drugs.

The K_p values of these drugs in all tissues, except muscle and fat, were significantly reduced after treatment with NH_4Cl, the ratios of decrease of K_p in the lung, heart and kidney being more than approximately 0·5 (Table 2). The volume of distribution at steady state (Vd_{SS}) for biperiden and trihexyphenidyl in the presence or absence of NH_4Cl treatment was estimated by the method of Chen & Gross (1979). The equation defining Vd_{SS} is:

$$Vd_{SS} = V_p + \Sigma\, K_{p,i} V_{t,i}$$

where V_p and $V_{t,i}$ are the volumes of plasma and tissue, respectively. The volumes of plasma and tissue in the rat were taken from the report of Yokogawa et al (1990a). The Vd_{SS} after NH_4Cl treatment was approximately 70% of the control value (4·58 to 3·41 L kg^{-1} for biperiden and 5·47 to 3·61 L kg^{-1} for trihexyphenidyl). The blood–ammonia concentration 8 h after infusion of NH_4Cl was nine times greater than the control value. NH_4Cl treatment did not affect the CL_{tot} of the drugs. Although it is known that reduction in the unbound fraction in the serum cause decreases in K_p, the unbound fraction was unaffected by NH_4Cl treatment. This suggests that a reduction in tissue affinity caused the decrease in the K_p of these basic drugs.

Biperiden and trihexyphenidyl have a very high affinity for lung and fat tissues (Yokogawa et al 1990b). As shown in Table 2, although the K_p value in the lung was almost equal to that in the fat, the K_p value decreased substantially with NH_4Cl treatment only in the lung, not in the fat. Because NH_4Cl increases the pH in acidic organelles (e.g. lysosomes), it is widely used as a lysosomal inhibitor in in-vitro uptake studies (Poole & Ohkuma 1981; Daniel & Wójcikowski 1997). We have previously reported an in-vitro study in which the uptake of biperiden into lysosomes was inhibited by the addition of NH_4Cl (Ishizaki et al 1996). Because the lysosome content is high in the lung, liver and kidney (MacIntyre & Cutler 1988; Daniel & Wójcikowski 1997), it is possible that the elevation of lysosomal pH by NH_4Cl caused the K_p values of biperiden and trihexyphenidyl to decrease.

We then examined the influence of NH_4Cl on the subcellular distribution of basic drugs in the lung. As shown in Table 3, the $K_{p,sf}$ of the P_2 fraction was highest in the control tissue, and was reduced by approximately 75 to 80% on treatment with NH_4Cl. The specific activity of acid phosphatases, a marker of lysosomes, was high. The ranking of acid phosphatase activity in each fraction decreased in the order $P_2 > P_3 > P_1 > S_1$. There was high correlation between the specific activity of acid phosphatases in the control study and the ratio of decrease of $K_{p,sf}$ in each fraction upon treatment with NH_4Cl (Figure 1). These results suggest that lysosomes determine K_p and contribute substantially to the distribution kinetics of basic drugs in the lung.

We have previously reported that the contribution of lysosomes to the subcellular distribution of basic drugs after intravenous administration is approximately 10% in rat liver (Ishizaki et al 1996). On the other hand, in this study the K_p values of biperiden and trihexyphenidyl decreased in the lung, heart, kidney and brain by approximately 50% or more upon treatment with NH_4Cl. In in-vitro studies, the effective concentration of NH_4Cl used as a lysosomal inhibitor is usually 10 mM or more (Poole & Ohkuma 1981). However, in this study, lysosomal inhibition was apparent at a blood–ammonia concentration of merely 0·5 mM. Measurement of lung lysosomal pH in-vivo is very difficult, and no data are yet available. The inhibitory effect of this low concentration of NH_4Cl might be because of different NH_4Cl concentrations in the plasma and cells or the long intravenous infusion time of NH_4Cl (a total dose of approximately 360 mg NH_4Cl administered during 8 h), or both, which might have had some secondary pH-perturbing effect on lysosomes.

Daniel & Wójcikowski (1997) have suggested, from studies using tissue slices in-vitro, that lysosomal trapping is an important factor determining the subcellular distribution of basic psychotropic drugs in the lung and liver. They have also suggested that the uptake of these drugs in other tissues (kidney, brain, heart, muscle and adipose tissue) depends more on phospholipid binding than on lysosomal trapping because of the low level of suppression by NH_4Cl or monensin. However, they measured drug uptake for 1 h using tissue slices in buffered salt solution (Krebs–Henseleit buffer) devoid of an energy source; under such conditions the vacuolar pH might be raised, resulting in

underestimation of the role of the vacuolar system. In our previous study, we reported the effect of fat-tissue volume on the distribution kinetics of biperiden in rats and demonstrated that Vd_{SS} was reduced by approximately 40% with a decrease of the whole-body-fat volume from 17 to 10% (Yokogawa et al 1990a). In this work we found that acidic organelles participate in the distribution kinetics of basic drugs and that Vd_{SS} was reduced by approximately 30% by treatment with NH_4Cl.

Our observations suggest that administration of lysosomal inhibitors might accelerate the excretion of basic drugs by reducing drug accumulation within lysosomes; this might result in the avoidance of some side effects.

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