Characterization of human erythrocyte choline transport in chronic renal failure


Renal Group, Department of Medicine, King’s College School of Medicine and Dentistry, London, UK

Abstract

Background. Membrane transport of choline cations is elevated in renal failure in erythrocytes and cerebral tissue but the origins and clinical importance of this are unknown.

Methods. The membrane transport changes have been characterized using erythrocytes from patients on maintenance haemodialysis (HD), patients on continuous ambulatory peritoneal dialysis (CAPD), and control subjects. Data were obtained from cells depleted of intracellular choline to create zero-trans (ZT) conditions for choline influx. $[^{14}\text{C}]$-choline influx measurements provided a kinetic description of choline flux as the sum of a saturable transport system (defined by $V_{\text{max}}$ and $K_{m}$) and an apparent diffusion pathway. Inhibition of choline transport by hemicholinium-3 (HC-3), quinine and N-ethylmaleimide (NEM) has been studied. Actions of three cationic polyamine putative uraemic toxins (putrescine, spermidine, spermine) were tested in control erythrocytes.

Results. Mean ($\text{SEM}$) $V_{\text{max}}$ (ZT) was increased in HD at 45.0 (3.0) $\mu$mol/l cells/h and in CAPD at 46.6 (2.5) $\mu$mol/l cells/h compared to controls (30.0 (2.0) $\mu$mol/l cells/h). Mean $K_{m}$ (ZT) was not significantly altered in HD or CAPD (HD: 6.1 (1.6) $\mu$M; CAPD: 5.5 (0.7) $\mu$M; control: 5.1 (0.9) $\mu$M). The sensitivity of choline transport to the inhibitors tested was not altered in HD. 1.0 mM quinine, 2.0 mM NEM and 1.0 mM HC-3 caused 75–90% inhibition of transport in both HD and controls. For inhibition of ZT influx of 25 $\mu$M choline the mean IC$_{50}$ of quinine was 90 (9) $\mu$M in HD and 101 (13) $\mu$M in controls (n.s.). The ZT influx of 200 $\mu$M choline was not altered by any of the polyamines at concentrations up to 1.0 mM.

Conclusions. Membrane choline transport in CRF remains protein-mediated and exhibits normal substrate and inhibitor affinities; high values of $V_{\text{max}}$ seem to occur through increased surface expression of an active normal choline transporter. Increases in plasma polyamines cannot explain the choline transport changes in CRF.

Key words: choline; chronic renal failure; erythrocyte; membrane transport; polyamines; uraemia

Introduction

Renal failure is associated with abnormal membrane transport of choline [1–5]. Increased transmembrane choline flux has been reported both in erythrocytes from patients with chronic renal failure and in cerebral synaptosomes isolated from a rat model of renal failure [1,4]. The abnormalities appear to reverse within a few days of successful renal transplantation [3]. Membrane transport of choline is vital for the subsequent synthesis of choline-containing structural and bioactive phospholipids such as phosphatidylcholine, sphingomyelin and platelet-activating factor. Choline transport is also an essential prerequisite for the biosynthesis of acetylcholine in cholinergic neurones. Abnormal choline transport has been associated with altered T-cell function and cell proliferation and with a genetically determined cardiomyopathy [6–8]. Abnormal choline flux and metabolism has also been reported in Alzheimer’s disease. Accordingly, changes in choline transport have been proposed as playing a role in some of the clinical manifestations of uraemia, and particularly in neurological, muscular, and immunological deficits [5].

At least three membrane transport systems for choline transport have been identified on the basis of substrate affinity, $\text{Na}^+$ dependence, tissue distribution, and sensitivity to inhibition by hemicholinium-3 (HC-3) [1,9,10]. Genes for a yeast Na-independent choline transporter and a rat Na-dependent choline transporter have been cloned and sequenced [11,12]. In this paper the abnormal membrane choline transport in erythrocytes from patients with renal failure has been characterized with respect to the kinetics of transport and transporter sensitivity to inhibitors HC-3 and quinine. The sensitivity of choline transport to the thiol-active agent N-ethylmaleimide (NEM) and to altered $\text{Na}^+$ have also been examined. In previous work the kinetics of choline transport have been estimated using a 2-parameter Michaelis–Menten fit of the concentration-dependence of flux (defined by $V_{\text{max}}$ and
In the present work an improved 3-parameter model has been employed in order to include an apparent diffusion component of flux, defined by a diffusion constant $K_d$. Cellular influx of choline is enhanced by the presence of intracellular choline through the phenomenon of trans-acceleration, which occurs because the choline carrier reorients within the membrane at a faster rate when loaded with choline so that choline–choline exchange is faster than unidirectional choline flux [13]. The importance of trans-acceleration in the abnormal choline transport of renal failure has been examined by experiments performed after depletion of intracellular choline to create zero trans (ZT) conditions for choline influx.

The transport of choline in CRF appears to be modified by plasma factors. The polycationic putative uraeic toxins termed polyamines are candidate molecules for interaction with the transporter for cationic choline. Accordingly actions of the polyamines putrescine, spermidine, and spermine on choline transport have also been studied.

**Subjects and methods**

**Patients and materials**

Thirty-one patients on maintenance haemodialysis (HD), 10 patients on continuous ambulatory peritoneal dialysis (CAPD), and 15 normal controls were studied. Clinical parameters for the patients studied are summarized in Table 1. The mean age of the controls was 46 years (SD 7) and was not significantly different from the patients studied. Blood samples (non-fasting) were taken immediately before a dialysis session for HD patients or at a routine clinic visit for CAPD patients.

$[^{14}C]$-Choline chloride (specific activity 40–60 mCi/mmol) was obtained from New England Nuclear (Stevenage, Herts, UK). Ecoscint A was purchased from National Diagnostics (Hessle, Hull). Free base forms of the cationic polyamines putrescine $(\text{NH}_2^+(\text{CH}_2)_2\text{NH}_2)$, spermidine $(\text{NH}_3^+(\text{CH}_2)_3\text{NH}_2^+)$, and spermine $(\text{NH}_4^+(\text{CH}_2)_4\text{NH}_3^+)$ were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Choline chloride, three times recrystallized, was also obtained from Sigma Chemical Co. as were all other chemicals unless otherwise stated. The cell washing and choline flux procedures were performed in saline of the following composition: NaCl, 140 mM; KCl, 5 mM; glucose, 10 mM; MOPS, 10 mM; pH 7.4. A zero-Na solution was made by substituting N-methyl D-glucamine (NMDG) chloride for NaCl. Cell washing after flux incubation was performed in isotonic MgCl$_2$, of composition MgCl$_2$, 107 mM; MOPS, 10 mM, pH 7.4.

### Cell preparation and measurement of choline influx

Choline uptake was measured using established methods for erythrocyte transmembrane flux experiments [1]. Briefly, 10 ml of blood was taken into a heparinized tube and kept on ice for less than 2 h before erythrocyte separation by four centrifugation washes (5 min, 3000 g) at 4°C in saline. Half of the washed erythrocytes were immediately used for choline flux measurements (IU experiments) and the rest were depleted of intracellular choline by incubation at 37°C for 5 h in saline at a haematocrit of 0.02. This depletion created cells in which the influx of choline could be measured in zero trans (ZT) conditions.

The initial rate of erythrocyte choline uptake was measured in duplicate in 1-ml cell suspensions of haematocrit 0.03–0.06 by incubation for 5 min at 37°C with extracellular choline at concentrations of 0–500 μM in the presence of tracer amounts of $[^{14}C]$-labelled choline. The flux incubation was started from and stopped on ice. Cells were then separated from extracellular radiolabelled choline by three rapid centrifugation-washes in ice-cold MgCl$_2$ buffer. After haemolysis by 0.5 ml 0.1% Triton X-100 and precipitation of proteins by 0.5 ml 5% trichloroacetic acid, the $[^{14}C]$ content of the cell lysate was measured by liquid scintillation counting in Ecoscint A in a LKB 1209 Rackbeta liquid scintillation counter. The haematocrit of the cell suspension used for the flux measurements was measured in triplicate and the choline influx rates were calculated in units of μmoles of choline per litre of cells per hour.

### Clinical parameters for the patient groups studied

<table>
<thead>
<tr>
<th></th>
<th>CAPD</th>
<th>HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>31</td>
</tr>
<tr>
<td>Age (years)</td>
<td>57 (19)</td>
<td>53 (9)</td>
</tr>
<tr>
<td>Sex (m/f)</td>
<td>5/5</td>
<td>17/31</td>
</tr>
<tr>
<td>Time on dialysis (months)</td>
<td>27 (8)</td>
<td>24 (5)</td>
</tr>
<tr>
<td>On antihypertensive treatment</td>
<td>7/10</td>
<td>24/31</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>9.9 (1.6)</td>
<td>9.1 (0.8)</td>
</tr>
<tr>
<td>Creatinine (μmoles/l)</td>
<td>480 (93)</td>
<td>560 (45)</td>
</tr>
<tr>
<td>On erythropoietin treatment</td>
<td>3/10</td>
<td>19/31</td>
</tr>
<tr>
<td>Dialysis dose</td>
<td>Median 4 exchanges of 2 l/day</td>
<td>Median 4 h dialysis 3 times/week</td>
</tr>
<tr>
<td>Renal diagnosis</td>
<td>Chronic GN 3</td>
<td>Chronic GN 11</td>
</tr>
<tr>
<td></td>
<td>Diabetic nephropathy 3</td>
<td>Diabetic nephropathy 7</td>
</tr>
<tr>
<td></td>
<td>Interstitial disease 2</td>
<td>Intestinal disease 5</td>
</tr>
<tr>
<td></td>
<td>APKD 1</td>
<td>APKD 3</td>
</tr>
<tr>
<td></td>
<td>Other 1</td>
<td>Other 5</td>
</tr>
</tbody>
</table>

Means are shown where appropriate with standard deviations in parentheses. There were no significant differences between the groups with respect to these parameters. The HD was performed with cuprophone membranes. GN, glomerulonephritis; APKD, adult polycystic kidney disease.
conditions for choline influx) and cells were preincubated at 37°C with inhibitor for 30 min. Choline influx was then measured in the presence of the same concentration of inhibitor at an extracellular choline concentration of 25, 100 or 200 μM. In addition the possible effects of intracellular polyamines were examined by 37°C incubation of cells at a low haematocrit (0.05) with polyamine at 0–1.0 mM for 4 h to allow intracellular accumulation, followed by rapid cell washing and immediate measurement of choline influx. Separate experiments were performed to examine the Na-dependence of choline influx. For measurements in Na-free solution, ZT cells were given their final two washes in NMDG solution and then choline influx was measured in NMDG. Duplicates flux measurements were made in NaCl for comparison.

Analysis of flux data

The observed relationship between choline influx rate (V) and extracellular choline concentration (S) was fitted with a model incorporating two components of the flux. One component is saturable at high concentrations and can be described by Michaelis–Menten kinetics using the maximal flux at high concentrations (Vmax) and the concentration of choline giving half maximal flux (Km). The second component is not saturable and is linearly related to the extracellular choline concentration (S) by an apparent diffusion constant (Kd). The total choline flux is the sum of these two components and is given by the relationship:

\[ V = \frac{V_{\text{max}} S}{S + K_m} + K_d S \]  

(1)

The sensitivity of choline transport to HC-3 and quinine were quantified using the IC50 for inhibition which was determined graphically as the concentration of inhibitor corresponding to the flux rate V0.5, where:

\[ V_{0.5} = 0.5 \left( V_{\text{max}} + V_{\text{min}} \right) \]  

(2)

Vmax was the maximal rate of choline influx without inhibitor and Vmin was the rate of choline influx in the presence of high concentrations of inhibitor.

Statistical significances were examined using Student’s t tests, paired where appropriate.

Results

The kinetics of choline transport in CAPD patients and controls are shown in Figure 1 where the mean observed rate of choline influx (V) is plotted as a function of the extracellular choline concentration (S) in ZT conditions. The data were well-fitted with equation (1) and the lines of fit are shown in the Figure. Choline flux rates were higher in CAPD than in controls both in IU and ZT conditions. The fitted parameters Vmax, Km and Kd are summarized in Table 2 for CAPD patients, HD patients, and controls. It is clear that both CAPD and HD are associated with significant increases in Vmax and in Kd compared with controls with no significant alterations in ZT Kd.

Quinine 1.0 mM, NEM 2.0 mM, and HC-3 1.0 mM caused 75–90% inhibition of transport in both HD and controls. The effects of these inhibitors are summarized in Table 3. The values of IC50 for quinine and HC-3 were unaltered in HD patients’ erythrocytes when compared with controls and the NEM sensitivity of choline transport was also unaltered in the HD group.

The polyamines tested did not significantly affect measured choline influx. The data obtained with 0–1.0 mM extracellular putrescine, spermidine, and spermine is summarized in Figure 2. Even at the highest polyamine concentrations tested, the mean rates of choline transport were unaltered with respect to controls. Cells that had been incubated with polyamines for 4 h also showed no significant alterations in choline influx (data not shown). The substitution of NMDG for sodium did not cause inhibition of choline flux in control cells or in cells from HD patients, and erythrocyte choline flux remained sodium independent.

Discussion

The major finding of the current work is that the increased capacity for choline transport in renal failure arises through an increase in the maximal transport capacity of the cell membrane (Vmax) and not through alterations in the substrate affinity of the transporter (defined by Km). It has been suggested that recruitment of a new low-affinity transporter in addition to the existing high-affinity system could explain the observed increase in Vmax as, alternatively, would changes in the

Table 2. Summary of the mean kinetic parameters of choline transport in erythrocytes from controls and from patients on CAPD and HD

<table>
<thead>
<tr>
<th></th>
<th>CAPD (n=10)</th>
<th>HD (n=31)</th>
<th>Controls (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IU conditions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vmax μmol/(l.h)</td>
<td>151** (12)</td>
<td>128** (11)</td>
<td>87.8 (9.0)</td>
</tr>
<tr>
<td>Km μM</td>
<td>9.22* (1.4)</td>
<td>8.70* (1.0)</td>
<td>5.80 (0.3)</td>
</tr>
<tr>
<td>Kd h⁻¹</td>
<td>0.26*(0.05)</td>
<td>0.18*(0.03)</td>
<td>0.09 (0.02)</td>
</tr>
<tr>
<td>ZT conditions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vmax μmol/(l.h)</td>
<td>46.6** (2.5)</td>
<td>45.0** (3.0)</td>
<td>30.0 (2.0)</td>
</tr>
<tr>
<td>Km μM</td>
<td>5.5 (0.7)</td>
<td>6.1 (1.6)</td>
<td>5.1 (0.9)</td>
</tr>
<tr>
<td>Kd h⁻¹</td>
<td>0.200** (0.015)</td>
<td>0.11* (0.004)</td>
<td>0.045 (0.009)</td>
</tr>
</tbody>
</table>

Parameters defined by and obtained from fits of equation (1). IU, immediate use (cells not depleted of choline); ZT, zero-trans (cells depleted of choline). SEM are shown in parentheses. Significant differences from control data are indicated by * (P<0.05) and ** (P<0.01) using unpaired Student’s t tests. There were no significant differences between the CAPD and HD data with the exception of Kd (ZT) which was significantly higher in the CAPD group (P<0.05).
Fig. 1. Kinetics of choline transport. The concentration dependence of choline influx (V) in zero-trans conditions is plotted as a function of extracellular choline concentration (S) for erythrocytes from CAPD patients (open symbols) and from controls (filled symbols). Each point is the mean of data obtained from 10–15 subjects; error bars are SEM. The lines are those of best fit to the data using equation (1).

Table 3. Summary of the actions of quinine, HC-3, and NEM on ZT choline transport in HD and controls

<table>
<thead>
<tr>
<th></th>
<th>HD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC_{50} quinine (μM)</td>
<td>90 (9)</td>
<td>101 (13)</td>
</tr>
<tr>
<td>IC_{50} HC-3 (μM) at 25 μM choline</td>
<td>44 (6)</td>
<td>47 (8)</td>
</tr>
<tr>
<td>IC_{50} HC-3 (μM) at 100 μM choline</td>
<td>102 (5)</td>
<td>102 (4)</td>
</tr>
<tr>
<td>% inhibition by 2 mM NEM</td>
<td>74</td>
<td>78</td>
</tr>
</tbody>
</table>

None of the differences (HD vs control) was significant; SEM are shown in parentheses, n = 3.

characteristics of the existing transport system [1]. This study aimed to distinguish these possibilities by studying choline transport in normal and uraemic erythrocytes under zero-trans conditions and comparing (a) the apparent dissociation constant of the choline transport system (K_{m}); (b) the effect of the presence or absence of sodium; (c) the effect of the specific competitive inhibitors hemicholinium-3 and quinine; and (d) the effects of the thiol-reactive reagent N-ethylmaleimide (NEM) [9].

In earlier work the kinetics of choline transport had been fitted with a 2-parameter model and this analysis had suggested a decrease in substrate affinity in HD patients i.e. an increase in K_{m} [1]. The 3-parameter fit used here includes an apparent diffusion component for choline influx and provides a better description of the data. Inspection of Figure 1 reveals that the choline influx does not saturate at high substrate concentrations and a linear diffusion component is necessary for an adequate fit of the data. The earlier conclusion of an increase in K_{m} in renal failure was an artefact arising from the presence of a variable diffusion component of choline flux. The present analysis provides good evidence that the value of K_{m} measured in ZT conditions does not alter and that substrate affinity of the transporter is unaltered in HD and CAPD patients (Table 2). The variation in K_{m} found in the IU experiments can be explained by the increased intracellular content of choline in uraemia, which increases the apparent substrate dissociation constant for influx measurements [13,14].

The studies with the inhibitors quinine, HC-3 and NEM provide a consistent picture. In HD patients erythrocyte choline transport exhibits unaltered sensitivity to these agents, suggesting that the molecular properties of the transporter are normal. The unchanged inhibitory activity of the thiol-reactive agent NEM demonstrates that the transport of choline remains protein mediated. Taken with the normal substrate affinity, these results indicate that the increased choline transport seen in HD and CAPD patients (and in non-dialysed chronic renal failure [2]) arises from an increased surface expression of a normal active choline transport protein, rather than through a non-specific membrane leak or from expression of an abnormal or new choline transporter protein. This conclusion is also consistent with the recent results of Flanagan et al., who demonstrated that the increased ZT V_{max} for erythrocyte choline influx could not be accounted for by altered intracellular substrate analogues [14]. The hypothesis of increased surface expression of an active choline transporter cannot be
confirmed with direct ligand binding studies at present, as no specific high-affinity ligand or antibody is available.

It is also possible, but less likely, that the apparent upregulation of the transporter arises from an increased turnover number of each transporter molecule. An increase in turnover could result from the changes in erythrocyte lipid environment displayed in uraemia [15]. Certain phospholipid species and free fatty acids are altered both in plasma and in erythrocytes, and it has been suggested that these substances affect membrane Na,K-ATPase pump function [16,17]. Changes in lipid composition may likewise alter choline transport by modulating bulk bilayer properties or through specific membrane interactions [18]. However, the measured alterations in lipid composition are small and appear unlikely to cause such dramatic changes in the turnover of a specific transport system. The upregulation of the surface expression of the choline transporter does not appear to arise from altered rates of

---

**Fig. 2.** Polyamines and choline transport. Summary of the effects of extracellular polyamines at 0–1.0 mM on zero-trans choline influx at an extracellular choline concentration of 100 μM. A, putrescine; B, spermidine; C, spermine. For each experiment the choline influx has been calculated as a ratio: the value in the presence of polyamine divided by the result obtained in duplicates in the absence of polyamine. Means for five experiments are shown, error bars are SEM. The horizontal lines are drawn at a ratio of 1.0. In control conditions the mean absolute value of choline flux was 27.3 μmol.(l cells.h)$^{-1}$. 
transcription or translation. The reversal of the high choline transport $V_{\text{max}}$ of renal failure within a few days of renal transplantation in cells which do not possess transcriptional or translational machinery is strong evidence for post-translational modulation of the activity of the transporter [3]. The evidence points to variability in the surface expression of a functional form of the transport protein. The signals that generate this variation and the mechanism of the modulation are unclear.

Flanagan et al. [14] examined the effect of a single HD session on erythrocyte choline uptake. They found that the IU choline influx $V_{\text{max}}$ was reduced by 23% by a session of HD but that the ZT $V_{\text{max}}$ was not significantly affected. This result suggests that the IU $V_{\text{max}}$ in HD patients is partly the result of trans-acceleration by high intracellular concentrations of substrates for the choline transporter, including choline itself, but that the ZT $V_{\text{max}}$ differences cannot be accounted for in this manner. Elevated plasma levels of choline are found in patients on HD [19] and these could have long-term effects, including the observed increase in ZT $V_{\text{max}}$ for choline influx, but there is no direct evidence for this.

In the present work there was no significant correlation between $V_{\text{max}}$ and plasma creatinine or urea. The degree of uraemia has been correlated with elevations in erythrocyte choline $V_{\text{max}}$ in chronic renal failure patients (not on dialysis) and in renal transplant patients with different levels of renal function [1,2].

As a cation transporter the choline transport system might be expected to interact with a variety of cationic solutes. The polyamines are a group of polycationic solutes which accumulate in renal failure. The physiological plasma concentrations of putrescine and spermidine are in the range 0.1–0.2 μM; for spermine the range is 20–50 nM [20]. The lack of activity of the very high polyamine concentrations employed in this study (0–1.0 mM, i.e. up to 10-fold higher than substrate choline), at both the extracellular and intracellular surfaces of the membrane, demonstrates that the choline transport system is not a polyamine transporter and does not appear to interact at all with the polyamines [21]. Flanagan et al. [14] also report insensitivity of the choline transporter to a range of amines including putrescine, spermidine, spermine and trimethylamine-N-oxide. Trimethylamine at 0.2–1.0 mM did interact with the transporter but was not found to accumulate to remotely similar levels in uraemia. Accordingly, the accumulation of polyamines and amines cannot readily account for the abnormal regulation of the choline transport system in renal disease.

Further work on this problem is needed to characterize the direct actions of whole plasma (from normal individuals and renal failure patients) on choline transport. In addition, the possible modulation of choline transport by renal failure in other cell types needs to be explored, particularly in neuronal and epithelial tissue. The work of Ni et al. [4] suggests that renal failure is associated with abnormal choline transport in rat brain tissue. The mechanisms by which altered membrane choline transport might be pathogenic remain to be defined. It is possible that an abnormally high capacity for Na-independent choline uptake into cells close to a synaptic region might deplete the supply of choline available for the Na-dependent presynaptic reuptake that is linked to acetylcholine synthesis. In this manner, for example, an elevated $V_{\text{max}}$ for Na-independent choline transport could result in a relative failure of cholinergic neurotransmission.

Acknowledgements. This work was supported by the National Kidney Research Fund and the MRC.

References

8. Rabkin SW, Cheng KM. A genetic abnormality of cardiac myocytes from the blind mutant (RC) chick heart: abnormalities of cardiac structure and choline transport. Basic Res Cardiol 1992; 87: 610–617
17. Kelly RA, Canessa ML, Steinman TI, Mitch WE. Hemodialysis


Received for publication: 7.10.96
Accepted in revised form: 20.5.97