Human erythrocyte choline uptake in uraemia: the role of intracellular substrate and an investigation into the effects of haemodialysis

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INTRODUCTION

Choline is essential for human growth; it is a major constituent of cell membranes and is the precursor of the neurotransmitter acetylcholine [1]. There are specific membrane transporters for choline which enable this biologically important molecule to enter cells. In the erythrocyte this transporter is of the simple facilitated diffusion type, with transport following Michaelis-Menten kinetics. In common with many transporters of this type, the erythrocyte choline carrier shows the phenomenon of trans-stimulation, i.e. increased intracellular substrate enhances uptake [2, 3]. In patients with chronic renal failure there is a 2–3-fold increase in human erythrocyte choline transport compared with normal subjects. After successful renal transplantation, erythrocyte choline transporter activity rapidly falls to normal levels [4, 5]. Plasma choline levels are known to be two to three times higher in patients with chronic renal failure who are on maintenance dialysis, and there are similar increases in plasma levels of other related molecules such as aliphatic amines and certain amino acids [6–8]. It would be expected that intracellular levels of substrate would come into equilibrium with plasma levels in vitro, leading to trans-stimulation of choline influx. The changes in transporter Vmax seen in renal failure have been attributed to either the intracellular accumulation of trans-stimulatory molecules, such as choline, amines or free fatty acids, or to an increase in transporter number. Intracellular or plasma levels of such potential substrates have not been measured in other studies of membrane transport in uraemic individuals. Synaptosomal choline transport in a rat model of chronic renal failure has also shown an increase in choline uptake [9], and this suggests that altered choline transport may be a more generalized phenomenon of the uraemic syndrome.

In the present work, we study choline transport in erythrocytes and the effects of prior haemodialysis, subsequent washing in vitro and autologous plasma. Erythrocytes from uraemic patients were compared with those from individuals with normal renal func-
ton. We also examine the effect of a number of putative uraemic toxins on choline uptake and efflux in normal erythrocytes. We found that a number of molecules, present in raised concentrations in plasma from uraemic individuals, were able to alter choline transport; these included trimethylamine (TMA), dimethylamine (DMA) and trimethylamine-N-oxide (TMA-O). NMR spectroscopy was then performed on erythrocyte extracts from uraemic patients and compared with extracts from normal subjects to establish whether the intracellular accumulation of these molecules could explain the increased erythrocyte choline transport found in uraemia.

SUBJECTS AND METHODS

Subjects

This research has been carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and was approved by the ethics committee of the Oxford Renal Unit. With informed consent, blood was obtained either by venesection or by removal from arterial dialysis lines. In the haemodialysis experiments, 10 patients (six male and four female) with end-stage renal failure receiving maintenance haemodialysis gave blood samples immediately before and after a 4 h session of haemodialysis. These were compared with 10 control subjects (seven male and three female) with normal renal function. Patients with diabetes or intercurrent illness were excluded from the study. In incubation studies and NMR studies, blood was obtained during routine clinic appointments of patients. NMR was used to examine erythrocyte extracts from nine control subjects with normal renal function, 32 individuals with renal impairment and nine samples from haemodialysis patients.

Measurement of choline transport

Samples of fresh heparinized blood (10 ml) were diluted with four volumes of saline solution (NS), containing 145 mmol/l NaCl, 4 mmol/l KCl, 10 mmol/l Mops, 5 mmol/l glucose, pH 7.4, centrifuged (3000 g for 5 min) and the plasma and Buffy coat were aspirated and discarded. The remaining erythrocyte pellet was resuspended in 15 volumes of NS and fluxes were measured. This was repeated every hour for 3 h and then resuspended in NS and fluxes were measured.

[^14]Choline influx

Choline flux was measured by the method of Fervenza et al. [5]. A 1 ml erythrocyte suspension with a haematocrit of ≈5% was incubated for 10 min at 37°C in [^14]Choline solutions with a concentration range of 5–250 μmol/l (12 concentrations). Samples were then placed on ice for 2 min, centrifugated (10,000 g for 15 s), the supernatant aspirated and the erythrocyte pellet resuspended in 0.75 ml of ice-cold isotonic MgCl₂ washing solution (107 mmol/l MgCl₂, 10 mmol/l Mops, pH 7.4 with Tris base). Washing and centrifugation were repeated four times and then erythrocyte pellets were lysed with 0.5 ml of 0.1% Triton X-100, mixed and protein precipitated by adding 0.5 ml of 5% trichloroacetic acid. Samples were again mixed and then centrifugated (10,000 g, 10 min). The supernatant was then transferred to a scintillation vial, 2 ml of Liquiscint was added and [^14]C was measured on a Packard 2000CA B-scintillation spectrometer. Fluxes are quoted as μmol h⁻¹ l⁻¹ of erythrocytes. In preliminary experiments, erythrocytes from two patients with chronic renal failure were placed in a 1 mmol/l [^14]Choline solution in NS and incubated at 37°C for 1 h; cells were then washed three times and treated as per the preparation of choline-depleted cells. Samples were removed for intracellular [^14]Choline estimation at hourly intervals for 4 h, confirming that less than 2% of the intracellular choline remained after 2 h incubation.

Investigation of putative uraemic toxins

Influx studies. In four normal subjects, erythrocytes were obtained and washed as before and 0.25 and 1 mmol/l TMA, DMA, TMA-O, spermine, spermidine and putrescine were examined for their effect on choline influx at a choline concentration of 10 μmol/l.

Efflux studies. After washing, erythrocytes from normal volunteers were placed in a solution containing NS and [^14]Choline, incubated (1 h at 37°C) and then washed to remove any extracellular choline. Erythrocytes were then placed in NS at 37°C, containing 200 μmol/l of one of TMA, DMA, TMA-O, spermine, spermidine or putrescine or 30 μmol/l choline, and measurements of extracellular and intracellular radioactivity were determined at 1 min intervals over 10 min. This enabled a rate constant to be determined for choline efflux in the presence of each amine at 200 μmol/l.

Effect of autologous plasma

In patients with chronic renal failure and normal control subjects, blood samples were obtained by
venesection, heparinized and plasma was separated and kept at 4°C. The cells were then suspended in NS and prepared for estimation of transporter activity both immediately and after extensive washing in vitro. After washing in vitro, erythrocytes were then resuspended in autologous plasma and incubated at 37°C for a period of either 2, 6 or 20 h. Erythrocytes were then separated from the plasma, washed to remove residual plasma, and choline transport characteristics were determined. All assays were performed in triplicate.

Preparation of samples for analysis by NMR spectroscopy

Erythrocyte extracts were prepared following the method of Quirk et al. [10]. Briefly, samples of heparinized blood (10 ml) were taken and immediately diluted with four volumes of ice-cold NS, centrifuged (3000g for 5 min) and the plasma and Buffy coat were aspirated and discarded. The remaining erythrocyte pellet was resuspended in 15 volumes of NS and the process was repeated five times. After the washing procedure, 1 ml of packed erythrocytes was taken and resuspended in 4.5 ml of sterile, deionized water and left for 1 h at 4°C to lyse cells. Lysed cells were then treated with 2.75 ml of 8% perchloric acid, mixed and centrifuged (3000g for 5 min). The supernatant was then placed on ice and back-titrated with 5 mol/l KOH to pH 7.4, centrifuged (3000g for 5 min), the precipitated KClO₄ removed and the supernatant lyophilized and resuspended in 700 μl of 2H₂O, containing 0.114 mmol/l 2,2,3,3-tetradeutero-(3)trimethylsilyl propionic acid (TSP) as an internal intensity and chemical shift reference.

Acquisition of spectra

All Varian INOVA spectrometers (Varian Associates Ltd, Palo Alto, CA, U.S.A.) using a 5 mm pulsed-field gradient inverse probe. All spectra represented the sum of 32 transients, collected across 32 K data points, with a 20s delay to obtain ‘fully relaxed’ resonances. Spectra were transformed using one degree of zero-filling and an exponential multiplication factor of 0.5 Hz. Line-widths were typically in the order of 0.5-1 Hz. Assignments were verified by reference to standard spectra, by addition of authentic compounds to the sample and by acquisition of a 2D-quantum filtered, two-dimensional correlation spectra. Concentrations were calculated by fitting Lorentzian lineshapes to the resonances of interest using standard Varian software and comparing these with those from the intensity reference (TSP). One sample, from an individual with renal impairment, was not included for statistical analysis as the choline peak was thought to be co-resonant with another molecule which gave a spurious result for the choline concentration, two orders of magnitude greater than for the other samples.

Statistics and maximal flux estimation

\( V_{\text{max}} \) and \( K_m \) were estimated using an ‘enzfitter’ program based on non-linear least squares regression using the formula \( V = V_{\text{max}}/(1 + K_m/S) \), where \( V \) is flux and \( S \) is the concentration of substrate. This analysis assumes non-carrier-mediated transport is negligible up to choline concentrations of 0.25 mmol/l. Comparison between pre- and post-dialysis samples was performed with the paired \( t \)-test and between controls and other samples with the unpaired \( t \)-test.

RESULTS

Choline uptake in fresh cells

When choline uptake in freshly drawn human erythrocytes from 10 control subjects with normal renal function was measured over the substrate concentration range 5-250 μmol/l, the results were consistent with a single saturable component of choline influx, with a mean \( K_m \) value of 29 μmol/l and \( V_{\text{max}} \) of 55 μmol h⁻¹ l⁻¹ of cells (Table 1).

Similarly, data for erythrocytes from pre-dialysis samples from 10 patients with chronic renal failure tested immediately after venesection produced a similar value for \( K_m \) (17 μmol/l) but a 3-fold higher \( V_{\text{max}} \) (146 μmol h⁻¹ l⁻¹ of cells) than normal cells. If \( V_{\text{max}} \) values for choline transport in erythrocytes from patients with chronic renal failure are compared immediately before and then after 4 h of haemodialysis (Fig. 1), it is apparent that in every case transport was lower in the post-dialysis sample. The extent of the fall in transport varied between individuals but there was a mean decrease in \( V_{\text{max}} \).

| Table 1. Mean values of human erythrocyte choline transport characteristics from 10 subjects with chronic renal failure (pre- and post-dialysis), and 10 control subjects. Comparison between samples processed immediately and after 4h of extensive washing in vitro (choline-depleted). Statistical significance: *P<0.05 compared with controls; **P<0.005 pre-compared with post-dialysis; †P<0.005 compared with control. ‡Not significant (pre-compared with post-dialysis); ‡‡, not significant compared with control. |
|---|---|---|---|
| | Immediate use | Depleted | |
| \( V_{\text{max}} \) (μmol h⁻¹ l⁻¹ of cells) | Pre-dialysis | Post-dialysis | Control |
| 146 ± 20†‡ | 113 ± 13† | 55.3 ± 7 |
| 16.7 ± 2 | 17.9 ± 5‡‡ | 29.1 ± 7 |
| Pre-dialysis | Post-dialysis | Control |
| 62.4 ± 6* | 59.5 ± 6* | 34.3 ± 4 |
| 14.2 ± 2†‡ | 15.1 ± 2‡‡ | 21.3 ± 4 |
Effect of extensive washing on choline uptake

To deplete intracellular choline and other organic non-electrolytes (amines, amino acids), cells from control subjects with normal renal function were washed extensively with NS in vitro over a period of 4 h. Choline uptake experiments with these cells were consistent with a single Michaelis-Menten component, with no change in $K_m$, but a 38% decrease in the choline transport $V_{\text{max}}$ to 34 $\mu$mol h$^{-1}$ l$^{-1}$ of cells (Table 1). When cells from either pre- or post- dialysis samples were similarly washed extensively in vitro, there was no longer a difference in choline transport $V_{\text{max}}$ between individual pre- and post-dialysis samples, both being reduced to a mean of approximately 60 $\mu$mol h$^{-1}$ l$^{-1}$ of cells (Fig. 2, Table 1).

Effect of autologous plasma

Figure 3 demonstrates the effect of autologous plasma on choline transport; choline influx was measured in erythrocytes from 17 patients with chronic renal failure and from eight normal subjects. Choline influx was measured in fresh cells, after extensive washing procedures in vitro and after the incubation of those 'washed cells' in autologous plasma for 2, 6 or 20 h. After extensive washing in vitro, as shown earlier, erythrocyte choline transport $V_{\text{max}}$ from uraemic subjects fell to 54 ± 5% of initial values. With subsequent incubation in autologous plasma, this value increased to 60% and then 67% after 2 and 6 h respectively, finally reaching 97 ± 18% after 20 h incubation. Parallel experiments with erythrocytes and plasma from normal subjects gave similar results (50 ± 10%, 62 ± 4%, 61 ± 8%), although after 20 h of incubation the recovery was less marked (88 ± 8%).

Investigation of putative uraemic toxins

In choline influx studies, TMA, DMA, TMA-O, spermine, spermidine and putrescine were investigated. Only TMA and DMA had any appreciable effect on choline influx, both being inhibitory (Fig. 4). When erythrocytes were loaded with $[1^{14}C]$choline and then placed in a solution containing 0.2 mmol/l amine, TMA and TMA-O are capable of increasing choline efflux (Fig. 5).

NMR studies

Acquisition of NMR spectra of erythrocyte extracts from control subjects and individuals with renal impairment confirmed that intracellular choline levels were not significantly different between normal samples (40 ± 9 $\mu$mol/l) and samples freshly taken from dialysis-dependent individuals (47 ± 10 $\mu$mol/l). As expected, the washing of cells taken from dialysis-dependent individuals depleted cells of choline (30 ± 7 $\mu$mol/l), but this failed to
reach statistical significance \(P = 0.08\). In extracts from individuals with renal impairment who were non-dialysis-dependent, choline was present in higher concentrations \(71 \pm 10 \, \mu\text{mol/l}\) than in extracts from normal subjects \(P = 0.03\), but not from those who were dialysis-dependent \(P = 0.11\). No relationship was demonstrated between serum creatinine and intracellular choline (Fig. 6). Trimethylamine was present in trace amounts in samples from patients with chronic renal failure, whether from dialysis-dependent patients or from individuals who had never been dialysed.

**DISCUSSION**

The principal aim of this work was to determine how much of the increased choline transport rate observed in erythrocytes from patients with chronic renal failure is explained by exchange fluxes stimulated by increased intracellular substrate. A number of putative substrates, known to accumulate in renal failure, were examined for their effect on choline transport, and erythrocyte extracts were examined by NMR for the presence of these molecules.

Plasma choline and aliphatic amines have been measured during the course of haemodialysis. Choline is present in higher concentrations in plasma from uraemic subjects than from non-uraemic subjects but falls to lower levels during the first hour of haemodialysis. There is a slow increase in plasma choline after this, but it fails to reach pre-dialysis levels. This 'buffering effect' on plasma choline levels presumably reflects release of choline from the tissues into the plasma as it is lost during dialysis [6]. We found that aliphatic amines such as TMA, DMA and TMA-O, may inhibit choline uptake and/or trans-stimulate choline efflux. These amines, as well as choline, are increased in the plasma of uraemic subjects and fall after dialysis [8, 11, 12]. Choline transport in freshly drawn erythrocytes from uraemic patients decreased after dialysis (22.5\%), but not to the extent produced by extensive washing \textit{in vitro} (47–58\%). A fall in intracellular levels of choline or these amines, after their loss in dialysate, could have accounted for the reduction in erythrocyte choline uptake after dialysis. We measured choline, TMA, DMA and TMA-O in erythrocyte extracts from patients with chronic renal failure and unaffected individuals before and after washing \textit{in vitro}. Erythrocyte choline levels in individuals with normal renal function were in accordance with previous studies but choline was not significantly different in extracts from dialysis patients [13]. Of the amines tested, only TMA was present in uraemic erythrocytes and this was only in trace amounts that would be insufficient to trans-stimulate choline uptake.

Our results indicate that, apart from choline, no other trans-stimulatory amines were present in any significant amounts, and the reduction in erythrocyte choline uptake that followed washing \textit{in vitro} is due
to a fall in intracellular choline. The recovery in choline transport after the incubation of choline-depleted erythrocytes with autologous plasma also suggests that in both normal and uraemic erythrocytes, intracellular choline is the major determinant of exchange fluxes. Choline uptake in uraemic erythrocytes is markedly increased compared with normal erythrocytes; this disparity persists after washing in vitro and occurs despite similar levels of intracellular choline. This suggests that the increased choline uptake of uraemic erythrocytes is explained by a combination of trans-stimulation by intracellular substrate, predominantly choline, and a second effect, still evident after depletion of intracellular substrate. The second effect may reflect either an increase in transporter number as a compensatory response to circulating inhibitors of choline uptake, such as TMA and DMA, or the presence of other molecules which are capable of increasing the choline transporter activity but are more slowly reversed, such as free fatty acids. The crucial biochemical role of choline means that although the consequence of disturbances in its transport are not known, it may be of profound significance in the aetiology of the uraemic syndrome.

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REFERENCES