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^{23}Na NMR DETECTS PROTECTION BY GLYCINE AND ALANINE AGAINST HYPOXIC INJURY IN THE ISOLATED PERFUSED RAT KIDNEY

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SUMMARY Protection against hypoxic injury by supraphysiological glycine and alanine concentrations was investigated in the isolated perfused rat kidney (IPRK). ^{23}Na NMR detects consistent increases in total renal Na in IPRK during hypoxic perfusion. Increasing the concentration of glycine and alanine to 5 mM each produced a 34% ($p<0.001$) reduction in the increase in total renal Na following 30 minutes of hypoxia compared to a matched control group supplemented with 5 mM each of serine and glutamine. There was also a trend ($p=0.067$) to improvement in the fractional excretion of sodium (FE_{Na}) in the glycine plus alanine treated group. Hypoxic alterations of other physiological parameters were not prevented by supraphysiological glycine plus alanine. This suggests that monitoring total renal Na is a more sensitive method of defining renal injury and protection than monitoring changes in FE_{Na}, fractional excretion of potassium (FE_K) and inulin clearance.

Physiological concentrations of the amino acids glycine and alanine protect isolated rabbit proximal tubules from hypoxic injury (1-3). The mechanism of protection is unknown but appears not to require normal ATP or reduced thiol concentrations (1-4). Glycine has been less promising in vivo. Infusion of glycine (but not alanine) into cisplatin treated rats reduces tubular injury and improves renal function (5) but renal damage in vivo due to ischemia (6, 7) or contrast agents (8) is not prevented by glycine.

The isolated perfused rat kidney (IPRK) allows the well controlled study of renal function in intact kidney (9-12). Graded hypoxia in the IPRK, as might occur in low flow ischemia, allows investigation of the induction phase of hypoxic injury, which is not possible in arterial clamp models of acute renal failure. IPRK perfusion with cell-free buffered salt solution supplemented only with albumin and glucose leads to progressive morphological damage to medullary thick

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Abbreviations Used: NMR, nuclear magnetic resonance; FE_{Na}, fractional excretion of sodium; FE_K, fractional excretion of potassium; C_{In}, inulin clearance; IPRK, isolated perfused rat kidney; mTAL, medullary thick ascending limb of Henle's loop.

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ascending limbs (mTAL) and a decline in renal function (10, 13) which are reduced by addition of a mixture of 20 amino acids (14). Glycine alone, and to a lesser extent alanine help to prevent damage to the mTAL (15) and improve proximal tubular brush border function (16) during normoxic perfusion. Under hypoxic conditions there is marked reduction in renal function and damage to mTAL and proximal tubules especially the S3 segment (12, 15, 17, 18), which are alleviated by addition of glycine and alanine (15) or by reduction in transport activity (17). The mechanism of protection is unclear but appears not to involve amino acid metabolism (16, 19).

Damage to the mTAL in the cell-free perfused kidney results from low oxygen delivery (10) and is related to changes in cellular ATP levels and FE_{Na} (10, 12). Intracellular Na reflects both the energy state of the cell and cell membrane permeability to Na. Monitoring Na by ^{23}Na NMR is thus useful as a non-invasive method of assessing viability in intact organs and tissues. In the IPRK we recently demonstrated with ^{23}Na NMR that hypoxia induces a reproducible rise in total renal sodium (20), which is partially inhibited by dimethylthiourea and dimethylsulfoxide. In the present study, we monitored total renal sodium in the IPRK during induction of hypoxia both in the presence and absence of elevated perfusate glycine and alanine.

MATERIALS AND METHODS

Experimental: Right kidneys from male Wistar rats (300-400 g) were perfused at 37°C with Krebs-Henseleit buffer (KHB) containing bovine serum albumin (6.7 g/dl), glucose 5 mM, 20 amino acids (14) and gassed with 95% O_2: 5% CO_2 as described previously (11, 20). After 60 min normoxic perfusion, hypoxia was induced by switching the perfusate gas mixture to 95% N_2: 5% CO_2. The perfusion medium was modified by increasing the concentration of two amino acids: Group 1 (n=4), glycine and alanine concentrations were 5 mM each (increased from 1.18 and 2.30 mM respectively); Group 2 (n=4), serine and glutamine concentrations were 5 mM each (increased from 1.07 and 3.26 mM respectively) while glycine and alanine remained normal (1.18 and 2.30 mM). Perfusion conditions and composition of media, including osmolality, were otherwise identical. Renal artery perfusion pressure was held constant at 90-110 mm Hg and perfusate oxygen tension monitored in-line by a Clark-type oxygen electrode. Kidney function was estimated from plasma and urine samples collected at 5 min intervals for ^{14}C-inulin clearance (C_{In}) and fractional excretion of sodium (FE_{Na}) and potassium (FE_K). Only kidneys with an initial (30-45 min) C_{In} greater than 0.5 ml/min were accepted for analysis.

^{23}Na NMR Measurement: Kidneys were freely suspended within a 22 mm diameter Helmholtz coil, tuned to ^{23}Na (79.36 MHz), in a custom-built NMR probe in a Bruker CXP 300 vertical bore magnet as previously described (20). ^{23}Na spectra were averaged over 2 minute intervals using a pulse length of 80 microsec, a sweep width of 15,000 Hz and a repetition interval of 0.56 or 1.0 sec. A reference capillary containing saturated NaDy(PPP) was located within one loop of the Helmholtz pair on the outside of the plastic shell housing the suspended kidney. The intensity of each renal ^{23}Na peak was measured by integration and calibrated against the intensity of the low field reference ^{23}Na peak, which did not change following the induction of hypoxia. To facilitate comparison between experiments, the ^{23}Na intensity in each was normalised to the average intensity of the initial five ^{23}Na spectra collected during baseline normoxic perfusion. NMR "visibility" of ^{23}Na is 100% in this preparation (20).

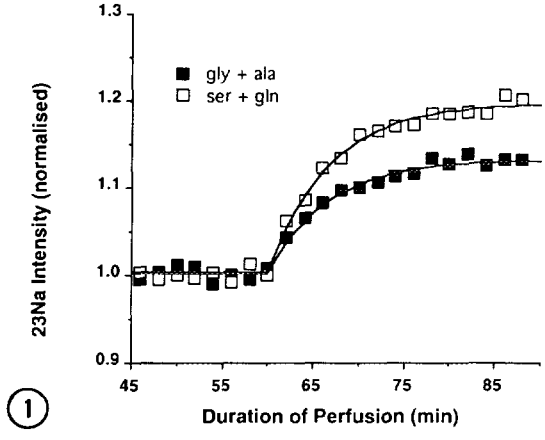
Statistical Analysis: Differences in the post hypoxic increase in total renal Na were analysed by fitting a model function (a discontinuous function with linear and exponential components) to the time-averaged data by non-linear least squares regression and comparing the residual sum of squares by variance ratio analysis (F-Test). The analytical method was described recently and illustrated with the NMR data detailed here (21). This yielded estimates and standard errors for

two hypoxic parameters for comparison; in this case, the first order rate constant and the asymptotic intensity change following hypoxia. For estimates of C_{In}, FE_{Na} and FE_K the Kolmogorov-Smirnov test was used to test for normality of the distribution. Repeated measures analysis of variance (ANOVA) was used to consider differences by time or group. Where significant group or time differences were noted in the overall ANOVA, the Student's t-test was used to compare differences in pairs of means. The significance level was set at 95%.

RESULTS

Control experiments have demonstrated that the preparation is stable for at least 90 min and that ^{23}Na intensity remains constant (20). Hypoxia induced an increase in ^{23}Na NMR intensity in both groups (Fig 1). Model fitting yielded equivalent rate constants for the post-hypoxic increase in total renal ^{23}Na for Group 1 and 2 of 0.161±0.025 and 0.144±0.010 min^{-1} respectively. The amplitude of increase was reduced in the presence of glycine and alanine (Group 1) 0.130± 0.006 compared to 0.198± 0.005 for Group 2 ($F=41.15$, $p<0.001$).

The effect of the perfusion solutions on physiological function are shown in Fig 2. The mean C_{In} differed significantly with time (ANOVA, $F=13.25$, $p<0.001$). From 65 min there was a significant decrease in mean C_{In} ($p<0.05$ in all comparisons). The trend with time was similar in both groups. FE_{Na} differed significantly with time ($F=73.03$, $p<0.001$), with a steady increase in mean FE_{Na} from 65 to 85 min ($p<0.05$ for all comparisons). Although the trend with time



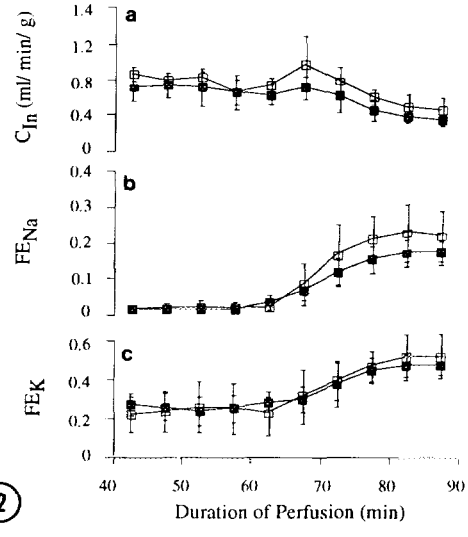


Figure 1. Averaged normalized total renal ^{23}Na NMR intensity measurements from perfused rat kidney. The closed squares are mean values for perfusate supplemented with glycine and alanine to 5 mM each (n=4) and the open squares perfusate supplemented with serine and glutamine to 5 mM each (n=4). Hypoxia was induced at 60 min while perfusion continued.

Figure 2. Kidney function data for kidneys perfused as in Fig 1. (a) Inulin clearance. (b) Fractional excretion of sodium. (c) Fractional excretion of potassium. Error bars represent (± 1) SD.

appeared to differ between the two groups (F=1.93, p=0.067), this was not supported by pairwise comparisons. Mean FE_K differed with time (F=24.27, p<0.001). After 70 min the mean FE_K increased (p<0.05 for all comparisons). The trend with time was similar for both groups. Summarizing, the difference in FE_{Na} between the two groups approached but did not reach statistical significance, while C_{In} and FE_K remained identical.

DISCUSSION

Physiological concentrations of glycine and supraphysiological concentrations of alanine protect isolated rabbit proximal tubules from hypoxic damage (1-4). Glycine at 2 mM reduces deterioration in renal function during normoxic perfusion in the IPRK and also alleviates but does not completely prevent hypoxic injury (15). An increase in total renal sodium occurs during hypoxia using standard perfusate with 1.18 mM glycine, 2.3 mM alanine (20). With supraphysiological concentrations of glycine and alanine (5 mM each, Group 1) the maximum amplitude ^{23}Na NMR signal was 34% lower compared to the group supplemented with serine and glutamine (Group 2) during hypoxia (Fig 1). Comparison with hypoxic increases in Na in non-filtering kidneys also indicates that the observed increases reflect mainly intracellular and interstitial compartments (20). Previously we showed a close correlation between decreases in ATP (^{32}P NMR) and K (by ^{87}Rb NMR) with the increase in Na (^{23}Na NMR) during hypoxic perfusion (22) suggesting that changes in total renal sodium reflect intracellular changes. Direct measurement of increased intracellular sodium by electron microprobe in ischemic kidney (23) and hypoxic cortical tubules (24) support this conclusion. Aqueous shift reagents to distinguish intra- from extracellular Na were avoided in this study because available shift reagents are nephrotoxic (25), however newer shift reagents such as Tm(DOTP) (26) may prove useful. Renal function was reduced during hypoxic perfusion (Fig 2). There were no significant differences between the two groups although there was a trend (p=0.067) towards a lower FE_{Na} in the glycine plus alanine treated group. Measurement of cellular injury by ^{23}Na NMR (Fig 1 and ref 20) therefore appeared more sensitive than conventional parameters of physiological function. ^{23}Na NMR spectra can be acquired on the subminute time scale which allows rapid detection of change and provides more data for analysis, for example by the curve fitting technique (21), than measurements of C_{In}, FE_{Na} or FE_K which require 5-15 min for collection of urine samples.

In the intact kidney the actions of glycine and alanine are likely to be more complex than in isolated cortical tubules, since amino acids have direct effects on renal perfusion (27), filtration and oxygenation. Glycine increases oxygen consumption by the IPRK (28) and isolated medullary tubules (15) during normoxia. A tendency to enhance oxidative metabolism runs counter to the protective actions of the amino acids on the hypoxic filtering IPRK . Since different tubular segments vary in transport function, membrane permeability and metabolic activity, they may respond differently to protective factors. Whereas isolated proximal tubules concentrate glycine several fold that in the extracellular fluid (29), isolated medullary tubules do

not (15). Comparison with isolated tubules suggests that cellular glycine levels in vivo should provide maximal protection (29) during ischemia. However the presence or absence (7) or even augmentation (30) of ischemic injury in intact kidney in vivo depends on the actual plasma glycine concentration, a host of interactive regional and biochemical factors, and on the method of detection. Our studies suggest that monitoring more sensitive parameters of cell viability in the intact kidney, such as total renal Na, can reveal protection by supraphysiological concentrations of perfusate glycine.

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