^{23}Na-NMR Detects Hypoxic Injury in Intact Kidney: Increases in Sodium Inhibited by DMSO and DMTU

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Hypoxic injury in the isolated perfused rat kidney (IPRK) was monitored using ^{23}Na-NMR in the presence or absence of 1.5 and 15 m*M* dimethylthiourea (DMTU) or 15 m*M* dimethylsulphoxide (DMSO) before and after inducing hypoxia. Hypoxia induced a prompt exponential increase in total renal $^{23}Na^+$, renal vascular resistance, and sodium excretion and decreased inulin clearance and adenine nucleotides and reduced glutathione concentrations. Lipid peroxide metabolites were unaltered. The increase in $^{23}Na^+$ was significantly reduced ($P < 0.001$) by both DMTU and DMSO although hypoxic perturbations of function and biochemical parameters were not. Posthypoxic increases in renal $^{23}Na^+$ include approximately 10% from the intratubular compartment, but principally reflect the intracellular and interstitial compartments. The results demonstrate that ^{23}Na-NMR is a sensitive indicator of hypoxic renal injury in intact kidney and suggest that DMTU and DMSO protect against hypoxic injury by a mechanism independent of free radical-binding.

Key words: ^{23}Na-NMR; perfused rat kidney; hypoxic renal injury; variance ratio analysis.

INTRODUCTION

Studies of experimental acute renal failure (ARF) using ^{31}P NMR have documented both the prompt and expected fall in renal ATP with ischemia *in vivo* (1) and with hypoxia *in vitro* (2). *In vitro*, these ^{31}P NMR studies showed that the extent of depletion of total renal ATP was linearly correlated with the volume of cells injured by hypoxia (2) and *in vivo* that the final renal ATP content after ischemia correlated well with the rate of ATP recovery and with recovery of renal function (3). The low NMR sensitivity of the ^{31}P nucleus limits the time resolution of ^{31}P NMR investigations of acute renal injury, even at high magnetic fields, with spectra often taking 10 to 20 min to acquire. ^{23}Na-NMR studies are complementary to ^{31}P NMR, because they allow ATP-dependent transcellular $^{23}Na^+$ gradients to be investigated. The higher NMR sensitivity of the ^{23}Na nucleus allows adequate ^{23}Na-NMR signal to be acquired within 1 or 2 min, so that a time course can be monitored more closely after a given perturbation. Although several studies have demonstrated the feasibility of ^{23}Na-NMR studies in isolated renal tubules (4, 5) and in the isolated perfused rat kidney (6, 7), there has been no systematic application of this technique to the study of experimental acute renal failure.

Along with other groups (8, 9), we have demonstrated (2, 10) that graded hypoxia in the IPRK is a useful model for studying hypoxic injury in experimental ARF. In this model the changes in ATP and the fractional excretion of sodium (FE_{Na}) are closely correlated with oxygen delivery and with the extent of morphological injury to renal tubules, specifically to proximal tubules and medullary thick ascending limbs of Henle's loops. We have also studied this model using ^{23}Na-NMR and demonstrated that changes in total renal sodium rapidly follow the induction of hypoxia and are closely related to changes in ATP and K^+ (6). In contrast to the renal clamp model of ARF, hypoxia in the IPRK is not associated with interruption of perfusion during the induction phase of injury but low levels of oxygen continue to perfuse the kidney, as might occur in "low flow ischemia." Because this oxygen could act as a source of OFR, we investigated whether a component of injury in the IPRK after hypoxia could be attributed to OFR and whether monitoring changes in total renal sodium by ^{23}Na-NMR could be used as a sensitive means of detecting damage caused by OFR. ^{23}Na-NMR determinations of total renal $^{23}Na^+$, kidney concentrations of adenine nucleotides, oxidized and reduced glutathione, and lipid peroxide metabolites were measured after hypoxia in the IPRK in the presence and absence of scavengers (DMTU and DMSO) protective against reperfusion injury in IPRK (11).

METHODS

Experimental

Right kidneys from male Wistar rats weighing 300 to 400 g were used in all experiments. Rats were fed on Norco Rat and Mouse Cubes (Norco Cooperative Ltd., Lismore, Australia) and supplied with water ad libidum. Renal artery perfusion was begun without intervening ischemia by cannulation through the superior mesenteric artery (12). Kidneys were perfused with Krebs-Henseleit buffer (KHB), (13) at 37°C. The medium was supplemented with bovine serum albumin (6.7 g/100 ml), glucose 5 m*M*, and 20 amino acids (14), and gassed with 95% O_2/5% CO_2 (Carbogen, CIG Ltd. Australia) as described previously (2). Hypoxia was induced after 60 min of perfusion by switching the gas mixture to 95% N_2/5% CO_2.

Renal artery perfusion pressure was measured directly within the renal artery using a Statham type pressure

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gauge (model P23, Gould, UK) through a polyethylene line contained within the glass cannula perfusing the artery. Pressure was held constant between 90 to 110 mmHg by a process controller (model 2073, West Division, Gulton Industries Inc., Schiller Park, IL) that regulated the speed of the peristaltic pump (Watson and Marlow model 501U; Smith and Nephew, Watson-Marlow, England). Perfusate oxygen tension was monitored in-line by a Clark-type oxygen electrode, attached to a blood gas analyzer (PHM 72, Radiometer, Copenhagen). Perfusate flow was monitored by wide beam ultrasound using a Transonic T206 flow meter with an in-line "cannulating" flow probe (SN22; Transonic Systems Inc., Ithaca, NY). Analog outputs from the pressure recorder (Gould RS 3400, Gould Inc., Cleveland, OH), flow meter and oxygen meter were connected to separate channels of a 16-channel A/D converter board (DT2801; Data Translation, Marlboro, MA) and converted to real-time data display and recording using "The Real Environment" software (Laboratory Software Associates Pty. Ltd., Melbourne, Australia) on an IBM AT-compatible computer (Fig. 1).

The ureter was cannulated by a 3-cm length of 0.61 mm OD polyethylene tubing (ID 0.28 mm, Dural Plastics & Engineering, Auburn, Australia) connected in turn to an 80-cm length of 0.96-mm polyetheylene tubing (ID 0.58 mm). This urinary drainage line was located vertically within the NMR probe. Even at high urinary flow rates, this arrangement provides negligible resistance to urine flow. Kidney function was estimated from urine and plasma samples collected every 5 min for measurement of ^{14}C-inulin (Amersham, UK) clearance and electrolyte excretion (Na^+, K^+). ^{14}C-inulin activity was measured in an LKB 1217 Rackbeta scintillation counter. Na^+ and K^+ were measured by flame photometry (FLM3, Radiometer, Copenhagen).

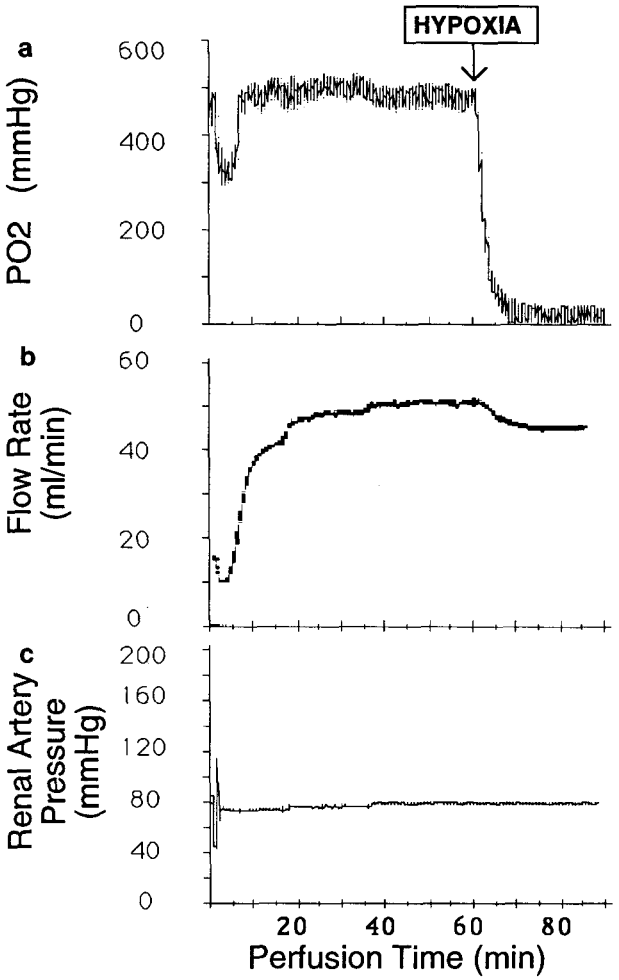

FIG. 1. Real time recording of PO_2 (mmHg), flow rate(ml/min), and mean arterial pressure (mmHg) in the isolated perfused rat kidney from a typical control group experiment in the absence of OFR scavengers demonstrating (a) the rapid decrease in perfusate PO_2 as the gassing mixture was switched to (95%/5%) N_2/CO_2 (b) the immediate and sustained reduction in flow after induction of hypoxia, and (c) tight control of perfusion pressure.

Experimental Protocol

Kidneys were perfused both within the NMR magnet and outside the magnet (on the bench) using an identical protocol. Renal function was monitored throughout in both groups. Following cannulation (zero time), kidneys were allowed to stabilize for 30 min before measurement of function was begun. The OFR scavengers DMTU (15 or 1.5 m*M*) or DMSO (15 m*M*) were added at 45 min. Hypoxia was begun at 60 min by switching to a 95% N_2/5% CO_2 gas source and was continued for a further 30 min. The experiment was then terminated. After bench experiments, kidneys were rapidly freeze-clamped in preparation for measurements of adenine nucleotides and glutathione metabolites. After NMR experiments, the kidneys were weighed and homogenized for measurement of Na^+ by flame photometry. The group sizes (n) for ^{23}Na-NMR experiments were: control normoxia, $n = 3$; control hypoxia, $n = 7$; 1.5 m*M* DMTU, $n = 7$; 15 m*M* DMTU, $n = 5$; and 15 m*M* DMSO, $n = 5$. For bench experiments $n = 5$ in each treatment group.

Nonfiltering Kidneys

Additional experiments were performed to examine the contribution of the urinary ^{23}Na signal to the increase in total renal $^{23}Na^+$ after the induction of hypoxia. Kidneys were perfused as described above, but were rendered nonfiltering by perfusing with hyperoncotic medium (bovine serum albumin 11 g/100 ml, $n = 4$). The results were compared with filtering kidneys (albumin 6.7 g/100 ml, perfusion pressure 90 to 100 mmHg, $n = 8$, including the same hypoxic controls noted above).

^{23}Na-NMR Measurement

Kidneys were perfused 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. The uncannulated venous effluent flowed over the kidney keeping it warm and moist, and both the venous effluent and the urine drained from the probe by gravity. ^{23}Na spectra were averaged over 2-min intervals using a pulse length of approximately 80 μs, a sweep width of 15,000 Hz, and a repetition interval of 0.56 or 1.0 s. A reference capillary containing saturated sodium

dysprosium tripolyphosphate (Na-Dy(PPP)) was located within one loop of the Helmholtz pair on the outside of the plastic shell housing the suspended kidney and over which the coil was wound.

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 after the induction of hypoxia. To facilitate comparison between experiments, the ^{23}Na intensity in each was normalized to the average intensity of the initial five ^{23}Na spectra collected during normoxic perfusion. In addition, the ^{23}Na signal was calibrated against latex phantoms containing known concentrations and volumes of NaCl. NMR "visibility" of $^{23}Na^+$ was determined by comparing the calibrated sodium intensity measured by ^{23}Na NMR with the absolute mass of sodium determined by flame photometry.

Biochemical Assays

At the end of each perfusion, kidneys were freeze-clamped and stored in liquid nitrogen until assayed. In addition, unperfused control kidneys were obtained for some assays by rapidly freeze-clamping unperfused left and right kidneys (n = 5). For the measurement of metabolites of lipid peroxidation, frozen tissue was weighed and homogenized at high-speed in 10 volumes of cold 0.05 M phosphate buffer, pH 7.4, before processing for assay of the individual metabolites. For adenine nucleotide and total glutathione measurements, kidney tissue was pulverized in liquid nitrogen and extracted into 0.02 M HCl in methanol at −30°C as described by Lowry and Passonneau (15). When fully extracted the tissue was diluted into cold (4°C) 0.02 M HCl and the protein precipitated with 3 M $HClO_4$. Extracts were neutralized with 2 M KOH/0.4 M KCl/0.4 M imidazole and the precipitated $KClO_4$ was removed prior to analysis. Oxidized glutathione (GSSG) was assayed by adding 2-vinyl pyridine to the extraction buffers to bind reduced glutathione (GSH) and maintain *in vivo* ratios of GSH:GSSG. Assays for both GSH and GSSG and for adenine nucleotides were performed within 24 h of tissue extraction.

For adenine nucleotide determination, 10 µl of neutralized extract were injected onto a Waters' 5-Bondapak HPLC column and chromatographed by reversed-phase ion-pair chromatography (16). The mobile phase was 0.1 M KH_2PO_4, 2 mM tetrabutylammonium phosphate, 10% methanol at pH 6.0, and flow rate 0.8 ml/min. The purity of the nucleotide peaks was confirmed by selective peak removal (16). Peaks were quantified by comparison with injections of standard solutions of ATP, ADP, and AMP (Sigma Chemical Co., St. Louis, MO). Values for the total adenine nucleotides (TAN), as (ATP+ADP+AMP) and the energy charge (EC) as {([ATP]+ 0.5 [ADP])/TAN} were calculated from the measured levels of ATP, ADP, and AMP (after Atkinson, 17).

Levels of both GSH and GSSG in prepared extracts were measured by the glutathione reductase method (18). Changes in absorption produced by tissue extracts were compared with standard curves prepared using fresh solutions of GSH and GSSG (Sigma Chemical Co.).

The concentrations of Schiff bases and diene conjugates were determined by the method of Lunec and Dormandy (19). One-milliliter aliquots of kidney homogenate in phosphate buffer were extracted in 4 ml of chloroform:methanol (2:1 v/v) containing 2.5 mM 2,6 di-tert-butyl-p-cresol as an antioxidant. Formation of Schiff bases was determined in the aqueous phase by monitoring the fluorescence maximum between 400 and 450 nm when excited at 355 nm. Diene conjugates were estimated in the chloroform phase by measuring absorbance at 240 nm. Malondialdehyde was measured as thiobarbituric acid reactive substances (TBARS) assayed by the modified fluorescence method of Suematsu and Abe (20) with tetramethoxypropane as a standard. All results were related to the protein concentration of the kidney homogenate.

Statistical Analysis

The functional and biochemical data were analyzed initially by ANOVA, and individual treatment differences were compared by the Student's t-test with Bonferroni correction of the critical value for multiple group comparisons. Changes in total renal sodium measured by ^{23}Na NMR were analyzed for significance by the variance ratio (F test) method as recently described in detail (21). Briefly, a model function was fitted by a nonlinear regression program, DNRP53 (22), to the time-averaged data from each group of experiments to yield estimates and standard errors for each parameter, including rate constants, plus a residual sum of squares (RSS), which represents the weighted square of the difference between the fitted and experimental values, summed over all data points. Evaluation of the suitability of the model function itself, analyses of various individual data sets and various combinations of data sets were compared using the values of RSS in an F test (21, 23–25).

Mathematical Model for NMR Measured ^{23}Na Intensity

Control experiments (see normoxic control, Fig. 4) demonstrated that the preparation was stable over at least 90 min and that ^{23}Na intensity was constant prior to inducing hypoxia at 60 min. Starting from 60 min there was an exponential increase in ^{23}Na intensity. Mathematically this can be described as:

$$\mathbf{y} = \begin{cases} \mathbf{y}_0 & \mathbf{t} \le 60 \\ \mathbf{y}_0 + \mathbf{A}(1 - \mathbf{e}^{-\mathbf{k}(\mathbf{t}-60)}) & \mathbf{t} > 60 \end{cases} \quad [1]$$

where $\mathbf{y}_0$ is the baseline intensity; $\mathbf{A}$, the asymptotic intensity change after hypoxia; and $\mathbf{k}$, the first order rate constant. Fitting more complex functions to the data such as a double exponential function and the integrated Michaelis-Menten equation resulted in a fit, which was both visually and statistically indistinguishable from the fit to a single exponential (21). Thus, a single exponential increase in intensity after hypoxia (Eq. [1]) was taken to be a sufficient description of the data. Adding either free radical scavenger to the perfusion medium at 45 min prior to the induction of hypoxia provided a further potential perturbation. To address whether the scavenger itself altered ^{23}Na intensity, an expanded form of Eq. [1], with discontinuities at 45 and 60 min was fitted to the

data.

$$y = \begin{cases} y_0 & t \leq 60 \\ y_0 + A_1(1 - e^{-k_1(t-45)}) & 45 < t \leq 60 \\ y_0 + A_1(1 - e^{-k_1(t-45)}) + A_2(1 - e^{-k_2(t-60)}) & t > 60 \end{cases} \quad [2]$$

Comparison of fits of Eqs. [1] and [2] to the data in the presence of the scavengers indicated that the discontinuity between 45 and 60 min was required only when 15 m*M* of DMTU was added (see below). Fitted curves to the three sets of scavengers and to the control with hypoxia alone (hypoxic control, Fig. 4) were then combined and analyzed systematically. An exponential component beginning at 60 min was included in the analysis of all four curves. An additional exponential component at 45 min was included only for the 15 m*M* DMTU curve.

RESULTS

$^{23}Na^+$ in Filtering Kidneys

The consistent and rapid rise in total renal $^{23}Na^+$ induced by hypoxia is illustrated for a control experiment in Fig. 2. The very small but constant resonance immediately upfield of the main *in vivo* ^{23}Na resonance was present (and constant) in some experiments and absent from others. This probably represented a shimming artifact rather than a second form of tissue sodium. Although included in the peak integration for analysis, this signal was unchanged with time and therefore had no quantitative significance and was automatically corrected for by the normalization procedure when within- and between-group comparisons were made. ^{23}Na measurements by ^{23}Na-NMR at the end of each experiment were compared with sodium analysis of filtered kidney homogenates by flame photometry. NMR detected 107 ± 23% (n = 12, r = 0.823) of sodium detected by flame photometry; this difference was not statistically significant, indicating that $^{23}Na^+$ was 100% NMR detectable in this model.

Individual and time-averaged data for a single group (15 m*M* DMTU group, n = 6) are illustrated in Fig. 3. Fits of Eqs. [1] and [2] to the average time points of this group by nonlinear regression (DNRP53) were compared by the F test, which showed that Eq. [1] did not give an adequate description of the data for this particular group (F = 48.56 for 2, 20 degrees of freedom, $P < 0.001$). Inclusion of the additional exponential process at 45 min coincident with the addition of 15 m*M* DMTU, i.e., Eq. [2], was necessary to describe the data adequately. For both 1.5 m*M* DMTU and 15 m*M* DMSO, fitting Eq. [2] to the data

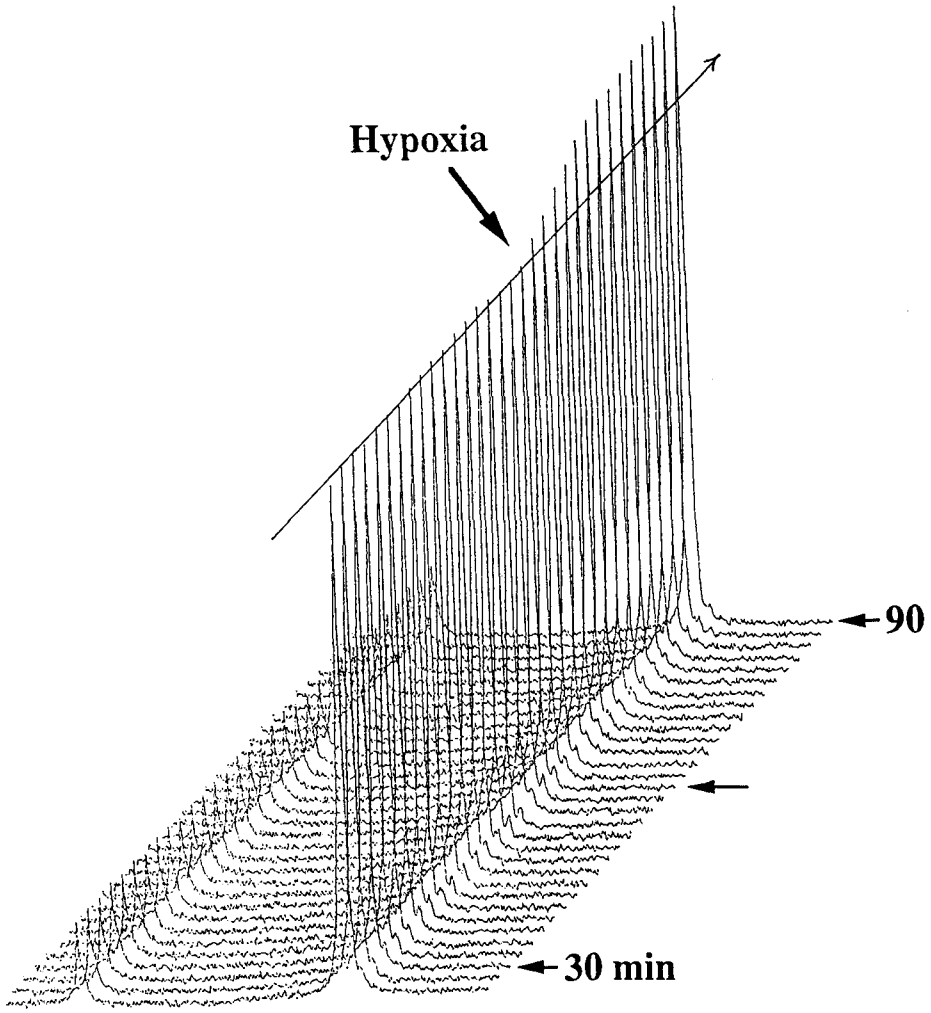

FIG. 2. Stack plot of ^{23}Na spectra from perfused rat kidney before and after the induction of hypoxia at 60 min (arrowed). Each spectrum represents 128 free induction decays averaged over 2 min. The small unmarked and unchanging peak on the left shows ^{23}Na from the reference capillary containing NaDy(PPP).

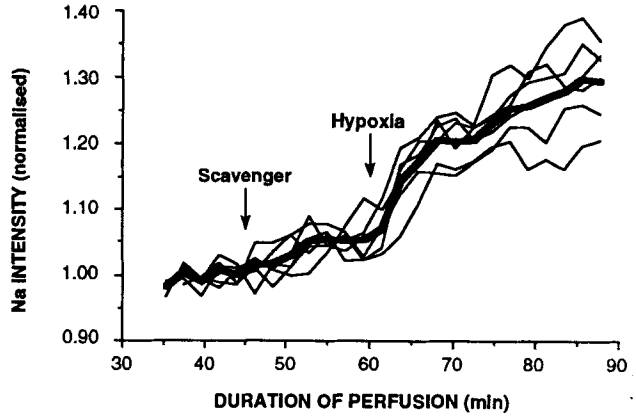

FIG. 3. Illustration of the effect of pretreatment with 15 m*M* DMTU on the hypoxia-induced increase in total renal ^{23}Na. Each continuous thin line represents a single experiment with a value of ^{23}Na normalized to the average intensity value obtained for the first five spectra. The thick line represents normalized ^{23}Na intensity for the whole group obtained by averaging intensity estimates at each 2-min time point.

gave no significant improvement ($P > 0.05$ and $P > 0.2$, respectively). Thus, the introduction of the scavengers 1.5 m*M* DMTU and 15 m*M* DMSO did not perturb ^{23}Na intensity, whereas 15 m*M* DMTU did.

The time-averaged data for all groups are shown in Fig. 4a. Statistical analysis of these data (21) showed that the rate constant and amplitude of the rise in $^{23}Na^+$ at 60 min was the same for all scavenger-treated groups. The hypoxic control was also described by the same rate constant; however, the amplitude was 49% greater ($P < 0.001$). This analysis confirmed that all three scavenger treatments had a similar effect on sodium intensity. The only difference between the effects of the scavengers themselves should thus arise from the additional discontinuity introduced at 45 min in Eq. [2] in order to fit the 15 m*M* DMTU data. If this assumption is correct, subtracting the increase introduced by this perturbation (i.e., by subtracting the value of $A_1(1-e^{-k1t})$ from each of the averaged values after 45 min) should cause the ^{23}Na intensity measurements after hypoxia in the presence of 15 m*M* DMTU to coincide with those of the other scavenger groups. The effect of analysing the data in this way is illustrated in Fig. 4b, confirming the similarity of effect DMTU and DMSO in the presence of hypoxia. Thus, the effect of pretreatment with any of the free radical scavengers tested was to attenuate the maximum increase in ^{23}Na intensity after the induction of hypoxia without affecting the rate constant for the increase. Pretreatment with 15 m*M* DMTU induced an independent increase in ^{23}Na intensity prior to the induction of hypoxia.

$^{23}Na^+$ in Nonfiltering Kidneys

No urine was detected before or after hypoxia in nonfiltering kidneys. The effect of hypoxia on the total renal ^{23}Na signal is shown in Fig. 5a. Both the final amplitude (control 0.291 ± 0.011, nonfiltering 0.50 ± 0.08) and the rate constant (control 0.110 ± 0.011 min^{-1}, nonfiltering

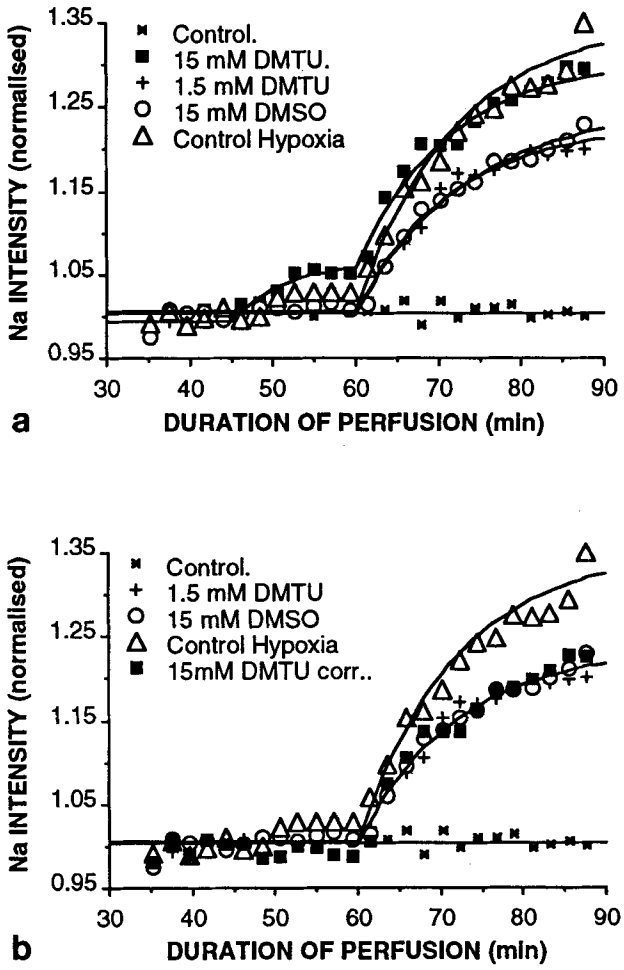

FIG. 4. Hypoxia-induced increases in total renal sodium in IPRK illustrating the effect of adding free radical scavengers prior to hypoxia. Data points indicate time-averaged results for each group. (a) Individual lines have been fitted separately to each group. The fitted lines represent Eq. [1] except for 15 m*M* DMTU group, where Eq. [2] was used (see Methods section). (b) The same data and fitted lines are shown except for the 15 m*M* DMTU group, where the projected value of the prehypoxic (45–60 min) component of Eq. [2] has been subtracted from each of the subsequent data points. See Results section for details. The single solid line passing through the scavenger treatment group represents the fit of Eq. [1] to the combined data set. The only difference from the control hypoxia group (broken line) is the greater amplitude (A) value. The final parameters obtained by nonlinear regression for these lines were: Control y_0 = 1.0034 ± 0.0011; Control Hypoxia y_0= 1.006 ± 0.003, A = 0.35 ± 0.04, k = 0.084 ± 0.012; Combined scavengers y_0= 1.006, A = 0.234 ± 0.017, k = 0.084 ± 0.012.

0.026 ± 0.006 min^{-1}) were significantly different by variance ratio analysis. Thus, urinary $^{23}Na^+$ in renal tubules and in the intrarenal pelvis appeared to make a small but significant contribution to the total renal ^{23}Na signal after the induction of hypoxia. This was quantified by calculating the fraction of the total ^{23}Na intensity in nonfiltering kidneys and subtracting this from unity (1 – NFK/Hypoxic control; Fig. 5b). This suggested that, after hypoxia, intrarenal urinary $^{23}Na^+$ contributed a variable amount to the total renal ^{23}Na signal, usually less than 10%. Consistent with this, subtracting the posthypoxic

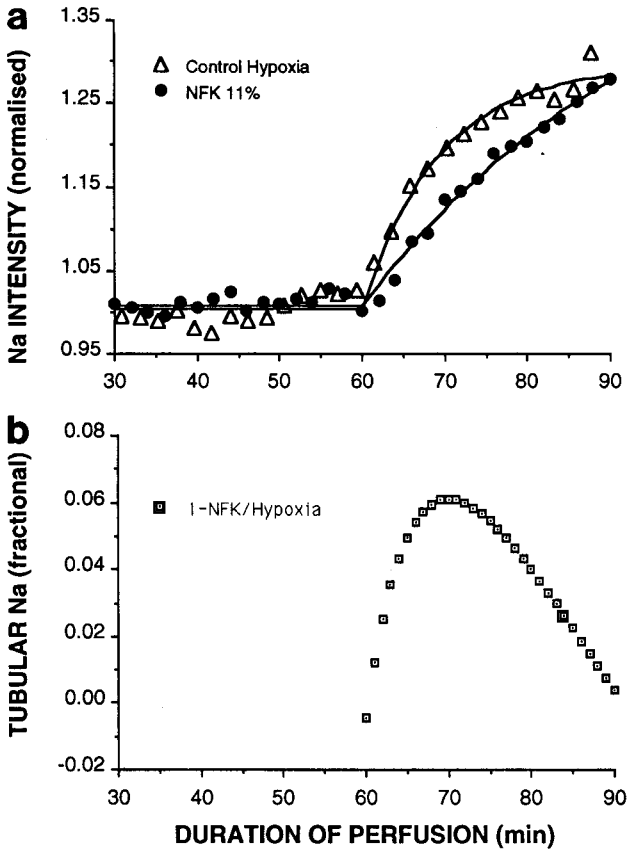

FIG. 5. Comparison of the effect of hypoxia on the accumulation of ^{23}Na in filtering and nonfiltering rat kidneys. Kidneys were rendered nonfiltering by perfusion with hyperoncotic buffer, containing 11% albumin as described in the text. (a) Parameters obtained by nonlinear regression of Eq. [1] to the filtering (control hypoxia) data are given in the legend to Fig. 4; for the the nonfiltering kidneys these were: $y_0 = 1.0076 \pm 0.0024$, $A = 0.50 \pm 0.08$, $k = 0.026 \pm 0.006$. (b) The fractional difference after hypoxia between the sodium intensities in filtering and nonfiltering kidneys shown in (a).

area under each curve in the upper panel of Fig. 5 gives a value of 33.4 – 29.8 = 3.55 units^2, that is, 10.64% of the total posthypoxic renal signal. In filtering kidneys over the same period, absolute urinary sodium exretion ($U_{Na}V$) increased from 6 ± 3 μmol/min prior to hypoxia to a maximum of 23 ± 12 μmol/min between 75 and 80 min, declining slightly thereafter (Table 1). Further evidence that the urinary contribution to the total renal ^{23}Na intensity was small is provided by the observed reduction in ^{23}Na intensity after hypoxia with both DMTU and DMSO (Fig. 4) despite the absence of a reduction in $U_{Na}V$ (Table 1).

Caution is required in any exact comparison between $U_{Na}V$ and the difference shown in Fig. 5b. The amount of detectable urinary ^{23}Na signal is changing constantly after hypoxia, presumably as both urine flow and sodium concentration are changing continuously. However, both the relative magnitudes and the different patterns of increase suggest that the bulk of the renal ^{23}Na signal did not come from the urinary space within the kidney. The vascular ^{23}Na concentration was constant in this recirculating perfusion system. Because ^{23}Na visibility was 100% in this preparation, this suggests that, although the bulk of the ^{23}Na signal prior to inducing hypoxia comes from extracellular sodium, the increase in total renal sodium after hypoxia reflects sodium increasing primarily in the intracellular and interstitial compartments.

It was not possible to compare the prehypoxic ^{23}Na signals because these data required normalization for comparison. However, the significant increase in $U_{Na}V$, which followed the addition of 15 m*M* DMTU (but not 1.5 m*M* DMTU or 15 m*M* DMSO), as well as the increase in ^{23}Na noted already, suggests that urinary $^{23}Na^+$ may have contributed to the increase in total renal $^{23}Na^+$. As indicated above, the comparison shown in Fig. 5b suggests the contribution of urinary $^{23}Na^+$ was probably small.

Renal Function

Initial kidney function from experiments conducted either inside or outside the NMR magnet was identical and the data sets for physiological function were combined. The pooled physiological data is summarized in Fig. 6. In control kidneys, 30 min of hypoxia produced a marked deterioration in function (Fig. 6) as shown by the significant decrease in GFR (50 ± 24%; $P < 0.05$ compared with the decrease in the normoxic group of 16 ± 18%), an increase in renal vascular resistance, RVR, (of 14 ± 9 %; $P < 0.01$ compared with normoxic kidneys –0.2 ± 2.6% i.e., a decrease) and a large increase in FE_{Na} (1282 ± 510%; n.s.) compared with normoxic kidneys (–18 ± 30%, a decrease).

The time course for changes in physiological function was also reproducible. FE_{Na} increased within the first

Table 1
Na Excretion ($U_{Na}V$, μmol/min) before and after Hypoxia

Time (min)	Control Hypoxia (μmol/min)	1.5 m*M* DMTU (μmol/min)	15 m*M* DMTU (μmol/min)	15 m*M* DMSO (μmol/min)
30–45	6 ± 3	2.0 ± 1.5	5.7 ± 0.5	1.2 ± 0.8
50–60	6 ± 5	1.9 ± 1.2	10.7 ± 2.8*	1.2 ± 0.7
60–65	9 ± 9	1.9 ± 1.3	13.0 ± 2.8	1.2 ± 1.8
65–70	13 ± 11	7 ± 4	25.0 ± 2.8	7.0 ± 2.2
70–75	20 ± 11	14 ± 5	40 ± 6	16 ± 6
75–80	23 ± 12	17 ± 7	28 ± 3	20 ± 5
80–85	22 ± 10	18 ± 7	39 ± 5	18 ± 6
85–90	20 ± 11	19 ± 9	31 ± 5	16 ± 5

The 45- to 50-min period has been omitted because this represents the washout phase following the addition of DMTU or DMSO.* $P < 0.01$ compared with 30- to 45-min time period.

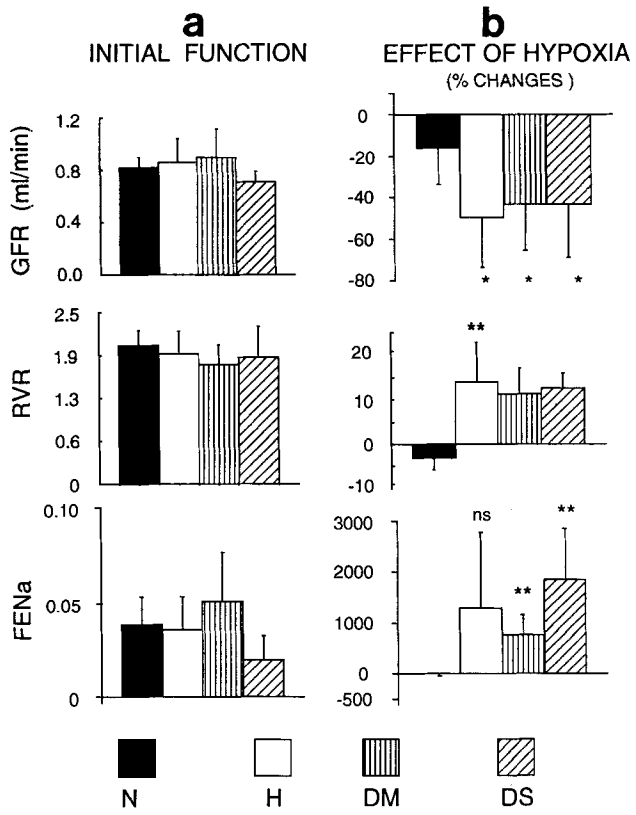

FIG. 6. Effect of hypoxia on renal function in the presence and absence of 15 m*M* of DMTU or DMSO. The results are pooled from both NMR and bench experiments and show (a) initial GFR, RVR, and FE_{Na} calculated from averaged values between 30- and 45-min baseline perfusion. (b) Change in function (as % change in initial value) comparing values averaged between 80 and 90 min with those between 50 and 60 min (immediately prior to induction of hypoxia at 60 min). Values represent mean 1 SD. Significance: $^{*}P < 0.05$; $^{**}P < 0.01$, compared with normoxic perfusion. Key: N, normoxia; H, hypoxia alone; DM, hypoxia plus DMTU (15 m*M*); DS, hypoxia plus DMSO (15 m*M*).

5-min collection after switching gas mixtures and while the PO_2 was still falling rapidly. There was a simultaneous increase in urine volume and total sodium excretion ($U_{Na}V$) increased within the first 5 min after the onset of hypoxia (Table 1). RVR began to increase within 1 to 3 min as shown by the measured decrease in perfusate flow rate (Fig. 1). A decrease in GFR was detectable beginning 10 to 15 min after the induction of hypoxia.

Addition of OFR scavengers at 45 min did not affect renal function prior to hypoxia, except for 15 m*M* DMTU, which produced an immediate and sustained increase in FE_{Na} and $U_{Na}V$ (Table 1). Changes based on $U_{Na}V$ are independent of inulin clearance and thus simpler to interpret. Comparison of $U_{Na}V$ for the 50- to 60-min with the 30- to 45-min collection period for each group showed a significant increase after 15 m*M* DMTU ($P < 0.01$) but not for any other group.

Inclusion of scavengers of OFR did not change the rate or magnitude of decline in renal function after the induction of hypoxia (Fig. 6).

Renal Biochemistry

Adenine Nucleotides. The energy charge of normoxic control kidneys was identical with that of nonperfused kidneys (Fig. 7) suggesting a normal phosphorylation potential. Compared with normoxic controls, ATP, total adenine nucleotides, and energy charge were reduced significantly in the hypoxic kidneys ($P < 0.01$ for each measurement). After 30 min of hypoxia, there was no difference between hypoxia alone and hypoxia plus DMTU or DMSO. There was a significant rise in AMP concentration in all groups exposed to hypoxia compared with the normoxic group. ADP was present in a concentration of 0.45 ± 0.17 m*M* prior to hypoxia and was unchanged by hypoxia or by pretreatment with DMTU or DMSO. The significant reduction of ATP and TAN concentrations confirms the extent of hypoxic stress induced by the experimental procedure.

Reduced and Oxidized Glutathione Levels. GSH in IPRK was 97.8 ± 1.2% (SD) of total glutathione in normoxic kidneys. The concentration of GSH was significantly decreased by hypoxia (Fig. 8a, $P < 0.02$). There was no difference between hypoxia and hypoxia plus DMTU or DMSO, suggesting that these scavengers were noncompetitive in the mechanism that decreased GSH. Levels of glutathione disulfide (GSSG) were the same in normoxic

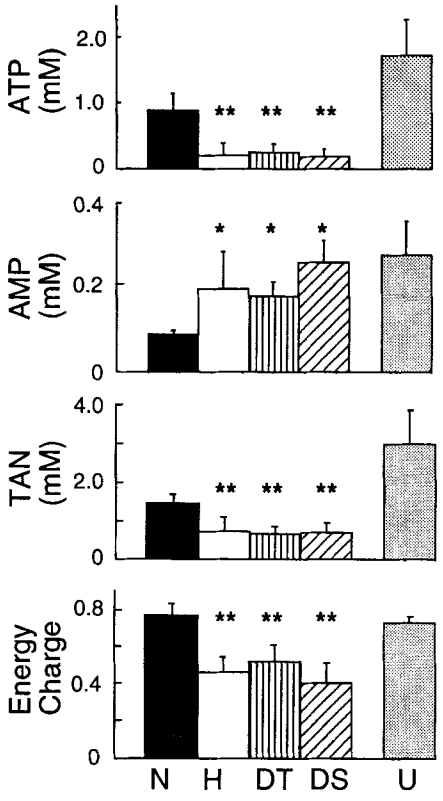

FIG. 7. Effect of hypoxia on adenine nucleotides (ATP, AMP, TAN) and energy charge in the presence and absence of 15 m*M* of DMTU or DMSO. For comparison, panel U (unperfused) illustrates nucleotide concentrations in unperfused or briefly perfused (5 min) kidneys ($n = 5$). Values represent mean 1 SD. Significance: $^{*}P < 0.05$; $^{**}P < 0.01$, compared with normoxic perfusion. Key: as for Fig. 6; U, unperfused kidney.

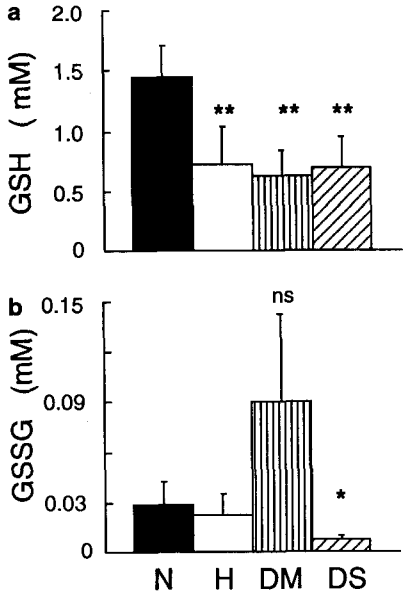

FIG. 8. Effect of hypoxia on GSH and GSSG in the presence and absence of 15 m*M* of DMTU or DMSO. Values represent mean 1 SD. Significance: $*P < 0.05$; $**P < 0.01$, compared with normoxic perfusion. Key: as for Fig 6.

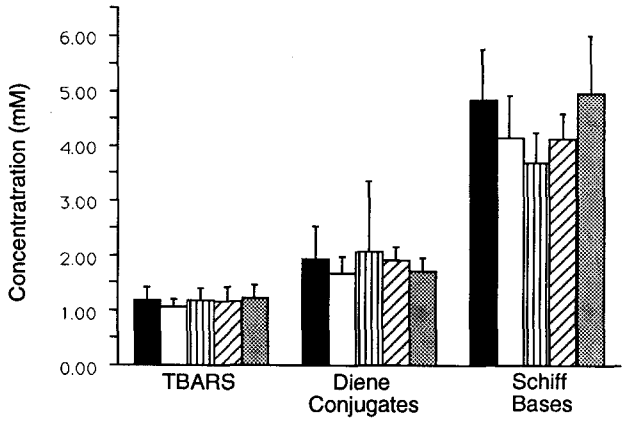

FIG. 9. Effect of hypoxia on lipid peroxide metabolites in the presence and absence of 15 m*M* of DMTU or DMSO. Units: TBARS (malondialdehyde), nmole/mg protein; Diene conjugates, A_{240}/mg protein ($\times 10^{-1}$); Schiff bases, fluorescence u/mg protein. Values represent mean ±SD. No significant differences were observed between control and treatment groups. Key: as for Fig. 7.

(0.029 ± 0.013 m*M*), hypoxia alone (0.022 ± 0.013 m*M*), and with hypoxia plus 15 m*M* DMTU (0.090 ± 0.053 m*M*) treatments (Fig. 8b). This suggests either that measurable conversion of GSH to GSSG did not occur during the hypoxic period or that GSSG did not accumulate to a significant extent in the tissue, perhaps because of washout or transport. Levels of GSSG were significantly decreased with DMSO (0.007 ± 0.003 m*M*) compared with levels in normoxic kidneys (0.029 ± 0.013 m*M*; $P < 0.03$), suggesting that GSSG levels were independently modified in some way by 15 m*M* DMSO.

Metabolites of Lipid Peroxidation. All three measured indices of lipid peroxidation were unchanged by any treatment compared with normoxic perfused kidneys and to unperfused kidneys (Fig. 9). There was therefore no measurable lipid peroxidation as a result of perfusion, or as a result of 30 min of hypoxia.

DISCUSSION

In the current study, ^{23}Na NMR was used to monitor total renal sodium in a hypoxic model of acute renal failure. In addition, the potential involvement of OFR in the injury seen in this model was assessed by examining the effects of adding OFR scavengers on the biochemical, physiological, and ^{23}Na NMR parameters of injury. Kidneys perfused without cellular elements were made hypoxic while perfusate flow rate was maintained. Kidney function was predictably impaired in filtering kidneys during the induction of hypoxia as reflected by the decrease in inulin clearance, the increase in FE_{Na} and the increase in renal vascular resistance. In addition adenine nucleotides and total energy charge were markedly diminished. With the commencement of hypoxia, there was an immediate and progressive increase in total renal $^{23}Na^+$ as measured by ^{23}Na NMR (Figs. 2–4).

The site of sodium accumulation detected by ^{23}Na NMR is uncertain. Paramagnetic shift reagents were not used to separate extra- from intracellular $^{23}Na^+$, because of their apparent nephrotoxicity (26). Because $^{23}Na^+$ was 100% detected by NMR in our preparation, as has been shown previously (7), an increase in any compartment should contribute to the total renal ^{23}Na signal. Vascular $^{23}Na^+$ remained constant in this recirculating system. Absolute urinary sodium excretion increased from 6 ± 3 to 23 ± 12 μmol/min in control kidneys after hypoxia. The difference in total renal $^{23}Na^+$ between filtering and nonfiltering kidneys (Fig. 5) suggests that urinary $^{23}Na^+$ contributed approximately 10% of the total increase in total $^{23}Na^+$ detected by NMR. However, the urinary contribution was not constant. Furthermore, because DMTU and DMSO reduced the increase in total renal $^{23}Na^+$ without reducing urinary sodium excretion after hypoxia (Table 1), it is likely that the increase in total renal $^{23}Na^+$ reflects the urinary compartment to only a very limited extent. By analogy with other studies, an increase in intracellular $^{23}Na^+$ seems certain. We have previously shown that increases in total renal $^{23}Na^+$ are inversely related to ATP and K (measured as ^{87}Rb) concentrations in this preparation (6). Electron microprobe analysis after ischemia *in vivo* (27) has shown a fivefold increase in intracellular Na^+ occurred in proximal tubular cells within 20 min. Interstitial Na^+ could also increase and presumably does, because light microscopy of kidneys fixed with glutaraldehyde after hypoxia reveals some separation of the tubules, indicating interstitial edema. However, the interstitial compartment is difficult to quantitate directly. It is reasonable to conclude that the observed increase in total renal $^{23}Na^+$ reflected predominantly intracellular and interstitial compartments.

Studies of rat kidneys subjected to ischemia *in vivo* (28) or to applied oxidant stress (11, 29, 30) as well as studies in several species of kidneys subjected to warm

ischemia and reperfusion prior to transplantation including the pig (31, 32) and the rabbit (33) suggest that OFR are involved in the loss of function associated with ischemia followed by reperfusion. Studies in perfused kidneys subjected to ischemia *in situ* prior to *in vitro* perfusion (34) and in isolated proximal tubules (35) also support a role for OFR in producing renal tubular injury in acute renal failure. OFR have also been implicated in the etiology of various forms of glomerulonephritis (36) and in damage induced by some toxins, for example glycerol (37).

Perfusion of the IPRK with free radical-generating systems, such as glucose plus glucose oxidase (34) and xanthine plus xanthine oxidase (29), has resulted in changes in renal function. In a study of glomerular permeability in the IPRK, Tay *et al.* (38) suggested that the progressive increase in proteinuria observed in their preparation in the absence of deliberate hypoxia was due to OFR generated from increased xanthine oxidase as the kidney became hypoxic during prolonged perfusion (for more than 90 min).

Despite the evidence provided by these studies, the extent of the role of OFR in acute renal failure has remained controversial. In studies using partial aortic ligation or models of hemorrhagic shock or cardiopulmonary shock to reduce kidney blood flow (39–41) and in the renal artery clamp model of complete ischemia (42, 43) Zager and colleagues were unable to demonstrate any action of OFR, based on the absence of protective effects of scavengers and on the absence of increased lipid peroxidation. Although Galat *et al.* (29) found that the renal dysfunction caused by hypoxia paralleled that caused by free radical generation during perfusion with xanthine plus xanthine oxidase, they could not confirm that the damage in ARF was due to OFR (44).

Rather than focus on reperfusion injury, we attempted to discern whether OFR could contribute to the induction phase of hypoxic injury, because a source of oxygen, albeit in greatly reduced amount, remains present because flow continues in this perfused kidney preparation. Injury of this type would also be relevant to other organs subjected to reduced but continued blood flow, i.e., "low flow ischemia." In the case of the kidney, intrarenal diffusional shunting of oxygen keeps both cortical and medullary oxygen tensions low despite high renal blood flow and low arteriovenous oxygen extraction (e.g., 8, 10, 45). This predisposes metabolically active cells dependent on aerobic metabolism to severe hypoxia and perhaps generation of OFR with only a modest reduction of blood flow. Low flow ischemia could provide the basis for a hypoxic stimulus to the release of proteolytic enzymes or uptake of calcium to induce conversion of xanthine dehydrogenase to xanthine oxidase (46) and provide a potent free radical generating system.

Physiological and biochemical parameters, including lipid peroxide metabolites, reduced and oxidized forms of glutathione and adenine nucleotides and total energy charge, were not modified by pretreatment with OFR scavengers reported to prevent reperfusion injury after ischemia in a similarly perfused model of rat kidney (34). However, ^{23}Na NMR detected a highly significant reduction in the amplitude of the hypoxia-induced increase in total renal sodium when these same free radical scavengers were present (Fig. 4b). Because of the absence of positive indications of lipid peroxidation and oxidation of GSH, there are two plausible explanations for why these scavengers prevented the increase in total renal $^{23}Na^+$ without demonstrating protection against other features expected to accompany OFR injury.

First, it is possible that the scavengers, DMTU and DMSO, are protective by a mechanism that is unrelated to the binding of free radicals; this alternative mechanism would appear to be equally effective for both scavengers. An obvious mechanism would be the promotion of an osmotic diuresis, which could be protective by preventing tubular obstruction and by reducing cell swelling and interstitial edema. The latter effect would presumably also reduce the total renal ^{23}Na signal. Surprisingly, an identical reduction in total renal $^{23}Na^+$ followed both 15 m*M* DMTU and the 10-fold lower dose of 1.5 m*M* DMTU (Fig. 4). However, because posthypoxic sodium excretion was not significantly different between these two concentrations (Table 1), this mechanism remains a strong possibility. Alternatively, both scavengers could be protective by some intracellular biochemical or biophysical action. For example, at high concentrations (~1.2 *M*), DMSO is known to inhibit Na^+/K^+–ATPase *in vitro* (47). However, this should exacerbate the rise in cellular $^{23}Na^+$. DMSO permeates membranes rapidly and, by affecting hydrophilic bonding, interrupts the association of protein subunits and the interaction of enzymes with their substrates (48). However, extremely high concentrations of DMSO of 20–40% (2.5–5.0 *M*) are required, and it is not known whether DMTU has these properties. How such properties of DMSO and DMTU could contribute to protect the kidney remains speculative.

Second, because of the apparent protection offered by the scavengers, it is possible that OFR are reponsible for some of the increase in renal $^{23}Na^+$ measured by NMR and that it is this increase that is ameliorated by the scavengers. This would be consistent with the observation that activity of renal Na^+/K^+-ATPase is reduced as a result of OFR activity, during reperfusion after ischemia (49), perhaps through direct oxidation of the ATPase enzyme or oxidation of membrane lipids adjacent to the enzyme. Assuming that the production of OFR in this model is small, lipid peroxide metabolites could remain below the level of detection of the assays used, especially given the short time course of injury in this model. This could be exacerbated if the production or effect of the OFR were limited to small regions. In unit time the production of OFR is likely to be less in this model than *in vivo*, both because of the lower available oxygen and because reperfusion after ischemia will produce a burst of free radicals. A further reason may be the absence of neutrophils from our perfusion system. In some studies in the perfused rat kidney, neutrophils have been reported to exascerbate injury by releasing OFR and elastase (50, 51), although this effect has not been confirmed *in vivo* (52). The time course for lipid peroxidation and the numbers of OFR required to perturb renal cell $^{23}Na^+$

balance in the IPRK is not known. Any suggestion that OFR may be producing hypoxic cellular injury needs to be interpreted cautiously in the absence of evidence of lipid peroxidation. More direct estimates of OFR generation would resolve such questions.

Some comment should be made regarding the apparent absence of protection by the scavengers against glutathione depletion, loss of function and against changes in adenine nucleotide levels. Reduced glutathione (GSH) is capable of preventing or limiting membrane lipid peroxidation and maintaining intracellular protein sulfhydryl groups. Decreases in tissue GSH similar to those found in our experiments have been reported after renal ischemia *in vivo* (53), in the IPRK (11), and also after oxidant damage in the IPRK (34). These reductions have been interpreted to indicate oxidative damage. Our experiments showed no demonstrable increase in GSSG, and OFR scavengers did not prevent the loss of GSH. This suggests that oxidant damage plays only a small role in the large loss of GSH in the IPRK after hypoxia. Although the precise fate of GSH is unknown, cellular glutathione is maintained through a dynamic equilibrium between glutathione biosynthesis and consumption or release (54). The reduction of GSH in the hypoxic IPRK may result from cell damage allowing leakage of GSH from cells (53) and subsequent catabolism by γ-glutamyl transpeptidase and dipeptidase associated with the basolateral and luminal cell membranes (55, 56). Because mitochondrial ATP synthesis is inhibited during hypoxia, resynthesis of GSH from glycine, cysteine, and γ-glutamate is impaired. Even without leakage of GSH, the normal rapid turnover of GSH, with a reported half-life of 29 min in rat kidney (57), will lead to rapid depletion of GSH when resynthesis is inhibited in the hypoxic kidney. It is possible that any GSSG produced during hypoxic stress is catabolized in the same manner as GSH (55). Conversion of GSH to GSSG would then be masked. Reliance on a single measurement of GSH and GSSG, at the end of 30 min of hypoxia, may also be misleading as an index of oxidant damage occurring in the early phase of hypoxia.

The absence of a protective effect of the scavenger on Na^+ excretion and inulin clearance in our model despite limiting the increase in the total $^{23}Na^+$ could result from two factors. First, 15 m*M* DMTU provoked an increase in both total renal $^{23}Na^+$ and in urinary Na^+ excretion (Table 1). A small protective reduction by DMTU of the increase in FE_{Na} after hypoxia could be obscured by the increase in FE_{Na} provoked by the scavenger itself as a result of an induced osmotic diuresis. A similar explanation could account for the lack of protection against the decrease in inulin clearance. In this model hypoxia provokes a transient increase in inulin clearance, which is presumed to result from washout of more concentrated urine as a result of the hypoxia-induced natruriesis. Thus, changes in physiological function assessed from urinary parameters must be interpreted with caution, particularly near the time of the hypoxic perturbation. The same caveat does not hold for the ^{23}Na NMR data. Changes in $^{23}Na^+$ reflect changes occurring in the kidney at the time of spectral acquisition, regardless of the compartment involved, rather than a late downstream manifestation of tubular or glomerular function or integrity. Furthermore, the variance ratio method for evaluating the significance of these changes utilizes the whole post-hypoxic data set of ^{23}Na values, allowing small but consistent changes to be detected as significant.

Concentrations of adenine nucleotide metabolites, measured after 30 min of hypoxic perfusion, confirm a severe degree of tissue hypoxia. The rate of decline in ATP and EC in our model was not measured but is known to be rapid (2, 6) and is reflected here in the rapid changes in FE_{Na} and in total renal $^{23}Na^+$. Accumulation of intracellular $^{23}Na^+$ would be accompanied by an increase in cell water and in cell volume. Cell swelling could produce a rise in tissue pressure that would constrict arteries and renal tubules (58) producing the observed decreases in renal perfusate flow. However, the time course of this reduction in flow is very brief (5–10 min, Fig. 1), while $^{23}Na^+$ continues to rise exponentially. Vasoconstriction is a likely alternative explanation for the observed decrease in perfusate flow. On balance, the pattern of kidney failure in our model may be explained through the effects of a decrease in ATP, TAN, and EC after hypoxia. The immediate increase in urine sodium and in total renal $^{23}Na^+$ suggests that the activity of membrane Na^+/K^+-ATPase is impaired within minutes of the beginning of hypoxia. A component of this injury may be mediated by OFR because it is reduced by the presence of scavengers of OFR (Figs. 4a and 4b), but this suggestion awaits confirmation.

In summary, the predictable increase in total renal sodium detected by ^{23}Na NMR demonstrates the usefulness of this technique in revealing acute disturbances within the intact kidney. Total renal $^{23}Na^+$ was sensitive to hypoxia, and changes were detected by ^{23}Na-NMR before changes in other parameters of renal function. OFR scavengers prevented a significant component of the hypoxia-induced increase in renal sodium without modifying markers of lipid peroxidation. Although the mechanism is uncertain, the findings suggest a role for DMTU and DMSO in protection against renal cell injury during the induction of hypoxia.

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