An anatomically unbiased approach for analysis of renal BOLD magnetic resonance images

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Submitted 28 February 2013; accepted in final form 8 July 2013

RENAL TISSUE HYPOXIA IS IMPLICATED in the pathogenesis of chronic kidney disease (CKD), with low partial pressure of oxygen (PO2) causing activation of a profibrotic cascade (27). Even under physiological conditions, the PO2 within the kidney is thought to have a marked cortico-medullary gradient, with the medulla being poorly oxygenated (9). Cells of the medulla have a higher anaerobic capacity than those of the cortex and paracrine signaling pathways also provide some resistance against hypoxia (25). Nevertheless, the medulla is susceptible to hypoxic injury and chronic hypoperfusion, which can initiate a vicious cycle of microvasculature injury, inflammation, and fibrosis (21).

A causal link between defects in renal oxygenation and renal disease is supported by immunohistochemical detection of pimonidazole adducts (24), formed when PO2 is <10 mmHg. This method is, however, insensitive and nonquantitative (30). O2-sensitive microelectrodes offer a quantitative and sensitive approach (10), but measurements made at the electrode tip cannot give global insights into the distribution of oxygen. Moreover, being invasive, microelectrodes are not usually amenable to longitudinal studies of renal function.

Blood oxygen level-dependent magnetic resonance imaging (BOLD MRI) is emerging as a technique through which to assess renal oxygen bioavailability (20). BOLD imaging exploits the paramagnetic properties of deoxyhemoglobin, generating images based on the dephasing of spin relaxation rate from an applied electromagnetic field pulse. This relaxation rate (R2* = 1/T2*) is proportional to the level of deoxyhemoglobin, and the R2* reflects the oxygenation status of red blood cells. This can be associated with tissue PO2 and indeed much of the physiological utility of BOLD MRI rests on the assumption that tissue PO2 is in responsive equilibrium with red blood cell PO2. In pigs the spatial gradients of oxygenation observed by BOLD MRI are consistent with those measured in the contralateral kidney by O2 microelectrodes (28).

Although the use of BOLD MRI to rapidly and noninvasively define renal hypoxia is clinically attractive (14), interpretation of images is often challenging. Most postacquisition analyses rely on manual selection of small regions of interest (ROI) to generate anatomically informative R2* maps. However, kidneys are subject to respiratory and cardiovascular motions that might be difficult to gate against, particularly in experimental models. Unless image registration is employed, time-series data within an individual ROI are unlikely to be acquired from exactly corresponding anatomical regions. The selection of small segments that are well delineated within the cortex and medulla has been advocated (12), but this approach will discard from the analysis biological information contained within the data set. Moreover, this approach is subjective and risks a selection bias towards areas at the extremes of signal intensity (14).

In biomedical research, the use of algorithms that cluster individual data points based on concepts of quantitative ‘nearness’ or ‘similarity’ are more commonly associated with analysis of gene expression data sets (6) but are broadly applicable to large data sets, such as those generated through BOLD MRI. The present study applied k-means clustering as an anatomically unbiased approach to BOLD MRI analysis. The central tenet of this approach is that voxels clustered on
quantitative nearness of the R2* signal share a commonality of biological process. Importantly, quantitative similarity does not necessarily equate to close anatomical proximity of voxels or compartmentalization within a given region of the kidney.

We employed two complementary strategies to affect R2* in an acute and chronic time frame. Acutely, we manipulated pharmacologically nitric oxide bioavailability. The maneuvers were designed to change whole kidney blood flow, either rapidly and reversibly with acetylcholine, or with sustained effect through nitric oxide synthesis inhibition, without altering autoregulatory capacity (2). To cause a chronic change in R2* we infused angiotensin II: over this time frame we anticipated no major change in renal vascular resistance but an increase in renal tubular sodium reabsorption (1, 32, 38). These complementary experiments provided the means to dissect the physiologically complex R2* signal, resolving the influence of nitric oxide bioavailability and renal blood flow.

METHODS

All experiments were performed under a UK Home Office license following ethical approval by The University of Edinburgh. Male F344/IcoCrl rats (Charles River), aged 12–16 wk, were given free access to water and commercial rat chow (0.3% sodium by weight; Special Diet Services) and housed under controlled conditions of temperature (24 ± 1°C), humidity (50 ± 10%), and light/dark (light 7 AM–7 PM) during the experiments.

BOLD MRI. Measurements were performed using a 7 Tesla preclinical MRI scanner (Agilent Technologies). Rats (n = 6) were anesthetized with 1.5-2% isoflurane in oxygen-enriched air (0.5 l/min air and 0.5 l/min oxygen). Rectal temperature was maintained at (37°C). Respiration and ECG were monitored for stability throughout the scanning protocol. A birdcage volume coil (72-mm diameter) and a 4-channel phased array surface coil (Rapid Biomedical) were used for radio frequency transmission and signal reception, respectively.

Image acquisition used a multiple echo gradient-recalled BOLD MRI pulse sequence of ten images weighted in T2*; TE = 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 ms; TR = 100 ms and flip angle of 30° at an 83 kHz bandwidth. An axial slice through the centre of the right kidney was selected with 50 × 40 mm field of view containing a 192 × 128 acquisition matrix (in-plane resolution = 0.26 × 0.31 mm). A single axial slice, aligned parallel with the renal artery identified by rapid scout scanning (fast gradient echo, 3 slices in coronal orientation), ensured slice position encompassed the most representative section of the kidney regions. Slice thickness was 2 mm with 14 signal averages. Temporal resolution was 3 min for each BOLD scan.

Scans obtained under control conditions (days 6 and 4) were compared statistically and then combined into one group. On day 0, osmotic minipumps (model 2002; Alzet, Charles River, UK), adapted for MRI by replacement of the stainless steel flow moderator with a polyetheretherketone equivalent (part no. 2496, PEEK, Charles River, UK), containing angiotensin II (60 ng/min) were surgically implanted under isofluorane anaesthetic. Rats were scanned again after 3 days of angiotensin II infusion.

Image selection and registration. Motion correction was performed by complimentary measures to ensure voxels in different frames overlapped; global registration (consistent over entire image), intensity-based registration (using grey-level image values), and rigid registration (only allowing translation and rotation) were used on each rat and on each day separately.

Initial outlier detection was assessed by a Hampel identifier, used to detect scans having an intensity profile significantly different from the median (X0.5) or outside the 90th percentile (X0.9): scan imprecision largely reflected significant motion artefact and these outliers were rejected.

Rigid registration was performed on the remaining images using an exhaustive search. The third baseline scan was selected and all other scans in the sequence were translated and rotated until the mutual information (gradient2) was maximized. This improved stability across sequences: 96% of scans required less than one voxel transla-

![Fig. 1. Analysis pipeline of blood oxygen level-dependent magnetic resonance imaging (BOLD MRI) data. Following registration of the images, a kidney quadrant was manually selected. The k-means clustering of the data for time series analysis was then performed automatically, using the MATLAB code provided (https://github.com/andrewzm/BOLD_Kidney) and two compartments identified with statistically distinct mean R2* time series. For clarity blue arrows indicate manual steps and red arrows automation. ROI, regions of interest; BL, baseline; L-NAME, N\textsuperscript*-nitro-l-arginine methyl ester hydrochloride.](https://github.com/andrewzm/BOLD_Kidney)
tional correlation or less than 1° rotation, indicating that rigid registration was sufficient for the present dataset.

$k$-Means clustering analysis. Automated image segmentation was performed using a $k$-means clustering algorithm (with $k$, the number of clusters). The $k$-means clustering identifies $k$-clusters within a multi-dimensional space using Euclidean distance (for details, Ref. 3, chapter 9.1). Given a set of points, the target of the algorithm is to find $k$ cluster-centers such that the sum of square distances of each point to its closest cluster centre is a minimum. The (local) minimum is searched for in an iterative manner, the two steps of which are 1) the association of the points with their closest cluster centers, and 2) the updating of the cluster centers such that the sum of square distances to the associated points is minimized. The final cluster configuration can be dependent on the initial cluster configuration. To validate our approach, the appropriate number of initial conditions was therefore determined to establish the lowest number insensitive to the starting conditions. The present dataset found 10 random initial conditions to fit this condition, thus for each scan set we ran the algorithm and saved the final configuration as that with the lowest sum of intracluster distances.

To select the number of clusters ($k$), we performed pilot analysis using $k = 1, 2, 3, \ldots 13$ to identify the value of $k$ such that the increase in explained variance of $k + 1$ clusters was $<50\%$ of the additional variance explained by the $k$th cluster. With the use of this approach $k = 2$ was chosen, as the addition of a third cluster did not contribute sufficiently to an increase in explained variance. Each time series required $\sim 50$ ms to converge run in MATLAB on a standard desktop computer.

Pharmacological protocol. The effect of acetylcholine (5 $\mu$g/kg; Sigma-Aldrich) on the R2* signal was determined before and following administration of N$^\text{e}$-nitro-L-arginine methyl ester hydrochloride (l-NAME; 10 mg/kg; Sigma-Aldrich). Both compounds were administered via the tail vein in a volume of $\sim 0.15$ ml. Immediately following injection, the catheter was flushed through with a volume of saline equal to the catheter volume. The potential effects of water loading must be considered (15). However, in the present study, the volume of saline injected for each animal over $\sim 1$ h did not exceed 0.95 ml in total.

Measurement of blood pressure and renal blood flow. The effects of acetylcholine and the nitric oxide synthase inhibitor l-NAME on blood pressure and renal blood flow were measured in a parallel study using the protocol described above. Renal blood flow data are normalized to total kidney weight. Control rats ($n = 4$) and rats receiving angiotensin II (60 ng/min; $n = 5$) were anesthetized (120 mg/kg ip thiobutabarbital). The right jugular vein was cannulated for infusion of 0.9% NaCl containing 1% bovine serum albumin. Rats were infused at 100 $\mu$L/min until a total volume of 1.25 ml/100 g body wt was reached and then 30 $\mu$L/min maintenance rate. The left femoral artery was cannulated for blood pressure measurement (MLT844; AD Instruments); a tracheotomy was performed to maintain a clear airway. A midline laparotomy was performed, and a Doppler transit time probe (MA1PRB; Transonic) was placed around the left renal artery. Core body temperature was servo-maintained at 37°C.

Statistics. Data are presented as means $\pm$ SE. Statistical analysis was performed by repeated-measures ANOVA, unless otherwise stated. Post hoc significance testing was performed by the Bonferroni method unless otherwise stated.

RESULTS

Postacquisition generation of R2* maps. The clustering approach was used to generate R2* maps within a kidney quadrant in each of six control rats, using the postacquisition pipeline shown in Fig. 1. Two compartments of distinct mean R2* intensities were created (cluster 1 $= 70.96 \pm 1.48$; cluster 2 $= 79.00 \pm 1.50$; means $\pm$ SE; $n = 18$ scans in 6 rats; $P < 0.01$). Each rat underwent three consecutive baseline scans on control days 6 and 4 and following 3 days of angiotensin II infusion. In control rats, baseline scans repeated sequentially on separate days or on different rats did not vary significantly for either cluster 1 (Fig. 2A) or cluster 2 (Fig. 2B). Consecutive baseline scan reproducibility was also observed following chronic angiotensin II infusion. Baseline scans were therefore combined for both groups.

In control rats, the cluster having a low R2* mean ("higher"
oxygenation) had anatomical coordinates that largely overlaid regions of the renal cortex and the higher R2* mean ("lower"
oxygenation) lay within regions of the renal medulla. Thus, in the control setting, regions of quantitative oxygen homogeneity were also spatially proximate, mapping to distinct anatomical regions of the kidney. The spatial oxygenation gradient established here is consistent with that reported using ROI selection and with direct PO2 measurement by microelectrodes.

Nitric oxide bioavailability and renal R2*. Systemic injection of acetylcholine significantly attenuated the R2* signal ($P < 0.01$, ANOVA) in both clustered compartments (Fig. 3A), suggesting an increase in Po2 throughout the kidney. This effect was transient; reaching its nadir in the scan performed 6 min postinjection. In a parallel study, acetylcholine initially caused a rapid fall in mean arterial pressure and renal blood flow. This effect was short lived (<1 min), and during the BOLD MRI scan protocol, renal blood flow was $\sim 30\%$ higher than at baseline (Fig. 3B). Importantly, the average renal blood

![Fig. 2. Reproducibility of baseline BOLD MRI data. Rats were scanned in triplet (baseline 1, 2, and 3) and in 2 cohorts on day 6 (solid grey lines) or day 4 (broken grey lines). No significant difference between any of these measurements was observed (black line); thus baseline scans were assimilated into one baseline value.](image-url)
flow for the entire period remained unchanged (Table 1) suggesting intact autoregulation.

Administration of L-NAME caused a slowly progressive increase in signal intensity in the high R2* compartment (P ≤ 0.01, ANOVA) but was without effect in the low R2* compartment (Fig. 3C). This is consistent with previous observations showing no effect of nitric oxide synthesis inhibition on R2* intensity in cortical ROI (29). L-NAME also caused a reduction in renal blood flow over this time course (Fig. 3D).

The effects of L-NAME on both R2* and renal vascular resistance reached steady state after ~20 min in general agreement with the reported inhibitory effect on nitric oxide bioavailability (16). Acetylcholine was again injected. The effect on blood pressure and renal blood flow persisted, but the attenuation of R2* signal by acetylcholine was no longer observed (data not shown).

**Effect of chronic angiotensin II infusion.** Angiotensin II was infused by osmotic minipump over a 3-day period and BOLD-

Table 1. Mean arterial blood pressure and left renal artery blood flow in control rats or rats receiving a chronic infusion of angiotensin II

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>ACh1</th>
<th>L-NAME</th>
<th>ACh2</th>
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<tbody>
<tr>
<td>BP, mmHg</td>
<td>89 ± 2</td>
<td>89 ± 1</td>
<td>114 ± 4*</td>
<td>113 ± 4</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>90 ± 3</td>
<td>91 ± 3</td>
<td>110 ± 2*</td>
<td>109 ± 2</td>
</tr>
<tr>
<td>RBF, ml·min⁻¹·g kidney wt⁻¹</td>
<td>2.70 ± 0.3</td>
<td>3.04 ± 0.4</td>
<td>1.73 ± 0.2*</td>
<td>1.74 ± 0.2</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
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<tr>
<td>Angiotensin II</td>
<td>3.19 ± 0.4</td>
<td>3.41 ± 0.5</td>
<td>1.63 ± 0.2*</td>
<td>1.60 ± 0.2</td>
</tr>
</tbody>
</table>

Mean arterial blood pressure (BP) and left renal artery blood flow (RBF) in control rats (n = 4) or rats receiving a chronic infusion of angiotensin II (60 ng/min) for 3 days (n = 5). Data are means ± SE and taken during steady state at baseline and during injection of acetylcholine (ACh), either before or after administration of N⁶-nitro-l-arginine methyl ester hydrochloride (l-NAME). *P < 0.05, baseline vs. l-NAME.
MRI scans were once again obtained. Blood pressure and renal blood flow were not significantly affected by this infusion (Table 1). The k-means clustering approach resolved the data into distinct compartments; indeed, convergence is assured by this algorithm, but the magnitude of the difference in intensity between the two compartments was much reduced (cluster 1 = 71.30 ± 2.00; cluster 2 = 72.48 ± 1.27; mean ± SE; NS). Critically, baseline means of the two clustered compartments were less distinct during baseline and no longer mapped to discrete anatomical regions of the kidney (see Fig. 5 for example images). This suggests that a short exposure to angiotensin II disrupts the oxygenation gradient through the kidney, creating areas of high and low PO2 in both cortex and medulla. As stated previously, the R2* signal actually reflects oxygenation of red blood cells and thus altered patterns of renal perfusion may also account for the disrupted gradient. Following angiotensin II infusion, the R2* was no longer significantly affected by administration of either acetylcholine or L-NAME (Fig. 4A). Nevertheless, acetylcholine still induced a transient reduction in renal blood flow and blood pressure (Fig. 4, B and C) and L-NAME significantly increased renal vascular resistance.

**DISCUSSION**

BOLD MRI is an attractive tool for clinical research since it does not require exogenous contrast agent and has a rapid acquisition time (14). BOLD MRI is increasingly used to quantify renal “oxygenation” in a variety of disease settings, and studies typically focus on the medulla, which is vulnerable to hypoxic insult. Hypoxia of the cortex is also evident in severe renovascular disease (13) and in diabetic and nondiabetic CKD (19). A global map of renal R2* could therefore be a valuable diagnostic/prognostic tool for ischemic renal disease. However, the relationship between R2* and tissue hypoxia is complex (20). R2* reports oxygenation of the red blood cells.
cells, and the signal reflecting local perfusion is also influenced by pH and hydration status. In CKD, BOLD MRI studies have produced conflicting data (22, 37). This partially reflects the complexity of CKD; the future diagnostic utility of renal BOLD MRI is nevertheless contentious (17).

One major challenge for the field is standardization of protocols, particularly for postprocessing, that would facilitate meaningful cross-comparison of data sets. Most studies quantify renal oxygenation by measuring R2* signal intensity in small manually selected ROI. A strength of this approach is that the generation of time-series data are based firmly on anatomical knowledge of renal structure. Reliable placement can be difficult, however, and averaging across several ROI can mask the heterogeneity of oxygenation. Importantly, the ROI approach discards from analysis much of the biological information contained within an image and the power of BOLD MRI to assess renal oxygenation on a global scale is often underexploited.

Data-led segmentation of datasets has previously been used for analysis of brain (4) and kidney (7, 39). MRI and our work complements the recent compartmentalization approach to analysis of Ebrahimi et al. (7). Our method has two main differences. First, our cluster is defined from time series since voxel variation in time is informative and more easily controlled than maps defined in a separate computed tomography scanner where renal orientation might be difficult to replicate. Second, we were not able to resolve distinct and separate cortical and medullary R2* distributions and therefore assume no anatomically defined distribution functions. We have incorporated this approach, developing a semi-automated postacquisition pipeline for analysis of BOLD MRI images: k-means (where k = 2) clustering was used to assign individual voxels into one of two statistically distinct compartments. The advantages of the method are that it: 1) does not require user-led selection of small anatomical ROI but rather a gross quadrant of the whole kidney to be segregated into two compartments in a user-independent manner; 2) obviates the need for voxel-tracking through a time-series stack; and 3) is anatomically unbiased, identifying on a global scale clusters of O2 homogeneity for each patient or experimental subject. The analysis pipeline is shown in Fig. 1 and our MATLAB code is available freely (https://github.com/andrewzm/BOLD_Kidney).

In control rats, the clustering analysis delineated compartments that largely mapped to discrete anatomical regions of the kidney (Fig. 5). The low R2* cluster was located predominantly in the cortex, and the high R2* cluster localized primarily to the outer medulla. Under control conditions the clustering approach supports the notion of a cortico-medullary PO2 gradient and perhaps offers little interpretive gain over ROI selection. However, the clustering analysis indicated a dissipation of the cortex-to-medulla renal PO2 gradient following angiotensin II infusion (Fig. 5). This global map of oxygenation captures nuances of regional gradients.

This has clinical relevance: a recent study, averaging R2* across multiple ROI, demonstrated cortical hypoxia in a small number of CKD patients (19). The effect size was small and the variation large, suggesting that constraints of statistical power will make comparisons of absolute R2* across patients and between studies difficult (22, 37). Qualitatively, Manno- cath et al. (19) noted that the R2* signal was more heterogeneous and liable to rapid decay in CKD patients than in controls. We suggest that this global disruption of a spatially constrained PO2 gradient may be a hallmark of the defects in renal oxygenation associated with renal injury.

Acute administration of angiotensin II causes a rapid increase in R2* in healthy subjects, attributed to a fall in renal perfusion (31). Conversely, acute blockade of AT1 receptors increases PO2 in the renal cortex of CKD patients (19). An increase in cortical oxygenation following AT1 receptor blockade has also been observed in normal (26) and hypertensive rats (34), effects attributed to improved blood flow and efficiency of O2 usage respectively. In our study, a 3-day infusion of angiotensin II did not increase blood pressure, consistent with previous data (5), and gross renal blood flow was unchanged. Tubular sodium reabsorption is increased within this timeframe (1, 38), and we found evidence for disruption of the regional homogeneity of PO2. This may reflect a local mismatch of delivery/consumption or a reduction in the efficiency of O2 utilization, as reported in the angiotensin II-dependent Goldblatt model (36).

Most MRI scanners are not calibrated directly for PO2. The absolute R2* value is often therefore less informative for cross-comparison than is the dynamic response to maneuvers affecting perfusion or sodium transport. In this study, injection
of acetylcholine suppressed the R2* signal throughout the kidney. This was probably not dependent on whole kidney perfusion, there being temporal separation between the reduction in blood flow and the reduction in R2*. The attenuation of R2* was dependent on NO generation, being inhibited by L-NAME, and had a delayed onset, being evident only in the second scan postinjection. A previous study also reported no immediate effect of NO on R2* intensity (31).

We recognize that NO reacts irreversibly with both oxy- and deoxygenated moieties (8). These reactions are rapid, and hemoglobin and deoxyhemoglobin levels should be equivalently affected over each 3-min BOLD scan. We therefore ascribe the reduced R2* to an NO-dependent increase in PO2 throughout the kidney, most probably reflecting inhibition of tubular sodium transport (11). The effect of acetylcholine was lost following chronic angiotensin II infusion. Prolonged exposure to angiotensin II causes oxidative stress in rats (18), and our data plausibly reflect accumulation of superoxide anion leading to NO deficiency and defects in renal oxygenation (35).

There are two important limitations to our study. First, it was not possible to measure renal blood flow and BOLD signal simultaneously. The invasive surgery required for Doppler measurements of renal arterial blood flow also mean that measurements were not made in the same animals: we chose instead to obtain repeated R2* measurements in a longitudinal study. Second, the BOLD MRI and renal blood flow data were obtained under differing anesthetic regimens. Maintenance of anesthesia within the MRI scanner required ECG monitoring under gas anesthesia (isoflurane) while stability of renal perfusion following the invasive abdominal surgery is best obtained with a long-lasting barbiturate (33). Renal hemodynamics may be differentially affected by the anesthetics, but it is unlikely that this accounts for the temporally distinct dynamic response to acetylcholine of the R2* signal and blood flow.

In summary, we have developed an anatomically unbiased method for the assessment of renal function by BOLD MRI, employing signal analysis to remove errors inherent in manual ROI selection. These data indicate that protocols assessing the dynamic response of R2* to acetylcholine can provide information relating to renal NO bioavailability and offer temporal insight into renal oxygenation homeostasis.

ACKNOWLEDGMENTS

We thank Paul O’Connor and Chris Kenyon for critical reading of the manuscript. This work has been published in abstract form at The Experimental Biology 2013 Meeting.

GRANTS

This work was funded through the University of Edinburgh Preclinical Imaging Initiative (to M. A. Bailey, J. W. Dear, J. J. Mullins, and D. J. Webb), an Engineering and Physical Sciences Research Council Shaping Capability Award (to G. Sanguinetti and M. A. Bailey), and a British Heart Foundation Imaging Initiative (to M. A. Bailey, J. W. Dear, J. J. Mullins, and D. J. Webb), respectively.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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