Effects of Renal Perfusion Pressure on Renal Medullary Hydrogen Peroxide and Nitric Oxide Production

Chunhua Jin, Chunyan Hu, Aaron Polichnowski, Takefumi Mori, Meredith Skelton, Sadayoshi Ito, Allen W. Cowley, Jr

Abstract—Studies were designed to determine the effects of increases of renal perfusion pressure on the production of hydrogen peroxide (H$_2$O$_2$) and NO$_2^-$+NO$_3^-$ within the renal outer medulla. Sprague-Dawley rats were studied with either the renal capsule intact or removed to ascertain the contribution of changes of medullary blood flow and renal interstitial hydrostatic pressure on H$_2$O$_2$ and NO$_2^-$+NO$_3^-$ production. Responses to three 30-minute step changes of renal perfusion pressure (from $\approx$85 to $\approx$115 to $\approx$145 mm Hg) were studied using adjustable aortic occluders proximal and distal to the left renal artery. Medullary interstitial H$_2$O$_2$ determined by microdialysis increased at each level of renal perfusion pressure from 640 to 874 to 1593 nmol/L, as did H$_2$O$_2$ urinary excretion rates, and these responses were significantly attenuated by decapsulation. Medullary interstitial NO$_2^-$+NO$_3^-$ increased from 9.2 to 13.8 to 16.1 $\mu$mol/L, with parallel changes in urine NO$_2^-$+NO$_3^-$, but decapsulation did not significantly blunt these responses. Over the range of renal perfusion pressure, medullary blood flow (laser-Doppler flowmetry) rose 30% and renal interstitial hydrostatic pressure rose from 7.8 to 19.7 cm H$_2$O. Renal interstitial hydrostatic pressure and the natriuretic and diuretic responses were significantly attenuated with decapsulation, but medullary blood flow was not affected. The data indicate that pressure-induced increases of H$_2$O$_2$ emanated largely from increased tubular flow rates to the medullary thick-ascending limbs of Henle and NO largely from increased medullary blood flow to the vasa recta. The parallel pressure–induced increases of H$_2$O$_2$ and NO indicate a participation in shaping the “normal” pressure-natriuresis relationship and explain why an imbalance in either would affect the blood pressure salt sensitivity. (Hypertension. 2009;53:1048-1053.)

Key Words: renal medullary oxidative stress ▪ hydrogen peroxide ▪ nitrate and nitrite ▪ nitric oxide ▪ pressure natriuresis ▪ renal medullary blood flow

Renal oxidative stress is enhanced in many animal models of hypertension and renal disease and is associated with renal fibrosis, vasoconstriction, apoptosis, and a reduction of urinary excretion of sodium (UNaV). It has been demonstrated that either a reduction of NO production or an increase in renal oxidative stress within the renal medulla can produce hypertension and renal injury. The mechanisms leading to excess production of reactive oxygen species within the kidney are beginning to be understood. It is evident, eg, that both elevations of hormones such as angiotensin II and increased tubular or extracellular sodium concentrations can stimulate the production of superoxide (O$_2^-$) within the medullary thick ascending limbs of Henle (mTALs) and contribute to hypertension and renal injury.

The elevation of renal perfusion pressure (RPP) with hypertension can contribute importantly to the progressive renal injury generally observed in hypertension, as demonstrated in 2 rat models of hypertension: infusion of low pressor angiotensin II plus a high-salt diet and the Dahl salt-sensitive rat strain, in which RPP to 1 kidney was chronically protected from elevated pressures using a computerized servocontrolled balloon occluder implanted between the distal left and proximal right renal arteries. In the kidneys exposed to high RPP, molecules related to pathways of oxidative stress, eg, transforming growth factor-$\beta$ and nuclear factor-$\kappa$B, exhibited enhanced expression compared with the pressure-protected kidney. Kidneys exposed to the higher perfusion pressure also exhibited greater glomerular and medullary tubular sclerosis and interstitial fibrosis with an exaggerated expression of genes related to pathways of oxidative stress and apoptosis when compared with the pressure-protected kidney. These results indicate that RPP in some manner stimulated oxidative stress and contributed to the progression of hypertension and renal injury.

Because it was found that increased delivery of NaCl to the mTAL by microperfusion results in increased production of O$_2^-$ in this tubular segment of the outer medulla, we hypothesized that elevations of RPP with a resulting in-

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1048
increased delivery of NaCl would produce oxidative stress in this region of the kidney. Given that elevations of RPP are known to increase medullary blood flow (MBF) and renal interstitial hydrostatic pressure (RIHP), these parameters were also determined to indirectly assess the mechanisms responsible for observed changes of H$_2$O$_2$ in the outer medulla. The study, therefore, determined whether kidneys of normal Sprague-Dawley rats, when subjected to acute increases of RPP (85 to 115 to 145 mm Hg), responded with increased levels of H$_2$O$_2$ within the outer medulla and whether these responses were driven by increases of MBF and RIHP by comparing responses in kidneys with intact renal capsules with responses in decapsulated kidneys. Also, changes in NO production in response to elevations of RPP were also studied to determine how these events may be interrelated.

Methods

Experimental Animals

Male Sprague-Dawley (SD) rats were used in all of the protocols (protocols 1 and 2 used Harlan Sprague-Dawley; protocol 3 used SLC Sprague-Dawley rats) aged 10 to 11 weeks. Rats were anesthetized with ketamine (20 mg/kg, IM) and inactin (50 mg/kg, IP) and placed on a temperature-controlled surgical table to maintain body temperature at 37°C. All of the procedures were approved by the Institutional Animal Care and Use Committee.

Surgical Preparation

In studies to determine the relationship among 3 levels of RPP (80 to 85, 110 to 115, and 140 to 145 mm Hg) and H$_2$O$_2$, or NO$^{-}$, NO$^{+}$, NO$^-$ (NOx) production in a single kidney, the left kidney was isolated, the renal artery denervated, and an adjustable micro-Blalock clamp placed around the aorta proximal and distal to the renal arteries to raise or lower RPP. Superior mesenteric and celiac arteries were tied off the celiac and superior mesenteric arteries and adjusting the final elevation of RPP with a ligature placed around the aorta distal to both renal arteries. Urine was collected bilaterally from catheters inserted into both ureters for measurement of changes of urinary H$_2$O$_2$ excretion rates associated with the increase of RPP.

Protocol 1: Effects of Step Increases in RPP on Production of Renal Medullary H$_2$O$_2$ or NOx in Rats With Intact Kidney Capsule and in Rats With the Capsule Removed

In these studies, a linear microdialysis fiber (320 µm OD, 5-mm membrane window, LM-5, BAS Inc) was inserted longitudinally using a 30-gauge needle from the lower to the upper pole of the kidney to pass through the outer medulla and anchored in place on the kidney surface with cyanoacrylate adhesive. This dialysis fiber was perfused with 0.9% NaCl at a rate of 2 µL/min throughout the study. Dialysate was collected continuously throughout each 30-minute period for determination of either H$_2$O$_2$ or NOx. The placement of the fiber in the outer medulla was confirmed at the end of the experiment by careful visual examination, and rats with incorrectly placed fibers were discarded from the study. In one group of rats, the renal capsule remained intact, whereas in a separate group of rats, the capsule was removed as described previously. Two separate groups of rats were surgically prepared in the identical manner, but RPP was maintained constant at control levels throughout the entire study. H$_2$O$_2$ was determined in one time-control group and NOx in the second time-control group (time control; Table S1A and S1B, available in the online data supplement at http://hyper.ahajournals.org).

Protocol 2: Effect of Step Increases of RPP on Medullary RIHP or MBF in Rats With Intact Kidney Capsule and in Rats With the Capsule Removed

Because simultaneous implantation into the same kidney of the microdialysis fiber, the implanted polyethylene catheter for RIHP measurement, and the optical fiber for measurement of MBF was too disruptive to normal function, separate groups of rats were studied. For the determination of RIHP, rats were prepared as described above except, rather than implanting a microdialysis fiber, a polyethylene catheter (PE50) with a polyethylene matrix in the tip was implanted into the outer medulla of the kidney as described previously. For rats in which MBF was determined, an optical fiber was inserted into the outer medulla as described previously. RIHP or MBF was measured continuously during the step changes in RPP. These measurements were made in groups of rats with the kidney capsule either intact or removed.

Protocol 3: Urinary H$_2$O$_2$ Responses of the Left and Right Kidneys Within the Same Rat

In another group of rats, the capsule of the left kidney remained intact, whereas that of the right kidney was removed. RPP was then increased from 119 to 145 mm Hg to both kidneys by tying off the celiac and superior mesenteric arteries and adjusting the final elevation of RPP with a ligature placed around the aorta distal to both renal arteries. Urine was collected bilaterally from catheters inserted into both ureters for measurement of changes of urinary H$_2$O$_2$ excretion rates associated with the increase of RPP.

Protocol 4: Effect of Step Increases of RPP on Glomerular Filtration Rate

Glomerular filtration rate was determined by inulin clearance of fluorescein isothiocyanate inulin. Fluorescein isothiocyanate inulin (5 mg/mL; Sigma) dissolved in BSA and saline was infused at 3 mL/h for 60 minutes before the collection of the first urine sample. Blood samples were taken at the midpoint of each urine collection period. Collected samples were diluted with PBS (pH 7.4) and the fluorescence measured with a microplate reader.

Biochemical Measurements

H$_2$O$_2$ concentration was determined in interstitial fluid collected by microdialysis and urine using a fluorescence spectrometric assay (Amplex Red Hydrogen Peroxide Assay kit, Molecular Probes), as described previously. NOx was determined in interstitial fluid collected by microdialysis and urine with an absorbance spectrophotometer using the Greiss reaction (Nitrate/Nitrite Colorimetric Assay kit, Cayman Chemical Company), as described previously. Because all of the nitrite in the sample is converted by the reaction to nitrate, the final determination is the sum of the converted nitrite plus the nitrate already in the sample. This sum is designated as NOx in this article. Urine volume for calculation of urine flow rate was determined gravimetrically and urine sodium for calculation of UNaV measured by flame photometry.

Statistical Analysis

Data are presented as mean±SE. For statistical comparisons, 2-way ANOVA with repeated measures was used, followed by a Duncan’s posthoc test. All of the statistical analyses were performed on the raw data. P<0.05 was considered to be statistically significant.
Results

Relationship of RPP to Sodium and Water Excretion, Renal Medullary Interstitial Concentrations, and Urinary Excretion of H$_2$O$_2$ and NO$_x$ in Rats With Intact Renal Capsule and in Rats With the Renal Capsule Removed

Figure 1A summarizes changes of UNaV and medullary interstitial and urine H$_2$O$_2$ in response to RPP adjusted to 3 levels in one group of rats (n=7) with an intact renal capsule compared with a group with the renal capsule removed (n=8). In rats with the renal capsule intact, RPP was adjusted downward from resting control levels of 112±2 to 84±0.8 mm Hg and then to 111±0.3 mm Hg (not different from pressure setting of time control group) and finally to 143±0.3 mm Hg. Associated with these step increases of RPP, UNaV rose significantly from 0.36±0.1 to 2.88±0.5 to 9.86±0.6 μmol/min per gram of kidney weight and urine volume from 4.3±0.5 to 18.3±3.1 to 54.4±4.9 μL/min per gram of kidney weight (data not shown). Elevation of RPP significantly increased medullary interstitial H$_2$O$_2$ at each pressure step (640±47, 874±53, and 1593±120 nmol/L; P<0.05). Parallel increases of urinary H$_2$O$_2$ excretion were observed in these rats increasing significantly from 0.18±0.01, 0.44±0.02, to 0.92±0.11 nmol/min per gram of kidney weight with the increases of RPP.

In a separate group of rats (n=8), GRF was determined and found to autoregulate in a manner similar to that reported by others. With RPP fixed at 85±0.5 mm Hg, glomerular filtration rate averaged 0.50±0.05 mL/min per gram of kidney weight in the left kidney. When RPP was increased to 109.0±1.1 mm Hg, glomerular filtration rate rose significantly to 0.69±0.05 mL/min per gram of kidney weight. Glomerular filtration rate was not further increased (0.78 mL/min per gram) at the highest RPP fixed at 139.0±2.1 mm Hg. RPP in the time control group (n=7) was adjusted to remain constant at 110.0±4.9, 111.0±5.0, and 110.0±4.8 mm Hg, respectively, for the 3 collection periods. No significant changes in any measured variable were observed in the time-control group (Table S1A).

Similar step increases of RPP were evaluated in the decapsulated group (83±0.6 to 109±1.0 and then 141±0.8 mm Hg; n=8; Figure 1A). Elevation of RPP from 83 to 109 mm Hg increased renal interstitial H$_2$O$_2$ in the decapsulated kidney significantly and to nearly the same amount seen in the group with the intact capsule. However, when RPP was elevated from 109 to 141 mm Hg, medullary interstitial H$_2$O$_2$ was significantly attenuated in this group, increasing only 15% compared with the 82% increase observed in the group with the intact capsule. Similarly, urinary excretion of H$_2$O$_2$ increased with each pressure step, with the excretion in the intact kidney being significantly greater than the increase measured in the decapsulated kidney at the highest pressure step.

Figure 1B summarizes changes of medullary and urine NO$_x$ in response to similar changes of RPP in renal intact (n=7) and decapsulated (n=6) rats. With the renal capsule intact, RPP was adjusted downward from resting control levels of 116.0±3.2 to 83.0±0.5 mm Hg, then to 111.0±0.6 mm Hg, and finally to 140.0±2.3 mm Hg (n=7). Associated with the step changes of RPP, rising from 83 to 111 to 140 mm Hg, UNaV increased significantly from 0.22±0.10 to 2.42±0.70 to 10.28±1.60 μmol/min per gram of kidney weight and UV from 3.4±0.5 to 20.4±3.5 to 53.2±5.3 μL/min per gram of kidney weight (data not shown).

Medullary interstitial NO$_x$ increased significantly when RPP...
was increased from 83 to 111 mm Hg (9.2±2.8 to 13.8±3.9; P<0.05). As RPP was increased further to 140 mm Hg, medullary interstitial NOx rose to 16.1±3.9 μmol/L. Similarly, urinary NOx excretion increased from 1.6±0.9 to 7.0±2.9 to 11.1±4.8 nmol/min per gram of kidney weight at the 3 respective pressure steps. In decapsulated kidneys, the pressure-natriuresis response was significantly reduced compared with the intact group (UNaV: 0.25±0.10 to 3.15±0.60 to 6.24±0.90 μmol/min per gram of kidney weight; urine volume: 3.1±0.7 to 18.1±3.7 to 31.4±5.2 μL/min per gram of kidney weight; data not graphed). In contrast to the H2O2 responses, renal decapsulation did not significantly attenuate the relationship between RPP and medullary interstitial NOx (8.9±2.4, 15.9±3.2, and 16.9±3.3 μmol/L) or urinary excretion of NOx (1.1±0.3 to 6.6±0.9 to 7.8±1.3 nmol/min per gram of kidney weight). No significant changes in any measured variable were observed in the time-control group (Table S1B).

Effect of Step Increases of RPP on Medullary RIHP and MBF Responses to Decapsulation

In rats with intact renal capsules and an implanted catheter for determination of RIHP (n=7), as RPP was adjusted from 82.0±0.6 to 111.0±0.8 to 142.0±0.6 mm Hg, RIHP increased significantly from 7.8±0.8 to 12±0.7 to 19.7±0.5 cm H2O (Figure 2A). UNaV increased from 0.10±0.04 to 2.20±0.50 to 7.20±0.90 μmol/min per gram of kidney weight, whereas UV increased from 2.5±0.4, 15.5±2.7 to 54.8±3.4 μL/min per gram of kidney weight with the 3 pressure steps. With decapsulation (n=7), the increase of RIHP was significantly attenuated, rising from 4.4±0.5 to 6.5±0.4 to 11.1±0.4 cm H2O for the same pressure steps. The increase in UNaV was significantly blunted in the kidneys that were decapsulated compared with the kidneys with intact renal capsules (4.5±0.6 versus 7.2±0.9 μmol/min per gram of kidney weight; P<0.05). Similarly, UV was also attenuated by decapsulation, averaging 2.7±0.4 to 12.3±1.9 to 30.4±5.1 μL/min per gram of kidney weight.

In rats with intact renal capsules and optical fibers implanted for the measurement of MBF (n=5), as RPP was adjusted from 84.0±0.5 to 111.0±0.9 to 141.0±2.1 mm Hg, MBF increased 13±6% above the lowest pressure at the intermediate step and then 25±5% at the highest pressure step (Figure 2B). UNaV increased from 0.3±0.1 to 3.4±0.4 to 10.0±0.8 μmol/min per gram of kidney weight, whereas UV increased from 4.2±0.6 to 22.2±2.5 to 72.2±11.2 μL/min per gram of kidney weight with the 3 pressure steps. In contrast to the RIHP, MBF was not significantly attenuated by decapsulation (n=6). However, because the rise of RIHP was clearly blunted by decapsulation, increases in UNaV and UV were also blunted in this group of rats.

Changes in Urinary Oxidative Stress With Differing RPP Between 2 Kidneys

The relationship among RPP, urinary H2O2 excretion, and the influence of RIHP was further confirmed in another group of rats (N=7) by comparing responses within the same rat. The renal capsule was removed from the left kidney, whereas the capsule of the right kidney remained intact. In these rats, the aortic occluder was placed below both of the renal arteries so that RPP increased from 119.0±3.6 to 145.0±5.3 mm Hg in both kidneys, as shown in Figure 3. Urinary H2O2 excretion of the intact right kidney increased from 0.12±0.02 to 0.48±0.09 nmol/min per gram of kidney weight (P<0.05), an increase significantly greater than the response of the decapsulated left kidney in which H2O2 excretion was increased slightly with the pressure step and did not reach significance. These data support the results shown above and
emphasize the importance of changes of RIHP on the production of renal H$_2$O$_2$.

Discussion

Renal Medullary Oxidative Stress as Driven by Acute Increases of RPP

The concept of pressure-natriuresis and the relationships among RPP, MBF, RIHP, and UNaV has been well established. It was the goal in the present study to establish how these factors were involved in the observed increases of H$_2$O$_2$ and NO in response to elevations of RPP. The present study provides the first evidence that acute increases of RPP elevate the production of H$_2$O$_2$ in the outer medulla and that this response is triggered by an increase of RIHP.

Because blood flow to the renal medulla of the rat is poorly autoregulated, increases of RPP are transmitted to the vasa recta circulation, and as RPP and vasa recta flow increase, autoregulated increases of RPP are transmitted to the vasa recta of the outer medulla. A rise of RPP with an increase of RIHP signals the release of oxygen free radical (O$_2^-$) production, and increased luminal flow and NaCl delivery to the mTAL can stimulate O$_2^-$ production. It is of interest that changes of urinary H$_2$O$_2$ excretion in the present study were remarkably parallel to those seen in the microdialysis samples of the outer medullary interstitial fluid. This suggests that urinary H$_2$O$_2$ excretion could serve as a good marker of renal medullary oxidative stress, because H$_2$O$_2$ is relatively stable in aqueous solutions and a more specific assay than other currently used markers of renal oxidative stress, eg, 8-isoprostan, determined by enzyme immunoassay, and products of lipid peroxidation, as determined by a colorimetric assay for thiobarbituric acid reactive substances.

Finally, it should also be noted that, although we have previously used microdialysis techniques to measure NO, NOx, O$_2^-$, and H$_2$O$_2$ in the renal medulla, these earlier studies used a needle type microdialysis probe (BR-2, BAS, Inc) that provided a 2-mm length of membrane for dialysis. The linear microdialysis fiber (with nearly a 5-mm length of membrane) used in the current study provided a greater membrane surface area and enhanced the dialysis efficiency from 32% seen with the needle probe to 52%. The linear fiber maintained a more stable position in the face of increases of RPP. Because fluorescence units were not corrected for the efficiency of the membranes, the absolute values are greater than those reported previously from our laboratory.

Effects of Increases of RPP on Renal Medullary NO Production

Medullary interstitial NOx concentrations and urinary NOx excretion levels also increased as RPP was increased, confirming observations of others. This, however, was mostly associated with the increases of RPP from the lowest (85 mm Hg) to the intermediate pressure step (110 mm Hg). Changes in MBF after renal decapsulation have not been reported previously, and a novel finding of the present study is that, in contrast to the changes observed with RIHP, changes of MBF with RPP were unaffected by decapsulation. Microperpufusion studies by Zhang and Pallone found flow-dependent increases of NO production in isolated perfused vasa recta of the outer medulla of the rat. A rise of RPP with an increase of MBF would, therefore, be expected to produce an increased endothelial release of NO and not be affected by renal decapsulation. Although we have reported that increased tubular flow and delivery of Na$^+$ to mTAL reduce NO production within isolated perfused mTAL because medullary interstitial NOx was increased in the present study, it appears that these levels were dominated by NO produced by the vasa recta vessels. These relationships are clearly complex, and, because medullary interstitial NO did not rise substantially between the RPPs of 110 to 140 mm Hg, although MBF did rise significantly over this range of RPPs, it is evident that something else is going on at these pressure

Figure 3. Urinary oxidative stress determined as urinary excretion of H$_2$O$_2$ (UH$_2$O$_2$V) increases with an increase in RPP in the intact right kidney (○) but the decapsulated left kidney (●) of the same rat showed significantly less excretion with the same change of pressure. Values are mean±SE. *Significantly different from the baseline pressure in the same kidney (P<0.05), †Significantly different from the decapsulated left kidney (P<0.05).
levels. It is possible that as RPP and MBF are increased to these higher levels, further increases of interstitial NO concentrations may be attenuated by medullary washout.

We conclude that elevations of RPP stimulate the release of both NO and H$_2$O$_2$ within the renal outer medulla. The mechanisms responsible for the parallel release of these important vasoactive molecules differ, whereby NO appears to be driven by increases of MBF whereas increases of H$_2$O$_2$ result from increased mTAL delivery of NaCl. The balance of H$_2$O$_2$ and NO production in the outer medulla are, therefore, important considerations in understanding the interrelationships among RPP, sodium excretion, and the long-term control of arterial blood pressure. The parallel increases of H$_2$O$_2$ and NO produced by elevations of RPP appear to be important determinants of the “normal” pressure-natriuresis relationship.

**Perspectives**

Pressure-natriuresis appears to come with the price of also producing oxidative stress within the outer medulla of the kidney unless offset by a parallel production of NO. This would appear to explain why excess levels of medullary H$_2$O$_2$ can lead to a salt-sensitive form of hypertension, whereas greater levels of medullary NO can reduce salt-sensitivity and lower arterial pressure. One can also speculate that, in the chronic state, if the production of both O$_2^-$ and NO occurs with elevations of RPP, greater amounts of peroxynitrite would also be produced in the renal outer medulla that may be associated with greater tissue fibrosis and injury. The present data suggest that the development of antioxidant agents that could effectively reduce medullary O$_2^-$ and H$_2$O$_2$ production may be of greater clinical benefit than those that target greater production of NO, eg, L-arginine.

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**Disclosures**

None.

**References**

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EFFECTS OF RENAL PERFUSION PRESSURE ON
RENAL MEDULLARY H₂O₂ AND NO PRODUCTION

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Running title: Pressure-induced renal oxidative stress

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**Table S1:** Summary of results for Time Control studies run for three periods of 30 minutes to correspond to the periods of step changes in pressure.

A. Time control for H$_2$O$_2$ measurements (n=7):

<table>
<thead>
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<th>Measurement</th>
<th>Period 1</th>
<th>Period 2</th>
<th>Period 3</th>
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<td>MAP (mmHg)</td>
<td>110±4.9</td>
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<td>UNaV (µmol/min per g kwt)</td>
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<td>UV (µL/min per g kwt)</td>
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<td>Medullary Interstitial H$_2$O$_2$ (nmol/L)</td>
<td>928±33</td>
<td>978±34</td>
<td>1011±54</td>
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<td>UH$_2$O$_2$V (nmol/min per g kwt)</td>
<td>0.53±0.05</td>
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B. Time control for Nox measurements (n=7):

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<th>Measurement</th>
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<th>Period 3</th>
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<td>111±3.8</td>
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