Effects of Salt Depletion on the Kidney: Changes in Medullary Oxygenation and Thick Ascending Limb Size

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ABSTRACT

Previous studies have shown that salt depletion enhances the susceptibility of the kidney to nephrotoxins (amphotericin, cyclosporine, and contrast). To study the renal response to salt depletion, Sprague-Dawley rats were fed a sodium-deficient diet (N=12) with paired controls (N=13) for 4 wk. In addition, rats from each group underwent 24-h water deprivation studies (N=9; four salt deprived, five normal). Plastic 1-µm horizontal sections of mid-inner stripe were examined, and cross-sectional areas of the medullary thick ascending limb (mTAL) were analyzed. The mTAL of the salt-deprived rats were smaller (P=0.04) and showed greater variance in size (P=0.02) than control (618 ± 106 versus 693 ± 50 µm²). However, mean glomerular and collecting duct cross-sectional areas were unaffected by salt intake. Cross-sectional areas of long- and short-loop mTAL were significantly different, regardless of group (518 ± 78 versus 732 ± 92 µm²). Maximal urinary concentrating ability was found to correlate with mTAL cross-sectional area (r = 0.85; P = 0.004) and with long-loop mTAL size (r = 0.77; P = 0.016). However, it did not significantly correlate with short loop mTAL size (r = 0.53; P = 0.14). Thus, mTAL size varied with dietary salt availability and correlated with concentrating ability. In a parallel study of intrarenal oxygenation with Clark-type O₂ microelectrodes, medullary O₂ tension was markedly increased from 29 ± 2 (in controls) to 49 ± 2 mm Hg in salt-depleted rats (P < 0.0001), whereas cortical P₀₂ was reduced from 50 ± 2 to 27 ± 2 mm Hg, consistent with reduced transport activity in the inner stripe and/or the corticomedullary redistribution of blood flow. Thus, chronic salt depletion both diminishes mTAL size and reverses the normal corticomedullary O₂ gradient, perhaps reflecting a reduced mTAL workload in conjunction with the reallocation of blood flow.

Key Words: Concentration, inner stripe, cortical P₀₂, loop of Henle, transport

The medullary thick ascending limb (mTAL) plays an important role in the countercurrent concentrating mechanism. Active chloride reabsorption along this water-impermeable part of the nephron, with the sodium ion following passively along the electrical gradient, enables the mammalian kidney to concentrate and dilute urine (1). This sodium-potassium ATPase-dependent ion transport system is heavily dependent on aerobic metabolism (1). Because of relatively low blood flow and the countercurrent exchange, a low oxygen tension normally exists within the renal medulla (2-5), rendering the inner stripe associated with polyene antibiotics vulnerable to hypoxic injury (6). Increased mTAL metabolic activity might increase medullary oxygen insufficiency. This may explain the enhancement of mTAL hypoxic damage in hypertrophic kidneys, noted in vivo (7,8) and in vitro (9), the injury to the outer stripe associated with polyene antibiotics (10), and the prevention of mTAL damage produced by the reduction of energy requirements (11,12).

Salt depletion is a well-established risk factor for the syndrome of ATN and is known to potentiate nephrotoxins, both under experimental conditions (7,13,14) and in clinical practice (15-19). These studies were designed to evaluate alterations in medullary morphology and oxygen balance in chronically salt-depleted rats.

METHODS

Male Sprague-Dawley rats weighing 329 ± 8 g were used for all experiments and were randomized to two experimental groups: control and salt depletion. All animal experimentation was conducted in accord with the NIH Guide for the Care and Use of Laboratory
Animals. Chronic salt depletion was induced over 4 wk with a sodium-deficient diet (*902902; ICN Nutritional Biochemicals, Cleveland, OH). Rats in the salt-depleted group were given tap water ad libitum. Control rats, paired with the same chow, were given 0.25% NaCl distilled water as their ad libitum drinking water. The rat size was chosen to avoid the effect of salt deprivation on the developing kidney (20).

Functional Studies

At the conclusion of the experiment, all animals were subjected to a 24-h urine collection period, and urine and plasma samples were analyzed for the determination of creatinine, sodium, potassium, and urine osmolality. Standard laboratory techniques were used for all of these measurements. Creatinine clearance, fractional tubular reabsorption of sodium, and fractional potassium excretion were calculated by the use of standard formulas. In addition, in the fourth week of the experiment, randomly selected rats (N = 4 salt deprived and five control) from each group were placed in metabolic cages (Nalge Co., Rochester, NY) for 24 h of water deprivation and urine collection for the determination of sodium and potassium excretion, creatinine, and maximal osmolality. These rats were given a 24-h recovery period, before the initiation of terminal functional studies.

Measurements of Renal Parenchymal PO2

In a parallel study, intrarenal oxygen tension was measured, as previously described (21,22), in a different group of 15 salt-depleted rats, weighing 376 ± 48 g, after 14 ± 3 days of a salt-free diet; these results were compared with those obtained in 52 control rats, as previously published (21). In brief, sensitive O2 glass microelectrodes of the Clark type (handmade by M.E. Traube for Omega Technologies, Haifa, Israel) with fine- (5- to 15-μm) tip diameter were used, polarized at −0.75 V, connected to a picoampermeter and a recorder, and calibrated at 37°C (with N2 and 12% O2) at the start and at the end of each experiment.

The rats were anesthetized with Inactin (BYK Gulden, Konstanz, Germany; 100 mg/kg body wt). Tracheostomy was performed, and the femoral vein and artery were cannulated with PE50 catheters (Clay-Adams, Parsippany, NJ) for the infusion of normal saline with BSA (4 g/dL) (at a rate of 0.09 mL/min) during the administration of indomethacin and for the monitoring of the blood pressure by a pressure transducer. The left kidney was exposed by a mid-abdominal incision and mechanically fixed. The temperature of the kidney was monitored by a needle copper probe connected to a type T thermocouple (Omega Engineering, Stamford, CT) and was kept at 37°C with a heating lamp and by dripping warm saline and mineral oil. Two or more electrodes, mounted on micromanipulators, were inserted at different depths of the kidney parenchyma.

The experiment was carried out in two phases, after an equilibration period of over 30 min, for the stabilization of parenchymal oxygen tensions. At first, repeated measurements of PO2 (averaging 13 per animal) were carried out with the electrodes placed at various depths for the acquisition of PO2 profiles in salt-depleted and control rats (total number of measurements, 193 and 205, respectively). In the second phase of the experiment, cortical and outer medullary electrodes were positioned at depths of 1.5 ± 0.2 and 4.3 ± 0.4 mm, respectively. Blood pressure and cortical and outer medullary oxygen tension were monitored continuously at baseline and over 30 min after the iv injection of indomethacin (10 mg/kg body wt; Sigma Chemical Co., St. Louis, MO).

Morphometric Studies

At the end of the functional studies, the rats were anesthetized with Inactin. Renal perfusion fixation in vivo was performed through the aorta at a pressure of 140 mm Hg with 1.25% glutaraldehyde (Eastman Kodak Co., Rochester, NY) in 0.1 M phosphate buffer (pH 7.4). The kidney that appeared more completely fixed was selected, weighed, measured in three dimensions, and cut in horizontal (i.e., parallel to the corticomedullary axis) cross-sections. Selected 4 × 4-mm tissue slices were postfixed in buffered 2% OsO4, dehydrated, and embedded in an araldite–Epon 812 mixture; sections were cut at 1 μm and stained with methylene blue.

Morphometric studies were performed with a Leitz Aristoplan microscope (Wild Leitz USA, Inc., Rockleigh, NJ), equipped with a color video camera and connected to a color video monitor. The Bioquant System IV (versions 11/6/89 and 7/30/91) was used for morphometric analysis (R & M Biometrics, Nashville, TN).

Horizontal sections of the inner stripe of the outer medulla was studied, equivalent to the mid-inner stripe, or level 2, as defined by Bubby et al. (23). To standardize the level of the examined section, we evaluated the percentage of inner stripe cross-sectional area that was taken up by vascular bundles, for each slide studied. That value was not significantly different between salt-depleted and control rats (means of 17 and 18%, respectively).

Sections of the inner stripe were examined at 50× (field area 9.9 × 104 μm2), and the cross-sectional area of each mTAL and collecting duct (excluding the lumen) was quantified. Three full fields were examined per kidney, containing together 125 mTAL and 24 collecting ducts on average.

Long- and short-looped mTAL were measured at a
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separate session, with the same sections. They were defined, respectively, by direct proximity to a vascular bundle and juxtaposition to a collecting duct. Thus, an mTAL was considered to be long looped if it was both in contact with a vascular bundle and did not touch a collecting duct. Conversely, a short-looped mTAL was defined as one that touched a collecting duct but did not lie adjacent to a vascular bundle. These strict criteria resulted in many of the mTAL not being counted as either a short- or long-looped mTAL.

Superficial and deep cortical sections were examined as well, and all glomeruli with clearly defined vascular poles were measured. Of these, the 20 largest measurements were retained for analysis.

Preliminary Studies

The morphometric study presented here followed two previous observations suggesting a smaller sized mTAL in salt-depleted rats. The first observation was made in a study designed to investigate the synergistic effects of chronic amphotericin administration and salt depletion (24). Morphometric analysis disclosed that the average mTAL size was 1,195 ± 48 and 922 ± 45 μm² in control and salt-depleted animals, respectively (P < 0.01 by analysis of variance [ANOVA]). To validate this observation prospectively, a second pilot study was performed where two groups of animals were evaluated—control and salt-depleted—after 4 wk of treatment. Morphometric analysis again revealed that the average mTAL size was smaller after salt depletion (887 ± 120 versus 1,079 ± 279 μm², salt-depleted rats versus controls). However, the study did not attain statistical significance because of variability among the animals of both groups. In addition, the animals were not pairfed. Thus, we were concerned that the findings were, perhaps, related to protein and/or caloric malnutrition. This study was therefore designed to verify the effect of salt depletion on mTAL size in pairfed groups of animals.

Statistics

Results are presented as the mean ± SD, in the tables and text, and as the mean ± SE in the figures. Comparisons between the control and salt-depleted groups were done with the independent group t test with two-tailed P values. ANOVA with the Newman-Keuls test was applied for comparisons of repeated Po₂ measurements. A P value of less than 0.05 was considered to be statistically significant. Computations were performed with the CRUNCH program (CRUNCH Software Corp., Oakland, CA).

RESULTS

Functional Studies

Despite initially identical body weights and rigorous pairfeeding, by the end of the experiment, salt-depleted rats weighed significantly less than controls (413 ± 15 versus 443 ± 21 g; P < 0.001). Nevertheless, kidney weight and volume were virtually identical. Salt deprivation in salt-depleted rats was confirmed by 24-h sodium excretion (25) (Table 1). Both groups differed by their terminal plasma concentrations of sodium and potassium but not by plasma urea nitrogen, creatinine, or creatinine clearance. Maximal urinary osmolality tended to be reduced in salt-depleted rats, but the difference between the groups fell short of statistical significance (probably because of the small number of observations).

Po₂ Measurements

The results of multiple measurements of renal parenchymal oxygen tension at different structural levels are displayed in Figure 1. In control rats, a steep gradient of tissue oxygenation was noted at the level of the outer medulla, between a 3- and 3.5-mm depth of micropuncture (2,21,22). The mean cortical Po₂ of about 50 mm Hg fell to 20 to 30 mm Hg at this level. The great heterogeneity of cortical measurements, probably reflecting random locations in the cortical labyrinth or medullary rays, was transformed into a

<table>
<thead>
<tr>
<th>Group</th>
<th>Terminal Plasma Sodium (mEq/L)</th>
<th>Terminal Plasma Potassium (mEq/L)</th>
<th>Terminal Plasma BUN (mg/dL)</th>
<th>Terminal Plasma Creatinine (mg/dL)</th>
<th>Creatinine Clearance (mL/min per 100 g)</th>
<th>24-h Urinary Sodium (mEq)</th>
<th>Maximal Urinary Osmolarity (mosm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>145 ± 2</td>
<td>3.1 ± 0.1</td>
<td>13 ± 3</td>
<td>0.5 ± 0.1</td>
<td>0.38 ± 0.09</td>
<td>2.3 ± 1.3</td>
<td>2426 ± 242</td>
</tr>
<tr>
<td>N</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>SD</td>
<td>143 ± 3</td>
<td>3.6 ± 0.2</td>
<td>13 ± 3</td>
<td>0.5 ± 0.1</td>
<td>0.43 ± 0.08</td>
<td>0.5 ± 0.1</td>
<td>2124 ± 226</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
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<tr>
<td>P &lt;a</td>
<td>0.04</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.001</td>
<td>0.1</td>
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</table>

*a NS, not significant.
uniform tracing of low tissue oxygen tension within the deeper structures. In general, \( P_{O_2} \) decreased with the depth of the micropuncture.

This pattern of renal parenchymal oxygenation was remarkably reversed in chronically salt-depleted rats. Mean cortical oxygen tension measurements were as low as 30 mm Hg, whereas tissue \( P_{O_2} \) gradually rose at the corticomedullary junction, reaching mean values of about 50 mm Hg. The mean oxygen tension measurements were significantly different \( (P < 0.0001) \) at every millimeter depth, except for the third, where there was no significance. ANOVA (two-way) for salt and depth of \( P_{O_2} \) gave an \( F \) value of 31 \( (P < 0.0001 \) for salt plus depth; \( P < 0.02 \) for salt only; \( P < 0.001 \) for depth only). Thus, in salt-depleted rats, as opposed to controls, \( P_{O_2} \) directly increased with depth of micropuncture.

Repeated \( P_{O_2} \) measurements at fixed depths at cortical and outer medullary regions are displayed in Figure 2, at baseline and 20 to 30 min after the administration of indomethacin. Although cortical oxygenation was not consistently affected, outer medullary \( P_{O_2} \) significantly declined, from 43 ± 20 to 22 ± 18 mm Hg \( (P < 0.001) \).

**Morphometric Studies**

Table 2 lists the results of the morphometric evaluation. Although different in body weight, kidney volume and weight were identical in both groups. No abnormalities were noted by light microscopy, and glomerular sclerosis was absent in both salt-depleted and control rats.

Although glomerular and collecting duct cytoplasmic cross-sectional areas were unaffected by salt intake, the mean mTAL area was significantly reduced in salt-depleted rats \( (618 \pm 106 \text{ versus } 693 \pm 51 \mu m^2 \text{ in control animals}; \ P < 0.04) \). From the standpoint of light microscopy, there appeared to be equivalent mTAL mitochondrial density. As shown in Table 2, long-looped mTAL were significantly smaller than short-looped mTAL in both groups \( (P < 0.01) \).

Maximal urinary concentrating ability was found to correlate with mTAL cross-sectional area \( (r = 0.85; \ P = 0.004) \) and with long-loop mTAL size \( (r = 0.77; \ P = 0.016) \); however, it did not correlate with short-loop mTAL size \( (r = 0.53; \ P = 0.14) \). In addition, the variance in mTAL size was significantly different between the two groups \( (P = 0.02) \).

**DISCUSSION**

Salt depletion potentiates nephrotoxins and is a known risk factor for the development of acute renal failure under various experimental and clinical conditions \( (7,13-19) \). Because the renal medulla normally functions under low oxygen tension \( (6) \), augmentation of the physiologic outer medullary hypoxia might be expected in chronically salt-depleted rats. Unexpectedly, our experiments revealed the opposite: in contrast to control rats, cortical \( P_{O_2} \) was markedly reduced, whereas outer medullary oxygen tension
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Figure 2. The effect of indomethacin (10 mg/kg body wt) on renal parenchymal \( pO_2 \) in control and salt-depleted rats. Repeated measurements were performed by the use of two electrodes simultaneously placed at cortical and inner stripe levels (1.5 ± 0.2 and 4.3 ± 0.4 mm depth, respectively). Cortical \( pO_2 \) is unaffected by indomethacin, whereas medullary \( pO_2 \) declines to the same magnitude in salt-depleted and control rats.

<table>
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<th>Group</th>
<th>Kidney Wt (g)</th>
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<td>Control (N = 13)</td>
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<td>13,581 ± 1,100</td>
<td>975 ± 78</td>
<td>693 ± 51</td>
<td>543 ± 52</td>
<td>729 ± 78</td>
</tr>
<tr>
<td>SD (N = 12)</td>
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<td>2.79 ± 0.2</td>
<td>13,833 ± 1,361</td>
<td>925 ± 131</td>
<td>618 ± 106</td>
<td>518 ± 78</td>
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</tr>
<tr>
<td>( P &lt; )</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.036</td>
<td>NS</td>
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was substantially increased, reversing the normal corticomedullary oxygenation gradient.

These findings could be explained by several mechanisms. Salt depletion increases the concentrations of circulating vasoconstrictors, such as angiotensin II and catecholamines (26–28). Under such circumstances, a redistribution of intrarenal blood flow occurs: outer cortical RBF decreases (29) with a shift of blood supply toward the inner cortex (30), presumably designed to preserve medullary perfusion.

These hemodynamic changes have not been consistently found (31,32), perhaps because of the technical difficulties inherent in the measurement of intrarenal blood flow. Local stimulation of prostaglandin release by salt deprivation (28) would contribute to the relative preservation of medullary blood flow. In the rats, the mTAL of long-looped nephrons, adjacent to the vasa recta, are less hypoxic than those of short-looped nephrons, located away from vessels. Thus, a redistribution of glomerular filtration from the superficial to the deep nephrons would improve medullary hypoxia not only by enhancing medullary blood flow (which derives from juxtamedullary glomeruli) but also by shifting the reabsorptive transport workload to better-oxygenated long-looped mTAL. The intrarenal redistribution of blood flow could therefore play a role in the reversed corticomedullary gradient of oxygenation observed in salt-depleted rats.

An additional, possibly major, physiologic event underlying this altered intrarenal oxygenation is a shift of the transport workload from the distal to the proximal tubules. Salt depletion is consistently associated with enhanced proximal tubular reabsorption of sodium (33,34), decreased distal delivery, and reduced work of the distal tubule (35).

The inverted pattern of renal oxygenation under these circumstances could result from the reduction in cortical oxygen supply associated with increased proximal tubular reabsorption and from decreased distal delivery, metabolic workload, and oxygen consumption at the level of the outer medulla (6). The local production of prostaglandins, which down-regulate tubular transport activity (36,37), may contrib-

### Table 2. Morphometric evaluation of kidneys from salt-depleted and control rats

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ute to this improvement in medullary oxygen balance. As illustrated in Figure 2, the inhibition of prostaglandin synthesis by indomethacin reduced outer medullary $P_{O_2}$, suggesting that increased prostaglandin release may participate in the reversed profile of intrarenal oxygenation noted in chronically salt-depleted rats. However, it is to be noted that prostaglandin secretion is important in the normal state because the medullary $P_{O_2}$ in the controls was reduced as well.

We have recently observed a similar reversal of the renal parenchymal oxygenation profile during acute hypotension (38). Paradoxically, acute reductions in renal perfusion (produced by aortic ligation, hemorrhage, or nitroprusside infusion) increased medullary $P_{O_2}$, while reducing cortical $P_{O_2}$. In the presence of furosemide (which inhibits solute reabsorption in the mTAL and increases medullary $P_{O_2}$), acute hypotension did not further increase $P_{O_2}$ in the medulla. These data suggest that the hypotension-induced reversal of the corticomedullary oxygen gradient derives at least in part from a reduction in transport workload in the outer medulla.

Our morphometric findings suggest an additional mechanism for decreased oxygen consumption in the outer medulla. The mean cross-sectional area of mTAL cells at the level of the inner stripe of the outer medulla was smaller in salt-deprived rats, as compared with that in controls. Although both groups did not differ by mitochondrial density, as assessed by light microscopy, it is conceivable that oxygen consumption may correlate with the reduction in mTAL cellular mass. Supporting this hypothesis is the correlation found between mTAL cellular mass and maximal urinary concentration. Because the latter is the product of energy-dependent ion transport that takes place in mTAL, a lower oxygen requirement is anticipated when cellular mass is reduced.

The association of chronic salt depletion with trophic changes in the inner stripe is an additional example of the remarkable plasticity of the mTAL in response to various pathophysiologic changes (39). Vasopressin or water restriction (23), thyroid hormones (40), and a high-protein diet (41) have been observed to increase mTAL cell size, often disproportionately to trophic changes in the remainder of the nephron. In addition, the activity of the Na-K-ATPase in mTAL is increased by antidiuretic hormone (42), mineralocorticoid (43), and high protein intake (44,45). Variation of salt intake may also have trophic effects on the mTAL. Increased dietary salt load has been shown to stimulate the Na-K-ATPase of mTAL segments in the rabbit (46). Sodium deprivation in the mouse did not cause a clear change in mTAL enzyme activity, probably reflecting the result of opposing influences of decreased sodium delivery and increased endogenous aldosterone (43). This effect contrasted with the stimulation of enzyme activity in the cortical collecting tubule (known to respond to mineralocorticoids).

Several studies have indicated a trophic role of salt on the kidney. A high-salt diet increased renal size and protein content, indicating stimulation of renal growth due to hyperplasia in the rat (47,48). Salt intake may be particularly important in modulating the hypertrophic response of remnant kidneys. Recent studies have shown that dietary salt restriction lessens renal damage by inhibiting compensatory kidney growth after severe renal ablation (49–51). The mechanism of protection, apparently independent of changes in intraglomerular hypertension, may relate to some trophic effect of sodium chloride on the renal parenchyma. In this study, the reduction in epithelial volume after salt depletion was more readily apparent in mTAL than in other nephron segments, perhaps because of the greater plasticity of the mTAL in response to various stimuli (22,39–41). Recent insights into the regulation of gene expression by extracellular tonicity in the renal medulla (52) may help in the future to clarify the mechanisms of this trophic modulation of mTAL by sodium chloride.

The trophic effect of salt upon the kidney may be mediated through growth factors. Augmenting distal tubular workload with furosemide induced hypertrophy (53) and increased immunoreactivity for insulin-like growth factor (IGF-1) and its binding proteins in the distal nephron (54). An altered IGF-1 signal, largely confined to the outer medulla (55), would secondarily affect receptors locally and throughout the renal cortex (55) in an autocrine or paracrine fashion to induce hemodynamic and growth signals. Looking at the stimulatory effect of sodium on kidney growth after injury (56), Finn and Yang recently suggested the presence of a circulating factor modified by salt intake and able to influence tubular epithelial cell growth. Studies of the effects of dietary salt upon renal tissue and circulating IGF and/or other growth factors would be of great interest.

As previously reported (39), regardless of salt intake, mTAL cell volume in short-looped nephrons was consistently larger than in long-looped nephrons. Bankir has suggested (personal communication) that epithelial cell size gradually decreases as the thick limb ascends from its origin. Short-looped mTAL would, therefore, be larger at any level simply because the cells are closer to the start of the segment.

In conclusion, chronic salt depletion inverts the profile of renal tissue oxygenation. Cortical $P_{O_2}$ falls, while medullary hypoxia is ameliorated. This process maintains inner stripe oxygenation under conditions that would otherwise have resulted in substantial hypoxic injury. The increase in medullary $P_{O_2}$ may
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result from the redistribution of RBF and the reduction of oxygen requirements, in part due to reduced mTAL mass.

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