Renal angiotensin II concentration and interstitial infiltration of immune cells are correlated with blood pressure levels in salt-sensitive hypertension

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A SERIES OF INVESTIGATIONS have documented that infiltration of immune cells, oxidative stress, and increased numbers of infiltrating inflammatory cells and proximal tubular cells that express angiotensin II (AII-positive cells), are present in the kidney in experimental models of salt-sensitive hypertension (SSHTN). The functional relevance of these findings is indicated by a series of experiments that demonstrate that suppression of the inflammatory infiltrate or oxidative stress results in amelioration or prevention of hypertension (reviewed in 17, 26). It is well known that inflammatory reactivity is inextricably linked with oxidative stress, as these conditions induce and maintain one another (13); however, the participation of intra-

renal AII activity as an integral part of the tubulointerstitial inflammatory reactivity in the pathophysiology of SSHTN has not been investigated. AII-positive cells have been identified in the models of salt-sensitive hypertension and double staining studies have shown that the cells staining positive for AII are both proximal tubular cells and infiltrating immune cells (1, 12, 14). On the basis of this evidence, we have postulated that in models of SSHTN, the tendency to salt retention is driven by intrarenal inflammation, oxidative stress, and AII activity (13, 17), and results from other laboratories have confirmed that intrarenal inflammation plays a role in the pathogenesis of hypertension in several experimental conditions (5, 8, 10, 24).

The relevance of the intrarenal AII activity in the pathogenesis of hypertension has been emphasized in the experiments of Navar and his associates (reviewed in Ref. 7), who have elegantly shown that intrarenal AII functions as a separate compartment that is not physiologically modulated by influences, such as plasma volume expansion, which reduce plasma AII levels (9). Investigations from our group in the cellophane-wrapped Page kidney model (25) demonstrated the critical role of interstitial inflammation and intrarenal, rather than plasma, AII activity in the long-term maintenance of hypertension, and more recently, micropuncture studies (3) showed that interstitial inflammation is likely responsible for the cortical vasoconstriction that is present in salt-sensitive hypertension. While the accumulated evidence is compelling, a demonstration that increased concentration of AII in the kidney is, in fact, correlated with the severity of hypertension, with the increased numbers of AII-positive cells and with immune cell infiltration has been lacking. Furthermore, concomitant evaluation of the renal and the plasma AII concentration in relation to the blood pressure levels has not been made in experimental models of SSHTN.

Therefore, the present work was done to define the following questions: 1) What is the relationship between blood pressure, renal AII, and plasma AII concentrations in the salt-driven hypertension that follows AII infusion?; and 2) Is there an association between the concentration of AII in the kidney, the number of AII-positive cells, and the severity of the inflammatory infiltrate?

Our studies demonstrate that blood pressure levels are negatively correlated with plasma AII and positively correlated with renal AII concentrations. In addition, we found that renal...
AII concentration is correlated with the number of AII-positive cells and infiltrating immune cells in tubulointerstitial areas of the kidney in this experimental model.

METHODS

Male Sprague-Dawley rats from the animal facilities of the Instituto Nacional de Cardiología (Mexico, D.F) weighing (340–360 g) were used in the experiments. The animals were housed and handled in accordance with institutional guidelines of animal care. We discuss below the groups of rats studied.

Post-AII infusion salt-sensitive hypertension. The salt-sensitive hypertension (AII-SSHTN) group (n = 10) was studied following a protocol used in previous publications from our group (4, 14). Briefly, AII (Sigma, St. Louis, MO) was infused during 2 wk by subcutane-
ously implanted osmotic pumps (Alzet model 2002, Alza Corp., Palo Alto CA) at a rate of 435 ng/kg/min. At the end of 2 wk, pumps were withdrawn, and after a period, a washout of 5 days on a normal sodium diet (0.4% NaCl), they were placed in a high-salt diet (4% NaCl, Harlan) for 5 wk. At the end of this period, the rats were prepared for the studies of interstitial AII concentration by microdi-
alysis as described afterward, and when those studies were concluded, kidneys were harvested for immunohistology (see later).

Mycophenolate mofetil-treated AII-infused rats. Rats in the myco-
phenolate mofetil group (AII-MMF, n = 11) received the AII infusion, as described in the AII-SSHTN group above, and they also received MMF (30 mg·kg⁻¹·day⁻¹ by gastric gavage) during the 2-wk period of AII infusion, as described in earlier studies (4, 14). The rest of the experiment was similar to the AII group described above, including the washout period and the 5 wk of a high-salt diet.

Control rats with a high-salt diet. Rats in the control-high salt diet (c-HSD) group (n = 10) were housed in similar conditions and were fed a 4% salt diet for 5 wk.

Control rats with normal salt diet. Rats in the normal salt diet control group (c-NSD, n = 11) were housed in similar conditions and were given a normal (0.4%) salt diet for 5 wk before the studies.

Additional experiments were done to determine AII renal and plasma concentrations after the AII infusion was discontinued and before the induction of SSHTN with the administration of a HSD. These experiments were done 5 days after removal of the osmotic minipumps in 10 additional rats of the AII-SSHTN group and in nine additional rats of the AII-MMF group. Control studies were done in eight rats with NSD.

Blood pressure. Systolic blood pressure (SBP) measurements were performed at baseline and every 2 wk in conscious, restrained rats by tail-cuff plethysmography (Narco Biosystems, Austin, TX). Before the studies, all rats were conditioned to the procedure as described in previous communications (4, 14). Determinations of the SBP at the time when the studies were done were used in the correlation analyses.

Plasma and renal AII determinations. For determinations of renal AII, rats were prepared as for micropuncture studies, as described in previous communications (3, 4, 21). Rats were anesthetized with pentobarbital sodium (30 mg/kg ip); supplementary doses were ad-
ministered as required. The rats were placed on a thermoregulated table, and the temperature was maintained at 37°C. The left kidney was exposed and placed in a Lucite holder, and mean arterial pressure was continuously monitored with a pressure transducer placed in the femoral artery.

Renal AII concentration was determined in the renal cortex by a microdialysis technique, as described by Nishiyama et al. (9), Fox et al. (2), and Siragy et al. (23), as well as reported by our group in a previous communication (3). Preliminary studies were done to test the microdialysis probe in vitro, testing AII concentrations from 2 to 10 nMol, and correlation coefficient was $r^2 = 0.94$. At a perfusion rate of 1.5 ml/min, the relative equilibrium rate was 66 ± 2%, which did not deteriorate with time. In vivo calibrations were performed from 0 to 180 min to test the stability of the AII concentrations, according to the method described by Nishiyama et al. (9). Thirty minutes after implantation of the probe, the concentrations of AII reach a steady state and remain stable up to 180 min. Previous studies have deter-
mined that AII derived from tubular fluid does not contaminate the dialysate (9).

For determination of AII, a 10-mm polysulfone microdialysis probe (Fresenius Medical Care, San Mateo, CA), with a 33,000-D trans-
membrane diffusion cut-off was implanted into the renal superficial cortex. Steel needles were inserted in both ends of the fiber. The inflow of the probe was connected with a polyethylene tubing (PE-10) to a microinfusion pump (Harvard Apparatus, Holliston, MA). The probe was perfused with Ringer’s solution containing 1% bovine serum albumin (pH 7.4), at a rate of 1.5 μl/min. The dialysate was collected directly from the outflow steel tubing into a tube containing a solution of inhibitors (30 μl of 500 mM EDTA, 15 μl of 1 mM enalaprilat, and 30 μl of 125 M o-phenanthroline and 0.2 mM pepstatin A in 95% ethanol), to allow the dialysate effluent to be immediately mixed with the inhibitors. After 90 min of the microdialysis probe implantation, three 60-min sample collections were taken. Immedi-
ately thereafter, each sample was vortex-mixed, and 100 μl of the solution was transferred to a tube containing 1 ml chilled 100% methanol. Blood was collected at the end of the experiment into a prechilled syringe containing the inhibitors solution. After centrifugation at 4°C plasma was separated.

All assay. Determination of plasma and interstitial fluid AII by enzyme-linked competitive immunoassay using a commercial Kit (SPL Bio; Bertin Group, Montigny-le-Bretonneux, France), following the procedure recommended by the manufacturer. All assays were carried out by one researcher to avoid interobserver variability.

The dialysate samples were reconstituted with 100 μl of 0.9% saline solution and extracted with methanol. Methanol was evaporated to dryness with nitrogen; samples were reconstituted in assay buffer, extracted in phenyl cartridges, and assayed in duplicate using an 8-point standard curve (125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.625 pg/ml, 7.81 pg/ml, 3.9 pg/ml, 1.95 pg/ml, and 0.98 pg/ml). Absor-
bance at 415 nm was determined in a microplate reader (Bio-Rad; Hercules, CA). When all concentration levels of the sample were above 125 pg/ml in the angiotensin standard curve, dilutions (1:5, 1:10, and 1:25) were done to obtain lectures between 7.81 and 62.5 pg/ml.

Cross-reactivity with the following angiotensin peptides was deter-
ned: (A1-9; Bachem, H-5038.0025), AII (Bachem, H-1705.0025), A1-7 (Bachem, H-1715.0025), and AI/II3-8 (Bachem, H-8125.0025). A 100 pg/ml standard was prepared for each peptide. Readings were done at 415 nm. Cross-reactivity results were as follows: AI = <0.01%; AII = 99.9%; A1-7 = <0.01%, and A3-8 = 34.2%. Cross-reactivity with AIII (H-Phe-Arg-Val-Tyr-Ile-His-Pro-Phe-OH) was not tested because reagent was unavailable but is assumed to be 100% (Bachem Online Catalog) Results obtained in our laboratory shown above were similar or better than those reported by the manufacturers of the commercial kit used in these studies (SPL Bio, Bertin Group).

Recovery using [3H]-labeled AII was 88 ± 16% in six samples (range from 82.16 to 94.23%). The intra- and interassay variations (three different assays each one in duplicate) were 3.93 ± 0.56 and 0.56 and 10.7 and 0.58, respectively. The minimal detectable concentration was 0.99 pg/ml.

Histological studies. Coronal sections were used for histological studies. The fragments were fixed in 10% formalin and embedded in paraffin. Immunoperoxidase methodology was used to identify lymphocytes (CD5-positive cells), macrophages (ED1 positive cells), as detailed previously (11). Cellular infiltration was evaluated sepa-
rately in the glomeruli and in tubulointerstitial areas and expressed as positive cells per glomerular cross section (gcs) or positive cells per square millimeter, respectively.

The primary antibodies used were anti-CD5 (mouse monoclonal anti-rat thymocytes and lymphocytes) and anti ED-1 (mouse monoclonal anti-rat monocytes and macrophages), purchased from Bio-
source International (Camarillo, CA). Rabbit anti-human AII antiserum with cross-reactivity to rat AII (Peninsula Laboratories) was used to identify AII-positive cells. Specificity of the AII staining was validated by the demonstration that staining was prevented by preincubating the antibody with human AII, as described in a previous communication (15). Secondary biotin-conjugated affinity-pure antibodies with minimal reactivity to rat serum proteins were purchased from Accurate Chemical and Scientific (Westbury, NY). Nonrelevant antibodies were used for negative control studies. Immunohistochemical techniques have been reported previously (1, 12, 15, 16).

Immunohistology was evaluated with an Olympus BX51 System Microscope and DP70 microscope Digital camera, with Image Analysis software of Sigma Pro (Leesburgh, VA), as described in previous communications (1, 15, 16). Immunohistological analyses were done without previous knowledge of the experimental group of the biopsy or the results of other determinations.

**Statistical analysis.** Differences between groups were examined with multigroup ANOVA and Tukey-Kramer post hoc tests. Correlations between variables were analyzed with Pearson’s tests. Data are shown as mean ± SD. Two-tailed P values <0.05 were considered statistically significant.

**RESULTS**

The mean values of plasma AII, renal AII concentration, cellular immune infiltration in tubulointerstitial areas and the SBP in the experimental groups are shown in Table 1. CD5 + cells and ED1 + cells were rare (0–2/gcs), and AII + cells were not found in the glomeruli in all the experimental groups that were studied.

As shown in Table 1, the experimental groups covered a range of blood pressure that went from mean values of 160 mmHg in the SSHTN group to 118 mmHg in the c-NSD group, and all the experimental and control groups had mean blood pressure levels significantly different from one another. Correspondingly, the mean values of plasma and renal AII were significantly different from one another in all experimental groups. The infiltrating CD5 (lymphocytes) and ED1 (macrophages) cells and AII-positive cells in tubulointerstitial areas were similar in the AII-MMF group and the c-HSD groups that had blood pressures of 140 mmHg and 129 mmHg, respectively and significantly different (P < 0.001) in the SSHTN group and c-NSD groups compared between themselves and to the AII-MMF and c-HSD groups (Table 1).

The plasma levels of AII were negatively correlated (P < 0.0001) with the blood pressure (Fig. 1A) in sharp contrast with the positive correlation (P < 0.0001) that existed between renal AII and blood pressure (Fig. 1B). Positive correlations also existed between CD5-positive cells and blood pressure (Fig. 2A) and ED1-positive cells and blood pressure (Fig. 2B). Renal AII concentration was correlated with the intensity of the inflammatory ED1 infiltration (Fig. 3A) and with the number of tubulointerstitial cells that stained positive for AII (Fig. 3B). All of these correlations were highly significant (P < 0.0001). A representative microphotograph of AII-positive cells in the biopsy of a rat in the SSHTN group is shown in Fig. 4. As

<table>
<thead>
<tr>
<th></th>
<th>SSHTN Group (n = 11)</th>
<th>AII-MMF Group (n = 10)</th>
<th>c-HSD Group (n = 10)</th>
<th>c-NSD Group (n = 11)</th>
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<tbody>
<tr>
<td>SBP, mmHg≤8</td>
<td>160±7.24</td>
<td>140±8.36</td>
<td>129±4.74</td>
<td>118±5.64</td>
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<td>Plasma AII, pg/ml≤9</td>
<td>2.37±1.38</td>
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<td>40.74±3.12</td>
<td>114.4±54.4</td>
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<td>Renal AII, pg/ml≤13</td>
<td>1310±208.8</td>
<td>454±128.2</td>
<td>154.95±91.0</td>
<td>121±30.0</td>
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<td>AII +, cells/mm²≤10</td>
<td>51.9±7.78</td>
<td>25.7±4.80</td>
<td>25.49±2.42</td>
<td>10.9±2.17</td>
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<td>CD5 +, cells/mm²≤10</td>
<td>61.4±9.43</td>
<td>30.2±4.31</td>
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<tr>
<td>ED1 +, cells/mm²≤10</td>
<td>34.6±5.73</td>
<td>18.9±2.86</td>
<td>24.18±3.64</td>
<td>10.7±1.40</td>
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SBP, systolic blood pressure; SSHTN, salt-sensitive hypertension; AII-MMF, angiotensin II mycophenolate mofetil; c-HSD, control high-salt diet; c-NSD, control normal salt diet. AII + cells, lymphocytes (CD5 + cells) and macrophages (ED1 + cells) are given as positive cells for mm² in cortical tubulointerstitial areas. Plasma AII determinations were done in 9 rats from the AII group, 8 rats from the AII-MMF group, 6 rats from the c-HSD group and 9 rats from the c-NSD group. *Experimental groups are P < 0.01 or P < 0.001 versus one another. **Experimental groups are P < 0.01 to P < 0.001 versus one another. Experimental groups are P < 0.001 versus one another except for AII-MMF and HSD. *Experimental groups are P < 0.001 versus one another except for AII-MMF versus HSD that are P < 0.05.

Fig. 1. Negative correlation between plasma angiotensin II (AII) (log 2 scale) and systolic blood pressure in salt-sensitive hypertension (SSHTN; A) contrasting with the positive correlation present between blood pressure and renal AII concentration (B; log 2 scale). HSD, high-salt diet; NSD, normal salt diet; AII-MMF, AII-mycoconenate mofetil.
shown in the figure, both infiltrating cells and proximal tubular cells stain positive for AII in a pattern that includes cell membrane and cytoplasm.

Table 2 shows SBP and plasma and interstitial AII 5 days after AII infusion was discontinued (osmotic minipumps removed) and before the induction of SSHTN with a high salt diet. At this time, SBP and plasma AII had returned to normal, whereas renal AII remains high in the AII-SSHTN group. In contrast, the AII-MMF group presents renal AII concentration that is not significantly different from the control group.

DISCUSSION

We had previously shown that experimental salt-sensitive hypertension is associated with inflammatory infiltration and increased numbers of AII-positive cells in tubulointerstitial areas of the kidney (1, 3, 12, 14, 16). As in previous studies, we did not find AII-positive cells within the glomerular tuft, despite the fact that AII may be produced in the glomerulus, albeit in lesser quantity than in tubulointerstitium. The present studies demonstrated a relationship between increased intrarenal AII concentration, the number of tubulointerstitial AII-positive cells, and the severity of the inflammatory infiltration in the post-AII SSHTN model. The relationship between AII-positive cells and AII activity in interstitial fluid raises the possibility that these cells are potential sources of interstitial AII. Because both proximal tubular cells and immune cells are capable of producing AII, it is conceivable that AII produced by infiltrating cells and by proximal tubular cells may be released in the interstitial fluid. This possibility needs to be addressed in subsequent studies. However, it should be noted that AII concentration in the kidney is modulated by physiological mechanisms independent of interstitial inflammation, and therefore, conclusions based on the correlation with the immune cell accumulation are applicable only to the post-AII model of SSHTN.

Determinations of interstitial AII concentrations in the rats after the infusion of angiotensin confirmed previous studies that showed that renal AII is increased after exogenous angiotensin administration (27, 29). In fact, the values found 5 days after AII infusion was stopped (Table 2), are higher than the values found 5 wk later, during the period of SSHTN (Table 1). From these data, it is apparent that normal blood pressure and high concentrations of renal AII coexist in the post-angiotensin infusion period. Increased renal angiotensin concentration appears to be relevant to hypertension specifically in the context of a HSD when a tendency to sodium retention would result in SSHTN. Relevant to this issue, Kobori et al. (6) showed that intrarenal angiotensinogen production is markedly increased in Dahl salt-sensitive hypertensive rats, suggesting that augmentation of renal angiotensin activity may be involved in salt-dependent hypertension. Of note, MMF treatment during the administration of exogenous angiotensin prevented the increment in renal AII concentration induced by exogenous AII (Table 2). The present studies do not allow discerning whether MMF is suppressing AII production directly or as a result of the drug’s anti-inflammatory effects.

To study a wide range of values, we choose to study experimental groups in which we knew from previous studies (4, 14) that the immune cell infiltration, the number of AII-

![Fig. 2. Systolic blood pressure is positively correlated with lymphocyte infiltration (CD5 + cells; A) and macrophage infiltration (ED1 + cells; B) in tubulointerstitial areas.](#)

![Fig. 3. Tubulointerstitial inflammation, as represented by macrophage infiltration (ED1 + cells), is correlated with renal AII concentration (A). Correlation between the renal AII concentration and the number of cells staining positive for AII in tubulointerstitial areas (B).](#)
was increased AII concentration in the kidney (Table 2). It is in fact, 5 days after the AII infusion was discontinued, there that AII infusion results in increased renal AII (20, 27, 28), and before the studies were done. Other investigators have shown potential direct effects of MMF on the experimental variables AII infusion, prior to the SSHTN phase. In this manner, significantly reduced by MMF treatment administered during (AII infusion model) and that all of these characteristics were positive cells, and blood pressure were significantly increased (AII infusion model) and that all of these characteristics were significantly reduced by MMF treatment administered during AII infusion, prior to the SSHTN phase. In this manner, potential direct effects of MMF on the experimental variables were unlikely since the drug was stopped more than 5 wk before the studies were done. Other investigators have shown that AII infusion results in increased renal AII (20, 27, 28), and in fact, 5 days after the AII infusion was discontinued, there was increased AII concentration in the kidney (Table 2). It is undefined for how long the intrarenal AII remains elevated after AII administration in the absence of a high-salt diet, but since AII infusion had been stopped 40 days before the SSHTN studies were conducted (5 days of washout period plus 5 wk of high-salt diet), it seems unlikely that intrarenal AII levels would be solely due to a carried-over effect of exogenous angiotensin administration. MMF-treated rats were exposed to similar amounts of exogenous AII but had significantly less intrarenal AII prior to and after SSHTN developed. Consequently, the findings in our studies are likely independent of both exogenous AII and MMF.

The observation that plasma AII was suppressed, whereas intrarenal AII was high in SSHTN does not completely rule out the possibility that plasma AII is not participating in this model, as it may not be suppressed as much as it should be from the effects of the high-salt diet and elevated blood pressure. Indeed, Sealey et al. (22) have proposed that intrarenal microvascular disease (which occurs in this model) may lead to uneven renal perfusion (nephron heterogeneity) that may result in some nephrons to overproduce renin, whereas others may show an opposing effect. While we did not measure renin levels, the observation that rats with SSHTN had lower (and not higher) plasma AII than c-HSD rats suggests that plasma AII was even more suppressed in SSHTN. Furthermore, the linear negative correlation between plasma AII and blood pressure (Fig. 1) would also argue against a prohypertensive role of incomplete suppression of plasma AII, because if such were the case, one would expect that the plasma AII and blood pressure levels in the SSHTN rats would be positively correlated.

The results of the present studies are in agreement with previous investigations from our group, mentioned before, and others (5, 8, 10, 24), showing an association between immune cell infiltration and the development of SSHTN. In fact, a recent authoritative review suggests that the beneficial effects of AII blockers may be related in part to anti-inflammatory effects (19). To be noted, the associations of blood pressure levels with the intensity of the inflammatory infiltrate and the numbers of AII-positive cells are not evident in the middle range of the findings. As shown in Table 1, the AII-MMF group and the c-HSD group do not differ significantly in the number of infiltrating cells or in the number of AII-positive cells and, nevertheless, these groups have significantly different mean blood pressure levels (140 ± 8.4 mmHg and 129 ± 4.7 mmHg, respectively, P < 0.001 in Table 1). Consequently, other factors are also playing a role in the development of SSHTN, and it is reasonable to consider that these factors are also responsible for driving the immune infiltrate from the low levels found in c-NSD to the high levels found in the SSHTN.

Table 2. SBP and AII levels 5 days after AII infusion and before the induction of salt-sensitive hypertension

<table>
<thead>
<tr>
<th></th>
<th>All Group (n = 10)</th>
<th>All + MMF Group (n = 9)</th>
<th>cNSD Group (n = 8)</th>
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</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>138.1 ± 20.8</td>
<td>126.7 ± 12.9</td>
<td>126.0 ± 7.35</td>
</tr>
<tr>
<td>Plasma AII, pg/ml</td>
<td>115.1 ± 48.4 (9)</td>
<td>111.8 ± 23.4</td>
<td>116.3 ± 42.0</td>
</tr>
<tr>
<td>Renal AII, pg/ml</td>
<td>1637 ± 345.0 (8)</td>
<td>359.7 ± 153.2 (8)</td>
<td>147.8 ± 107.2</td>
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</table>

***P < 0.001 versus All + MMF and cNSD. Renal AII in the All group is also higher (P < 0.05) than the value found at the end of the SSHTN period shown in Table 1. Numbers in parenthesis indicate the number of animals studied when it was different from that shown for the total group.

Fig. 4. Microphotographs showing angiotensin-positive cells that are both proximal tubular cells (arrowheads), as well as infiltrating interstitial cells (arrows). Positive staining is observed in cell membranes and cytoplasm. Increased numbers of AII-positive cells are present in a biopsy from the SSHTN group (A) in contrast with the near absence of these cells in a biopsy of a rat from the NSD group (B). (immunoperoxidase technique, original magnification ×400).
REFERENCES

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DISCLOSURE

Dr. Johnson is a consultant for Nephromics, Inc.

REFERENCES