Kidney

Isoforms and Functions of NAD(P)H Oxidase at the Macula Densa

Rui Zhang, Pamela Harding, Jeffrey L. Garvin, Ramiro Juncos, Ed Peterson, Luis A. Juncos, Ruisheng Liu

Abstract—Macula densa cells produce superoxide (O$_2^-$) during tubuloglomerular feedback primarily via NAD(P)H oxidase (NOX). The purpose of the present study was to determine NOXs expressed by the macula densa and the role of each one in NaCl-induced O$_2^-$ production. To identify which isoforms are expressed, we applied single-cell RT-PCR to macula densa cells isolated by laser capture microdissection and to MMDD1 cells (a macula densa-like cell line). The captured cells expressed neuronal NOS (marker of macula densa), NOX2, and NOX4 but not NOX1. Expression of the NOXs and neuronal NOS was essentially identical in the MMDD1 cells. Thus, we used MMDD1 cells to investigate which isoform is responsible for NaCl-induced O$_2^-$ production. We used small-interfering RNA to knock down NOX2 or NOX4 in MMDD1 cells and measured O$_2^-$ exposed to low-salt solution (LS; 70 mmol/L of NaCl) or high-salt solution (HS; 140 mmol/L of NaCl). Exposing control cells (scrambled small-interfering RNA) to HS increased O$_2^-$ concentrations from 0.75±0.28 to 1.48±0.46 U/min per 10$^5$ cells in LS and HS, respectively (P<0.001). Inhibiting NOX2 blocked the HS-induced increase in O$_2^-$ (0.62±0.39 versus 0.76±0.31 U/min per 10$^5$ cells in LS and HS groups, respectively). Blocking NOX4 did not affect HS-induced O$_2^-$ levels. O$_2^-$ levels in the control cells during LS and HS were 0.80±0.30 and 1.56±0.49 U/min per 10$^5$ cells, respectively (P<0.001); whereas O$_2^-$ levels in NOX4-small-interfering RNA-treated cells during LS and HS were 0.40±0.25 and 1.26±0.51 U/min per 10$^5$ cells, respectively (P<0.001). We conclude that, whereas macula densa cells express the NOX2 and NOX4 isoforms, NOX2 is primarily responsible for NaCl-induced O$_2^-$ generation. (Hypertension. 2009;53:556-563.)

Key Words: NAD(P)H oxidase ■ superoxide ■ macula densa ■ tubuloglomerular feedback

Macula densa cells are modified epithelial cells located at the end portion of the thick ascending limb, at the hilus of its own glomerulus, where they are in close contact with the glomerular arterioles. In response to changes in luminal NaCl delivery, they initiate signaling pathways that adjust glomerular filtration rate (a process called tubuloglomerular feedback [TGF]) and regulate renin release. These 2 processes are among the most important mechanisms that regulate the renal microcirculation and sodium excretion. However, which isoforms of NAD(P)H oxidase (NOXs) are produced at the macula densa and the function of each isoform are unknown. Performing such studies has been difficult, because macula densa cells represent a very small percentage of renal cells and are located in very small clusters. However, the recent development of laser capture microdissection (LCM),15-17 a novel technique that facilitates separating and harvesting of specific cells, now allows us to isolate and study specific renal cells, including macula densa cells.

In the present study, we isolated macula densa cells from frozen rat kidney using LCM. We identified the NOXs expressed by these captured macula densa cells and compared response to increased NaCl delivery.6,13 Macula densa-derived O$_2^-$ can modulate single nephron glomerular filtration rate during infusion of angiotensin II,14 and we reported recently that O$_2^-$ in the macula densa augments the TGF response, primarily by scavenging NO.6 Accordingly, O$_2^-$ is an important modulator of TGF, renal hemodynamics, and sodium excretion. However, which isoforms of NAD(P)H oxidase (NOXs) are produced at the macula densa cells and the function of each isoform are unknown. Such studies have been difficult, because macula densa cells represent a very small percentage of renal cells and are located in very small clusters. However, the recent development of small interfering RNA technology that facilitates separating and harvesting of specific cells, now allows us to isolate and study specific renal cells, including macula densa cells.

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them with those expressed by a macula densa-like cell line (MMDD1). In addition, we investigated which isoform is responsible for NaCl-induced \( \text{O}_2^- \) generation in the macula densa. We found that captured macula densa and MMDD1 cells express NOX2 and NOX4 and that the NOX2 is the isoform primarily responsible for NaCl-induced \( \text{O}_2^- \) production by the macula densa.

**Materials and Methods**

All of the experiments were approved by the Henry Ford Hospital Animal Care and Use Committee before performing any procedures on animals. Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the Guidelines of the Animal Welfare Act. Experiments were undertaken on renal tissue obtained from male Sprague-Dawley rats and in MMDD1 cells, a renal epithelial cell line with properties of macula densa cells (kindly provided by Dr J. Schnermann, National Institutes of Health). All of the chemical compounds were purchased from Sigma, except DMSO and lucigenin, which were obtained from Invitrogen.

**LCM of Macula Densa Cells**

Sprague-Dawley rats weighing between 100 and 120 g were anesthetized with ketamine (50 mg/kg IP) and xylazine (50 mg/kg IP). The abdomen was opened and the kidneys removed. Sections of kidney were snap frozen in optimal cutting temperature medium and lucigenin, which were obtained from Invitrogen.

**MMDD1 Cells**

We used MMDD1 cells, a renal epithelial cell line with properties of macula densa cells, developed and kindly supplied by Dr J. Schnermann (National Institutes of Health). These cells were derived from SV40 transgenic mice and acquired using fluorescence-activated cell sorting of renal tubular cells labeled with segment-specific lectins. This cell line has been shown to express well-known macula densa markers, eg, cytochrome oxidase 2 (COX-2), nNOS, ROMK, and NKCC2. In the present studies, MMDD1 cells at passages 15 to 20 were routinely trypsinized and suspended in DMEM nutrient mixture-Ham’s F-12, supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 \( \mu \)g/mL). The cells were plated onto culture dishes and incubated at 37°C in a humidified atmosphere of 95% room air-5% \( \text{CO}_2 \). The medium was changed every 2 days, and once the cells reached confluence (typically in 3 to 4 days), the cells were ready for small-interfering RNA (siRNA) and \( \text{O}_2^- \) experiments.

**RT-PCR for Macula Densa Cells Isolated by LCM**

We used the single-cell RT-PCR kit (Ambion), as described below. Despite the potential for measuring mRNA from single cells, our pilot experiments suggested that \( \geq 20 \) cells captured from the frozen slide were required to extract enough mRNA for RT-PCR. Total RNA was extracted using a Picopure RNA isolation kit (Arcturus) according to the manufacturer’s instructions. Five microliters of RNA were reverse transcribed for 30 minutes at 45°C using 50 \( \mu \)mol/L of random primers (Invitrogen) and a MessageSensor reverse transcription (RT) kit (Ambion) and then heated for 10 minutes at 95°C and subsequently placed on ice. The resultant RT product was then amplified by PCR using the following protocol. Five \( \mu \)L of RT product and 0.5 \( \mu \)mol/L of gene-specific primers were added to 1 U of SuperTag (Ambion) and heated to 95°C for 5 minutes. The samples were then cycled 40 times as follows: 15 seconds at 95°C, 30 seconds at 58°C, and 1 minute at 72°C. The final extension was for 10 minutes at 72°C.

**RT-PCR for MMDD1 Cells**

Total RNA was extracted using the RNeasy Micro kit (Qiagen) according to the manufacturer’s instructions. Briefly, 0.5 \( \mu \)g of total RNA were reverse transcribed for 1 hour at 37°C using 10 \( \mu \)mol/L of random primers (Invitrogen) and an Omniscript RT kit (Qiagen). The resultant RT product was then amplified by PCR by adding 5 \( \mu \)L of the RT reaction and 1 \( \mu \)mol/L of the gene-specific primers to the PCR Master Mix kit (Promega). The mixed samples were then heated to 94°C for 5 minutes and cycled at 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 1 minute for 35 cycles. Final extension was for 10 minutes at 72°C.

The amplified products of the single-cell RT-PCR and MMDD1-RT-PCR were run on 1.5% agarose gels containing ethidium bromide (0.5 \( \mu \)g/mL) and visualized under UV light. GAPDH, as a housekeeping gene, was set up as an internal loading control. All of the steps for RT-PCR are the same according to the manufacturer’s instruction. Samples that were not reverse transcribed were used as a negative control, and samples from kidney cortex were used as a positive control. A 100-bp DNA ladder marker was used to identify the molecular weight of the targeted DNA. Primer sequences, expected band, and GenBank No. are listed in the Table.

**Preparations for siRNA**

All of the siRNAs were designed and synthesized by Santa Cruz Biotechnology. siRNA transfection was performed using a siRNA Reagent System (Santa Cruz Biotechnology) according to the manufacturer’s instructions. Scrambled siRNAs were synthesized and

### Table. Primer Sequences, Expected Band, and GenBank Nos

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>GenBank No.</th>
<th>PCR Length, bp</th>
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<tr>
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<tr>
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</table>

F indicates forward; R, reverse.
used as negative controls. To achieve optimal transfection efficiency, various parameters, including the amounts of transfection reagent, RNA, and trans-mRNA complexes, the cell density, and the length of exposure of cells to trans-mRNA complexes, were optimized. At 24 hours before transfection, MMDD1 cells were transferred onto 6-well plates (5×10⁵ cells per well) and transfected with 0.8 μg each of siRNA duplex using trans-mRNA transfection reagent for 4 hours in medium devoid of serum and antibiotics. This procedure does not affect cell viability. Macula densa cells were then washed once with PBS and grown in complete medium. Gene silencing was monitored by measuring RNA after incubation for 24 to 72 hours.

Measurement of O₂⁻ With Lucigenin

We measured O₂⁻ production in the MMDD1 cell line using a lucigenin-enhanced chemiluminescence assay, as described previously. Briefly, confluent MMDD1 cells were rinsed twice in PBS solution. The cells were then trypsinized and suspended in 5 mL containing either high- or low-NaCl solutions. The high-NaCl solution contained (in mmol/L): 140 NaCl, 10 HEPES, 1.0 CaCO₃, 0.5 K₂HPO₄, 4.0 KHCO₃, 1.2 MgSO₄, 5.5 glucose, 0.5 Na acetate, and 0.5 Na lactate (pH 7.4). The low-NaCl solution was the same as high-NaCl solution except that the NaCl was reduced to 70 mmol/L and mannitol was used to maintain the same osmolarity as the high-NaCl solution. Lucigenin (5 μmol/L) was added to each of the samples, which was then placed in a 1.6-mL polypropylene 8×50-mm tubes (Evergreen Scientific). After allowing the samples to equilibrate with lucigenin at 37°C for 15 minutes, the tubes were placed in a luminometer (TD-20e; Turner Designs) with the light chamber maintained at 37°C. Luminescence measurements were integrated for 30-second periods and the cycle repeated 9 times, averaging 10 values. At the end of each experiment, the cell-permeant O₂⁻ scavenger Tiron (10 mmol/L) was added and 15 more cycles read; the final 8 values were averaged. O₂⁻ was expressed as units per minute per 10⁵ cells. All of the O₂⁻ measurements were performed in the presence of 10⁻⁴ mmol/L N-nitro-L-arginine methyl ester, a NOS inhibitor, to eliminate O₂⁻ quenching by NO.

Statistics

We used a 2-way ANOVAs to assess whether a NOX or the level of salt affected O₂⁻ production and whether the NOX affects salt-induced changes. The design had 2 main effects, NOX and salt concentration, and one 2-way interaction. If the interaction was significant, we checked for salt effects using paired t-tests on each isoform separately and for NOX effects using Student t-tests on each salt concentration separately.

The examination of 3 repeated measures was accomplished with ANOVA for repeated measures. The interest in this analysis was primarily directed at the 3 pairwise comparisons. These were done using paired t-tests with a Hochberg’s adjustment for multiple testing. Data are expressed as the means plus or minus the SE, and an adjusted P value <0.05 was considered significant.

Results

NOXs Expressed in LCM-Captured Macula Densa and MMDD1 Cells

We first demonstrated the feasibility of isolating and capturing macula densa cells using LCM. Figure 1 shows a representative example of a glomerulus with its macula densa, before and after the macula densa has been captured with LCM; the isolated macula densa cells are shown in Figure 1C. As seen in the figure, macula densa cells were readily identifiable by their anatomic location and morphology. These captured cells expressed nNOS (see below and Figure 2) but not endothelial NOS (expressed by the thick ascending limb and vasculatures), thus further confirming that the captured cells were macula densa cells and not contaminated by surrounding cells.

We next used RT-PCR techniques to identify which NOXs are expressed by the macula densa cells. Figure 2 shows representative blots for the NOX1, NOX2, and NOX4 isoforms, as well as for nNOS, in renal cortex, laser-captured macula densa cells, and MMDD1 cells. Figure 2A shows that the macula densa cells collected using LCM clearly expressed the NOX2 and NOX4, as well as nNOS (they did not express NOX1), indicating that NOX2 and NOX4 are the main isoforms present in macula densa cells.

Because the MMDD1 cell line may have some differences with macula densa cells isolated from the in vivo renal cortex, yet we would be using this cell line to study the function of each NOX in these cells, we tested whether the MMDD1 cells expressed the same NOXs as the laser-captured macula densa cells. The representative blot depicted in Figure 2B shows that the expression profile for the NOXs and nNOS in the MMDD1 cells was essentially identical to that of the laser-captured macula densa cells. Thus, these results demonstrate that the laser-captured macula densa cells and MMDD1 cells exhibit the same NAD(P)H oxidase expression profile, and together they provide strong evidence that macula densa cells express the NOX2 and NOX4 isoforms. Figure 2C shows that the renal cortex expressed all 3 of the NOXs and nNOS, thus verifying the efficacy of all of our primers to detect the NOXs and nNOS.

Figure 1. Isolating macula densa cells with LCM. A, Macula densa cells were identified by their anatomic location and morphology with the LCM microscope in a frozen slide of kidney cortex from a Sprague-Dawley rat. B, Macula densa cells were captured with LCM, using a beam width of 7.5 μm and a beam intensity of 50 mW. C, Captured macula densa cells on the cap.
Comparative Function of Macula Densa–Derived NOX2 and NOX4

To study the functions of individual NOXs, we used siRNA to knock down the NOX2 and NOX4 mRNA in the MMDD1 cell line. We first determined the efficacy of the siRNA in reducing their target NOX mRNA. Figure 3A shows a representative blot for NOX2 mRNA in MMDD1 cells with the siRNA-NOX2 or the scramble siRNA control, whereas Figure 3B shows an analogous blot for NOX4 mRNA. The bottom graphs in this figure show the corresponding densitometric data. Incubating the MMDD1 cells for 48 hours with 0.8 μg of siRNA duplex transfection reagent was very effective at knocking down the mRNA of its intended target; the siRNA-NOX2 knocked down NOX2 mRNA by 91±0.5%, NOX2 mRNA knocked down NOX4-siRNA mRNA only by 6±5.1% (as compared with scramble NOX2; Figure 3), and the siRNA-NOX4 knocked down NOX4 mRNA by 86±2.1%. To test the specificity of the siRNAs that we used, we measured NOX2 mRNA in siRNA-NOX4–treated cells and measured NOX4 mRNA in siRNA-NOX2–treated cells. In siRNA-NOX4–treated cells, NOX2 mRNA was 94.5±5.2% compared with the cells treated with scrambled siRNA. In siRNA-NOX2–treated cells, NOX4 mRNA was 103.1±5.6% compared with the control (Figure 3; n=5). Accordingly, we used this dose and incubation time in the remaining experiments.

One of the primary stimuli for NOX-derived O$_2^-$ production in macula densa cells is the increased luminal NaCl. To determine which NOX isoform is responsible for this increase in O$_2^-$, we tested whether knocking down either NOX2 or

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**Figure 2.** Isoforms of NAD(P)H oxidase expressed by the macula densa identified by RT-PCR. A, NOX2 and NOX4 are detected in laser-captured macula densa cells. nNOS as a positive marker of the macula densa cells. B, In cultured macula densa cells (MMDD1), NOX2, NOX4, and nNOS are detected. C, All of the primers were tested in renal cortex.

**Figure 3.** siRNA knocking down NOX2 and NOX4 mRNA. Top, Representative RT-PCR of scrambled and siRNA NOX2 and NOX4. Bottom, Quantitative densitometry of the bands (n=5; ‘*’<0.01).
NOX4 mRNA in MMDD1 cells prevented high-NaCl-induced increases in $O_2^-$. The effect of knocking down NOX2 on $O_2^-$ concentration levels in MMDD1 cells is shown in Figure 4. A high-NaCl solution caused $O_2^-$ concentration to increase in control MMDD1 cells (treated with scrambled NOX2 siRNA); the $O_2^-$ concentration was 0.88±0.11 and 1.74±0.17 U/min per 10^5 cells in the low- (70 mmol/L) and high- (140 mmol/L) NaCl solutions, respectively (P<0.001). Knocking down NOX2 did not alter basal $O_2^-$ levels but blocked high-NaCl–induced increases in $O_2^-$. $O_2^-$ concentrations were 0.73±0.20 and 0.90±0.15 U/min per 10^5 cells in the low- and high-NaCl solutions, respectively; n=7). On the other hand, knocking down NOX4 had no effect on high-NaCl–induced $O_2^-$ production (Figure 5). The $O_2^-$ concentration in the control cells (treated with scrambled NOX4 siRNA) was 0.94±0.12 and 1.82±0.17 U/min per 10^5 cells in the low- and high-NaCl groups, respectively (P<0.001). The high-NaCl solution caused a similar increase in $O_2^-$ concentration in NOX4 siRNA-treated cells; $O_2^-$ was 0.51±0.12 and 1.58±0.24 U/min per 10^5 cells in the low- and high-NaCl groups, respectively (P<0.001; n=9). These data indicate that NOX2 is the primary isoform responsible for NaCl-induced $O_2^-$ generation in the macula densa, and NOX4 is an isoform responsible for basal $O_2^-$ generation.

We reported previously that NaCl-induced $O_2^-$ generation in isolated perfused MD cells is mainly attributable to NAD(P)H oxidase.13,25 To establish whether NAD(P)H oxidase was also the main source of $O_2^-$ in MMDD1 cells, we determined the relative contribution of NAD(P)H oxidase, xanthine oxidase, and COX-2 to NaCl-induced $O_2^-$ generation in MMDD1 using an antagonist of NAD(P)H oxidase, xanthine oxidase, and COX-2 (Figure 6). First, we tested the role NAD(P)H oxidase in NaCl-induced $O_2^-$ generation in MMDD1. The $O_2^-$ concentration in MMDD1 cells on low- and high-NaCl solution was 0.50±0.05 and 1.36±0.09 U/10^5 cells (n=7; P<0.01). Adding the NAD(P)H oxidase inhibitor apocynin (10^{-5} M) for 30 minutes to high-NaCl MMDD1 cells caused the $O_2^-$ concentration to decrease to 0.68±0.05 U/10^5 cells (n=19; P<0.01). Thus, blocking NAD(P)H oxidase blunted NaCl-induced increases in $O_2^-$ concentration, suggesting that NAD(P)H is an important source of $O_2^-$ production in these cells. In contrast, blocking xanthine oxidase with oxypurinol did not significantly alter $O_2^-$ concentrations. In these experiments, the $O_2^-$ concentrations in the cells maintained in low- and high-NaCl solutions were 0.59±0.05 and 1.32±0.12 U/10^5 cells, respectively (P<0.001), and 1.14±0.12 U/10^5 cells in the cells treated with oxypurinol (10^{-4} mmol/L) for 30 minutes. Finally, blocking COX-2 with NS-398 also did not affect the NaCl-induced increase in MMDD1 $O_2^-$ concentration. $O_2^-$ concentrations were 0.52±0.03 and 1.36±0.09 U/min per 10^5 cells during low- and high-NaCl solutions, respectively (n=14; P<0.01) and 1.25±0.09 U/min per 10^5 cells in the cells treated with NS-398 (10^{-5} mmol/L) for 30 minutes. These data indicate that NaCl-induced increases in $O_2^-$ in MMDD1 cells, like that in freshly isolated and perfused macula densa cells, are predominantly via NAD(P)H oxidase.13,25
Discussion

In the present study, we successfully applied LCM to isolate macula densa cells from a frozen rat kidney cortex. These cells expressed NOX2 and NOX4 but not NOX1. This expression profile was essentially identical to that of the MMDD1 cells, further verifying the similarity of these 2 cell types and their suitability for macula densa research. Finally, using the MMDD1 cells, we found that the NOX2 is the main source of NaCl-induced $O_2^-$, and NOX4 is responsible for basal $O_2^-$ production.

The macula densa plays a major role in NaCl-dependent regulation of glomerular arteriolar tone and renin release, and, thus, has been the subject of much study. However, it is difficult to acquire macula densa cells in quantities sufficient enough to perform the necessary biochemical analysis required to study cellular and molecular mechanisms. The recent development of LCM provides us with a tool that can be used to isolate macula densa. LCM is a novel technique based on the adherence of visually selected cells to a thermoplastic membrane (overlying the dehydrated tissue section), which is focally melted by triggering a low-energy infrared laser pulse. The melted membrane forms a composite with the selected tissue area that is removed by simply lifting off the membrane. The size of the laser spot can be selected as 30.0, 15.0, or 7.5 μm, facilitating dissection of groups of cells or even single cells. Consequently, LCM can be applied to a wide range of cell and tissue preparations, including frozen-tissue sections. The first objective of the present study was to use LCM to harvest macula densa cells from frozen rat kidneys. We identified macula densa cells in the tissue sections and found that we were readily able to dissect and harvest them using LCM. We then confirmed that the captured cells were macula densa cells by verifying that they expressed nNOS, demonstrating that LCM can be used effectively to harvest macula densa cells. Thus, we next used LCM-harvested macula densa cells to investigate the source of NaCl-induced $O_2^-$ generation.

Evidence suggests that $O_2^-$ produced by the macula densa plays an important role in regulating TGF. We reported recently that increasing tubular NaCl induced $O_2^-$ production by the macula densa primary from NAD(P)H oxidase. $O_2^-$ produced by the macula densa augmented TGF by scavenging NO. This contention is further supported by Chabrashvili et al, who found that the macula densa expresses the main components of NAD(P)H oxidase, some of which are overexpressed in the spontaneously hypertensive rat, an experimental model of hypertension that has enhanced oxidant stress and TGF. These studies indicate the existence and functional importance of NAD(P)H oxidase at the macula densa.

These different roles for the NOXs are not unexpected, for the following reasons. First, NOX2 and NOX4 localize to different subcellular compartments. Because $O_2^-$ cellular signaling likely depends on both the amount and site of $O_2^-$ production, it seems likely that NOX2 and NOX4 may mediate distinct signaling pathways. In addition, they are regulated differently. Activation of NOX2 requires agonist stimulation to induce translocation of cytoplasmic p47^phox subunit. Our present study has established that, whereas NOX4 contributes to basal $O_2^-$ production in the macula densa, NOX2 is the primary source for $O_2^-$ induced by NaCl.
arginine derivatives dictates O$_2^-$ production. In the present study, we cannot exclude the possibility of enhanced O$_2^-$ production from nNOS in MMDD1 cells when we used N6G, nitro-L-arginine methyl ester. However, we believe that the O$_2^-$ from nNOS is not significant, because inhibition of NAD(P)H oxidase with apocynin blocks O$_2^-$ production (see Figure 6).

In summary, we found that NOX2 and NOX4 are the isoforms of NAD(P)H oxidase expressed at the macula densa. NOX2 is the main source of O$_2^-$ induced by NaCl, whereas NOX4 is responsible for basal O$_2^-$ production.

**Perspectives**

We used a novel approach that combined LCM of macula densa cells with functional studies in MMDD1 cells, a macula densa-like cell line, to facilitate the study of macula densa cellular function. We applied LCM to isolate and harvest macula densa cells from frozen kidney tissue and then identified that these captured macula densa cells express the NOX2 and NOX4 isoforms of NAD(P)H oxidase. The expression profile of these cells was essentially the same as that of the MMDD1 cells. The NOX2 is the main source for NaCl-induced O$_2^-$, whereas the NOX4 contribute to the basal O$_2^-$ production. Thus, conditions in which the activity of either of these NOXs is altered may contribute to enhanced TGF and abnormal NaCl homeostasis.

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**Disclosures**

None.

**References**


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