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# Changes of Cell Volume and Nitric Oxide Concentration in Macula Densa Cells Caused by Changes in Luminal NaCl Concentration

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*Abstract.* The luminal NaCl concentration ([NaCl]) at the macula densa (MD) controls both tubuloglomerular feedback (TGF) and renin release. Nitric oxide (NO) inhibits TGF sensitivity to a great extent. The NO concentration in the MD cells is not known. This study measured this concentration in MD cells with confocal microscopy in the isolated perfused thick ascending limb using a NO-sensitive fluorophore 4,5-diaminofluorescein (DAF-2). Calcein was used to measure cell volume changes. The loop perfusion fluid was a modified Ringer solution containing 10, 35, or 135 mM NaCl with a constant total osmolarity (290 mOsm), and the bath was perfused with the 135 mM NaCl solution. The results show that MD cell volume and NO concentration measured with DAF-2 DA increased considerably with increasing luminal [NaCl] and with calcium-free solutions in the lumen and bath. L-arginine (5 mM) increased NO concentration in the MD cells by 30%. 7-nitroindazole could totally inhibit the NO production caused by L-arginine and by increased luminal [NaCl]. In conclusion, the MD cell volume changes caused by the changes of luminal [NaCl] were quantitatively measured, and it was found that increasing the luminal [NaCl] resulted in an increase in cell volume. It was also found that NO formation in MD cells could be measured with DAF-2 and that NO production was increased through neuronal NO synthase activation with an increased luminal [NaCl]. An increased NO production will inhibit the vasoconstriction induced by the TGF and at the same time will reduce TGF sensitivity.

The juxtaglomerular apparatus (JGA) is a complex assembly of specialized structures related to each other anatomically, forming the vascular pole of the glomerulus. The JGA is comprised of the macula densa (MD), the extraglomerular mesangium, and the afferent and efferent arterioles. The MD is a plaque of 20 to 30 specialized epithelial cells belonging to the end portion of the thick ascending limb. The luminal NaCl concentration ([NaCl]) at the MD has two established effects: (*1*) regulation of glomerular arteriolar resistance through tubuloglomerular feedback (TGF); and (*2*) control of renin release (1,2). The first step in these signal transmissions involves NaCl transport by the MD, which is relatively well understood. NaCl uptake occurs primarily through the $Na^+$-$K^+$-$2Cl^-$ cotransporter, which has been demonstrated both on functional and transcriptional levels (3,4). The next step is not yet clear. The possible mediators and modulators of the information transmitted between the MD and its target cells include the ion concentration, ATP, angiotensin II, adenosine, arachidonic acid metabolites, and nitric oxide (NO) (5–7). Among these, NO appears to play an important role in the vascular response to changes in luminal [NaCl]. Many studies have been done on the effects of NO on TGF regulation (8–14), but there has been no report on instantaneous NO concentration changes caused by changes in the luminal [NaCl]. In the present study, an NO-sensitive probe was used for direct measurement of NO concentration in the MD during changes in luminal [NaCl].

## Materials and Methods

### *Animal Preparation and Microperfusion*

Individual cortical thick ascending limbs (cTAL) with attached glomeruli were dissected and perfused as described previously (15). In short, female New Zealand White rabbits**,** weighing 1.0 to 1.5 kg**,** were killed, and the left kidney was removed and cut into several 1.5-mm to 3-mm transverse slices. These slices were placed in chilled 35 mM NaCl buffer solution containing 35 mM NaCl, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 1.6 mM $KH_2PO_4$, 5 mM glucose, and 20 mM Hepes, with the pH adjusted to 7.4 and osmolarity adjusted to 290 mOsm with sucrose. Glomeruli with their attached cTAL and containing the MD plaque were isolated by microdissection at 4°C under a dissection microscope and then transferred to a chamber fixed to the stage of a Nikon microscope attached to a laser confocal system (Noran, Middleton, WI). The cTAL was cannulated and perfused with the 35 mM NaCl buffer solution. The preparation was bathed continuously in a 135 mM NaCl buffer solution containing 135 mM NaCl, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 1.6 mM $KH_2PO_4$, 5 mM glucose, and 20 mM Hepes, with the pH adjusted to 7.4 and osmolality adjusted to 290 mOsm with sucrose.

### *Fluorescence Probe Loading*

A cell-permeable fluorescence NO indicator, 4,5-diaminofluorescein diacetate (DAF-2 DA) or DAF-2 FM, was used to detect NO

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production in MD cells. The cells were loaded with 10 $\mu$M DAF-2 DA or DAF-2 FM (in 0.5% DMSO) from the luminal side for 40 to 50 min. Calcein was used to measure cell volume changes. The MD cells were loaded with 5 $\mu$M calcein dissolved in 0.1% DMSO from the luminal for 10 to 15 min. A Nikon ×60/1.2 water-immersion objective lens was used to visualize MD cells. The image size was set to 640 × 480 pixels. The confocal slit was set at a width of 15 nm. Photobleaching was kept to a minimum by maintaining the laser intensity below 30% of the maximum and using a shutter so that the preparation was exposed to laser light only during the collection of images. Date collection, with the confocal system is controlled by a Silicon Graphics workstation (Mountain View, CA). Image acquisition was limited to 30 frames per second, and, when necessary, image noise was reduced by averaging or summing 16 to 32 individual images. The sampling time for each pixel was 100 ns. Calcein or DAF-2 was excited at 488 nm with an argon-ion laser, while emitted fluorescence was recorded at wavelengths of 510 to 550 nm. Square-shaped regions of interest (ROI) were set inside the cytoplasmic area of MD cells, and the mean intensities within the ROI were recorded every 5 s. Figure 1 shows the confocal loading image of calcein with [NaCl] at 10 and 35 mM and a laser-transmitted image. Figure 2 shows DAF-2 DA loaded cells and a laser-transmitted image of the perfused thick ascending limb of the loop of Henle.

## *Calculation*

Calculations were based on the following equations.

In Equation 1, the $V_{c1}$ is cell volume and $C_{c1}$ is concentration of calcein during resting condition; $V_{c2}$ and $C_{c2}$ are changed cell volume and changed concentration of calcein, respectively. The concentration

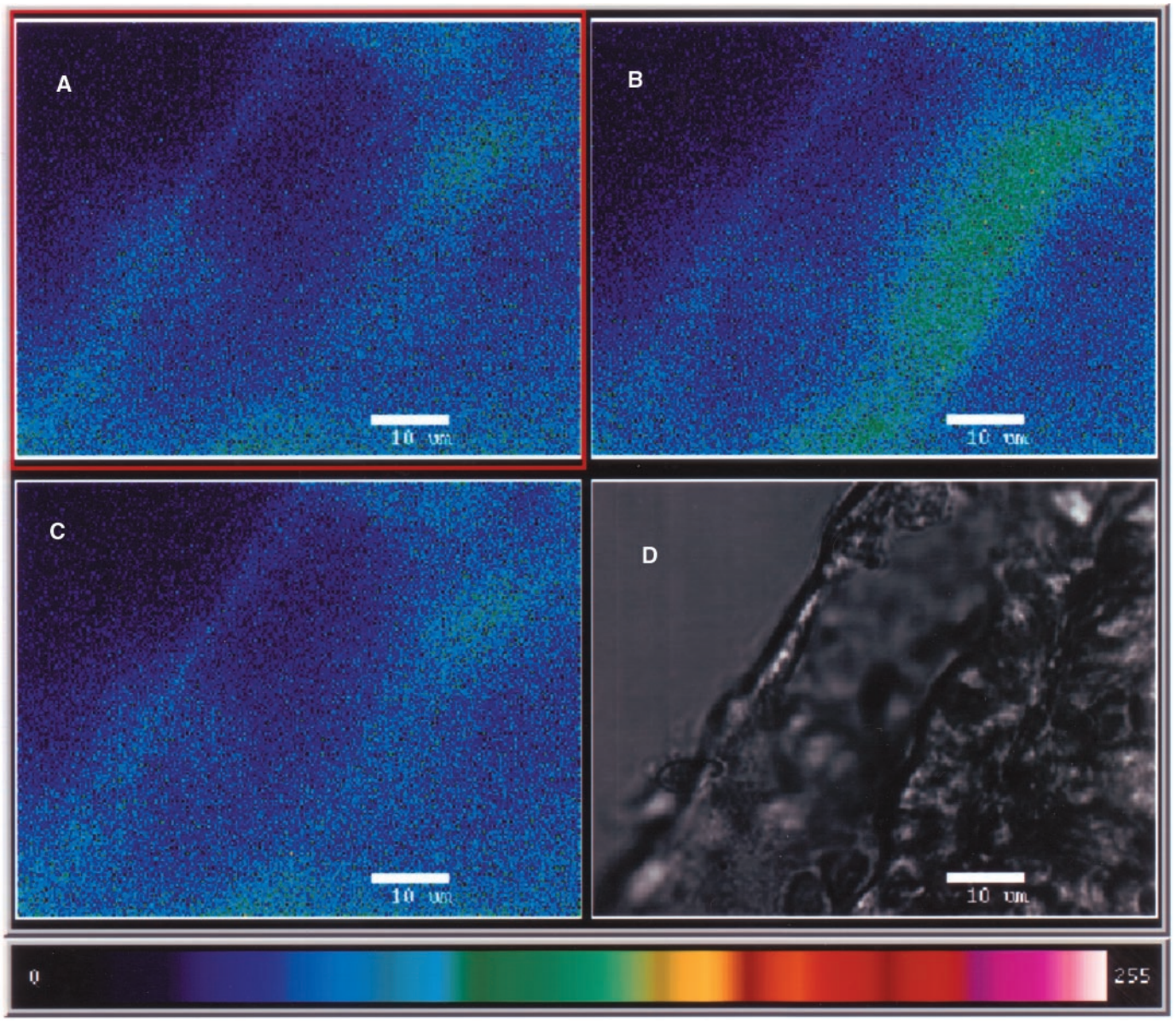


*Figure 1.* Calcein-loaded microperfused macula densa (MD) cells. (A) Tubule perfused with 35 mM NaCl solution. (B) Tubule perfused with 10 mM NaCl solution. The calcein intensity was increased, which means that the cell volume has decreased. (C) The tubule was again perfused with the 35 mM NaCl solution, and the intensity almost regained basal level. (D) Laser-transmitted image.

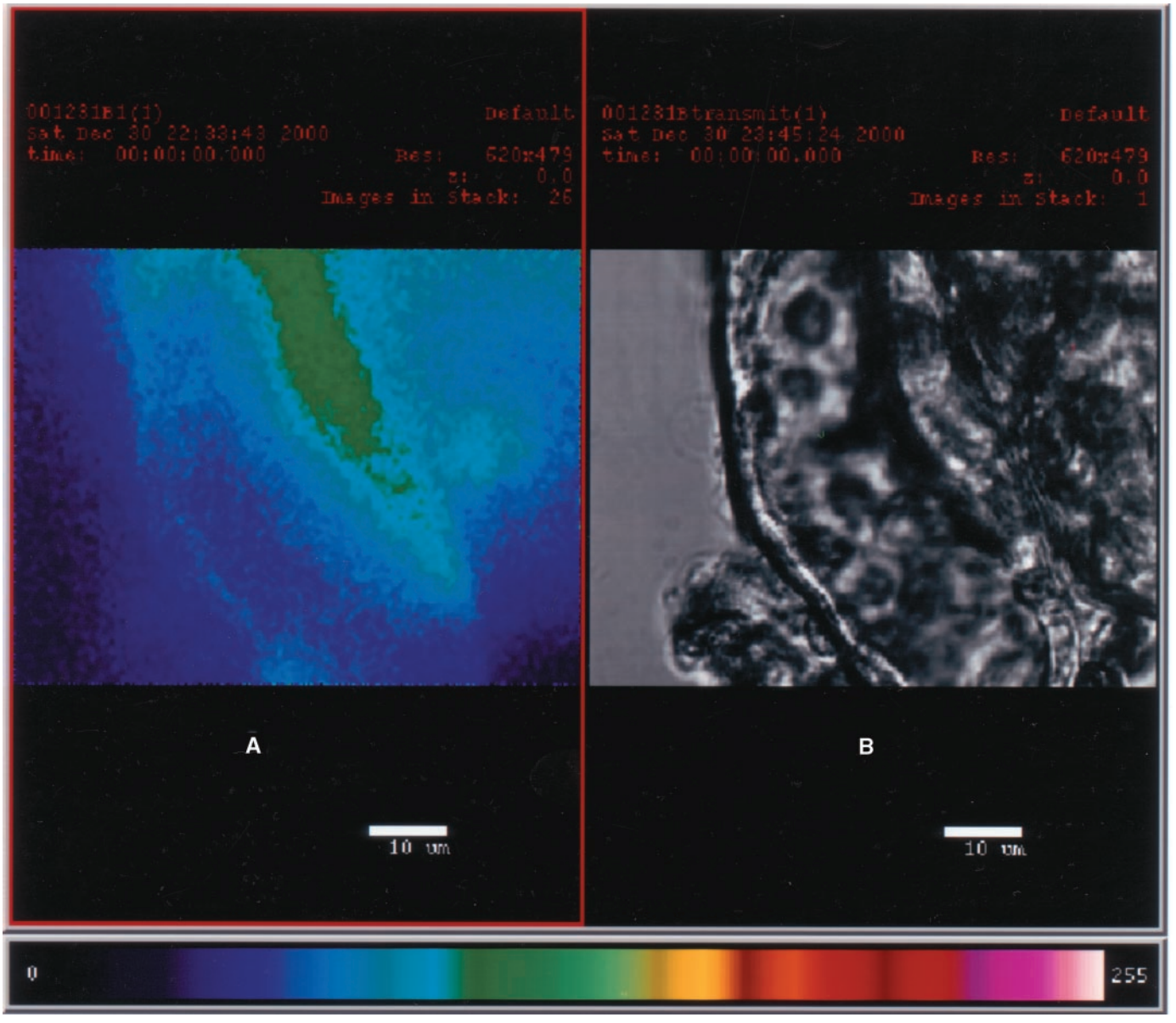


*Figure 2.* 4,5-diaminofluorescein (DAF-2)–loaded MD cells. (A) DAF-2 loaded fluorescent image. (B) Laser transmitted image.

of calcein is proportional to its fluorescence intensity; therefore, the ratio of concentrations is equal to the ratio of intensities. Assuming that $V_{c1}$ is 1, the changed cell volume could be expressed with the changed calcein intensity ($F_{c2}$) and the intensity at basal level ($F_{c1}$) (equation 2).

Delta-relative changes of cell volume can be calculated with equation 3.

The triazolofluorescein of DAF-2 (DAF-2T) is the fluorescence form after DAF-2 selectively traps NO between two amino groups in its molecule. The relative changes of the amount of DAF-2T in MD cells can be calculated by the changed cell volume ($V_{d2}$) multiplied with changed concentration ($C_{d2}$) of DAF-2T divided by the basal level of cell volume ($V_{d1}$) multiplied with concentration ($C_{d1}$) of DAF-2T (equation 4).

$V_{d2}$ and $V_{c2}$ are the same, and $V_{d1}$ is regarded as 1; therefore, the $C_{d2}/C_{d1}$ could be expressed by the ratio of DAF-2T intensities in changed conditions ($F_{d2}$) and in basal level ($F_{d1}$). The delta-relative changes of DAF-2T amount can be expressed by equation 5.

$$V_{c1} * C_{c1} = V_{c2} * C_{c2} \quad (1)$$

$$V_{c2} = V_{c1} * C_{C1}/C_{c2} = F_{c1}/F_{c2} \quad (2)$$

$$(V_{c2} - V_{c1})/V_{c1} = F_{c1}/F_{c2} - 1 \quad (3)$$

$$(V_{d2} * C_{d2})/(V_{d1} * C_{d1}) \quad (4)$$

$$[(V_{d2} * C_{d2}) - (V_{d1} * C_{d1})]/(V_{d1} * C_{d1}) = V_{c2} * (F_{d2}/F_{d1}) - 1 \quad (5)$$

Equations 3 and 5 were used to calculate the cell volume and DAF-2T amount of MD cells in the present study.

## *Calibrations*

**DAF-2 Calibration.** The calibration was made with DAF-2, a non–cell permeable form of the fluorophore, dissolved in the following groups of solutions: (*1*) Phosphate-buffered saline (PBS) (without $Ca^{2+}$, $Mg^{2+}$); (*2*) PBS with 1.3 mM $CaCl_2$; (*3*) PBS with 1.3 mM

$MgSO_4$; (*4*) PBS with 1.3 mM $CaCl_2$ and 1.3 mM $MgSO_4$; (*5*) PBS with 5 mM EGTA; (*6*) 135 mM NaCl solution with 1.3 mM $CaCl_2$; (*7*) 135 mM NaCl solution with 5 mM EGTA. The final concentration of DAF-2 in the above solutions was 10 $\mu$M. Fluorescence was detected with the same settings as in the experiments. The NO standard solution was freshly prepared by gassing the PBS (without $Ca^{2+}$, $Mg^{2+}$) at room temperature with 100% $N_2$ gas for 30 min and subsequently with an 1.8% NO + 98.2% $N_2$ gas mixture for 30 min. The NO concentration in the standard was 3.7 $\mu$M at room temperature (16). Solutions with different concentrations of NO (0, 50, 100, 200, 400, and 800 nM) were made by adding the NO standard.

**Calcein Calibration.** In these experiments, the cTAL was carefully removed, leaving the MD plaque attached to the glomerulus. MD cells were loaded in the 35 mM NaCl buffer solution using 5 $\mu$M calcein with 0.1% DMSO for 10 min at room temperature. Each preparation was then transferred to a chamber attached to the confocal system. Glass holding pipettes (outer tip diameter, 30 to 40 $\mu$m), connected to micromanipulators (MM3, Narishige, Japan), were used to position the glomerulus so that the MD plaque was clearly visible (Figure 4). Experiments were performed at 37°C with continuous perfusion of the 35 mM NaCl buffer solution at a rate of 6 to 7 ml/min. Different osmolality solutions (150, 290, 450, 600, and 750 mOsm adjusted with sucrose respectively) with the 35 mM NaCl buffer solution) were used to evaluate the correlation between the cell volume and calcein intensity. In some experiments, the cell heights were measured after changes in osmolarity.

### *Experimental Protocol*

Solutions with [NaCl] of 10 mM (containing 10 mM NaCl, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 1.6 mM $KH_2PO_4$, 5 mM glucose, and 20 mM Hepes, pH adjusted to 7.4, and osmolality adjusted to 290 mOsm with sucrose), 35 mM, and 135 mM were perfused from lumen. Experiments were performed at 37°C with continuous perfusion in the bath with the 135 mM NaCl buffer solution at a rate of 6 to 7 ml/min. When $Ca^{2+}$-free solutions were used, $CaCl_2$ was replaced by 5 mM EGTA both in bath and in luminal solutions. In some other experiments, 7-nitroindazole (7-NI) was added to the bath and luminal solutions for 30 min before administration of either 5 mM L-arginine or an increase in luminal [NaCl] from 35 to 135 mM.

### *Chemicals*

DAF-2 DA, DAF-2 FM, and 7-NI were obtained from Calbiochem, San Diego, CA. PBS was from Biochrom KG, Berlin, Germany. Calcein was from Molecular Probes, Eugene OR. All other chemicals were obtained from Sigma, St. Louis, MO.

### *Statistical Analyses*

Non-paired, paired *t* test (two-tail) and one-way ANOVA were used where appropriate. $P < 0.05$ was set as the significance level. Data are presented as mean ± SEM.

## Results

The intensities of DAF-2 at the same NO concentrations among different groups of solutions with or without calcium and/or magnesium were not significantly different (Table 1). Figure 3A shows a corresponding increased response curve with stepwise increased concentrations of NO. Figure 3B shows a linear relationship between DAF-2 intensity and NO concentration ($n = 24$).

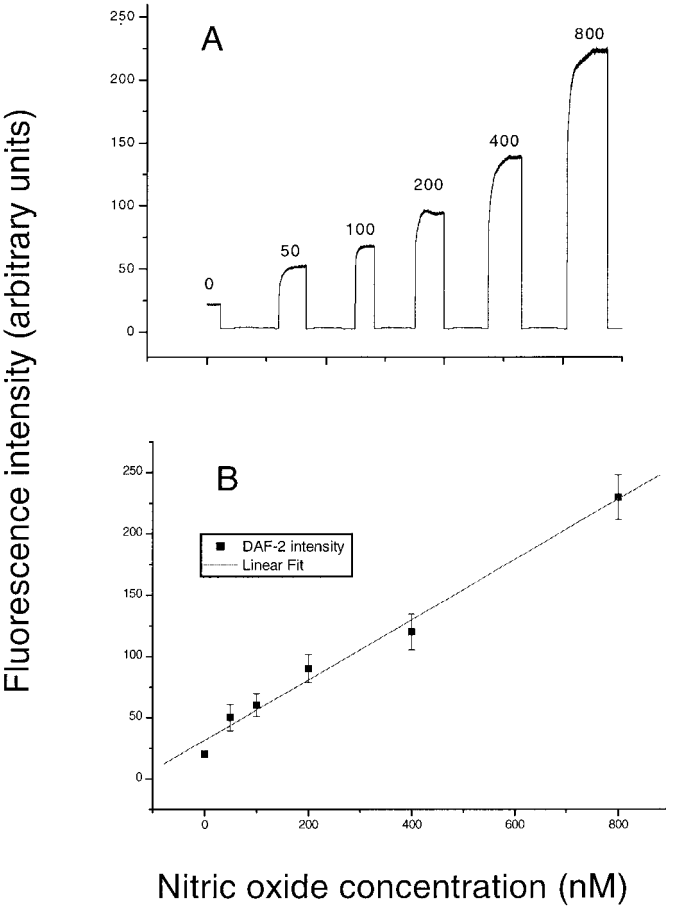


*Figure 3.* Calibration curve of NO concentration and DAF-2 intensity. (A) Corresponding increase of DAF-2 intensity with stepwise increased NO concentration. (B) Linear fit curve between DAF-2 intensity and NO concentration.

Macula densa cell volume could be measured by direct measurements of the cell height when changing total osmolarity. Figure 4 shows an increase in cell height when the osmolarity was changed from 290 to 150 mOsm in a preparation where the cTAL was removed. When total osmolarity was changed stepwise from 150 to 700 mOsm using calcein to measure cell volume, we found a linear volume change that, was in essential agreement with an osmometric behavior of the macula densa cells ($n = 19$) (Figure 5).

When the luminal solution was changed from 10 mM or 35 mM [NaCl] to 135 mM [NaCl], the cell volume was increased by 19.0 ± 1.2% ($n = 69$) or 14.6 ± 1.9% ($n = 18$), respectively ($P < 0.05$). When the luminal solution was changed from 135 mM or 35 mM [NaCl] to 10 mM [NaCl], the cell volume was reduced by 21.2 ± 1.7% ($n = 55$) or 9.6 ± 1.5% ($n = 13$), respectively ($P < 0.05$). When the luminal [NaCl] was changed, there were no significant differences in cell volume changes between the use of $Ca^{2+}$-free solutions both in bath and lumen and that containing 1.3 mM $CaCl_2$ solutions (Figure 6).

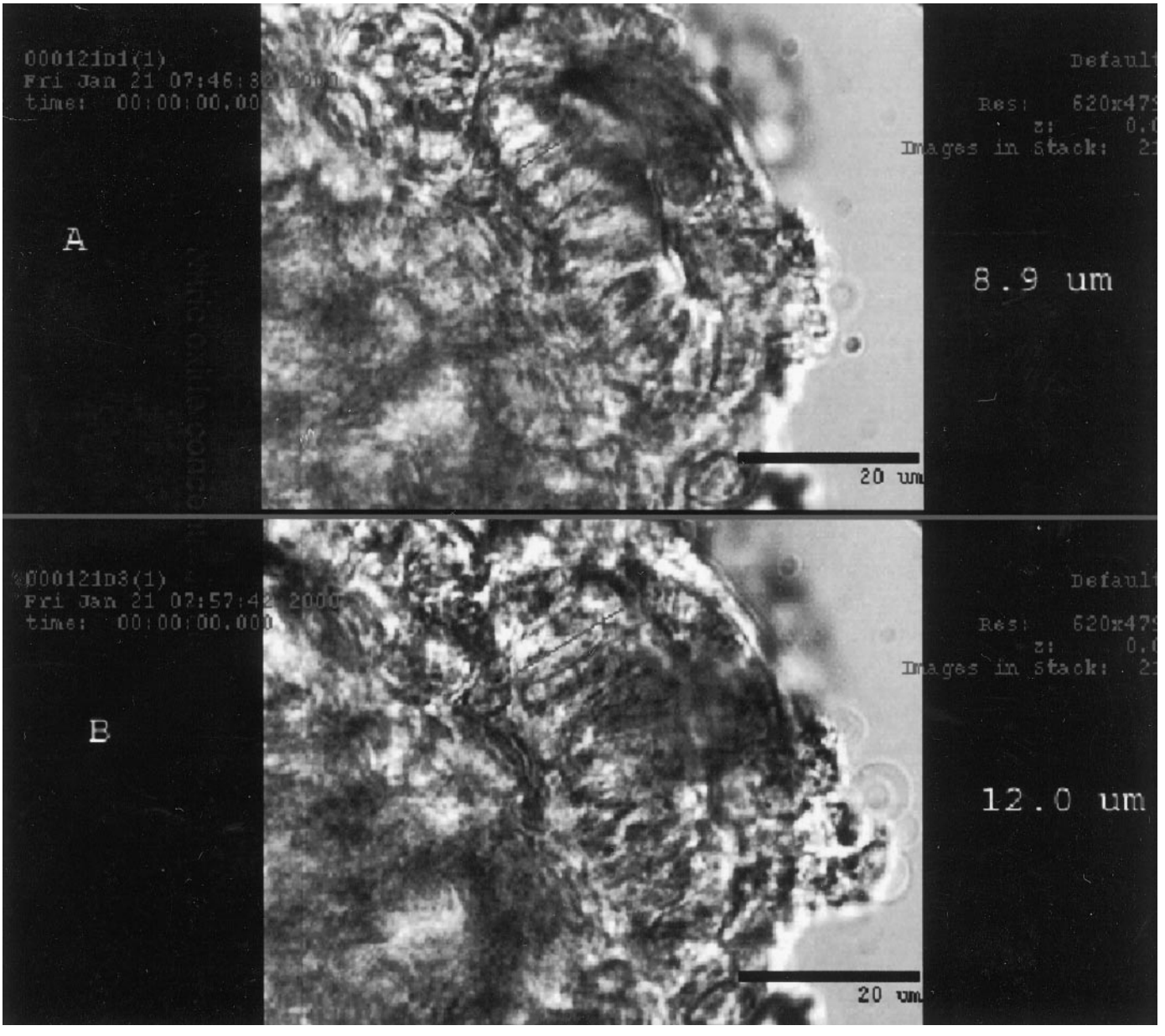


*Figure 4.* Changes in length of MD cells in hypotonic solution. (A) MD cells after the cTAL removed in 35 mM buffer solution (290 mOsm). (B) The MD cells in 150 mOsm solution. A cell height was changed from 8.9 $\mu$m to 12.0 $\mu$m. Osmolarity was adjusted with sucrose.

*Table 1.* Results of DAF-2 calibration[a]

| Group | Contents | 0 | 50 | 100 | 200 | 400 | 800 nM NO | *n* |
|---|---|---|---|---|---|---|---|---|
| 1 | PBS without $Ca^{2+}$ and $Mg^{2+}$ | 20.8 ± 0.3 | 50.7 ± 5.4 | 60.5 ± 4.1 | 90.1 ± 10.5 | 121.8 ± 13.1 | 229.4 ± 15.8 | 4 |
| 2 | PBS with 1.3 mM $Ca^{2+}$ | 20.1 ± 0.7 | 51.5 ± 4.7 | 61.4 ± 5.9 | 91.7 ± 9.6 | 122.8 ± 11.7 | 233.8 ± 19.7 | 3 |
| 3 | PBS with 1.3 mM $Mg^{2+}$ | 21.6 ± 0.6 | 52.3 ± 3.2 | 61.6 ± 2.4 | 94.9 ± 8.8 | 122.5 ± 14.6 | 233.0 ± 20.7 | 3 |
| 4 | PBS with 1.3 mM $Ca^{2+}$ and $Mg^{2+}$ | 21.3 ± 0.7 | 51.8 ± 3.1 | 60.8 ± 6.5 | 93.7 ± 9.9 | 123.9 ± 13.2 | 233.4 ± 18.2 | 4 |
| 5 | PBS with 5 mM EGTA | 20.5 ± 0.4 | 52.1 ± 4.6 | 60.7 ± 5.3 | 94.3 ± 7.6 | 122.2 ± 12.6 | 232.8 ± 15.1 | 4 |
| 6 | Buffer solution with 1.3 mM $Ca^{2+}$ | 20.6 ± 0.6 | 51.4 ± 5.1 | 63.7 ± 5.1 | 94.4 ± 8.5 | 119.0 ± 13.2 | 229.8 ± 19.8 | 3 |
| 7 | Buffer solution with 5 mM EGTA | 20.0 ± 1.1 | 53.8 ± 5.7 | 63.2 ± 6.4 | 93.2 ± 9.5 | 121.1 ± 14.1 | 230.9 ± 21.3 | 3 |
| Total | 20.8 ± 0.2 | 51.9 ± 3.9 | 61.5 ± 4.1 | 93.2 ± 7.9 | 122.0 ± 9.2 | 231.9 ± 15.6 | 24 | |

[a] One-way ANOVA analysis showed no significant differences among the seven groups ($P > 0.05$).

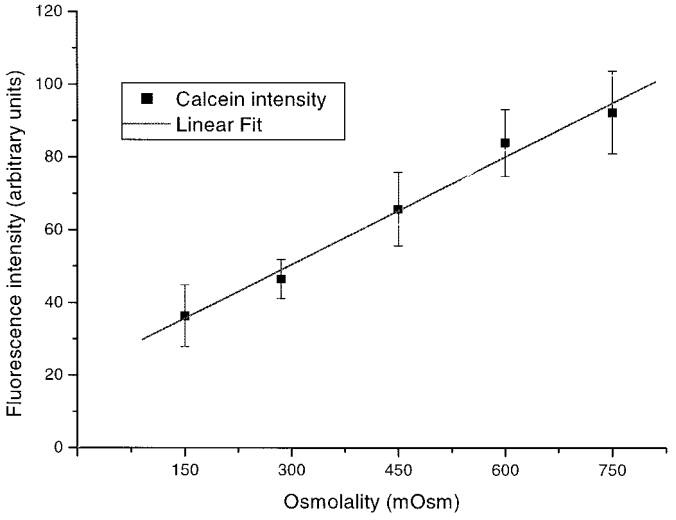


*Figure 5.* Cell volume and calcein intensity. The intensity of calcein is in a linear relationship with the MD cell volume caused by different osmolality ($n = 19$).

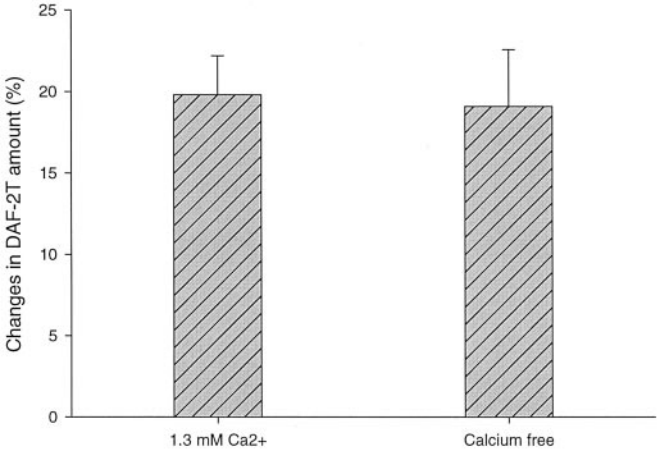


*Figure 7.* Changes in NO induced by changes in the luminal [NaCl]. When the luminal [NaCl] was increased from 35 mM to 135 mM, the NO concentration in the MD cells increased significantly. There was no significant difference in the results between normal calcium and calcium-free solution.

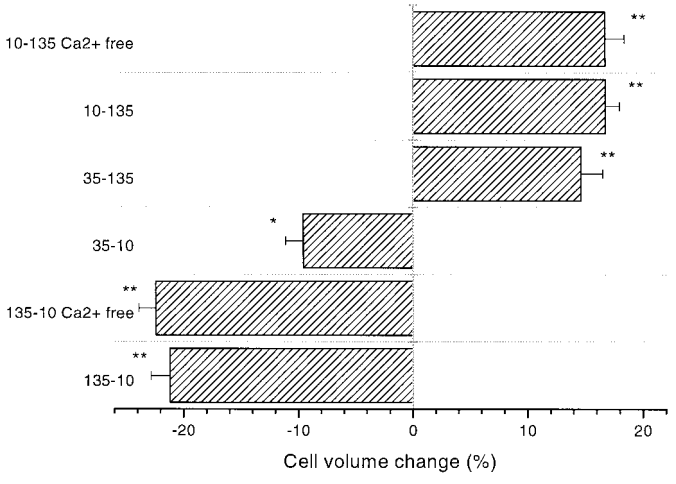


*Figure 6.* Cell volume changes caused by changes in luminal NaCl concentrations ([NaCl]). 10–135 means that the luminal NaCl solution is changed from 10 to 135 mM; the other numbers indicate analogous changes. When the luminal [NaCl] increased, the volumes of the MD cells significantly increased. When the luminal [NaCl] decreased, the cell volumes decreased significantly. There was no significant difference in changes in cell volume between experiments with normal calcium solution and those with calcium-free solution ($Ca^{2+}$-free). * $P < 0.05$; ** $P < 0.01$.

In the DAF-2 DA–loaded experiments corrected for volume changes, when the luminal [NaCl] was changed from 35 mM to 135 mM, the delta changes in the amount of DAF-2 DA were significantly increased by 19.8 ± 2.4% ($n = 24$) ($P < 0.01$). In the experiments with $Ca^{2+}$-free solutions, the changes in the amount of DAF-2 DA did not significantly differ from those in which a 1.3 mM $CaCl_2$ solution was used (Figure 7). DAF-2 FM, a new isoform of DAF-2 much less influenced by pH changes (17,18), was also used in a few experiments. The results showed that when the luminal [NaCl] was changed from 35 mM to 135 mM, the delta changes in the amount of DAF-2 FM were significantly increased by 24.76 ± 4.1% ($n = 6$) ($P < 0.01$).

Addition of 5 mM L-arginine from the bath and luminal side resulted in an increase in the changes in DAF-2 DA intensity by 17.3 ± 2.9% ($n = 18$) and 30.3 ± 5.3% ($n = 15$), respectively ($P < 0.01$). 100 $\mu$M 7-NI was perfused both from the bath and lumen for 30 min, after which 5 mM L-arginine was added either from the bath ($n = 3$) or lumen ($n = 5$). No significant change in DAF-2 DA intensity was observed (0.3 ± 0.6% bath, 0.2 ± 1.7 lumen; $P > 0.05$). When the luminal [NaCl] was changed from 35 mM to 135 mM after addition of 7-NI ($n = 3$), no significant change in DAF-2 DA intensity could be detected (0.8 ± 1.1%; $P > 0.05$).

## Discussion

The finding of a constitutive isoform of NO synthase (NOS), neuronal NOS (nNOS), in MD cells (8,19,20) has raised the question as to a possible role of NO in the MD cell function. The addition of NOS inhibitors to the luminal fluid enhanced MD-dependent vasoconstriction in both *in vivo* studies of the TGF response and *in vitro* experiments using an isolated double-perfused JGA preparation (9,10,13,14). It has also been reported that use of 7-NI, a blocker of the nNOS, sensitized TGF in a degree similar to that obtained with a general inhibitor (10). This result indicated that most of the effect of NO on the TGF was exerted through activation of the neuronal isoform of NOS. It was concluded from these results that NO, generated by nNOS in MD cells and released in either a constitutive or transport-regulated fashion, acts tonically to suppress the full effect of the vasoconstrictor mediator in the TGF. In the present study, the use of confocal microcopy made it possible to directly measure the NO generation in MD cells.

DAF-2 is newly developed for real-time measurement of NO

with a detection limit of 5 nM (17,18,21). DAF-2 selectively traps NO between two amino groups in its molecule and yields stable triazolofluorescein (DAF-2T), which emits green fluorescence when excited at about 490 nm. DAF-2T is not formed in the absence of NO. Stable forms of NO (*e.g.*, $NO_2^-$ and $NO_3^-$), reactive oxygen species, such as superoxide ($O_2^-$), $H_2O_2$, and peroxynitrite ($ONOO^-$) do not react with DAF-2 to yield a fluorescence product (17). However, the fluorescence intensity of DAF-2 DA is pH-dependent (17,18). The fluorescence of DAF-2 DA is comparatively stable in media above pH 7, but its fluorescence substantially decreases below a pH of 7. If, for example, intracellular pH dropped to 6.5, the DAF-2 DA intensity would almost decrease by 50% only as an effect of pH (17,18). DAF-2 FM, a new isoform of DAF-2, has the property of being much less influenced by pH changes, which intensity keeps constant in media above pH 5 (17,18). It has earlier been found that increased luminal [NaCl] elevated pH, whereas decreased luminal [NaCl] lowered the pH in MD cells through the apical Na:H exchangers (22,23). Whereas luminal [NaCl] changed during 35 to 135 mM, the intracellular pH in MD cells keeps above 7, which will have no significant influence on DAF-2 intensity (22,23). In the present study, when luminal [NaCl] changed during 35 to 135 mM, the influence of pH on the DAF-2 DA intensity should be minor. When luminal [NaCl] decreased to 10 mM, the pH value in MD cells decreased below 7, and this probably influenced the intensity of DAF-2DA significantly (22,23)). It is also reported that calcium and magnesium enhance the DAF-2 signal detection of NO released by NO donors up to 200 times (24). In the present calibration, NO gas instead of NO donors was used. We found that neither $Ca^{2+}$ nor $Mg^{2+}$ showed any influence on the intensity of DAF-2, and there was a good linear relationship between the NO concentration and the DAF-2 intensity. The reason for this difference might be due to the other factors, such as the reactions with the NO donors, other than divalent cations really influenced the intensity of DAF-2T. The results of $Ca^{2+}$-free experiments in the present study also support our findings, in which $Ca^{2+}$-free solutions were used in both bath and luminal sides. This would inhibit the cytosolic calcium changes in MD cells (6,7). However, the same increase in DAF-2 DA with increased luminal [NaCl] was received with and without 1.3 mM $Ca^{2+}$ solutions. This indicated that $Ca^{2+}$ had no significant influence on the intensity of DAF-2 DA. A very recent report also got similar results (25).

DAF-2 is a single-wavelength measurement probe, which is affected by cell volume; therefore, it is necessary to determine cell volume to be able to calculate the changes of the amount of DAF-2T. We used the intracellularly trapped dye, calcein, measured within a defined intracellular volume (set by the properties of the confocal microscope and the ROI) as a reflection of cytoplasmic volume changes. Calcein is insensitive to changes in intracellular calcium and pH (26) and is only minimally affected by variations in ionic strength (27). Swelling or shrinkage of the cell will be accompanied by a decrease or an increase, respectively, in the dye concentration in the cell (28). Thus, changes in cell volume are expected to be reflected in changes in the fluorescence intensity, with decreased intensity during cell swelling and increased intensity during cell shrinkage (29,30). Since this is the first time calcein has been used to measure cell volume in MD cells, a calibration procedure was performed using solutions with different osmolality. When the cTAL was carefully removed, both the apical and basolateral MD cells can be challenged simultaneously with the same solution (Figure 4). This method is not unlike those methods used in the cultured cells. We found linear changes in calcein concentration similar to those expected of an osmometric behavior. Therefore, our data indicate that confocal determination of calcein concentration could be used to measure the MD cell volume.

In our experimental data, where total osmolarity was constant but the luminal [NaCl] solution was decreased, MD cell volume decreased significantly; when luminal [NaCl] was increased, cell volume increased considerably. Furthermore, we found that the cell volume change was independent of the extracellular $Ca^{2+}$ concentration. These cell volume changes were probably also independent of the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), because changes in $[Ca^{2+}]_i$ in MD cells caused by alteration in the luminal [NaCl] are dependent on the extracellular $Ca^{2+}$ concentration and $[Ca^{2+}]_i$ changes could be abolished in $Ca^{2+}$-free solution (6,7).

The amount of DAF-2 DA, corrected for by changes in cell volume as described previously in the Materials and Methods section, could be determined in MD cells. We found that when the luminal [NaCl] was increased from 35 mM to 135 mM and the total osmolarity was kept constant, the amount of DAF-2 DA increased by 19.8% (Figure 7). As discussed above, the DAF-2 DA amount in such cases reflected the NO productions in MD cells. So, the NO production in MD cells increased significantly after an increase in luminal [NaCl]. Results regarding the distal [NaCl] and the production of NO in MD in earlier studies are somewhat conflicting. In studies concerning the influence of sodium intake on NOS expression, it has been found that sodium deficiency stimulates nNOS expression, as do other conditions that are likely to result in reduced MD [NaCl] or transport, such as furosemide administration and unilateral stenosis of the renal artery (31,32). In contrast, high-salt diets and hyperfusion significantly inhibited nNOS mRNA expression (31,32). But in the other experiments in which the urinary excretions of nitrate and nitrite were measured, Shultz and Tolins (33) found that high salt intake generated more NO in MD cells. Wilcox and Welch (34) also reported that NO in MD cells was increased in rats maintained on a high-sodium diet. The results of recent studies (11,35,36) indicate that increased distal tubular flow stimulates nNOS activity at the MD to increase NO formation. Furthermore, the regulation of local NO production may not depend only on the level of NOS activity. The delivery of the NO substrate L-arginine to MD cells may also influence the amount of NO generated by nNOS. It has been proposed that the uptake or availability of arginine may become rate limiting in the formation and release of NO (37,38).

In the current study, L-arginine significantly increased the DAF-2 DA intensity in MD cells from both these sides. 7-NI, a selective nNOS inhibitor, totally inhibited the increase of

DAF-2 DA intensity caused by L-arginine and increased luminal [NaCl]. These results showed that the DAF intensity reflected the NO generation in MD cells and that the NO produced by L-arginine and changes in luminal [NaCl] were exerted through activation of nNOS located in MD cells. However, it is interesting to note that the effect of L-arginine was more effective on NO production when administered from the luminal side than from the bath. The reason for this is not clear.

Our results also showed that with the use of $Ca^{2+}$-free solution, the changes in NO intensity were not significantly different from those with the normal $Ca^{2+}$ solution (Figure 7). A calcium-free solution will abolish intracellular calcium changes in the MD cells (6,7), NO is still released to the same extent as with calcium changes induced by alteration in luminal [NaCl]. One would expect that a constitutive NOS would be dependent on intracellular calcium and calmodulin (39). Our results showed that the NO release rate in MD cells is not significantly related to the $Ca^{2+}$ concentration. However, it is well known from studies with endothelial cells that an increase in shear stress can increase NO release in a non–calcium-dependent way (40,41). And a similar mechanism could possibly exist in the MD cells. In addition, the changes in MD $[Ca^{2+}]_i$ caused by alterations of the luminal [NaCl] were only in a range of 20 to 40 nM (6,7). Such a small change might not be enough to overwhelm other influential factors. Furthermore, as discussed above, the local NO production could also be regulated below the level of NOS activity. Regarding the concentration of dye in the cells, it has been reported that the amount of DAF-2 is not a rate-limiting factor in this reaction, since very little DAF-2 is consumed compared with the amount of NO (42). Furthermore, in the present experiments, we were able to repeat the experiment three to four times with a similar response of DAF-2 DA increase each time. This also indicated that the intracellular DAF-2 concentration was high enough to bind the generated NO in MD cells.

We found that when the luminal [NaCl] was changed from 135 mM or 35 mM to 10 mM, the changes in the amount of DAF-2 DA decreased significantly (data not shown). Decreased luminal [NaCl] would lower the pH in MD cells (23), which could decrease the DAF-2 DA substantially (17,18). The significant decrease of DAF-2 DA fluorescence in the present study is probably caused by the falling intracellular pH in MD cells. A decrease in pH makes it difficult to evaluate the actual NO production in MD cells with DAF-2 DA. However, the results of a limited number of experiments with DAF-2 FM were similar to that of with DAF-2 DA. This also indicated that the changes of DAF- 2 intensity while the luminal [NaCl] was increased from 35 mM to 135 mM indeed reflect the NO concentration and not influenced by pH changes.

In conclusion, for the first time, we could quantitatively measure the MD cell volume changes caused by the changes of luminal [NaCl], and we found that increasing the luminal [NaCl] resulted in an increase in cell volume. We also found that NO formation in MD cells could be measured with DAF-2 and that NO production was increased through nNOS activation with an increased luminal [NaCl]. An increased NO production in MD could directly inhibit the vasoconstriction induced by the TGF and as well adjust TGF through its modulatory role.

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