Dietary salt enhances glomerular endothelial nitric oxide synthase through TGF-β1

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Ying, Wei-Zhung, and Paul W. Sanders. Dietary salt enhances glomerular endothelial nitric oxide synthase through transforming growth factor-β1 (TGF-β1). Am. J. Physiol. 275 (Renal Physiol. 44): F18–F24, 1998.—Dietary salt controls production of nitric oxide (NO), a potent paracrine relaxation factor involved in glomerular filtration and salt excretion. We hypothesized that glomerular NO production was enhanced through endothelial nitric oxide synthase (NOS3). Rats in metabolic cages were studied after 4 days on 0.3% (Lo-salt) or 8.0% (Hi-salt) NaCl diet. Steady-state mRNA and protein levels of NOS3 and calcium-dependent NO production of isolated glomeruli from Hi-salt animals were greater than those values observed in glomeruli from Lo-salt rats. Because dietary salt enhanced glomerular production of transforming growth factor-β1 (TGF-β1) ([W.-Z. Ying and P. W. Sanders. Am. J. Physiol. 274 (Renal Physiol. 43): F635–F641, 1998], studies were then conducted to examine the interaction between NOS3 and TGF-β1. Glomerular steady-state levels of mRNA of NOS3 and TGF-β1 were directly correlated (r² = 0.946; P < 0.0001). A neutralizing antibody to TGF-β1 reduced NOS3 protein and NO production in cultured glomeruli from Hi-salt animals to levels seen in the Lo-salt glomeruli. Thus dietary salt increased glomerular expression of TGF-β1, which in turn augmented NO production through NOS3.

METHODS

Animal preparation. Studies were conducted using 48 male Sprague-Dawley rats, 28 days of age, obtained from Charles River Laboratories (Wilmington, MA). Animals were chosen at this age because of our previous experience which showed normal renal function and blood pressure responses to dietary salt over 2 wk of observation (8). The rats were housed under standard conditions and given 0.3% NaCl diet (AIN-76A with 0.3% NaCl; Dyets, Bethlehem, PA) and water ad libitum for 4 days before initiating the experiment. The animals were then placed in metabolic cages and allowed free access to water and rat diet, which contained either 0.3% (termed “Lo-salt” group) or 8.0% (AIN-76A with 8.0% NaCl, Dyets; termed “Hi-salt” group) NaCl. Urine was collected under oil to prevent desiccation. Food consumption, urine flow, and body weight were recorded daily, and the experiment was concluded after the 4th day. The collected urine sample from each rat was filtered to remove any particulate matter and centrifuged at 325 g for 2 min at 4°C. The supernatant was collected and immediately frozen at −80°C until use. Urine samples were analyzed for sodium and potassium, using flame photometry (model IL-943; Instrumentation Laboratories, Lexington, MA). On the day of study, rats were anesthetized with a pentobarbital sodium (50 mg/kg ip) injection. Both kidneys were harvested under sterile conditions for isolation of glomeruli for in vitro incubation experiments and obtaining protein for Western blotting and total RNA for Northern hybridization.

Isolation of glomeruli. Glomeruli were isolated using a graded sieving technique. This protocol has been shown to produce pure and viable glomeruli for study of transforming growth factor-β (TGF-β) and NO production (6, 20, 35, 38). The kidneys were perfused in situ through the aorta with cold isotonic heparinized saline until blanched (50–60 ml saline over 2 min). The renal cortices from each rat were individually dissected and minced to a pastelike consistency. The homogenate was passed successively through a 106-μm metal sieve that excluded blood vessels and a 75-μm nylon sieve that retained the glomeruli and allowed cells and small tubular segments to pass through. Glomeruli were washed three times with ice-cold PBS at 120 g for 5 min. The pelleted glomeruli were then used as described below for incubation studies, Western blotting, and Northern analysis. All sodium chloride; nitric oxide; glomerulus; endothelial nitric oxide synthase; transforming growth factor-β1

TO EXCRETE SALT in appropriate amounts, several systems monitor variations in dietary salt intake and effect changes in renal salt excretion. The renin-angiotensin-aldostrone axis is one such system. Recently, another factor, nitric oxide (NO), has been suggested to play an important role in salt balance (8–10, 16, 28, 33, 37). Dietary salt controls NO production (6, 20, 35, 38). The current studies sought to determine whether glomerular production of NO was enhanced by an increase in dietary salt and further to identify the potential mechanism involved.

SYNTHETIC CURVES

The rats were housed under standard conditions and given 0.3% NaCl diet (AIN-76A with 0.3% NaCl; Dyets, Bethlehem, PA) and water ad libitum for 4 days before initiating the experiment. The animals were then placed in metabolic cages and allowed free access to water and rat diet, which contained either 0.3% (termed “Lo-salt” group) or 8.0% (AIN-76A with 8.0% NaCl, Dyets; termed “Hi-salt” group) NaCl. Urine was collected under oil to prevent desiccation. Food consumption, urine flow, and body weight were recorded daily, and the experiment was concluded after the 4th day. The collected urine sample from each rat was filtered to remove any particulate matter and centrifuged at 325 g for 2 min at 4°C. The supernatant was collected and immediately frozen at −80°C until use. Urine samples were analyzed for sodium and potassium, using flame photometry (model IL-943; Instrumentation Laboratories, Lexington, MA). On the day of study, rats were anesthetized with a pentobarbital sodium (50 mg/kg ip) injection. Both kidneys were harvested under sterile conditions for isolation of glomeruli for in vitro incubation experiments and obtaining protein for Western blotting and total RNA for Northern hybridization.

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glomerular preparations consisted of more than 95% glomeruli with minimal tubular contamination, as assessed visually at ×40 magnification.

Glomerular incubation studies. The pelleted glomeruli were resuspended at 5 × 10³ glomeruli/ml of serum-free medium (RPMI 1640; Life Technologies, Grand Island, NY). Simultaneously obtained samples of glomeruli from Hi-salt and Lo-salt animals were placed in 24-well plates. Samples were initially incubated with medium alone or medium that contained 3 mM tetraethylammonium chloride (TEA; Sigma Chemical, St. Louis, MO) or 10 µg/ml rabbit polyclonal antibody that specifically neutralizes TGF-β (catalog no. AB-100-NA; R & D Systems, Minneapolis, MN) in serum-free RPMI media at 37°C. TEA in this dose specifically inhibits potassium channels but is relatively nonselective (7, 12, 13, 25, 26) and has been used previously to examine TGF-β production by endothelial cells (25). In some experiments, nonspecific rabbit IgG (10 µg/ml; Southern Biotechnology Associates, Birmingham, AL) was added as another control. After a 30-min incubation period, the medium was removed and replaced with serum-free medium alone or serum-free medium that contained the same concentrations of TEA or neutralizing antibody. Human TGF-β1 (0–10 ng/ml, R & D Systems) was added in some experiments. As described by others (18), to determine calcium-dependent NO production, some samples of each preparation also contained the calcium ionophore A-23187 (1 µM, Sigma Chemical). All samples were incubated for 24 h at 37°C; samples of conditioned media were then harvested stored at −20°C until assayed for nitrate, nitrite, and TGF-β1. The glomeruli were then processed for total protein assay and Western blotting, as described below.

Measurement of glomerular NO production. Concentrations of nitrate and nitrite, collectivly termed NOx, in conditioned media were determined by methods described previously (10). All reagents were from Sigma. To determine NOx concentration, samples of medium, diluted 1:5 in deionized water and sodium nitrate standards (0–200 µM), were simultaneously reduced for 1 h at 37°C by Escherichia coli ATCC no. 25922; American Type Culture Collection, Rockville, MD) grown previously under anaerobic conditions. After centrifugation for 10 min at 1,000 g, 50 µl of supernatant were added to 50 µl of 0.1% sulfanilamide in 30% acetic acid and 50 µl of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 60% acetic acid (Griess reagent). After mixing, the optical density was read at 540 nm, using a microplate reader (THERMOmax, Molecular Devices, Menlo Park, CA).

TGF-β1 immunoassay. Active TGF-β1 in the medium was determined using an enzyme immunoassay (TGF-β1 Emax Immunoassay System; Promega, Madison, WI), following the protocol provided by the manufacturer. Briefly, plates containing 96 flat-bottom wells (Falcon; Becton Dickinson, Oxnard, CA) were coated overnight at 4°C with 100 µg of anti-TGF-β1 monoclonal antibody diluted 1:1,000 in buffer that contained 0.025 M sodium bicarbonate and 0.025 M sodium carbonate, pH 9.7. Thereafter, unbound sites in the wells were blocked using 270 µl of the blocking reagent supplied in the kit for 2 h at room temperature, then 100 µl of undiluted medium or TGF-β1 standard in sample buffer were added to wells. After incubation for 3 h at room temperature with vigorous shaking, the wells were washed five times with TBS wash buffer (20 mM Tris·HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20), then 100 µl of polyclonal anti-TGF-β1 diluted in sample buffer were added. The plates were again incubated overnight at 4°C. After five washes with TBS buffer, wells were filled with 100 µl of antibody conjugated with horseradish peroxidase and incubated for 3 h at room temperature with shaking. After additional washes as in the previous steps, color was developed by adding 100 µl of peroxidase substrate in 3,3′,5,5′-tetramethylbenzidine solution. After an ~10-min incubation at room temperature, 100 µl of 1 M phosphoric acid were added to stop the color reaction. Optical density was determined at 450 nm using a microplate reader (THERMOmax, Molecular Devices). Standards were performed in duplicate using TGF-β1 (7.8–1,000 pg/ml in sample buffer) and were used to construct standard curves from which the concentrations of the samples were determined.

Western blot analysis. Glomeruli that were freshly isolated from the Hi-salt and Lo-salt rats and glomeruli that were treated as described in the incubation studies described above were washed with PBS, then centrifuged at 200 g for 5 min at 4°C. The pellet was dissolved in RIPA buffer (in mM: 50 Tris·HCl, 150 NaCl, 1 disodium EDTA, 0.1 EGTA, 1.0% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate). Phenyldimethylsulfonil fluoride (PMSF, 1 mM), 10 µg/ml apro- tinin, and 10 µg/ml leupeptin were added as protease inhibitors. All of these inhibitors were from Sigma Chemical. Total protein of each sample was determined using a kit (Micro biciniconidnic acid protein assay reagent kit; Pierce, Rockford, IL), and the volume of each sample was adjusted to allow loading of 30 µg into each well. The samples were boiled in SDS-Laemmli sample buffer for 5 min, then the proteins were resolved using a 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. After incubation in blocking buffer (10 mM Tris, pH 7.5, containing 5% nonfat dry milk, 100 mM NaCl, and 0.1% Tween 20), the membranes were probed with anti-human NOS3 monoclonal antibody (Transduction Laboratories, Lexington, KY), 1:1,000 dilution, in blocking buffer for 2 h at room temperature. The membranes were then washed five times with TBST and were incubated with horseradish peroxidase-conjugated anti-IgG antibody (Bio-Rad, Hercules, CA), 1:2,000 dilution, in blocking buffer. After three additional washes using TBST, the membrane was developed using ECL enhanced chemiluminescence Western blotting system and Hyperfilm (Amersham International, Buckinghamshire, UK). The films were scanned using a densitometer to quantify NOS3 (model 620 Video Densitometer, Bio-Rad).

Northern hybridization. Total RNA was isolated from freshly isolated glomeruli in standard fashion by single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction (11). The concentration and purity of RNA in each sample was determined using optical density at 260 and 280 nm. Twenty micrograms of total RNA from each sample was resolved in 1.0% agarose gels containing 2.2 M formaldehyde and 0.2 M MOPS, pH 7.0, then transferred to a nylon membrane (Genescreen Plus Hybridization Transfer Membrane; NEN Life Science Products, Boston, MA) by vacuum blotting (model 785, Bio-Rad) for 2 h in 10× standard sodium citrate (SSC). Nucleic acids were cross-linked by ultraviolet light. The films were exposed to X-ray film for 24 h.

Table 1. Comparison of physiological parameters of animals in this study

<table>
<thead>
<tr>
<th>Hi-Salt</th>
<th>Lo-Salt</th>
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<tbody>
<tr>
<td>No. of animals</td>
<td>24</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>152±5</td>
</tr>
<tr>
<td>U Na, mEq/day</td>
<td>60±2*</td>
</tr>
<tr>
<td>U Na V, µEq/min</td>
<td>20.6±1.4*</td>
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<td>Values are means ± SE. Hi-salt; 8% NaCl diet; Lo-salt, 0.3% NaCl diet; U Na, urinary flow rate; U Na V and U K V, urinary and potassium excretion rates, respectively. * P &lt; 0.05, greater than corresponding mean data obtained from the Lo-salt group.</td>
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irradiation (Stratagene, La Jolla, CA). The membranes were prehybridized for 20 min at 68°C in standard hybridization solution (QuikHyb, Stratagene). They were then hybridized at 68°C for 4 h with cDNA probes for rat TGF-β1 (kindly provided by Dr. Thomas S. Winokur, University of Alabama at Birmingham) and for bovine NOS3 (generously provided by Dr. William C. Sessa, Yale University School of Medicine). The cDNA probes, which consisted of an 1-kb fragment that was produced by digestion of the TGF-β1 plasmid with Hind III and Xba I and included the entire coding region (17) and an 4-kb fragment produced by digestion of the NOS3 plasmid with EcoR I, were labeled with [α-32P]dCTP by random oligonucleotide priming (Prime-a-Gene Labeling System, Promega). The blots were washed in 2× SSC with 0.1% SDS at room temperature for 30 min and in 0.1× SSC with 0.1% SDS at 60°C for 20–30 min. Membranes were exposed to XAR-5 film (Kodak) at −80°C. The blots were then stripped in solution containing 1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and 0.1× Denhardt’s at 75°C for 2 h and rehybridized with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe obtained through the American Type Culture Collection. Autoradiographs were scanned using a densitometer (model 620 Video Densitometer, Bio-Rad). The density of the GAPDH band in the same lane was used to normalize mRNA loading. For quantification, densities of the bands of TGF-β1 and NOS3 were individually divided by the density of band for GAPDH in the same lane.

Statistical analysis. All data are presented as means ± SE. Significant difference among data sets was determined using either the unpaired t-test or one-way analysis of variance with standard posthoc testing (Statview, version 4.5; Abacus Concepts, Berkeley, CA), where appropriate. P < 0.05 was statistically significant.

RESULTS

Mean body weight, food intake, urinary flow rate, urinary sodium excretion rate, and urinary potassium excretion rate of the 32 rats in this study were shown in Table 1. As expected, urinary flow rate and urinary sodium and potassium excretion rates of the Lo-salt group were less (P < 0.05) than the corresponding Hi-salt group.

Steady-state mRNA and protein levels of NOS3 were determined using freshly isolated glomeruli from rats

Fig. 1. Northern hybridization showing steady-state mRNA levels of endothelial nitric oxide synthase (NOS3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in freshly isolated glomeruli from high (Hi)-salt (n = 4) and low (Lo)-salt (n = 4) rats. Graph (bottom) is mean densities of NOS3 relative to GAPDH and shows increase (0.534 ± 0.05 vs. 0.161 ± 0.01, P = 0.0006) in relative mRNA levels of NOS3 in glomeruli of animals maintained on 8.0% NaCl (Hi-salt) diet, compared with those animals maintained on the 0.3% NaCl (Lo-salt) diet.

Fig. 2. Western blot of NOS3 expression in freshly isolated glomeruli from Hi-salt (n = 4) or Lo-salt (n = 4) rats. Mean relative NOS3 expression in Hi-salt glomeruli was greater (1.058 ± 0.071 vs. 0.507 ± 0.89, P = 0.003) than Lo-salt glomeruli.

Fig. 3. Steady-state mRNA levels of NOS3 and transforming growth factor-β1 (TGF-β1) obtained from freshly isolated glomeruli of eight rats (4 in each group) were directly correlated (r² = 0.946, P < 0.0001).
on the 8.0% and 0.3% NaCl diets. Northern analysis and Western blotting confirmed increased expression of NOS3 in glomeruli from the Hi-salt group (Figs. 1 and 2). Mean steady-state mRNA of TGF-β1 was also greater (0.632 ± 0.037 vs. 0.326 ± 0.032, P < 0.0008) in freshly isolated glomeruli from the Hi-salt group, compared with the Lo-salt group. Glomerular expressions of mRNA of NOS3 and TGF-β1 were linearly correlated (r² = 0.946, P < 0.0001) (Fig. 3). With glomeruli in culture, production of active TGF-β1 was shown to be increased in rats on the Hi-salt diet (Fig. 4). Consistent with our previous results (42) and that published by Ohno et al. (25), the increase in production of TGF-β1 was abrogated by addition of TEA to the medium. NOS3 protein expression of isolated glomeruli in culture was enhanced by dietary salt (Fig. 5). Addition of either TEA or a neutralizing antibody to TGF-β1 to the medium reduced these values to levels found in the Lo-salt group (Fig. 6). To ensure that the effect of the antibody to TGF-β was specific, additional Western blotting experiments were performed following incubation with either the neutralizing antibody or nonspecific rabbit IgG, 10 µg/ml (Fig. 7). Nonspecific rabbit IgG had no effect on NOS3 protein expression. In another study, TGF-β1 was added to the medium in concentrations between 0 and 10 ng/ml. Addition of TGF-β1 to glomeruli from the Hi-salt animals increased expression of NOS3 but had no effect on glomeruli from the Lo-salt animals (Fig. 8). Thus, although TGF-β1 was essential in stimulation of NOS3, dietary salt produced another factor that permitted TGF-β1 to promote NOS3 expression in glomeruli.

**DISCUSSION**

TGF-β1 belongs to a family of five multifunctional peptide growth factors (29). TGF-β1 is produced in the glomerulus (2, 40, 43) and has come under increasing scrutiny because of the important role this growth factor plays in glomerular senescence (4, 5, 19, 24, 29, 38–41, 44). We recently examined the potential role of dietary salt in modifying TGF-β1 expression in the kidney (42). An increase in dietary salt produced sustained increases in TGF-β1 mRNA and protein expression in the glomerulus. This effect was inhibited by TEA, suggesting a shear stress-related mechanism (25, 27). In addition, previous studies have shown that TGF-β1 directly regulates expression of NOS3 in bovine endothelial cells in culture (18). Based on these data and other observations that showed plasma volume increased in rats on a Hi-salt diet (14), we hypothesized an increase in NO mediated through TGF-β1 in glomeruli of rats on a Hi-salt diet. One expected compensation by which NO facilitates salt excretion is the ability of NO to increase Kᵋ (15). In the current study, an increase in dietary salt increased NOS3 mRNA and protein expression in freshly isolated glomeruli. Calcium-dependent NO production was also increased, confirming augmented NOS3 activity. NOS3

![Fig. 4](http://ajprenal.physiology.org/) Production of active TGF-β1 by glomeruli isolated from Hi-salt (n = 4) and Lo-salt (n = 4) rats. Increase in TGF-β1 was abrogated by addition of 3 mM tetraethylammonium chloride (TEA) to the medium, as we showed previously (42).

![Fig. 5](http://ajprenal.physiology.org/) Western blot of NOS3 expression in glomeruli from Hi-salt (n = 4) or Lo-salt (n = 4) rats. Top: glomeruli had been incubated in medium alone or medium containing 3 mM TEA or a neutralizing antibody to TGF-β (Ab) for 24 h prior to processing. Con, positive control. Bottom: results of quantification of the image. Addition of either TEA or Ab to the medium reduced (P = 0.0124) mean NOS3 expression in glomeruli from Hi-salt rats, compared with NOS3 expression from untreated glomeruli from Hi-salt rats, to levels similar to that seen in Lo-salt glomeruli. Neither TEA nor Ab reduced NOS3 expression in glomeruli from the Lo-salt rats.
mRNA expression correlated linearly with TGF-β mRNA (Fig. 3). Importantly, both NOS3 protein expression and function were inhibited by addition of a neutralizing antibody to TGF-β. Thus the combined data confirmed that dietary salt enhances expression of TGF-β1 through a TEA-sensitive mechanism, probably shear stress. In turn, TGF-β1 stimulated glomerular NO production through NOS3 expression.

The present data provided support for the work of Deng and Baylis (15), who showed a role of NO in glomerular hemodynamics and glomerular function. The current studies also suggested one potential source of augmented production of NO on a Hi-salt diet (33). Enhanced glomerular NO production produces favorable glomerular hemodynamics to facilitate salt excretion (3), presumably by relaxation of the mesangial cells. Certainly, other NOSs, in the kidney and other organs, are also involved in the increase in NO production by dietary salt (9, 21). Although our current studies were consistent with the role of changes in NO in response to dietary salt (8, 16, 33, 37), the data disagree with two previous reports (21, 34). Mattson and Higgon (21) used Western blotting to demonstrate that dietary salt increased expression of NOS3, along with NOS1 and NOS2, in the medulla, but an increase in NOS3 was not seen in the cortex. As suggested by the authors, one limitation of their study was the low expression of NOS3 in the cortex and use of cortical tissue; isolated glomeruli were not examined. Potentially, these factors obscured any potential differences that occur in glomeruli in response to dietary salt intake. In the second study, Singh and associates (34) utilized only semiquantitative RT-PCR to detect mRNA of NOS3 in microdissected glomeruli. Although this approach is technically very difficult and contains many pitfalls, we cannot otherwise explain their negative results. However, pooling large numbers of isolated glomeruli allowed successful comparison of steady-state mRNA of NOS3 using standard Northern hybridization analysis. Although differences between our data
and the literature exist, our study used three different assays to show that steady-state mRNA, protein levels, and functional activity of NOS3 were all increased in glomeruli from rats on the Hi-salt diet. Our work provided in vivo correlation with the previously described role of TGF-β1 in expression of NOS3 in endothelial cells in culture (18).

The mechanism of regulation of NOS3 by TGF-β1 has been partially elucidated. Previous studies have shown that TGF-β1 upregulates mRNA expression of mouse α2(I) collagen gene through activation of a nuclear factor-1 (NF-1) binding site in the promoter region (30). Inoue et al. (18) further demonstrated that TGF-β1 stimulated gene transcription directly by activation of a similar element in the NOS3 promoter. A recent intriguing study showed that TGF-β1 regulated CCAAT-binding transcription factor (CTF-1), the prototype member of the NF-1 family of transcription factors, in part, through mobilization of intracellular calcium stores and activation of calcineurin and calmodulin-dependent protein kinase IV. Another unidentified pathway also participates in the process of induction of CTF-1 transcription by TGF-β1 (1). The role of Smad proteins in this signal cascade is currently unclear. Interestingly, although supplemental TGF-β1 increased NOS3 in glomeruli from rats on the Hi-salt diet, TGF-β1 had no effect on NOS3 production in glomeruli from rats on the Lo-salt diet. The reason for this difference is uncertain at present but may relate to a direct alteration in the signal transduction cascade known to stimulate NOS3 transcription by changes in dietary salt.

In summary, dietary salt enhanced glomerular production of TGF-β1 through a TEA-sensitive mechanism. TGF-β1 directly increased NOS3 expression and calcium-dependent NO production. Almost four decades ago, pioneering work of Meneely and associates demonstrated a direct link between dietary salt and life span in rats. As dietary salt was increased from 0.15 to 21%, blood pressure progressively increased and life span fell. Autopsy studies further suggested findings compatible with arteriolsclerosis and atherosclerosis, particularly in the kidneys, as well as glomerular damage. Enhanced glomerular production of TGF-β1 by dietary salt is one potential mechanism of damage, although the concomitant increase in NO mitigates this response. Thus a delicate balance in the glomerulus between TGF-β1 and NOS3 ensures an appropriate hemodynamic action without untoward effects. However, with endothelial dysfunction, NO production might fail and facilitate glomerulosclerosis. These concepts provide the foundation for further investigation.

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