Alterations in Renal Endothelin-1 Production in the Spontaneously Hypertensive Rat

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Endothelin-1 inhibits sodium and water transport systems in the inner medullary collecting duct. Endothelin-1 levels are reduced in the medulla of spontaneously hypertensive rats (SHR), raising the possibility that decreased inner medullary collecting duct production of endothelin-1 could contribute to inappropriate sodium and water retention. In the current study, immunoreactive endothelin-1 was measured in the urine, blood, and eluates from cortex and outer and inner medulla of SHR before (age 3–4 weeks) and after (age 8–9 weeks) the development of hypertension and in age-matched Wistar-Kyoto (WKY) controls. There was no difference in endothelin-1 levels between prehypertensive SHR and WKY rats. In contrast, 8–9-week-old SHR had significantly reduced endothelin-1 in the urine and outer and inner medulla, but not in the cortex or serum compared with those of WKY controls. Furthermore, inner medullary collecting duct cells from 8–9-week-old SHR, either acutely isolated or cultured, released less endothelin-1 than did those from WKY rats. Finally, the level of endothelin-1 messenger RNA was only reduced in the inner medulla and in inner medullary collecting duct cells from 8–9-week-old SHR. In summary, renal medullary, and in particular terminal collecting duct, endothelin-1 production is reduced in SHR only after the development of hypertension. Such decreases in inner medullary collecting duct endothelin-1 production may contribute to the hypertensive state in SHR. (Hypertension 1992;20:666–673)

KEY WORDS: • kidney • hypertension, renal • RNA • kidney tubules, collecting
ET-1 excretion is believed to reflect renal production of ET-1, these findings also suggest that renal production of ET-1 is reduced in the setting of hypertension. Such a decrease in nephron ET-1 production could theoretically lead to reduced autocrine inhibition of sodium and water reabsorption and potentially contribute to the development or maintenance of the hypertensive state. The current studies were undertaken, therefore, to directly determine if renal ET-1 production is altered in hypertension. Using the SHR as a model, renal production of immunoreactive ET-1 and ET-1 messenger RNA (mRNA) were assessed before and after the development of hypertension in SHR and in age-matched WKY controls. We report that ET-1 synthesis is markedly lower in the kidney of hypertensive rats, that the IMCD cell is the primary site of this reduced ET-1 production, and that such changes occur only after the development of hypertension.

Methods

Blood Pressure Measurements

Arterial blood pressure was measured in male SHR and WKY rats (Harlan Sprague Dawley, Madison, Wis.), aged 3-4 and 8-9 weeks. Rats were placed in a restraining box and warmed until a pulse was detected in the tail. Systolic pressure was assessed using a tail-cuff and pressure transducer in conjunction with an automated pressure delivery system and chart recorder (Narco BioSystems, Austin, Tex.).

Tissue Isolation and Culture

For cultured IMCD cell studies, 3-4- and 8-9-week-old SHR and WKY rats were killed and the kidneys immediately removed. IMCD cells were isolated as previously described. The inner medulla was dissected out, finely minced, and incubated in 0.1% collagenase (type II, Worthington Diagnostics Systems, Freehold, N.J.) and 0.01% deoxyribonuclease (type I, Sigma Chemical Co., St. Louis, Mo.) in Krebs buffer for 1 hour at 37°C. The deoxyribonuclease was added to reduce cell clumping. Distilled water was added to give an osmolality of 120 mosm, a procedure shown to disrupt all cells except those of the collecting duct. The cells were washed in phosphate-buffered saline containing 10% albumin and suspended in modified medium KL10 (50:50; Dulbecco's modified Eagle media:Ham's F12 media). Measurement of Immunoreactive Endothelin-1

The following tissues and cells from 3-4- and 8-9-week-old SHR and WKY rats were analyzed for ET-1 concentration: 1) acutely isolated and minced cortex, inner medulla, and outer medulla; 2) acutely isolated and minced cortex, inner medulla, and outer medulla; and 3) cultured IMCD cells. Each of these tissues were incubated with PRF-DMEM for 6 hours at 37°C in a 5% CO2 environment. PRF-DMEM was used since phenol red interferes with ET-1 extraction. At the end of the incubation, the supernatant discarded and the cells resuspended in Krebs buffer. This low speed centrifugation was repeated two more times, and the pellet was either suspended in guanidinium thiocyanate (GITC) and immediately homogenized. For ET-1 release studies, the cortex, outer medulla, and inner medulla were dissected out, minced until no pieces larger than 1 mm remained, and placed into separate tubes using PRF-DMEM as described below.

Measurement of Immunoreactive Endothelin-1

The following tissues and cells from 3-4- and 8-9-week-old SHR and WKY rats were analyzed for ET-1 release: 1) acutely isolated and minced cortex, inner medulla, and outer medulla; 2) acutely isolated and enzyme-purified IMCD cells; and 3) cultured IMCD cells. Each of these tissues were incubated with PRF-DMEM for 6 hours at 37°C in a 5% CO2 environment. The cells were mixed with l/10th volume 5% bovine serum albumin and l/40th volume 0.4% Triton X-305 and applied to a Bond Elut C4 cartridge (Analytichem Int., Harbor City, Calif.) prewashed successively with 100% acetonitrile (AcCN)/0.1% trifluoroacetic acid (TFA), 100% methanol (MeOH)/0.1% TFA, and 0.1% TFA. After applying the sample, the column was washed with 0.1% TFA, then 40% MeOH/0.1% TFA, and endothelin eluted with 70% MeOH/0.1% TFA/0.01% Triton X-305. The eluate was dried and suspended in radioimmunoassay (RIA) buffer. This procedure resulted in recovery of 85±8% (n=4) of 128 pg and 81±9% (n=4) of 8 pg ET-1 standards added to 2 ml incubation media.

ET-1 was measured using a kit purchased from Peninsula Laboratories, Inc., Belmont, Calif., as previously described. Standards or samples in RIA buffer were incubated with rabbit anti-ET-1 antibody (1:20,000 dilution of sera) overnight at 4°C.
day $^{32}$P-ET-1 (10,000–15,000 cpm) was added and incubated overnight at 4°C. The next morning goat anti-rabbit globulin and normal rabbit serum were added, the precipitate centrifuged, and counts per minute determined on a Micromedic 4/600 gamma counter. The lower limit of sensitivity for ET-1 detection was 2 pg. Intra-assay variation was less than 9%; interassay variation was less than 15%. Competitive binding inhibition curves for ET-1 antibody showed less than 5% cross-reactivity with unlabeled endothelin-3 and less than 3% cross-reactivity with unlabeled endothelin-2. Reactivity with big endothelin has not been evaluated.

All cells from which supernatants were obtained for ET-1 measurement were analyzed for total protein content. Cells were solubilized with 0.1N NaOH, an aliquot was removed and mixed with Bradford reagent (Bio-Rad, Richmond, Calif.), and absorbance at 590 nm was determined.19

Isolation of RNA

RNA was obtained from acutely isolated renal cortex, outer medulla, and inner medulla; acutely isolated IMCD cells; and cultured IMCD cells from 3–4- and 8–9-week-old SHR and WKY rats. As described above, cortex, outer medulla, and inner medulla were snap-frozen and placed in GITC; acutely isolated IMCD cells were centrifuged and placed in GITC; and cultured IMCD cells were overlaid with GITC. The GITC solution contained 4 M GITC, 25 mM sodium citrate, 1% β-mercaptoethanol, and 1% sarcosyl (pH 7.0). After homogenization, each sample was subjected to cesium chloride density gradient centrifugation. The RNA pellet was washed in 70% ethanol, chloroform extracted, and ethanol precipitated. The integrity of each RNA sample was verified by agarose/formaldehyde gel electrophoresis and quantified spectrophotometrically.

Quantitation of RNA

Total RNA (2.5 μg) from each sample was reverse transcribed by incubating with 100 pmol/μl random hexamers (Boehringer), 4 mM MgCl₂, 10 units/μl murine Maloney Leukemia Virus reverse transcriptase (Gibco), 2 units/ml RNAsin (Promega), 500 μM deoxynucleotide triphosphates (dNTP, Perkin-Elmer), 1 mM dithiothreitol, 50 mM KCl, 10 mM Tris-Cl, and 0.01% gelatin (final buffer pH 8.3) for 1 hour at 37°C. The reverse transcriptase was inactivated by heating for 5 minutes at 95°C. The resultant complementary DNA (cDNA) was stored at -20°C for up to 1 month.

The cDNA was amplified by polymerase chain reaction (PCR). Each sample was measured for ET-1 and B-actin cDNA in separate tubes using specific primers. The upstream and downstream primers for ET-1 were GC-CAAGCAGACAAAGAACTCCGAG and GCTCTG-CAAGCAGACAAAGAACTCCGAG and GCCTCTG-TAGTCAATTGTCGTCGGTTT, respectively. These yielded a 247–base pair fragment that is complementary to position 371–618 in rat ET-1 cDNA.20 PCR of rat genomic DNA yielded a 1,300–base pair product, indicating that these primers spanned an intron. The upstream and downstream primers for B-actin were TGGAGAAGAG-CTATGAGCTGCTG and GTGCCAAGAGACTGTTGTTG, respectively, which yielded a 201–base pair cDNA fragment. PCR of genomic DNA yielded a 289–base pair product that is complementary to position 2,499–2,788 in the B-actin gene, confirming that this primer set also spans an intron. PCR was performed by incubating 5 μl (approximately 0.25 μg) of sample cDNA with 50 mM KCl, 10 mM Tris-Cl, 0.01% gelatin, 1.5 mM MgCl₂, 2.5% formamide, 2 units Thermus aquaticus (Taq) polymerase (Boehringer), 200 μM dNTP, 100 pmol of B-actin or ET-1 primers, and 1 μCi $^{32}$P-dCTP (Amersham) in 50 μl final volume (final pH 8.3 at room temperature). PCR using B-actin primers was carried out for 25 cycles (15 seconds at 94°C, 15 seconds at 65°C, 30 seconds at 72°C) using a Perkin-Elmer Cetus 9600 GeneAmp System. PCR using ET-1 primers was carried out for 30 cycles under identical conditions. ET-1 and B-actin primers were never combined in the same tube. Twenty microliters of the final PCR reaction was electrophoresed using 1% ME agarose (FMC), 2.5% NuSieve GT agarose gel in 1× TAE buffer containing 1 μg/ml ethidium bromide. The bands corresponding to the cDNA product were excised, mixed with scintillation cocktail, and counts per minute determined on a Beckman beta counter.

ET-1 and B-actin cDNA obtained from PCR of reverse-transcribed RNA were used to generate standard curves. The cDNA was amplified by PCR, and the resultant amplified product was divided into small fractions that were, in turn, reamplified. The purity of the final product was confirmed by electrophoresis. If a single band of the appropriate size was obtained, the final product was cleaned using Magic PCR Prep (Promega) to remove the primers. The cleaned product was again electrophoresed to confirm that it contained only the desired cDNA. If pure, the cDNA was quantitated spectrophotometrically. Standard curves for B-actin or ET-1 were made by simultaneously amplifying sample cDNA and, in separate tubes, standard cDNA (10⁻³ to 10⁻⁷ nanograms per tube). Every PCR amplification included a standard curve.

To facilitate comparison between ages and species, each PCR amplification included samples from both 3–4- and 8–9-week-old WKY rats and SHR. A given PCR amplification always contained grouped cortex, outer medulla, inner medulla, or acutely isolated or cultured IMCD cells. To facilitate comparison between regions of the kidneys, some PCR amplifications included all regions of the kidney from 3–4- and 8–9-week-old SHR and WKY rats. However, similar results were obtained when RNA from cortex, outer medulla, and inner medulla were measured in the same or separate PCR reactions. Finally, all PCR consisted of simultaneous amplification (in separate tubes) of cDNA for ET-1 and B-actin.

All results are expressed as nanograms ET-1 cDNA/nanograms B-actin cDNA to control for the amount of RNA initially reverse transcribed.

Statistics

All data were compared by analysis of variance. Results are expressed as mean±SEM.

Materials

Insulin, transferrin, and selenium were purchased from Collaborative Research, Bedford, Mass. Unless otherwise stated, all other reagents were purchased from Sigma Chemical Co., St. Louis, Mo.
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week-old SHR and WKY rats also released similar amounts of ET-1; however, the outer and inner medulla was not different between WKY rats and SHR in either age group. Urinary ET-1 excretion was also not different between WKY rats and SHR at 8–9 weeks of age (n=6 in all groups).

In the first set of experiments, plasma and urine ET-1 levels in SHR and WKY rats were assessed to determine if renal ET-1 excretion was altered in hypertensive animals and if such alterations might be due to differences in the amount of circulating ET-1. As shown in Figure 1, serum ET-1 levels were not different between WKY (105±4 mm Hg) and SHR (110±5 mm Hg) at 3–4 weeks of age, whereas systemic blood pressure was significantly elevated in SHR (155±7 mm Hg) compared with that of WKY rats (110±4 mm Hg) at 8–9 weeks of age (n=6 in all groups).

FIGURE 1. Bar graph shows concentration of immunoreactive endothelin-1 (ET-1) in the serum of 3–4- and 8–9-week-old spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats. n=6 each data point.

Results

SHR typically develop hypertension after 6–7 weeks of age. Consequently, SHR were studied at 3–4 weeks (before the onset of hypertension) and 8–9 weeks (after development of hypertension) of age along with normotensive WKY controls. To confirm that hypertension occurred at the predicted time, tail arterial systolic pressure was measured. Systolic blood pressure was not different between WKY (105±4 mm Hg) and SHR (110±5 mm Hg) at 3–4 weeks of age, whereas systemic blood pressure was significantly elevated in SHR (155±7 mm Hg) compared with that of WKY rats (110±4 mm Hg) at 8–9 weeks of age (n=6 in all groups).

To localize any renal site (or sites) of altered ET-1 production, ET-1 release by dissected and minced renal cortex, outer medulla, and inner medulla from 3–4- and 8–9-week-old SHR and WKY rats was examined. As shown in Figure 3, ET-1 release from cortex, outer medulla, and inner medulla was not different between 3–4-week-old SHR and WKY rats. The cortex of 8–9-week-old SHR and WKY rats also released similar amounts of ET-1; however, the outer and inner medulla obtained from SHR released significantly less ET-1 than the same areas of the kidney obtained from WKY rats (Figure 3). These data suggested that the outer and inner medulla contained cells that were producing less ET-1 once hypertension developed.

Since the medullary collecting duct is the predominant nephron site of ET-1 production11 and is confined exclusively to the areas where ET-1 production was reduced in hypertensive SHR, the production of ET-1 by collecting duct cells was examined. IMCD cells were isolated from 3–4- and 8–9-week-old SHR and WKY rats and examined for ET-1 release. Similar to medullary tissue, IMCD cells from 3–4-week-old SHR and WKY rats released small but comparable amounts of ET-1 (Figure 4). In contrast, IMCD cells freshly isolated from 8–9-week-old SHR released markedly less ET-1 than did IMCD cells from age-matched WKY rats (Figure 4). These studies suggest that the decrease in ET-1 release by the outer and inner medulla of hypertensive rats was due, at least in part, to decreased production of ET-1 by collecting duct cells. This decrease in ET-1 release could be either a result of hypertension or an intrinsic alteration in IMCD cell function. To assess IMCD cell ET-1 production independent of the immediate influence of hypertension, IMCD cells from 3–4- and 8–9-week-old SHR and WKY rats were purified and grown in culture to confluence. As before, IMCD cells obtained from 3–4-week-old SHR and WKY rats produced similar amounts of ET-1 (Figure 5). Conversely, cultured IMCD cells obtained from 8–9-week-old SHR released far less ET-1 than did cultured IMCD cells from 8–9-week-old WKY rats (Figure 5). These data suggest that IMCD cells develop an intrinsic alteration in ET-1 production that persists in the absence of exposure to the hypertensive state. Interestingly, IMCD cells from 8–9-week-old rats produced significantly more ET-1 than did the same cells from 3–4-week-old animals, regardless of strain. The mechanism (or mechanisms) by which IMCD ET-1 production increases with age is unknown but would be of interest since derangements in the factors responsible for age-related changes in ET-1
production may be relevant to understanding the alterations in ET-1 synthesis in SHR.

Although the above studies assessed immunoreactive ET-1 release by renal cells, they did not absolutely rule out the possibility that differences in ET-1 production could be due, at least in part, to alterations in ET-1 receptor number on the cells, variable binding of circulating ET-1, and subsequent different release of bound ET-1 into the media. This possibility seemed remote, particularly in view of the relative irreversibility of ET-1 binding to its receptor. Furthermore, it is highly unlikely that the differences in ET-1 production observed in the cultured IMCD cells were due to carryover of in vivo
The results of reverse transcription and PCR of RNA from cortex and outer and inner medulla are shown in Figure 6. When care was taken to amplify by PCR the same amount of cDNA, the levels of B-actin were not significantly different between SHR and WKY rats. Thus, the ET-1/B-actin ratio reflected changes only in ET-1 mRNA. There was no significant difference in the amount of ET-1 mRNA in the inner medulla of 3–4-week-old WKY rats and SHR (Figure 6). In contrast, there was a marked reduction in ET-1 mRNA in the inner medulla of 8–9-week-old SHR compared with age-matched WKY controls (interassay variability of 15.3% for WKY rats and 16.5% for SHR). To determine if this reduction in inner medullary ET-1 mRNA reflected ET-1 mRNA in the IMCD, acutely isolated IMCD were examined. Unfortunately, we were unable to get measurable quantities of RNA from acutely isolated cells. It is probable that this failure was due to release of substantial amounts of RNases during the process of mincing and digesting the inner medulla. Nonetheless, the finding of substantially reduced ET-1 release by acutely isolated IMCD cells from 8–9-week-old SHR argues in favor of decreased ET-1 synthesis by this group since it is highly unlikely that significant amounts of ET-1 would remain attached to the cells during the process of enzymatic digestion and multiple washings. Finally, to confirm the findings on ET-1 release by cultured IMCD cells, ET-1 and B-actin mRNA were quantitated in cultured IMCD cells obtained from 3–4- and 8–9-week-old WKY rats and SHR (Figure 7). There was a tendency for ET-1 mRNA to be lower in the cultured IMCD cells from 3–4-week-old SHR compared with age-matched WKY rats; however, this difference was not significant. Conversely, there was a marked decrease in ET-1 mRNA in IMCD cells from 8–9-week-old SHR compared with age-matched WKY rats and SHR, confirming that whatever mechanism (or mechanisms) are responsible for decreased ET-1 production by IMCD cells in hypertensive SHR persist in culture.

Discussion

Abnormal renal handling of sodium and water has been shown to be important in the development and maintenance of hypertension. The proximal tubule has been thought to be the primary site responsible for the impaired pressure–natriuresis response in genetic hypertension; however, other nephron segments have been implicated. Micropuncture studies have identified increased chloride reabsorption in the loop of Henle in Dahl salt-sensitive (DS) rats compared with Dahl salt-resistant (DR) rats when the kidneys were perfused at the same pressure. The collecting duct, although less well studied, may also undergo functional changes in

![FIGURE 6. Bar graphs show ratio of endothelin-1 (ET-1) messenger RNA (mRNA) to B-actin mRNA in cortex (panel A), outer medulla (panel B), and inner medulla (panel C) from 3-4- and 8-9-week-old spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats. Each data point represents the mean of triplicate determinations on RNA obtained from eight rats. *p<0.001.](image-url)
animals with genetic hypertension. Husted and Stokes noted that monolayers of cultured IMCD cells obtained from DS rats had higher short circuit currents than did IMCD cells from DR rats. Furthermore, IMCD cells from DS rats are less responsive (as determined by cyclic GMP accumulation) to atrial natriuretic factor and nitroprusside. IMCD cells from DS rats also have lower basal and stimulated PGE2 production compared with those from DR rats. Thus, IMCD cells from DS rats appear to have a higher basal sodium reabsorption rate, to produce less endogenous inhibitors of sodium transport, and to have a reduced response to exogenous inhibitors of sodium transport. Our finding that ET-1 production by IMCD cells is reduced in SHR provides further evidence that collecting duct, and in particular IMCD, function is altered in animal models of hypertension. Since ET-1 potently inhibits Na,K-ATPase activity and AVP-stimulated water permeability in IMCD cells, these results raise the possibility that, like DS rats, sodium reabsorption pathways in the IMCD of SHR may be under less endogenous inhibition.

The mechanism by which ET-1 levels are reduced in the kidney of SHR is unknown. These changes occur only after the development of hypertension, suggesting that the hypertensive state in SHR, per se, may influence ET-1 production by IMCD cells. It is possible that alterations in medullary blood flow, interstitial pressures, or local hormonal changes could affect production of ET-1 by IMCD cells in SHR, although no such relation between these factors and ET-1 synthesis by IMCD cells has been described. Reduced IMCD ET-1 production could conceivably be due to alterations in circulating plasma ET-1 concentration; however, neither the present study nor previous ones detected changes in plasma ET-1 in SHR. It is also possible that the reduced ET-1 production is caused by an intrinsic change in IMCD cells. Such a conclusion is supported by the observation that cultured IMCD cells from SHR continue to synthesize less ET-1 than do IMCD cells obtained from age-matched WKY rats. Why this difference in IMCD ET-1 production should be manifest only after the SHR develops hypertension is unknown; however, it may relate to age-associated changes in ET-1 synthetic capability. In the current study, a substantial increase in renal ET-1 production (per cell) was observed as both the SHR and WKY rats aged. This suggests that whatever process is responsible for the rise in ET-1 synthesis with age may be deranged in SHR. Clarification of these mechanisms awaits further studies.

Decreased ET-1 production by IMCD cells, as noted above, may ultimately contribute to enhanced sodium and water reabsorption by this nephron segment in SHR. Several issues need to be considered, however, before this speculation can be confirmed. First, alterations in ET-1 release by IMCD cells from SHR could be accompanied by changes in endothelin receptors. Such a possibility has not been directly investigated; however, a previous study has detected no difference between SHR and WKY rats in whole kidney Kmax or Bmax for ET-1. Furthermore, in this same study, whole kidney ET-1 was also found to be markedly reduced in hypertensive SHR compared with WKY controls. It is also possible that ET-1 receptor activation results in an altered signal in the IMCD of SHR. Enhanced and diminished responsiveness of blood vessels to ET-1 has been described in the SHR; thus it is not possible to predict a change in IMCD ET-1 receptor signaling. A second consideration is the possibility of altered ET-1 production by IMCD cells of SHR in response to locally produced or circulating stimulatory or inhibitory factors. The current study assessed only basal immunoreactive ET-1 release; thus it is conceivable that IMCD cell responsiveness to regulatory agents could be modified. It has been noted that AVP enhances ET-1 release from renal medullary slices and that this increase is greater in DS rats than in DR rats. We have not been able to detect a stimulatory effect of AVP on ET-1 release by IMCD cells, whether cultured or acutely isolated (unpublished observation from our laboratory). AVP can augment ET-1 secretion by cultured endothelial cells, suggesting that the enhanced responsiveness to AVP noted above was due to endothelial cell ET-1 production. This also raises the possibility that endothelial cells in the renal medulla of rats with genetic hypertension may have altered production of ET-1. Finally, sodium and water transport by the IMCD of SHR has not been directly evaluated. Before a role for ET-1 in altered IMCD function in SHR could be confirmed, such studies would need to be done, if possible, in the presence and absence of ET-1 receptor antagonists or anti–ET-1 antibodies.

In summary, the production of ET-1 by the inner medulla is markedly reduced in SHR compared with age-matched WKY controls. This reduction is most likely due to decreased synthesis and release of ET-1 by IMCD cells. Furthermore, the reduced ET-1 production by IMCD cells from SHR persists in culture. Since ET-1 inhibits sodium and water reabsorption pathways in the IMCD, these findings raise the possibility that reduced ET-1 in the inner medulla of hypertensive rats may contribute to the hypertensive state. Further studies are needed to define the role that ET-1 in the renal medulla plays in the development and maintenance of hypertension.
Acknowledgment

We thank Eva Padilla for her excellent technical assistance.

References


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Hypertension. 1992;20:666-673
doi: 10.1161/01.HYP.20.5.666

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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