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Renal nuclear Angiotensin II receptors in normal and hypertensive rats

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Accumulation of Angiotensin II (Ang II) in the kidneys of hypertensive rats infused chronically with Ang II occurs by AT_1 receptor mediated internalization of Ang II, which may interact with intracellular targets, including nuclear binding sites. The aims of this study were to determine if kidney cell nuclei have specific Ang II binding sites and if chronic infusion of Ang II (70 ng/min; n=9) influences the nuclear Ang II binding capacity. Kidneys were harvested from control and Ang II infused rats and the renal cortexes were homogenized to obtain crude membrane preparations and nuclear fractions. Ang II binding sites were measured with a single point assay by incubating each fraction with 10 nM ^{125}I-Sar-Ile-Ang II in the absence (total binding sites) or presence of either 2.5 M Sar-Leu-Ang II or 25 μM losartan to detect specific AT or AT_1 binding sites. Both fractions exhibited specific Ang II binding sites that were displaced by both saralasin and losartan. In control rats, crude membrane preparations had 792±218 and the nuclear fraction had 543±222 fmol/mg protein AT_1 receptors. AT_1 receptor levels in membrane (885±170 fmol/mg protein) and nuclear fractions (610±198 fmol/mg protein) were not significantly different in Ang II infused rats. These data support the presence of nuclear Ang II receptors predominantly of the AT_1 subtype in renal cells. Chronic Ang II infusion did not alter overall Ang II receptor densities.

Keywords: hypertension, Angiotensin II infusions, AT_1 receptors, nuclear Ang II receptors

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The infusion of Angiotensin II (Ang II) at low doses leads to progressive hypertension and accumulation of Ang II in kidneys, and this effect is associated with compromised renal function and reduced sodium excretory capability (30, 36, 37). Recent observations indicate that part of the renal accumulation of Ang II is mediated by AT_1 receptor-dependent endocytosis with some of the internalized Ang II remaining intact in the endosomal fraction (35). These data support the concept that the internalized Ang II–AT_1 receptor complex may exert an intracellular signaling function that could contribute to the renal actions of the hormone in hypertension.

Membrane receptor endocytosis, commonly associated with receptor down-regulation and desensitization, may also contribute to continued activation of the signaling process (16). However, there is evidence that the internalized Ang II may activate intracellular targets and the hypothesis of an "intracrine" Ang II system has been postulated to describe the actions of peptide hormones within its cell of synthesis (23, 24). In this regard, AT_{1A} receptors, Ang II peptides, and ACE have been identified in renal endosomes (13). There is also experimental evidence in support of the existence of nuclear Ang II receptors in several non-renal tissues (2, 7, 8, 15, 25, 26, 28), as well as nuclear accumulation of Ang II (10, 27) and nuclear translocation of AT_{1A} receptors upon agonist exposure (4). The AT_1 mediated accumulation of Ang II in kidneys of Ang II infused rats may redistribute Ang II and its plasma membrane receptor to the nucleus, however there is little evidence regarding the presence of nuclear receptors or Ang II binding sites in kidney cells. Therefore, the aims of this study were to determine if kidney cells have specific nuclear Ang II binding sites and if chronic infusion of Ang II changes the Ang II receptor density or affinity in the rat renal cortex.

Materials and Methods

Male Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 250 to 350 g were housed in temperature- and light-controlled room. The animals were fed standard Purina Rat Chow (Ralston-Purina, St. Louis, MO) and had free access to tap water. On day 0, the animals were anesthetized with pentobarbital sodium (50 mg/kg) ip, and osmotic mini-pumps (Alzet model 2002; Alza Corporation, Palo Alto, CA) were inserted subcutaneously (middle dorsum of the neck and the upper interscapular region) to infuse Ang II at 70 ng/min (n=9) or the saline vehicle (n=9). Infusion of Ang II at 60–80 ng/min in normal rats (11, 29, 31) for two weeks has been shown to elicit a pattern of hypertension that resembles the pattern observed during development of Goldblatt hypertension. Systolic blood pressures (SBP) were monitored with a Blood Pressure Monitor model 52-0338 (Harvard Apparatus) connected to a tail cuff photoelectric transducer. Blood pressures were measured between 3.00 and 6.00 PM

before implanting the mini-pumps as well as on day 3, 7, 10 and 13 after implantation. The average SBP was calculated from 3 to 5 consecutive stable blood pressure traces. The rats were weighed the same days that SBP was recorded.

Preparation of subcellular fractions

On day 14 after implantation, the rats were sacrificed with an overdose of pentobarbital sodium and both kidneys were harvested. The renal cortexes were dissected out, weighed and placed in ice cold saline, transferred to a 0.25 M sucrose buffer (250 mM sucrose, 50 mM HEPES, pH 7.4) at 4 °C at 5:1 v:w, minced and kept at 4 °C during the procedures to isolate the subcellular fractions. The enzyme inhibitors phenylmethysulfonyl fluoride (PMSF, 100 M), 1,10-phenanthroline (50 M), pepstatin (0.5 g/ml), and calpain inhibitor I (1 g/ml) were added to the mixture (6) before homogenization with a Tissue Tearor homogenizer model 985–370 (six strokes, 10 sec each at 30,000 rpm). Crude membrane preparations were obtained by centrifugation of the homogenates twice at 48,000 g, 20 min. The supernatants were discarded and the final pellet was suspended (5:1 v:w) in incubation buffer (120 mM NaCl, 5 mM $MgCl_2$, 0.5% bovine serum albumin 50 mM Tris, pH 7.4 at 20 °C). The nuclear fractions were obtained by a method developed to isolate intact functional nuclei (17) 0.5 ml of the homogenates were placed at the top of a 3 ml cushion of 1.8 M sucrose buffer (1.8 M sucrose, 0.5 mM spermidine, 0.15 mM spermine, 10 mM HEPES, pH 7.4) followed by centrifugation at 100,000 g, for 60 min. The nuclear pellet was suspended in incubation buffer (3.1 v/w) (17). The plasma membrane fraction was prepared by centrifugation of the renal cortex homogenates at 2,000 g for 5 min, the supernatant was collected and centrifuged twice at 20,000 g for 20 min, and the pellet was suspended in incubation buffer as above (33). The dilution of each of the subcellular fractions was subsequently adjusted to 0.5 mg protein/ml. Alkaline phosphatase activity (ALP reagent kit 245-20, Sigma Diagnostics) was used as an index of contamination of the subcellular fractions with brush border membranes of proximal tubule cells. ALP values were 14- and 10-fold higher in the plasma membrane and crude membrane fractions than in the nuclear fraction. Protein concentration was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories) (3).

Ang II receptor binding assay

The procedures described by Pulin et al. (6) and Ernsberger et al. (9) were modified for the initial studies to optimize the Ang II receptor binding assay. Enzyme inhibitors (100 M bacitracin, 100 M PMSF, 50 M 1,10-phenanthroline, 10 M phosphoramidon, 130 M bestatin, 1 M leupeptin, 5 g/ml pepstatin A, and 1 g/ml calpain

inhibitor I) were added to the isolated subcellular fractions (0.5 mg protein/ml). To determine total binding, crude membrane fractions (50 g protein) were incubated in triplicate for 40 min at room temperature (optimized in preliminary studies) with 0.1–10 nM Sar^1-Leu^8-Ang II and 1000 cpm ^{125}I-Sar^1-IIe^8-Ang II in a total volume of 250 l using polypropylene tubes. Nonspecific binding was measured in parallel in the presence of either 2.5 M Sar^1-Leu^8-Ang II or 25 M losartan. The reaction was stopped with the addition of 2 ml cold incubation buffer and bound ligand was separated from free by vacuum filtration on a Millipore manifold on glass fiber filters (Poretics GF-75) presoaked in 1 mg/ml BSA (6). The filters were rinsed twice with 2 ml incubation buffer and dried, the radioactivity was counted in a gamma counter at 73% efficiency. In addition, the Ang II receptor densities were investigated in crude membrane and nuclear fractions of the renal cortex in saline (n=5) and Ang II infused rats (n=4) by single point assay.

Analytical procedures and statistical analysis

Specific binding was calculated by subtracting nonspecific (in the presence of excess competitor) from total binding. The specific binding calculated from that displaced by 2.5 M of unlabeled Sar^1-Leu^8-Ang II was defined as the total Ang II AT receptor complement and that displaced by 25 M losartan was defined as the Ang II receptor subtype 1 (AT_1). Data were expressed as fmol/mg protein and were analyzed by nonlinear regression analysis of the competition curve to calculate IC_{50} and the Hill coefficient. Nonlinear regression of the binding isotherms and linear fitting of the Scatchard plots were used to calculate Kd and B_{max} (Graph Pad Prism) (21). The results of individual experiments were averaged and the difference between groups was tested with one-way ANOVA and Newman Keuls post-test or with the paired t-test. In addition, the average binding curves and Scatchard plots of each group were also plotted and analyzed by nonlinear and linear regression analysis to calculate IC_{50}, Kd and B_{max}; there were no significant differences between the parameters determined by these separate calculation procedures.

Chemicals

Ang II was purchased from Calbiochem–Novabiochem, Corp. (La Jolla, Ca), ^{125}I-Sar^1-Ile^8-Ang II from Sigma Chemical Co (St. Louis, MO), Merck Pharmaceutical Co (Wilmington, DE) supplied losartan. Unless otherwise noted, all other chemicals used in this study were of analytical grade and were purchased from Sigma Chemical Co (St. Louis, MO).

Results

Systolic Blood Pressure and body weight

A significant increase in SBP was detected in the Ang II infused group after three days of infusion (Fig. 1). The average daily increase in SBP in the Ang II infused group was 5.9 mm Hg/day and continued to increase during the 13 days of treatment. The SBP in the saline infused rats remained between 109 and 117 mmHg (Fig. 1).

Body weights increased from 227±7 to 294±7 g in the control group, and from 265±7 to 324±8 g in the Ang II infused rats after 13 days of treatment. Thus, a weight difference of 35 g between both groups was maintained along the study; and Ang II infusion did not alter the body weight gain.

IC_{50} and Hill slope

The average competition plots and transformations to saturation binding curves are depicted in Figs 2 and 3. The IC_{50} of Sar^1-Leu^8-Ang II or losartan competition with ^{125}I-Sar^1-Ile^8-Ang II for the total AT and the AT_1 subtype binding sites in crude membrane fractions of the renal cortex of saline infused rats were 3.3±0.5 and 2.9±0.4 nM, respectively; while the corresponding values in the Ang II infused rats were 3.8±0.6 and 3.4±0.5 nM, respectively. The Hill slopes of the competition curves for the AT and AT_1 binding sites were –0.84±0.07 and –0.97±0.07 in the saline-treated group, and –0.91±0.06 and –1.01±0.07 in the Ang II infused group.

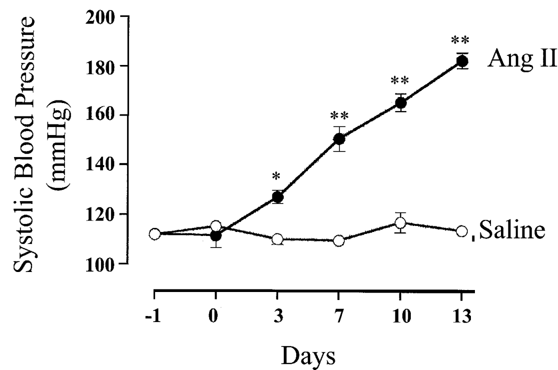

Fig. 1. Hypertensive effect of chronic infusion of Ang II. Osmotic mini-pumps containing Ang II (70 ng/day) or saline were implanted sc at day 0. Data are presented as mean ± SE for n=9 animals/group. *p<0.05, **p<0.01 by ANOVA

The Hill slope values close to –1 and a close fit of the data to the equation for one single binding site ($Y = Nonspecific + (Total\text{-}Nonspecific)/(1+10^{(X\text{-}LogEC_{50})(Hill\ slope)})$) support the hypothesis of one predominant type of Ang II receptor in the rat renal cortical cells.

B_{max} and Kd

As shown in Table I, using ^{125}I-Sar^{1}-Leu^{8}-Ang II for Scatchard analysis, the B_{max} and the Kd of the AT_1 receptor subtype were slightly ($P<0.05$) lower compared to the total AT receptor complement in the renal cortex. Thus, 21–23% of the total AT receptor complement in the renal cortex represent AT_2 or other AT binding components not displaced by the AT_1-specific competitor losartan. The B_{max} and Kd values calculated by linear regression of the individual or averaged Scatchard were consistent with the results obtained by nonlinear regression fitting of the binding isotherms. In addition, since the Kd of the total AT receptor populations was significantly ($P<0.05$) higher than the Kd of the AT_1 subtype, the remaining non-AT_1 receptor population must be of significantly lower affinity. Importantly, chronic infusion of Ang II did not alter the densities or affinities of the renal AT or the predominant AT_1 subtype binding sites in crude membranes of the renal cortex (Table I).

Table I

AT and AT_1 Specific Binding in Crude Membrane Preparations

	AT		AT_1	
	Saline	Ang II	Saline	Ang II
Crude membranes:				
IC_{50} (nM)	3.93 ± 1.11	2.82 ± 1.04	3.33 ± 1.11	3.04 ± 1.02
Kd (nM)	4.10 ± 0.48	3.97 ± 0.43	2.82 ± 0.36*	3.29 ± 0.41*
B_{max} (fmol/mg protein)	1352 ± 175	1308 ± 132	1045 ± 239*	1038 ± 99*

Data represent mean ± SE for n=5 (saline-treated) or n=4 (Ang II-treated) animals.
*p<0.05 vs. AT receptor group (paired one-tailed t-test).

Nuclear fraction: B_{max}

Ang II receptor binding was assessed in nuclear fractions (17) of the renal cortex using a single point binding assay (Fig. 4). In the nuclear fractions from control rats, the corresponding AT and AT_1 specific binding values were 610±198 and 543±222 fmol/mg protein, while the corresponding values for AT and AT_1 specific

binding in Ang II infused rats were 698±152 and 594±158 fmol/mg protein. These binding values are substantial being 60–70% of the binding values in crude membrane fractions. However, there were no significant differences in the number of AT or AT_1 binding sites among treatment groups. Similarly in crude membrane fractions, there was no change in the level of AT or AT_1 binding sites with Ang II infusion (Fig. 4). These results are consistent with the data obtained using the nine-point assay and further confirm that Ang II infusion for the 13 days does not significantly alter the Ang II receptor density in crude membranes of the rat renal cortex.

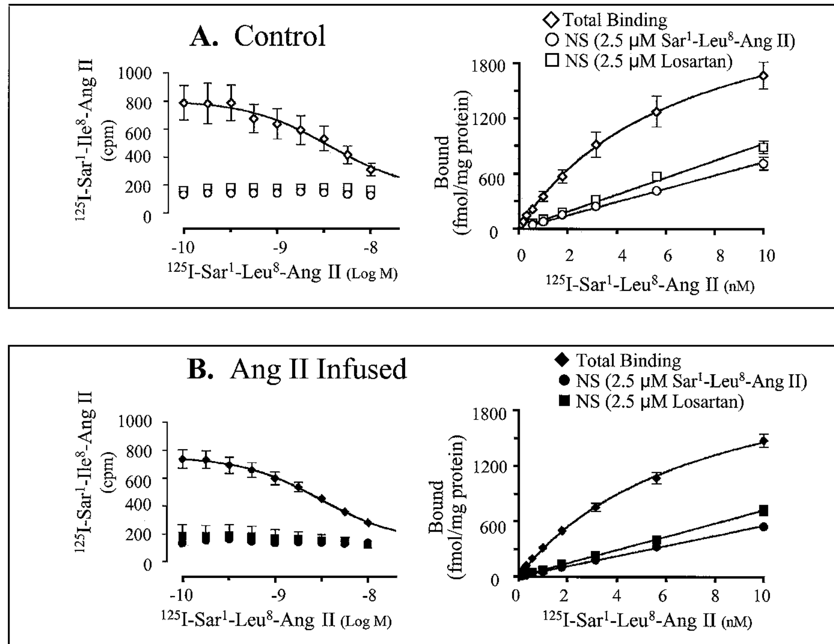

Fig. 2. Competition and saturation binding curves of crude membranes of the renal cortex incubated with ^{125}I-Sar^1-Ile^8-Ang II/Sar^1-Ile^8-Ang II

Discussion

The results of the present study demonstrate Ang II specific binding in isolated cell nuclei harvested from the rat renal cortex and indicate that the majority of the nuclear AT receptors are AT_1 binding sites. Furthermore, in the kidney total AT and AT_1 receptor densities were not altered in either crude membrane nor nuclear preparations after 13 days of chronic exposure *in vivo* to Ang II, a treatment sufficient to elicit hypertension.

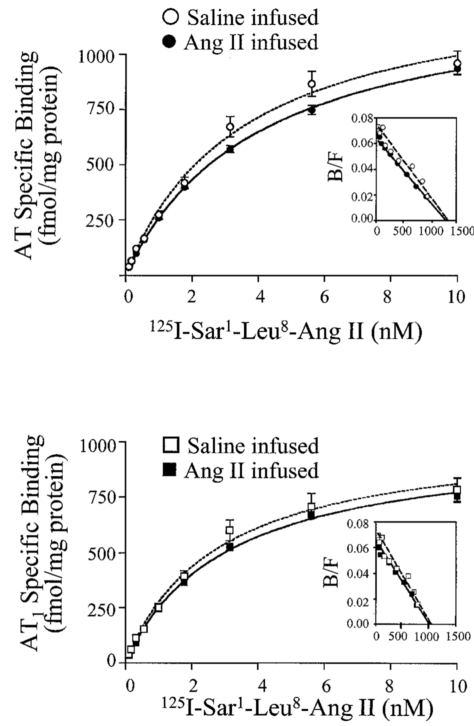

Fig. 3. Ang II receptor Bmax and Kd in crude membranes of the renal cortex in rats chronically infused with Ang II compared with saline vehicle infused rats

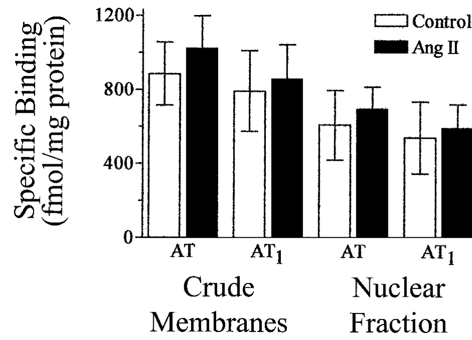

Fig. 4. Ang II specific binding to nuclear fractions and crude membranes of the renal cortex in rats chronically infused with Ang II compared with saline infused rats

While it is possible that the isolated nuclear fraction may be contaminated with endosomes or plasma membranes containing AT receptors, we observed only a slight difference in the AT receptor densities between the crude membrane and the nuclear fractions while the activity of the brush border membrane marker ALP was 14 and 10 times higher in the plasma membrane and crude membranes than in the nuclear fraction. The yield of nuclei with this classical technique developed to provide intact functional nuclei is between 50 and 90% (18), 3.5% contamination with plasma membranes, using 5'-nucleotidase activity as an enzymatic marker, was obtained adding preparative and washing centrifugation steps.

As observed before, the progressive increase in arterial pressure in the Ang II infused model of hypertension follows a pattern similar to that seen in Goldblatt hypertension (30). The hypertension produced by Ang II is modulated by sympathetic influences since renal sympathectomy decreases the slope of the hypertensive effect produced by Ang II infusion, is delayed by diuretics such as hydrochlorothiazide and is completely prevented by concomitant treatment with AT_1 receptor blockers (31, 35, 36). These data support the predominant role of renal AT_1 receptors in mediating the hypertensive response to Ang II infusions. AT_1 receptors are widely distributed in glomeruli, proximal tubules, renal vasculature, distal tubules, macula densa cells and collecting ducts and also in medullary structures like thick ascending limb of the loop of Henle, vasa recta bundles and medullary collecting ducts (11, 19, 34), thus explaining the powerful and synergistic actions of Ang II to decrease sodium excretion (20, 22, 31). Interestingly, Ang II may play an important role in regulating its own receptors; however there is controversy concerning the effect of Ang II at various sites within the kidney. In this regard, it was shown that AT_1 receptor mRNA expression and specific ^{125}I-Ang II binding were upregulated in proximal tubule cells incubated with Ang II (5). In contrast, the infusion of Ang II *in vivo* did not change glomerular or vascular AT_1 receptors (1) but decreased total AT receptors measured in whole kidney membranes (32). The present results show that the chronic administration of Ang II does not change the Ang II receptor density in the renal cortex and thus support previous findings that the AT_1 receptor is well maintained in Ang II dependant hypertension thus allowing continued action of the elevated Ang II levels (12).

Ang II binding sites sensitive to losartan were also predominant in the nuclei of kidney cells suggesting that nuclear AT_1 receptors may be involved in genomic actions of Ang II. However, no significant differences in Ang II binding sites were found between the control and the hypertensive Ang II infused rats. Thus, the present data demonstrate the presence of AT_1 binding sites in the nuclei of kidney cells and adds to previous evidence from non-renal tissue indicating the existence of nuclear AT_1 receptors. Further studies are needed to determine the functions regulated through nuclear AT receptors. Moreover, our studies do not differentiate between Ang II

binding sites on nuclear membranes (or other membranes closely associated with nuclei) vs Ang II binding sites at intranuclear locales. It also remains unclear if the nuclear actions of Ang II can be implemented independently from the classical signal transduction mechanisms activated via the plasma membrane receptors.

Nuclear localization of polypeptide ligands subsequent to receptor-mediated internalization, such as insulin, platelet-derived growth factor (PDGF), epithelial growth factor, nerve growth factor, prolactin, interleukin-1 and somatostatin suggests the existence of a non-classical and parallel mechanism of intracellular signaling for peptide hormones. After endocytosis and processing at the plasma membrane, activated receptor-ligand complexes may translocate to the nucleus to modulate gene regulation directly (14).

The liver is a target tissue for Ang II, which stimulates gluconeogenesis, glycogenolysis and production of angiotensinogen. Ang II plasma membrane and nuclear binding sites in hepatic cells were different in pH sensitivity. Hepatic nuclear sites exhibited higher binding affinities for Ang I, II and III with IC_{50} 33.1, 1.4 and 2.1 nM, while plasma membrane receptors showed 279, 4.8 and 24.4 nM respectively (2). However, losartan inhibited Ang II binding to both sites with a similar affinity, consequently they were classified as AT_1 (2, 28). Ang II binding density in the hepatic nuclei preparation was 10.6 fmol/mg protein compared to 92.5 fmol/mg in hepatic plasma membranes (28). An increase in the production of mRNA for renin, angiotensinogen, platelet-derived growth factor and oncogene *c-myc* was observed when isolated nuclei were challenged with Ang II (7, 8, 25) suggesting that long-term regulation of growth factors may be the function of nuclear receptors.

It is not clear whether the Ang II binding sites in the plasma membrane and nuclei are identical. There is no clear evidence for the existence of a mechanism to redistribute activated AT receptors as Ang II–AT receptor complexes from the plasma membrane to the nucleus. However, nuclear accumulation of ^{125}I-labelled Ang II was shown after exposing isolated hepatocytes to the radiolabelled hormone. This effect was insensitive to the acidotrophic agent, chloroquine, known to alter intracellular processing of ligand-receptor complexes by preventing the acidification of endosomes, nor to the disruption of the cytoskeleton by colchicines. Furthermore, monensin significantly reduced nuclear accumulation of Ang II suggesting that disruption of the Golgi apparatus affecting intracellular transport of newly synthesized molecules may interfere with the Ang II accumulation in the nucleus (15). Interestingly, however, the B_{max} reported for hepatic AT_1 sites (28) was substantially lower (10 fmol/mg protein) than determined here (500–600 fmol/mg protein), which may reflect either a higher overall renal Ang II receptor content or perhaps a greater role for nuclear AT_1 receptors in the kidney than in the liver. More recently, a study by Chen et al. (4) demonstrated in Chinese hamster ovary cells transfected with an AT_1 receptor linked to green fluorescent protein (GFP)

that exposure with Ang II led to internalization of the GFP and migration to the nucleus. Colocalization of the fluorescence with that of the nuclear stain, TOTO-3, was increased suggesting that the membrane receptor does indeed migrate to the nucleus. Collectively these results support the possibility that nuclear receptors for Ang II may exert transcriptional actions distinct from those mediated via the classical pathways activated by cell membrane receptors.

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