Adrenomedullin in experimental congestive heart failure: cardiorenal activation

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Adrenomedullin (ADM) is a newly identified peptide hormone that has potent vasodilating and natriuretic activities (9, 13). ADM consists of 52 amino acids and contains a COOH-terminal amide structure, which is the common structure of the calcitonin gene-related peptide superfamily (13). ADM circulates in plasma (2, 3, 8, 11, 12, 18, 19) and when injected intravenously causes hypotension in association with a decrease in systemic vascular resistance (4, 13). We have recently reported that intrarenal infusion of ADM produces an increase in urinary sodium excretion (9), which is mediated in part by the renal prostaglandin system (7). Vascular endothelial cells and smooth muscle cells are known to produce and secrete ADM (25, 26), and vascular smooth muscle cells have specific ADM receptors that are functionally coupled to adenylate cyclase (1, 5). These findings indicate that ADM elicits its action as an endocrine factor as well as an autocrine and/or paracrine factor. Although ADM was originally discovered in the extracts of human pheochromocytoma, ADM gene message and its immunoreactivity have also been detected in the heart and kidney (2, 9, 10, 15). Presently, ADM is considered to be a newly discovered cardiorenal hormone with potent vasodilating and natriuretic activities that may participate in cardiorenal and body fluid homeostasis.

Chronic congestive heart failure (CHF) is a pathophysiological state in which the failing ventricle inadequately provides peripheral perfusion to meet the metabolic needs of the peripheral tissues. In CHF, neurohumoral activation plays an important role in the progression of ventricular dysfunction (20). Activated vasodilating and natriuretic factors are opposed by coactivation of local and circulating vasoconstrictive and sodium-retaining factors. Recently, we first reported that circulating ADM is increased in human CHF (11). We have also demonstrated that ADM increases in proportion to the severity of New York Heart Association (NYHA) functional classification and that there is a significant step-up in the concentration of ADM between aorta and anterior interventricular vein (AIV), suggesting that the failing human ventricle secretes ADM, contributing to the elevation of circulating ADM in human CHF (8). Our immunohistochemical study confirmed the increased immunostaining in the failing human ventricle (11). More recently, other investigators reported that cardiac overload produced by angiotensin II infusion stimulates ventricular ADM gene expression in rats (22). To date, however, the plasma and cardiorenal tissue concentrations of ADM in an experimental animal model of CHF remain undefined. Second, whether or not ADM immunohistochemical staining is augmented in experimental CHF also remains unknown. Third, the relationships between myocardial ADM and left ventricular mass as well as between circulating ADM and ventricular function are not known. Our hypothesis is that, like atrial natriuretic peptide (ANP), which is a cardiac peptide hormone with potent vasodilating and natriuretic activities, circulating and cardiorenal tissue ADM is increased in an experimental CHF and may have an important role in the pathophysiology of CHF. Therefore, the first objective of the present study was to determine circulating and cardiorenal tissue ADM concentrations in experimental canine model of CHF produced by rapid ventricular pacing for 38 days. The
second objective was to investigate the localization of ADM in the cardiorenal tissues in the presence and absence of CHF by immunohistochemistry. The third objective was to examine the relationship between myocardial ADM and left ventricular mass as well as between circulating ADM and ventricular function. We also sought to determine the relationship between renal tissue ADM and urinary ADM in this model.

METHODS

Experimental canine model of CHF. Fourteen male mongrel dogs weighing 18–24 kg were examined in the current study. Experimental CHF was produced by progressive rapid ventricular pacing for 38 days as previously reported (17, 24). Nine dogs underwent implantation of a programmable cardiac pacemaker (Medtronic, Minneapolis, MN). Under pentobarbital sodium anesthesia (30 mg/kg iv) and artificial ventilation (Harvard respirator; Harvard Apparatus, Millis, MA) with 5 l/min supplemental oxygen, left lateral thoracotomy and pericardiotomy were performed. The heart was exposed, and a screw-in epicardial pacemaker lead was implanted into the right ventricle. The pacemaker generator was implanted subcutaneously into the left chest wall and connected to the pacemaker lead. In addition, these dogs underwent implantation of a chronic femoral artery catheter (model GPV Vascular-Access Port; Access Technologies, Skokie, IL) via the left femoral artery and subcutaneously connected to a port above the left upper hind limb. All dogs were allowed to recover for at least 10 days after surgery.

After recovery from the surgery, the pacemaker was started with a stepwise increase of stimulation frequencies over 38 days. During the first 10 days, animals were paced at 180 beats/min, and the pacing rate was then increased weekly to 200, 210, 220, and 240 beats/min to produce overt CHF (17, 24). All pacemakers were checked at the time of programming, then weekly and at the day of death for proper pacing. CHF was defined as pacing-induced systolic dysfunction with avid sodium retention and clinical signs of congestion. At baseline before pacing and after being paced at 240 beats/min for 7 days (overt CHF), a two-dimensional guided M-mode echocardiogram was obtained. Mean arterial blood pressure monitoring in the conscious state at baseline before pacing and at the end of the pacing at 240 beats/min. Cardiac output was measured by thermodilution method using Cardiac Output model 9520-A computer (Edwards Laboratories, Santa Ana, CA) in triplicate and averaged. Systolic vascular resistance and pulmonary capillary wedge pressure, and cardiac output were measured via a percutaneously placed flow-directed balloon-tip catheter (model 93131–7F; American Edwards Laboratories, Anasco, PR) in the conscious state at baseline before pacing and at the end of the pacing at 240 beats/min. Cardiac output was measured by thermol dilution method using Cardiac Output model 9520-A computer (Edwards Laboratories, Santa Ana, CA) in triplicate and averaged. Systolic vascular resistance and pulmonary vascular resistance were calculated according to the standard formula. At the end of the pacing at 240 beats/min for 7 days, the dogs were killed and the cardiac and renal tissues were rapidly harvested (Sleepaway intravenous euthanasia solution; Fort Dodge Laboratories, Fort Dodge, IA). This study was approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and conducted in accordance with the Animal Welfare Act.

Preparation for tissue ADM quantification and immunohistochemical staining. Cardiac and renal tissues were obtained from six out of nine overt CHF dogs after pacing at 240 beats/min for 7 days. A second group of five age-matched normal dogs served as tissue donor for the control group. Cardiac tissues included both atria and ventricles. The heart was rapidly trimmed and left ventricle was weighed for the calculation of left ventricular mass index (left ventricular weight (in grams) divided by body weight (in kilograms)). Atrial sections were taken from the appendages and free walls of both atria, and full thickness sections of ventricular myocardi um were obtained from the middle third of the free wall of both left and right ventricles from both normal and overt CHF dogs. Renal tissues were dissected into cortex and medulla. Each tissue was divided into two parts; one was frozen in liquid nitrogen and stored at −80°C for the measurement of tissue ADM concentration, and the other was immediately fixed with 10% buffered Formalin and embedded in paraffin and used for the immunohistochemical staining.

Tissue extraction procedure. Cardiac and renal tissues were boiled for 5 min in 10 vol of 1 M acetic acid containing 20 mM hydrochloric acid to abolish intrinsic proteolytic activity. They were homogenized with a Polytron homogenizer (PT 1200, Polytron). The homogenate was then ultracentrifuged at 27,000 g at 4°C, and the supernatant was stored at −80°C until radioimmunoassay.

Quantification of plasma, urinary, and tissue ADM. Plasma, urinary, and tissue ADM quantifications were performed using a specific and sensitive radioimmunoassay for ADM as previously reported (8, 11). In brief, blood samples for ADM assay were collected in chilled tubes containing EDTA and immediately placed on ice. After centrifugation at 2,500 revolutions/min (rpm) at 4°C for 15 min, the plasma was decanted and stored at −20°C until analysis. Plasma and each tissue supernatant were extracted on C18 Bond Elute Cartridges and eluted with 75% methanol containing 1% trifluoroacetic acid. Concentrated eluates were then assayed using a specific and sensitive radioimmunoassay for ADM (Phoenix Pharmaceuticals, Mountain View, CA). Urinary ADM was measured directly by this radioimmunoassay without extraction. Samples and standards were incubated with 1:100 dilution of antibody raised against human ADM (1–52) at 4°C for 24 h. 125I-labeled ADM (100 µl) was added and incubated another 24 h at 4°C. Free and bound fractions were then separated by addition of a second antibody and centrifuged. Radioactivity of the pellet was measured with a gamma counter. Minimal detectable concentration for the assay is 0.5 pg per tube, and the half-maximal inhibition dose of radioiodinated ligand binding by ADM was 10 pg per tube. Recovery is 72 ± 2%, and intra-assay and interassay variations are 10 and 12%, respectively.

Immunohistochemical staining. Immunohistochemical studies were performed using the indirect immunoperoxidase method as described previously (9–11). In brief, the tissues were immediately fixed with 10% buffered Formalin. These tissues were embedded in paraffin, and 6-µm-thick sections were cut and mounted on slide glasses treated with silica. The slides were incubated at 60°C and deparaffinized with graded concentrations of xylene and ethanol. To block the activity of endogenous peroxidase, the slides were incubated with 0.6% hydrogen peroxide in methanol for 20 min at room temperature. After washing, they were subsequently incubated with 5% goat serum (Dako, Santa Barbara, CA) for 30 min at room temperature to reduce nonspecific background staining and were then incubated with rabbit polyclonal anti-ADM antiserum (Peninsula Laboratories, Belmont, CA) at a dilution of 1:800 in humidified chambers for 24 h at room temperature. All the treated slides were incubated for 30 min with second antibody-horseradish peroxidase conjugate (Tago, Burlingame, CA) at a dilution of 1:100. The final reaction was achieved by incubating the sections with freshly prepared 3,3-diaminobenzidine hydrochloride and hydrogen peroxide in Tris-HCl buffer.
reagent containing 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) dissolved in dimethylformamide and sodium acetate. The sections were counterstained with hematoxylin, mounted, and reviewed with an Olympus microscope.

Absorption tests were performed to confirm the immunohistochemical specificity of the reaction between antiserum and tissue. The anti-ADM antiserum was preincubated with 10−6 M of human ADM-(1–52) (Phoenix Pharmaceuticals) overnight. After centrifugation at 4,500 rpm for 10 min, the supernatant was used instead of primary antiserum. The specificity was further confirmed by substitution of nonimmune rabbit serum (NRS) (Dako) or phosphate-buffered saline (PBS) for primary antiserum.

Statistical analysis. Results of the values are expressed as means ± SE. Statistical comparisons in the group were performed by using analysis of variance for repeated measures followed by Fisher's least-significant difference test of repeated measures when appropriate, and statistical comparisons between groups were performed by Student's unpaired t-test. The correlations between plasma ADM concentration and pulmonary capillary wedge pressure, between plasma ADM and left ventricular ejection fraction, between plasma ADM and cardiac output, and between left ventricular mass index and ventricular tissue ADM concentration were performed using linear regression analysis. Statistical significance was accepted for P < 0.05.

RESULTS

Hemodynamics. Table 1 summarizes the cardiovascular hemodynamic parameters and urinary sodium excretion in six dogs at baseline before rapid ventricular pacing and during induction of overt CHF. In overt CHF, mean arterial blood pressure and cardiac output significantly increased (−16 and −57%, respectively), whereas cardiac filling pressure (right atrial pressure, +160% pulmonary capillary wedge pressure, +120%) and systemic vascular resistance (+94%) significantly increased compared with baseline. Pulmonary vascular resistance tended to increase (+71%), although not significantly. Left ventricular dimension significantly increased (+21%) in left ventricular end-diastolic diameter and +56% in left ventricular end-systolic diameter and ejection fraction significantly decreased (−57%). Urinary sodium excretion significantly decreased in overt CHF (−72%) compared with baseline. Six CHF dogs in which cardiac and renal tissues were obtained had pulmonary edema and pleural effusion. Among them, all but one had ascites. These results indicate that overt CHF dogs had cardiac dysfunction with avid sodium retention and signs of congestion.

Circulating and urinary ADM. Figure 1 illustrates the change of plasma ADM concentration in nine dogs at baseline before pacing, at 180 beats/min pacing, at 220 beats/min pacing, and at 240 beats/min pacing. Plasma ADM concentration was 5.4 ± 0.4 pg/ml at baseline before pacing and increased to 10.5 ± 2.1 pg/ml at 180 beats/min pacing (P < 0.05 vs. baseline), 13.7 ± 2.3 pg/ml at 220 beats/min pacing (P < 0.05 vs. baseline), and 14.5 ± 2.5 pg/ml at 240 beats/min pacing (P < 0.05 vs. baseline). Urinary ADM concentration (n = 5) was 22.2 ± 3.9 pg/ml at baseline before pacing and tended to decrease to 13.5 ± 0.8 pg/ml at 180 beats/min pacing, 11.0 ± 1.7 pg/ml at 220 beats/min pacing, and 16.5 ± 2.0 pg/ml at 240 beats/min pacing during induction of overt CHF; however, these values were not significant.

Cardiac and renal tissue ADM. Figure 2 illustrates cardiac ADM concentration in normal and overt CHF dogs. Atrial ADM concentrations were significantly higher than the ventricular ADM concentrations in both normal (n = 5) and overt CHF dogs (n = 6). No significant differences were observed in left atrial ADM between normals (859 ± 165 pg/g) and the overt CHF dogs (688 ± 124 pg/g), nor in right atrial ADM between normals (919 ± 157 pg/g) and CHF dogs (721 ± 81 pg/g). Left ventricular ADM was significantly increased in overt CHF dogs (279 ± 53 pg/g) compared with normals (134 ± 32 pg/g) (Fig. 2). Right ventricular ADM was also significantly increased in overt CHF dogs (297 ± 58 pg/g) compared with normals (129 ± 16 pg/g).

Table 1. Cardiovascular hemodynamics and urinary sodium excretion at baseline before pacing and during overt CHF

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Overt CHF</th>
</tr>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td>108 ± 3</td>
<td>91 ± 6*</td>
</tr>
<tr>
<td>RAP, mmHg</td>
<td>5 ± 1</td>
<td>13 ± 2*</td>
</tr>
<tr>
<td>PAP, mmHg</td>
<td>18 ± 1</td>
<td>27 ± 2*</td>
</tr>
<tr>
<td>PCWP, mmHg</td>
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<td>22 ± 2*</td>
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<td>CO, l/min</td>
<td>5.1 ± 0.4</td>
<td>2.2 ± 0.3*</td>
</tr>
<tr>
<td>SVR, mmHg·l−1·min</td>
<td>20.7 ± 1.9</td>
<td>40.2 ± 5.6*</td>
</tr>
<tr>
<td>PVR, mmHg·l−1·min</td>
<td>1.7 ± 0.3</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>39 ± 1</td>
<td>47 ± 1*</td>
</tr>
<tr>
<td>LVDs, mm</td>
<td>27 ± 1</td>
<td>42 ± 1*</td>
</tr>
<tr>
<td>EF, %</td>
<td>53 ± 1</td>
<td>23 ± 2*</td>
</tr>
<tr>
<td>UNaV, meq/24 h</td>
<td>40.0 ± 4.2</td>
<td>11.2 ± 4.7*</td>
</tr>
</tbody>
</table>

Data were obtained in the conscious animal and are expressed as means ± SE; n = 6. CHF, congestive heart failure; MAP, mean arterial pressure; RAP, right atrial pressure; PAP, pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; CO, cardiac output; SVR, systemic vascular resistance; PVR, pulmonary vascular resistance; LVDD, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; EF, ejection fraction; UNaV, urinary sodium excretion. *P < 0.05 vs. baseline.

Fig. 1. Plasma concentrations of adrenomedullin (ADM) in dogs at baseline before pacing, at 180 beats/min pacing, at 220 beats/min pacing, and at 240 beats/min pacing. P < 0.05 vs. baseline.
Since the discovery of ADM in human pheochromocytoma, accumulating evidence has shown that ADM is a new member of cardiorenal peptides with potent vasodilating and natriuretic actions. In our previous reports (8, 11), we have demonstrated that circulating ADM is increased in patients with CHF, and its circulating concentration is increased in proportion to the severity of NYHA functional classification. However, no reports...
have demonstrated the elevation of circulating ADM in an experimental model of CHF to date. The current study confirms for the first time that plasma ADM is increased in experimental CHF induced by rapid ventricular pacing, that its ventricular and renal medullary immunoreactivity are also increased, and that circulating and ventricular ADM are respective markers for alterations in ventricular function and structure.

Fig. 4. Immunohistochemical staining for ADM in atrium and ventricle in normal and overt CHF dogs. NRS, non-immune rabbit serum. Original magnification ×400.

Fig. 5. Immunohistochemical staining for ADM in kidney cortex and medulla in normal and overt CHF dogs. Original magnification ×400.
The mechanism of the increase in circulating ADM in experimental CHF is unknown. Although synthesis, secretion, and metabolism of ADM are not fully understood, there may be an increase in biosynthesis and secretion of ADM and/or a decrease in clearance of ADM in experimental CHF. Because ADM immunoreactivity and ADM mRNA are present in the heart, like ANP, ADM may be released from the heart in association with hemodynamic changes, including atrial stretch or increased ventricular wall stress. Indeed, as illustrated in Fig. 6, there was a positive correlation between pulmonary capillary wedge pressure and plasma ADM. The facts that ADM immunoreactivity is increased in the failing ventricle (11) and that there is a significant step-up in ADM concentration between aorta and AIV (8) suggest that the failing ventricle secretes ADM, therefore contributing to the elevation of circulating ADM in CHF.

Another possible mechanism of elevated circulating ADM in experimental CHF may be due to cytokines or other vasoactive hormones that have a stimulating effect on the production of ADM within vascular smooth muscle cells and/or endothelial cells. Tumor necrosis factor-α is reported to be increased in plasma of patients with CHF (16), and the gene and protein of tumor necrosis factor-α are increased in failing human hearts (28). Recently, tumor necrosis factor-α and interleukin-1 are reported to stimulate production of ADM in vascular smooth muscle cells (26). On the other hand, angiotensin II and endothelin-1 are also reported to stimulate production of ADM in vascular smooth muscle cells, and both peptides are known to be increased in this model of experimental CHF (17, 24). Increased circulating and tissue cytokines and/or vasoactive hormones may also stimulate synthesis of ADM from the failing hearts.

We have already reported that ADM is present in the atrial and ventricular myocytes of the heart (10, 11), glomeruli, cortical distal tubules, and medullary collecting duct cells of the kidney in normal dogs (9). To date, however, no reports have described the immunohistochemical alterations during the progression of CHF. Tissue quantification of ADM in the present study demonstrated that tissue ADM is increased in the ventricle and renal medulla in overt CHF dogs compared with normals. Our immunohistochemical examination confirmed this result, demonstrating that intensities of immunoreactivity in the ventricular myocytes of the heart and medullary collecting duct cells are increased in the overt CHF dogs. Immunohistochemistry also depicted an increase in the intensity of ADM in the glomeruli and distal tubules in the overt CHF dogs compared with normals. Although radioimmunoassay in the present study failed to show a statistically significant increase in the renal concentration of ADM in the cortex of the overt CHF dogs, ADM in the kidney cortex tended to increase in the overt CHF dogs. An alternative explanation of ADM immunoreactivity in cardiorenal tissue would be internalization and/or binding of ADM to these known sites of action. Further investigations such as studies using in situ hybridization are necessary to elucidate this issue.

It is known that ADM is present in the kidney and that it has natriuretic and diuretic actions (9). The current study confirms and extends our previous study, demonstrating that glomeruli, distal tubules, and medullary collecting duct cells express ADM in the kidney in CHF.
ullary collecting duct cells are immunopositive for ADM and that their immunoreactivities are increased in CHF. ADM may be synthesized and secreted from the kidney in response to the circumstances associated with CHF. In our previous study, ADM-mediated natriuresis was associated with increases in glomerular filtration rate and decreases in distal tubular sodium reabsorption (9). More recently, we have reported that ADM-natriuresis is mediated in part by the renal prostaglandin system (7). Additionally, in a preliminary study, we investigated the natriuretic effects of ADM in acute CHF dogs and found that exogenous ADM is natriuretic in acute CHF at concentrations that mimic pathophysiological concentrations (6). Taken together, one may speculate that increased ADM activity in CHF may provide natriuretic properties even in CHF. Despite the increase in renal ADM tissue immunoreactivity in overt CHF, urinary ADM was not increased, suggesting a more local autocrine and/or paracrine role of ADM in renal regulation.

In the present study, there was a positive correlation between left ventricular mass index and left ventricular ADM concentrations. The fact that ADM is increased in the ventricles in accordance with ventricular mass suggests that ventricular ADM expression may be increased by the ventricular hypertrophy. This is analogous to the known increase in ventricular production of the natriuretic peptides, ANP and brain natriuretic peptide (BNP), secondary to ventricular dilatation and myocyte hypertrophy in patients with CHF (29, 30). In the same fashion as these natriuretic peptides ANP and BNP, ventricular expression of ADM may be enhanced in patients with CHF, and ventricular ADM may contribute, at least in part, to the rise in the plasma concentrations of ADM in patients with CHF. ADM may therefore be considered as an additional tissue marker for the left ventricular hypertrophy like ANP. In addition, because ADM mediates its biological actions via generation of adenosine 3',5'-cyclic monophosphate, the elevation of ADM in CHF may enhance myocardial contractility to improve CHF. Although the direct action of ADM on myocardial contractility has not been well described, previous investigators reported that systemic administration of ADM results in an increase in cardiac output and stroke volume in mammals (4, 21). A more recent study demonstrated that ADM has a potent inotropic action in the perfused rat heart (27). ADM may thus play a compensatory role in CHF to not only maintain sodium balance and systemic vascular tone because of its natriuretic and vasorelaxing activities but also have a direct action on the ventricular myocardium. Further studies are clearly warranted. In 1993, Kitamura et al. (15) isolated and sequenced cDNA clone encoding human ADM precursor. Porcine ADM has been isolated (14), and the cDNA encoding a precursor for rat ADM has also been cloned and sequenced (23). These reports indicate that the structure of mammalian ADM is well conserved among species and raise the possibility that antiserum to human ADM could also detect ADM in other mammalian species, such as in dogs. In the present study, we used human ADM radioimmunooas-

say for plasma, urinary, and tissueADM determination as well as polyclonal anti-human ADM antiserum to detect immunohistochemical ADM localization in the dogs. Although canine ADM has not been isolated, radioimmunooassay and immunohistochemistry in the current study could detect “canine ADM” using the cross-reactivity between human ADM and canine ADM.

In summary, the current study demonstrates that circulating ADM is increased in experimental CHF and that ventricular and renal ADM is activated in the progression of CHF, suggesting that ventricular and renal ADM expression may be influenced by the circumstances associated with CHF. Tissue and circulating ADM also are markers for the alterations in myocardial structure and function. This study supports a potential role for ADM in the neurohumoral activation in the progression of CHF.

Perspectives

The present study was designed to advance our understanding of the role of ADM in CHF. The activations of certain cardiac hormones are important to the adaptation to the fall in cardiac function in CHF. In particular, ANP released from the heart to promote salt excretion is now well known as a compensatory mechanism in the early stages of CHF to preserve sodium homeostasis. In 1993, ADM, a potent endogenous vasodilating and natriuretic peptide, was found in the extracts of human pheochromocytoma. Unlike ANP, little is known regarding the function of ADM as an adaptive mechanism in CHF. Our hypothesis was that ADM synthesis and release are activated in CHF in a similar fashion as ANP. To address this hypothesis, a canine model of CHF was produced by rapid ventricular pacing for 38 days. In the present study, we have demonstrated that plasma concentrations of ADM are increased in experimental CHF, particularly in early CHF like ANP. Furthermore, ventricular and renal ADM are activated in CHF as well. Tissue and circulating ADM also serve as markers for the alterations in myocardial structure and function. Furthermore, increased ADM synthesis and secretion may also serve to delay the progression of CHF by mediating vasodilatory and natriuretic actions that delay the onset and magnitude of systemic and renal vasoconstriction and sodium retention.

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