Original Paper

Cholecystokinin Expression in the Development of Postinfarction Heart Failure

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Key Words

Cholecystokinin • Myocardial infarction • Heart failure • Brain natriuretic peptide • Gastrointestinal hormone

Abstract

Background/Aims: Cholecystokinin (CCK) is expressed in cardiomyocytes and may also play an important role in cardiovascular regulation. Clinical studies have shown that plasma CCK levels are an independent marker of cardiovascular mortality in cardiac disease. However, whether the development of postinfarction heart failure is associated with changes in CCK expression is unknown. **Methods:** To investigate CCK expression patterns and the association between CCK expression and heart functional parameters, we randomized male Sprague-Dawley rats into myocardial infarction (MI) or sham operation (SO) groups. CCK expression levels were assessed by western blotting, immunohistochemistry, real-time polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) at different time points (2, 4 or 6 weeks) after surgery. Brain natriuretic peptide (BNP) concentrations were determined using Western blotting and ELISA, myocardial morphology was assessed by microscopy. **Results:** Plasma CCK and BNP levels were significantly increased in all the MI groups compared with the corresponding SO groups. However, the degree to which myocardial CCK mRNA and protein expression levels were increased the MI groups compared with the SO groups was greater in the 4- and 6-week groups than in the 2-week group. Furthermore, plasma CCK levels were positively correlated with BNP concentrations and left ventricular end-systolic diameter (LVDs) and significantly negatively correlated with the ejection fraction (EF) and shortening fraction (SF) in model animals. **Conclusions:** Heart failure progression after infarction is associated with upregulated CCK levels; thus, CCK may be useful as a novel marker of heart failure.

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Introduction

Chronic heart failure (CHF) is not merely a complex syndrome. Rather, CHF is a complicated pathophysiologic process whose development has been attributed to several mechanisms. Hormonal imbalances may be important contributors to worse prognoses in patients with heart failure [1, 2]. Researchers have become increasingly interested in using appetite-regulated hormones, such as orexigenic peptides (ghrelin)[3-5] and anorexigenic peptides (leptin)[6], as biomarkers of cardiac and systemic abnormalities and as potential drugs in the treatment of heart failure, especially in cardiac cachexia [4, 7].

Cholecystokinin (CCK) is another appetite-regulated hormone that is widely distributed throughout various organs and tissues [8-10] and exists in various molecular forms (CCK-58, CCK-33, CCK-22, CCK-8, and CCK-4)[11]. However, the variation in bioactive CCKs is small in among species. In addition to mediating gallbladder contraction, pancreatic secretion, memory, and satiation [12, 13], CCK has also been shown to exert protective effects in the lung [14], kidney [15], liver [16] and heart through its receptors (CCK-AR/BR) in recent studies [17]. The multifunctionality of CCK suggests that it may have potential as a target in the evaluation and treatment of various diseases [18-20]. A recent study demonstrated that CCK was expressed at high concentrations in cardiac myocytes [21] despite being synthesized predominantly in the gastrointestinal tract and central nervous system [10]. These findings indicate that CCK has a variety of effects as a novel cardiovascular hormone. Previous studies have shown that CCK regulates blood pressure [22-24] and increases cardiac contractility [25]. Moreover, clinical studies have reported that plasma CCK levels are linked to cardiovascular mortality; thus, CCK concentrations may be useful as valuable biomarkers in elderly females with symptoms of cardiac disease, as shown in a previous study [26]. These findings suggest that CCK may play an important role in the development of cardiovascular diseases. However, to our knowledge, whether the development of heart failure is associated with changes in CCK expression is unknown. In addition, the relationships between plasma CCK expression and heart functional parameters and brain natriuretic peptide (BNP) concentrations remain unknown. Thus, the present study was conducted to analyse CCK expression patterns in the animal model of left anterior descending coronary artery (LAD) ligation, and to determine the correlation between CCK expression and heart functional parameters. This work demonstrated that CCK expression is correlated with myocardial injury in postinfarction heart failure.

Materials and Methods

Animals

Male Sprague-Dawley rats (n=60, weighing 200-220 g, 6-7 weeks of age) were obtained from the Laboratory Animal Center of the First Affiliated Hospital of Harbin Medical University. Four rats were housed per cage under standard conditions (temperature 22-24°C humidity 50±5%, light from 8 a.m. to 8 p.m.) and allowed access to food and water ad libitum. All investigations conformed to the Guide for the Care and Use of Laboratory Animals of the National Research Council, and the protocol for this study was reviewed and approved by the ethics committee of our hospital.

Rat model of heart failure

After a 7-day acclimation period, the animals were randomly subjected to LAD ligation (MI) or sham operation (SO). The models were established as previously described [27], with minor modifications. Briefly, the rats were anaesthetized with 10% chloral hydrate (3 ml/kg, intraperitoneal injection), endotracheally intubated with a 14-gauge angiocath, and then mechanically ventilated with a ventilator. LAD ligation was performed through the fourth intercostal space, and the proximal LAD was ligated with 6-0 sutures. Successful LAD ligation was signified by the presence of changes consistent with ST-segment elevation on electrocardiography and colour changes in the ischaemic area. SO animals underwent the same procedure but did not undergo ligation. All surgeries were performed using sterile techniques. Three rats died during

surgery. Post-operatively, the surviving MI and SO rats were randomly assigned to the following groups: (A) a 2-week group (n=9/9, MI/SO), (B) a 4-week group (n=9/10) and (C) a 6-week group (n=10/10).

Echocardiography

Echocardiography was performed under 10% chloral hydrate anaesthesia that was injected intraperitoneally. The rats were placed in the appropriate position after their fur was shaved and their skin was cleaned, and then two-dimensional M-mode echocardiography was performed to assess cardiac function and to measure several structural parameters in both the parasternal long- and short-axis views using an ultrasound machine (SONOS 7500, Philips) fitted with a 12-MHz transducer. Left ventricular end-systolic diameter (LVDS) and left ventricular end-diastolic diameter (LVDD) were measured in three consecutive cardiac cycles, after which the average value of each parameter was recorded. Left ventricular fractional shortening (LVFS) and the left ventricular ejection fraction (LVEF) were calculated using the following formulas: $LVFS(\%) = (LVDD - LVDS) / LVDD \times 100\%$ and $LVEF(\%) = (LVDD^3 - LVDS^3) / LVDD^3 \times 100\%$ [28].

Sample collection

After the above echocardiographic parameters were measured, blood was collected from the abdominal aorta and centrifuged to obtain plasma. The rats were subsequently euthanized, and their hearts were rapidly removed and irrigated with cold saline solution. The weight of each heart was measured, and the ratio of heart weight to body weight (HW/BW) was calculated. A portion of the myocardium was fixed in 4% paraformaldehyde for further histological analysis, while duodenal samples, which ultimately served as a positive control in subsequent protein and gene expression analyses, and the remaining myocardial samples were quickly frozen in liquid nitrogen and stored at -80°C.

Morphological changes in the myocardium

Transverse sections of the cardiac apex were cut into 5-μm-thick sections and stained with haematoxylin and eosin (H&E) to analyse LAD ligation-induced changes in myocardial morphology and Masson's trichrome to evaluate the severity of myocardial fibrosis. Three slides each were randomly selected from three rats in each group for morphological analysis.

Real-time PCR

CCK mRNA expression levels in the noninfarcted area of the left ventricle were detected by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted with an RNA Extraction Kit (Takara Bio, Otsu, Japan), and cDNA was synthesized using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio, Otsu, Japan), according to the manufacturer instructions. CCK amplification was performed with SYBR Green on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). The following primer sequences were used for this experiment: CCK: forward: 5'-CCC GAT ACA TCC AGC AGG TC-3' and reverse: 5'-AAA TCC ATC CAG CCC ATG TAG TC-3'; and GAPDH: forward: 5'-GGC ACA GTC AAG GCT GAG AAT G-3' and reverse: 5'-ATG GTG GTG AAG ACG CCA GTA-3'. GAPDH was used as an internal standard. The relative expression levels of CCK were determined using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

To measure endogenous CCK and BNP expression levels in cardiomyocytes, we extracted proteins from noninfarcted left ventricular tissue samples, diluted them with 5× buffer and measured protein concentrations using a protein assay kit (BCA, Beyotime Institute of Biotechnology, Haimen, China). The proteins were then subjected to heat denaturation before being separated on 12% SDS-polyacrylamide gels and then transferred to PVDF membranes, which were blocked for 1 hour at room temperature before being incubated with primary antibodies against CCK(1:800; Sigma-Aldrich, Saint Louis, USA), BNP(1:4, 000; WanLeiBiotechnology, ShengYang, China), and GAPDH(1:4, 000; ZhongShan, Beijing, China) overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG 1:2, 000 or anti-rabbit IgG 1:2, 000; ZhongShan, Beijing, China) for 1 hour at room temperature. All images were captured and analysed using Image Lab software (Bio-Rad Universal Hood II, USA). The expression levels of the above proteins were normalized to those of GAPDH. All experiments were performed three times.

Enzyme immunoassay (EIA) for CCK and BNP expression measurements

Plasma CCK and BNP levels were measured with commercially available enzyme-linked immunoassay kits (RayBiotech, Inc., Norcross, GA), according to the manufacturer's protocol. The lower limits of detection for CCK and BNP were 0.2 pg/ml and 4.7 pg/ml, respectively. The coefficient of variation was <10%.

Immunohistochemistry

For the CCK immunostaining experiments, myocardial tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin and then sectioned at a thickness of 4 μ m. The sections were subsequently de-paraffinized, rehydrated and then incubated with a rabbit anti-rat antibody against CCK (1:100, Cloud-Clone Corp, Wuhan, China) overnight at 4 °C. The sections were then counterstained with haematoxylin and observed under a fluorescence microscope (OLYMPUS DP73, Japan).

Statistical analysis

The results are presented as the mean $\pm$ standard deviation (SD). Comparisons between two groups were performed using independent samples t-tests (two-sided). ANOVA of a factorial design was used to assess the differences among groups. If the interactions among the groups were not significant, the ANOVA was followed by a least significant difference (LSD) test; however, if the interactions among the groups were significant, the ANOVA was followed by a simple effect analysis. Pearson's correlation coefficient was used to study the associations between CCK expression and heart functional parameters. Statistical analysis was performed using SPSS 20.0 software (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant. To reduce subjective bias when assessing the results, the data collectors, outcomes adjudicators, and data analysts were blinded by labeling the groups with nonidentifying terms (A or B) until the entire analysis was completed.

Results

Experimental model

Forty-two surviving animals (8 animals in the 2-week SO group, 9 animals in the 4-week SO group, 8 animals in the 6-week SO group, 5 animals in the 2-week MI group, 6 animals in the 4-week MI group, and 6 animals in the 6-week MI group) completed this study.

Body weights and cardiac functional and structural parameters

The cardiac morphological and functional characteristics of the rats were measured via echocardiography (Table 1). Body weight was significantly lower ($P < 0.001$), and IVSTd was thinner ($P < 0.01$) in the 6-week MI group than in the corresponding SO group. LA ($P < 0.05$), LVDd ($P < 0.001$), and the HB/BW ratio ($P < 0.001$) were significantly higher in the 4-week MI group than in the 4-week SO group, and LVDs was significantly greater in all the MI groups than in the corresponding SO groups. However, LVFS and LVEF were significantly decreased at all time points in the MI group compared with the SO group. No significant differences in LVPWTd were observed between two groups at any of the above time points.

Myocardial degeneration and fibrosis

H&E and Masson's trichrome staining showed (Fig. 1) that significant inflammatory infiltrates, cardiomyocyte derangement, and collagen fibre deposition were present in the MI groups at all time points. Moreover, leukocytic infiltration was more pronounced in the 2-week MI group than in the 4- and 6-week MI groups. More severe cardiomyocyte hypertrophy and degeneration and myocardial interstitial fibrosis were observed in the 6-week MI group than in the other groups.

Measurement of plasma CCK and BNP levels

Plasma CCK and BNP levels were measured by ELISA (Fig. 2), which showed that plasma BNP levels were significantly higher in the MI groups than in the corresponding SO groups ($P < 0.001$). Plasma CCK concentrations were also increased in the MI groups compared with

Table 1. Body weights and cardiac functional and structural parameters in the rat models at the end of the study. BW: body weight; HW: heart weight; LA diameter: left atrial diameter; IVSTd: end-diastolic interventricular septal thickness; LVd: left ventricular end-diastolic diameter; LVdS: left ventricular end-systolic diameter; LVPWTd: left ventricular end-diastolic posterior wall thickness; LVEF: left ventricular ejection fraction; LVFS: left ventricular short-axis fractional shortening; MI: myocardial infarction group. Data are expressed as the mean $\pm$ standard deviation (SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the time-matched SO groups

Parameters	2 Weeks		4 Weeks		6 Weeks	
	Sham	MI	Sham	MI	Sham	MI
BW(g)	288.20 $\pm$ 20.33	297.60 $\pm$ 23.93	317.20 $\pm$ 23.49	293.40 $\pm$ 18.08	385 $\pm$ 14.58	325.00 $\pm$ 16.25***
HW/BW(mg/g)	3.19 $\pm$ 0.20	3.38 $\pm$ 0.15	3.06 $\pm$ 0.18	3.86 $\pm$ 0.22***	2.61 $\pm$ 0.09	3.41 $\pm$ 0.12***
LA(mm)	2.91 $\pm$ 0.20	3.04 $\pm$ 0.27	2.99 $\pm$ 0.19	3.60 $\pm$ 0.43*	3.04 $\pm$ 0.18	3.12 $\pm$ 0.33
IVSTd(mm)	0.85 $\pm$ 0.11	0.88 $\pm$ 0.05	0.83 $\pm$ 0.03	0.72 $\pm$ 0.10	0.86 $\pm$ 0.02	0.75 $\pm$ 0.05**
LVd(mm)	6.18 $\pm$ 1.29	7.91 $\pm$ 1.16	5.42 $\pm$ 0.45	8.15 $\pm$ 0.73***	7.15 $\pm$ 0.69	8.14 $\pm$ 1.08
LVdS(mm)	3.61 $\pm$ 0.91	5.94 $\pm$ 0.90**	2.95 $\pm$ 0.19	7.30 $\pm$ 1.22***	4.44 $\pm$ 0.59	6.75 $\pm$ 1.03**
LVPWTd(mm)	0.93 $\pm$ 0.07	0.94 $\pm$ 0.09	0.94 $\pm$ 0.05	0.94 $\pm$ 0.40	1.00 $\pm$ 0.03	0.97 $\pm$ 0.04
LVEF(%)	77.98 $\pm$ 5.30	49.96 $\pm$ 13.80**	81.82 $\pm$ 4.00	28.46 $\pm$ 9.57***	72.92 $\pm$ 5.52	40.98 $\pm$ 4.20***
LVFS(%)	40.92 $\pm$ 6.10	24.72 $\pm$ 6.36**	45.36 $\pm$ 4.41	13.46 $\pm$ 5.60***	37.990 $\pm$ 3.90	17.26 $\pm$ 3.29***

Fig. 1. Pathological examination of the rat cardiac apex. H&E (upper panels) and Masson's trichrome (lower panels) staining of myocardial tissues at 2, 4, and 6 weeks after surgery. MI: myocardial infarction group. Images were photographed at x200 magnification.

the corresponding SO groups ($P < 0.001$). However, the degree to which CCK levels were increased in the MI groups compared with the SO groups was greater in the 4-week group than in the 2- and 6-week groups ($P < 0.01$). There was no significant difference in BNP levels among the three MI groups.

CCK mRNA and protein expression levels and BNP protein expression levels in the noninfarcted area of the left ventricle

The CCK to GAPDH ratio was not different between the 2-week MI and SO groups; however, CCK expression levels were significantly higher in the 4- and 6-week MI groups than in the corresponding SO groups ($P < 0.001$). Additionally, CCK expression levels were

Fig. 2. Changes in plasma CCK (A) and BNP (B) concentrations at different time points after surgery. All values are expressed as the mean $\pm$ SD ($n=5$). MI: myocardial infarction groups. * $P < 0.001$ versus the time-matched sham group, # $P < 0.01$ versus the 2- and 6-week MI groups.

Fig. 3. The noninfarcted area of the left ventricle were collected at the end of treatment and analyzed by RT-PCR and Western blotting. (A) Analysis of BNP, CCK and GAPDH protein expression from the noninfarcted area of the left ventricle samples by Western blot. (B-C) Bar graphs show relative intensity of BNP to GAPDH and CCK to GAPDH respectively. (D) The bars indicate the CCK mRNA levels; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; CCK: cholecystokinin; BNP: brain natriuretic peptide; MI: myocardial infarction groups. The data were summarized from 3 independent experiments. All values are represented as the mean $\pm$ SD ($n=3$). * $P < 0.05$, ** $P < 0.001$, compared with the time-match sham surgery groups; # $P < 0.01$, ## $P < 0.001$, versus the 2-week MI group; && $P < 0.001$, versus the 2-week and 6-week MI groups.

higher in the 4- and 6-week MI groups than in the 2-week MI group. BNP expression levels were significantly increased in 4-week MI rats compared with 4-week SO rats and 2- and 6-week MI rats ($P < 0.001$). However, BNP expression levels were not different between the 2-week MI and SO groups, nor were they different between the 6-week MI and SO groups. Quantitative analysis of cardiac CCK mRNA expression levels in the myocardium, which was performed by real-time PCR, showed that CCK expression levels were significantly different between the MI and corresponding SO groups at 4 and 6 weeks after surgery ($P < 0.05$ and $P < 0.001$, respectively); however, CCK mRNA expression levels were not different between the MI and SO groups at 2 weeks after surgery. Moreover, the degree to which CCK mRNA

Fig. 4. CCK expression levels were detected by immunohistochemical staining (x400 magnification). MI: myocardial infarction group.

Fig. 5. Correlations between plasma CCK and BNP levels and cardiac functional parameters in model rats. LVEF: left ventricular ejection fraction; LVFS: left ventricular short-axis fractional shortening; LVDs: left ventricular end-systolic diameter; BNP: brain natriuretic peptide. Table 1. Body weights and cardiac functional and structural parameters in the rat models at the end of the study. BW: body weight; HW: heart weight; LA diameter: left atrial diameter; IVSTd: end-diastolic interventricular septal thickness; LVDd: left ventricular end-diastolic diameter; LVDs: left ventricular end-systolic diameter; LVP-WTd: left ventricular end-diastolic posterior wall thickness; LVEF: left ventricular ejection fraction; LVFS: left ventricular short-axis fractional shortening; MI: myocardial infarction group. Data are expressed as the mean $\pm$ standard deviation (SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the time-matched SO groups.

levels differed between the MI and SO groups was greater in the 4- and 6-week groups than in the 2-week group (Fig. 3).

Detection of CCK expression by immunohistochemical staining

CCK was expressed at low levels in the myocardial tissues of the SO groups. Staining for CCK was significantly increased in the infarcted and noninfarcted areas of the left ventricle in the 4- and 6-week MI groups compared with those in the corresponding SO groups (Fig. 4).

Associations between plasma CCK levels and heart failure parameters

The correlations between CCK levels and BNP levels and cardiac functional parameters are shown in the indicated figure (Fig. 5). Plasma CCK levels were positively correlated with BNP levels ($P < 0.001$) and LVDs ($P < 0.001$) and significantly negatively correlated with the EF ($P < 0.001$) and SF ($P < 0.001$) in the SO and MI groups.

Discussion

Increased plasma CCK levels are associated with higher cardiovascular mortality [26]; however, the changes in CCK expression that occur during the development of postinfarction heart failure are unknown. The present study showed for the first time that the development of postinfarction heart failure was associated with significant changes in both CCK mRNA expression and CCK protein expression. Furthermore, plasma CCK levels were positively correlated with BNP concentrations and LVDs and significantly negatively correlated with the EF and SF in rats after surgery.

As a classical brain-gut peptide, CCK and its receptors can be found in a variety of tissues and organs. In addition to having the ability to exert regulatory effects on digestion and the central nervous system [19], CCK also appears to have several effects on the cardiovascular system, although the mechanisms underlying these effects remain unknown [29]. Cholecystokinin octapeptide (CCK-8) has been considered a minor biologically active fragment and has been shown to significantly improve cardiac function and hypotension in rats with lipopolysaccharide-induced endotoxic shock [30], most likely through the CCK-AR/BR, which is expressed on cardiac myocytes [25]. A recent clinical study investigated the prognostic value of plasma CCK levels in patients with symptoms of cardiac disease and suggested that plasma CCK levels are an independent biomarker of cardiovascular mortality in elderly female patients [26].

In the present study, we assessed CCK expression levels at multiple time points during the development of heart failure induced by LAD ligation in rats. Ischemic heart disease is the most prevalent cause of HF in humans, and the model enables precise observations of the timing of endocrine and heart function changes that are associated with heart failure progression. We observed higher CCK levels in the plasma in all the MI groups compared with the corresponding SO groups. We also observed that increases in plasma CCK levels were associated with corresponding changes in heart functional parameters and BNP levels. Plasma CCK levels are elevated in rats with heart failure, suggesting that CCK may be an indicator of myocardial injury. Unfortunately, measurements of the levels of the different molecular forms of CCK in plasma were not possible in the present study. The mechanism by which plasma CCK concentrations increase in rats with heart failure is unclear. Our results support the hypothesis that the rise in plasma CCK levels that occurs in heart failure may be due to the release of CCK from myocardial tissue; however, we cannot exclude the possibility that other factors involved in regulating CCK release may participate in the development of MI. Additionally, whether circulating CCK levels reflect myocardial CCK levels remains to be determined. Plasma BNP concentrations in the MI groups were significantly higher than those in the SO groups; however, BNP protein expression levels in the myocardium were not significantly different between the 2- and 6-week groups. These divergent results may indicate that BNP located in ventricular myocardial granules is rapidly secreted or degraded.

In this study, we demonstrated that CCK protein expression levels were significantly increased in the noninfarcted area of the left ventricle in the MI groups compared with the matched SO groups at 4 and 6 weeks after MI. We also measured CCK mRNA expression levels by qRT-PCR to determine whether the changes in CCK mRNA expression levels that occurred after MI were consistent with those that occurred in CCK protein expression levels in model animals. We found that CCK mRNA expression levels were significantly increased in the 4- and 6-week MI groups compared with the time-matched SO groups. Furthermore, immunohistochemical staining of the rat myocardium showed that CCK expression levels in the left ventricle were significantly increased in the 4- and 6-week MI groups compared with

the corresponding SO groups. A previous report demonstrated that cardiac CCK expression is significantly regulated by isoprenaline [21]; thus, the significant increases in CCK mRNA and protein expression in the noninfarcted area of the left ventricle that were noted herein may reflect adaptations that increase sympathetic activity during heart failure progression. Upregulated CCK may stimulate aldosterone secretion [31, 32]. Therefore, upregulated CCK may have autocrine or paracrine effects in the pathogenesis of heart failure.

This study did not describe infarct sizes, which may affect CCK expression levels. However, our combined immunohistochemical (IHC) staining, western blotting, real-time PCR and ELISA data enabled us to perform an accurate assessment of the changes in CCK expression that are associated with the development of postinfarction heart failure. The results obtained from the heart failure rat models provide the basic experimental data and evidence for further studies in other species and clinical patients. Future studies should elucidate the exact mechanisms and physiological targets of the CCK system in the heart.

Conclusion

In conclusion, this study showed that the development of postinfarction heart failure is associated with changes in CCK expression and that upregulated CCK levels, which were correlated with heart functional parameters and BNP levels, may play a role in heart failure progression after infarction. Thus, CCK may serve as a promising target for the evaluation and treatment of heart failure.

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Disclosure Statement

The authors have no financial conflicts of interest.

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