The expression and role of β3AR protein in myocardial ischemia/reperfusion in rats

Zi-Long Wang[1], Xiao-Chen Sun[1], Rong Luo[1], Dong-Ye Li[2], Hao-Chen Xuan[2]

[1]Department of Cardiology, QingPu Branch of Zhongshan Hospital Affiliated to Fudan University, Shanghai, China
[2]Department of Cardiology, Affiliated Hospital of XuZhou Medical University, Jiangsu Province, China

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Corresponding author:
Zi-Long Wang
Department
of Cardiology
QingPu Branch of
Zhongshan Hospital
Affiliated to Fudan
University
1688 Easten Garden Road
QingPu District
Shanghai 201700
China
Phone: +86 15921735040
Fax: +86 69719190
E-mail: wamgzilomg@21cn.com

Abstract

Introduction: The aim of this study is to explore serum norepinephrine (NE) concentration and β3-adrenoceptor (β3AR) protein expression at different times during myocardial ischemia/reperfusion (I/R) in rats and to examine the role of β3AR in I/R.

Material and methods: A total of 28 Sprague-Dawley (SD) rats were randomly divided into one sham group and six I/R groups (6 h, 12 h, 18 h, 24 h, 72 h, and 120 h). The rats in the I/R groups were subjected to ischemia for 45 min. After reperfusion, the serum NE concentration and the β3AR protein expression in the myocardial tissue of the left ventricular injury region were detected. Another 18 SD rats were randomly divided into a sham group, I/R groups, and an I/R + BRL37344 group. The myocardial infarct size (MIS) and the expression of apoptotic and anti-apoptotic proteins in the left ventricular myocardium of each group were measured.

Results: Compared with the sham group, the serum NE concentration of rats in the I/R groups was significantly higher at 6 h ($p < 0.001$); the expression of myocardial β3AR protein started at 12 h. The serum NE concentration and myocardial β3AR protein expression were both highest at 72 h but remained high in the 120-hour group. Compared with the sham group, the expression levels of the pro-apoptotic proteins Bax and cleaved caspase-3 after I/R were significantly higher ($p < 0.01$, $p < 0.001$, respectively), and the expression of anti-apoptotic protein Bcl-2 was significantly lower ($p < 0.01$). Compared with I/R groups, the expression levels of Bax and cleaved caspase-3 in the I/R + BRL37344 group were significantly lower ($p < 0.05$, $p < 0.01$, respectively), and that of Bcl-2 was up-regulated ($p < 0.05$); MIS was significantly decreased ($p < 0.001$).

Conclusions: With the prolongation of myocardial I/R in rats, serum NE concentration and β3AR protein expression showed a significant increasing trend and reached a peak at 72 h. Specific β3AR agonist BRL37344 can reduce myocardial I/R injury *in vivo* in rats, alleviate apoptosis, reduce infarct size, and improve cardiac function.

Key words: I/R injury, β3-adrenoceptor, norepinephrine, apoptotic protein, anti-apoptotic protein

Introduction

Myocardial ischemic injury is a pathological state of myocardial energy metabolism and necrosis caused by a coronary blood supply disor-

der [1–3]. Myocardial injury is exacerbated within a period of time after myocardial ischemia/reperfusion (I/R), i.e., myocardial I/R injury, which is one of the main factors affecting the prognosis of acute myocardial infarction [4]. The β3-adrenoceptor (β3AR), a member of the G protein-coupled receptor family, differs from the well-known β1AR and β2AR in that it mediates the negative inotropic action of the myocardium and the relaxation of vascular smooth muscle in the cardiovascular system [5]. It has recently attracted much attention in the fields of heart protection [6–9].

However, there are few reports on the pathophysiological characteristics, roles, and mechanisms of β3AR action in myocardial I/R. In this study, the changing trend of β3AR and its endogenous activator norepinephrine (NE), along with the prolongation of I/R to 120 h, was observed by measuring the serum NE concentration and β3AR protein expression at different times of I/R. As is well known, apoptosis often occurs during IR. Bcl-2, Bax, and cleaved caspase-3 are proteins related to apoptosis. The study also explored whether the specific β3AR agonist BRL37344 can reduce I/R injury in rats, improve cardiac function, and alleviate myocardial apoptosis and its related mechanisms *in vivo* in rats suffering from I/R injury. It is hoped that this study will provide new targets and theoretical and experimental bases for the clinical intervention of I/R injury.

Material and methods

Rats, reagents, and instruments

Rats

A total of 64 healthy male adult Sprague-Dawley (SD) rats, weighing 200–250 g, were provided by the Experimental Animal Center of Xuzhou Medical College. This study was approved by the Ethics Committee of the QingPu Branch of Zhongshan Hospital Affiliated to Fudan University and performed according to the Principles of Laboratory Animal Care [10].

Key reagents

BRL37344 (Sigma, USA), BSA (Amresco, USA), dimethyl sulfoxide (DMSO) (Gibco, USA), triphenyl tetrazolium chloride (TTC) staining fluid (Sigma Aldrich, USA), RIPA pyrolysis liquid (strong), phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein-loading buffer (5X), bicinchoninic acid (BCA) protein assay kit, developing and fixing kit (Beyotime Biotechnology, China), β3-adrenoceptors (CST, USA), β-actin antibodies (Zsbio Commerce Store, China), rat NE enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Bioengineering Institute, China), polyvinylidene fluoride (PVDF) membrane (Amersham, USA), alkaline phosphatase-labeled goat anti-rabbit/mouse IgG (Zsbio Commerce Store, China), nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) color reagent (Promega, USA), and enhanced chemiluminescence (ECL) immunoblotting substrate (Thermo Scientific Pierce, USA).

Key instruments

Rodent ventilator and BL-420S biological function experimental system (Chengdu Techman, China), pressure steam sterilizer (Shanghai Huaxian, China), Multiskan Spectrum (Thermo Scientific, Finland), protein electrophoresis and electric transfer device (Beijing Liuyi, China), Bio-Rad 550 microplate reader (Bio-Rad, USA)

Data collection

In vivo observation of β3AR expression and changes in NE at different time modes of I/R

A total of 28 male SD rats were divided into a sham group (n = 4) and an I/R group (n = 24). The I/R group was further divided into six groups (n = 4, 80% power at the alpha level of 0.05) according to different reperfusion times (6 h, 12 h, 18 h, 24 h, 72 h, and 120 h). Rats in the sham group received thoracotomy only, without ligation. Rats in the I/R groups were modeled using modified traditional left anterior descending (LAD) ligation. After being subjected to ischemia for 45 min, these rats received reperfusion according to their respective time modes [11]. The blood flow was blocked using modified traditional LAD ligation [3]. Along a vertical incision made parallel to the midclavicular line around the left sternum, the skin was cut open and the subcutaneous tissue separated. The pectoralis major and pectoralis minor were bluntly dissected and then pulled to both sides. The intercostal muscle was carefully cut open from the point with the strongest beat (generally between the third and fourth ribs). The ribs were separated up and down with forceps to fully expose the heart. After thoracotomy, the parietal pericardium was lifted, cut open, and peeled off. An 8-0 suture needle with a suture line was inserted through the deep LAD 1–2 mm below the region between the pulmonary artery cone and the inferior margin of the left atrial appendage. The insertion depth of the needle was about 1 mm through the myocardium, and the width between inserting and withdrawing the needle around the artery was 2.0–2.5 mm. The ligatures on both sides of the LAD were withdrawn from a plastic cannula (although a needle cannula could also have been used for clinical blood collection or infusion needles), and the taut ligatures were clamped using bulldog clamps on the upper side

to initiate ischemia. At the same time, needle-type electrodes were inserted into the subcutaneous layer of all four limbs, and the tail ends of the electrodes were connected to the corresponding electrocardiogram (ECG) leads. Standard ECG lead II was used for tracing. The signs of successful myocardial ischemia caused by ligation were cyanosis on the left ventricular anterior wall and obvious elevation of the ST segment and integration with the towering T-wave to form a convex mono-phasic curve (as shown by the standard ECG lead II). After ischemia, the bulldog clamps were released, the plastic cannula was removed, and the ligature was withdrawn. The success of reperfusion was marked by the ST segment decreasing by more than 50% and cyanosis disappearing in the ischemic region of the left ventricular anterior wall. After reperfusion, serum NE concentration (ELISA) and β3AR protein expression (Western blot) were measured in each group.

In vivo observation of the effect of β3AR on I/R

A total of 18 male SD rats were randomly divided into three groups (n = 6): a sham group, an I/R group, and an I/R + BRL37344 (specific β3AR agonist) group. After 45 min of ischemia, the rats in the IR group and the I/R + BRL37344 group received reperfusion for 24 h. Normal saline and BRL37344 (1 μg/kg) were respectively given via the jugular vein 5 min before reperfusion according to the modified method of Harms *et al.* [12]. After ischemia, the skin on the neck was preserved and iodophor was used for disinfection. The incision was located at the midpoint between the sternum and clavicle and was extended 1.5 cm vertically to the neck. The skin was cut open to expose the subcutaneous connective tissue. Blunt dissection was performed in layers until the jugular vein was exposed (mild pulsation was observed at the thoracic inlet). The jugular vein was slightly separated and its distal end lifted with smooth forceps. A small scalp needle was linked to the front of a syringe, which was filled with NS, and the needle was inserted into the jugular vein to withdraw visible blood. Injectable drugs or NS were used for administration, after which NS was used again and the remaining drugs in the scalp needle tube were injected into the jugular vein. After drug administration, the scalp needle was withdrawn and a cotton swab used for hemostasis by compression for 1 min. The tissue and skin were then sutured.

The self-made carotid artery intubation and pressure transducer (filled with heparin liquid) were connected to the BL-420S biological function experimental system, and the cardiac function of the rats in each group was measured *in vivo* by a cardiac catheter. After reperfusion, the left ventricular myocardial tissue was removed, and the expression levels of pro-apoptotic proteins Bax and cleaved caspase-3 and anti-apoptotic protein Bcl-2 were determined by Western blot. Myocardial infarct size (MIS) (MIS = mass of infarct size/left ventricular mass × 100%) was measured according to the report by Fang *et al.* [13]. After reperfusion, the heart was removed and rinsed with phosphate-buffered saline (PBS) solution, and filter paper was used to absorb excess moisture. The tissue was then wrapped in tin foil and immediately placed in a –80°C refrigerator or liquid nitrogen. After 2 h or 20 min, the heart was cut into 5, 6, or 7 slices, each about 2 mm thick, along the direction perpendicular to the longitudinal axis. These slices were then placed in 1% (TTC) staining fluid preheated to 37°C and stained while being agitated for 15–20 min. The slices were then removed and immediately fixed in 10% formaldehyde solution overnight. The necrotic and non-necrotic regions were separated and weighed. The infarct size was expressed as the percentage of the necrotic region mass to the left ventricular mass (LVM). Western blotting and infarct size measurements cannot be done using the same hearts. We took another 18 SD rats and proceeded step by step.

Results

Changes in serum NE concentration in rats of different groups at different times of reperfusion

With the prolongation of reperfusion, the serum NE concentration of rats in the I/R 6 h group began to increase significantly ($p < 0.001$) compared with the sham group. It reached a peak at 72 h, and remained at a high level at 120 h (Figure 1).

Changes in myocardial β3AR protein expression in rats of different groups at different times of reperfusion

With the prolongation of reperfusion, the expression of myocardial β3AR protein in rats increased after I/R for 12 h ($p < 0.05$) and gradually increased with time ($p < 0.001$), reaching a peak at 72 h. The elevating trend remained until 120 h after reperfusion. Compared with the 6 h and 12 h groups, the expression of myocardial β3AR protein was significantly increased upon reperfusion in the 24 h, 72 h, and 120 h groups after 45 min of ischemia ($p < 0.001$) (Figure 2).

Comparison of MIS between I/R and I/R + BRL37344 groups

Compared with the I/R group (46.20 ±3.65%), MIS was significantly lower in the I/R + BRL37344 group (33.48 ±2.08%) ($p < 0.001$) (Table I, Figure 3).

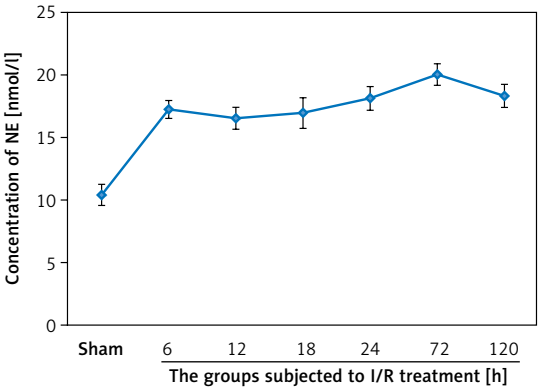

Figure 1. Changes in serum NE concentration of rats at different time points of reperfusion

Changes in apoptotic proteins after drug intervention

Compared with the sham group, the expression levels of Bax and cleaved caspase-3 after I/R were significantly higher, and the expression of anti-apoptotic protein Bcl-2 was significantly lower ($p < 0.01$). Compared with the I/R group, the expression of Bax and cleaved caspase-3 in the I/R + BRL37344 group was significantly lower and the expression of Bcl-2 was up-regulated ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. I/R group) (Figure 4).

Discussion

In this study, serum NE concentration and expression of β3AR protein at different times of I/R reperfusion were measured and their trends of change from the beginning of I/R reperfusion to 120 h later were clarified. Upregulation of the serum level of NE after cardiac I/R has been reported [14, 15]. Consistently, we also found a significant

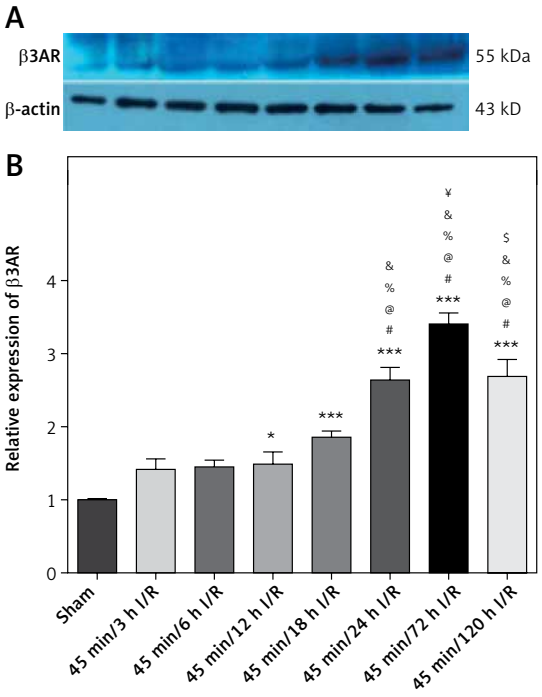

Figure 2. Changes in β3AR protein expression of rats at different time points of reperfusion. **A** – Western blot bands; **B** – Relative expression quantity of β3AR in each group

$^{*}p < 0.05$, $^{***}p < 0.001$ vs. sham; $^{\#}p < 0.001$ vs. 45 min/3 h I/R; $^{@}p < 0.001$ vs. 45 min/6 h I/R; $^{\%}p < 0.001$ vs. 45 min/12 h I/R; $^{\&}p < 0.001$ vs. 45 min/18 h I/R; $^{¥}p < 0.001$ vs. 45 min/24 h I/R; $^{\$}p < 0.001$ vs. 45 min/120 h I/R.

Table I. Comparison of MIS between I/R and I/R + BRL37344 groups

Groups	LV weight [mg]	Infarct size [mg]	Infarct size/LV (%)
I/R (n = 6)	393.47 ±8.36	181.75 ±11.34	46.20 ±3.65
I/R + BRL37344 (n = 6)	401.65 ±9.18	134.67 ±14.87	33.48 ±2.08***

Comparison of MIS between I/R + BRL37344 and I/R groups, $^{***}p < 0.001$.

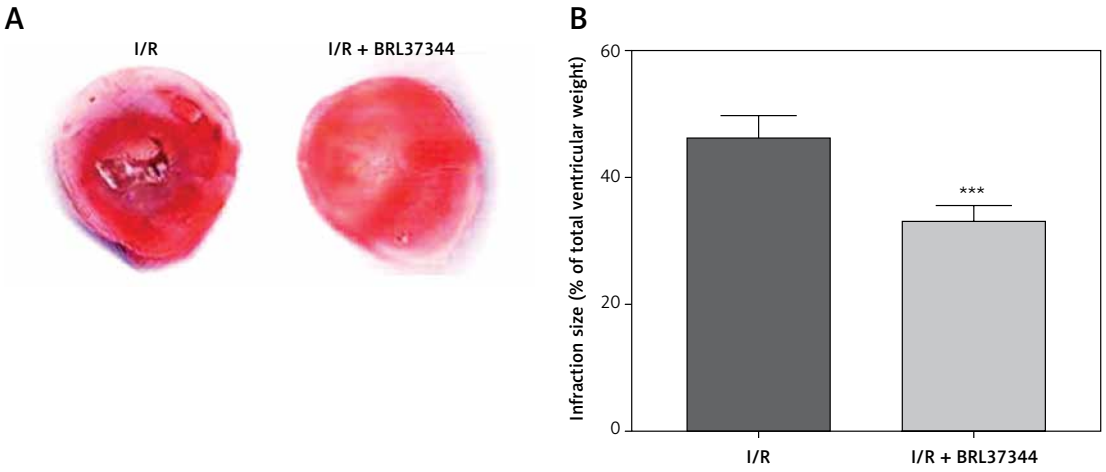

Figure 3. Comparison of MIS between I/R and I/R + BRL37344 groups. **A** – Myocardial sample, **B** – comparison of MIS in each group. $^{***}p < 0.001$ vs. I/R group

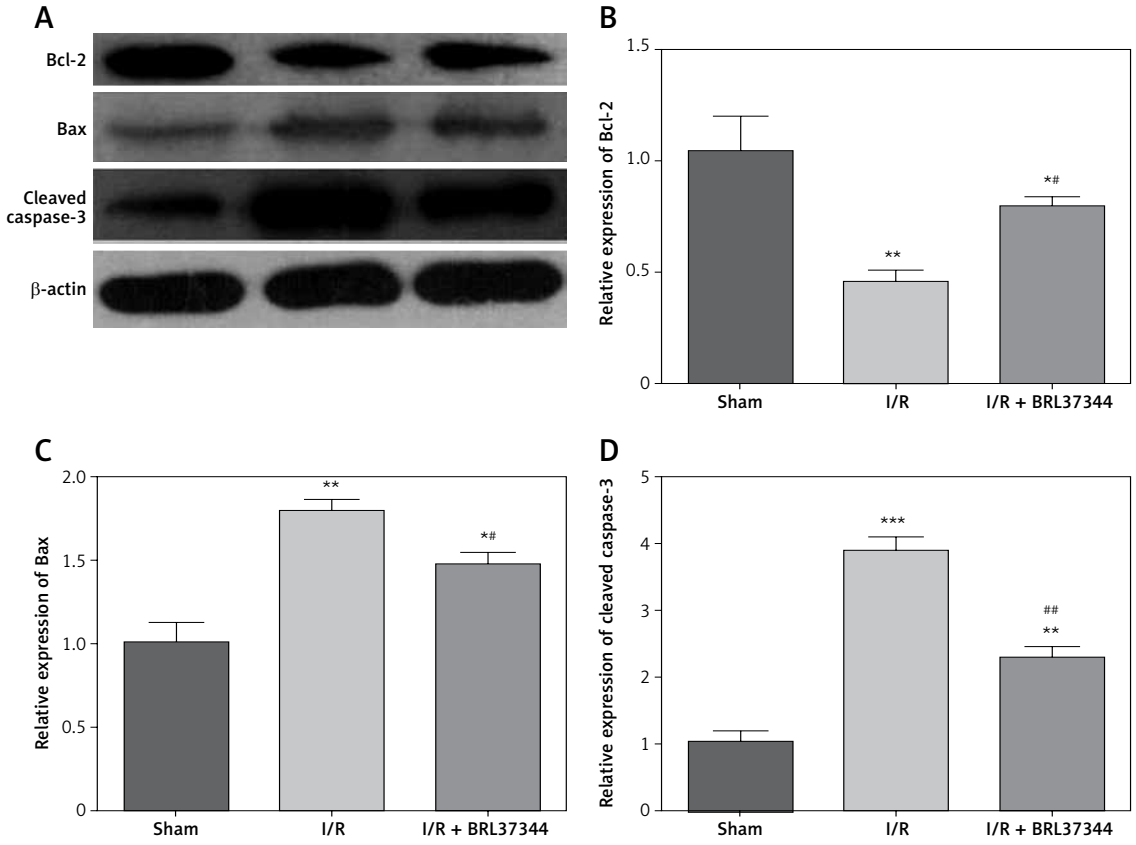

Figure 4. Comparison of changes in apoptotic proteins after drug intervention. **A** – Protein bands of Bcl-2 and Bax and cleaved caspase-3 in the myocardial tissues of rats detected by Western blot. **B**–**D** – the quantitative analysis of Bcl-2 and Bax and cleaved caspase-3 proteins, respectively

*N = 6, mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 vs. sham group; #p < 0.05, ##p < 0.01 vs. I/R group.*

increasing trend in the serum NE concentration (Figure 1). Serum NE is significantly increased 6 h into I/R. More importantly, we also found that the myocardial β3AR protein expression was significantly increased. A significant increase in the protein expression of β3AR occurred at 12 h into I/R (Figure 2). Both reach their peaks at 72 h and decline at 120 h. As one of the ligands of βAR, NE activates βAR *in vivo*, and the ability of NE to excite β3AR is ten times that of epinephrine [16–18]. Therefore, the similarity of the NE curve and the β3AR protein expression change curve further illustrates the close relationship between NE and β3AR in I/R, indicating that they may be synergistic in the occurrence and progression of I/R injury.

Previous studies have shown that β3AR is activated in the early stages of chronic cardiac failure to antagonize the excessive activation of β1AR and β2AR, reduce NE concentration in cardiac tissue, inhibit the activity of sympathetic nerves, slow the heart rate, regulate oxygen consumption, and prevent calcium overload by means of negative inotropic and chronotropic effects [19, 20]. This negative feedback regulation is a remedial action to prevent myocardial cell injury. When cardiac failure progresses to an advanced stage, β1 and β2 receptor failure is down-regulated, while β3AR is increased persistently, which breaks the balance between positive and negative inotropic effects of the heart regulated by catecholamine and causes further cardiac dysfunction [21–23].

In recent years, there have been reports about the role of β3AR in I/R. Relevant studies have shown that the use of specific β3AR agonists in I/R can significantly reduce infarct size and that β3AR can reduce acute I/R injury through the oxidative stress mechanism by increasing the expression of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) [24, 25]. It was noted in the current study that intervention and treatment with the specific β3AR agonist significantly reduced MIS (Figure 3), significantly decreased the expression of pro-apoptotic proteins Bax and cleaved caspase-3, and up-regulated the expression of anti-apoptotic Bcl-2 (Figure 4). The ratio of Bax and cleaved caspase-3 and Bcl-2 in cells determines whether apoptosis occurs [26]. When Bax is excessive, Bax/Bax homodimers are formed to induce apoptosis. On this basis, it can be deduced that the activation of β3AR inhibits apoptosis to some extent and plays a role in alleviating I/R injury.

The current study has several limitations. Firstly, due to experimental constraints, the activity of β3AR protein in I/R could not be detected, so it is not convincing enough to clarify the role of β3AR in I/R. Secondly, evidence that the activation of β3AR alleviates I/R was not sought from the molecular transduction pathway, which will be the focus of subsequent studies. Thirdly, whether β3AR would affect other apoptosis-related proteins or apoptosis signaling needs to be explored in future studies.

In conclusion, in the current study serum NE concentration and β3AR protein expression significantly increased with the prolongation of myocardial I/R in rats. A specific β3AR agonist, BRL37344, alleviated apoptosis, reduced infarct size, and improved cardiac function in rats, suggesting that the activation of β3AR could reduce myocardial I/R injury *in vivo*. These findings highlighted the protective property of β3AR and elucidated the close relationship between NE and β3AR in myocardial I/R injury.

Acknowledgments

Zi-Long Wang and Xiao-Chen Sun contributed equally to this study.

Conflict of interest

The authors declare no conflict of interest.

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