Antioxidants attenuate myocyte apoptosis in the remote non-infarcted myocardium following large myocardial infarction

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Abstract

Objective: Increased oxidative stress and myocyte apoptosis co-exist in the remote non-infarcted myocardium (RM) following a large myocardial infarction. We proposed that these phenomena are causally related. Methods and results: On day 3 after induction of myocardial infarction, Sprague–Dawley rats were randomized to receive probucol and pyrrolidine dithiocarbamate (MI-T), or vehicle only (MI) for 7 weeks. Control rats (C) received vehicle. At 7 weeks, lipid peroxidation within the RM was assessed by measuring thiobarbituric acid reactive substances, which were significantly increased in MI vs. C, while MI-T was not different from C. There was a significant increase in cardiac myocytes positive for in situ TdT-UTP nick-end labeling within the RM in MI vs. C, which was inhibited in MI-T. Furthermore, internucleosomal DNA fragmentation was clearly demonstrated on agarose gels from RM in the MI group, while it was much less apparent on gels from RM in the C and MI-T groups. Western blot analysis showed a significant increase in p53, Bax and caspase-3 protein expression within the RM of MI vs. C, all of which were inhibited in the MI-T group. Furthermore, there was evidence for an increase in caspase-3 activity within the RM from MI vs. C, which was normalized in the MI-T group. Conclusions: Long-term treatment with the antioxidants probucol and pyrrolidine dithiocarbamate attenuates oxidative stress, myocyte apoptosis, caspase-3 like activity and the expression of p53, bax and caspase-3 within RM in rats after a large myocardial infarction. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Increased myocyte apoptosis occurs in various cardiac pathologies, including different types of cardiomyopathy [1–6], cardiac allograft rejection [7] and following myocardial ischemia–reperfusion injury [8,9]. Recently, increased myocyte apoptosis was also found within the remote non-infarcted part of the heart (RM) following a large myocardial infarction [10,11]. This is an intriguing observation in light of the remodeling process known to occur within the RM, which is characterized by myocyte slippage, elongation, hypertrophy, and a significant net loss of cardiac myocytes [12]. It seems reasonable to propose that apoptosis may contribute significantly to the loss of cardiomyocytes observed in the RM [13], but the molecular mechanisms responsible remain largely unknown. Nevertheless, increased oxidative stress has recently been shown to occur concomitantly with increased cardiomyocyte apoptosis within the RM [11]. This is a provoking observation since oxidative stress is a powerful inducer of programmed cell death [14–18], including cardiac myocyte apoptosis [17,18]. Furthermore, pro-apoptotic proteins, such as p53, Bax and caspase-3/CPP-32 are upregulated and activated during oxidative stress [18–21].

In this study, we tested the hypothesis that the coexistence of oxidative stress and myocyte apoptosis in the RM after myocardial infarction are causally related. We...
show for the first time that long-term treatment with the antioxidants probucol and pyrrolidine dithiocarbamate (PDTC) in rats, starting on day three after a large myocardial infarction, reduces oxidative stress, attenuates myocyte apoptosis, blocks the increased expression of p53, Bax and caspase-3/CPP-32 and inhibits the activation of caspase-3/CPP-32 within the RM.

2. Methods

2.1. Animal model and study groups

Antero-lateral myocardial infarction (MI) was produced by coronary ligation [22] in male Sprague–Dawley (SD) rats (180–200 g). Control animals underwent sham operation. Three days later the infarcted rats were randomly divided into two groups: (1) eight rats received active treatment (MI-T): probucol 37 mg/kg/week and pyrrolidine dithiocarbamate (PDTC) 280 mg/kg/week, in three divided doses into the peritoneal cavity (i.p.); (2) six rats received coconut oil (disolvent for probucol and PDTC) as vehicle only, i.p., three times per week (MI). Six control rats (C) were also given vehicle i.p. three times per week. All three groups were provided with food and water ad libitum. Some infarcted rats died during the protocol and some had only minimal infarct and were not included in the final analyses. Surviving rats in the MI and MI-T groups with infarction >15% of the LV based on 2D echocardiographic criteria (see below), were included in final analysis. The Institutional Review Boards at University of Iowa and Iowa City Veterans Administration Hospital approved this animal research protocol and the investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Determination of infarct size

Each animal was anesthetized with an intraperitoneal injection of brevital. The animal was positioned supine in the palm of the sonographer’s hand. Gel (Aquasonic, Parker Labs, Fairfield, NJ) was applied to improve the acoustic interface. Two-dimensional imaging was performed using an Acuson (Mountain View, CA) Sequoia C256 unit, equipped with a 13.2-MHz linear array transducer. Frame rate varied depending on the size of the field of view, averaging approximately 40 per second. Systolic function and estimation of infarct size was determined as follows. Cross-sectional (‘short-axis’) views were obtained at the level of the chordae tendinae using a parasternal approach. Images were stored digitally and recalled in slow motion. End-diastole was identified visually and designated as the frame with the largest left ventricular endocardial area. End-systole was identified as the frame with the smallest endocardial area. An area ejection fraction was computed for each animal using the formula: ejection fraction=(end-diastolic area–end-systolic area)/end-diastolic area. The fraction of the left ventricle which had undergone infarction was estimated using a method previously shown to provide reasonable determinations in dogs two days following coronary occlusion [23]. Briefly, echocardiographic studies were played back in cine-loop format. The fraction of the end-diastolic circumference which did not undergo systolic thickening was identified visually and traced manually using standard on-line image analysis tools, and was expressed as ‘%MI’. Results for the infarct groups and for the non-infarct controls are shown in Table 1.

2.3. Tissue preparation

After 7 weeks the rats were sacrificed, the hearts excised and placed on ice, the myocardium flushed with ice-cold Krebs buffer via the aortic root and the right and left ventricles separated and weighed. The left ventricle was sliced into segments along the short-axis. One segment from the mid-ventricle was fixed in 4% phosphate buffered formalin and embedded in paraffin. The remainder of the heart slices were quickly divided into three parts and stored under liquid nitrogen for further analysis. The three parts were defined as follows: (a) the infarcted myocardium, which was macroscopically mostly a white scar tissue, (b) border zone, which was 2 mm into the scar and 2 mm into the non-infarcted tissue, (c) the remote non-infarcted myocardium which was defined as the unscarred tissue between the border zones, at least 2 mm away from the macroscopic scar. In this model of antero-lateral myocardial infarction, this area tends to be the inferior and septal walls of the left ventricle. Hematoxylin and eosin staining revealed normal-appearing myocardium 2 mm beyond the scar boundary.

Table 1

Results for the infarct groups and for the non-infarct controls

<table>
<thead>
<tr>
<th></th>
<th>% MI size, day 3</th>
<th>% Area EF, day 3</th>
<th>Final body weight (g)</th>
<th>LV/BW (mg/g)</th>
<th>RV/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>N/A</td>
<td>65±4*</td>
<td>324±12*</td>
<td>2.0±0.08</td>
<td>0.66±0.01</td>
</tr>
<tr>
<td>MI (n=4)</td>
<td>22±2</td>
<td>32±5</td>
<td>301±24</td>
<td>2.37±0.14</td>
<td>1.49±0.13**</td>
</tr>
<tr>
<td>MI+T (n=5)</td>
<td>27±5</td>
<td>25±4</td>
<td>298±23</td>
<td>2.33±0.12</td>
<td>1.08±0.10***</td>
</tr>
</tbody>
</table>

* The first two columns show echo data obtained on day 3, while the other three columns show post-mortem data, 7 weeks. C, control; MI, infarcted treated with vehicle only; MI-T, infarcted, treated with probucol and PDTC.

** P<0.05 vs. MI and MI+T; *** P<0.05 for control vs. MI and MI+T; ** P<0.05 for MI vs. control and MI+T; *** P<0.05 for MI+T vs. control.
2.4. In situ TdT-UTP nick-end labeling (TUNEL staining)

TUNEL staining was performed as described previously [11]. Briefly, after deparaffinization and rehydration, 5-μm thick tissue sections were incubated in PBS, containing 0.1% saponin and 1 mM EGTA for 30 min. Subsequently, sections were covered with a solution containing 0.1 U/μl of terminal deoxynucleotidyl transferase, 2.5 mM CoCl₂, 0.2 M sodium cacodylate, 25 mM Tris–HCl, 0.25% BSA and 0.5 mM 2’-biotin-16-UTP. Sections were incubated in this solution for 45 min at 37°C in a humidified chamber. After incubating with HRP-Avidin (0.5 mg/ml in PBS) 30 min at 37°C, the sections were stained with DAB (diaminobenzidine). This process yields deep brown staining of positive nuclei and clear light brown staining of myocyte cytoplasm; nuclei were counterstained with methyl green and yielded green negative nuclei (representative color figure in Ref. [11]). Four sections of each sample, each cut at 20-μm intervals from the paraffin block, were used for staining. All of the stained areas were counted. Only tissue that was at least 2 mm from the infarct margins was considered to be remote myocardium and included in the analysis. Results were presented as the number of apoptotic nuclei within myocytes per 10⁵ total nuclei counted.

2.5. DNA gel electrophoresis

For detection of DNA laddering, agarose gel electrophoresis was performed as described before [11,24]. Briefly, DNA was extracted from remote myocardium by DNAzol (Gibco BRL, Gaithersburg, MD) according to supplied protocols. One μg of DNA was treated with 5 U of Klenow polymerase in 10 mM Tris–HCl (pH 7.5) buffer containing 0.5 mM of α32P-dCTP. The reaction is incubated for 10 min at room temperature and terminated by addition of 10 mM EDTA. The unincorporated nucleotides were removed by Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). 4000 Cerenkov counts of each labeled DNA sample was applied to a 1.8% agarose gel and electrophoresed for 2 h at 100 V. After drying on Whatman 3 MM paper, the gel was exposed for autoradiography.

2.6. Thiobarbituric acid reacting substances assay

Lipid peroxide content in myocardium was determined by using the thiobarbituric acid reactive substances (TBARS) method as described previously [25]. Briefly, Tissue was homogenized 1:5 in PBS. Homogenate was centrifuged at 10,000×g for 30 min, and 0.1 ml of the supernatant was mixed with 0.8 ml of assay solution containing 0.1 M NaCl, 0.7 M trichloroacetic acid and centrifuged at 8000×g for 10 min. Then, 800 μl supernatant was mixed with 100 μl 0.1 M 2-thiobarbituric acid and heated at 75°C for 30 min. After cooling, absorbance was read at 535 nm in a spectrophotometer. An MDA standard was prepared from 1,1,3,3-tetraethoxypropane.

Protein concentrations were measured using a Bio-Rad protein assay kit according to the manufacturer’s instructions; bovine serum albumin was employed as a standard.

2.7. Caspase-3 activity assay

Caspase-3 activity was determined by Enzchek Caspase-3 assay kit from Molecular Probes (Eugene, OR). Briefly, the myocardium was homogenized in lysis buffer, 50 μl homogenate was mixed with 50 μl reaction buffer (10 mM PIPES, pH 7.4, 2 mM EDTA and 0.1% CHAPS) containing 200 μM Z-DEVD-AMC substrate. Substrate cleavage to release free AMC (340 nm; 460 nm) was monitored at room temperature. Fluorescent units were converted to pmol AMC using a standard curve generated with free AMC.

2.8. Western blot

The myocardium was homogenized in PBS containing a protease inhibitor cocktail, the homogenate subjected to SDS–PAGE (Fig. 4D demonstrates equal protein loading) and western blotting performed as described previously [11]. The membranes were probed using mouse monoclonal anti-P53 antibody and rabbit anti-CPP32 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-Bax antibody (Zymed Laboratories, San Francisco, CA). Products were visualized using an enhanced chemiluminescent system (NEN, Boston, MA) after incubating the blot with horseradish peroxidase-conjugated anti-rabbit IgG. The resulting films were scanned by placing the films directly on a scanner (BioRad, Hercules, CA), then they were projected on to a computer screen, the blot of interest was encircled. After the background was automatically substracted, optical density (OD) was measured and analyzed with the NIH image software.

2.9. Statistical analysis

Data presented as mean±S.E.M. and ANOVA used for analysis. A P value <0.05 was considered significant.

3. Results

The initial infarct size and area ejection fraction as estimated by 2D echocardiography on day 3, was similar in both infarct groups (Table 1). Infarcted animals gained less weight than control rats, but there was no difference between the treated and untreated rats (Table 1).

At the time of sacrifice, LV/body weight ratio was higher in the infarcted rats vs. control rats, despite a significant loss of myocardial tissue within the infarcted
area, suggesting hypertrophy of the RM. There was not a significant difference between the treated and untreated infarct groups (Table 1).

Both treated and untreated infarct rats showed an increase in RV/body weight ratio compared to control rats, indicating right ventricular hypertrophy. There was a significant attenuation in the hypertrophic response among the treated infarcted rats vs. untreated (Table 1).

3.1. Treatment effect on lipid-peroxidation

TBARS assay was used to compare the level of lipid-peroxidation within the RM from each group. As shown in Fig. 1, there was a significant increase in TBARS within the RM from infarcted rats vs. control hearts, which was completely inhibited in the RM from the treated infarcted rats.

3.2. Apoptosis

There was a nearly three-fold increase in the number of TUNEL positive myocyte nuclei within the RM from the untreated infarct group vs. control, which was significantly reduced in the treated infarct group (Fig. 2A). Furthermore, agarose gel electrophoresis on DNA isolated from the RM of untreated infarcted hearts showed a typical pattern for internucleosomal DNA fragmentation, which was much less apparent in DNA derived from the RM of control hearts and treated infarcted hearts (Fig. 2B).

3.3. p53

p53 is an important mediator of apoptosis [26,27]. Western blot analysis showed a 2.2-fold increase in the expression of p53 within the RM from untreated infarcted rats vs. control, which was effectively inhibited in the RM from treated infarcted rats (Fig. 3A) (In relative OD units: C = 1 ± 0.37, MI = 2.2 ± 0.13*, MI+T = 1.05 ± 0.29. * P < 0.05 vs. C and MI-T).

3.4. Bax

p53 is a powerful inducer of Bax expression [28]. Western blot analysis showed a 2.3-fold increase in Bax within the RM of untreated infarcted rats, which was effectively inhibited in RM from the treated infarcted rats (Fig. 3B) (In relative OD units: C = 1 ± 0.22, MI = 2.36 ± 0.12*, MI+T = 1.25 ± 0.29. * P < 0.05 vs. C and MI-T).
3.5. Caspase-3/CPP32

Caspase-3/CPP32 plays a critical role in the signal transduction pathway leading to apoptosis [29]. Western blot analysis showed a 3.3-fold increase in the expression of caspase-3/CPP32 protein within the RM of untreated infarcted rats, which was almost completely inhibited in the treated infarct group (Fig. 3C) (In relative OD units: C=1±0.38, MI=3.28±0.28*, MI+T=1.36±0.1. *P<0.05 vs. C and MI-T). Furthermore, caspase-3/CPP32 like activity, as measured by the cleavage of the substrate Z-DEVD-AMC, was also shown to be increased two-fold within the RM from the infarcted untreated hearts vs. control hearts, while this was inhibited in the RM from the treated infarct group (Fig. 4).

4. Discussion

This study shows, for the first time, that long term therapy with the antioxidants probucol and PDTC, started 3 days after induction of a large myocardial infarction in rats, reduces oxidative stress, decreases cardiomyocyte apoptosis, attenuates the expression of p53, Bax and caspase-3/CPP-32 and inhibits the activation of caspase-3/CPP32 within the remote non-infarcted myocardium (RM).

4.1. Oxidative stress in the RM

The results of this study confirm previous reports that have shown evidence for increased oxidative stress within the RM after large myocardial infarction [11,30,31] while it is the first study to show that the oxidative stress within the RM can be significantly attenuated with antioxidant therapy.

We propose that the chronic hemodynamic stress which the RM is exposed to following a large myocardial infarction, with ventricular dilation, increase in wall stress and heart rate, leads to an unrelenting upregulation of oxidative metabolism in the surviving myocardium and, therefore, a sustained increase in the production of mitochondrial derived oxygen free radicals. This proposed connection between myocardial work load and oxidative stress is supported by recent data which show that myocard-
dial stretch increases superoxide radical production in the myocardium [32]. Other models of unabating long term hemodynamic stress on the heart, such as tachycardia pacing [33] and aortic coarctation [34], have also shown evidence of oxidative stress within the myocardium, which was attenuated by antioxidant therapy [33,34]. This concept is further supported by a recent study in which an aggressive afterload reduction following a large MI, which decreases the hemodynamic load on the heart, reduced the degree of oxidative stress within the RM [35].

Importantly, we show in this study that the initial infarct size, as assessed by echocardiography on day 3, was similar in both infarct groups, assuring similar hemodynamic strain on the RM in both infarct groups at baseline. Furthermore, the treatment with antioxidants was started 3 days after the induction of the heart attack itself. It was felt, that by that time, it was rather unlikely that the treatment protocol per se, would directly influence the final infarct size, and therefore modify the strain on the RM.

Probucol and PDTC are both well established antioxidants that cross cell membranes easily, which allows them to scavenge oxygen free radicals formed within cells. Their effectiveness in protecting the myocardium from oxidative injury has previously been clearly demonstrated [33,36]. Importantly for the purposes of this study, we found that when these drugs were given to healthy control rats for 2 weeks, they did not have significant effect on blood pressure or heart rate (measured as previously described [37], data not shown). Thus, the inhibition of lipid-peroxidation within the RM observed in the treatment group of this study is most likely mediated by the antioxidant properties of probucol and PDTC but not via direct hemodynamic effects.

In this study, TBARS assay was used to measure the degree of lipid-peroxidation within the RM as an indicator of oxidative stress. The validity of the TBARS assay to assess the level of oxidative stress in this experimental model is strongly supported by recent studies by Hill and coworkers [30,31] who showed close correlation between changes in TBARS and several other sophisticated indicators of oxidative stress in the myocardium.

4.2. Myocyte apoptosis and oxidative stress

This study confirms previous reports showing increased myocyte apoptosis within the RM from untreated infarcted hearts [10,11]. However, this is the first study to show that long term treatment with antioxidants can significantly attenuate myocyte apoptosis in a failing heart.

Since programmed cell death has been shown to be induced by oxidative stress [14–18] and to be strongly associated with lipid peroxidation [38–40], we propose that the inhibition of apoptosis observed in this study, is at least in part, due to the antioxidant effect of the treatment given. However, based on these data alone, one can not exclude that either probucol or PDTC might have inhibited apoptosis by other mechanism(s) than their antioxidant properties, an issue that would be very difficult to resolve in an in vivo experiment like this. Nevertheless, other recent studies that employed different approaches to prevent or reduce oxidative stress, provide further support for this concept [9,41].

4.3. Apoptosis, p53, Bax and caspase-3/CPP32

To assess the degree of cardiac myocyte apoptosis in this study we used the TUNEL test, the results of which were confirmed by internucleosomal DNA fragmentation detected by agarose gel electrophoresis.

While TUNEL staining has been criticized for lack of specificity when used on tissue where concomitant necrosis occurs along with apoptotic cell death, recent studies [42,43] have demonstrated that in a tissue where necrotic cells are not present to any significant degree, such as in the RM, good correlation is found between the TUNEL test and other more sophisticated methods.

In addition to increase in TUNEL positive myocytes nuclei and DNA laddering, this study showed also an upregulation of p53, Bax and caspase-3/CPP32 protein expression within the RM from untreated infarcted hearts. Importantly, all these parameters were significantly inhibited in the RM from the treated infarct group. Furthermore, increased caspase-3/CPP32 like activity, as assessed by cleavage of the Z-DEVD-AMC substrate, was found to be increased within the RM of the untreated infarct rats, while it was inhibited in the treated group. While originally, this substrate was felt to be fairly specific for the proteolytic activity of caspase-3/CPP32, recent evidence suggest that other members of the caspase family can also cleave this substrate. However, it is fair to state that the enzymatic activity measured by this assay is likely to reflect on the activity of the program cell death machinery within the tissue specimen. Collectively, these findings suggest that oxidative stress leads to cardiac myocyte apoptosis within the RM, at least in part, via upregulation and activation of these pro-apoptotic molecules.

Previous data have indeed shown that oxidative stress can activate p53 both in vivo and in vitro models [18–20] and oxidative stress has recently been shown to induce caspase-3/CPP32 activity in vitro studies [21]. The importance of p53 is related to its down stream effects, where it increases the expression of various other pro-apoptotic proteins [26], including Bax [28]. Furthermore, p53 mediated programmed cell death is, at least under certain conditions, dependent on the activation of caspase-3/CPP32 [27], and it is intriguing that recent data suggest that p53 mediated upregulation of Bax may in fact be an important intermediate step that leads to subsequent activation of caspase-3/CPP32 [44]. This may be mediated via Bax induced leak in the mitochondrial membranes, releasing cytochrome c, which in turn causes the activation of caspase-3/CPP32 [45].
Finally, recent data strongly suggest the involvement of these promoters of apoptosis in other in vivo models of myocardial pathology. p53 and Bax have been shown to be upregulated in myocytes isolated after tachycardia-pacing induced cardiomyopathy [43], which is an experimental model associated with oxidative stress [33] and increased myocyte apoptosis [5]. Furthermore, the critical role of caspase-3/CPP32 in the signal-transduction pathway leading to myocyte apoptosis following ischemia–reperfusion injury, a classic model of oxidative stress, was also recently demonstrated by Yaoita et al. [46].

4.4. The significance of this study

This study shows that long term treatment with antioxidants in vivo can modify the degree of oxidative stress and cardiomyocyte apoptosis which occurs over time in the RM.

The importance of cardiac myocyte apoptosis in cardiac pathology remains mostly speculative. Nevertheless, it appears intuitive that progressive loss of cardiac myocytes in a heart that is already struggling is only going to lead to further deterioration of cardiac function. While recent data suggest that this can, in part, be compensated for by cardiomyocyte proliferation [47], net loss of myocytes seems to occur over time [12]. Furthermore, in a recent report, an intriguing association was found between the degree of cardiomyocyte apoptosis and the severity of left ventricular dilation and systolic dysfunction in acromegaly associated cardiomyopathy in humans [48]. Another report shows that the transition of concentric left ventricular hypertrophy to left ventricular dilatation and systolic dysfunction was associated with increased degree of myocyte apoptosis [49]. Therefore, the finding that the process of programmed cell death can be attenuated by antioxidant therapy in the failing heart, may have a potential therapeutic importance in treatment of congestive heart failure. In this context, it is tempting to speculate that some of the recently observed beneficial effects of carvedilol treatment in heart failure may in part be attributed to its antioxidant effect and its ability to inhibit apoptosis [9,50]. This is definitely an area of investigation that needs further attention.

While this study was not designed to study the hemodynamic effects of the antioxidant therapy, oxidative stress has previously been shown to have adverse effects on myocardial function [51]. Thus, attenuation of oxidative stress within the myocardium is likely to inhibit deterioration of contractility in the RM over time. In other experimental models of cardiac disease in which oxidative stress appears to play a significant pathophysiologic role, such as tachycardia pacing [33], aortic banding [34] and high-dose adriamycin [36], long term treatment with antioxidants clearly attenuates the development of cardiac dysfunction. This study may also provide indirect evidence for partial preservation of cardiac function in the treated infarct group. The impressive right ventricular hypertrophy observed in all the infarcted hearts was significantly less pronounced in the active treatment group. While many factors may contribute to the development of right ventricular hypertrophy in this model, pulmonary hypertension secondary to left ventricular failure is likely to be an important factor. Thus, since the infarct size at baseline was similar in both groups (although larger in the treated infarct rats if anything), one way to interpret these results, is that over time, the overall left ventricular function was better preserved in the actively treated group of infarcted rats, and therefore resulted in less right ventricular hypertrophy.

4.5. Conclusion

Long term treatment with probucol and PDTC decreases oxidative stress in the remote non-infarcted myocardium after a large myocardial infarction. This treatment concomitantly results in decrease in cardiac myocyte apoptosis, inhibits the upregulation of p53, Bax and caspase-3/CPP32 protein expression and inhibits caspase-3/CPP32 like enzyme activity. Treatment with antioxidants may have a future role in the treatment of congestive heart failure.

Acknowledgements

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