Original Article

Heme oxygenase-1 overexpression exacerbates heart failure with aging and pressure overload but is protective against isoproterenol-induced cardiomyopathy in mice

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Abstract

Introduction: Heme oxygenase-1 (HO-1) is a cytoprotective enzyme induced by stress. Heart failure is a condition of chronic stress-induced remodeling and is often accompanied by comorbidities such as age and hypertension. HO-1 is known to be protective in the setting of acute myocardial infarction. The role of HO-1 in heart failure is not known, particularly in the setting of pressure overload.

Methods: Mice with alpha-myosin heavy chain restricted expression of HO-1 were aged for 1 year. In addition, mice underwent transverse aortic constriction (TAC) or were infused with isoproterenol (ISO) to induce heart failure.

Results: HO-1 transgenic mice developed spontaneous heart failure after 1 year compared to their wild-type littersmates and showed accelerated cardiac dysfunction 2 weeks following TAC. Wild-type mice undergoing pressure overload demonstrated extensive interstitial fibrosis that was prevented by HO-1 overexpression, yet HO-1 transgenic mice had reduced capillary density, contractile reserve, and elevated end-diastolic pressure. However, HO-1 transgenic mice had significantly attenuated ISO-induced cardiac dysfunction, interstitial fibrosis, and hypertrophy compared to control. Isolated cardiomyocytes from HO-1 transgenic mice treated with ISO did not show evidence of hypercontracture/necrosis and had reduced NADH oxidase activity.

Conclusions: HO-1 is an effective mechanism for reducing acute myocardial stress such as excess beta-adrenergic activity. However, in our age and pressure overload models, HO-1 showed detrimental rather than therapeutic effects in the development of heart failure.

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1. Introduction

Heme oxygenase-1 (HO-1) is an enzyme induced by stress to provide cytoprotection by conversion of heme to the metabolites carbon monoxide (CO), ferrous iron, and biliverdin. The preemptive cytoprotective benefits of elevated HO-1 expression in context of acute stress are well established. Enzymatic activity of HO-1 attenuates oxidative stress and inflammation, promotes neovascularization, and is negatively correlated with cardiovascular disease [1–10]. The proposal to target the HO-1 enzymatic pathway in clinical strategies for heart failure has been highlighted [11], and indeed, HO-1–related clinical proposals/trials for cardiovascular and other pathological conditions are already engaged (clinicaltrials.gov #NCT00483587; #NCT00531856; #NCT00122694; #NCT01140685; #NCT00682370; #NCT01430156; #NCT01206582). In the context of acute myocardial infarction (MI), the benefit of preemptively elevated HO-1 activity is unequivocal [12–15], demonstrating significantly improved myocardial function up to 1 year post MI [6]. The homeostatic expression of HO-1 in the myocardium however is not constitutive, and it is not yet clear whether sustained HO-1 activity can alleviate the sequelae associated with heart failure. Further validations of HO-1 strategies are needed before engaging them in aged patients with chronic cardiovascular pathologies.
Two common comorbidities of heart failure are age and chronic hypertension. It has been estimated that ~8% of the population over 65 years are in heart failure and ~70% of these cases are attributed to hypertension [16]. Pressure overload (PO) increases ventricular wall tension and transmural pressure impairing diastolic coronary perfusion. Increased afterload in PO heart failure is accompanied by increases in sympathetic activity resulting in myocardial redox imbalance, hypertrophy, and fibrosis. The initial concentric hypertrophy response to PO is a compensatory adaptation to maintain cardiac output but in time leads to contractile dysfunction, increased afterload in PO heart failure has not been investigated. Here, we show that the α-myosin heavy chain (α-MHC)-restricted overexpression of HO-1 in the heart can protect against acute sympathetic stress. However, our results also demonstrate that HO-1 overexpression exacerbates PO heart failure after transverse aortic constriction (TAC) and induces age-dependent cardiomyopathic heart failure. These results suggest that HO-1-directed therapy could alleviate symptomatic heart failure as a result of redox imbalance in the acute setting, but more investigation is required to fully understand the role of HO-1 in the aged myocardium and conditions of high afterload such as hypertension.

2. Materials and methods

2.1. Animals

Experiments were approved by the Animal Care Committee of Queen's University and in conformity with the guidelines of the Canadian Council on Animal Care. Young (8–10 weeks) and aged (52–58 weeks) mice were used. Cardiac-specific human HO-1 transgenic (Tg) mice were obtained from Dr. Shaw-Fang Yet (Harvard Medical School) and have been previously characterized [13]. We used HO-1 Tg M mice (FVB genetic background) which express four copies of human HO-1 transgene under the control of the α-MHC promoter [13]. Wild-type (non-Tg) littermates were used as controls. Myocardial transgene expression was confirmed by PCR using GoTaq™ (Fisher, Ottawa, Ontario, Canada) according to the manufacturers’ protocol using the following specific primers hHO-1 (F): 5'-CCCAATTCTTCCAGATTTC-3'; hHO-1 (R): 5'-GCTGTCGTCGTCGTCGTCGCT-3' (GeneBank accession number: NM_002133.2).

2.2. Transverse aortic constriction (TAC)

Eight-week-old male mice (25–30 g body weight) were anesthetized with 1.5% isoflurane, intubated, connected to a ventilator (Harvard Apparatus, St. Laurent, PQ, Canada) at a tidal volume of ~230 ul and 135 breaths/min. The thoracic cavity was opened, and a 1F microtipped catheter (SPR-1000; Millar Instruments, Houston, TX, US) was inserted into the apex of the left ventricle. All signals (left ventricle pressure and conductance) were digitized at a sampling rate of 1 kHz and acquired to a computer using Cambridge Electronic Design Spike 2 software (Cambridge, UK) as previously described [17]. Contractile reserve was assessed with infusion of dobutamine, a β-adrenergic agonist, injected iv to determine the heart’s inotropic response. Following hemodynamic measurements, hearts were fixed in situ or snap frozen in liquid nitrogen, and stored at ~−80°C.

2.5. Histology

Hearts were fixed in diastole by infusion of 1% KCl in PBS, followed by Carnoy’s solution overnight and embedded in paraffin. Sections (5 μm) were prepared and stained with hematoxylin and eosin stain, Masson’s-Trichrome or isolectin. Sections were photographed using bright-field and fluorescence microscopy with an inverted microscope (Leica DM-IRB, JH Technologies, Fremont, CA, US). To determine capillary density, sections were stained with isolectin-B4 conjugated to Alex 488 (Invitrogen, Burlington, ON, Canada) for 48 h at 4°C in a humidified, airtight opaque container. To control for autofluorescence, sections were then blocked in 0.3% Sudan Black. Images were analyzed using Image-J; five individual samples were analyzed for each genotype.

2.6. Measurement of heme oxygenase (HO) enzyme activity

Mouse heart microsomal fractions were prepared from left ventricles, and total microsomal HO activity was determined by quantifying the formation of CO from the degradation of methemalbumin (heme complexed with albumin) according to a previously published method [18]. Briefly, reaction mixtures (150 μl) consisted of 100-mM phosphate buffer (pH, 7.4), 50-μM methemalbumin, 0.5–1.0 mg/ml protein, 1.5-mM β-NADH, and the incubations were carried out for 30 min at 37°C. Reactions were stopped by instantly freezing the reaction mixture on pulverized dry ice, and CO formation was determined by gas chromatography using a TA 3000R Process Gas Analyzer (Trace Analytical, AMETEK Process Instruments, Calgary, AB, Canada).

2.7. Isolation and culture of adult mouse cardiomyocytes

Eight-week-old mice were given heparin 10 IU/g intraperitoneally and then humanely sacrificed by cervical dislocation. The heart was rapidly excised, and Ca2+-tolerant mouse ventricular myocytes were isolated by modified Langendorff perfusion and Type B collagenase digestion as previously described [19]. Cell viability was typically 75–80%, as assessed by trypan blue exclusion. Cells were plated at a density of 104 rod-shaped cells/cm2 in Minimum Essential Media (MEM) culture medium containing 2.5% fetal bovine serum (FBS) and 1% penicillin–streptomycin. After 6 h of culture in 5% CO2 incubator at 37°C, the medium was changed to FBS-free MEM supplemented with albumin 0.2%, L-carnitine 2 mM, creatine 5 mM, taurine 5 mM, L-glutamine 1.3 mM, insulin 0.1 mM, triiodothyronine 0.1 nM, pyruvate 2.5 mM, and butanedione monoxime 10 mM. ISO treatment was implemented after 6 h of culture to allow for myocyte attachment and recovery. The culture medium along with unattached myocytes was aspirated and replaced with medium containing 0.5% NaCl (control) or ISO dissolved in 0.9% NaCl at two different final concentrations (0.1 and 1 μM). Attached cardiac myocytes were then cultured for an additional 12 h. The

2.4. Hemodynamic measurements

Mice were anesthetized with 1.5% isoflurane and placed in a supine position, and body temperature was maintained at 37°C. Animals were tracheotomized, connected to a small rodent ventilator (Harvard Apparatus, St. Laurent, PQ, Canada) at a tidal volume of ~230 ul and 135 breaths/min. The thoracic cavity was opened, and a 1F microtipped catheter (SPR-1000; Millar Instruments, Houston, TX, US) was inserted into the apex of the left ventricle. All signals (left ventricle pressure and conductance) were digitized at a sampling rate of 1 kHz and acquired to a computer using Cambridge Electronic Design Spike 2 software (Cambridge, UK) as previously described [17]. Contractile reserve was assessed with infusion of dobutamine, a β-adrenergic agonist, injected iv to determine the heart’s inotropic response. Following hemodynamic measurements, hearts were fixed in situ or snap frozen in liquid nitrogen, and stored at ~−80°C.
criteria for viable myocytes: rod shape, clearly defined sarcomeric striations and quiescent-contractile waves <5 min during observation.

2.8. NADH oxidase assay

To assess NADH oxidase activity as a marker for superoxide-induced oxidative stress, microsomal fractions were prepared from saline (control) and ISO-treated primary cardiac myocytes. NADH oxidase activity was measured from microsomal fractions resuspended and incubated in Krebs-HEPES buffer (pH 7.4) containing 10-μmol/L lucigenin at 37°C for 1 min, and then NADH (100 μmol/L, final concentration) was added to the reaction mixture. Luminescence was measured with a luminescence reader, and NADH oxidase activity was expressed as relative luminescence units/mg of protein.

2.9. Immunoblotting

Total protein extracts from tissues were prepared by homogenization in radioimmunoprecipitation assay buffer (RIPA buffer) (50-mM Tris- HCl, pH=7.5, 150-mM NaCl, 2-mM EDTA, 1% NP40, 0.5%-Na deoxycholate, 0.1% SDS) containing a protease and phosphatase inhibitor cocktail (20-mM NaF, 1-mM Na3VO4, 40-μg/ml PMSF, 2-μg/ml pepstatinA, 20-μg/ml leupeptin, and 15-μg/ml aprotinin). Homogenates were centrifuged at 15,000 × g for 15 min at 4°C and supernatants collected. Lysates (10-μg protein) were resolved by 1D SDS-PAGE, and HO-1 was detected with a polyclonal HO-1 antibody (SPA-895, StressGen, Enzo Life Sciences, Brockville, ON, Canada) as previously described [20–22].

2.10. Statistical analysis

All values are expressed as mean±S.D. or S.E.M. where appropriate. Comparisons of two parameters used unpaired t test, and parameters among three to five groups were analyzed by one-way analysis of variance followed by Newman–Keuls or multiple comparisons. Differences were considered statistically significant at a value of P<.05.

3. Results

3.1. Characterization of cardiac-specific HO-1 Tg mice

Human HO-1 is constitutively expressed in the heart downstream of the 5’-UTR of the cardiomyocyte-specific α-MHC promoter [13]. As expected, the hHO-1 gene was detectable only in the hearts of these Tg-mice (Supplemental Fig. 1a). HO-1 protein expression was likewise only detectable in the Tg-heart compared to Wild Type (WT) heart (Supplemental Fig. 1b). Expression of HO-1 in the spleen, which has the highest basal endogenous HO-1 expression levels, was unaffected in the Tg-mice, consistent with restriction of the transgene to cardiomyocytes (Supplemental Fig. 1b). HO activity in ventricle and liver homogenates were detected in both groups of mice. Consistent with PCR and Western blot analysis, HO activity was elevated only in the Tg-hearts (Supplemental Fig. 1c).

3.2. Stress determines the cardiac effect in Tg-mice and induction of heart failure

We evaluated three factors associated with heart failure: age, hypertension, and adrenergic stress. One-year-old mice were evaluated for cardiac function. To induce pathological hypertrophy, we used both TAC and continuous ISO infusion for 2 weeks to impose a pressure and catecholamine dysregulation, respectively, which are commonly associated with heart failure. Hypertrophy was assessed at study end-point by determining heart weight (HW), heart-to-body weight, and heart-weight-to-tibia-length. Aging in Tg-mice led to a significant increase in total HW, increased ventricular and atrial mass, and profoundly enlarged left atrial chambers (Fig. 1a, arrow) often containing visible thrombi at sacrifice (Fig. 1a and b; Supplemental Table 1).

Following 2 weeks of TAC, a significant increase in HW to tibia length ratio was present in Tg-mice when compared with WT controls (Fig. 1b). Critically, a similar duration of ISO infusion resulted in reduced HW in Tg-mice when compared with WT controls with either body weight or tibia length normalization (Fig. 1b; Supplemental Table 1).

3.3. ISO treatment

In contrast to the aging and TAC models, 2 weeks of a subpressor ISO infusion increased hypertrophy and EDP in...
WT animals but not Tg mice, indicating cytoprotection against β-adrenergic stress with HO-1 overexpression. To further examine the effect of ISO, isolated adult cardiomyocytes from biological replicates of Tg and WT animals were stimulated with low (0.1 uM) and high (1.0 uM) concentrations of ISO for 12 h. WT cardiomyocytes exposed to ISO for 12 h underwent morphological changes consistent with necrosis, namely, hypercontracture (sudden conversion from rod-shaped to rounded cardiomyocyte morphology; Fig. 3a). These changes were not observed in the Tg cardiomyocytes. We then examined whether Tg mice had reduced ISO-induced NADH oxidase activity, which is associated with redox-imbalance in cardiomyocytes. When compared with vehicle control, WT cardiomyocytes showed dose-dependent increase in NADH oxidase activity from ISO (Fig. 3b). In contrast, Tg cardiomyocytes show no increase in NADH oxidase activity with either dose of ISO treatment, further lending support that overexpression of HO-1 confers a stress-specific cardioprotection. These results reveal an intricacy in the role of HO-1 in the cardiomyocyte that has not previously been recognized.

In all three models, the Tg animals had significantly lower heart rates than our WT controls (Supplementary Table 2). It is well documented that measures of ionotropy (i.e., +dp/dtmax) are sensitive to changes in preload, afterload, and heart rate. However, Georgakopolos and Kass (2001) found that, for murine heart rates in a physiological range (400–700 bpm), there were only modest effects on contractility for even large changes in heart rate [23]. When taken in context with these
data, the observed depressions in contractility seen in the Tg mice far exceed what would be attributable to heart rate alone. Indeed, bradycardia is a prognostic indication of exacerbated heart failure progression [24–27].

4. Discussion

We analyzed cardiac-specific (α-MHC promoter) regulated overexpression of HO-1 in response to three different stresses (ISO infusion, TAC, and aging). In the present study, we demonstrate that cardiac HO-1 overexpression can be either protective or detrimental in the heart depending on the type of stress context. Following ISO infusion, cardiac HO-1 overexpression demonstrated protective effects in the myocardium by attenuating the damaging effects of sympathetic stress. In contrast, cardiac HO-1 overexpression was detrimental in aging and TAC. In aging, Tg mice exhibited bradycardia, hypotension, left ventricular systolic, and diastolic dysfunction and reduced contractile reserve compared to age-matched control animals. Following 2 weeks of TAC, cardiac HO-1 overexpression exacerbated heart failure, resulting in an increased HW and profound systolic and diastolic dysfunction when compared with wild-type animals. No detrimental effects associated with cardiac HO-1 overexpression in the aged myocardium or following TAC are reported in the literature. These findings emphasize the need to further explore the role of HO-1 in the pathophysiology of heart failure to fully determine its viability as a therapeutic agent. The level of HO-1 expression, its substrate heme, and the by-products of heme catabolism require careful evaluation, particularly in aged patients and those with chronic cardiovascular pathology. This is especially urgent as clinical studies are currently underway promoting HO-1 overexpression in aged patients.

Following ischemia/reperfusion, cardiac HO-1 overexpression improves cardiac function in a dose-dependent manner [13]. Preemptive intramyocardial HO-1 gene delivery reduces infarct size and improves long-term survival [15]. In a permanent ligation model of myocardial injury, cardiac HO-1 overexpression preserves cardiac function, reduces pathological remodeling, oxidative damage, and improves survival [11]. In a related systemic model of HO-1, overexpression (α-actin promoter) induced cardiac hypertrophy and interstitial fibrosis [28]. Interestingly, despite the morphological
changes, cardiac function remained comparable between the systemic HO-1 Tg and WT animals, differing from our findings. The pressure stress by TAC in our study was significantly higher than that described by Chen et al. (2011). We constricted the transverse aorta with 7/0 suture tied around a 27G needle. In contrast, Chen et al. performed their constriction using a larger 26G needle, resulting in a less severe constriction. Our two groups also examined different time points as a result, with Chen et al. performing analysis at 4 weeks, compared to our analysis at 2 weeks. These significant differences made it challenging to draw direct comparisons between the mechanical stress models employed. In addition, the Tg models employed had noticeable differences. The background strains and promoter regulation were different: C57Bl/6 strain and α-actin promoter (Chen et al.) and FVB and α-MHC promoter, in the current work. Strain differences are known to have slightly different TAC responses [29]; the α-actin promoter is expressed in striated muscle and in other tissue types (i.e., liver, kidney). Though we both examined HO-1 protein levels, in the study, we also assessed the HO-1 protein activity. These conditional and compartmental differences may account for the difference in the observed experimental outcomes. However, taken together with our findings, these data demonstrate that overt cardiac dysfunction in systemic HO-1 overexpression with TAC could develop.

HO-1 is widely considered a robust cytoprotective enzyme, though several important studies have identified pathological roles involving extended HO-1 expression. Astrocyte-restricted overexpression of HO-1 resulted in a neurophenotype similar to that of human schizophrenia, with increased oxidative stress, mitochondrial damage, and reduced levels of dopamine and serotonin [30]. Also affecting the brain, systemic overexpression of HO-1 promoted tau accumulation at levels similar to Alzheimer’s disease [31]. By 14 months of age, the brains of HO-1 Tg animals present with tauopathy and increases in tau aggregation within the neurons of the hippocampus and cerebral cortex [31]. This increase in tau phosphorylation was attributed to excessive HO-1-derived iron accumulation [31]. Up-regulation of HO-1 may potentially lead to over accumulation of iron within the heart similar to that shown in the brain. Interestingly, decreased cardiac contractility and bradycardia, as observed in our model, are the salient features of iron-overload cardiotoxicity [32]. In prostate cancer, epithelial HO-1 overexpression reinforced the loss of the pro-apoptotic tumor suppressor PTEN resulting in adverse patient outcomes [33]. In the vasculature, overexpression of HO-1 may promote endothelial dysfunction and vascular smooth muscle apoptosis [34–36]. In the Dahl-rat salt-sensitive model of hypertension, HO-1 expression increases after 4 weeks of hypertension accompanied by endothelial dysfunction and impaired vasodilation [36]. Dysfunction was mediated by an increase in HO-1-derived CO production that inhibited eNOS-nitric oxide production [36]. Often, HO-1 is universally coexpressed in pathology but distinguishing between correlative adaptation to stress and disease distinct from coacausation of pathology is rarely, if ever, considered.

In cell culture, increased expression of HO-1 inhibits vascular smooth muscle proliferation and growth, which is restorable via administration of HO-1 inhibitors [35]. Adenovirus-mediated overexpression of HO-1 in aortic smooth muscle increased proapoptotic p53 protein levels, inhibiting proliferation in a dose-dependent manner [34]. Though HO-1 is cytoprotective against oxidative stress-induced apoptosis [37], excessive adenosine overproduction [34] or external administration of biliverdin and bilirubin, but not CO or iron, promoted smooth muscle apoptosis and inhibited proliferation in vitro [34]. The findings of Peyton et al. further demonstrate that exogenous administration of CO directly blocks cell growth through suppression of cyclin A activity, supporting the need to discern more clearly between the affects of HO-1 enzyme and its metabolites [35].

These data strongly suggest that HO-1 overexpression in heart, brain, and vasculature may not always be of therapeutic value and should be contextually validated in greater detail. A potential explanation for these discrepancies could be differences in experimental design and methodologies. Different Tg models of HO-1 overexpression vary in the method of gene delivery as well as the degree of overexpression. Importantly, additional future studies are needed to investigate, not only the effects of HO-1 overexpression, but also the degree of overexpression, the time resolved influences of HO-1 expression in different models of cardiovascular disease, and the heme content and other regulators of heme metabolism in different disease contexts. Variations in surgical interventions and animal models could also potentially account for the differences in the effects of HO-1 overexpression. However, this is unlikely as our findings demonstrate a divergence in HO-1 effects within the same Tg strain of cardiac-specific HO-1 overexpression. Observed discrepancies may also be due to the artificial genetic modifications utilized for overexpression, rather than the intrinsic biochemistry of HO-1 itself. In 2010, Huang et al. found that Tg overexpression of green fluorescent protein, a molecule purported to be biologically inert, induced dilated cardiomyopathy under the α-MHC promoter [38]. The α-MHC promoter is extensively used in Tg models investigating cardiomyocyte-specific expression [38]. The study by Huang et al. was the first to suggest that pathologies observed in Tg models could be due, instead, to the promoter rather than the investigated gene. We do not believe this is the case in our study as the reported cytoprotective benefit (ISO) and pathological exacerbation (PO) occurred in the same strain, under similar timelines; though we cannot rule it out as a possible alternate explanation in our aging study at this time. When investigating Tg overexpression of HO-1, experiments should include additional controls to conclude, with certainty, which effects are specifically attributed to overexpression of the gene of interest. A set of future studies is needed to better establish the long-term genotoxicity of HO-1 using α-MHC promoter-driven scrambled sequences and alternate tissue and temporal promoter regulation. In addition, therapeutic efficacy or genotoxicity (HMOX1) should not be considered synonymously with substrate (hemin) or metabolite (CO) pharmacotoxicological studies.

The effects of HO-1 overexpression demands further investigation before it can be explored as a viable therapeutic agent, either directly or indirectly, using disease- and age-appropriate models. It is critical to reconcile the literature and fully define the conditions and pathologies in which HO-1 overexpression is beneficial, detrimental, or already proportionally coincident with disease; thereby, rendering it effectively inert. We have evolved to maintain set limits for inducible proteins, such as HO-1, that differ from those proteins that are constitutively active. It may be that HO-1 overexpression is beneficial in conditions where HO-1 protein levels are acutely induced. Following an acute myocardial infarction (AMI), HO-1 protein expression increases in the myocardium [39] and in human serum samples [40,41]. Similarly, acute treatment with ISO also induces HO-1 expression [42]. However, aged animals decreased HO-1 protein and mRNA expression in the carotid body [43] and in cardiac myocytes [44]. Our lab has shown that, after 2 weeks of TAC, HO-1 levels decrease in the myocardium (data not shown). This suggests that in conditions where HO-1 levels are natively induced (i.e., AMI, ISO), further overexpression is beneficial, whereas in conditions where HO-1 is down-regulated (i.e., aging, TAC), overexpression can be detrimental. Chronic disease states and chronic HO-1 therapies may result in detrimental effects and requires careful consideration. Furthermore, pathologies are associated with a high incidence of comorbidities and there are certainly individual physiological differences within a population, which include the regulation of the HO-1 gene [45,46]. HO-1 overexpression has been investigated in a wide range of pathologies [11,13,20,28,31] and time points [11,13,31,36], both systemically [28] and localized [11,13,34]. The findings of this study further support the cardioprotective properties of HO-1. In our study, overexpression of HO-1 reduces acute sympathetic stress. However, we also identify potential limitations to the cytoprotective effects of HO-1 with chronic comorbidities, such as age or PO. These data highlight the importance for further cardiovascular examinations of heme metabolism. Our data also provide important information to further advance HO-1-based therapies in comorbid or chronic disease conditions.
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.carpath.2014.03.007.

Acknowledgments

The authors thank the generosity of Dr. Shaw-Fang Yet of the National Research Health Institutes, Taiwan, for the provision of the Tg mice.

Authorship


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