Right heart failure chronically stimulates heat shock protein 72 in heart and liver but not in other tissues

Laura Comini a, Giuseppina Gaia a, Salvatore Curello b, Claudio Ceconi b, Evasio Pasini a, Massimo Benigno a, Tiziana Bachetti a, Roberto Ferrari b,∗

a Fondazione Salvatore Maugeri, Centro di Fisiopatologia Cardiovascolare, Gussago, Brescia, Italy
b Cattedra di Cardiologia, Università degli Studi di Brescia, c/o Spedali Civili, P. le Spedali Civili, I-25123 Brescia, Italy

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Abstract

Objectives: During cardiac failure several ontogenically developed adaptational mechanisms are activated. Among these, heat-shock proteins (HSP) are expressed in response to stress. The aim of the present study was to investigate the HSP72 protein expression in lungs, liver, cardiac and skeletal muscles during congestive heart failure (CHF). Methods: CHF was induced in Sprague-Dawley rats by a single intraperitoneal injection of monocrotaline (50 mg/kg). Two groups of animals emerged: a CHF group (n = 10) with right ventricular hypertrophy, pleural and peritoneal effusions, and an Hypertrophy group (n = 12) with right ventricular hypertrophy without CHF. The data for each group were compared with those of control (saline infused) age matched rats. Lungs, liver, right and left ventricles, soleus, extensor digitorum longus and tibialis anterior muscles were excised and analyzed for HSP72 concentration by Western blot analysis using a specific monoclonal antibody. Noradrenaline levels in the heart were also measured using HPLC. Results: The CHF group showed: (1) reduced right (0.460 ± 0.090 vs 0.830 ± 0.070 nmol/ventricle, P < 0.01) and left (1.10 ± 0.09 vs 2.10 ± 0.130 nmol/ventricle, P < 0.001) ventricular content of noradrenaline compared to the control; (2) significant activation of HSP72 concentration in right and left ventricles (39.4 ± 1.6 vs 5 ± 0.9% and 13 ± 1.2 vs 3.15 ± 0.6%, P < 0.001 both) and in the liver (39.8 ± 11 vs 6 ± 2%, P < 0.001); (3) no modification in HSP72 concentration in lungs and all of the peripheral muscles considered. The Hypertrophy group showed: (1) unchanged total noradrenaline tissue content as compared to the control; and (2) unmodified HSP72 concentration in all tissues analyzed. Conclusions: The present study demonstrates that CHF, but not compensatory hypertrophy, is a specific stimulus for chronic HSP72 induction in the heart and liver. On the contrary, CHF does not affect HSP in lungs and peripheral muscles. HSP 72 induction represents an intracellular marker of stress reaction which can persist chronically.

Keywords: Heat shock protein; Heart failure; Monocrotaline; Hypertrophy; Rat, skeletal muscle; Rat, heart

1. Introduction

Cardiac failure is a complex syndrome involving several adaptational mechanisms. The composition of contractile proteins in the heart muscle is modified, its mass increases and it enlarges [1,2]; the myofibrillar composition of the skeletal muscle changes [3,4], the neurohumoral system is chronically activated [5,6]. All these mechanisms, ontogenically developed as a stress reaction, may be useful in the short term, but become detrimental in the long term, and affect the evolution of the disease [7,8].

Among the many proteins expressed in the heart during the stress reaction, there is the inducible form of the heat-shock protein 70 family (HSP72). These proteins play a pivotal role in protecting the cells from subsequent stresses and are involved in the recovery from different injuries [9,10]. Several stress conditions such as hyperthermia [11], ischaemia [12,13], hypoxia [14] and acute cardiac overload [15] induce the expression of HSP72 in the heart. In addition, noradrenaline promotes the gene expression of HSP [16]. The effects of heart failure on the level of HSP72, however, have not been studied.
The aim of the present study was to investigate the protein expression of HSP72 in rats subjected to heart failure by treatment with monocrotaline. Monocrotaline is a pyrrolizidine alkaloid that causes epithelial proliferation of the small pulmonary arteries. It induces pulmonary hypertension to varying degrees, depending on the dose and individual susceptibility [17]. Consequently, a fraction of the animals develops ventricular hypertrophy with no signs of heart failure. Others present a measurable accumulation of liquids in the pleural and peritoneal spaces and a neuroendocrine activation, thus resembling the condition of CHF in humans [17,18]. We measured levels of HSP72 and noradrenaline in both right and left ventricles of rats with hypertrophy and overt heart failure. We also determined the concentrations of HSP72 in the lungs, liver and skeletal muscles, as these tissues undergo important biochemical, morphological and structural changes during heart failure.

2. Materials and methods

2.1.1. Experimental model

The experiments were carried out in accordance with the use of laboratory animals of the University of Brescia and the “Guide for the care and use of laboratory animals” [DHHS publication (NIH) 85-23, revised 1985, Animal Resources Programm, DRR/NIH, Bethesda, MD 20205]. Female Sprague-Dawley rats weighing 50-80 g were given a single intraperitoneal injection of 50 mg/kg of monocrotaline (Sigma Chemical, St. Louis, MO, USA). Control rats were injected with the same volume of saline (2 ml). Each group was fed on the standard rat-cubes diet of the University of Brescia. The rats given monocrotaline had a free supply of food. Control rats received each day only the quantity of food consumed by the treated animals the previous day. This attention to diet was necessary because of the systemic effects of the alkaloid, which soon causes a generalized illness and lack of appetite that appear to be due to an effect on the liver [17]. Within 2 weeks, 8% of our treated rats died of these early toxic effects. Those that survived recovered from the generalised ill effects; their appetite returned and their weight increased. Approximately 4 weeks after the injection, 76% started to show signs of illness which, from our previous experience [18,19], were known to be due to the development of CHF. At this stage, sick animals were killed. The animals that survived this second phase of mortality ate well and developed normally. They were sacrificed 6 weeks after the injection of monocrotaline. They had no signs of CHF and were considered to be resistant to the drug.

2.1.2. Experimental groups

Two groups of animals were studied: the first group (referred to as CHF) comprised 16 animals which survived the early systemic effects of the alkaloid but became seriously ill in week 4 after the treatment and were sacrificed in the terminal stages. All of these animals had severe right ventricular hypertrophy and pleural and peritoneal effusions. Ten were used for HSP72 measurements. Six were used for routine haemodynamic measurements. The second group (referred to as Hypertrophy) comprised 18 animals which survived the second period of mortality from CHF. These animals had substantial right ventricular hypertrophy but no pleural or peritoneal effusions and were sacrificed 6 weeks after the injection of monocrotaline. Six were used for haemodynamic determination and the remainder for HSP72 measurements. Separate control groups were necessary for each experimental group, since age and body weight substantially differed in the two experimental groups. The CHF control group consisted of 11 animals and the Hypertrophy control group consisted of 12 animals.

All the animals were anaesthetized by pentobarbital (45 mg/kg, Sigma Chemical, St. Louis, MO, USA). Soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were accurately excised from the left leg of each animal. The thorax was rapidly opened, the effusion of fluid measured by a syringe, and either the whole heart or the ventricles were removed. Individual tissues (including heart, lung and liver) were separated. The interventricular septum was discharged. The samples were immediately stored in liquid nitrogen and then kept at -80°C. The specimens were weighed and used for assaying noradrenaline and HSP72. The lungs and the livers were also weighed and stored. Pulmonary pressure was measured by positioning a catheter in the pulmonary artery through the jugular vein. Cardiac output values were obtained from the thermodilution curve using a Cardiotherm-II-R cardiac output computer (Columbus Instruments, Ohio, USA). Other haemodynamic parameters were calculated by using the standard equations.

2.1.3. Noradrenaline analysis

Tissue catecholamines were quantitated by reverse-phase, ion-pair high pressure liquid chromatography with electrochemical detection as previously described [18].

2.1.4. Heat-shock protein analysis

Frozen portions (250 mg) of different tissues were ground into liquid nitrogen and then homogenized as previously described [20]. Total protein concentration of the supernatant was assessed following Bradford’s method [21]. Resolution of HSP72 was performed on 8% SDS-PAGE gel according to the procedure described by Laemmli [22]. Western blot analysis was performed as previously described [20]. Accuracy of loading and transfer was confirmed by optical observation of the actin band using Coomassie blue staining of the gel, and by Ponceau staining of the membrane. Autoradiographs of the blots were densitometrically quantified with respect to a fixed amount (0.3 μg) of the stress protein standard (SP70).
This standard is a mix of constitutive HSC73 and HSP72 proteins, purified from bovine brain. The final amount of HSP72 was evaluated by the absorption of the HSP72 band compared to that of the standard fixed amount and expressed in percentage. Assay of HSP72 was repeated at least four times for each experiment.

2.1.5. Statistical evaluation

Final values are expressed as the mean ± s.e. The significance was assessed by one-way analysis of variance and group to group comparison was performed by Student’s t-test, using Bonferroni’s correction. Results are considered significant if $P < 0.05$.

2.1.6. Chemicals

All of the chemicals were obtained from Sigma, St. Louis, Missouri, USA. The mouse monoclonal antibody for high inducible HSP72 (C92P3A-5) was used for immunoblot analysis. The stress protein standard (SP70) and the primary antibody were obtained from Stressgen Biotechnologies, Corp., Victoria, Canada. The horseradish peroxidase-conjugated rabbit anti-mouse antibody was provided by Dako, Glostrup, Denmark.

3. Results

In total, 114 animals were treated with monocrotaline. As expected, 61% of them ($n = 69$) died before the protocol could be accomplished. A number of rats were sacrificed after 4 weeks ($n = 27$), 59% of them showing liver and lung congestion with pleural and peritoneal effusions. Ten of these were included in the CHF group. The remaining 6 were used for haemodynamic measurements. Their mean cardiac index and pulmonary resistances were $360 ± 16$ ml/min · kg and $123 ± 12$ Wood U/kg, respectively. Systolic pulmonary artery pressure was $56 ± 9$ mmHg. Rats without effusion were discharged. Twelve animals of the remaining resistant rats ($n = 18$) were sacrificed 6 weeks after the drug injection and constituted the Hypertrophy group. Haemodynamics were measured in the remaining 6 rats. Their mean cardiac index and pulmonary resistances were $470 ± 26$ ml/min · kg and $77 ± 14$ Wood U/kg ($P < 0.001$ vs CHF group). Mean pulmonary artery pressure was $49 ± 11$ mmHg (NS vs CHF).

| Weight of animals and organs | CHF control $n = 11$ | CHF $n = 10$ | Hypertrophy control $n = 12$ | Hypertrophy $n = 12$
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<tbody>
<tr>
<td>Body</td>
<td>206.47 ± 11.95</td>
<td>178.34 ± 18.50 *</td>
<td>242.7 ± 13.30 ○○</td>
<td>251 ± 9.60 #</td>
</tr>
<tr>
<td>Whole heart</td>
<td>0.714 ± 0.041</td>
<td>1.024 ± 0.079 **</td>
<td>0.783 ± 0.022 ○○</td>
<td>1.096 ± 0.160 **</td>
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<tr>
<td>Right ventricle</td>
<td>0.133 ± 0.029</td>
<td>0.372 ± 0.075 **</td>
<td>0.143 ± 0.017</td>
<td>0.356 ± 0.089 **</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>0.561 ± 0.043</td>
<td>0.598 ± 0.057</td>
<td>0.586 ± 0.035</td>
<td>0.621 ± 0.087</td>
</tr>
<tr>
<td>Lungs</td>
<td>1.324 ± 0.125</td>
<td>2.149 ± 0.392 **</td>
<td>1.343 ± 0.132</td>
<td>1.403 ± 0.086</td>
</tr>
<tr>
<td>Liver</td>
<td>7.898 ± 1.332</td>
<td>7.625 ± 1.267</td>
<td>7.279 ± 0.593</td>
<td>7.480 ± 1.017</td>
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Fig. 1. Noradrenaline concentration (A) and content (B) of the right and left ventricles of the CHF and Hypertrophy groups in comparison with their age-matched control groups. Results are expressed as mean ± s.e.

Values are expressed as mean ± s.d. (in g).

* $P < 0.01$; ** $P < 0.001$ experimental groups vs relevant control.
○○ $P < 0.001$ CHF control vs Hypertrophy control.
# $P < 0.001$ CHF vs Hypertrophy.
Fig. 2. Representative autoradiograms of HSP72 western blot. Right (RV) and left (LV) ventricular, soleus, extensor digitorum longus (EDL), tibialis anterior (TA) muscles, lung and liver of rats with monocrotaline-induced congestive heart failure. Their age-matched controls refer to two groups: one fed ad libitum and the other receiving the amount of food consumed by the treated animals the previous day. An aliquot (0.3 μg) of the shock protein standard (SP70) was loaded onto each gel. Position of HSP72 is indicated by the arrow on the left.

Fig. 3. Densitometric scanning of HSP72 content in the ventricles of the CHF and Hypertrophy group. Data (mean ± s.e.) for each sample are expressed as the percentage of the shock protein standard (0.3 μg) loaded onto the relative gel.
3.1. Organ weights

Table 1 shows how body weight of CHF rats was significantly less than controls, despite the presence of pleural effusion and ascites. Body weight of the Hypertrophy group was unchanged. Right ventricular weight was increased in both groups: 2.81 and 2.56 times, respectively. Left ventricle weights were unaffected in all of the groups considered. The lungs of CHF rats were significantly heavier than controls and hypertrophic rats. Liver weight was unchanged in all of the groups.

3.1.2. Noradrenaline

Noradrenaline values are given in Fig. 1. They are expressed either as ventricular concentration (Fig. 1A) or content (Fig. 1B). In control rats, noradrenaline concentration was substantially lower in the left than in the right ventricle ($P < 0.001$, for each control group). Due to the greater size of the left ventricle, the total content of noradrenaline was greater in the left than in the right ventricle ($P < 0.001$). Noradrenaline concentration in the right ventricle of CHF rats was only 20% that of controls ($P < 0.001$). In the left ventricle it was also reduced, but only to 51% of control ($P < 0.001$). In failing rats, the diminished right and left ventricular noradrenaline concentration was not simply due to the increased size of the chambers, since the ventricular content of noradrenaline was also reduced to 55% and 52% for right ($P < 0.01$) and left ventricle ($P < 0.001$) respectively.
Both ventricles of the Hypertrophy group showed a significant decrease in their noradrenaline concentration: to 57% (P < 0.001) for the right ventricle and to 75% (P < 0.01) for the left ventricle, whilst ventricular contents of noradrenaline do not differ significantly from the control values.

3.1.3. HSP72

Fig. 2 shows a typical Western Blot in which HSP72 protein was evaluated in all tissues considered. Figs. 3 and 4 and 5 report the mean data obtained on HSP72 (expressed as percentage of SP70 standard). In both ventricles of CHF rats, there was significant expression of HSP72, more evident in the right (39.4 ± 1.6% vs 5 ± 0.9%, P < 0.001) than in the left ventricle (13 ± 1.2 vs 3.5 ± 0.6%, P < 0.001) (Figs. 2 and 3). A mild signal for HSP72 was also present in control groups. Nonetheless, such expression was not present in a group of rats injected with saline and fed ad libitum (Fig. 2). We could not find any increase in HSP72 concentrations in the ventricles of hypertrophic animals.

As expected, in the soleus muscle HSP72 was constitutively expressed in both CHF and hypertrophic age-matched controls, while only a minor amount could be detected in EDL and TA muscles (Figs. 2 and 4) [23]. Fig. 4 shows that CHF or hypertrophy does not affect the HSP72 expression in these skeletal muscles. Figs. 2 and 5 also show that expression of HSP72 was selectively induced in the liver of the CHF rats. No expression could be detected in the liver of the Hypertrophy group. In the lungs, HSP72 was constitutively expressed and unchanged in all studied groups.

4. Discussion

These data show that right CHF is a selective stimulus for the induction of HSP72 in the heart and liver, whilst chronic hypertrophy without signs of heart failure does not increase the concentration of this protein. The expression in the lungs and peripheral muscles is not affected by either CHF or hypertrophy.

4.1.1. Experimental model

The main advantages of this model are: (1) it avoids surgical operations and is therefore ideal for determination of the neuroendocrine response to CHF; (2) it provides large numbers of small, standard animals; (3) monocrotaline itself has no direct effects on the heart or peripheral muscles, as shown by morphological studies using both light and electron microscopy [17]; (4) there is an intrinsic control in the un hypertrophied left side of the heart; (5) it allows the study of two distinct animal groups: one with right adequate compensatory ventricular hypertrophy alone and one in which the burden on the right ventricle is so severe that it is accompanied by drastic haemodynamic changes and by a gradual water retention and development of the syndrome of CHF [17]; (6) in animals with CHF, an alteration of metabolism and function of the peripheral muscles occurs [24].

The major disadvantage of the preparation is that CHF due to pure right ventricular overload is a relatively rare event in patients. The model is not suitable for blood sampling for determination of catecholamines, as chronic cannulation of awake rats is practically not feasible and yields artificially high levels of catecholamines [25]. Equally, manipulation of the animals and the effects of anaesthesia strongly affect the labile and transient plasma levels of catecholamines. For these reasons, in the present study we have determined sympathetic activation only in terms of ventricular store of noradrenaline.

4.1.2. Noradrenaline

The striking decrease in noradrenaline concentration in the right ventricle and the substantial decrease in the left ventricle of CHF rats are similar to previous findings [18,26,27]. In the right ventricle, the decline in noradrenaline concentration was due to a decrease in the absolute content per ventricle. The mechanism by which myocardial concentration and content of noradrenaline become reduced in CHF is debatable. Evidence has been presented that depletion of noradrenaline is due to a limitation of tyrosine hydroxylase activity [28], dopamine β-hydroxylase activity [29] and altered neuronal uptake [30,31]. Whatever the intrinsic mechanism, it seems likely to be determined by the high extracellular concentrations of catecholamines which are found in CHF, since a similar depletion in myocardial noradrenaline stores can be induced by prolonged infusions of isoprenaline [32]. Similarly, in CHF there is evidence of a decrease in the density of β-adrenergic receptors [33,34]. We did not succeed in measuring blood content of noradrenaline in CHF rats, without artefacts, for the reasons previously discussed.

Noradrenaline concentration in both right and left ventricles of hypertrophied rats was also reduced, but this could be accounted for by changes in ventricular weights, since the total ventricular content of noradrenaline was not significantly different from the control values. In these apparently healthy rats, without ascites or pleural effusion, there was a 2.6-fold increase in right ventricular mass, causing a "dilution" of the concentration but no real depletion of noradrenaline content.

4.1.3. Heat shock protein 72

The most relevant messages from our results are the following.

First, HSP72 was expressed in the heart and liver of animals with CHF, but not in those with hypertrophy, without signs of heart failure. In the Hypertrophy group, the weight of right and left ventricles as well as the liver was similar to that of CHF group, but HSP72 was increased only in the latter.
Previous data had shown acute pressure overload induced in rats by aortic banding to cause a reprogramming of gene expression, including HSP70, which in the left ventricle was clearly evident 2 days after operation, and then disappeared at days 7 and 21 [15]. Similarly, acute hypertension is a potent and prompt stimulus for transient HSP72 synthesis [35]. On the contrary, several authors have shown that development of hypertrophy in spontaneously hypertensive rats with chronic pressure overload does not activate HSP72 synthesis [20,36], thus suggesting that chronic pressure overload is not a stimulus for HSP expression. In our study HSP72 expression could not be evidenced in the hypertrophied group, where monocrotaline caused chronic pressure overload and right ventricular hypertrophy in the absence of surgical stress. It is, however, possible that in this group a transient increase in HSP concentrations occurred during early phases of pulmonary hypertension, thereafter disappearing when adequate compensatory hypertrophy developed.

Second, in CHF animals the HSP72 concentration was higher in the right than in the left ventricle. This suggests that ventricular overload and abnormal chronic stretch due to increased pulmonary resistances is a stimulus for HSP synthesis. It has been shown that in isolated blood perfused rabbit heart a single stretch induces expression of HSP72 mRNA [37]. Unfortunately, we have no data on transcriptional state of myocardial cells (i.e. HSPmRNA), which could contribute to the understanding of the kinetics of HSP synthesis stimulation.

Third, in CHF animals, HSP72 was also increased in the left, unhypertrophied ventricle. This indicates that ventricular overload and enlargement is not the only stimulus for the synthesis of HSP72. Other factors are likely to be involved. A possible candidate is a neuroendocrine circulating factor activated in CHF, such as noradrenaline. Moalic has shown that injection of noradrenaline is able to induce HSP68 mRNA in rat ventricles within 2 h [16]. Fig. 6 shows that depletion of myocardial stores of noradrenaline below 5 nmol/g is concomitant with increased expression of HSP72. We have further investigated the meaning of this finding by treating 6 rats with a single injection of reserpine (0.3 mg/kg, intraperitoneally) as reported by Bond [38]. As a result, after 48 h, norepinephrine ventricular content decreased from 7.4 ± 0.9 to 0.4 ± 0.08 nmol/g (P < 0.001). HSP72 expression, however, did not change. These data suggest that a depletion of norepinephrine stores alone is not a stimulus for HSP72 protein expression. In the context of CHF, however, a ventricular depauperation of norepinephrine is an indirect index of generalized sympathetic activation and of high plasma level of catecholamines which, in turn, are likely to be responsible for HSP72 concentration.

Fourth, HSP72 protein expression was clearly evident in the liver of CHF animals, showing peritoneal effusion. Most likely the hepatic circulatory overload consequent to right ventricular failure is the stimulus for HSP expression. Alternatively, HSP72 might increase as a general reaction to monocrotaline liver toxicity. This hypothesis is less likely, as liver toxicity is usually responsible for the well described early mortality of this model. In addition, the protein expression was specifically increased in CHF but not in hypertrophied rats which also received monocrotaline. Thus, these data support the concept that HSP concentration is increased in the two organs (right heart and liver) which, in the present model, are predominantly affected by the CHF syndrome. Furthermore, we found HSP72 to be constitutively expressed even in the lungs of the saline-treated rats. The entire issue of HSP protein expression in the lungs is very controversial and it is quite difficult to make any statement at present.

Fifth, HSP72 expression was not increased in the peripheral muscles which we studied. The three muscles were chosen according to their different fiber composition: primarily slow twitch oxidative fiber I for the soleus; fast twitch fiber IIB for the EDL; and a mixture of type I, IIA and IIB for the TA [39]. The soleus muscle expresses HSP72 spontaneously, while the other ones do not. This reflects its high percentage of type I oxidative fibers with high catalase and ubiquitin levels [23]. During heart failure, these muscles undergo important morphological and metabolic alterations consisting of a reduction of sarcoplasm, development of interstitial fibrosis and shifting of fibers from type I to type IIB, thereby from aerobic to anaerobic metabolism [4]. These changes result partly from muscle underperfusion and partly from an adaptational process to the pathological condition, resulting in a deconditioning of the muscles [4]. From our previous data we know that monocrotaline-induced CHF in rats causes a reduction in peripheral circulation and a depletion of ATP and CP content in the soleus muscle [24]. Despite this, HSP72 was not increased in the soleus nor was it expressed in either EDL or TA.
Sixth, a minor HSP expression was observed in the ventricles and peripheral muscles (EDL and TA) in both control groups. This is explained by the fact that control rats received only the (reduced) amount of food consumed by treated animals the previous day. The relative fasting of these animals causes a mild stimulus for HSP induction, as these proteins could not be detected in sham-treated rats not subjected to food restriction [40].

5. Conclusion

The present study shows that CHF, but not chronic hypertrophy, due to monocrotaline injection in rats is a specific stimulus for the induction of HSP72 in the heart and in the liver. The presence of CHF seems to specifically influence the cardiac HSP72 gene and it appears that activation of the sympathetic system and HSP in heart failure represent, at two separate levels, a stereotyped reaction to stress.

The pathophysiological role of HSP in CHF remains unknown. It is clear that HSP72 plays no protective role against the development of CHF, since the syndrome was not prevented and hypertrophied rats without CHF have no overexpression. Probably, in analogy with the neuroendocrine response, HSP expression in the organs predominantly affected by the disease represents an extreme and unsuccessful form of intracellular adaptation to the new condition. These proteins could be overexpressed to counteract the deleterious effects of oxygen free radicals, whose production is increased in CHF [41] or could act as chaperons of protein folding, thus preventing the formation of irreversible aggregates of polypeptides [42], that may cause increased proteins turnover.

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