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Heat shock treatment induces expression of several heat shock proteins and subsequent post-ischemic myocardial protection. Correlations exist between the degree of stress used to induce the heat shock proteins, the amount of the inducible heat shock protein 70 (HSP70) and the level of myocardial protection. The inducible HSP70 has also been shown to be protective in transfected myogenic cells. Here we examined the role of human inducible HSP70 in transgenic mouse hearts. Overexpression of the human HSP70 does not appear to affect normal protein synthesis or the stress response in transgenic mice compared with nontransgenic mice. After 30 min of ischemia, upon reperfusion, transgenic hearts versus nontransgenic hearts showed significantly improved recovery of contractile force (0.35 +/- 0.08 versus 0.16 +/- 0.05 g, respectively, P < 0.05), rate of contraction, and rate of relaxation. Creatine kinase, an indicator of cellular injury, was released at a high level (67.7 +/- 23.0 U/ml) upon reperfusion from nontransgenic hearts, but not transgenic hearts (1.6 +/- 0.8 U/ml). We conclude that high level constitutive expression of the human inducible HSP70 plays a direct role in the protection of the myocardium from ischemia and reperfusion injury.

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Transgenic Mice Expressing the Human Heat Shock Protein 70 Have Improved Post-Ishemic Myocardial Recovery

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Abstract

Heat shock treatment induces expression of several heat shock proteins and subsequent post-ischemic myocardial protection. Correlations exist between the degree of stress used to induce the heat shock proteins, the amount of the inducible heat shock protein 70 (HSP70) and the level of myocardial protection. The inducible HSP70 has also been shown to be protective in transplanted myogenic cells. Here we examined the role of human inducible HSP70 in transgenic mouse hearts. Overexpression of the human HSP70 does not appear to affect normal protein synthesis or the stress response in transgenic mice compared with nontransgenic mice. After 30 min of ischemia, upon reperfusion, transgenic hearts versus nontransgenic hearts showed significantly improved recovery of contractile force (0.35±0.08 versus 0.16±0.05 g, respectively, P < 0.05), rate of contraction, and rate of relaxation. Creatine kinase, an indicator of cellular injury, was released at a high level (67.7±23.0 U/ml) upon reperfusion from nontransgenic hearts, but not transgenic hearts (1.6±0.8 U/ml). We conclude that high level constitutive expression of the human inducible HSP70 plays a direct role in the protection of the myocardium from ischemia and reperfusion injury. (J. Clin. Invest. 1995, 95:1854–1860.) Key words: heat shock protein-physiology • ischemia • myocardium-pathology • transgenic animal • hypoxia.

Introduction

It seems we are on the threshold of an era of molecular and genetic therapeutics (1–3). Transgenic mice expressing high levels of specific gene products have increased resistance to cerebral ischemia (4, 5) or have enhanced myocardial function (6). In fact, heat shock–induced changes in gene expression have been associated with cellular protection and improved function for more than a decade (7–9). More recently, in isolated and perfused heart studies, heat shock treatment and expression of heat shock proteins were associated with improved post-ischemic myocardial contractile recovery (10–12). In in vivo experiments, myocardial infarct size was significantly reduced in heat shock–treated animals (13–15). Indeed, a direct correlation has now been shown between the amount of the highly inducible member of the heat shock protein 70 (HSP70) family of stress-induced proteins and the degree of myocardial protection, as measured by reduction in infarct size (16).

Although heat shock treatment is associated with myocardial protection, it is difficult to know whether the protection is due to the overexpression of one or several proteins, or to some other unrelated adaptive process. Overexpression of HSP70 alone was demonstrated to protect cells from thermal injury and increase cell survival (17, 18). We decided therefore to use transgenic mice engineered to express constitutively high levels of human inducible HSP70, to investigate the contribution of this protein to myocardial protection.

Methods

Transgenic mice. Mice were cared for in accordance with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Preparation of the DNA fragments for microinjection. The Apr-HS70 expression vector was constructed as described previously (17). It contains the human inducible hsp70 gene under the regulation of the β-actin promoter. A 6.6-kb EcoRI–Hind III fragment containing the human β-actin promoter, IVS1 (intervening sequence 1 of the β-actin gene), and the human hsp70 gene was made devoid of vector sequences by preparative electrophoresis, Gene-clean purification (RI0101, UK), and passage through an Elutrap-D column (Schleicher & Schuell, Germany). Fragments for microinjection were dissolved in 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA to a final concentration of 2–4 μg/ml.

Generation of transgenic mice. Original inbred CBA, C57BI/6, and outbred NMRI mice were obtained from IFFA-CREDO (France) and maintained in the Hellenic Pasteur Institute’s facilities. CBA × C57BI/6 hybrid mice (F1) were used for the backcrossings throughout the study. NMRI vasectomized males were used to prepare pseudopregnant F1 females for oviduct transfers. To obtain fertilized F2 zygotes for microinjection, 3–4-week-old F1 females were superovulated as described (19) and mated with F1 males. Microinjection was performed essentially as described elsewhere (20, 21). Viable eggs were transferred into oviducts of pseudopregnant females and allowed to develop to term.

To identify transgenic founder mice, DNA was isolated from tail biopsies at 10–15 d of age (19), digested with BglII, and analyzed by Southern blot (22) using the microinjected fragment as a probe. Approximate copy numbers were estimated by comparison of signal

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intensity with that of an endogenous Thy-1 single-copy gene. Transgenic progeny from founder mice were identified by either Southern or slot-blot hybridization analysis.

RNA analysis. Total RNA was prepared from animal tissues by guanidinium thiocyanate extraction as described (23). S1 nuclease mapping was performed using as a probe a 692-bp Sall–BglII fragment, derived from the PAT-HS70 plasmid (24). This fragment contained 277 bp of vector sequences, 63 bp of the human hsp70 gene 5′ untranslated region, and 352 bp of the human hsp70 gene 5′ coding sequences. The probe was 5′ end labeled using [γ-32P]ATP and T4 polynucleotide kinase, purified though a G-50 spin column, denatured by boiling, and allowed to hybridize with 30 μg of RNA at 30°C overnight. S1 nuclease digestion was performed at 37°C for 1 h with an enzyme concentration of 600 U/ml. Under these conditions, only the fully homologous fragments were protected; these were subsequently analyzed under denaturing conditions on 5% sequencing gels (25).

Protein analysis. Hearts, livers, and kidneys from four transgenic and four nontransgenic mice were analyzed by one- and two-dimensional gel electrophoresis. Hyperthermic treatment (42°C rectal temperature for 15 min) was applied to two transgenic and two nontransgenic mice. The hyperthermia-treated mice were allowed to recover for 30 min before injection of radiolabel. Hyperthermia-treated and control mice were given an intraperitoneal injection of 0.25 mCi of L-[^35S]methionine (Dupont-NEN, Boston, MA; sp act > 1,000 Ci/mmol) and overdosed with sodium pentobarbital 2 h later, and organs were excised. Mice receiving hyperthermic treatment were injected with radioactive label 30 min after the end of the heat shock.

One-dimensional gels. Proteins (40 μg protein per lane) were separated by 7.5% SDS-PAGE. Gels were stained with Coomasie brilliant blue R, destained, and photographed. Gels were then processed for fluorography and exposed to X-OMat AR film (Eastman Kodak Co., Rochester, NY).

Two-dimensional gels and Western blot analysis. Protein samples from heart ventricular muscle were analyzed by two-dimensional SDS-PAGE (26) as previously described (27). For Western blotting, proteins were transferred to Immobilon-P membranes (Millipore Corp., Toronto, Ontario) at 200 mA overnight, according to the method of Towbin et al. (28). Blots were incubated in PBS containing 5% skim milk powder to block nonspecific binding sites on the membranes (29). Blots were immunoreacted first with a 1:7,500 dilution of a rabbit (#799) anti–human inducible 70-kd polyclonal antibody graciously provided by Dr. R. M. Tanguay (Université Laval, Québec). Second, blots were incubated in a 1:50 dilution of a peroxidase-conjugated goat anti–mouse IgG. 4-Chloro-1-naphthol was used as the substrate for the visualization of the reaction product. Blots were counterstained with amido black to show other proteins.

Langendorff perfusion protocol. Adult mice (25–35 g) were anesthetized with sodium pentobarbital (50 mg/kg body wt intraperitoneally). Hearts were excised rapidly and immersed to ice-cold buffer, and the aorta was cannulated on a Langendorff apparatus. Hearts were retrogradely perfused with Krebs-Henseleit buffer consisting of 120 mM NaCl, 20 mM NaHCO3, 4.63 mM KCl, 1.17 mM KH2PO4, 1.2 mM MgCl2, 1.25 mM CaCl2, and 8 mM glucose, pH 7.4, at a flow of 3 ml/min for 30 min. The perfusion system was regulated during the preischemic, ischemic, and reperfusion periods at 37°C, using a water-jacketed chamber and coil. Hearts were made ischemic by turning off the buffer flow for 30 min and then reperfused with 3 ml/min buffer for 30 min. Hearts were paced at 390 beats per min (6.7 Hz, 3.3 V, 3 ms) with a stimulator (model S44, Grass Instruments, Quincy, MA). Mechanical activity was measured throughout each perfusion experiment according to previously described methods (10, 11). Briefly, apical-cobal displacement was obtained by attaching a strain gauge transducer (model FT.03, Grass Instruments) to the heart apex. The transducer was positioned to yield an initial resting tension of 0.3 g. Perfusion pressure was measured using a pressure transducer (model P23 ID, Gould Inc., Cleveland OH). Recordings of mechanical activity and pressure were recorded on a polygraph (model 7, Grass Instruments).

Creatine kinase analysis. Creatine kinase release from hearts were measured in the effluent buffer. Samples were collected during the last minute of pre-ischemic perfusion and at 1, 5, 30, 60, and 20, and 30 min of reperfusion. Creatine kinase content was determined by the spectrophotometric method of Rosalki (30) using creatine kinase kits from Sigma Chemical Co. (St. Louis, MO).

 Catalase assay. Catalase activity was determined in hearts using a modification of previously described methods (31, 32). Briefly, 100 mg of heart was homogenized in 0.5 ml of ice-cold Tris sucrose buffer containing 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA, 0.5 mM DTT, and 0.1 mM PMSF, pH 7.5. Samples were centrifuged at 3,000 g for 15 min, and the supernatants were recovered. Protein concentration of the supernatants was determined by the method of Lowry et al. (33). Samples of heart supernatants (40 μg of protein) were brought to 1-ml volume with an assay buffer of 35 mM phosphate buffer, pH 7.2, 0.02% Triton X-100. The enzymatic reaction was started by adding 30 μl of 1% H2O2 to the assay mixture. Catalase activity was estimated by measuring the rate of H2O2 consumption at 240 nm in a spectrophotometer (model DU 50, Beckman Instr., Fullerton, CA). Optical density was recorded at 15, 25, 35, 45, and 55 s, and the velocity of the reaction (slope) was determined.

 Statistical analysis. All values expressed are mean±SEM. Differences were determined by comparison of the calculated test statistic with the two-tailed significance limits of Student’s distribution.

Results

Transgene expression. Four transgenic mouse lines (namely 2906, 2633, 2702, and 2710) were obtained as determined by Southern blot hybridization analysis of DNA from tail biopsies (data not shown). Expression of the transgene was monitored using an S1 nuclease mapping assay. To distinguish expression of the transgene from that of the endogenous mouse hsp70, we took advantage of a divergence in the sequences of the 5′ untranslated region in these two genes and used a 692-bp probe containing human HSP70 coding sequences plus 63 bp of the human 5′ untranslated region. Under stringent conditions of S1 digestion, different length fragments of this probe were protected with mouse and human hsp70 mRNAs. Using this assay, hsp70 mRNA levels in the four transgenic mouse lines were tested in heart tissues (Fig. 1). We found that the 2906 transgenic line expressed large amounts of human hsp70 mRNA at normal temperature, compared with the barely detectable levels of the endogenous mouse hsp70 mRNA. We estimated the copy number of the transgene to be ~50. Offspring of founder mouse 2906 were selected by Southern blot or slot-blot hybridization for transgene expression. Nontransgenic littermates as well as normal mice of the same strain were used as controls.

Protein analysis. One-dimensional gels stained with Coomasie brilliant blue R and fluorograms of livers and kidneys of transgenic and nontransgenic mice and of heat-shocked transgenic and nontransgenic mice are presented in Fig. 2. Comparison of liver or kidney from nontransgenic (Fig. 2A) and transgenic (Fig. 2B) mice revealed no apparent difference in the steady-state level of proteins separated by one-dimensional gel electrophoresis. After hyperthermic treatment (lanes 2 and 4), there appears to be a similar increased accumulation of several protein bands in the livers and kidneys of nontransgenic and transgenic mice (compare Fig. 2A with B). De novo protein synthesis was assessed by l-[^35S]methionine radiolabeling of heat-shocked and non–heat-shocked transgenic and nontransgenic mice. l-[^35S]methionine incorporation was similar for livers and kidneys of transgenic and nontransgenic mice (data not shown). Fluorography revealed no apparent difference
in protein synthesis between the nontransgenic (Fig. 2 C) and transgenic (Fig. 2 D) mice. Both nontransgenic and transgenic mice appeared to respond in a similar fashion to the hyperthermic treatment (lanes 2 and 4). Several protein bands have increased incorporation of L-[35S]methionine in liver and kidney (compare Fig. 2 C with D).

L-[35S]methionine–labeled proteins from hearts were separated by two-dimensional gel electrophoresis and processed to reveal the proteins synthesized during the 2-h labeling period. Fluorograms of L-[35S]methionine–labeled proteins revealed no detectable basal level of synthesis of either mouse inducible HSP70 or human HSP70 (transgene product) in the nontransgenic or transgenic mouse hearts (Fig. 3, A and B). After the hyperthermic treatment, mouse inducible HSP70 was easily detectable in the nontransgenic mouse hearts (Fig. 3 C). In the transgenic mouse hearts there was also an increase in mouse inducible HSP70, whereas there was little or no increase in human hsp70 gene product (Fig. 3 D; compare with Fig. 4 D).

Western analysis of two-dimensional gels of normal and hyperthermia-treated nontransgenic and transgenic hearts is presented in Fig. 4. A small amount of mouse inducible HSP70 was detectable in the nonstressed nontransgenic mouse hearts (Fig. 4, A and A'). After hyperthermic treatment, the accumulation of mouse inducible HSP70 was visibly increased (Fig. 4, B and B') in nontransgenic mouse hearts. Interestingly, from the nonstressed transgenic mouse hearts, mouse inducible HSP70 and human HSP70 fractionated into discrete spots (Fig. 4, C and C'). A small accumulation of mouse inducible HSP70 was detectable adjacent to a larger accumulation of human HSP70. After hyperthermic treatment, the accumulation of mouse inducible HSP70 was visibly increased, whereas there was no discernible change in the amount of human hsp70 transgene product (Fig. 4, D and D').

Contraction. Contractile data for isolated hearts from both groups of animals is illustrated in Fig. 5. None of the parameters differed during the equilibration period (t = 0–30 min). The resting tension was adjusted to 0.3 g during and at the end of the equilibration period. Ischemia produced a complete reduction in contractility within 15 min (t = 45 min), with a progressive increase in resting tension. The responses during ischemia did not differ significantly between the nontransgenic and transgenic hearts, although the rate of relaxation and the rate of contraction appeared to decrease more slowly in transgenic hearts. However, substantial differences in contractile recovery
were evident after restoration of the normal flow. After 30 min of reperfusion (t = 90 min), hearts of transgenic animals had significantly increased recovery of force (0.35±0.08 versus 0.16±0.05 g), rate of contraction (+dF/dt; 90.9±9.98 versus 47.08±13.99 g/s), and rate of relaxation (−dF/dt; 94.09±10.34 versus 52.33±13.04 g/s) when compared with nontransgenic hearts. Relative to pre-ischemic values, the recovery of force was 70 versus 29%, +dF/dt was 87 versus 48%, and −dF/dt was 83 versus 50% for the transgenic versus control hearts. In addition, the differences tended to increase according to the reperfusion time. Resting tension changed in a similar fashion for both groups.

Creatine kinase release. Creatine kinase release during the pre-ischemic perfusion was undetectable in hearts from transgenic and nontransgenic mice. Upon reperfusion, a high level of creatine kinase was released from nontransgenic hearts (67.7±23.0 U/ml) as compared with transgenic hearts (1.6±0.8 U/ml) (Fig. 6). At 5 min of reflow, the level of creatine kinase was considerably reduced in nontransgenic hearts and was similar to that in transgenic hearts (13.7±8.8 versus 19.9±10.1 U/ml, respectively).

Catalase activity. Catalase activity was not significantly different in the transgenic and nontransgenic hearts (1.75±6.46 [4] versus 0.83±4.84 [6] U per mg of protein; mean ±1 SD [n]; P = NS, respectively).

Perfusion pressure. Fig. 7 illustrates pressure data from transgenic and nontransgenic mouse hearts. Before ischemia, no significant difference was noted between the two groups, although nontransgenic hearts appeared to have a higher perfusion pressure (107±12 mmHg) than transgenic hearts (73±14 mmHg) (t = 30 min). Upon reperfusion (t = 60 min), perfusion pressure appeared to be higher for nontransgenic hearts than transgenic hearts, and the two experimental groups (nontransgenic versus transgenic hearts) differed significantly (109±12 versus 69±11 mmHg; respectively) after 10 min of reflow (t = 70 min).

Discussion

Persistent overexpression of the human hsp70 gene product in mice does not result in observable changes in basal protein content or synthesis. After hyperthermic treatment, the transgenic mouse liver, kidney, and heart have a similar increase in synthesis of a small number of proteins as the nontransgenic mouse tissues. However, overexpression of the human hsp70 gene product conveys a significant protection to isolated hearts after ischemic injury, during reperfusion. The transgenic hearts had significantly improved post-ischemic contractile recovery and significantly lower release of creatine kinase.

In previous studies, elevated levels of the highly inducible member of the HSP70 family of stress-induced proteins has been associated with improved post-ischemic recovery (10–12) and with reduction in infarct size in hearts (13–15). As mentioned earlier, there appears to be a direct correlation between the amount of the inducible HSP70 and the amount of myocardial protection (16). With transgenic mice overexpressing inducible human HSP70, assessment of the contribu-
tion of inducible HSP70 to cellular protection could be examined.

Overexpression of a single protein may induce drastic changes in cell morphology, physiology, and cell and animal survival (34, 35). However, in the case of the mice studied here, there was no obvious developmental or morphological differences between nontransgenic and transgenic mice. At the biochemical level, protein analysis revealed no clear differences in protein profiles of tissues from nontransgenic and transgenic mice. Even after heat shock treatment, the transgenic tissues respond in a similar fashion as the nontransgenics, with the increased synthesis of several heat shock proteins (Figs. 2 and 3). Comparison of the two-dimensional gel fluorograms (Fig. 3) and the Western blots (Fig. 4) revealed that after heat shock treatment, the human transgene is not induced whereas the endogenous mouse gene is induced. It should also be noted that a small amount of mouse inducible HSP70 is present in the non–heat-shocked nontransgenic and transgenic mouse hearts. The primary antibody used for the Western analysis is extremely sensitive and detects very small accumulations of the protein. Although mouse inducible HSP70 is detectable by Western analysis, its synthesis, as revealed by fluorography, is below a clearly detectable level in the non–heat-shocked hearts. At the protein level, it seems that transgenic and nontransgenic mice do not differ except for the presence of human HSP70.

HSP70 has been reported to autoregulate its own expression in a negative feedback loop (36). hsp70 transcription depends on the availability of heat shock transcription factor (HSF). HSP70 binds to HSF, thereby maintaining HSF in an inactive form. In our transgenic model, however, high levels of human HSP70 did not alter mouse HSP70 constitutive expression in hearts (Figs. 3 and 4). Moreover, synthesis of mouse inducible HSP70 after heat shock appeared in transgenic and nontransgenic mice. It may be that human HSP70 was not able to bind mouse HSF and regulate mouse hsp70 transcription. Indeed, mouse HSP70 and human HSP70 are not identical. These proteins can be resolved into discrete spots by two-dimensional gel electrophoresis.

The contractile data revealed no significant difference dur-
ing the pre-ischemic perfusion period. There were no significant differences in contractility during the ischemic period; however, the contractility of the transgenic hearts appeared to decline more slowly compared with the nontransgenic hearts. Although the temperature of the perfusion apparatus was regulated at 37°C, the temperature of the hearts may have decrease slightly during the ischemic period. However, it is unlikely that such a decrease in temperature would have contributed to the apparent difference in contractility during the ischemic period since conditions were similar for the transgenic and nontransgenic hearts. Significant differences between transgenic and nontransgenic hearts appeared upon reperfusion. The earliest indication of protection was at 1 min of reperfusion. The nontransgenic hearts released a large amount of creatine kinase, indicating cell and membrane disruption, whereas the transgenic hearts released almost no creatine kinase (Fig. 6). The contractile force and \( \pm dF/dt \) recovered stronger in mice overexpressing human HSP70 (Fig. 5). Almost all hearts in both groups recovered contractility with 1 min of reperfusion. For both groups, initial contractile force was strong and then declined at 5 min of reperfusion. This decline in contractile force between 1 and 5 min of reperfusion may be indicative of free radical injury. The transgenic hearts clearly had better recovery of contractility, both initially and over the 30-min reperfusion period.

Perfusion pressure, although not significantly different during the pre-ischemic perfusion period, appeared to be higher in nontransgenic than in transgenic mice (Fig. 7). During the reperfusion period, when flow rate was regulated at 3.0 ml/min, the transgenic hearts had a significantly lower perfusion pressure. At present we are uncertain whether the lower perfusion pressure is a result of the transgene product in the cells of the microvessels. However, it was previously reported that prior heat shock treatment did tend to lower the perfusion pressure in isolated rat hearts (11). On the other hand, other experiments suggest that heat shock pretreatment elevated the product of heart rate multiplied by the mean arterial pressure (rate – pressure product) in rabbits (14).

The presence of human HSP70 in the transgenic hearts seems to play a role in the improved post-ischemic contractile recovery. With perfusion and the introduction of oxygen, oxygen free radicals are generated, and membranes are disrupted by lipid peroxidation. The release of creatine kinase appears to be the result of such injury. Although human HSP70 seems to be involved in reducing this injury, it is unclear whether human HSP70 is involved directly or indirectly. Studies of human hsp70-transfected myocytes have shown increased resistance to hypoxic or metabolic stresses mimicking ischemia (37, 38). One possibility is that human HSP70, by its abilities to renature protein, is modifying the activity of enzymes such as antioxidants, which can protect cells from free radical injury. Although there is no direct evidence that heat shock proteins can change the enzymatic activity of antioxidants, catalase activity (11) and reduced glutathione (39) are reported to be increased after heat shock treatment. In the present experiments, catalase activity did not appear to be increased in the transgenic compared with nontransgenic hearts. However, conclusions about this low catalase activity should be made cautiously, since these measurements were determined in transgenic and nontransgenic hearts that were stored at –80°C for several weeks.

Insights on the role of human HSP70 can be suggested by analyzing the myocardial contractility during reperfusion. First, the apparent stronger contractile force recovery at 1 min of reperfusion suggests that there are more myocytes surviving after the 30-min ischemic interval. Second, contraction rate \( (+dF/dt) \) and relaxation rate \( (-dF/dt) \) of transgenic hearts progressively increased during reperfusion, whereas the nontransgenic hearts progressively decreased. This suggests that reperfusion injury is less in the transgenic hearts. One might argue that human HSP70 protects myocytes against oxidant-induced cell death and increases the resistance of myocytes to oxidative injury. HSP70 may bind to proteins denatured during ischemia and promote refolding or renaturation to normal configurations upon reperfusion.

These experiments with transgenic mice strongly suggest that an elevated level of inducible HSP70 plays a role in cell survival and recovery after ischemic injury. In addition, it seems that alterations in gene expression, whether induced or engineered (as in this study), can play an important role in the survival of cells. Understanding the role played by gene expression and how to regulate such expression after infarction and other ischemic injuries will lead to new ways of intervening therapeutically in cardiovascular injury.

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References


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