

Analysis of the variation in the *hsp70-1* and *hsp90 α* mRNA expression in human myocardial tissue that has undergone surgical stress

Simona Storti, Simona Vittorini, Maria Rosaria Iascone, Monica Sacchelli, Alessandra Baroni, Vincenzo Stefano Luisi, Adrian Crucean, Vittorio Vanini, Andrea Biagini, and Aldo Clerico

Clinical Physiology Institute, G. Pasquinucci Hospital, National Research Council, Massa, Italy

Abstract In the present work we reported a semiquantitative detection of messenger ribonucleic acids (mRNAs) encoding the human heat shock proteins Hsp70-1, the stress inducible member of the HSP70 family, and hsp90 α , the inducible member of the HSP90 family. We investigated the change in the expression of these mRNAs in tissue samples taken from the right atrium of 48 pediatric patients, soon after the ischemic period during surgery to correct congenital heart diseases, in which a crystalloid cold cardioplegic solution was used. No significant variations were found for either *hsp70-1* or *hsp90 α* expressions. Moreover, we searched for an association between the *hsp70-1* promoter region polymorphism and the expression of the *hsp70-1* in a smaller group of these patients ($n = 27$). The $-110AA$ genotype was on average significantly associated with a decrease in the *hsp70-1* mRNA level ($P < 0.05$), whereas the other genotypes $-110AC$ or $-110CC$ did not seem to be associated with the *hsp70-1* expression level. The lack of any observed increase in the hsp70-1 expression level may be due to the high basal level of the Hsp70 protein in the tissues examined.

INTRODUCTION

In response to adverse environmental changes, cells from many organisms increase the expression of a large class of proteins known as heat shock or stress proteins (Hsps), each named according to its molecular weight (Benjamin and McMillan 1998). Numerous studies have shown that synthesis of HSPs is enhanced to protect cells against various stress conditions, such as elevated temperature, exposure to heavy metals, as well as ischemia (Knowlton et al 1991). Indeed, HSPs act in protecting nascent or denatured proteins from aggregation, assisting their folding or refolding into the correct conformation or their degradation after an ir-

reversible damage, as well as in the translocation of damaged proteins.

The induction of *hsp70* gene expression by ischemia as well as an inverse correlation between the gene expression of *hsp70* and the infarct size have been previously demonstrated in some animal models, as recently reviewed (Benjamin and McMillan 1998). In a preliminary study, we investigated whether ischemic stress, induced by cardiopulmonary bypass (CPBP), can affect the expression of *hsp70-1* in the right atria of pediatric patients undergoing surgery for congenital heart defect (CHD) (Storti et al 2001). Our results showed no significant changes in the *hsp70-1* messenger ribonucleic acid (mRNA) expression, the stress-inducible gene of the HSP70 family, in atrial tissue after aortic cross-clamping (CC).

To better clarify whether the Hsps could have a cardioprotective role during surgical stress, we also mea-

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Correspondence to: Dr. Simona Storti, Tel: 39-0585-493621; Fax: 39-0585-493601; E-mail: kingle@ifc.pi.cnr.it.

sured the expression of *hsp90 α* , the inducible member of the HSP90 family, together with that of *hsp70-1* in human right atrium tissue samples, collected before and soon after the end of the aortic CC in 48 pediatric patients who had undergone surgery for CHD. Furthermore, we searched for an eventual association between the polymorphism of the *hsp70-1* promoter region and the mRNA expression level to explain the lack of expression variations in the *hsp70-1* gene during cardiac surgery. For this reason, we also genotyped 27 of these patients for this polymorphism.

MATERIALS AND METHODS

Patients

We examined 48 pediatric patients (ages ranging from 3 months to 15 years) undergoing elective surgical correction of CHD, 25 being affected by tetralogy of Fallot (TOF), 7 by ventricular septal defect (VSD), 1 by atrial septal defect (ASD), 4 by atrioventricular canal defect (AVCD), 4 by mitralic insufficiency (MI), 1 by double outlet right ventricle (DORV), 2 by ASD + VSD, 3 by aortic stenosis (AoSt), and 1 by anomalous partial pulmonary venous return (APPVR). Before the surgical procedure no patient was taking drugs nor was exposed to maneuvers known to increase blood pressure. CPBP was established with a crystalloid cold cardioplegic solution. During surgery, the mean CC temperature was $28.15^{\circ}\text{C} \pm 4.04$ (average \pm standard deviation). Mean CPBP time was 120 ± 33 minutes and mean CC time was 75 ± 26 minutes. Patients were divided into 4 groups according to their clinical condition and the gravity of their disease. These groups are shown in Table 1 along with the patients' ages, diagnoses, CPBP and CC times and temperatures, and pO_2 (mmHg) at the beginning of surgery under ventilation.

Two sequential samples were taken from a myocardial site in the right atrium discarded during the surgical intervention: the first specimen (right atrium 1 [RA1]) was obtained on average 20 minutes after the sternotomy, before any surgical manipulation of the heart. The second specimen (RA2) was obtained adjacent to the first one, outside the purse string, immediately after the end of the aortic CC. The duration of anesthesia up to the time that the RA1 specimen was taken as well as the total volume of the fluids administered to each patient are shown in Table 1 as a surrogate marker of anesthesia and intraoperative stress. The specimens were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Informed consent was obtained from parents because all children were under 18 years of age, and the study protocol was approved by the local Ethics Committee.

Semiquantitative reverse transcriptase–polymerase chain reaction

Total RNA was extracted from each specimen by the Tripure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany) in a procedure based on the guanidinium thiocyanide method (Chomczynski and Sacchi 1987). The integrity of each RNA preparation was determined by electrophoretic fractionation through an agarose-formaldehyde gel; 1 μg of RNA was treated with a DNase RQ1 (Promega, Madison, WI, USA) and 300 ng underwent reverse transcription by means of an oligo (dT)_{12–18} priming (Gibco BRL Life Technologies, MD, USA), in a mix containing also 2 μL of 5 \times Reverse Transcriptase buffer (Promega), 0.5 μL of deoxynucleoside triphosphates 1.25 mmol/L concentration of each, (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 5 U of RNasin (Promega), and 20 U of Moloney Murine Leukemia Virus (M-MLV RT) (Promega) and water up to a final volume of 10 μL . Another contemporary reaction in which no RT was added (No RT) was performed. The complementary deoxyribonucleic acids (cDNA) of glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) and each *hsp* target were coamplified in a multiplex polymerase chain reaction (PCR), using 2 couples of specific primers. Primer sequences are summarized in Table 2 (Tang et al 1995). The PCR solution (final volume: 25 μL) contained: 5 μL of the first strand cDNA solution, 1 μL of 1.25 mmol/L dNTP, 2.5 μL of 10 \times PCR buffer, 0.8 μL of 50 mmol/L magnesium chloride, 0.3 $\mu\text{mol/L}$ of forward and reverse *GAPDH* and *hsp70-1* or *hsp90 α* primers. The annealing temperature was 58°C for *hsp70-1* and 63°C for *hsp90 α* and 30 cycles were done in both cases. For each sample, and for each target gene, 2 separate PCRs were performed from the same RT. The PCR products were resolved on a 6% polyacrylamide gel (19:1 acrylamide–*N,N*-methyl-bis-acrylamide) stained by silver nitrate. The densities of the *GAPDH* and *hsp70-1* or *hsp90 α* bands were analyzed by the NIH Image 1.60 software (developed at the US National Institute of Health and available on line at <http://rsb.info.nih.gov/nih-image/>). The calculations were made as follows: first, the ratios between the *hsp70-1* or *hsp90 α* and *GAPDH* band densities were calculated; second, the variation of the *hsp70-1* or *hsp90 α* expression in RA2, referred to as RA1 expression taken as baseline, was evaluated for each sample and for the 2 different PCRs; third, the average of the 2 results was calculated.

hsp70-1 Promoter polymorphism detection

DNA was extracted from myocardial tissue from 27 patients by the Tripure Isolation Reagent (Roche Molecular Biochem-

Table 1 Characterization of patients and changes in hsp70-1 and hsp 90a mRNA expression

Id	Ages	Sex	Diagnosis	Disease severity	Geno- type	RA 2 hsp70-1	RA 2 hsp90 α	CPBP time (min)	CC time (min)	CC temp (°C)	Anes-thesia duration (h)	VFI (ml)	pO ₂ (mmHg)
1	3m	F	ASD+VSD	II		0.6	no 90	106	48	27	5.5	190	367
2	6m	M	ASD+VSD	II		0.7	1.7	116	69	24	4.5	490	206
3	3y	F	MI	II	aa	0.2	1.4	163	113	24	6.6	900	167
4	9y	F	AVCD	II	cc	2.4	2.5	95	60	26	4.2	1900	251
5	8m	M	TOF	IV	ac	0.3	0.6	135	75	20	5.0	260	283
6	3y	M	TOF	IV	aa	1.0	1.8	195	105	23	6.0	1330	75
7	14y	F	MI	II	ab	0.8	1.1	155	90	20	6.0	2100	66
8	2y	F	MI	II	ac	0.9	1.2	105	70	24	4.0	300	525
9	2y	F	TOF	IV	cc	0.8	0.6	140	70	25	4.8	400	43
10	12y	M	TOF	III	aa	0.4	1.2	130	85	13	5.5	1100	168
11	10m	F	TOF	IV	aa	0.9	0.3	145	94	25	5.0	470	42
12	4y	F	AoSt	II	cc	1.1	2.6	126	88	30	4.6	550	148
13	11y	M	TOF	III		0.5	1.1	124	80	33	5.0	900	63
14	1y	M	TOF	IV	aa	0.8	no 90	113	72	35	5.0	250	239
15	1y	M	TOF	IV	aa	0.7	no 90	113	63	27	4.0	500	70
16	4m	F	AVCD	II	aa	1.0	1.5	187	107	26	6.0	125	157
17	3y	M	TOF	IV	cc	1.7	no 90	113	66	31	4.5	520	71
18	3y	M	TOF	IV	ac	0.7	0.9	136	87	25	5.0	960	33
19	7y	F	TOF	IV	ac	0.8	no 90	139	57	30	5.0	2000	130
20	1y	F	TOF	III	ac	1.7	0.7	136	87	25	5.5	880	47
21	7m		TOF	III		0.4	0.9	128	71	32	4.5	510	401
22	6m	F	TOF	III	aa	0.7	1.6	120	83	31	4.5	265	62
23	6m	M	TOF	IV		2.3	0.7	160	95	20	5.5	315	120
24	9m	F	TOF	IV		1.0	0.9	130	87	28	5.0	440	149
25	4m	M	VSD	IV		0.8	0.3	75	55	24	4.0	280	118
26	10m	M	TOF	III		0.5	1.2	95	62	28	5.7	360	67
27	2y	M	TOF	III		0.4	0.9	130	71	27	5.0	410	129
28	8m	F	TOF	III		1.9	0.8	145	105	29	6.0	470	29
29	3y	M	DORV	IV		0.6	1.0	116	83	31	5.8	650	255
30	3y	M	VSD	II		0.7	0.7	91	53	33	4.3	800	557
31	13y	F	TOF	III		0.6	1.0	153	42	24	6.8	1500	117
32	3m	F	VSD+PI	II		2.6	1.1	126	68	28	5.6	460	287
33	8m	M	AVCD	II		0.8	0.9	142	84	31	5.0	800	180
34	7y	M	AoSt	II		1.4	1.0	57	28	30	3.5	3000	318
35	15y	M	AoSt	IV		1.3	1.0	219	132	30	7.5	3400	328
36	11y	M	TOF	II		1.3	0.76	129	69	20	5.8	1140	76
37	2y	M	TOF	III	cc	1.6	1.1	124	73	20	5.7	1220	49
38	2y	F	TOF	III		2.6	1.0	161	87	32	5.0	830	48
39	3y	F	ASD	I		0.8	1.5	46	13	31	3.7	350	203
40	3m	M	VSD	IV	bc	1.2	1.2	143	105	31	6.0	300	215
41	3m	F	AVCD	III	ac	1.1	0.7	123	78	26	5.5	410	99
42	10m	F	VSD	II		1	0.8	68	35	32	4.2	220	215
43	9y	M	APPVR	II	bc	1.8	1.6	94	56	32	5.0	900	672
44	13y	M	MI	II	bc	0.2	1.0	166	97	32	6.6	700	178
45	7m	F	TOF	IV	ac	0.7	0.9	169	138	22	5.5	330	120
46	8m	M	TOF	III	ac	1.3	0.9	130	68	20	5.8	250	108
47	2y	F	VSD	II	ac	1.1	0.8	68	38	27	4.6	610	185
48	10m	F	VSD	II	ac	0.7	0.7	96	56	29	6.0	220	380
	3m-15y				average	1.07	1.01	125	75	28.15	5.2	754	175
					st dev	0.59	0.41	35	26	4.04	0.84	696	142

y = years, m = months, TOF = Tetralogy of Fallot, DORV = Double Outlet Right Ventricle, AoSt = Aortic Stenosis, APPVR = Anomalous Partial Pulmonary Return, ASD = Atrial Septal Defect, VSD = Ventricular Septal Defect, MI = Mitral Insufficiency, AVCD = Atrioventricular Canal Defect, genotype = hsp70-1 promoter region genotype; VFI, volume of intraoperative fluid; h, hour, RA2 hsp70-1 = changes in mRNA hsp70-1 level, RA2 hsp90 α = changes in mRNA hsp90 α level.

icals). The genotyping protocol for the promoter region polymorphism was taken from Cascino (Cascino et al 1993, Table 2), with some modifications. Briefly, 50 ng of DNA was amplified with 10 mol/L of each specific primer in 25 μ L of total reaction volume, with an annealing temperature

of 55°C. Five microliters of PCR product was loaded on a 10% polyacrylamide (29% acrylamide, 1% bis-acrylamide in Tris Boric EDTA-TBE-buffer (TBE buffer) gel vertical plate 20 cm in length, run at room temperature for 22 hours, at 18 mA in TBE buffer 1 \times , and then stained by silver nitrate.

Table 2 Primer sequences and polymerase chain reaction product size

Gene	Primer sequences		Product size (bp)
	Primer F	Primer R	
<i>hsp90α</i>	ACAAGCACATATGGCTGGACAGCA	TTCAGTTACAGCAGCACTGGTATC	342
<i>hsp70-1</i>	CTAGCCTGAGGACTGGCTGCGACA	GTTCCCTGCTCTGTGTCGGCTCGG	205
<i>GAPDH</i>	GAGTCAACGGATTGGTTCGT	GGTGCCATGGAATTTGCCAT	156
<i>hsp70-1 Promoter polymorphism</i>	CGCCATGGAGACCAACACCC	GCGGATCCGCGTTCCCTGCTCTC	496

RESULTS

DNase 1 treatment

DNase 1 treatment is usually done after total RNA extraction to assure that no genomic DNA is contaminating the RNA solution (Grillo and Margolis 1990). In the absence of genomic DNA contamination, the cDNA synthesis reactions, performed without RT (No RT), and the subsequent PCR gave no products (data not shown).

Expression of *hsp70-1*

The results of *hsp70-1* expression changes in the RA2 biopsy, compared with the mRNA expression in the RA1 biopsy considered as baseline in all 48 pediatric patients, are shown in Table 1. On average, no change in the mRNA level was found in *hsp70-1* expression (1.07 ± 0.59 -fold the RA1 baseline value). No relationship between changes in the *hsp70-1* expression and patient age distribution, disease severity classes, or CPBP duration was found (Figs 1A, 2A, and 3A). Furthermore, no significant correlation was found between the expression changes and the CPBP time ($R^2 = 0.001$, $P = 0.8201$) or CC time ($R^2 = 0.0001$, $P = 0.9435$) or temperature ($R^2 = 0.004$, $P = 0.6886$).

Expression of *hsp90α*

The results of the measurement of *hsp90α* mRNA expression in the 48 patients are shown in Table 1. On average, no variation in the mRNA level was found in *hsp90α* expression when the values obtained after the aortic CC were compared with those measured before CC (1.01 ± 0.41 -fold the RA1 baseline value). No relationship was found between changes in *hsp90α* expression and patient age distribution, disease severity classes or CPBP duration (Figs 1B, 2B, and 3B). No significant correlation was found between the expression changes of *hsp90α* mRNA and the CPBP time ($R^2 = 0.001$, $P = 0.8680$) or the CC time ($R^2 = 0.001$, $P = 0.8146$) or temperature ($R^2 = 0.025$, $P = 0.3130$).

Analysis of the *hsp70-1* promoter region polymorphism

Using the primers described by Cascino (Cascino et al 1993), a single 496-base pair product was obtained. On

the polyacrylamide gel, 3 bands of different mobility were interpreted as corresponding to different alleles and were named: a (slow), b (fast), c (intermediate). Variations among the 3 alleles were restricted to 2 sites: -110 and +120. At -110 the a allele differed from b and c for the presence of an adenine instead of a cytosine. At +120 the b allele differed from a and c for the presence of a cytosine instead of a thymine.

Twenty-seven patients were analyzed for this polymorphism, and the results are shown in Table 1. Individuals could exhibit either 1 or 2 bands; the combinations found were the following: "ab" (-110AC, +120TC) (1/27, $f_{ab} = 0.037$), "ac" (-110AC, +120TT) (10/27 $f_{ac} = 0.370$), "bc" (-110CC, +120CT) (3/27 $f_{bc} = 0.111$), "aa" (-110AA, +120TT) (8/27 $f_{aa} = 0.296$), and "cc" (-110CC, +120TT) (5/27 $f_{cc} = 0.185$). The "bb" (-110CC, +120CC) genotype has not been encountered, as previously reported by Cascino (Cascino et al 1993). The genotype assortment was as expected from the Hardy-Weinberg equilibrium in the 3 allelic system; the calculated frequencies of the 3 alleles being as follows: $f_a = 0.500$, $f_b = 0.074$, and $f_c = 0.426$.

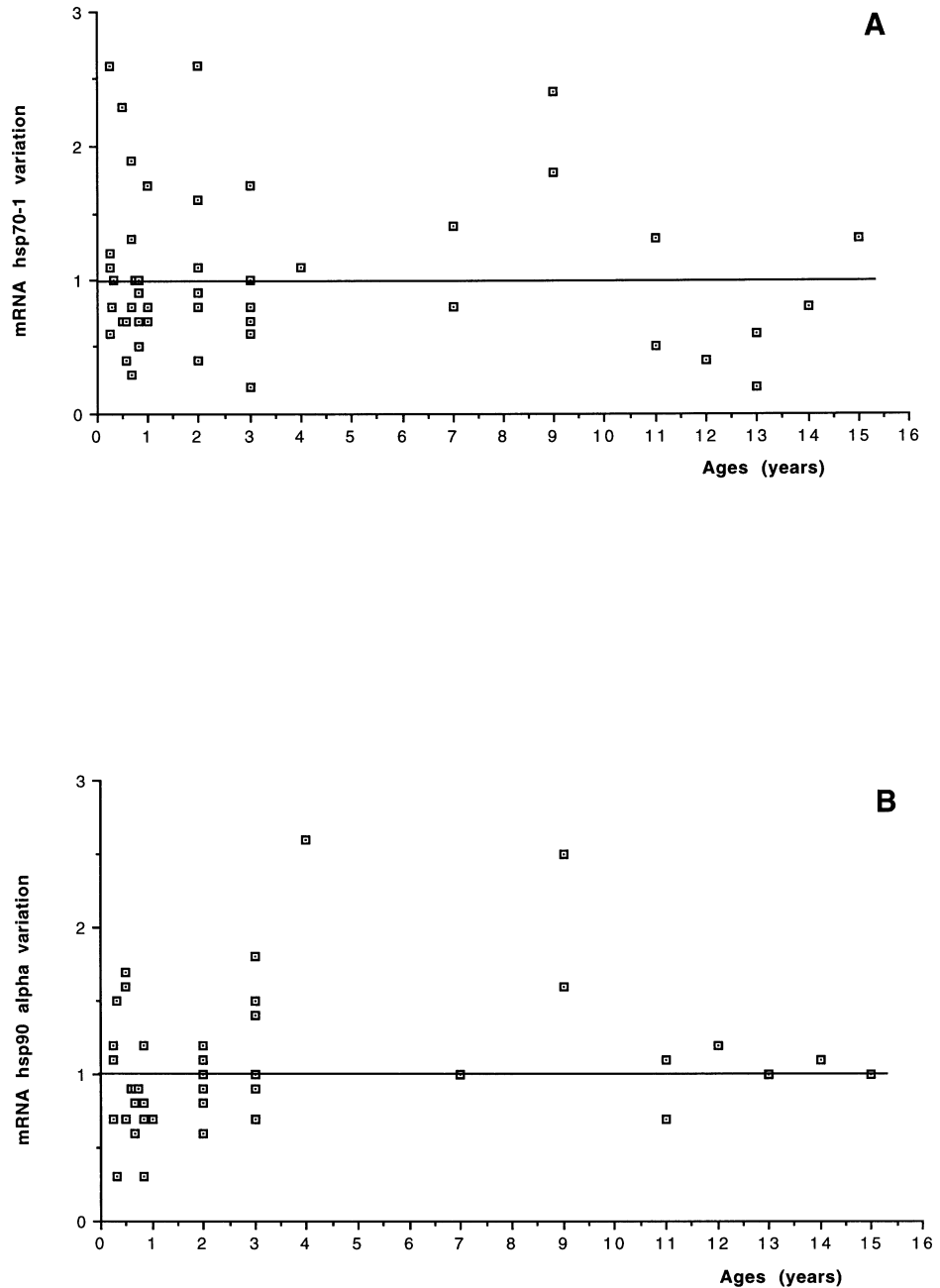
Relation between *hsp70-1* promoter genotype and *hsp70-1* mRNA expression

The "aa" genotype, found in 8 patients (3, 6, 10, 11, 14, 15, 16, 22), showed on average a significant decrease in the *hsp70-1* mRNA level after the ischemic period (on average 0.71 ± 0.29 , t -test P -value = 0.025, Table 1). Moreover, the only patient whose genotype was "ab" showed a decrease in the *hsp70-1* mRNA level, whereas the other patients, characterized as "ac," "bc," or "cc," had no significant changes of *hsp70-1* mRNA expression before and after the ischemic period. Data from genotypic analysis are presented in Figure 4.

DISCUSSION

In our study we evaluated the change in *hsp70-1* mRNA expression in biopsies from right atria of pediatric patients undergoing surgery for CHD correction. During surgery, CPBP was established by means of a cold crystalloid cardioplegic solution. Because of the young age of the patients, the samples collected were very small (8–20

Fig 1. Age distribution: relationship between age distribution of patients and changes and *hsp70-1* (A) or *hsp90 α* (B) expression.



mg). Consequently, a previously described semiquantitative method of quantifying mRNA expression from only 300 ng of total RNA (Storti et al 2001) was set up using the housekeeping *GAPDH* gene as an internal standard, on the assumption that its level would remain unchanged before and after the ischemic period. Before reverse-transcription, total RNA was treated with a DNase 1 and a No RT control was included (Grillo and Margolis 1990). The DNase I pretreatment is essential because *hsp70-1* is an intronless gene (Wu et al 1985) and also because in genomic DNA there is a *GAPDH* pseudogene. Indeed, DNase I pretreatment eliminates the false positives that

can result from any preexisting DNA and could confuse the interpretation of results.

In our study, on average no variation of *hsp70-1* mRNA expression was found after the ischemic period (1.07 ± 0.59 -fold the baseline value). However, a limitation of this study is that it has not been possible to have a truly baseline cardiac preparation with which to compare the intraoperative surgical samples because the samples were taken from individuals who had severe cardiac disease and who often experienced varying degrees of pathophysiological stresses before surgery. In fact, the first sample represents a time point in which a patient has under-

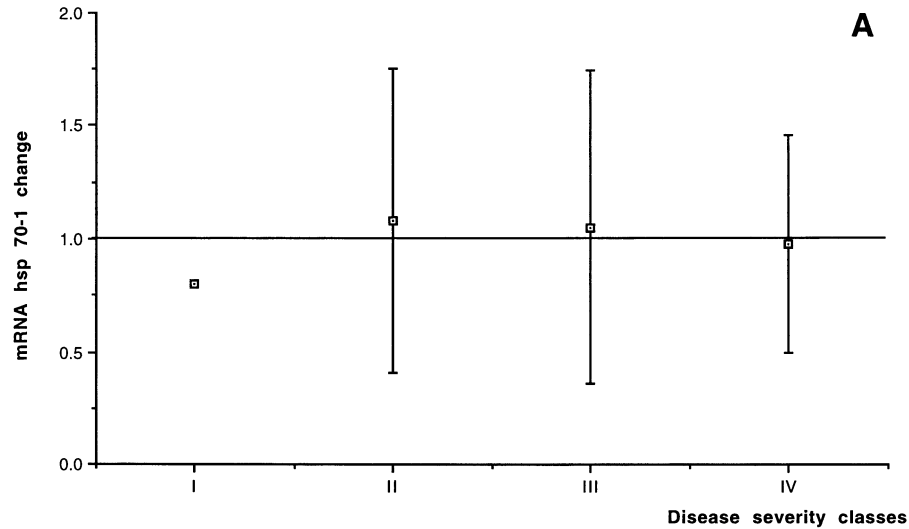
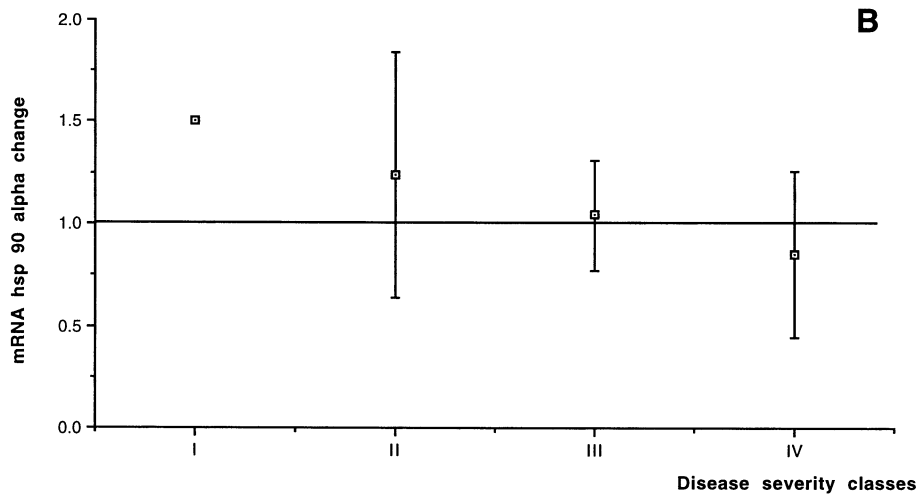


Fig 2. Disease severity classes: relationship between the severity of patient disease, classified from I to IV according to the minor or major gravity of the congenital heart disease, and changes in *hsp70-1* (A) or *hsp90 α* (B) expression.



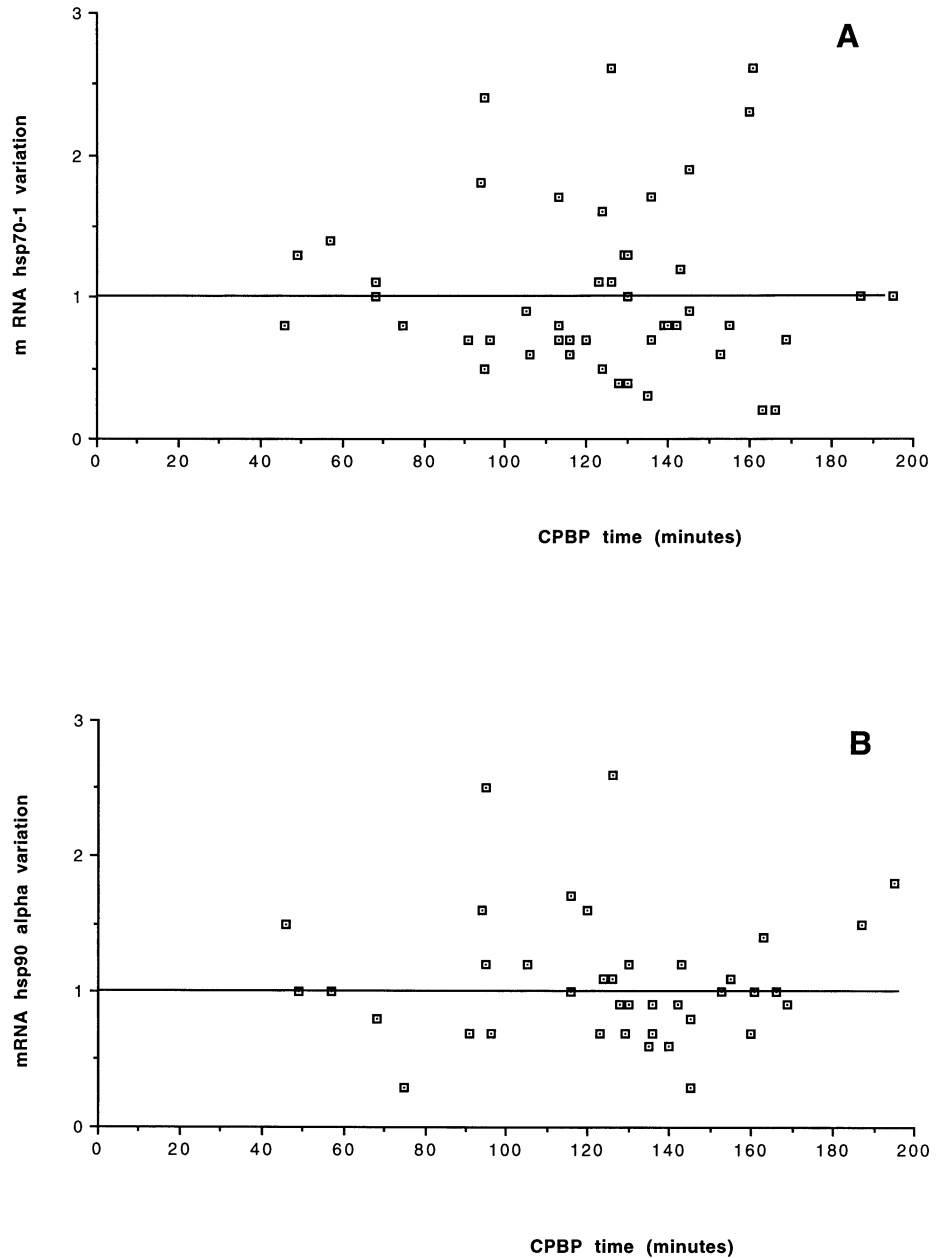
gone an open sternotomy and this initial sample may reflect a significant degree of stress already experienced by the patient. However, this protocol of study was the best we could set up because it was not possible to obtain a completely unstressed human cardiac preparation to use as baseline.

In our opinion, among the hypotheses that could explain our result, 2 appear very interesting.

A first consideration may be done about the basal high level of the Hsp70 protein (Shi et al 1998) in patients before cardiosurgery. In fact, it has been noted that in higher eukaryotes, the stress signal leads to the elevated expression of heat shock genes; stress induced

transcription requires activation of heat shock factors (HSFs) that bind to the heat shock promoter element. In unstressed cells, HSFs are maintained in an inert non-DNA-binding state. Upon exposure of cells to stress conditions, HSFs become activated to a DNA-binding, transcriptionally active state, which results in the preferential transcription of the heat shock genes. In stress conditions, Hsp70 stably associates with the heat shock factor 1 (HSF1), and so it functions as a repressor of transcriptional activity of the heat shock genes (Shi et al 1998). According to this hypothesis, a preexisting state of physiological stress, perhaps due in our patients to the congenital heart disease, can pro-

Fig 3. Cardiopulmonary bypass (CPBP) duration. Relationship between the duration of CPBP and changes and *hsp70-1* (A) or *hsp90 α* (B) expression.



voke an inhibition of the further transcription of all heat shock genes. In the present study, this hypothesis has been tested by the evaluation of the change in the expression of *hsp90 α* , the inducible member of the HSP90 family. It has been shown, in fact, that in *hsp70* overexpressing cells the transcription of the *hsp90 α* gene was not induced after heat shock, whereas in cells uninduced for *hsp70* there was a dramatic heat shock induction of the *hsp90 α* gene transcription (Shi et al 1998). We found on average no induction of *hsp90 α* mRNA too (1.01 ± 0.41 -fold the baseline value). In conclusion, we can suggest that a negative feedback

regulation by Hsp70 acting as an expression repressor on *hsp* genes transcription is possible.

A second hypothesis involves the *hsp70-1* promoter region polymorphism. The method used for detecting the promoter polymorphism yields stereoscopic images of the DNA superhelical conformation, which can be directly related to the electrophoretic behavior. In effect, the introduction of an adenine at position -110 (ie, allele a) increases the curvature of the molecule and provides an adequate explanation of the electrophoretic retardation of this allele; the modification of the DNA curvature by introducing a cytosine at the $+120$ site (allele b) is much

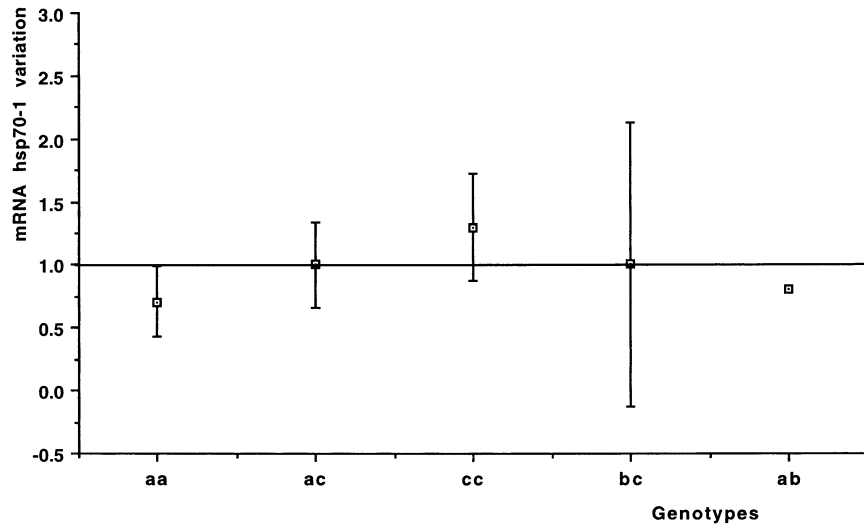


Fig 4. *hsp70-1* Promoter region polymorphism. Relationship between the mean change of mRNA *hsp70-1* for each observed genotype.

less marked. Moreover, the adenine-cytosine interchange at position -110 , which gives the strongest effect on DNA curvature, is located between the first 2 of a series of 5 consecutive regulatory elements involved in the binding of HSF1 (Cascino et al 1993). It is possible that this interchange causes a variation of *hsp70-1* expression after stress stimuli, owing to the intimate connection between the local curvature of a DNA segment and its function in regulatory processes. Indeed, our data indicate that the $-110AA$ genotype seems to be significantly associated with an inactivation of the *hsp70-1* expression, whereas the other genotypes do not seem to be associated with the *hsp70-1* mRNA level. Unfortunately, we obtained the genotype data only from a minority of patients; our result, although suggestive, needs to be confirmed in a larger population, and so further studies are necessary to better clarify the pathophysiological importance of this association.

In conclusion, on average, no increase in the *hsp70-1* mRNA expression was found in biopsies from human right atria taken soon after the ischemic period during heart surgery in the present study. It is possible that the lack of any observed increase in *hsp70-1* expression may be due to the high basal level of Hsp70 protein because *hsp90* failed to induce in the same tissue. Furthermore, our data revealed an association between the *hsp70-1* promoter polymorphism and the expression of the *hsp70-1*: in fact, the $-110AA$ genotype seems to be

significantly associated with an inactivation of the *hsp* transcription.

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