Increased Myocardial GRP94 Amounts During Sustained Atrial Fibrillation

A Protective Response?

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Background—Structural and phenotypic changes of cardiomyocytes characterize atrial fibrillation. We investigated whether changes in the glucose-regulated protein GRP94, which is essential for cell viability, occur in the presence of chronic atrial fibrillation.

Methods and Results—Samples of fibrillating atrial myocardium obtained from both goat and human hearts were analyzed for GRP94 expression by an immunologic approach. In goats, atrial fibrillation was induced and maintained for 2, 4, 8, and 16 weeks. After 16 weeks of atrial fibrillation, cardioversion was applied and followed by 8 weeks of sinus rhythm. GRP94 levels doubled in goat atrial myocytes after 4 to 16 weeks of fibrillation with respect to normal atria and returned to control levels in atrial myocardium of cardioverted goats. Immunohistochemical analyses confirm that GRP94 increase occurred within cardiomyocytes. Significantly, increased levels of GRP94 were also observed in samples from human fibrillating atria. In the absence of signs of myocyte irreversible damage, the GRP94 increase in fibrillating atria is comparable to GRP94 levels observed in perinatal goat myocardium. However, calreticulin, another endoplasmic reticulum protein highly expressed in perinatal hearts, does not increase in fibrillating atria, whereas inducible HSP70, a cytoplasm stress protein that is expressed in perinatal goat hearts at levels comparable to those observed in the adult heart, shows a significant increase in chronic fibrillating atria.

Conclusions—Our data demonstrate a large, reversible increase in GRP94 in fibrillating atrial myocytes, which may be related to the appearance of a protective phenotype. **(*Circulation*. 2001;103:2201-2206.)**

Key Words: fibrillation ■ atrium ■ myocytes ■ sarcoplasmic reticulum ■ stress

Long-term atrial fibrillation (AF) is accompanied by structural changes within viable cardiomyocytes, characterized by loss of myofibrils, presence of glycogen granules, and organelle aggregates both in the human heart and in experimental chronic AF in goats.[1,2] Fibrillating atrial myocytes display a fetal cardiomyocyte phenotype, because α-smooth muscle actin immunoreactivity is detected and immunostaining for titin and cardiotin is reduced in goat fibrillating atria,[3] and the β-myosin heavy chain isoform is expressed in fibrillating human auricles.[4] On the basis of this evidence, the phenotypic change that occurs in fibrillating atrial myocytes has been called dedifferentiation.[2,3]

Furthermore, fibrillating atrial myocytes display signs of intracellular calcium overload,[5,6] possibly because of rapid atrial depolarization and Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) stores. A thorough cytochemical analysis of calcium distribution in fibrillating goat atria showed increased sarcolemma-bound and mitochondrial calcium deposits after 1 to 2 weeks of fibrillation.[6] The increase in intracellular calcium in AF is associated with or followed by reduced accumulation of transcripts and protein for the sarcolemmal L-type Ca^{2+} channel and SR Ca^{2+} pump,[7,8] whereas the expression of other SR proteins involved in calcium handling, such as calsequestrin, ryanodine receptor, and phospholamban, appears to be unaffected.[7]

The glucose-regulated protein GRP94 belongs to a class of stress proteins that localize in the endoplasmic reticulum (ER).[9,10] It acts as a molecular chaperone[11] and is involved in the maintenance of cell survival because it exerts a specific protection against stresses due to Ca^{2+} depletion from the ER.[12] GRP94 is a low-affinity–high-capacity Ca^{2+}-binding protein, a property it has in common with other ER resident proteins, such as calreticulin and protein disulfide isomerase.[13] GRP94 is expressed in the SR of adult rabbit cardiomyocytes.[14] Although its precise functional role in cardiac myocytes remains to be determined, it is upregulated after

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exposure to bacterial lipopolysaccharide and during prenatal development.[14] SR Ca^{2+} depletion and consequent intracellular Ca^{2+} overload have been recognized as stimuli for GRP94 upregulation.[9,12] In the present study, we analyzed how GRP94 expression is affected in the presence of AF, using both an experimental model of chronically induced yet reversible AF in the goat and atrial samples from patients suffering from chronic AF.

Methods

Goat Model of Chronic AF

As previously described,[15] an Itrel pacemaker (Medtronic) was implanted in the neck of adult female goats under general anesthesia (1% to 2% halothane and N_2O). A bipolar screw-in electrode was inserted through the jugular vein and fixed to the right atrial appendage. One week after surgery, the pacemaker was switched on, and AF was induced and maintained by bursts of stimuli (50 Hz, 2 seconds, 4 times diastolic threshold). Because in subsequent days the duration of paroxysms of AF became longer, the interburst period was gradually prolonged.[15] Thirty-six goats were included in the present study. They were divided into the following groups: sinus rhythm (n=6); AF of 2, 4, 8, and 16 weeks' duration (n=6 each); and 8 weeks postcardioversion after 16 weeks of AF (n=6). Animal handling was carried out according to the Dutch law on animal experimentation and the European Directive for Protection of Vertebrate Animals.

At the end of each protocol, the goats were anesthetized, and the left and right atrial appendages were excised. Left ventricular samples and biopsy samples from limb skeletal muscles were taken from the control group. Additionally, atrial and ventricular samples were obtained from 2 prenatal goats (1 to 2 weeks before birth) and 5 neonatal goats (3 days after birth). The samples were frozen immediately after excision in isopentane precooled with liquid nitrogen.

Human Heart Samples

Samples from the left atrial appendage were obtained from 13 human hearts. Control samples were excised at the time of heart explantation from 5 healthy individuals who died because of subarachnoid hemorrhage (mean age±SE 38±2.9 years). Eight fibrillating atrial samples were provided during cardiac surgery for mitral valve replacement. Patients (mean age±SE 45±9 years) displayed sustained AF for >6 months and were undergoing digitalis treatment. Five of them received additional calcium-antagonist medication. Each sample was collected after procedures in accordance with institutional guidelines from the Heart Surgery Center of the University of Padova.[4,16]

Antibodies

Monoclonal antibody 3C4 reacted specifically with rabbit GRP94[14]; monoclonal antibody BN-59 reacted with both cardiac and skeletal troponin T of several different species.[17] Monoclonal anti-HSP70 inducible form (SPA810) and polyclonal anti-calreticulin antibodies (SPA-600) were from Stressgen Biotechnologies Corp. Anti-desmin antibody (clone DE-B-5) was from Chemicon. Peroxidase-conjugated antibodies were from BioRad and Dako. Fluorescein-conjugated antibody was from Cappel.

Western Blotting

Cryostat sections from tissue samples were homogenized in electrophoresis sample buffer in the absence of blue bromophenol. After heating for 5 minutes and centrifugation at 15 000*g* for 15 minutes at 4°C, protein concentration was determined as described by Lowry et al[18] with bovine serum albumin used as standard.[19] Equal amounts of samples were run either in 6% to 12% gradient or in 10% linear SDS-polyacrylamide gel together with molecular weight standards (BioRad) at 5 mA, transferred to nitrocellulose, saturated with ovalbumin, and incubated with primary antibodies. After being washed with TBST (10 mmol/L Tris-HCl, pH 8.00, 150 mmol/L NaCl, 0.05% Tween 20), filters were incubated with anti-immunoglobulins conjugated with peroxidase and revealed by diaminobenzidine[14] or chemiluminescence[19] when the same blot was stained for both GRP94 and desmin.

For troponin T cross-linking studies, homogenization was preceded by incubation of sections of control goats with propionate buffer for 30 minutes at 37°C in the presence or absence of 10 mmol/L $CaCl_2$, as described previously.[17,20] Additional experiments were performed in the presence of the calpain inhibitor calpeptin (Z-Leu-Nle-OH, Novabiochem).

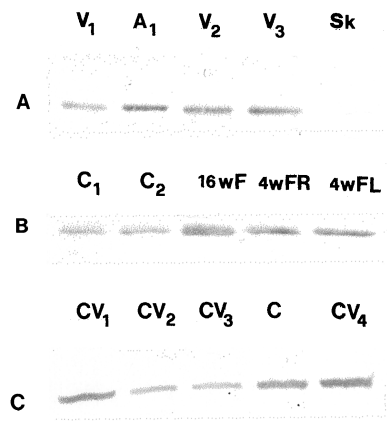

Figure 1. GRP94 expression in control, fibrillating, and cardioverted goat atria. A, B, and C correspond to representative Western blots reacted with anti-GRP94 antibody 3C4. Equal amounts (40 μg) of whole homogenates obtained from ventricular (V) and atrial (A) myocardium and skeletal muscle (Sk) from 3 adult control (C) goats are represented in A. B and C illustrate GRP94 immunoreactivity on 40 μg of whole homogenates obtained from 2 control left atria (C), 16-week fibrillating left atria (16wF), 4-week fibrillating right and left atria (4wFR and 4wFL, respectively), and left atrium of 4 goats after 16 weeks of fibrillation plus 8 weeks in sinus rhythm (CV).

Quantitative and Statistical Analysis

Quantitative densitometry of Western blots was achieved with a Shimadzu chromatoscanner CS-930, as described previously.[14,19] Variability among different experiments was compensated for by use of the same control sample as internal reference. Sample values were also normalized to the corresponding densitometric value of desmin immunoreactivity for control and 4- and 16-week-AF samples. Statistical analysis was performed with the unpaired Student's *t* test.

Immunocytochemistry

Ten-micrometer cryosections were fixed for 10 minutes with 4% freshly prepared buffered paraformaldehyde, rinsed with PBS (10 mmol/L Na phosphate, 150 mmol/L NaCl, pH 7.4), permeabilized with 0.1% Triton X-100, and incubated with 3C4 monoclonal antibody for 1 hour. After being washed in PBS and incubated with secondary antibodies, peroxidase was revealed with diaminobenzidine.[14] Controls were performed with nonimmune mouse immunoglobulins. For confocal microscopy analysis (BioRad MRC 1024ES), a fluorescein-conjugated secondary antibody was used, with the addition of 1 μmol/L propidium iodide.

Results

Goat Fibrillating Atria Display Increased GRP94 Levels

Western blotting showed reactivity of 3C4 antibody with a single polypeptide with the apparent molecular weight of 99 kDa, corresponding to goat GRP94, in both atrial and ventricular homogenates (Figure 1A). Because we demon-

TABLE 1. Percentage of GRP94 Levels in Goat and Human Fibrillating Atria

	Goat Heart Samples	
	Left Atrium	Left Ventricle
Perinatal	242.6±16.3 (7)*	199.6±24.0 (5)†
Adult control	111.3±8.6 (6)	90.6±14.2 (3)
2-wk AF	92.2±14.0 (6)	
4-wk AF	297.2±32.5 (6)*	
8-wk AF	354.1±44.2 (6)*	
16-wk AF	233.8±20.4 (6)*	
16-wk AF+8-wk SR	106.7±11.9 (6)	
	Human Left Atrium Samples	
Donors	104.77±12.6 (5)	
AF	178.0±12.2 (8)†	

SR indicates sinus rhythm. Values are mean±SE of percentage of protein relative to control sample used as internal reference. Number in parentheses indicates number of goats studied.

*$P<0.0003$ vs control atria.

†$P<0.003$ vs control atria.

strated that GRP94 is highly expressed in the heart conduction system,[14] we restricted our quantitative analysis to regions such as atrial appendages and left ventricular free wall, where working myocardium predominates. GRP94 amount did not differ significantly between atrial and ventricular samples of control goats (Table 1). Analysis was extended to left and right goat atrial appendages obtained at 2, 4, 8, and 16 weeks after onset of AF. No significant increase in GRP94 amount was observed in 2-week-AF samples, whereas a 2- to 3-fold increase was detected after 4, 8, and 16 weeks of AF (Figure 1B; Table 1). Comparable results were obtained when GRP94 content in control and 4- and 16-week AF was normalized for desmin (not shown). Conversely, samples obtained from goat hearts that were in normal sinus rhythm for 8 weeks after 16 weeks of AF showed normal GRP94 levels (Figure 1C; Table 1).

GRP94 is expressed in every heart cell.[14] Immunohistochemistry revealed that the GRP94 increase detected in 4-, 8-, and 16-week-AF samples was due to the stronger GRP94 immunoreactivity within cardiomyocytes (Figure 2). Conversely, reactivity of cardioverted myocytes was similar to that of controls (Figure 2D). Confocal microscopy (Figure 3) showed that the increase in GRP94 immunoreactivity was characterized by the presence of a diffuse dotted staining, which almost masked the striped pattern at the Z-line level detectable in control cardiomyocytes.[14]

GRP94 Increase, Myocyte Damage, and Dedifferentiation

Irreversible myocyte damage can determine increased GRP94 expression. We previously showed that myocyte death due to intracellular calcium overload or apoptosis is accompanied by troponin T proteolysis due to calpain and subsequent cross-linking to myofibrillar proteins by transglutaminase.[17,20] Consequently, Western blot displays high-molecular-weight polypeptides that are immunoreactive with anti-troponin T antibodies corresponding to cross-linked troponin T. In vitro experiments, performed by exposing control goat atrium

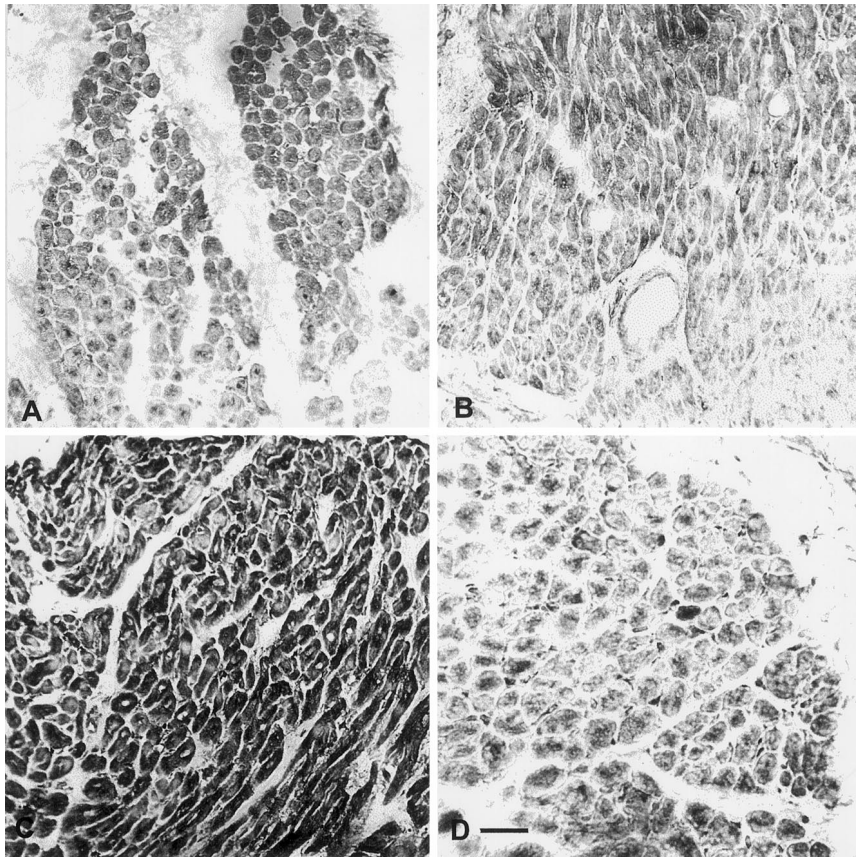

Figure 2. GRP94 immunoreactivity in fibrillating goat atria. Indirect immunoperoxidase with anti-GRP94 was performed on cryosection from left atrium of control (A), 2-week AF (B), 16-week AF (C), and 16-week AF plus 8-week cardioversion (D). Note that in C, myocytes display stronger staining intensity than myocytes of control, 2-week AF, and cardioverted atria samples. Bar=50 μm.

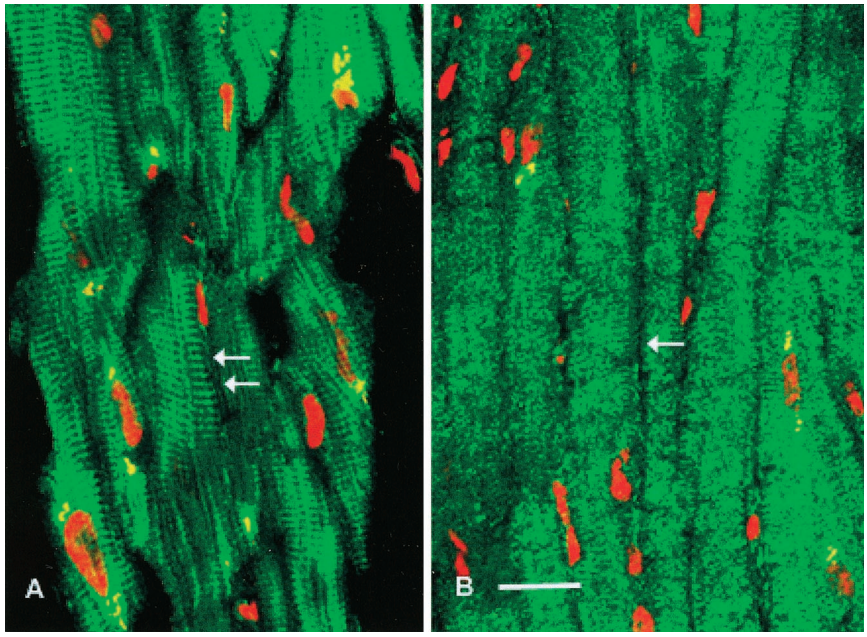

Figure 3. Confocal analysis of GRP94 immunoreactivity. Cryosections from left atrium of control (A) and 16-week-AF goats (B) were stained in indirect immunofluorescence with anti-GRP94 (green). Nuclei (red) were labeled with propidium iodide. In A, immunolabeling is distributed with Z-line periodicity (arrows), consistent with corbular SR localization,[14] whereas in B, additional dotted immunoreactivity, attributable to rough ER expansion,[3] is detectable. Yellow fluorescence corresponds to lipofuscin deposits. Bar=20 μm.

homogenates to millimolar amounts of calcium, showed the appearance of 66- and 80-kDa molecular weight polypeptides that were reactive with anti-troponin T antibodies (Figure 4A). Coincubation with calpeptin, a calpain inhibitor, confirmed the specificity of the process (not shown). Fibrillating goat atrial samples were negative for the presence of high-molecular-weight, troponin T–immunoreactive polypeptides, indicating the absence of irreversible damage (Figure 4B).

Reexpression of fetal cardiac proteins appears to be a feature of fibrillating atrial myocytes. GRP94 expression in goat newborn atrial and ventricular myocardium is significantly higher than in the adult myocardium (Figure 5 and Table 1) and comparable to the levels detected in fibrillating atria. However, when we analyzed calreticulin expression, we found that it was present in newborn samples, whereas it was barely detectable in both adult control and fibrillating samples (Figure 5).

We then investigated whether the expression of another stress protein, inducible HSP70, changes during AF. The amount of inducible HSP70 increases significantly in fibrillating atria and returns to control levels in cardioverted atria (Figure 5 and Table 2). However, the amount of inducible HSP70 in the newborn heart did not differ significantly from that observed in the adult heart (Figure 5 and Table 2).

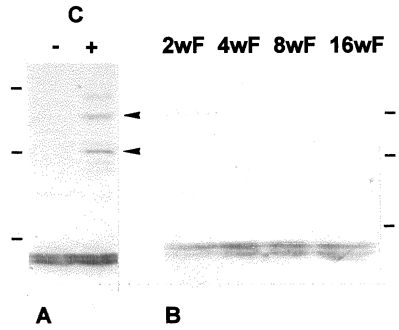

Figure 4. Absence of troponin T cross-linking in fibrillating goat atria. Both control (C, panel A) and 2-, 4-, 8-, and 16-week fibrillating (wF) goat left atrial samples (panel B) were analyzed by 6% to 12% gradient gel electrophoresis and Western blot with anti-troponin T antibody BN-59. In A, cryosections from control atria were incubated in presence (+) or absence (−) of 10 mmol/L $CaCl_2$. Two immunoreactive bands of apparent molecular weight ratio (M_r) of 42 000 to 43 000, corresponding to cardiac troponin T isoforms, were detected in each sample of both panels. Additional immunoreactive bands corresponding to higher M_r polypeptides (≈66 and 80 kDa) were observed only in control sample exposed to calcium. Migration of protein M_r standard (97 400; 66 200; 45 000) is indicated.

GRP94 and HSP70 Expression in Human Fibrillating Atria

We extended the analysis for GRP94 and inducible HSP70 expression to samples obtained from patients suffering chronic AF to ascertain whether this condition also affected the expression of these proteins in the human heart. GRP94 level was significantly increased in fibrillating atria with

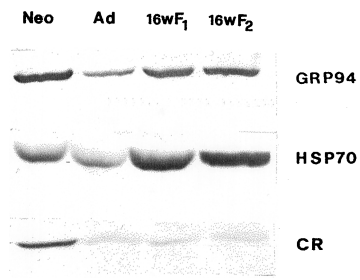

Figure 5. GRP94, HSP70, and calreticulin expression in developing, adult, and fibrillating goat atria. Western blot was performed on whole homogenates from neonate (Neo), control adult (Ad), and two 16-week-AF samples (16wF) with anti-GRP94, anti-inducible HSP70, and anti-calreticulin (CR) antibodies. Calreticulin immunoreactivity is detectable only in neonatal sample; GRP94 immunoreactivity is increased in neonatal and fibrillating atrial samples; inducible HSP70 labeling is similar in both neonatal and adult samples and is increased in fibrillating atria.

TABLE 2. Percentage of Inducible HSP70 Levels in Goat and Human Fibrillating Atria

	Goat Heart Samples	
	Left Atrium	Left Ventricle
Perinatal	112.8±35.4 (7)	163.4±24.2 (5)
Adult control	106.7±12.4 (5)	167.3±17.3 (3)*
16-wk AF	189.9±28.9 (6)*	
16-wk AF+8-wk SR	76.3±12.6 (6)	
	Human Left Atrium Samples	
Donors	114.7±13.8 (4)	
AF	75.9±6.5 (8)*	

SR indicates sinus rhythm. Values are as described in Table 1.
*$P<0.03$ vs adult control atria.

respect to controls (Figure 6 and Table 1), whereas the amount of inducible HSP70 appeared reduced (Figure 6 and Table 2).

Discussion

The present study shows that myocytes from chronic fibrillating atria display approximately a 2-fold increase in GRP94, which returns to the control level after cardioversion. The increase in GRP94 that occurs within cardiomyocytes was observed in an experimental model of AF in the goat and in human samples obtained from patients with chronic AF. The consistent findings outline the relevance of obtaining and characterizing an experimental model of sustained yet electrically reversible atrial fibrillation.

GRP94 level increases significantly in goat samples only after ≥4 weeks of AF, and this occurs in the absence of extensive irreversible myocyte damage, indicating that the upregulation of the GRP94 gene is not part of a generic stress response. At present, we cannot exclude that synthesis of stress proteins may occur very early after the initiation of AF by burst pacing; nevertheless, the amount of GRP94 after 2 weeks of AF is not different from the value observed in control atria. Furthermore, we could rule out death of fibrillating myocytes as an additional stimulus for a stress response; consistent with previous observations that did not detect degenerative changes in fibrillating myocytes,[2] we obtained no evidence for troponin T cross-linking, which has been characterized as an early marker for irreversible myocyte damage due to intracellular calcium overload.[17,20]

Dedifferentiation of fibrillating myocytes, namely, the switch to a fetal-like phenotype,[3] is a possible explanation for GRP94 increase in AF. Indeed, newborn goat atrial and ventricular myocardium displays higher GRP94 levels than the adult heart, as do fibrillating atrial samples. However, a similar upregulation does not occur for calreticulin, another ER calcium-binding protein that is highly expressed in developing cardiomyocytes.[21] Such a finding not only indicates that the dedifferentiation process occurs only partially in fibrillating atrial myocytes but reveals GRP94 as the only SR calcium-binding protein that is increased during chronic AF, because no change was reported for calsequestrin.[7] Mechanisms responsible for the GRP94 increase in fibrillating atria remain speculative. Perturbations of ER/SR are followed by increased synthesis of GRP94,[9,10] and SR disorganization has been described within myolytic areas of fibrillating goat atrial myocytes[2]; interestingly, a significant increase of myolysis occurred from 4 weeks of AF onward.[6] Using confocal microscopy, we now demonstrate that the distribution of GRP94 within the SR/ER is greatly modified in the presence of chronic AF, in conjunction with the appearance of SR disorganization.

In this context, the increased accumulation of GRP94 might have important consequences: it may prevent SR protein aggregation through its chaperoning function[9,10,22] and/or may cooperate to restore calcium homeostasis through its calcium binding sites. Fibrillation is accompanied by intracellular calcium overload,[23] and consistent ultrastructural changes have been described.[5,6] Interestingly, sarcolemmal and mitochondrial calcium deposits, which increased in 1- to 2-week-AF goat atria, slowly returned to control levels in 4- to 8-week-AF samples,[6] ie, when the GRP94 amount increased. We presently do not know whether a comparable time course in the recovery of calcium deposits also occurs in human fibrillating atria. Although calcium-blocker treatment may variably affect atrial electrical remodeling,[24,25] we report significantly increased GRP94 levels irrespective of the type of therapy. Thus, we speculate that GRP94 may improve survival of fibrillating atrial myocytes due both to chaperoning and calcium-binding properties. To date, GRP94 has been implied in the maintenance of cell viability after exposure to ER calcium depletion or other ER stresses.[12] Goat fibrillating atrial myocytes, but not human fibrillating samples, also display significantly higher levels of inducible HSP70, a cytoplasm stress protein that improves myocyte survival after ischemic stress[26,27]; nevertheless, it has been shown that cell viability is maintained even in the absence of HSP70 upregulation if ER stress proteins are increased.[28] Although the present study does not provide evidence for the functional consequence of GRP94 increase in AF, preliminary observations reveal that muscle cell lines overexpressing GRP94[19] show increased survival with respect to cells with normal levels of GRP94 when exposed to intracellular calcium overload (M. Vitadello and L. Gorza, unpublished data, 2000).

Taken together, our findings suggest that sustained AF induces the appearance of a protective phenotype in myocytes. The increase in GRP94 expression that occurs in both experimental and human chronic AF and the return to control levels after cardioversion in the goat may be part of a cell protective program that can be switched on and off. Such a

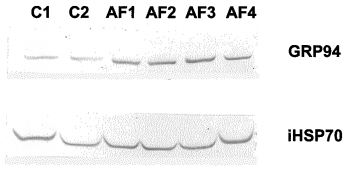

Figure 6. GPR94 and inducible HSP70 expression in control and fibrillating human left appendages. Western blots reacted with anti-GRP94 and anti-iHSP70 antibodies. Lanes were loaded with equal amounts (40 μg) of whole homogenates of samples obtained from hearts of 2 different donors (C) and 4 different patients with AF.

hypothesis is not in contrast with the dedifferentiated phenotype proposed thus far: embryonic and neonatal hearts not only display higher resistance to ischemic injury with respect to adult hearts[29] but are also more calcium tolerant.[30]

Acknowledgments

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