Rabbit cardiac and skeletal myocytes differ in constitutive and inducible expression of the glucose-regulated protein GRP94

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The glucose-regulated protein GRP94 is a stress-inducible glycoprotein that is known to be constitutively and ubiquitously expressed in the endoplasmic reticulum of mammalian cells. From a rabbit heart cDNA library we isolated four overlapping clones coding for the rabbit homologue of GRP94 mRNA. Northern blot analysis shows that a 3200 nt mRNA species corresponding to GRP94 mRNA is detectable in several tissues and it is 5-fold more abundant in the heart than in the skeletal muscle. Hybridization analysis *in situ* shows that GRP94 mRNA accumulates in cardiac myocytes, whereas in skeletal muscles it is not detectable in myofibres. A monoclonal antibody raised by using a 35 kDa recombinant GRP94 polypeptide as immunogen detects a single reactive polypeptide of 94 kDa in a Western blot of liver and heart homogenates and does not react with skeletal muscle homogenates. Conversely, GRP94 mRNA and protein are detectable in both cardiac and skeletal muscle myocytes of fetal and neonatal rabbits. After 24 h of endotoxin administration to adult rabbits, GRP94 mRNA accumulation increases 3-fold in both heart and skeletal muscle and it is followed by a comparable increase in protein accumulation. However, hybridization and immunohistochemistry *in situ* do not reveal any change in the expression of GRP94 mRNA and protein in skeletal muscle myocytes after endotoxin treatment. Thus skeletal muscle fibres display a unique regulation of the GRP94 gene, which is up-regulated during perinatal development, whereas in the adult animal it is apparently silent and not responsive to endotoxin treatment.

INTRODUCTION

Mammalian cells respond to particular metabolic insults by increasing the synthesis of a small number of polypeptides (heat shock or stress proteins) with a concomitant decrease in production of the normal array of cellular proteins [1]. For example, in situations where extracellular glucose is low in concentration or absent, intracellular Ca^{2+} stores are depleted or glycosylation is inhibited, the increased synthesis of a class of proteins referred to as glucose-regulated proteins (GRPs) occurs [2]. This class of proteins comprises several members (GRP58, GRP78, GRP94 and GRP170) [2–4], which are localized in the endoplasmic reticulum (ER). The most studied member of this group, GRP78 or BiP, is expressed constitutively and is involved in protein translocation into the ER and in their subsequent folding and assembly [5]. GRP94 has other important roles in the ER related to nuclear signalling, protein folding, sorting and secretion [6]; in addition it exerts a specific protection against Ca^{2+} depletion stress [7] and is involved in antigen presentation [8]. Furthermore GRP94 has a low affinity for Ca^{2+} ions but a high capacity to bind them [9] and has been described in the sarcoplasmic reticulum (SR) vesicles of cardiac and skeletal muscles [10].

The potential involvement in Ca^{2+} storage of GRP94 prompted us to investigate in more detail the expression of this protein in striated muscle. From a rabbit heart cDNA library we isolated several incomplete clones specific for rabbit GRP94 mRNA and used a GRP94 recombinant polypeptide to raise specific monoclonal antibodies (mAbs). In the present paper we demonstrate that GRP94 expression differs between cardiac and skeletal muscles, not only quantitatively but also qualitatively. Whereas GRP94 mRNA and protein are expressed constitutively in cardiac myocytes and accumulate after exposure *in vivo* to bacterial lipopolysaccharide (LPS), GRP94 expression in adult skeletal muscle seems to be restricted to interstitial cells and such a distribution is not changed by exposure to LPS. Nevertheless GRP94 is detectable in the skeletal muscle myocytes of fetal and newborn rabbits, suggesting a role for this protein in the control of Ca^{2+} homoeostasis of the skeletal muscle cell before the attainment of a mature ER organization.

MATERIALS AND METHODS

Tissue source and experimental protocols

Rabbit embryos (15 days old), 4-, 7- and 10-day-old and adult (2.5 kg) New Zealand male rabbits were used for this study.

Because there is little information on rabbit responses to LPS, preliminary experiments were undertaken in three adult rabbits to assess the proper means of administration and the dose that would raise body temperature over 40 °C but be non-lethal. Then a single intramuscular injection of LPS from *Salmonella typhimurium* (4 mg/kg; Sigma) was performed in six adult animals; control animals ($n = 3$) were injected with saline. At 6 h after LPS injection, rabbits were checked for hyperthermia and killed 24 or 48 h after the injection.

Samples from central nervous system (brain, cerebellum and medulla), liver, kidney, hindlimb muscles and diaphragm, aorta, bladder and heart [i.e. the atrial and the ventricular myocardium, the sino-atrial node region, the atrio-ventricular (AV) node and AV bundle regions] were obtained from adult control rabbits. Samples of cardiac and skeletal muscles were collected from three 4-day-old, two 7-day-old and two 10-day-old rabbits.

Abbreviations used: AV, atrio-ventricular; ER, endoplasmic reticulum; GRP, glucose-regulated protein; LPS, bacterial lipopolysaccharide; mAb, monoclonal antibody; SR, sarcoplasmic reticulum; SSPE, saline sodium phosphate EDTA (0.18 M NaCl/0.01 M sodium phosphate/1 mM EDTA); TBS, Tris-buffered saline.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF001631.

From LPS-treated animals only samples from liver, skeletal muscles and heart were collected. All specimens were collected far from the injection site, frozen immediately in liquid nitrogen and stored at −80 °C until use.

cDNA isolation and probe preparation

A rabbit heart cDNA library was prepared from poly(A)$^+$ RNA obtained from the AV node region and AV septum of 120 adult rabbit hearts, with the λ Unizap phage as a vector (Stratagene) and following the procedures described by Vitadello et al. [11]. Four incomplete and overlapping GRP94 cDNA clones were isolated. Clone 2.5 was first isolated by expression screening, performed as described previously [11], with the mAb 1H2 (M. Vitadello and L. Gorza, unpublished work). In brief, clone 2.5 was isolated to purity and the cDNA insert was rescued from Unizap lambda phage together with the pBluescript phagemid by using co-infection of *Escherichia coli* strain SolR with ExAssist helper phage (Stratagene). Sequence analysis was performed on both strands with the dideoxy chain termination method [12] by using modified T7 DNA polymerase (Pharmacia) on the isolated clone and on subclones obtained after restriction endonuclease digestion with the aid of sequence-specific oligonucleotides as primers. The 750 bp insert present in clone 2.5 was then utilized for further screening; 2×10^5 plaque-forming units of the same cDNA library were transferred to 137 mm diameter nylon filters (Hybond N; Amersham) and hybridized overnight at 65 °C in the presence of 5 × SSPE (where SSPE is 0.18 M NaCl/0.01 M sodium phosphate/1 mM EDTA)/5 × Denhardt's/0.5 % SDS/sonicated herring sperm DNA with 5×10^5 c.p.m./ml clone 2.5 insert, labelled by random priming with [α-^{32}P]dCTP (3000 mCi/mmol). High-stringency washes were performed at 65 °C with 0.1 × SSPE. Filters were exposed to X-ray film for between 6 h and 2 days. Clones 2.52 and 2.53 (2305 bp) and 2.54 (600 bp) were isolated to purity and sequenced on both strands as described above (Figure 1, upper panel).

Northern blotting

Total RNA was isolated by following the procedure described by Chomczynski and Sacchi [13]. Equal amounts (approx. 10 μg) of each sample, as determined by ethidium bromide staining of formaldehyde/agarose gels, were transferred to nylon filters (Hybond N$^+$; Amersham) by capillary elution with 20 × SSPE. Blotted RNA was fixed by following the manufacturer's instructions and hybridized overnight at 61 °C in the presence of 50 % formamide, 5 × SSPE, 5 × Denhardt's, 0.5 % SDS and 5×10^5 c.p.m./ml anti-sense cRNA, transcribed *in vitro* in the presence of [α-^{32}P]UTP (800 mCi/mmol) from clones 2.5 and 2.53, with linearized templates for probe transcription as described for hybridization *in situ*. High-stringency washes were performed at 65 °C with 0.1 × SSPE. Filters were exposed to X-ray film for 6 days; they were then stripped with boiling 0.1 % SDS and hybridized in some cases with the other GRP94 probe and either with an 18 S RNA-labelled probe of 80 nt or with anti-sense mouse β-actin RNA probe of 245 nt (Ambion) for normalization. Hybridization with 18 S or mouse β-actin RNA probes was performed overnight at 55 °C under the conditions described above. Filters were exposed from 30 min to 6 h after hybridization with 18 S RNA and overnight after hybridization with anti-sense mouse β-actin.

Hybridization *in situ*

Radiolabelled anti-sense and sense cRNA probes were transcribed from clone 2.5 after linearization with *Bam*HI or *Xho*I (Promega) respectively, with T7 or T3 RNA polymerase (Epicentre) and [^{35}S]UTP, as previously described [11]. Both probes corresponded to the length of the insert and for purposes of hybridization *in situ* they were digested to 50–100 nt by mild alkaline hydrolysis. Cryosections were fixed with formaldehyde and digested with 20 μg/ml proteinase K. Sections were hybridized overnight at 52 °C with 10 μl of probe at a concentration of 10^5 c.p.m./μl, and washed at 65 °C with 50 % formamide/0.3 M NaCl/0.03 M sodium citrate/0.1 M dithiothreitol. After dehydration, sections were dipped in autoradiographic emulsion (Kodak NTB-2) diluted 1:1 with water and exposed for 5–7 days at 4 °C. Slides were developed with D 19 (Kodak) for 3.5 min and fixed, then examined with a Zeiss Axioplan microscope equipped with dark-field optics. Serial sections were processed for immunohistochemical staining.

Preparation and characterization of mAbs

The insert of clone 2.5 was gel-purified after restriction nuclease digestion with *Eco*RI and *Pst*I (Promega) and subcloned in the bacterial expression vector pQE-30 (Qiagen), which carries at the 5′ end a nucleotide sequence coding for six histidine residues. Expression of the recombinant polypeptide was induced in transformed *E. coli* strain M15 in the presence of 2 mM isopropyl β-D-thiogalactoside. SDS/PAGE was used to identify colonies positive for the presence of the recombinant protein, which displayed an apparent molecular mass of 35 kDa. Purification of the recombinant GRP94 polypeptide was achieved with a Ni$^+$-Sepharose column (Qiagen) in the presence of 8 M urea and 10 mM 2-mercaptoethanol. Protein bound to the column was eluted with 250 mM imidazole, checked for purity by PAGE and dissolved in 10 mM Tris/HCl, pH 8.00, by stepwise dialysis. Balb/c mice were injected intramuscularly with 30 μg of the recombinant protein in an emulsion with Freund's adjuvant. After receiving a boost injection, mice were killed 1 month later and spleen cells were fused with NS0 myeloma cells by following the procedures described by Vitadello et al. [14]. Screening of positive hybridomas was performed by testing 50 μl of culture medium in an ELISA after coating each well with 100 ng of recombinant polypeptide. Bound antibody was revealed with anti-mouse immunoglobulins conjugated with peroxidase (Dakopatts) and *o*-dianisidine as substrate. Positive hybridomas were cloned twice by limiting dilution. Ascites cells were obtained from pristane (2,6,10,14-tetramethylpentadecane; Sigma)-primed mice injected intraperitoneally with 10^6 positive hybridoma cells.

Western blotting

Tissue samples were homogenized in electrophoresis sample buffer, heated for 5 min in boiling water and centrifuged (15000 ***g***) for 15 min at 4 °C, as described previously [15]. Samples were run in a 10 % (w/v) polyacrylamide gel together with commercially available preparations of molecular mass standards (Bio-Rad, Richmond, CA, U.S.A.) at constant current (5 mA) overnight. Comparable amounts of each sample, as determined by densitometric analysis of the actin band in Coomassie Blue-stained gels, were transferred to nitrocellulose paper at 400 mA for 2 h. Paper was saturated with 1 % (w/v) ovalbumin in TBS [10 mM Tris/HCl (pH 8.0)/150 mM NaCl/0.05 % (v/v) Tween 20] for 1 h at room temperature and subsequently incubated with adequate dilution of ascites fluid of the anti-GRP94 mAb 3C4 (1:1000 dilution). For quantitative analysis, different amounts of each sample were loaded in the gel and, after being blotted, the region of the nitrocellulose sheet corresponding to the mobility

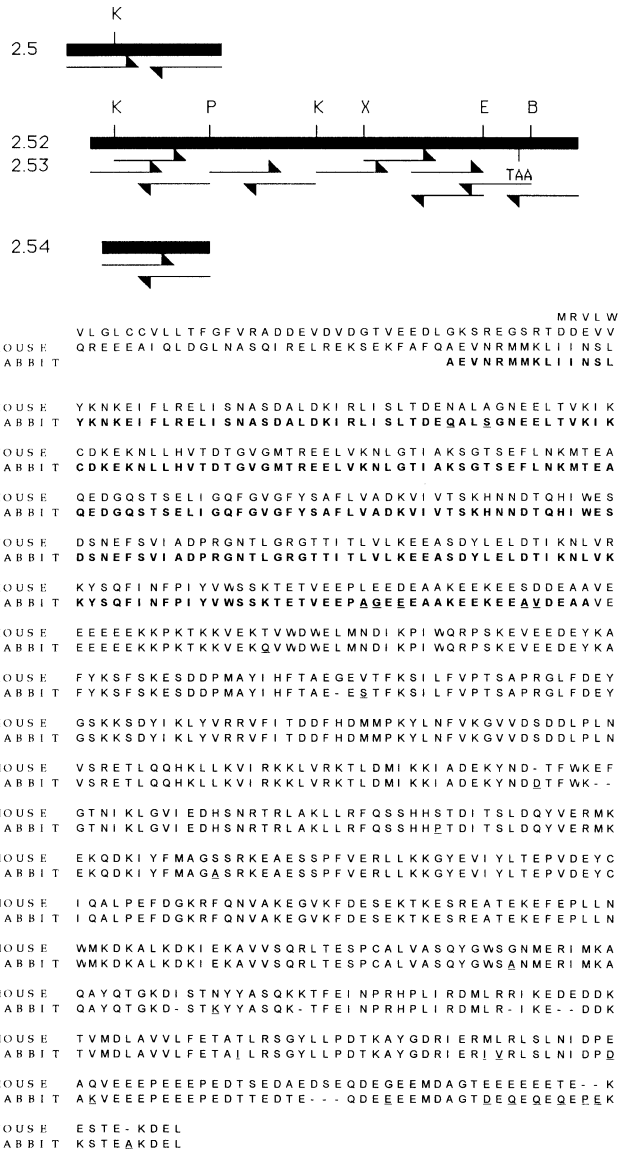

Figure 1 DNA sequencing strategy of rabbit GRP94 cDNA clones (upper panel) and deduced amino acid sequence (lower panel)

Upper panel: arrows indicate the directions of the different sequencing reactions. The translation stop codon is labelled. Only the restriction sites used for subcloning are shown. B, *Bam*HI; E, *Eco*RV; K, *Kpn*I; P, *Pst*I; X, *Xba*I. Lower panel: comparison of rabbit GRP94 with murine GRP94 [17]. The sequences have been aligned for maximum similarity. Non-conserved amino acid are underlined. Bold letters indicate the region of the cDNA chosen for the preparation of GRP94 recombinant polypeptide.

of polypeptides below 66 kDa was incubated with anti-desmin mAb (1:320 dilution; Boehringer Mannheim, Germany). Unbound antibody was removed after extensive rinses with TBS, and filters were incubated with secondary anti-mouse immunoglobulin conjugated with peroxidase. Peroxidase activity was revealed with the use of diaminobenzidine, as described previously [15].

Samples from heart and skeletal muscle were homogenized (10% wet w/v) in buffer containing 80 mM potassium acetate, 25 mM K/Mes, pH 5.5, with 1 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 2 μg/ml aprotinin as protease inhibitors. After 10 min of centrifugation at 12000 ***g***, 10 μl of homogenate was incubated with 5 μ-units of endoglycosidase H (EC 3.2.1.96; Boehringer Mannheim) for 60 min at 37 °C. The reaction was stopped with boiling electrophoresis sample buffer. Polypeptides were separated by SDS/PAGE [7.5% (w/v) gel], transferred to nitrocellulose paper and incubated with mAb 3C4, as described above.

In another set of experiments, polypeptides from liver, heart and skeletal muscle samples were separated by SDS/PAGE and blotted to nitrocellulose paper. Strips were incubated with calf alkaline phosphatase (EC 3.1.3.1 type VII; Sigma) by following the procedures described previously [16] and then decorated with mAb 3C4. Dephosphorylation of neurofilament proteins, followed by decoration with the anti-neurofilament mAb clone 1G2 directed against a phosphorylated epitope [16], was used as a control.

Immunohistochemistry

Cryosections (10 μm thick) were cut and stained on the same day. Sections were fixed for 10 min at room temperature with 4% (w/v) freshly prepared buffered paraformaldehyde, rinsed twice for 15 min with PBS [10 mM sodium phosphate/150 mM NaCl (pH 7.4)] and incubated with 3C4 mAb diluted 1:500 with 0.5% in PBS. Incubation was performed in a humidified chamber at room temperature for 30 min. After three 10 min rinses in PBS, sections were incubated with appropriate dilutions of secondary antibodies coupled with peroxidase, with the use of diaminobenzidine as a substrate [11]. For double immunofluorescence staining, labelling with 3C4 was performed by incubation with rhodamine-conjugated anti-mouse immunoglobulins (1:40 dilution; Dakopatts), followed either by staining with polyclonal anti-desmin antibodies (1:20 dilution; Sigma) and anti-rabbit immunoglobulins conjugated with fluorescein (1:30 dilution; Cappel) or by incubation with an anti-(myosin heavy chain) mAb conjugated directly with fluorescein, as previously described [15]. After immunofluorescence staining, to label nuclei, sections were mounted with glycerol buffered with PBS and containing 2 μg/ml 4,6-diamino-2-phenylindole (Sigma).

Statistical analysis

Quantitative densitometry was performed on Northern and Western blots of samples obtained from newborn, adult and LPS-treated rabbits. Autoradiographic bands were analysed with a Shimadzu dual-wavelength chromatoscanner CS-930 at a wavelength of 600 nm; immunoreactivity on Western blots was analysed at 530 nm. Densitometric profiles were cut from paper and weighed. To compensate for differences in probe-specific activity between experiments, a cardiac or a skeletal muscle RNA sample from an adult untreated rabbit was used as an internal reference. Sample values were normalized to the corresponding amount in either 18 S or β-actin RNA. For Western blot experiments, the GRP94 immunoreactivity of each sample was normalized to the immunoreactivity of desmin, and variability between different experiments was compensated for with a sample from adult rabbit ventricle in each experiment as an internal reference.

Statistical analysis was performed with the unpaired Student *t* test.

RESULTS

Analysis of rabbit GRP94 cDNA clones

Four overlapping cDNA clones (2.5, 2.52, 2.53 and 2.54) were isolated from the library screening. A 2385 nt sequence obtained

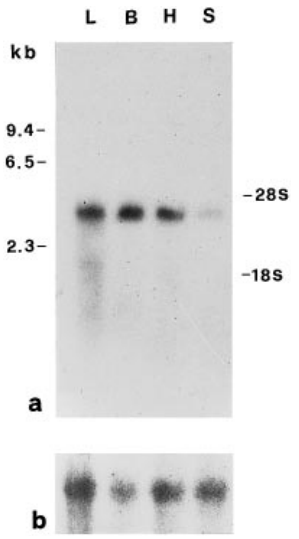

Figure 2 Northern blot analysis of rabbit GRP94 mRNA

Rabbit total RNA was hybridized with GRP94 2.5 anti-sense cRNA probe (**a**) and with 18 S probe (**b**). Lanes were loaded with 10 μg of liver (L), brain (B), heart (H) and skeletal muscle (S) total RNA. The migrations of λ *Hin*dIII DNA markers (Pharmacia) and 28 S and 18 S RNA are indicated at the left and the right respectively.

after the sequencing of both strands is available from GenBank, and the deduced amino acid sequence is illustrated in Figure 1 (lower panel). Sequence comparison shows that our cDNA clones code for the greater part of the coding sequence and the 3′ untranslated region of the rabbit homologue of GRP94 mRNA and display an average 83% identity to mouse Erp99 mRNA [17] and 89.4% to human Gp96 mRNA [18]; the identity increases to 90% when the deduced amino acid sequences of mouse and rabbit GRP94 are compared [17]. As indicated in Figure 1 (lower panel), regions of higher diversity are detectable close to the C-terminus, which nevertheless maintains the amino acid sequence KDEL, the ER retention signal [19].

Expression of GRP94 mRNA and protein in cardiac and skeletal muscles

Northern blot analyses performed on total RNA from different rabbit tissues show that both probes derived from clone 2.5 and clone 2.53 hybridized with a single mRNA species of approx. 3200 nt (Figure 2). This mRNA is easily detected in liver, heart, brain (Figure 2), kidney and aorta (results not shown), whereas the level in skeletal muscle is one-fifth of that in the heart (Figure 2 and Table 1). Hybridization analysis *in situ* of cardiac and skeletal muscles shows that such a difference is related to the distribution of the transcript. In addition to interstitial cells, cardiomyocytes are positive for the accumulation of GRP94 mRNA (Figure 3a, label H), which seems to be more concentrated in myocytes of the heart conduction system such as the AV bundle (results not shown). In constrast, hybridization signals in skeletal muscle fibres apparently do not differ from the background (Figures 3a and 3b, label S), whereas weak hybridization signals are detectable only in the interstitial space, presumably due to the accumulation of the transcript in fibroblasts (Figure 3a, arrowhead).

Studies performed at the protein level confirmed the difference in GRP94 expression observed at the mRNA level between cardiac and skeletal muscle myocytes. Specific mAbs were raised by using a recombinant GRP94 polypeptide, which corresponds to the insert of clone 2.5 and to the protein region shown in bold letters in Figure 1 (lower panel); mAb 3C4 was selected for this study. Immunohistochemistry shows that, in the heart, staining is detectable in both fibroblasts and cardiomyocytes and it is strikingly evident in Purkinje myocytes of the heart conduction system (Figure 3c, label P and arrows). Immunofluorescence analysis of longitudinal sections shows that in cardiac myocytes GRP94 immunoreactivity displays a sarcomeric pattern of distribution (Figures 3d to 3e), which, by comparison with anti-myosin (Figure 3f) or anti-desmin staining (results not shown), corresponds to the Z-line region, namely the region enriched in junctional SR in the heart. Conversely, in skeletal muscle, GRP94 immunoreactivity is not detectable in myocytes (Figures 3c, 3g and 3h), whereas it is detectable in all the other non-myocyte cells or structures present there, such as endothelial and vascular muscle cells (Figure 3g), nerve axons (results not shown) and fibroblasts (Figure 3h).

Western blot analysis showed reactivity with a single polypeptide of molecular mass 94 kDa in whole homogenates from liver and from ventricular myocardium (Figure 4), as well as from atrial myocardium and from strands of ventricular Purkinje myocytes (Figure 5b). In contrast, only a faint reactivity was detectable with skeletal muscle homogenates (Figures 4 and 5b). Such a difference was indeed observed with four other anti-GRP94 mAbs (results not shown). To exclude the possibility that post-translational modifications such as phosphorylation and glycosylation affected epitope accessibility of GRP94 in skeletal muscle and thus masked GRP94 immunoreactivity, skeletal and cardiac muscle samples were treated with alkaline phosphatase or with endoglycosidase H before immunostaining. Figure 4 shows that neither dephosphorylation (Figure 4a) nor deglycosylation (Figure 4b) changed the pattern of mAb 3C4 immunoreactivity in skeletal muscle samples.

At variance with adult samples, hybridization analysis *in situ* of fetal rabbit embryos revealed intense hybridization signals for GRP94 mRNA in both cardiac and skeletal muscle cells (Figures 6a and 6d). Immunohistochemical analysis of serial sections with 3C4 mAb showed positive staining for GRP94 in both cardiac and skeletal muscle myocytes (Figures 6c and 6f), which were diffusely labelled and occasionally displayed a striated pattern of immunoreactivity (double arrows in Figures 6c and 6f). Immunohistochemistry of newborn rabbit skeletal muscle showed variable intensity of staining for GRP94 among skeletal muscle myocytes (Figure 6g), which was characterized by a discrete punctate pattern of labelling in transverse section (Figure 6h), suggesting that a decrease in GRP94 expression in skeletal muscle cells takes place during the first weeks after birth. Quantification of GRP94 mRNA and protein in cardiac and skeletal muscles of newborn rabbits showed that in both tissues the levels of mRNA and protein were higher than in the adult animals (Table 1). Similar results to those obtained with 18 S RNA for normalization, and illustrated in Table 1, were achieved when a tissue-specific amount of β-actin mRNA was chosen as an internal reference (Figure 5a): the normalized concentration of GRP94 mRNA of 4-day-old rabbit heart was 1.42 ± 0.14 (mean $\pm$ S.E.M.; $n = 7$) in comparison with 0.92 ± 0.15 ($n = 4$) observed in the adult heart ($P < 0.05$); in 4-day-old rabbit skeletal muscle the normalized concentration of GRP94 mRNA was 2.41 ± 0.27 ($n = 6$) in comparison with 0.97 ± 0.07 ($n = 3$) in adult skeletal muscle samples ($P < 0.01$). In addition, protein levels were significantly higher in both cardiac and skeletal muscle samples of 4-day-old

Table 1 GRP94 mRNA and protein accumulation in cardiac and skeletal muscles during postnatal development and after LPS treatment

Densitometric values were normalized and expressed in arbitrary units (means ± S.E.M.). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ with respect to control adult values. Abbreviation: n.d., not detectable.

			LPS-treated	
Sample source and type	4-day-old rabbits	Adult rabbits	After 24 h	After 48 h
Heart				
mRNA	$1.63 \pm 0.08^{***}$ ($n = 3$)	0.96 ± 0.034 ($n = 4$)	$3.5 \pm 1.24^{*}$ ($n = 3$)	2.0 ± 0.79 ($n = 3$)
Protein	$8.96 \pm 2.60^{*}$ ($n = 3$)	1.00 ± 0.56 ($n = 3$)	$1.94 \pm 0.16^{**}$ ($n = 8$)	$2.26 \pm 0.16^{***}$ ($n = 5$)
Skeletal muscle				
mRNA	$0.50 \pm 0.11^{*}$ ($n = 3$)	0.21 ± 0.05 ($n = 4$)	$0.62 \pm 0.04^{***}$ ($n = 3$)	$0.60 \pm 0.04^{*}$ ($n = 3$)
Protein	1.83 ± 0.53 ($n = 3$)	n.d. ($n = 4$)	n.d. ($n = 3$)	n.d. ($n = 3$)

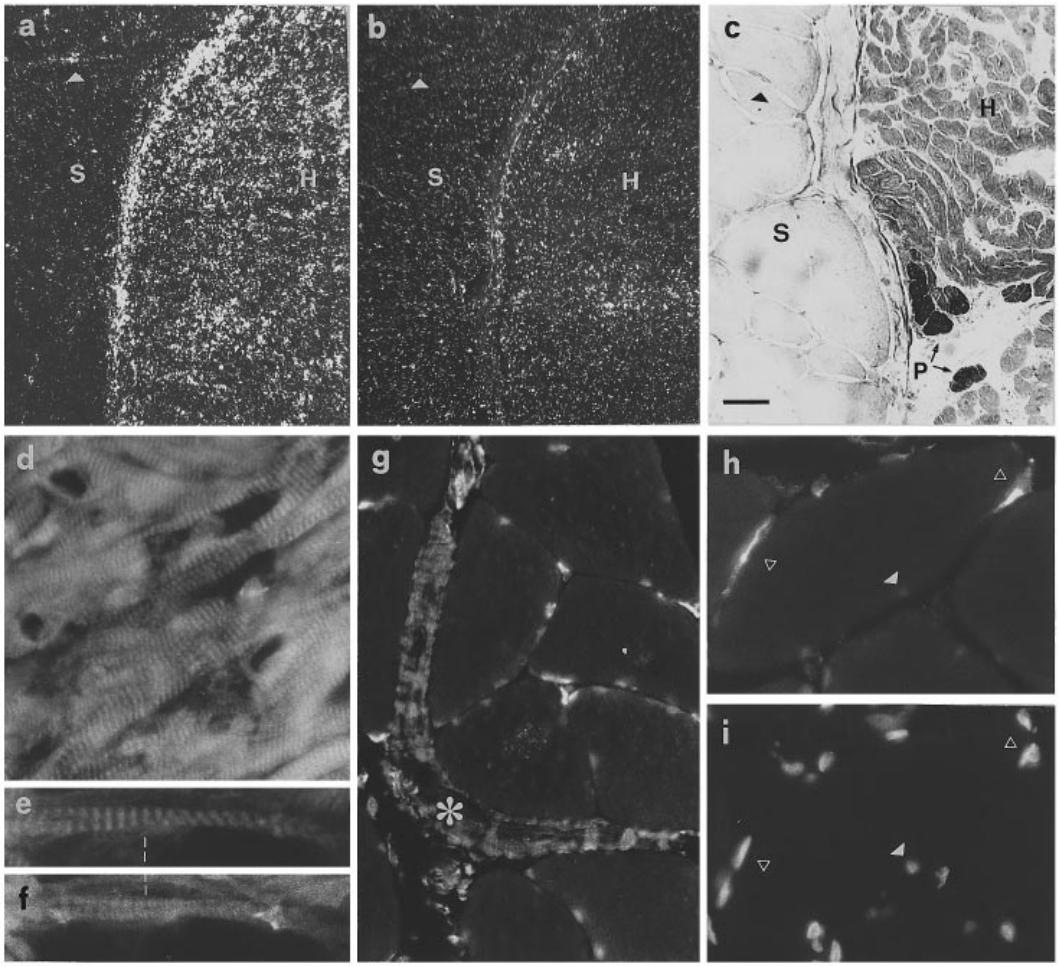

Figure 3 Hybridization and immunohistochemistry of GRP94 *in situ* in rabbit adult skeletal and cardiac muscles

(**a**–**c**) Serial cryosections of rabbit diaphragm (S) and ventricular myocardium (H), which were juxtaposed before freezing. (**a**,**b**) Hybridization with anti-sense GRP94 cRNA (clone 2.5) and the sense probe respectively. (**c**) A serial section stained with mAb 3C4 with indirect immunoperoxidase. Note weak hybridization signals with anti-sense GRP94 probe in skeletal muscle fibres, whereas strong signals are detected with cardiac myocytes and interstitial cells of skeletal muscle (arrowhead). Cardiac myocytes and Purkinje ventricular myocytes (P, arrows) show intense reactivity towards GRP94 in immunohistochemistry. (**d**–**f**) A longitudinal section, 6 μm thick, of adult rabbit myocardium doubly labelled in immunofluorescence for GRP94 (**d**,**e**) and myosin (**f**). GRP94 immunoreactivity in cardiac myocytes shows a striated pattern of distribution with Z-line periodicity. (**g**–**i**) A section of adult skeletal muscle doubly stained in immunofluorescence for GRP94 (**g**,**h**) and the nuclear stain 4,6-diamino-2-phenylindole (**i**). GRP94 immunoreactivity is detectable in endothelial and smooth muscle cells of vessels (**g**, asterisk) and in fibroblasts of the endomisium (**h**, **i**, open arrowheads), whereas nuclei of GRP94-negative cells correspond to skeletal myocyte nuclei (**h**,**i**, filled arrowhead). Scale bars: (**a**–**c**) 40 μm, (**d**,**h**,**i**) 12 μm, (**e**–**f**) 6 μm, (**g**) 20 μm.

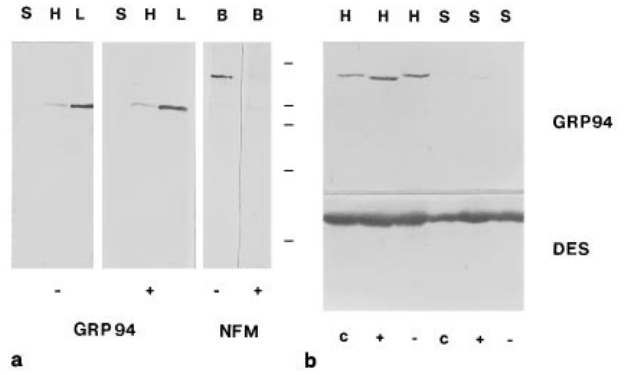

Figure 4 Specificity of anti-GRP94 mAb 3C4 and effects of dephosphorylation and endoglycosidase H treatment on GRP94 immunoreactivity

(**a**) Immunoreactivity for GRP94 on whole homogenates of skeletal muscle (lanes S), ventricular myocardium (lanes H) and liver (lanes L) in Western blots untreated (−) or treated (+) with alkaline phosphatase. A control experiment was performed on a Western blot of whole brain (lanes B) homogenates with an anti-neurofilament M antibody directed against a phosphorylated epitope [17]. Migration of high-molecular-mass standards (molecular masses 200, 116.25, 92.5, 66.2 and 45 kDa; Bio-Rad) is indicated on the right. Anti-GRP94 mAb 3C4 labels a polypeptide of apparent molecular mass of 94 kDa in heart and liver homogenates, whereas only a faint reactivity is observed in skeletal muscle homogenates. Note that alkaline phosphatase does not modify the immunoreactivity pattern of GRP94, whereas it abolishes the immunoreactivity of the anti-neurofilament M antibody. (**b**) Ventricular myocardium (lanes H) and skeletal muscle (lanes S) were incubated in the presence (+) or the absence (−) of endoglycosidase H, blotted together with whole homogenate preparations (c) and stained with anti-GRP94. The bottom of the blot was stained with anti-desmin antibodies (DES) to evaluate differences in loading. The sensitivity of GRP94 to endoglycosidase H is demonstrated by the increased mobility of the protein [19], which did not show differences in immunoreactivity.

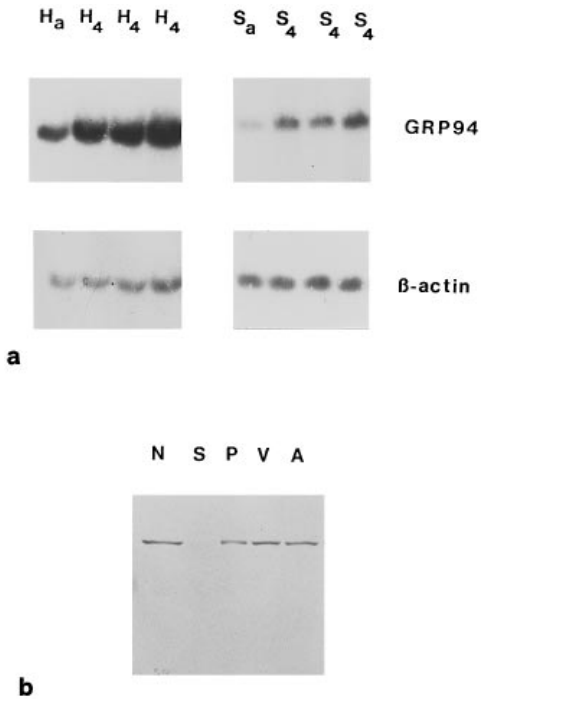

Figure 5 GRP94 expression in newborn skeletal muscle

(**a**) Northern blot analysis of total RNA prepared from ventricles (lanes H) and skeletal muscle (lanes S), from three 4-day-old rabbits, hybridized with GRP94 anti-sense cRNA probe 2.5. The same blots were stripped and hybridized with β-actin probe for normalization. H_a corresponds to a sample of adult ventricle and S_a to a sample of adult skeletal muscle. (**b**) Western blot of whole homogenates of 7-day-old skeletal muscle (lane N), adult skeletal muscle (lane S), strands of ventricular Purkinje myocytes (lane P), ventricular myocardium (lane V) and atrial myocardium (lane A) stained with 3C4 mAb. Note the strong immunoreactivity of GRP94 polypeptide in the neonatal skeletal muscle sample.

rabbits (Table 1); in skeletal muscle samples, GRP94 immunoreactivity could still be detected in Western blot homogenates of 7-day-old (Figure 5b) and 10-day-old rabbits (results not shown).

Effect of LPS treatment on GRP94 expression in cardiac and skeletal muscle

We therefore investigated whether GRP94 expression in skeletal muscle cells of the adult rabbit might be induced by conditions known to affect GRP94 synthesis. From the various experimental protocols used to up-regulate the GRP94 gene [2], we chose LPS, which was shown to increase GRP94 expression 10-fold in lymphocytes *in vitro* [20] and has the great advantage of being applicable *in vivo*. Our results show that GRP94 mRNA accumulation increased significantly in both cardiac and skeletal muscles 24 h after LPS administration and that such a difference was still significant in the skeletal muscle after 48 h (Figure 7 and Table 1). However, hybridization analysis *in situ* did not reveal any change in the distribution of the transcript in both cardiac and skeletal muscle between untreated and treated animals. Whereas both treated and untreated animals showed detectable signals within cardiac myocytes (results not shown), only background level signals were observed in skeletal muscle fibres. In contrast, interstitial cells showed evident hybridization signals, which seemed slightly more intense in treated than in untreated muscles (compare Figures 8a and 8d). The 2–3-fold increase in GRP94 mRNA was followed by a 2-fold increase in the protein, which it was possible to quantify only in cardiac samples (Table 1). Immunohistochemistry of samples obtained from LPS-treated animals confirmed the observations performed with hybridization *in situ*: the immunoreactivity was detectable within myocytes in the cardiac muscle (results not shown), whereas in the skeletal muscle it was localized only in the interstitial cells (Figures 8c and 8f).

DISCUSSION

In the present study we demonstrate that GRP94, a constitutive ER-resident protein, is differentially expressed between cells of rabbit striated muscles in a developmentally regulated manner and that such a difference is not changed after exposure to stress *in vivo*. These results were obtained through the isolation of incomplete cDNA clones that code for the homologues of GRP94 mRNA in the rabbit, whose sequence was previously unknown, and the use of a recombinant GRP94 polypeptide as an antigen for the production of mAb. The latter strategy was chosen to select antibodies with high affinity and to avoid possible cross-reactivity with heat shock protein 90, which shares approx. 50% identity with GRP94 [17].

In adult rabbit tissue, GRP94-specific probes recognize only a single mRNA species whose relative amount seems to occur in skeletal muscle samples at one-fifth the abundance of that in heart and liver samples. No evidence for other minor mRNA species was observed in our total RNA preparations, at variance with what has been reported for canine striated muscles [10]. The smaller amount of GRP94 mRNA in skeletal muscle is consistent with the observation of very small amounts of the protein, below the limit of detection. The difference in GRP94 immunoreactivity observed in Western blots between rabbit skeletal and cardiac

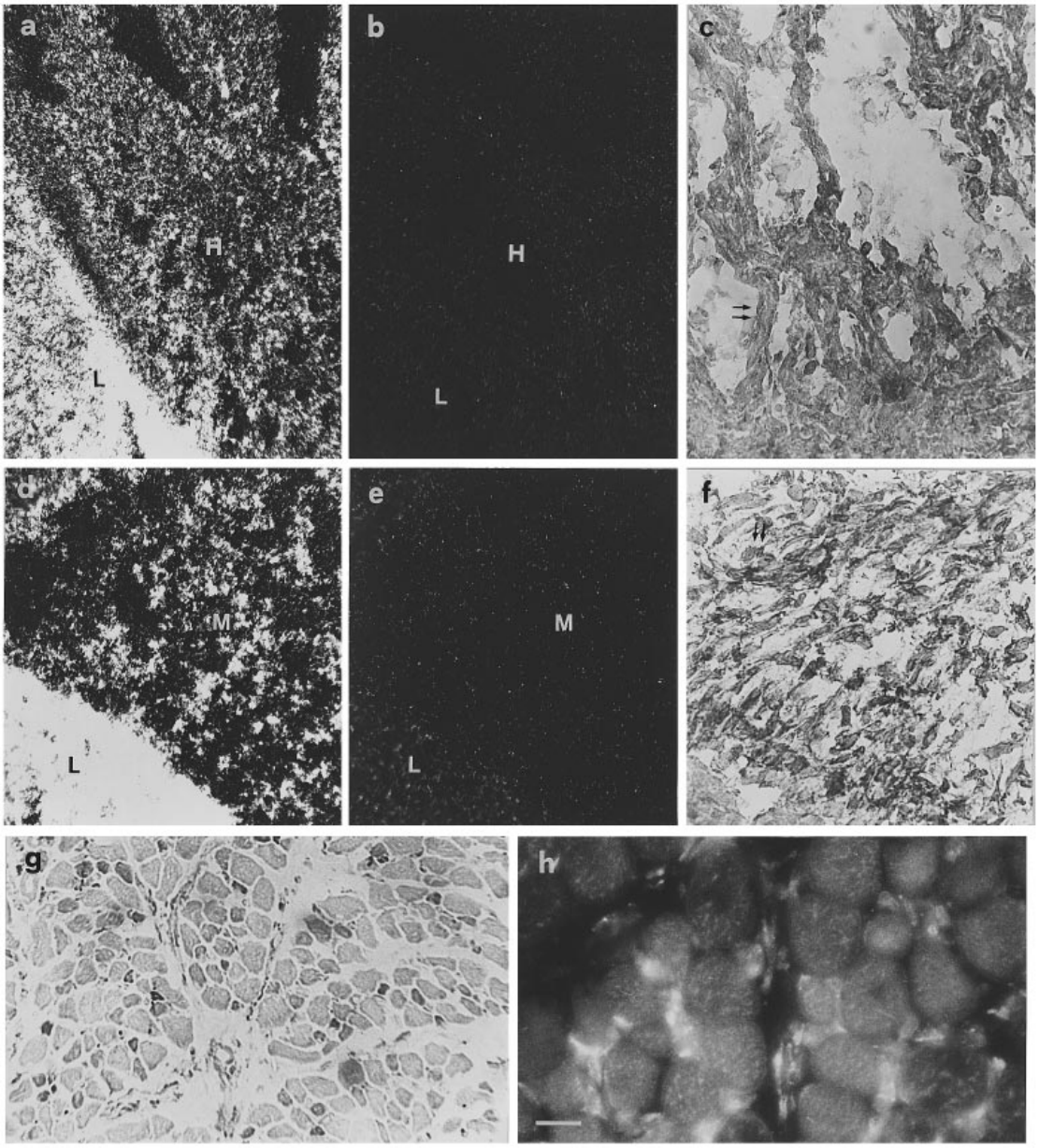

Figure 6 GRP94 mRNA and protein distribution in cardiac and skeletal muscles of the developing rabbit

(**a**–**f**) Cryosections of cardiac (**a**–**c**) and abdominal muscle (**d**–**f**) regions from a 15-day embryo rabbit. (**a**,**d**) Hybridization with anti-sense GRP94 cRNA (clone 2.5); (**b**,**e**) the same fields of a consecutive section hybridized with the sense probe. Abbreviations: L, liver; H, heart; M, abdominal muscle. Note positive hybridization signals in both cardiac and skeletal muscle myocytes. (**c**,**f**) Enlargement of the previous regions stained with mAb 3C4 with indirect immunoperoxidase. Both cardiac and skeletal myocytes are positive for GRP94, which also shows a striated pattern of distribution (double arrows). (**g**,**h**) Transverse cryostat sections from a limb skeletal muscle of a 7-day-old rabbit stained with 3C4 with indirect immunoperoxidase (**g**) or immunofluorescence (**h**). GRP94 staining of skeletal muscle fibres is still detectable at this stage, together with staining of skeletal interstitial cells. At higher magnification, GRP94 immunoreactivity shows a punctate distribution in skeletal muscle myocytes (**h**). Scale bars, (**a**,**b**) 80 μm, (**c**,**f**,**g**) 20 μm, (**d**,**e**) 40 μm, (**h**) 12 μm.

muscle samples was also detected between the same tissues from mouse and rat (L. Gorza and M. Vitadello, unpublished work). The lack of GRP94 immunoreactivity in skeletal muscle could be explained by masking of epitope(s) by post-translational modifications such as phosphorylation and glycosylation. Such a hypothesis was ruled out because the same pattern of reactivity was detected with five different mAbs and was not changed by treatment with alkaline phosphatase or endoglycosidase H. Thus the tissue-specific expression of GRP94 is apparently not due to molecular heterogeneity, as otherwise suggested in the case of methylcolantrene-induced fibrosarcomas [21]. Besides, our *in situ* and immunohistochemical observations show that in skeletal muscle GRP94 mRNA and protein are not apparently detectable within myofibres but only in interstitial cells, namely fibroblasts, nerve axons, endothelial and vascular muscle cells, in contrast with the heart, where GRP94 mRNA and protein are also observed within cardiomyocytes. Thus the small amount of GRP94 mRNA and protein observed in Northern and Western blots of rabbit skeletal muscle should be interpreted as being of a non-myocyte origin.

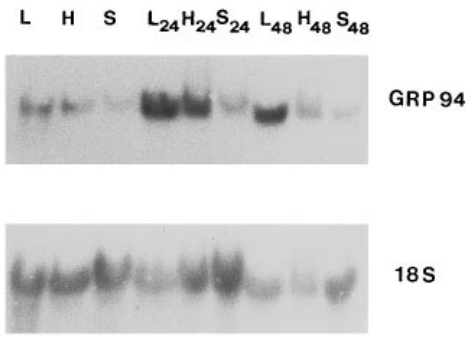

Figure 7 LPS treatment and GRP94 mRNA accumulation

A 10 μg sample of each of liver (L), heart (H) and skeletal muscle (S) total RNA was prepared from control and LPS-treated rabbits, 24 and 48 h after endotoxin administration. The Northern blot was hybridized with the GRP94 anti-sense cRNA probe 2.5 and with the 18 S probe for normalization.

The apparent absence of GRP94 in skeletal muscle fibres might be explained by the higher complexity of SR in these muscle cells in comparison with SR architecture in cardiomyocytes [22]. Consistent with our finding, a recent report showed a lower mRNA accumulation of another member of GRPs, BiP, in *Xenopus* adult skeletal muscle in comparison with cardiac muscle [23]. The analysis of crude microsomal preparations of rabbit skeletal muscle during post-natal development have shown that both BiP and calreticulin, two non-muscle-specific ER Ca^{2+}-binding proteins, can be detected before the expression of the muscle-specific SR Ca^{2+}-binding protein calsequestrin [24]. Comparable results have been described in the fetal rat heart, where calreticulin is detected before the appearance of cardiac calsequestrin [25]. These results are consistent with our evidence for GRP94 expression in skeletal muscle fibres of the fetal and newborn rabbit. However, our study shows that the GRP94 gene is down-regulated in adult skeletal muscle fibres, whereas no comparable evidence has been reported for BiP and calreticulin content in adult rabbit skeletal muscle [24]. This finding suggests that GRP94 might have a more focused role in skeletal muscle fibres during development, possibly in relation to the presence of immature SR. Indeeed, the persistent expression of GRP94 in the heart and its concentration in myocytes of the conduction system, whose SR is characterized by an unusual combination of Ca^{2+}-release channels and pumps [26], might be explained by cardiac SR architecture, i.e. the abundance of extended junctional SR and the presence of numerous peripheral couplings [22], a feature characteristic of immature skeletal muscle [27].

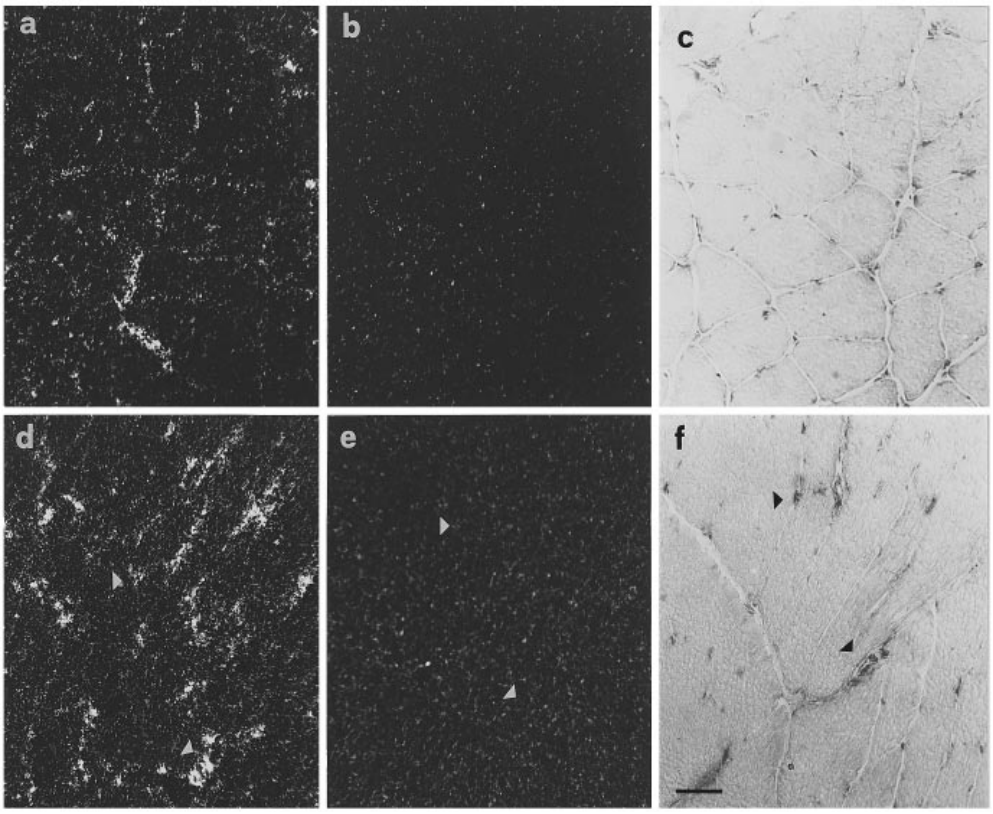

Figure 8 Hybridization *in situ* and immunohistochemistry of GRP94 in LPS-treated skeletal muscles

Cryostat sections of skeletal muscles, excised from sham-treated rabbits (**a**–**c**) and from LPS-treated rabbits 48 h after injection (**d**–**f**), were hybridized with anti-sense GRP94 cRNA (clone 2.5) (**a**,**d**) or sense probe (**b**,**e**) or stained with mAb 3C4 with indirect immunoperoxidase (**c**,**f**). No difference in intensity of hybridization with anti-sense GRP94 probe or in immunoreactivity for GRP94 is detectable between treated and untreated skeletal muscle myocytes. In contrast, slightly stronger hybridization signals are detected in interstitial cells of treated muscles (**d**, arrowhead). Scale bar, 40 μm.

During embryonic development, stress response genes can be active before they gain the ability to be up-regulated by stress [28,29]. Because GRP94 is a stress protein, in addition to being an ER-resident Ca^{2+}-binding protein and a chaperonin [2], we tested whether the exposure of adult rabbits to stress would up-regulate the GRP94 gene, especially in skeletal muscle fibres. For this purpose we chose LPS, which represents a potent stimulus for the elaboration and release of several inflammatory cytokines [30] and was shown to up-regulate the GRP94 gene in lymphocytes *in vitro* [20]. Interestingly, exposure to LPS *in vivo* down-regulated genes central to Ca^{2+} homoeostasis, such as the voltage-dependent Ca^{2+} channel, the SR Ca^{2+} channel and pump, in the rat heart [31]. Our results show that exposure to LPS *in vivo* up-regulates the GRP94 gene in cardiac and skeletal muscles and that such an effect is followed by comparable accumulation of the protein in the heart, but not in the skeletal muscle. However, our hybridization analysis *in situ* did not demonstrate GRP94 mRNA accumulation within skeletal muscle fibres of LPS-treated rabbits, at variance with fetal skeletal muscle myocytes. Whereas the lack of evidence for GRP94 mRNA accumulation in LPS-treated skeletal muscle fibres cannot rule out the possibility of high mRNA instability in this cell type, it allows the exclusion of either failure of mRNA translation or increased protein turnover as mechanisms involved in the absence of increase in GRP94 accumulation. Thus, despite the responsiveness of the GRP94 gene to the stress condition induced by exposure to LPS observed in the heart (this study) and in other cell types [20], no detectable consequence occurs in skeletal muscle fibres. We speculate that such a response could be the consequence of a tight control on GRP94 gene down-regulation in skeletal muscle myocytes that takes place a few days after birth. In contrast, it has been demonstrated that tissue-specific differences in the expression of the constitutive or inducible forms of heat shock protein 70 in the adult animal might influence the level of induction of the same protein in the stress response, such that the lower the accumulation of the protein before the exposure to the stress, the higher the level of the response [32,33]. This is not consistent with the apparent absence of GRP94 expression in adult skeletal muscle fibres before and after exposure to a stress condition and suggests a diverse, prominent role for GRP94 in skeletal muscle fibres during development.

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