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ORIGINAL PAPER

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Oxidative and cold shock cause enhanced induction of a 50 kDa stress protein in *Trichinella spiralis*

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Abstract The anti-heat shock protein (Hsp)-90 monoclonal antibody AC-16 reacts on blots with Hsp90 and a 50 kDa protein (prot-50) from infective-stage (L_1) larvae of the nematode *Trichinella spiralis*. We examined Hsp90 and prot-50 levels by densitometric analysis of immunoblots of *T. spiralis* larval extracts prepared before (time 0, 37°C) and after oxidative [hydrogen peroxide (H_2O_2)] stress, or cold shock at 4°C. Extracts from H_2O_2-exposed L_1 were obtained after 2 h; the others at 2, 4, and 8 h after the temperature shift. After H_2O_2 shock, the constitutive Hsp90 and prot-50 were both significantly induced and appeared as slower migrating inducible isoforms. However, whereas Hsp90 levels decreased after cold shock, prot-50 levels immediately and persistently increased after shock at 4°C. These data present compelling evidence that the prot-50 described here functions as a Hsp and a cold shock protein.

Introduction

One of several events occurring in cells and tissues undergoing stress is the enhanced production of stress proteins. Two functionally different groups of stress proteins, inducible by different stress conditions, have been identified; these include heat shock and cold shock proteins (Hsps and Csps). Originally termed a heat shock (HS) response due to increased Hsp induction following hyperthermia (Ritossa 1962), the mechanism that initiates HS gene transcription is sensitive to many stressors. Such possible stimuli can include hypoxia, inflammation, oxidative stress, glucose deprivation and metals (Schlesinger 1990; Polla et al. 1993; Goering et al. 2000). However, when exposed to cold shock cells react differently. This condition acts first as a potent (transient) depressor of ongoing Hsp production and second, by inducing synthesis of Csps. In addition to in the highly studied *Escherichia coli*, major Csps have thus far been found in several prokaryotic and eukaryotic organisms (Jones and Inouye 1994; Horton et al. 2000). It has been reported that Csps, unlike Hsps, are rarely expressed constitutively (Etchegaray and Inouye 1999) and serve cytoprotective roles in, for example, cell growth at low temperature (for review, see Jones and Inouye 1994). Here, we describe a 50 kDa protein from infective-stage (L_1) larvae of the helminth *Trichinella spiralis*, which accumulates therein both after oxidative stress and cold shock at 4°C.

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Materials and methods

One batch of L_1 of *T. spiralis*, MFEL/SP/62/GM1 (La Rosa et al. 1992) was used. The procedure for obtaining bacteria-free, viable (>99%) L_1 was described previously (Martinez et al. 1999), with the additional precaution that L_1 were at no time exposed to ≤ 35°C. Pooled larvae were counted (3×) using a standard protocol for counting large numbers of worms (Wood et al. 1995). This was followed by addition of aliquots (0.5 ml) containing 5,000 L_1 to tubes with 6.5 ml prewarmed (37°C) medium 199 (Sigma). Successful allotting of ±5,000 L_1 to tubes was verified using the standard method referred to above: 5,003.4 ± 20.5 L_1/tube (mean ± SD; $n = 45$). Following this step, tubes were exposed to either oxidative or cold stress (three tubes used per condition). Oxidative stress experiments involved storage at 37°C for 2 h in the presence of 0 (controls), 20, 100, or 200 mM hydrogen peroxide (H_2O_2). For cold stress studies, the cultures were shifted from 37°C to 4 ± 1°C and then stored for 2, 4, or 8 h.

The mAb to Hsp90 (AC-16) that crossreacts with prot-50 (Martinez et al. 1999) and the peroxidase secondary Ab were purchased from Sigma. In blotting experiments prior to this study, mAb AC-16 was found to produce linear response curves to Hsp90 and prot-50 ($r > 0.95$, three repeat studies).

Proteins of interest in *T. spiralis* larval extracts were detected by Western blotting. In brief, after appropriate stimuli as indicated, extracts were obtained from the three tubes incubated per condition [plus protease inhibitors (Sigma)] by sonication at 4°C. After removal of insoluble material (14,000 *g*, 20 min at 4°C), supernatants were prepared for gel electrophoresis. Proteins (5 µl, in triplicate) were separated on 10% resolving gels (Laemmli 1970) and transferred to Immobilon-P membranes (Millipore). Molecular weight standards were included on each gel. After incubation in blocking buffer [10 mM phosphate-buffered saline, pH 7.2 plus 0.05%Tween 20 (PBS-T) plus 5% non-fat dry milk (Nestlé)], blots were incubated with mAb AC-16 diluted 1/10,000. Positive bands were detected by incubation with 1/6,000 diluted peroxidase secondary Ab and 0.06% 3,3′diaminobenzidine as substrate. The incubation periods for AC-16 and secondary Ab were overnight at 4°C and 2 h at room temperature, respectively. Three washes with PBS-T were performed after each step. Protein bands were quantified using an image analyser and 1D image analysis software (Program Quantity One, Bio-Rad). The statistical differences were evaluated by the Dunnet test. Probability (*P*) values <0.05 were considered significant.

Results

We evaluated prot-50 and Hsp90 immunoreactivity in extracts from *T. spiralis* L_1, after stress as described above, and from controls. While prot-50 activity is the prime interest here, we shall focus on Hsp90 results also, as these indicate normal behaviour of an Hsp under the conditions studied.

Figure 1 shows the immunoblot and densitometric analysis data obtained for Hsp90 and prot-50 after exposure to 0, 20, 100 and 200 mM H_2O_2. As can be seen (Fig. 1A), the constitutive expression of Hsp90 was changed as a result of H_2O_2 exposure. All doses caused Hsp90 levels to visibly increase and the migration of the protein was also retarded, shifting from its normal position to a position 1–2 kDa higher. This result is consistent with Hsp characteristics after stress: (1) significantly induced and/or (2) altered migration, shifting 1–2 kDa upwards or downwards (Polla et al. 1993; Goering et al. 2000). Note that such changes also occurred in the prot-50 expression as a result of H_2O_2 exposure (see 100 and 200 mM data). This result thus establishes evidence for prot-50 acting as an Hsp. By densitometric analysis of Fig. 1A, the levels of production of Hsp90 at 20, 100 and 200 mM H_2O_2 increased significantly ($P<0.05$, $P<0.01$ and $P<0.01$, respectively; Fig. 1B). For prot-50 levels, under these conditions, increases expressed as *P* values were $P<0.05$, $P>0.05$, and $P>0.05$, respectively (Fig. 1C).

We next extended studies of prot-50 and Hsp90 levels to *T. spiralis* L_1 exposed to cold shock. Figure 2 shows immunoblot and densitometric data from controls and samples at time points 2, 4, and 8 h after cold shock. When analysed as above, Hsp90 levels decreased from 2 to 8 h after cold shock whereas those of prot-50 surprisingly, and markedly, accumulated to above control levels (Fig. 2A). Densitometric analysis of Fig. 2A revealed that levels of Hsp90 and prot-50 8 h after the shift significantly decreased and increased, respectively, with respect to control levels (Fig. 2B, C). This result is consistent with the findings of others (Jones and Inouye 1994) showing that a temperature downshift is followed by a short period (acclimation phase) of downregulation of normal cellular proteins, including Hsps, with concomitant induction of Csps. We thus conclude that prot-50, when circumstances so dictate, also functions as a Csp.

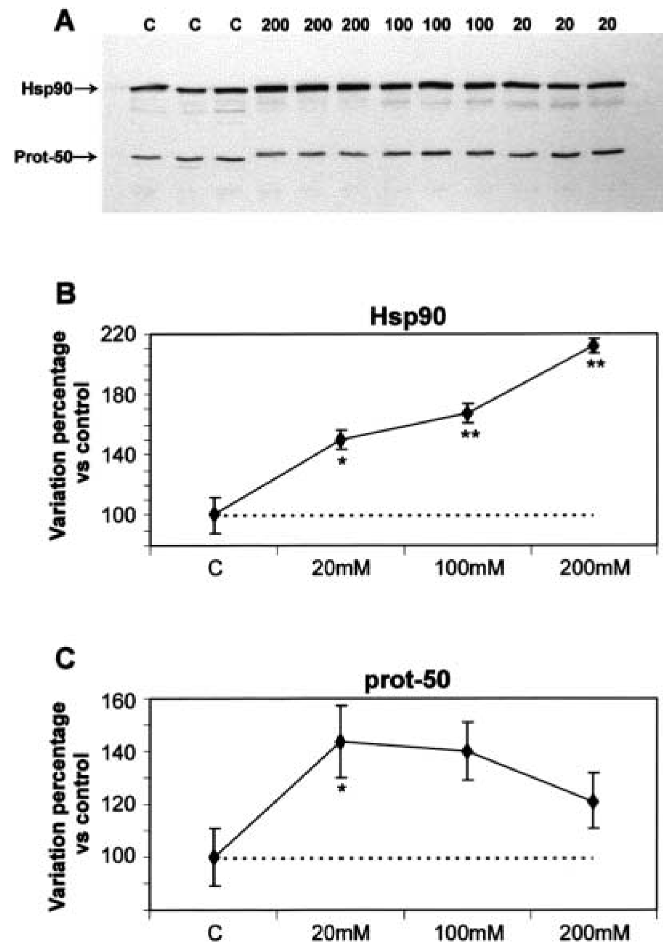

Fig. 1A–C Prot-50 and heat shock protein (Hsp)-90 expression levels in *Trichinella spiralis* L_1 before and after oxidative stress. **A** Immunoblot from specimens before (controls) and after exposure to hydrogen peroxide (H_2O_2) at the concentrations (mM) noted. **B, C** Densitometric analysis, expressed as variation percentage versus time-0 control, of Hsp90 and Prot-50 bands, respectively. Data are presented as mean ± SE of triplicate tests (*$P<0.05$, **$P<0.01$). *Broken line* Time-0 value, taken as 100% and used as control value in the study

To complete the study, we evaluated overt injurious effects by the two stressors on *T. spiralis* L_1 during each condition by direct microscopy. For this study, viable and dead L_1 were defined according to Jenkins and Carrington (1981). In brief, viable L_1 are coiled, uncoiled (incompletely), or assume an undulating configuration, whereas dead L_1 are completely uncoiled, assuming a "comma" shape (typical of dead *T. spiralis* L_1). The time-0 viability score was the criterion used for identification of earliest change(s). Viability in *T. spiralis* L_1 exposed to 20, 100 and 200 mM H_2O_2,

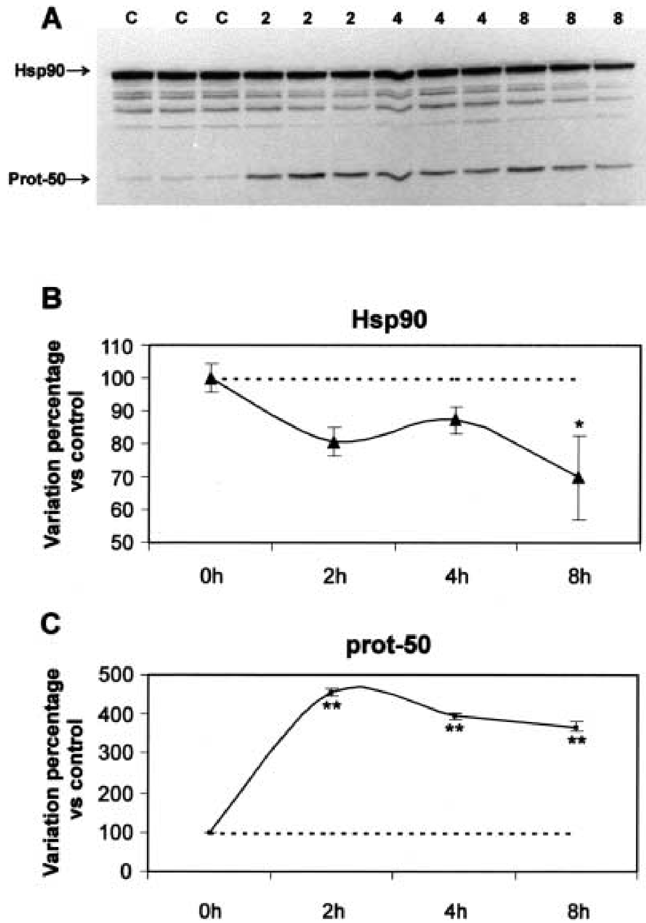

Fig. 2A–C Prot-50 and Hsp90 levels in *T. spiralis* L_1 before and after cold stress (4°C). **A** Immunoblot of samples before (*C* controls) and at 2, 4 and 8 h. **B, C** Densitometric analysis expressed as variation percentage vs time-0 controls from **A**. Data are presented as mean ± SE of triplicate tests ($*P<0.05$, $**P<0.01$). *Broken line* Time-0 value, taken as 100% and used as control value in the study

after 90 min, decreased 0%, 50%, and 100%, respectively, and 0%, 100% and 100%, respectively, after 120 min. In populations stored at 4°C for 2, 4, and 8 h, viability was 100%.

Discussion

This is, to our knowledge, the first report to describe the presence of a 50 kDa protein in L_1 of the nematode *T. spiralis* that is significantly induced in both oxidative shock (a stressor which induces Hsps) and cold shock (which induces Csps). The protein was detected using a crossreactive Hsp90 mAb in a quantitative Western blotting assay (this assay was confirmed to detect Hsp90 and the 50 kDa protein in *T. spiralis* extracts linearly). Densitometric analysis of immunoblots showed significant induction of Hsp90 in test extracts after oxidative shock as expected. Interestingly, this approach also detected induced synthesis of the prot-50 in such samples. In general, prot-50 induction in the samples followed that of Hsp90, raising the possibility that degradation of (induced) Hsp90 might account for the observed phenomenon. However, the results obtained in L_1 stored at 4°C during 8 h strongly argue the opposite. While Hsp90 levels decreased in response to cold, prot-50 levels increased. This differential regulation suggests that prot-50 is not a product of Hsp90 degradation.

In addition, the data strongly indicate this stress protein to be unique in another important aspect. Whereas in cold shocked organisms an immediate, but transient suppression of synthesis of classical Hsps ensues (Jones and Inouye 1994), as we show here for Hsp90, prot-50 exhibited a different pattern of production under such conditions. Baseline production of prot-50 did not drop after cold shock but increased immediately and markedly. These findings are interesting both from a practical/biological standpoint, and in relation to their (apparent) genetic basis. It would seem that in the eukaryotic organism studied here, unlike in e.g. bacteria, regulatory mechanisms for prot-50 synthesis occur that enable its immediate switch, if circumstances so require, from Hsp to Csp activity. However, it would be necessary to obtain a microsequence of prot-50 to prove that it belongs to a Hsp or Csp protein family.

That this 50 kDa protein with a possible cryoprotective function was detected in a *T. spiralis* strain of Spanish origin is very intriguing as Spain is not a very cold country. However, the occurrence of the dual-purpose 50 kDa protein in a parasite species with a world-wide distribution, is rather remarkable and may be of evolutionary significance for the parasite. We are presently conducting experiments to determine whether prot-50 also has a role in cold-resistant *Trichinella nativa*.

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References

Etchegaray J-P, Inouye M (1999) CspA, CspB, and CspG, major shock proteins of *Escherichia coli*, are induced at low temperature under conditions that completely block protein synthesis. J Bacteriol 181:1827–1830

Goering PL, Fisher BR, Noren BT, Papaconstantinou A, Rojko JL, Marler RJ (2000) Mercury induces regional and cell-specific stress protein expression in rat kidney. Toxicol Sci 53:447–457

Horton AJ, Hak KM, Steffan RJ, Foster JW, Bej AK (2000) Adaptive response to cold temperatures and characterization of cspA in *Salmonella typhimurium* LT2. Antonie van Leeuwenhoek 77:13–20

Jenkins DC, Carrington TS (1981) An in vitro screening test for compounds active against the parental stages of *Trichinella spiralis*. Tropenmed Parasitol 32:31–34

Jones PG, Inouye M (1994) The cold shock response-a hot topic. Mol Microbiol 11:811–818

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

La Rosa G, Pozio E, Rossi P, Murell KD (1992) Allozyme analysis of *Trichinella* isolates from various host species and geographical regions. J Parasitol 78:641–646

Martinez J, Perez-Serrano J, Bernadina WE, Rodriguez-Caabeiro F (1999) In vitro stress response to elevated temperature, hydrogen peroxide and mebendazole in *Trichinella spiralis* muscle larvae. Int J Parasitol 29:1457–1464

Polla BS, Perin M, Pizurki L (1993) Regulation and function of stress proteins in allergy and inflammation. Clin Exp Allergy 23:548–556

Ritossa FM (1962) A new puffing pattern induced by heat shock and DNP in *Drosophila*. Experientia 18:571–573

Schlesinger MJ (1990) Heat shock proteins. J Biol Chem 265:12111–12114

Wood IB, Amaral NK, Bairden K, Duncan JL, Kassai T, Malone JB, Pankavich JA, Reinecke RK, Slocombe O, Taylor SM, Vercruysse J (1995) World Association for the Advancement of Veterinary Parasitology (WAAVP) second edition of guidelines for evaluating the efficacy of anthelminthics in ruminants (bovine, ovine, caprine). Vet Parasitol 58:181–213