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Expression of Hsp90, Hsp70 and Hsp60 in *Trichinella* species exposed to oxidative shock

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Abstract

Stress response and phosphorylation of heat shock proteins (HSPs) 60, 70 and 90 were studied in *Trichinella nativa*, *T. nelsoni*, *T. pseudospiralis* and *T. spiralis* larvae at 30-min intervals following exposure to 20, 100 and 200 mM H_2O_2. There was a time- and dose-dependent differential survival for the infective stage larvae (L1) of these four *Trichinella* species. Immunoblotting analysis revealed that constitutive Hsp60 and Hsp70, but not Hsp90, from test *Trichinella* species are constitutively phosphorylated on serine/threonine residues as they converted to forms with increased sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) mobility by treatment with alkaline phosphatase. After exposure to H_2O_2, while there was a time-related occurrence of the three HSPs with decreased SDS–PAGE mobility, these HSPs were insensitive to alkaline phosphatase except in the case of exposure to 20 mM H_2O_2 for Hsp60 from all *Trichinella* species and Hsp70 from *T. spiralis* and *T. nelsoni*. The synthesis of HSPs forms with decreased SDS–PAGE mobility is a susceptibility signal because the lower concentration of peroxide (20 mM) did not cause a decrease on HSPs SDS–PAGE mobility in *T. spiralis* and *T. nelsoni*, the two more resistant selected *Trichinella* species.

Introduction

Today, there is no doubt that heat shock proteins (HSPs) are key factors in the induction and maintenance of many important cellular processes. Heat shock proteins play regulatory roles in, for example, prevention of stress injury, protein synthesis and degradation, antigen processing, immune response generation and apoptosis. Of all such cellular functions some require constitutive HSP involvement whereas others depend upon 'activated' inducible HSPs (see Lindquist, 1986; Polla *et al.*, 1993; Mehlen *et al.*, 1996; Bukau & Horwich, 1998). Several mechanisms can be postulated to account for inducing activated HSPs. Because phosphorylation and dephosphorylation of proteins are major processes for regulating cellular functions, they are potential candidates for the activation event (Edelman & Blumenthal, 1987).

Components of antioxidant defences are divided into enzymatic and non-enzymatic groups (Murray *et al.*, 1998). Enzymatic components (peroxidase, catalase and superoxide dismutase) have been studied thoroughly in parasites, including species of *Trichinella* (Hadas *et al.*, 1994). High cellular levels of antioxidative enzymes have been proposed as a parasitic mechanism of resistance against oxidative environment caused by the host's immune response. However, there are few studies on the role of heat shock proteins in protection against oxidative stress injury.

In our laboratory, studies on stress responses in parasite models have been a subject of great interest over the past

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few years. Using infective-stage larvae (L1) of the nematode *Trichinella spiralis*, we established that HSP levels increased after peroxide (H_2O_2) shocks (Martinez *et al.*, 1999). In the ideal situation, i.e. exposure of L1 to 0.2% (v/v) H_2O_2 (20 mM H_2O_2), HSP production grew in larvae manifesting no decrease in viability. As lower concentrations of H_2O_2 are lethal for other parasites (Ou *et al.*, 1995; Murray *et al.*, 1998), we suggest that in *T. spiralis* increased induction of HSPs and/or activation of HSPs may have a regulatory role in the maintenance of tolerance to the toxic oxidant H_2O_2.

The present study approaches the oxidative stress response in four *Trichinella* species (*T. spiralis*, *T. nelsoni*, *T. nativa* and *T. pseudospiralis*) examining both L1 survival and levels of HSPs 60, 70 and 90 at 30-min intervals for 2 h. In addition, phosphatase treatments with subsequent analyses were used to determine phosporylation of HSPs (whether constitutive or induced) therein.

Reported evidence indicating that *Trichinella* species followed different adaptative pathways (Pozio *et al.*, 1992) suggests that differential dealing by test species' L1 with 20, 100 and 200 mM H_2O_2 shocks might occur.

Materials and methods

Trichinella L1 were collected as described (Brand *et al.*, 1952) from CD1 mice inoculated 30 days before with four individual *Trichinella* species. The *Trichinella* species were: (i) *T. spiralis*, strain MFEL/SP/62/ISSN48, T1; (ii) *T. nativa* strain MPAN/SU/87/ISSN71, T2 (La Rosa *et al.*, 1992); (ii) *T. nelsoni*, strain MCRO/KE/75/ISS29, T7K; and (iv) *T. pseudospiralis*, strain MPRO/SU/72/ISSN13, T4 (Pozio *et al.*, 1989). Following this step, the washed L1 were selected for high (> 99%) viability, pooled and counted. Larvae were then aliquoted to Leighton tubes, each containing 5000 L1 in 7 ml medium 199 (Sigma). All experiments were carried out in duplicate but data are shown in single in order to reduce the number of figures except for HSPs phosphorylation data at 120 min. Experiments involved exposure of larvae at 37°C to medium 199 containing 20, 100 or 200 mM H_2O_2 for 30, 60, 90 and 120 min. After each incubation period the L1 were given two rinses in saline solution (0.9%) and scored for test-induced mortality. The mortality of L1 was quantified microscopically according to the typical 'comma' shape (Jenkins & Carrington, 1981).

All procedures used to obtain proteins for analysis of HSP production were carried out at 4°C. These included subjecting the viable and/or non-viable *Trichinella* L1 in (0.1 ml) H_2O with protease inhibitors (Sigma, P2714) added to repeated sonication to disrupt all cells and centrifugation at $14000 \times g$ for 20 min. The resulting supernatant was collected, analysed for protein content by the Bradford procedure (Bio-Rad) and stored in liquid nitrogen until needed.

In order to test the phosphorylation stage of the HSPs, the total protein concentration of all samples was adjusted to 2 mg ml^{-1}. Then 20 μl of one of each duplicated sample (unless otherwise stated) were mixed with alkaline phosphatase (AP) solution and dephosphorylation buffer (Roche) according to the manufacturer's instructions. The mixture was incubated for 30 min at 37°C and then stored in liquid nitrogen.

Larval proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 8% running gels (and a 4% stacking gel). Equal amounts of protein of each sample were loaded in the lanes and electrophoresis was performed in the cold room (6°C) at a constant current of 40 mA for 2 h (Miniprotean III, Bio-Rad). After electrophoresis, the separated polypeptides were electroblotted to PVDF membranes (Immobilon P, Millipore) as previously described (Martinez *et al.*, 2000). The PVDF membranes were then washed with phosphate-buffered saline + 0.05% Tween 20 (PBS-Tw) for 10 min, a procedure repeated between all incubations. Blots were blocked using PBS-Tw + 5% (w/v) defatted milk (Nestle) and incubation for 60 min. After blocking, they were incubated overnight at 4°C with individual HSP primary Abs. These were: 1/1000 diluted anti-Hsp70 (clone BRM-22, Sigma) and anti-HSP-60 (clone LK-2, Sigma) and 1/10000 diluted anti-Hsp90 (clone AC-16, Sigma) mAbs. Positive bands were identified with peroxidase conjugated anti-mouse serum (1/6000 diluted, Sigma) and 0.06% 3,3′-diaminobenzidine plus H_2O_2 as a substrate.

Results

Prior to detailed HSP analyses, we assessed L1 survival rate following H_2O_2 shocks. Table 1 summarizes the time- and dose-dependent changes in viability. *Trichinella spiralis* and *T. nelsoni* were equally more resilient than *T. nativa* and *T. pseudospiralis*; both survived 20 mM H_2O_2 and become non-viable in 100 and 200 mM H_2O_2 after comparable incubation periods. Although both *T. nativa* and *T. pseudospiralis* were non-viable after 2 h-incubation

Table 1. Viability time course in *Trichinella spiralis* (Ts), *T. nelsoni* (Tnel), *T. nativa* (Tnat) and *T. pseudospiralis* (Tps) larvae exposed to different doses of H_2O_2*.

	20 mM H_2O_2				100 mM H_2O_2				200 mM H_2O_2			
Species	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min
Ts	+[a]	+	+	+	+	+	+/− [c]	−	+	+	−	−
Tnel	+	+	+	+	+	+	+/−	−	+	++/−	−	−
Tnat	+	+	++/− [d]	−[b]	+	+	−	−	+	+/−	−	−
Tps	+	++/−	+/−−[e]	−	+	−	−	−	+/−	−	−	−

*Data are viability scores from duplicate cultures assayed in triplicate at the end of the incubation periods indicated. [a](+) 100% viable L1; [b](−) 100% non-viable L1; [c](+/−) ≥ 50% viable L1; [d](++/−) ≥ 90% viable L1; [e](+/−−) ≥ 90% non-viable L1.

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in 20 mM H_2O_2, the time- and dose-dependent survival rate in 100 and 200 mM H_2O_2 was in the order *T. nativa* > *T. pseudospiralis*.

In the four *Trichinella* species, both Hsp60 and Hsp70 were constitutively phosphorylated, as treating control samples with AP converted both to forms with increased SDS–PAGE mobility (fig. 1). However, no identifiable change occurred in the migration pattern of Hsp90 from the same species when controls were treated with AP.

When *T. spiralis* and *T. nelsoni* larvae were treated with 20 mM H_2O_2 they had Hsp90, Hsp70 and Hsp60 bands with constitutive-like SDS–PAGE mobility at all time points before (fig. 2a,b,c,d) and after treatment with AP (fig. 1). However, in samples from *T. nativa* we detected the HSPs with slower SDS–PAGE mobility at 120 min of exposure (fig. 2e,f) and at 90 min for *T. pseudospiralis* (fig. 2g,h). In addition, the slower forms of Hsp60, but not Hsp70, treated with AP showed changes in their mobility like the constitutive forms (fig. 1c,d). It would, therefore, seem that under our experimental conditions, only the phosphorylation of inducible Hsp70 changed in *T. nativa* and *T. pseudospiralis* after exposure to 20 mM H_2O_2.

Exposure to 100 and 200 mM H_2O_2 causes in all *Trichinella* species the occurrence of slower moving forms of Hsp60 in particular, but also of HSPs70 and 90 in a dose- and time-dependent way (figs 3 and 4). The forms of the three HSPs so-induced (higher relative molecular weight) were insensitive to treatment with AP.

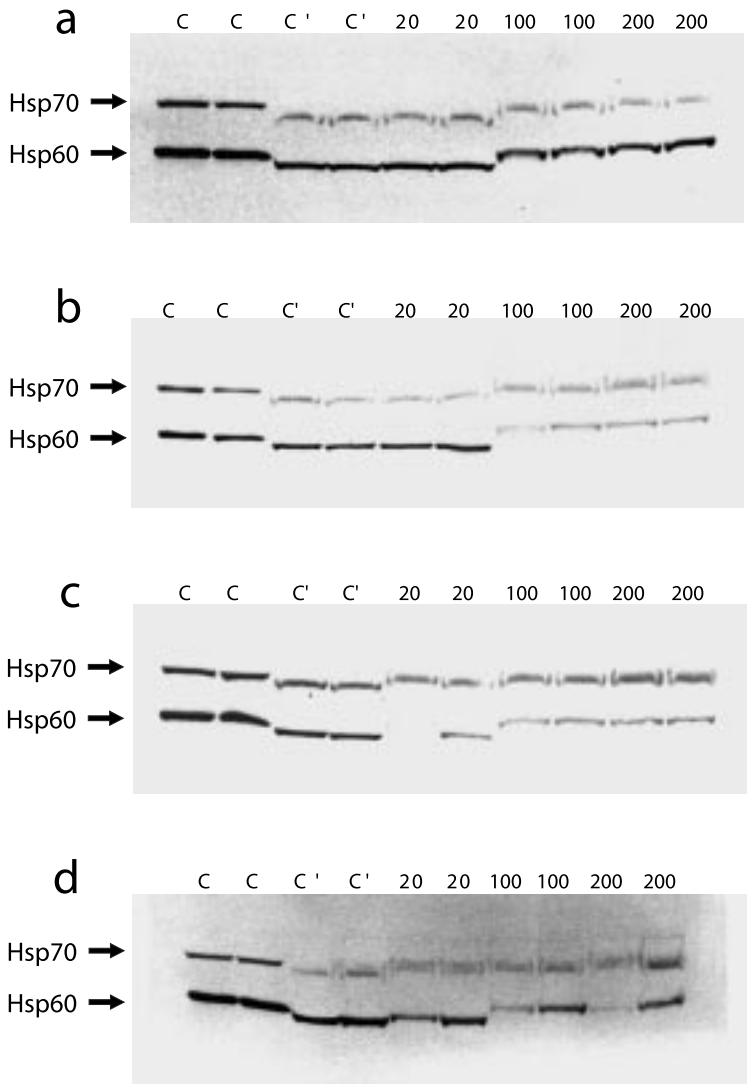

Fig. 1. Effect of alkaline phosphatase treatment on Hsp60 and Hsp70 from larvae of *Trichinella* species exposed for 120 min at 20, 100 and 200 mM H_2O_2. Analysis was carried out by means of Western blot. (a) *T. spiralis*; (b) *T. nelsoni*; (c) *T. nativa*; (d) *T. pseudospiralis*. C untreated controls; C′, control samples treated with alkaline phosphatase.

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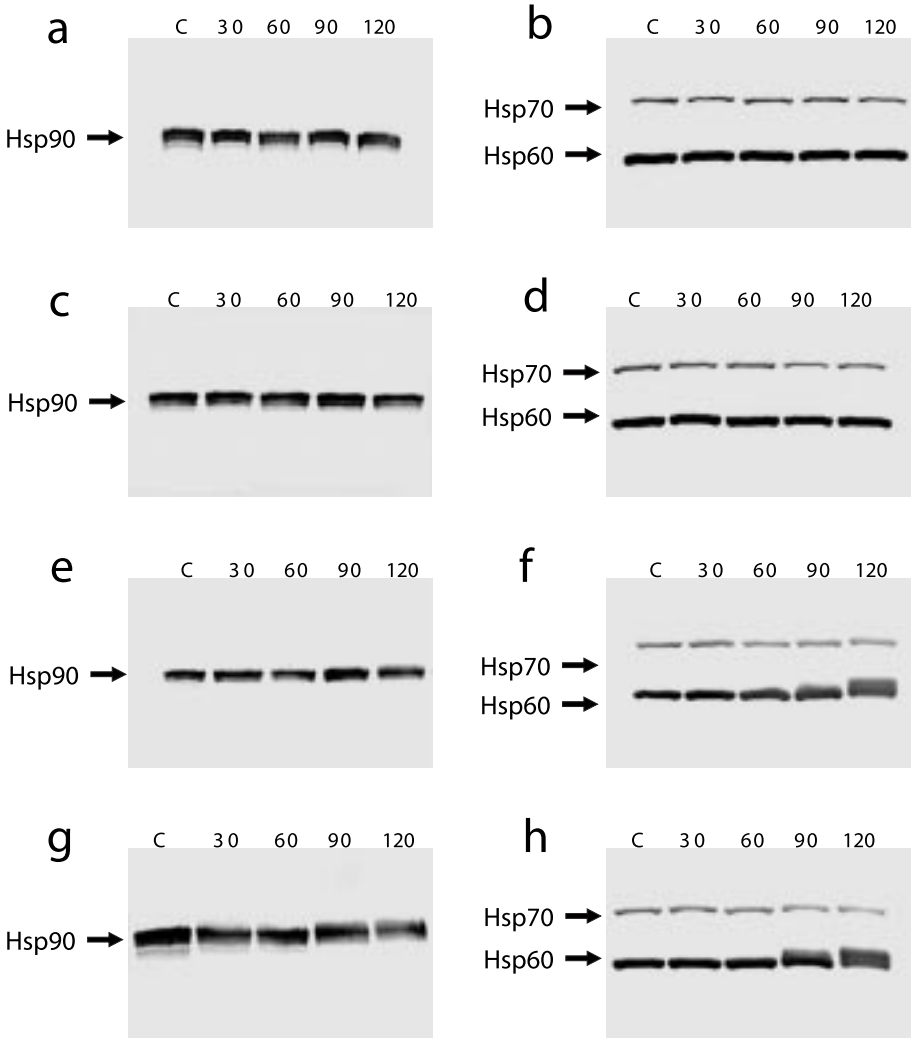

Fig. 2. Expression of HSPs 60, 70 and 90 in *Trichinella* species exposed at 20 mM H_2O_2 for 30, 60, 90 and 120 min. (a, b) *T. spiralis*; (c, d) *T. nelsoni*; (e, f) *T. nativa*; (g, h) *T. pseudospiralis*.

Discussion

For *T. spiralis*, the concurrence in hosts of stages readily killed as new-born larvae (NBL) up to 2 h old or not as L1 and adults by phagocytes, is well known (Kazura & Grove, 1978; Buys *et al.*, 1981). Further studies have provided insights into some mechanisms of *T. spiralis* resistance to killing by leukocytes (Kazura & Meshnick, 1984). However, the *in vitro* optimum conditions (cell/larvae ratio and time of exposure) used to demonstrate that NBL are readily killed by phagocytes did not appear to occur naturally *in vivo* (Bell, 1998). Subsequent studies provided new oxidant resistance mechanisms or extended responses previously described for *T. spiralis* larval stages to some other *Trichinella* species (Hadas *et al.*, 1994; Bruschi *et al.*, 2000). The present study, the first one involving four classical *Trichinella* species, also indicates a possible reparative role for HSPs. The *Trichinella* species studied are interesting both from a clinical standpoint and in relation to the apparent genetic basis for their differential susceptibility to H_2O_2 stress.

In *Trichinella* biology, the transit through homeothermic hosts may have been used as an evolutionary advantage for these helminths in two ways. It has been shown that although some NBL up to 2 h old are killed by oxidants, not all are killed during *Trichinella* infections (Bell & Wang, 1987; Wang & Bell, 1988). Thus, the migration of NBL through the host serves as a 'quality control' mechanism by which less resistant NBL are culled. New-born larvae that overcome this barrier may survive in low responder hosts or may acquire increased resistance in individuals, with a high response to the infection (Wakelin, 1984). Despite overall uniform host defences, the proposed resistance development scenario has not produced similar capabilities in the four *Trichinella* species under study. The resistance grade of *Trichinella* species seem polarized towards two different endpoints: *T. spiralis* and *T. nelsoni* with comparable higher resistance

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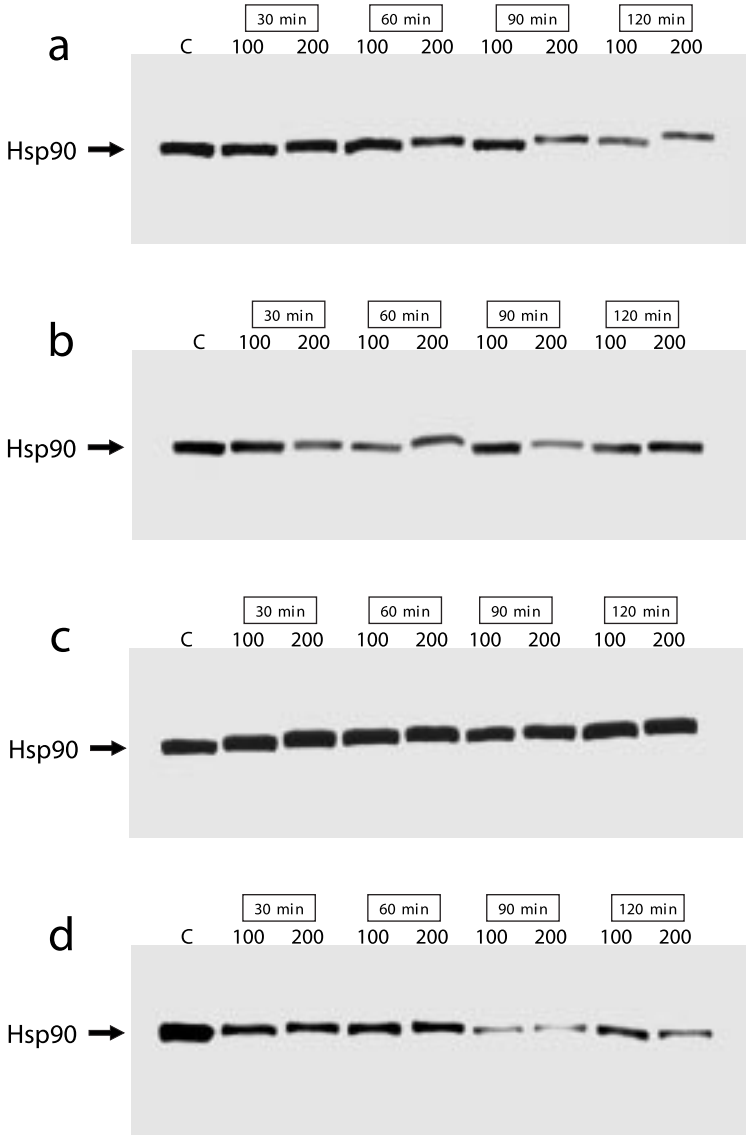

Fig. 3. Expression of Hsp90 in *Trichinella* species exposed at 100 and 200 mM H_2O_2 for 30, 60, 90 and 120 min. (a) *T. spiralis*; (b) *T. nelsoni*; (c) *T. nativa*; (d) *T. pseudospiralis*.

to H_2O_2 vs. *T. nativa* and *T. pseudospiralis* with comparable lower resistance to H_2O_2. Obviously, the use of test concentrations of up to 200 mM H_2O_2 enabled this discovery. How and why the *Trichinella* species came to be resistant to toxic conditions far exceeding those they would ever encounter in their life-time is unclear. However, the phenomenon *per se* is not exceptional as similar findings have been reported for worms of *Brugia malayi*, although this species is less resistant to oxidative shock than any *Trichinella* species (Ou *et al.*, 1995). This observation may have important consequences for current efforts to develop vaccines to control parasitic (helminth) diseases. *In vivo* killing of parasites is associated with the release by leukocytes of, at the most 25 μM H_2O_2, (Weiss *et al.*, 1986) but this concentration is not lethal for a very long time for *Trichinella* larvae (L1).

Recovery from acute stress injury involves the rapid synthesis of HSPs and (re)programming of biological activity to prevent further damage and restore homeostasis after cessation of physiological stress (Schlesinger, 1990). We believe that phosphorylation of HSPs has an important role to play in this process. Because the present study intentionally stressed the test *Trichinella* species far beyond their limits of resistance, there was obviously no realistic reparative role for HSPs herein. Indeed, all test L1 died during the experiments with the exception of *T. nelsoni* and *T. spiralis* larvae exposed to 20 mM H_2O_2. However, our time-course studies indicate that *Trichinella* species preserve life for some time at all H_2O_2 doses (i.e.

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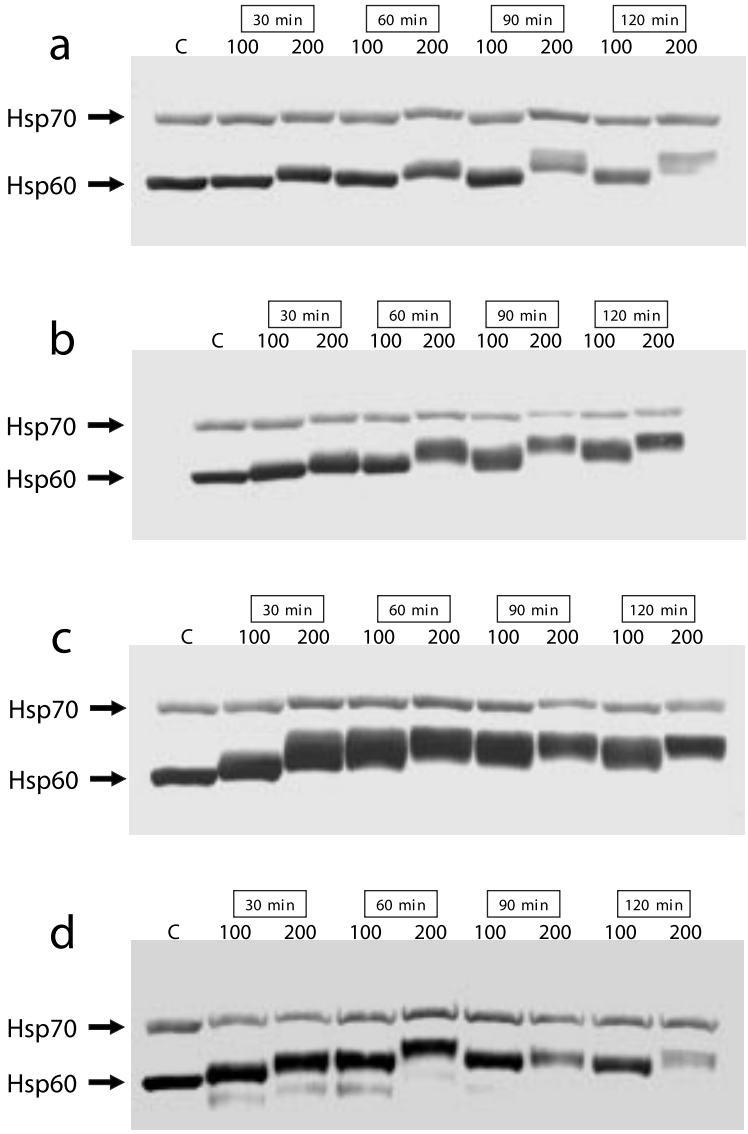

Fig. 4. Expression of Hsp60 and Hsp70 in *Trichinella* species exposed at 100 and 200 mM H_2O_2 for 30, 60, 90 and 120 min. (a) *T. spiralis*; (b) *T. nelsoni*; (c) *T. nativa*; (d) *T. pseudospiralis*.

minimally 60 min for all except *T. pseudospiralis*) before irreversible cell injuries caused their death. Such conditions of overt toxicity would be responsible for HSP induction and sequential attempts to preserve life or a consequence of an apoptotic process. Moreover, under our test conditions, residual effects of processes triggered off in the previously viable L1 would still be detected in larvae considered dead.

Data obtained using AP to examine HSPs' phosphorylation states rendered some interesting observations. Firstly, HSPs 60 and 70 of all the *Trichinella* species were constitutively phosphorylated on serine/threonine residues. As phosphorylation is the main mechanism in activating proteins, it is possible that enzymatic digestion made to harvest the larvae was responsible for this process. Moreover, the natural digestion of infected meat could prepare larvae for future incidents. Secondly, our 20 mM-H_2O_2-exposure data indicate that HSPs 60 and 70 in *T. nelsoni* and *T. spiralis* remain AP-identifiably as phosphorylated during the study, whereas in *T. nativa* and *T. pseudospiralis* this was only the case for the Hsp60. The basis for these differences is not yet known, but it may be significant that *T. nativa* and *T. pseudospiralis* are the only *Trichinella* species succumbing to 20 mM H_2O_2, *T. pseudospiralis* being the most susceptible. In spite of this, *T. pseudospiralis* has a higher amount of antioxidant enzymes than other *Trichinella* species (Hadas *et al.*, 1994). Therefore, it seems clear that other homeostatic mechanisms must be used to repair oxidative stress injury. Thirdly, while a time- and dose-dependent synthesis of

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inducible HSPs 60, 70 and 90 occurred in all *Trichinella* species at 100 and 200 mM H_2O_2, most dramatic changes were evident in *T. nativa* and *T. pseudospiralis*, which died first under these conditions. Inasmuch as constitutive HSPs 60 and 70 in all test *Trichinella* species seem to be phosphoproteins (sensitive to AP treatment), the present results imply that exposure to lethal H_2O_2 induces dephosphorylation of constitutive-type HSPs 60 and 70. We are currently trying to obtain tools to examine whether inducible forms of HSPs insensitive to AP treatment are related to tyrosine phosphorylation or with other molecular modifications such as acetylation and methylation.

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References

Bell, R.G. & Wang, C.H. (1987) The *Trichinella spiralis* newborn larvae: production, migration and immunity *in vivo*. *Wiadomosci Parazytologiczne* **4–5**, 453–478.

Bell, R.G. (1998) The generation and expression of immunity to *Trichinella spiralis* in laboratory rodents. *Advances in Parasitology* **41**, 149–217.

Brand, T., Weistein, P., Mehlan, B. & Weinbach, E. (1952) Observation on the metabolism of bacteria free larvae of *Trichinella spiralis*. *Experimental Parasitology* **1**, 245–255.

Bruschi, F., Carulli, G., Azzarà, A., Homan, W., Minnucci, S., Rizzuti-Gillaci, A., Sbraa, S. & Angiolini, C. (2000) Inhibitory effects of human neutrophil functions by the 45–kD glycoprotein derived from the parasitic nematode *Trichinella spiralis*. *International Archives of Allergy and Applied Immunology* **122**, 58–65.

Bukau, B. & Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines. *Cell* **92**, 351–366.

Buys, J., Wever, R., Van Stigt, R. & Ruitenberg, E.J. (1981) The killing of newborn larvae of *Trichinella spiralis* by eosinophil peroxidase *in vitro*. *European Journal of Immunology* **11**, 843–845.

Edelman, A.M. & Blumenthal, D.K. (1987) Protein serine/threonine kinases. *Annual Review of Biochemistry* **56**, 567–613.

Hadas, E., Rodriguez-Caabeiro, F. & Jimenez-Gonzalez, A. (1994) Oxidant defenses in different isolates of *Trichinella*. *Acta Parasitologica* **39**, 32–36.

Jenkins, D.C. & Carrington, T.S. (1981) An *in vitro* screening test for compounds active againts the parenteral stages of *Trichinella spiralis*. *Tropenmedizin und Parasitologie* **32**, 31–34.

Kazura, J.W. & Grove, D.I. (1978) Stage-dependent antibody-dependent eosinophil-mediated destruction of *Trichinella spiralis*. *Nature* **274**, 588–589.

Kazura, J.W. & Meshnick, S.R. (1984) Scavenger enzymes and resistance to oxygen mediated damage in *Trichinella spiralis*. *Molecular and Biochemical Parasitology* **10**, 1–10.

La Rosa, G., Pozio, E., Rossi, P. & Murrell, K.D. (1992) Allozyme analysis of *Trichinella* isolates from various host species and geographical regions. *Journal of Parasitology* **78**, 641–646.

Lindquist, S. (1986) The heat shock response. *Annual Review of Biochemistry* **55**, 1151–1191.

Martinez, J., Perez-Serrano, J., Bernadina, W.E. & Rodriguez-Caabeiro, F. (1999) *In vitro* stress response to elevated temperature, hydrogen peroxide and mebendazole in *Trichinella spiralis* muscle larvae. *International Journal for Parasitology* **29**, 1457–1464.

Martinez, J., Perez-Serrano, J., Bernadina, W.E. & Rodriguez-Caabeiro, F. (2000) Detection of heat shock protein-70 from *Trichinella spiralis* larvae using a modification of the routine Western blotting procedure. *Journal of Parasitology* **86**, 637–639.

Mehlen, P., Schultze-Osthoff, K. & Arrigo, A.P. (1996) Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1 and staurosporine-induced cell death. *Journal of Biological Chemistry* **271**, 16510–16514.

Murray, E.S., Smith, V.P., Thomas, G.R. & Gounaris, K. (1998) Resistance of filarial nematode parasites to oxidative stress. *International Journal for Parasitology* **28**, 1315–1332.

Ou, X., Thomas, G.R., Chacón, M.R., Tang, L. & Selkirk, M.E. (1995) *Brugia malayi*: differential susceptibility to a metabolism of hydrogen peroxide in adults and microfilariae. *Experimental Parasitology* **80**, 530–540.

Polla, B.S., Perin, M. & Pizurki, L. (1993) Regulation and function of stress proteins in allergy and inflammation. *Clinical and Experimental Allergy* **23**, 548–556.

Pozio, E., La Rosa, G. & Rossi, P. (1989) Present status of the taxonomy of the *Trichinella* genus. *Wiadomosci Parazytologiczne* **5**, 483–484.

Pozio, E., La Rosa, G., Murell, K.D. & Lichtenfels, J.R. (1992) Taxonomic revision of the genus *Trichinella*. *Journal of Parasitology* **78**, 654–659.

Schlesinger, M.J. (1990) Heat shock proteins. *Journal of Biological Chemistry* **265**, 12111–12114.

Wakelin, D. (1984) Evasion of the immune response: survival within low responder individuals of the host population. *Parasitology* **88**, 639–657.

Wang, C.H. & Bell, R.G. (1988) Antibody-mediated in-vivo cytotoxicity to *Trichinella spiralis* newborn larvae in immune rats. *Parasite Immunology* **10**, 293–308.

Weiss, S.J., Test, S.T., Eckmann, C.M., Roos, D. & Regiani, S. (1986) Brominating oxidants generated by human eosinophils. *Science* **234**, 200–203.

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