Dermal exposure to immunostimulants induces changes in activity and proliferation of coelomocytes of *Eisenia andrei*

**Joanna Homa · Anna Zorska · Dawid Wesolowski · Magdalena Chadzinska**

Received: 7 February 2012 / Revised: 23 August 2012 / Accepted: 28 August 2012 / Published online: 27 September 2012

© The Author(s) 2012. This article is published with open access at Springerlink.com

**Abstract** Due to the specific habitat conditions in which they live, earthworms are constantly exposed to pathogens. Consequently, they have evolved various immuno-defense mechanisms, including cellular (coelomocytes) and humoral responses, which may help to eliminate deleterious micro-organisms but also repair and/or protect host cells and tissues. Similar to mammalian phagocytes, coelomocytes can kill ingested pathogens with reactive oxygen species (ROS) and nitric oxide. In the present work, we studied the effects of the dermal exposure of *Eisenia andrei* earthworms to different immuno-stimulants: phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS) or concanavalin A (ConA). After 3 days of treatment with all immuno-stimulants, decreased numbers and changed composition of the coelomocytes were observed. The immuno-stimulants also induced numerous changes in bactericidal activity, including ROS production. Furthermore, all stimulants increased cell proliferation while only LPS-treatment significantly elevated apoptosis of coelomocytes. These results demonstrate that in vivo treatment of earthworms with immuno-stimulants induces various changes in their coelomocyte response.

**Keywords** Coelomocytes · Immunostimulants · Proliferation · Heat shock protein 72 · Nitric oxide · Respiratory burst

**Introduction**

Earthworm innate immunity is maintained by immuno-competent cells—coelomocytes, localized in the coelomic cavity. Depending on the classification, they can be further divided into three different cell populations: hyaline amoebocytes, granular amoebocytes and eleocytes/chloragocytes (Adamowicz 2005; Cooper et al. 2002; Kurek et al. 2007) or five: leucocytes type I (basophilic) and II (acidophilic), neutrophils, granulocytes and eleocytes (Calisi et al. 2009). All types of coelomocytes can recognize foreign materials (e.g. pathogens) and carry out phagocytosis and encapsulation (Engelmann et al. 2004; Kalac et al. 2002; Popović et al. 1998). Similar to vertebrates, invertebrate immuno-competent cells also possess the capacity to combat pathogens using highly reactive metabolites, such as superoxide radicals ($O_2^{−}$), hydroxyl radicals ($OH^·$), hydrogen peroxide ($H_2O_2$) and nitric oxide (NO) (Rivero 2006; Valembois et al. 1994; Valembois and Lassegues 1995). In addition, chloragogen tissue derived eleocytes are responsible for maintaining the constant pH of coelomic fluid and the storage of glycogen and lipids (Affar et al. 1998; Fischer and Molnár 1992; Prentø 1979). An interesting feature of eleocytes is their ability to autofluorescence, originating mainly from riboflavin which is located in the chloragosomes of these cells (Cholewa et al. 2006; Koziol et al. 2006; Peeters-Joris 2000; Płtycz et al. 2007). Apart from cellular components, coelomic fluid contains numerous humoral immune factors, such as lysozyme, agglutinins (e.g. lectins), fetidins, lysenins and calcium-binding proteins (e.g. calreticulin) (Bilej et al. 2000; Engelmann et al. 2004; Kauschke et al. 2007; Slírova et al. 2007). Some of these humoral factors function as opsonins facilitating phagocytosis (Bilej et al. 1990; Kalac et al. 2002) while some others, e.g. the coelomic cytolytic...
factor (CCF), act as pattern recognition molecules. The latter molecules bind pathogen associated molecular patterns (PAMPs), including the peptidoglycan of Gram positive bacteria but also β-1,3-glucan from yeast or lipopolysaccharide (LPS) from Gram negative bacteria, and initiate serine protease cascades activating the prophenoloxidase (pro-PO) system (Bilej et al. 2001).

In vertebrates some PAMPs can act as mitogens, i.e. molecules that induce the polyclonal overreaction of lymphocytes. In particular, LPS induces strong stimulation of B and T cells (Goodman and Sultzer 1979; Tough et al. 1997); however, it has also been shown that LPS-induced production of nitric oxide (NO) inhibits macrophage proliferation (Vadiveloo et al. 2001). Some effects on cell proliferation have also been reported for phorbol-12-myristate-13-acetate (PMA) and for lectin concanavalin A (ConA) extracted from the jack-bean, Canavalia ensiformis. In leukocytes, PMA enhances proliferation via a protein kinase C (PKC)-dependent mechanism; however, the proliferation of cancer cell lines is inhibited by PMA (Cao et al. 2009). Also, ConA was found to increase the number of coelomocytes due to cell proliferation in the coelomic epithelium, axial organ and Tiedemann body. Moreover, in snails (Biomphalaria glabrata) LPS injection, as well as in vitro exposure of amebocyte-producing organ to PMA, resulted in an increase in the number of hemocytes (Salamat and Sullivan 2009; Sullivan et al. 2011).

It was shown previously that ConA and LPS can also enhance programmed cell death (apoptosis) (Grant et al. 1995). Thus far, clear evidence of apoptosis for earthworm coelomocytes has only been shown upon treatment with heavy metals (e.g. Reinecke and Reinecke 2004) and our group showed that heavy metals (Cu, Cd, Pb) stimulate expression of the apoptosis executor protein (caspase-3) (Homa et al. 2007). Moreover, the same heavy metals stimulate expression of heat shock proteins (HSP70 and 72). HSPs are highly conserved proteins, which are constitutively expressed in all cell types, both in invertebrates and vertebrates, where they are essential for protein folding and protection from denaturation in physiological conditions, e.g. during cell cycle and cell differentiation (Kiang and Tsokos 1998). Furthermore, HSPs can protect cells from stress-induced caspase-dependent apoptosis (Parcellier et al. 2003). In addition, it has been suggested that in mammals heat shock proteins function as proteins enhancing binding and recognition of LPS (Wallin et al. 2002).

As the effects of mitogens on the invertebrate immune response are still unclear, we aimed to investigate the impact of in vivo LPS, PMA and ConA stimulation on the number and composition of Eisenia andrei coelomocytes, as well as their activity (respiratory burst and NO production), apoptosis and proliferation. Moreover, we verified the expression of heat shock proteins in coelomocytes retrieved from earthworms exposed to those immunostimulants.

### Materials and methods

#### Animals and exposure condition

Adult (clitellate) earthworms (0.41–0.83 g body weight) of Eisenia andrei (Sav.) were collected from the stockbreeding maintained in the Institute of Zoology of the Jagiellonian University, kept in controlled laboratory conditions (16 ± 1 °C; 12:12 LD) in commercial metal-free soil (PPUH Biowita, Poland) samples in plastic boxes for at least 2 weeks for acclimatization. The experiments were conducted by the filter paper contact method (Homa et al. 2005; OECD 1984). Vijver and co-workers (2003) showed that dermal exposure may be considered as a significant route of toxicant/stimuli uptake in earthworms (Vijver et al. 2003).

After 3 h on moist filter paper (Whatman, International Ltd, UK), each worm was washed, dried and placed individually for 3 days in 15 ml vials with filter papers, that were soaked either with water (control) (as described previously in Homa et al. 2007) or with immunostimulants: lipopolysaccharide - LPS from Escherichia coli 0111:B4 (Sigma-Aldrich Co., St. Louis, MO, USA) (0.5, 1, 2 and 5 mg/ml), phorbol 12-myristate 13-acetate-PMA (Sigma-Aldrich Co., St. Louis, MO, USA) (0.01, 0.05, 0.1, 0.5, 1, 5 and 10 µg/ml) or concanavaline A – ConA (1, 2.5, 5 and 10 µg/ml) (Sigma-Aldrich Co., St. Louis, MO, USA).

All mention immunostimulants are widely used in immune response studies, both in vertebrates and invertebrates (e.g. Cao et al. 2009; Fortier et al. 2008; Salamat and Sullivan 2009). In addition some control animals were kept in a commercial soil.

#### Harvesting of coelomocytes

After 3 days of exposure to the immunostimulants the earthworms were stimulated for 1 min with a 4.5 V electric current to expel coelomic fluid with coelomocytes through the dorsal pores according to the procedure described previously (Homa et al. 2008; Roch 1979).

#### Flow cytometric measurement and analysis

To determine a cell composition of coelomocytes, the coelomic fluid samples were analyzed with a FACScalibur.
flow cytometer (BD Biosciences). During analytical experiments, 10,000 threshold events per worm sample were collected and analyzed on the basis of their forward scatter (FSC) (for cell size) and sideward scatter (SSC) (cell complexity) properties. Fluorescence FL1-H was recorded for estimation of autofluorescence of eleocytes. Data were analyzed using WinMDI 2.9 software (Joe Trotter, http://facs.scripps.edu).

Respiratory burst

The respiratory burst activity of coelomocytes was measured with the nitroblue tetrazolium (NBT) as described previously (Chadzinska et al. 2009). Suspension of coelomocytes, 1 × 10⁶/ml was incubated with NBT (1 mg/ml, Sigma–Aldrich Co., St. Louis, MO, USA) and after 1 h incubation, the reaction was stopped with methanol. The plates were air-dried and 120 μl of 2 N potassium hydroxide and 140 μl of dimethyl sulphoxide were added to each well. The optical density (O.D.) was recorded in an ELISA reader (Perkin Elmer Luminescence Spectrometer LS50B) at 540 nm.

Nitric oxide release

Nitrite/nitrate production, an indicator of nitric oxide synthesis, was measured in cell culture supernatants as described previously (Chadzinska et al. 2009). Briefly, 100 μl of cell culture supernatant was added to 50 μl of 1 % (w/v) sulphanilamide in 2.5 % (v/v) phosphoric acid and 50 μl of 0.1 % (w/v) N-naphthyl-ethylenediamine in 2.5 % (v/v) phosphoric acid (all from Sigma–Aldrich Co., St. Louis, MO, USA). The O.D. reading was taken at 540 nm.

Cell cycle analysis

In order to quantify percentage of the proliferating and apoptotic cells among free-floating coelomocytes, flow cytometry analysis was performed on the cells stained with propidium iodide where intensity of propidium iodide-derived FL-2 fluorescence is proportional to DNA content in the cells (Blacklidge and Bidwell 1993). The cell suspension was incubated with “DNA buffer”: 5.1 mg propidium iodide (Sigma Chemical Co., St. Louis, MO, USA), 10 mM Tris-base (Sigma Chemical Co.) 10 mM NaCl (POCH, Gliwice, Poland), 700 U/l RNase (Sigma Chemical Co.), 0.1 ml/100 ml Nonidet P-40 (Sigma Chemical Co., pH 8.0) and then analyzed by flow cytometry to assess the cell cycle DNA profile.

Coelomocytes (500 μl of 1 × 10⁶ cells/ml suspension) were mounted on slides by cytopsin (5 min; 1900 rpm), fixed in 4 % paraformaldehyde, cytopsines, mounted in Vectashield with DAPI (Vector Labs), and analyzed in fluorescence microscopy (Axio Imager. M2, Zeiss, Germany) with imaging system AxioCam MRm (monochrome variant).

Immuno-blot detection of heat shock protein HSP72

To examine expression of stress proteins, dot-blot assays were performed in a 96-well plate format using a Bio-dot microfiltration manifold (Bio-Rad, Hercules, CA, USA). Lysates of tissues were prepared according to the manufacture’s protocols (Roche Diagnostic GmbH, Mannheim, Germany) as described by Homa et al. (2005). The protease inhibitor cocktail PMSF (Roche Applied Diagnostic GmbH, Mannheim, Germany) was used to prepare coelomocyte extracts. The amount of protein was determined by the BCA (Sigma–Aldrich Co., St. Louis, MO, USA) method and samples were diluted to the same concentration. For analyses, 50 μl of protein was added to each well of microfiltration apparatus. Samples microfiltration blotting was performed according to the Bio-Rad protocol on to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 45 min at 37 °C in a blocking buffer containing 5 % non-fat powder milk (Gostyn, Poland) in TBS (20 mM Tris–HCl, 500 mM NaCl, pH 7.5). Then membranes were incubated with monoclonal anti-HSP72 biotin conjugate antibody (Stressgen, San Diego, CA, USA) diluted 1:4,000 in TTBS (TBS with 0.05 % Tween 20) containing 1 % non-fat milk and incubated overnight at 4 °C. Next day membranes were washed at room temperature by continuous shaking in TTBS. The presence of HSP72 was immunodetected with Streptavidin–Alkaline Phosphatase SAv-AKP, BD Pharmingen, San Diego, USA) after 30 min incubation at room temperature. Then the reaction was developed by BCIP/NBT (Bio-Rad, Hercules, CA, USA). The membranes were air-dried and a densitometric analysis of protein dots was performed using of the UVISoft-UVIMap program (UVItec, Ltd.).

Data analysis and statistics

Results are expressed as means ± standard errors (X ± SE). Significant differences between means were evaluated using one-way ANOVA with post hoc Tukey test. The level of significance was established at p < 0.05.

Results

Coelomocyte number and composition

The number of cells obtained from animals kept on the filter paper soaked with PMA (0.01 and 0.05 μg/ml), LPS
(1 and 5 mg/ml) or ConA (1–10 µg/ml) was lower compared to the control groups (soil and H2O). In animals treated with PMA and LPS cell reduction was particularly visible in the case of eleocytes, while ConA decreased numbers of both amoebocytes and eleocytes (Fig. 1). The highest concentration of PMA (0.5 µg/ml) did not change total coelomocyte numbers, but induced changes in cell composition, i.e. it increased the number of amoebocytes and reduced the number of eleocytes (Fig. 1). The changes observed under the microscope were additionally confirmed by flow cytometric analyses. These revealed that LPS, ConA, and especially PMA-treatment, significantly reduced the numbers of highly granular and autofluorescent cells (Fig. 2a). Moreover, flow cytometry analysis showed changes in the amoebocyte composition, as all stimulants increased in terms of the percentage of granular amoebocytes (AG), but not of hyaline amoebocytes (AH) (Fig. 2ab).
Production of reactive oxygen species and nitric oxide

NBT reduction significantly increased production of reactive oxygen species and nitric oxide in coelomocytes derived from animals treated with PMA (0.01, 0.1 and 0.5 µg/ml) and LPS (1–5 mg/ml), whereas in animals treated with ConA only the highest concentration (10 µg/ml) significantly increased the NBT reduction (Fig. 3a).

Also, the production/release of NO increased in coelomocytes retrieved from animals treated with PMA (0.01 and 0.05 µg/ml) and ConA (5 and 10 µg/ml); however, LPS did not induce significant changes in the NO level (Fig. 3b).

Coelomocyte proliferation and apoptosis

The percentage of proliferating coelomocytes was comparable in the case of control animals, i.e. the earthworms obtained from soil, and those kept for 3 days on water-soaked filters (approximately 5 %), while both PMA and ConA increased the percentage of proliferating cells in the coelomic cavity to approximately 20 and 10 %, respectively. Only the highest concentration of LPS (5 mg/ml) induced significant coelomocyte proliferation (Fig. 4a, b).

Moreover, LPS in a concentration of 2 mg/ml increased coelomocyte apoptosis. In animals treated for 3 days with PMA a tendency to increased coelomocyte apoptosis was observed, while ConA did not affect coelomocyte apoptosis at the concentrations employed (Fig. 4c).

Expression of heat shock proteins HSP72

Both PMA (0.5 µg/ml) and LPS (5 mg/ml) treatments, but not ConA, induced significant up-regulation of HSP72 expression in earthworm coelomocytes (Fig. 6).

Discussion

The internal defense mechanisms of annelids involve phagocytosis, nodule formation, encapsulation, blood coagulation and wound repair (Cooper 1996; Valembois et al. 1992). All these processes as well as reactions to
toxicants (e.g., heavy metals) lead to a reduction in the effector cell population which is followed by the reconstruction of their resources (Homa et al. 2007, 2008). In the present study, we revealed that in vivo dermal exposition of the earthworm *Eisenia andrei* to different immuno-stimulants induces changes in the number, composition and activity of coelomocytes. Moreover, we found that a reduction in the total number of coelomocytes increased cell proliferation. The most potent compounds causing this process are PMA and ConA, while LPS induces much weaker cell proliferation.

Currently, we cannot clearly state whether the reduction in the number of cells in the coelom results from their spontaneous release after dermal irritation with immuno-stimulants or whether it is related to aggregation and/or formation of brown bodies inside the cavity. Valembois and co-workers (1992) demonstrated that in earthworms the formation of brown bodies and their melanisation in the coelomic cavity may result from aggregation of coelomocytes around foreign cells, such as bacteria and gregarines (Valembois et al. 1992). Thus, it might be that also in the present experiments the activated coelomocytes were

![Flow cytometric analysis](https://example.com/flow-cytometry.png)

**Fig. 4** Flow cytometric analysis of the percentage of proliferating and apoptotic coelomocytes derived from earthworms (*Eisenia andrei*) kept in soil (n = 18) or exposed to filter paper soaked either with distilled water (0 mg/ml, n = 18) or PMA, LPS or ConA (n = 9–12). a Representative DNA histograms of coelomocytes from worms exposed to filter paper soaked with distilled water (control) or PMA (0.5 μg/ml), LPS (5 mg/ml) or ConA (5 μg/ml). b Percentages of proliferating coelomocytes. c Percentages of apoptotic coelomocytes. X ± SE. Different letters indicate the values that are significantly different according to ANOVA (e.g., “A” and “B”) while the values sharing the same letter (e.g., “A” and “AB”, “A” and “A”) are similar i.e. not statistically different.
“arrested” in capsules/aggregates and could not be retrieved by electric shock stimulation. This hypothesis may be supported by the observation that after 48 h of in vitro incubation with mitogens (especially ConA) both amoebocytes and eleocytes can form aggregates (data not shown).

Interestingly, dermal exposure of earthworms to all stimulants resulted in changes in cell composition. In the case of animals treated with stimulants, a reduction in the percentage of eleocytes and an increase in the percentage of granular amoebocytes was observed. Based on the observations of cell morphology and analysis of their autofluorescence (FL-1 fluorescence) we can exclude the possibility that cells described here as granular amoebocytes are partially degranulated eleocytes. However, we cannot exclude the possibility that the observed increase in amoebocyte granularity, demonstrated by the increase in side scatter (SSC), results from the cell activation and elevation of their lysosome numbers. A similar effect was observed in the case of human peripheral blood mononuclear cells (PBMC) after PMA treatment (Oppen et al. 2010). It was also shown that bacterial infection of earthworms Eisenia fetida increased the numbers of lysosomes in coelomocytes (Engelmann et al. 2004). The increased number of granular amoebocytes might alternatively result from their enhanced proliferation. In the earlier experiments, we showed that experimental coelomocyte depletion from Dendrobaena veneta stimulates proliferation of coelomocytes (Homa et al. 2008). In the present experiments, amoebocytes, consist the main cell-proliferating population in the coelom of immuno-stimulant-treated earthworms. Moreover, our previous experiments revealed that proliferation of eleocytes/chloragocytes can be found mainly in the typhlosole (Olchawa et al. 2006) suggesting that the main population of cells proliferating in the coelomic cavity are amoebocytes. In addition, in vitro tests showed that ConA, LPS or PHA stimulate proliferation of E. fetida coelomocytes (Cooper et al. 1995; Roch et al. 1975).

Furthermore, the data presented here suggest that the elevated contribution of the granular amoebocytes to the pool of coelomic cells influences the killing activity of the cells as measured by the respiratory burst and the release of nitric oxide. In vertebrates, it is well known that immuno-stimulants, such as LPS and PMA induce production of proinflammatory mediators such as NO and ROS, respectively (O’Neill 2011; Yamada et al. 2006). Our results also demonstrate that PMA and LPS stimulated

---

**Fig. 5** Representative microphotographs of *Eisenia andrei* DAPI-stained coelomocytes (amoebocytes - A, eleocytes - E) retrieved from animals exposed to filter paper soaked with distilled water (control) or PMA (0.01 µg/ml). Bright-field (left panel) and corresponding fluorescence microphotographs (right panel). Arrow indicates cell division.

**Fig. 6** Fold increase of heat shock protein (HSP72) expression (measured densitometrically) in coelomocyte lysates derived from earthworms (*Eisenia andrei*) kept in soil (n = 18) or exposed to filter paper soaked either with distilled water (0 mg/ml, n = 18) or PMA.

LPS or ConA (n = 9–12). X ± SE. Different letters indicate the values that are significantly different according to ANOVA (e.g. “A” and “B”) while the values sharing the same letter (e.g. “A” and “AB”, “A” and “A”) are similar i.e. not statistically different.
ROS generation in *E. andrei* coelomocytes. Previously, differences in ROS generation upon treatment with different immuno-stimulants have been observed in hemocytes of the American lobster (*Homarus americanus*) stimulated *inter alia* with ConA, LPS or PMA (Anderson and Beaven 2005). In this study, exposure to PMA induced a strong ROS response, while ConA and LPS failed to stimulate O2- generation (Anderson and Beaven 2005). In the case of earthworm coelomocytes, elevation of the respiratory burst was observed in vitro upon zymosan stimulation (Valembois and Lassègues 1995) and in vivo during brown body formation (Valembois et al. 1994). Several reports have described NO synthesis/release by invertebrate hemocytes originating from e.g. echinoder-mates (Beck et al. 2001), insects (Nappi et al. 2000) and snails (Wright et al. 2006), thus indicating that NO is the evolutionally conserved product of phagocytes (Beck et al. 2001). In our experiments, PMA and ConA, but surprisingly not LPS, stimulated production/release of NO by coelomocytes, while production of ROS was up-regulated in cells from animals treated with different doses of LPS and PMA. On the other hand, a significantly elevated percentage of apoptotic cells was observed only in coelomocytes retrieved from animals treated with LPS (2 mg/ml). One should keep in mind that production of reactive oxygen species, viewed as one of the first defense lines against invading pathogens, might also damage host cells, e.g. by inducing cell apoptosis. Therefore, the increased ratio of apoptotic coelomocytes upon LPS treatment could explain the simultaneously decreased NO release and weak cell proliferation.

Furthermore, elevated production of reactive oxygen species might stimulate a protective HSP response (Jacquier-Sarlin et al. 1994), which subsequently can interfere with the apoptotic pathway stimulation and protect cells from the stress-induced caspase-dependent apoptosis (Parcellier et al. 2003). For this expression of HSPs, expression such as the HSP72 protein is a good marker of cell stress (Kiang and Tsokos 1998). In our earlier experiments, we showed that exposure to heavy metals and high temperature induces expression of the HSP72 protein in coelomocytes (Homa et al. 2005, 2007; Kurek et al. 2001). In the present work, an increased level of heat shock proteins was observed in coelomocytes retrieved from animals treated with high concentrations of PMA or LPS, but not ConA. A similar increase in HSP72 expression was observed in U937 monocytes differentiated to macrophage-like cells upon PMA treatment (Twomey et al. 1993), while in lymphocytes HSP expression was induced with ConA (Ghassemi et al. 1991). However, in turn the increase in HSP levels in macrophages inhibited the release of cytokines, oxygen free radicals or nitric oxide and reduced the bactericidal capacity of the organism (Kiang and Tsokos 1998, Mochida et al. 2007). In the present work, HSP72 levels did not correlate with the synthesis of ROS and NO, while the potential anti-apoptotic role of HSP72 could only be seen in the case of earthworms treated with 5 mg/ml of LPS. At this concentration, the expression of HSP72 was significantly higher, while the percentage of apoptotic cells was low.

It should be stated here that mammalian leukocytes, including phagocytes, can be activated in vitro with very low LPS concentrations, down to 10 pg/ml (e.g. Mészáros et al. 1994), while cold-blooded vertebrates and invertebrates are much less sensitive to LPS. For example, insect hemocytes show significant in vitro response only when treated with 1–10 mg/ml of LPS (Wittwer et al. 1997). Consequently, in the current study we used concentrations of LPS and PMA that were higher in comparison to those used in vertebrates, while in the case of ConA we based our approach on earlier in vitro experiments (Cooper et al. 1995). The lower sensitivity of earthworms to LPS may be connected with their role *inter alia* in organic matter degradation and their continuous contact exposure to soil bacteria and other micro-organisms. The natural behavior of earthworms is increasingly being utilized for vermicomposting, and the extent to which earthworms promote the survival and dispersal of the bacterium (Williams et al. 2006).

In the present work we showed for the first time that dermal exposure of earthworms to various immuno-stimulants causes a broad range of changes in the number and activity of coelomocytes, including alternations in the production of oxygen free radicals and nitric oxide, and the expression of heat shock proteins, as well as cell proliferation and apoptosis. Furthermore, we demonstrated a different sensitivity of earthworms/earthworm coelomocytes to various stimulants. However, further work aimed to explain mechanisms of these processes is still required.

Acknowledgments The present study was supported by a grant BW/11/IZ/2010 and KZDS/001955.

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

Adamowicz A (2005) Morphology and ultrastructure of the earthworm *Dendrobena veneta* (Lumbricidae) celomocytes. Tissue Cell 37:125–133

Affar EB, Dufour M, Poirier GG, Nadeau D (1998) Isolation, purification and partial characterization of chloragocytes from the earthworm species *Lumbricus terrestris*. Mol Cell Biochem 185:123–133
Anderson RS, Beaven AE (2005) In vitro activation of hemocytes from the American lobster, Homarus americanus, as measured by production of reactive oxygen species. J Shellfish Res. 24:699–703

Beck G, Ellis T, Haiyan Z, Wenyu L, Beauregard K, Habicht GS, Truong N (2001) Nitric oxide production by coelomocytes of Asterores fasci. Dev Comp Immunol 25:1–10

Bilej M, Vetvicka V, Tuckova L, Trebichavsky L, Koukal M, Sima P (1990) Phagocytosis of synthetic particles in earthworms. Effect of antigenic stimulation and opsonisation. Folia Biol 36:273

Bilej M, De Baetselier P, Beschin A (2000) Antimicrobial defense of the earthworm. Folia Microbiol. (Praha) 45:283–300

Bilej M, De Baetselier P, Van Dijck E, Stijlemans B, Colige A, Beschin A (2001) Distinct carbohydrate recognition domains of an invertebrate defense molecule recognize Gram-negative and Gram-positive bacteria. J Biol Chem 49:45480–45487

Blackledge KH, Bidwell Cha (1993) Protocol for DNA measurement in fishes by flow cytometry. In: Robinson JP, Darzynkiewicz P, Dean I, Dressler H, Tanke H, Wheelless L (eds) Handbook of flow Flow cytometric quantification of proliferating coelomocytes 13 cytometry methods, 2nd edn. Wiley-Liss New York, NY, pp 225–228

Calisi A, Lionetto MG, Schettino T (2009) Pollutant-induced alterations of granulocyte morphology in the earthworm Eisenia foetida. Ecotoxicol Environ Saf 51:1369–1377

Cao HJ, Lin H-Y, Luidens MK, Keating T, Grandis JR, Davis FB (2009) Cooper EL (1996) Earthworm immunity. In: Rincevich B, Müller-Cholewa J, Feeney GP, O’Reilly M, Sturzenbaum SR, Morgan AJ, Chadzinska M, Savelkoul HF, Verburg-van Kemenade BML (2009) O’Neill LA (2011) A critical role for citrate metabolism in LPS signalling. Biochem J 438:433–436

Olchawa E, Schürenbaum MR, Morgan AJ, Płytczy B (2005) Early-phase immunodetection of metallothionein and heat shock proteins in extruded earthworm coelomocytes after dermal exposure to metal ions. Environ Pollut 135:275–280

Homa J, Sturzenbaum SR, Morgan AJ, Płytczy B (2007) Disrupted homeostasis in coelomocytes of Eisenia fetida and Allobophora chlorophora exposed dermally to heave metals. Europ. J Soil Biol. 43:273–280

Homa J, Browska M, Klimek M, Płytczy B (2008) Flow cytometric quantification of proliferating coelomocytes non-invasively retrieved from the earthworm, Dendrobaena veneta. Dev Comp Immunol 32:9–14

Jaqcuer-Sarlin MR, Fuller K, Dinh-Xuan AT, Richard MJ, Polla BS (1994) Protective effects of hsp70 in inflammation. Experientia 50:1031–1038

Kalač Y, Kimaran A, Ulakoglu G, Çotuk A (2002) The role of opsinin in phagocytosis by coelomocytes of the earthworm Dendrobaena venata. J. Cell Mol. Biol. 1.7:1–14

Kauschke E, Mohrig W, Cooper EL (2007) Coelomic fluid proteins as basic components of innate immunity in earthworms. Eur J Soil Biol. 43:110–115

Kiang JG, Tsokos GC (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol Ther 1998(80):183–201

Kozioł B, Markowicz M, Kruk J, Płytczy B (2006) Riboflavin as a source of autofluorescence in Eisenia fetida coelomocytes. Photochem Photobiol 82:570–573

Kurek A, Pyza E, Płytczy B (2001) Thermal shock induced expression of HSP70 and HSP72 proteins in coelomocytes of the earthworm Eisenia fetida. Cent Eur J Immunol 26:88–92

Kurek A, Homa J, Kauschke E, Płytczy B (2007) Characteristic of coelomocytes of the stubby earthworm, Allobophora chlorotica (Sav.). Eur J Soil Biol 43:1–6

Mészáros K, Aberle S, Dedrick R, Machovich R, Horwitz A, Birr C, Theofan G, Parent JB (1994) Monocyte tissue factor induction by lipopolysaccharide (LPS): dependence on LPS-binding protein and CD14, and inhibition by a recombinant fragment of bacterial/lipopolysaccharide-permeability-increasing protein. Blood 83:2516–2525

Mochida S, Matsura T, Yamashita A, Horie S, Ohata S, Kusumoto C, Nishida T, Minami Y, Inagaki Y, Ishibe Y, Nakada J, Ohya Y, Yamada K (2007) Geranylgeranylationcetone ameliorates inflammatory response to lipopolysaccharide (LPS) in murine macrophages: inhibition of LPS binding to the cell surface. J Clin Biochem. Nutr. 41:115–123

Nappi AJ, Vass E, Frey F, Carton Y (2000) Nitric oxide involvement in Drosophila immunity. Nitric Oxide 4:423–430

OECD (1984) Guidelines for the testing of chemicals. Earthworm acute toxicity tests, No 207

Olchawa E, Światkiwicz I, Homa J, Browska M, Płytczy B (2006) Restoration of coelomocytes in Dendrobaena veneta after experimental extrusion, in: “Molecular and physiological aspects of regulatory processes of the Organism”, 4–9th September. Krakow, Poland 106

O’Neill LA (2011) A critical role for citrate metabolism in LPS signalling. Biochem J 438:433–436

Oppor B, Németh P, Engelmann P (2010) Calcium is required for coelomocyte activation in earthworms. Mol Immuno 47:2047–2056

Parcellier A, Gurbuxani S, Schmitt E, Solary E, Garrido C (2003) Heat shock proteins, cellular chaperones that modulate...
mitochondrial cell death pathways. Biochem Biophys Res Commun 304:505–512
Peeters-Joris Ch (2000) The lysosomes of earthworm chloragocytes: biochemical and morphological characterization. Comp Biochem Physiol 126:323–340
Płtycz B, Klimek M, Homa J, Tylko G, Kolaczkowska E (2007) Flow cytometric measurement of neutral red accumulation in earthworm coelomocytes: novel assay for studies on heavy metal exposure. Eur J Soil Biol 43:116–120
Popović M, Hrzeňjak T, Grdisa M, Vuković S (1998) Adhesins of immunoglobulin-like superfamly from earthworm Eisenia fetida. Gen Pharmacol 30:795–800
Prenito P (1979) Metals and Phosphate in the Chloragosomes of Lumbricus terrestris and Their Possible Physiological Significance. Tissue Cell 196:123–134
Reinecke SA, Reinecke AJ (2004) The comet assay as biomarker of heavy metal genotoxicity in earthworms. Arch Environ Contam Toxicol 46:208–215
Rivero A (2006) Nitric oxide: an antiparasitic molecule of invertebrates. Trends in Parasitology 22:219–225
Roch P (1979) Protein analysis of earthworm coelomic fluid: polymorphic system of the natural hemolysin of Eisenia fetida andreii. Dev Comp Immunol 3:599–608
Roch P, Valenbois P, Du Pasquier L (1975) Response of earthworm leukocytes to Concanavalin A and transplantation antigens. Adv Exp Med Biol 64:45–54
Salamat Z, Sullivan JT (2009) Involvement of protein kinase C signalling and mitogen-activated protein kinase in the amebocyte-producing organ of Biomphalaria glabrata (Mollusca). Dev Comp Immunol 33:725–727
Silerova M, Kauschke E, Procházková P, Josková R, Tucková L, Bilej M (2007) Characterization, molecular cloning and localization of calreticulin in Eisenia fetida earthworms. Gene 397:169–177
Sullivan JT, Bulman CA, Salamat Z (2011) Effect of crude lipopolysaccharide from Escherichia coli O127:B8 on the amebocyte-producing organ of Biomphalaria glabrata (Mollusca). Dev Comp Immunol 35:1182–1185
Tough DF, Sun S, Sprent J (1997) T cell stimulation in vivo by lipopolysaccharide (LPS). J Exp Med 185:2089–2094
Twomey BM, McCallum S, Isenberg DA, Latchman DS (1993) Elevation of heat shock protein synthesis and hsp gene transcription during monocyte to macrophage differentiation of U937 cells. Clin Exp Immunol 93:178–183
Vadiveloo PK, Keramidaris E, Morrison WA, Stewart AG (2001) Lipopolysaccharide-induced cell cycle arrest in macrophages occurs independently of nitric oxide synthase II induction. Biochim Biophys Acta 1539:140–146
Valembois P, Lassègues M (1995) In vitro generation of reactive oxygen species by free coelomic cells of the annelid Eisenia fetida andreii: an analysis by chemiluminescence and nitro blue tetrazolium reduction. Dev Comp Immunol 16:95–101
Valembois P, Seymour J, Lassègues M (1994) Evidence of lipofuscin and melanin in the brown body of the earthworm Eisenia fetida andreii. Cell and Tissue Res 227:183–188
Vijver MG, Vink JPM, Miermans CJH, van Gestel CAM (2003) Oral sealing using glue: a new method to distinguish between intestinal and dermal uptake of metals in earthworms. Soil Biol Biochem 35:125–132
Wallin RP, Lundqvist A, Moré SH, von Bonin A, Kiessling R, Ljunggren HG (2002) Heat-shock proteins as activators of the innate immune system. Trends Immunol 23:130–135
Williams AP, Roberts P, Avery LM, Killham K, Jones DL (2006) Earthworms as vectors of Escherichia coli O157:H7 in soil and vermicomposts. FEMS Microbiol Ecol 58:54–64
Wittwer D, Weise C, Götz P, Wiesner A (1997) LPS (lipopolysaccharide)-activated immune responses in a hemocyte cell line from Estigmene acraea (Lepidoptera). Dev Comp Immunol 21:323–336
Wright B, Lachini AH, Davies AJ, Walker AJ (2006) Regulation of nitric oxide production in snail (Lymnaea stagnalis) defence cells: a role for PKC and ERK signalling pathways. Biol Cell 98:265–278
Yamada H, Arai T, Endo N, Yamashita K, Fukuda K, Sasada M, Uchiyama T (2006) LPS-induced ROS generation and changes in glutathione level and their relation to the maturation of human monocyte-derived dendritic cells. Life Sci 78:926–933