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Review

Cell signalling in the immune response of mussel hemocytes

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Abstract

In this work data on immune cell signallling in the circulating hemocytes of the edible bivalve, the mussel *Mytilus spp,* are summarized. Studies with different bacterial species and strains, heterologous cytokines and natural hormones, as well as with organic environmental chemicals, led to the identification of the role of conserved components of kinase-mediated transduction pathways, including cytosolic kinases (such as MAPKs and PKC) and kinase-activated transcription factors (such as STATs, CREB, NF-kB), in the immune response. From these data a general scenario emerged indicating that close similarities exist in the signalling pathways involved in cell mediated immunity in bivalve and mammalian immunocytes. In particular, the results indicate that both the extent and duration of activation of components of kinase-mediated cascades are crucial in determining the hemocyte response to extracellular stimuli. The identification of the basic mechanisms of immunity and its modulation in mussels can give important information for the possible utilization of these species as an invertebrate model for studies on innate immunity. Moreover, the application of this knowledge to the understanding of the actual adaptive responses of bivalves when exposed to micro-organisms in their natural environment can represent significant ecological, economical and public health-related interest.

Key words: *Mytilus*; hemocytes; innate immunity; kinase-mediated cell signalling

Introduction

Innate immunity has recently received a renewed interest, in particular due to genetic and molecular evidence supporting the hypothesis that it represents an ancient and evolutionary conserved defense system (Cooper ***et al.***, 2002, 2006). In particular, studies from the invertebrate model *Drosophila* have been crucial in the identification of key molecular components (such as the Toll-like receptors) of cell-mediated immunity also in vertebrates (Miester and Lagueux, 2003; Nappi ***et al.***, 2004).

However, invertebrates represent about 95 % of total species in animal kingdom and only for a few other invertebrate models information is becoming available on sequences coding for immune genes and genes involved in immune cell signalling (Gueguen ***et al.***, 2003; Shida ***et al.***, 2003; Venier ***et al.***, 2003; Kim and Ausuvel, 2005). Therefore, for many invertebrate species, information on the mechanisms of activation of the innate immune response, and in particular on the signall transduction pathways involved, has been largely obtained by indirect means, in functional studies utilizing antibodies and pharmacological inhibitors directed towards the mammalian counterparts of signalling components. The scenario emerging from these studies indicates that a high degree of conservation occurs between the pathways involved in the activation of immunocytes from molluscan species (mainly gastropods and bivalves) and those of mammals. In bivalves (mussels, oysters, etc.) a few molecular studies have identified sequences potentially involved in immune signalling (Escoubas ***et al.***, 1999; Gueguen ***et al.***, 2003; Montagnani ***et al.***, 2004).

Here we will summarize the results obtained on the signall transduction pathways involved in mediating the immune function of the edible marine

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bivalve, the mussel *Mytilus galloprovincialis* Lamk. Mussels are adapted to the natural exposure to a variety of microorganisms, and can accumulate in their tissues huge quantities of bacteria, mainly Gram negative enterobacteria and vibrios, that can be pathogenic both to the mussels and to humans. Although mussels are extremely simple organisms, they have a long life cycle (about 10 years) during which they are constantly exposed to microbes, and thus probably require rather complex homeostatic mechanisms to integrate both environmental and endogenous signalls. Functional and molecular characterization of the basic mechanisms underlying the innate immune response in bivalves can give important comparative information on the immune response and on its modulation in this invertebrate group that can be applied to the understanding of the actual adaptive responses of these molluscs exposed to microorganisms in their natural environment.

The immune system of bivalve mollusks

The immune system of bivalves consists of the blood cells, the hemocytes, and of soluble hemolymph factors, that operate in a co-ordinated way to provide protection from invading micro-organisms (Renwrantz, 1990; Rinkevich and Muller, 1996; Hine, 1999). Bivalve hemocytes are responsible for cell-mediated immunity through phagocytosis and various cytotoxic reactions (such as lysosomal enzyme and antimicrobial peptide release and oxyradical production); hemolymph serum contains humoral defense factors, such as soluble lectins, hydrolytic enzymes and antimicrobial peptides (Canesi *et al.*, 2002a; Pruzzo *et al.*, 2005) (Fig. 1). Bivalve hemocytes are extremely heterogeneous; classifications proposed for different bivalve species are reported elsewhere (Fryer and Bayne, 1996; Ottaviani *et al.*, 1998; Hine 1999; Wootton *et al.*, 2003). In *Mytilus spp.* granular hemocytes represent the dominant cell type in the hemolymph; they are characterised by a low nucleus/cytoplasm ratio, high phagocytic activity and capacity for oxyradical production (Wottoon *et al.*, 2003).

Survival of bacteria to the hemolymph microbicidal activity may depend on their different ability to attract phagocytes, to interact with opsonizing molecules, to bind to hemocyte surface, thus favouring or inhibiting the response of the host (Canesi *et al.*, 2002a; Pruzzo *et al.*, 2005). Bacteria could evade hemocyte killing by avoiding phagocytosis, generally by inducing damage in the hemocyte, as well as by preventing the oxidative burst associated with the phagocytic process. Finally, in analogy with host-pathogen interactions in mammalian models of infection (Rosenberger and Finlay, 2003), bacteria may undermine the hemocyte function through disregulation of the signalling pathways involved in hemocyte activation.

Cell signalling involved in the responses of *Mytilus* hemocytes to bacterial challenge: the role of MAPKs and PKC

In our first studies on the immune response of *Mytilus* hemocytes to bacterial challenge we utilized an *in vitro* model of hemocyte monolayers incubated in the presence of the soluble hemolymph fraction (hemolymph serum) with *E. coli* MG155, a wild type (wt) strain carrying the type 1 fimbriae (Canesi *et al.*, 2001a). This strain was shown to adhere to and to be internalized by hemocytes through mannose-sensitive interactions that were dependent on the presence of soluble serum components. The extent and time course of the hemocyte bactericidal were similar *in vitro* and *in vivo* (Canesi *et al.*, 2001a); therefore, the *in vitro* model was subsequently utilized for investigating the signall transduction pathways involved in the immune response.

In mammalian systems, activation of a complex network of signalling pathways, that include protein-kinase and lipid-kinase driven cascades, is critical in innate immunity. In particular, one of these pathways, involving members of the highly conserved Mitogen Activated Protein Kinase (MAPK) superfamily, plays a key role in both macrophage and neutrophil activation (Caffrey *et al.*, 1999). MAPKs are a family of serine-threonine kinases that are activated by phosphorylation in responses to different extracellular stimuli, with each member being activated by a distinct kinase cascade (Caffrey *et al.*, 1999). The Extracellularly regulated MAPKs (ERK MAPKs) are regulated by mitogens and growth factors; the stress-activated p38 and the c-Jun N-terminal kinases (JNKs) are activated by stress signalls such as UV, cytokines, heat and osmotic shock, endotoxin. A conserved role of MAPK members in mediating the pleiotropic response to growth factors was demonstrated in *Mytilus* cells (Canesi *et al.*, 2001b)

Cell signalling involved in the hemocyte response to bacterial challenge was first investigated by use of pharmacological inhibitors. Hemocyte bactericidal activity (i.e. percentage of bacterial killing) towards wt *E. coli* MG155 was prevented by cell pre-treatment with specific inhibitors of the stress-activated p38 MAPK and of PI-3kinase (phosphatidyl inositol 3 kinase), SB203580 and wortmannin, respectively. A significant decrease in the hemocyte response was also observed with inhibitors of enzymes involved in arachidonic acid production and metabolism, indicating a role for prostaglandins and leukotrienes in mediating the hemocyte response to bacterial challenge (Canesi *et al.*, 2002b).

Therefore, we first focused our attention on the involvement of MAPKs in hemocyte immune signalling. The presence and phosphorylation state (activation) of different MAPK-like proteins were evaluated by SDS-PAGE electrophoresis and Western blotting of hemocyte protein extracts with specific anti-phospho-antibodies directed against mammalian ERK MAPKs and p38 and JNK MAPKs. The key role of MAPKs, in particular of the stress-activated MAPKs, in the immune response of mussel hemocytes towards the wt *E. coli* strain was confirmed by the rapid increase in phosphorylation of p38 and JNK MAPKs induced upon bacterial challenge in the presence of hemolymph serum; activation of ERK MAPKs was also observed (Canesi *et al.*, 2002c).

Further information on the kinase-mediated pathways involved in the hemocyte response came from studies on the interactions with different bacterial species and strains; *Vibrio cholerae* strains were utilized as a model of autoctonous marine

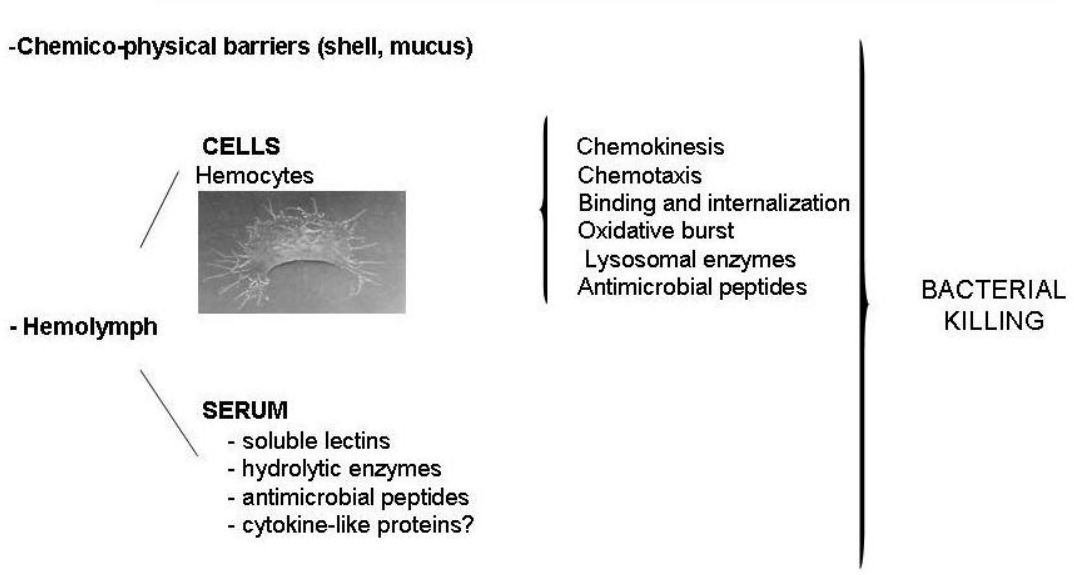

Fig. 1 Mechanisms involved in the bactericidal activity of bivalve hemolymph.

vibrios that are particularly accumulated in mussel tissues, where they can persist after depuration processes in controlled waters (Pruzzo *et al.*, 2005). Different strains of *E. coli* and *V. cholerae* incubated with hemocytes showed differences in adhesion and internalization (Canesi *et al.*, 2001a; Zampini *et al.*, 2003). These differences were associated with different sensitivities to the overall hemocyte bactericidal activity, with wt *E. coli* > Mutant *E. coli* ≅ wt *V. cholerae* > Mutant *V. cholerae.* We subsequently showed that different strains of *E. coli* and *V. cholerae* induced distinct patterns of MAPK phosphorylation in mussel hemocytes within a narrow time range (5-60 min) (Canesi *et al.*, 2005a). Although both wt and mutant *E. coli* strains induced an increase in the phosphorylation of the stress-activated p38 and JNK MAPKs, a different time course was observed in hemocytes incubated with the mutant *E. coli* strain compared to that observed with the wt *E. coli*: the increase in p38 MAPK phosphorylation was delayed, and JNK phosphorylation was only transient. These differences may be ascribed to differences in adhesion between the two strains to hemocytes in the presence of serum; since the higher adhesion of the wild strain was due to serum opsonins (Canesi *et al.*, 2001a), different mechanisms in binding to hemocytes of the two strains may involve differential activation of receptors, receptor complexes and signalling pathways, this resulting in different patterns of MAPK phosphorylation and leading to differential activation of the hemocyte response.

Interestingly, wt *V. cholerae* had little effect on MAPK phosphorylation, in particular on the stress-activated MAPKs; on the other hand, this strain induced a rapid and large Protein Kinase C (PKC) phosphorylation. A distinct scenario was observed with the *V. cholerae* mutant, that, among the tested strains, showed the lowest sensitivity to hemocyte microbicidal activity (Zampini *et al.*, 2003). Moreover, this strain induced severe cellular stress in the hemocytes, as indicated by the large destabilisation of lysosomal membranes, and the decrease in the phosphorylation state of all MAPK members (ERK, p38, JNKs), as well as of PKC (Canesi *et al.*, 2005a). These data suggested that the inability of mussel hemocytes to mount an efficient defense response against the *V. cholerae* mutant may be related to down-regulation of the hemocyte signalling pathways, in particular through interruption of signalling cascades upstream of MAPK activation, as previously described in mammalian host cells infected with pathogens (Rosemberger and Finlay, 2003). Overall, the results demonstrated that, in bivalve hemocytes like in mammalian cells, different bacteria and bacterial strains can differently modulate the host signalling pathways. Moreover, these studies indicated that not only the extent, but also the time course of bacteria-induced phosphorylation (activation) of different cytosolic kinases (MAPKs and PKC) is crucial in determining the hemocyte response.

Phosphorylation of transcription factors

In mammalian immunocytes different stimuli can activate a number of cytosolic kinases, including MAPKs and PKC, leading to phosphorylation of different transcription factors that can modulate the expression of various genes involved in the immune response (Su and Karin, 1996; Guha and Mackman, 2001). Among these, STAT proteins (Signall Transducers and Activators of Transcription) are a family of transcription factors conserved in vertebrates and invertebrates unique in that they act both as signalling molecules and as transcription factors (Decker and Kovarik, 1999; Horvath, 2000); moreover, they are the only transcription factor specifically activated by tyrosine phosphorylation (Decker and Kovarik, 1999; Horvath, 2000). Initially identified in interferon signalling (the Jak/STAT

pathway), STATs have been recognised as essential components of both adaptive and innate immunity (Stark *et al.*, 1998). In particular, STAT1 activation plays a critical role in the macrophage response against Gram negative bacteria (Ohmori and Hamilton, 2001).

In mussel hemocytes wt *E. coli* MG155 induced rapid and persistent tyrosine phosphorylation of immunoreactive STAT1-, STAT3-, and STAT5-like proteins (Canesi *et al.*, 2003a); the time course of STAT phosphorylation was consistent with that of the bactericidal activity, suggesting a physiological role for STAT-like proteins in mediating the immune function. We have recently observed that also the wt *V. cholerae* strain induced significant tyrosine phosphorylation of STAT1 and the results are reported in Fig. 2A. Interestingly, a distinct pattern of phosphorylation of STAT1 was observed in response to different bacteria; *V. cholerae* rapidly induced a large but transient increase in STAT1 phosphorylation, whereas the level of p-STAT1 steadily increased with time in response to *E. coli*.

We also investigated the effect of bacterial challenge on the phosphorylation state of the transcription factor CREB (c-AMP Responsive Element Binding protein), whose activation is mediated by serine/threonine kinases. CREB activation is an early response transcription factor whose role in inflammatory response has been well established; CREB can be phosphorylated at Ser133 by both PKA and MAPKs; interactions between CREB and other transcription factors such as STATs can modulate transcriptional activity (Decker and Kovarik, 1999). A CREB-like protein was identified in mussel hemocytes (Canesi *et al.*, 2005b). As shown in Fig. 2B, wt *E. coli* induced a rapid and dramatic increase in CREB phosphorylation; the level of p-CREB peaked at 5 min after addition of bacteria (reaching about a maximal ten-fold increase with respect to controls) and remained high up to 60 min. Also wt *V. cholerae* induced a significant CREB phosphorylation; however, the effect was transient and much smaller than that observed with wt *E. coli*. In macrophages, differential activation of CREB by different bacteria was shown to be dependent on differential activation of both PKA and p38 MAPK, with consequent effects on Tumor necrosis factor α (TNF α) production (Roach *et al.*, 2005). These data further support the hypothesis that, like in mammalian macrophages, conserved kinase-mediated signalling cascades lead to phosphorylation of transcription factors, this possibly resulting in modulation of transcriptional responses in the hemocyte. Moreover, the results further support the hypothesis that different bacteria have distinct effect on components of kinase-mediated cell signalling, this resulting in differential activation of the immune response.

Cytokine signalling

Many studies indicate that host defense mechanisms in different invertebrate groups, including molluscs, can be modulated by a cytokine network as in vertebrates; however, most information relies on functional assays, using heterologous cytokines and antibodies directed towards vertebrate cytokines and their receptors (reviewed by Beschin *et al.*, 2003; Ottaviani *et al.*, 2004). Although no molecular evidence for the presence of full length cytokine receptor or cytokine homologues has been provided in the genome of model invertebrates like *Drosophila melanogaster* and *Caenorhabditis elegans*, genes and corresponding proteins expressing domains found in vertebrate cytokines, cytokine receptors and components of cytokine signalling have been described in invertebrates (Beschin *et al.*, 2003). In *Mytilus spp*, heterologous cytokines have been shown to modulate the activity of the hemocytes, and functional analogues of different cytokines and cytokine receptors have been described in this species (Hughes *et al.*, 1990, 1993; Ottaviani *et al.*, 1995, 2000; Cao *et al.*, 2004).

In mammalian macrophages, complete activation results from stimulation with both bacterial products (LPS) and the cytokine interferon γ (IFNγ) that act in concert to generate a maximal capacity to ingest and kill the microbes through activation of signalling pathways, including MAPKs and STATs, leading to modulation of gene expression (Su and Karin, 1996, Guha and Mackman, 2001). We therefore tested the possibility that heterologous IFNs may modulate mussel hemocyte signalling and function. We observed that short-term hemocyte pre-treatment with human recombinant IFNγ, but not with IFNα, significantly increased the bactericidal activity of mussel hemocytes towards *E. coli* (Canesi *et al.*, 2003a). IFNγ stimulated tyrosine phosphorylation of different STAT-like members STAT1, STAT3 and STAT5. In particular, IFNã lead to persistent phosphorylation of immunoreactive STAT1; moreover, hemocyte pretreatment with IFNã, but not with IFNα, significantly increased STAT1 phosphorylation induced by bacterial challenge with *E. coli*.

IFNγ also affected the phosphorylation state of different MAPKs; in particular, activation of ERK_2 MAPK and slow downregulation of stress-activate MAPKs were observed. The extent and time course of MAPK phosphorylation induced by IFNγ were distinct from those elicited by either IFNα or bacterial challenge, again indicating a specificity of the hemocyte response to IFNγ. These results indicate that the hemocyte function can be modulated by heterologous cytokines and bacterial signals that act in concert through tyrosine kinase-mediated transduction pathways involving both MAPK- and STAT-like members. In particular, in mussel hemocytes, like in mammalian macrophages, both bacterial signals and IFNã converge on activation of STAT1, a transcription factor that plays a critical role in the response towards Gram negative bacteria.

In previous studies, mussel hemocytes were shown to be responsive to heterologous TNFα and produce TNFα–like molecules in response to bacterial components (Hughes *et al.*, 1990, 1993; Ottaviani *et al.*, 1995).TNFα is a pleiotropic cytokine that plays a pivotal role in orchestrating innate immune responses, as well as in regulation of cell proliferation, differentiation and apoptosis in vertebrates (Baud and Karin, 2001). TNFα signalling involves binding to members of TNF receptor superfamily (TNFRs) and recruitment of a complex of adapter proteins; among these, TNF-receptor-associated factors (TRAFs) activate several

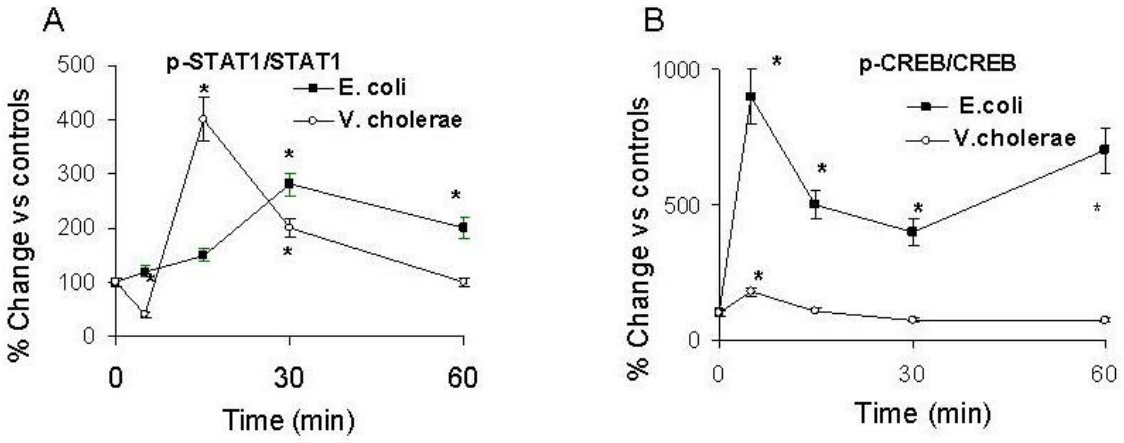

Fig. 2 Effects of bacterial challenge on the phosphorylation state of transcription factors of mussel hemocytes. Changes in phosphorylation of STAT1 (A) and CREB (B) are shown in hemocytes incubated with wt *E. coli* (strain MG155) and *V. cholerae* (O1 El Tor biotype strain N16961) in the presence of hemolymph serum for different periods of time. Hemocyte protein extracts were subjected to 12 % and 10 % SDS-PAGE, respectively, followed by western blotting using polyclonal phosphospecific antibodies directed against phosphorylated STAT1 (Tyr701) and CREB (Ser133) as previously described (Canesi *et al*., 2003a, 2005b). Bands were stripped and reprobed with antibodies to the corresponding unphosphorylated STAT1 and CREB forms. Data (mean±SD) of three independent experiments are expressed in % changes in p-STAT1/STAT1 and p-CREB/CREB with respect to controls. * = P 0.05, Mann Whitney U test. Relative increases in band optical densities (arbitrary units) were normalised for the control band in each series.

intracellular signall transduction pathways, in particular MAPKs and NF-kB, that lead to modulation of gene expression by different transcription factors (Baud and Karin, 2001). Members of TNFα transduction machinery have been characterized in invertebrates; in *Drosophila* a TNF superfamily ligand, *Eiger*, has been identified, that triggers JNK-dependent apoptosis (Cha *et al.*, 2003). In *Drosophila*, dTRAF1 is essential for endogenous JNK activation, whereas dTRAF2 is required for NF-kB signalling.

When we first assayed hemocyte functional parameters in response to heterologous TNFα, distinct effects were observed in the presence and absence of hemolymph serum (Betti *et al.*, 2006). When added in the absence of serum (in ASW-artificial sea water) TNFα induced cellular stress, as indicated by large lysosomal destabilization and decreased phagocytosis; on the other hand, in the presence of serum, TNFα did not affect lysosomal stability and significantly stimulated phagocytosis. TNFα induced rapid and large phosphorylation of the stress-activated p38 and JNK MAPKs, as well as of STAT1; activation of p38 and JNKs in mediating the effects of TNFá was confirmed by the use of specific MAPK inhibitors. However, the effects on MAPKs and STAT1 were persistent in ASW but transient in serum, a difference that had not been previously observed with IFNã. Flow cytometric analysis indicated that TNFα in the presence of serum induced transient phosphatidylserine exposure on the haemocyte surface, evaluated as annexin V binding; in ASW, the cytokine resulted in a stable increase in the percentage of both annexin V- and propidium iodide-positive cells, indicating possible apoptotic/necrotic processes.

More recent data indicate TNFα can also affect NF-*k*B signalling in the hemocyte.

Transcription factors of the NF-*k*B/Rel family play a pivotal role in the inflammatory and immune response (Gosh *et al*., 1998). NF-*k*B/Rel is composed of a set of structurally related and evolutionary conserved DNA binding proteins: members of class I (p100 and 105 in mammals and Relish in *Drosophila*) and members of class II RelA/p65, c-Rel, RelB in mammals, Dorsal, Dif in *Drosophila* and Gambif in *Anopheles*). A Rel homolog, *Cg*-Rel, has been characterized in oysters (Montagnani *et al*., 2004). P105 and p100 are activated by proteasomal degradation to p50 and p52 products that form dimeric complexes with Rel proteins, which are then able to bind DNA and regulate transcription. Activation and nuclear translocation of NF-kB is modulated through both phosphorylation and ubiqutination induced by different stimuli (Karin and Ben-Neriah, 2000).

Western blots of mussel hemocyte extracts with anti-NF-*k*B p105/p50 and anti-phospho-NF-*k*B-p65 antibodies showed the presence of immunoreactive protein bands corresponding to the p105 precursor and its cleavage product p50; moreover, a constitutively Ser536 phosphorylated p65-like protein was identified (Fig. 3). As shown in the figure, addition of TNFα to the hemocytes in the presence of serum did not apparently affect the level of p105 and p50; however, TNFá induced a significant, rapid and transient increase in the level of phosphorylated p65. Inducible phosphorylation of p65 at Ser536 has been described in mammalian

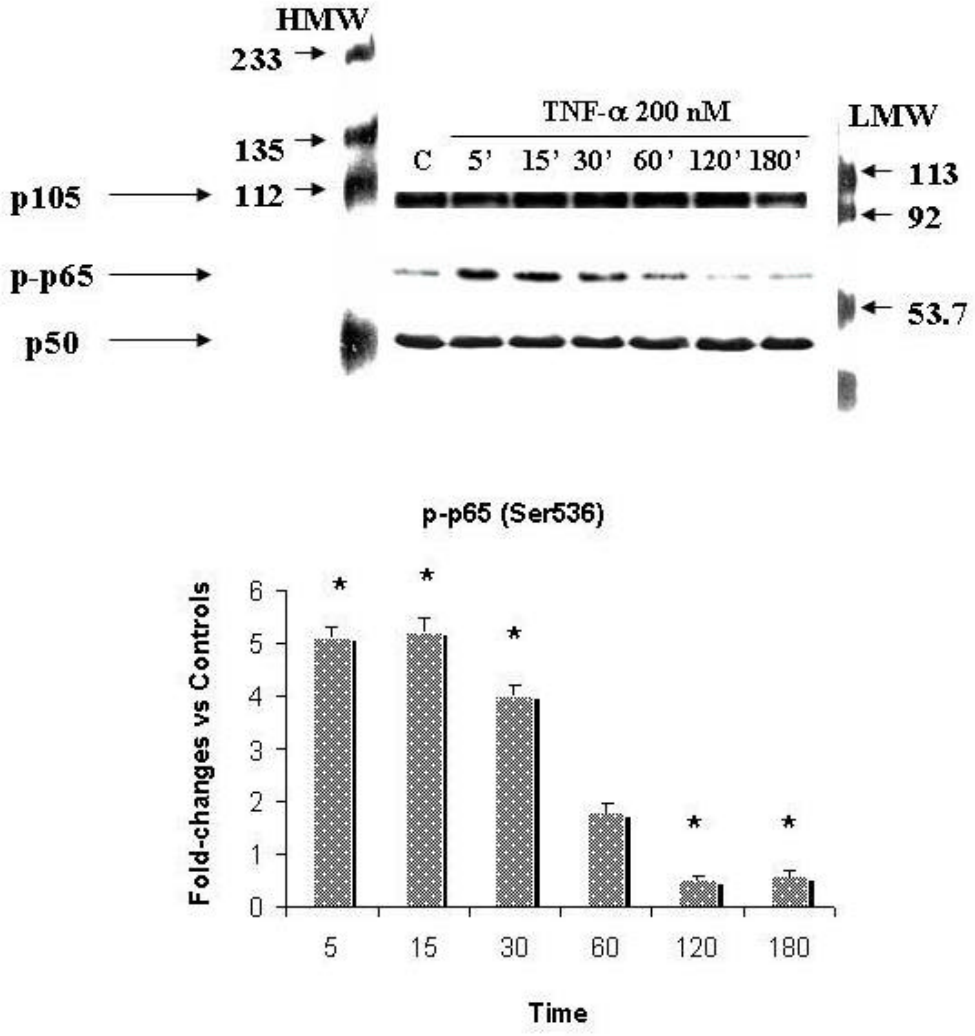

Fig. 3 Effect of TNFα (200 nM) on the level and phosphorylation state of NF-*k*B components in mussel hemocytes. Protein extracts from control and TNFα-treated hemocytes were subjected to 12 % SDS-PAGE followed by Western blotting using polyclonal antibodies to p105 and p50 (A), or polyclonal phosphospecific antibodies to p65 (Ser536) (B). Bands were detected using enhanced chemioluminescence reagents. Results are representative of three independent experiments. C = control. HMW and LMW protein standards. *Inset*: densitometric analysis of blots (p-p65) from three independent experiments (mean±SD). * = P≤0.05, Mann Whitney U test. Relative increases in band optical densities (arbitrary units) were normalised for the control band in each series.

cells in response to TNF and LPS, leading to increase in NF-kB transcriptional activity (Sasaki *et al.*, 2005). Our data support the hypothesis that components of NF-*k*B signalling are present in *Mytilus* hemocytes and that their activity may be modulated by heterologous TNFα.

Overall, the results indicate that TNFα can affect the function of bivalve haemocytes through conserved transduction pathways involving stress-activated MAPKs, STATs, and components of NF-kB signalling and suggest that the haemocyte response to this cytokine is influenced by soluble hemolymph components.

Endogenous immunomodulators: 17β-estradiol signalling

In bivalve molluscs, and *Mytilus* in particular, a few endocrine and immune modulators have been identified so far (Lacoste *et al.*, 2001; Stefano *et al.*,

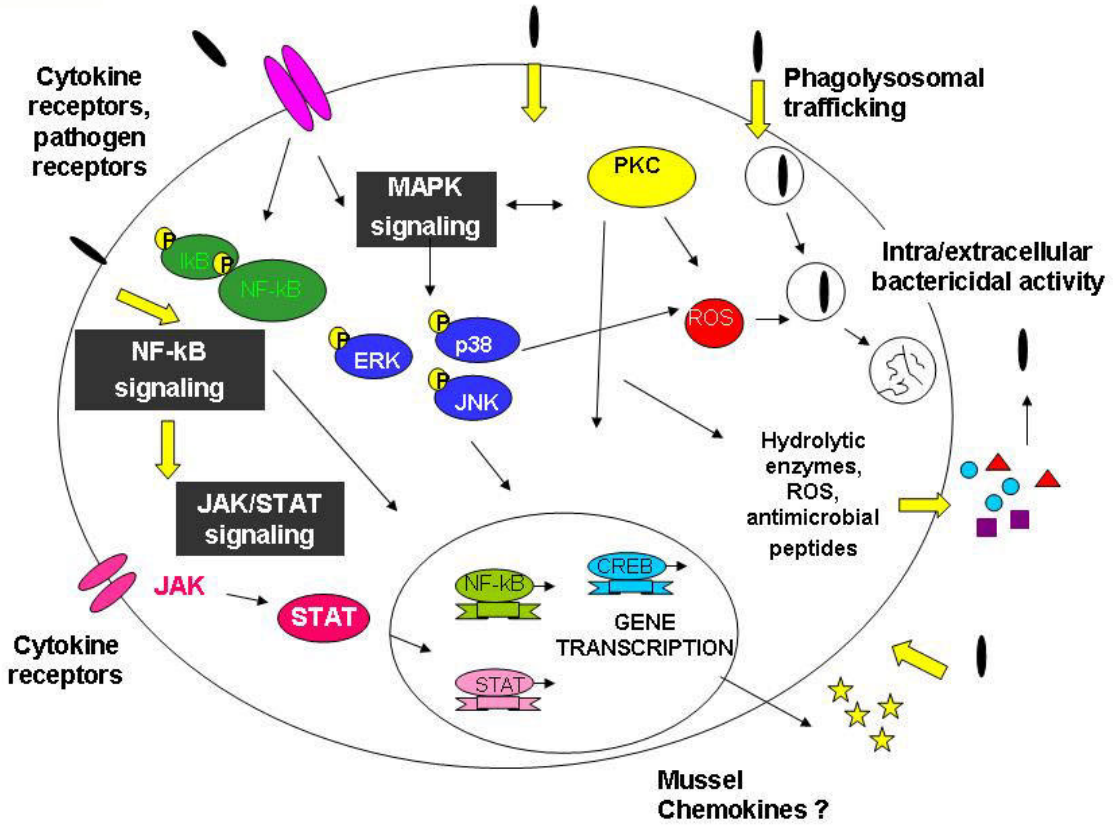

Fig. 4 Signalling pathways involved in the immune response of *Mytilus* hemocytes. The main signalling components activated by both bacterial challenge and heterologous cytokines are reported. MAPKs = Mitogen Activated Protein Kinases; ERK = Extracellularly Regulated Kinase; p38 = stress-activated p38 MAPK; JNK = c-Jun N-terminal kinase; PKC = Protein Kinase C; JAK = Janus Activated Kinase; STAT = Signall Transducer and Activators of Transcription; CREB =c-AMP Responsive Element Binding protein; NF-*k*B = nuclear Factor *k*B; ROS = Reactive Oxygen Species.

2003a). Estrogens have been identified in bivalves, where their role has been mainly investigated in the control of gametogenesis (Osada *et al.*, 2004; Gauthier-Clerc *et al.*, 2006). However, evidence has been provided that estrogen can represent an important signalling molecule involved in roles other than reproduction in these organisms. In neural tissues of *Mytilus* spp., 17β-estradiol (E_2) has been shown to downregulate ganglionic microglial cells after surgical insult; the effect was mediated by rapid induction of a Ca^{2+}-induced nitric oxide (NO) release by nervous tissue and were antagonized by classical antiestrogens (Stefano *et al.*, 2003b).

In *Mytilus*, E_2 was also shown to affect the digestive cells and circulating hemocytes, in particular at the level of the lysosomal function (Moore *et al.*, 1978; Burlando *et al.*, 2002). Addition of E_2 (in the low nM range) to hemocyte monolayers induced a moderate increase in cytosolic [Ca^{2+}], destabilisation of lysosomal membranes, morphological changes, hydrolytic enzyme release and stimulated the bactericidal activity towards *E. coli* (Canesi *et al.*, 2004a); all these effects were rapid, occurring from seconds to minutes from E_2 addition and were prevented by the antiestrogen tamoxifen (Canesi *et al.*, 2004a). The effects of E_2 were mediated by rapid activation of the stress-activated p38 MAPK. Moreover, in mussel hemocytes, E_2 induced increased tyrosine phosphorylation of STAT3- and STAT5-like members, as previously observed in mammalian cells. When the effects of E_2 on components of the immune response were investigated in more detail, we observed that E_2, in a narrow concentration range (5-25 nM), rapidly stimulated phagocytosis and oxyradical production; higher concentrations inhibited phagocytosis (Canesi *et al.*, 2006). E_2-induced oxidative burst was prevented by the nitric oxide synthase inhibitor L-NMMA and by SOD, indicating the involvement of both NO and $O_2^{\cdot-}$; NO production was confirmed by nitrite accumulation. Also these effects of E_2 were prevented by the antiestrogen tamoxifen and by SB203580, further supporting a receptor-mediated event and involvement of the p38 MAPK. E_2-induced

stimulation of phagocytosis and oxidative burst were also prevented by the PKC inhibitor GF109203X, indicating a role also for PKC in mediating the effects of E_2. In fact, E_2 induced rapid and transient increases in the phosphorylation state of PKC, and in particular of a PKCα/βII-like isoform, as well as of the transcription factor CREB. The results demonstrated that the signalling components that play a key role in the immune response of mussel hemocytes represent significant targets for the action of E_2. The effects of E_2 on the immune function were confirmed *in vivo*. For longer exposure times (6 and 24 hrs) in the hemocytes of E_2-injected mussels lower concentrations resulted in immuno-stimulation, whereas higher concentrations had inhibitory effects (Canesi *et al.*, 2006). Overall, the obtained data indicate that E_2 signalling appears to be conserved from invertebrates to mammals.

Immune signalling as a target for environmental contaminants

Different environmental contaminants known as Endocrine Disrupting Chemicals (EDCs) (Witorsch, 2002) have been shown to affect the hemocyte function through modulation of the signall transduction pathways involved in the activation of the immune response. First data were obtained with polychlorinated biphenyls (PCBs): different *ortho*-substituted, non coplanar PCB congeners were shown to affect the immune function through disregulation of MAPK and STAT signalling (Canesi *et al.*, 2003b). In particular, the di-*ortho*-substituted PCBs P47 (2,2',4,4'-tetrachlorobiphenyl) and P153 (2,2',4,4',5,5'-hexachlorobiphenyl) were shown to rapidly increase the phosphorylation state of both MAPKs and STAT members. Among the tested congeners P47 showed the strongest immunotoxic effect, resulting in lysosomal destabilization, inhibition of *E. coli*-induced lysozyme release, decrease in the overall bactericidal activity; the effects were mainly due to a large and persistent phosphorylation of the stress-activated MAPKs p38 and JNKs.

Other EDCs, including synthetic estrogens, phytoestrogens and estrogenic chemicals that represent significant contaminants of the aquatic environment were shown to induce destabilization of lysosomal membranes in mussel hemocytes (Canesi *et al.*, 2004b). The effects were prevented by different kinase inhibitors, indicating that the effect of each compound was mediated by activation of different signalling components. When the effects of different EDCs on the phosphorylation state of MAPKs and STATs were evaluated, certain synthetic estrogens, like DES (diethylstilbestrol) were shown to induce activation of p38 MAPK and STATs, with effects similar to those observed with the natural estrogen E_2, although at higher concentrations; other estrogenic chemicals, such as the alkylphenols bisphenol A and nonylphenol, induced a large decrease in phosphorylation of p38 MAPK and STAT5. The effects of BPA on hemocyte function and signalling were confirmed *in vivo*, in the hemocytes of mussels sampled at different times post-injection with the compound (Canesi *et al.*, 2005b). The results showed that also *in vivo*, and at longer exposure times, BPA induced hemocyte lysosomal destabilization and decrease in phosphorylation state of p38 MAPK, STAT5, and CREB, indicating down-regulation of cell signalling and possible immunodepression.

Another class of contaminants identified as potential hemocyte immunomodulators are certain brominated flame retardants (Binmbaum and Staskal, 2004). TBBPA (tetrabromobisphenol A) was shown to induce activation of ERK, p38 and JNK MAPKs and PKC. These effects on cell signalling resulted in stimulation of the overall microbicidal activity through increase in phagocytosis and oxidative burst (Canesi *et al.*, 2005c).

The results so far obtained indicate that different organic contaminants known as endocrine disruptors in vertebrate system can also act as immune disruptors in mussel hemocytes through disregulation of different components of kinase mediated cell signalling, resulting in distinct effects depending on the compound tested.

Conclusions

The signalling pathways involved in the innate immune response show common features in bivalve hemocytes and mammalian immunocytes. From the results obtained so far, the main signalling components that play a role in the immune function of *Mytilus* hemocytes can be summarized as shown in Fig. 4.

The results obtained with different stimuli (bacteria, cytokines, hormones, environmental chemicals) support the hypothesis that both the extent and duration of activation of components of kinase-mediated cascades are crucial in determining the hemocyte response to extracellular stimuli. In general, sustained but transient phosphorylation of both cytosolic kinases and transcription factors seems to be associated with efficient activation of the immune response; on the other hand, large and persistent activation of signalling components leads to cellular stress and irreversible damage. Conversely, down-regulation of hemocyte signalling observed in response to certain bacteria of contaminants impairs hemocyte activation with different consequences ranging from immunodepression to citotoxicity.

However, the study of immune signalling in bivalve molluscs, compared to that in other invertebrate models, is still at its infancy. The scheme depicted in Fig. 4 does not include components whose involvement in mussel hemocytes has been suggested by indirect observations, such as the cAMP/protein kinase A (PKA) pathway, PI-3 kinase, or enzymes involved in arachidonic acid metabolism. Moreover, information immune-related receptors (Copper, 2006), on signalling components upstream of MAPK activation (Caffrey, 1999), as well as on the role of the Rho family GTPases, that are involved in many processes essential to the coordination of the complex machinery underlying innate immunity (Bokoch, 2005), is still lacking.

The identification of the basic mechanisms of immunity and its modulation in mussels can give important information for the possible utilization of this species as an useful invertebrate model for

studies on innate immunity. Moreover, the application of this knowledge to comprehension of the actual adaptive responses of bivalves when exposed to micro-organisms in their natural environment can represent significant interest not only from an ecological, but also a financial point of view. These studies can provide the basis for better understanding the reasons for the persistence of certain pathogenic agents in bivalve tissues, and possibly to prevent diseases in the molluscs themselves, with obvious advantages for the conservation of natural populations. The identification of the processes that influence the elimination of microorganisms by edible bivalves can also give useful information to devise strategies to ameliorate the depuration processes utilized in shellfish farming that are needed to lower the microbial load to levels acceptable for human consumption; these data can therefore be of interest for human health, since they contribute to prevent the risk of disease. A great effort is being dedicated by different groups at identifying immune- and signall transduction-related sequences in different molluscan species, including edible bivalves such as mussels and oysters. In this light, research carried out by the Bivalve Activity Group, within the activities of the ongoing project “Improved immunity of Aquacultured Animals- IMAQUANIM” (Sixth Framework EU Programme - Area T6.4, project n. 007103), will represent a crucial advance in the identification of the molecular and functional aspects of the immune function in these organisms.

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