The CRH-ACTH-biogenic amine axis in invertebrate immunocytes activated by PDGF and TGF-β

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Abstract In immunocytes from the mollusc *Mytilus galloprovincialis*, the major pathway followed by platelet-derived growth factor (PDGF)-AB and transforming growth factor (TGF)-β1 in provoking the release of norepinephrine, epinephrine and dopamine into cell-free hemolymph (serum) is mediated by a corticotropin-releasing hormone-adrenocorticotropin hormone (CRH-ACTH) biogenic amine axis. This axis not only annulled the inhibiting properties of PDGF-AB, it even reversed the latter's effect, while the inducing effect of TGF-β1 was amplified. These findings show that non-classical immune-neuroendocrine molecules, such as PDGF-AB and TGF-β1, are involved in building stress response, using the same conserved mechanisms present from invertebrates to vertebrates.

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Key words: Platelet-derived growth factor AB; Transforming growth factor-β1; Adrenocorticotropin hormone; Corticotropin-releasing hormone; Interleukin-1α; Biogenic amine; Invertebrate immunocyte; Stress response; High-performance liquid chromatography

1. Introduction

Stress response is a coordinated series of metabolic events that enable the body to cope with a variety of agents threatening life. We have shown that in invertebrates an ancestral type of stress response is present, and that the basic mechanisms and molecules involved are well preserved and fundamentally similar throughout evolution. As in vertebrates, the cascade follows the same order and pattern: corticotropin-releasing hormone (CRH) → adrenocorticotropin hormone (ACTH) → biogenic amines. The invertebrate immunocyte contains the same actors which are involved in a different, more simplified scene [1]. The cytokine interleukin (IL)-2 provokes a significant release of biogenic amines from the immunocytes, while this release is reduced when hemolymph is pre-incubated with IL-1α, IL-1β, IL-2, tumor necrosis factor (TNF)-α or TNF-β before the addition of CRH [2,3].

Recently, we have found that growth factors, such as platelet-derived growth factor (PDGF)-AB and transforming growth factor (TGF)-β1, also intervene in biogenic amine activation, the former inhibiting and the latter inducing the release of these molecules, suggesting an involvement in molluscan stress response [4].

In the present paper we report on the relationship between PDGF-AB and TGF-β1 and the classical mediators of stress response, with the aim of clarifying how the growth factors participate in the construction of this adaptive type response originally described by Selye [5].

2. Materials and methods

2.1. Animals

Specimens of *Mytilus galloprovincialis* Lmk. were collected from rocks in the Adriatic sea around Cattolica (Rimini, Italy) and maintained in the laboratory. The hemolymph was collected from the posterior adductor muscle with a sterile 2-ml syringe.

2.2. Sample preparation

For each experiment, which was repeated three times, 18 ml of hemolymph was divided into 12 parts (1.5 ml each) and placed in plastic tubes. To 6 tubes, the following substances were added: marine solution (MS) [6] (control sample), PDGF-AB (Sigma Chemical Co., St. Louis, MO, USA) (20 ng/ml final concentration) [6], TGF-β1 (Sigma Chemical Co., St. Louis, MO, USA) (5 pg/ml final concentration) [6], recombinant human interleukin (IL)-1α (Genzyme Co., Cambridge, MA, USA) (1000 U/ml) [3], CRH (Sigma) (10^{-8} M final concentration) [7] and ACTH (Sigma) (10^{-8} M final concentration) [7]. Of the 6 remaining tubes, two were sequentially pre-incubated with ACTH (40 min), CRH (40 min) and IL-1α (40 min), two with ACTH (40 min) and CRH (40 min), and two with ACTH (40 min) alone prior to the addition of PDGF-AB or TGF-β1. The tubes were then placed in the dark on a revolving mixer, incubated for 20 min at room temperature and immediately centrifuged ($600 \times g$ for 15 min). The biogenic amines norepinephrine (NA), epinephrine (A) and dopamine (DA) were determined on the supernatant (cell-free hemolymph) (serum) using high performance liquid chromatography.

In order to verify whether the NA, A and DA values are time-dependent, these catecholamines were determined at different time points in the samples incubated singularly with MS, ACTH, PDGF-AB and TGF-β1. The time points for MS were 20, 40, 60, 80, 100, 120 and 140 min, for ACTH 20, 40 and 60 min, and for PDGF-AB or TGF-β1 20 and 60 min. The samples were prepared following the procedure previously described.

2.3. Determination of biogenic amines

The procedure is described in detail in a previous paper [4]. Briefly, the serum was pre-purified using a Clin-Rep-Catecholamine Kit and the extracted sample was analyzed with a rapid and simple isocratic simultaneous determination of NA, A and DA. Monoamine peaks were identified by comparing their retention times in the sample (serum extracts) solution with those of a standard solution. Each biogenic amine in the serum was quantified using the internal standard (DHBA) method with a correction factor.

2.4. Apparatus

The HPLC system consisted of a Isocratic LC Pump (mod. 250 Perkin Elmer) equipped with a degasser (ERC-3512-Erma), an automatic injector (Rheodyne 7125, 100-μl loop), an electrochemical detector (mod. 460 Waters) and a Data System PE Nelson (mod. 1020 Perkin Elmer).

2.5. Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) of logarithmic values, followed by the Student-Newman-Keules multiple comparison test.

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Table 1
Concentrations of biogenic amines determined by HPLC in cell-free hemolymph (serum) of *Mytilus galloprovincialis* in the presence of 20 ng/ml PDGF-AB, 5 pg/ml TGF-β1, 1000 U/ml IL-1α, 10^{-8} M CRH and 10^{-8} M ACTH, respectively

	MS	PDGF-AB	TGF-β1	IL-1α	CRH	ACTH
NA	151.33 ± 5.69	107.33 ± 8.62*	275.33 ± 11.68*	368.33 ± 11.06*	265.67 ± 12.90*	229.00 ± 14.18*
A	33.03 ± 0.99	b.s.	50.10 ± 3.12*	49.93 ± 2.46*	47.64 ± 2.24*	43.40 ± 1.28*
DA	108.33 ± 6.10	118.20 ± 7.45	115.30 ± 7.35	141.20 ± 7.30*	107.85 ± 5.10	117.00 ± 5.00

NA, norepinephrine; A, epinephrine; DA, dopamine; b.s., below the sensitivity of the HPLC assay. The detection limit is 20 pg. The mean ± S.D. of three experiments is shown. Data are expressed as pg/ml. Statistical analysis was performed by ANOVA. *$P < 0.01$ vs. MS, marine solution (control).

Table 2
Concentrations of biogenic amines determined at different times by HPLC in cell-free (serum) hemolymph of *Mytilus galloprovincialis* in the presence of MS, 10^{-8} M ACTH, 20 ng/ml PDGF-AB, 5 pg/ml TGF-β1, respectively

Time (min)		0	20	40	60	80	100	120	140
MS	NA	151.30 ± 5.69	151.30 ± 3.14	147.20 ± 5.0	153.70 ± 5.0	156.30 ± 4.0	154.30 ± 5.10	158.60 ± 3.50	152.30 ± 3.50
	A	33.03 ± 0.99	35.0 ± 2.0	30.30 ± 1.50	30.0 ± 1.0	35.30 ± 2.10	34.0 ± 2.60	34.30 ± 4.0	33.30 ± 0.60
	DA	108.33 ± 6.10	108.70 ± 6.10	111.0 ± 3.60	111.70 ± 5.50	115.0 ± 4.0	110.70 ± 5.50	109.0 ± 2.0	113.30 ± 6.10
ACTH	NA	229.0 ± 14.18	230.0 ± 14.10	238.30 ± 11.37	226.0 ± 24.70				
	A	43.40 ± 1.30	43.80 ± 0.95	44.30 ± 1.25	44.90 ± 0.85				
	DA	117.0 ± 5.0	123.30 ± 4.20	118.0 ± 7.0	121.70 ± 3.50				
PDGF-AB	NA	107.33 ± 8.62	100.30 ± 4.72		107.0 ± 10.50				
	A	b.s.	b.s.		b.s.				
	DA	118.20 ± 7.45	123.0 ± 5.0		119.0 ± 8.20				
TGF-β1	NA	275.33 ± 11.68	280.30 ± 8.50		279.0 ± 12.0				
	A	50.10 ± 3.12	52.0 ± 1.73		54.30 ± 1.15				
	DA	115.30 ± 7.35	118.0 ± 6.50		116.10 ± 7.50				

NA, norepinephrine; A, epinephrine; DA, dopamine; b.s., below the sensitivity of the HPLC assay. The detection limit is 20 pg. The mean ± S.D. of three experiments is shown. Data are expressed as pg/ml. Statistical analysis was performed by ANOVA.

3. Results

Chromatographic analysis of hemolymph samples from the mollusc *M. galloprovincialis* pre-incubated with TGF-β1, IL-1α, CRH and ACTH demonstrated, as expected, a significantly greater release of biogenic amines with respect to controls. The opposite response was observed with PDGF-AB (Table 1). The more intense increase in amine concentration was observed with NA, in particular in the presence of IL-1α. The values of A in the presence of PDGF-AB were below the sensitivity of the method, while in the presence of TGF-β1, IL-1α, and CRH a significant release occurred. The values of DA both in the treated samples and the controls were practically unchanged. The only significant difference was observed in the presence of IL-1α.

Amine concentrations in samples incubated with MS at different times showed no significant modifications at the various time points, suggesting that the release of biogenic amines was time-independent (Table 2). The same behavior was also observed when the single effects of ACTH, PDGF-AB or TGF-β1 were analyzed. Indeed, the coefficient of variation (cv %) is always ≤ 6% (Table 2).

As reported in Table 3, the inhibiting effect of PDGF-AB on NA and A release was annulled when the hemolymph was pre-incubated with ACTH followed by CRH. Even higher concentrations were observed after pre-incubation with ACTH alone. However, DA concentration was elevated only after pre-incubation only with ACTH.

The effect of TGF-β1 on amine release increased significantly when the hemolymph was pre-incubated with either ACTH followed by CRH and IL-1α or with ACTH followed by CRH. A reverse response was observed after pre-incubation with ACTH alone (Table 4).

The responses elicited by PDGF-AB and TGF-β1 on hemolymph pre-incubated with ACTH alone deserve particular attention. As reported in Tables 3 and 4, PDGF-AB provoked

Table 3
Concentrations of biogenic amines determined by HPLC in cell-free hemolymph (serum) of *Mytilus galloprovincialis* following addition of 20 ng/ml PDGF-AB alone and after pre-incubation with 10^{-8} M ACTH followed by 10^{-8} M CRH and by 1000 U/ml IL-1α (PDGF-AB I), or with 10^{-8} M ACTH followed by 10^{-8} M CRH (PDGF II), or with 10^{-8} M ACTH (PDGF-AB III)

	PDGF-AB	PDGF-AB I	PDGF-AB II	PDGF-AB III
NA	107.33 ± 8.62	102.68 ± 4.12	149.67 ± 9.50*	245.77 ± 17.75*
A	b.s.	36.22 ± 2.64*	53.46 ± 2.90*	91.58 ± 2.60*
DA	118.20 ± 7.45	105.00 ± 10.53	104.33 ± 13.0	5143.67 ± 10.02*

NA, norepinephrine; A, epinephrine; DA, dopamine; b.s., below the sensitivity of the HPLC assay. The detection limit is 20 pg. The mean ± S.D. of three experiments is shown. Data are expressed as pg/ml. Statistical analysis was performed by ANOVA. *$P < 0.01$ vs. PDGF-AB.

Table 4
Concentrations of biogenic amines determined by HPLC in cell-free hemolymph (serum) of *Mytilus galloprovincialis* following addition of 5 pg/ml TGF-β1 alone and after pre-incubation with 10^{-8} M ACTH followed by 10^{-8} M CRH and by 1000 U/ml IL-1α (TGF-β I), or with 10^{-8} M ACTH followed by 10^{-8} M CRH (TGF-β1 II), or with 10^{-8} M ACTH (TGF-β III)

	TGF-β1	TGF-β1 I	TGF-β1 II	TGF-β1 III
NA	275.33 ± 11.68	342.10 ± 16.05*	365.33 ± 13.05*	148.00 ± 11.13*
A	50.10 ± 3.12	77.25 ± 1.87*	137.02 ± 4.62*	49.96 ± 1.52
DA	115.30 ± 7.35	234.67 ± 11.93*	136.00 ± 9.16*	100.63 ± 10.50

NA, norepinephrine; A, epinephrine; DA, dopamine; b.s., below the sensitivity of the HPLC assay. The detection limit is 20 pg. The mean ± S.D. of three experiments is shown. Data are expressed as pg/ml. Statistical analysis was performed by ANOVA. *$P < 0.01$ vs. TGF-β1.

an unexpected increase in biogenic amine concentrations. The opposite behavior was seen with TGF-β1, where amine concentrations fell.

4. Discussion

PDGF-AB or TGF-β1 added to *M. galloprovincialis* immunocytes alone or after pre-incubation with various substances, such as ACTH, CRH and IL-1α, affect biogenic amine release.

The inhibiting effect of PDGF-AB and the inducing effect of TGF-β1 is evidently a general phenomenon, as the same behavior has been reported in two other molluscan species, i.e. *Planorbarius corneus* and *Viviparus ater* [4].

Concerning the action of the two growth factors after pre-incubation with other substances there seems to be a preferential pathway from CRF to ACTH, which in turn stimulates biogenic amine release. This axis seems to be the principal route of the amine release.

PDGF-AB shows a high capability in down-regulating serum biogenic amine concentration, which is reversed after pre-incubation with CRH and/or ACTH. On the other hand, while pre-incubation with both hormones enhances the stimulatory effect of TGF-β1, the effect of ACTH alone decreases the TGF-β1-mediated biogenic amine release.

At present, it is not easy to explain the effect of PDGF-AB and TGF-β1 on biogenic amine release after pre-incubation of the hemolymph with ACTH alone. However, Rainey et al. [8] have reported that TGF-β decreases the number of ACTH binding sites in ovine adrenocortical cells and completely blocks the ability of ACTH to increase the number of its receptor. Further studies are needed for the elucidation of the interplay between growth factors and classical hormones at receptor and post-receptor level in invertebrate immunocytes.

IL-1α exerts a profound effect on NA, A and DA release, and, as reported for IL-2 [2,3], is involved in a prototype stress response in molluscs. In an evolutionary perspective, this new finding is not unexpected, bearing in mind the major role that cytokines such as IL-1 are known to play in the stress response in vertebrates and in the activation of the hypothalamus-pituitary-adrenal axis [9,10].

As reported before, another aspect to underline from these results, is the importance of the order of combination of the molecules [4]. While in the present experiments CRH and ACTH have a deep effect on the action of PDGF-AB and TGF-β1, we previously found that the addition of CRH or ACTH to hemolymph pre-incubated with PDGF-AB or TGF-β1 did not influence the inhibiting or inducing properties of the two growth factors [4].

The different responses from the two growth factors used alone or in combination with other molecules suggest greater flexibility in the system which neutralizes stimuli in order to maintain soma integrity. According to different demands, one pathway may prevail over the others. The basic constraint is the efficiency of the combination with respect to its compatibility with the environment.

From an evolutionary point of view, we have proposed three different levels of organisation of the stress response [11]. The first level is that of the single, mobile and diffuse cell, such as the invertebrate immunocytes and the vertebrate blood cells. The second level (local stress) involves cells found singularly or in small groups within an endocrine gland, such as the thymus. Finally, a third and more centralized level is represented by the nervous system. At all these levels, stress mediators are present. Bearing in mind this hypothesis and the data presented in this paper, we can also surmise an involvement of growth factors not only in a single cell, but also in local stress response. A similar hypothesis has already been proposed for CRH, ACTH and IL-1α [1,11]. For instance, it is well known that mammalian anterior pituitary cells produce hormones such as ACTH, releasing factors such as CRH, cytokines such as IL-1 and growth factors such as PDGF and TGF-β [12–15]. However, the biological activity of some of these molecules is still little known [15]. As the pituitary gland is both the target and the producer of peptides and their related receptors, auto- and paracrine mechanisms may be involved [14,15]. Thus, the findings presented here could be explained, at least in part, by the adaptation of pituitary function to altered physiological conditions, such as stress, pregnancy and disease.

In conclusion, this study shows that growth factors participate in the complex relationship between immune and neuroendocrine interactions, and must therefore be included with the classical mediators of immune and neuroendocrine systems among the components of the integrated mechanism devoted to the maintenance of body homeostasis. Indeed, it is well known that PDGF-AB and TGF-β1 play a role in cell migration [6,16], phagocytosis [16] and stress response [4].

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