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INVOLVEMENT OF HUMAN BETA-DEFENSIN-2 IN INTRACELLULAR SIGNALING: *IN VITRO* STUDY

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Aim: **To analyze involvement of human beta-defensin-2 (hBD-2) in intracellular signaling *in vitro*. *Materials and Methods:* A431cells were cultured in the presence of 1 μg/ml of recombinant hBD-2 and/or 10 ng/ml EGF. For evaluation of expression of mRNAs for p70S6 kinase, isoforms alpha and beta, RT-PCR analysis was applied. Expression and activity of p70S6K, phosphorylation of PDK1, ERK, JNK, p38 kinases and EGF receptor (EGFR) was evaluated using Western blot analysis. *Results:* 30 min incubation of A431 cells with 1 μg/ml of hBD-2 didn't influence autophosphorylation level of EGFR, but resulted in activation of p70S6K, 12 h treatment – in prominently increased level of mRNA for alpha and beta-isoforms of p70S6 kinase, whilst 24 h treatment – in elevation of p70S6K synthesis on protein level. Up-stream kinase phosphorylating p70S6K, PDK1, is also phosporylated upon influence of exogenous hBD-2 *in vitro*. *Conclusion:* Our data point on the involvement of PDK1-p70S6K pathway in mediation of action of hBD-2 in A431 cells.**

Key Words: **human beta-defensin-2, EGF, EGF receptor, p70S6 kinase, PDK1.**

Human defensins — small antimicrobial peptides — are in the focus of numerous researches aimed on evaluation of their structure, biological functions and patterns of expression *in vitro* and *in vivo* in normal state and upon pathology [1, 2]. Such interest to these biomolecules is due to recently established involvement of defensins in a number of processes important for cell behavior — proliferation, differentiation, growth and death [3–6]. On multicellular level, these antimicrobials are taking part in the protection of host from invading pathogens, functioning of immune system, tissue repair and tumorigenesis [1, 2, 5, 7–13].

It is established also that in *in vitro* models production of defensin and/or exogenous addition of defensins to the cells results in elevation of their proliferation rate [3, 4, 11]. At the same time the mechanisms mediating defensin-dependent cell proliferation remains poorly studied yet as well as potential intracellular targets of these antimicrobials.

Up to date the data on involvement of defensins in intracellular signaling are scarce. One may speculate that unique molecular properties of defensins (amphipatic chain, high cationic charge, low molecular weight) favor their interactions with subcellular components, plasma membranes, as well as with discrete membrane-bound and cytoplasmic proteins — components of intracellular signaling pathways. In few publications the role of defensins in activation of intracellular signaling cascades *in vitro* has been analyzed. It has been reported that alpha-defensin may interact with LRP receptor expressed in smooth muscle cells (SMC), undergo internalization and specifically inhibit protein kinase C [14]. In another research it was demonstrated that treatment of airway SMC with HNP1–3 leads to phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 [15]. In human keratinocytes, hBD-2, -3, and -4-dependent secretion of IL-18 was shown to be mediated by p38 and ERK1/2 MAPK pathways [16]. Human cathelicidin LL-37 was shown to activate MAPK/ERK pathway in airway epithelial cells and transactivate EGFR via metalloproteinase-mediated cleavage of membrane-anchored EGFR-ligands [17]. In airway epithelial cells treatment with HNP1–3 caused activation of ERK1/2, and the process of HNP-dependent wound repair seems to be dependent on EGFR activation [9].

Our previous studies have shown increase of cell proliferation *in vitro* upon treatment of A431 cells with exogenous hBD-2 [4]. The present research was aimed on identification of possible molecular targets of hBD-2 in A431 cells that could be involved in mediation of its pro-proliferative action.

MATERIALS AND METHODS

Cell cultures and cultivation. A431 (human vulval epidermoid carcinoma) cells were received from the Institute of Molecular Biology of Russian Academy of Sciences (Moscow, Russia).

A431 cells were cultivated in 6–well plates in DMEM culture medium supplemented with 5% FBS (Gibco BRL, UK) in atmosphere of 5%CO_2 at 37 °C until cells reached 70% confluence; then the medium was replaced with DMEM without FBS and cultivation continued for 24 h. Then the cells (5×10^5 cells per well) were incubated with EGF (10 ng/ml) and/or recombinant hBD-2 (1 μg/ml) [18] for 30 min, 12 h, and 24 h for kinase assays, RT-PCR analysis of expression of p70S6K on mRNA and protein levels, respectively.

RT-PCR-analysis. For detection of expression of p70S6Kα and p70S6Kβ semiquantative RT-PCR analysis was applied as described in [19]. The next

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Abbreviations used: EGF – epidermal growth factor; EGFR – receptor of epidermal growth factor; hBD-2 – human beta-defensin-2; PDK1 – phosphoinositide-dependent kinase 1; RT-PCR – reverse transcription polymerase chain reaction.

primers were used: direct ones — 5`-ttggggcatttacatcaaaaggg – for p70S6Kα and 5`-ggatcgccgccctttaccgca — for p70S6Kβ; reverse primer 5`-cccag(a/g)aag(a/g)cctg(a/g)ttggcact — for both genes. 30 cycles of amplification at regimen (94 °C – 20 s, 64 °C — 30 s, 72 °C — 45 s) were performed. Expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (direct primer — 5′-tgaaggtcggagtcaacggatttggt, reverse primer — 5′-catgtgggccatgaggtccaccac) served as the control (25 cycles at the regimen 95 °C — 20 s, 64 °C — 30 s, 72 °C — 45 s). The products of RT-PCR were routinely analyzed by electrophoresis in agarose gel.

Western blot analysis, autophosphorylation of EGF-receptor, kinase assays and cross-linking technique. Western-blot analysis was performed by standard procedure with the use of the next antibodies: MoAbs against full size molecule of p70S6Kα [20], polyclonal antibodies against C-terminal fragment of p70S6Kβ (Institute of Molecular Biology and Genetics, NAS of Ukraine, Kyiv, Ukraine), MoAbs against phosphorylated thyrosine (PY20, Santa Cruz Biotechnology INC, USA), MoAbs against EGFR (Sigma, USA), phospho-PDK1 mAbs (Ser241), phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 MARK (Thr180/Tyr182), phospho-MARK/JNK (Thr183/Tyr185) (CellSignaling Tech, USA), and polyclonal antibodies against ribosomal S6 protein phosphorylated at the position Ser235/236 (CellSignaling Tech, USA). The data of Western-blot analysis were analyzed by densitometry using TotalLab v1.10 programme (the results were recalculated by loading control values). Each experiment was repeated in triplicate.

Kinase assay was performed as follow: A431 cells were cultivated in 6–well plates (5 x 10^5 cells per well) for 24 h in DMEM without FBS; then cells were washed with serum-free medium and incubated in HEPES-PBS buffer with 10 ng/ml EGF and/or 1 μg/ml hBD-2 for 30 min. Then buffer was aspirated and the cells were immediately lysed by hot Laemmly sample buffer. Then the samples were subjected to 10 or 7–22% SDS-PAGE gradient electrophoresis and Western-blot analysis was carried out by standard schedule using abovementioned antibodies and developed using ECL kit (Amersham, UK).

For cross-linking experiment for evaluation of formation of EGFR dimers, after incubation of cells with hBD-2 the medium was removed and replaced with BS^3-containing buffer (Pierce, USA) for 30 min at 37 °C according to the instructions of manufacturer. Then 100 ml of the sample buffer were added, and the proteins were analyzed by Western blot analysis.

RESULTS

Influence hBD-2 on activation of EGFR and down-stream kinases. The first task of the present research was to analyze possible influence of hBD-2 on activation of EGFR receptor *in vitro*. For that purpose we have analyzed the autophosphorylation level of EGF receptor in A431 cells treated with recombinant hBD-2 at the concentration causing maximal increase of cell proliferation *in vitro* [4] and compared the content of monomers and dimers of EGF receptor in A431 cells upon influence of 1 μg/ml hBD-2 and/or 10 ng/ml EGF (used as positive control). Our data have shown (Fig. 1) that 30 min treatment of A431 cells with hBD-2 did not influence autophosphorylation level of EGFR, but decreased the level of formation of EGFR dimers in the presence of EGF, in particular if 15 min pretreatment with hBD-2 was applied prior to addition of EGF (Fig. 1, line 5).

As we have reported earlier [21], the treatment of A431 cells with hBD-2 does not cause activation of EGFR-dependent downstream cascade, typical for classic EGFR ligand. However, in A431 cells incubated with hBD-2 for 30 min, a band of 3–4 phosphorylated proteins with molecular weights of 40–60 kDa appears, and accumulation of these products lasts till 24 h of incubation. Using anti-phospho-ERK1/2, -MEK1/2, -JNK1/2, and -p38 mAbs, we have estimated that incubation of A431 cells with hBD-2 didn't influence phosphorylation of p38 and JNK kinases (data not shown), but resulted in phosphorylation of MEK1/2 and ERK1/2 kinases (Fig. 2).

Influence hBD-2 on activation of PDK1-p70S6K pathway. We have find out that 12 h incubation of A431 cells with 1 μg/ml hBD-2 resulted in a marked

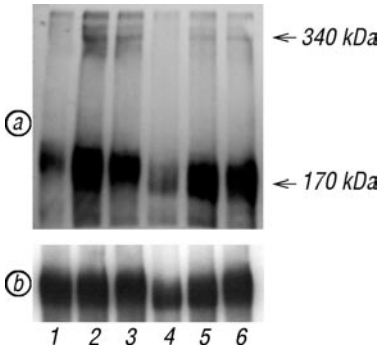

Fig. 1. Western blot analysis of autophosphorylation of EGF receptor and EGFR-dimer formation in A431 incubated with EGF and/or hBD-2 (A, pY20 MoAbs). Expression of EGFR (B, anti-EGFR-MoAbs) served as loading control. A431 cells (2 x 10^5/well, 24-well plate) were cultured 24 h without FBS, then EGF (10 ng/ml) and/or hBD-2 (1 μg/ml) were added for 15–30 min. In case (*a*) cells were treated with BS^3 crosslinker. Lines: 1 — control cells; 2 — + EGF, 30 min; 3 — + EGF for 15 min, then + hBD-2 for 15 min; 4 -+ hBD-2 for 30 min; 5 -+ hBD-2 for 15 min, then + EGF for 15 min; 6 — EGF + hBD-2 simultaneously for 30 min

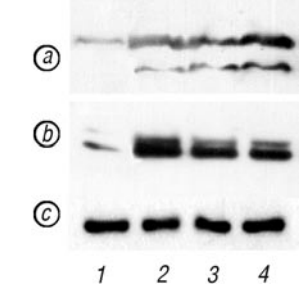

Fig 2. Western blot analysis of phosphorylation of MEK1/2 (*a*) and ERK1/2 (*b*) kinases in A431 cells incubated for 30 min with 1 μg/ml hBD-2 (line 2), 10 ng/ml EGF (line 3) or hBD2+EGF (line 4). Line 1 — control A431 cells without inductors. β-actin expression (anti-β-actin-MoAbs (Sigma, USA) (*c*) served as control

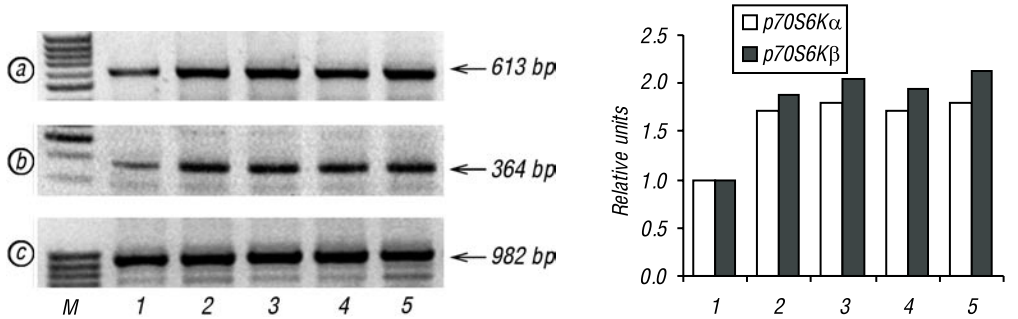

Fig. 3. RT-PCR analysis of expression of mRNA for p70S6 kinase, isoform alpha (*a*) and p70S6 kinase, isoform beta (*b*) in A431 cells. G3PDH expression (*c*) served as control. A431 cells were grown in 6-well plates (5 x 10^5 cells per well) till 70% monolayer, than the medium was replaced by serum-free one for 24 h. Then cells were treated with 10 ng/ml) EGF and/or 1 μg/ml hBD-2 for 12 h. Lanes: 1 — control; 2 — + EGF; 3 -+ FBS; 4 — EGF + hBD-2; 5 — hBD-2; M — DNA 1 kb ladder

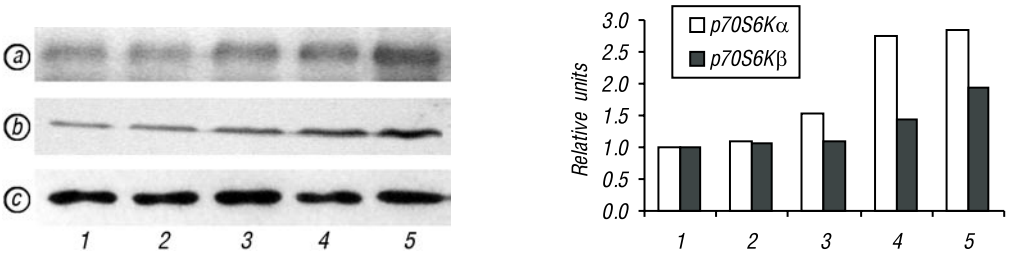

Fig. 4. Western blot analysis of expression of p70S6 kinase, isoform alpha (*a*), p70S6 kinase, isoform beta (*b*). β-actin expression (anti-β-actin-MoAbs (Sigma, USA) (*c*) served as control. A431 cells were grown in 6-well plates (5 x 10^5 cells per well) till 70% monolayer, than the medium was replaced by serum-free one for 24 h. Then the cells were treated with 10 ng/ml EGF and/or 1 μg/ml hBD-2 for 24 h. Lanes: 1 — control; 2 — + EGF; 3 -+ FBS; 4 — EGF + hBD-2; 5 — hBD-2

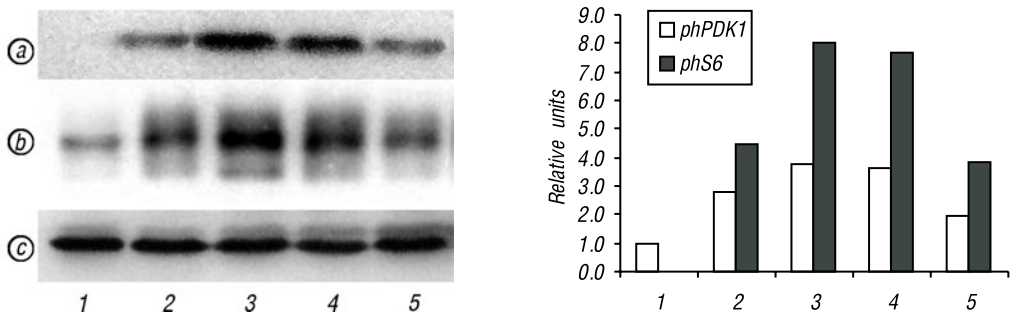

Fig. 5. Western blot analysis of phosphorylation level of ribosomal S6 protein (Ser235/236) (*a*) and PDK1 (Ser241) (*b*) in A431 cells. β-actin expression (anti-β-actin-MoAbs (Sigma, USA)) (*c*) served as control. A431 cells were grown in 6-well plates (5 x 10^5 cells per well) till 70% monolayer, than the medium was replaced by serum-free one for 24 h. Then the cells were treated with 10 ng/ml EGF and/or 1 μg/ml hBD-2 for 30 min. Lanes (*a* and *c*): 1 — control; 2 — + EGF; 3 -+ FBS; 4 — EGF + hBD-2; 5 — hBD-2; *b*: 1 — control; 2 — + EGF; 3 — hBD-2 (15 min) + EGF for 15 min; 4 — EGF (15 min) + hBD-2 for 15 min; 5 — hBD-2

(nearly 2-fold) increase of expression of mRNAs for alpha- and beta-isoforms of p70S6 kinase that was close to that upon treatment of cells with 10 ng/ml EGF (Fig. 3). Western blot analysis of expression of p70S6K isoforms in A431 cells after 24 h incubation of the cells with 1 μg/ml hBD-2 has demonstrated the similar pattern — defensin strongly influenced production of alpha- and beta-isoforms of p70S6 in A431 cells (by 3-fold and 2-fold, respectively) and elevation of the activity of the p70S6 kinase (Fig. 4, Fig. 5, *a*). Moreover, PDK1 — the up-stream kinase phosphorylating p70S6K – is also found to be nearly 2-fold higher phosphorylated in A431 cells incubated with 1 μg/ml hBD-2 for 30 min compared to that in control cells (Fig. 5).

DISCUSSION

In the present research we aimed to evaluate possible involvement of EGFR in hBD-2 dependent cell signaling. Our previous data [4] have demonstrated that in micromolar concentration range exogenous hBD-2 promotes proliferation of cultured cells *in vitro* and the rate of hBD-2–dependent proliferation correlates with the content of EGFR in given cells. Moreover, we have observed that simultaneous stimulation of A431 cells with EGF and hBD-2 resulted in the decrease of incorporation of [^{3}H]-thymidine compared with that for cells treated separately with hBD-2 or EGF (unpublished data). That's why we tried to analyze the autophosphorylation level of EGF receptor in A431 cells treated with micromolar concentration of hBD-2 and to evaluate the content of monomers and dimers of EGF receptor in A431 cells upon influence of hBD-2 and/or EGF. Our data have shown that short-term treatment of A431 cells with hBD-2 did not influence the level of autophosphorylation of EGFR, but seems too decrease the level of formation of EGFR dimers in the presence of EGF. We hypothesize that there could be at least two possible explanations of such phenomenon: 1) hBD-2 may penetrate/intercalate cell membrane due to well-

known membrane-permebealizing properties of antimicrobial pepties thus preventing dimer's formation; 2) hBD-2 may non-specifically bind to EGF-binding site of EGF receptor due to its steric homology with EGF molecule [22], however, without further dimerization/activation of receptor. Our data have shown that action of hBD-2 *in vitro* is different from that of HNP-1 on airway epithelial cells [17], where transient activation of EGFR realized via activation of metalloproteinase-mediated cleavage of membrane-anchored EGFR-ligands has been observed.

Taking into account the data of other authors [15, 16] on possible involvement of MAP-kinase pathway, in particular ERK1/2 kinase, in mediation of action of defensins *in vitro*, we carried out the research of the phosphorylation state of some kinases of MAP-kinase pathway in A431 cells treated with hBD-2 and have shown hBD-2-dependent activation of MEK1/2 and ERK1/2 kinases. Mentioned kinases are evolutionary conservative proteins that link surface cell receptors with key regulatory molecules and control cell survival and adaptation. It is known that phosphorylation of MEK1/2 and ERK1/2 leads to activation of different transcriptional factors (in particular AP-1 and NF-kB), and initiation of proliferation, growth and differentiation of cells [23, 24]. Our data points on involvement of MEK1/2 and ERK1/2 in mediation of action of hBD-2 in A431 cells. Unfortunately, we are unable yet to determine up-stream kinases mediating hBD-2-dependent phosphorylation of ERK 1/2.

Mitogenic properties of hBD-2 make it reasonable to research its influence on expression patterns and activity of p70S6 kinase — key enzyme of the system of protein biosynthesis, that is hyperactivated upon mitogenic influence of growth factors *in vitro*. We have find out that incubation of A431 cells with micromolar concentrations of hBD-2 resulted in a marked increase of expression of mRNAs and respective protein products of alpha- and beta-isoforms of p70S6 kinase as well as activity of the kinase. Such up-regulation of p70S6 kinase expression and activity may be in part explained by the detected defensin-dependent elevation of phosphorylation of PDK1 — the up-stream kinase that phosphorylates p70S6K [25].

As far as we know it is a first report on involvement of defensins in regulation of enzymes controlling protein biosynthesis. PDK1 is playing a central role as activator of multiple signaling pathways, that may be affected upon carcinogenesis, in particular protein synthesis. The obtained data do not allow us yet to propose the exact mechanism of interaction between hBD-2 and PDK1-signaling cascade. However, one may speculate that such effect possibly may relay in direct interaction between plasma-membrane bound PDK1 and defensin — according to data-base (www.scansite.mit.edu) there is extremely high affinity between hBD-2 and PDK1 molecules. Our preliminary data based on pull-down technique for PDK-1-hBD-2 (data not shown) also point on the possibility of such interactions. We hope that new studies with the use of recombinant defensins will put new insight on the role of these multifunctional antibiotics in cell signaling and behavior.

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УЧАСТИЕ БЕТА-ДЕФЕНСИНА-2 ЧЕЛОВЕКА В ПЕРЕДАЧЕ ВНУТРИКЛЕТОЧНЫХ СИГНАЛОВ *IN VITRO*

Цель: **проанализировать участие бета-дефенсина-2 человека (hBD-2) в механизмах передачи внутриклеточных сигналов в модели *in vitro*. *Материалы и методы:* клетки линии A431 культивировали в присутствии 1 μг/мл рекомбинантного hBD-2 и/или 10 нг/мл ЭФР. Экспрессию мРНК альфа- и бета-изоформ p70S6 киназы оценивали методом полуколичественного ОТ-ПЦР анализа. Экспрессию и активность p70S6K, фосфорилирование PDK1, ERK, JNK, p38 киназ и рецептора ЭФР (ЭФРР) исследовали методом Вестерн-блот анализа. *Результаты:* 30 мин инкубация клеток A431 с 1 μг/мл hBD-2 не оказывала влияния на уровень аутофосфорилирования ЭФРР, но препятствовала образованию димеров рецептора в присутствии ЭФР. В то же время 30 мин обработка клеток hBD-2 приводила к активации p70S6K, 12 ч — к значительному повышению уровня мРНК альфа- и бета-изоформ p70S6 киназы, а 24 ч — к повышению синтеза p70S6K на уровне белка. PDK1-киназа, фосфорилирующая p70S6K, также подвергалась фосфорилированию в присутствии экзогенного hBD-2 *in vitro*. *Выводы:* данные свидетельствуют об участии каскада PDK1-p70S6K в опосредовании действия hBD-2 в клетках A431.**

***Ключевые слова*: бета-дефенсин-2 человека, ЭФР, рецептор ЭФР, p70S6 киназа, PDK1.**