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Interaction between the antagonist of the *N*-methyl-*D*-aspartate receptor (NMDAR) and elastin-derived peptide (VGVAPG) in primary astrocytes

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Aim. The aim of the present study was to determine the impact of the elastin derived peptide (VGVAPG) alone and in co-treatment with MK-801 (N-methyl-D-aspartate receptor (NMDAR) antagonist) on the expression of the aryl hydrocarbon receptor (AhR) in mouse primary astrocytes *in vitro*. **Methods.** Primary astrocytes were cultured in DMEM/F12 medium without phenol red supplemented with 10 % fetal bovine serum. The cells were exposed to 10 nM and 1 μM of the VGVAPG peptide and in co-treatment with the antagonist MK-801. After 48 h of exposition to the peptide, the expression of AhR was measured. **Results.** In this study, significant changes in the astrocyte co-treatment were observed only in a group treated with 10 nM VGVAPG peptide and MK-801, which is probably an effect of the "N" or "U" shaped response to the VGVAPG peptide concentrations. This study is the first one to show an impact of the VGVAPG peptide on the AhR protein level in mouse astrocytes *in vitro*. It is the first to describe changes in the AhR protein level under treatment with MK-801 agent acting as the NMDAR antagonist. **Conclusions.** Taking into account the postulated important role of AhR in the extracellular matrix metabolism, the obtained data suggest that the VGVAPG peptide may cause changes in the extracellular matrix in the NMDAR-dependent manner.

K e y w o r d s: elastin-derived peptides; VGVAPG; astrocytes; AhR; NMDAR

Introduction

Elastin is a natural biological polymer determining the elasticity and flexibility of many tissues and organs, e.g. brain, lungs, and large blood vessels [1]. As a result of the natural aging process, elastin is degraded and elastin derived peptides (EDPs) are released, which may produce different tissue-dependent biological effects [2, 3]. To date, EDP with the Val-Gly-Val-Ala-Pro-Gly (VGVAPG) amino acid sequence has been described to affect the

cellular concentration of calcium (Ca^{2+}) [4]. Moreover, it has been described that certain Ca^{2+} calcium channel antagonists, such as nifedipine, verapamil, and MK-801 (N-methyl-D-aspartate receptor (NMDAR) blocker), are able to inhibit the VGVAPG peptide-induced influx of Ca^{2+} into astrocytes [4]. Further studies have shown that the main mechanism of the impact of the VGVAPG peptide on the Ca^{2+} level is the interaction with NMDAR [4, 5].

NMDAR is a receptor mainly for glutamate with an important role in the neurotransmission in the brain [6]. Moreover, NMDARs play an integral role in synaptic plasticity and determine Ca^{2+}-dependent excitotoxicity [7]. To date, NMDARs have been described to be able to affect the activation of another important receptor involved in toxicity, namely the aryl hydrocarbon receptor (AhR), through different molecular pathways [8]. AhR was originally identified as a receptor for environmental contaminants, but now, a number of diverse endogenous and exogenous ligands have been described [9]. Moreover, it has currently been found that AhR pathways play an important regulatory role in cell adhesion and matrix metabolism [10]. Interestingly, AhR ligands (e.g.2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)) induce excitotoxicity through the NMDAR-dependent Ca^{2+} influx [11].

Data are available that AhR is involved in primary astrocyte proliferation after stimulation using the VGVAPG peptide [12]. Moreover, as mentioned before, NMDARs are involved in the Ca^{2+} ion influx in astrocytes [4]. However, the impact of the NMDAR blocker together with the VGVAPG peptide on the AhR protein expression has not been studied before.

Therefore, the aim of the present study was to determine the impact of the VGVAPG peptide alone and in co-treatment with MK-801 on the expression of AhR in mouse primary astrocytes.

Materials and Methods

Reagents

Trypsin, streptomycin, penicillin, and dizocilpine (MK-801) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The radioimmunoprecipitation assay (RIPA) buffer and fetal bovine serum (FBS) were purchased from EURx (Gdańsk, Poland). The VGVAPG peptide was synthesized by LipoPharm.pl (Gdańsk, Poland). The AhR (EM0590) ELISA assay was obtained from WuHan Fine Biotech (WuHan, China). Stock solutions of the VGVAPG peptide and tool compounds were prepared in DMSO and added to the DMEM/F12 medium. The final concentration of DMSO in the culture medium was 0.1 %.

Primary Astrocyte Cell Culture

All procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Bioethics Commission (No.46/2014) as compliant with a law accepted in Poland. In the experiments, culture of mouse astrocyte was used. The detailed procedure and preparation of the primary culture of astrocytes were described previously [13]. The experiments were performed on mouse glial cells isolated from the fetuses (17/18 embryonal day) of pregnant female Swiss mice. Isolated cells were cultured

in phenol red-free DMEM/F12 medium supplemented with 10 % of FBS, 100 U/mL penicillin, 0.10 mg/mL streptomycin and 250 ng/mL amphotericin B (amphotericin B — eliminate proneuronal cells). The cells were seeded at a density of 20×10^6 cells per T75 culture flasks. The cultures of the glial cells were maintained at 37 °C in atmosphere containing 5 % CO_2. In the logarithmic phase, after reaching 90 % confluence, the cells were collected and frozen in liquid nitrogen (passage and freezing — eliminate proneuronal cells). After thawing the cells were cultured in flasks (pure astrocytes culture) and then seeded in the first passage in 6-well culture plates at a density of 11×10^4 per well and initially cultured for 24 h before the start of experiment. The culture medium was changed prior to treating cell cultures with the VGVAPG peptide.

ELISA for AhR

The level of the AhR protein was determined *via* ELISA after 24- and 48-h treatment with 10 nM and 1 μM of the VGVAPG peptide and/or in co-treatment with 1 μM of MK-801. After the experiment was finished, the samples were collected using RIPA buffer. The assay was conducted according to the manufacturer's instructions from Elabscience Biotechnology and Wuhan Fine Biotech Co., Ltd. (WuHan, China) and as described in previous study [5]. The absorbance was measured at 450 nm using a microplate reader (FilterMax F5) and this value was proportional to the amount of the AhR protein. Protein concentration was measured in each sample and determined in triplicate for each sample using the Thermo Fisher NanoDrop device (USA).

Statistical analysis

The data are presented as the mean ± standard deviation (SD) of three independent experiments. The treatment was repeated twice for the ELISA method (n = 6). The results were used in the statistical analysis performed in the GraphPad Prism 8.0 Statistical Analysis Mode. The data were analyzed *via* one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure and denoted as $***p < 0.001$ and $**p < 0.01$ vs. the control. Moreover, the t-test was used to compare statistical differences between the MK-801 alone group and the group co-treated with MK-801 and peptides as # at $p < 0.05$.

Results and Discussion

In previous study,we have found that the VGVAPG peptide acted *via* an AhR-dependent pathway, which was confirmed with the use of the AhR predesigned siRNA [12]. In the cited study, the knock-out of AhR resulted in a decrease in the proliferation of astrocytes stimulated by 10 nM of the VGVAPG peptide. However, the protein expression of this receptor was not investigated. Therefore, here we assessed the AhR protein expression under treatment of astrocytes with VGVAPG peptide. The present data showed that, after the 24-h exposure to 10 nM or 1 μM of the VGVAPG peptide, only the 1 μM concentration increased the AhR protein expression by 0.28 ng/mL, compared to the control (Fig. 1A). Moreover, MK-801 (NMDAR antagonist) alone increased the level of AhR by 0.51 ng/mL, compared to the control (Fig. 1A). It should be noted that the cell co-treatment with MK-801 and 10 nM of the VGVAPG peptide decreased the AhR protein level by 0.22 ng/mL, compared to MK-801

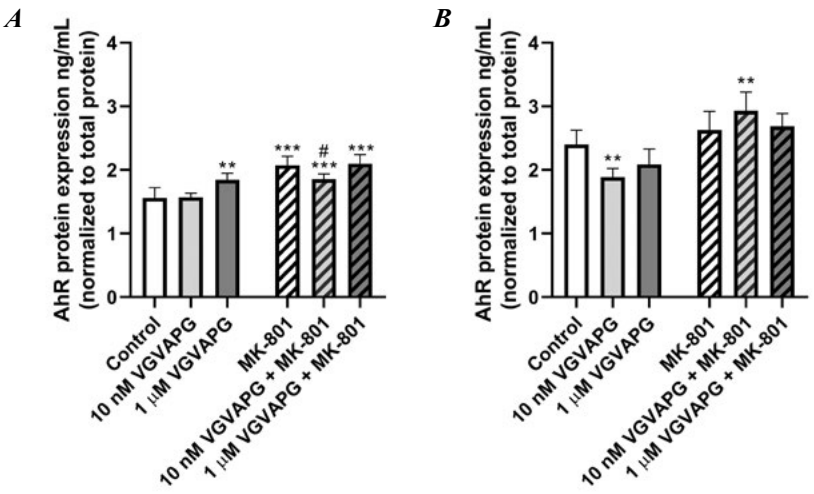

Fig. 1. Effect of treatment of mouse primary astrocytes *in vitro* with 10 nM or 1 μM of VGVAPG alone and/or the co-treatment with 10 nM or 1 μM of VGVAPG and 1 μM MK-801 on the level of AhR protein expression after 24h (*A*) and 48h (*B*). The data are presented as the mean ± standard deviation (SD) of three independent experiments. The treatment was repeated twice for the ELISA method (n = 6). **p < 0.01 and ***p < 0.001 vs. the control cells. #p < 0.5 vs. the group treated with the MK-801 alone and groups co-treated with MK-801 and peptides.

(Fig 1A).The cells co-treated with 1 μM of the VGVAPG peptide and MK-801 did not differ significantly from the MK-801 treated group (Fig. 1A). On the other hand, after the 48-h exposure of the primary astrocytes to 10 nM and 1 μM of the VGVAPG peptide, a decrease in the AhR protein level was observed only in the cells treated with 10 nM of the VGVAPG peptide (decrease by 0.51 ng/mL, compared to the control) (Fig. 1B). An increase in the AhR protein expression was observed in the group co-treated with MK-801 and 10 nM of the VGVAPG peptide, compared to the control (increase by 0.53 ng/mL) (Fig. 1B).

As shown by previously published data, the result of theVGVAPG peptide action is cell-, dose-, and time-dependent [3]. Moreover, the potency of the peptide does not increase in direct proportion to the concentration, but it was the strongest for the nM concentrations [2, 12]. Probably, in view of the aforementioned dose-effect relations found in our study, only 10 nM of the VGVAPG peptide sufficiently interfered with MK-801 (NMDAR antagonist) and affected the AhR protein level. To date, it has been described that 1-h exposure to 10 μM of MK-801 alone induced a slight but statistically insignificant increase in the *AhR* mRNA expression in mouse neurons *in vitro* [14]. In turn, the AhR agonist TCDD caused an increase in *AhR* mRNA expression, but no changes in *AhR* mRNA expression were observed in a group co-treated with TCDD and MK-801 [14]. Lin *et al.* did not study AhR protein expression, nevertheless, they have reported that the mRNA and protein expression are not always correlated [15]. Moreover, very often, the mRNA expression is opposite to the level of the studied protein as a result of the negative feedback [15]. Thus, we can assume that our data are consistent with the results described by Lin *et al.* [14]. Taking into account the present data and results of previous studies, we concluded that the VGVAPG peptide interfered with both AhR and NMDAR pathways [4, 12].

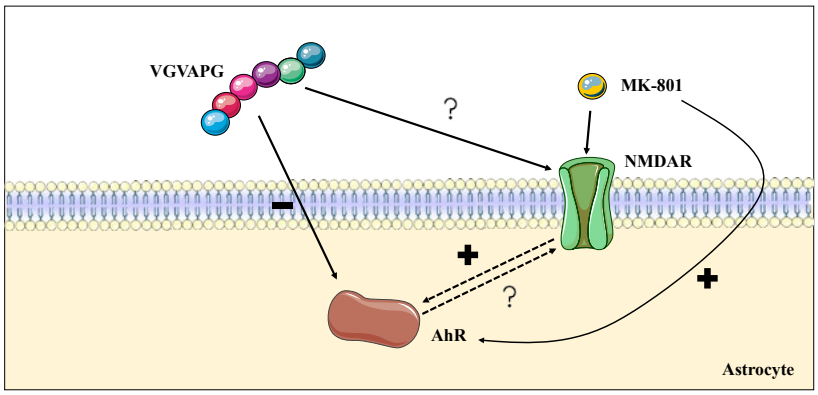

Fig. 2. Interaction between the VGVAPG peptide, the aryl hydrocarbon receptor (AhR), MK-801 and the N-methyl-D-aspartate receptor (NMDAR) in astrocytes. "+" or "-" — the direction of the effects; "?" — effect unknown

Conclusion

In the present study, significant changes in the astrocyte co-treatment were observed only in the group treated with 10 nM of the VGVAPG peptide and MK-801, which is probably an effect of the "N" or "U" shaped response to concentrations of the tested peptide. The present study is the first one to show an impact of the VGVAPG peptide on the AhR protein level in mouse astrocytes *in vitro*. Moreover, the experiments are the first to describe changes in the AhR protein level under treatment with MK-801 (NMDAR antagonist) mechanism of action. Taking into account an important role of AhR in cell matrix metabolism, the present data suggest that the VGVAPG peptide may cause changes in the extracellular matrix in the NMDAR-dependent manner (Fig 2).

Acknowledgments

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Дослідження взаємодії між антагоністом рецептора *N*-метил-*D*-аспартату (NMDAR) і пептидом похідним від еластину (VGVAPG) у первинних астроцитах

К. А. Шиховський

Мета. Метою даного дослідження було визначити вплив пептиду, отриманого з еластину (VGVAPG) окремо та в комбінації з МК-801 (антагоніст *N*-метил-*D*-аспартатного рецептора (NMDAR)) на експресію білка арилового вуглеводневого рецептора (AhR) в первинних астроцитах миші *in vitro*. **Методи.** Первинні астроцити зберігали в DMEM/F12 без фенолового червоного з додаванням 10 % фетальної бичачої сироватки. Клітини піддавалися впливу 10 нМ і 1 мкМ пептиду VGVAPG та МК-801. Після 48 годин експозиції вимірювали експресію пептиду AhR. **Результати.** У цьому дослідженні значні зміни в астроцитах при сумісному введенні препаратів спостерігалися лише в групі, яка отримувала 10 нМ пептиду VGVAPG і МК-801, що, ймовірно, є ефектом нелінійного механізму дії пептиду VGVAPG. Це перше дослідження, яке показало вплив пептиду VGVAPG на рівень білка AhR в астроцитах миші *in vitro*. Крім того, дане дослідження є першим, у якому описано зміни рівня білка AhR після введення агенту МК-801 (антагоніст NMDAR). **Висновки.** Враховуючи важливу роль AhR у метаболізмі позаклітинного матриксу, отримані дані свідчать про те, що пептид VGVAPG може викликати зміни в позаклітинному матриксі, які залежні від рівня NMDAR.

Ключові слова: еластин-похідні пептиди; VGVAPG; астроцити; AhR; NMDAR

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