Calcium channel antagonists interfere with the mechanism of action of elastin-derived peptide VGVAPG in mouse cortical astrocytes in vitro

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ABSTRACT

Elastin-derived peptides (EDPs) contain replications of the Val-Gly-Val-Ala-Pro-Gly (VGVAPG) hexapeptide. It has been described that the VGVAPG peptide induces reactive oxygen species (ROS) production in murine monocytes and astrocytes, human fibroblasts, and the human neuroblastoma (SH-SYSY) cell line. To date, there is growing evidence that calcium channel blockers (CCBs) reduce oxidative stress and development of inflammation in the nervous system. Therefore, the aim of the present study was to evaluate the impact of such CCBs as Nifedipine, Verapamil, and MK-801 on the expression of peroxisome proliferator-activated receptor (Ppar γ), i.e. ROS-related and inflammation-related proteins, in mouse astrocytes exposed in vitro to the VGVAPG peptide. The experiments showed that Nifedipine or MK-801 used in co-treatment with the VGVAPG peptide potentiated the effect of this peptide on the Ppar γ level after the 24-h and 48-h treatment. Moreover, all studied compounds decreased the VGVAPG-induced caspase-1 activity in both time intervals. The data also showed that the VGVAPG peptide decreased the interleukin 1 beta (IL-1β) level in both studied time intervals. Upon a short-time exposure, the use of CCBs intensified the decrease in IL-1β stimulated by the VGVAPG peptide, opposite to the longer treatment. Moreover, the VGVAPG peptide decreased the IL-1R1 level in both studied time intervals. After 24 h, Nifedipine and Verapamil potentiated the effect of the VGVAPG peptide. The VGVAPG peptide decreased the catalase (Cat) protein expression only after 24 h, whereas CCBs did not affect the expression of Cat induced by the VGVAPG peptide. The VGVAPG peptide increased the expression of the superoxide dismutase 1 (Sod1) protein. After 24 h of exposure, Nifedipine and Verapamil potentiated the increase in the Sod1 protein expression. Finally, our data showed that VGVAPG did not change the level of estradiol (E2) in the astrocytes. Interestingly, Nifedipine and Verapamil in co-treatment with VGVAPG increased the E2 level. Summarizing, it can be assumed that increased amounts of the VGVAPG during lifetime can play a certain role in calcium channel functioning in neurodegenerative diseases.

1. Introduction

Elastin-derived peptides (EDPs) are products of physiological and pathological degradation of elastin (Baranek et al., 2007; Szychowski et al., 2021). EDPs are detected in the healthy human brain but their level significantly increases after ischemic stroke (Nicoloff et al., 2008; Tzvetanov et al., 2008). As described, EDPs may be formed in a reactive oxygen species (ROS)-dependent and/or ROS-independent manner and as a product of the activity of elastases, such as serine proteases, cysteine proteases, and matrix metalloproteinases (reviewed by Szychowski and Skóra (2021)). Interestingly, the slow degradation of elastin correlates with its long half-life and results in EDP release after about 70 years (Shapiro et al., 1991). Thus, EDPs may be a cause of such neurodegenerative diseases as Alzheimer’s disease (AD) (Ma et al., 2019, 2020), whose first symptoms usually develop in over 60- year-old patients ( Ramos-Cejudo et al., 2018). EDPs contain repeated sequences of the

Abbreviations: AD, Alzheimer’s disease; CAT, catalase; CNS, central nervous system; DMSO, dimethyl sulfoxide; E2, estradiol; EDPs, elastin-derived peptides; FBS, fetal bovine serum; ICE, interleukin-1-beta–converting enzyme; IL-1β, interleukin-1 beta; IL-1βR1, interleukin 1 beta receptor 1; MK-801, dizocilpine; MMPs, matrix metalloproteinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PBS, phosphate-buffered saline; PPARγ, peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; T2DM, type 2 diabetes mellitus; VGCCs, voltage-gated calcium channels; VGVAPG, Val-Gly-Val-Ala-Pro-Gly; VGPGA, Val-Val-Gly-Pro-Gly-Ala.

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Val-Gly-Val-Ala-Pro-Gly (VGVAPG) hexapeptide, which has a broad range of biological activities (Adair-Kirk and Senior, 2008). The effects of the VGVAPG action are mainly mediated by the elastin-binding protein (EBP), which is an alternatively spliced, enzymatically inactive form of the GLB1 gene (β-galactosidase) known as β-Gal (Privitera et al., 1998). The production of EDPs can be induced by a number of chemical, enzymatic, and physical factors and, as described above, directly by ROS (Szychowski et al., 2021). EDPs can increase ROS production through positive feedback [reviewed in (Szychowski and Skóra, 2021)]. It has been described that EDPs and/or the VGVAPG peptide induce ROS production in murine monocytes and astrocytes, human fibroblasts, or human neuroblastoma cell lines (SH-SY5Y) (Gayral et al., 2014; Robert et al., 1984; Scandolera et al., 2015; Szychowski et al., 2019a; Szychowski and Gmínski, 2019a). As shown in the reports, the VGVAPG peptide can also be internalized into the cell by galecin-3 and integrin avb3; moreover, this hexapeptide affects the intracellular receptor, e.g. the peroxisome proliferator-activated receptor-gamma (PPARγ) (Lucie et al., 2009; Szychowski and Gmínski, 2019b).

PPARγ is a ligand-activated nuclear receptor playing a crucial role in the coordination of lipid, glucose, and energy metabolism. Its elevated levels are found in the brains of AD patients (Jiang et al., 2008). Moreover, the engagement of PPARγ in the regulation of the redox homeostasis in the cell is well-described, showing that this receptor controls e.g. the expression of such antioxidant enzymes as catalase (Cat) or superoxide dismutase 1 (Sod1) (Kim and Yang, 2013). It has also been shown that, among intracellular ligands, such lipids as 9-hydroxyoctadecadienoic acid (9-HODE) or 13-hydroxyoctadecadienoic acid (13-HODE), which are products of lipid oxidation (present also in many pathological states) inside the cell, are characterized by high affinity to PPARγ and cause its activation (Itoh et al., 2008; Yuan et al., 2013). As reported, the constant redox imbalance in the cell leads to inflammation, in which PPARγ plays a modulatory role (Villapol, 2018). Interestingly, voltage-gated calcium channels (VGCCs) are listed in the neurogenic inflammation pathway and their ligands are characterized by therapeutically potential in the inflammation pain treatment (Hiben et al., 2020; Sekiguchi et al., 2018). Moreover, Ding et al. showed that PPARγ agonists inhibit the influx of extracellular calcium as well as ischemic nerve damage, showing some cross-talk between these structures (Ding et al., 2020). Interestingly, in the nervous system, high ROS levels usually correlated with impaired calcium homeostasis (Zündorf and Reiser, 2011). It is well known that calcium influx stimulates ROS production and thus leads to development of inflammation, which finally results in AD (Hernández-Fonseca et al., 2008; Leng and Edison, 2013). It has been described that tropoelastin, EDPs, or the VGVAPG peptide increase the Ca²⁺ level in human umbilical venous endothelial cells (HUVEC) and in different glial cell lines (C6, CB74, CB109, and CB191) (Coquerel et al., 2009; Faury et al., 1998a, 1998b). Additionally, disturbance in the estradiol (E2) level is associated with AD and calcium channel functioning (Mahmoodzadeh and Dwaratzek, 2019; Slooter et al., 1999). Moreover, the PPARγ agonist affects the estradiol (E2) level, which is an important neurosteroid in astrocyte function (Szychowski et al., 2020; Verkratsky et al., 2014).

Caspase-1, which cleaves inactive pro-interleukin 1 beta (pro-IL-1β) into the active form IL-1β, is a marker of inflammation (Gallacher-Beckley, 2013). The activation of this protein requires the presence of Ca²⁺ and inflammasome formation (Murakami et al., 2012). Moreover, nowadays calcium channel blockers (CCBs) have been widely used for many indications such as hypertension, coronary spasm, angina pectoris, supraventricular dysrhythmias, hypertrophic cardiomyopathy, and pulmonary hypertension (McKeever and Hamilton, 2021). Growing evidence also shows the engagement of CCBs in reduction of oxidative stress and development of inflammation in the nervous system (Huang et al., 2014). However, there are no reports elucidating the role of PPARγ and calcium channel blockers in the mechanism of VGVAPG action. This is necessary, as disturbances in these relationships may lead to dysfunction of nervous cells and development of neurodegenerative diseases.

Therefore, the aim of the present study was to evaluate the impact of such calcium channel antagonists as Nifedipine, Verapamil, and MK-801 on the expression of Pparg, i.e. ROS-related and inflammation-related proteins in mouse astrocytes exposed in vitro to the VGVAPG peptide.

2. Materials and methods

2.1. Reagents

Dulbecco’s Modified Eagle’s Medium/Hams F-12 (DMEM/F12) without phenol red as well as trypsin, penicillin, streptomycin, amphotericin B, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 4-(2-hydroxyethyl)perazine-1-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), DL-dihithreitol (DTT), N-a-acetyl-Tyr-Val-Ala-Asp-p-nitroanilide (Ac-YVAD-pNA), Nifedipine, Verapamil, Dizocilpine (MK-801), and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Eurotx (Gdańsk, Poland). ELISA kits for Sod1 (M2398), IL-1β (M0037), IL-1R1 (M0017), Cat (M2605), and Pparγ (M0893) were purchased from Elabscience Biotechnology (Wuhan, China). The ELISA kit for estradiol (E2) (EIA-2693) was purchased from DRG MedTek (Marburg, Germany). The VGVAPG and VGPGPA were synthesized by Lipopharm.pl. The stock solutions of the peptides were prepared in DMSO and added to the DMEM/F12 medium. The final concentration of DMSO in the culture medium was always 0.1%.

2.2. Astrocyte-enriched cell culture and treatment

The experiments were conducted on mouse astrocyte cell cultures. All experimental procedures were approved by the Bioethics Commission (approval no. 46/2014; First Local Ethical Committee on Animal Testing at the Jagiellonian University in Krakow) as compliant with the laws of the European Union. The cell cultures were prepared from fœtuses of pregnant female Swiss mice on Day 17/18 of gestation. After the isolation and digestion processes, the cells were centrifuged and the pellet was suspended in DMEM/F12 1:1, without phenol red, which was supplemented with 10% FBS, 100 U/mL penicillin, 0.10 mg/mL streptomycin, and 250 ng/mL amphotericin B. Isolation was undertaken according to a previously described method for separation of an almost pure culture of astrocytes (>98% astrocytes) (Szychowski et al., 2019b, supplementary data). The cultures of glial cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. In the logarithmic phase after reaching 90% confluence, the cells were trypsinized with 0.25% trypsin/0.05% EDTA and passaged onto an experimental plate. The astrocytes were seeded at densities of 5 × 10⁵ per well in a 96-well plate for colorimetric analysis and 60 × 10⁵ per well in a six-well plate for protein analysis. The culture medium was changed prior to the experiment with the VGVAPG and VGPGPA peptides and tool compounds (10 μM Nifedipine, 10 μM Verapamil, 1 μM MK-801) selected for this study. The cells were exposed to the peptides and tool compounds for 24 and 48 h. The VGPGPA peptide (negative control peptide) in both time intervals (24 and 48 h) and concentrations 10 nM and 1 μM did not affect the analyzed parameters (caspase-1 activity, level of E2, and expression of Sod1, IL-1β, IL-1R1, Cat, and Pparγ proteins). Therefore, we decided not to show such data to make the figures clearer.

2.3. Caspase-1 activity

Caspase-1 activity was assessed according to the method described by Nicholson et al. (1995) with some modifications. The glial cells treated with the tested compounds were lysed using lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM DTT) at 10 °C for 10 min. After initial incubation, the lysates were incubated with the caspase-1 substrate Ac-YVAD-pNA at 37 °C.
After 30 min, absorbance of the lysates at 405 nm was measured on a microplate reader (FilterMax F5 Multi-Mode microplate reader). The quantity of the colorimetric product was monitored continuously for 120 min.

2.4. ELISA for Sod1, Cat, II-1β, II-1β/R1, and Pparγ

Before the analysis, the amount of protein was normalized to the total protein level. The total protein concentration was determined in triplicate in each sample using a Thermo Fisher NanoDrop device. The levels of Pparγ, Sod1, Cat, II-1β, and II-1β/R1 proteins were determined with the use of ELISA after 24- or 48-h treatment with the tested compounds. The expression of Sod1, Cat, and IL-1β was determined in the cell-culture lysate, whereas IL-1β release was measured in the cell-culture medium. These proteins were specifically detected with ELISA and subsequently subjected to a quantitative sandwich enzyme immunoassay, which was conducted according to the manufacturer’s instructions (Elabscience Biotechnology, Wuhan, China). Briefly, a 96-well plate was pre-coated with monoclonal antibodies specific to Pparγ, Sod1, Cat, II-1β, and II-1β/R1. Standards and collected cell extracts were added to the wells and incubated for 90 min at 37 °C. Next, the liquid was removed, and 100 μL of biotinylated detection antibodies were added to the cultures for 60 min. The cells were washed three times to remove any unbound substances, and then horseradish peroxidase-conjugated avidin was added. The cells were washed again, and 90 μL of the substrate solution was added to the wells for 15 min. Then, 50 μL of the stop solution was added to terminate the reaction and absorbance was measured at 450 nm using a FilterMax F5 Multi-Mode microplate reader. The value obtained was proportional to the amount of Pparγ, Sod1, Cat, II-1β, and II-1β/R1.

2.5. Enzyme-linked immunosorbent assay (ELISA) for estradiol

Before the analysis, the amount of protein was normalized to total protein level. The total protein concentration was determined in triplicate in each sample using a Thermo Fisher NanoDrop device. The level of ε2 was determined after 24- and 48-h treatment with the tested compounds using ELISA. The determination was performed in the cell culture lysate and in the culture medium. Specific detections of steroids were performed using ELISA based on the principle of competitive binding. Briefly, 25 μL of each standard, control, and sample was added to appropriate wells on a 96-well plate. Then, 200 μL of enzyme conjugate was added to each well and thoroughly mixed. The 96-well ELISA plate was incubated for 60 min at room temperature. Following incubation, the plate was washed three times, and 200 μL of the substrate solution was added to each well. After 15 min of incubation at room temperature, 100 μL of the stop solution was added to each well, and the absorbance was measured at 450 nm using a FilterMax F5 Multi-Mode microplate reader. The absorbance value was inversely proportional to the level of ε2.

2.6. Statistical analysis

The data are presented as means ± SD of three independent experiments. Each treatment in the experiment was repeated three times. Therefore, the number “n” from all experiments was 9. The experimental data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Statistical significance was determined at ***p < 0.001, **p < 0.01, and *p < 0.05 vs. the control. Moreover, statistical differences between certain groups were determined at ###p < 0.001, ##p < 0.01, and #p < 0.05.

3. Results

3.1. Expression of PPARγ

After the 24-h cell treatment with 1 μM VGVAPG, a 1.31 ng/mL decrease in Pparγ protein expression was observed, compared to the control (Fig. 1A). The cell treatment with Nifedipine, Verapamil, and MK801 alone decreased Pparγ protein expression by 2.31, 1.48, and 2.16 ng/mL, respectively, compared to the control. The co-treatment of the astrocytes with Nifedipine and 10 nM or 1 μM VGVAPG decreased Pparγ protein expression by 2.10 and 1.00 ng/mL, respectively.
compared to 10 nM or 1 μM VGVAPG administered alone. The co-treatment of the astrocytes with Verapamil and 10 nM or 1 μM VGVAPG decreased Ppary protein expression by 0.77 and 0.67 ng/mL, respectively, compared to the treatment with 10 nM or 1 μM VGVAPG alone. Similarly, the cell co-treatment with MK-801 and 10 nM or 1 μM VGVAPG decreased Ppary protein expression by 1.92 and 1.52 ng/mL, respectively, compared to the doses of 10 nM or 1 μM VGVAPG administered alone (Fig. 1A).

The 48-h cell treatment with 10 nM and 1 μM VGVAPG resulted in an increase in Ppary protein expression by 1.78 and 4.65 ng/mL, respectively, compared to the control (Fig. 1B). The treatment with Nifedipine and Verapamil alone increased Ppary protein expression by 8.88 and 2.99 ng/mL, respectively, compared to the control. The co-treatment of the astrocytes with Nifedipine and 10 nM VGVAPG yielded a 6.34 ng/mL increase in Ppary protein expression, compared to the variant with 10 nM VGVAPG alone. In turn, the co-treatment of the astrocytes with Nifedipine and 1 μM VGVAPG decreased Ppary protein expression by 3.95 ng/mL, compared to the administration of 1 μM VGVAPG alone. The cells co-treated with Verapamil and 1 μM VGVAPG exhibited a 3.93 ng/mL decrease in Ppary protein expression, compared to the treatment with 1 μM VGVAPG alone. In the astrocytes co-treated with MK-801 and 10 nM VGVAPG, a 5.04 ng/mL increase in Ppar protein expression, compared to the administration of 10 nM VGVAPG alone, was observed (Fig. 1B).

3.2. Caspase-1 activity

The 24-h cell treatment with 10 nM and 1 μM VGVAPG resulted in a 71.42 and 66.05% increase in caspase-1 activity, respectively, compared to the control (Fig. 2A). The cell treatment with Nifedipine, Verapamil, and MK801 alone did not change caspase-1 activity. The co-treatment of the astrocytes with Nifedipine and 10 nM or 1 μM VGVAPG decreased caspase activity by 30.12 and 32.00%, respectively, compared to the variants with 10 nM or 1 μM VGVAPG alone. The co-treatment of the astrocytes with Verapamil and 10 nM or 1 μM VGVAPG yielded a decrease in caspase activity by 75.77 and 50.11%, respectively, compared to the administration of 10 nM or 1 μM VGVAPG alone. Similarly, the cells co-treated with MK-801 and 10 nM or 1 μM VGVAPG exhibited a 63.73 and 79.10% decrease in caspase activity, respectively, compared to the variants with 10 nM or 1 μM VGVAPG alone (Fig. 2A).

After the 48-h cell treatment with 10 nM and 1 μM VGVAPG, there was a 76.47 and 66.66% increase in caspase-1 activity, respectively, compared to the control (Fig. 2B). As after 24 h, the cell treatment with Nifedipine, Verapamil, and MK801 alone did not change caspase-1 activity in the 48-h time interval. The 48-h astrocyte co-treatment with Verapamil and 10 nM or 1 μM VGVAPG resulted in a 84.17 and 58.97% decrease in caspase activity, respectively, compared to the variants with 10 nM or 1 μM VGVAPG alone. The astrocyte co-treatment with Verapamil and 10 nM or 1 μM VGVAPG decreased caspase activity by 76.47 and 51.28%, respectively, compared to the administration of 10 nM or 1 μM VGVAPG alone. Similarly, cells co-treated with MK-801 and 10 nM or 1 μM VGVAPG exhibited a 63.73 and 79.10% decrease in caspase activity, respectively, compared to the variants with 10 nM or 1 μM VGVAPG alone (Fig. 2A).

3.3. Expression of IL-1β

After the 24-h cell treatment with 10 nM and 1 μM VGVAPG, both concentrations of the peptide contributed to a 0.28 and 0.30 pg/mL decrease in IL-1β expression, respectively, compared to the control (Fig. 3A). The cell treatment with Nifedipine and Verapamil alone decreased the IL-1β protein expression by 0.81 and 0.40 pg/mL, respectively. In the astrocytes co-treated with Nifedipine and 10 nM or 1 μM VGVAPG, a 0.65 and 0.63 pg/mL decrease in IL-1β expression, respectively, was observed, compared to the group treated with the VGVAPG peptide only (Fig. 3A). The astrocyte co-treatment with Verapamil and 10 nM or 1 μM VGVAPG yielded a 0.53 and 0.51 pg/mL decrease in IL-1β expression, respectively, compared to the doses of 10 nM or 1 μM VGVAPG administered alone. Similarly, cells co-treated with MK-801 and 10 nM or 1 μM VGVAPG decreased IL-1β protein expression by 0.81 and 0.40 pg/mL, respectively, compared to the doses of 10 nM or 1 μM VGVAPG administered alone (Fig. 1A).

Verapamil and 10 nM or 1 μM VGVAPG resulted in a 0.50 and 0.48 pg/mL decrease in IL-1β expression, respectively, compared to the group treated with the VGVAPG peptide only. Although the administration of MK-801 alone had no effect on the level of IL-1β protein expression in the cells, the co-treatment of the astrocytes with MK-801 and 10 nM or 1 μM VGVAPG resulted in a 0.53 and 0.51 pg/mL decrease in the IL-1β expression, respectively, compared to the group treated with the VGVAPG peptide only (Fig. 3A).

After the 48-h cell treatment with 10 nM and 1 μM VGVAPG, both concentrations of the peptide decreased IL-1β expression by 0.18 and 0.12 pg/mL, respectively, compared to the control (Fig. 3B). The cell
treatment with Nifedipine, Verapamil, and MK-801 alone increased IL-1β protein expression by 0.89, 1.06, and 0.23 pg/mL, respectively. In the astrocytes co-treated with Nifedipine and 10 nM or 1 μM VGVAPG, an increase in IL-1β expression by 1.19 and 1.13 pg/mL, respectively, compared to the group treated with the VGVAPG peptide only (Fig. 3B).

### 3.4. Expression of IL-1βR1

After the 24-h cell treatment with 10 nM and 1 μM VGVAPG, both concentrations of the peptide decreased IL-1βR1 expression by 175.05 and 115.90 pg/mL, respectively, compared to the control (Fig. 4A). The cell treatment with Nifedipine, Verapamil, and MK-801 alone decreased IL-1βR1 protein expression by 366.67, 496.07, and 162.32 pg/mL, respectively. The astrocytes co-treated with Nifedipine and 10 nM or 1 μM VGVAPG exhibited a 350.07 and 389.59 pg/mL decrease in IL-1βR1 expression by 10 nM or 1 μM VGVAPG and cell co-treatment with 10 nM or 1 μM VGVAPG and 1 μM Verapamil, 1 μM Nifedipine, or 1 μM MK-801 on the level of IL-1β protein after 24 (A) and 48 (B) h in mouse primary astrocytes in vitro. The additional negative control (10 nM or 1 μM VVGPGA peptide) did not affect IL-1β protein expression - data not shown. Data are expressed as means ± SD of three independent experiments, each of which comprised six replicates per treatment group. The number of repetitions n = 9; *p < 0.5; **p < 0.01 and ***p < 0.001 vs. the control cells. #p < 0.5; ##p < 0.01; ###p < 0.001 vs. the group treated with the VGVAPG peptide alone.

Fig. 3. Effect of 10 nM or 1 μM VGVAPG and cell co-treatment with 10 nM or 1 μM VGVAPG and 1 μM Verapamil, 1 μM Nifedipine, or 1 μM MK-801 on the level of IL-1β protein after 24 (A) and 48 (B) h in mouse primary astrocytes in vitro. The additional negative control (10 nM or 1 μM VVGPGA peptide) did not affect IL-1β protein expression - data not shown. Data are expressed as means ± SD of three independent experiments, each of which comprised six replicates per treatment group. The number of repetitions n = 9; *p < 0.5; **p < 0.01 and ***p < 0.001 vs. the control cells. #p < 0.5; ##p < 0.01; ###p < 0.001 vs. the group treated with the VGVAPG peptide alone.

Fig. 4. Effect of 10 nM or 1 μM VGVAPG and cell co-treatment with 10 nM or 1 μM VGVAPG and 1 μM Verapamil, 1 μM Nifedipine, or 1 μM MK-801 on the level of IL-1β receptor 1 (IL-1βR1) protein after 24 (A) and 48 (B) h in mouse primary astrocytes in vitro. The additional negative control (10 nM or 1 μM VVGPGA) did not affect IL-1βR1 protein expression - data not shown. Data are expressed as means ± SD of three independent experiments, each of which comprised six replicates per treatment group. The number of repetitions n = 9; *p < 0.5; **p < 0.01 and ***p < 0.001 vs. the control cells. #p < 0.5; ##p < 0.01; ###p < 0.001 vs. the group treated with the VGVAPG peptide alone.
expression, respectively, compared to the group treated with the VGVAPG peptide only (Fig. 4A). In the astrocytes co-treated with Verapamil and 10 nM or 1 μM VGVAPG, there was a 286.37 and 235.96 pg/mL decrease in IL-1β expression, respectively, compared to the group treated with the VGVAPG peptide only (Fig. 4A).

The 48-h cell treatment with 10 nM and 1 μM VGVAPG induced a 136.37 and 158.02 pg/mL decrease in IL-1β expression, respectively, compared to the control (Fig. 4B). The cell treatment with Nifedipine and Verapamil alone did not affect the level of IL-1β in the cells. Only MK-801 decreased the level of IL-1β by 99.68 pg/mL, compared to the control group (Fig. 4B). The cell co-treatment with Nifedipine, Verapamil, or MK-801 and 10 nM or 1 μM VGVAPG did not affect the level of IL-1β, compared to the administration of the VGVAPG peptide alone (Fig. 4B).

3.5. Expression of the Cat protein

After the 24-h cell treatment with 10 nM and 1 μM VGVAPG, both concentrations of the peptide decreased Cat expression by 13.66 and 13.31 ng/mL, respectively, compared to the control (Fig. 5A). The cell treatment with Nifedipine and Verapamil alone decreased Cat protein expression by 18.29 and 19.91 ng/mL, respectively. In the astrocytes co-treated with Verapamil and 10 nM VGVAPG, a 7.13 ng/mL increase in Cat expression was observed.

Interestingly, after the 48-h exposure to 10 nM and 1 μM VGVAPG, no statistically significant changes were observed, compared to the control (Fig. 5B). After the cell treatment with Nifedipine alone, an increase in Cat protein expression by 13.69 ng/mL, compared to the control, was observed. However, MK-801 alone decreased Cat protein expression by 8.13 ng/mL, compared to the control. The cell co-treatment with Nifedipine and 10 nM VGVAPG decreased Cat protein expression by 11.60 ng/mL, compared to the administration of 10 nM VGVAPG. Similarly, in the cells co-treated with MK-801 and 10 nM VGVAPG, an 8.13 ng/mL decrease in Cat protein expression, compared to the treatment with 10 nM VGVAPG, was observed (Fig. 5B).

3.6. Expression of the Sod1 protein

After the 24-h cell treatment with 10 nM and 1 μM VGVAPG, both concentrations of the peptide increased Sod1 expression by 0.16 and 0.31 pg/mL, respectively, compared to the control (Fig. 6A). The cell treatment with Verapamil and MK-801 alone increased Sod1 protein expression by 0.36 and 0.56 pg/mL, respectively, compared to the control. The astrocytes co-treated with Nifedipine and 10 nM VGVAPG exhibited a 0.36 pg/mL increase in Sod1 expression, compared to the treatment with 10 nM VGVAPG. Interestingly, in the group co-treated with Nifedipine and 10 nM VGVAPG, there was a decrease in Sod1 expression by 0.11 pg/mL, compared to the variant with 1 μM VGVAPG. In the astrocytes co-treated with Verapamil and 10 nM or 1 μM VGVAPG, an increase in Sod1 protein expression by 0.40 and 1.30 pg/mL, respectively, compared to the exposure to 10 nM or 1 μM VGVAPG alone, was observed (Fig. 6A).

The 48-h cell treatment with 10 nM VGVAPG only contributed to a significant 0.48 pg/mL increase in Sod1 expression, compared to the control (Fig. 6B). The cell treatment with Nifedipine and Verapamil alone increased Sod1 protein expression by 0.27 and 0.33 pg/mL, respectively, compared to the control. In the astrocytes co-treated with Verapamil and 1 μM VGVAPG, a 0.42 pg/mL decrease in Sod1 protein expression, compared to the variant with 1 μM VGVAPG alone, was observed (Fig. 6B).

3.7. Production of estradiol

After the 24-h cell treatment with 10 nM and 1 μM VGVAPG, no changes in E2 level were observed (Fig. 7A). The cell treatment with Verapamil and MK801 alone increased the E2 level by 244.88 and 126.76 ng/mL, respectively, compared to the control. The co-treatment of the astrocytes with Nifedipine and 10 nM or 1 μM VGVAPG resulted in a 505.37 and 341.74 ng/mL increase in the E2 level, respectively, compared to the administration of 10 nM or 1 μM VGVAPG alone. The astrocyte co-treatment with Verapamil and 10 nM or 1 μM VGVAPG yielded a 616.22 and 276.66 ng/mL increase in the E2 level, respectively, compared to the variant treated with 10 nM or 1 μM VGVAPG alone. The cells co-treated with MK-801 and 10 nM VGVAPG exhibited an 82.81 ng/mL increase in the E2 level, compared to the treatment with 10 nM VGVAPG alone (Fig. 7A).

After the 48-h cell treatment with 10 nM and 1 μM VGVAPG, a slight
but not statistically significant increase in the E_2 level was observed (Fig. 7B). Moreover, none of the tool compounds used alone or in co-treatment with the VGVAPG peptide exerted an effect on the E_2 level (Fig. 7B).

4. Discussion

The half-life of elastin is estimated as 70 years (Shapiro et al., 1991). This phenomenon correlates with the first symptoms of certain neurodegenerative diseases such as Alzheimer’s disease (AD). One of the products of elastin degradation is the VGVAPG hexapeptide, which is internalized in cells by EBP, galectin-3, and avß3 integrin. However, there are reports on the interaction between VGVAPG and Pparγ (Szychowski and Gmiński, 2019b). PPARγ is an important receptor participating in degenerative processes in the brain and in peripheral systems (Villapol, 2018). The described intracellular functions of this receptor in cells have been associated e.g. with the control of anti-inflammatory mechanisms, response to oxidative stress, neuronal death, neurogenesis, differentiation, and angiogenesis (Villapol, 2018). Our experiments show that the 24-h exposure to the VGVAPG peptide decreases the level of Pparγ in astrocytes. In turn, after the 48-h of exposure to the
GVGAPG peptide, an increase in the Ppary protein level was observed. Moreover, our present data show that the use of Nifedipine or MK-801 in co-treatment with the VGVAPG peptide potentiated the effect of the VGVAPG peptide in both time intervals. Data from our previous publication show that the VGVAPG peptide lowered the Ppary expression in astrocytes after 24 h; however, the Ppary expression increased after 48 h, which is most likely the effect of receptor activation (Szychowski and Gmiński, 2019a). A similar expression pattern of PPARγ after a VGVAPG peptide treatment was observed in the SH-SYSY cell line (Szychowski et al., 2019a). To date, such CCBs as Nifedipine have been reported to increase PPARγ protein expression in mouse peritoneal macrophages and human platelets (Ishii et al., 2010; Shih et al., 2014). Similarly, the Verapamil CCB was found to increase the level of Ppary expression in C57BL mice (Tang et al., 2019). Therefore, these papers confirm our hypothesis that the analyzed VGVAPG peptide and CCBs works in an additive way. PPARγ activation has generally been shown to have anti-inflammatory effects (Clark, 2002). Moreover, our previous studies showed that the Ppary activation by the VGVAPG peptide in co-treatment with rosiglitazone (Pparγ agonist) was accompanied by a decrease in caspase-1 activity and decreased the levels of inflammatory markers (Szychowski and Gmiński, 2020a). Changes in the morphology of astrocytes are very important in the course of the inflammatory process (Verkhratsky et al., 2017). Moreover, the crosstalk between astrocytes and microglia is crucial in this process (Liddelow et al., 2017; Verkhratsky et al., 2017). However, our previous studies show that 10 nM and 1 μM of the VGVAPG peptide do not change the morphology of astrocytes (Szychowski et al., 2019; Szychowski and Gmiński, 2020). In light of the mentioned paper by Liddelow et al. (2017), this may be an effect of the absence of microglia in our culture (Liddelow et al., 2017). Therefore, in the further part of this study, we investigated the activity of caspase-1 and expression of IL-1β and IL-1βR1.

Caspase-1 plays a pivotal role in cell immunity as an inflammatory response initiator through activating pro-interleukin-1 beta (pro-IL-1β) to active IL-1β (Scheer, 2013). Moreover, caspase-1 can be activated in many nonimmune cell types, such as keratinocytes, astrocytes, hepatocytes, and cardiomyocytes (Ganz et al., 2011; Yazdi et al., 2010). Thus, this protein is considered a well-established indicator of active inflammation in many cell types. Our study is the first to investigate the impact of well-established inhibitors of certain calcium channels (Nifedipine, Verapamil, and MK-801) on VGVAPG-treated astrocytes. The experiment shows that all the studied CCBs decreased VGVAPG-induced caspase-1 activity in both time intervals (24 and 48 h of exposure). To date, available data have described that the VGVAPG peptide per se increases caspase-1 activity in mouse astrocytes (Szychowski and Gmiński, 2020a). Additionally, Szychowski and Gmiński (2020) described that caspase-1 activity initiated by the VGVAPG peptide was inhibited by rosiglitazone, i.e. a Ppary agonist; this was accompanied by an increase in Ppary expression, which suggests the engagement of this receptor in caspase-1 activity induction (Szychowski and Gmiński, 2020a). It is known that Ca2+ is required for caspase-1 activation and inflammasome formation (Murakami et al., 2012), which can explain why Nifedipine, Verapamil, and MK-801 decreased VGVAPG-stimulated caspase-1 activity in a similar way. Since caspase-1 activates pro-IL-1β to active IL-1β, we investigated IL-1β and IL-1βR1 in the further part of our study.

IL-1β is a key pro-inflammatory cytokine in the central nervous system (Moynagh, 2005). It also stimulates the production of adhesion molecules and chemokines in astrocytes (Bourke and Moynagh, 1999; Moynagh et al., 1994). IL-1 is a secreted protein and its receptor type 1 (IL-1R1) is a membrane-bound protein which may mediate the biological effects of IL-1α and IL-1β (Színga et al., 2009). Moreover, dysregulation of both IL-1β and IL-1βR1 is associated with AD (Martini et al., 2019). Our data show that the VGVAPG peptide decreased the IL-1β protein level in both time intervals. In the short-time exposure variant, the CCBs (Nifedipine, Verapamil and MK-801) intensively decreased IL-1β stimulated by the VGVAPG peptide. Opposite results were shown by Matsumori et al., who proved that Nifedipine did not cause significant changes in the IL-1β secretion in human PBMC cells. However, the tested organisms were different and the PBMC cells used were not pretreated with the VGVAPG peptide (Matsumori et al., 2010). Interestingly, in the longer period, CCBs contributed to an increase in IL-1β. Moreover, our experiments showed that the VGVAPG peptide decreased the level of IL-1βR1 in both time intervals. After 24 h, Nifedipine and Verapamil potentiated the effect of the VGVAPG peptide. In turn, after the 48-h exposure, CCBs did not have any effect on the level of IL-1βR1. Italiani et al. showed that the levels of IL-1βR1 were increased only in AD patients, compared to patients with mild cognitive impairment and memory complaints, which may have some diagnostic application (Italiani et al., 2018). In turn, our results showed that VGVAPG alone or in the co-treatment with CCBs exerted an opposite effect on IL-1βR1, which may indicate no engagement of the peptide in AD progression. However, as evidenced in our previous work, VGVAPG was able to decrease the proliferation of neuronal cell line SH-SYSY and induce oxidative stress, which is in accordance with the present theory of AD induction (Szychowski et al., 2019a). Moreover, different authors agree that EDPs induced the production and/or secretion of IL-1α, IL-1β, and IL-6 in ligamentum flavum cells, synovial cells, and melanoma cell lines (Debrot et al., 2006; Kobayashi et al., 2017; Satta et al., 1998). Unfortunately, the data concerning EDPs and IL-1β and/or IL-1βR1 are limited. To date, only one paper described the role of the VGVAPG peptide on the IL-1β and IL-1βR1 decrease in mouse astrocytes (Szychowski and Gmiński, 2020a). Szychowski and Gmiński (2020) reported that 10 nM VGVAPG induced a decrease in IL-1β and IL-1βR1 protein expression (Szychowski and Gmiński, 2020a). Moreover, the authors described that the Ppary agonist rosiglitazone potentiated the decrease in the IL-1β level caused by the VGVAPG peptide. Interestingly, rosiglitazone strongly increases IL-1βR1 protein expression as well (Szychowski and Gmiński, 2020a). On the other hand, in mouse macrophage cell line RAW264.7, Nifedipine did not increase IL-1β with an increase in Ppary expression, but such an effect could be cell and tissue specific (Choe et al., 2021). It is well described that SOD1 is necessary for the production of mature IL-1β activity (Harjihit et al., 2014; Meissner et al., 2008). Moreover, PPARγ controls the expression of such antioxidant enzymes as CAT or SOD1 (Kim and Yang, 2013). Therefore, in the next part of our study, we decided to measure the expression of two important antioxidant enzymes Cat and SOD1 in mouse astrocytes.

Cat and SOD1 are two main enzymes of the intracellular antioxidant system whose expression can be regulated by Ppary. Our data showed that the VGVAPG peptide contributed to a decrease in the Cat protein expression only after 24 h. Interestingly, CCBs did not influence the expression of Cat affected by the VGVAPG peptide in our experimental model. In turn, the VGVAPG peptide increased the Sod1 protein expression. However, only after the 24-h exposure to Nifedipine and Verapamil, the drugs potentiated the increase in Sod1 protein expression initiated by the VGVAPG peptide. Our data are consistent with our previous study, which showed that 10 nM VGVAPG increased the expression of Sod1 but decreased the expression of Cat in astrocytes (Szychowski and Gmiński, 2020a). Our present data show that Nifedipine and Verapamil exert a similar impact on the Sod1 level in astrocytes. Previously published data demonstrated that Nifedipine did not completely reduce VGVAPG peptide-activated ROS production, in contrast to MK-801 and Verapamil (Szychowski and Gmiński, 2019c). In our previous study, co-treatment of astrocytes with rosiglitazone and VGVAPG increased the levels of Sod1 and Cat, compared to their expression in the group treated with the VGVAPG peptide alone (Szychowski and Gmiński, 2020a). This is in line with the current state of knowledge of the ability of PPARγ to activate the expression of SOD1 and CAT. Interestingly, in the human neuroblastoma SH-SYSY cell line, 50 nM VGVAPG decreased the SOD1 protein expression but the expression of CAT was unchanged (Szychowski et al., 2019a). On the other hand, in a longer time interval, 50 nM VGVAPG peptide produced no changes in the expression of SOD1 although the expression of CAT was decreased (Szychowski et al., 2019a). The results obtained in the
SH-SY5Y cell line may differ from those from mouse astrocytes, since different concentration of the VGVAPG peptide were used in the experiments and SH-SY5Y cells have features of some stem cells (Walton et al., 2004).

Estradiol is an important neurosteroid that may have both beneficial and undesirable effects on the human brain and AD progression depending on the concentration, brain region, and time of exposure (Slooter et al., 1999; Song et al., 2020). As shown above, the VGVAPG hexapeptide exerts a certain impact on the Ppar gamma receptor and thus affects the inflammation indicators used in this study. Interestingly, the crosstalk between E2 and PPARgamma is well described (LIN et al., 2013).

Therefore, the last part of our manuscript was devoted to evaluation of the impact of CCBs on the E2 level in mouse astrocytes. Our data showed that VGVAPG did not change the level of E2 in the tested cells. However, after the 48-h exposure to 1 μM VGVAPG, the peptide slightly but not statistically significantly increased the E2 level. Interestingly, Nifedipine and Verapamil in the co-treatment with VGVAPG increased the E2 level. Our previous data have shown that the level of E2 after VGVAPG stimulation depends on the level of FBS in the medium (Szychowski et al., 2020; Szychowski and Gmizinski, 2020b). It is known that the doses of 10 nM and 1 μM VGVAPG increase the E2 level in medium with 1% FBS. In turn, the studied VGVAPG peptide concentrations do not affect the E2 level in medium with 10% FBS (Szychowski and Gmizinski, 2020b).

Previous experiments with the use of 10% of FBS demonstrated that the level of E2 in the tested cells, independent of NF-kappa B pathway in melanoma cells. Since from mouse astrocytes, our data showed (Szychowski et al., 2020b).

However, in the absence of Ppar gamma, the amount of E2 was increased along with a simultaneous decrease in astrocyte proliferation (Szychowski and Gmizinski, 2020b).

5. Conclusion

Our study is the first to investigate the impact of Nifedipine, Verapamil, and MK-801 on astrocytes treated with the VGVAPG peptide. The experiments showed that Nifedipine or MK-801 used in co-treatment with the VGVAPG peptide potentiated the effect of the VGVAPG peptide on the Ppar gamma level in both time intervals. Moreover, the obtained results showed that, depending on the concentration of VGVAPG, the tested CCBs can change the impact of such EDPs on the antioxidant enzymes Cat and Sod1 through interaction with PPARgamma. In addition, Nifedipine, Verapamil, and MK-801 affected the VGVAPG-induced IL-1β production and the level of IL-1βR1, which may suggest a role of such a mechanism in AD progression, which is characterized by enhanced IL-1 secretion. Furthermore, the results prove the previous findings of the impact of VGVAPG on the intracellular enzymatic anti-oxidant system, which is enhanced after treatment with CCBs, and suggest the occurrence of oxidative stress in the cell due to increased calcium and redox imbalance.

Author contribution statement

KS conceived and designed the research. KS conducted the experiments. KS analyzed the data. KS, BS and AT wrote the manuscript. All authors have read and approved the manuscript.

Compliance with ethical standards

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 a Bioethics Commission (No. 46/2014) as compliant with Polish law.

Declaration of competing interest

The authors declare that they have no conflict of interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2022.105405.

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