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Neuropeptide Y reduces migration capacity of human choriocarcinoma cell line by altering oxidative/antioxidative status

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Abstract: Reduced migration capacity of trophoblast cells leads to poor placentation and correlates with severe pregnancy disorders such as intrauterine growth restriction and preeclampsia. Neuropeptide Y (NPY) is sympathetic cotransmitter involved in various physiological processes and its levels are significantly increased in preeclamptic pregnancy compared to healthy pregnancy. In this study the prooxidative role of NPY and its effects on migration capacity of human trophoblast cell line JEG-3 were investigated together with the effects of nitric oxide (NO) depletion, a molecule that was shown to play an important role in promoting cell migration. The cells were treated for 24 h (short-term stimulation) and 72 h (long-term stimulation) respectively with 1 nM NPY. Oxidative/antioxidative status and the migration index of cells were measured. The results showed increased concentrations of oxidative stress parameters ($O_2^{\bullet-}$, H_2O_2) and molecules of the antioxidant defense system (reduced glutathione and oxidized glutathione), while the levels of intracellular nitrites (indicators of NO) and cell migration index were significantly decreased in trophoblast cells treated with NPY (both at 24 h and 72 h of exposure) compared to the control cells. These results suggest that NPY may significantly contribute to reduced migration capacity of trophoblast cells by generating oxidative stress and reducing the bioavailability of NO.

Key words: Neuropeptide Y, choriocarcinoma trophoblasts, oxidative stress, nitric oxide, migration

1. Introduction

The essential role in placentation belongs to trophoblast cells, which arise from the outer layer of blastocysts (Pennisi et al., 2012). The cytotrophoblast stem cells undergo either the villous or the extravillous pathway of differentiation. In the villous pathway, cytotrophoblasts fuse to form multinucleated syncytiotrophoblasts or aggregate to form anchoring villous trophoblasts, which give rise to extravillous trophoblasts (Loke et al., 1995). Villous trophoblasts cover the chorionic villi, creating the placental–blood barrier, involved in gas exchange and nutrient supply between the mother and the fetus. Extravillous trophoblasts deeply invade the uterine connective tissue, degrading the extracellular matrix (interstitial route), and place themselves between decidual and myometrial cells and then invade the uterine spiral arteries (endovascular route) (Loke et al., 1995). At this stage the extravillous trophoblasts remodel the maternal spiral arteries, replacing smooth muscle and endothelial cells forming vessels with a larger diameter, and increase blood flow and reduce vascular resistance, making these maternal vessels independent of maternal vasoconstriction. This remodeling invasion is essential in establishing and developing the placenta as well as in maintaining normal pregnancy, considering that the fetal requirement for blood supply is much higher in the later stages of pregnancy (Zhou et al., 1997). Since the process strongly depends on an optimal trophoblast invasion of the maternal tissue environment, failure of extravillous trophoblasts to fully invade and modify the uterine tissue leads to shallow placentation and is often associated with serious pregnancy complications such as preeclampsia and intrauterine growth restriction (Chaddha et al., 2004).

Neuropeptide Y (NPY) is a neuropeptide present in significant levels in the peripheral and central nervous systems and it has a role in various processes such as anxiety, stress-related behaviors, food intake, digestion, metabolism, immune response, memory, and learning (Heilig, 2004; Ferreira et al., 2010; Farzi et al., 2015). Literature data show that levels of NPY in patients with preeclampsia and eclampsia, both caused by inadequate placentation, are 2- to 4-fold higher than in women with normal pregnancies (Khatun et al., 2000).

The production of reactive oxygen species is a physiological process inherent to aerobic organisms due to mitochondrial respiratory chain activity and phagocytic

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actions (Nazıroğlu et al., 2015). Oxidative stress represents an imbalance between the production of reactive oxygen species and the compensating antioxidant mechanisms, which can lead to multiple cellular damages (Li et al., 2013).

Nitric oxide (NO) is a multifunctional signal molecule and has been reported as a mediator of many physiological and pathological processes in a variety of tissues, including those involved in human reproduction. NO is one of the most pleiotropic signaling molecules at systemic and cellular levels, participating in vascular tone regulation, cellular respiration, proliferation, apoptosis, and gene expression (Krause et al., 2011). Nitric oxide is generated from L-arginine by the enzyme nitric oxide synthase (NOS). Two of the three known isoforms of NOS are constitutively expressed in different cell types (NOS1 or neuronal; NOS3 or endothelial NOS - eNOS), whereas the expression of the third isoform (NOS2 or inducible NOS - iNOS) can be induced with cytokines and other agents (Moncada et al., 1991). Both iNOS and eNOS are present in human trophoblasts (Rossmanith et al., 1999; Hambartsoumian et al., 2001). The quantification of NO metabolites (like nitrites and nitrates) in biological samples provides valuable information with regard to in vivo NO production, bioavailability, and metabolism (Bryan et al., 2007). The production of NO by iNOS in the myometrium and placenta increases throughout pregnancy until delivery, when it decreases rapidly (Von Mandach et al., 2003). One of the main roles of NO increase during pregnancy is to stimulate vasodilatation and to increase blood flow subsequently (Benest et al., 2008). Nitric oxide activates protein kinase G (PKG), which phosphorylates multiple protein substrates including integrins and cell adhesion molecules. NO-dependent phosphorylation induces conformational changes of the integrins, altering their ligand-binding affinity, which affects cell motility (Cartwright and Balarajah, 2005; Chigaev et al., 2011).

It was shown in mice and rats that the stimulation of macrophages with NPY increased the production of reactive oxygen species such as peroxide and superoxide (De la Fuente et al., 2001; Dimitrijevic et al., 2005) and that these effects are mediated via Y1 and Y2 receptors (Bedoui et al., 2008). By stimulating the generation of these reactive oxygen molecules, which can alter levels of free NO (Jaimes et al., 2001; Hsieh et al., 2014), NPY could be an important factor in depletion of cell migration dependent on NO-mediated pathways. In this study we investigated the modulatory capacity of NPY on the mobility of a human choriocarcinoma cytotrophoblastic cell line by examining its effects on the oxidative/antioxidative balance and bioavailability of NO.

2. Materials and methods

2.1. Cell culture and treatment

The cytotrophoblastic human choriocarcinoma cell line JEG-3, purchased from the American Type Culture Collection, was used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with heat-inactivated 10% fetal bovine serum (FBS), penicillin, and streptomycin (GIBCO). The cells were grown to 80% confluence in 75-cm^2 flasks at 37 °C in a normal atmosphere containing 5% CO_2. This cell line is easy to culture and represents a convenient model for cell migration. NPY concentrations of 1 nM, 10 nM, and 100 nM were tested for cytotoxicity. In order to investigate the effects of NPY on oxidative/antioxidative status and cell migration capacity, 1 nM NPY was used as treatment. The stock solution of NPY was prepared in phosphate-buffered saline (PBS) and then the NPY stock was serially diluted in DMEM to final concentrations of 1 nM, 10 nM, and 100 nM. The control cells were cultured in conditioned medium only (DMEM) to acquire the basal levels of oxidative stress parameters and migration index.

2.2. MTT assay

The cells were seeded into a 96-well plate (10,000 cells per well) and incubated at 37 °C and 5% CO_2 for 24 h. The medium was replaced with 100 µL of medium containing various concentrations of NPY (1 nM, 10 nM, and 100 nM) and cells were incubated for 24 h. Untreated cells were used as the control. After 24 h of NPY treatment, the cell viability was determined by MTT assay (Mosmann, 1983). The test was based on the color reaction of mitochondrial dehydrogenase in living cells by MTT. At the end of the treatment period, MTT (final concentration: 5 mg/mL of phosphate buffer solution) was added to each well, which were then incubated at 37 °C in 5% CO_2 for 3 h. The colored crystals of produced formazan were dissolved in 150 µL of DMSO. The absorbance was measured by a microplate reader. The cell viability was calculated as a ratio of absorbance of the treated group divided by the absorbance of the control group and multiplied by 100 to give a percentage of viability.

2.3. Transwell assay for cell migration

The cell migration capacity was determined by the ability of cells to cross the pores of polycarbonate membranes (pore size 8 µm; Greiner Bio-One, Switzerland) fitted to the bottom of transwell chambers. The migration test was based on a protocol described by Chen (2005). The cells were exposed to 1 nM NPY for 24 h and 72 h, respectively. In another set of experiments the cells were incubated with 1 mM NG-nitro-L-arginine methyl ester (L-NAME) for 24 h and 72 h. The initial solution of L-NAME in PBS was used for the study and then the solution was diluted in conditioned medium (DMEM) to acquire the

1 mM concentration. The control cells were cultured only in conditioned medium (DMEM, GIBCO). Each measurement was performed in triplicate (3 wells for each experimental cell group). After the exposures, all three groups of cells were trypsinized and placed in the upper chambers at a density of 1×10^5 cells/well in 500 µL of DMEM with 10% FBS. The lower chambers of the control cells contained 750 µL of DMEM supplemented with 10% FBS whereas the lower chambers with treated cells were filled with 1 nM NPY or 1 mM L-NAME diluted in 750 µL of DMEM supplemented with 10% FBS. After 6 h of incubation at 37 °C, the cells from the upper surface of the filter were completely removed with gentle swabbing. The remaining migrated cells were fixed for 20 min at room temperature in 4% paraformaldehyde and stained with 0.1% crystal violet in 200 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 6.0) for 10 min. After adding 10% acetic acid, absorbance was measured by an ELISA microplate reader. The migration index was calculated as the ratio of absorbance of the treated group divided by the absorbance of the control and multiplied by 100 to give the percentage.

2.4. Cell extraction

After 24 h or 72 h of NPY treatment, cells were trypsinized, transferred from each flask into a separate test tube, and centrifuged for 10 min at 1000 × *g* and 4 °C in order to remove the culture medium (discarded supernatant). The cells were washed with ice-cold PBS (GIBCO), and after recentrifugation (10 min, 1000 × *g*, 4 °C) they were resuspended in a volume of PBS in which the number of cells was 10^6/mL. This cell suspension was used for further analysis.

2.5. The determination of superoxide anion ($O_2^{\bullet-}$)

NPY-treated cells adjusted to 1×10^6 cells/mL in PBS were plated at 100 µL/well in a 96-well flat-bottomed plate and mixed with 100 µL of 0.1% nitro blue tetrazolium (NBT). The determination of superoxide anion ($O_2^{\bullet-}$) was based on the reduction of NBT in the presence of $O_2^{\bullet-}$ (Auclair and Voisin, 1985). The concentration was expressed as nmol/L in 10^6 cells/mL.

2.6. NO_2^- and H_2O_2 determination

To determine the concentration of NO_2^- and H_2O_2, cell membranes were broken by sonication of the cell suspension on ice with a series of 10 successive acoustic shocks. The samples were then centrifuged for 20 min at 10,000 × *g* and 4 °C. The concentrations of nitrites and hydrogen peroxide were determined in the supernatant. The determinations of nitrites (NO_2^-, indicator of NO concentration) were performed using the Griess method (Green et al., 1982). The determination of the hydrogen peroxide (H_2O_2) concentration was based on oxidation of phenol red in the presence of horseradish peroxidase as a catalyst (Pick and Keisari, 1980). The concentrations of both parameters were determined by ELISA microplate reader. The concentrations of NO_2^- were expressed as µmol/L in 10^6 cells, while the levels of H_2O_2 were expressed as nmol/L in 10^6 cells/mL.

2.7. Glutathione determination

For measuring the concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG), the used supernatant was obtained by the following procedure: the cell suspension was centrifuged for 10 min at 1000 × *g* and 4 °C, and after the removal of supernatant, the pellet was resuspended in 2.25% sulfosalicylic acid. Cell membranes were lysed by alternate freezing (-80 °C) and thawing (37 °C) in 3 cycles for 15 min followed by 30 min of centrifugation at 1000 × *g*. The concentration of GSH was determined based on GSH oxidation with 5.5-dithiobis-6.2-nitrobenzoic acid (Beutler, 1975). The concentration of GSSG was determined based on GSH determination assay using glutathione reductase (Beutler, 1975) after inhibition of spontaneous GSH oxidation by 4-vinylpyridine. Glutathione concentration was expressed as µmol/L in 10^6 cells/mL.

2.8. Statistical analysis

All groups of data were analyzed with SPSS 13.0 for Windows. The results are presented as the mean ± SEM using the independent t-test to evaluate significant differences between experimental groups versus the control group. Probability values of less than 0.05 were considered significant.

3. Results

3.1. MTT viability assay

For the assessment of the appropriate concentration of NPY to be used for the treatment, the effect of this peptide on cytotrophoblast cell viability was examined. To investigate the acute effects of NPY on oxidative stress parameters and migration capacity, the highest noncytotoxic concentration of NPY was used in our study. The results of the MTT test showed that only the concentration of 1 nM was not cytotoxic for the JEG-3 cell line (more than 95% of cells survived). The higher tested concentrations of NPY (10 nM and 100 nM) significantly caused the decrease of the number of living cells (Figure 1).

3.2. Concentrations of oxidative/antioxidative status parameters ($O_2^{\bullet-}$, H_2O_2, and NO_2^-)

The concentrations of oxidative stress parameters after exposure to 1 nM NPY were measured in our experiment. The results showed that the concentrations of $O_2^{\bullet-}$ and H_2O_2 were significantly increased in cells treated with NPY for both 24 h and 72 h compared to the control cells ($P < 0.05$) (Figure 2). In this study the concentrations of NO_2^- (the indicator of NO) after 1 nM NPY treatment of trophoblast

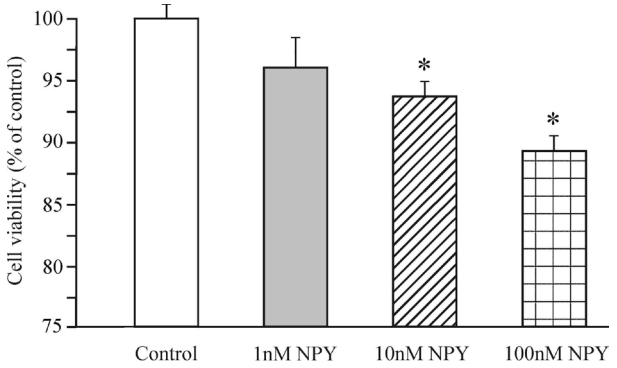

Figure 1. The results of MTT viability assay of human choriocarcinoma cell line JEG-3 exposed to various concentrations of NPY. Data are representative of three independent experiments and values are expressed as mean ± SEM. *: Statistically significant compared to the control group ($P < 0.05$).

cells were also examined. The results showed that NPY induced significant decrease in the concentrations of NO_2^- in the cells treated with NPY for 24 h and even stronger decrease in long-term (72 h) treatment as compared to control cells ($P < 0.05$) (Figure 2).

3.3. Concentrations of antioxidant defense system parameters (GSH and GSSG)

The concentrations of GSH in cells treated with NPY both for 24 h and 72 h were significantly increased compared to control cells ($P < 0.05$) (Figure 3). Glutathione is one of the strongest antioxidative components and its increased concentration may indicate de novo synthesis induced by NPY. No significant changes in the concentration of GSSG were recorded in the cells treated with NPY for 24 h compared to the control cells (Figure 3), which could indicate the initial high activity of glutathione reductase (GR) as one of the major enzymes of the antioxidant defense system in trophoblasts. However, the GSSG level in cells treated with NPY for 72 h was significantly elevated compared to control cells (Figure 3).

3.4. Transwell migration assay (Boyden chamber assay)

To examine the effects of NPY on the migration capacity of a human trophoblast cell line, a transwell migration assay was performed. Results showed 1.38-fold and 1.58-fold decrease in the cell migration index of JEG-3 cells exposed to NPY for 24 h and 72 h, respectively, compared to the control cells. Long-term exposure to NPY induced

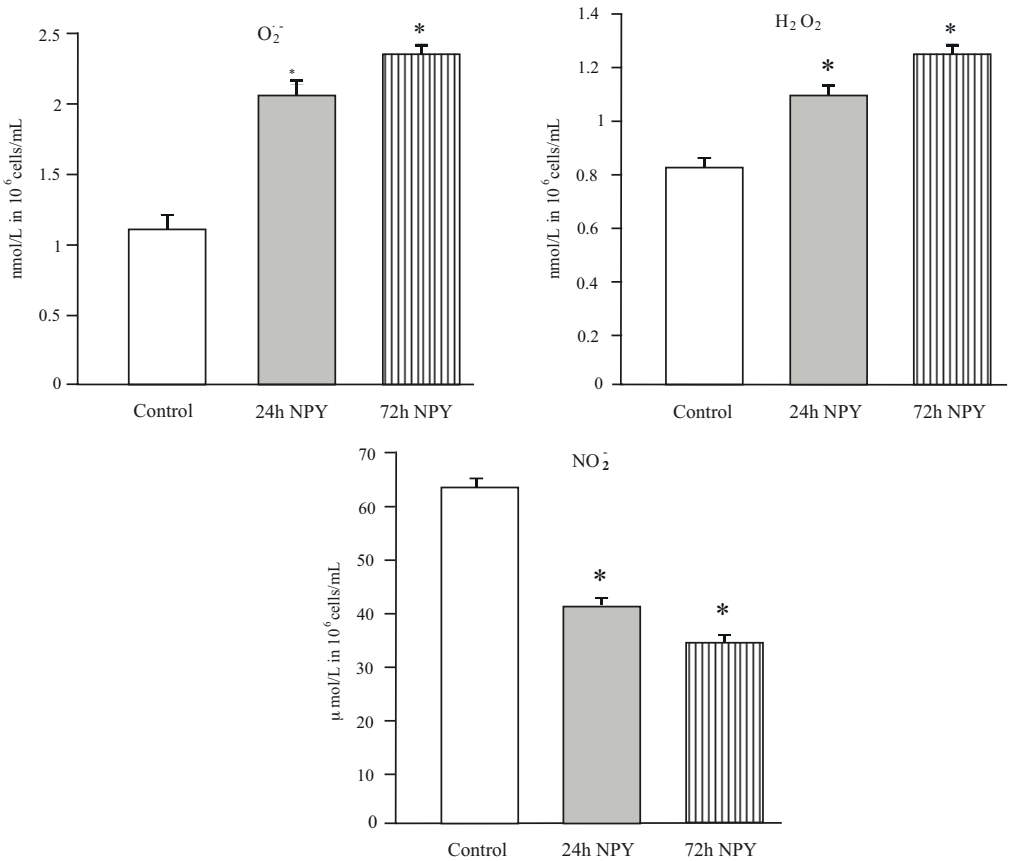

Figure 2. The effects of short-term (24 h) and long-term (72 h) exposure to NPY on parameters of oxidative/antioxidative status in human choriocarcinoma cell line JEG-3. Data are representative of three independent experiments and values are expressed as mean ± SEM. *: Statistically significant compared to the control group ($P < 0.05$).

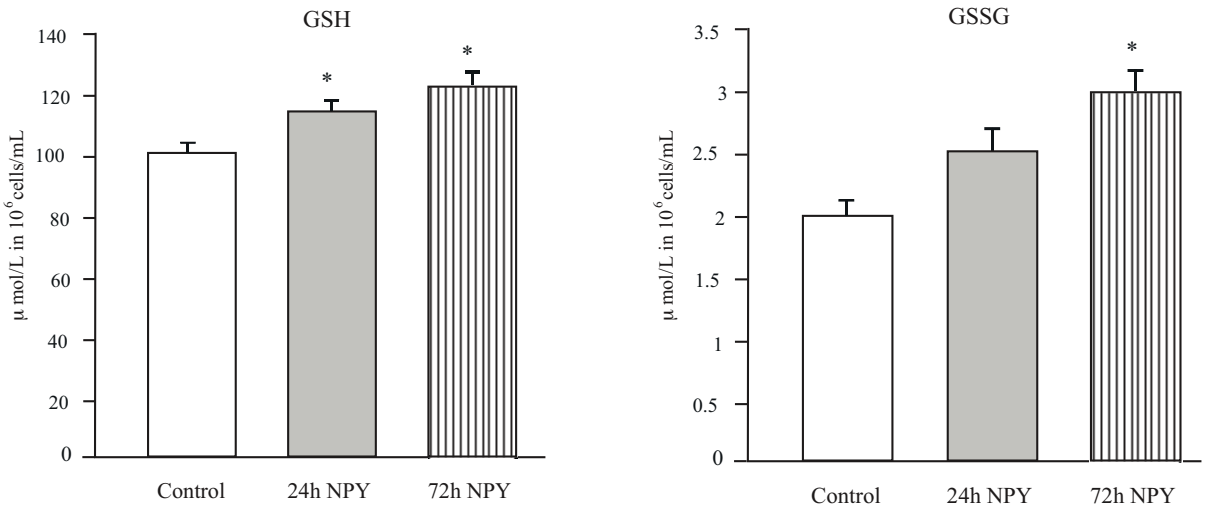

Figure 3. The effects of short-term (24 h) and long-term (72 h) exposure to NPY on reduced glutathione (GSH) and oxidized glutathione (GSSG) levels in human choriocarcinoma cell line JEG-3. Data are representative of three independent experiments and values are expressed as mean ± SEM. *: Statistically significant compared to the control group ($P < 0.05$).

stronger reduction of trophoblast migration compared to 24 h of treatment. These data indicate that treatment with 1 nM NPY significantly reduced the migratory capacity of human choriocarcinoma trophoblast cells (Figure 4). We proposed that NPY-induced reduction of migration capacity was related to a decrease in NO concentration during NPY treatment, so we exposed cells to 1 mM L-NAME (NOS inhibitor) for 24 h and 72 h. Our results showed 2.36-fold decrease in cell migration index in cells treated with L-NAME for 24 h and 2.14-fold decrease in cells treated with L-NAME for 72 h as compared to control cells (Figure 5). By comparing the migration index of the cells between NPY treatment and L-NAME treatment we could conclude that there was 1.76-fold decrease in cell migration index between short-term NPY and L-NAME exposures (24 h) (Figure 6a) and 1.36-fold decrease in cell migration index between long-term NPY and L-NAME exposures (72 h) (Figure 6b). These data indicate that

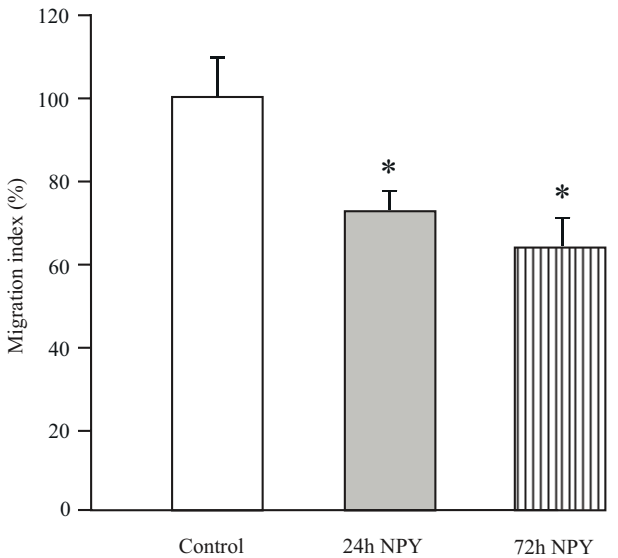

Figure 4. The effects of short-term (24 h) and long-term (72 h) exposure to NPY on migration capacity of human choriocarcinoma cell line JEG-3. Data are representative of three independent experiments and values are expressed as mean ± SEM. *: Statistically significant compared to the control group ($P < 0.05$).

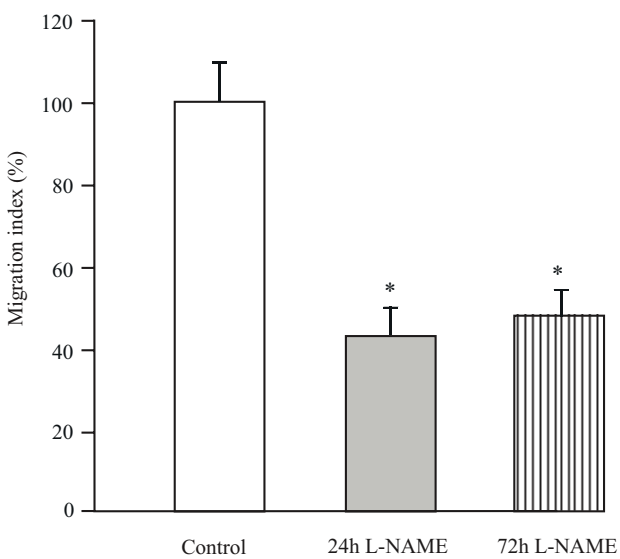

Figure 5. The effects of short-term (24 h) and long-term (72 h) exposure to L-NAME on migration capacity of human choriocarcinoma cell line JEG-3. Data are representative of three independent experiments and values are expressed as mean ± SEM. *: Statistically significant compared to the control group ($P < 0.05$).

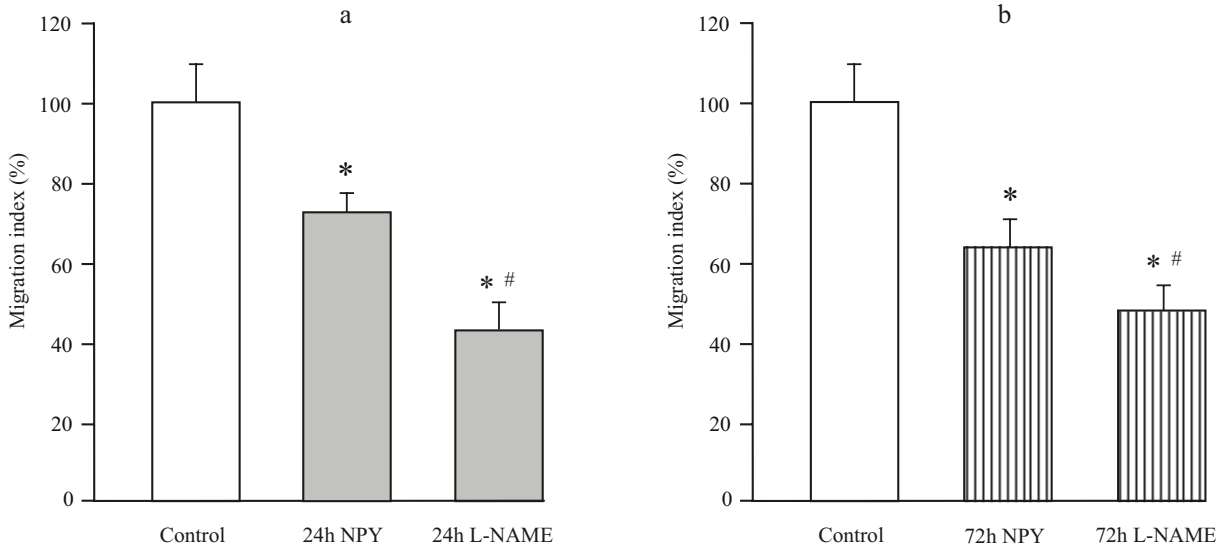

Figure 6. The ratio between the effects on migration capacity of human choriocarcinoma cell line JEG-3 of short-term (24 h) and long-term (72 h) exposure of these cells to 1 nM NPY and 1 mM L-NAME, respectively. Data are representative of three independent experiments and values are expressed as mean ± SEM. *: Statistically significant compared to the control group ($P < 0.05$).

NO depletion by L-NAME, and to a lesser extent by NPY, is responsible for decreased cell migration capacity. Together, these results suggest that reduced migration of human trophoblast cell line JEG-3 could be mediated by decreased levels of NO.

4. Discussion

Trophoblast migration is an essential step in successful implantation and formation of the placenta (Pijnenborg et al., 1983; Knöfler and Pollheimer, 2013). Khatun et al. (2000) showed that high concentrations of NPY may contribute to the development of preeclampsia, which is characterized by shallow trophoblast invasion. We proposed that NPY, regarding its increased level in preeclampsia, could be one of the factors that affect the migratory capacity of trophoblast cells by inducing oxidative stress. Various studies reported signal pathways that can affect the oxidative/antioxidative balance in cells. NPY has been shown to trigger the phospholipase C-protein kinase C (PLC-PKC) pathway, activating nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) via its receptors in human syncytiotrophoblasts and hamster suprachiasmatic nuclei cells (Robidoux et al., 1998; Schak et al., 2001). NADPH oxidase is an important respiratory chain enzyme and represents the main source of superoxide anion radical ($O_2^{\bullet-}$) in neutrophils, vascular endothelial cells, smooth muscle cells, and trophoblasts (Griendling et al., 2000; Halliwell and Gutteridge, 2007). So far, the Y_1 and Y_3 receptors for this peptide have been described in human trophoblasts as a mixed population (Robidoux et al., 1998, 2000). Prooxidative effects of neuropeptide Y mediated via Y_1 and Y_2 receptors were recorded in macrophages (Dimitrijevic et al., 2005; Bedoui et al., 2008), suggesting that elevated levels of reactive oxygen species in trophoblasts exposed to NPY could be caused by the elevated activity of NADPH oxidase in these cells. However, the possible activity alterations of NADPH oxidase induced by NPY exposure in trophoblasts are yet to be determined.

In this study we measured both the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG). Although these molecules might not directly interfere with nitric oxide, they are accepted as important markers of the oxidative/antioxidative status of the cell. Glutathione levels were measured in order to elucidate the intensity of oxidative stress induced by neuropeptide Y in trophoblasts, and to examine some antioxidative performances of these cells as well. The results showed increased levels of glutathione in both NPY treatments (24 h and 72 h). Reduced glutathione, a tripeptide consisting of cysteine, glutamate, and glycine, is one of the strongest antioxidative components in cells, maintaining intracellular thiol status and detoxicating various metabolites, essential for optimal activity of some enzymes and other cellular macromolecules (Chung et al., 2016). Since GSH is a potent electron donor, it represents the major nonenzymatic antioxidant component and one of the most important scavengers of free radicals, converting itself to the oxidized dimeric form, GSSG (Jones, 2002). Increased concentrations of reduced glutathione (GSH) recorded in our experiment suggest de novo synthesis of GSH induced by NPY. GSH is synthesized in cells de

novo and rapid induction of intracellular GSH synthesis occurs in response to various stressors (Kondo et al., 1993; Shi et al., 1994). Oxidative stress has been shown to induce transcriptional upregulation of the enzyme in the GSH biosynthesis pathway, γ GCS-gamma-glutamyl-cysteine synthetase (Rahman and MacNee, 1999; Ray et al., 2002). The cell culture medium (DMEM) contains a significant amount of cystine, which is one of the components used in GSH biosynthesis. Our results show the elevated levels of glutathione (GSH), which might be caused by de novo synthesis. The increased activity of GSH biosynthesis enzymes could be due to the high concentrations of reactive oxygen species generated in trophoblasts by NPY. Elevated GSH levels appear to be an important defense mechanism in human cytotrophoblasts in response to aggressive oxidative stress radicals induced by NPY. An increase in GSSG levels and reduction of the GSH/GSSG ratio indicates glutathione antioxidative activity in response to oxidative stress, which could lead to numerous cell damages (Lu, 2009). No significant changes in the concentrations of GSSG were recorded in cells treated with NPY for 24 h compared to the control cells (Figure 3), which could indicate the initial high activity of glutathione reductase (GR) as one of the major enzymes of the antioxidant defense system in trophoblasts. Since trophoblasts have stem cell features, the basal antioxidative activity of these cells may be higher compared to the other highly differentiated cell types (Aplin et al., 1999; Baczyk et al., 2006; Shenghui et al., 2009; Kobayashi and Suda, 2012). However, after long-term NPY exposure, trophoblast cells express increased levels of oxidized glutathione (GSSG). This elevation might be caused by prolonged production of reactive oxygen species generated by long-term NPY exposure, exceeding the reductive capacity of the GR enzyme and leading to accumulation of GSSG. The activity of GR and other antioxidant defense enzymes in JEG-3 cells is yet to be determined. Our results indicate the high basic antioxidant potential of trophoblasts, which could mean that NPY has the ability to generate an abundant burst of reactive oxygen species in trophoblast cells. The increased levels of H_2O_2 and $O_2^{\bullet-}$ during NPY exposure demonstrated in our study suggest that this peptide is a significant inducer of oxidative stress in this cell line.

In human trophoblasts both iNOS and eNOS are present, so these cells synthesize NO (Rossmanith et al., 1999; Hambartsoumian et al., 2001). Various actions of nitric oxide are known to contribute to the maintenance of pregnancy. Numerous data show that oxidative stress contributes to altered NO bioavailability (Pandey and Rizvi, 2010; Prokić et al., 2015). Superoxide anion radical, when produced in high concentrations, scavenges nitric oxide and forms cytotoxic peroxynitrite ($ONOO^-$) (Jourd'heuil et al., 2001; Calabrese et al., 2007). Not only does the superoxide anion radical oxidize lipids and proteins in cell membranes and cytoplasm, but the formation of peroxynitrites also results in reduced NO bioavailability, affecting NO-mediated pathways. Nitric oxide has been shown to affect cell migration and invasion in a variety of cell types (Murohara et al., 1999; Giordano et al., 2006; Li et al., 2009). In vitro studies showed that motility and invasion of trophoblast cells also depend on trophoblast-derived NOS (Cartwright et al., 1999; Harris et al., 2008). Nitric oxide has significant roles in cell migration via regulation of integrin phosphorylation (Chigaev et al., 2011). NO appears to be involved in the regulation of the phosphorylated state of two adhesion molecules, VCAM-1 (vascular cell adhesion molecule-1) and α4β1 integrin. These adhesion molecules are important in trophoblast interaction with endothelial cells in uterine arteries and their ligand binding affinity depends on phosphorylation performed by NO-dependent protein kinase G (Cartwright and Balarajah, 2005). In this study, the effects of NO depletion in the JEG-3 cell line on cell migration capacity were examined by treating the cells with 1 mM L-NAME, which is the inhibitor of NOS enzymes. Our data showed over 2-fold decrease in migration capacity (Figure 5), suggesting the essential role of nitric oxide in the migration mechanisms of these cells. The results obtained in a study by Harris et al. suggest that nitric oxide could also promote trophoblast invasion by mechanisms other than phosphorylation; S-nitrosylation of proteins at the leading edge of trophoblasts contributes to the elevated migration of these cells (Harris et al., 2008). The results of our experiment show that NPY treatment increased $O_2^{\bullet-}$ and decreased NO levels, suggesting that the formation of peroxynitrite ($ONOO^-$) is one of the possible mechanisms of reducing NO bioavailability in JEG-3 cells, contributing to the reduction of NO-dependent migration capacity. Moreover, regarding the presence of NO-producing enzymes (eNOS and particularly iNOS) in human trophoblasts (Rossmanith et al., 1999; Hambartsoumian et al., 2001), we assume that long-term (72 h) NPY treatment might affect expression patterns of these enzymes. Stronger reduction in NO bioavailability, as well as more severe reduction of migration capacity recorded in long-term NPY-treated cells in comparison to short-term NPY treatment, could be caused by prolonged production of reactive oxygen species (especially $O_2^{\bullet-}$) and/or by changes in expression levels of NO-generating enzymes. In further research, we plan to investigate mRNA levels and protein quantification of iNOS in long-term NPY treatment of the same cell line.

Although the majority of studies have reported invasion-promoting effects of NPY on various cell types (Ruscica et al., 2007; Sheriff et al., 2010), there are a number of data that indicate its antimigratory and antiproliferative effects.

NPY administration to a variety of invasive cancers results in reduced invasive potential and/or decreased proliferation of cells of these tumors in a concentration-dependent manner. NPY administration to a number of prostate cancer cell lines inhibited the proliferation through an NPYR1-dependent mechanism, while NPY administration to cholangiocarcinoma cells in vitro reduced the invasion potential of these tumor cells in a concentration-dependent manner (Ogasawara et al., 1997; DeMorrow et al., 2011). One study indicated NPY as an inhibitor of migration of monocytes to the tissue (Nave et al., 2004). The results of our investigation also indicate an antimigratory role of NPY on human cytotrophoblast cells.

According to the results we conclude that NPY induces oxidative stress in human trophoblast cell line JEG-3 by stimulating increased production of oxygen reactive species such as $O_2^{\bullet-}$ and H_2O_2 both in short-term (24 h) and long-term (72 h) treatments. The levels of GSH, as one of the main antioxidative components in trophoblasts, are increased probably due to its elevated de novo biosynthesis in response to NPY exposure, which implies considerable basal antioxidative capacity of human trophoblasts. Nevertheless, the increased levels of oxidized glutathione (GSSG) after long-term NPY exposure may suggest that NPY is a significant generator of oxidative stress, which can surpass the inherent trophoblast antioxidative capacity. The concentrations of nitric oxide are significantly decreased in trophoblasts exposed to NPY for both 24 h and 72 h of treatment compared to the control cells, as well as the migratory capacity of trophoblasts, with more severe reduction during long-term (72 h) exposure. Based on these data, we proposed that 24 h and 72 h of NPY treatment reduced the migratory potential of human choriocarcinoma trophoblast cells by reducing the bioavailability of NO. The short-term and long-term treatments with a specific inhibitor of NOS enzymes (L-NAME) reduced the migration index of these cells. Accordingly, we assume that this motility reduction is caused by depleted bioavailability of NO, which affects NO-dependent migration pathways. NO is involved in the regulation of the function of cell adhesion molecules, presumably the integrins, important for cell motility. Since NO reduction is mediated by superoxide anion radical scavenging of free nitric oxide and possibly by altering the activities and expression patterns of NO-generating enzymes, we assume that NPY is responsible for depleted migration capacity of human trophoblasts by generating considerable oxidative stress in these cells. In future studies, we plan to examine the effects of NPY on the other antioxidant defense system components in trophoblasts, as well as on the expression patterns and activities of oxidative stress generating enzymes, such as NADPH oxidase. Furthermore, the impact of NPY on the activities and expressions of iNOS enzymes, and on the expression and distribution of both adhesion and invasion proteins, will be examined as well. These data should contribute to a better understanding of underlying mechanisms of placental disorders.

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