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Metabolic Reprogramming of Trophoblast Cells in Response to Hypoxia

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Hypoxia of trophoblast cells is an important regulator of normal development of the placenta. However, some pathological states associated with hypoxia, *e.g.* preeclampsia, impair the functions of placental cells. Oxyquinoline derivative inhibits HIF-prolyl hydroxylase by stabilizing HIF-1 transcription complex, thus modeling cell response to hypoxia. In human choriocarcinoma cells BeWo b30 (trophoblast model), oxyquinoline increased the expression of a core hypoxia response genes along with up-regulation of *NOS3*, *PDK1*, and *BNIP3* genes and down-regulation of the *PPARGC1B* gene. These changes in the expression profile attest to activation of the metabolic cell reprogramming mechanisms aimed at reducing oxygen consumption by enabling the switch from aerobic to anaerobic glucose metabolism and the respective decrease in number of mitochondria. The possibility of practical use of the therapeutic properties of oxyquinoline derivatives is discussed.

Key Words: *BeWo b30; placenta; hypoxia; oxyquinoline; barrier*

The placenta plays a key physiological role in the fetal nutrition during pregnancy [5]. During embryogenesis, the embryo is implanted into the uterine wall at the blastocyst stage and then the inner cell mass (embryoblast that subsequently forms the fetus) and the outer cell layer (trophoblast that forms one of the basic elements of the placental barrier between the maternal and fetal blood) are formed. At the early stages of pregnancy, the trophoblast is under physiological hypoxia, which largely determines the natural processes occurring in it. At the same time, after complete formation of the blood supply system to the placenta by maternal blood, hypoxia of the trophoblast occurs only under pathological conditions, *e.g.* in preeclampsia [6,8]. Modeling the trophoblast cell response to hypoxia is necessary to study both physiological and pathophysiological processes in the placenta.

The molecular mechanism of cell response to hypoxia is common for all multicellular organisms. In response to low oxygen level, the cells increase the level of hypoxia-inducible factor (HIF), a highly conserved transcription factor responsible for the physiological and pathophysiological responses to hypoxia. This factor consists of a constitutive HIF-β subunit and a regulatory α-subunit (HIF-1α, HIF-2α, or HIF-3α). Hypoxia increases the level of α-subunit in the cell cytoplasm by inhibiting HIF-prolyl hydroxylases. Normally, these enzymes hydroxylate α-subunit followed by its degradation in proteasomes. Inhibition of prolyl hydroxylase prevents this process [15].

As previously shown, oxyquinoline derivatives with various substituents are able to interact with the active site of HIF-prolyl hydroxylases and inhibit hydroxylation of HIF-α subunits [9,10]. This leads to stabilization of the level of HIF factor transcription and activation of a number of target genes in the cells. In this case, activation of HIF in trophoblast cells can lead to metabolic reprogramming of cells, which manifests in a decrease in the number of mitochondria, a decrease in the activity of the electron transport chain,

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and transition from aerobic to anaerobic glucose metabolism, which ultimately increases glucose consumption by cells, but reduces oxygen consumption and increases oxygen supply to the fetus [3].

We studied the effect of oxyquinoline on a clone of choriocarcinoma BeWo b30 cells used to model trophoblast in the placental barrier. To this end, we studied the permeability for glucose of the cellular monolayer after incubation with oxyquinoline, as well as the expression of the genes involved in response to hypoxia and metabolic reprogramming under conditions of HIF activation.

MATERIALS AND METHODS

BeWo cells (clone b30) were obtained from Prof. Christiana Albrecht (University of Bern, Switzerland) with permission of Dr. Alan Schwartz (Washington University in St. Louis, USA). The cells were cultured in DMEM without L-glutamine and pyruvate with glucose content of 4.5 g/liter (Gibco) supplemented with 10% One Shot fetal calf serum (Gibco), L-glutamine (PanEco), non-essential amino acids MEM NEAA (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The cells were cultured at 37°C and 5% CO_2. We used cells with passage number no higher than 35.

For assessing oxyquinoline effect, the cells were seeded at a density of 2.1×10^5/cm^2 in polyester Transwell culture inserts (pore size 1 μ, growth area 0.143 cm^2, recommended volumes of the upper and lower chambers 70 and 235 μl, respectively; Corning Costar). The cell medium was daily replaced in recommended volumes after measuring transepithelial electrical resistance (TEER) with an EVOM2 instrument and a STX100C96 electrode (World Precision Instruments). On day 2 (in 42 h after cell seeding in Transwell inserts), when the cell monolayer reached confluence, 75 μl medium containing 1.5 μl 500 μM oxyquinoline derivative solution (hereinafter oxyquinoline) in DMSO was added to the upper chamber; final concentration of oxyquinoline in the medium was 10 μM. In the control, 75 μl medium containing 1.5 μl DMSO without oxyquinoline was added to the upper chamber. In 6 and 24 h after addition of oxyquinoline (48 and 66 h after cells seeding into Transwell inserts, respectively), TEER was measured.

Twenty-four hours after addition of oxyquinoline, the medium in the upper and lower chambers was replaced with the recommended volume of fresh medium. DMEM with low glucose content (1 g/liter), 1 mM sodium pyruvate, without L-glutamine and phenol red (Gibco) supplemented with 0.5% One Shot fetal calf serum (Gibco), L-glutamine (PanEco), and penicillin/streptomycin (100 U/ml+100 μg/ml; Gibco) was added to the lower chamber of Transwell system, while the same medium with glucose level adjusted to 4.5 g/liter with sterile D-(+)-glucose solution (Sigma-Aldrich) was added to the upper chamber. The cells were incubated for 1 h at 37°C and 5% CO_2 in a cell culture CO_2 incubator. Thereafter, the culture medium was sampled from the lower chambers to analyze glucose transport through the BeWo b30 monolayer. To do this, the Transwell inserts were neatly transferred to a new stand without medium in the lower chambers to prevent further transfer of glucose, then 10 μl medium was sampled from the lower chambers after careful mixing by pipetting and mixed with 990 μl Glucose LiquiColor reagent (Human GmbH). Standard glucose sample with a concentration of 1 g/liter and medium samples from the upper and lower chambers before incubation with the cells with expected glucose concentrations of 4.5 and 1 g/liter were also mixed with the reagent. Samples were incubated in a water bath at 37°C for 5 min, 200 μl of each sample was transferred to a Costar Ultra-Low Attachment 96-well plate (Corning), and the samples were read at λ=500 nm. Glucose concentrations in the samples were measured relative to the standard sample with a glucose concentration of 1 g/liter. Permeability for glucose was expressed as a percentage of the amount of glucose passed through the monolayer to the initial glucose content in the upper chamber of the Transwell system. The distribution of the permeability coefficients of glucose were compared to normal distribution by the Kolmogorov–Smirnov test. The distribution of the values did not differ significantly from the normal distribution; therefore, standard Student's t test was used to compare the permeability values.

After medium sampling for the glucose permeability assay, the membranes with BeWo b30 cells were neatly separated with tweezers and transferred to a tube containing 1000 μl of Qiazol Lysis Buffer (Qiagen). Nine membranes with cells were placed in one tube to obtain a sufficient amount of total RNA; in total, we obtained 3 tubes with lysates of cells incubated with oxyquinoline and 3 tubes with control cell lysates (all 54 culture inserts were cultured under the same conditions throughout the experiment). In each tube with the lysate, GlycoBlue Coprecipitant (Thermo Fisher Scientific) was added to increase the yield of RNA due to expected small amount of RNA [2,4,13]; then, total RNA was extracted using miRNeasy Micro Kit (Qiagen) according to manufacturer's protocol. The RNA concentration was measured on a NanoDrop ND-1000 instrument (Thermo Fisher Scientific) [11]. The quality of the isolated RNA was assessed on a Bioanalyzer 2100 using RNA 6000 Nano Kit (Agilent), all isolated RNA samples had RIN quality score >9.5.

For transcriptome analysis on Human Transcriptome Array 2.0 microarrays (Affymetrix), 500 ng total RNA isolated from each cell lysate was used [7,12]. The normalization and analysis of expression measurement were carried out by the Transcriptome Analysis Console version 4.0.1.36 (Thermo Fisher Scientific) using the eBayes statistical method. The changes were considered significant, if fold change of the expression was no less than 2 and false discovery rate (FDR) adjusted p value was <0.01.

RESULTS

Analysis of the permeability of the BeWo b30 cell monolayer for glucose after incubation for 24 h in the presence of oxyquinoline showed that glucose passage from the upper to lower chamber of the Transwell system (% of the total amount of glucose present in the upper chamber over 1 h) was 13±3% under control conditions and 9±3% after cell incubation with oxyquinoline ($p<0.05$). These changes can be explained by transition of trophoblast cells from aerobic to anaerobic glucose metabolism upon activation of HIF-1 [3]. To confirm activation of HIF-1, the expression of HIF-1 target genes and genes activated by hypoxia were analyzed.

In a previous study [1], HIF-1 target genes forming the core of the response to hypoxia were analyzed using an integrated genomic approach. At the first stage, the authors performed a search for studies that assessed the effect of hypoxia on the gene expression profile in various cells using Affymetrix chips. The authors noted that the lists of genes altered by hypoxia in various types of cells are significantly different, and a fairly small number of genes in these lists overlap. Using the integrated genomic approach the authors compiled the list of genes that reliably enter the core of the response to hypoxia [1]. We studied the change in the expression of these genes in BeWo b30 cells exposed to oxyquinoline that should mimic hypoxia due to inhibition of prolyl hydroxylases and accumulation of HIF-1. The changes were considered significant, if fold change of the expression was no less than 2 and FDR adjusted p value was <0.01. Transcriptional activation of the expression of these genes (Table 1) confirms stabilization of the HIF-1 transcription complex after exposure of BeWo b30 cells to oxyquinoline.

It was also reported [3] that metabolic reprogramming that takes place in the trophoblast cells *in vitro* under conditions of hypoxia leads to reduced oxygen consumption by mitochondria due to both transition from aerobic to anaerobic glucose metabolism and regulation of activity of the electron transport chain (ETC) directly and/or through NO synthesis that competes with oxygen in the ETC. In our experiment, exposure to oxyquinoline led to an increase in the expression of NO synthase gene *NOS3* in BeWo b30 cells by 2.1 times ($p<0.001$), which can indicate activation of NO synthesis aimed at reduction of oxygen consumption in ETC.

In our experiment, the expression of the *PDK1* gene encoding pyruvate dehydrogenase kinase 1 was increased by 3.1 times. It is known that HIF-1 is capable of increasing the level of PDK1, which leads to inhibition of the regulatory subunit E1 of the pyruvate dehydrogenase complex and transition from aerobic to anaerobic metabolism of pyruvate, the final product of glycolysis [3].

Another possible mechanism of trophoblast reaction to hypoxia is a HIF-1-dependent decrease in the number of mitochondria and the corresponding reduction of oxygen consumption by the trophoblast. This process is achieved through several mechanisms. The decrease in the expression of the *PPARGC1B* gene encoding PPAR-γ co-activator 1β suppresses biogenesis of mitochondria. At the same time, enhanced expression of the *BNIP3* gene leads to a hypoxia-induced increase in mitochondrial autophagy [3]. In our experiment, the expression level of *BNIP3* increased by 5.8 times ($p=0.001$), and the expression of *PPARGC1B* gene decreased by 2.6 times ($p<0.001$), which can attest to compensatory activation of the decrease in the number of mitochondria in BeWo b30 cells in response to exposure to oxyquinoline.

In BeWo b30 cells, the transcription of the *NFE2L2* gene encoding transcription factor Nrf2 also doubled upon exposure to oxyquinoline. This protein plays a key role in the regulation of the antioxidant program in cells and provides the protective cell response to hypoxia [14]. This led to a slight (by 1.5 times) increase in the expression of the thyroredoxin reductase gene 1 *TXNRD1* ($p<0.001$) and superoxide dismutase gene *SOD1* ($p=0.007$), as well as to a marked increase in the expression of glutathione reductase gene *GSR* by 6.3 times ($p<0.001$) and hemoxygenase 1 gene *HMOX1* by 3.1 times ($p<0.001$).

Thus, chemical activation of HIF-1-associated response to hypoxia in the BeWo b30 choriocarcinoma cells used to model the trophoblast in the placental barrier led to an increase in the expression of HIF-1 target genes forming the core of the response to hypoxia. We unveiled a preventive change in the expression of genes responsible for metabolic reprogramming in the trophoblast cells aimed at reduction of oxygen consumption through a decrease in the number of mitochondria and transition from aerobic to anaerobic glucose metabolism. These changes are accompanied by a decrease in the permeability of the BeWo b30 monolayer for glucose, probably due to higher glucose consumption by cells with predominance of

TABLE 1. Genes of the Core of the Response to Hypoxia [1], the Expression of Which Is Enhanced in BeWo b30 Cells upon Exposure to Oxyquinoline

Gene	$\log_2$ level of expression after oxyquinoline exposure	$\log_2$ level of expression without oxyquinoline	Multiplicity of the increase in expression	p
AKAP12	8.7	7.4	2.5	0.002
KDM3A (JMJD1A)	12.1	10.3	3.4	<0.001
P4HA1	12.2	9.8	5.2	<0.001
SEC24A	13.6	12.6	2.0	0.001
DHX40	13.8	12.8	2.1	0.001
GYS1	9.2	8.1	2.2	0.007
SEC61G	10.4	9.0	2.5	0.003
SRP19	10.2	9.2	2.0	0.005
NAMPT	10.2	8.5	3.2	<0.001
RRAGD	9.0	6.8	4.4	0.001
FAM162A (C3orf28)	7.2	6.2	2.0	0.004
MXI1	8.8	7.7	2.1	0.002
CIART (C1orf51)	10.1	7.5	6.2	0.001
FGFR1OP2	10.4	9.3	2.1	0.005
NDRG1	8.7	6.8	3.7	<0.001
STIP1	14.4	13.0	2.6	<0.001
DDIT3	10.6	7.7	7.5	<0.001
BNIP3	12.8	10.3	5.8	0.001
ATF3	11.8	9.1	6.2	<0.001
STAT3	11.6	9.8	3.4	<0.001
GADD45B	11.0	7.0	16.3	<0.001
NXF1	13.8	11.9	3.6	<0.001
MXD1	12.9	11.9	2.0	<0.001
ATP6V1D	11.7	10.8	2.0	0.001
TMEM39A	11.5	10.3	2.4	<0.001
PPME1	10.4	9.2	2.3	0.003
CLK3	11.4	9.5	3.7	<0.001
TIPARP	8.5	7.4	2.1	0.006
SERPINE1	11.3	9.7	3.0	<0.001
OSER1 (C20orf111)	11.3	9.2	4.2	<0.001

anaerobic metabolism. Moreover, we observed activation of the antioxidant system of cells at the expression level after 24-h incubation with oxyquinoline. The obtained results indicate the possibility of using oxyquinoline derivatives in the treatment of pathological conditions caused by reduced oxygen supply to the placenta and fetus at various stages of pregnancy. The pulse application for rapid increase in the concentration of HIF-1 protein is possible followed by the launch of the "cascade" of genes that form the core response to hypoxia. In addition, preventive "reprogramming" of trophoblasts can considerably facilitate tolerability of the pathological process caused by hypoxia. The possibility of the therapeutic effects of oxyquinoline on fetal cells depends on the effectiveness of transcellular transport through the placental barrier. Further research is needed to better understand the underlying processes.

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REFERENCES

1. Benita Y, Kikuchi H, Smith AD, Zhang MQ, Chung DC, Xavier RJ. An integrative genomics approach identifies Hypoxia Inducible Factor-1 (HIF-1)-target genes that form the core response to hypoxia. Nucleic Acids Res. 2009;37(14):4587-4602.
2. Fomicheva KA, Osip'yants AI, Knyazev EN, Samatov TR, Shkurnikov MY. Detection of potential Metastatic prostate cancer circulating biomarkers by comparison of miRNA profiles in DU145 cells and culture medium. Bull. Exp. Biol. Med. 2017;162(6):792-796.
3. Illsley NP, Caniggia I, Zamudio S. Placental metabolic reprogramming: do changes in the mix of energy-generating substrates modulate fetal growth? Int. J. Dev. Biol. 2010;54(2-3):409-419.
4. Knyazev EN, Fomicheva KA, Mikhailenko DS, Nyushko KM, Samatov TR, Alekseev BY, Shkurnikov MY. Plasma levels of hsa-miR-619-5p and hsa-miR-1184 differ in prostatic benign hyperplasia and cancer. Bull. Exp. Biol. Med. 2016;161(1):108-111.
5. Knyazev EN, Mal'tseva DV, Zacharyants AA, Zakharova GS, Zhidkova OV, Poloznikov AA. TNFα-induced expression of transport protein genes in HUVEC cells is associated with enhanced expression of transcription factor genes RELB and NFKB2 of the non-canonical NF-κB Pathway. Bull. Exp. Biol. Med. 2018;164(6):757-761.
6. Knyazev EN, Mal'tseva DV, Zakharyants AA, Zakharova GS, Zhidkova OV, Poloznikov AA. Expression of microRNA genes MIR221, MIR222, and MIR181B1 increases during induction of inflammation in the endothelial barrier model. Bull. Exp. Biol. Med. 2018;164(6):749-752.
7. Knyazev EN, Nyushko KM, Alekseev BY, Samatov TR, Shkurnikov MY. Suppression of ITGB4 gene expression in PC-3 cells with short interfering RNA induces changes in the expression of β-integrins associated with RGD-receptors. Bull. Exp. Biol. Med. 2015;159(4):541-545.
8. Macklin PS, McAuliffe J, Pugh CW, Yamamoto A. Hypoxia and HIF pathway in cancer and the placenta. Placenta. 2017;56):8-13.
9. Osipyants AI, Smirnova NA, Khristichenko AY, Hushpulian DM, Nikulin SV, Chubar TA, Zakhariants AA, Tishkov VI, Gazaryan IG, Poloznikov AA. Enzyme-substrate reporters for evaluation of substrate specificity of HIF prolyl hydroxylase isoforms. Biochemistry (Mosc). 2017;82(10):1207-1214.
10. Poloznikov AA, Zakhariants AA, Nikulin SV, Smirnova NA, Hushpulian DM, Gaisina IN, Tonevitsky AG, Tishkov VI, Gazaryan IG. Structure-activity relationship for branched oxyquinoline HIF activators: Effect of modifications to phenylacetamide "tail". Biochimie. 2017;133:74-79.
11. Rudimov EG, Knjazev EN, Khaustova NA, Grigorieva OV, Buravkova LB. Transcriptomic changes in human umbilical cord blood endothelial cells under simulated microgravity. Dokl. Biochem. Biophys. 2017;472(1):1-4.
12. Shkurnikov MY, Knyazev EN, Wicklein D, Schumacher U, Samatov TR, Tonevitskii AG. Role of L1CAM in the Regulation of the canonical Wnt pathway and class I MAGE genes. Bull. Exp. Biol. Med. 2016;160(6):807-810.
13. Skurnikov MY, Makarova YA, Knyazev EN, Fomicheva KA, Nyushko KM, Saribekyan EK, Alekseev BY, Kaprin AD. Profile of microRNA in blood plasma of healthy humans. Bull. Exp. Biol. Med. 2016;160(5):632-634.
14. Smirnova NA, Kaidery NA, Hushpulian DM, Rakhman II, Poloznikov AA, Tishkov VI, Karuppagounder SS, Gaisina IN, Pekcec A, Leyen KV, Kazakov SV, Yang L, Thomas B, Ratan RR, Gazaryan IG. Bioactive flavonoids and catechols as Hif1 and Nrf2 protein stabilizers — implications for Parkinson's disease. Aging Dis. 2016;7(6):745-762.
15. Thompson CB. Into thin air: How we sense and respond to hypoxia. Cell. 2016;167(1):9-11.