Growth Factors, Cytokines, and Cell Cycle Molecules

Oxygen Modulates the Response of First-Trimester Trophoblasts to Hyperglycemia

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Pregestational diabetes retards early embryonic growth. Placental and fetal growth are closely associated, suggesting that placental growth is also impaired. During the first trimester of gestation, oxygen tension rises steeply, leading to excessive production of reactive oxygen species (ROS), which is exacerbated in diabetes and may affect placental development. We hypothesized that oxygen modifies hyperglycemic effects on ROS formation, resulting in decreased first-trimester trophoblast growth. This was tested using a first trimester trophoblast-derived cell line (ACH-3P). Normoglycemia did not alter ACH-3P proliferation at 2.5%, 8%, and 21% oxygen. Hyperglycemic conditions for up to 3 days reduced cell number by 65% and resulted in cell cycle (G₁- and S-phase) changes but only at 21% oxygen. Proliferation reduction could be partially restored by an inhibitor of mitogen-activated protein kinase (MAPK) ERK1/2 but not of Akt/PkB. Intracellular ROS elevation under hyperglycemia was oxygen independent, whereas mitochondrial superoxide levels were enhanced under hyperglycemia only at 21% oxygen. Intervention to modulate cytosolic and mitochondrial ROS, using ROS formation inducers and inhibitors, did not alter cell growth under hyperglycemia at 21% oxygen. The combination of hyperglycemia and high oxygen levels (21%) reduces proliferation of human first-trimester trophoblasts in a ROS-independent manner involving MAPK. This may account for reduced placental growth and, therefore, also for embryonic growth during the first-trimester pregestational diabetic pregnancies when the oxygen tension increases. (*Am J Pathol* 2012, 180:153–164; DOI: 10.1016/j.ajpath.2011.09.012)

The human placenta is the key organ for sustaining fetal growth and development. It transports maternal nutrients and oxygen to the fetus and synthesizes a variety of

hormones. Pathological pregnancies, such as those complicated by early pregnancy failure,¹ preeclampsia,² and intrauterine growth restriction,³ are associated with inadequate placental development.

In pregnancies of diabetic women, the incidence of these pathologies is increased.^{4–6} Maternal diabetes is often associated with high plasma glucose levels, which in turn lead to fetal hyperglycemia.⁷ During the first trimester of a diabetic pregnancy, embryonic growth may be transiently reduced, a condition known as early embryonic growth delay.^{8,9} The strong association between fetal and placental weight^{10,11} makes it tempting to speculate that early embryonic growth delay in diabetes is associated with impaired placental development. Lower maternal serum levels of the human trophoblast-specific hormones placental lactogen and pregnancy-associated plasma protein A support this notion.¹² Trophoblast proliferation is the key driver of placental growth in early pregnancy. However, little is known about potential changes of placental development in first trimester diabetic pregnancies and the underlying molecular mechanisms.

Early in pregnancy, the placenta and embryo develop in a state of low oxygen tension, and nutrients are supplied by maternal plasma and by secretion of maternal endometrial glands. The placental oxygen tension steeply rises between 8 to 12 weeks of gestation, reflecting the onset of maternal blood flow. Concomitantly, establishment of placental blood flow is associated with a burst of oxidative stress in this organ. Increasing oxygen concentrations lead to generation of reactive oxygen species (ROS), thus exposing the placenta to oxidative stress even in normal pregnancies.^{13–15} Physiological ROS levels can promote and control cellular fate, playing a crucial role in placental development through cellular signaling. Under normal conditions, the placental antioxidant defense systems, which have the capacity to induce conversion of ROS to water and molecular oxygen,

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prevent ROS overproduction as a consequence of increased metabolic activity of placental mitochondria throughout gestation.¹⁶

In several tissues, hyperglycemia appears to be directly linked to ROS production.¹⁷ Thus, high glucose concentrations as found in maternal diabetes can increase oxidative stress and may lead to biochemical disturbances in the complex processes of fetal development and growth.¹⁸ Several studies have reported that oxidative stress appears to be a key event in diabetic complications.^{19–21} This notion is supported by the findings of decreased vitamin C and vitamin E levels and reduced antioxidant capacity of superoxide dismutase (SOD) in diabetic women.²²

In most mammalian cells, ROS can be generated in the cytosol by xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cytochrome P450 oxidoreductase, and other NADPH-dependent oxidoreductases, and in mitochondria by the electron transport chain. These mechanisms are also operative in the human placenta.²³ Cytosolic NADPH oxidase, xanthine oxidase, or cytochrome P450 in the endoplasmic reticulum produces superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by transferring electrons from NADPH or NADH, respectively, to molecular oxygen.^{24,25} Mitochondrial ROS is produced as a result of electron transport in the mitochondrial respiratory chain incorrectly coupled to oxidative phosphorylation. Incomplete reduction of oxygen yields O_2^- , which is eventually converted to H_2O_2 and hydroxyl radical.^{26,27} Recent work reported enhanced mitochondrial O_2^- production under hyperglycemic conditions compared to cells cultured at low glucose levels.²⁸

The present study tested the hypothesis that diabetes-associated hyperglycemia *in vitro* leads to a ROS-induced reduction of placental growth during the first trimester of pregnancy in an oxygen-tension-dependent manner. Because placental growth early in pregnancy is mainly driven by trophoblast growth, we focused on the first-trimester trophoblast. Primary human trophoblasts do not proliferate in culture; therefore, we recently generated a first trimester trophoblast-derived cell line, ACH-3P, which closely resembles the primary trophoblasts.²⁹ Because ACH-3P cells proliferate *in vitro*, they represent a particularly suitable model for the purpose of the present study.

Materials and Methods

Cell Culture

ACH-3P cells were cultured at 37°C, 5% CO_2 , and 21% O_2 in 175-cm² flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium (DMEM) containing low D(+)-glucose, sodium pyruvate, and L-glutamine (Gibco, Invitrogen, Paisley, UK). Medium was supplemented with 10% fetal calf serum (FCS; Gibco) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria). Cells were grown in 75-cm² flasks (5×10^5 cells/flask; Nunc), 6-well dishes (5×10^4 cells/well; Iwaki, Tokyo, Japan), or 24-well dishes (1×10^4 cells/well; Corning, Lowell, MA). The day before any treatment, medium was replaced with fresh DMEM low

in D(+)-glucose, without FCS, supplemented with 1% penicillin/streptomycin overnight. On the following day, the cells were treated with DMEM low (normoglycemia; 5.5 mmol/L) or high (hyperglycemia; 25 mmol/L) in D(+)-glucose, supplemented with 2% FCS and 1% penicillin/streptomycin, for up to 3 days at 37°C, 5% CO_2 , and 2.5%, 8%, or 21% oxygen in an XVIVO incubation system Model G300C (BioSpherix, Lacona, NY). For osmotic control, 19.5 mmol/L L(–)-glucose (Invitrogen, Eugene, OR) was added to DMEM low in D(+)-glucose. Media were replaced every 24 hours in the XVIVO incubation system.

Quantification of Intracellular ROS

Intracellular ROS generation was measured using nonfluorescent 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA ; Invitrogen), which becomes fluorescent through oxidation and remains in the cytosol after enzymatic intracellular hydrolysis. Relative increase in fluorescence intensity was used as indicator for oxidative stress. ACH-3P cells were cultured in 24-well dishes under normoglycemia, hyperglycemia, and osmotic control conditions at 2.5%, 8%, and 21% oxygen for up to 3 days. After treatment, cells were loaded with 10 μ mol/L H_2DCFDA for 15 minutes, and the dye was replaced by PBS (Gibco) subsequently. Fluorescence intensity was determined (485 nm/520 nm excitation/emission) using a FluoSTAR Optima 413 spectrofluorimeter (BMG Labtechnologies, Offenburg, Germany). For normalization to cell numbers after fluorescence measurements, cells were washed once with Hank's balanced salt solution (Gibco) and loaded with 1 μ mol/L CellTrace Calcein Violet AM (Invitrogen) for 30 minutes at 37°C to determine cell viability. Wells were measured in staining solution (355 nm/460 nm excitation/emission). Nonfluorescent Calcein Violet AM permeates cell membranes and is enzymatically hydrolyzed to a fluorescent charged form, retained in living cells. We used it as a proxy for the number of viable cells, accounting for proliferation changes at various culture conditions. Subsequently, the signal obtained by intracellular ROS generation was normalized to that of Calcein Violet for each well.

Quantification of Hydrogen Peroxide

H_2O_2 released from ACH-3P cells after 3 days under hyperglycemia at 21% oxygen was measured using Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) in combination with horseradish peroxidase. Cells were cultured in 24-well dishes under normo- and hyperglycemic conditions. After incubation, cells were washed once and incubated with Hank's balanced salt solution containing 100 μ mol/L Amplex Red and 1 U/mL horseradish peroxidase (Sigma Aldrich, Steinheim, Germany). The increase in resorufin fluorescence was immediately measured at 544 nm/580 nm (excitation/emission) for 20 minutes using monochromatization and top excitation. To account for proliferation changes at various culture conditions, resorufin fluorescence readings were normalized to cell numbers using DAPI staining as a proxy for the number of viable cells. To this end, the cells were washed once and subsequently fixed with 4% formaldehyde for 15 minutes at

37°C, washed again with Hank's balanced salt solution, and then stained with 500 μ L DAPI (2.5 μ g/mL; Invitrogen) for 10 minutes at 37°C. Cell fluorescence was measured in PBS at 355 nm/460 nm (excitation/emission). Subsequently, the signal representing released H₂O₂ was normalized to that of DAPI staining for each well.

Real-Time RT-PCR

Total RNA was isolated using TRI Reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. RNA concentration was determined by a NanoDrop ND-1000 spectrophotometer (Peglab Biotechnologies, Erlangen, Germany), and RNA quality was assessed by red staining of denaturing agarose (Biozym, Vienna, Austria) gels. RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Briefly, 2 μ g of RNA from cells cultured under normo- or hyperglycemia were incubated with the kit components, in a total volume of 20 μ L, for 10 minutes at 25°C, 120 minutes at 37°C, and 10 seconds at 85°C in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems).

Real-time RT-PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen, Germantown, MD) for glutathione reductase 1 (Hs_GPX1_1_SG, QuantiTec Primer Assay; Qiagen), nuclear factor (erythroid-derived 2)-like 2 (Hs_NFE2L2_a_SG, QuantiTec Primer Assay), and heme oxygenase 1 (Hs_HMOX1_1_SG, QuantiTec Primer Assay). Ribosomal L30 (Hs_RPL30_1_SG, QuantiTec Primer Assay) served as internal control. Briefly, cDNA templates were diluted 1:10, and 13.2 μ L of cDNA was mixed with 13.2 μ L of SYBR Green (2 $\times$) and 3.3 μ L of Primer (10 $\times$). A LightCycler 480 (Roche, Mannheim, Germany) was used to analyze gene expression levels in triplicate. The appropriate program involves initial denaturation of 5 minutes at 95°C and a subsequent two-step cycle at 95°C for 10 seconds and at 60°C for 30 seconds for 40 cycles. The Ct values were automatically calculated by the LightCycler 480 software, and relative gene expression was calculated by the standard $\Delta\Delta$ Ct method using the expression level of RPL30 as reference.

Visualization of Mitochondrial Superoxide

Mitochondrial superoxide was assayed with MitoSOX Red (Invitrogen) that permeates living cells and targets mitochondria, where it is oxidized by superoxide, yielding red fluorescence. After 3 days under normo- or hyperglycemia, cells were loaded with 5 μ mol/L MitoSOX Red for 10 minutes at 37°C. Microscopy fluorescence images were taken (Eclipse TE2000-U; Nikon, Vienna, Austria) at fixed exposure time. Subsequently, cells were incubated with 2.5 μ mol/L CellTracker Green CMFDA (Invitrogen) for 20 minutes at 37°C to visualize living cells.

Proliferation Assay

Cell proliferation was determined by automatic counting of viable and dead cells using CASY Model TT (Schärfe System, Reutlingen, Germany). Proliferation of ACH-3P cells was determined after 1 to 3 days of treatment. For CASY

measurements, cells were enzymatically detached, and 50 μ L of cell suspension were diluted (1:200) in 10 mL CASY-ton (Roche), and viable and dead cells were determined.

Cell Cycle Analysis

Cell cycle was analyzed by flow cytometry at the Flow Cytometry Core Facility at the Center for Medical Research of the Medical University of Graz. After 3 days of treatment, the cells were harvested, centrifuged, resuspended in 500 μ L PBS and fixed with 5 mL of ice-cold 70% ethanol (Merck, Darmstadt, Germany) at 4°C for 10 minutes. After fixation, cells were centrifuged and washed twice with 5 mL PBS. Pellets were resuspended in 250 μ L of PI-staining buffer (Beckman Coulter, Brea, CA), incubated for 15 minutes at 37°C, and kept on ice until analysis. Cell cycle distribution was determined by FACSCalibur (BD Bioscience, San Jose, CA). The proportion of cells in each cell cycle phase (G₁, S, and G₂/M) was determined by Moefit Software (Verity Software House, Topsham, ME).

Exposure of ACH-3P Cells to Varying Antioxidants Such as Ascorbate, Trolox, and N-Acetylcysteine

For antioxidant studies, culture medium was supplemented with ascorbate (100 μ mol/L), Trolox (100 μ mol/L), and N-acetylcysteine (NAC) (1 mmol/L; Sigma-Aldrich). Stock solutions of ascorbate and NAC were prepared in bidistilled water; Trolox was prepared in ethanol. As vehicle control, ethanol was added to the medium and incubated under the same conditions for 3 day.

Exposure of ACH-3P Cells to Allopurinol, Apocynin, VAS-2870, and BCNU

For inhibition of xanthine and NADPH oxidases, culture medium was supplemented with varying concentrations of inhibitors; 0.1 to 1 mmol/L allopurinol, 100 μ mol/L and 250 μ mol/L apocynin (Sigma-Aldrich), and 1 μ mol/L VAS-2870 (Enzo Life Science, Plymouth Meeting, PA) were used. Stock solution of allopurinol was prepared in bidistilled water. Apocynin was diluted in ethanol, and VAS-2870 stock was prepared in DMSO (Merck). BCNU [1,3-bis-(2-chloroethyl)-1-nitrosourea; Sigma-Aldrich] inactivates glutathione reductase and interrupts recycling of glutathione disulfide to glutathione. It was dissolved in PBS and added at final concentrations of 5 μ mol/L, 10 μ mol/L, and 15 μ mol/L. As vehicle, ethanol or DMSO was added to medium and incubated under the same conditions for 3 day.

Exposure of ACH-3P Cells to Antimycin A, Oligomycin, and FCCP

Antimycin A is an inhibitor of complex III of the mitochondrial electron transport chain. Oligomycin was used as ATPase (complex V) inhibitor and FCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Sigma Al-

drich] as an uncoupler of oxidative phosphorylation, inhibiting mitochondrial O_2^- production. Antimycin A and oligomycin were added at a final concentration of 10 $\mu\text{mol/L}$ to the medium. FCCP was used at concentrations of 0.1 $\mu\text{mol/L}$, 0.3 $\mu\text{mol/L}$, and 0.5 $\mu\text{mol/L}$. Stocks were prepared in ethanol and DMSO, which were also used as vehicle controls. Cells were treated for 3 days.

Exposure of ACH-3P Cells to U0126 and Wortmannin

U0162 (Calbiochem, San Diego, CA) is a potent and specific inhibitor of MEK1 and MEK2; hence, it blocks the extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling pathway. Wortmannin (Calbiochem) was used as protein kinase B (Akt/PkB) inhibitor. U0126 and wortmannin were added to the medium at a final concentration of 10 $\mu\text{mol/L}$ and 100 nmol/L, respectively. Stocks were prepared in DMSO, which was also used as vehicle control. Cells were treated for 3 days.

Statistical Analysis

Statistical analysis used Prism 5 (GraphPad Software, La Jolla, CA). Differences between two treatment conditions were tested with unpaired *t*-test. Differences between three or more groups were analyzed with one-way analysis of variance followed by Bonferroni test. Data were expressed as mean $\pm$ SD. *P* values of <0.05 were considered statistically significant.

Results

Basal Proliferation under Normoglycemia Is Oxygen-Independent

We first investigated the effect of varying oxygen concentrations on cell proliferation. To this end, ACH-3P cells were cultured for up to 3 days under normoglycemia at 2.5%, 8%, and 21% oxygen. Subsequently, viable and dead cells were counted. Neither number of viable nor dead ACH-3P cells was significantly altered after 1 to 3 days under normoglycemic conditions at different oxygen concentrations (Figure 1, A and B). The proportion of dead cells after 3 days of incubation amounted to 17%, 19%, and 20% of total cells at 2.5%, 8%, and 21% oxygen, respectively. These data suggest that basal proliferation of ACH-3P cells under normoglycemia is oxygen-independent.

Proliferation Is Reduced and Cell Cycle Is Altered under Hyperglycemia Only at 21% Oxygen

Under hyperglycemic conditions, the number of viable cells did not differ after 1 to 3 days at 2.5% and 8% oxygen, compared to normoglycemic conditions. Likewise, the number of dead cells also was not affected by these conditions (Figure 2, A and C). The absence of changes in cell number corresponded to cell cycle anal-

Figure 1. Proliferation of ACH-3P cells for up to 3 days under normoglycemia at 2.5%, 8%, and 21% oxygen. Number of viable cells at 2.5%, 8%, and 21% oxygen (A); number of dead cells at 2.5%, 8%, and 21% oxygen (B); mean $\pm$ SD (*n* = 6) of three independent experiments.

ysis, which did not show significant alterations in the G₁-, S-, and G₂/M-phase (Figure 2, B and D). Hence, hyperglycemia did not affect cell growth and cell cycle at 2.5% and 8% oxygen.

However, at 21% oxygen, hyperglycemia led to decreased cell proliferation compared to normoglycemia and osmotic control. Two days under hyperglycemic conditions resulted in 40% fewer viable ACH-3P cells. The number of viable cells was even more decreased (up to 65%) after 3 days under hyperglycemia (*P* < 0.001) compared to cells cultured under normoglycemic and osmotic control conditions. No alterations in dead cell count were observed (Figure 2E). Cell cycle analysis showed a significant increase in cells in S-phase and a tendency of decreased G₁-phase (*P* = 0.07) under hyperglycemia at 21% oxygen. Compared to cells treated under normoglycemia, the G₂/M-phase was unchanged (Figure 2F). These results indicate that reduced proliferation under hyperglycemia is caused by changes in G₁- and S-, but not in the G₂/M-phase.

Intracellular ROS Generation Is Oxygen-Independent under Hyperglycemia

Hyperglycemia can directly cause excessive production of ROS.^{16,30} Thus, to determine whether hyperglycemia-induced proliferation changes at 21%, but not at

Figure 2. Proliferation and cell cycle analysis of ACH-3P cells cultured for up to 3 days under normoglycemia (NG) and hyperglycemia (HG) at 2.5%, 8%, and 21% oxygen. **A, C, and E:** Number of viable (gray bars) and dead (black bars) cells; mean $\pm$ SD ($n = 18$); **(B, D, and F)** cell cycle analysis (G_1 -, S-, and G_2 /M-phase); mean $\pm$ SD ($n = 6$) from three (**A, C, and E**) and four (**B, D, and F**) independent experiments, respectively. * $P < 0.05$, *** $P < 0.001$ versus NG; and $^{†††}P < 0.001$ versus osmotic control. n.s., not significant. Based on 18 replicates, the coefficient of variation for 1 day, 2 days, and 3 days under NG was 17%, 35%, and 23% at 2.5% oxygen; 19%, 20%, and 18% at 8% oxygen; and 25%, 37%, and 10% at 21% oxygen, respectively. After 1 day, 2 days, and 3 days under HG, the coefficient of variation was 16%, 43%, and 27% at 2.5% oxygen; 20%, 27%, and 38% at 8% oxygen; and 21%, 88%, and 14% at 21% oxygen, respectively.

2.5% and 8% oxygen, are paralleled by changes in oxidative stress, intracellular and mitochondrial ROS levels were measured. HepG2 cells served as a positive control³⁰ for increased ROS production under hyperglycemia. Intracellular ROS levels measured by H₂DCFDA were significantly ($P < 0.001$) elevated (1.9-fold) after 48 hours at high glucose concentrations compared to cells cultured at normal glucose levels (data not shown).

Under hyperglycemia, 2.1- and 2.2-fold more ROS were produced at 2.5% ($P < 0.001$) and 8% oxygen ($P < 0.01$ versus normoglycemia; 0.05 versus osmotic control), respectively, compared to controls. Interestingly, at 21% oxygen, ACH-3P cells generated 35% less ROS under hyperglycemia ($P < 0.001$). Under normoglycemia, the highest ROS generation was measured at 21% oxygen ($P < 0.001$). ROS production under hyperglycemia at 21% oxygen was not significantly different from

Figure 3. Intracellular ROS levels of ACH-3P cells under hyperglycemia (HG) at 2.5%, 8%, and 21% oxygen and expression levels of stress response genes at 21% oxygen. **A:** ACH-3P cells were cultured for 3 days under normoglycemia (NG; black bars), hyperglycemia (gray bars), and osmotic control conditions (white bars) at different oxygen concentrations; **(B)** ROS levels of ACH-3P cells after 0, 1, 2, and 3 days under hyperglycemia at 21% oxygen. ROS levels were normalized to cell number and are presented as arbitrary units (a. u.); the ROS levels under normoglycemia (control) were set to 100%; ($n = 9$). **C:** H₂O₂ release by ACH-3P cells after 3 days under hyperglycemic conditions. H₂O₂ levels were normalized to cell number and are presented as arbitrary units ($n = 12$). **D:** mRNA expression of *HMOX1*, *GPX1*, and *NFE2L2* after 3 days under hyperglycemia at 21% oxygen using quantitative real-time RT-PCR. Expression levels were normalized to RPL30 ($n = 3$); data are presented as mean $\pm$ SD of four (**A** and **B**), two (**C**) and three (**D**) independent experiments, respectively. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus NG; and $^{\dagger}P = 0.05$ and $^{+++}P = 0.001$ versus osmotic control.

ROS levels under the same conditions at 2.5% and 8% oxygen. This suggests that ROS formation under long-term hyperglycemia is independent of oxygen, whereas under normoglycemia, higher oxygen tension (21%) augments ROS formation (Figure 3A). Already after 1 day under hyperglycemia, ROS levels were decreased ($P <$

0.05) by up to 10% compared to normoglycemic conditions at 21% oxygen. They decreased even further by up to 30% (Figure 3B) after 2 days under these conditions. The lowest ROS levels ($P < 0.001$, decreased by 36%) were reached after 3 days under hyperglycemia at 21% oxygen (Figure 3, A and B).

To corroborate the changes in intracellular ROS production found by H₂DCFDA, H₂O₂ release from ACH-3P cells was monitored using the Amplex Red assay. After 3 days under hyperglycemia at 21% oxygen, H₂O₂ release was reduced by 28% ($P < 0.001$) (Figure 3C). Taken together, these results indicate decreased ROS levels in ACH-3P cells at high glucose and high oxygen concentrations.

Hyperglycemia Up-Regulates Antioxidant Genes

Expression levels of several antioxidant-response related genes were analyzed with quantitative real-time RT-PCR. Hyperglycemia significantly increased mRNA levels of *HMOX1* (1.6-fold), *GPX1* (1.9-fold), and *NFE2L2* (2.3-fold) compared to normoglycemic conditions at 21% oxygen (Figure 3D), indicating an up-regulation of these cellular antioxidant systems under hyperglycemia.

Mitochondrial Superoxide Levels Are Enhanced under Hyperglycemia at 21% Oxygen

In addition to cytosolic ROS sources, mitochondria produce ROS as a result of incomplete reduction of oxygen during electron transport in the mitochondrial electron transport chain.²⁷ Mitochondrial superoxide levels were determined using MitoSOX Red. Under hyperglycemic conditions (Figure 4D), the cells showed elevated levels of superoxide compared to cells under normoglycemia (Figure 4B). Addition of two electron transport chain inhibitors, antimycin A and oligomycin, served as positive controls. Both inhibitors enhanced superoxide levels under normo- (Figure 4, B versus F) and hyperglycemic (Figure 4, D versus H) conditions at 21% oxygen. In contrast to total intracellular ROS levels, which were reduced under hyperglycemic conditions compared to control cells, mitochondrial superoxide levels were increased by hyperglycemia as compared to normoglycemia at 21% oxygen. This suggests that hyperglycemia differently alters ROS levels in mitochondria and the cytosol under the experimental conditions.

Addition of Antioxidants Does Not Restore Proliferation under Hyperglycemia at 21% Oxygen

To investigate the mechanism behind decreased cell proliferation under hyperglycemia at 21% oxygen, we assessed the effects of antioxidants (ascorbate, Trolox, and NAC) on proliferation. Whereas the glucose effect on cell number was confirmed again, addition of antioxidants to the culture medium had no effect on cell proliferation (Figure 5, A to C). The number of dead cells was also not altered in any treatment conditions (data not

Figure 4. Fluorescence microscopy of mitochondrial superoxide levels after 3 days under hyperglycemia (HG) at 21% oxygen. After treatment, ACH-3P cells were loaded with CellTracker Green CMFDA (**A**, **C**, **E**, and **G**) and MitoSOX Red (**B**, **D**, **F**, and **H**). **A** and **B**: Normoglycemia (NG); (**C** and **D**) hyperglycemia; (**E** and **F**) normoglycemia in the presence of two mitochondrial electron transport chain (ETC) inhibitors (antimycin A and oligomycin); (**G** and **H**) hyperglycemia in the presence of antimycin A and oligomycin.

shown). Thus, scavenging ROS by ascorbate, Trolox, and NAC did not restore proliferation of ACH-3P cells under hyperglycemia, suggesting no association between ROS and proliferation under these conditions.

To confirm our hypothesis that proliferation of ACH-3P is ROS independent, we further increased and decreased ROS levels using specific inhibitors. The effects of the reagents used here on cytosolic and mitochondrial ROS formation are shown in Figure 6.

Manipulating Cytosolic ROS Levels Does Not Restore Proliferation under Hyperglycemia at 21% Oxygen

Cytosolic ROS levels were manipulated using allopurinol, apocynin, VAS-2870, and BCNU, and measured how this affects cell proliferation. Xanthine and NADPH oxidases are known to generate cytosolic ROS.^{31,32} Inhibition of NADPH oxidase with apocynin (100 μ mol/L and 250 μ mol/L) or VAS-2870 (1 μ mol/L), a novel NADPH oxidase inhibitor, did not influence the number of viable cells

under hyperglycemia (Figure 7, A and B). The inhibition of xanthine oxidase using allopurinol under hyperglycemic conditions also did not increase cell proliferation to normoglycemic levels (Figure 7C). The number of dead cells was not altered under the conditions used here (data not shown). Hence, decreasing cytosolic ROS generation did not restore proliferation of ACH-3P cells, indicating that impaired proliferation under hyperglycemia does not depend on ROS formation.

Further experiments were performed using an inhibitor of glutathione reductase (BCNU), which eventually leads to enhanced ROS generation. Addition of BCNU significantly reduced proliferation under normo- and hyperglycemic conditions in a dose-dependent manner (Figure 7D). The number of dead cells was not altered under any of the conditions (data not shown). In the absence and presence of 5 μ mol/L and 10 μ mol/L BCNU, proliferation was still significantly decreased under hyperglycemia compared to control cells. Thus, BCNU treatment did not restore the hyperglycemic levels to those of normoglycemia. Collectively, decreasing and increasing cytosolic ROS generation did not restore proliferation of ACH-3P cells under hyperglycemia at 21% oxygen.

Figure 5. Addition of antioxidants had no effect on cell proliferation under hyperglycemia (HG) at 21% oxygen. ACH-3P cells were cultured for 3 days under normoglycemia (NG; black bars) and hyperglycemia (gray bars) in the presence and absence of antioxidants at 21% oxygen. **A**: Ascorbate (100 μ mol/L), (**B**) Trolox (100 μ mol/L), and (**C**) NAC (1 μ mol/L); number of viable cells are presented as mean $\pm$ SD ($n = 6$) of three independent experiments. n.s., not significant. Based on 18 replicates, the coefficient of variation under NG and HG was 45% and 40%, respectively.

Figure 6. Effect of inhibitors on increasing and decreasing cytosolic (c) and mitochondrial (m) ROS generation. Allopurinol blocks xanthine oxidase and, therefore, reduces cytosolic ROS generation. Apocynin and VAS-2870 are inhibitors for membrane-bound NADPH oxidase and lead to decreased ROS formation in the cytosol. BCNU inhibits glutathione reductase (GR) and thereby prevents recycling of the oxidized glutathione disulfide (GSSG) to the reduced form of glutathione (GSH), which may cause increased intracellular ROS production. FCCP disrupts the tight coupling of the oxidative phosphorylation and reduces mitochondrial superoxide anion ($O_2^{\bullet-}$) generation; antimycin A blocks complex III; and oligomycin inhibits ATPase (complex V) in the mitochondrial electron transport chain, leading to enhanced superoxide levels. CAT, catalase; CuZn-SOD, copper-zinc superoxide dismutase; GPX, glutathione peroxidase; Mn-SOD, manganese superoxide dismutase.

Enhanced and Reduced Mitochondrial Superoxide Levels Do Not Restore Proliferation under Hyperglycemia at 21% Oxygen

Hyperglycemia had a different effect on cellular and mitochondrial ROS levels. Therefore, we investigated the effect of mitochondrial ROS on cell proliferation by manipulating mitochondrial superoxide levels. Cells were treated with FCCP, antimycin A, and oligomycin to reduce (FCCP) or elevate (antimycin A, oligomycin) superoxide levels under hyperglycemia at 21% oxygen. Varying concentrations of FCCP could not restore proliferation (Figure 8A). After the addition of 0.5 $\mu\text{mol/L}$ FCCP, significantly fewer viable cells were observed under normo- and hyperglycemic conditions. Lower FCCP concentrations reduced the number of viable cells under hyperglycemia (Figure 8A). The number of dead cells did not change under any of the conditions (data not shown). Hence, decreased superoxide levels via mitochondria could not improve proliferation under hyperglycemia at 21% oxygen.

Forced elevation of superoxide levels with antimycin A³³ (Figure 8B) decreased the number of viable cells under normo- ($P < 0.001$) and hyperglycemia compared to vehicle controls, whereas the number of dead cells was not changed (data not shown). Oligomycin had a similar effect on proliferation (Figure 8C).

Cell growth under hyperglycemia was still reduced after the addition of antimycin A and oligomycin compared to cells treated under normoglycemic conditions. These results suggest that mitochondrial ROS formation does not restore cell proliferation under hyperglycemia at 21% oxygen.

Inhibition of the ERK1/2, but Not the Akt/PKB, Signaling Pathway Increase Cell Proliferation under Hyperglycemia at 21% Oxygen

The number of viable cells was increased ($P < 0.001$) in the presence of ERK1/2 inhibitor U0126 (10 $\mu\text{mol/L}$) un-

Figure 7. Increased and decreased cytosolic ROS generation did not alter ACH-3P proliferation under hyperglycemia (HG) at 21% oxygen. ACH-3P cells were treated for 3 days under normoglycemia (NG; black bars) and hyperglycemia (gray bars) in the presence and absence of NADPH oxidase (apocynin and VAS-2870), xanthine oxidase (allopurinol), and glutathione reductase inhibitors (BCNU) at 21% oxygen. **A:** Apocynin, **B:** VAS-2870, **C:** allopurinol, **D:** BCNU. Number of viable cells are presented as mean $\pm$ SD ($n = 6$). *** $P < 0.001$ versus NG or HG and NG + vehicle or HG + vehicle. Based on six replicates, the coefficient of variation under NG and HG was (**A** and **B**) 16% and 10%, (**C**) 19% and 21%, (**D**) 10% and 22%, respectively.

Figure 8. Reduced or enhanced mitochondrial superoxide levels did not restore ACH-3P proliferation under hyperglycemia (HG) at 21% oxygen. ACH-3P cells were treated for 3 days under normoglycemia (NG; black bars) and hyperglycemia (gray bars) in the presence and absence of an uncoupler (FCCP) and two different mitochondrial electron transport chain (ETC) inhibitors (antimycin A and oligomycin) at 21% oxygen. Number of viable cells after addition of (A) FCCP, (B) antimycin A, and (C) oligomycin. Number of viable cells are presented as mean $\pm$ SD ($n = 6$). *** $P < 0.001$ versus NG + vehicle or HG + vehicle. Based on six replicates, the coefficient of variation under NG and HG was (A) 10% and 22%, (B) 16% and 10%, and (C) 33% and 10%, respectively.

der hyperglycemia at 21% oxygen compared to cells cultured with vehicle under the same conditions, suggesting that inhibition of ERK1/2 partially abolished the high glucose effect. Blocking the Akt/PkB signaling pathway by addition of wortmannin (100 nmol/L) did not restore hyperglycemia-reduced ACH-3P proliferation under these conditions (Figure 9). The combination of both inhibitors resulted in the same effect as U0126 alone. This indicates that ERK1/2, but not Akt/PkB, is involved in the hyperglycemia-mediated effect. The partial effect of the ERK1/2 inhibitor suggests that other mitogen-activated protein kinases such as ERK5, JNK, and p38, also might be involved. The number of dead cells under hyperglycemic conditions was also affected by ERK1/2 and Akt/PkB inhibition. Significantly more dead cells were found

using U0126 and wortmannin either singly or in combination compared to cells treated with vehicle control (data not shown).

Discussion

The effects of maternal diabetes on the placenta at the end of gestation have received much attention.³⁴ However, only little information is available on placental development in the first trimester of a diabetic pregnancy. In the present *in vitro* study, we focused on the trophoblast, the key placental cell type in early pregnancy, and investigated its growth in response to the diabetic environment. We used long-term hyperglycemic conditions *in vitro* to represent one component of the diabetic environment *in vivo*. The experiments were performed under varying oxygen conditions (2.5%, 8%, and 21% O_2) to mimic the increasing oxygen concentrations *in vivo* during the first trimester of pregnancy. The characteristics of ACH-3P cells closely resemble many characteristics of different aspects of primary first-trimester trophoblasts but are combined with the potential to proliferate, which the primary trophoblasts do not do *in vitro*.²⁹

To the best of our knowledge, this is the first study in which oxygen was used as a modulator of the hyperglycemic response. The key findings of our study were: i) long-term hyperglycemia reduced ACH-3P cell proliferation at 21% oxygen, whereas high glucose conditions did not affect cell growth under lower oxygen concentrations; ii) hyperglycemia increased trophoblast ROS levels independently of oxygen; and iii) the hyperglycemic effect on proliferation was independent of ROS levels. In none of the experiments was the number of dead cells altered. Thus, we conclude that hyperglycemia did not influence cell apoptosis and cell survival.

Figure 9. Inhibition of ERK1/2 signaling pathway leads to increased ACH-3P cell proliferation under hyperglycemia (HG) at 21% oxygen. ACH-3P cells were treated for 3 days under normoglycemia (NG; black bars) and hyperglycemia (gray bars) in the presence and absence (DMSO vehicle) of 10 $\mu\text{mol/L}$ U0126 (ERK1/2 inhibitor) or 10 nmol/L wortmannin (Akt/Pkb inhibitor) at 21% oxygen. Number of viable cells are presented as mean $\pm$ SD ($n = 6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus NG + vehicle, NG + U0126, NG + wortmannin or NG + U0126/wortmannin, respectively; † $P = 0.01$, †† $P = 0.001$ versus NG + vehicle or HG + vehicle, respectively. Based on six replicates, the coefficient of variation under NG + vehicle and HG + vehicle was 13% and 12%, respectively.

Oxygen-Dependent Proliferation under Hyperglycemia

At about week 12 of gestation, maternal blood flow toward the placenta is fully established, leading to enhanced oxygen tension within the organ.¹³ The rapid increase of placental oxygen concentrations is associated with increased oxidative stress. ROS production occurs in normal pregnancies, but is even more pronounced in pregnancies complicated with diabetes.³⁵

It is tempting to speculate that this could be an explanation for our findings that hyperglycemia-reduced proliferation is oxygen-dependent, whereas basal proliferation is not modified by oxygen concentrations. Previous proliferation studies under hyperglycemic conditions in different cell types and tissues showed inconsistent findings. Decreased proliferation,^{11,36} as well as enhanced cell growth,^{37,38} was observed, indicating that the hyperglycemic effect on proliferation is cell-specific.

Cell cycle analysis showed, at 21% oxygen, modifications in specific cell cycle phases. Long-term hyperglycemia resulted in reduced G₁-phase and significantly increased S-phase cell populations, whereas G₂/M-phase was not affected under these conditions. This may reflect an arrest at some point past S-phase of the cell cycle. Hyperglycemia may affect cyclins and cyclin-dependent kinase inhibitors³⁹; however, the specific mechanisms underlying cell cycle changes in our setting are still unknown.

Oxygen-Independent ROS Formation under Hyperglycemia

In placental explants, an increase of oxidative stress markers after hypoxia-reoxygenation has been reported,⁴⁰ suggesting that ROS formation is oxygen dependent. Furthermore, a large body of evidence shows increased ROS levels caused by hyperglycemia in different cell types.^{41,42} Thus, previous observations indicate that both oxygen and high glucose concentrations can lead to oxidative stress in cells and tissues.

To examine the underlying mechanisms leading to decreased trophoblast proliferation at 21% oxygen, we determined ROS levels and H₂O₂ release in these cells. Intracellular ROS production was enhanced under hyperglycemia at 2.5% and 8% oxygen compared to cells cultured under normoglycemic conditions. This set of data indicates that at 2.5% and 8% oxygen under hyperglycemia, ACH-3P cells produced significantly more ROS, which did not affect proliferation. At 21% oxygen, the situation was different: ACH-3P cells showed higher ROS generation and H₂O₂ release under normoglycemia. Indeed, ROS levels under hyperglycemia were not significantly altered at 21% oxygen compared to cells after 3 days under high glucose concentrations at 2.5% and 8% oxygen. These findings, independently obtained by two different methods, indicate that ROS production under normoglycemia is oxygen-dependent, but oxygen-independent under hyperglycemia.

In a likely scenario, reduced ROS levels under hyperglycemia at 21% oxygen can be the consequence of cell adaption to high glucose concentrations by up-regulating cellular antioxidant systems, which may have an impact on proliferation and the cell cycle. Such effects of hyperglycemia on antioxidant systems have been reported previously^{43,44} and were confirmed here. Hyperglycemia up-regulated heme oxygenase 1, glutathione peroxidase 1, and nuclear factor (erythroid-derived 2)-like 2 compared to normoglycemic conditions. All three genes encode proteins with a special function as antioxidants, strongly supporting the notion that ACH-3P cells had adjusted to the diabetic environment (Figure 3D).

In addition, mitochondrial mass in the human placenta increases with gestational age, making possible an increase in superoxide production by mitochondria throughout the course of pregnancy.²⁴ It had been shown that first-trimester trophoblast mitochondria are resistant against hyperglycemia-induced high-amplitude swelling.⁴⁵ Recent work highlighted the influence of mitochondrial superoxide on cell proliferation.⁴⁶ As shown in Figure 4D, superoxide levels were increased in cells treated under hyperglycemia and 21% oxygen. Therefore, we further hypothesized that elevated mitochondrial ROS formation contributes to decreased proliferation.

Cytosolic versus Mitochondrial ROS and Their Influence on Proliferation

To investigate whether cytosolic and mitochondrial ROS influence proliferation at 21% oxygen, we modulated ROS generation in both cell compartments (Figure 6). Addition of antioxidants, such as ascorbate,⁴⁷ Trolox,⁴⁸ and NAC,⁴⁹ which are known to generally decrease intracellular ROS levels, had no beneficial impact on proliferation under hyperglycemic conditions.

Proliferation could not be restored by supplementation of any antioxidant. Our findings were in line with previous observations in endothelial progenitor cells where addition of antioxidants (SOD, catalase, ascorbate, NAC) did not alter proliferation under hyperglycemic conditions.⁵⁰ This may represent a cell-specific effect since catalase completely reversed the proliferation effect of high glucose treatment in aortic smooth muscle cells.⁵¹ Interestingly, decreasing and increasing cytosolic and mitochondrial ROS levels did not modify ACH-3P proliferation under hyperglycemia, suggesting ROS and cell proliferation are not associated in ACH-3P cells under the conditions used here, which further corroborates the lack of effect of the antioxidants applied.

Our results indicate that high glucose levels do not control proliferation by using intracellular ROS as signaling molecules. To gain insight into potential pathways involved, we focused on two key kinases, Akt/PkB and ERK1/2. Akt/PkB inhibition did not restore proliferation under hyperglycemia at 21% oxygen, indicating that Akt/PkB is not involved. However, inhibition of ERK1/2 partially abolished reduced proliferation under hyperglycemia at 21% oxygen. Thus, signaling through the MAP kinase pathway contributes to the effect of hyperglyce-

mia, but the involvement of other mechanisms cannot be excluded.

Pathophysiological Implications

Early in pregnancy, the oxygen tension in the intervillous space and within the placenta is low. At this stage, hyperglycemia may not affect the development and proliferation of the trophoblast, and hence, placental growth. Later in the first trimester, when the oxygen tension rises, the trophoblast becomes sensitive to the hyperglycemic environment and reduces its proliferation. This may result in diminished nutrient delivery to the fetus, ultimately resulting in reduced growth of the fetus proper.⁸ In this scenario, the trophoblast would be the primary target of the diabetic environment, and the fetus would respond with some delay. Some support for this speculation comes from the observation that fetal growth was delayed only at week 12 after menstruation, but not at week 8 after menstruation, although the oxygen tension rises the most between week 8 and 10 after menstruation.⁹

Here, we demonstrate for the first time that a ROS-independent decrease of proliferation under hyperglycemia can be caused by elevated oxygen concentrations. These findings suggest that in diabetes, reduced trophoblast proliferation may have an impact on embryonic growth at the time of oxygen increase at the end of the first trimester of pregnancy. Thus, we propose that maternal diabetes alters placental development during the first trimester of gestation. Whether the increased risk for preeclampsia, miscarriage, and intrauterine growth restriction in diabetic pregnancies^{4–6} is related to the impaired trophoblast growth remains an open question.

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