Effects of vitamins C and E, acetylsalicylic acid and heparin on fusion, beta-hCG and PP13 expression in BeWo cells

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ABSTRACT

Preeclampsia is one of the leading causes for maternal and fetal morbidity. Placental protein 13 (PP13) is a placenta specific protein and with its decreased maternal serum levels in the first trimester it is one of the most promising markers to predict the syndrome in early pregnancy. In clinical trials attempts to prevent preeclampsia have already been made using low-dose aspirin, low-molecular-weight heparin, and antioxidants such as vitamins C and E. Here we investigated the effect of these agents on PP13 and beta-hCG levels using choriocarcinoma cell lines as surrogates for primary villous trophoblast. Five different cell lines were triggered with forskolin and cultured for 48 h. Amongst the five tested cell lines BeWo cells showed the strongest increase in PP13 mRNA after forskolin treatment compared to controls. Hence these cells were used to investigate the effect of varying concentrations of vitamin C, acetylsalicylic acid (ASA), Trolox® and heparin on cell fusion and PP13 and beta-hCG levels. The response to vitamin C was a dose-dependent increase in protein expression, while the other drugs showed only modest effects. Since first trimester PP13 has been shown to be significantly decreased in women subsequently developing preeclampsia, this data might point to a beneficial effect of very early vitamin C treatment of such women already in the early first trimester of pregnancy.

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1. Introduction

Preeclampsia remains one of the leading causes for maternal and fetal/neonatal mortality and morbidity affecting 2–5% of all pregnancies. It is characterized by new onset hypertension and proteinuria after 20 weeks of gestation in previously normotensive women [1]. However, no adequate conservative medical treatment is available, and so far the only therapeutic intervention is termination of pregnancy by inducing delivery or Caesarean section. Hence, it is becoming increasingly important to find a way to predict and consequently prevent preeclampsia thus improving maternal and fetal health. So far, a primary prophylaxis is not available, as the underlying cause leading to this syndrome is not fully understood yet [2].

Agents that have been discussed as candidate therapeutics for secondary prophylaxis of preeclampsia include low-dose aspirin, low-molecular-weight heparin, folic acid, antioxidants such as vitamins C and E, and calcium [3]. In clinical trials the effects of some of these drugs on the development of preeclampsia have already been investigated in vivo. In randomized placebo-controlled studies administration of aspirin throughout the first trimester or of heparin starting at 10–11 weeks of gestation lowered the risk of developing hypertensive pregnancy complications such as preeclampsia [4,5]. However, the prophylactic benefit of vitamins C and E is more controversially discussed. In a small randomized trial a beneficial effect of these vitamins was suggested for women at high risk of developing preeclampsia [6]. Unfortunately, this finding could not be confirmed in two multicenter randomized clinical trials where administration of vitamins C and E started between 14 and 21 weeks of gestation [7,8].

Several biochemical markers have been detected in maternal peripheral blood or urine. Such markers alone or in combination with the uterine artery Doppler pulsatility index have already been tested to evaluate the risk to develop preeclampsia [9–13]. One of the most promising biochemical markers is placental protein 13 (PP13), a member of the beta-galactoside binding S-type galectin superfamily [14]. In the human PP13 is only expressed in placental tissues, and within the villous trophoblast it can only be found in the multinucleated syncytiotrophoblast [14]. PP13 is expressed and released into the intervillous space, where it enters the maternal circulation and can be detected in peripheral blood. While in normal pregnancy serum concentrations of PP13 rise from the first to the second half of pregnancy, women who develop preeclampsia
show significantly lower serum levels of PP13 in early pregnancy, then rising above normal in the second half of pregnancy [15]. Serum levels of beta-hCG are also elevated in second and third trimester in cases suffering from preeclampsia compared to controls, while in first trimester serum beta-hCG is not significantly altered between both groups [16]. Accordingly, alterations of PP13 serum concentrations could serve to assess the risk and progression of preeclampsia as early as mid first trimester.

Here we established a stable cell culture model to investigate how preeclampsia prevention candidates such as vitamins C and E, aspirin and heparin affect the expression of PP13 and beta-hCG in fusing trophoblastic cells. To mimic the multinucleated syncytio-trophoblast, we used several choriocarcinoma cell lines and treated them with forskolin to trigger cell fusion [17–19]. Quantification of cell fusion as well as PP13 and beta-hCG RNA and protein levels were used to assess the agents’ effects on trophoblastic cells.

2. Materials and methods

2.1. Cell culture

BeWo cells (ECACC, ACT-M59 cells (provided by H.G. Frank, Aachen, Germany) and ACH-3P cells (provided by C. Desoye, Graz, Austria) [20] were cultured at 37 °C and 20% O₂ in DMEM (Gibco, Austria) and Ham’s F12K (Gibco) (ratio 1:1) supplemented with 10% FCS, 1% penicillin/streptomycin and 1% amphotericin B. Jar cells (ATCC, USA) were cultured under the same conditions in RPMI 1640 (PAA, Austria) supplemented with 2% γ-glutamine, 1% Na-pyruvate, 1% HEPES, 10% FCS, 1% penicillin/streptomycin and 15% amphotericin B. Jeg-3 cells (provided by H.G. Frank) were cultured as mentioned above in EMEM with γ-glutamine (PAA) supplemented with 10% FCS, penicillin/streptomycin and amphotericin B.

Cells were plated in 12 well dishes (BeWo: 2.3 × 10³ cells/well, ACH-M59: 6 × 10³ cells/well, ACH-3P: 2 × 10³ cells/well, Jar: 9 × 10³ cells/well, Jeg-3: 9 × 10³ cells/well) and incubated over night. The following day medium was replaced by fresh medium with or without 20 μM forskolin (Sigma–Aldrich, Austria) or 0.2% DMSO (as vehicle control). After 48–72 h culture medium was collected and centrifuged at 2300 g for 10 min at 4 °C. Supernatant was used immediately or stored at −80 °C for further use. Human fibroblasts (Detroit 551; ATCC) served as negative control.

For morphological analysis and quantification of fusion, BeWo cells were seeded onto chamber slides (Nunc, Austria) (1 × 10⁵ cells/chamber) or detached by treatment with 120 μl/well Accutase (PAA) supplemented with 0.01 mg/ml DNase I (Roche, Germany) for 15 min at 37 °C and spun onto glass slides at 57 g for 5 min (Shandon “Cytospin 2”, Histocom, Austria).

2.2. RNA-isolation

Total RNA was isolated with Tri Reagent (Applied Biosystems, Austria) according to the manufacturer’s instructions. The quality of total RNA was assessed by ethidium bromide staining of denaturing agarose gels.

2.3. Real-time RT-PCR

Reverse transcription of total RNA was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's manual. In brief, 1.5 μg total RNA of each sample was mixed with the Kit components in a total reaction volume of 20 μl and incubated for 10 min at 25 °C, 150 min at 37 °C and 5 s at 85 °C in a thermocycler.

Real-time RT-PCR was performed using the QuantiFast SYBR Green PCR Kit (Qiagen) and QuantiTect Primer Assays (Qiagen) for PP13 (Hs_LGALS13_1_SG QuantiTect Primer Assay), beta-hCG (Hs_CGB_1_SG QuantiTect Primer Assay) and ribosomal protein PO (RPLP0). The latter was used as internal reference. 8.8 μl of cDNA (15 ng/μl) were mixed with 2.2 μl primers and 11 μl SYBR green master mix. Expression levels were analyzed in triplicates in a thermocycler. The amplification protocol comprised an initial 5 min denaturing step at 95 °C and a subsequent two-step cycling including 10 s at 95 °C and 30 s at 60 °C for 45 cycles. Ct values were automatically generated by the Light Cycler 480 Software (Roche) and relative quantification of gene expression was calculated by standard ΔΔct method using expression of RPLP0 as reference.

2.4. Exposure of BeWo cells to vitamin C, acetylsalicylic acid (ASA), Trolox® and heparin

Culture medium in presence or absence of 20 μM forskolin or 0.2% DMSO was supplemented with increasing concentrations of vitamin C (Herba Chemosan, Austria) [30–200 μM], acetylsalicylic acid (ASA; Sigma–Aldrich) [1–10 μg/ml, equivalent to 5.6–56 μM], low-molecular-weight heparin (Fragmin®, Pfizer, Austria) [1–6 IU/ml] and the vitamin E derivative Trolox® (Sigma–Aldrich) [10–100 μM]. Concentration ranges of vitamin C, Trolox® and heparin were chosen according to previous studies [6,21,22] and of ASA according to a personal communication by Dr. Uwe Gessner (Bayer Vital GmbH, Germany). Stock solutions of vitamin C and heparin were prepared in double distilled water, acetylsalicylic acid was dissolved in culture medium and stock solutions of Trolox® were prepared in ethanol. Culture media of controls were supplemented with respective volumes of solvents. Cells were incubated for 48 h or 72 h under the same conditions as mentioned above.

2.5. Cell lysis and protein determination

Cells were washed once in PBS and lysed with 80 μl/well RIPA-buffer (Sigma–Aldrich) with Complete protease inhibitor cocktail, Roche) for 5 min on ice. Lysates were centrifuged at 16,000g for 10 min at 4 °C. Supernatant was used immediately or stored at −80 °C for further analysis. Total protein concentration was assessed by Lowry protein assay.

2.6. DELFIA assay

PP13 and beta-hCG concentrations of cell lysates were determined using time-resolved fluoroimmunoassay kits (PerkinElmer, Finland). Cell lysates were diluted 1:3 and 1:5 in DELFIA diluents I and II, respectively. The fluorescent signal of each sample was converted by MultiCalc (PerkinElmer) into the respective protein concentration calculated from a standard curve. PP13 and beta-hCG concentrations were normalized to total protein concentration in cell lysates.

2.7. Cell viability assessment

Cell viability was assessed by determination of LDH in culture supernatant using a LDH Cytotoxicity Detection Kit (Takara, Japan) according to the manufacturer’s manual.

2.8. Immunofluorescence staining and quantification of apoptosis and fusion

Prior to staining cells were fixed in acetone for 7 min. UV-Block (Lab-Vision, Thermo Scientific, Austria) was supplemented with 10% human AB-serum and used to block non-specific background staining. Primary antibodies (Table 1) were diluted in antibody diluent (Dako, Austria) and incubated for 30 min at room temperature alone or in combination. Fluorescent-labeled secondary antibodies (Alexa Fluor® 555 goat anti-mouse IgG or Alexa Fluor® 488 goat anti-rabbit IgG, 1:200; Invitrogen, Austria) were incubated for 30 min at room temperature. Nuclei were counterstained with DAPI (1:2000) in 1% DAPI. Slides were dried, mounted with ProLong Gold antifade reagent (Invitrogen) and analyzed by fluorescent microscopy using an AxioCam microscope connected to an AxioCam HRc digital camera (Zeiss, Germany) or using a Leica DM6000B microscope connected to an Olympus DP72 digital camera (Olympus, Austria).

Apoptosis rates of BeWo cells were determined by analyzing M30 staining with DAPI counterstaining in a total of 15 randomly selected microscopic fields of each condition in three independent experiments. The ratio of M30 positive areas to total area of nuclei in each picture was automatically determined (Visiomorph software, Visiopharm, Denmark). The apoptosis rate of the forskolin control at 48 h was set to one and the rates of all other conditions were calculated accordingly.

BeWo cell fusion was determined by analyzing alpha-fodrin and desmosomal protein staining in a total of at least 20 randomly selected microscopic fields of each condition, analyzed in three independent experiments (newCAST software, Visiopharm). Number of nuclei in synctia and total number of nuclei were counted and fusion index was calculated (number of nuclei in synctia/total number of nuclei × 100). The fusion index of the forskolin control was set to one and the indices of the other conditions were calculated accordingly.

2.9. Statistical analysis

Real-Time RT-PCR and DELFIA data are expressed as mean ± SD, fusion data are presented as “Box-and-Whisker-Plot”. Differences between two groups (as displayed
in Fig. 1C–D) and 2) were tested with t-test or Mann–Whitney Rank Sum Test, as appropriate. Differences between three or more treatment conditions (as displayed in Fig. 1A–E, 3 and 5) were tested with ANOVA or Kruskal–Wallis Test, as appropriate, followed by Tukey post hoc tests. When comparing the control group with each treatment condition (as displayed in Figs. 3A–H and 5C) many-to-one testing procedures (Dunnett’s or Dunn’s Test, as appropriate) were used. Statistical analysis was performed using SigmaPlot (SigmaPlot 11.0, Systat Software, Germany). P-Values of < 0.05 were considered significant.

3. Results

3.1. PP13 and beta-hCG mRNA and protein are increased in response to forskolin treatment of trophoblast derived cell lines

In five different trophoblast derived cell lines — the choriocarcinoma cell lines BeWo, Jar and Jeg-3 and the hybridoma cell lines AC1-M59 and ACH-3P — PP13 and beta-hCG mRNA and protein levels were determined following treatment with 20 μM forskolin. All five cell lines significantly increased PP13 and beta-hCG mRNA after forskolin stimulation compared to non-treated or DMSO treated control cells (Fig. 1A and B). BeWo cells showed the strongest increase of PP13 and beta-hCG mRNA after forskolin stimulation compared to controls (Fig. 1A and B). After forskolin stimulation PP13 and beta-hCG levels significantly increased in most of the cell lines, except for Jeg-3 cells with no changes in PP13 (Fig. 1C and D). The strongest increase of PP13 was found in BeWo cells. Thus, these cells were used for subsequent experiments.

3.2. In forskolin treated BeWo cells PP13 is decreased and apoptosis rates are increased after 72 h compared to 48 h

In a time-course experiment from 48 h to 72 h BeWo cells showed a significant decrease in PP13 with extended forskolin exposure time which was not evident in the presence of vitamin C (Fig. 2A). Differences in exposure time did not lead to changes in beta-hCG (Fig. 2B). However, addition of vitamin C significantly increased beta-hCG (Fig. 2B). In the forskolin controls as well as the vitamin C treated cells an increase in cultivation time from 48 h to 72 h both significantly increased the rate of apoptosis (Fig. 2C). Therefore an exposure time of 48 h was used for further experiments.

3.3. Candidate therapeutics affect PP13 and beta-hCG levels in BeWo cells

PP13 as assessed by DELFIA assays increased significantly after supplementation with physiological (≤50 μM) and above physiological (≥100 μM) serum concentrations of vitamin C in the presence of forskolin compared to controls (Fig. 3A). Highest PP13 values were found at 100 μM vitamin C (1.8-fold compared to forskolin control). In the presence of forskolin vitamin C also affected beta-hCG levels with a significant increase of 1.9-fold at 30 μM vitamin C up to 2.4-fold at 200 μM vitamin C compared to controls (Fig. 3B).

A negative effect on PP13 was found after addition of acetylsaliclycic acid (ASA). One μg/ml ASA decreased PP13 significantly to about 60% of the respective forskolin control. Higher concentrations of ASA did not decrease PP13 any further (Fig. 3C). ASA did not show to have any effect on beta-hCG (Fig. 3D).

Supplementation of the culture medium with the vitamin E derivative Trolox® slightly decreased PP13 in a low concentration (10 μM), whereas a significant increase of beta-hCG was only found at above physiological concentrations (100 μM) (Fig. 3E and F). The low-molecular-weight heparin Fragmin® did not show any effect on PP13 and beta-hCG levels (Fig. 3G–H).

The combined treatment with vitamin C and ASA did not show a different effect compared to vitamin C alone (Fig. 3I–K). Only vitamin C significantly altered PP13 and beta-hCG levels while an additive effect of both drugs could not be detected (Fig. 3I–K).

Cell viability as tested by LDH release was not affected in any of the conditions (data not shown).
against beta-hCG (Fig. 5A and B, green) was used to quantify fusion events. Cell fusion analysis revealed that fusion rates in the presence of 2.5 µg/mL ASA or 100 µM Trolox increased significantly compared to forskolin treated controls. The median of the fusion rates of forskolin controls was set to 1.0, and the medians of ASA and Trolox treated cells were 1.44 and 1.82, respectively (Fig. 5C).

4. Discussion

First we investigated the potential use of five different trophoblast derived cell lines to serve as a surrogate for syncytialisation of placentral trophoblast by means of PP13 and beta-hCG expression as well as direct assessment of fusion. The choriocarcinoma cell lines BeWo, Jar and Jeg-3 as well as the hybridoma cell lines AC1-M59 (hybrid with third trimester trophoblast; [23]) and ACH-3P (hybrid with first trimester trophoblast; [20]) were treated with forskolin to enhance differentiation and trigger cell fusion. This was performed to mimic the in vivo situation of cell differentiation and fusion of mononucleated cytrophoblasts to generate and maintain the multinucleated syncytiotrophoblast. In the presence of forskolin an increase of PP13 and beta-hCG levels in combination with an increased fusion rate was specifically seen in BeWo cells. Thus, this cell line was again shown to be the best-suited in vitro cell line model to study villous trophoblast differentiation and fusion.

Various candidate therapeutics have been suggested to have beneficial effects in preeclampsia, including low-dose aspirin, low-molecular-weight heparin, folic acid, antioxidants such as vitamins C and E, and calcium. Four of them—aspirin, heparin and vitamins C and E—were chosen to assess their effects on PP13 and beta-hCG expression as well as fusion of BeWo cells. Concentrations of the agents chosen for this study reflect physiological and high physiological plasma levels of the respective substances during treatment in vivo according to previous studies [6,21,22] and a personal communication (Dr. Uwe Gessner, Bayer Vital GmbH, Germany). Vitamins C and E have been thought to be useful in preventing preeclampsia. However, nowadays they are discussed controversially regarding their importance in the prevention of this pregnancy related syndrome [7,24,25]. Clinical studies on the use of anti-coagulants such as heparin or anti-platelet reagents such as low-dose aspirin during pregnancy showed a possible benefit in the prevention of preeclampsia or other hypertensive pregnancy disorders. However, data on such drugs are considered to be only preliminary and need to be confirmed in large-scaled, multi-centered, randomized clinical trials [4,5].

Here we tested the impact of these four drugs on cell fusion and the expression of two syncytiotrophoblast-specific proteins, PP13 and beta-hCG. An effect of heparin could not be seen in any of the used concentrations neither on PP13 nor on beta-hCG expression. Vitamin C, low-dose acetylsalicylic acid and the vitamin E derivative Trolox affected the expression of these proteins, although partly in opposite directions. Whilst the expression of both proteins was significantly increased in the presence of vitamin C in a dose-dependent manner, the addition of low-dose aspirin resulted in a significant decrease of PP13. Beta-hCG expression was not altered in the presence of aspirin. The effect of Trolox was very weak with a slight increase of beta-hCG at higher concentrations. BeWo cell fusion was significantly increased only in the presence of ASA and Trolox (Fig. 5C).

Tannetta et al. used isolated primary trophoblasts from term placenta to study the effect of combined vitamins C and E [26]. These authors described a non-significant decrease in syncytiotrophoblast formation at 48 h of culture in the presence of vitamins C and E with no difference in hCG secretion at 48 h culture [26]. They also suggested that the combined treatment with vitamins C and E does

3.4. Vitamin C in combination with forskolin results in an increase of beta-hCG staining

While cells treated with DMSO were mainly negative for beta-hCG in immunofluorescence staining (Fig. 4A and B), the intensity of beta-hCG staining increased in the presence of forskolin (Fig. 4C and D). It even further increased in the presence of forskolin combined with vitamin C (Fig. 4E–H).

3.5. ASA and Trolox affect the fusion rate of BeWo cells

Staining using an antibody against a desmosomal protein (Fig. 5A and B, red) or alpha-fodrin in combination with an antibody

![Fig. 2. Differences between 48 h (grey bars) and 72 h (black bars) forskolin treatment on PP13 (A) and beta-hCG (B) expression and apoptosis (C) in BeWo cells in the presence and absence of 100 µM vitamin C (mean ± SD). (A) Expression of PP13 was assessed in pg/ml by PP13 DELFIA assay and related to mg/ml total protein. (B) Expression of beta-hCG was assessed in ng/ml by free beta-hCG DELFIA assay and related to mg/ml total protein. (C) Ratio of M30 positive stained areas to total area of nuclei. All data were normalized to the forskolin control. Significant differences between 48 h and 72 h treatment are marked with an asterisk (*). Data were obtained from three independent experiments.

![Diagram](image-url)
Fig. 3. Effects of vitamin C (A, B), ASA (C, D), Trolox® (E, F), low-molecular-weight heparin (G, H) and the combination of vitamin C and ASA (I, K) on the protein expression of PP13 and beta-hCG in BeWo cells after 48 h (mean ± SD). Expression of PP13 (dark grey bars) was assessed in pg/ml by PP13 DELFIA assay and related to mg/ml total protein. Expression of beta-hCG (light grey bars) was assessed in ng/ml by free beta-hCG DELFIA assay and related to mg/ml total protein. All data were normalized to the respective forskolin control. Significant differences to controls are marked with an asterisk (*). Significant differences to the treatment condition of 2.5 μg ASA/ml (Fig. 3I) are marked with a hash (#). Data shown here represent protein concentrations of forskolin treated cells in presence and absence of the agents. Data were obtained from three to four independent experiments.
not stimulate beta-hCG secretion directly but rather leads to improved syncytiotrophoblast viability. There is striking similarity of data between the primary cells [26] and BeWo cells (this study). The vitamin C effects shown here (higher PP13 and beta-hCG with no changes in the fusion rate) point to stabilization and increased syncytiotrophoblast differentiation rather than increased trophoblast fusion.

Low levels of antioxidant proteins such as copper/zinc superoxide dismutase (SOD-1) and catalase in the syncytiotrophoblast are correlated with syncytial fusion [27]. In cases with trisomy
21 higher levels of such proteins are related to defective trophoblast fusion [28]. It has been speculated that high activity of antioxidant proteins in the trophoblast impairs syncytial fusion. Hence, it is tempting to speculate that there is a certain threshold of antioxidants where a beneficial concentration turns into a detrimental effect.

We have already used BeWo cells in combination with villous explants to assess the effect of serum from IVF failure patients [29]. In this study we could demonstrate a direct positive effect of heparin and aspirin on the viability of villous trophoblast. Hence, caution is recommended when extrapolating such in vitro results to the in vivo situation. At the same time it is tempting to speculate on the value of such an in vitro test system. It may be a useful tool to compare the effect of serum from low and high risk patients on marker expression followed by the evaluation of the impact of agents as evaluated here. May be a respective cell culture system could be used as a platform to individually test pregnant women’s serum on its effects on trophoblast derived cells and which treatment is best to minimize such effects.

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