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Activation of adenosine A_{2B} receptor impairs properties of trophoblast cells and involves mitogen-activated protein (MAP) kinase signaling

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ABSTRACT

Introduction: Shallow trophoblast invasion of the maternal spiral arteries contributes to impaired placental perfusion and is hypothesized to be involved in the pathophysiology of preeclampsia. Hypoxia is a potent stimulus for the release of adenosine.
Methods: We investigated the effects of hypoxia and A_{2B} adenosine receptor signaling on migration, invasion, proteolytic activity of matrix metalloproteinase (MMP)-2, expression of MMP-2 and vascular endothelial growth factor (VEGF) mRNA, and production of human chorionic gonadotropin (hCG) in trophoblast cells (HTR-8/SVneo, BeWo).
Results: The adenosine A_{2B} receptor agonist 5-N-ethylcarboxamidoadenosine (NECA) reduced trophoblast (HTR-8/SVneo and BeWo) migration at 2%, 8% and 21% O_2 compared to untreated control cells. A_{2B} adenosine receptor stimulation decreased phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) at all three O_2 concentrations. ProMMP-2 activity, MMP-2 mRNA levels and hCG levels were markedly decreased after A_{2B} adenosine receptor activation in trophoblast cells. Adenosine receptor A_{2B} stimulation decreased VEGF expression at 2% and 8% O_2 but led to increased levels at 21% O_2.
Conclusions: These data indicate A_{2B} receptor activation blunts trophoblast migration possibly as a result of reduced activation of the MAPK signaling pathway and lower proMMP-2 levels. These data suggest a role for adenosine receptor A_{2B} in placental development and possibly in the pathophysiology of preeclampsia.

1. Introduction

Adenosine is a purine nucleoside and involved in numerous physiological processes [1]. Extracellular adenosine levels increase in response to hypoxia, ischemia and inflammation, preventing tissue damage during instances of cellular stress or injury [2,3]. Adenosine receptors are transmembrane spanning G protein-coupled receptors and include A_1, A_{2A}, A_{2B}, and A_3 subtypes [4]. Adenosine and adenosine receptors are involved in angiogenesis, endothelial cell proliferation, migration and blood vessel formation in various vascular beds [5,6]. Adenosine also promotes neovascularization [2,5] and under hypoxic conditions human endothelial and smooth muscle cells modulate adenosine receptors toward an A_{2B} "angiogenic" phenotype [7].

Preeclampsia is a pregnancy-specific syndrome that affects 3–5% of pregnant women worldwide [8] and a leading cause of maternal and fetal morbidity and mortality [9]. Preeclampsia is clinically characterized by new onset hypertension and proteinuria after 20 weeks of gestation [10]. The pathophysiology of the syndrome is not fully understood. However, placental hypoxia as a result of impaired trophoblast invasion is proposed to be a central component of the pathophysiology of preeclampsia [11,12]. Hypoxia is a potent stimulus for the release of adenosine [3,4] and patients with preeclampsia exhibit higher concentrations of adenosine in the maternal and fetal circulation compared to uncomplicated pregnancies [13,14]. Maternal adenosine concentrations increase with the severity of the syndrome [15]. Furthermore, adenosine receptor expression in the human placenta is higher in pregnancies complicated by preeclampsia [16]. However, the role of adenosine receptor A_{2B} in the placenta, a receptor influenced by hypoxic conditions, is unclear.

The focus of this study was to investigate the effect of adenosine receptor A_{2B} stimulation in placental function using a model system of trophoblast cells (HTR-8/SVneo and BeWo trophoblast cell line),

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and specifically to investigate the role of adenosine receptor A_{2B} in trophoblast cell migration, invasion and the cell signaling pathways. We hypothesized that hypoxia through adenosine receptor A_{2B} activation may adversely affect trophoblast function and thereby contribute to placental dysfunction and the pathophysiology of preeclampsia.

2. Materials and methods

2.1. Cell culture

We used the human, invasive extravillous cytotrophoblast HTR-8/SVneo cell line (HTR-8/SVneo, gift by Prof. Charles Graham, Queen's University, Kingston, ON, Canada), established from immortalized explant cultures of first trimester chorionic villi and the immortalized trophoblast cell line BeWo, which has the capability to fuse and is a well-established model for the syncytiotrophoblast (Cell Lines Service, Eppelheim, Germany). HTR-8/SVneo were cultured in RPMI 1640 media (Invitrogen, Germany) supplemented with 5% fetal bovine serum (FBS, Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Biochrom, Berlin, Germany) and BeWo in Ham's F12K medium (Life Technologies GmbH, Darmstadt, Germany) with 10% FBS and 1% penicillin/streptomycin at standard culture conditions (37 °C, 5% CO_2).

3. Treatments

Trophoblast cells were incubated in the presence or absence of adenosine A_{2B} receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA, 10 μM) or antagonist 8-[4-[((4-cyanophenyl) carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine hydrate (MRS 1754, 1 μM), both purchased from Sigma–Aldrich (Steinheim, Germany). The A_{2B} receptor is the least well-defined adenosine receptor subtype. NECA is the most potent nonselective adenosine A_{2B} receptor agonist, with a concentration producing a half-maximal (EC_{50}) dose for stimulation of adenyl cyclase of approximately 2 μM [1]. MRS 1754 is a selective antagonist ligand of A_{2B} adenosine receptors and a relatively hydrophobic molecule [17]. All experiments were performed in the presence of 1.0 U/ml adenosine deaminase (Calbiochem, Darmstadt, Germany) to remove endogenously produced adenosine. Experiments were performed simultaneously in three separate incubator chambers of an ex-vivo incubation system (Biospherix Inc., USA) at 2%, 8% and 21% O_2 all with 5% CO_2 respectively to simulate hypoxic (2% O_2) and standard tissue culture conditions (21%). The 8% O_2 group served as additional control to simulate physiologic oxygen conditions of placental tissue after the first trimester.

3.1. Real Time qRT-PCR

Total RNA was isolated from HTR-8/SVneo cells using the standard guanidinium thiocyanate (GT)-phenol-chloroform method by Chomczynski and Sacchi [18]. Two μg of total RNA were used for cDNA synthesis in a 20 μl reaction volume with high capacity reverse transcription kit (Invitrogen). Real-time RT-PCR for VEGF and MMP-2 cDNA was performed on the Rotor Gene 6000 PCR System (Corbett Research, Qiagen, Hilden, Germany). For normalization 18S rRNA and GAPDH were used as housekeeping genes. The PCR conditions were: initial denaturation (10 min at 95 °C) followed by 40 cycles of denaturation (30 s at 95 °C), annealing (45 s at 64 °C) and extension (45 s at 72 °C). The primer sequences can be found as Supplementary Table. Each sample was analyzed in triplicate. Inter-sample comparison of target gene amplification was achieved after normalization to housekeeping genes and to the untreated sample using the $\Delta\Delta C_T$ method.

3.2. Migration assay

The effects of A_{2B} adenosine receptor activation and inhibition on migration of HTR-8/SVneo and BeWo cells under different oxygen conditions were investigated using a “scratch wound healing” assay [19]. Cells were cultured to 90% confluence and incubated in serum reduced medium with 0.2% FBS for 4 h. Subsequently, monolayers were scratched with a sterile P1000 pipette tip and cells were incubated at 2%, 8% or 21% O_2 in the presence or absence of 10 μM NECA or 1 μM MRS 1754. Photographs of the scratch were taken at the beginning of the experiment ($T = 0$) and after 22 h ($T = 22$) by a phase contrast microscope at 2.5× magnification (Leica CTR 6000, Leica Microsystems CMS GmbH, Wetzlar, Germany) and quantitatively analyzed using Image J software (NIH Image). The ability of the cells to migrate into the wound in the presence or absence of effectors in growth media was analyzed using Image J software and calculated as percent of closed wound area.

3.3. siRNA transfection

Trophoblast cells were seeded at 30–50% density and transfected with either ADORA2B (adenosine receptor A_{2B}) siRNA duplex (Silencer® Select Validated siRNA s1093; Ambion®) or control siRNA (Silencer negative control #1 siRNA AM4630; Ambion®) using Lipofectamine 2000 transfection reagent (Invitrogen™, Darmstadt, Germany) per manufacturer's instructions. Trophoblast cells transfected with siRNA specific for human ADORA2B resulted in an 80% reduction in ADORA2B mRNA and was verified by Real Time qRT-PCR (data not shown).

3.4. Matrigel invasion assay

The Matrigel invasion assay was performed in 24 well-fitted inserts with membranes (8.0 μm pore size, Corning Cornstar Corp., NY, USA). Inserts were pre-coated with Matrigel and 5×10^4 HTR-8/SVneo cells were plated in the upper chamber. The lower wells were filled with medium (5% FBS) with or without 10 μM NECA or 1 μM MRS 1754 respectively and incubated at 2%, 8% or 21% O_2 for 48 h. After fixation in 3.7% formaldehyde, the cells were permeabilized with 100% methanol and stained with Giemsa. Invaded cells were counted under a light microscope (Olympus, Hamburg, Germany) and four fields per chamber were analyzed (200× magnification). Experiments were repeated six times in duplicate.

3.5. Gelatin zymography

The effect of A_{2B} adenosine receptor activation or inhibition under different oxygen concentrations on proteolytic activity of proMMP-2 was investigated using gelatin zymography [19]. HTR-8/SVneo trophoblast cells were incubated in serum reduced medium (0.1% FBS) and in growth medium, with either 10 μM NECA or 1 μM MRS 1754 for 24 h at 2%, 8% or 21% O_2. Twenty five μg of total protein extracted from cell culture media were loaded on a 10% SDS polyacrylamide gel containing 2.3 mg/ml gelatin (Sigma–Aldrich, Steinheim, Germany). Following electrophoresis, the gel was washed in 2.5% Triton-X-100, incubated in a collagenase buffer (50 mM Tris–HCl (pH 7.0), 5 mM $CaCl_2$), stained in 0.4% Coomassie brilliant blue (SERVA, Heidelberg, Germany) and destained in methanol:acetic acid:water mixture (30:10:60). Proteolytic activity of proMMP-2 was identified as a clear band on the gel. Quantitative analysis was conducted with Image J software.

3.6. Western blot

Western blot analysis was performed as described previously with minor modifications [19]. Primary antibodies were ERK1/2 (p42/44, mouse mAb, Cell Signaling, New England Biolabs, Frankfurt am Main) or phosphoERK1/2 (phospho p42/44, mouse mAb,