Cyclosporin A Promotes Growth and Invasiveness In Vitro of Human First-Trimester Trophoblast Cells Via MAPK3/MAPK1-Mediated AP1 and Ca\(^{2+}\)/Calcineurin/NFAT Signaling Pathways

Mei-Rong Du, Wen-Hui Zhou, Lin Dong, Xiao-Yong Zhu, Yin-Yan He, Jin-Ying Yang, and Da-Jin Li

Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, China

Department of Obstetrics and Gynecology, The Affiliated Hospital, Hainan Medical College, Haikou 570102, China

ABSTRACT

Cyclosporin A (CsA) has provided the pharmacologic foundation for organ transplantation as a calcineurin inhibitor blocking T-cell activation. We have demonstrated that CsA promoted trophoblast viability/proliferation and invasion in vitro. In the present study, we further investigated the intracellular signalling pathways involved in enhancing cell viability/proliferation and invasiveness of the human trophoblast induced by CsA. We showed that blocking mitogen-activated protein kinase 3 (MAPK3)/MAPK1 signaling by U0126 attenuated CsA-increased cell viability and invasiveness of trophoblasts. Cyclosporin A inhibited ionomycin-stimulated nuclear factor of activated T-cells (NFAT) transactivation in JAR cells and reversed the ionomycin-inhibited trophoblast invasiveness. However, either activating calcineurin by ionomycin, resulting in NFAT transactivation, or inhibiting NFAT using an NFAT inhibitor had no effect on trophoblast cell viability/proliferation and apoptosis in vitro. Hence, the CsA-induced promotion of trophoblast growth and invasion occurred by overlapping but independent pathways. The MAPK3/MAPK1 pathway was essential for both trophoblast growth and invasion, whereas the Ca\(^{2+}\)/calcineurin/NFAT pathway was only involved in the CsA-promoted trophoblast invasiveness. Finally, potential cross-talk between MAPK3/MAPK1 and Ca\(^{2+}\)/calcineurin/NFAT and its relationship to activator protein 1 activation was investigated. Our findings explored possible signal transduction pathways modulated by CsA, which may lead to the expansion of the clinical applications of this drug.

cyclosporine, placenta, signal transduction, trophoblast

INTRODUCTION

The calcineurin inhibitor cyclosporin A (CsA) is a potent immunosuppressive that has formed the pharmacologic cornerstone for solid organ transplantation. Cyclosporin A prevents the activation of lymphokine genes essential for T-cell proliferation by disrupting calcium-dependent signal transduction pathways in leukocytes [1]. Although pharmacologic studies of CsA have focused mainly on T-cell responses, there is emerging evidence that this agent may exert potent effects on wide range of cell types, including endothelial cells [2], vascular smooth muscle cells [3], cardiac myocytes [4], epithelial cells [5], and tumor cells [6], regulating disparate biological functions depending on the cell type and dosage of CsA. Our previous study showed that CsA in vitro at low concentrations could promote proliferation and invasion and could inhibit apoptosis of human first-trimester trophoblasts. However, high concentration of CsA suppressed trophoblast proliferation and invasion and induced trophoblast apoptosis [7, 8]. A study in a mouse model has also demonstrated that administration with low dosage of CsA improved fetal viability in abortion-prone matings to that of normal pregnant matings [9]. Obviously, CsA exerted a beneficial regulatory role in the maternofetal relationship, but the signal transduction pathway by which CsA stimulated proliferation and invasion of human trophoblast cells has not been elucidated.

The current understanding of the immunosuppressive action of CsA focuses on cyclophilins and inhibition of the calcineurin/nuclear factor of activated T-cells (NFAT) signal [10], but it is unknown whether the stimulatory actions on trophoblast cells operate through the similar mechanisms. Transcription factors of the NFAT family are the major mediators of the intracellular effects of calcineurin activation. Recently, accumulating evidence has shown that NFAT transcription factors are present in a wide range of cell types and tissues that are not related directly to the immune response, and NFAT transcription factors have been implicated in the regulation of functional genes that control cell cycle progression, development and differentiation, angiogenesis and, possibly, tumorigenesis [11]. Given the importance of these processes in embryo implantation and placentation, which are similar to tumorigenesis, it is of considerable interest to postulate that calcineurin/NFAT signaling may be involved in the CsA-regulated trophoblast growth and invasion in vitro.

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved signaling modules through which cells transduce extracellular signals into intracellular response. The MAPK3/MAPK1 (also known as ERK1/ERK2) pathway has been associated with the regulation of cellular proliferation, differentiation, angiogenesis, embryo development, and tumor
invasion [12–14]. Recently, it was reported that MAPK3 and MAPK1 were widely expressed and markedly activated in the villous cytotrophoblasts throughout early-stage embryos, especially in the villous cytotrophoblasts and extravillous cytotrophoblasts of the first-trimester pregnancy. Disruption of the MAPK1 locus leads to embryonic lethality early in mouse development after implantation. The MAPK1 is involved in a specific regulation of trophoblast cell proliferation and invasion [15, 16]. It has also been reported that CsA can activate the MAPK3/MAPK1 pathway in several cell types [5, 17]. So we hypothesize that CsA may promote trophoblast growth and invasion in vitro by activating the MAPK3/MAPK1 signal pathway.

Almost all signals play roles by their downstream transcription factors, and regulatory transcription factors are of central importance in mediating cellular responses to environmental stimuli by coordinating the regulation of genes encoding proteins and enzymes that implement the responses. Activator protein 1 (AP1) is a transcription factor that has been implicated in diverse cellular processes, including growth, differentiation, tissue remodeling, migration, and invasion. In human placenta, AP1 is widely expressed and activated [18], suggesting that AP1 is involved in the regulation of placental and fetal development.

In the present study, we first demonstrated regulating effects of CsA on growth and invasion of human first-trimester trophoblasts, and then elucidated signaling pathways and transcription factor activation mediating the regulation of growth and invasion in the trophoblasts cells by CsA. In addition, we have investigated a potential cross-talk between the MAPK3/MAPK1 and Ca2+/calcinerin/NFAT pathways, and their association with AP1 activation. These results may contribute to a better understanding of the underlying mechanisms by which CsA promotes cell growth and invasion, and they may also suggest a potential for CsA therapy in recurrent miscarriage and other pregnant complications where decreased proliferation and invasion of trophoblasts occurred.

**MATERIALS AND METHODS**

**Isolation and Cultivation of Human First-Trimester Trophoblast Cells**

The first-trimester human placenta (6–9 wk of gestation) were obtained at each time from four to five clinical normal pregnancies, which were terminated for nonmedical reasons, at the Hospital of Obstetrics and Gynecology of Fudan University. The study has been approved by the Human Research Ethics Committee of the Obstetrics and Gynecology Hospital, Fudan University, and each patient completed a signed, written consent form.

The trophoblast cells were isolated by the trypsin-DNase I digestion and discontinuous Percoll gradient centrifugation, as described by our previous study [19]. With this method, a 95% purity of trophoblast cells was prepared. The isolated human primary trophoblast cells were cultured in Dulbecco modified Eagle medium (DMEM)-high glucose complete medium (2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and incubated in 5% CO2, pH 37°C. The human chorionicarcinoma cell line JAR was cultured in DMEM complete medium supplemented with 10% FBS in 5% CO2 at 37°C. The human chorionicarcinoma cell line JAR was cultured in DMEM complete medium supplemented with 10% FBS in 5% CO2 at 37°C. Serum-reduced media were prepared by substitution of serum-free complete medium DMEM supplemented with 15% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and incubated in 5% CO2 at 37°C. The cells were decomposed and incubated in the dark for 15 min at room temperature. At the end of the incubation, a further 400 μl binding buffer was added, and the cells were analyzed immediately by flow cytometry (BD Biosciences, Franklin Lakes, NJ). Control tubes of unstained cells, cells stained with PI alone, and cells stained with annexin V only were included for setting up the flow cytometric compensation. Apoptosis index was calculated as the percentage of annexin V+PI- cells of all tested cells.

**Transwell Invasion Assay**

The invasiveness of the cells was determined by their ability to cross the 8-μm pores of polycarbonate membranes (6.5-mm filters; Corning, Corning, NY) coated with Matrigel (BD Biosciences) fitted to the bottom of transwell chambers. In brief, the purified human first-trimester trophoblasts and JAR cells were plated in upper chambers at a density of 1.0 × 105 cells/well in 200 μl DMEM with 2% FBS or different concentrations of CsA (0, 0.0001, 0.001, 0.01, 0.1, 1.0, and 10 μM), respectively. The lower chamber was filled with 600 μl DMEM with 10% FBS. After incubation of 48 h at 37°C, the cells from the upper surface of the filter were completely removed with gentle swabbing, and the remaining invaded cells were fixed in methanol for 10 min at room temperature and stained with hematoxylin. Cell invasive ability was determined by counting the number of stained cells on the membranes in 10 randomly selected, nonoverlapping fields at 200× magnification using a light microscope. The invasion index was calculated as the ratio of the percentage of invasion of the CsA-treated cells to that of the vehicle.

**Western Blot**

The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, and phosphatase inhibitors). Protein samples were separated by 10% SDS-PAGE (50 μg/lane) and transferred onto nitrocellulose membranes. The blots were first incubated with primary antibodies phosphorylated MAPK3/MAPK1 (1:1000 dilution; Cell Signaling Technology, Beverly, MA), and then incubated with horseradish peroxidase secondary antibodies. The bands were visualized using an ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) following the manufacturer’s protocol. The membranes were stripped and reblotted with anti-total MAPK3/MAPK1 monoclonal antibody (Cell Signaling Technology) to normalize the amount of protein loaded on gels. The scanning densitometry was performed after exposure to x-ray film. The relative band intensities of MAP3/MAPK1 in each lane were measured by quantitative scanning densitometer and image analysis software Bio-ID, version 97.04 (Froebel, Wasserburg, Germany).

**MAPK Activity Assay**

The trophoblasts were serum starved overnight to synchronize kinase activity to almost zero, and they were treated with 30 μM U0126 for 30 min prior to treatment with CsA. The cells were lysed in RIPA buffer, and the assay of kinase activity was carried out using nonradioactive MAPK (MAPK3/MAPK1) activity assay kits (Chemicon, Temecula, CA) according to instructions. The MAPK activity is expressed as the ratio of absorbance in the treatment group to the vehicle control group.

**Transient Transfection and Reporter Gene Assay**

To determine the transcriptional activity of AP1 and NFAT, JAR cells were transiently cotransfected with 200 ng pAP1-luciferase or pNFAT-luciferase and 10 ng pRLSV40 (used for normalization of transfection efficiency) using LipofectAMINE (Life Technologies, Grand Island, NY) according to the
manufacturer’s instructions. In the 24 h following transfection, the JAR cells were treated with CsA and/or 1.0 nM ionomycin and/or NFAT inhibitor, which was used to inhibit NFAT1, NFAT2, and NFAT4 transcription activity; some wells were pretreated with 30 μM U0126 for 20 min. As for NFAT transcription activity, ionomycin was added as an activator for 6 h, and the cells were then harvested in passive lysis buffer. Luciferase activity was measured by luminometer using the dual luciferase assay system (Promega, Madison, WI) according to the manufacturer’s instructions. Change in luciferase activity with respect to control was calculated. The results were expressed as a percentage of luciferase activity.

Statistical Analysis

Data are expressed as mean ± SEM, and statistical evaluation was performed using one-way ANOVA followed by a Dunnett test. Differences were accepted as significant at P < 0.05.

RESULTS

CsA Promoted Viability and Invasiveness of Human First-Trimester Trophoblast Cells

As shown in Figure 1, A, B, and C, within the range of 0.0001–1.0 μM, CsA increased trophoblast cell viability while inhibiting serum deprivation-induced apoptosis of human first-trimester trophoblast cells in a concentration-dependent manner. However, at the higher concentration of 10 μM, CsA reduced cell viability and enhanced cell apoptosis. These results suggested that exposure of human trophoblast cells to CsA induced a different response depending on the concentration. These responses ranged from cytoprotection/antiapoptosis to proapoptosis.

Thereafter, we examined the effects of CsA on human trophoblast invasion. Using the Transwell assay, we observed that the number of cells invading through the polycarbonate membranes appeared to increase in a concentration-related manner with the CsA concentration, peaking around 1.0 μM and then decreasing. The invasion index of human first-trimester trophoblasts was 2.19-fold, 2.2-fold, and 2.42-fold higher, respectively, in the CsA concentrations of 0.01, 0.1, and 1.0 μM (P < 0.01) compared with that of the vehicle control (Fig. 1D).

We also observed the effects of cell proliferation suppression on cell invasion. The data demonstrated that treatment with mitomycin C inhibited cell replication by 90% and reduced the number of cells invading through the polycarbonate membranes, but it had no effect on the invasion index in either control or the CsA-treated cultures (data not shown). Hence, the number of invading cells, but not the invasion index, depended on cell proliferation.

CsA Activated MAPK3/MAPK1 and Inhibited Ca2+/Calcineurin/NFAT Signal of Human Trophoblasts in Time- and Concentration-Dependent Manners

As shown in Figure 2A, phosphorylation of MAPK3/MAPK1 in the primary trophoblasts was observed as early as 10 min following CsA (1.0 μM) treatment, with a peak at 30 min. Moreover, the CsA-induced MAPK3/MAPK1 phosphorylation remained at a higher level for at least 1 h, and then decreased significantly in 2 h following CsA treatment. The amount of the total MAPK3/MAPK1 remained unchanged during the experiment. In addition, we also evaluated the MAPK3/MAPK1 phosphorylation level upon stimulation with varying concentrations of CsA for 30 min. The MAPK3/
MAPK1 was activated in a dose-dependent manner up to 1.0 μM CsA (Fig. 2B). These results indicated that fairly low concentrations of CsA were sufficient to upregulate MAPK3/MAPK1 signaling. Our other experiment also confirmed by ELISA that treatment with CsA significantly enhanced MAPK activity in a concentration- and time-dependent manner (data not shown). The MAP3K1/2 inhibitor U0126 abolished MAPK3/MAPK1 phosphorylation and activation in trophoblasts in a concentration-dependent manner, and at the concentration of 30 μM it completely inhibited the CsA-induced MAPK3/MAPK1 phosphorylation and activation (Fig. 2, C and D).

We then examined whether Ca²⁺/calcineurin/NFAT existed in human trophoblasts. To verify this possibility, we undertook a transient transfection and reporter gene assay. Thinking of the difficulty of transfection in primary trophoblasts, we used a trophoblast-like cell line, human choriocarcinoma JAR cell, and monitored NFAT transcription activity. The results showed that NFAT-luciferase activity increased 5.6-fold after treatment with ionomycin, an activator of calcineurin, and treatment with CsA inhibited NFAT-luciferase activity in a dose-dependent manner; 1.0 μM CsA was sufficient to inhibit the ionomycin-induced NFAT transactivation in JAR cells completely (Fig. 2E).

CsA Activated MAPK3/MAPK1 and Inhibited Ca²⁺/Calcineurin/NFAT Signaling Pathway Independently

Since CsA could inhibit the ionomycin-induced, calcineurin-dependent NFAT transcriptional activity (Fig. 2E) and also activate MAPK3/MAPK1 in trophoblasts (Fig. 2, A and B), we wondered whether the CsA-induced activation of MAPK3/MAPK1 and the inhibition of Ca²⁺/calcineurin/NFAT were direct or dependent on each other. The results in Figure 3A show that activating calcineurin by ionomycin resulted in activation of NFAT but had no effect on the induction of phosphor-MAPK3/MAPK1 level or MAPK activity induced by CsA. Furthermore, inhibition of NFAT transactivation with NFAT inhibitor did not affect the dual phosphorylated MAPK3/MAPK1 level and MAPK activity. These data supported the notion that CsA activated MAPK3/MAPK1 independently of the inhibition of Ca²⁺/calcineurin/NFAT signaling. In the same way, we pretreated JAR cells with U0126 and observed NFAT transcription activity. As shown in
blockade of MAPK3/MAPK1 signaling pathway did not change the ionomycin-induced NFAT transcription activity or reverse the CsA-inhibited NFAT activity. Taken together, our data suggest that the CsA-induced activation of MAPK3/MAPK1 and inhibition of Ca\(^{2+}\)/calcineurin/NFAT signaling pathway were independent of each other.

MAPK3/MAPK1, Not Ca\(^{2+}\)/Calcineurin/NFAT Signaling, Was Involved in the CsA-Promoted Growth of Trophoblast Cells

If CsA improved cell viability and antiapoptosis of trophoblast cells via MAPK3/MAPK1 activation, inhibition of MAPK3/MAPK1 should lead to blocking the increased viability and antiapoptosis of trophoblast cells induced by CsA. Our data in Figure 2, C and D, clearly showed that U0126 inhibited the CsA-induced MAPK3/MAPK1 phosphorylation and activation in trophoblasts. The concentration of 30 \(\mu\)M U0126 abolished completely the CsA-induced effect at 30 min. Meanwhile, U0126 inhibited the CsA-increased cell viability of trophoblasts and reversed the protective effect of CsA on serum deprivation-induced apoptosis in trophoblasts (Fig. 4). These results provide direct evidence that CsA stimulated survival and rescued the serum deprivation-induced apoptosis in trophoblasts (Fig. 4). These data strongly suggest that the CsA-induced survival and antiapoptosis of trophoblast cells are not through the classical Ca\(^{2+}\)/calcineurin/NFAT pathway.

CsA Stimulated Invasiveness of Human Trophoblasts Via Activation of MAPK3/MAPK1 and Inhibition of Ca\(^{2+}\)/Calcineurin/NFAT Signaling Pathway

We analyzed the role of MAPK3/MAPK1 in CsA-regulated trophoblast invasiveness. To this end, we blocked the MAPK3/ MAPK1 pathway in trophoblast cells by pretreatment with U0126. As shown in Figure 5, U0126 efficiently inhibited the CsA-induced increase in invasiveness of the trophoblasts, suggesting that CsA stimulated invasion of human trophoblast cells via MAPK3/MAPK1 signaling pathway. Since the inhibition of the CsA-induced increase in invasiveness by U0126 was incomplete, it was speculated that other molecular mechanisms participated in the upregulation of CsA in the invasiveness of trophoblasts.
To examine whether Ca\textsuperscript{2+}/calcineurin/NFAT cascade was involved in the CsA-regulated invasion of trophoblast cells, we conducted Transwell assay under the activating conditions of this signaling by ionomycin stimulation and inactivation with CsA or NFAT inhibitor. As shown in Figure 5, pretreatment with ionomycin inhibited invasiveness of human trophoblast cells. Administration with CsA or NFAT inhibitor reversed the ionomycin-inhibited trophoblast cell invasiveness. Interestingly, the effect of NFAT inhibitor was lower than that of CsA, and the combination of CsA with NFAT inhibitor showed no synergistic effect. These results were consistent with the fact that other signals, such as MAPK3/MAPK1 signal, contribute to the CsA-induced invasive improvement of trophoblast cells, and NFAT is a downstream molecule to the Ca\textsuperscript{2+}/calcineurin signaling pathway.

**AP1 Was Involved in the CsA-Increased Cell Viability and Invasiveness of Human JAR Cells**

We next examined whether AP1 was involved in the CsA-promoted proliferation and invasiveness of human trophoblasts. The JAR cells were cotransfected with a luciferase reporter construct (pAP1-Luc) and its control plasmid (pRL-SV), and then treated with CsA. The results in Figure 6, A, B, and C, showed that CsA stimulated AP1 transactivation in human JAR cells in concentration- and time-dependent manners, and the maximal transcriptional activity was at 1.0 \( \mu \text{M} \) CsA for 4 h, which was inhibited by pretreatment with U0126. These data indicated that the MAPK3/MAPK1 signaling activation was necessary for the CsA-induced AP1 transactivation of JAR cells. U0126 inhibited both the basal and CsA-induced AP1 activity, suggesting that the basal AP1 activity is also mediated by MAPK3/MAPK1 signaling.

**The CsA-Induced, MAPK3/MAPK1-Mediated AP1 Transactivation Was Not Due to Inhibition of Ca\textsuperscript{2+}/Calcineurin/NFAT**

Calcineurin (phosphoprotein phosphase 2B) has been found to specifically downregulate the transcriptional activity of transcription factor ELK1, a target of activated MAPK required for c-fos (one important component of transcription factor AP1) transcription [20]. So there is a negative cross-talk between calcineurin and MAPK-mediated AP1 transactivation. It is proposed that the CsA-induced, MAPK3/MAPK1-mediated
activation of AP1 may be due to the inhibition of calcineurin. To exclude this possibility, we examined the effect of ionomycin (activator of calcineurin) on AP1 in JAR cells. If CsA activated AP1 through inhibiting calcineurin, activation of calcineurin by ionomycin should lead to blocking of AP1 transactivation.

In fact, treatment with 1.0 nM ionomycin had no effect on the CsA-induced AP1 transactivation (Fig. 7). Furthermore, treatment with ionomycin or NFAT inhibitor did not change MAPK activation or AP1 activity in the CsA-treated and untreated JAR cells (Figs. 3A and 7). All these data suggested that the CsA-induced, MAPK3/MAPK1-mediated AP1 transactivation was independent of the inhibition of Ca2+/calcineurin/NFAT signaling pathway.

DISCUSSION

During early pregnancy, the embryonic trophoblast cells proliferate and then differentiate into tumor-like cells that invade the uterus and its vasculature, remodeling the maternal spiral arteries. This is an essential step in establishing and maintaining a normal pregnancy, and it is necessary for the higher blood requirement of the fetus later in gestation. Therefore, adequate proliferation and invasion of trophoblast cells are basic requirements for a successful pregnancy without complication. In preeclampsia, intrauterine growth restriction, and spontaneous miscarriage, there seems to be decreased proliferation and invasion and an increased apoptosis of villous trophoblasts [21–25]. The mechanisms underlying trophoblast proliferation and invasion are still poorly understood, which is important for our understanding of the early events of pregnancy, and may provide insights into the processes that occur in complications of pregnancy.

Cyclosporin A is a potent immunosuppressant widely used to prevent rejections following organ transplantation. Our previous study demonstrated that treatment of CBA/J pregnant females with low doses of CsA only significantly reduced the resorption rate and reversed the observed decrease in proliferation and invasiveness of the trophoblast cell from the early gestation in the abortion-prone matings to that of normal pregnancy [9]. Likewise, administration with CsA could promote the proliferation and invasiveness of human first-trimester trophoblasts [7, 8]. These studies provided the first evidence that the CsA-regulated proliferation and invasiveness of trophoblast cells might contribute to the improvement of trophoblast cell growth and invasion induced by CsA.

The potential applications of this immunosuppressant. In this study, we further explored the signaling mechanism by which CsA regulated human trophoblast cell viability and invasiveness.

Although pharmacologic research of CsA has focused primarily on T cell response, there is emerging evidence that this agent may exert potent effects on varieties of cells, regulating wide biological functions [2–5]. It was reported that the impact of CsA on signal transduction pathways in nonimmune cell types is distinct from its well-characterized immunosuppressive effect on immune cells [26]. However, the effect of CsA on human trophoblasts has not been defined.

It was reported that the MAPK3 and MAPK1 were expressed and activated in human trophoblasts, especially in villous and extravillous trophoblasts during the first trimester in human placenta, suggesting that the activated MAPK3/MAPK1 cascade might be involved in the proliferation and invasion of these cells [16]. Consistent with these studies, our data suggested that CsA regulated human trophoblast cell viability and invasiveness.

Since inhibiting calcium-mediated calcineurin/NFAT signaling was the classical way by which CsA exerted wide regulation in multiple cell types, we analyzed this pathway in human trophoblast cells. Our results have demonstrated that the classical calcineurin/NFAT signaling is not involved in the
regulation of viability and apoptosis in trophoblasts by CsA. In contrast, Ca\(^{2+}\)/calmodulin/NFAT signaling downregulated trophoblast invasiveness induced by CsA. The effect of NFAT inhibitor was lower than that of CsA, so it may be postulated that other signaling pathways are involved in the CsA-regulated invasion of trophoblast cells, which is consistent with the fact that MAPK3/MAPK1 signaling contributes to the process.

Although MAPK3/MAPK1 and Ca\(^{2+}\)/calmodulin/NFAT are two different signaling cascades, calmodulin is a protein phosphatase, which participates in the dephosphorylation of protein kinases. Some research groups reported that there was a reciprocal yet reinforcing signaling relationship between calmodulin and MAPK3/MAPK1, such that calmodulin activation promotes MAPK3/MAPK1 activation, and Ras-MAPK activation enhances NFAT activation through an unknown mechanism [27, 28]. Some studies indicated a negative regulatory role of calmodulin in the MAPK3/MAPK1 pathway [20, 29], whereas others showed there was no relation between calmodulin and MAPK3/MAPK1 [30]. Thus, the relationship between the CsA-induced activation of MAPK3/MAPK1 and inhibition of calmodulin-NFAT pathway must be determined. Here, we clearly demonstrated that neither activation of NFAT by ionomycin nor inhibition of NFAT by its inhibitor influenced MAPK3/MAPK1 phosphorylation and activation. In addition, the inhibition of MAPK3/MAPK1 pathway by U0126 also failed to change the transcriptional activity of NFAT. Therefore, the calmodulin-NFAT signaling and MAPK3/MAPK1 signaling regulated by CsA were independent of each other in human trophoblast cells. Taken together, MAPK3/MAPK1 signaling can either stimulate or inhibit calmodulin-NFAT activity, depending on the cell type and the time course that was employed.

Since MAPK3/MAPK1 phosphorylation has been implicated in the transcriptional regulation of c-fos and activation of AP1 [31], we explored the transcriptional mechanisms mediating improvement of trophoblast viability and invasion by CsA. The activator protein AP1 is mainly composed of c-Jun homodimers or c-Jun/c-fos heterodimers. The members of the AP1 family of transcription factors are key regulators of cellular proliferation, differentiation, and invasion in multiple systems. It has been demonstrated that AP1 transcriptional activity is increased with the induction of the transformed phenotype and with neoplastic progression [32, 33]. In human placenta, the AP1 transcription factors are specifically expressed in the intermediate (extravillous) trophoblast, where they could be implicated in regulating proliferation and expression of invasion-specific molecules, such as matrix metalloproteinases [18]. Growing evidence also indicated that CsA could activate AP1 [34, 35]. Our results have demonstrated that CsA can induce AP1 transcription in human trophoblasts, and this induction required activation of MAPK3/MAPK1 signaling, suggesting CsA regulated trophoblast proliferation and invasion through activating MAPK3/MAPK1-mediated AP1 transcription pathway. Recently, a glucocorticoid receptor-mediated AP1 activity was reported [36]. Maybe the CsA induction of AP1 activity could also occur by inhibition of P-glycoprotein (P-gp)-mediated glucocorticoid efflux, which leads to elevated intracellular glucocorticoids and activated AP1 transcription.

Despite the evidence that Ca\(^{2+}\)/calmodulin-dependent calcineurin is a major phosphatase of the Ets family of transcription factor Elk-1 and plays a negative role in induction of c-fos transcription [20], it remains to be established whether calmodulin/calcineurin constantly inhibits c-fos activity in human trophoblasts. In fact, our present study has demonstrated that 1.0 nM ionomycin is efficient to activate calcineurin while not changing AP1 activity, suggesting activation of calcineurin does not always inhibit c-fos activity in human trophoblasts. The data were different from the CsA-induced AP1 activation in T cells [35] mediated by calcineurin but showed striking similarities to the CsA-induced AP1 activation in human aortic smooth muscle cells [37] and endothelial cells [34].

In summary, we have demonstrated that the MAPK3/MAPK1 and Ca\(^{2+}\)/calmodulin/NFAT pathways are differentially involved in the regulation of growth and invasion of human trophoblast cells by CsA. On one hand, CsA promoted growth of trophoblast cells through activating the MAPK3/MAPK1 pathway; on the other hand, CsA enhanced invasiveness of the cells via activating MAPK3/MAPK1 and inhibiting Ca\(^{2+}\)/calmodulin/NFAT pathways. In addition, we have shown that the CsA-induced, MAPK3/MAPK1-mediated transactivation of AP1 is responsible for the growth and invasion of trophoblasts. A model depicting the role of CsA in regulating growth and invasion of human trophoblasts is presented in Figure 8. Our results contribute to the understanding of mechanisms of trophoblast cell growth and invasion regulated by CsA, and thus they may lead to a novel strategy for therapeutic intervention for certain pathological pregnancy, such as alloimmune pregnancy wastage and preeclampsia.

**ACKNOWLEDGMENT**

The authors would like to thank Dr. Qing Qiu from University of Ottawa for his helpful revision on the manuscript.

**REFERENCES**


