Protein kinase C dependent and independent mechanisms controlling rat trophoblast cell DNA synthesis and differentiation

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Trophoblast giant cells are the steroidogenic cells of the rat placenta. In this study, the role of protein kinase C signalling pathways in the control of DNA synthesis and differentiation-dependent progesterone biosynthesis by trophoblast cells were investigated. Rho-1 trophoblast cells, derived from a rat choriocarcinoma, can be experimentally manipulated to proliferate or differentiate and provide a useful model for studying trophoblast giant cell endocrine differentiation. The role of protein kinase C signal transduction was examined through the treatment of Rho-1 trophoblast cells with isoquinolinesulfonamide derivatives (H7, a protein kinase C inhibitor; HA1004, a control compound), chelytherine (a protein kinase C inhibitor), and phorbol esters (protein kinase C activators). Treatment with H7 significantly attenuated DNA synthesis in proliferating and differentiating trophoblast cells and accelerated the acquisition of progesterone biosynthetic capabilities by trophoblast cells. Treatment with HA1004, the related but functionally distinct isoquinolone, did not significantly affect trophoblast DNA synthesis or proliferation and only weakly increased progesterone accumulation. Chelytherine significantly inhibited trophoblast cell proliferation but failed to influence trophoblast progesterone production significantly. The phorbol ester, 12-O-tetradecanoylphorbol acetate, did not significantly influence progesterone accumulation. H7 did not significantly influence the concentration of either P450scc or the mRNA encoding it in Rho-1 trophoblast cells, or the transcriptional activity of the P450scc gene. The results indicate that signalling pathways sensitive to protein kinase C are involved in the control of trophoblast cell proliferation. Differentiation-dependent production of progesterone is sensitive to H7 but appears to be independent of protein kinase C and occurs at a stage other than P450scc expression.

Introduction

Trophoblast giant cells of the rat placenta possess specialized abilities in coordinating embryonic and maternal activities through the secretion of hormones (Soares et al., 1991, 1993, 1996). The acquisition of endocrine function by trophoblast giant cells is poorly understood. Rho-1 trophoblast cells, derived from a rat choriocarcinoma, can be experimentally manipulated to proliferate or differentiate and provide a useful model for studying trophoblast giant cell endocrine differentiation (Faria and Soares, 1991; Hamlin et al., 1994; Yamamoto et al., 1994, 1995, 1996). Differentiation is characterized by endoreduplication and the expression of genes encoding members of the placental prolactin gene family and enzymes involved in steroidogenesis (Faria and Soares, 1991; Yamamoto et al., 1994, 1995, 1996). Progesterone biosynthesis has been demonstrated to be a useful marker of Rho-1 trophoblast cell differentiation (Yamamoto et al., 1994). Furthermore, cytochrome P450 (the rate-limiting enzyme of steroidogenesis) side chain cleavage (P450scc) has been found to be transcriptionally activated during trophoblast giant cell differentiation (Yamamoto et al., 1994, 1995).

Protein kinase C has been implicated in regulatory pathways controlling cell proliferation and differentiation of a number of different cell lineages (Nishizuka, 1992; Olson, 1993; Liu, 1996). Although there are reports suggesting that protein kinase C pathways are involved in the regulation of human and bovine trophoblast cell endocrine function (Shemesh et al., 1984, 1994; Ritvos, 1988; Ritvos and Voutilainen, 1992), the role of protein kinase C in the regulation of the trophoblast giant cell differentiation pathway is unclear. In the present study, we examined the involvement of protein kinase C in the control of Rho-1 trophoblast cell proliferation and differentiation. The role of protein kinase C signalling pathways was examined through the treatment of Rho-1 trophoblast cells with isoquinolinesulfonamide derivatives (H7, a protein kinase C inhibitor; HA1004, a control compound) and phorbol esters (protein kinase C activators).

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Materials and Methods

Reagents

Fetal bovine serum (FBS) and donor horse serum (HS) were purchased from JRH Bioscience (Lenexa, KS). H7 and HA1004 were purchased from Seikagaku America, Inc. (Rockville, MD) and chelerythrine was purchased from LC Services Corp. (Woburn, MA). Supplies for polyacrylamide gel electrophoresis were purchased from Bio–Rad Chemicals (Richmond, CA). Nitrocellulose was obtained from Schleicher and Schuell (Keene, NH). Immunoassay kits for progesterone and human growth hormone (hGH) were acquired from Diagnostic Products Corporation (Los Angeles, CA) and Nichols Institute (San Juan Capistrano, CA), respectively. The pSV2–Neo plasmid was obtained from American Type Culture Collection (ATCC, Rockville, MD). Liposome-mediated transfection kits were purchased from Life Technologies, Inc. (Gaithersburg, MD). Radiolabelled nucleotides were obtained from DuPont–NEN (Boston, MA). Radiolabelled thymidine was acquired from ICN Biomedicals Inc. (Irvine, CA). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Maintenance of the Rho-1 trophoblast cell line

The Rho-1 trophoblast cell line was routinely maintained in subconfluent conditions with NCTC-135 culture media containing 20% FBS as reported by Faria and Soares (1991) and Hamlin et al. (1994). Differentiation was induced by growing the cells to near confluence in NCTC culture medium supplemented with 20% FBS and then replacing the serum supplementation with 1–10% HS. High cell density and the absence of growth stimulation (removal of FBS) facilitates giant cell formation (Hamlin et al., 1994).

Measurement of DNA synthesis

Incorporation of [3H]thymidine was used as a measure of DNA synthesis in proliferating and differentiating Rho-1 trophoblast cells. Cells were plated at 2.5 × 10⁴ cells per well in 24-well plates and maintained in NCTC culture medium supplemented with 20% FBS until day 2 of culture when the medium was changed to NCTC supplemented with 10% HS. On day 1 or day 8 of culture, cells were washed with serum-free RPMI–1640 culture medium to remove serum factors. Cultures were then incubated for 24 h in serum-free RPMI–1640 culture medium before addition of treatments in fresh RPMI–1640 culture medium for an additional 24 h incubation. Treatments were added in the presence of 10% FBS (day 2 cells) or under serum-free conditions (day 9 cells). Proliferating cells (day 2) maximally incorporate [3H]thymidine in the presence of 10–20% FBS, whereas endoreplicating cells (day 9) maximally incorporate [3H]thymidine in serum-free conditions (Hamlin and Soares, 1995). At the end of the incubation, cells were pulsed with [3H]thymidine (1 μCi ml⁻¹) for 6 h. Measurement of [3H]thymidine incorporation by liquid scintillation counting was performed as described by Soares et al. (1989).

Progesterone radioimmunoassay

Progesterone was assayed with 125I-labelled radioimmunoassay kits and normalized to cell number as described by Yamamoto et al. (1994, 1995).

Western blot analysis

Western blot analyses for the detection of P450scc protein were performed according to the procedures of Roby et al. (1991) and Yamamoto et al. (1994). Mitochondrial lysates were prepared, protein concentrations determined, and samples, equivalent in protein amount, separated by electrophoresis in 10% polyacrylamide gels, transferred to nitrocellulose and probed with antibodies to P450scc (Roby et al., 1991). Protein measurements followed the dye-binding assay procedure of Bradford (1976). Antipeptide antibodies directed to two different domains of rat P450scc were used in the experiments: an internal domain of rat P450scc (amino acids 421–441) and the carboxy terminal domain of rat P450scc (amino acids 509–526). Each of the antibodies was used at a final dilution of 1:1000.

Northern blot analysis

Expression of P450scc was evaluated by northern blot analysis (Yamamoto et al., 1994) using a cDNA for rat P450scc (Golding et al., 1987). Total RNA from cultured cells was isolated, separated on agarose gels, blotted onto nitrocellulose and processed for hybridization as described by Soares et al. (1987), Faria et al. (1990) and Faria and Soares (1991). After hybridization, filters were autoradiographed with Kodak X–Omat AR X-ray film (Eastman Kodak, Rochester, NY) at −80°C. A P450scc pGEM plasmid was used as a template for the synthesis of 32P-labelled cRNA probes.

Transfection of Rho-1 trophoblast cells

Rho-1 trophoblast cells were transfected using a liposome-mediated delivery system as described by Shida et al (1993) and Yamamoto et al. (1994). Trophoblast cells were stably transfected by co-transfection of an 894 bp rat P450scc promoter–human growth hormone (hGH) reporter construct (9 μg; Oonk et al., 1990) with pSV2–Neo DNA (1 μg). Stable transfectants were selected by exposure to G418 (250 μg ml⁻¹). Concentrations of hGH were measured from conditioned medium by immunoradiometric assay (Yamamoto et al., 1995).

Statistical analysis

The data are presented as the mean ± SEM and were analysed by the nonparametric Mann–Whitney U test (Keppel, 1973).

Results

The role of protein kinase C in the control of trophoblast cell growth and differentiation was investigated with a series of
isoquinolinesulfonamide derivatives. Treatment with H7, a protein kinase C inhibitor, inhibited DNA synthesis in both proliferating and differentiating (endoreduplication) trophoblast cells. Proliferating cells were more sensitive to H7 than were differentiated cells (Fig. 1). The minimal effective inhibitory doses of H7 for proliferating and differentiating cells were 20 μmol l⁻¹ and 50 μmol l⁻¹, respectively. Treatment with HA1004, a related compound used as a control, did not significantly influence DNA synthesis in either proliferating or differentiating trophoblast cells (Fig. 1).

The inhibitory effects of H7 on DNA synthesis prompted an investigation of the effects of the protein kinase inhibitor on progesterone biosynthesis, an indicator of trophoblast giant cell differentiation. Treatment with H7 stimulated Rcho-1 trophoblast cell progesterone accumulation in a dose- and time-dependent manner (Figs 2 and 3). Treatment with HA1004 showed a modest stimulatory effect on progesterone accumulation by Rcho-1 trophoblast cells when used at 20 μmol l⁻¹ and 50 μmol l⁻¹ (Fig. 2). For further examination of the involvement of protein kinase C in trophoblast differentiation, Rcho-1 trophoblast cells were treated with another protein kinase C inhibitor, chelytherine, and a phorbol ester known to activate protein kinase C, 12-O-tetradecanoylphorbol acetate (TPA). Chelytherine significantly inhibited trophoblast cell proliferation (Table 1, P < 0.05) but failed to significantly influence trophoblast cell progesterone production (data not shown). Similarly, TPA did not significantly influence trophoblast progesterone production (data not shown).

The mechanism of inhibition of the isoquinolinesulfonamide derivatives on progesterone secretion was further explored through investigations on the regulation of the P450scc gene which encodes for a key enzyme controlling steroidogenesis. Treatment with H7 and HA1004 had no effect on P450scc protein (Fig. 4) and mRNA expression in Rcho-1 trophoblast cells (data not shown). In addition, treatment of
Rcho-1 trophoblast cells with H7 did not affect P450scc promoter activity (Fig. 5). HA1004 modestly inhibited P450scc promoter activity (Fig. 5).

**Discussion**

DNA synthesis in proliferating trophoblast cells is dependent upon exogenous signals activating protein tyrosine kinase and protein kinase C mediated pathways, whereas DNA synthesis in differentiating cells is dependent upon autocrine-activated pathways involving these same classes of signal transduction pathways (Hamlin and Soares, 1995; present study). The nature of the extracellular activators or the specific kinases involved in the control of DNA synthesis in proliferating and differentiating trophoblast cells have yet to be determined. Collectively, the results of the present study suggest a role for a pathway involving protein kinase C in trophoblast cell proliferation but not in the differentiation-dependent activation of trophoblast giant cell progesterone biosynthesis. In contrast, the protein kinase C signalling pathway has been implicated in the control of steroidogenesis in bovine trophoblast cells (Shemesh et al., 1984, 1994). The H7-mediated stimulation of trophoblast
progesterone accumulation, observed in the present study, appears to be the result of activation or interference of another signalling pathway. Boulton et al. (1995) have identified an H7-sensitive serine-threonine kinase distinct from protein kinase C that is involved in a cytokine signalling pathway. Possible nonprotein kinase C regulatory pathways in rat trophoblast cells possessing sensitivity to H7 are not known at present. The site of regulation of this unknown pathway appears to be at locations other than those involved in the regulation of P450sc expression. These mechanisms may include the uptake and utilization of cholesterol precursors or the metabolism of progesterone.

In summary, protein kinase C signalling pathways participate in the control of trophoblast proliferation and endoreduplication; however, their involvement in the endocrine differentiation of trophoblast giant cells is not evident from the present experimentation. Differentiation-dependent production of progesterone is sensitive to treatment with H7 and occurs at a stage other than P450sc expression.

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References


Olson EN (1993) Signal transduction pathways that regulate skeletal muscle gene expression Molecular Endocrinology 7 1369–1378


Ritvos O (1988) Modulation of steroidogenesis in choriocarcinoma cells by cholera toxin, phorbol ester, epidermal growth factor and insulin-like growth factor I Molecular and Cellular Endocrinology 59 125–133

Ritvos O and Voutilainen R (1992) Regulation of aromatase cytochrome P-450 and 17β-hydroxysteroid dehydrogenase messenger ribonuclease acid levels in choriocarcinoma cells Endocrinology 130 61–67


