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UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

FACULTAD DE MEDICINA

LA PLACENTA, SITIO EXTRARRENAL DE SÍNTESIS Y EXPRESIÓN DE LA 25-HIDROXIVITAMINA D_3 1α-HIDROXILASA

T E S I S

que para obtener el grado de:

DOCTORA EN CIENCIAS

Presenta la Bióloga:

LORENZA DIAZ NIETO

Programa de Doctorado en Ciencias Biomédicas

Director de tesis: Fernando Larrea Gallo

México, D.F. **Noviembre de 2002**

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FACULTAD DE MEDICINA

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ING. LEOPOLDO SILVA
DIRECTOR GENERAL DE ADMINISTRACION
ESCOLAR DE LA UNAM.
PRESENTE.

Por medio del presente me permito informar a usted que en la reunión del Subcomité Académico de Doctorado en Ciencias Biomédicas que se llevo a cabo el día 17 de junio del presente año, **se acordó designar el siguiente jurado para examen de Doctorado en Ciencias Biomédicas** de la BIOL. DIAZ NIETO LORENZA con número de expediente 12982703 y número de cuenta 98860032 con la tesis titulada: "*LA PLACENTA, SITIO EXTRARRENAL DE SÍNTESIS Y EXPRESIÓN DE LA 25-HIDROXIVITAMINA D3 1α-HIDROXILASA*", dirigida por el Dr. Fernando Larrea Gallo.

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Facultad de Medicina

Dr. Abel Moreno Cárcamo
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Tutor(a) Dr. Fernando Larrea Gallo.
Coordinación de Doctorado

Teléfonos: 622-38-92 y 622-38-37 Fax 622-38-92 e-mail: pdcb@servidor.unam.mx Internet: www.pdcb.unam.mx

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ING. LEOPOLDO SILVA
DIRECTOR GENERAL DE ADMINISTRACION
ESCOLAR DE LA UNAM
PRESENTE.

Por medio del presente le informamos que el candidato al grado de doctor (a) LORENZA DIAZ NIETO con número de expediente 12982703 y número de cuenta 98860032, inscrita en el Doctorado en Ciencias Biomédicas, ha cubierto satisfactoriamente todas las actividades académicas establecidas en el plan de estudios del programa.

En consecuencia y de acuerdo al artículo 7 de las normas operativas del programa y del artículo 33 del Reglamento General de Estudios de Posgrado, **se autoriza al candidato para solicitar su examen de grado Doctoral** haciendo defensa de la tesis, "*LA PLACENTA, SITIO EXTRARRENAL DE SÍNTESIS Y EXPRESIÓN DE LA 25-HIDROXIVITAMINA D3 1α-HIDROXILASA*", dirigida por el Dr. Fernando Larrea Gallo.

Agradeciendo de antemano la atención que se sirva prestar a la presente le reiteramos nuestra consideración más distinguida.

Atentamente
"Por mi raza hablará el espíritu"
Cd. Universitaria, D.F., a 18 de junio de 2002.

Dr. Gabriel Roldán Roldán
Responsable del Programa en la Entidad

Dr. Abel Moreno Cárdamo
Coordinador del Programa

C.c.p. Biol. Francisco Javier Incera, Jefe de la Unidad de Administración del Posgrado.
Dr.(a) Fernando Larrea Gallo.
Coordinación Doctorado

Teléfonos: 622-38-92 y 622-38-37 Fax 622-38-92 e-mail: pdcb@servidor.unam.mx Internet: www.pdcb.unam.mx

INCMNSZ

El presente trabajo se llevó a cabo en el Departamento de Biología de la Reproducción del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán bajo la dirección del Dr. Fernando Larrea Gallo

DEDICATORIA

A Dios y a María,
Por todas sus bendiciones

A mi familia, Juan, Sofía y Nicolás,
Por su amor y apoyo incondicional
Por ser fuente de esperanza y motivo de lucha

A mis padres, Luis Porfirio y Teresa,
Por estar siempre presentes
Por su ayuda y su cariño
Por ser el mejor ejemplo de vida

A mis hermanos Laura y Porfirio,
Por haber compartido toda esta vida juntos

A mis compañeros y amigos, especialmente a Cecilia, Euclides, Angélica, Isabel, Rocío, y Mayel
Por los buenos y los malos momentos
Por compartir las angustias y las alegrías
Por hacer el trabajo más divertido

A mi tía Laura,
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INDICE

I. RESUMEN

El riñón es el sitio principal de conversión de la 25-hidroxivitamina D_3 (25-(OH)D_3) en calcitriol (1,25-$(OH)_2D_3$)[1,2]. Esta reacción es catalizada por una enzima perteneciente a la familia de las oxidasas mitocondriales, la 25-(OH)D_3-1α-hidroxilasa (1α-(OH)asa, CYP27B1)[3,4]. Durante la gestación las células deciduales sintetizan a la 1α-(OH)asa, la cual es funcionalmente activa y proviene del mismo gen que la del riñón[5]. Sin embargo, en la parte fetal de la placenta la hidroxilación del precursor de la 1,25-$(OH)_2D_3$ es controversial y la información en la literatura indica que el calcitriol placentario es producto de una reacción química[6]. En este trabajo se reporta por primera vez la expresión de la 1α-(OH)asa en sinciciotrofoblastos humanos en cultivo. Además, se describe la clonación y expresión del DNA complementario (DNAc) de la 1α-(OH)asa de placenta en una línea celular eucarionte, lo cual resultó en la síntesis constitutiva de la enzima catalíticamente activa. Estos resultados demostraron que el trofoblasto de la placenta humana contiene la maquinaria enzimática necesaria para llevar a cabo la producción del metabolito activo de la vitamina D_3. Adicionalmente se determinó que la síntesis de calcitriol en la placenta es regulada por el factor de crecimiento relacionado con la insulina (IGF-I). Se observó que la expresión de IGF-I se incrementa progresivamente conforme la célula adopta el fenotipo endocrino, y este proceso fue similar tanto en cultivos de sinciciotrofoblastos normales como en los de placentas de embarazos complicados con preeclampsia (PE). Finalmente, se estudió la síntesis y expresión de la 1α-(OH)asa en las placentas PE. A este respecto la capacidad de los sinciciotrofoblastos de placentas PE para biotransformar 25-(OH)D_3 en 1,25-$(OH)_2D_3$ fue significativamente menor que la observada en cultivos de placentas normales. Resultados similares se obtuvieron cuando se analizó el RNA mensajero de CYP27B1. Estos datos sugieren que las anormalidades del calcio y de la vitamina D observadas en la preeclampsia tienen relación con la baja expresión y actividad de la 1α-(OH)asa de las placentas PE.

II. ABSTRACT

The limiting step in the biosynthesis of the active form of vitamin D is hydroxylation at position 1-alpha of 25-OH-D. This reaction is catalyzed by a member of the mitochondrial inner membrane oxydases: 25-(OH)D_3-1α-hydroxylase (1α-(OH)ase, CYP27B1). The kidney is the main site of conversion of 25-hydroxyvitamin D_3 (25-(OH)D_3) into calcitriol (1,25-$(OH)_2D_3$), but during gestation decidual cells also synthesize 1α-(OH)ase, which is a product of the same gene than kidney's 1α-(OH)ase, and is functionally active. Nevertheless, 1,25-$(OH)_2D_3$ production by the fetal portion of the placenta has been controversial, since some data in the literature have shown that placental calcitriol is the product of a chemical reaction rather than an enzymatic process. In the present work CYP27B1 expression in syncytiotrophoblast maintained in culture is reported for the first time. Cloning and expression of placental 1α-(OH)ase complementary DNA (cDNA) into an eukaryotic cell line is also described. This resulted in the constitutive synthesis of a fully active 1α-(OH)ase. Additionally, we demonstrated that calcitriol synthesis in placental cultures is up-regulated by insulin like growth factor-I (IGF-I). Placentas from preeclamptic complicated pregnancies (PE) were also studied. The PE syncytiotrophoblasts synthesized significantly less 1,25-$(OH)_2D_3$ than control trophoblasts when incubated in the presence of equimolar concentrations of [^{3}H]25-(OH)D_3. CYP27B1 expression was also found to be reduced in PE syncytiotrophoblasts. On the other hand, IGF-I expression in PE syncytiotrophoblast cultures was not different from normal syncytiotrophoblasts; and increased linearly as differentiation occurred. Taken together these results suggest that low expression and activity of 1α-(OH)ase in PE placentas might be related to calcium abnormalities that are characteristic of PE.

III. INTRODUCCIÓN

1. Generalidades

El nombre genérico de la vitamina D (VD) abarca un grupo de esteroides íntimamente relacionados y que se caracterizan por presentar abierto el anillo B de su núcleo esteroide. La VD se encuentra en dos formas diferentes en la sangre: la vitamina D_2 o ergocalciferol de origen vegetal y la vitamina D_3 o colecalciferol que se sintetiza en la piel gracias a la acción de la luz ultravioleta. Esta última también se puede obtener de la dieta a partir del aceite de hígado de pescado y del huevo. La VD (VD sin subíndice se refiere a ambas formas D_2 y D_3) presenta en su estructura el núcleo del ciclopentanoperhidrofenantreno; sin embargo, el anillo B se encuentra abierto entre los carbonos 9 y 10 lo que permite identificarla como un secoesteroide. Adicionalmente, a diferencia de otras hormonas como el estradiol o la testosterona, la VD conserva 8 carbonos de la cadena lateral del colesterol. La diferencia estructural entre la VD_2 y la VD_3 es que la primera presenta un grupo metilo en el carbono 24 y un doble enlace entre el carbono 22 y 23. (Fig. 1)

2. Síntesis de la vitamina D_3

En la piel la VD_3 se sintetiza a partir del 7-dehidrocolesterol. La radiación ultravioleta rompe el anillo B dando lugar a la formación de un intermediario inestable (previtamina D), que a través de un proceso fototérmico se biotransforma en colecalciferol. Este metabolito circula en la sangre unido a su proteína transportadora hasta el hígado, en donde es hidroxilado en el carbono 25 para convertirse en 25-hidroxivitamina D_3 (25-$(OH)D_3$)[7]. Este metabolito, si bien es el más abundante en el plasma, no representa la forma activa. En el riñón la 25-$(OH)D_3$ es hidroxilada en el carbono 1 para obtener la 1,25-dihidroxivitamina D_3 (1,25-$(OH)_2D_3$) también conocida como calcitriol. (Fig. 2)

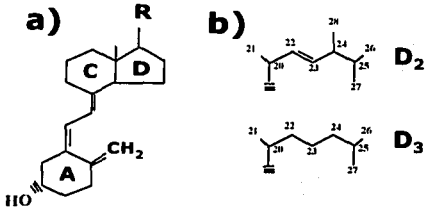


Figura 1. a) Estructura química de la vitamina D. b) Grupo alifático (R) que caracteriza a cada una de sus dos formas D_2 y D_3

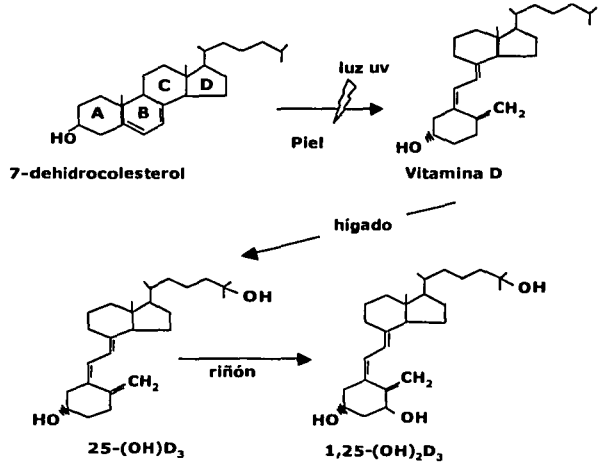


Figura 2. Síntesis de $1,25\text{-}(OH)_2D_3$ a partir del 7-dehidrocolesterol. La luz solar rompe el anillo B para formar la vitamina D_3 que al hidroxilarse en el hígado por la acción de la enzima vitamina D 25-hidroxilasa se convierte en $25\text{-}(OH)D_3$. La forma activa se obtiene tras una segunda hidroxilación en el riñón por efecto de la 1α-(OH)asa.

3. Mecanismo de acción y funciones de la vitamina D

Actualmente se sabe que el calcitriol no se limita a mantener la homeostasis del calcio. De hecho es una hormona muy versátil capaz de generar respuestas biológicas por la vía genómica a través de receptores intracelulares (iVDR), o por la vía no genómica a través de receptores membranales (mVDR). En la primera vía participan receptores nucleares o citoplásmicos. El iVDR activado por la hormona se dimeriza con otro receptor nuclear conocido como el receptor retinoide X (RXR) y el heterodímero así formado se une a elementos de respuesta para la vitamina D (VDRE) en la región promotora de los genes blanco. El heterodímero puede reclutar coactivadores y componentes del complejo de iniciación de la transcripción con la finalidad de modular la transcripción de genes y provocar respuestas biológicas[8]. Los genes de la osteocalcina, osteopontina, hormona paratiroidea (PTH) y 25-(OH)D 24-hidroxilasa (CYP24) contienen VDREs en sus regiones promotoras[9].

La segunda vía para generar efectos biológicos es a través de receptores membranales. Se ha sugerido a la anexina 2 (36 kDa) como posible candidato para el mVDR; sin embargo, es factible que exista más de un solo receptor membranal[8]. La unión del calcitriol a su mVDR provoca la activación de la proteína cinasa C (PKC), de la fosfolipasa C, de la adenilato ciclasa y/o la apertura de canales de calcio mediados por voltaje[10]. Esta vía de acción rápida se relaciona con los efectos no genómicos, e involucra cambios en las concentraciones de segundos mensajeros. Algunos ejemplos de este sistema de señalización son el del transporte rápido de calcio en el duodeno[11] también conocido como transcaltaquia (estimulación del transporte de calcio en 2-3 minutos)[12] y la estimulación de la síntesis y secreción de la insulina en los islotes pancreáticos[13]. En ambos modelos participan canales de calcio sensibles al voltaje que se activan una vez que el ligando interactúa con el mVDR. De manera general, las funciones que se atribuyen a la $1,25\text{-}(OH)_2D_3$ son: mantener la homeostasis del calcio y fósforo, regular la transcripción de ciertos genes, promover la diferenciación celular, regular la proliferación de células normales y malignas, regular el sistema inmunológico así como los procesos relacionados con el transporte de calcio. Además, se han localizado receptores para esta hormona en tejidos tan diversos como la placenta, cerebro, músculo, intestino y muchos sitios más[9].

4. Estructura, regulación y distribución de la 1α-(OH)asa

El paso limitante en la biosíntesis de la forma activa de la vitamina D es la hidroxilación en la posición 1 alfa de la 25-(OH)D_3. Esta reacción es catalizada por una enzima perteneciente a la familia de las oxidasas de la membrana interna mitocondrial: la 25-(OH)D_3 1α-hidroxilasa (1α-(OH)asa) o CYP27B1. La 1α-(OH)asa, que pertenece a la superfamilia de los citocromos P450 se acopla a dos transportadores de electrones para hidroxilar al carbono 1 del sustrato en la orientación α. Las proteínas acopladas son una flavoproteína (NADPH-ferredoxina reductasa) y una ferredoxina. Cada hidroxilación catalizada por un citocromo P450 se lleva a cabo mediante la siguiente reacción general:

$$\text{esteroide-H} + \text{NADPH} + H^+ + O_2 \Rightarrow \text{esteroide-OH} + NADP^+ + H_2O$$

Esta reacción consume dos electrones, un protón y una molécula de oxígeno. Uno de los átomos del oxígeno es incorporado al sustrato, mientras que el otro es reducido hasta agua[14]. Los electrones son transferidos del NADPH al citocromo P450 por la NADPH-ferredoxina reductasa y la ferredoxina.

Regulación de la 1α-(OH)asa

La 1α-(OH)asa renal es regulada por diversos factores tales como el fósforo, el factor de crecimiento similar a la insulina (IGF-I), el calcio, la PTH, la calcitonina y la misma 1,25-$(OH)_2D_3$[15,16,17]. La proteína transportadora de la vitamina D (DBP) también regula a la 1α-(OH)asa limitando la disponibilidad del sustrato. Recientemente se descubrió otra proteína involucrada en la homeostasis de la vitamina D: la megalina. La megalina es una proteína localizada en los túbulos proximales del riñón y es considerada de vital importancia para mantener concentraciones adecuadas de 25-(OH)D_3 en la sangre, constituyendo por lo tanto un eslabón importante en la vía de activación de la 25-(OH)D_3 en el riñón[8].

Adicionalmente, la enzima CYP24 también regula la síntesis del calcitriol al competir por la 25-(OH)D_3 y al inactivar al 1,25-$(OH)_2D_3$ mediante la hidroxilación de su cadena lateral en el C-24. De hecho, CYP24 es muy importante pues al haber un exceso de calcio en suero esta enzima es activada y constituye la vía metabólica más importante de depuración del calcitriol[18]. Cabe señalar que los estudios de

regulación de la 1α-(OH)asa han sido realizados sólo en términos de su actividad, pues la clonación de su DNA complementario (DNAc) es reciente[19,20,21]. Sin embargo, actualmente se han llevado a cabo diversos trabajos que toman en consideración la estructura recientemente descrita del gen de la 1α-(OH)asa[22]. A este gen se le ha asignado el nombre oficial de CYP27B1[23].

Distribución de la 1α-(OH)asa

Durante mucho tiempo la 1α-(OH)asa se detectó exclusivamente en los túbulos proximales del riñón[1,2] y este órgano fue considerado el único sitio de conversión de la 25-(OH)D_3 en 1,25-$(OH)_2D_3$. Sin embargo, estudios posteriores demostraron la conversión extrarrenal de 25-(OH)D_3 a su forma hormonal en otros tejidos tales como el hueso[24,25], la próstata[26], los queratinocitos[27] y en células del sistema inmunológico[8]. La síntesis de calcitriol en la placenta ha sido demostrada,[28,29,30,31] sin embargo, no ha sido posible aún esclarecer de manera clara y específica si se trata de un proceso enzimático o de otro tipo[6].

5. La vitamina D y el embarazo

El embarazo plantea grandes retos al organismo materno, y uno de ellos es el de proveer de calcio al feto sin alterar la homeostasis de dicho mineral. Al término del embarazo el feto llega a acumular hasta 30 gramos de calcio, y para satisfacer esta demanda el metabolismo de la vitamina D sufre ajustes que se traducen en el aumento de las concentraciones de 1,25-$(OH)_2D_3$ en el suero de la madre, favoreciendo así un incremento en la absorción intestinal de calcio[32].

Además del riñón, la placenta contribuye de manera importante en mantener la homeostasis del calcio durante la gestación, metabolizando 25-(OH)D_3 en 1,25-$(OH)_2D_3$[28,29,30]. La primera evidencia que se tuvo de la síntesis extrarrenal de calcitriol fue en ratas preñadas, en donde la nefrectomía bilateral reducía, mas no eliminaba, la conversión de 25-(OH)D_3 en calcitriol[33]. La producción placentaria de esta hormona se localiza en mayor proporción en las células deciduales, donde se ha corroborado la existencia de la maquinaria enzimática necesaria para dicha síntesis, y más aún, se ha determinado que la 1α-(OH)asa renal y la decidual provienen del mismo gen[5]. Por otro lado, estudios *in vitro* han mostrado evidencias de que la decidua humana[5] y el tejido placentario de roedores[31] producen

1,25-$(OH)_2D_3$; sin embargo, hasta antes del presente trabajo no se había demostrado una producción consistente de dicho metabolito por parte de los trofoblastos[34,35]. A este respecto, Hollis[6] sugirió que la 1,25-$(OH)_2D_3$ producida *in vitro* por la placenta humana era el resultado de un proceso mediado por radicales libres y no una hidroxilación enzimática. En contraste, otros estudios sugieren que existe actividad de 1α-(OH)asa en los trofoblastos y que las propiedades bioquímicas de esta hidroxilasa placentaria la señalan como una enzima de origen mitocondrial, muy similar a la renal[28,29].

6. El calcitriol en la placenta y el feto

Las concentraciones de calcitriol en el suero del feto son más bajas que en el suero de la madre, lo que probablemente se explique por el hecho de que el feto es hipercalcémico cuando se le compara con la madre. Esta situación demanda la presencia de mecanismos que permitan el transporte de calcio madre-feto en contra de un gradiente de concentración. La placenta es el órgano encargado de transportar el calcio de la madre al feto, y si bien este sistema no está aún bien caracterizado, diversos mecanismos que dan cuenta del transporte activo requerido han sido localizados en la placenta. Tal es el caso de los canales de calcio[36] y las proteínas acarreadoras de este nutrimento, como la calbindina D9K[37] y la ATPasa del calcio[38]. El calcitriol tiene un papel importante en este sistema de transporte estimulando el paso transplacentario del calcio a manera dosis-dependiente[39] y promoviendo la producción del RNAm de las proteínas acarreadoras de calcio[40,41], así como favoreciendo el transporte activo de este mineral.

7. La placenta

La placenta es un órgano transitorio que media el intercambio fisiológico entre la madre y el feto y modula el metabolismo materno mediante la secreción hormonal en diferentes etapas de la gestación. Al término del embarazo la placenta pesa entre 300 y 500 g, tiene forma de disco y deriva de las células del blastocisto que no formaron embrión. La placenta se compone de una cara materna y una fetal y por lo tanto presenta células de distinto genotipo. Las células deciduales son de genotipo materno y las trofoblásticas de genotipo fetal. La cara materna se caracteriza por presentar de 12-20 subdivisiones llamadas cotiledones, altamente

vascularizados. La placenta provee una amplia superficie para el intercambio de gases y el paso de nutrimentos y funciona como órgano endocrino complejo. De su adecuado funcionamiento depende el proceso de implantación, mantenimiento del embarazo, protección inmunológica del feto y parto. Las vellosidades coriónicas representan la unidad funcional de la placenta y están compuestas de una matriz laxa que contiene fibroblastos, macrófagos y múltiples vasos y capilares fetales, rodeados de una capa de citotrofoblastos interna y una de sinciciotrofoblastos externa.

El trofoblasto es el precursor de todas las células placentarias y de acuerdo a un gradiente de la hormona gonadotropina coriónica humana (hCG) éstas se diferenciarán en: 1) sinciciotrofoblasto, cuya función principal es secretar la mayoría de las hormonas placentarias; 2) trofoblasto invasivo, encargado de la penetración de las arterias espirales maternas en el útero, y 3) trofoblasto de anclaje, que secreta fibronectina para promover la sujeción de la placenta al útero[42].

La placenta sintetiza principalmente hCG, lactógeno placentario (hPL), progesterona, estradiol, prolactina, hormona liberadora de gonadotropinas (GnRH) y hormona liberadora de corticotropina (CRH), entre otras[43]. La hormona hCG es secretada desde el cuarto día posterior a la fertilización y prepara al endometrio para que sea receptivo al embrión, asegurando así la implantación. La hCG rescata al cuerpo lúteo de la involución manteniendo la secreción de progesterona por las células lúteas[43]. El cuerpo lúteo es el responsable de la producción temprana de estrógenos y progesterona, pero posteriormente la placenta asume su biosíntesis utilizando precursores de origen materno y fetal ya que los trofoblastos carecen de la 17 α-hidroxilasa y no sintetizan colesterol *de novo*[44].

8. El factor de crecimiento similar a la insulina

La diferenciación adecuada y el buen funcionamiento de la placenta es vital para el correcto desarrollo fetal. La placenta sintetiza factores de crecimiento similares a la insulina, así como sus proteínas transportadoras y el receptor para IGF-I[45]. La expresión del RNAm del IGF-I ha sido demostrada en el sinciciotrofoblasto, decidua, amnios y el corion[46]. Los IGFs son polipéptidos secretados preferencialmente por el

hígado, y la regulación de su producción se encuentra bajo la influencia de la hormona de crecimiento (GH) y factores nutricios tales como el aporte proteínico, energético y el zinc; mientras que la regulación de la interacción con sus receptores específicos esta modulada por una familia de seis proteínas transportadoras (IGFBPs)[47]. El IGF-I mimetiza las acciones de la insulina y sus efectos más conocidos son la estimulación de la síntesis de DNA y la replicación celular, la estimulación del transporte de aminoácidos, la inducción de la diferenciación celular y la regulación de la secreción hormonal. Estudios *in vivo* e in *vitro* han mostrado que el IGF-I es un regulador de la producción renal de 1,25-$(OH)_2D_3$[15,16]; y en experimentos en ratones normales tratados con IGF-I se observó un incremento del calcitriol en suero independiente de las concentraciones de calcio, fósforo o glucosa[15]. Estos estudios señalan al IGF-I como un estimulador importante de la producción de 1,25-$(OH)_2D_3$.

Por otro lado, las concentraciones de IGF-I en el suero de la mujer embarazada aumentan progresivamente conforme avanza la edad gestacional,[48,49,50] lo cual sugiere un papel importante de este factor en el crecimiento y el desarrollo intrauterino.

9. Preeclampsia

Definición: La preeclampsia (PE) y la eclampsia son complicaciones del embarazo que se caracterizan por hipertensión, proteinuria, edema repentino generalizado y en los casos más graves, crisis convulsivas. Este cuadro clínico se hace evidente generalmente después de la semana 28 de la gestación.

Etiología: Las causas de este padecimiento son desconocidas; sin embargo, la evidencia señala que se trata de una patología multifactorial[51]. Por otro lado se han identificado varios elementos de susceptibilidad tales como los de carácter genético, inmunológico, de tipo nutricional o disfunciones orgánicas previas en el sistema cardiovascular o renal. Los síntomas clínicos desaparecen rápidamente tras la remoción de la placenta, lo cual apoya la idea de que este órgano juega un papel importante en el desarrollo de la patogénesis de la PE.

Fisiopatología y placenta PE: Diversos cambios morfológicos y funcionales han sido reportados en la placenta PE[52]. Se han observado infartos, hematomas y

proliferación del citotrofoblasto entre otros[53]. Como resultado de la sinergia de varias condiciones patogénicas no bien identificadas aún, se genera una situación de hipoxia-isquemia uteroplacentaria con daño trofoblástico[54]. El flujo sanguíneo se encuentra disminuido en la placenta y la invasión de las arteriolas maternas por el trofoblasto invasivo es insuficiente o ausente[55]. En la PE se ha reportado disminución de antioxidantes, aumento en el proceso de peroxidación lipídica, aumento en los tromboxanos, disminución de las prostaciclinas[56], disminución del IGF-I y de su proteína transportadora 3 (IGFBP3)[57] y aumento en la IGFBP1[58], entre otras alteraciones. En relación con esto último, se ha demostrado que la IGFBP1 inhibe la invasión trofoblástica[59]. Adicionalmente la PE se ha asociado con bajo peso del recién nacido, hipocalciuria, bajas concentraciones plasmáticas de 1,25-$(OH)_2D_3$ así como de IGF-I[60,61,62,57].

Todos estos datos señalan a la placenta como un órgano involucrado en el desarrollo de la PE y hacen factible que existan alteraciones en la función endocrina de la placenta que expliquen la reducción en las concentraciones de IGF-I y de 1,25-$(OH)_2D_3$ en el suero de la mujer con preeclampsia.

IV. JUSTIFICACIÓN

La placenta sintetiza calcitriol durante la gestación[6,28,29,30,31] y su contribución se presume importante[29,33]. Sin embargo, se ha generado controversia en lo que respecta a la síntesis de 1,25-$(OH)_2D_3$ en la parte fetal de la placenta y existen datos contradictorios en la literatura. Durante el embarazo se duplican las concentraciones de 1,25-$(OH)_2D_3$ en el suero materno,[32,63] sin asociarse con hipocalcemia, hipofosfatemia o incremento en las concentraciones circulantes de la PTH[64], por lo que se supone la participación de otros factores que regulen su síntesis. El IGF-I es un estimulador de la producción renal de 1,25-$(OH)_2D_3$ y sus concentraciones en el suero materno aumentan de manera paralela a la de calcitriol durante el embarazo[48,49,50]. El IGF-I es también regulador de la esteroidogénesis en citotrofoblastos en cultivo.[65,66] Por lo anterior, el presente trabajo justifica sus objetivos al pretender: 1) determinar si el trofoblasto de la placenta humana sintetiza calcitriol y si es dependiente de un proceso enzimático; y 2) establecer si el IGF-I regula la síntesis de 1,25-$(OH)_2D_3$ en la placenta.

Por otro lado, utilizaremos a la PE como modelo para estudiar la relación entre las bajas concentraciones circulantes de calcitriol e IGF-I en el suero durante el embarazo y la secreción placentaria de ambas moléculas. La preeclampsia constituye un problema de salud pública mundial y es causa importante de morbilidad y mortalidad materno-infantil[51]. Una de las alteraciones ampliamente descritas en la PE reside en el metabolismo del calcio[61,62]. El beneficio que aportaría el estudio de la placenta PE es determinar si una disfunción endocrina de dicho órgano pudiera estar en relación con la hipocalcemia, como probable consecuencia de las bajas concentraciones en suero de 1,25-$(OH)_2D_3$[57,60] y la disminución en la absorción intestinal de calcio resultante.

Por lo anterior, el estudio de la placenta PE permitirá conocer si las bajas concentraciones de 1,25-$(OH)_2D_3$ en el suero de la mujer con preeclampsia se deben a una menor producción placentaria de esta hormona, dependiente o independientemente del IGF-I.

V. OBJETIVOS

Objetivo general

1. Conocer si el trofoblasto humano en cultivo produce 1,25-$(OH)_2D_3$ y estudiar su regulación por el IGF-I.

Objetivos específicos

1. Determinar si CYP27B1 se transcribe en el trofoblasto de la placenta humana normal, y en su caso, comparar esta expresión en placentas de mujeres con PE.
2. Estudiar la actividad de la 1α-(OH)asa en cultivos de sinciciotrofoblastos de mujeres NT y PE.
3. Estudiar el efecto del IGF-I sobre la síntesis placentaria de 1,25-$(OH)_2D_3$ en cultivos de sinciciotrofoblastos de mujeres NT y PE.

VI. HIPÓTESIS

1. La síntesis de calcitriol por las células trofoblasticas es un proceso enzimático similar al que ocurre en el riñón y es mediado, al menos en parte, por la 1α-(OH)asa.
2. El IGF-I estimula la actividad y expresión de la 1α-(OH)asa placentaria.
3. Los cambios en las concentraciones circulantes de 1,25-$(OH)_2D_3$ en la PE son secundarios a la disminución en la síntesis y/o actividad de la 1α-(OH)asa placentaria.

VII. MATERIAL Y MÉTODOS

Este protocolo fue aprobado por el Comité de Ética del Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán".

A) Reactivos. El medio de Dulbecco alto en glucosa (DMEM-HG), el medio modificado de Dulbecco con $CaCl_2$ 1 mM y 1 mM NaH_2PO_4 (DMEM-F12), las sales balanceadas de Hank (HBSS), el suero fetal de ternera (SFT), la penicilina, la estreptomicina, la fungizona, el estuche comercial *"SuperScript II®"* para la reacción de transcriptasa reversa, el estuche comercial para marcaje *Rad Prime,* todos se obtuvieron de Gibco, Life Technologies (Grand Island, NY, EUA). El Percoll, monofosfato 3'5'-cíclico de 8-bromoadenosina (8-bromo-AMPc), desoxirribonucleasa I (DNasa I), albúmina de suero bovino (BSA) y la cicloheximida se compraron de Sigma Chemical Co. (St Louis, MO, EUA). Todos los solventes (grado HPLC) son de Mallinckrodt Baker, (Kentucky,EUA). El estándar de 1,25-$(OH)_2D_3$ fue donado por los Doctores E.M. Gutknecht y P. Weber (F. Hoffmann-La Roche LTD, Basel, Suiza). La 25-hidroxi-[26,27-metil-^{3}H] colecalciferol ([^{3}H]25-$(OH)D_3$) con actividad específica de 30 Ci/mmol se compró a Amersham (Amersham, Inglaterra). La hCG utilizada en los radioinmunoensayos (RIA) fue generosamente donada por el *"National Institute of Diabetes and Digestive and Kidney Diseases"* (NIDDK) (Rockville, MD, EUA). La Taq polimerasa y el estuche comercial *"ABI PRISM Dye Terminator Cycle Sequencing Kit®"* para la secuenciación del DNAc se compró a Perkin Elmer (EUA). El estuche comercial CONCERT® *High purity plasmid purification* y el vector de expresión pcDNA 3.1/V5-His Topo TA® son de Invitrogen (EUA). El reactivo de transfección Fugene® es de Roche (Alemania).

B) Materiales diversos. Se utilizaron cajas de cultivo de 6 pozos de 35 mm cada uno, Nunclon (Dinamarca). Las membranas de nylon fueron marca Zeta Probe® de Bio Rad (Hercules CA, EUA). Las columnas para purificación de DNA (Centricon® y Sentri-sep®) son de Amicon, Beverly, MA (EUA).

C) Material biológico.

Las líneas celulares de riñón humano (HEK 293) y de coriocarcinoma humano (JEG-3) se obtuvieron de Microbix Biosystems, (Ontario, Canada).

Las placentas humanas se colectaron de acuerdo a los siguientes criterios:

Se consideraron como criterios de inclusión para la recolección de las placentas aquellas provenientes de mujeres embarazadas con edad biológica entre 18 a 35 años y gestacional mayor de 35 semanas, que presentaron presión arterial menor de 140/90 mm Hg. Este grupo se denominó control (NT). Se excluyeron las placentas de las mujeres que padecieran cualquier enfermedad.

Para estudiar las placentas de mujeres con preeclampsia, se consideraron los siguientes criterios: placentas de mujeres con edad biológica entre 18 a 35 años y gestacional mayor de 35 semanas; que presentaron presión arterial sistólica/diastólica ≥ 140 y/o ≥ 90 mm Hg y proteinuria superior o igual a 100 mg/dL. Este grupo se denominó PE. Se excluyeron las placentas de mujeres que presentaron cualquier enfermedad diferente de la PE.

1. Cultivo celular

Las placentas a término (38-42 semanas de gestación) se obtuvieron de mujeres que cursaron embarazos normales y/o que cumplieron con los criterios que definen a la preeclampsia previamente descritos. Las placentas fueron transportadas al laboratorio para procesarlas. El aislamiento de citotrofoblastos se llevó a cabo según Kliman *et al.*[67] Brevemente: Se pesaron 30 g de cotiledones placentarios sin tejido fibroso ni vasos. Se lavó el exceso de sangre con NaCl 0.9% a temperatura ambiente y se llevaron a cabo 3 digestiones enzimáticas sucesivas con tripsina y DNasa I en HBSS libre de calcio y magnesio 30 minutos por cada incubación, a 37°C (ver tabla en el Anexo 1). La suspensión celular resultante se centrifugó a 1000 X *g* 10 minutos, se aspiró el sobrenadante y la pastilla celular se resuspendió en DMEM-HG. Las células se separaron en un gradiente 5-70% de Percoll (Vol/Vol) con HBSS (Anexo 2). El gradiente se centrifugó a 1200 X *g* a temperatura ambiente por 20 minutos sin freno, después de lo cual la banda correspondiente a los citotrofoblastos (densidad 1.048-1.062 g/mL) se separó, y las células se lavaron con DMEM-HG y se diluyeron a razón de 2 x 10^6 células/mL en

medio para cultivo: (DMEM-HG acondicionado con L-glutamina 4mM, 100 unidades de penicilina/mL, estreptomicina 100 μg/mL, fungizona 0.25 μg/mL, y 20 % de SFT inactivado a 56°C durante 45 minutos). Las células se cultivaron en placas de 6 pozos y se incubaron en atmósfera húmeda con CO_2 5% - aire 95% a 37 °C. Diariamente se cambió el medio y se adicionó nuevo. A las 48 horas del cultivo se cambió el medio por DMEM-F12 sin SFT. A las 72 horas del cultivo se estudió el metabolismo de la 25-(OH)D_3 como se describe posteriormente.

Diariamente se examinaron el aspecto y la morfología de los citotrofoblastos. Como control de viabilidad celular se cuantificó mediante radioinmunoanálisis[68] (RIA) la secreción de hCG en presencia y ausencia de 8-Bromo-AMPc[69] en los cultivos de sincicíotrofoblastos normales y PE. El anticuerpo utilizado esta dirigido contra la subunidad β de la hCG y se utilizó a una dilución final de 1:150 000. La sensibilidad del análisis fue de 0.025 ng/tubo. Adicionalmente se analizó la expresión de la subunidad β de la hCG en los diferentes días del cultivo (24-96 horas) tanto en células que provenían de mujeres NT como de las que padecieron preeclampsia.

La proteína total se cuantificó por el método de Bradford[70] utilizando BSA como estándar.

2. Estudios del metabolismo de la 25-(OH)D_3

Se estudió la capacidad de los sincicíotrofoblastos y de las células del coriocarcinoma humano "JEG-3" para convertir al [^{3}H]25-(OH)D_3 en [^{3}H]1,25-(OH)$_2D_3$. Al tercer día de cultivo de los trofoblastos y en 2 mL de medio DMEMF-12 libre de SFT se adicionó [^{3}H]25-(OH)D_3 (180 nCi/pozo) a una concentración final de 3 nM, después de lo cual se incubó durante una hora y se colectaron los medios de cultivo en tubos de borosilicato. La extracción clorofórmica de los metabolitos tritiados de la vitamina D_3 se llevó a cabo según el método de Bligh y Dyer[71]. Este método consiste en lavar las células con 1 mL de metanol, el cual se recupera en los tubos de borosilicato correspondientes, se adicionan 3 mL más de metanol y 4 mL de cloroformo. Se agita vigorosamente y la fase clorofórmica se seca con flujo de nitrógeno, para resuspender el extracto lipídico en 100 μL de solvente cromatográfico (hexano-isopropanol 92:8). Posteriormente se inyecta en un

cromatógrafo de líquidos de alta presión (HPLC) con un flujo de 1.6 mL/min utilizando como marcador interno de elusión 100 ng de 1,25-$(OH)_2D_3$ auténtica. Este proceso se llevó a cabo en un HPLC Beckman equipado con una columna Ultrasphere Si de 5 μm, 4.6 x 250 mm. De las fracciones separadas por este método se tomaron las que coeluyeron con el marcador y se evaporaron para someterlas a una segunda separación cromatográfica utilizando como fase móvil diclorometano-isopropanol 95:5, con una velocidad del flujo de 1 mL/min. Finalmente se cuantificó la radiactividad de cada fracción con 4 mL de líquido de centelleo (Liquidfluor Dupont 4.4%, alcohol etílico 2.1% y tolueno 93.5%) en un contador de centelleo líquido y se determinó el porcentaje de sustrato radiactivo convertido en calcitriol tritiado. Los resultados se expresaron como femtomolas de [^{3}H]1,25-$(OH)_2D_3$/mg proteína, y los cálculos se realizaron como se indica a continuación. Las células JEG-3 se procesaron de igual forma que los trofoblastos, una vez que alcanzaron la confluencia.

La radiactividad total (**rt**) es aquella que se recupera después de la primera HPLC. R1 equivale al porcentaje de rt que eluye dentro de la región de la 1,25-$(OH)_2D_3$ auténtica en la primera HPLC. R2 es el porcentaje de R1, que eluye dentro de la región de 1,25-$(OH)_2D_3$ auténtica, después de la segunda HPLC.

Si se incubaron 6000 femtomolas de sustrato por pozo, entonces:

R1 x R2 x 0.6 = femtomolas totales producidas/pozo

3. Efectos del IGF-I sobre la síntesis de calcitriol

Para determinar el efecto del IGF-I sobre la actividad de la 1α-(OH)asa placentaria se preincubaron sinciciotrofoblastos en presencia de dicho factor de crecimiento a diferentes tiempos (0, 2, 8 y 16 horas) antes de la adición de [^{3}H]-25-$(OH)D_3$ 3 nM. Posteriormente se estimó la biotransformación del sustrato mediante la metodología descrita anteriormente.

La curva dosis-respuesta se llevó a cabo preincubando los sinciciotrofoblastos con distintas concentraciones de IGF-I (0- 6.5 nM) durante dos horas previas a la adición del [^{3}H]-25-$(OH)D_3$.

Por otro lado, el efecto del IGF-I sobre la producción de [^{3}H]-1,25-$(OH)_2D_3$ se evaluó en presencia de cicloheximida 30 μM, el cual es un inhibidor de la síntesis de

proteínas. Las incubaciones con cicloheximida se realizaron una hora previa a la adición de IGF-I.

4. Estudios del RNA

Se colectaron 13 placentas de mujeres normotensas a término y 13 de mujeres PE. Las placentas se lavaron con NaCl 0.9% y se obtuvieron muestras de cotiledones que fueron inmediatamente congelados en nitrógeno líquido. El tejido colectado se mantuvo a −70 °C hasta el momento de su uso. Posteriormente se extrajo el RNA de los tejidos por medio de la técnica descrita por Chomczynsky y Sacchi[72].

4.1 Oligonucleótidos y reacción de RT-PCR

El RNA total (3 μg) se utilizó como templado para la síntesis de DNAc con transcriptasa reversa (200 U), oligo (dT) (1 μg), y dNTP's (0.5 mM) del estuche comercial *SuperScript*®. La amplificación de cada DNAc por PCR se llevó a cabo con Taq polimerasa (2.5 U) en un volumen final de 25 μL y los iniciadores específicos (2 μM).

Con base en la secuencia de la 1α-(OH)asa previamente descrita[19] se diseñaron y sintetizaron iniciadores que generaron diferentes fragmentos del DNAc de la 1α-(OH)asa para estudios de Northern y Southern blot, así como el fragmento completo codificante de la 1α-(OH)asa. Los pares de oligonucleótidos utilizados se muestran en las siguientes tablas:

Oligonucleótidos para la 1α-(OH)asa	Orientación
OH1: 5'-GTT GCT ATT GGC GGG AGT GGA C-3'	Sentido
OH2: 5'-GTG ACA CAG AGT GAC CAG CAT AT-3'	Antisentido
OH3: 5'-TTG GGG ATA ATA TAG TCA CCC AC-3'	Sentido
OH4: 5'-CCA CTC AGA GAT CAC AGC TGC-3'	Antisentido
OH5: 5'-ACG CTG TTG ACC ATG GC-3'	Sentido
VDF5: 5´-C CTG AAC CAG ACC ATG ACC C-3´	Sentido
VDR3: 5´-C TTA TCC CTA TGA TGA ATG AAA GG-3´	Antisentido
RACED: 5'-GTT AGA CCC AAG ACC CGG ACT GTC-3'	Sentido
PRO 3: 5'-CGT GCT GAG TGG TAC TCT CGG TAG C-3'	Antisentido

Pares de oligonucleótidos utilizados	Tamaño del fragmento obtenido
OH1/OH2	298 pb
OH3/OH4	183 pb (sonda interna)
OH5/OH2	543 pb
VDF5/VDR3	1592 pb (región codificadora completa)

Las amplificaciones se llevaron a cabo en un termociclador Perkin Elmer Cetus 9600 (Norwall, CT) bajo las condiciones siguientes: un ciclo a 94ºC por 1 minuto, seguido de 30 ciclos de 94 ºC 50 s; 60 ºC 50 s; y 72 ºC 1 min. Finalmente, se dio un periodo de extensión de 7 min a 72 ºC.

La sonda para la 1α-(OH)asa se generó con los iniciadores OH1/OH2 que se obtuvo a partir del RNA de células de riñón humano HEK-293 por RT-PCR como se describió previamente. La sonda interna para Southern blot de la 1α-(OH)asa se obtuvo con los iniciadores OH3/OH4.

Para la amplificación de la secuencia codificante completa de la 1α-(OH)asa se utilizaron 30 ciclos de 94 °C por 45 s, 57 °C 45 s y 72 °C 45 s, seguidos de un último ciclo a 72 °C por 3 minutos.

Por otro lado se sintetizaron distintos iniciadores que generaron dos diferentes fragmentos del DNAc de IGF-I:

Oligonucleótidos para el IGF-I	Orientación
IF1: 5'-TCA CAT CGG CCT CAT AAT ACC-3'	Sentido
IF2: 5'-AAA TAA AAG CCC CTG TCT CCA-3'	Antisentido
IF3: 5'-AGC TCT GCC ACG GCT GGA CCG GAG-3'	Sentido
IF4: 5'-CAC GAA CTG AAG AGC ATC CAC CAG-3'	Antisentido

Pares de oligonucleótidos utilizados	Tamaño del fragmento obtenido
IF1/IF2	229 pb
IF3/IF4	66 pb (sonda interna)

La sonda para IGF-I se obtuvo a partir de la amplificación de DNAc de placenta humana con los iniciadores IF1/IF2. La sonda interna de IGF-I se sintetizó con los iniciadores IF3/IF4. Se utilizó el siguiente programa para las amplificaciones de los fragmentos de IGF-I: 1 ciclo (94°C durante 1 min, 56.5° durante 45 s, 72°C durante 30 s); 28 ciclos (94°C durante 45 s, 56.5°C durante 45 s, 72°C durante 30 s) y finalmente 1 ciclo (94°C durante 45 s, 56.5°C durante 45 s, 72°C durante 3 minutos).

También se sintetizaron distintos iniciadores que generaron cuatro diferentes fragmentos del DNAc del gen constitutivo de la ciclofilina:

Oligonucleótidos para la ciclofilina	**Orientación**
CF1: 5'-CCC CAC CGT GTT CTT CGA CAT-3'	Sentido
CF2: 5'-AGG TCC TTA CCG TTC TGG TCG-3'	Antisentido
CF3: 5'-CAC ACG CCA TAA TGG CAC TGG TGG-3'	Sentido
CF4: 5'-AAA GAC CAC ATG CTT GCC ATC CAG C-3'	Antisentido

Pares de oligonucleótidos utilizados	**Tamaño del fragmento obtenido**
CF1/CF2	453 pb
CF3/CF4	187 pb (sonda interna)

Se sintetizaron los siguientes oligonucleótidos que generaron un fragmento del DNAc de la subunidad β de la hCG:

Oligonucleótidos para hCG	**Orientación**
hCG1: 5'-CGC ACC AAG GAT GGA GA-3'	Sentido
hCG2: 5'-AGG AGG GTG TTA TTT CCG-3'	Antisentido

Pares de oligonucleótidos utilizados	**Tamaño del fragmento obtenido**
hCG1/hCG2	494 pb

Los programas de tiempos y temperaturas para las amplificaciones de ciclofilina y hCG fueron los mismos que los utilizados para la 1α-(OH)asa.

Las sondas moleculares se separaron en geles de agarosa 1.2%, se eluyeron, reamplificaron y finalmente se purificaron en columnas de Centricon®-30. La identidad de cada sonda se confirmó mediante secuenciación como se describe posteriormente. El marcaje radiactivo de las sondas se llevó a cabo utilizando [^{32}P]-dCTP y un estuche comercial basado en la técnica de hexámeros al azar.

4.2 Obtención del DNAc de la 1α-(OH)asa

Con la finalidad de conocer la secuencia del DNA complementario de la 1α-(OH)asa del sinciciotrofoblasto, se secuenciaron los productos de RT-PCR obtenidos con los iniciadores VDF5 y VDR3. De manera similar las sondas obtenidas fueron secuenciadas antes de ser utilizadas. Con esta finalidad se reamplificó cada fragmento para la incorporación de los nucleótidos cromogénicos utilizando el estuche comercial ABI PRISM® siguiendo las indicaciones del fabricante y el siguiente programa de amplificación: 25 ciclos de 96°C x 10 s; 50°C x 5 s; y 60°C x 4 minutos. Posteriormente se separaron los nucleótidos no incorporados con columnas Sentrisep® y las muestras se liofilizaron para resuspenderlas en solución de carga. Posteriormente los productos de la reacción fueron desnaturalizados a 95° C x 5 minutos y finalmente separados en geles de poliacrilamida 4.75%. La secuenciación de bases se llevó a cabo en un secuenciador automático modelo 373-01 (Applied Biosystems-Perkin Elmer, EUA).

4.3 Southern blot

Para obtener los fragmentos de RT-PCR de la 1α-(OH)asa utilizados en estos estudios se usaron los iniciadores OH1/OH2 u OH5/OH2; para los de IGF-I los oligonucleótidos IF1/IF2, para hCG: hCG1/hCG2, y para la ciclofilina: CF1/CF2. Estos productos de RT-PCR se separaron en geles de agarosa 1.2% y se transfirieron a membranas de nylon por capilaridad, en presencia de SSC 10 X (citrato de sodio 0.3 M, NaCl 3 M). Los ácidos nucleicos se fijaron covalentemente a la membrana mediante la exposición por 30 segundos a la luz ultravioleta. La membrana se prehibridó en Na_2HPO_4 0.25 M y SDS 7% a 65°C por 1 hora y posteriormente se adicionaron las sondas internas específicas para la 1α-(OH)asa, IGF-I, hCG o ciclofilina según el caso, marcadas con [^{32}P]-dCTP. La hibridación continuó 18 horas al cabo de las cuales se lavaron las membranas como se describe

a continuación: 20 minutos en 50 mL de SSC 2X, SDS 0.1% a temperatura ambiente, y 1 lavado de 10 min a 65°C en 50 mL SSC 0.1 X y SDS 0.1%. Posteriormente se expusieron las membranas a placas de rayos X de 3-10 minutos y se realizó el análisis densitométrico. Para controlar la eficiencia de la reacción de RT, se amplificaron todos los DNAc paralelamente, pero utilizando los iniciadores de la ciclofilina. Se realizaron incubaciones en ausencia de reacción de RT como control de la RT-PCR.

4.4 Northern blot

El RNA total se separó en geles de agarosa al 1.2% y formaldehído 6%; se transfirió por capilaridad a membranas de nylon con SSC 10X, se fijó con luz ultravioleta y se prehibridó con Na_2HPO_4 0.25 M y SDS 7% a 65°C. Este proceso duró una hora, después de lo cual se llevó a cabo la hibridación 18 h más a 65°C o a 60°C con una sonda específica para la 1α-(OH)asa o para el IGF-I respectivamente; previamente desnaturalizada a 95°C por 10 min. Los lavados de la membrana se llevaron a cabo de manera similar a como se hizo en el apartado previo. La exposición de las membranas a una placa de rayos X se realizó durante 18-24 horas, después de lo cual se realizaron las densitometrías.

Tras exponer las membranas a las placas de rayos X se analizaron las bandas de hibridación mediante un analizador de imágenes (Eagle-Eye system; Stratagene, EUA).

4.5 Clonación y expresión de la 1α-(OH)asa

La región completa codificante para la 1α-(OH)asa se obtuvo a partir de RNA de sinciciotrofoblastos como se describió en la sección 4.1. Este DNAc se insertó en el vector de expresión pcDNA 3.1/V5-His Topo TA® mediante una topoisomerasa. El mapa del vector utilizado se muestra en el Anexo 3. Una vez construido el vector recombinante con el inserto se procedió a transformar células *E. coli* One Shot TOP10 químicamente competentes. Las bacterias transformadas se seleccionaron por su capacidad de crecer en medio LB sólido con ampicilina, como se describe a continuación: se sembraron las *E. coli* transformadas en cajas Petri con medio LB (Triptona 1 %, extracto de levadura 0.5%, NaCl 1%, pH 7.0) con agar (15 g/L) y ampicilina (amp) 100 μg/mL. Las colonias portadoras de plásmidos se aislaron y crecieron en medio LB líquido + amp por 24 h a 37°C. La purificación del plásmido

se llevó a cabo utilizando el estuche comercial CONCERT® *High purity plasmid purification* siguiendo las indicaciones del fabricante. El DNA plasmídico se utilizó para determinar la presencia y orientación del DNAc de la 1α-(OH)asa mediante PCR utilizando oligonucleótidos que hibridan con secuencias del vector y del inserto. Los iniciadores que reconocen secuencias del vector se describen a continuación:

Oligonucleótidos para el vector de expresión pcDNA 3.1/V5-His Topo TA®	**Orientación**
T7: 5'-TAATACGACTCACTATAG-3'	Sentido
BGH: 5'-TAGAAGGCACAGTCGAG-3'	Antisentido

Pares de oligonucleótidos utilizados	**Tamaño del fragmento obtenido**
T7/PRO3	210 pb
OH5/BGH	1060 pb
RACED/BGH	297 pb

Además, el DNAc se secuenció en un equipo automático, como se describió en el inciso 4.2. La transfección de las células HEK-293 con el vector plasmídico se llevó a cabo utilizando el sistema de los lípidos catiónicos Fugene® y medio DMEM F-12 libre de SFT. Se transfectaron 1.5, 3.0 y 4.5 μg de plásmido. Las células permanecieron 48 horas en presencia del reactivo de transfección y del DNA recombinante. Posteriormente, y con la finalidad de establecer si las células transfectadas expresaban al DNAc de la 1α-(OH)asa, se procedió a incubarlas en presencia de [^{3}H]-25-(OH)D_3 (0.45 μCi) y 25-(OH)D_3 a una concentración final de 1 μM durante 1 a 3 horas. Los productos de biotransformación del sustrato fueron identificados por doble HPLC como se describió en la sección 2.

5. Análisis estadístico

Los resultados se expresaron como la media ± la desviación estándar (SD) o el error estándar (SE). Las diferencias estadísticas se establecieron mediante la prueba de *t* de Student. Un valor de $P \leq 0.05$ se consideró como estadísticamente significativo.

VIII. RESULTADOS

1. Aspectos morfológicos y funcionales del cultivo de trofoblastos

Después de la purificación con gradientes de Percoll se obtuvo una población uniforme de células mononucleares que fue sembrada en medio de cultivo DMEM-HG + SFT 20% a 37°C. Diariamente se examinaron al microscopio las cajas de cultivo. Al momento de sembrar las células se observó una población básicamente mononuclear y redonda (Fig. 3a); sin embargo, a las 24 h los citotrofoblastos se adhirieron al fondo de la caja de cultivo y su citoplasma comenzó a desarrollarse con proyecciones hacia la periferia (Fig. 3b). A las 48 horas las células se agregaron de manera consistente como resultado de la migración de los citotrofoblastos mononucleares, apreciándose sincicios multinucleares producto de las fusiones de las membranas (Fig. 3c). El fenotipo celular predominante en este estadio fue el sinciciotrofoblasto. A las 72 horas se observaron únicamente sincicios bien diferenciados (Fig. 3d) conteniendo un promedio de 10 núcleos en su interior, pero también se detectaron sincicios con mayor número de ellos.

Por otro lado, la concentración de hCG en el medio de cultivo de los trofoblastos (Fig. 4) aumentó progresivamente conforme las células adoptaron el fenotipo de sincicios. Este hecho fue acompañado de un incremento en la expresión de la subunidad β de la hCG (Fig. 5). Además, con la finalidad de determinar la capacidad de los trofoblastos de responder a estímulos, se llevaron a cabo incubaciones en presencia de 8-Br-AMPc. La adición de este análogo del AMPc incrementó de manera significativa la síntesis y expresión de la hCG (Fig. 6)

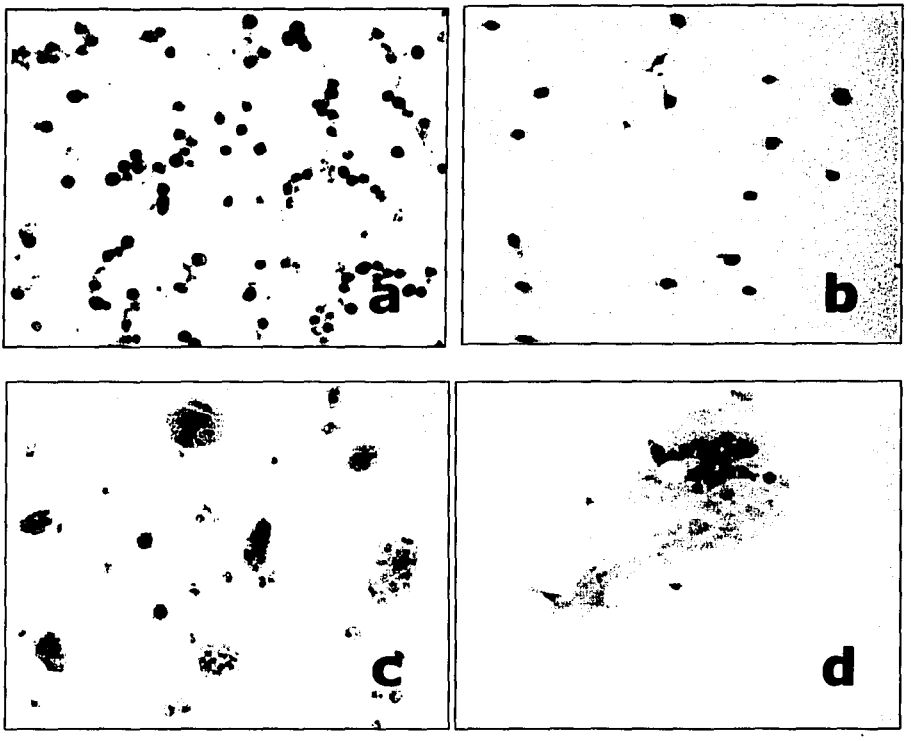


Figura 3. Diferenciación de los citotrofoblastos en cultivo. Se aprecia la morfología a los diferentes tiempos de incubación: 0 h (a), 24 h (b), 48 h (c) y 72 h (d). Tinción hematoxilina-eosina, 20 X.

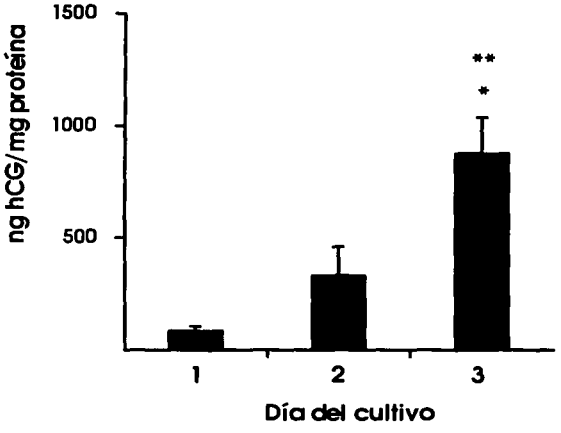


Figura 4. Concentración de la hCG en los medios de cultivo de los trofoblastos mantenidos durante tres días en incubación. Los medios de cultivo fueron cambiados diariamente para la determinación de la hCG por radioinmunoanálisis. Datos expresados como la media ± el error estándar.
* $P < 0.001$ vs día 1; ** $P = 0.02$ vs día 2. n = 8

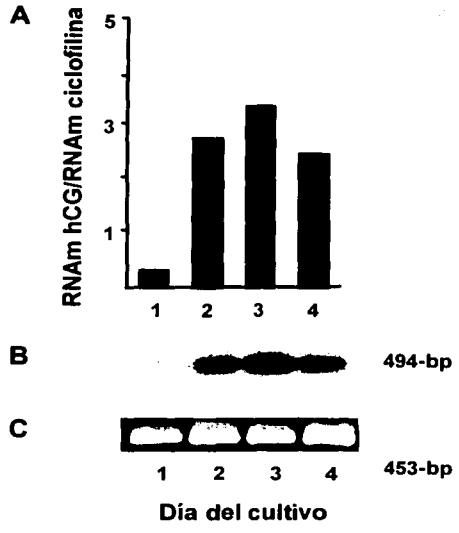


Figura 5. Expresión de la subunidad β de la hCG en diferentes etapas de diferenciación del trofoblasto. La abundancia relativa del RNAm de la subunidad β de la hCG se estimó por RT-PCR tal y como se describe en Material y Métodos utilizando los oligonucleótidos hCG1 y hCG2. Adicionalmente se utilizaron iniciadores de la ciclofilina con la finalidad de normalizar los resultados. (A): Gráfica de la relación entre la densidad óptica de hCG vs la ciclofilina. (B): Southern blot de productos de RT-PCR para hCG en diferentes días del cultivo. (C): Productos de RT-PCR amplificados con iniciadores para la ciclofilina y teñidos con bromuro de etidio. La figura corresponde a los resultados de un cultivo representativo.

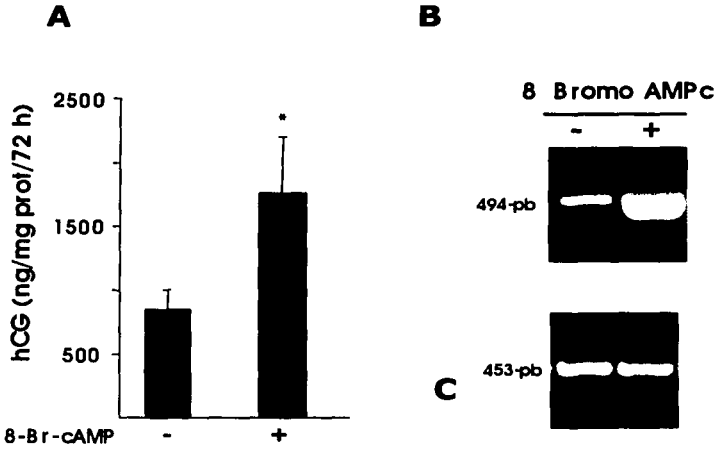


Figura 6. Síntesis y secreción de hCG por los sinciciotrofoblastos incubados en presencia (+) o ausencia (-) de 8-bromo-AMPc 1.5 mM. (A): Área bajo la curva de la β-hCG secretada en el intervalo de 0-72 h. Los datos se expresan como la media ± el error estándar de 8 diferentes placentas. $*P < 0.05$. (B) Expresión de la subunidad β de la hCG por sinciciotrofoblastos incubados en presencia (+) o ausencia (-) de 8-bromo-AMPc durante 24 h. Se muestran fragmentos de 494-pb del DNAc de la β-hCG obtenidos por RT-PCR a partir del RNA de los sinciciotrofoblastos, separados en un gel de agarosa y teñidos con bromuro de etidio. (C) Productos de RT-PCR de la ciclofilina teñidos con bromuro de etidio, obtenidos a partir del mismo DNAc que en el inciso B.

2. Actividad de la 1α-(OH)asa en sinciciotrofoblastos

Se estudió la capacidad de los trofoblastos en cultivo de sintetizar calcitriol. Los estudios de la actividad de la 1α-(OH)asa demostraron que los sinciciotrofoblastos son capaces de convertir [^{3}H]25-(OH)D_3 en [^{3}H]-1,25-(OH)$_2D_3$. Lo anterior se demostró mediante la incubación de las células de 72 h en presencia de [^{3}H]-1,25-(OH)$_2D_3$ como se describió en Material y Métodos. Después de la extracción clorofórmica se separaron los metabolitos de la vitamina D por doble HPLC. En la primera HPLC se recuperaron las fracciones coincidentes con la elusión del estándar auténtico de calcitriol, el cual presentó un tiempo de retención de 16 minutos en fase móvil hexano:isopropanol (92:8). En la segunda HPLC se detectaron varios metabolitos pero únicamente se consideró para su análisis el que presentó un tiempo de retención de 13 minutos coincidente con el estándar sintético en fase móvil de diclorometano:isopropanol (95:5). El patrón de elusión típico se muestra en la Figura 7. La cantidad de calcitriol sintetizado por los sinciciotrofoblastos incubados en presencia de [^{3}H]-25-(OH)D_3 (3 nM) durante 1 hora fue de 215.6 ± 114.3 fmol de [^{3}H]-1,25-(OH)$_2D_3$/mg de proteína. Estos resultados corresponden al análisis de 10 diferentes placentas.

2.1 Efectos del IGF-I sobre la síntesis placentaria de calcitriol

La preincubación con IGF-I a diferentes tiempos resultó en la bioconversión significativa del sustrato tritiado a [^{3}H]-calcitriol; a diferencia de las células incubadas en ausencia de IGF-I. La máxima bioconversión a calcitriol se obtuvo en cultivos preincubados por dos horas en presencia de IGF-I (2.6 nM), como lo muestra la Figura 8.

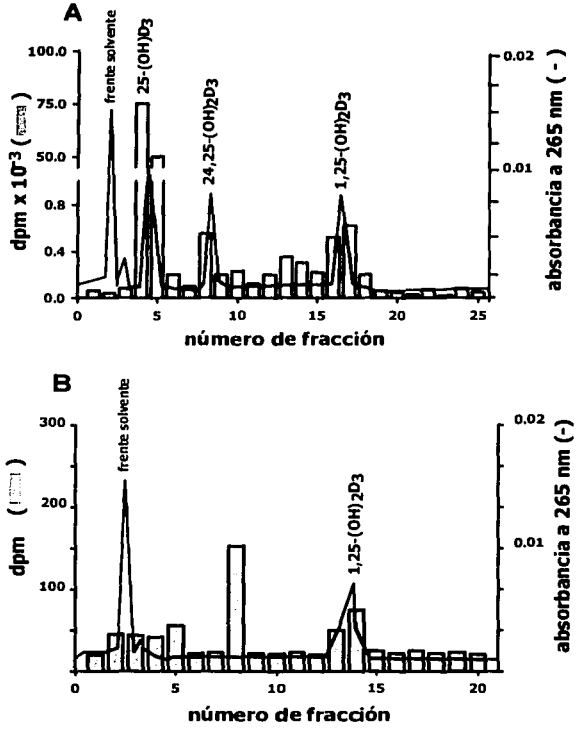


Figura 7. Tiempos de retención de los productos de biotransformación de [^{3}H]25-(OH)D_3 por los sinciciotrofoblastos cultivados. En la primera HPLC (A) uno de los metabolitos separados coeluyó con el estándar auténtico de calcitriol con un tiempo de retención de 16 minutos en fase móvil hexano:isopropanol (92:8). En la segunda HPLC (B) se detectaron varios metabolitos pero únicamente el que presentó un tiempo de retención de 13 minutos coeluyó con el estándar auténtico con una fase móvil diclorometano:isopropanol (95:5). La figura muestra un cromatograma representativo de la primera y segunda HPLC.

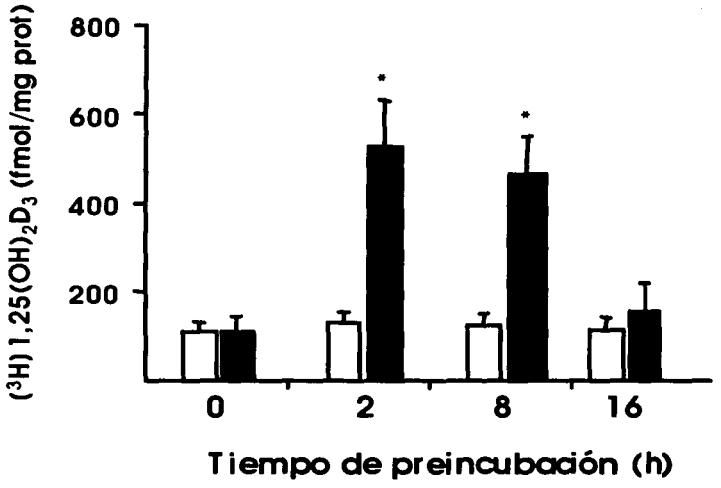


Figura 8. Producción de [^{3}H]-1,25-$(OH)_2D_3$ por sinciciotrofoblastos en cultivo. Las células fueron preincubadas por diferentes tiempos en presencia de IGF-I ■ (2.6 nM) o vehículo □ Posteriormente se adicionó [^{3}H]25-$(OH)D_3$ y se incubó durante una hora adicional. Los datos se graficaron como la media ± la desviación estándar. * $P < 0.01$ vs vehículo. (n = 3).

Por otro lado la curva dosis-respuesta mostró que la concentración de IGF-I con mayor efecto sobre la síntesis de 1,25-$(OH)_2D_3$ fue de 2.6 nM, (Fig. 9).

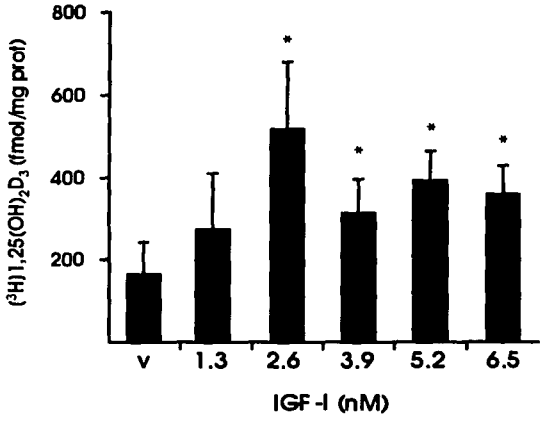


Figura 9: Curva dosis respuesta del efecto del IGF-I sobre la síntesis de $[^3H]1,25\text{-}(OH)_2D_3$ en sinciciotrofoblastos en cultivo. Las células se preincubaron en presencia de diferentes concentraciones de IGF-I durante dos horas antes de la adición del sustrato tritiado. $*P < 0.01$ vs vehículo (v). Los datos se graficaron como la media ± la desviación estándar. (n = 3)

Adicionalmente, y con la finalidad de esclarecer si el efecto estimulador ejercido por el IGF sobre la síntesis de $1,25\text{-}(OH)_2D_3$ involucra la síntesis de proteínas, se llevaron a cabo incubaciones en presencia de IGF-I y cicloheximida (chx). Estos experimentos demostraron la inhibición significativa ($P < 0.001$) del estímulo provocado por el IGF-I sobre la síntesis de $1,25\text{-}(OH)_2D_3$ (Fig. 10). Incubaciones control se realizaron en presencia de 8-Br-AMPc, con o sin chx y los efectos se analizaron por la cuantificación de la hCG en los medio de cultivo (Fig. 10).

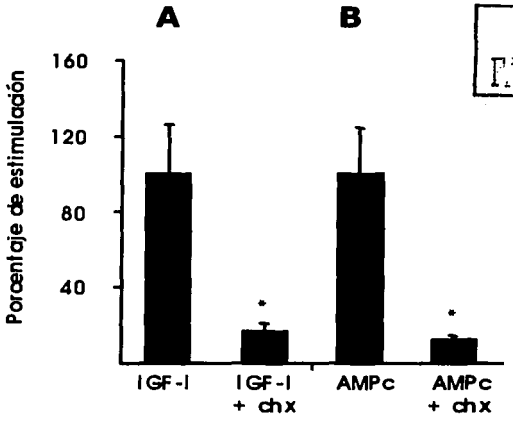


Figura 10: Efecto de la adición de chx en el estímulo del IGF-I sobre la síntesis de calcitriol (A) y del 8 bromo-AMPc sobre la síntesis de hCG (B). La chx abolió en un 80% ambos estímulos cuando se preincubó a los sinciciotrofoblastos durante una hora en presencia del inhibidor de síntesis proteínica. Los datos se graficaron como la media ± la desviación estándar $P < 0.001$. (n = 3)

3. Expresión de CYP27B1 en el trofoblasto

El día previo a la extracción del RNA el medio de cultivo de los trofoblastos se cambió por medio conteniendo calcio 1 mM. Se extrajo el RNA a las 72 horas y se analizó por Northern blot utilizando la sonda obtenida a partir de las células HEK-293 (Fig. 11 A). La hibridación con la sonda específica reveló un producto de transcripción con características similares al de riñón y la decidua (Fig. 11 B).

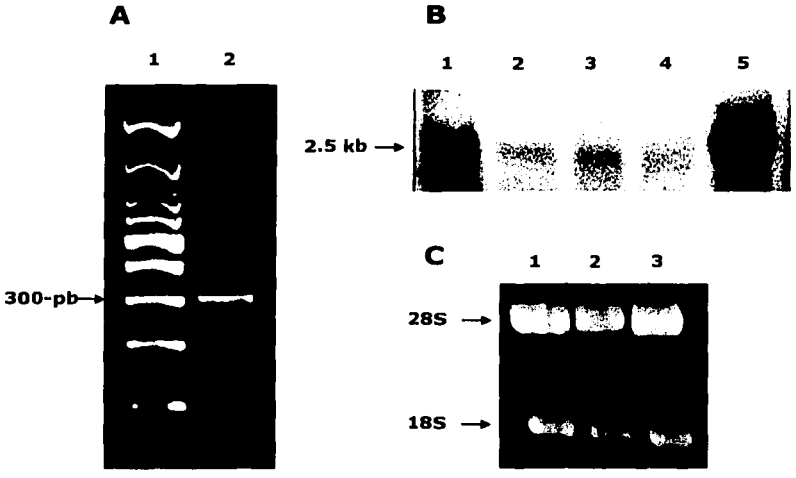


Figura 11. (A) Análisis del producto de RT-PCR utilizando RNA de HEK-293 como templado y teñido con bromuro de etidio. Después de su secuenciación el producto de RT-PCR se purificó y se marcó con [^{32}P]-dCTP para ser utilizado como sonda para los Northern blots. (B) Northern blot de RNA de HEK-293 (1), sinciciotrofoblastos (2-4) y decidua (5). (C) RNAs ribosomales teñidos con bromuro de etidio.

3.1 Clonación del DNAc de la 1α-(OH)asa de placenta

Con la finalidad de determinar la identidad del producto de transcripción que hibridó con la sonda específica para la 1α-(OH)asa, se reamplificaron los RNAs mensajeros del sinciciotrofoblasto por RT. El DNAc de la 1α-(OH)asa comprende 2469 pb.[20] En este trabajo se amplificó un fragmento que se localiza entre las bases 110–1702 de la secuencia informada por Monkawa y cols.[20] y entre las bases 1–1592 de la secuencia informada por Fu y cols.[19], utilizando los iniciadores VDF5/VDR3. Estos oligonucleótidos fueron diseñados para abarcar desde la señal de inicio (ATG) hasta la señal de terminación (TAA) de la traducción (1596 pb en total). Como puede observarse en la Figura 12 el producto obtenido por RT-PCR mostró la talla adecuada y resultó ser idéntico en la secuencia de bases flanqueadas por los iniciadores al DNAc descrito para el riñón humano (Tabla 1).

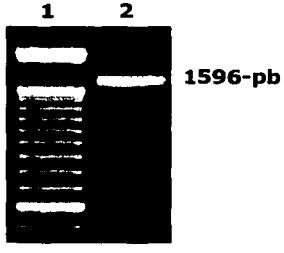


Figura 12. Análisis del producto obtenido por RT-PCR a partir de sinciciotrofoblastos, teñido con bromuro de etidio (carril 2). La amplificación se llevó a cabo según se describe en Material y Métodos. Carril 1: marcador molecular en escalera de 100 pares de bases.

1 ctgaaccaga ccatgacccа gaccctcaag tacgcctcca gagtgttcca tcgcgtccgc
61 tgggcgcccg agttgggcgc ctccctaggc taccgagagt accactcagc acgccggagc
121 ttggcagaca tcccaggccc ctctacgccc agctttctgg ccgaactttt ctgcaagggg
181 ggactgtcga ggctacacga gctgcaggtg cagggcgccg cgcacttcag gccggtgtgg
241 ctagccagct ttgggacagt gcgcaccgtg tacgtggctg cccctgcact cgtcgaggag
301 ctgctgcgac aggagggacc ccggcccgag cgctgcagct tctcgccctg gacggagcac
361 cgccgctgcc gccagcgggc ttgcggactg ctcactgcgg aaggcgaaga atggcaaagg
421 ctccgacagtc tcctggcccc gctcctcctc cggcctcaag cggccgcccg ctacgccgga
481 accctgaaca acgtagtctg cgaccttatg cggcgtctga ggcgccagcg gggacgtggc
541 acggggccgc ccgccctggt tcgggacgtg gcgggggaat tttacaagtt cggactggaa
601 ggcatcgccg cggttctgct cggctcgcgc ttgggctgcc tggaggctca agtgccaccc
661 gacacggaga ccttcatccg cgctgtgggc tcggtgtttg tgtccacgct gttgaccatg
721 gcgatgcccc actggctgcg ccaccttgtg cctgggccct gggaccgcct ctgccgagac
781 tgggaccaga tgtttgcatt tgctcagagg cacgtggagc ggcgagaggc agaggcagcc
841 atgaggaacg gagggacagcc cgagaaggac ctggagtctg gggcgcacct gacccacttc
901 ctgttccggg aagagttgcc tgcccagtcc atcctgggaa atgtgacaga gttgctattg
961 gcgggagtgg acacggtgtc caacacgctc tcttgggctc tgtatgagct ctcccggcac
1021 cccgaagtcc agacagcact ccactcagag atcacagctg ccctgagccc tggctccagt
1081 gcctacccct cagccactgt tctgtcccag ctgcccctgc tgaaggcggt ggtcaaggaa
1141 gtgctaagac tgtaccctgt ggtacctgga aattctcgtg tcccagacaa agacattcat
1201 gtgggtgact atattatccc caaaaatacg ctggtcactc tgtgtcacta tgccacttca
1261 agggaccctg cccagttccc agagccaaat tcttttcgtc cagctcgctg gctgggggag
1321 ggtcccaccc cccacccatt tgcatctctt ccctttggct ttggcaagcg cagctgtatg
1381 gggagacgcc tggcagaagct tgaattgcaa atggctttgg cccagatcct aacacatttt
1441 gaggtgcagc ctgagccagg tgcggcccca gttagaccca agacccggac tgtcctggta
1501 cctgaaagga gcatcaacct acagtttttg gacagatagt cccatggaaa gagactgtca
1561 tcatcaccct ttcattcatc atagggataa gatttttttgt aggcacaaga ccaaggtata
1621 catcttcccc taatgcctat ctgaccaaac tggatagaac caccatagtg aagtgtgagg
1681 cggcctgacc aatgtgtgaa gtatgcactt ggcctgactc aggaagccag gtgagaaaac
1741 catggtctct ctgcttgctt ggcccttctg atcatgtatg catccccaa ggatgaaatc
1801 agatttttaac taataatgct ggatgcgcct gaggaaagat tcaactgcct ctctttttgg
1861 gctttcatca gtgttcattg atgctgctgg ctaagcattt atcaaagcat aagctcagta
1921 actgtgcatc tggtctgtac ctggttggtc cttcgtcttt gcatgtaagc tctttgagag
1981 gaagggtgaa gccttatttg ttttttatgt cccctgccag ggcctgtctc tgactaggtg
2041 tcaccataca cattcttaga ttgaatctga accatgtggc agaagggata agcagcttac
2101 tagtaggctc tgtctacccc cttccttctt tgtcttgccc ctaggaaggt gaatctgccc
2161 tagcctggtt tacggtttct tataactctc ctttgctctc tggccactat taagtgggtt
2221 tgccccatca cttagttctc aggcagagac atctttgggc ctgtccctgc ccaggcctct
2281 ggctttttat attgaaaatt tttaaatatt cacaaatttt agaataaatc aaatattcca
2341 ttcttaaaaa aaaaaaaaa aaaaa

Tabla 1. Secuencia completa del RNAm de la 1α-(OH)asa humana[19] y localización del fragmento de 1596 pb amplificado, clonado y expresado en este trabajo (bases subrayadas).

Una vez que se obtuvo el DNAc de la 1α-(OH)asa placentaria se procedió a estudiar si se expresaba en una enzima catalíticamente activa. Con esta finalidad se insertó el DNAc de la 1α-(OH)asa (1596 pb) en el vector de expresión pcDNA 3.1/V5-His Topo TA® y se transformaron bacterias *E. coli*. Se seleccionaron las bacterias portadoras del plásmido creciéndolas en medio LB sólido + amp. Después de amplificar las colonias seleccionadas se extrajo el DNA plasmídico y se determinó la presencia y orientación del inserto mediante PCR utilizando oligonucleótidos que hibridaron con secuencias del vector y del inserto. Las preparaciones en donde se obtuvo el fragmento esperado se utilizaron para transfectar células HEK-293 y los productos de expresión se identificaron por Northern blot. La Figura 13 muestra los productos de transcripción reconocidos por la sonda de 1α-(OH)asa-[^{32}P]-dCTP, obtenidos de células transfectadas. La expresión endógena de CYP27B1 no fue posible detectarla en células no transfectadas debido muy probablemente al corto tiempo de exposición.

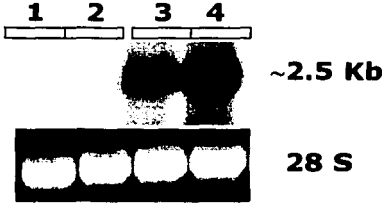


Figura 13. Northern blot de células HEK-293. (1-2): Control de células no transfectadas. (3-4): RNA de células transfectadas con plásmido + DNAc para la 1α-(OH)asa.

La transfección de células HEK-293 con el vector de expresión conteniendo el DNAc de la 1α-(OH)asa resultó en la síntesis constitutiva de la enzima activa y con capacidad de transportarse a las mitocondrias, según se deduce de los resultados que a continuación se presentan en la Figura 14.

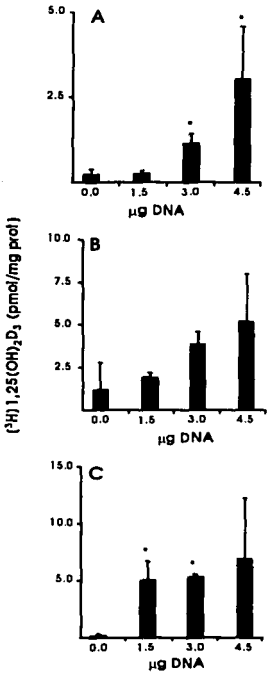


Figura 14. Actividad de la 1α-(OH)asa recombinante en células HEK-293 transfectadas con DNAc de la 1α-(OH)asa. La transfección de las células HEK-293 con el vector plasmídico se llevó a cabo como se describió en Material y Métodos. Las células se transfectaron con 1.5, 3.0 y 4.5 µg de plásmido. Las células permanecieron durante 48 horas con el reactivo de transfección y el DNA recombinante, después de lo cual se adicionaron 0.45 µCi de [^{3}H]-25-(OH)D_3 (30 Ci/mmol) y 25-(OH)D_3 a una concentración final de 1 µM. Las incubaciones se llevaron a cabo durante 1h **(A)**, 2h **(B)** y 3h **(C)** en presencia del sustrato, después de lo cual se practicó la extracción clorofórmica y la separación de los metabolitos tritiados de la vitamina D_3. Los datos se graficaron como la media ± la desviación estándar. (n = 3)
A: $^*P < 0.05$ vs control.
C: $^*P < 0.01$ vs control

Como puede observarse en la Figura 14, la transfección de las células HEK-293 con diferentes cantidades de DNA se acompañó del aumento en la bioconversión del sustrato a calcitriol. Este aumento fue dependiente de la dosis del DNA transfectado, con incrementos que variaron entre 4 y 13 veces en comparación con las células no transfectadas.

3.2 Patrón temporal de expresión de CYP27B1

Con la finalidad de conocer el patrón de expresión de CYP27B1 en los diferentes estados de diferenciación del trofoblasto, se extrajo el RNA a las 0, 24, 48, 72 y 96 horas del cultivo. Con este RNA se realizó un Northern Blot (Fig. 15) en el que se observa la presencia de señales de transcripción de la 1α-(OH)asa en todas las muestras analizadas.

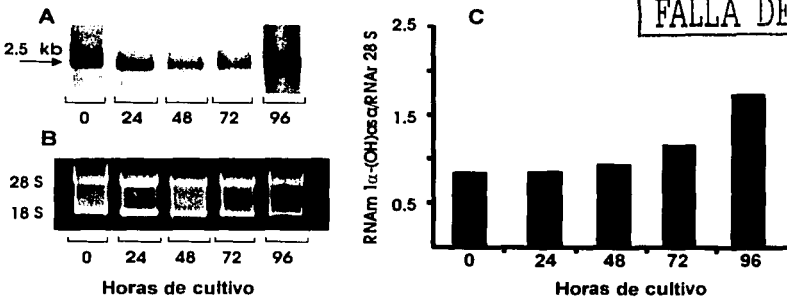


Figura 15. Expresión temporal del RNAm de la 1α-(OH)asa durante la diferenciación del trofoblasto en cultivo. (A) Northern blot. (B) Gel teñido con bromuro de etidio. (C) Abundancia relativa del RNAm de la 1α (OH)asa vs RNA 28 S. Se muestra un solo experimento de expresión temporal.

3.3 Efecto del IGF-I sobre la expresión de CYP27B1

Con la finalidad de estudiar los efectos del IGF-I sobre la transcripción de CYP27B1, los sinciciotrofoblastos fueron estimulados en presencia de IGF-I (2.6 nM) durante 24 horas en medio libre de SFT. Al terminar este periodo el RNA fue extraído y analizado. Los estudios de Northern blot demostraron que la transcripción del gen que codifica para la 1α-(OH)asa es estimulada por el IGF-I (Fig. 16). Estos datos sugieren que el IGF-I estimula la síntesis de calcitriol a nivel transcripcional.

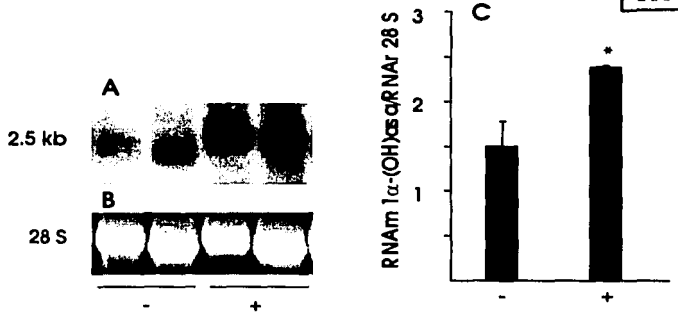


Figura 16. (A) Análisis por Northern blot de sinciciotrofoblastos incubados en presencia (+) o ausencia (-) de IGF-I (2.6 nM) durante 24 horas. (B) RNAs ribosomales 28S teñidos con bromuro de etidio. (C) Relación entre la densidad óptica relativa del transcrito para la 1α-(OH)asa y el RNA ribosomal 28S. La hibridación se llevó a cabo utilizando una sonda específica para la 1α-(OH)asa - [^{32}P]-dCTP. * $P < 0.05$ vs ausencia de IGF-I. Los datos se graficaron como la media ± la desviación estándar de dos experimentos que se muestran como duplicados en A y B.

4. Expresión de IGF-I en el cultivo de trofoblasto

Dado el importante efecto estimulador del IGF-I sobre la expresión de CYP27B1, decidimos estudiar el patrón de transcripción de este factor de crecimiento en el cultivo de sinciciotrofoblastos. Los resultados obtenidos se pueden apreciar en la Figura 17 A. Como puede observarse, el análisis de los productos de RT-PCR obtenidos a diferentes días del cultivo mostró una tendencia a aumentar conforme ocurre el proceso de citodiferenciación. Los resultados de este estudio fueron normalizados utilizando la expresión de un gen constitutivo, como es el caso de la ciclofilina (Fig. 17 B).

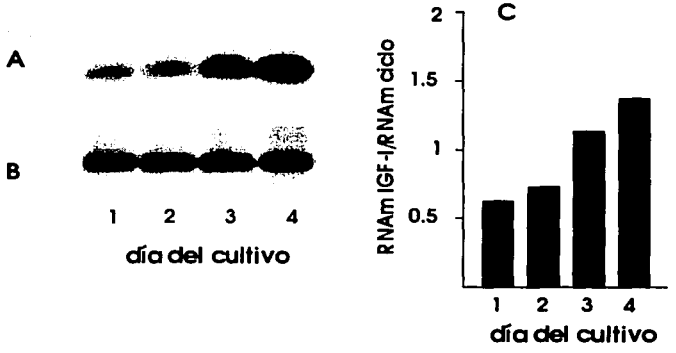


Figura 17. Patrón temporal de expresión del IGF-I en el cultivo de trofoblasto. (A): Southern blot de productos de RT-PCR de IGF-I (229-pb), obtenidos con los iniciadores IF1/IF2. (B): Southern blot de productos de RT-PCR de la ciclofilina (453-pb). Se utilizó el mismo DNAc para la PCR que en A, pero se amplificó usando los oligonucleótidos CF1/CF2. Las hibridaciones con sus respectivas sondas se realizaron como se describió en Material y Métodos. (C): Gráfica que muestra la diferencia entre la expresión del gen de IGF-I y de la ciclofilina, obtenida a partir de la relación entre la densidad óptica de las bandas de los Southern blots.

5. Estudios en células de coriocarcinoma

Los resultados obtenidos hasta este punto indicaron que el gen CYP27B1 de la placenta se expresa en una enzima capaz de bioconvertir [^{3}H]25-(OH)D_3 en [^{3}H]1,25-(OH)$_2D_3$. Debido a esto decidimos investigar si este proceso se repetía en un sistema celular relacionado con el trofoblasto. Con esta finalidad se eligió una línea celular inmortalizada, que tiene el mismo origen que los trofoblastos. La línea celular denominada JEG-3 proviene de coriocarcinoma humano, por lo que procedimos a analizar el RNA y el metabolismo de la 25-(OH)D_3 en estas células. Los resultados mostraron que las células del coriocarcinoma JEG-3 transcriben al gen CYP27B1 (Fig. 18) y tienen capacidad de convertir [^{3}H]25-(OH)D_3 en [^{3}H]1,25-(OH)$_2D_3$. La incubación de las células en presencia de [^{3}H]25-(OH)D_3 (3 nM) durante 1 h resultó en la producción de 45.1 ± 17 fmol/mg de proteína (n = 8 incubaciones).

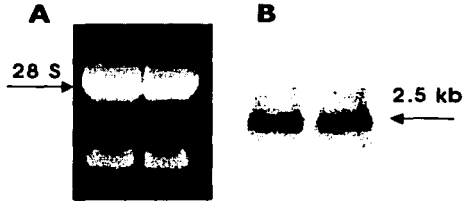


Figura 18. Expresión de CYP27B1 en la línea celular JEG-3. Se realizaron estudios de Northern blot de células JEG-3 que alcanzaron la confluencia en cajas de cultivo de 75 cm^2. (A): RNA ribosomal teñido con bromuro de etidio. (B): Hibridación con una sonda específica para la 1α-(OH)asa-[^{32}P]-dCTP. La figura muestra dos diferentes RNA's procedentes de 2 cultivos de células JEG-3.

6. Estudios en placentas de mujeres con preeclampsia

Con base en los resultados anteriores, nos preguntamos si la secreción placentaria de calcitriol contribuye al aumento en las concentraciones de esta hormona en el suero de la madre gestante. Para responder a esta pregunta se eligió la patología del embarazo asociada con bajas concentraciones de $1,25\text{-}(OH)_2D_3$ y de IGF-I en el suero materno, hipocalciuria e hipocalcemia, como modelo de estudio. Esta patología es la preeclampsia. Se colectaron 13 placentas NT y 13 PE, y las características clínicas de las madres se presentan en la tabla 2.

Variable	Grupo NT	Grupo PE	*P*
Presión arterial sistólica (mm Hg)	117 ± 10	160 ± 14	< 0.0001*
Presión arterial diastólica (mm Hg)	72 ± 6	103 ± 12	< 0.0001*
Proteinuria ♠	trazas	3 g/L	-
Edema	ausente	++	-
Edad de la madre	25.1 ± 5.8	23.2 ± 8	0.261
Edad gestacional (semanas)	39.4 ± 1.4	38.0 ± 2.0	0.09
Peso del recién nacido (kg)	3.2 ± 0.3	2.7 ± 0.4	0.01*
hCG (ng/mL suero)	925 ± 420	1337 ± 1104	0.2

Tabla 2.- Características clínicas de las madres donadoras de placentas
♠(+ = 30 mg/dL de albúmina; ++ =100 mg/ dL de albúmina, +++ = 300 mg/dL, ++++ = 1000 mg/dL).
Proteinuria se define como la presencia de albúmina en orina en concentraciones ≥ a 0.3 g/L[51]. Proteinuria de 3.0 – 5.0 g/L aunado a tensión arterial de 160/110 a 180/120 mm Hg es considerado preeclampsia grave. Proteinuria de 1.5 – 3.0 g/L aunado a una tensión arterial de 140/90 a 160/110 mm Hg es considerado como preeclampsia moderada[51]; por lo tanto, las mujeres incluidas en este estudio se clasifican como preeclámpticas moderadas a graves. * Es considerado significativo estadísticamente.

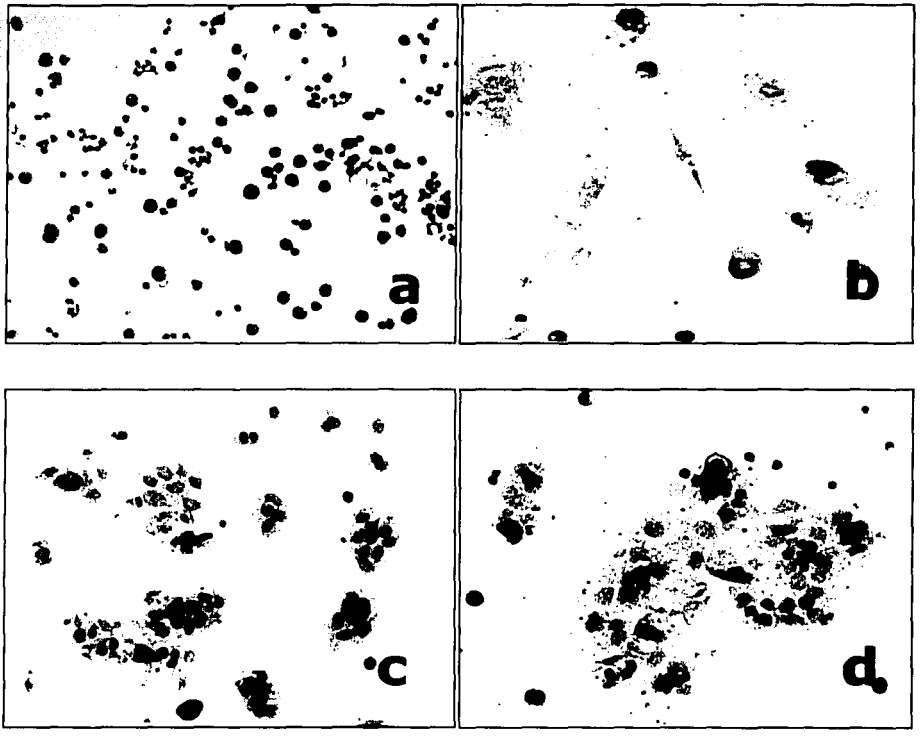


Figura 19. Diferenciación de los citotrofoblastos PE en cultivo. Se aprecia la morfología a los diferentes tiempos de incubación: 0 h (a), 24 h (b), 48 h (c) y 72 h (d). Tinción hematoxilina-eosina, 20 X.

6.1 Aspectos morfológicos y funcionales del trofoblasto PE

Se aislaron los trofoblastos de igual forma que en las placentas normales y se mantuvieron con las mismas condiciones de cultivo. El proceso de diferenciación celular se llevó a cabo en el transcurso de 72 horas (Fig. 19) y fue similar al de las células obtenidas de placentas normales.

Adicionalmente, la secreción de hCG al medio de cultivo por parte de los trofoblastos PE (Fig. 20) se incrementó conforme avanzó el proceso de citodiferenciación, de manera semejante a lo que ocurrió en los cultivos de células de placentas normales (Fig. 4).

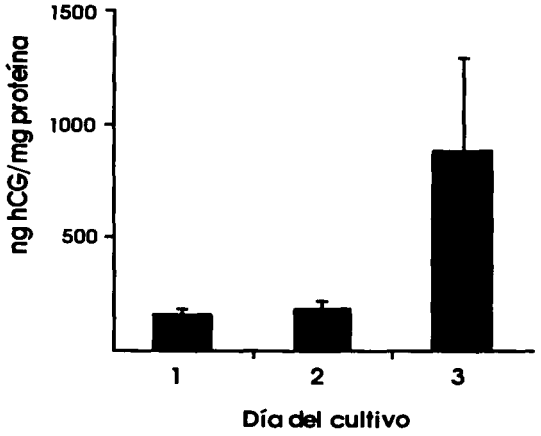


Figura. 20 Secreción de hCG por trofoblastos PE mantenidos en cultivo durante tres días. Diariamente se cambió el medio de cultivo y se determinó la concentración de hCG por radioinmunoanálisis. Datos expresados como la media ± el error estándar. (n = 8)

De acuerdo con lo anterior, la expresión de la subunidad β de la hCG también se incrementó conforme avanzó el proceso de diferenciación de los trofoblastos (Fig. 21) de manera similar a lo que ocurrió con los cultivos NT (Fig. 5).

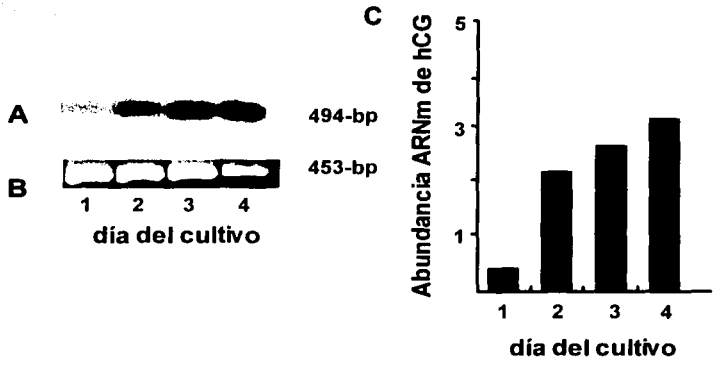


Figura 21. Expresión de la subunidad β de la hCG en diferentes etapas de diferenciación del trofoblasto PE. (A): Southern blot de productos de RT-PCR con los iniciadores hCG1/hCG2 (494-pb). (B): Productos de RT-PCR amplificados con iniciadores para la ciclofilina y teñidos con bromuro de etidio (453-pb). (C): Densidad relativa de la abundancia de productos de transcripción de la hCG con relación al gen constitutivo ciclofilina.

Con la finalidad de conocer la respuesta de los sinciciotrofoblastos PE a un estímulo conocido de la secreción de hCG[69], se incubaron células de placentas de mujeres PE en presencia de 8-bromo-AMPc. La adición de dicho análogo del AMPc incrementó significativamente la secreción de hCG al medio de cultivo (Fig. 22), sin diferir de los resultados obtenidos a partir de cultivos de células normales (Fig. 6).

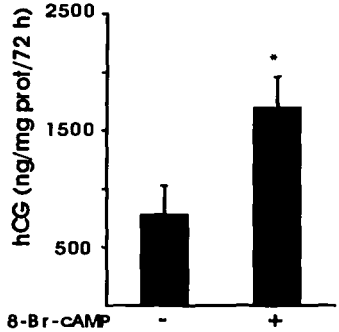


Figura 22. Secreción de hCG por sinciciotrofoblastos PE incubados en presencia (+) o ausencia (-) de 8-Bromo-AMPc (1.5 mM). La gráfica representa el área bajo la curva de la hCG secretada en el intervalo de 0-72 h. Los datos se expresan como la media ± el error estándar de 8 diferentes placentas. $P < 0.05$ vs control.

6.2 Síntesis de 1,25-$(OH)_2D_3$

La capacidad de los sinciciotrofoblastos obtenidos de placentas PE para biotransformar [^{3}H]25-$(OH)D_3$ al metabolito activo de la vitamina D fue estudiada bajo las mismas condiciones experimentales descritas para los cultivos de placentas NT. Los resultados demostraron que los trofoblastos de las placentas de mujeres con PE secretaron 1,25-$(OH)_2D_3$ cuando se les incubó durante una hora en presencia de [^{3}H]25-$(OH)D_3$ (3 nM), pero en menor cantidad que los de cultivos

control. Las células PE produjeron 19.4 ± 11.5 fmol de [^{3}H]1,25-(OH)$_2$D$_3$/mg de proteína. Estos resultados correspondieron al análisis de 8 diferentes placentas PE.

6.3 Expresión de CYP27B1 y regulación por IGF-I

Adicionalmente se estudió al gen que codifica para la 1α-(OH)asa en sinciciotrofoblastos PE, y su regulación por el IGF-I. Las células de placentas PE transcribieron al gen de la 1α-(OH)asa (Fig. 23). Por otro lado, al incubar sinciciotrofoblastos PE en presencia de IGF-I (2.6 nM) durante 24 horas se encontró que el IGF-I estimuló la transcripción de CYP27B1 (Fig. 23). Esta regulación se comportó de manera dosis-dependiente (Fig. 24).

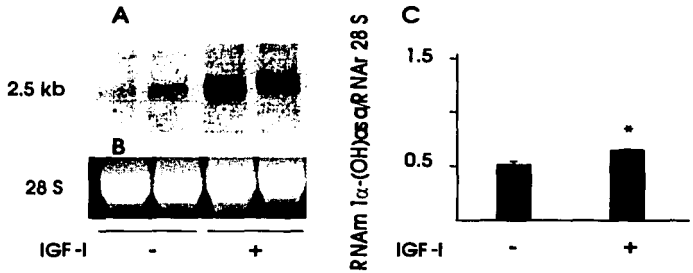


Figura 23.Expresión de CYP27B1 en sinciciotrofoblastos PE. (A): Northern blot de sinciciotrofoblastos PE incubados en presencia (+) o ausencia (-) de IGF-I (2.6 nM) durante 24 horas. (B): RNA ribosomal 28S teñido con bromuro de etidio. (C) Relación entre la densidad óptica relativa del transcrito para la 1α-(OH)asa y el RNA ribosomal 28S, graficados como la media ± desviación estándar. La figura muestra dos experimentos por cada tratamiento. La hibridación se llevó a cabo utilizando una sonda específica para la 1α-(OH)asa -[^{32}P]-dCTP. * $P < 0.05$ vs ausencia de IGF-I.

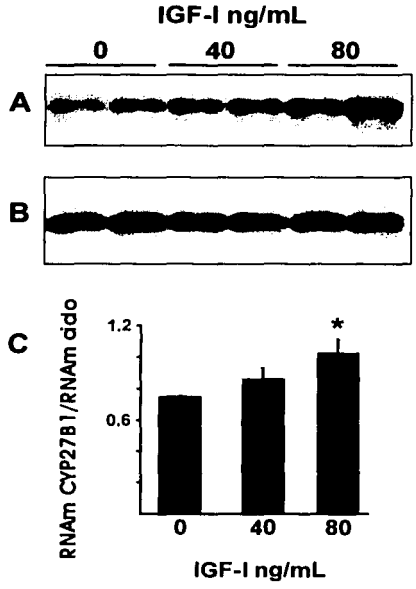


Figura 24. Regulación de la transcripción de CYP27B1 por el IGF-I. Se incubaron sinciciotrofoblastos PE por duplicado en presencia de 0, 40 u 80 ng/mL de IGF-I durante 24 horas, al cabo de lo cual se extrajo el RNA. (A): Southern blot con productos de RT-PCR amplificados con los iniciadores OH1/OH2 (298-pb). La hibridación se realizó en presencia de la sonda interna de 183-pb para la 1α-(OH)asa-[^{32}P]-dCTP, como se describió en Material y Métodos. (B): Productos de RT-PCR de ciclofilina utilizando los mismos DNAc que en A, pero amplificados con los iniciadores CF1/CF2 e hibridados con la sonda interna para la ciclofilina-[^{32}P]-dCTP. (C) Relación de la densidad óptica de los productos de RT de la 1α-(OH)asa vs la ciclofilina. La gráfica muestra la media ± la desviación estándar expresada en unidades arbitrarias de abundancia relativa del RNAm de la 1α-(OH)asa.* $P < 0.05$ vs control.

Los resultados anteriores demostraron que el IGF-I estimuló la transcripción de CYP27B1 tanto en sinciciotrofoblastos NT como PE; sin embargo, la síntesis de 1,25-$(OH)_2D_3$ en los cultivos de células PE no se incrementó cuando se preincubaron las células en presencia de dicho factor de crecimiento (Tabla 3).

Placenta	vehículo	IGF-I	Incremento sobre el control (número de veces)
1	9.9 ± 2.7	18.5 ± 7.0	1.8
2	18.0 ± 6.0	16.2 ± 5.0	0.0
3	6.1 ± 3.0	6.1 ± 2.8	0.0
Media	11.3 ± 6.0	13.5 ± 6.6	Promedio: 0.6 ± 1.0 veces sobre el vehículo. *P* no es significativa.

Tabla 3. Efecto del IGF-I en la síntesis de calcitriol en los sinciciotrofoblastos PE. Sinciciotrofoblastos provenientes de placentas PE fueron incubaron en presencia de IGF-I (2.6 nM) o su vehículo (BSA 0.1% y ácido acético 0.1N) durante dos horas, después de lo cual se adicionó [^{3}H]25-$(OH)D_3$ (3 nM) y se incubó durante una hora más. La extracción de los metabolitos de la vitamina D y su separación se realizó como se indicó en Material y Métodos. Los resultados se expresan como fmol 1,25-$(OH)_2D_3$/mg prot. Se muestran los resultados obtenidos en cultivos de tres diferentes placentas PE (n = 3).

6.4 Expresión de IGF-I en el trofoblasto PE

Con la finalidad de determinar si la expresión de IGF-I en los sinciciotrofoblastos PE se encontraba alterada, se estudió el patrón de transcripción de dicho factor de crecimiento en el cultivo de células de placentas PE. Para ello se trabajó con RNA de sinciciotrofoblastos PE extraído en diferentes momentos del cultivo y se realizó RT-PCR. Los resultados mostraron que la transcripción de IGF-I se incrementó progresivamente conforme el trofoblasto se diferenció en sinciciotrofoblasto (Fig. 25), de manera semejante a lo que ocurrió en el cultivo de placentas normales (Fig. 17).

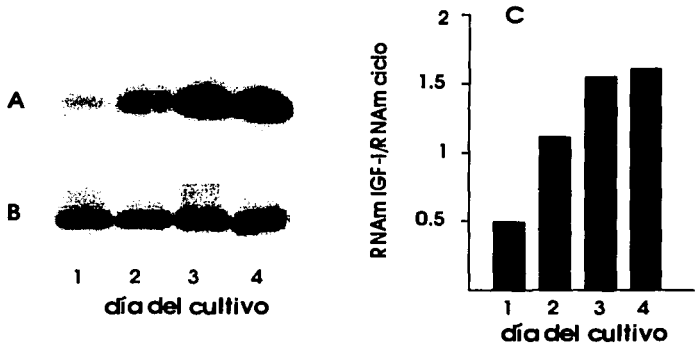


Figura 25. Patrón temporal de expresión de IGF-I en el cultivo de trofoblasto PE. (A): Southern blot de productos de RT-PCR de IGF-I. Después de extraer el RNA de sinciciotrofoblastos a diferentes días del cultivo, se obtuvieron los DNAc y se amplificaron con los iniciadores IF1/IF2 (229-pb). (B): Southern blot de productos de RT-PCR con iniciadores para la ciclofilina (453-pb). Las hibridaciones con sus respectivas sondas se realizaron como se describió en Material y Métodos. (C): Gráfica que muestra la diferencia entre la expresión del gen de IGF-I y el de la ciclofilina, obtenida a partir de la relación entre la densidad óptica de las bandas de los Southern blots y expresada como unidades arbitrarias de abundancia relativa del RNAm del IGF-I. Se muestra un experimento representativo.

7. Comparación entre placentas NT y PE

El trofoblasto de la placenta PE se comportó de manera semejante al de la placenta normal cuando se cultivaron ambos bajo las mismas condiciones de cultivo. Lo anterior se observó en el proceso de diferenciación (Figs. 3 y 19), secreción de hCG al medio de cultivo (Figs. 4 y 20), respuesta al 8-bromo-AMPc (Figs. 6 y 22), y regulación de CYP27B1 por el IGF-I (Figs. 16 y 23). Sin embargo, hubo algunas diferencias. A continuación se muestran algunos experimentos comparativos entre las placentas NT y PE.

7.1 Síntesis de 1,25-$(OH)_2D_3$

La Figura 26 compara la actividad de la 1α-(OH)asa en cultivos de sinciciotrofoblastos NT y PE. Esta actividad enzimática se encontró significativamente disminuida en los cultivos PE en comparación con los cultivos NT. Los resultados se expresan como fmol 1,25-$(OH)_2D_3$/mg prot.

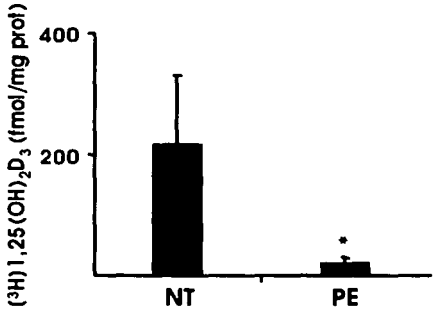


Figura 26: Secreción de 1,25-$(OH)_2D_3$ en cultivos de sinciciotrofoblastos NT y PE. Se cultivaron células de 10 diferentes placentas NT y 8 PE. Se incubaron durante 48 h en medio suplementado con SFT al cabo de lo cual se cambió por DMEMF-12 sin suero. A las 72 horas se adicionó [^{3}H]25-$(OH)D_3$ (3 nM) y se incubaron durante una adicional. Posteriormente se separaron los metabolitos de la vitamina D_3 como se describió en Material y Métodos. Los datos se graficaron como la media ± la desviación estándar.* $P<0.001$ vs NT.

7.2 Regulación de la síntesis de 1,25-$(OH)_2D_3$ por IGF-I

Se comparó el efecto del IGF-I sobre la secreción de calcitriol en cultivos de sinciciotrofoblastos NT y PE con la finalidad de determinar si este factor de crecimiento estimula la síntesis de 1,25-$(OH)_2D_3$ en las células PE, como lo hizo con las NT. Sin embargo, el IGF-I no aumentó la secreción de calcitriol en los cultivos PE (Fig. 27).

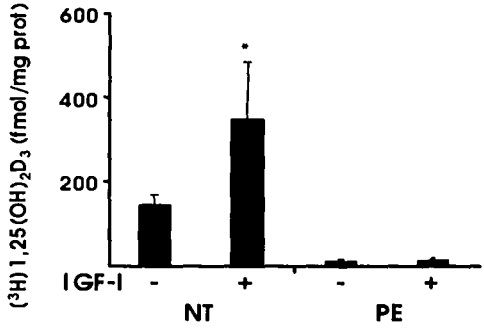


Figura 27. Secreción de 1,25-$(OH)_2D_3$ en cultivos de sinciciotrofoblastos de placentas NT y PE. Las células fueron incubados en presencia o ausencia de IGF-I (2.6 nM) o su vehículo antes de la adición de [^{3}H]25-$(OH)D_3$ (3 nM). Los resultados se muestran como la media ± la desviación estándar. n = 5 placentas NT y 3 PE. *$P < 0.001$ vs vehículo.

7.3 Expresión de CYP27B1 en placentas NT y PE

Con la finalidad de comparar la transcripción de CYP27B1 en tejido placentario total de mujeres NT y PE, se purificó el RNA de 13 placentas de cada grupo y se analizó por RT-PCR y Southern blot (Fig. 28). Los resultados obtenidos mediante esta técnica mostraron la presencia de productos transcripcionales de CYP27B1 en todas las muestras estudiadas. Sin embargo, en los análisis de Northern blot (Fig. 29) se observó que la transcripción de CYP27B1 es significativamente menor en el grupo PE $P<0.05$.

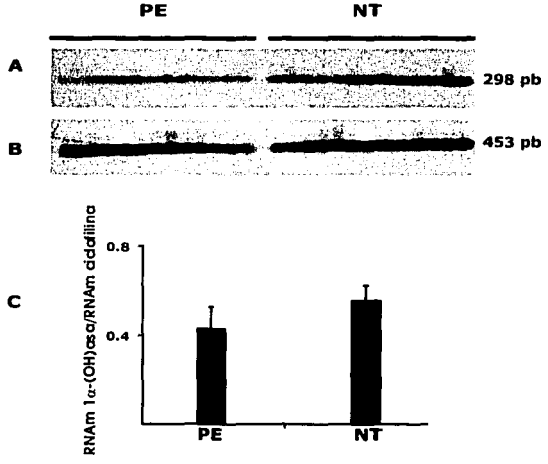


Figura 28. (A) Southern blot de productos de RT-PCR de la 1α(OH)asa. Se extrajo el RNA de tejido placentario y se hizo RT-PCR con los iniciadores OH1/OH2 (298-pb). Las hibridaciones se realizaron con las sondas internas respectivas. (B) Los DNAc se amplificaron con los iniciadores de la ciclofilina CF1/CF2 (453-pb). (C) La gráfica esquematiza la relación de la densidad óptica de la 1α-(OH)asa vs la de la ciclofilina, media ± desviación estándar. (n = 13)

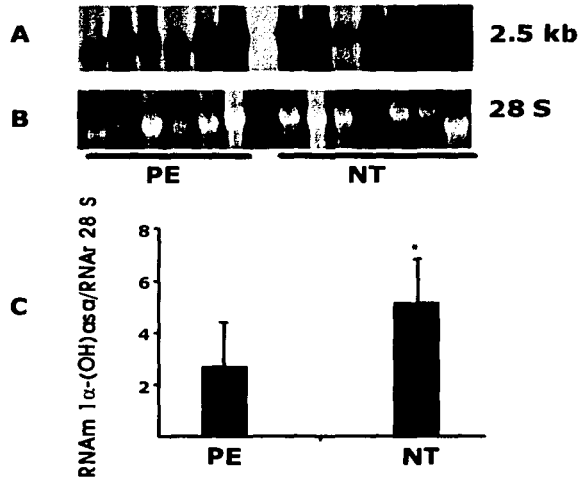


Figura 29. (A) Northern blot utilizando RNA total de 6 placentas PE y 7 NT. (B) Se muestran los RNA ribosomales 28 S teñidos con bromuro de etidio. (C) La gráfica esquematiza la relación de la densidad óptica de la 1α-(OH)asa vs la del RNA ribosomal 28S, media ± desviación estándar.
* $P< 0.05$

7.4 Expresión de IGF-I en placentas NT y PE

En los análisis de RT-PCR para el IGF-I en cultivo de sinciciotrofoblastos NT y PE (Fig. 17 y 25 respectivamente) se observó que no existía diferencia en la transcripción del gen que codifica para dicho factor de crecimiento en los dos grupos estudiados. Con la finalidad de investigar si este resultado se repetía cuando se utilizaba RNA de cotiledones placentarios, se colectaron muestras de tejido de la cara fetal de la placenta recién expulsada y se congelaron en

nitrógeno líquido inmediatamente. Posteriormente se llevó a cabo la extracción del RNA y el análisis por RT-PCR utilizando los iniciadores para el IGF-I. Los resultados confirmaron lo encontrado en el cultivo de trofoblasto pues mostraron que no existen diferencias en la transcripción de IGF-I en placentas de mujeres NT y PE (Fig. 30).

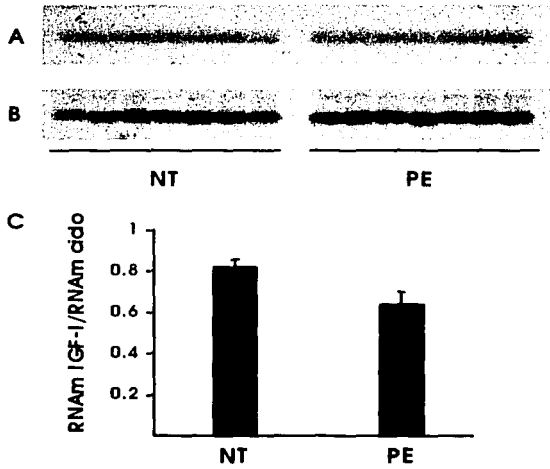


Figura 30. Estudio de la transcripción de IGF-I en tejido de cotiledones placentarios de mujeres normales y con PE. (A): Southern blot de productos RT-PCR con iniciadores para el IGF-I (229-pb). (B): Southern blot de productos de RT-PCR con los iniciadores de la ciclofilina (453-pb). Las hibridaciones se realizaron con las sondas internas respectivas como se describió en Material y Métodos. (C): Relación entre las densidades ópticas IGF-I vs ciclofilina expresada como unidades arbitrarias de abundancia relativa del RNAm de IGF-I. Media ± desviación estándar, (n= 7 placentas NT y 7 PE)

IX. DISCUSIÓN

La demanda fetal de calcio durante la gestación provoca modificaciones en el metabolismo del calcio materno que implican directamente al calcitriol. El calcitriol en el suero de la mujer embarazada se incrementa independientemente del calcio, el fósforo y la PTH[64], por lo que además de la producción renal, el aumento gestacional de 1,25-$(OH)_2D_3$ se ha atribuido a la placenta. La primera evidencia que se tuvo de la síntesis placentaria de calcitriol fue en ratas preñadas anéfricas, las cuales metabolizaron 25-$(OH)D_3$ en 1,25-$(OH)_2D_3$[33], señalando a la placenta como el órgano responsable de este proceso. Sin embargo, la contribución placentaria de calcitriol ha sido controversial. Zerwekh y Breslau[29] demostraron la síntesis de 1,25-$(OH)_2D_3$ en homogenados y preparaciones mitocondriales de la placenta e informaron que en mujeres con alteraciones en el metabolismo de la vitamina D, la producción de calcitriol durante el embarazo fue suficiente para restaurar la homeostasis del calcio. De acuerdo con estos estudios, Whitsett y cols.[28] encontraron producción de calcitriol por el trofoblasto pero no por la decidua. Por el contrario, Weismann y cols.[30] informaron que las células deciduales y no las trofoblasticas sintetizaron 1,25-$(OH)_2D_3$; y Rubin y cols.[34] demostraron la síntesis de calcitriol en cultivos de sinciciotrofoblastos pero únicamente en presencia de concentraciones suprafisiológicas de sustrato. Finalmente Hollis y cols.[6] sugirieron que la producción de 1,25-$(OH)_2D_3$ por homogenados de placenta o por la fracción mitocondrial es el resultado de un proceso asociado a radicales libres, cuestionando la existencia de un mecanismo enzimático en el trofoblasto. La discrepancia de los resultados aquí expuestos resulta confusa; sin embargo, pudiera explicarse por la cantidad de factores involucrados en las condiciones experimentales tales como la concentración del sustrato, el sistema biológico utilizado, los periodos de incubación o la composición de los medios de cultivo. Cabe destacar que los trabajos mencionados se centraron únicamente en el estudio de la conversión de 25-$(OH)D_3$ en metabolitos más activos y carecían de análisis molecular pues el DNAc de CYP27B1 se clonó hasta 1997 y 1998[19,20,21]. En resumen era evidente la necesidad de estudios determinantes para aclarar la confusión en torno a este tema.

Los hallazgos del presente trabajo indican claramente que la producción trofoblástica de calcitriol involucra a la 1α-(OH)asa y que este es un proceso enzimático similar al que tiene lugar en el riñón. Se presenta suficiente evidencia para asegurar que la enzima placentaria proviene del mismo gen que la renal ya que las secuencias de bases de los respectivos DNAc son idénticas. Por otro lado, los estudios de Northern y Southern blot demostraron la existencia de productos de transcripción de CYP27B1 en las diferentes etapas de diferenciación del trofoblasto y con la expresión del DNAc de la 1α-(OH)asa placentaria se obtuvo una proteína funcional capaz de bioconvertir activamente [^{3}H]25-(OH)D_3 en [^{3}H]1,25-$(OH)_2D_3$.

Los datos presentados aunados al hecho de que la línea celular JEG-3 convierte [^{3}H]25-(OH)D_3 en calcitriol y transcribe a CYP27B1, demuestran la participación placentaria en la síntesis gestacional de 1,25-$(OH)_2D_3$ como un proceso dependiente de la 1α-(OH)asa y permiten sugerir un papel primordial del calcitriol en los procesos asociados al manejo del calcio en la placenta humana desde etapas tempranas del embarazo.

1α-(OH)asa en la placenta

Debido a que algunos investigadores informaron que CYP27B1 no se expresa en la placenta[19,20], se intentó incrementar la señal de transcripción de la 1α-(OH)asa mediante dos estrategias. La primera fue elegir un modelo biológico que permitiera el desarrollo de trofoblastos cuya función principal es la secreción hormonal. Los sinciciotrofoblastos constituyen una población terminal en el proceso de diferenciación y secretan diversas proteínas para preservar el embarazo[42], razón por la cual se eligió este tipo celular para buscar productos de transcripción de CYP27B1. La segunda estrategia consistió en disminuir la concentración de calcio y fosfato en el medio de cultivo con la finalidad de inducir la expresión de CYP27B1, como lo hicieron previamente Fu y cols.[19] Mediante esta estrategia se detectó RNAm de la 1α-(OH)asa en el sinciciotrofoblasto.

IGF-I y el sinciciotrofoblasto

El IGF-I ha sido postulado como factor promotor de la diferenciación: Milio y cols.[73] informaron que el IGF-I participa en el proceso de diferenciación del trofoblasto a través de la unión con su receptor membranal[73], y Smith y cols.[74] demostraron que el IGF-I estimula la diferenciación celular en adipositos. En el

presente trabajo se encontró que el IGF-I se transcribe progresivamente conforme las células adquieren el fenotipo sincicial, lo cual señala a este factor como uno de los elementos que participa en el proceso de citodiferenciación del trofoblasto.

Por otro lado, si bien se sabe que el IGF-I estimula la expresión y la síntesis de enzimas esteroidogénicas[15,65,66,75], hasta la fecha no se conoce el mecanismo mediante el cual se lleva a cabo este proceso. A este respecto, los resultados del presente estudio demostraron que el IGF-I es un regulador positivo de la 1α-(OH)asa y que esto se debe al incremento en la transcripción de CYP27B1. Tomando en cuenta estos resultados, aunados a que el calcitriol favorece la diferenciación celular[8] y estimula la expresión y secreción de hPL por los sincitiotrofoblastos[76], se sugiere la participación del IGF-I y del calcitriol en la especialización a sincitiotrofoblasto y consecuentemente, en el establecimiento de la unidad fetoplacentaria.

Estudios de la placenta de embarazos con preeclampsia

De manera interesante, los resultados obtenidos en el presente trabajo demostraron la ausencia de actividad de la 1α-(OH)asa en cultivos de sincitiotrofoblastos de embarazos complicados con PE. Esta enfermedad se asocia con alteraciones en el metabolismo del calcio, crecimiento intrauterino reducido y bajas concentraciones de calcitriol en el suero materno; todo esto como consecuencia probable de las alteraciones de la placenta, del riñón materno o de ambos. Puesto que existe evidencia de que el calcitriol de origen placentario alcanza la circulación materna[31], es factible suponer que las bajas concentraciones del calcitriol en el suero de la madre con PE sean consecuencia de la actividad subóptima de la 1α-(OH)asa de la placenta. En este estudio se presentan evidencias que apoyan esta hipótesis. Se encontraron alteraciones en la transcripción de CYP27B1 en las placentas PE y sus trofoblastos produjeron significativamente menos calcitriol en comparación con el grupo control; observaciones que representan los hallazgos más significativos de esta tesis. Cabe destacar que los sincitiotrofoblastos PE se diferenciaron normalmente en cultivo y secretaron hCG en respuesta al 8-bromo-AMPc de manera similar a como lo hicieron las células control. Adicionalmente, tanto los sincitiotrofoblastos NT como los PE desarrollaron el mismo patrón transcripcional de IGF-I. Estas observaciones permiten descartar

alteraciones generalizadas de los trofoblastos PE en cultivo, en lo que concierne a las funciones estudiadas, como probable causa de la restricción en la secreción de calcitriol. Otros investigadores han cultivado trofoblastos PE y sus resultados difirieron de los nuestros ya que en su estudio el trofoblasto invasivo de las placentas PE no se diferenció adecuadamente *in vitro*[77]. Estas diferencias pudieran explicarse por el fenotipo celular obtenido, indicando que es más probable que sea el trofoblasto invasivo el que se encuentre alterado, y no así el sinciciotrofoblasto endocrino.

Se sugiere entonces la participación de factores reguladores de la actividad enzimática en los cultivos de trofoblastos PE, que contribuyan en abatir la actividad de la 1α-(OH)asa. A este respecto, se demostró que el IGF-I estimuló la secreción de calcitriol y la transcripción de CYP27B1 en el sinciciotrofoblasto. Sin embargo; si bien el IGF-I incrementó la transcripción de CYP27B1 en NT y PE, en lo que se refiere a la actividad únicamente logró estimular a la 1α–(OH)asa en el grupo NT. Se decidió entonces estudiar la transcripción de IGF-I en el trofoblasto en cultivo con la finalidad de descartar alteraciones a este nivel, pero los estudios de RT-PCR revelaron que este proceso se lleva a cabo de igual forma tanto en los cultivos NT como en los PE. Para explicar lo anterior se sugieren dos razonamientos 1) Es probable que exista un estado de resistencia a la insulina, lo cual ha sido informado en la preeclampsia[78]; si éste fuera el caso, se requerirían de dosis mayores de IGF-I para alcanzar el efecto equiparable a las placentas NT, y 2) Dado que existe un aumento de hasta 6 veces en la concentración de IGFBP1 en el suero de la madre PE[58], y que esta proteína inhibió las acciones biológicas del IGF-I en células de coriocarcinoma[79], es factible que este proceso suceda en el modelo utilizado en este trabajo y en la mujer PE, disminuyendo así la biodisponibilidad del IGF-I.

En realidad, la pregunta fundamental que aun queda sin respuesta, es ¿cuál es la causa de una menor producción de calcitriol en los trofoblastos PE? Para contestar esta pregunta es necesario considerar que la producción de 1,25-$(OH)_2D_3$ depende en gran medida de la cantidad de 1-α(OH)asa presente y catalíticamente activa; por lo que además de necesitarse estudios de la cinética enzimática se requieren análisis de inmunodetección. Sin embargo, existen otros factores que regulan la producción de calcitriol y que se deben considerar para estudios

posteriores. Estos factores son las proteínas transportadoras de electrones ferredoxina reductasa y ferredoxina[80], el oxígeno molecular, el sustrato de la reacción, la proteína intracelular de unión a la vitamina D[81] y la CYP24. La CYP24 es una enzima clave del metabolismo de la vitamina D ya que es la responsable de iniciar la vía de degradación del calcitriol que se efectúa principalmente por oxidación de la cadena lateral. Este proceso de depuración genera como productos intermedios a metabolitos 24-hidroxilados y como producto final al ácido calcitroico[18], metabolito soluble en agua y que se filtra en el riñón para ser excretado. La importancia de estudiar el catabolismo de la 1,25-$(OH)_2D_3$ radicaría en la posibilidad de que las bajas concentraciones de esta hormona detectadas en los sincitiotrofoblastos en cultivo del grupo PE sean consecuencia de un catabolismo acelerado.

Una pregunta adicional surge con base en los resultados obtenidos en este trabajo; ¿cuál es la consecuencia de la producción insuficiente de calcitriol por parte de los trofoblastos de la placenta? Considerando la función inmunosupresora de la 1,25-$(OH)_2D_3$[8,82], se sugiere que las alteraciones del establecimiento de la unidad fetoplacentaria características de la preeclampsia se deban en parte a las bajas concentraciones de esta hormona producida por los sincitiotrofoblastos PE. El calcitriol inhibe la secreción de interleucina 2 (IL-2) así como la proliferación linfocitaria[83], pudiendo entonces contribuir a evitar el rechazo del feto por parte de la madre. En efecto existen trabajos que demuestran que la decidua de las madres PE secreta IL-2, y ésta a su vez reduce la secreción de sustancias angiogénicas derivadas del trofoblasto, vitales para una irrigación adecuada[84,85], por lo que al haber menos 1,25-$(OH)_2D_3$ en la unidad fetoplacentaria, habría consecuentemente menor aporte sanguíneo.

Significado biológico de la síntesis de 1,25-$(OH)_2D_3$ en la placenta

En la actualidad se ignora el significado biológico de la síntesis de calcitriol en la placenta y por consiguiente, cual sería la consecuencia de su carencia. Algunas observaciones sugieren que el calcitriol tiene funciones específicas en la unidad fetoplacentaria: 1) La presencia de receptores para 1,25-$(OH)_2D_3$ en la placenta[86]; 2) La estimulación de la expresión y secreción de hPL por la 1,25-$(OH)_2D_3$ en los

sinciciotrofoblastos[76]; 3) La estimulación del transporte transplacentario de calcio por el calcitriol a manera dosis-dependiente[39] y 4) La participación del calcitriol en la decidualización del endometrio[87]. Sin embargo, para poder determinar con certeza la importancia biológica del calcitriol placentario se requiere analizar los casos de mujeres que durante la gestación presentaron bajas concentraciones de esta hormona, o bien contar con algún modelo animal carente de vitamina D. Con respecto a lo primero, aún cuando existen estudios que indican que es posible el desarrollo de productos sanos en madres que cursaron su embarazo con bajas concentraciones de calcitriol[88], también hay muchos otros trabajos que presentan casos en donde esta deficiencia en la madre tuvo consecuencias para sus bebés[89,90,91,92]. Con respecto a lo segundo, existen ya modelos animales genéticamente modificados que involucran alteraciones del sistema endocrino de la vitamina D. Dos laboratorios independientes generaron ratones en los cuales se modificó el dominio de unión al DNA en el iVDR, lo cual permitió estudiar los efectos de la ausencia de actividad biológica del calcitriol *in vivo*. Los ratones homocigotos (-/-) nacieron completamente normales pese a la deficiencia del receptor para la vitamina D durante toda la gestación; sin embargo, durante el primer mes de vida desarrollaron hipocalcemia, hiperparatiroidismo, hipoplasia uterina y alopecia total[93,94]. Es importante destacar que si bien estos trabajos introdujeron un modelo animal útil para estudiar la patología en el humano conocida como raquitismo resistente a la vitamina D (VDRR II), solamente estudiaron los efectos de la hormona mediados por el receptor intracelular, y no consideran la amplia gama de efectos biológicos producidos por la interacción con el receptor de membrana, y que pueden ser igualmente importantes. Por otro lado, existen también dos modelos animales del raquitismo pseudodeficiente de la vitamina D (PDDR)[95,96]. Esta patología es consecuencia de una alteración hereditaria que se asocia con la síntesis deficiente de calcitriol y que es ocasionada por una mutación en la estructura primaria de la 1α-(OH)asa[19,97]. En los modelos animales de PDDR[95,96] se obtuvieron resultados que son interesantes desde el punto de vista del presente trabajo: se demostró que en ausencia de la actividad de 1α-(OH)asa durante la gestación los ratones (-/-) nacieron perfectamente normales, tal como sucede con los niños PDDR, pero revelaron alteraciones en la reproducción femenina y en el sistema

inmunológico. Tomando en cuenta que el trofoblasto de los ratones (-/-) presenta el mismo genotipo que el producto, se puede suponer entonces que la $1,25\text{-}(OH)_2D_3$ sintetizada por la placenta no participa en eventos cruciales del desarrollo del feto o de la placenta misma; o bien, que el calcitriol producto de la decidua o riñón maternos suplen la carencia de esta hormona.

En resumen, la importancia biológica del calcitriol placentario no se conoce, pero es probable que el calcitriol de origen trofoblástico se encuentre implicado en el adecuado establecimiento de la unidad fetoplacentaria, modulando la respuesta inmune de la madre y promoviendo el crecimiento de la placenta y del feto, como un efecto mediado por el hPL, o bien actuando directamente sobre la diferenciación celular y el control de la expresión génica.

La contribución del presente trabajo señala únicamente la participación de la 1α-(OH)asa trofoblástica en la secreción gestacional de calcitriol y su regulación por el IGF-I; y destaca la insuficiencia endocrina de las placentas PE en lo que respecta a la síntesis de calcitriol.

X. CONCLUSIONES

- Los trofoblastos humanos hidroxilan a la 25-(OH)D_3 como un efecto mediado, al menos en parte, por la 1α-(OH)asa.
- La placenta contribuye en la síntesis extrarrenal de calcitriol durante la gestación.
- La secuencia del DNAc que codifica para la 1α-(OH)asa en el trofoblasto es idéntica a la del riñón humano, lo cual sugiere que provienen del mismo gen.
- El IGF-I estimula la transcripción de CYP27B1 cuando se expone a los sincicíotrofoblastos en cultivo a concentraciones nanomolares de dicho factor de crecimiento
- La transcripción del IGF-I se incrementa progresivamente durante el proceso de diferenciación del trofoblasto, tanto en los cultivos NT como en los PE.
- La transcripción y actividad de la 1α-(OH)asa en placentas PE se encuentra disminuida en comparación con las placentas NT. Se sugiere que las bajas concentraciones del calcitriol en el suero de la madre con PE sean en parte consecuencia de la actividad subóptima de la 1α-(OH)asa de su placenta.
- El IGF-I estimula la transcripción de CYP27B1 en sincicíotrofoblastos NT y PE cultivados, pero en lo que se refiere a la actividad únicamente estimula la síntesis de calcitriol en el grupo NT.

XI. PERSPECTIVAS

Con base en los resultados obtenidos en el presente trabajo, se proponen los siguientes objetivos para estudios posteriores:

- Estudiar la abundancia de la 1α-(OH)asa en tejido y trofoblastos cultivados provenientes de placentas normales y de embarazos con PE mediante técnicas de inmunohistoquímica.
- Analizar la secuencia del DNA complementario de la 1α-(OH)asa en trofoblastos PE.
- Estudiar si mayores dosis de IGF-I estimulan la actividad de la 1α-(OH)asa en los cultivos de trofoblastos PE.
- Estudiar el efecto de 1,25-$(OH)_2D_3$ sobre el proceso de diferenciación trofoblástica.
- Debido a que no se conoce el origen de las altas concentraciones de IGFBP1 en la interfase decidua-trofoblasto[98], se recomienda estudiar la síntesis de esta proteína transportadora en el trofoblasto PE y NT, y compararla con la decidua.
- Realizar estudios para determinar factores involucrados en el abatimiento de la actividad de la 1α-(OH)asa en cultivos PE. Para ello se sugiere:
    1. Estudiar la actividad de CYP24 en cultivos de trofoblastos NT y PE.
    2. Estudiar la síntesis de calcitriol en el cultivo PE en presencia de antioxidantes o moléculas que atrapen radicales libres con la finalidad de descartar la participación de especies reactivas de oxígeno.
    3. Estudiar la expresión de la ferredoxina reductasa, ferredoxina, CYP24 y el receptor de la vitamina D en trofoblastos cultivados NT y PE.

XII. ANEXOS

Anexo 1

Digestiones enzimáticas para el cultivo de citotrofoblastos

Incubaciones	HBSS 1x (mL)	Tripsina (mg)	DNasa (mg)	Tiempo (min.)
Primera	150	190	30	30
Segunda	100	125	20	30
Tercera	75	100	15	30

Anexo 2

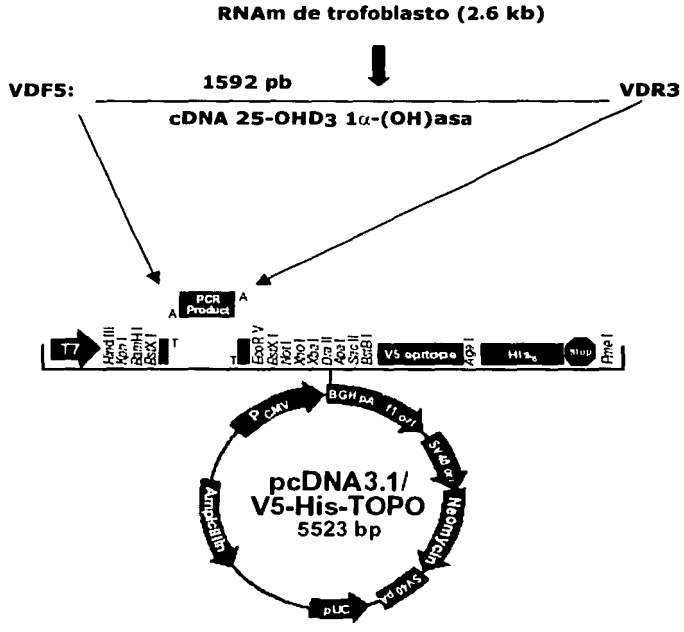
Gradiente de Percoll para la separación celular.

Para el Percoll 90%: A 18 mL de Percoll adicionar 2 mL HBSS 10 X.

Tubo	Percoll (%)	Percoll 90 % (mL)	HBSS 1X (mL)
1	70	2.33	0.67
2	65	2.17	0.83
3	60	2.00	1.00
4	55	1.83	1.17
5	50	1.67	1.33
6	45	1.50	1.50
7	40	1.33	1.67
8	35	1.17	1.83
9	30	1.00	2.00
10	25	0.83	2.17
11	20	0.67	2.33
12	15	0.50	2.50
13	10	0.33	2.67
14	5	0.17	2.83

Anexo 3

Mapa del plásmido pcDNA 3.1 V5-His-TOPO



CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 902-1019
TOPO® Cloning site: 953-954
V5 epitope: bases 1020-1061
Polyhistidine tag: bases 1071-1088
BGH reverse priming site: bases 1111-1128
BGH polyadenylation signal: bases 1110-1324
f1 origin of replication: bases 1387-1800
SV40 promoter and origin: bases 1865-2190
Neomycin resistance gene: bases 2226-3020
SV40 polyadenylation signal: bases 3039-3277
pUC origin: bases 3709-4382
Ampicillin resistance gene: bases 4527-5387
Hind III
Kpn I

XIII. BIBLIOGRAFÍA

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Expression and Activity of 25-Hydroxyvitamin D-1α-Hydroxylase Are Restricted in Cultures of Human Syncytiotrophoblast Cells from Preeclamptic Pregnancies

LORENZA DÍAZ, CELESTE ARRANZ, EUCLIDES AVILA, ALI HALHALI, FELIPE VILCHIS, AND FERNANDO LARREA

Department of Reproductive Biology, National Institute of Medical Sciences and Nutrition Salvador Zubirán, México Distrito Federal, 14000, México

The human placenta synthesizes 1,25-dihydroxyvitamin D_3 and expresses the vitamin D receptor. Because preeclampsia (PE) is associated with low circulating levels of maternal 1,25-dihydroxyvitamin D_3 and IGF-I, it is possible that alterations in calcium metabolism seen in PE could occur at the level of the fetoplacental unit. In this study, the patterns of gene expression and enzyme activity of 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) and the abundance of IGF-I mRNA in placentas from normal (NT) and PE-complicated pregnancies were investigated. Cultured syncytiotrophoblast cells from preeclamptic placentas had only one tenth the activity of 1α-hydroxylase and did not respond to IGF-I, when compared with NT cultures. Similarly, the levels of 1α-hydroxylase mRNA in syncytiotrophoblast cells from PE placentas under basal and IGF-I-stimulated conditions were significantly reduced. In contrast, IGF-I mRNA levels were found to increase during the differentiation process, with no differences between NT and PE cultures. These results support the role of placenta as a contributor to the abnormalities observed in calcium metabolism in PE. (*J Clin Endocrinol Metab* 87: 3876–3882, 2002)

ABNORMALITIES IN CALCIUM metabolism have been involved in the pathophysiology of pregnancy-induced hypertension (1, 2), which have also been linked to preeclampsia (PE)/eclampsia (3–5). PE is a common disease and remains as a major cause of maternal morbidity and mortality in developing countries. We previously reported that PE is associated with low circulating levels of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and IGF-I in both maternal and umbilical cord compartments (6). Furthermore, results from this laboratory have established that placenta expresses the mitochondrial cytochrome P_{450} 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) gene, which agreed with the capability of the placenta to produce $1,25(OH)_2D_3$ (7). In addition, placental 1α-hydroxylase activity, as in the case of kidney (8, 9), was stimulated in a dose-dependent manner by IGF-I (10), suggesting that this growth factor acts as a physiological regulator of placental $1,25(OH)_2D_3$ production. Therefore, we hypothesized that alterations in calcium metabolism that occur in PE as a result of low $1,25(OH)_2D_3$ are attributable, in part, to an altered 1α-hydroxylase expression and/or regulation at the level of the fetoplacental unit.

Herein, we report a study aimed at investigating the hormonal basis for low $1,25(OH)_2D_3$ circulating levels in PE. We determined the activity and expression of the 1α-hydroxylase enzyme in cultured placental syncytiotrophoblast cells obtained from normal (NT) and PE complicated pregnancies. In addition, expression of IGF-I gene, during trophoblast differentiation in both NT and PE cell cultures, was also studied.

Abbreviations: AUC, Area under the curve; 8-Br-cAMP, 8-bromo adenosine 3′5′-cAMP; HBSS, Hanks' balanced salt solution; hCG, human chorionic gonadotrophin; 1α-hydroxylase, 25-hydroxyvitamin D-1α-hydroxylase; NT, normal; [^{3}H]25(OH)D_3, 25-hydroxy-[26,27-methyl-^{3}H]cholecalciferol; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PE, preeclampsia.

Subjects and Methods

Subjects

The study protocol was approved by the Human Ethical Committee of the National Institute of Medical Sciences and Nutrition Salvador Zubirán. Subjects were considered to be PE when their blood pressure was found to be at least 140/90 mm Hg in two different time intervals of 6 h apart. In addition, hypertension should have been associated with excretion of more than 300 mg urinary protein per 24 h. Patients with chronic hypertension, diabetes mellitus, and renal, and other systemic illnesses were excluded from the study. Normotensive controls were selected from the prenatal clinic and admitted to the study at the time of delivery.

Materials

Hanks' balanced salt solution (HBSS), DMEM and DMEM-F12, fetal calf serum, penicillin-streptomycin mixture, and Fungizone were obtained from Life Technologies, Inc. (Grand Island, NY). Percoll, 8-bromo adenosine 3′5′-cAMP (8-Br-cAMP), deoxyribonuclease I, BSA, trypsin, and glutamine were purchased from Sigma (St. Louis, MO). All solvents (HPLC grade) were obtained from Merck & Co., Inc. (Darmstadt Germany). Unlabeled authentic 25(OH)D_3 and $1,25(OH)_2D_3$ were a generous gift from Dr. E. M. Gutknecht and Dr. P. Weber (F. Hoffmann-La Roche Ltd., Basel, Switzerland). The 25-hydroxy-[26,27-methyl-^{3}H]cholecalciferol ([^{3}H]25(OH)D_3; specific activity, 30 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Human chorionic gonadotrophin (hCG) RIA was kindly provided by NIDDK (Rockville, MD). Human embryonic kidney cells (HEK-293)

were purchased from ATCC (CRL-1573; Microbix Biosystems, Ontario, Canada). All other reagents were of analytical grade.

Tissue preparation and cell culture

The isolation and culture of cytotrophoblasts was performed as described by Kliman *et al.* (11). Briefly, term placentas (35–42 wk of gestation) were obtained from NT and PE women. Tissues were brought immediately to the laboratory, where several cotyledons were removed and rinsed thoroughly in 0.9% NaCl at room temperature. Soft villous tissue (30 g), free of connective tissue and vessels, was collected. Tissue was coarsely minced and digested with 0.125% trypsin and 0.2 mg/ml deoxyribonuclease I (1,500 Kunitz units/mg) in warmed calcium and magnesium-free HBSS, containing 25 mM HEPES (pH 7.4), for 30 min at 37 C. Cell suspensions were pooled, centrifuged at 1000 × *g* for 10 min, and resuspended in DMEM containing 25 mM HEPES and 25 mM glucose (DMEM-HG). The resultant cell suspension was placed on 5–70% Percoll (vol/vol) gradients made up in HBSS. Gradients, which consisted of 5% steps of 3 ml each, were centrifuged at 1200 × *g* at room temperature for 20 min. After centrifugation, the middle band (containing the cytotrophoblasts) was removed, washed once with DMEM-HG, and resuspended in medium for tissue culture. Percoll gradient-purified cytotrophoblasts were diluted to a concentration of 2×10^6 cells/ml with DMEM-HG containing 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone, and 20% heat-inactivated fetal calf serum; plated in 35-mm culture dishes (Nunc, Roskilde, Denmark); and incubated in humidified 5% CO_2–95% air at 37 C. Unless otherwise indicated, after 2 d in culture, cells were incubated in serum-free DMEM-F12 with low calcium and phosphate concentrations.

Daily, the morphological aspects of cell cultures were examined. The concentration of hCG in the culture media was measured, as previously described (7), by specific RIA using antibody anti-hCG-H80, at a final working dilution of 1:150,000. This antibody exhibits 1.2% and 3.2% cross-reactivity with free hCGα- and β-subunits, respectively. The sensitivity of the assay was 0.025 ng/tube, and the inter- and intraassay coefficients of variation were less than 10% and less than 6%, respectively. Total protein content of cell cultures was measured by the method of Bradford (12), in a 30-μl aliquot per duplicate, using BSA as standard.

Activity of 1α-hydroxylase in cultured human syncytiotrophoblast cells

The ability of syncytiotrophoblast cells to convert 25(OH)D_3 into 1,25(OH)$_2D_3$ was studied individually in 8 placentas from PE women and 10 placentas from NT subjects, as previously described (7, 10). Briefly, on the third day of culture, medium was changed, and cells were incubated in 2 ml serum-free medium (DMEM-F12) in the presence of [^{3}H]25(OH)D_3, at a final concentration of 3 nM, during 60 min. Culture medium was then transferred to glass tubes, and cells were washed with 1 ml methanol. Protein cell content was determined after addition of 0.5 ml 1-N NaOH. Vitamin D_3 metabolites were extracted from the medium with an additional 3 ml methanol, followed by 4 ml chloroform. The chloroform phase was dried down under N_2, and lipidic extracts were redissolved in chromatographic solvent. Authentic unlabeled 1,25(OH)$_2D_3$ was used as elution marker, and samples were separated on an HPLC fitted with a photodiode array detector (Model 996; Waters, Milford, MA), using an ultrasphere Si, 5 μm, 4.6 × 250-mm column (Beckman, Palo Alto, CA). A second straight phase HPLC was used to finally separate the vitamin D_3 metabolites (10, 13). The conversion of [^{3}H]25(OH)D_3 into putative [^{3}H]1,25(OH)$_2D_3$ was determined by estimating the percentage of radioactivity coeluting with authentic unlabeled 1,25(OH)$_2D_3$ after the 2 successive HPLC's. Results were expressed as femtomoles per milligram protein.

cDNA synthesis and PCR amplifications

Total RNA, isolated from cultured syncytiotrophoblast cells (14), was used as template for cDNA synthesis, using the SuperScript II preamplification system (Life Technologies, Inc.). PCR amplifications were then performed with *Taq* polymerase using the following sense and antisense primers: 1α-hydroxylase, (5′-GTTGCTATTGGCGGGAGTGGAC-3′ and 5′-GTGACACAGAGTGACCAGCATAT-3′); IGF-I, (5′-TCACATCGGCCTCATAATACC-3′ and 5′-AAATAAAAGCCCCTGTCTCCA-3′); and hCG, (5′-CGCACCAAGGATGGAGA-3′ and 5′-GCCTTTATTGTGGAGGA-3′), which yielded a 298-bp, 229-bp, and 494-bp RT-PCR product, respectively. Normalization was performed by the amplification of cyclophilin mRNA (CF) with the following sense and antisense primers: 5′-CCCCACCGTGTTCTTCGACAT-3′ and 5′-AGGTCCTTACCGTTCTGGTCG-3′, which yielded a 453-bp RT-PCR product. Incubations, in the absence of reverse transcriptase, were used as controls for the RT-PCR reactions. The PCR products were resolved on agarose gels, blotted onto nylon membranes (15) and hybridized with human 1α-hydroxylase or IGF-I cDNAs nested probes (183 bp and 66 bp, respectively) radiolabeled with [^{32}P]-deoxy-CTP by random priming. The probe for 1α-hydroxylase was obtained from HEK-293 cells as previously described (7). The IGF-I and cyclophilin nested probes were generated from human placental tissue by RT-PCR using the following primers: IGF-I (5′-AGCTCTGCCAACGCCTGGACCGGAC-3′ and 5′-CACGAACTGAAGAGCATCCACCAG-3′) and cyclophilin (5′-CACACGCATAATGGCACTGGTGG-3′ and 5′-AAAGACCACATGCTTGCCATCCAGC-3′) for sense and antisense primers, respectively. In the case of the β-subunit of hCG mRNA, the cDNA probe was obtained using the primers for PCR amplifications. The probes were sequenced for identity by ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Corp., Foster City, CA), as previously described (7).

For Northern blots, 30 μg total cellular RNA was size-fractionated on a 1.2% formaldehyde-agarose gel. After electrophoresis, RNA was transferred onto nylon membranes by capillary diffusion, fixed by UV crosslinking, and probed with the corresponding [^{32}P]-deoxy-CTP-labeled cDNA. After prehybridization for 1 h at 68 C, the radioactive probe was added and hybridized in 0.25 M Na_2HPO_4 and 7% sodium dodecyl sulfate during 24 h at 68 C.

Statistical analysis

The area under the curve (AUC) of hCG secretion in culture media was calculated by the trapezoid method, with the aid of a computer program (SigmaStat; Jandel Scientific Software, Chicago, IL). Statistical significance among comparisons was established using Student's *t* test. A P value ≤ 0.05 was considered statistically significant

Results

Functional trophoblast cell differentiation

Microscopic examination of placental cultures from both NT and PE women showed, after 3 d of culture, the presence of cell aggregates containing multiple nuclei with very little, if any, single mononuclear cells. Cultures from PE placentas were microscopically indistinguishable from those from NT pregnancies. In all cases, syncytiotrophoblasts appeared not isolated, but forming a network structure of multinucleated cells with clusters containing an average of more than 10 nuclei. Figure 1 shows the AUC of hCG released from NT and PE trophoblasts during the first 72 h of culture. As depicted, both NT and PE placentas released similar amounts of hCG during the 72-h period, either in the absence or presence of 8-Br-cAMP. In both groups, addition of the cyclic nucleotide analog significantly increased the amount of hCG in the culture media without significant differences between them. Figure 2 shows the temporal expression pattern of hCG mRNA isolated from NT and PE nonstimulated trophoblast cells at different days from plating (d 1–4). Total RNA was extracted from cultured cells and subjected to RT-PCR, using specific primers, as outlined under *Subjects and Methods*. From the Southern blot analysis, a temporal pattern for the amount of hCG mRNA was demonstrated that closely corresponded to the temporal pattern for released hormone (Fig. 1). The relative abundance of hCG mRNA (Fig. 2A) was obtained by normalizing the 494-bp band intensity (Fig. 2B)

with that generated for the constitutive gene cyclophilin (Fig. 2C).

Synthesis of 1,25(OH)$_2$D$_3$ by syncytiotrophoblast cells from NT and PE pregnancies

To demonstrate the ability of PE syncytiotrophoblasts in culture to synthesize 1,25(OH)$_2$D$_3$, cells at the third day of culture were incubated in the presence of [^{3}H]25(OH)D$_3$ during 1 h. As shown in Fig. 3A, cells from control placentas (n = 10), tested at the third day of culture, actively synthesized 1,25(OH)$_2$D$_3$ from the labeled precursor (215 ± 114 fmol/mg protein). By contrast, only one tenth the activity observed in control cells was obtained when syncytiotrophoblasts isolated from PE placentas (n = 8) were incubated under identical conditions (19 ± 11 fmol/mg protein; $P < 0.001$ *vs.* control). In all instances, cultures of syncytiotrophoblast cells isolated from PE placentas released hCG nonsignificantly different from control cultures.

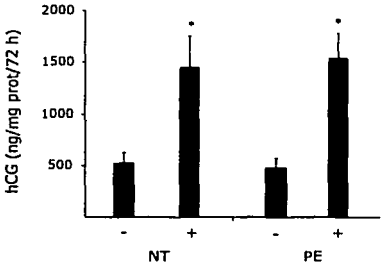

FIG. 1. hCG secretion by NT and PE cultured syncytiotrophoblasts in the absence (−) or presence (+) of 8-Br-cAMP. *Bars*, AUC of hCG (mean ± SE.), released during 72 h, of 10 cultured placentas, respectively. *, $P < 0.001$ *vs.* without 8-Br-cAMP.

Preincubation of NT syncytiotrophoblasts with 6.5 nM IGF-I resulted in a 243% increase ($P < 0.01$) in 1α-hydroxylase enzyme activity (Fig. 3A). On the contrary, cultures from PE placentas did not significantly increase the conversion of [^{3}H]25(OH)D$_3$ into [^{3}H]1,25(OH)$_2$D$_3$ in the presence of IGF-I (Fig. 3B).

To determine whether the low basal and IGF-I-stimulated metabolic conversion of [^{3}H]25(OH)D$_3$ into [^{3}H]1,25(OH)$_2$D$_3$ in cultured syncytiotrophoblasts from PE placentas was a result of an altered expression and/or regulation of placental 1α-hydroxylase, the expression of this enzyme was investigated by RT-PCR/Southern blot and Northern blots with and without the addition of IGF-I. Because initial attempts to identify placental 1α-(OH)ase gene products from total RNA isolated from fresh placental tissue were unsuccessful, we decided to use Percoll gradient-purified cytotrophoblast cells kept in culture as a source of placental RNA. Figure 4 shows a representative Southern blot of RT-PCR products of 1α-hydroxylase mRNA isolated from 72-h cultured syncytiotrophoblasts. A reduction in the relative abundance of 1α-hydroxylase mRNA (Fig. 4B), after normalizing with the band obtained for the constitutive gene cyclophilin (Fig. 4C), was observed in PE placentas, compared with controls (Fig. 4A). Similar results were obtained by Northern blots of total RNA extracted from fresh placental tissue (data not shown). The effects of preincubation with 6.5 nM IGF-I on 1α-hydroxylase expression were studied by Northern blots in cultured NT and PE syncytiotrophoblast cells. Three-day cultures were treated with IGF-I or vehicle alone for 24 h. As shown in Fig. 5, a 2.5-kb 1α-hydroxylase transcript was detected at low levels in untreated cells, with expression being

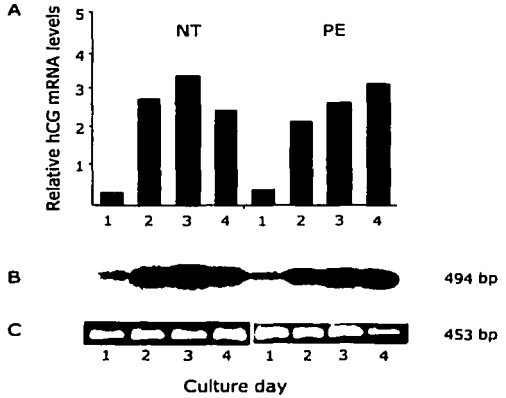

FIG. 2. Temporal pattern of expression of β-hCG mRNA in syncytiotrophoblast cells from NT and PE placentas. Daily, total RNA was obtained from cultured cells and subjected to RT-PCR as described in *Subjects and Methods*. The Southern blot was probed with β-hCG cDNA (B). Control RT-PCR amplifications in the same samples, using cyclophilin, are shown (C). Normalization of relative optical densities of RT-PCR products of β-hCG and cyclophilin are shown in A.

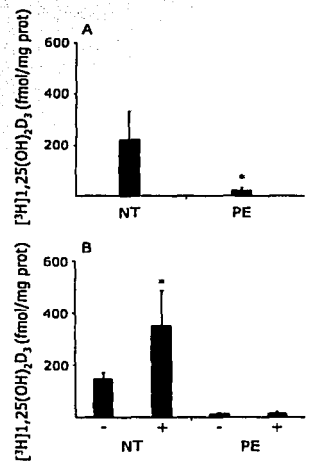

FIG. 3. A, [^{3}H]1,25(OH)$_2$D$_3$ production by syncytiotrophoblast cells cultured from NT and PE placentas. At 72 h after plating, cells were incubated for 1 h in the presence of 3 nM [^{3}H]25(OH)D$_3$, and the conversion products were separated by two-step straight-phase HPLC. Each *bar* represents the mean ± SD of 10 NT and 8 PE placentas, respectively. *, $P < 0.001$ *vs.* NT. B, Effects of IGF-I on [^{3}H]1,25(OH)$_2$D$_3$ production by NT and PE syncytiotrophoblast cultures. Cells were incubated as described above but in the presence of 6.5 nM IGF-I (+) or the vehicle alone (−). *, $P < 0.01$ *vs.* control.

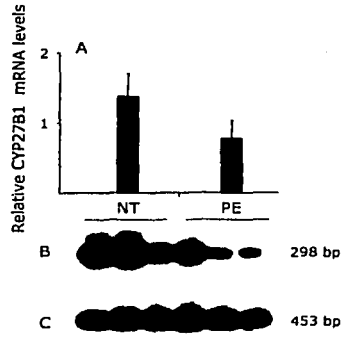

FIG. 4. Expression of 1α-hydroxylase mRNA (CYP27B1) in cultured syncytiotrophoblast cells from NT and PE placentas. Total RNA was obtained from 3-d cultured cells and then subjected to RT-PCR and Southern blot analysis using a specific 1α-hydroxylase cDNA probe (B) and cyclophilin probe (C), respectively. Normalization of relative optical densities of RT-PCR products of 1α-hydroxylase and cyclophilin is shown in A.

even lower in cells from PE placentas (Fig. 5A). IGF-I treatment increased the transcript levels in cultures from both NT and PE placentas; however, PE cultures showed a significantly lower transcriptional response to IGF-I.

IGF-I gene expression in NT and PE syncytiotrophoblast cultures

During differentiation of NT and PE cytotrophoblasts, total RNA was extracted at different times of plating and was used as template to generate a RT-PCR IGF-I 229-bp product. The results in Fig. 6 show that the level of IGF-I gene expression was not different in both NT and PE cultures; suggesting that IGF-I might not be the cause for lower 1α-hydroxylase activity in PE cell cultures. In addition, an increasing expression pattern of IGF-I gene was observed as trophoblast differentiation process progressed, and it was similar in NT and PE cultures. The relative abundance of IGF-I mRNA was established by normalizing the 229-bp band intensity with cyclophilin RT-PCR product (Fig. 6, C and A, respectively).

Discussion

Increase in maternal serum levels of 1,25(OH)$_2$D$_3$ has been considered as one of the mechanisms by which calcium absorption is enhanced during pregnancy (16). Although the factors involved in regulating maternal 1,25(OH)$_2$D$_3$ serum levels remains largely unknown, we have presented evidence that human placenta synthesized the active metabolite of vitamin D$_3$ (7, 10) through gene expression and activation of the cytochrome P$_{450}$ 1α-hydroxylase. It is also well known that circulating levels of 1,25(OH)$_2$D$_3$ are significantly lower in PE women than in normotensive and chronically hypertensive pregnant subjects (3, 4, 6, 17). In addition, these observations strongly raise the possibility that low levels of 1,25(OH)$_2$D$_3$ in PE could be the result of a deficient production of this active metabolite by the placenta.

The results presented herein demonstrated a clear significant difference in the ability of cultured syncytiotrophoblast cells isolated from PE placentas to convert [^{3}H]25(OH)D$_3$ into [^{3}H]1,25(OH)$_2$D$_3$. In all instances, cultures from NT placentas produced significantly higher proportions of 1,25(OH)$_2$D$_3$ than PE cultures, regardless of the time at which syncytiotrophoblast cells were tested. These results agreed also with those, herein presented, on the relative abundance of 1α-hydroxylase mRNA in the same cultured cells. The finding that PE placentas expressed less 1α-hydroxylase mRNA and 1α-hydroxylase activity than NT controls may indicate a specific alteration in placental ability to synthesize adequate amounts of 1α-hydroxylase. Our results showing that trophoblasts from PE pregnancies were able to normally differentiate into syncytiotrophoblasts and produced hCG in response to 8-Br-cAMP, in a manner similar to that of control

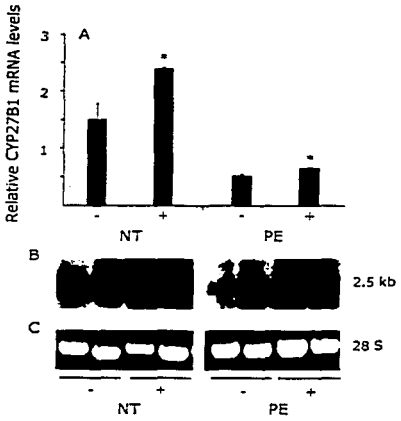

FIG. 5. Effects of IGF-I on 1α-hydroxylase gene expression in NT and PE syncytiotrophoblast cultures. Three-day cultures were incubated as described above, in the absence (-) or presence (+) of IGF-I. After 4 h incubation, total RNA was extracted and hybridized with the [^{32}P]-labeled 1α-hydroxylase cDNA probe (B). 28S ribosomal RNA was used to assess equal loading of placental RNA (C) and for normalization of 1α-hydroxylase mRNA levels (A). *, $P < 0.05$ *vs.* control.

cells, argued against an overall placental restricted metabolic capacity or a deficient cell differentiation process as factors responsible for low 1α-hydroxylase gene expression and enzymatic activity. This observation is in line with previous studies indicating that invasive trophoblast is more likely to be altered in PE than endocrine syncytiotrophoblast (18).

It has been an intriguing possibility that placental production of 1,25(OH)$_2$D$_3$ is altered in PE. Until now, there was no way to differentiate, by the current available methodology, between 1,25(OH)$_2$D$_3$ produced by the kidney *vs.* that produced by the placenta. In this study, we suggest that the etiology of vitamin D metabolic alterations seen in PE may reside, in part, in the placenta and is attributable to a deficient production of 1α-hydroxylase. The results presented herein clearly demonstrated that synthesis and activity of 1α-hydroxylase were indeed significantly restricted in cultured human syncytiotrophoblasts obtained from PE pregnancies. In this regard, PTH and 1,25(OH)$_2$D$_3$ are two of the major physiological factors among those involved in regulating 1,25(OH)$_2$D$_3$ production (19); and, until now, the molecular regulatory mechanisms of this enzyme have been partially clarified (20, 21). Interestingly, studies in humans have clearly indicated that both 1,25(OH)$_2$D$_3$ and IGF-I serum concentrations are significantly lower in PE than in NT pregnancies (6). These studies, linked to others indicating IGF-I as a unique calcium-dependent stimulator of renal (9) and probably placental 1,25(OH)$_2$D$_3$ production (10), prompted us to further investigate whether this factor was involved in the low 1α-hydroxylase activity in PE placentas. Although the role of IGF-II on placental 1α-hydroxylase cannot be ruled out, its relative abundance in the intermediate trophoblast

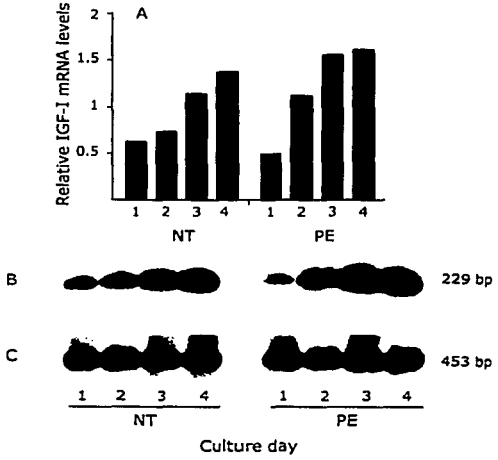

FIG. 6. Temporal expression of IGF-I mRNA in cultured syncytiotrophoblast cells from NT and PE placentas. Daily total RNA was obtained from cultured cells and then subjected to RT-PCR and Southern blot analysis using specific IGF-I probe (B) and cyclophilin (C), respectively. Normalization of relative optical densities of RT-PCR products of IGF-I and cyclophilin is shown in A.

blast, which seemed to increase as the cells invade into the maternal decidua (22), suggests that IGF-II would be involved rather in the process of invasion, growth, and differentiation of the trophoblast.

Expression of IGF mRNAs has been previously demonstrated in human placenta (23, 24). Both IGF-I and IGF-II mRNAs have similar distribution, but IGF-II mRNA is more abundant in placentas obtained at all gestational ages. These data, taken together with those demonstrating the presence of IGF-I receptors on placental membranes (25, 26), suggest an autocrine/paracrine mechanism of IGF-I, to regulate placental growth and metabolism, including 1α-hydroxylase expression and/or activity. Although, in this study, the role of other 1α-hydroxylase regulatory factors in the placenta cannot be ruled out, our results demonstrating that IGF-I expression in PE cultures was similar to that observed in NT cells, suggest that low 1α-hydroxylase expression and activity in PE were probably not the result of alterations in IGF-I locally produced at the placental level. However, an increase in the concentrations of IGF binding protein-1 in maternal serum and at the decidual-trophoblast interface, as described in PE (22), may decrease the bioavailability and biological activity of both systemic and locally produced IGF-I at the placental level. In addition, altered IGF-I and insulin action in erythrocytes of PE patients have been described (27), which may also be of relevance in many of the actions mediated by this growth factor in both maternal and fetal tissues. These observations agreed with our results on the effects of IGF-I on 1α-hydroxylase gene expression and enzyme activity.

On the other hand, it is also plausible that liver, rather than placental IGF-I, could be involved in regulating 1α-hydroxylase in the placenta, because variations of IGF-I in maternal serum during pregnancy parallel the changes in IGF-I mRNA in the liver (28). In addition, NT expression of IGF-I mRNA in placental cultures from PE pregnancies, during the time of syncytiotrophoblasts forming was coincident with changes in cell morphology and hormone secretion accompany trophoblast differentiation.

It is well known that IGF-I is primarily regulated by pituitary GH (29); however, during pregnancy, GH synthesis by the pituitary is inhibited, placental GH being the primary GH species in the maternal circulation (30). In PE, where placental invasion is compromised, it is possible that placental GH synthesis is also affected, thereby resulting in decreased liver IGF-I production. The consequence of reduced placental 1,25(OH)$_2$D$_3$, most probably as the result of the low plasma levels of IGF-I in PE, is still unclear. However, the presence of 1,25(OH)$_2$D$_3$ receptors in the human placenta (31) suggests the involvement of 1,25(OH)$_2$D$_3$ in the process of transport of calcium across the placenta. Indeed, the placenta is able to transport calcium actively even in the absence of fetal parathyroid glands (32), a mechanism that is most probably regulated by the locally produced 1,25-(OH)$_2$D$_3$ or other factors, such as PTHrP (33).

In summary, our results gave further support suggesting the placenta as a contributor of 1,25-(OH)$_2$D$_3$ during pregnancy. In addition, cultured syncytiotrophoblast cells from PE placentas expressed less 1α-hydroxylase mRNA and 1α-hydroxylase enzymatic activity than NT placental cells. These results may provide an explanation for a number of metabolic alterations in PE associated with changes in 1,25-(OH)$_2$D$_3$ production. Further studies on the elucidation of the mechanisms involved in the regulation of extrarenal 1,25-(OH)$_2$D$_3$ production will be of importance to our understanding of the tissue-specific functions of 1α-hydroxylase.

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Address all correspondence and requests for reprints to: Fernando Larrea, M.D., Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, México Distrito Federal, 14000, México. E-mail: larrea@sniconacyt.mx.

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Identification of a 25-Hydroxyvitamin D_3 1α-Hydroxylase Gene Transcription Product in Cultures of Human Syncytiotrophoblast Cells*

LORENZA DÍAZ, IRENE SÁNCHEZ, EUCLIDES AVILA, ALI HALHALI, FELIPE VILCHIS, AND FERNANDO LARREA

Department of Reproductive Biology, Instituto Nacional de la Nutrición Salvador Zubirán, México City 14000, México

ABSTRACT

Although accumulating data show that placenta is able to synthesize 1,25-dihydroxyvitamin D_3, the presence of cytochrome P_{450} enzyme capable of converting 25-hydroxyvitamin D_3 ($25OHD_3$) to the biologically active form of vitamin D in this tissue, has not been yet clearly established. In this study, we have investigated the presence of 25-hydroxyvitamin D_3 1α-hydroxylase (1α-(OH)ase) gene expression products in cultured human syncytiotrophoblast. Total RNA was isolated from cultured placental cells and subjected to Northern blots or RT-PCR by using 1α-(OH)ase-specific primers. The amplified complementary DNA fragments were analyzed by gel electrophoresis and nucleotide sequencing. Total RNA from kidney HEK 293 cells was subjected to reverse transcriptase reaction, and a 298-bp complementary DNA 1α-(OH)ase probe was generated by PCR. Primary cultures of human syncytiotrophoblasts exhibited 1α-(OH)ase activity, and a transcript for this gene could be demonstrated in these cells. Northern blot analysis revealed the presence of a 2.5-kb product, similar in size to that previously reported in kidney. RT-PCR analysis demonstrated the presence of a single transcript with nucleotide sequence identical to that previously reported for human 1α-(OH)ase complementary DNA clones. In addition, data are presented which suggest that differentiation of cytotrophoblast to the syncytial state was not necessary for this gene to be expressed, which may indicate a role of this enzyme all through pregnancy. The overall results of this study provide evidence for the presence of 1α-(OH)ase in the human placenta, suggesting that conversion of $25OHD_3$ to 1,25-dihydroxyvitamin D_3 in the trophoblast is most probably attributed to an enzymatic 1α-hydroxylation reaction. (*J Clin Endocrinol Metab* 85: 2543–2549, 2000)

THE KIDNEY represents the main source of circulating 1,25-dihydroxyvitamin D_3 (1, 25-$(OH)_2D_3$) or calcitriol (1, 2), the most potent naturally occurring metabolite of vitamin D_3. Synthesis of calcitriol is the result of a renal 25-hydroxyvitamin D_3 1α-hydroxylase (1α-(OH)ase), a mitochondrial cytochrome P_{450} enzyme, with a key role in calcium homeostasis. The first observation leading to the establishment of an extrarenal source of 1α-(OH)ase was in pregnant rats, where bilateral nephrectomy reduced, but did not completely eliminate, the serum concentrations of 1,25-$(OH)_2D_3$ (3). In fact, it has been shown that decidual cells represent a site of calcitriol synthesis during pregnancy (4). Although *in vitro* studies provided evidence that, in addition to human decidua, human and rodent placental trophoblasts produced 1,25-$(OH)_2D_3$ (3, 4), a number of investigators have been unable to demonstrate a consistent and detectable production of calcitriol by these cells (5, 6). Similarly, Hollis *et al.* (7) have suggested that 1,25-$(OH)_2D_3$ produced by human placenta, under *in vitro* conditions, is the result of a free radical chemistry, rather than an enzymatic-driven 1α-hydroxylation reaction. In contrast, and in agreement with earlier observations (8, 9), we have recently shown (10) that cultured human syncytiotrophoblast cells were able to produce 1,25-$(OH)_2D_3$ when incubated in the presence of physiological concentrations of 25-hydroxyvitamin D_3 ($25OHD_3$). This conversion was significantly stimulated, in a dose-dependent manner, by the presence of the insulin-like growth factor I (IGF-I) and inhibited with the protein synthesis inhibitor cycloheximide, suggesting the existence of a local protein-dependent regulatory effect. Taken together, these data suggest that human placenta is able to synthesize 1,25-$(OH)_2D_3$ from its endogenous precursor by an enzymatic 1α- hydroxylation mechanism. These findings are of importance, because 1α-(OH)ase gene expression has not yet been detectable in the human placenta (11–13). Nowadays, there is little, if any, information on the molecular mechanisms underlying placental 1,25-$(OH)_2D_3$ production and its hormonal regulation, including the understanding of its biological significance. Herein, we report the presence, in cultured human syncytiotrophoblast cells, of a 1α-(OH)ase gene transcription product with nucleotide sequence identical to that of transcripts previously characterized in the human kidney.

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Address correspondence and requests for reprints to: Fernando Larrea, M.D., Department of Reproductive Biology, Instituto Nacional de la Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, México 14000, México D.F. E-mail: larrea@mailer.main.conacyt.mx.

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Materials and Methods

Materials

DMEM and DMEM-F12, HBSS, FCS, HEPES, streptomycin, and Fungizone were obtained from Life Technologies, Inc (Grand Island, NY).

Percoll, 8-bromo adenosine 3',5'-cyclic monophosphate (8-Br-cAMP), deoxyribonuclease I, BSA, glutamine, cycloheximide, and trypsin were purchased from Sigma (St. Louis, MO). All solvents [high-pressure liquid chromatography (HPLC) grade] were obtained from Merck & Co., Inc. (Darmstadt, Germany). Unlabeled authentic 25OHD$_3$ and 1,25-(OH)$_2$D$_3$ were a generous gift from Dr. E. M. Gutknecht and Dr. P. Weber (F. Hoffmann-La Roche LTD, Basel, Switzerland). The 25-hydroxy-[26,(27)-methyl-^{3}H]cholecalciterol ([^{3}H]25-(OH)D$_3$; SA, 17 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). NIDDK (Rockville, MD) kindly provided human CG (hCG) RIA. All other reagents were of analytical grade.

Tissue preparation and cell culture

The study protocol was approved by the Human Ethical Committee of the Institute. Term placentae (38–42 weeks of gestation) were obtained from normal pregnant women after spontaneous vaginal delivery. Tissues were brought immediately to the laboratory, where several cotyledons were removed and rinsed thoroughly in 0.9% NaCl at room temperature. The isolation and culture of cytotrophoblasts was performed as described by Kliman *et al.* (14). Briefly, soft villous tissue (30 g), free of connective tissue and vessels, was collected. Tissue was coarsely minced and digested with 0.125% trypsin and 0.2 mg/mL deoxyribonuclease I (1, 500 Kunitz units/mg) in warmed calcium- and magnesium-free HBSS, containing 25 mmol/L HEPES (pH 7.4), for 30 min at 37 C. Cell suspensions were pooled, centrifuged at 1000 × *g* for 10 min, and resuspended in DMEM containing 25 mmol/L HEPES and 25 mmol/L glucose (DMEM-HG). The resultant cell suspension was placed on 5–70% Percoll (vol/vol) gradients made up in HBSS. Gradients, which consisted of 5% steps of 3 mL each, were centrifuged at 1200 × *g* at room temperature for 20 min. After centrifugation, the middle band (density, 1.048–1.062), containing the cytotrophoblasts, was removed, washed once with DMEM-HG, and resuspended in medium for tissue culture. Percoll gradient purified cytotrophoblasts were diluted to a concentration of 2×10^6 cells/mL with DMEM-HG containing 4 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg Fungizone/mL, and 20% heat-inactivated FCS, plated in 35-mm Nunclon culture dishes (Nunc, Roskilde, Denmark), and incubated in humidified 5% CO_2 95% air at 37 C. After 2 days in culture, or otherwise indicated, cells were incubated in serum-free DMEM-F12 with low calcium and phosphate concentrations.

Daily, the morphological aspects of cell cultures were examined. Human chorionic gonadotropin in the culture media was measured, as previously described (15), by specific RIA using reagents and protocols provided by the NIDDK. Anti-hCG-1480, at a final working dilution of 1:150 000, was used as antiserum. This antiserum exhibits 1.2 and 3.2% cross-reactivities with free hCG α- and β-subunits, respectively. The sensitivity of the assay was 0.025 ng/tube, and the inter- and intraassay coefficients of variation were < 10 and < 6%, respectively. Total protein content of cell cultures was measured by the method of Lowry *et al.* (16) using BSA as standard.

Complementary DNA (cDNA) synthesis and PCR amplifications

Total RNA was isolated from cultured syncytiotrophoblast cells, as described by Chomczynski and Sacchi (17). One μg of total RNA was used as template for cDNA synthesis using the SuperScript preamplification system (Life Technologies). PCR amplification was then performed using *Taq* polymerase and the following primers: 5'-ACGCTGTTGACCATGGC-3' for the sense primer, and 5'-GTGACACAGAGTGACCAGCGTAT-3' for the antisense primer. These primers generated a 543-bp 1α-(OH)ase RT-PCR product. The PCR products were resolved on agarose gels, eluted, subjected to further amplification, and purified through Centricon-30 membranes (Amicon, Beverly, MA) for sequence analysis. To monitor efficiency for RT reaction, we used, as a control, the amplification of the ubiquitous protein cyclophilin with the following sense and antisense primers: 5'-CCCCACCGTGTTCTTCGACAT-3', and 5'-ACGTCCTTACCGTTCTGGTCG-3' which yielded a 453-bp RT-PCR product. All oligonucleotides were synthesized in a DNA synthesizer Model 391 (PE Applied Biosystems, Perkin-Elmer Corp. Cetus Co., Norwalk, CT). PCR amplifications were performed on a Perkin-Elmer Corp. Cetus 9600 DNA Thermal Cycler using the following program: a denaturation step at 94 C for 1 min, followed by 30 cycles at 94 C for 50 sec, 60 C for 50 sec, and 72 C for 1 min. Finally, a 7-min extension period at 72 C was performed. Incubations, in the absence of reverse transcriptase, were used as controls for RT-PCR. The bands of predicted size (543-bp) were also confirmed as human 1α-(OH)ase, by Southern blot analysis of previously separated DNA on 1.2% agarose gels with a human 1α-(OH)ase cDNA probe (298-bp) radiolabeled with [^{32}P]deoxycytidine triphosphate ([^{32}P]dCTP) by random priming. This probe was obtained from human embryonic kidney cells (HEK 293, ATCC CRL-1573, Microbix Biosystems, Ontario, Canada) by RT-PCR, as described above, using the following sense and antisense primers: 5'-GTTCGCTATTGCCGCAGTGGAC-3' and 5'-GTGACACAGAGTGACCAGCGTAT-3', respectively.

Sequence analysis

Both DNA RT-PCR strands from human placenta and kidney cells were sequenced by ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Corp., Foster City, CA) with 25 cycles at 96 C for 10 sec, 50 C for 5 sec, and 60 C for 4 min on a Perkin-Elmer Corp. Cetus 9600 DNA Thermal Cycler. Then samples were dried in a speed-vac, resuspended in sequence loading buffer, denatured at 95 C for 5 min, and loaded on a 4.75% polyacrylamide gel. The sequence determination was carried out using the DNA sequencer model 373–01 (PE Applied Biosystems-Perkin-Elmer Corp.).

Characterization of the 1α-(OH)ase messenger RNA (mRNA)

For Northern blots, 30 μg of total cellular RNA was size-fractionated on a 1.2% formaldehyde-agarose gel. After electrophoresis, RNA was transferred into Zeta probe membranes (Bio-Rad Laboratories, Inc. New York, NY) by capillary diffusion, fixed by UV cross-linking, and probed with the 298-bp [^{32}P]dCTP-labeled cDNA fragment obtained from human HEK 293 cells. After prehybridization for 1 h at 68 C, the radioactive probe was added and hybridized in 0.25 mol/L Na_2HPO_4 and 7% SDS, during 18 h at 68 C.

Activity of 1α-(OH)ase in human placental cell cultures

To assess the ability of syncytiotrophoblast cells to convert 25OHD$_3$ into 1,25-(OH)$_2$D$_3$, we carried out experiments in incubations of placental cells on the third day of culture. At this time, medium was changed, and cells were incubated in 2 mL serum-free medium (DMEM-F12) in the presence of [^{3}H]25OHD$_3$ at a final concentration of 5 nmol/L, during 120 min. Culture medium was then transferred to glass tubes, and cells were washed with 1 mL methanol. Protein cell content was determined after addition of 0.5 mL of 1 mol/L NaOH. The [^{3}H]25OHD$_3$ and its metabolites were extracted from the medium with an additional 3 mL methanol, followed by 4 mL chloroform (10, 18). The chloroform phase was dried down under N_2, and lipidic extracts were redissolved in chromatographic solvent. The samples were cochromatographed with 0.1 μg unlabeled authentic 1,25-(OH)$_2$D$_3$ as elution marker on a Waters HPLC fitted with a photodiode array detector (PDA; model 996; Waters Corp. Associates, Milford, MA), using an ultrasphere Si, 5 μm, 4.6 × 250 mm column (Beckman Coulter, Inc., Palo Alto, CA). Two-step straight-phase HPLCs were used to separate the vitamin D$_3$ metabolites (10, 18). The conversion rate of [^{3}H]25OHD$_3$ into putative [^{3}H]1,25-(OH)$_2$D$_3$ was determined by calculating the percentage of radioactivity coeluting with authentic unlabeled 1,25-(OH)$_2$D$_3$ after the two successive HPLCs. Results were expressed as fmol/mg protein.

Statistical analysis

Data are presented as the mean ± SD. All experiments were performed at least three times. Statistical significance was established using Student's *t* test. $P \leq 0.05$ was considered statistically significant.

Results

Morphological and functional aspects of placental cell cultures

Microscopic examination of cell cultures showed that, within 3 days after plating, the cultured cytotrophoblasts

formed cell aggregates conformed mostly (99%) of larger areas containing multiple nuclei. In addition, single mononuclear cells were not observed at this time of culture. By day 3, multinucleated cells seemed to form a network (Fig. 1) that corresponded to functional syncytiotrophoblasts, in terms of their ability to secrete placental hCG. Figure 1 shows the data of three experiments on the temporal pattern of hCG release from cultured trophoblast cells. In each case, little or no detectable hormone was present in the culture medium during the first day of plating, regardless of the presence of 8-Br-cAMP. A detectable and significant ($P < 0.01$) increase in the level of hCG was observed by day 2 in 8-Br-cAMP-stimulated cultures, reaching peak values between days 3 and 4 and then decreasing daily throughout the remaining days. As can be seen, addition of 8-Br-cAMP significantly increased ($P < 0.001$) hCG secretion, when compared with cultures in the absence of the cyclic nucleotide analogue.

Expression of placental 1α-(OH)ase mRNA by syncytiotrophoblast cells

Because initial attempts to identify placental 1α-(OH)ase gene products from total RNA isolated from fresh placental tissue were unsuccessful, we decided to use Percoll gradient-purified cytotrophoblast cells kept in culture as a source of placental RNA. Gene expression was evaluated by Northern blot analysis using a 298-bp cDNA fragment for 1α-(OH)ase gene obtained from kidney HEK 293 cells, as described under *Materials and Methods* and shown in Fig. 2A. Figure 2B shows a Northern blot analysis of total cellular RNA isolated on day 3 of culture. From the Northern blot analysis, a signal that corresponds to 1α-(OH)ase mRNA was found in the syncytiotrophoblast cells (lanes 2–4). A similar-sized (2.5-kb) 1α-(OH)ase transcript was found in kidney HEK 293 cells (lane 1). Decidua total RNA was probed and used as positive control (lane 5). RT-PCR and DNA sequencing further confirmed these results.

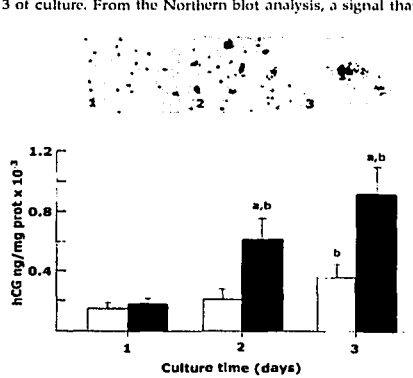

FIG. 1. hCG secretion by trophoblast cells in culture. At the designated times, medium from cultures incubated in the presence (■) or absence (□) of 8-Br-cAMP was removed and assayed for hCG, as described in *Materials and Methods*. *Bars* represent the mean ± SD of three separated cultures. The *top panel* shows the *in vitro* differentiation of cytotrophoblasts. Cells were fixed at the first, second, and third day of culture, respectively, and stained with hematoxylin. *a*, $P < 0.001$ *vs.* without 8-bromo-cAMP; b, $P < 0.01$ *vs.* day 1.

cDNA synthesis and PCR amplifications

RT-PCR was performed using primers based on human P_{450} 1α-(OH)ase cDNA sequence (11). RT-PCR of RNA from syncytiotrophoblast and HEK 293 cells yielded, on Southern blots, a single cDNA band of the expected size (543-bp) for the oligonucleotide primers used (Fig. 3, lanes 2 and 4, respectively). In the absence of RT, none of the RNA samples from syncytiotrophoblast cells subjected to PCR for 1α-(OH)ase gave positive results (Fig. 3, lane 3). Similar results were obtained when human genomic DNA was used instead of RNA (Fig. 3, lane 1).

DNA products generated by PCR were purified, amplified, and sequenced. As shown in Fig. 4, the nucleotide sequence of the 543-bp fragment from syncytiotrophoblast cells was found to be 100% identical, over all the nucleotides flanked by the PCR primers, to the nucleotide sequences of human 25-hydroxyvitamin D_3 1α-hydroxylase cDNA clones contained in GeneBank [accession numbers AB005038 (11), AF020192 (12), and AB005989 (13)]. The complete 1α-(OH)ase cDNA sequence consists of 2469 bp, so the fragment reported herein, in the placenta, is located within 706- and 1248-bp sections of the full-length cDNA. Identical results were obtained when the 543-bp RT-PCR fragment from HEK 293 cells was sequenced (data not shown).

Temporal expression of 1α-(OH)ase mRNA and enzyme activity in syncytiotrophoblasts cultures

To ascertain the pattern of expression of 1α-(OH)ase mRNA throughout culture, total cellular RNA was obtained at different times from plating (24, 48, 72, and 96 h) and prepared for RT and PCR amplifications using specific primers. Total RNA was also extracted from mononuclear Percoll gradient-purified cytotrophoblast cells and subjected to RT-PCR with the same set of primers (time zero of culture). As shown in Fig. 5, it was demonstrated that 1α-(OH)ase mRNA is expressed at all culture times studied, including those in the less-differentiated Percoll gradient-purified cytotrophoblast cells taken as representatives of day zero of culture. In addition, relative abundance of 1α-(OH)ase mRNA was obtained by normalizing the 543-bp band intensity (Fig. 5B) with that generated for the constitutive gene cyclophilin (Fig. 5C). Despite the absence of an apparent temporal pattern of expression of 1α-(OH)ase mRNA throughout culture (Fig. 5A), Northern blots of total cellular RNA, obtained at different days of plating, showed a gradual increase of expression, up to 96 h of culture (Fig. 6A). This difference may be explained by the fact that nonquantitative RT-PCR was used for temporal mRNA expression in cultured placenta.

Activity of 1α-(OH)ase was assessed in placental cultures by their ability to convert [^{3}H]25OHD$_3$ into [^{3}H]1,25-(OH)$_2$D$_3$. Cells at various times of plating were incubated in the presence of substrate. After 2 h of incubation, analysis of

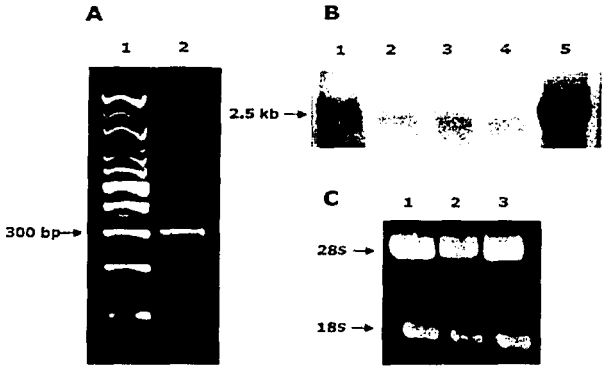

FIG. 2. A, RT-PCR analysis of 1α-(OH)ase transcripts in human embryonic kidney HEK 293, as described in *Materials and Methods*. The resulting DNA product was resolved by electrophoresis, on an agarose gel, and was stained with ethidium bromide. After nucleotide sequencing, this 298-bp cDNA was radiolabeled with [^{32}P]dCTP and used as probe (lane 2). The DNA size markers are shown in lane 1. B, Northern blot analysis of 1α-(OH)ase mRNA from HEK 293 cells (lane 1), syncytiotrophoblast cells (lanes 2–4), and decidua (lane 5) after hybridization with P^{32}-labeled 1α-(OH)ase 298-bp cDNA. C, Ethidium bromide-stained gel. Thirty micrograms of total RNA from HEK 293 cells (lane 1), cytotrophoblast cells (lane 2), and decidua (lane 3) were loaded onto each lane.

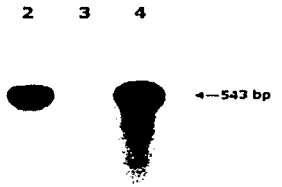

FIG. 3. RT-PCR analysis of 1α-(OH)ase mRNA in syncytiotrophoblast cells. Total RNA obtained from 3-day cultures was reverse-transcribed to cDNA. PCR amplifications were performed using specific 1α-(OH)ase primers in samples containing human genomic DNA (lane 1), cDNAs from syncytiotrophoblast and HEK 293 cells (lanes 2 and 4, respectively), and RNA in the absence of RT (lane 3). The resulting cDNA products were resolved by electrophoresis, on an agarose gel, and blotted. The Southern blot was probed with the human 1α-(OH)ase 298-bp cDNA under high-stringency conditions.

samples, using two-step straight-phase HPLC, showed the presence of a more polar metabolite that coeluted as a single peak with authentic unlabeled 1,25-$(OH)_2D_3$ (data not shown). As depicted in Fig. 6B, the amount of putative [^{3}H]1,25-$(OH)_2D_3$ formed by syncytiotrophoblast cells significantly increased ($P < 0.001$) at the second and third day of culture.

Discussion

The finding that anephric pregnant rats (3) and nephrectomized nonpregnant subjects (19) have detectable levels of serum calcitriol, suggested an independent vitamin D_3 metabolism in extrarenal tissues. In this regard, several studies have identified the human foreskin keratinocytes (20), lymphohematopoietic cells (21), and placenta (4, 5, 8, 10) as sources of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ synthesis. We have recently shown that human syncytiotrophoblast cells in culture were able to synthesize calcitriol from 25OHD_3 (10). This conversion was significantly enhanced by IGF-I and blocked by the protein synthesis inhibitor cycloheximide, which suggested a hormonally regulated protein-dependent hydroxylation reaction. Although it is not possible to ascertain whether calcitriol production by the placenta is, in part, dependent on a free radical chemistry reaction, as suggested by Hollis *et al.* (7), the importance of placental contribution to 1,25-(OH)2D3 increase observed during pregnancy (22–24) and/or its involvement in the transport of calcium across the fetoplacental unit has been suggested (25).

To prove that the P450 1α-(OH)ase gene is expressed in the human placenta, we sought to investigate the presence of P450 1α-(OH)ase mRNA in cultures of human syncytiotrophoblasts obtained from normal term placentas. This culture system has extensively been proven to form functional syncytiotrophoblasts free of mononuclear fibroblast cells (14, 16). RT-PCR was used in this study because the activity and, probably, the content of mRNA of placental 1α-(OH)ase, as in the case of kidney, are very low. In fact, in recent communications from two laboratories (11, 12), expression of this gene could not be detected by Northern blots using total RNA isolated from fresh human placental tissue. Herein, we report the presence of a transcriptional product of 1α-(OH)ase in cultures of human syncytiotrophoblast cells with a nucleotide sequence identical to human kidney 1α-(OH)ase (11).

Expression of human and rat P_{450} 1α-(OH)ase cDNA in mammalian cells has been reported (12, 26). Transfection of a plasmid expressing the full-length cDNA into cultured mouse Leydig MA-10 and monkey kidney COS-7 cells re-

706-acg ctg ttg acc atg gcg atg ccc cac tgg
ctg cgc cac ctt gtg cct ggg ccc tgg ggc cgc ctc
tgc cga gac tgg gac cag atg ttt gca ttt gct cag
agg cac gtg gag cgg cga gag gca gag gca gcc
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gag tct ggg gcg cac ctg acc cac ttc ctg ttc cgg
gaa gag ttg cct gcc cag tcc atc ctg gga aat gtg
aca gag ttg cta ttg gcg gga gtg gac acg gtg tcc
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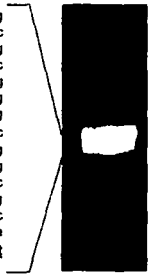

FIG. 4. Nucleotide sequence of the 543-bp RT-PCR product in syncytiotrophoblast cells. The RT-PCR product, generated as described in *Materials and Methods*, was subjected to DNA sequence analysis by the dideoxy chain termination method. The positions of primers are *highlighted* and *underlined*. *Flanking numbers* correspond to nucleotide positions relative to the transcriptional start site.

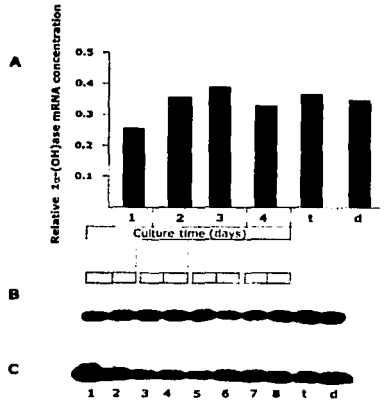

FIG. 5. Temporal pattern of expression of 1α-(OH)ase mRNA. Total RNA, obtained from cells cultured at the designated times or from mononuclear Percoll gradient-purified cytotrophoblasts (t) and decidual tissue (d), was reverse-transcribed, and cDNAs were amplified by PCR. The RT-PCR products were resolved by electrophoresis, on an agarose gel, and blotted. The Southern blot was probed with the human 1α-(OH)ase 298-bp cDNA under high-stringency conditions (B). Control RT-PCR amplifications in the same samples, using cyclophilin, are shown in the *bottom panel* (C). Normalization of relative optical densities of RT-PCR products, obtained with primers for 1α-(OH)ase and cyclophilin in syncytiotrophoblast cells, respectively, is shown in the *top panel* (A). Cultures were analyzed at the end of the first, second, third, and fourth days of plating per duplicate (lanes 1–8, respectively).

sulted in a marked 1α-(OH)ase activity, thus providing evidence that cloned cDNA encoded the P450 1α-(OH)ase, with robust enzymatic activity. The gene for human 1α-(OH)ase spans approximately 6 kb, is composed of nine exons, and is present in a single copy (11). In addition, it shares a relatively high homology with vitamin D_3 25-(OH)ase, and the deduced amino acid sequence shows 82% homology with the rat enzyme (26). Interestingly, although there have been few reports on the purification and antibody preparation against 1α-(OH)ase (27), there is not yet a subsequent definitive structural protein characterization, and availability of specific antibodies against human kidney 1α-hydroxylase is still lacking.

In this study, analysis of temporal expression of 1α-(OH)ase mRNA in cultured syncytiotrophoblast cells revealed the presence of a single expected-size RT-PCR product in either Percoll gradient-purified trophoblasts or in cultured differentiated syncytiotrophoblast cells. This finding indicates that, in this *in vitro* system, differentiation to a syncytial state was not necessary for this gene to be expressed, and this suggests a possible role of this enzyme all through pregnancy. Similar observations have been previously reported for other well-characterized trophoblast products under the same culture conditions (14). In addition, the expression of mRNA for human 1α-(OH)ase, determined by Northern blot analysis from different sources, demonstrated a major transcript of approximately 2.5 kb in kidney and decidua. Furthermore, Northern blot analysis of cultured syncytiotrophoblasts mRNA revealed a single transcript of similar size as, but in a considerably lower amount than, the one found in both human kidney and decidua. These findings agree with previous reports on the size, tissue distribution, and abundance of 1α-(OH)ase mRNA (11, 12), allowing us to establish, for the first time, the presence of an mRNA transcript of this enzyme in the human trophoblast, similar in size to that previously described in the human kidney. Furthermore, these observations strongly support the concept that, as in the case of kidney and decidua, the placenta enzyme is also encoded by the same gene.

Although transcriptional regulation of the 1α-(OH)ase gene has not been investigated in placental cells, it is possible, as reported recently (10), that expression of this gene in the placenta could be regulated similarly to the one present in the kidney. Thus, vitamin D status and those factors known to

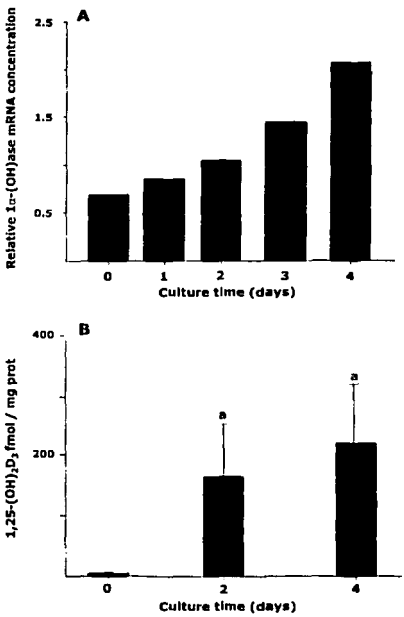

FIG. 6. A, Relative 1α-(OH)ase mRNA concentration in cultured placenta. At the end of each culture time, 1α-(OH)ase mRNA levels were determined by Northern blot hybridization. 28S ribosomal RNA was used to assess equal loading of placental RNA. The relative 1α-(OH)ase mRNA concentration on the autoradiograms was analyzed by densitometer and normalized with the 28S ribosomal RNA band. B, [^{3}H]1,25-$(OH)_2D_3$ production by syncytiotrophoblast cells at different times of culture. Cells were incubated for 2 h in the presence of 5 nmol/L [^{3}H]25OHD_3 and the conversion products were separated by two-step straight-phase HPLC, as described in *Materials and Methods*. Each *bar* represents the mean ± SD of three independent cultures. a, $P < 0.001$ *vs.* time zero.

influence the enzyme expression and activity in the kidney should be considered among potential candidates in regulating also placental 1α-(OH)ase. In addition, expression of 1α-(OH)ase mRNA in trophoblast and decidual cells, together with the recent purification and characterization of 1,25-$(OH)_2D_3$ receptor from human placenta (28), may suggest a local-tissue-specific function of calcitriol during pregnancy. Thus, the biological function of 1,25-$(OH)_2D_3$ in the fetoplacental unit may be considered either endocrine or autocrine/paracrine in nature, depending on its site of synthesis. Furthermore, that placenta contributes to 1,25-$(OH)_2D_3$ serum concentrations during pregnancy is derived from a number of case reports in patients with pseudohypoparathyroidism (PsHP) who remained normocalcemic without calcitriol treatment during pregnancy (29). These observations, taken together with those demonstrating that placental synthesis of 1,25-$(OH)_2D_3$ is not affected in patients with PsHP (30), may indicate that calcitriol treatment in both PsHP and hypoparathyroid patients during pregnancy should be adapted to physiological needs, to keep calcium levels in the normal range, as previously reported (31–33).

Inasmuch as the results presented herein should be only interpreted as indicating that human placenta expresses 1α-(OH)ase mRNA, the *in vitro*-observed 1α-hydroxylase activity in this and other studies may, in part, contribute to establish the local production of the protein. In addition, the overall data support and extend recent observations from our laboratory, suggesting that conversion of [^{3}H]25OHD_3 to [^{3}H]1,25-$(OH)_2D_3$ in cultured placenta is attributed to an enzymatic 1α-hydroxylation reaction.

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FELLOWS CORNER

Vitamin D: Implications in health and pregnancy

Lorenza Díaz*, Cecilia Cariño*, Isabel Méndez*

* Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Department of Reproduction Biology.

RESUMEN

La vitamina D cobró importancia desde que se descubrió la naturaleza esteroidea y carácter hormonal de uno de sus metabolitos: el calcitriol. Su participación en el mantenimiento de la homeostasis mineral, así como su capacidad para regular la transcripción génica y fomentar la diferenciación celular, han hecho de su estudio un tema de gran interés. Los recientes avances en el estudio de la enzima encargada de convertir la 25-hidroxivitamina D en 1,25-dihidroxivitamina D_3 (calcitriol), así como la descripción de su mecanismo de acción, han permitido ampliar el conocimiento de los factores reguladores que controlan el equilibrio del sistema endocrino de la vitamina D, y de las implicaciones del calcitriol en la salud en general y en el embarazo en particular.

Palabras clave. Placenta. *CYP27B1*. 1,25-$(OH)_2D_3$. 25-OH-D-1α hidroxilasa. Embarazo.

ABSTRACT

Vitamin D gained importance since the discovery of its steroid structure. Vitamin D participates in mineral homeostasis, regulation of gene expression, and cell differentiation. Recent advances in the study of the enzyme involved in the conversion of 25-hydroxyvitamin D_3 into 1,25-dihydroxyvitamin D_3 (calcitriol), as well as the discovery of it's hormone mechanism of action, have led to a better knowledge and understanding of vitamin D endocrine system, as well as it's implication in health and pregnancy.

Key words. *Placenta. CYP27B1. 1,25-$(OH)_2D_3$. 25-OH-D-1-α hydroxylase. Pregnancy.*

FORMATION AND EFFECTS OF VITAMIN D

The generic term vitamin D (VD) refers to a group of steroids characterized by an open B-ring in the steroid nucleus. Vitamin D occurs in blood in two forms: vitamin D_2 or ergocalciferol (VD_2), which is of plant source, and vitamin D_3 or cholecalciferol (VD_3), synthesized by the skin through UV radiation from the light. The former may also be obtained through the diet with fish liver oil, liver and eggs. VD (VD without sub-index it refers to both forms D_2 and D_3), which was discovered in 1920 by Mellanby,[1] was initially classified as a vitamin since it was known to play an essential role in skeleton formation, and to be obtained solely from food. At the beginning of the 1930's, however, Windaus[2] and Brockmann[3] definitely determined the chemical structure of VD, which allowed the demonstration of its steroid nature, placing it in the steroid hormones group. In fact, Windaus received the Chemistry Nobel Prize in 1928 for his studies on the VD structure, allowing their appliance in treatment of diseases such as rachitism and others due to deficiencies of this vitamin.

The VD structure consists of the cyclopentanoperhydrophenantrene nucleus of the steroid hormones, with an open B-ring between carbons 9 and 10, allowing its identification as secosteroid. VD conserves 8 carbons of the cholesterol lateral chain, in contrast with other steroid hormones (estradiol, testosterone), which do not have lateral chain. The difference between VD_2 and VD_3 is that the first has a methyl group in carbon 24 and a double bond between carbons 22 and 23 (Fig. 1).

98

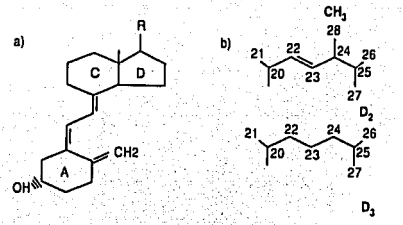

Figura 1. a) Chemical structure of vitamin D. b) Aliphatic group (R) that characterizes one of the two forms D_2 or D_3.

Formation and Metabolism of Vitamin D

The skin synthesizes VD from 7-dehydrocholesterol. Ultraviolet radiation breaks the B-ring forming an unstable intermediary (previtamin D), which through a photothermal process bio-transforms into cholecalciferol, which is transported in the blood, bonded to its carrier protein, to the liver, where it is hydroxylated at carbon 25 transforming into 25-hydroxyvitamin D_3 (25-OH-D).[4] This metabolite, although most abundant in plasma, does not represent the active form. In the kidney, 25-OH-D is hydroxylated at carbon 1 producing the hormonal form: 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (Fig. 2).

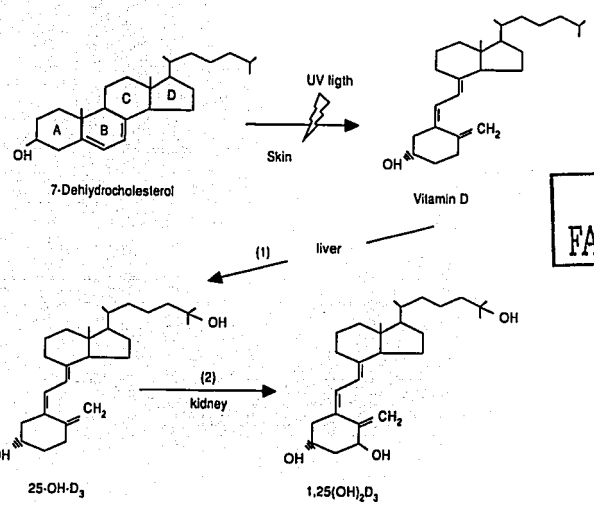

Figura 2. Synthesis of 1,25$(OH)_2D_3$ from 7-dehydrocholesterol. Light breaks B ring to form vitamin D, which becomes 25-OH-D_3 after a first hydroxylation relation in the live, by the 25-hydroxylase (1). The active form is obtained after a second hydroxylation reaction in the kidney by the 25-OH-D-1α hydroxylase (2).

99

Vitamin D Endocrine System

The endocrine nature of vitamin D was determined at the beginning of the 1970's based mainly on the discovery of nuclear receptors for 1,25-$(OH)_2D_3$ in the intestine,[5] and on the understanding of the role of the kidney in the production of this hormone, as well as on it's regulation by the parathyroid hormone (PTH). The vitamin D endocrine system is based on the fact that 1,25-$(OH)_2D_3$ is a hormone synthesized in the body, transported by blood and activated in certain organs, and has biological effects interacting with its specific receptors. The vitamin D endocrine system function depends then, on three main elements: occurrence of the respective cytochromes P450 in liver and kidney to bio-transform 25-OH-D into its active metabolite (1,25-$(OH)_2D_3$), occurrence of transport proteins to move these hydrophobic molecules to their target organs, and existence of specific receptors in a number of tissues. At present we know that 1,25-$(OH)_2D_3$ bonds to its receptors located in various target cells, and that its production and degradation are processes regulated by feedback mechanisms resulting from ionic (Ca++, P), polypeptidic (PTH, calcitonine), and steroid (1,25-$(OH)_2D_3$) factors. The main target organs of calcitriol are kidney, bone and intestine, and its main physiological functions are to maintain adequate concentration of calcium and phosphorus, and bone mineralization.

Mechanism of Action and Functions of Vitamin D

We currently know that calcitriol is not limited to the above mentioned functions, and that it is a very versatile hormone, capable of generating biological responses through genomic (intracellular receptors), or non-genomic (membrane receptors) pathways. In the first pathway, nuclear or cytoplasm receptors participate: once the receptor has been activated by the hormone, it dimerizes with another nuclear receptor known as retinoid X receptor (RXR), and the resulting heterodimer bonds to vitamin D response elements (VDRE) in the promoter region of target genes. The heterodimer may recruit co-activators and members of the transcription initiation complex, in order to modulate gene transcription and provoke biological responses.[6] These biological effects are achieved after a relatively long time (hours). The existence of VDREs in the promoter region of the genes coding for osteocalcine, osteopontine, PTH, and 25(OH)D-24-hydroxylase (CYP24) have been demonstrated.[7]

The second pathway to produce biological effects is through membrane receptors. Bond of calcitriol to membrane receptor may trigger protein kynase C (PKC), phospholipase C, adenylate cyclase (PKA) and/or voltage-mediated opening of calcium channels.[8] This rapid action pathway is related with non-genomic effects, and involves changes in second messenger concentration. An example of an adequate characterization of this pathway is the duodenum, and implies the calcium fast transport system.[9] This phenomenon is also know as transcaltachia (stimulation of calcium transport in 2-3 minutes), and it has been proposed that calcium channels, sensitive to voltage, participate in this particular system, which are triggered once the ligand interacts with its membrane receptor.[10]

In general, functions attributed to 1,25-$(OH)_2D_3$ are to maintain mineral ion homeostasis, regulation of certain gene transcription, promotion of cell differentiation, regulation of normal and malignant cell proliferation, stimulation of the immune system, and processes related with calcium transport. Receptors for this hormone have also been located in various tissues such as placenta, brain, muscle, intestine, bone, prostate, liver, ovary, breast, colon, epididymis, parathyroid gland, uterus, stomach, immune system cells, along with a number of cancer cell lines;[7] making the study of 1,25-$(OH)_2D_3$ functions a wide and interesting field.

STRUCTURE, REGULATION AND DISTRIBUTION OF 25-OH-D 1α HYDROXYLASE

The limiting step in the biosynthesis of the active form of vitamin D is hydroxylation at position 1 alpha of 25-OH-D. This reaction is catalyzed by an enzyme of the family of the mitochondrial inner membrane oxydases: 25-OH-D 1α hydroxylase (1α(OH)ase). This enzyme is formed by three elements: a flavoprotein (NADPH-ferredoxine reductase), a ferredoxine, and the cytochrome that confers specificity: cytochrome P450 1α, which is responsible of hydroxylizing the substrate in position a of carbon 1. Each hydroxylation catalyzed by a cytochrome P450 follows the general reaction:

$$\text{steroid-H} + \text{NADPH} + \text{H}^+ + \text{O}_2 \Rightarrow \text{steroid-OH} + \text{NADPH} + \text{H}_2\text{O}$$

This reaction consumes two electrons, a proton and an oxygen molecule, from which one of its atoms is incorporated to the substrate, while the other is reduced to water.[11] Electrons are transferred from NADPH to cytochrome P450 by the NADPH-ferredoxine reductase and ferredoxine.

Regulation

Renal 1α (OH)ase is regulated by different factors such as phosphorus, insulin-like growth factor I (IGF-I)[12-14] calcium, PTH, calcitonine[15] and 1,25-$(OH)_2D_3$ itself. We also know that the vitamin D transport protein (DBP) indirectly regulates the 1α(OH)ase limiting substrate availability, and that phospholipids also have a limiting role in this enzyme activity.[16] Moreover, some antimycotic agents inhibit 1α(OH)ase in a similar way as they do with other steroidogenic enzymes.[17] It must be mentioned that studies on 1α(OH)ase regulation have been performed basically in terms of its activity, since cloning of its cDNA is recent.[18-20] In present days, however, a number of studies are being conducted, which take into account the recently described structure of the 1a (OH)ase gene. This gene, know as CYP1α, $P450_{VD1\alpha}$, or P450c1α, has the official nomination of CYP27B1 in accordance with the nomenclature assigned by the Nomenclature Committee for the Cytochromes P450 Superfamily.[21] Works referring to the CYP27B1 structure, indicate that such gene contains response elements to cAMP (CRE) at its promoter region,[22] verifying previous observations concerning the stimulating factors of PTH over renal 1α(OH)ase, through intracellular generation of cAMP.[23] Kong[22] et al., studied the transcription activity pattern at region 5' of CYP27B1, building promoter portions and inserting them along with a reporter gene, in the kidney cell line AOK-B50. Although their studies showed occurrence of CREs in region 5' of CYP27B1, Kong and his group did not find specific response elements to IGF-I, or VDREs. The fact that no VDRE was located in such region is controversial, because 1,25-$(OH)_2D_3$ inhibited the stimulant effect produced by PTH on 1a(OH)ase, in this cellular type, suggesting that calcitriol suppress CYP27B1 expression through an indirect cascade interfering with the PTH signal pathway, without discarding the existence of other atypical or unknown VDREs, in the promoter region of CYP27B1. These findings and other works,[24] gave rise to the hypothesis that CYP27B1 inhibition by calcitriol is produced by a mechanism different to that of transcription. It is very probable that, as Henry et al.[24] mentioned, 1,25-$(OH)_2D_3$ has a repressive effect over its own synthesis, inhibiting a protein required for the P450 1α functioning, such as may be one of the proteins in charge of electron transport from NADPH to cytochrome P450 1α. The other option is that 1,25-$(OH)_2D_3$ does not directly repress transcription activity of CYP27B1, but masks its activity increasing the expression of gene CYP24 significantly.[25] CYP24 is usually triggered in the presence of high concentrations of 1,25-$(OH)_2D_3$ and/or high levels of serum calcium, as a regulation mechanism since it is a depuration metabolic pathway for calcitriol.[26] Other studies present, however, controversial results, demonstrating that negative regulation of the 1,25-$(OH)_2D_3$ synthesis by calcitriol itself is more complex than we currently suppose. Takeyama et al.,[27] in a series of experiments using mice strains, which lack the receptor to 1,25-$(OH)_2D_3$ (VDR), demonstrated that the calcitriol-VDR complex is responsible for the inhibition of the CYP27B1 expression. When they administered 1,25-$(OH)_2D_3$ to mice with wild phenotype, gene expression of CYP27B1 was repressed, but this did not happen with mice lacking receptors to vitamin D. Their results also suggested that VDR linked to 1,25-$(OH)_2D_3$, positively regulates CYP24 expression. Supporting these studies, Murayama et al.,[28] demonstrated that occurrence of VDR is essential for negative regulation of CYP27B1 by the 1,25-$(OH)_2D_3$, which highlights the need of more comprehensive studies concerning gene CYP27B1 regulation.

DISTRIBUTION

In general, 1α (OH)ase has been exclusively located in the kidney proximal tubules.[29,30] The kidney was considered as the sole site of conversion of 25-OH-D in 1,25-$(OH)_2D_3$; however, later studies demonstrated the extrarenal conversion of 25-OH-D to its hormone form in other tissues such as bone,[31,32] prostate,[33] placenta,[34-37] keratinocytes,[38] and immune system cells. Occurrence of 1α(OH)ase in these tissues confers to the hormone 1,25-$(OH)_2D_3$ a paracrine or autocrine nature, or both, and provides an enormous study and research field.

VITAMIN D AND HEALTH

The impact on health caused by vitamin D deficiency has been the matter of various studies. Rickets is the main child disease that results from vitamin D deficiency. Skeleton deformity due to deficient bone mineralization produced by a lack of calcium is reversible with administration of 1,25-$(OH)_2D_3$, calcium and phosphates. In adults, the pathological condition known as osteomalacia also occurs due to vitamin D deficiency, but with clinical and anatomorphic manifestations different to those of infantile rachitism. Generally, diseases produced

by vitamin D deficiencies occur due to dietary deficit, inadequate intestinal absorption, lack of sunlight, or renal damage. On the other hand, genetic pathologies associated with vitamin D imply mutations either in the gene coding for VDR or in CYP27B1 gene. The genetic disorder caused by mutations in the gene coding for the intracellular receptor of vitamin D is known as vitamin D-resistant rickets or rickets type II (VDDR II). The mutation produces changes in the DNA binding domain, or in the hormone binding domain,[39] thus this disease is associated with insensitivity of tissues to calcitriol,[40] and is characterized by hypocalcemia, secondary hyperparathyroidism,[40] high circulating concentration of serum $1,25\text{-}(OH)_2D_3$, and in some cases, alopecia.

On the other hand, the disorder known as pseudovitamin D-deficiency rickets (PDDR), also referred to as vitamin D-dependent rickets type I (VDDR I), is produced by a mutation of the 1α (OH)ase primary structure. This disease is the result of a hereditary defect associated with a deficient synthesis of $1,25\text{-}(OH)_2D_3$. PDDR is characterized by low serum calcium concentration, muscular weakness, secondary hyperparathyroidism, and decreased circulating concentration of $1,25\text{-}(OH)_2D_3$. The mutation responsible for the occurrence of PDDR was not discovered until recently, after cloning gene CYP27B1. All these studies determined that CYP27B1 is found in chromosome 12q13.1-q13.3,[41] and that 1α (OH)ase deficiency in PDDR is produced by a deletion in codons 211 or 231 of CYP27B1 gene18.

There are other pathological situations with symptomatology including hypercalcemia, that have been related with the significant increase of expression and/or activity of extrarenal 1α (OH)ase.[42] Of these, we can mention some diseases such as tuberculosis, sarcoidosis, candidiasis, and lymphoma,[43] where high concentration of serum calcitriol is detected. This increase has been associated with overproduction of $1,25\text{-}(OH)_2D_3$ by the immune system cells, either lymphocytes or macrophages.[43]

Concentration of $1,25\text{-}(OH)_2D_3$ also suffers modifications with age: various studies show that serum concentration of this hormone significantly decreases in the elderly, which is explained by various factors, but basically summarized in lower calcitriol skin production, lower ingestion in the diet, and alterations in health affecting absorption or renal function.[44-46] Also, menopause is associated with reduction in the concentration of $1,25\text{-}(OH)_2D_3$, as a probable result of the reduction of circulating estrogen.[47] There are some studies showing that estrogen promotes formation of $1,25(OH)_2D_3$ in vivo.[48,49] In fact, estrogen reduction favors bone mass loss, and their administration in postmenopausal women increases calcitriol concentration,[50] reducing the risk of bone fractures.

VITAMIN D AND PREGNANCY

Pregnancy involves great challenges for the mother's body, such as supplying calcium to the fetus with no homeostatic alteration of this mineral. At the end of pregnancy, the fetus has accumulated up to 30 grams of calcium, and to satisfy this demand, vitamin D metabolism adjusts increasing the blood concentration of $1,25\text{-}(OH)_2D_3$, favoring higher calcium intestinal absorption.[51] In fact, calcitriol concentration doubles from the first trimester of pregnancy, and remains so until the end.[52] The direct cause of circulating calcitriol increase is not easily explained, since this increase is not associated with low concentration of calcium or phosphorus, which are actually slightly high. Classical PTH regulation cannot be stated since in the case of pregnancy, these hormone levels are within a normal range.[53] Furthermore, the increase of 1,25-(OH)2D3 during gestation is independent from PTH.[52] These observations allow to suppose the existence of other factors involved in calcitriol regulation, which account for the increase of the circulating concentration of this hormone from the early stages of pregnancy. The probable candidates are estradiol, prolactine (hPRL), placental lactogen (hPL), IGF-I, and a PTH-related peptide (PTHrP). Estradiol, hPRL, IGF-I[12-14] and hPL promote activity of 1a (OH)ase in vitro,[53] and estradiol stimulates it in vivo.[48,50] On the other hand, PTHrP triggers the common receptor for PTH/PTHrP in kidney and bone, and it's concentration progressively increase as pregnancy continues, thus, it is possible that PTHrP promotes calcitriol synthesis independently from PTH during gestation. All of these factors may account for the increase of calcitriol circulating concentration during gestation. We must also consider the contribution of $1,25\text{-}(OH)_2D_3$ production by the fetal kidney, as well as the extrarenal production by the placenta. Supporting the former, experiments conducted in anephric pregnant rats demonstrated the occurrence of extrarenal-origin calcitriol in mother's circulation.[54] It is important to mention, however, that the increase of $1,25\text{-}(OH)_2D_3$ during pregnancy is considered the result of an increase in

the activity of mother's renal 1α (OH)ase53, therefore, extrarenal-origin calcitriol during this period may have a biological meaning of autocrine or paracrine nature.

Placental contribution of calcitriol

The first evidence concerning the extrarenal synthesis of $1,25\text{-}(OH)_2D_3$ was obtained in pregnant rats, in which bilateral nephrectomy reduced, but did not eliminate, the conversion of $[^3H]25\text{-}(OH)D_3$ to $[^3H]1\text{-}25\text{-}(OH)_2D_3$.[54] Later, some researchers verified the placental production of calcitriol in relation with 1a (OH)ase activity,[34-36] and the expression of gene CYP27B1 in decidual cells[55] and trophoblasts[37] was only recently confirmed. These works allowed to define that 1a(OH)ase from decidua, trophoblasts and kidney come from the same gene.

Calcitriol in placenta and fetus

Circulating concentration of calcitriol in the fetus is lower than in the mother, which may be explained by the fact that the fetus is hypercalcemic compared with the mother's serum calcium concentration. This situation requires mechanisms allowing the mother-fetus calcium transport against a concentration gradient. The placenta is the organ in charge of calcium transport from mother to fetus, and although this system is not fully characterized, some mechanisms for the required active transport have been found in the placenta. This is the case of calcium channels and transport proteins for this nutrient, such as calbindine D_{9K} and calcium ATPase.[56] The former is in charge of pumping calcium from basal layers from the fetal side of the placenta to the extracellular fetal fluid. Calcitriol has an important role in this transport system, since it promotes the transplacental passage of calcium in a dose-dependent way,[57] promotes mRNA production of calcium transport proteins, and favors active transport of this mineral maintaining maternal homeostasis, allowing calcium availability for the placenta, thus achieving an adequate mineralization of the fetal skeleton. On the other hand, presence of specific receptors for $1,25(OH)_2D_3$ in the placenta[58] suggests that this hormone has a specific function in this organ. The interaction $1,25(OH)_2D_3$-VDR promotes expression and secretion of hPL in human syncytiotrophoblasts[59] implying a roll of calcitriol in placental and fetal growth. Effects of $1,25(OH)_2D_3$ on cell differentiation and control of certain gene expression may contribute to the adequate establishment of the feto-placental unit during the first stages of gestation. Some studies conducted in rats support this, and they note the direct participation of $1,25(OH)_2D_3$ in the endometrium decidualization,[60] inducing differentiation of endometrial cells to decidual cells, which represents a key event in the blastocyst implantation process.

On the other hand, the availability of an animal model in which the DNA binding domain of the VDR has been modified (VDR knock-out mice), allowed the study in vivo of the biological activity of calcitriol. Homozygous mice (-/-) were born normal even though VDR absence during all of gestation, but during the first month of life, they developed hypocalcemia, hyperparathyroidism and complete alopecia. They survived the first six months.[61]

Consequences of deficiency and/or alteration of vitamin D metabolism during pregnancy

Even though some studies show the possible development of healthy newborns from mothers during pregnancy with low calcitriol concentration,[62] there are others demonstrating alterations. Some studies report birth of children with hypocalcemia, neonatal rickets, or who develop them later, and fetal growth retardation in cases where the mother had reduced vitamin D blood concentration.[63-65] There are also studies showing that low concentration of fetal serum calcitriol is associated with small size and low mineral content in the newborn.[66] Some reports have demonstrated alterations in heart development or neonatal heart failure as a result of mother's vitamin D deficiency,[67,68] , while others report convulsions and hypocalcemia in neonate, secondary to the mother's calcitriol deficiency.[69] Another study have suggested that mother's vitamin D deficiency adversely affects fetal brain development.[70]

Furthermore, the pregnancy pathology known as preeclampsia, is associated with low circulating concentration of $1,25\text{-}(OH)_2D_3$;[71,72] and although the causes of this are unknown, it is probable that this feature results from deficiencies in the endocrine functions of the placenta, mother's kidney, or both. In this disease, the neonate is usually born with low weight.

CONCLUSION

The knowledge of vitamin D has seen great advances in recent times, both in basic science and clinical medicine. The classical view of vitamin D as

a hormone whose effects were limited to calcium metabolism and bone homeostasis have been extensively revolutionized. Current studies on vitamin D consider the recently mentioned actions of this hormone on cell proliferation and differentiation, as well as on the immune system. This opens research fields in the health, disease and pregnancy processes, where vitamin D has gained importance. The therapeutic potential of vitamin D in various diseases has generated a search for calcitriol analogs with antiproliferative, immunosuppressing, and pro-differenciating effects.

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Address for correspondence:

Biol. Lorenza Díaz
Instituto Nacional de Ciencias Médicas y
Nutrición Salvador Zubirán
Vasco de Quiroga No 15, Tlalpan 14000, México, DF.
Tel: (525)573-11-60, Fax: (525) 655-98-59
E-mail: lorenzadiaz@hotmail.com

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Effects of IGF-I on 1,25-dihydroxyvitamin D_3 synthesis by human placenta in culture

Ali Halhali[1,4], Lorenza Diaz[1], Irene Sánchez[1], Michèle Garabédian[3], Héctor Bourges[2], and Fernando Larrea[1]

[1]Departments of Reproductive Biology and [2]Physiology of Nutrition, Instituto Nacional de la Nutricion Salvador Zubirán, Vasco de Quiroga No 15, Col. Tlalpan, CP 14000, México DF, México, and [3]Unité CNRS UPR 1524, Endocrinologie, Metabolisme et Développement, Hôpital Saint Vincent de Paul, Paris, France

[4]To whom correspondence should be addressed

The aim of the present study was to assess the effects of insulin-like growth factor I (IGF-I) upon the synthesis of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] by human placenta trophoblasts in culture. Cytotrophoblastic cells obtained from normal term human placentae were cultured in Dulbecco's modified Eagle's medium with HEPES and glucose (DMEM-HG) during 72 h and further incubated in serum-free DMEM-F12 in the presence of IGF-I prior to the addition of [^{3}H]-25-$(OH)D_3$ used as a precursor. The results showed that 2 h preincubation time with IGF-I was required for maximal production of [^{3}H]-1,25-$(OH)_2D_3$. Cultures in the presence of increasing concentrations of IGF-I (0–6.5 nmol/l), added 2 h before incubation with the labelled substrate, resulted in a dose-dependent response increment of [^{3}H]-1,25-$(OH)_2D_3$ production with a maximal conversion rate at the dose of 2.6 nmol/l. Higher doses of IGF-I did not result in further stimulatory effects. Co-incubations in the presence of cycloheximide significantly ($P < 0.0001$) inhibited the IGF-I-mediated effects upon [^{3}H]-1,25-$(OH)_2D_3$ production. Identity of putative [^{3}H]-1,25-$(OH)_2D_3$ produced by human placenta was confirmed by spectral and receptor binding analysis. These results demonstrate the ability of cultured human syncytiotrophoblast cells to convert 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$ and suggest a local protein-dependent regulatory effect of IGF-I upon this biotransformation.

Key words: 1,25-$(OH)_2D_3$/IGF-I/placenta/syncytiotrophoblasts

Introduction

Placenta is considered as an extrarenal source of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (Weisman *et al.*, 1979; Whitsett *et al.*, 1981; Zerwekh and Breslau, 1986; Hollis *et al.*, 1989). This extrarenal source may contribute to the high maternal serum concentrations of this hormone in pregnant women. It may also be important to the fetal pool of the hormone or as a local source for its action on the placenta (Delvin *et al.*, 1985; Zerwekh and Breslau, 1986; Kovacs and Kronenberg, 1997). However, the placenta contribution to vitamin D metabolism has been questioned. Indeed, synthesis of 1,25-$(OH)_2D_3$ by trophoblast homogenates requires supraphysiological amounts of 25-hydroxyvitamin D_3 [25-$(OH)D_3$] (Whitsett *et al.*, 1981; Zerwekh and Breslau, 1986; Hollis *et al.*, 1989) and 1,25-$(OH)_2D_3$ production by placental cells has been inconsistently found (Rubin *et al.*, 1993). Furthermore, it has been previously reported that, whereas synthesis of 1,25-$(OH)_2D_3$ in human decidua is an enzymatically-mediated process, trophoblast tissue might use an alternate non-enzymatic mechanism of hydroxylation (Hollis *et al.*, 1989; Glorieux *et al.*, 1995).

In any case, studies in maternal serum suggest that production of 1,25-$(OH)_2D_3$ during pregnancy is regulated by factors different from those acting in the non-pregnant state such as calcium, phosphate and parathyroid hormone (PTH) (Verhaegue and Bouillon, 1992; Ardawi *et al.*, 1997; Kovacs and Kronenberg, 1997). Numerous studies in cell cultures, rodents and humans suggest that insulin-like growth factor I (IGF-I) is an important regulatory factor of the activity and/or synthesis of the renal enzymatic complex hydroxylating 25-$(OH)D_3$ into 1,25-$(OH)_2D_3$ (Gray, 1987; Halloran and Spencer, 1988; Caverzasio *et al.*, 1990; Nesbitt and Drezner, 1993; Condamine *et al.*, 1994; Menaa *et al.*, 1995; Wong *et al.*, 1997; Bianda *et al.*, 1997, 1998; Wei *et al.*, 1998). These data, in addition to previous studies demonstrating that placenta is a source of IGF-I (Fant *et al.*, 1986; Han *et al.*, 1996), prompted us to investigate the effects of this growth factor upon the ability of cultured human placenta to convert 25-$(OH)D_3$ into 1,25-$(OH)_2D_3$.

Materials and methods

Materials and reagents

Dulbecco's modified Eagle's medium (DMEM and DMEM-F12), Hank's balanced salt solution (HBSS), fetal calf serum (FCS), HEPES, and gentamicine were obtained from Gibco (Grand Island, NY, USA). Percoll, 8-bromo adenosine 3',5'-cyclic monophosphate (8-Br-cAMP), deoxynbonuclease I (DNase I), bovine serum albumin (BSA), glutamine, and cycloheximide were purchased from Sigma Chemical Co (St Louis, MO, USA). All solvents were of high-performance liquid chromatography (HPLC) grade and were obtained from Merck (Darmstadt, Germany). Unlabelled 25-$(OH)D_3$

and 1,25(OH)$_2$D$_3$ were a generous gift from Dr E.-M.Gutknecht and Dr P.Weber (Hoffmann La Roche Ltd, Basel, Switzerland). The 25-hydroxy-[26,27-methyl-^{3}H]-cholecalciferol ([^{3}H]-25-(OH)D$_3$; specific activity: 17 Ci/mmol), 1α,25-dihydroxy-[26,27-methyl-^{3}H]-cholecalciferol ([^{3}H]-1,25-(OH)$_2$D$_3$; specific activity: 130 Ci/mmol) and recombinant human IGF-I were purchased from Amersham (Amersham, Bucks, UK). Human chorionic gonadotrophin (HCG) radioimmunoassay was kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (Rockville, MD, USA). All other reagents were of analytical grade.

Cytotrophoblastic cell isolation and culture

The study protocol was approved by the Human Ethical Committee of the Institute. Term placentae were obtained from normal pregnant women after spontaneous delivery. Tissues were brought immediately to the laboratory where several cotyledons were removed and rinsed thoroughly in 0.9% NaCl at room temperature. The isolation and culture of cytotrophoblasts were performed as previously described (Kliman *et al.*, 1986), with minor modifications. Briefly: soft villous tissue (30 g), free of connective tissue and vessels, was collected. Tissue was coarsely minced and digested with 0.125% trypsin, and 0.2 mg/ml DNAase I (1500 kunitz IU/mg) in warmed calcium- and magnesium-free HBSS containing 25 mmol/l HEPES, pH 7.4, at 37°C for 30 min. Cell suspensions were pooled, centrifuged at 1000 *g* for 30 min, and resuspended in DMEM containing 25 mmol/l HEPES and 25 mmol/l glucose (DMEM-HG). The resultant suspension was placed on 5–70% Percoll (v/v) gradients made up in HBSS. Gradients, which consisted of 5% steps of 3 ml each, were centrifuged at 1200 *g* at room temperature for 30 min. After centrifugation, the middle band (density 1.048–1.062) containing the cytotrophoblasts was removed, washed once with DMEM-HG, and resuspended in culture medium. Percoll gradient-purified cytotrophoblasts were diluted to a concentration of 1×10^6 cells/ml with DMEM-HG containing 4 mmol/l glutamine, 50 μg/ml gentamicin, and 20% heat-inactivated FCS, plated in 35 mm Nunclon culture dishes (Nunc, Roskilde, Denmark), and incubated for 72 h in humidified 5% CO_2 and 95% air at 37°C.

Morphological and functional aspects of placental cell cultures

Daily, the morphological aspects of cell cultures were examined. HCG in the culture media was measured as previously described (Diaz-Cueto *et al.*, 1994; Quelpo *et al.*, 1998) by specific radioimmunoassay using reagents and protocols provided by the NIDDK. Anti-HCG-H80, at a final working dilution of 1:150 000, was used as antiserum. This antiserum exhibits 1.2 and 3.2% cross-reactivities with free HCG α- and β-subunits respectively. The sensitivity of the assay was 0.025 ng/tube and the inter- and intra-assay coefficients of variation were <10 and <6% respectively. Total protein content of cell cultures was measured by a previously described method (Bradford, 1976), using BSA as standard.

Metabolism of 25-(OH)D$_3$ by placental cell cultures

On the third day of culture, medium was changed and the cells incubated in 2 ml serum-free DMEM-F12. To assess the ability of placental cell cultures to convert 25-(OH)D$_3$ into 1,25-(OH)$_2$D$_3$, a 3 nmol/l concentration of [^{3}H]-25-(OH)D$_3$ was added in fresh serum-free DMEM-F12 and incubations continued for 120 min. Culture medium was then transferred to glass tubes, and the cells were washed with 0.5 ml of methanol. Protein cell content was determined after addition of 0.5 ml of 1 mol/l NaOH. The [^{3}H]-25-(OH)D$_3$ and its metabolites were extracted from the medium with an additional 3.5 ml of methanol plus 4 ml of chloroform (Bligh and Dyer, 1959). The chloroform phase was dried down under N_2, and lipid extracts were redissolved in chromatographic solvent. The samples were co-chromatographed with 100 ng unlabelled 1,25-(OH)$_2$D$_3$ as an elution marker on a Waters HPLC fitted with a photodiode array detector (PDA; model 996; Waters Associates, Milford, MA, USA) using an ultrasphere Si, 5 μm, 4.6×250 mm column (Beckman Instruments, Palo Alto, CA, USA). Vitamin D$_3$ metabolites were separated by two-step straight phase HPLC as previously described (Sempere *et al.*, 1989; Kachkache *et al.*, 1993; Menaa *et al.*, 1995). Fractions (1 min) were collected and an aliquot of each was removed for radioactivity determination. Fractions co-eluting with unlabelled 1,25-(OH)$_2$D$_3$ were pooled and rechromatographed on a second straight phase HPLC using the same column and eluted in methylene chloride:isopropanol (95:5) at a flow rate of 1 ml/min. The conversion rate of [^{3}H]-25-(OH)D$_3$ into putative [^{3}H]-1,25-(OH)$_2$D$_3$ was determined by calculating the percentage of radioactivity co-eluting with unlabelled 1,25-(OH)$_2$D$_3$ after the two successive chromatographies. Results were expressed as fmol/mg protein.

Characterization of putative [^{3}H]-1,25-(OH)$_2$D$_3$

For this purpose, placental cells were incubated in the presence of 2.5 μmol/l unlabelled 25-(OH)D$_3$. The putative 1,25-(OH)$_2$D$_3$ produced by these cells was purified as described above with one exception: lipid extracts were co-chromatographed with 1 nCi [^{3}H]-1,25-(OH)$_2$D$_3$ instead of unlabelled hormone used as an elution marker. The amount of putative 1,25-(OH)$_2$D$_3$ was calculated from the absorbance at 265 nm of the single peak co-eluting with [^{3}H]-1,25-(OH)$_2$D$_3$ in the second HPLC. The purified 25-(OH)D$_3$ metabolite was tested for its ability to displace synthetic labelled [^{3}H]-1,25-(OH)$_2$D$_3$ from its specific calf thymus receptor (Reinhardt *et al.*, 1984) using a commercial radioreceptor assay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA).

Regulatory effects of IGF-I upon [^{3}H]-1,25-(OH)$_2$D$_3$ synthesis

In order to determine the effects of IGF-I on placental [^{3}H]-1,25-(OH)$_2$D$_3$ production, cell cultures were preincubated in the presence of IGF-I at different times previous to the addition of 3 nmol/l of tritiated [^{3}H]-25-(OH)D$_3$.

Dose–response curves of IGF-I effects on putative [^{3}H]-1,25-(OH)$_2$D$_3$ production were performed by preincubations with different amounts of IGF-I (0–6.5 nmol/l) during 2 h prior to the addition of [^{3}H]-25-(OH)D$_3$. Furthermore, the effects of IGF-I upon [^{3}H]-1,25-(OH)$_2$D$_3$ production were also assessed in the presence of the protein synthesis inhibitor cycloheximide (30 μmol/l). The IGF-I mitogenic effects were evaluated by measuring protein content in cell cultures.

Statistical analysis

Data are presented as mean ± SD. All experiments were performed at least three times and each of them consisted of six to nine culture replicates. Statistical significance between groups was established by one-way analysis of variance (ANOVA) using Fisher's protected least-square differences. $P < 0.05$ was considered to be statistically significant.

Results

Morphological and functional aspects of placental cell cultures

Microscopic examination of cell cultures showed that after 72 h, cytotrophoblast cells consistently aggregated forming well-differentiated syncytiotrophoblast structures. In addition, our placental cell cultures corresponded to functional

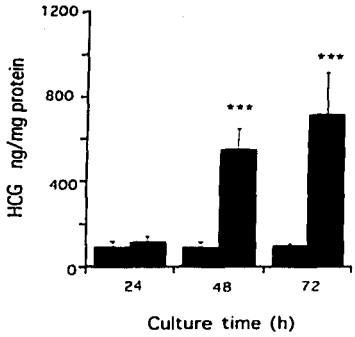

Figure 1. Human chorionic gonadotrophin (HCG) secretion by cytotrophoblast cells at different hours of culture in the presence of 8-bromo-cAMP (8-Br-cAMP; ■) or the vehicle alone (□). Each bar represents the mean ± SD from triplicate experiments. ***$P < 0.0001$ versus vehicle alone.

syncytiotrophoblasts since HCG secretion in the presence of 1.5 nmol/l 8-Br-cAMP increased significantly ($P < 0.0001$) when compared with cells cultured in the absence of the cyclic nucleotide analogue (Figure 1), as previously reported (Feinman *et al.*, 1986; Queenan *et al.*, 1987; Ulloa-Aguirre *et al.*, 1990; Díaz *et al.*, 1997; Queipo *et al.*, 1998).

Metabolism of 25-(OH)D$_3$ by placental cell cultures

Syncytiotrophoblast cells were able to convert 25-(OH)D$_3$ into more polar metabolites. As shown in Figure 2, one of these metabolites eluting as a single peak co-eluted with 1,25-(OH)$_2$D$_3$ during both the first (Figure 2A) and the second (Figure 2B) HPLC. The amount of this metabolite found after 2 h of incubation with 3 nmol/l [^{3}H]-25-(OH)D$_3$ ranged between 80 and 310 fmoles/mg protein (170 ± 80 fmoles/mg). When cells were incubated in presence of 2.5 µmol/l unlabelled 25-(OH)D$_3$, the amount of 1,25-(OH)$_2$D$_3$ produced by syncytiotrophoblasts averaged 40 ± 15 pmoles/mg protein. Putative 1,25-(OH)$_2$D$_3$ was identified by its spectral and binding properties. Indeed, putative and 1,25-(OH)$_2$D$_3$ presented similar maximum absorbance at 265 nm. Furthermore, the ability of putative 1,25-(OH)$_2$D$_3$ to displace [^{3}H]-1,25-(OH)$_2$D$_3$ from its specific calf thymus receptor was identical to that of 1,25-(OH)$_2$D$_3$ (Figure 3).

Regulatory effects of IGF-I upon 1,25-(OH)$_2$D$_3$ placental production

The simultaneous addition of IGF-I and [^{3}H]-25-(OH)D$_3$ to day 3 cultures did not result in a significant increase in the conversion of substrate to the active vitamin D metabolite (Figure 4). As shown in this figure, time-response experiments in cultures preincubated with IGF-I (3.3 nmol/l) at various times (0, 2, 8, 16 h) prior to the addition of [^{3}H]-25-(OH)D$_3$,

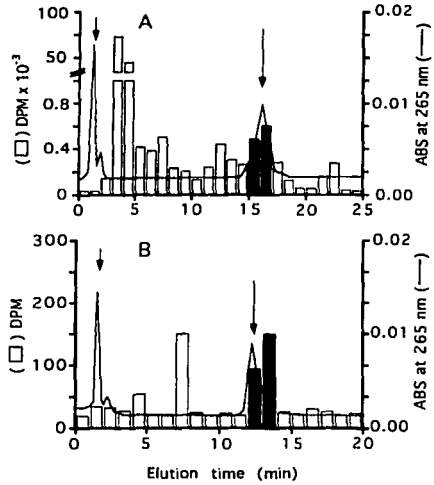

Figure 2. Typical high-performance liquid chromatography (HPLC) elution profiles of radioactivity extracted from human syncytiotrophoblast cells incubated with 3 nmol/l [^{3}H]-25-(OH)D$_3$, and absorbance (ABS)at 265 nm of unlabelled 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) during (A) the first and (B) the second HPLC. Solvent front and 1,25-(OH)$_2$D$_3$ elution times are shown by the arrows. Radioactivity co-eluting with 1,25-(OH)$_2$D$_3$ is shown in closed bars. DPM = disintegrations per minute.

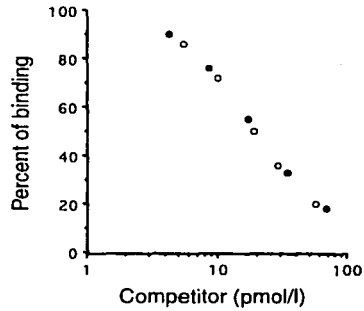

Figure 3. Ability of the putative 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) produced by syncytiotrophoblast cells (open circles) and 1,25-(OH)$_2$D$_3$ (closed circles) to compete with [^{3}H]-1,25-(OH)$_2$D$_3$ for binding to its specific calf thymus receptor. Each point represents the mean of three experiments.

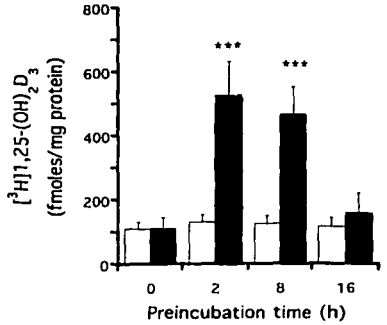

Figure 4. Time-course effect of insulin-like growth factor I (IGF-I) upon [^{3}H]-1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) production by syncytiotrophoblast cells. Cells were preincubated at the indicated times with 3.3 nmol/l IGF-I (■) or the vehicle alone (□) before the addition of [^{3}H]-25(OH)D_3. Each bar represents the mean ± SD from triplicate experiments. ***$P < 0.0001$ versus vehicle alone.

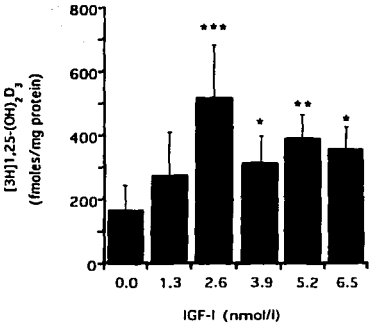

Figure 5. Dose–response curve of insulin-like growth factor I (IGF-I) on [^{3}H]-1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) production by syncytiotrophoblast cells. Cells were incubated for 2 h in the presence of various concentrations of IGF-I or the vehicle alone before the addition of [^{3}H]-25(OH)D_3. Each bar represents the mean ± SD from triplicate experiments. ***$P < 0.0001$, **$P < 0.001$, *$P < 0.005$ versus vehicle alone.

demonstrated that a minimum of 2–8 h preincubation period was required to significantly increase ($P < 0.0001$) the conversion of substrate to [^{3}H]-1,25-$(OH)_2D_3$. However, a significant decrease ($P < 0.0001$) in [^{3}H]-1,25-$(OH)_2D_3$ synthesis was observed with longer preincubation periods. This stimulatory effect of IGF-I was not associated with an increase in protein synthesis linked to the mitogenic action of this growth factor, since cell protein contents were similar in IGF-I-treated (81 ± 5 μg/well) and untreated cells (83 ± 5 μg/well).

Effects of increasing concentrations of IGF-I added to cultures 2 h prior to incubation with [^{3}H]-25-$(OH)D_3$ were also evaluated. As shown in Figure 5, a dose–response effect of IGF-I upon [^{3}H]-1,25-$(OH)_2D_3$ production was obtained with a maximal conversion rate at a concentration of 2.6 nmol/l ($P < 0.0001$ versus control). Higher doses of IGF-I did not further increase the stimulatory effect, but synthesis of [^{3}H]-1,25-$(OH)_2D_3$ remained significantly above controls ($P < 0.005$).

In addition, the stimulatory effects of IGF-I upon [^{3}H]-1,25-$(OH)_2D_3$ production were also evaluated in the presence of cycloheximide. As shown in Figure 6 (Panel A), the presence of 30 μmol/l of cycloheximide significantly inhibited ($P < 0.0001$) the production rate of [^{3}H]-1,25-$(OH)_2D_3$ in 2 h-preincubated cultures stimulated with IGF-I (2.6 nmol/l). A similar effect was observed on 8-Br-cAMP-stimulated HCG secretion (Figure 6B).

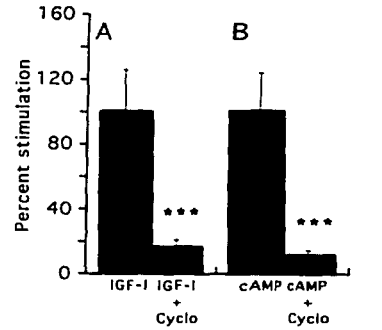

Figure 6. Effects of cycloheximide (cyclo) on [^{3}H]-1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and human chorionic gonadotrophin (HCG) production induced by insulin-like growth factor I (IGF-I) and 8-bromo-cAMP (8-Br-cAMP) in syncytiotrophoblast cells. Cultures were incubated in the presence of cycloheximide 1 h before the addition of IGF-I (Panel A) or 8-Br-cAMP (Panel B). Conversion to [^{3}H]-1,25-$(OH)_2D_3$ and the content of HCG in culture media were evaluated as described in the text. Each bar represents the mean ± SD from triplicate experiments. ***$P < 0.0001$ versus control.

Discussion

The results presented in this communication indicate that cultured human syncytiotrophoblasts are able to produce 1,25-$(OH)_2D_3$ when incubated in the presence of physiological concentrations of 25-$(OH)D_3$. The culture system used converted 25-$(OH)D_3$ into a metabolite with chromatographic behaviour, UV spectral patterns and binding abilities to thymus cytosol receptors identical to 1,25-$(OH)_2D_3$. Moreover, these data provide further evidence showing that not only decidual cells (Delvin *et al.*, 1985; Kachkache *et al.*, 1993) but also

syncytiotrophoblasts may contribute to the maternal or fetal pool of 1,25-$(OH)_2D$ concentrations during human pregnancy. These results are in agreement with other studies showing the ability of the trophoblast tissue or cells to produce 1,25-$(OH)_2D$ *in vitro* (Hollis *et al.*, 1989; Rubin *et al.*, 1993). In the present study, consistent production of 1,25-$(OH)_2D_3$ was detected using physiological amounts of 25-$(OH)D_3$, whereas in earlier similar studies (Hollis *et al.*, 1989; Rubin *et al.*, 1993) the results were inconsistent or required supra-physiological doses of the precursor. In addition, previous observations suggest that 1,25-$(OH)_2D_3$ production involves a different hydroxylation system than the 25-(OH)D-1α-hydroxylase (1αOHse) (Hollis *et al.*, 1989). Although it is not possible to ascertain whether the presently observed production of 1,25-$(OH)_2D_3$ involved the mitochondrial 1αOHse system, the conditions under which 1,25-$(OH)_2D_3$ production was observed [cell incubations with low doses of 25-$(OH)D_3$ during a short period of 2 h], make it likely. In addition, part of the discordance with previous studies may derive from differences in the placental tissues or cells tested, including trophoblast tissue (Hollis *et al.*, 1989; Rubin *et al.*, 1993) or cultured syncytiotrophoblast cells (Rubin *et al.*, 1993; and present work). It is also important to mention that differences in the culture medium and/or the concentration of FCS used may also have influenced the differentiation state of the cells and thus the ability to produce 1,25-$(OH)_2D_3$. Indeed, DMEM containing 3.75% FCS was used by Rubin *et al.* (1993) while DMEM supplemented with glucose and 20% FCS was used in the present work. Finally, the incubation of cells with DMEM or Roswell Park Memorial Institute (RPMI) 1640 medium may also have influenced the activity of the 1αOHse, as has been previously shown in cultures of decidual cells (Kachkache *et al.*, 1993).

In the culture system and under the experimental conditions used throughout this study, a clear and significant stimulatory effect of IGF-I on 1,25-$(OH)_2D_3$ production was observed. These data agree with previous observations that synthesis of 1,25-$(OH)_2D_3$ is under the control of a number of modulators. It is known that in addition to calcium and phosphate, and hormones such as PTH and calcitonin, including 1,25-$(OH)_2D$ itself, other factors may influence the activity and possibly the expression of renal 1αOHse (Garabédian *et al.*, 1972; DeLuca, 1978; Fraser, 1980; Kawashima and Kurokawa, 1986; Breslau, 1988; Reichel *et al.*, 1989). IGF-I is likely to be one of these additional physiological regulators, as it increases *in vivo* the serum concentrations of 1,25-$(OH)_2D$ and stimulates its renal production *in vitro* (Gray, 1987; Halloran and Spencer, 1988; Caverzasio *et al.*, 1990; Nesbitt and Drezner, 1993; Condamine *et al.*, 1994; Menaa *et al.*, 1995; Wong *et al.*, 1997; Bianda *et al.*, 1997, 1998; Wei *et al.*, 1998). These observations are of physiological importance since parallel increases in serum IGF-I and 1,25-$(OH)_2D$ concentrations have been observed during pregnancy (Petraglia *et al.*, 1996; Kovaks and Kronenberg, 1997), suggesting that the increase in serum maternal 1,25-$(OH)_2D$ results from an IGF-I-dependent stimulation of renal 1αOHse. However, based on the present findings, IGF-I may also stimulate the production of 1,25-$(OH)_2D$ in placenta, thus representing an additional source of maternal and/or fetal pools of this metabolite.

In the absence of precise kinetic data, it is not possible in this study to know whether the IGF-I-dependent increase in placental 1,25-$(OH)_2D_3$ production results from a stimulation of the 1αOHse activity or from a decreased catabolism of the produced 1,25-$(OH)_2D_3$. Yet kinetic studies with renal cells suggest that stimulation of the 1αOHse is a major component of the IGF-I effect on vitamin D metabolism (Nesbitt and Drezner, 1993; Menaa *et al.*, 1995). In so far as the mechanism of the IGF-I effect on trophoblast cells remains to be elucidated, results obtained with renal cells (Caverzasio and Bonjour, 1989, 1992; Caverzasio *et al.*, 1990; Quigley and Baum, 1991; Menaa *et al.*, 1995) indicate that this pathway may involve changes in phosphate transport or the stimulation of calcium uptake by placental cells. Whatever this mechanism, results of cell incubations with cycloheximide suggest that the IGF-I effect on 1,25-$(OH)_2D_3$ production by trophoblast cells involves de-novo protein synthesis. Recently IGF-II, rather than IGF-I, was shown to be preferentially expressed in the placenta trophoblast (Han *et al.*, 1996), thus suggesting that both local and/or systemic IGFs acting via the same or the specific receptor are involved in 1,25-$(OH)_2D_3$ production by the placenta. However, whether IGF-II is the relevant peptide deserves to be further investigated.

In summary, this study demonstrates a clear ability of cultured human syncytiotrophoblast cells to synthesize 1,25-$(OH)_2D_3$ with a marked stimulation of this production by physiological doses of IGF-I. The physiological relevance of these findings has not been evaluated, but interactions between IGF-I and 1,25-$(OH)_2D$ may play an important role in a tissue which expresses both the vitamin D and IGF-I receptors (Marshall *et al.*, 1974; Tanamura *et al.*, 1995). If the placenta appears not to be considered as an important contributor to the 1,25-$(OH)_2D$ concentrations in maternal blood, IGF-I may locally regulate the production of 1,25-$(OH)_2D$ by trophoblast cells and hence control some of the ion transport mechanisms, as in the case of renal cells (Caverzasio and Bonjour, 1989, 1992; Caverzasio *et al.*, 1990; Quigley and Baum, 1991; Menaa *et al.*, 1995), through the placental barrier or other vitamin D-dependent functions on this tissue. It may also contribute to the increase in the fetal pool of 1,25-$(OH)_2D$ during fetal growth.

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