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# INFLUENCIA DE DIVERSOS FÁRMACOS SOBRE LA FUNCIÓN ENDOTELIAL DE VENAS VARICOSAS HUMANAS


# T E S I S

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## I. RESUMEN

En segmentos de venas varicosas humanas, se exploró la función endotelial, al medir la relajación dependiente del endotelio inducida por acetilcolina, en preparaciones precontraídas con noradrenalina. También se realizaron curvas concentración-respuesta a acetilcolina antes y después de la incubación con agentes protectores de la función endotelial en arterias (captopril, losartán, troglitazona, pravastatina o simvastatina), así como con el agente antivaricoso escina. La relajación promedio inducida por acetilcolina fue de 13 %, al compararlo con controles históricos, como el observado en venas utilizadas para “bypass”, este porcentaje corresponde aproximadamente a un tercio del reportado. Las curvas concentración-respuesta a acetilcolina fueron en forma de “U”, esto como resultado de la relajación mediada por el endotelio en las concentraciones bajas y la sobreposición de la respuesta contráctil del músculo liso venoso en las concentraciones más altas. La relajación aumentó al incubar con los fármacos protectores del endotelio y con escina; los segmentos incubados con troglitazona obtuvieron la respuesta más discreta y los incubados con simvastatina la más grande. Se concluyó que las venas varicosas humanas presentan disfunción endotelial, que esta anomalía puede ser revertida por fármacos protectores del sistema cardiovascular y que esta disfunción puede jugar un papel importante en la patogénesis y el tratamiento de la insuficiencia venosa crónica.

## II. INTRODUCCIÓN.

### 2.1 Insuficiencia Venosa Crónica.

#### a. Generalidades.

La insuficiencia venosa crónica (IVC) es una entidad patológica que afecta principalmente al sistema venoso de los miembros inferiores. Clínicamente se caracteriza por signos como dolor, hiperpigmentación, edema, venas varicosas y en los casos más severos úlceras en los miembros afectados. Los pacientes con IVC reportan comúnmente pesadez, malestar y fatiga de los miembros pélvicos. El dolor es constante pero se exacerba por la tarde y después de periodos prolongados en posición erecta. Todas estas molestias disminuyen su intensidad al elevar las piernas (Nael y Rathbun, 2009). Es considerada como una de las enfermedades crónicas que originan más consultas médicas, estimándose que en los Estados Unidos de América más del 73% de las mujeres y más del 56% de los hombres cursan con este proceso (Carr, 2006), mientras que en poblaciones europeas como Londres, afecta al 17% de los hombres y 31% de las mujeres (London y Nash, 2000); varios factores se han asociado con la incidencia de este padecimiento, entre los que destacan la herencia, ser de sexo femenino, obesidad, permanecer periodos prolongados de pie y el embarazo (Bergan y col., 2006). Este último dato nos permite inferir que en nuestro país la IVC podría afectar a más de un millón de mujeres al año, según cifras de la Secretaría de Salud, ya que las causas obstétricas correspondieron a casi el 30% de los egresos hospitalarios en 2005.

#### b. Anatomía.

El conocimiento preciso de la anatomía venosa es esencial para la correcta estadificación y tratamiento de la IVC. El drenaje venoso de los miembros inferiores está compuesto de dos estructuras conectadas y paralelas: el sistema venoso profundo y el

sistema venoso superficial. La nomenclatura del sistema venoso de los miembros inferiores ha sido revisada recientemente y a continuación se enlistan los cambios (Tablas 1 y 2) (Caggiati y col., 2002; 2005).

**Tabla 1 – Venas superficiales**

| TERMINOLOGÍA ANATÓMICA | TERMINOLOGÍA PROPUESTA |
|---|---|
| Vena safena magna o larga. | Vena safena magna |
| | Venas inguinales superficiales |
| Vena pudenda externa | Vena pudenda externa |
| Vena circunfleja superficial | Vena iliaca circunfleja superficial |
| Vena epigástrica superficial | Vena epigástrica superficial |
| Vena dorsal superficial del pene o clítoris | Vena dorsal superficial del pene o clítoris |
| Vena labial anterior | Vena labial anterior |
| Venas escrotales anteriores | Venas escrotales anteriores |
| Vena safena accesoria | Vena safena magna accesoria anterior |
| | Vena safena magna accesoria posterior |
| | Vena safena magna accesoria superficial |
| Vena safena corta o menor | Vena safena pequeña |
| | Extensión craneal de la vena safena pequeña |
| | Vena safena pequeña accesoria superficial |
| | Vena circunfleja anterior del muslo |
| | Vena circunfleja posterior del muslo |
| | Venas intersafenas |
| | Sistema venoso lateral |
| Red venosa dorsal del pie | Red venosa dorsal del pie |
| Arco venoso dorsal del pie | Arco venoso dorsal del pie |
| Venas dorsales metatarsales | Venas superficiales metatarsales (dorsales y plantares) |
| Red venosa plantar | Red subcutánea venosa plantar |
| Arco venoso plantar | |
| Venas plantares metatarsales | Venas digitales superficiales (dorsales y plantares) |

| TERMINOLOGÍA ANATÓMICA | TERMINOLOGÍA PROPUESTA |
|---|---|
| Vena lateral marginal | Vena lateral marginal |
| Vena marginal medial | Vena marginal medial |

**Tabla 2 – Venas profundas**

| TERMINOLOGÍA ANATÓMICA | TRMINOLOGÍA PROPUESTA |
|---|---|
| Vena femoral | Vena femoral común |
| | Vena femoral |
| Vena femoral profunda o vena profunda del muslo | Vena femoral profunda |
| Vena circunfleja femoral medial | Vena circunfleja femoral medial |
| Vena circunfleja femoral lateral | Vena circunfleja femoral lateral |
| Venas perforantes | Venas femorales profundas comunicantes (venas que se acompañan de las arterias perforantes) |
| | Vena ciática |
| Vena poplítea | Vena poplítea |
| | Venas surales |
| | Venas del solium |
| | Venas de los gastrocnemios |
| | Vena medial de los gastrocnemios |
| | Vena lateral de los gastrocnemios |
| | Vena intergemelar |
| Venas geniculares | Plexo venoso genicular |
| Venas tibiales anteriores | Venas tibiales anteriores |
| Venas tibiales posteriores | Venas tibiales posteriores |
| Venas peroneales o fibulares | Venas peroneales o fibulares |
| | Vena plantar medial |
| | Vena plantar lateral |
| | Arco venoso plantar profundo |
| | Venas metatarsales profundas (plantares y dorsales) |
| | Venas digitales profundas (plantares y dorsales) |
| | Vena pedia |

i. Sistema venoso superficial.

Las venas superficiales de las extremidades inferiores forman una red vascular que conecta las venas dorsales superficiales del pie y las venas plantares profundas. El arco venoso dorsal, en el que desembocan las venas metatarsales dorsales, se conecta medialmente con la vena safena magna y lateralmente con la vena safena menor, como se muestra en la figura 1.

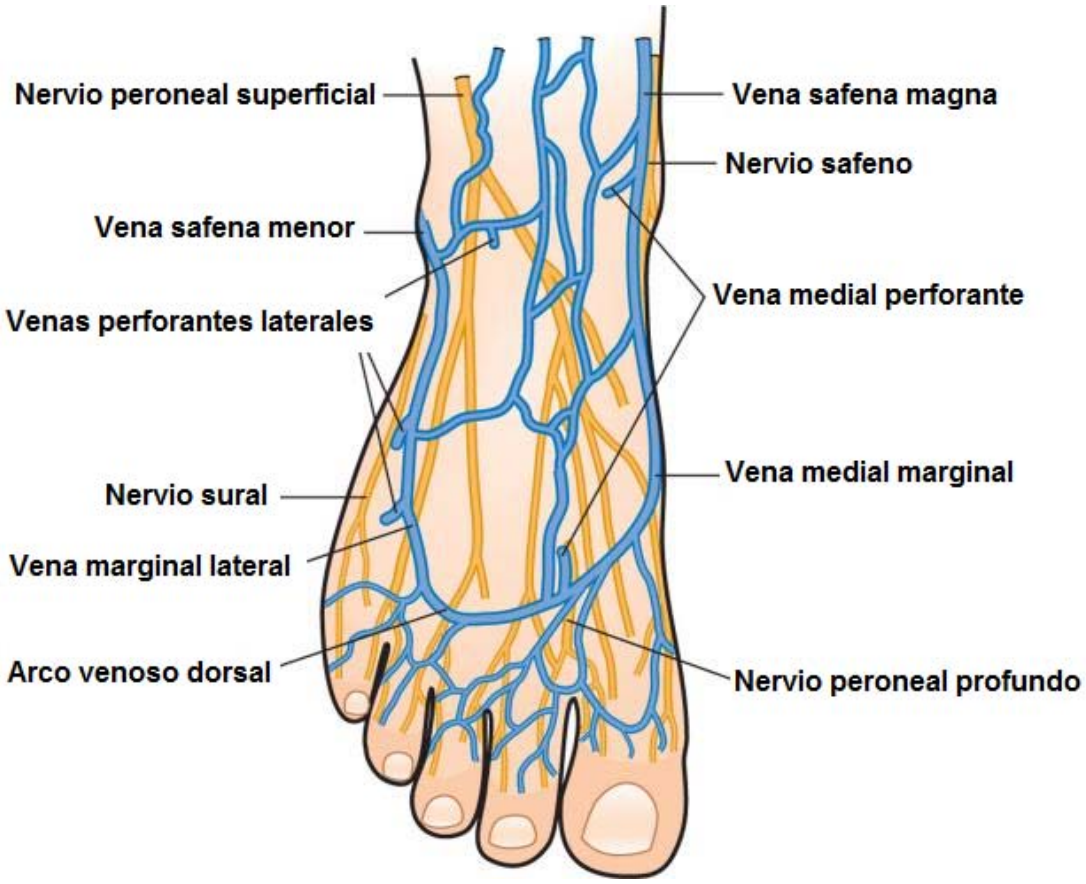


**Figura 1.** Sistema venoso superficial.

La vena safena magna, asciende anterior al maleolo medial, cruza, y luego asciende medial a la rodilla como se muestra en la figura 2. Asciende en el compartimiento superficial y desemboca en la vena femoral común antes de entrar a la *fossa ovalis*. Después de su unión con la vena femoral común recibe a las venas safenas accesorias medial y lateral, así como a pequeñas tributarias de las regiones inguinal y pudenda de la pared abdominal anterior. El arco venoso posterior drena el área que circunda el maleolo medial y este asciende posterior y medial con respecto a la parte posterior de la pierna, recibe a las venas perforantes mediales, llamadas perforantes de Cockett antes de su unión a la vena safena magna por debajo de la rodilla (Figura 2). La vena safena menor emerge del arco venoso dorsal y asciende posterior y lateral al maleolo, esta se une a la vena poplítea antes de penetrar la fascia.

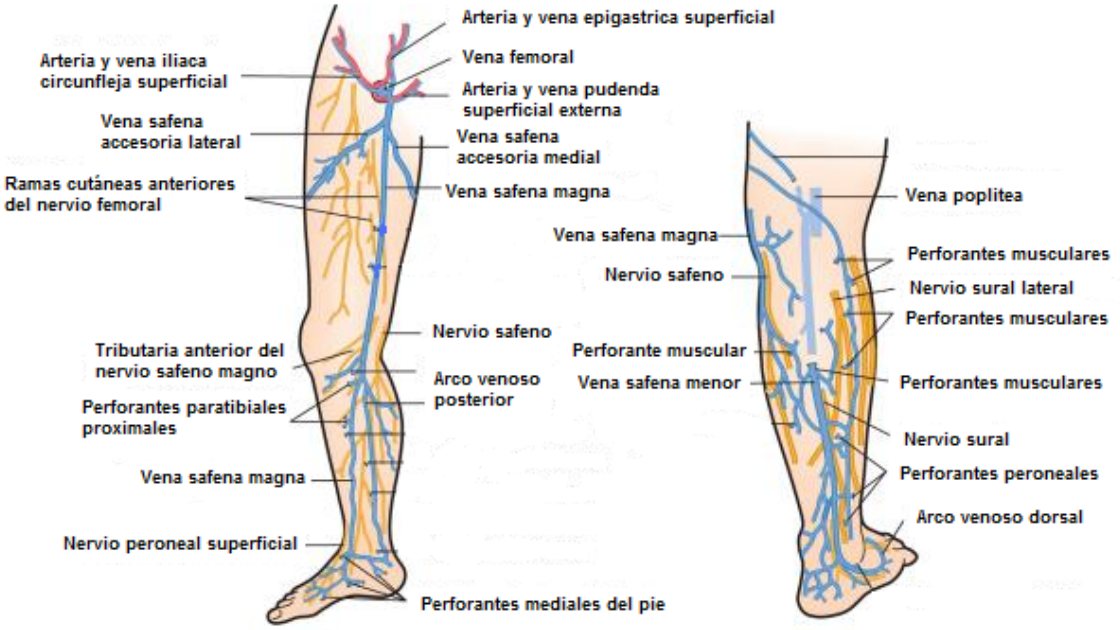


**Figura 2.** Sistema venoso superficial y profundo.

ii. Sistema venoso profundo.

Las venas digitales plantares son tributarias de la red venosa metatarsal que conforma al arco venoso plantar profundo. Estas continúan y drenan en las venas plantares mediales y laterales, que a su vez desembocan en las venas tibiales posteriores. Las venas pedias dorsales forman el par de venas tibiales anteriores en el tobillo, estas de forma adyacente y flanqueando a la arteria tibial posterior, corren por debajo de la fascia del compartimento posterior profundo. Estas venas entran al sóleo y se unen a la vena poplítea después de unirse al par de venas peroneas y tibiales anteriores, ahí forman grandes senos venosos – los senos soleales – que desembocan en las venas tibiales posteriores y peroneas. Las venas de los gastrocnemios se unen a la vena poplítea en un punto distal a la entrada de la safena menor.

La vena poplítea entra al aductor magno y se convierte en la vena femoral. La vena femoral asciende y recibe el flujo de la vena femoral profunda, y después de su confluencia, nace la vena femoral común. En cuanto la vena femoral común cruza el ligamento inguinal, se convierte en la vena iliaca externa.

Las venas perforantes conectan el sistema venoso superficial al sistema venoso profundo en varios puntos en los miembros pélvicos: el pie, medial y lateral en la pantorrilla, y en el centro y distal en el muslo (Figura 3). Estas ramas perforantes son extremadamente variables.

c. Histología normal venosa.

La pared venosa está compuesta por tres capas: la íntima, la media y la adventicia. La pared venosa tiene menos músculo liso y elastina que las arterias. La íntima posee una capa de células endoteliales; la media se compone de células de músculo liso y tejido

conectivo, elastina; la adventicia de la pared venosa contiene fibras adrenérgicas, particularmente en las venas cutáneas (Figura 3).

Las descargas simpáticas centrales y los centros termorreguladores del cerebro pueden alterar el tono venoso; otros estímulos, como cambios en la temperatura, dolor, estímulos emocionales, y cambios de volumen, también inciden sobre este tono.

Las características histológicas de las venas varían dependiendo del calibre de las mismas. Las vénulas, las venas más pequeñas con diámetro de 0.1 a 1 mm, contienen principalmente células de músculo liso, mientras que las más grandes contienen relativamente pocas células musculares. Estas venas de gran calibre tienen una capacidad contráctil limitada en comparación con venas de pared más gruesa como la vena safena magna. Las válvulas venosas impiden el flujo retrógrado y son más prevalentes en la porción distal de las extremidades inferiores.

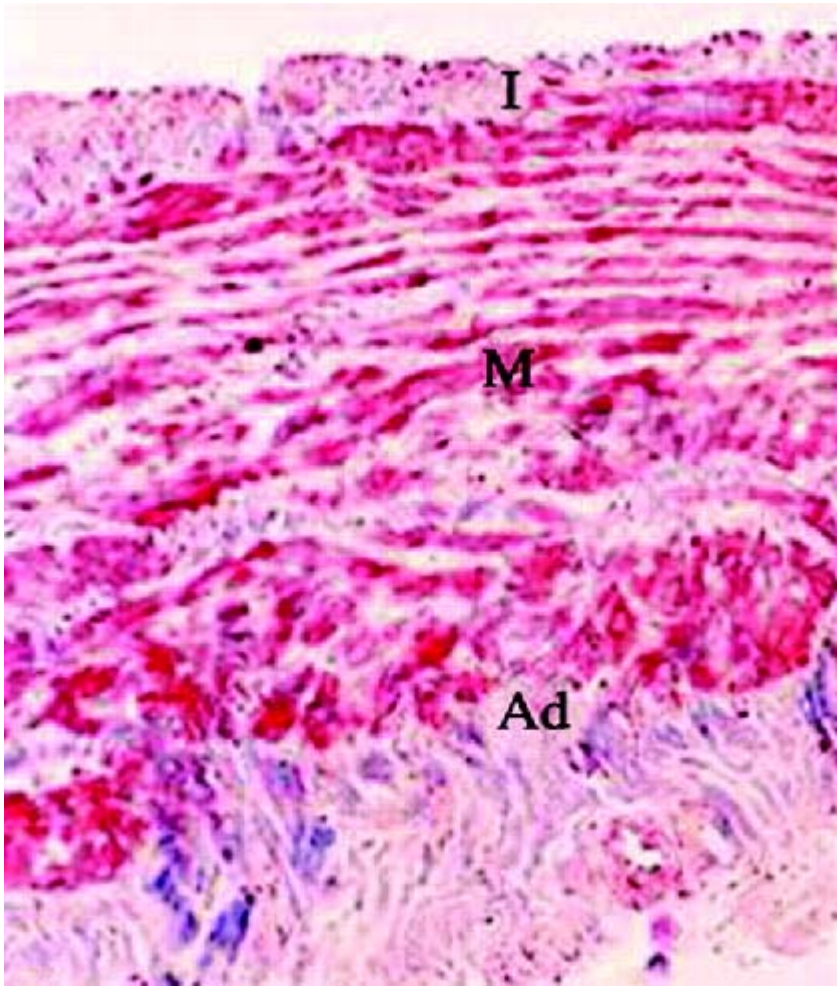


**Figura 3.** Histología normal venosa. Sección de un segmento normal de la vena safena mayor, donde se muestra en azul las fibras de colágeno en la capa subendotelial de la íntima (I), entre el músculo liso de la media (M) y en la adventicia (Ad). Tricrómico de Masson x 100.

La capacitancia de la red vascular es proporcionada principalmente por el sistema venoso, las venas pueden resistir grandes cambios en el volumen sanguíneo con cambios mínimos en su presión.

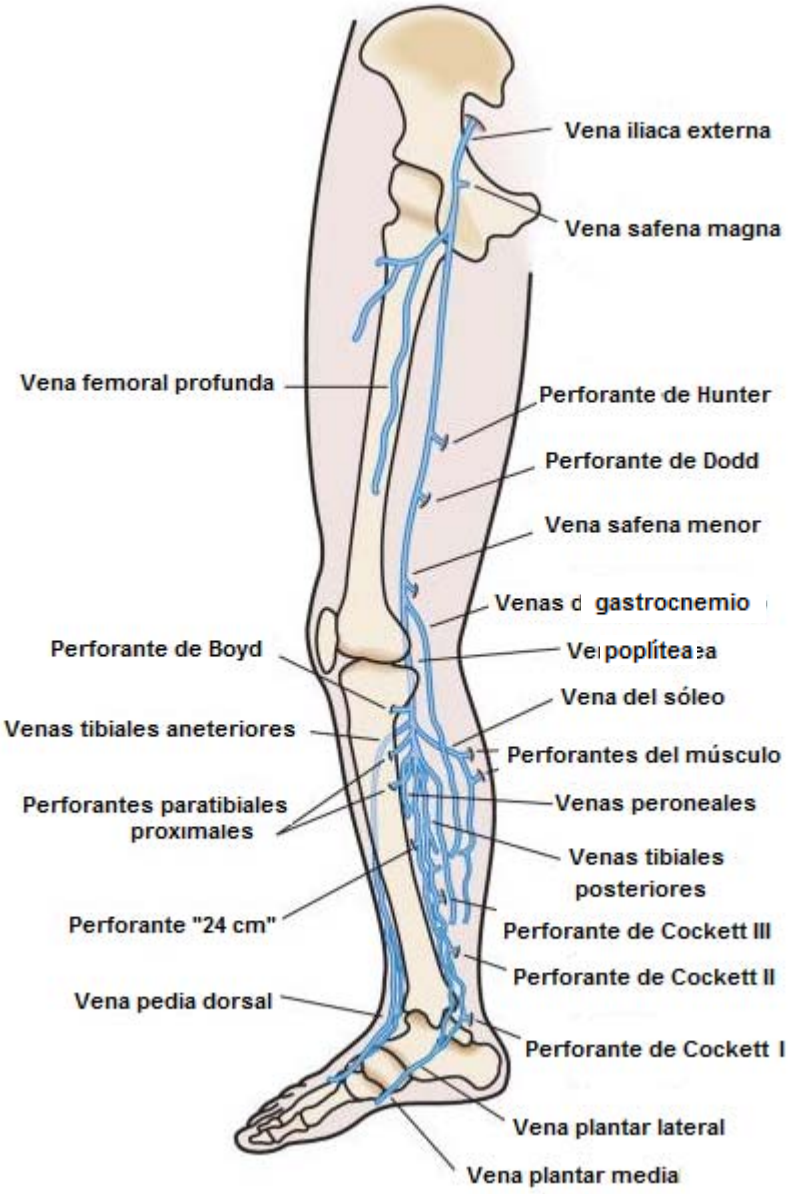


**Figura 4**. Perforantes y sistema venoso profundo.

Los músculos de la pantorrilla funcionan como una bomba y aumentan el flujo venoso de regreso al corazón. Las válvulas del sistema venoso previenen el flujo retrógrado.

d. Teorías fisiopatogénicas

i. Defectos de la pared venosa.

Los defectos de la pared venosa han sido objeto de análisis para explicar la IVC. Se ha comparado el contenido de colágeno y elastina de venas normales y varicosas y se ha observado un incremento significativo en la concentración de colágeno y una disminución en la concentración de elastina en venas con IVC (Figura 5) (Gandhi y col., 1993), (Travers y col., 1996), (Mohamed y col., 2007). Estos hallazgos fortalecen la teoría de que una debilidad primaria de la pared venosa propicia la aparición de venas varicosas.

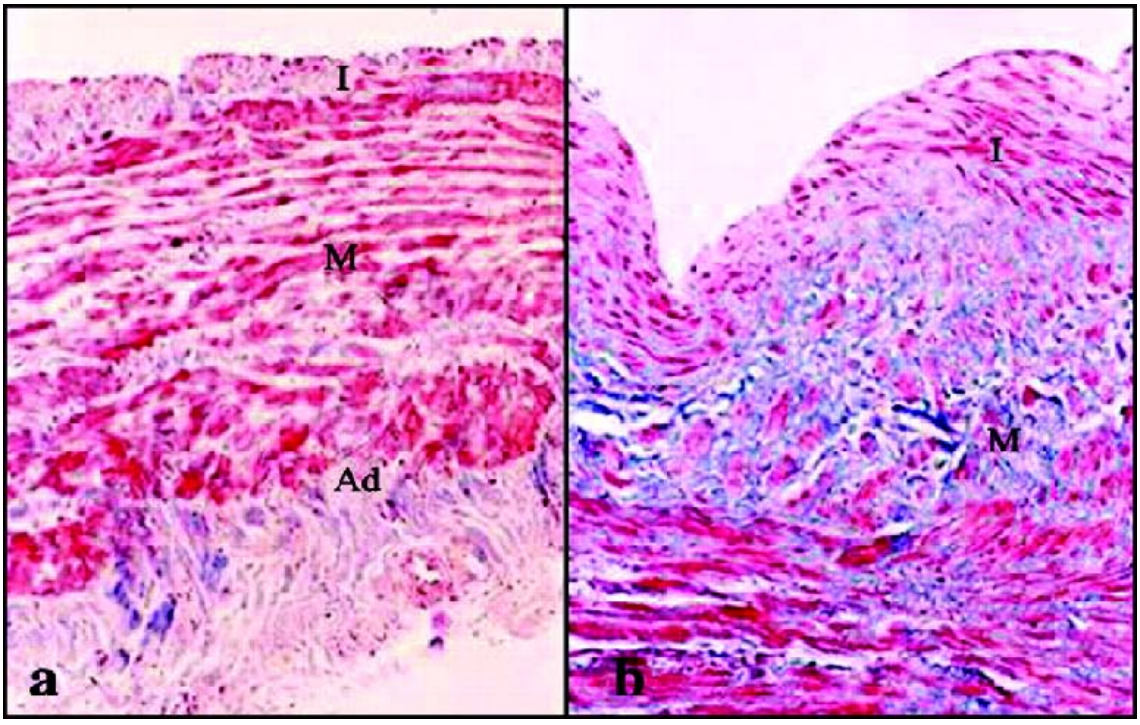


**Figura 5.** Comparación de una sección de vena safena mayor normal (a) y una sección de vena safena mayor con IVC (b). Se puede observar el aumento de las fibras de colágeno en azul fibras en la capa subendotelial de la íntima (I) y entre el músculo liso de la media (M). Tricrómico de Masson x 100.

Por otro lado, se describió la acumulación de matriz extracelular y células de músculo liso hipertrofiadas (Badier-Comander y col., 2001). Esta hipertrofia favorece la síntesis de citocinas como el factor de crecimiento tumoral β1 (TGFβ1), así como la síntesis, proliferación, migración y diferenciación de componentes de la matriz extracelular, específicamente colágeno, elastina, y factor de crecimiento básico de fibroblatos (bFGF), este último es un importante agente mitógeno de las células de músculo liso. Todo esto perpetúa las características morfológicas y patológicas de la insuficiencia venosa crónica. Wali y Eid (2002) observaron cambios importantes en la estructura venosa, caracterizados por degeneración y descamación de las células endoteliales, situación que promueve la migración intramural de los componentes sanguíneos, como leucocitos (Figura 6).

ii. Diferencias anatómicas en la posición de las venas superficiales.

Esta teoría se originó al observar que el tronco principal de la safena no siempre está afectado en la IVC, quizá debido a que este contiene una capa fibromuscular medial bien desarrollada y es sostenida por tejido conectivo fibroso que lo une a la facia profunda. En contraste las tributarias de la vena safena magna poseen menos soporte en la grasa subcutánea y se encuentran por arriba de la capa membranosa de la facia superficial (Figura 7). Estas tributarias también contienen menos masa muscular en su pared; entonces estas, y no los troncos principales, desarrollan varicosidades (Mashiah y col., 1991).

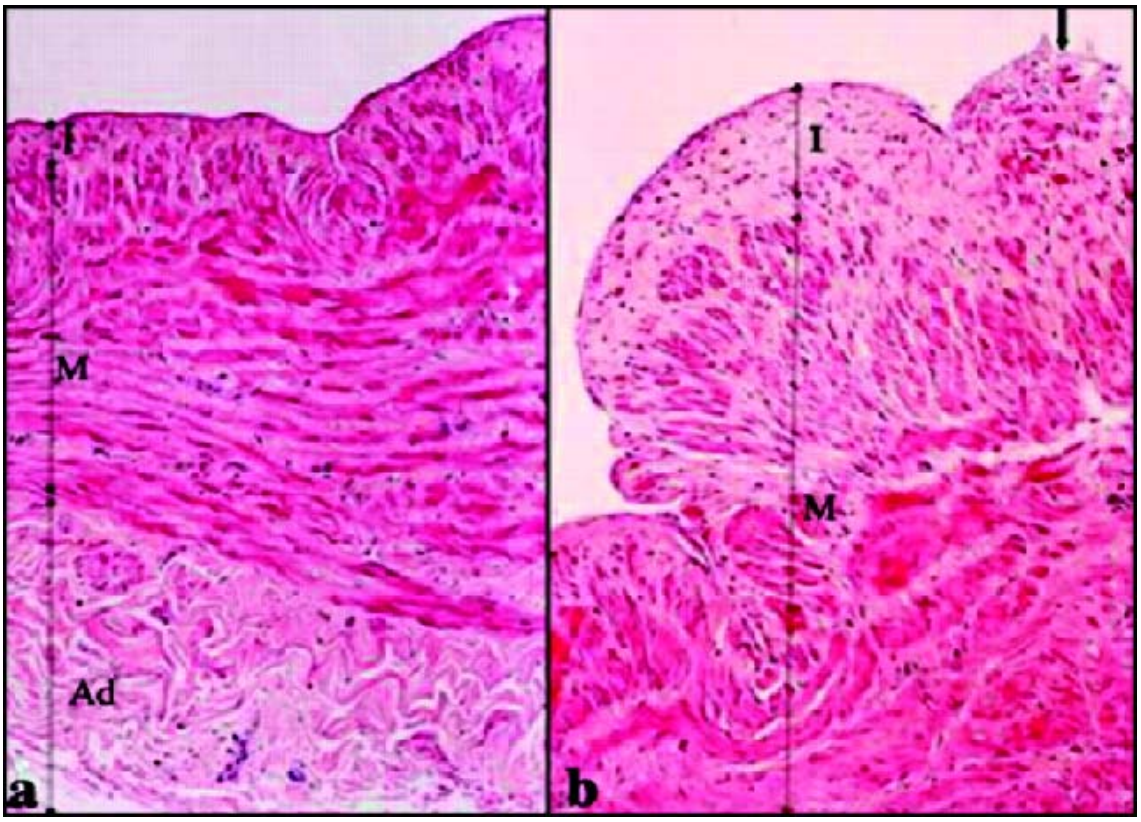


**Figura 6.** (a) Sección de un segmento normal de la vena safena mayor, donde se muestran las 3 túnicas que conforman su pared; túnica íntima (I), túnica media (M) y túnica adventicia (Ad). (b) Sección de un segmento de vena safena mayor con IVC en la que se observa un incremento en el tamaño de la túnica intima (I) y la túnica media (M). También se observa una descamación parcial de las células endoteliales de la íntima (flecha). Hematoxilina y eosina x100.

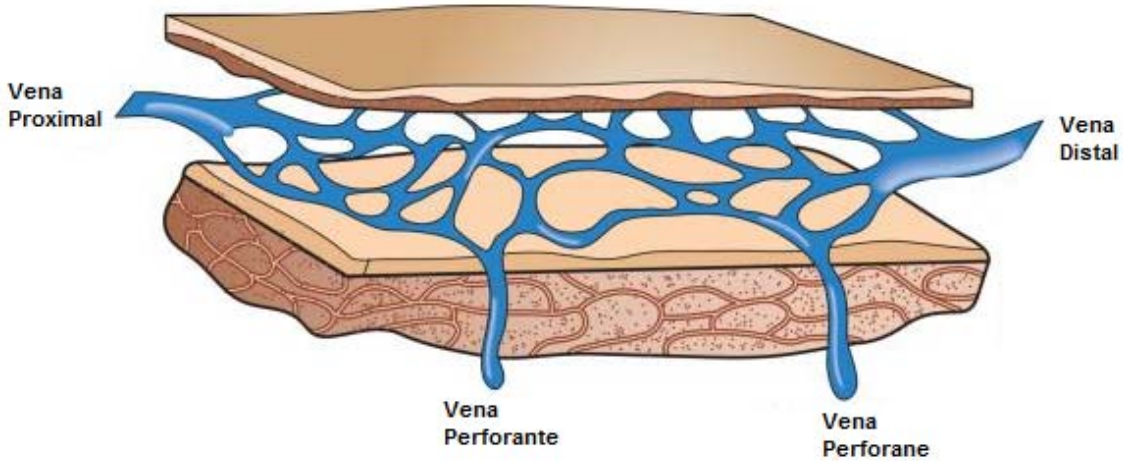


**Figura 7.** Dilatación de las venas tributarias superficiales por el aumento de volumen desde las venas perforantes.

iii. Incompetencia valvular.

La falla de las válvulas que protegen a las venas tributarias de la presión de la vena safena propicia el desarrollo de varicosidades. Estudios sobre la presión venosa han mostrado dos fuentes principales de hipertensión venosa: a) Presión hidrostática. Peso de la columna de sangre proveniente del atrio derecho, donde la presión más grande producida por este mecanismo se encuentra en el tobillo. b) Fuerza de la contracción muscular en los compartimentos de la pierna. Si una vena perforante falla, la presión venosa generada durante el ejercicio (150-200 mmHg) se transmite directamente al sistema venoso superficial, haciendo que se dilate y provocando que las válvulas venosas en estos plexos sean insuficientes y agraven el problema. En 1998, Sales y colaboradores, observaron diferencias en el número de válvulas en dos poblaciones: una con insuficiencia venosa (2.3 válvulas) y otra sin esta alteración (4.8 válvulas), proponiendo que un número suficiente de válvulas ayudan a mantener el flujo anterógrado venoso. En el mismo año, Ono y colaboradores, observaron que la alteración venosa no se relacionaba con el número de válvulas sino que estas estructuras presentaban deformidades y disminución en su tamaño, además observaron que tanto las válvulas como la pared venosa presentaban infiltración leucocitaria (monocitos y macrófagos).

iv. Disfunción endotelial.

Los sistemas venosos poseen dos características esenciales: así, transportan la sangre poco oxigenada de regreso a los pulmones para el intercambio gaseoso y son capaces de almacenar grandes volúmenes sanguíneos. Estas dos propiedades pueden provocar que este sistema desarrolle hipoxia celular, a la cual las células endoteliales son especialmente susceptibles. Debido a su localización estratégica entre el torrente sanguíneo

y el músculo liso vascular, el endotelio alterado no solo produce cambios en la regulación de la coagulación y activación de plaquetas y leucocitos, sino también regula la contracción y relajación del músculo liso al liberar moléculas vasoactivas como el óxido nítrico (NO), prostaglandina $I_2$ ($PGI_2$) o endotelina. Las células endoteliales también producen agentes mitogénicos como el factor de crecimiento derivado del endotelio (VEGF) o el bFGF, además de inhibidores de crecimiento celular como la heparina; ambos tipos de factores modulan la proliferación de células de músculo liso. Michiels y colaboradores (1994), en células de venas umbilicales humanas sometidas a hipoxia, demostraron la liberación endotelial de factores mitogénicos como factor de activación de plaquetas (PAF), prostaglandina $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandina $E_2$ y bFGF y sugieren que esa liberación podría explicar la morfología de las venas varicosas. Arnould y colaboradores (2001), demostraron liberación del PAF y $PGF_{2\alpha}$ en células endoteliales cultivadas y en hígado de rata, ambos en condiciones de isquemia; proponen que ya que estos dos factores son esenciales en la quimiotaxis y adhesión de neutrófilos al endotelio, podrían determinar la infiltración leucocitaria de las venas varicosas y el daño a los componentes de la matriz extracelular.

La hipoxia tiene activa a las células endoteliales disminuyendo la concentración de AMPc, que a su vez aumenta la concentración de calcio citosólico ($[Ca^{2+}]_i$) y este calcio elevado activa la fosfolipasa $A_2$ provocando la síntesis de prostaglandinas y del PAF, que junto con la $PGF_{2\alpha}$ parecen ser responsables de la actividad quimiotáctica de los neutrófilos. Por otro lado, la síntesis de prostanoides inhibe la producción de NO por retroalimentación negativa. La hipoxia también produce exocitosis dependiente de calcio en los organelos característicos de las células endoteliales, denominados cuerpos de Weibel-Palade, los cuales liberan factor de von Willebrand y P-selectina; esta última promueve

adherencia de neutrófilos y en conjunto con los prostanoides propicia una respuesta inflamatoria. La liberación de moléculas mitogénicas, como $PGF_{2\alpha}$, bFGF, endotelina 1 o el factor de crecimiento derivado de plaquetas (PDGF) también se ve favorecida por la hipoxia (Michiels y col., 2000) (Figura 8).

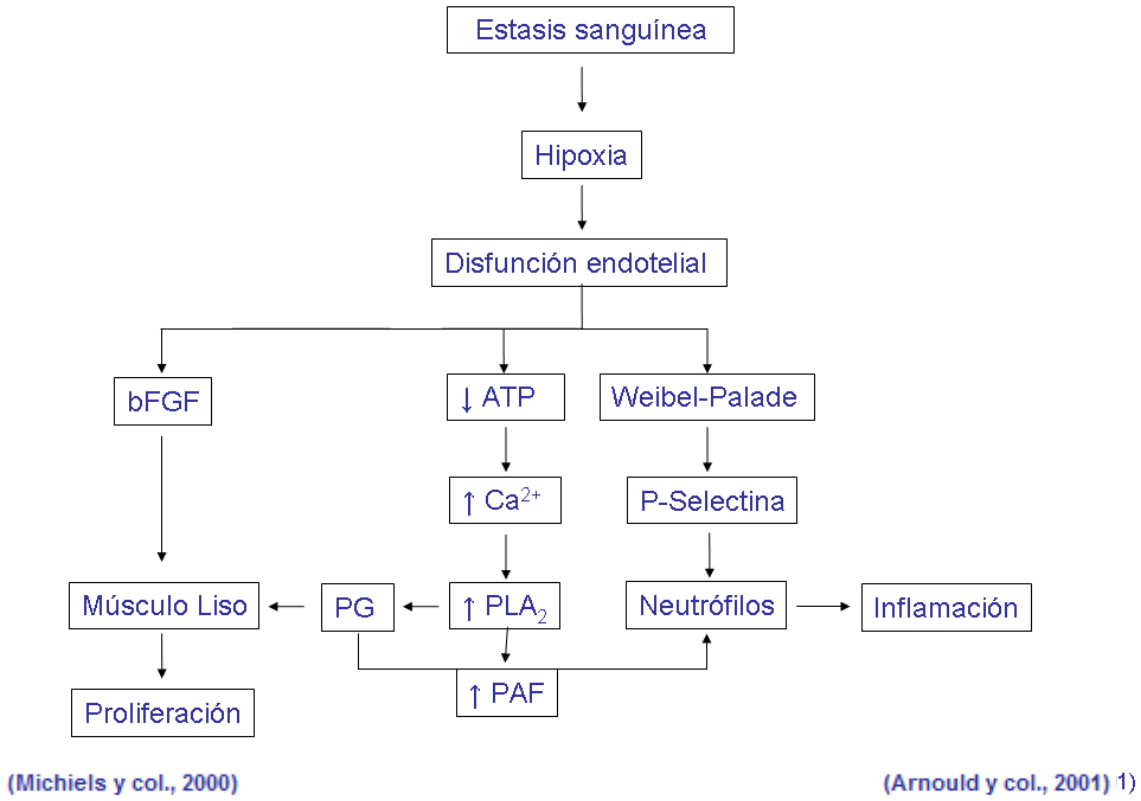


**Figura 8.** Cascada de eventos endoteliales provocados por la estasis sanguínea.
Factor de crecimiento de fibroblastos básico (bFGF). Adenosina Trifosfato (ATP).
Calcio citosólico ($[Ca^{2+}]_i$). Prostaglandinas (PG). Fosfolipasa $A_2$ ($PLA_2$).

b. Clasificación de la IVC.

i. Clasificación CEAP.

La clasificación CEAP estratifica a la enfermedad venosa basada en su presentación clínica, etiológica, anatómica y fisiopatológica (Kistner y col., 1996) (Tabla 3).

**Tabla 3. Clasificación CEAP**

| | |
|---|---|
| C | Signos clínicos (grado$_{0-6}$), complementados por "A" para asintomático y "S" para sintomático. |
| E | Clasificación etiológica (*c*ongénita, *p*rimaria, *s*ecundaria) |
| A | Distribución anatómica(*s*uperficial, *profunda (d)*, o perforante, solas o en combinación) |
| P | Disfunción fisiopatológica (*reflujo* obstrucción, solas o en combinación) |
| **Clasificación Clínica ($C_{0-6}$)** | |
| C 0 | No hay signos visibles o palpables de enfermedad venosa. |
| C 1 | Telangiectasia, venas reticulares. |
| C 2 | Venas varicosas. |
| C 3 | Edema sin cambios en la piel. |
| C 4 | Cambios en la piel (pigmentación, eczema, lipodermatosclerosis) |
| C 5 | Cambios en la piel con úlceras inactivas. |
| C 6 | Cambios en la piel con úlceras activas. |
| **Clasificación Etiológica ($E_C$, $E_P$, o $E_S$)** | |
| Congénita ($E_C$) Causa de IVC presente desde el nacimiento<br>Primaria ($E_P$) IVC de causa indeterminada<br>Secundaria ($E_S$) IVC asociada a una causa conocida (postrombótica, postraumática, etc.) | |
| **Clasificación Anatómica ($A_S$, $A_D$, o $A_P$)** | |
| *VENAS SUPERFICIALES ($A_{S1-5}$)* | |
| 1 | Vena safena magna |
| 2 | *Arriba de la rodilla* |
| 3 | *Debajo de la rodilla* |
| 4 | Vena safena menor |
| 5 | Vena que no es la safena |
| *VENAS PROFUNDAS ($AD_{6-16}$)* | |
| 6 | Vena cava inferior |

| | |
|---|---|
| 7 | *Iliaca común* |
| 8 | *Iliaca interna* |
| 9 | *Iliaca externa* |
| 10 | Pélvicas |
| 11 | *Femoral común* |
| 12 | *Femoral profunda* |
| 13 | *Femoral superficial* |
| 14 | Poplitea |
| 15 | Tibial (anterior, posterior, o peroneal) |
| 16 | Muscular (gastrocnemio, sóleo, otros) |
| ***Venas perforantes ($AP_{17,18}$)*** | |
| 17 | Muslo |
| 18 | Pantorrilla |
| **Clasificación fisiopatológica ($P_{R,O}$)** | |
| Reflujo ($P_R$)<br>Obstrucción ($P_O$)<br>Reflujo y Obstrucción ($P_{R,O}$) | |

De acuerdo a CEAP (Kistner y col., 1996).

2.2 Fármacos que mejoran la función endotelial.

a. Funciones del endotelio.

Las células endoteliales se localizan en la pared vascular en contacto íntimo con el flujo sanguíneo y forman una barrera selectiva para el transporte de moléculas entre la sangre y los tejidos. La pérdida de esta barrera puede desencadenar edema extracelular o en algunos casos, como en respuesta a estímulos inflamatorios, se vuelve menos restrictiva aumentando su permeabilidad al agua y algunas proteínas (Stevens y col., 2000). Ante estímulos como la histamina o la trombina se generan incrementos cortos y rápidos en la permeabilidad vascular, mientras que con estímulos causados por citocinas o VEGF la respuesta obtenida es sostenida.

El endotelio también secreta una variedad de moléculas que regulan la coagulación y la función plaquetaria. El NO y la $PGI_2$ son los principales agentes antiplaquetarios; ambos aumentan sinergísticamente el contenido plaquetario de AMPc, previniendo la agregación. El endotelio también libera ectonucleotidasas en la superficie luminar, que hidrolizan ATP y ADP, ambos potentes agentes de agregación plaquetaria, a AMP y adenosina (Pearson y col., 1980). El equilibrio endotelial puede pasar de un estado anticoagulante a uno procoagulante en respuesta a múltiples estímulos, como las citocinas o el daño vascular. Al menos dos mediadores liberados por las células endoteliales favorecen la activación plaquetaria, el primero es el PAF, inducido por trombina, histamina o citocinas, y el segundo es el factor de von Willebrand (vWF), que se une y estabiliza al factor de coagulación VIII, y facilita la unión de las plaquetas a componentes de la matriz extracelular cuando la pared vascular está dañada (Cines y col., 1998).

Furchgott y Zawadzki (1980) demostraron que la relajación del músculo liso vascular en respuesta a la acetilcolina depende de la integridad del endotelio; el factor relajante derivado del endotelio fue identificado después como el radical libre en estado gaseoso NO (Palmer y col., 1987), se sintetiza a partir de oxígeno y L-arginina por una familia de enzimas, las sintasas de óxido nítrico (NOS). Existen tres isoformas, la endotelial (eNOS) y neuronal (nNOS), que son constitutivas y dependen de calcio/calmodulina, y la inducible (iNOS) que se regula principalmente a nivel transcripcional y es independiente de estimulación por agonistas y por el calcio intracelular. Todas requieren los mismos cosustratos: oxígeno y nicotiamida-adenina dinucleótido fosfato (NADPH); y los mismos cofactores: mononucleótido de flavina (FMN), dinucleótido de flavina-adenina (FAD), tetrahidrobioterina, hemo, y calcio/calmodulina. El NO se difunde a las células del músculo

liso donde estimula a la guanilato ciclasa soluble, con el aumento de formación de guanosina monofosfato cíclico (GMPc) y la subsiguiente relajación (Moncada y Higgs, 1993; Stuehr y col., 2004) (Figura 9). Otros agentes vasodilatadores son la $PGI_2$ y el factor hiperpolarizante derivado del endotelio (EDHF). El endotelio también libera agentes vasoconstrictores, como las endotelinas, $PGF_{2\alpha}$ y tromboxano $A_2$, que en conjunto contribuyen a mantener el tono vascular basal y así determinar la tensión arterial. La estimulación mecánica que produce el flujo sanguíneo sobre el endotelio ("shear stress") activa y regula positivamente la sintasa de NO, además de inhibir la coagulación, la migración leucocitaria, y la proliferación de las células de músculo liso (Ranjan y col., 1995).

La respuesta inflamatoria se caracteriza por una compleja serie de eventos que incluyen dilatación de arteriolas, capilares y vénulas, lo cual desencadena aumento de la permeabilidad, aumento del flujo sanguíneo y migración de leucocitos al foco inflamatorio. Las células endoteliales son esenciales en este proceso ya que coordinan el reclutamiento de las células inflamatorias a los sitios de daño tisular o infección y producen y liberan citocinas y factores de crecimiento que sirven como señales de comunicación con los leucocitos (Muller, 2003).

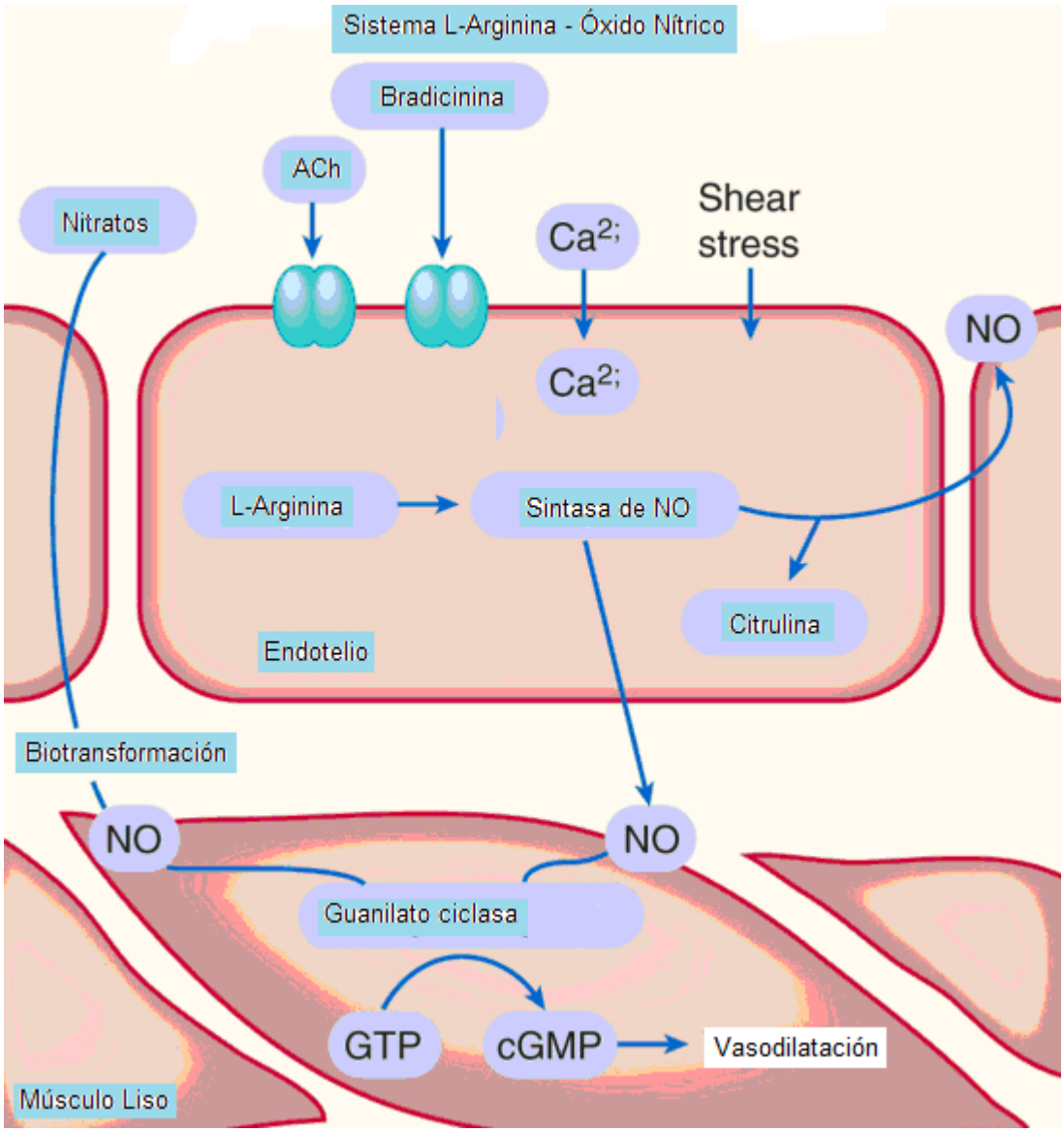


**Figura 9.** El óxido nítrico (NO) es sintetizado en las células endoteliales a partir del aminoácido L-Arginina por la sintasa del NO (eNOS). El NO en las células del músculo liso estimula a la guanilato ciclasa para formar monofofasto de guanosina cíclico (cGMP) resultando en vasodilatación.

Bajo algunas condiciones patológicas como hipertensión, diabetes o hipoxia, la activación de las células endoteliales puede conducir a la liberación de factores contráctiles como tromboxano $A_2$, $PGF_{2\alpha}$ o radicales libres, que son capaces de contrarrestar la actividad vasodilatadora y antitrombótica del NO (Katusic y Vanhoutte, 1989). Los

radicales libres dañan la función endotelial mediante la destrucción de NO (Gryglewski y col., 1986). Se ha detectado una disminución en la producción de NO en pacientes hipertensos (Forte y col., 1997), lo cual señala la importancia de la relajación dependiente del endotelio en el control de la tensión arterial. Uno de los principales mecanismos de disfunción endotelial en la hipertensión es la producción de anión superóxido, y otras especies reactivas de oxígeno que disminuyen la biodisponibilidad de NO. Existen tres principales fuentes enzimáticas del superóxido vascular: (1) la forma reducida de las NADPH oxidasas, que se expresan de manera universal en todos los tipos de células vasculares y son activadas por Angiotensina II; (2) la NOS, que produce superóxido solo cuando existe deficiencia del cofactor tetrahidrobiopterina; y (3) xantina oxidasa, que produce ácido úrico (Paravicini y Touyz, 2006). Por otro lado la hiperglucemia causa glucosilación de proteínas y fosfolípidos y al igual que la hipoxia incrementa el estrés oxidativo intracelular impidiendo la liberación o la actividad del NO (De Vriese y col., 2000).

Michiels y su grupo han propuesto que el medio hipóxico de los lechos venosos podría dañar a las células endoteliales generando disfunción de estas células e IVC (Michiels y col., 2000); en ese sentido, las venas con IVC deberán presentar signos de disfunción endotelial, estos podrían ser revertidos por fármacos que mejoran la función endotelial en arterias al igual que por fármacos que normalmente se utilizan para aliviar los síntomas de este padecimiento.

b. Antihipertensivos.

i. Inhibidores de la enzima convertidora de angiotensina.

El sistema renina-angiotensina. La renina separa 4 aminoácidos del angiotensiógeno circulante que es sintetizado en el hígado para formar un decapéptido biológicamente inactivo, angiotensina I. La enzima convertidora de angiotensina (ECA) separa dos aminoácidos de la angiotensina I para formar un octapéptido bioactivo, angiotensina II (Figura 10) (Weber, 2001).

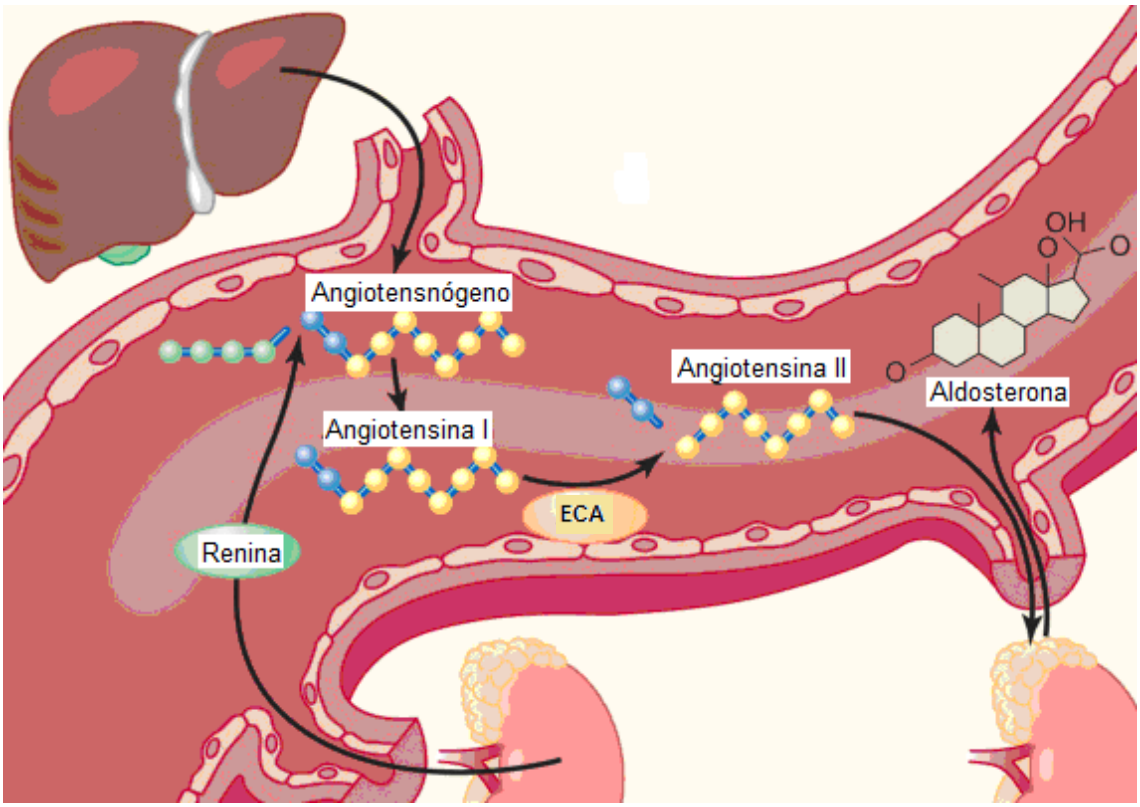


**Figura 10.** El sistema renina-angiotensina-aldosterona. El angiotensinógeno, precursor de los péptidos de angiotensina, se sintetiza en el hígado. En la circulación la renina, que es secretada al lumen de las arteriolas aferentes renales por las células yuxtaglomerulares, separa 4 aminoácidos del angiotensinógeno formado angiotensina I. La enzima convertidora de angiotensina (ACE), enzima unida a la membrana de las células endoteliales convierte a la angiotensina I en angiotensina II, esta última estimula la producción de aldosterona en la zona glomerulosa de la corteza adrenal.

La angiotensina II también puede ser sintetizada por una vía independiente de la renina. Esto es a través de la conversión enzimática de angiotensinógeno a angiotensina I por la calicreína y catepsina G (Figura 11) (Timmermans y col., 1993).

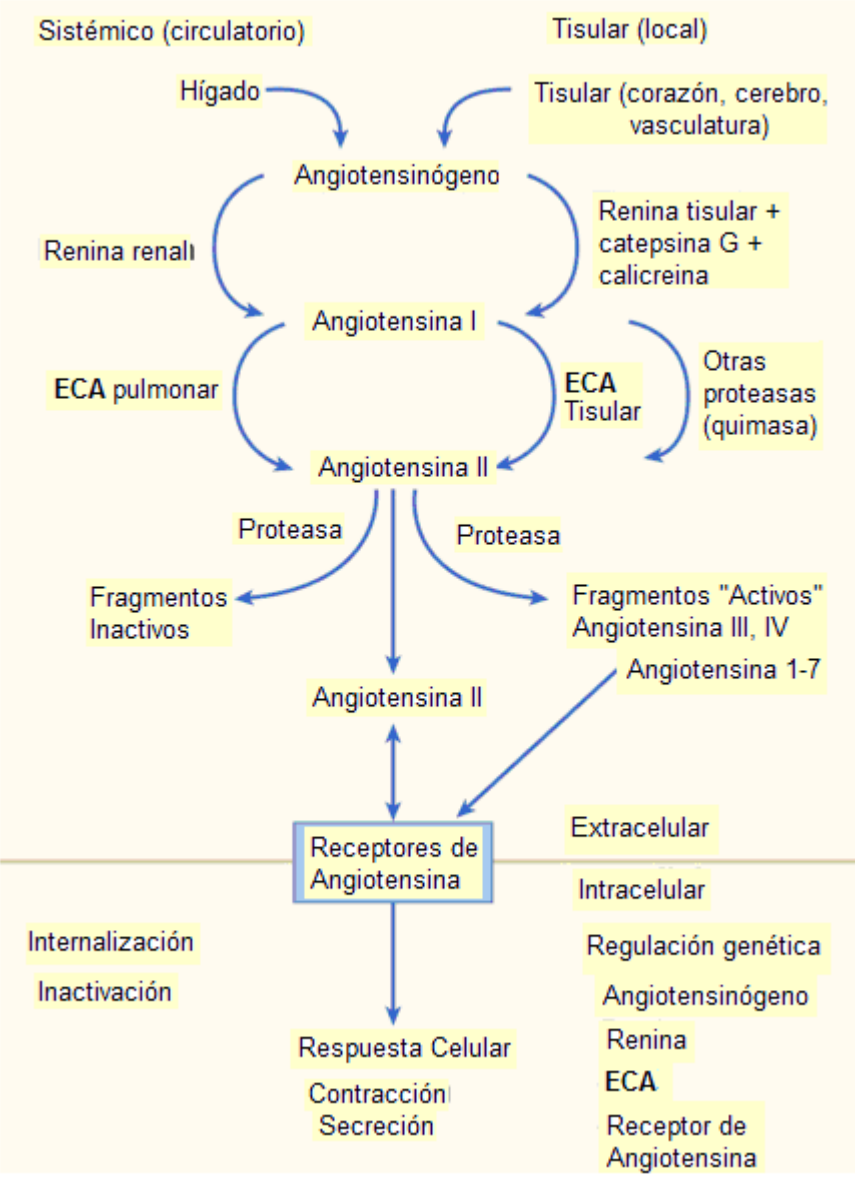


**Figura 11.** Componentes sistémicos y tisulares del sistema renina-angiotensina. Varios tejidos incluyendo al corazón, vasos sanguíneos, riñón, y cerebro tienen la capacidad de generar angiotensina II de forma independiente al sistema renina-angiotensina de la circulación.

La producción tisular de angiotensina II también puede ocurrir a través de vías independientes de ECA, por la activación de la quimasa. Esta vía alterna puede ser de gran importancia en el miocardio, particularmente cuando los niveles de renina y angiotensina I se elevan por el uso de inhibidores de la ECA. La angiotensina II se puede proteolisar para generar tres sustancias con actividad biológica: angiotensina III y angiotensina IV que promueven la vasoconstricción (Haulică y col., 2004), así como la angiotensina (1-7), que puede neutralizar los efectos dañinos de la angiotensina II sobre la función endotelial.

La angiotensina II ejerce sus efectos uniéndose a dos receptores acoplados a proteína G, angiotensina tipo 1 (AT1) y angiotensina tipo 2 (AT2). El receptor de angiotensina que predomina en los vasos es el AT1, a pesar de que ambos AT1 y AT2 están presentes en el miocardio. La activación del receptor AT1 promueve la contracción vascular, crecimiento celular, secreción de aldosterona, y liberación de catecolaminas, mientras que la activación del receptor AT2 promueve vasodilatación, inhibición del crecimiento celular, natriuresis, y liberación de bradicinina.

Los principales efectos farmacológicos de los inhibidores de la enzima convertidora de angiotensina (IECA) son debidos al bloqueo de la producción de angiotensina II, potente vasoconstrictor con actividad cronotrópica positiva (Mori y Hashimoto, 2006) y actividad citotóxica capaz de causar disfunción endotelial por inhibición de la actividad de la sintasa del NO o inducción de estrés oxidativo por activación de la NADPH oxidasa (Griedling y col., 1994). La acción endotelial de estos fármacos también depende de la acumulación local de bradicinina que promueve la liberación de $PGI_2$ endotelial (Gryglewski y col., 2001), el aumento en la actividad de la sintasa de NO y la modulación de la actividad de la NADPH oxidasa, todo lo cual le confiere actividad antioxidante (Rosenkranz y col., 2006). Algunos representantes de este grupo farmacológico como el captopril, poseen actividad

antioxidante *per se* debido a la presencia de un grupo sulfhidrilo en su estructura química (Nakagawa y col., 2006)

ii. Antagonistas de los receptores de angiotensina II.

Existe evidencia experimental de que la angiotensina II produce efectos negativos sobre la función endotelial y de que el bloqueo de los receptores correspondientes revierte dichos efectos. Por ejemplo, en 1996 Rajagopalan y colaboradores demostraron que la angiotensina II incrementa la producción de radicales libres mediante la activación de la NADH/NADPH oxidasa; también observaron que la administración simultánea de angiotensina II y losartán, un antagonista del receptor $AT_1$ de angiotensina II, normalizó la producción de anión superóxido y la relajación por acetilcolina. Prasad y colaboradores (2000) demostraron que en pacientes con aterosclerosis coronaria el losartán mejora la relajación dependiente del endotelio e incrementa la disponibilidad de NO, fenómeno que no ocurrió en los pacientes sin aterosclerosis, indicando que la mejoría en la función endotelial es más marcada entre mayor es el deterioro del endotelio. En el mismo año, Schiffrin y colaboradores reportaron que el antagonismo del receptor $AT_1$ de angiotensina II reduce la hipertrofia ventricular y mejora la relajación dependiente del endotelio en arterias subcutáneas de pacientes con hipertensión; atribuyeron este fenómeno al mismo mecanismo: aumento de NO por la disminución de radicales libres. Esta disminución es debida en parte al aumento en la actividad de la superóxido dismutasa extracelular, secundario al tratamiento con losartán (Horning y col., 2000).

c. Hipocolesterolemiantes.

i. Inhibidores de la 3-hidroxi-3-metil-glutaril-CoA reductasa.

Las estatinas son inhibidores reversibles de la enzima microsomal 3-hidroxi-3-metilglutaril coenzima A (HMG-CoA) reductasa, que convierte HMG-CoA a mevalonato, el paso que determina la velocidad de síntesis de colesterol (Goldstein y Brown, 1990). Esta inhibición lleva a la disminución de la biosíntesis intracelular de colesterol y a la regulación positiva de los receptores de lipoproteínas de baja densidad (LDL-C) en las membranas del hepatocito, favoreciendo la disminución de la circulación de las apolipoproteinas E y B que contienen lipoproteínas. Además de que las estatinas reducen las LDL-C de una forma particularmente efectiva también disminuyen las partículas remanentes, incluyendo las lipoproteínas de muy baja densidad del colesterol (VLDL-C) y las lipoproteínas de densidad intermedia (IDL-C). En consecuencia, la terapia con estatinas, disminuye efectivamente las concentraciones plasmáticas de las lipoproteínas que no son de alta densidad (Halcox y Deanfield, 2004).

Las estatinas proporcionan beneficios terapéuticos al restaurar la síntesis de NO endotelial bajo condiciones mórbidas (p.e. hiperlipidemia). Esto se logra al disminuir el colesterol de la membrana, que restaura el transporte normal de L-arginina, el sustrato de la eNOS. Estudios previos indican que el paso de L-arginina, por el transportador de aminoácidos en la membrana de las células endoteliales, puede estar alterado en la aterosclerosis cuando el colesterol membranal no esterificado esta elevado (Vergnani y col., 2000). Los cambios en el microambiente membranal alteran las propiedades del transporte activo de las proteínas de membrana, como los transportadores de aminoácidos. Así, las estatinas restauran la producción de NO mejorando la captura de L-arginina mientras el colesterol membranal retorna a la normalidad (Mason y col., 2004). Mas allá de la

reducción de las LDL séricas, las estatinas aumentan la liberación de NO a través de diversos mecanismos, como el incremento en la expresión de eNOS y la interferencia en la formación de superóxido. La restauración del NO es esencial, ya que su biodisponibilidad se reduce dramáticamente cuando hay hiperlipidemia, resultando en la pérdida de sus efectos benéficos vasodilatadores y cardioprotectores (Vane y col., 1990). El tratamiento a corto plazo con estatinas ha demostrado mejorar la función endotelial; en sujetos con niveles de colesterol moderadamente elevados (6.2 - 7.2 mmol/L), el tratamiento con simvastatina, 20 mg/día, comparado con placebo, incrementó significativamente ($p<0.0005$) la respuesta vasodilatadora a acetilcolina, determinada por el flujo sanguíneo en el antebrazo, a las cuatro semanas de tratamiento. A los tres meses de tratamiento el grupo de simvastatina mostró una mejoría adicional significativa ($p<0.005$), comparada con la observada a las cuatro semanas (O'Driscol y col., 1997). Un estudio realizado en hombres jóvenes y normocolesterolémicos demostró que el tratamiento con 80 mg de atorvastatina mejora la función endotelial a las 24 horas. El efecto ocurrió antes de que el colesterol y la proteína C-reactiva altamente sensible (hsCRP) séricos, disminuyeran. Estos hallazgos sostienen la teoría de que las estatinas pueden tener efectos benéficos sobre la disfunción endotelial, que son independientes de la disminución plasmática de colesterol (Laufs y col., 2001). La terapia con estatinas a largo plazo también mejora la función endotelial en pacientes con aterosclerosis. En un estudio se compararon un régimen dietético, un régimen de disminución de lipoproteínas de baja densidad (LDL) (lovastatina y colestiramina), y un régimen de disminución de LDL más antioxidantes, todos aplicados durante un año. Se comprobó que en el grupo de disminución de LDL y antioxidantes la vasoconstricción inducida por acetilcolina en arterias coronarias epicárdicas fue menor ($p=0.01$) (Anderson y col., 1995). Las estatinas han mostrado que previenen la regulación negativa de la eNOS,

enzima que cataliza la formación de NO a partir de L-arginina (Martínez y col., 2001). La regulación negativa de la eNOS puede estar mediada por la capacidad de las LDL de incrementar los niveles de caveolina-1, un inhibidor de la actividad de eNOS (Ju y col., 1997; Feron y col., 1998; 2001). La caveolina bloquea el acceso de la enzima a su cofactor, calcio/calmodulina, regulando la producción de NO en el endotelio. Niveles altos de caveolina se asocian con una reducción en la síntesis de NO, que contribuye al aumento de superóxido y pérdida de los efectos benéficos mediados por NO, incluyendo la inhibición de agregación plaquetaria, proliferación de células musculares lisas y adhesión leucocitaria.

Las estatinas también incrementan directamente la actividad de la eNOS constitutiva, aumentando la biodisponibilidad de NO (Romano y col., 2000). Varios mecanismos pueden estar involucrados, tales como disminución de caveolina-1 e incremento en Hsp90, que actúa como un chaperón molecular que facilita la activación a largo plazo de la eNOS. Otros mecanismos incluyen estabilización del RNA mensajero de la eNOS y menor producción de especies reactivas de oxígeno que inactivan el NO (Laufs y col., 1998; Wassmann y cols., 2001). Las estatinas también interfieren con la prenilación de Rho GTPasa por geranilgeranil pirofosfato (GGPP), previniendo su translocación a la membrana celular donde ésta regula negativamente la actividad de la eNOS (Laufs y Liao, 1998).

Las estatinas reducen la oxidación de LDL, evento desencadenante de disfunción endotelial. Los mecanismos de este efecto son: (1) bloqueo de la isoprenilación de rac 1, un importante componente del complejo NADPH oxidasa; (2) reducción de la expresión de las subunidades de la NADPH oxidasa; y (3) reducción de LDL séricos disponibles para la oxidación. Adicionalmente, se observó que la atorvastatina reduce los radicales libres al aumentar la expresión de catalasa, de manera independiente de los cambios de LDL

(Wagner y col., 2000; Bokoch y Prossnitz, 1992; Wassmann y col., 2002). La simvastatina y la pravastatina inducen la fosforilación de la eNOS en Ser 1179 o 1177 mediada por Akt, incrementando la rápida liberación de NO (Kureishi y col., 2000); el aumento en la fosforilación de la eNOS ocurre además en Ser 617 y en Ser 635 a través de las vías PI3-cinasa/ Akt y PKA, respectivamente, lo que contribuye a disminuir la dependencia de la eNOS por calcio-calmodulina. Todo esto explica el aumento en la liberación de NO en tan corto tiempo (Brennan Harris y col., 2004). Álvarez de Sotomayor y colaboradores (2000), demostraron que los beneficios vasculares de las estatinas son también debidos a la liberación de prostanoides endoteliales, al observar que la relajación dependiente del endotelio al construir una curva concentración-respuesta a la simvastatina es inhibida parcialmente por indometacina.

d. Antidiabéticos.

i. Tiazolidinedionas.Activadores de los receptores gamma activados por proliferador de peroxisoma.

Las tiazolidindionas (TZD) son una clase de antidiabéticos orales que aumentan la sensibilidad periférica a la insulina mediante la activación de los receptores gamma activados por proliferador de peroxisoma (PPARγ) (Carsten y col., 2002; Wang y Tafuri, 2003). Las TZD mejoran la función endotelial mediante varios mecanismos, entre ellos un incremento en la síntesis de NO mediada por citocinas, inhibiendo la degradación del mRNA de la iNOS (Hattori y col., 1999). El hecho de que la rosiglitazona no aumente la proteína de eNOS en células endoteliales de bovino, sugiere que la síntesis de NO no depende del aumento de esta isoforma enzimática (Goya y col., 2004). Este mismo hallazgo fue interpretado por Cho y colaboradores (2004) como indicador de que no es un efecto genómico, sino que interviene una regulación positiva del VEGF y de su receptor KDR/Flk-1, por fosforilación de la eNOS-Ser1179, y reduce la dependencia al complejo calcio-calmodulina e incrementa el flujo de electrones del dominio reductasa al de oxigenasa en la enzima. En el incremento en la producción de NO por VEGF también está involucrada la desfosforilación de la eNOS-Ser116 (Kou y col., 2002). Del mismo modo, Calnek y colaboradores (2003) observaron que los PPARγ aumentan la producción de NO por células endoteliales de diferentes sitios anatómicos, sugiriendo que estos efectos se pueden generalizar sin importar la especie o lecho vascular de donde provengan dichas células. Por otro lado los PPARγ suprimen la formación del ión superóxido y aumentan su degradación favoreciendo, así, la liberación de NO (Hwang y col., 2005).

## 2.3 Tratamiento de la Insuficiencia Venosa Crónica.

### a) Medidas conservadoras.

La primera medida terapéutica es la compresión externa utilizando medias elásticas; se han observado algunos efectos fisiológicos, entre ellos, la reducción de la presión venosa durante la deambulación y la mejoría de la microcirculación de la piel. El segundo aspecto de la terapia conservadora es elevar las extremidades inferiores por arriba del nivel del corazón por algunos minutos dos veces en el día.

### b) Tratamiento farmacológico.

#### i. Escina.

Es el principio activo de la semilla del castaño de indias, *Aesculus hippocastanum* L. Químicamente es una mezcla natural de saponinas triterpénicas; su forma β posee propiedades farmacológicas (venoconstrictoras, antiedematosas y antiinflamatorias), que le han colocado como uno de los principales fármacos en el tratamiento de la insuficiencia venosa crónica. Varias de estas propiedades se han atribuido a su capacidad de favorecer la permeabilidad de la membrana celular a los iones de calcio, lo que provoca la vasoconstricción de capilares que impide la formación de edema; por otro lado, favorece la liberación de PGF2α, lo que promueve venoconstricción y mejoría en el retorno venoso (Guillaume y Padioleau, 1994; Sirtori, 2001). Arnould y col. (1996), demostraron que la escina disminuye la activación endotelial inducida por hipoxia, evitando la disminución de ATP y el incremento de fosfolipasa $A_2$, ambas situaciones promotoras de inflamación. Su eficacia terapéutica ha quedado demostrada en varios estudios clínicos (Pittler y Ernst, 1998).

ii. Diosmina.

Es otro importante fármaco utilizado en el tratamiento sintomático de la insuficiencia venosa crónica, aislado de otro flavonoide llamado hesperidina contenido en una gran variedad de plantas como los cítricos. Se ha documentado que tanto la diosmina como la hesperidina prolongan el efecto vasoconstrictor de la noradrenalina, aumentan la sensibilidad a los iones de calcio, inhiben la activación y adhesión leucocitaria e inhiben la producción de radicales libres, propiedades que favorecen el aumento del tono venoso, disminuyen la permeabilidad capilar y en conjunto alivian la sintomatología de la IVC, como lo demuestran varios estudios clínicos (Katsenis, 2005).

c) Tratamiento quirúrgico y escleroterapia.

La escleroterapia es útil en venas con diámetro menor a 1 mm y que no requieren cirugía, la técnica consiste en inyectar una solución esclerosante (tetradecil sódico) directamente en la vena afectada. El uso para este fin de nuevas tecnologías, como el láser, no ha tenido la efectividad esperada.

La flebectomía ambulatoria es una técnica quirúrgica que preserva a las venas safena magna y menor y se utiliza cuando estas no tienen incompetencia valvular (Bishop y Jarrett, 1986) (Figura 12).

Cuando la vena safena magna o la safena menor se ven afectadas, el procedimiento quirúrgico utilizado es la safenectomía (Figura 13). Las indicaciones para realizarla pueden ser desde motivos estéticos hasta el tratamiento de una úlcera activa.

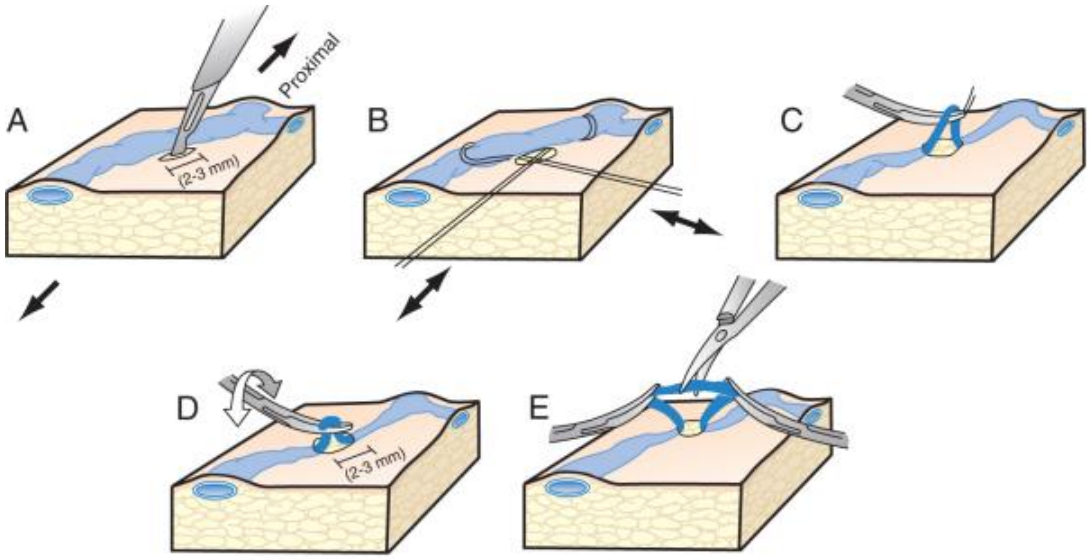


**Figura 12.** Técnica de la flebotomía ambulatoria.

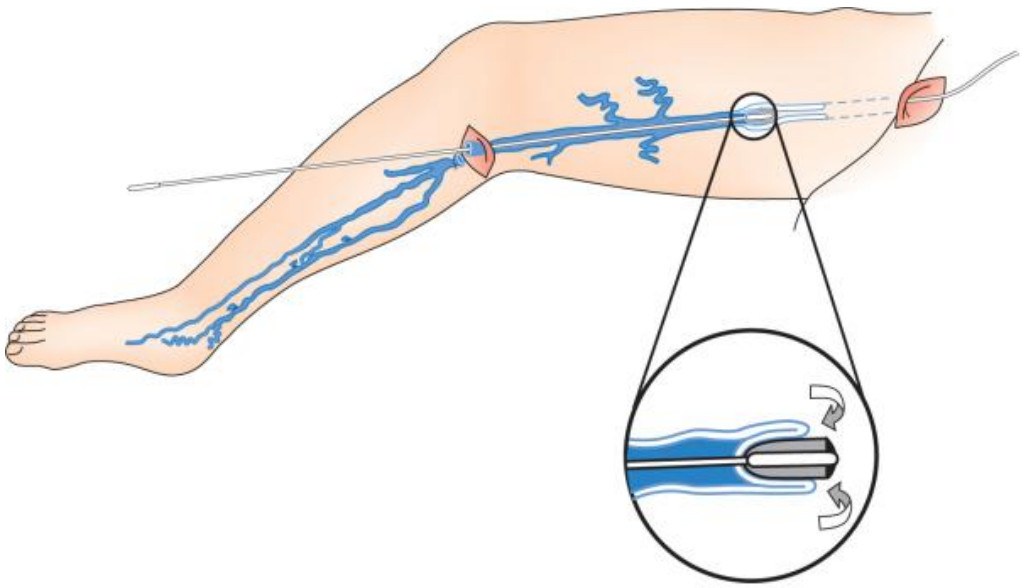

**Figura 13.** Safenectomía.

## III. Desarrollo científico.

### 3.1 Planteamiento del problema.

¿La disfunción endotelial interviene en la fisiopatogenia de la Insuficiencia Venosa Crónica?

### 3.2 Hipótesis

Si la disfunción endotelial es una causa de IVC, entonces la relajación dependiente del endotelio en segmentos de venas varicosas humanas estará comprometida. Este hecho fortalecerá esta teoría fisiopatogénica y podría postular otro blanco terapéutico para este padecimiento.

### 3.3 Estrategia experimental

Determinar la influencia de diversos fármacos (captopril 30 μM, losartán 10 μM, troglitazona 10 μM, pravastatina 100 μM, simvastatina 300 μM y escina 1 μM) sobre la función endotelial de segmentos de venas varicosas humanas colocadas en cámaras de tejido aislado, mediante la medición poligráfica de la relajación dependiente del endotelio.

### 3.4 Objetivo general

Demostrar mediante una técnica funcional, que la disfunción endotelial es una causa fisiopatológica de la insuficiencia venosa crónica.

## 3.5 Objetivos específicos

1. Demostrar que en venas obtenidas de pacientes con insuficiencia venosa crónica existe disfunción endotelial.

2. Demostrar que los fármacos captopril, losartán, troglitazona, simvastatina, pravastatina y escina mejoran la disfunción endotelial venosa.

3. Definir los mecanismos que alteran la función endotelial en la insuficiencia venosa crónica, con base en los mecanismos que se postula que intervienen en la protección endotelial por estos fármacos

## IV. Material y métodos

### 4.1 Aprobación del Comité de Ética.

Este protocolo fue aprobado por el Comité Institucional de Ética e Investigación del Hospital General "Manuel Gea González", México, D.F., con el número 12-67-2004. Todos los pacientes donantes de segmentos venosos firmaron una carta de consentimiento informado.

### 4.2 Descripción de los pacientes.

Se incluyeron un total de 39 pacientes con IVC que habían sido programados para safenectomía; 33 de ellos fueron mujeres y 6 fueron hombres, la edad promedio de todos los pacientes fue de 52 años (con un rango de 34 – 69). La paridad en el grupo femenino varió desde 0 a 6. En todos los casos el diagnóstico de IVC se confirmó clínicamente y por ultrasonografía doppler; todos fueron clasificados según la nomenclatura CEAP como $C_2$ $E_P$ $A_S$ $P_R$. Se excluyeron del estudio pacientes con hábito tabáquico y aquellos con co-morbilidades que dañaran al endotelio vascular (hipertensión, diabetes, hipercolesterolemia, etc.), también se excluyeron a aquellos que recibían medicamentos que modificaran la función endotelial (inhibidores de la ECA, estatinas, antioxidantes, anticonceptivos orales, etc.).

### 4.3 Segmentos venosos.

Los segmentos de vena safena fueron obtenidos de la porción distal a la unión safeno-femoral, durante el procedimiento quirúrgico de safenectomía. Inmediatamente después de la extracción, los segmentos fueron colocados en una solución de Krebs-Henseleit que se mantuvo a 4° C en un recipiente térmico, y se transportaron al laboratorio.

Todo este proceso invariablemente se llevó a cabo en menos de 30 minutos. La composición de la solución de Krebs-Henseleit fue 127 mM NaCl; 4.7 mM KCl; 1.1 mM MgSO4; 1.2 mM $KH_2PO_4$; 2.5 mM $CaCl_2$; 24 mM $NaHCO_3$; 11 mM glucosa; y 0.02 mM ácido etilen-diamino-tetraacético (EDTA).

4.5 Procedimiento experimental.

De cada segmento venoso se obtuvieron 4 anillos de 0.5 cm de longitud que se suspendieron en cámaras de incubación para tejido aislado, entre dos ganchos de niquel-cromo. Un gancho se fijó al fondo de la cámara y el otro a un transductor de tensión Grass FT03 conectado a un polígrafo Grass Modelo 79 (Grass Instrument Division, Astro-Med, West Warwick, RI). Las cámaras contenían 20 mL de solución de Krebs de la composición antes descrita, conservada a temperatura de 37 °C y con burbujeo constante con una mezcla de 95% $O_2$ y 5% $CO_2$; el pH fue de 7.4. Las preparaciones se sometieron a una tensión de reposo de 1 g, que se mantuvo constante durante los experimentos; esta tensión ha sido descrita como óptima para la realización de experimentos con este tipo de vasos humanos (Gavin y col., 1997). Se permitió la estabilización de los anillos por un periodo de al menos 60 minutos, durante el cual se les estimuló varias veces con 3 μM de noradrenalina, con el objeto de favorecer el proceso de estabilización y determinar la viabilidad del tejido; las preparaciones en la que no se observó una respuesta contráctil después de la estimulación fueron descartadas. Los procedimientos que se describen a continuación se aplicaron consecutivamente a los anillos viables.

a. Función endotelial.

Los anillos se estimularon con 3 μM de noradrenalina; una vez obtenida una contracción sostenida, se aplicó acetilcolina 1 μM y se observó la respuesta de relajación. Esta prueba se llevó a cabo en preparaciones provenientes de los 39 pacientes.

b. Reactividad a la noradrenalina.

Se realizaron curvas concentración-respuesta para noradrenalina, acumulativas (10 nM a 10 μM), para calcular la afinidad y eficacia de la catecolamina en estos segmentos. Los anillos que se utilizaron para este procedimiento fueron obtenidos de 37 pacientes.

c. Influencia de fármacos que mejoran la función endotelial.

Se realizaron curvas concentración-respuesta a acetilcolina, acumulativas (10 nM a 100 μM) en anillos precontraídos con 3 μM de noradrenalina. Después del lavado, los fármacos por probar se agregaron a las cámaras y se incubaron durante 30 minutos. Sin lavar la preparación, los anillos fueron contraídos nuevamente y se repitió la curva concentración-respuesta a acetilcolina. Los fármacos probados y sus concentraciones fueron captopril 30 μM, losartán 10 μM, troglitazona 10 μM, escina 1 μM, pravastatina 100 μM, y simvastatina 300 μM. Se ha reportado que estas concentraciones producen protección endotelial en arterias (Fu y col., 2003; Prasad y col., 2000; Laufs y col., 1998; Calnek y col., 2003; Carrasco y Vidrio, 2007). Se incluyeron dos grupos control, en uno se observó la influencia del tiempo en las venas, por lo que no se agregó ningún fármaco durante la incubación y un grupo más donde se incubaron los anillos con dimetilsulfóxido (DMSO) al 1 %. Cada grupo experimental consistió de 8 anillos obtenidos de pacientes diferentes.

## 4.6 Fármacos utilizados.

Los fármacos cloruro de acetilcolina, clorhidrato de (±)-noradrenalina, β-escina, captopril, troglitazona y pravastatina se obtuvieron de Sigma-Aldrich, Toluca, México. El losartán potásico y la simvastatina fueron obsequiados por Merck-Sharp & Dohme México. La noradrenalina se disolvió en ácido ascórbico al 0.1 % para retardar su oxidación; la troglitazona, pravastatina y simvastatina se disolvieron en dimetilsulfóxido (DMSO). Los demás fármacos fueron disueltos en Krebs; todas las soluciones, incluyendo la de Krebs, se prepararon el día del experimento.

## 4.7 Presentación de datos y análisis estadístico

Los resultados son presentados como media ± error estándar. La relajación con acetilcolina se expresó en porcentaje de la contracción producida 3 μM de noradrenalina. En las curvas concentración-respuesta a noradrenalina, la contracción producida por cada concentración se expresó como porcentaje de la respuesta máxima a esta catecolamina. En cada grupo que recibió los fármacos protectores, las respuestas a la acetilcolina en la segunda curva, se compararon con los de la primera con la prueba *t* de Student pareada; un nivel de probabilidad menor a 0.05 ($P<0.05$) se aceptó como indicador de significancia estadística. Para cada par de curvas a acetilcolina se calculó el área bajo la curva y estos valores también se compararon mediante una prueba de *t* de Student pareada. Las curvas concentración-respuesta a noradrenalina se evaluaron sometiendo cada curva a un análisis de regresión no lineal para calcular el valor de $pD_2$ (logaritmo negativo de la concentración efectiva 50 - CE50-) a la catecolamina. La evaluación estadística, las áreas bajo la curva y los análisis de regresión no lineal se realizaron con el paquete GraphPad Prism 4.02 (GraphPad Software, Inc., San Diego, CA).

## V. RESULTADOS

Los anillos venosos precontraídos con noradrenalina se relajaron a la concentración de prueba de acetilcolina un 13.0 ± 1.4%, con un rango entre 2.0% y 35.0% (Figura 14). Los valores de $E_{max}$ y $pD_2$ para la noradrenalina fueron 2.5 ± 0.2 g y 6.4 ± 0.2, respectivamente. La relajación por acetilcolina no fue diferente en mujeres u hombres y fue independiente de la edad, número de embarazos en el grupo de las mujeres o magnitud de la respuesta previa a noradrenalina. Esto fue determinado por análisis de correlación entre la relajación y los diferentes parámetros considerados, resultados que fueron no significativos.

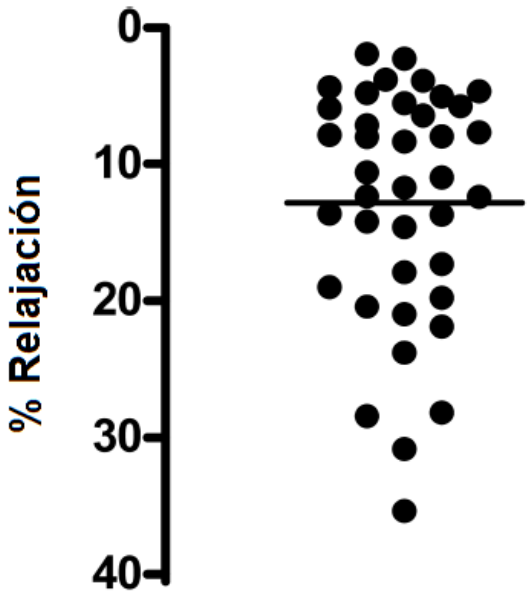


**Figura 14.** Efecto relajante de la acetilcolina (1 μM) sobre la respuesta contractil inducida por noradrenalina (3 μM) en anillos de venas varicosas obtenidas de 39 pacientes. Cada círculo corresponde a un paciente y representa la media de las preparaciones viables (de 1 a 4) obtenidas de ese paciente. La línea horizontal entre los círculos señala la media de las 39 observaciones. El eje vertical indica la relajación como porcentaje de la respuesta contráctil a noradrenalina.

Al administrar acetilcolina, en forma acumulativa, en anillos precontraídos con noradrenalina, se observó relajación progresiva en el rango de concentración de 10 nM - 1 o 3 μM (Figuras 15 a 17 líneas continuas); esta respuesta tendió a desaparecer al adicionar mayor concentración, dando lugar a una curva en "U". Este patrón no se modificó al construir una segunda curva concentración-respuesta 30 minutos después, en ausencia de los fármacos de prueba o ante la presencia del disolvente DMSO (Figura 15).

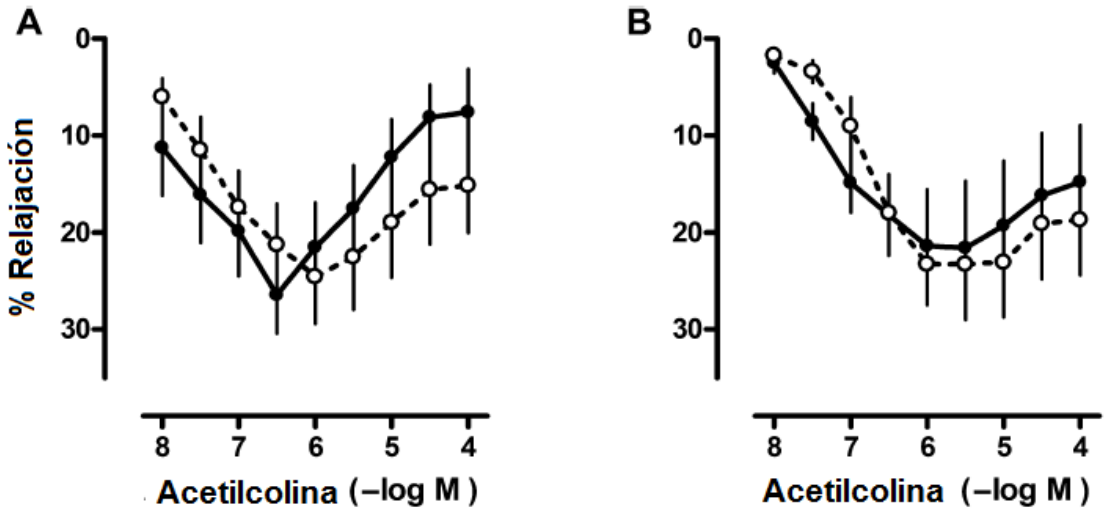


**Figura 15.** Curvas concentración-respuesta a acetilcolina en anillos de venas varicosas humanas, contraídas previamente con noradrenalina (3 μM) (A). Los círculos representan la media ± error estándar de respuestas obtenidas en 8 preparaciones antes (círculos llenos) y después de un periodo de espera de 30 min (círculos vacíos) sin ningún fármaco de prueba. (B) Los círculos representan la media ± error estándar de respuestas obtenidas en 8 preparaciones antes (círculos llenos) y después (círculos vacíos) de 30 minutos de incubación con dimetilsulfóxido (DMSO) al 1%. Los ejes horizontales indican el logaritmo negativo de la concentración molar de acetilcolina. Los ejes verticales corresponden a la relajación como porcentaje de la respuesta contráctil a noradrenalina 3 μM.

La incubación con captopril, losartán, troglitazona, o pravastatina, durante 30 minutos incrementó el efecto relajante de la acetilcolina a mayor concentración (Figuras 16 y 17). Las escina y la simvastatina, en cambio, aumentaron la respuesta de relajación a todas las concentraciones de acetilcolina (Figura 17). En todos los casos la naturaleza bifásica de la curva de acetilcolina permaneció sin cambios.

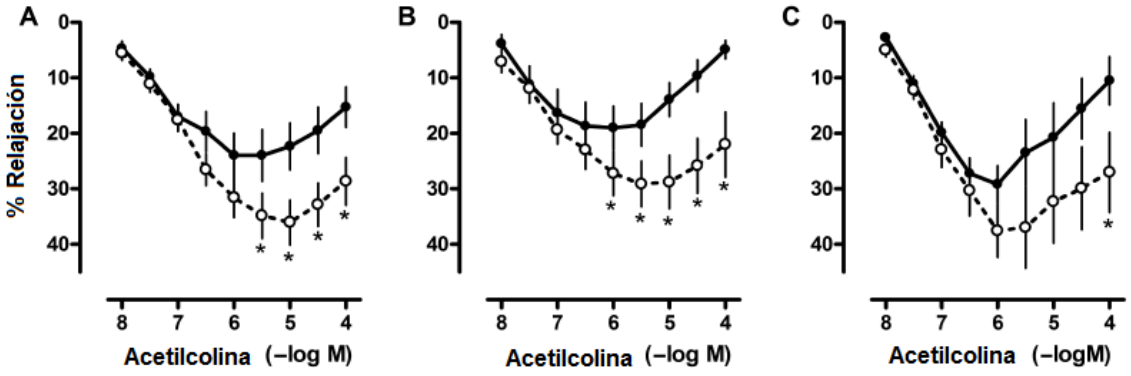


**Figura 16.** Curvas concentración-respuesta a acetilcolina en anillos de venas varicosas humanas contraídas previamente con noradrenalina (3 μM). Los círculos representan la media ± error estándar de respuestas obtenidas en 8 preparaciones antes (círculos llenos) y después de un periodo de 30 min de incubación (círculos vacíos) con 30 μM de captopril (A), 10 μM de losartán (B), o 10 μM de troglitazona (C). Los asteriscos denotan diferencias significativas con los puntos de las primeras curvas. Los ejes horizontales indican el logaritmo negativo de la concentración molar de acetilcolina; los ejes verticales corresponden a la relajación expresada como porcentaje de la respuesta contráctil a la noradrenalina.

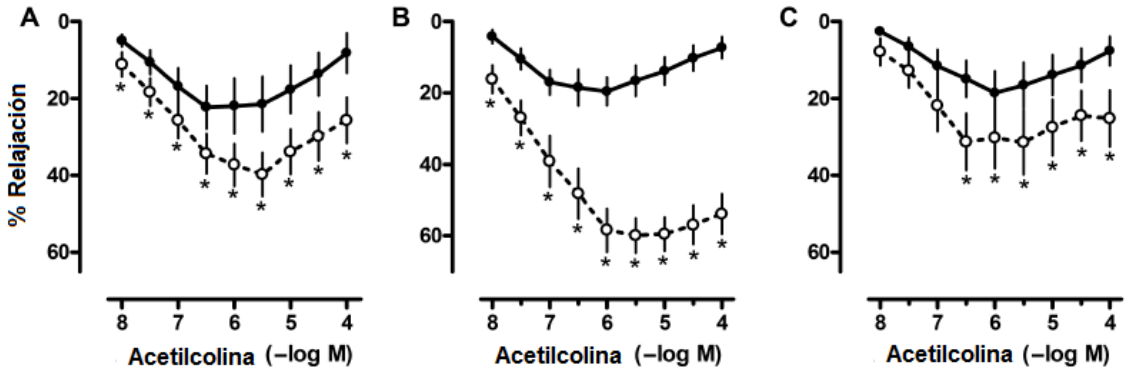


**Figura 17.** Curvas de concentración respuesta a acetilcolina en anillos de venas varicosas humanas contraídas previamente con noradrenalina (3 μM). Los círculos representan la media ± error estándar de respuestas obtenidas en 8 preparaciones antes (círculos llenos) y después de un periodo de 30 min de incubación (círculos vacíos) con 1 μM de escina (A), 300 μM de simvastatina (B), o 100 μM de pravastatina (C). Los asteriscos denotan diferencias significativas con los puntos de las primeras curvas. Los ejes horizontales indican el logaritmo negativo de las concentraciones molares de acetilcolina; los ejes verticales corresponden a la relajación expresada como porcentaje de la respuesta contráctil a la noradrenalina.

Las áreas bajo la curva de cada par de curvas concentración-respuesta a acetilcolina se muestran en la Figura 18. A pesar de que las áreas correspondientes a las primeras curvas de cada par (barras sombreadas) fueron variables, el análisis de varianza reveló que no existieron diferencias estadísticamente significativas entre ellas. Todos los fármacos probados incrementaron en forma significativa el área bajo la curva, siendo simvastatina la más potente.

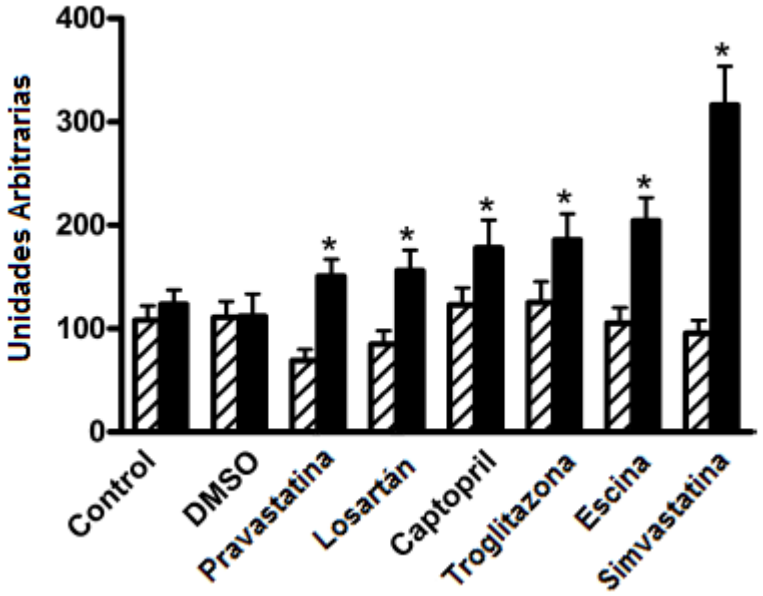


**Figura 18.** Áreas bajo la curva de las curvas concentración-respuesta a acetilcolina en los grupos experimentales descritos en las figuras 15, 16 y 17. Se muestran los valores para las áreas antes (barras sombreadas) y después (barras negras) de 30 minutos de incubación con los fármacos que se indican. Los asteriscos denotan diferencias significativas entre cada par de barras. Los ejes verticales corresponden a las áreas bajo la curva expresadas en unidades arbitrarias. DMSO = dimetilsulfóxido.

## VI. DISCUSIÓN

La estasis venosa que caracteriza a la IVC se ha atribuido, entre otros factores, a un defecto en la respuesta de los lechos venosos a agentes endógenos que promueven la vasoconstricción, uno de ellos es la noradrenalina. Esta teoría se fundamenta en experimentos con segmentos de venas varicosas humanas que muestran reducción en las respuestas contráctiles máximas ($E_{max}$) a las catecolaminas (Thulesius y col., 1991; Lowell y col., 1992). En el presente trabajo se exploraron estas respuestas y se calculó el valor de $pD_2$ para la noradrenalina, un índice de su afinidad por los adrenorreceptores $\alpha_2$ que median la contracción en las venas (Gavin y col., 1997). El resultado obtenido fue de 6.37, mayor que el de 5.93 reportado en venas no varicosas (Cracowski y col., 1999). Es posible que esta afinidad aumentada por la noradrenalina sea una fallida respuesta adaptativa de los adrenorreceptores a la dilatación persistente de las venas varicosas.

Los resultados muestran que la relajación dependiente del endotelio, inducida por acetilcolina, en venas varicosas es del orden de 13%, relajación que el presente estudio no difiere de la que se observa en venas normales (por ética no se obtuvieron venas no varicosas de sujetos sanos). Sin embargo, al comparar este porcentaje con el observado en venas safenas que se utilizaron como "bypass" (30%) (Desai y col, 2004; Rakici y col., 2005), se puede concluir que a pesar de la co-morbilidad que prevalece en los pacientes que se someten a una cirugía de "bypass" (hipertensión, hipercolesterolemia, diabetes, etc.), y a que estas patologías ocasionan algún grado de disfunción endotelial (Lakka y col., 2002), los datos encontrados sugieren que la IVC cursa con una grave alteración de la función endotelial venosa, y que esta condición puede ser un factor esencial en la génesis y evolución de esta patología. Michiels y su grupo han propuesto una hipótesis que vincula la disfunción endotelial y la IVC: el medio hipóxico de los lechos venosos podrían dañar a las células endoteliales, provocando una liberación desorganizada de mitógenos (bFGF) (Michiels y col., 2000) y moléculas proinflamatorias como prostaglandinas y Factor Activador de Plaquetas (PAF) (Michiels y col., 1993). Estas condiciones podrían ser las responsables de la expresión clínica clásica de la IVC: venas alargadas y tortuosas, con dolor y edema (Michiels y col., 2004).

Estas observaciones sobre la disfunción endotelial coinciden con las realizadas por Lowell y colaboradores (1992), quienes reportaron una drástica reducción de la relajación dependiente del endotelio inducida por el ionóforo de calcio A23187 en venas varicosas; también son compatibles con la disminución de NO plasmático observada por Hollingsworth y colaboradores (2001) en pacientes con IVC. En contra de estos hallazgos está la observación de un aumento en la producción de NO por células endoteliales de venas varicosas en cultivo (Schuller-Petrovic y col., 1997). Sin embargo, la relevancia de estos datos obtenidos de cultivos de células endoteliales, sustraídas del medio hipóxico que normalmente les rodea ha sido puesta en tela de juicio (Cines y col., 1998).

Otro indicio de disfunción endotelial en estas venas varicosas fue el hallazgo de las curvas de concentración-respuesta a acetilcolina en forma de "U". Una posible explicación es que a la concentración de hasta 3 μM, este agente produce relajación por la liberación de factores vasodilatadores endoteliales; sin embargo, a mayor concentración la relajación mediada por las células endoteliales dañadas es superada por la estimulación de los receptores muscarínicos en las células del músculo liso, dando lugar a contracción en sustitución a relajación como respuesta a acetilcolina.

Algunos fármacos usados en la terapéutica, se han identificado como protectores del endotelio vascular en las arterias. Este efecto se ha descrito para los inhibidores de la ECA como el captopril (Chen y col., 2008), los antagonistas de los receptores AT1 como el losartán (Bayorh y col., 2005), ligandos del PPAR-γ como la troglitazona (Watanabe y col., 2000), y los inhibidores de la HMG-CoA reductasa como la pravastatina (Nanayakkara y col., 2007) y la simvastatina (Török y col., 2007). Esta acción ha sido ligada a influencias sobre el sistema del NO por diversos mecanismos. Por ejemplo, el captopril posee efectos antioxidantes al limitar la actividad de la enzima NADPH oxidasa (enzima estimulada por la angiotensina II), que incrementa la formación del anión superóxido y éste disminuye la biodisponibilidad del NO (Rosenkranz y col., 2006), también funciona como atrapador de radicales libres de oxígeno através de su grupo sulfhidrilo (Nakagawa y col., 2006). El losartán reduce el estrés oxidativo aumentando la actividad de la superóxido dismutasa y de esta forma mejora la biodisponibilidad de NO (Horning y col., 2000). La troglitazona

regula positivamente el VEGF KDR/Flk-1, reduciendo la dependencia de la eNOS a calcio-calmodulina, por la fosforilación de Ser 1179 (Cho y col., 2004); también aumenta la biodisponibilidad de NO por reducción en la formación e incremento de la degradación del anión superóxido (Hwang y col., 2005). Las estatinas también aumentan la fosforilación de eNOS en Ser 1179 o 1177 activando la proteína cinasa Akt (Kureishi y col., 2000). De acuerdo con los resultados obtenidos, todos estos mecanismos de los fármacos estudiados parecen contribuir a la protección endotelial de las venas varicosas humanas.

La escina, el principio activo más importante de las semillas del *A. hippocastanum*, fue incluido en este estudio para determinar la posible correlación entre la eficacia clínica de este agente en IVC (Pittler y Ernst, 1998) y su efecto protector del endotelio en arterias (Carrasco y Vidrio, 2007). Esto último podría ser atribuido a su mecanismo atrapador de radicales libres (Guillaume y Padioleau, 1994) y a la activación de la eNOS inducida por el aumento de calcio intacelular (Carrasco y Vidrio, 2007). El hallazgo de que la escina mejora la relajación mediada por el endotelio en venas varicosas humanas, sugiere que la disfunción endotelial ocupa un lugar importante en la patogénesis de la IVC.

## VII. CONCLUSIONES.

Los resultados confirman la existencia de disfunción endotelial en segmentos de venas varicosas humanas, al haber una pobre relajación dependiente del endotelio. Además, los resultados muestran que esta disfunción puede ser revertida al exponer al vaso a agentes que protegen el endotelio vascular arterial, indicando que los efectos benéficos no se circunscriben solo a este lecho vascular sino también al sistema venoso.

El hecho de que la escina, un agente antivaricoso clínicamente efectivo, también mejore la función endotelial en las venas, constituye evidencia adicional para pensar en la disfunción endotelial como un participante importante en la patogénesis de la IVC y sugiere que mejorar la función endotelial podría representar un blanco terapéutico para el tratamiento de esta condición.

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# Endothelial Function Impairment in Chronic Venous Insufficiency: Effect of Some Cardiovascular Protectant Agents

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In segments of human varicose veins, endothelial function was assessed by measuring relaxation induced by acetylcholine in noradrenaline-precontracted preparations. In addition, concentration-response curves to acetylcholine were obtained before and after incubation with the arterial endothelium protectant agents captopril, losartan, troglitazone, pravastatin, or simvastatin. The antivaricose agent escin was also tested. Mean acetylcholine-induced relaxation of varicose venous rings was about 13%, approximately one third of that reported for control saphenous veins. Concentration-response curves to acetylcholine were "u" shaped, the result of endothelium-mediated relaxation at low concentrations, superseded by subsequent smooth muscle contractile responses. Relaxation was enhanced by the endothelium-protecting agents and by escin, troglitazone being the least, and simvastatin the most effective. It was concluded that endothelial dysfunction is present in varicose veins, that this anomaly can be reverted by cardiovascular protecting agents, and that it can play a role in the pathogenesis and treatment of chronic venous insufficiency.

**Keywords:** varicose veins; endothelial function; vasoactive drugs

## Introduction

Chronic venous insufficiency (CVI) is a clinical entity with an abnormally functioning venous system, manifested by a range of signs, the most apparent of which are varicose veins (tortuous, twisted, or lengthened veins) and venous ulcers. Other signs and symptoms may include aching, heaviness, leg-tiredness, cramps, itching, sensations of burning, swelling, edema, telangiectasia (spider veins), hyperpigmentation of skin of the ankle "atrophie blanche" (white scar tissue), and lipodermatosclerosis (induration caused by fibrosis of the subcutaneous fat).[1] Comprehensive diagnosis of CVI is achieved through the universally adopted Clinical, Etiological, Anatomical, and Pathophysiological (CEAP) classification system. Clinically, findings may range from no visible or palpable signs of venous disease to active ulceration; etiologically, CVI may be congenital, primary (undetermined cause), or secondary; anatomically, superficial, deep, or perforator veins may be involved, and finally, the pathophysiologic findings may explain the production of CVI by reflux, obstruction, or both (Table 1).[2] The incidence of varicose veins is higher among women than in men, up to 73% in women and 56% in men, with an increasing prevalence with increased age, the highest rates being in the age range of 40 to 49 years for women and 70 to 79 years for men.[3]

Most varicose veins are primary (unknown origin); only a minority are secondary to conditions such as deep vein thrombosis and occlusion, pelvic tumors, or arteriovenous fistulae.[4] One of the traditional theories of the pathogenesis of CVI attributes development of primary varicose veins to failure or incompetence of the valves leading to reflux of blood from the deep to the superficial system, venous hypertension, and dilation of the involved veins.[5]

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**Table 1.** Chronic Venous Insufficiency Classification[a]

| Clinical Findings | | Etiology | | Anatomy | | Pathophysiology | |
|---|---|---|---|---|---|---|---|
| $C_0$ | None | $E_C$ | Congenital | $A_S$ | Superficial | $P_R$ | Reflux |
| $C_1$ | Telangiectasia | | | | | | |
| $C_2$ | Varicose veins | $E_S$ | Secondary | $A_P$ | Perforator | $P_O$ | Obstruction |
| $C_3$ | Edema | | | | | | |
| $C_4$ | Skin changes | | | | | | |
| $C_5$ | Healed ulcers | $E_P$ | Primary | $A_D$ | Deep | $P_{R,O}$ | Both |
| $C_6$ | Active ulcers | | | | | | |

[a] According to Padberg.[2]

Recent observations indicating that varicose veins can develop without valvular incompetence[6] have stressed the importance of other pathogenic factors, such as local hormonal influences in pregnant women or mechanical disturbances and high venous pressure in the pelvic limbs, as observed after prolonged standing. All these factors, including the first-mentioned valvular defect, contribute to the development of venous blood stasis and consequently to a limited oxygen supply state in the venous media, particularly in the endothelial cells.[7] The venous endothelium constitutes the first barrier between blood and vascular tissue and is designed to prevent physical disruption of the vessel wall by trauma or other aggressions as well as to maintain intravascular volume and insure adequate oxygen delivery.[8] This protective function is achieved through the synthesis of vasoactive molecules such as nitric oxide (NO), prostaglandin $I_2$ ($PGI_2$), or endothelin; compounds regulating leukocytes, interleukin-8 (IL-8), or platelet-activating factor (PAF); and smooth muscle cell function regulators, platelet-derived growth factor (PDGF), endothelial cell-derived growth factor (ECDGF), basic fibroblast growth factor (bFGF), or heparin. Hypoxic conditions have profound effects on endothelial cells, activating them and leading to a disorganized release of the above chemotactic and mitogenic factors.[9-11] Such abnormal situation could explain some of the findings typical of CVI, such as vein inflammation and hypertrophy, leg pain, edema and itching, and fibrosis. Thus, endothelial vein dysfunction could play a major role in CVI as it does in arteries in important systemic diseases such as hypertension, diabetes, or hypercholesterolemia.[12-14]

The current study was carried out in segments of human varicose veins to explore the function of venous endothelium in CVI. Following the discovery of endothelium-dependent relaxation of isolated aorta by acetylcholine,[15] recording of this response has been widely used to study endothelial function in a variety of arteries. Although endothelium-dependent relaxation in veins is generally considered less than that in arteries, there are no systematic studies on this question.[16] This response has also been assessed in human varicose veins,[17] but whether endothelial dysfunction is present in these preparations cannot be ascertained, in view of the lack of availability of segments of normal human veins for comparison. The nonvaricose veins generally regarded as controls are segments used for coronary bypass procedures, obtained from patients suffering from advanced atherosclerotic processes and thus with some degree of endothelial dysfunction. A first objective of this work was to measure the extent and variability of relaxation of human varicose veins induced by a standard concentration of acetylcholine.

A number of drugs are known to improve endothelium-mediated arterial relaxation, mainly through an increase in NO availability. Among these are the antihypertensive agents angiotensin-converting enzyme (ACE) inhibitors[18] and angiotensin II receptor ($AT_1$) antagonists,[19] the cholesterol-lowering drugs 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors or statins,[20] and the antidiabetic adjuvants peroxisome proliferator-activated receptor-$\gamma$ (PPAR-$\gamma$) ligands or thiazolidinediones.[21] Accordingly, a second objective of this study was to determine whether members of these classes of drugs also improve acetylcholine-induced relaxation of segments of human varicose veins. The agents tested were the ACE inhibitor captopril, the $AT_1$-receptor antagonist losartan, the statins simvastatin and pravastatin, and the thiazolidinedione troglitazone. In addition, the effect of escin, the major active principle of extracts of the seeds of *Aesculus hippocastanum*, the horse chestnut tree, widely used in the treatment of CVI,[22] was evaluated. This product has recently been found to exert an endothelium-protectant action in rat aorta.[23]

## Materials and Methods

### Ethical Approval

The current study protocol, designated by code number 12-67-2004, Manuel Gea González General Hospital, Mexico City, was approved by the Institutional Ethics and Research Committee. All participants signed an informed consent form.

### Participants

A total of 39 patients with CVI scheduled for saphenectomy participated in the study; 33 were women and 6 were men, with a mean age for the whole group of 52 years (range 34-69). Parity among women ranged from 0 to 6. In all cases, diagnosis of CVI was confirmed clinically and by duplex scanning examination; all were classified as $C_2$ $E_P$ $A_S$ $P_R$ according to the CEAP nomenclature. Excluded from the study were smokers and patients with endothelial-damaging morbidity (hypertension, diabetes, hypercholesterolemia, etc) or receiving drugs modifying endothelial function (ACE inhibitors, statins, antioxidants, oral contraceptives, etc).

### Vein Segments

A segment of saphenous vein was obtained from each patient just distal to the saphenofemoral junction, prior to stripping of the remaining portion of the vein during the saphenectomy procedure. Immediately after retrieval, segments were placed in Krebs-Henseleit solution kept at 4°C in a thermos bottle and transported to the laboratory, a procedure always lasting less than 30 minutes. The composition of the physiological solution was 127 mmol/L NaCl; 4.7 mmol/L KCl; 1.1 mmol/L $MgSO_4$; 1.2 mmol/L $KH_2PO_4$; 2.5 mmol/L $CaCl_2$; 24 mmol/L $NaHCO_3$; 11 mmol/L glucose; and 0.02 mmol/L EDTA.

### Experimental Procedure

From each venous segment, 4 rings of 0.5 cm long were obtained. Rings were suspended in jacketed 20-mL organ chambers between 2 nickel–chromium wire hooks. One of the hooks was fastened to the bottom of the chamber and the other was attached to a Grass FT03 force transducer, which was connected in turn to a Grass Model 79 polygraph (Grass Instrument Division, Astro-Med, West Warwick, RI). The baths contained Krebs-Henseleit solution of the above composition, which was kept at 37°C and bubbled with a mixture of 95% $O_2$ and 5% $CO_2$; pH was 7.4. The preparations were subjected to a resting tension of 1 g, which was maintained constant throughout the experiments; this tension has been found by others to be optimal for tests of this type in human veins.[24] The rings were allowed to stabilize for a period of at least 60 minutes, during which they were stimulated several times with 3 μmol/L noradrenaline, both to aid the stabilization process and to determine viability of the tissue; preparations failing to contract upon such stimulation were discarded. The following procedures were then conducted in succession in viable rings:

1. *Endothelial function*. The relaxant response to 1 μmol/L acetylcholine was determined in rings previously contracted with 3 μmol/L noradrenaline. This assay was conducted in preparations from 39 patients.
2. *Reactivity to noradrenaline*. Complete cumulative concentration-response curves to noradrenaline (10 nmol/L to 10 μmol/L) were obtained to calculate the affinity and efficacy of the catecholamine in these preparations. Rings subjected to this procedure were obtained from 37 patients.
3. *Influence of endothelium-protecting drugs*. Cumulative concentration-response curves to acetylcholine (10 nmol/L to 100 μmol/L) were constructed in rings precontracted with 3 μmol/L noradrenaline. After washing, the test drug was added and incubated with the preparation for 30 minutes. Without washing, the ring was again contracted and the curve to acetylcholine was repeated. The test drugs were captopril 30 μmol/L, losartan 10 μmol/L, troglitazone 10 μmol/L, escin 1 μmol/L, pravastatin 100 μmol/L, and simvastatin 300 μmol/L. These concentrations have been reported to exert endothelium protection in arteries.[18-21,23] Control experiments included a time control with no drug added during incubation and incubation with 1% dimethylsulfoxide (DMSO). Each experimental group consisted of 8 rings obtained from different patients.

### Drugs

Acetylcholine chloride, (±)-noradrenaline hydrochloride, β-escin, captopril, troglitazone, and pravastatin were obtained from Sigma-Aldrich (Toluca, Mexico). Losartan and simvastatin were gifts from Merck-Sharp & Dohme Mexico. Noradrenaline was dissolved in 0.1% ascorbic acid to prevent oxidation;

troglitazone, pravastatin, and simvastatin were dissolved in DMSO. The rest of the drugs were dissolved in Krebs. Stock solutions of acetylcholine and noradrenaline were prepared daily and appropriate dilutions were made with Krebs.

### Data Presentation and Statistical Analyses

Results are presented as means ± SEM. Venous relaxation with acetylcholine is expressed as percentage of the contractile response to 3 μmol/L noradrenaline. Contraction with noradrenaline is expressed as percentage of the maximum response to this catecholamine. In each group receiving the test drugs, individual responses of the second acetylcholine concentration-response curve were compared with those of the first curve by a paired *t* test, with a *P* value of less than .05 considered significant. Each pair of acetylcholine curves was also compared by calculating the corresponding areas under the curve and subjecting them to a paired *t* test. Concentration-response curves to noradrenaline were evaluated by subjecting individual curves to nonlinear regression analysis to calculate $pD_2$ values (negative logarithm of $EC_{50}$) for the catecholamine. Statistical evaluation, areas under the curve, and nonlinear regression analyses were carried out with a GraphPad Prism 4.02 package (GraphPad Software, Inc, San Diego, Calif).

## Results

Venous rings previously contracted with noradrenaline relaxed to the test concentration of acetylcholine by 13.03 ± 1.35%, with a range of 2% to 35% (Figure 1). Values of $E_{max}$ and $pD_2$ for noradrenaline were 2.50 ± 0.21 g and 6.37 ± 0.20, respectively. Acetylcholine relaxation was not different in men or women and was independent of age, parity, or responsiveness to noradrenaline. This was determined by analysis of correlation between relaxation and the different parameters considered.

Acetylcholine, when applied cumulatively at concentrations between 10 nmol/L and 1 or 3 mol/L, induced progressively increasing relaxation of noradrenaline-contracted rings (Figures 2 through 4, solid lines). This response tended to subside upon application of higher concentrations. This pattern was not modified upon construction of a second concentration-response curve 30 minutes later, either in the absence of no other drug or in the presence of the solvent DMSO (Figure 2).

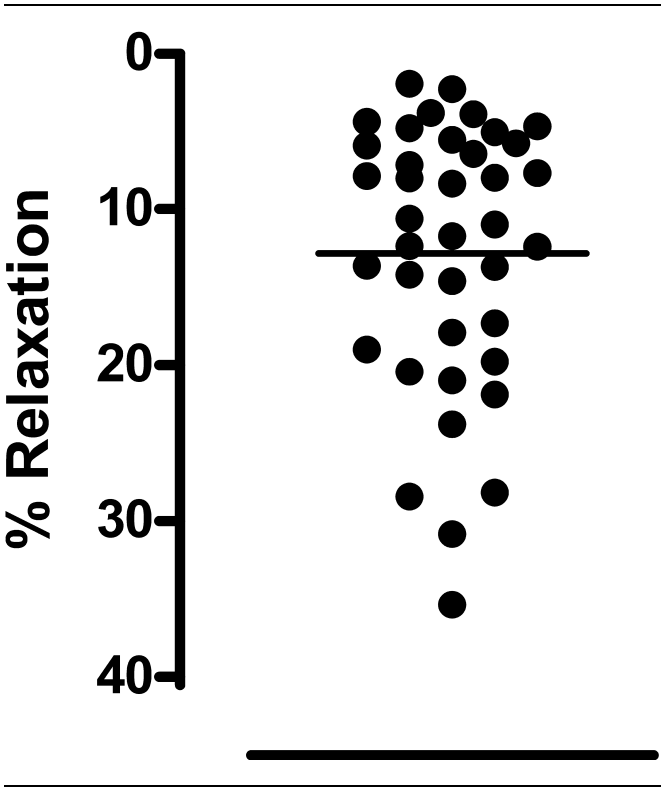


**Figure 1.** Relaxant effect of acetylcholine (1 μmol/L) in rings of human varicose veins precontracted with noradrenaline (3 μmol/L). Each circle represents 1 of 39 patients from which the rings were obtained and corresponds to the mean of the viable preparations (1-4) of that patient. The horizontal line across the circles denotes the mean of the 39 observations. The vertical axis indicates relaxation as percentage of the contractile response to noradrenaline.

Incubation for 30 minutes with captopril, losartan, troglitazone, escin, pravastatin, or simvastatin increased the relaxant effect of acetylcholine, specially of the higher concentrations of this agent (Figures 3 and 4). Escin and simvastatin, however, elicited this effect at all concentrations of acetylcholine (Figure 4). In all cases, the biphasic nature of the acetylcholine curve remained essentially unchanged.

Areas under each pair of acetylcholine concentration-response curves are shown in Figure 5. Although the areas corresponding to the first curves of each pair were somewhat variable, analysis of variance revealed no significant differences among them. All drugs tested significantly increased the area under the curve, the most potent being simvastatin.

## Discussion

The venous stasis characteristic of CVI has been attributed, among other factors, to a defect in venous

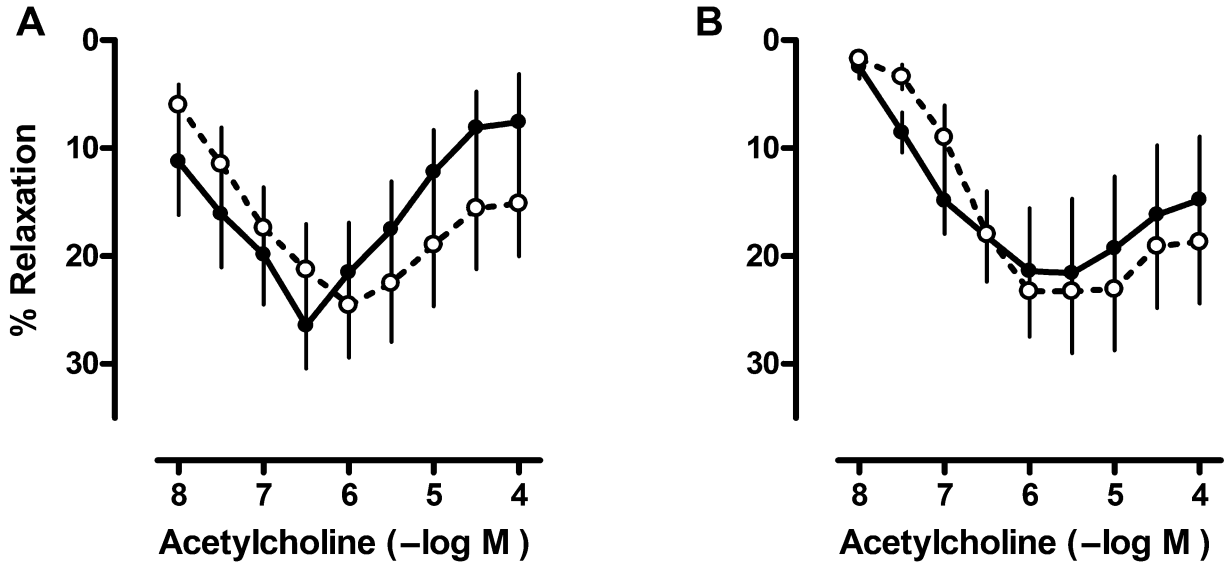


**Figure 2.** Concentration-response curves to acetylcholine in rings of human varicose veins precontracted with noradrenaline (3 μmol/L). Circles represent means ± SE of responses obtained in 8 preparations before (solid circles) and after 30 minutes of incubation (empty circles) with no drug (A) or with 1% dimethylsulfoxide (B). Abscissae indicate the negative logarithms of molar concentrations of acetylcholine; ordinates correspond to relaxation expressed as percentage of the contractile responses to noradrenaline.

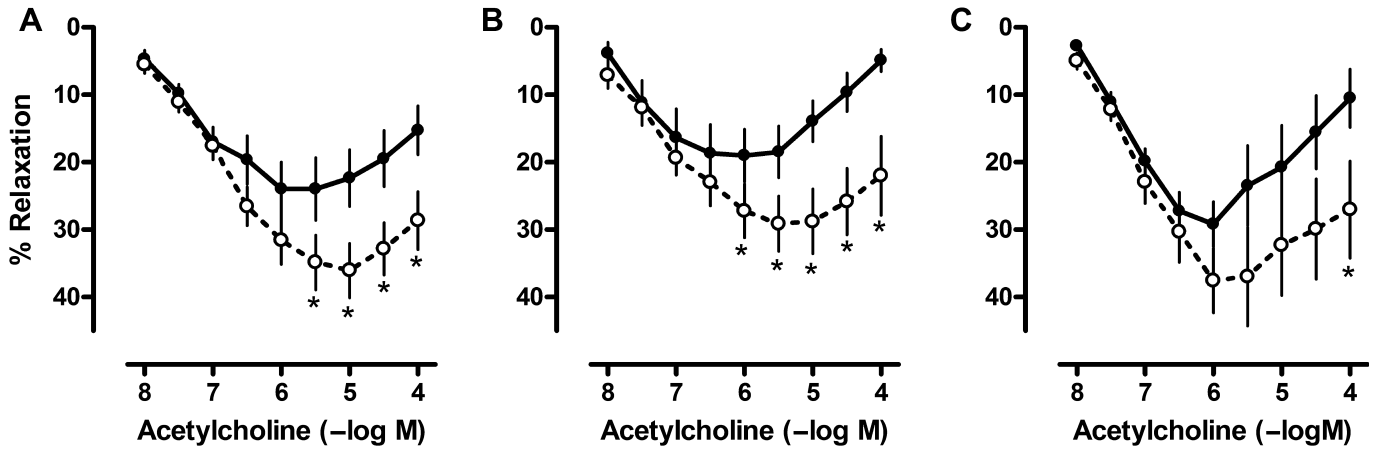


**Figure 3.** Concentration-response curves to acetylcholine in rings of human varicose veins precontracted with noradrenaline (3 μmol/L). Circles represent means ± SE of responses obtained in 8 preparations before (solid circles) and after 30 minutes of incubation (empty circles) with 30 μmol/L captopril (A), 10 μmol/L losartan (B), or 10 μmol/L troglitazone (C). Asterisks denote significant differences from points in the first curves. Abscissae indicate the negative logarithms of molar concentrations of acetylcholine; ordinates correspond to relaxation expressed as percentage of the contractile responses to noradrenaline.

responses to endogenous contractile agents, among them noradrenaline. This possibility is supported by experiments in human varicose vein segments showing reduced maximal contractile responses ($E_{max}$) to the catecholamine.[25,26] In the current study, we explored these responses and calculated the $pD_2$ value for noradrenaline, an index of its affinity for the $\alpha_2$ adrenoceptors mediating contraction in veins.[24] Unexpectedly, the resulting value of 6.37 was greater than that of 5.93 reported for non-varicose veins.[27] It is possible that as an adaptive response to the persistent dilatation of the varicose veins, the receptors therein undergo changes leading to the enhanced noradrenaline affinity observed.

The current results show that in varicose veins acetylcholine-induced endothelium-dependent

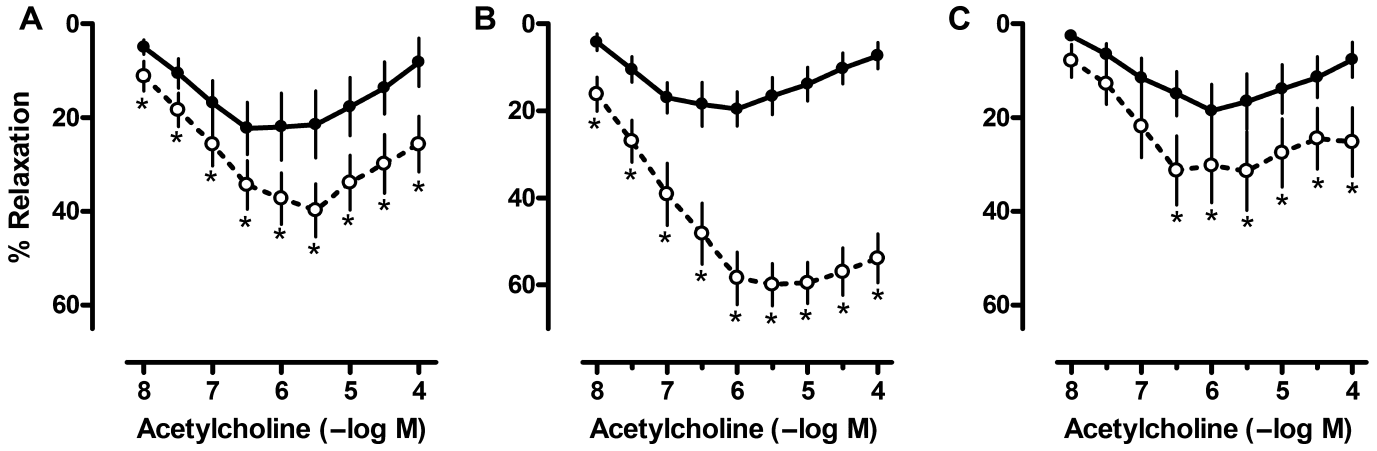


**Figure 4.** Concentration-response curves to acetylcholine in rings of human varicose veins precontracted with noradrenaline (3 μmol/L). Circles represent means ± SE of responses obtained in 8 preparations before (solid circles) and after 30 minutes of incubation (empty circles) with 1 μmol/L escin (A), 300 μmol/L simvastatin (B), or 100 μmol/L pravastatin (C). Asterisks denote significant differences from points in the first curves. Abscissae indicate the negative logarithms of molar concentrations of acetylcholine; ordinates correspond to relaxation expressed as percentage of contractile responses to noradrenaline.

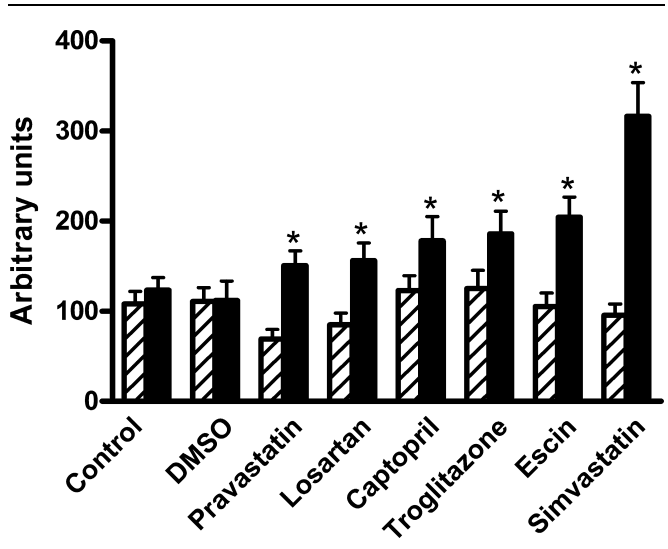


**Figure 5.** Areas under the curve of concentration-response curves to acetylcholine in the experimental groups depicted in Figures 2, 3, and 4. Shown are values for areas before (shaded bars) and after (black bars) 30 minutes of incubation with the test drugs indicated. Asterisks denote significant differences from the corresponding control bars. Ordinates correspond to areas under the curve expressed in arbitrary units. DMSO = dimethylsulfoxide.

relaxation is of the order of 13%. Although for ethical reasons healthy human saphenous veins were not included for comparison, endothelium-dependent relaxation in these vessels has been assessed in the quest for the most suitable vascular conduit for cardiac bypass.[28] Saphenous veins obtained from bypass patients showed a relaxation close to 30%,[16] much greater than that shown by varicose veins in the current experiments. Considering that the comorbility existing in bypass patients (hypertension, hypercholesterolemia, diabetes, etc) leads to some degree of endothelial dysfunction,[29] the current findings suggest that CVI courses with severe impairment of venous endothelial function and that this condition could be an important factor in the genesis and evolution of this pathology. A hypothesis linking endothelial impairment and CVI has been proposed by Carine Michiels and her group: the venous hypoxic medium would damage endothelial cells, leading to a disorganized release of mitogens (bFGF)[9] and proinflammatory molecules such as prostaglandins and platelet activating factor (PAF).[7] Such condition would be responsible for the classical clinical expression of CVI: elongated and tortuous veins with leg pain and edema.[10]

The above data on endothelial dysfunction agree with those of Lowell et al,[26] who report a drastically reduced endothelium-dependent relaxation induced by the calcium ionophore A23187 in varicose veins and are compatible with the decreased plasma NO observed by Hollingsworth et al in patients with CVI.[30] It should be noted, however, that NO production, as indicated by histamine-induced release of cyclic GMP, has been found to be increased in cultured endothelial cells obtained from varicose veins.[31] The relevance of results with cultured endothelial cells removed from the hypoxic environment such as that found in varicose veins has been questioned.[8]

Another indication of the endothelial dysfunction in varicose veins is the finding of "u" shaped concentration-response curves to acetylcholine. This can be interpreted as indicating that at concentrations up to approximately 3 μmol/L, this agent induces a progressively increasing relaxation as a consequence of release of endothelial relaxing factors. At higher concentrations, the response of the damaged endothelial cells is overwhelmed by contraction of smooth muscle cells stimulated by activation of muscarinic receptors therein.

Drugs used for a variety of therapeutic purposes are known to elicit endothelial protection in arteries. This effect has been described for ACE inhibitors such as captopril,[32] $AT_1$ receptor antagonists such as losartan,[33] PPAR-γ ligands such as troglitazone,[34] and HMG-CoA reductase inhibitors such as pravastatin[35] and simvastatin.[36] This action has been linked to influences on the NO system by diverse mechanisms. For example, captopril exerts an antioxidant action by limiting the activity of the angiotensin II-stimulated enzyme nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase that increases superoxide anion formation and thus diminishes vascular NO availability[37] as well as by scavenging oxygen free radicals through its sulfhydryl group.[38] Losartan reduces oxidative stress by enhancing superoxide dismutase activity and NO availability.[39] Troglitazone upregulates vascular endothelial growth factor (VEGF) KDR/Flk-1, thus reducing calcium-calmoduline dependence of the NO-synthesizing enzyme eNOS by favoring Ser 1179 phosphorylation[40]; NO availability is also enhanced through reduced formation and increased degradation of superoxide anion.[41] Statins also augment eNOS Ser 1179 or 1177 phosphorylation in this case by activating the protein kinase Akt.[42] According to the current results, all these mechanisms appear to contribute to the endothelium protection exerted by these drugs in human varicose veins.

Escin, the major active principle of the seeds of *A hippocastanum*, was included in this study to determine whether a correlation could be established between the documented clinical efficacy of this agent in CVI[22] and its recently described arterial endothelium-protectant effect.[23] The latter could be attributed both to free radical scavenging[43] and to increased calcium-induced activation of eNOS.[23] The finding that escin does improve endothelium-mediated relaxation of varicose veins suggests that endothelial dysfunction is indeed an important component in CVI pathogenesis.

In conclusion, the current results confirm the existence of endothelial dysfunction in segments of human varicose veins, as shown by a reduced endothelium-dependent relaxation. In addition, the results show that this condition can be reverted by exposure of these preparations to a variety of cardiovascular protectant agents, indicating that the beneficial effects of these drugs are not circumscribed to arteries. The finding that the clinically effective antivaricose agent escin also exerts venous endothelial protection provides further evidence for a role of endothelial dysfunction in the pathogenesis of CVI and suggests that such dysfunction could constitute a therapeutic target in the treatment of this condition.

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# Endothelium protectant and contractile effects of the antivaricose principle escin in rat aorta

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**Abstract**

The triterpene saponin escin is the active component of the extract of seeds of *Aesculus hippocastanum* used in the treatment of chronic venous insufficiency. Escin is also used experimentally to increase membrane permeability in isolated cells. Since endothelial dysfunction is postulated to be involved in venous insufficiency, the possible endothelium-protectant effect of escin was explored in rat aortic rings, a model widely used to study such effects with cardiovascular agents. Escin enhanced endothelium-dependent relaxation induced by acetylcholine when such relaxation had been reduced by exposure to the superoxide ion generator pyrogallol. This effect was attributed to enhanced nitric oxide production by endothelial nitric oxide synthase, a calcium-dependent enzyme, activated by the increased endothelial cell permeability to calcium induced by escin. Another effect of escin thought to contribute to its therapeutic activity is its ability to produce venous contraction. The compound was found to induce concentration-related contraction also in rat aortic rings. This response was partially inhibited by removal of the endothelium or by preincubation with indomethacin, and was completely abolished by incubation in a calcium-free perfusion fluid. Contraction was considered to be due mainly to the aforementioned effect on calcium permeability, with some mediation by release of endothelial vasoconstrictor prostanoids. It was concluded that, in rat aorta, escin possesses an endothelium-protectant action and a direct contractile effect. The former could contribute to its beneficial effect in the treatment of venous insufficiency, while the latter could constitute a limiting side effect.


*Keywords:* Escin; *Aesculus hippocastanum*; Endothelial dysfunction; Nitric oxide synthase; Calcium influx

## 1. Introduction

Escin is the major active principle of the seeds of *Aesculus hippocastanum*, the horse chestnut tree. Extracts of the seeds are widely used in the treatment of chronic venous insufficiency, a condition characterized by the appearance of varicose veins. Hippocastanum extracts have been subjected to detailed pharmacological studies, which provide the basis for such use (Sirtori, 2001), as well as to a number of clinical trials confirming their therapeutic activity (Pittler and Ernst, 1998; Sirtori, 2001). The antivaricose effect of the extract is attributed to its venoconstrictor, antiedematous, antiinflammatory and antioxidant properties (Guillaume and Padioleau, 1994).

Escin is a natural mixture of triterpene saponins; the aglycones are derivatives of protoescigenin, acylated by acetic acid at position 22 and by either angelic or tiglic acids at position 21 (Fig. 1). It exists in two forms, α and β, that can be distinguished by melting point, specific rotation, hemolytic index and solubility in water. The β form appears to be the active component of the mixture and was used throughout the present study.

Although venous valvular malfunction has traditionally been regarded as a major contributing factor in the physiopathology of chronic venous insufficiency, Michiels et al. (1993a) postulate that venous endothelial hypoxia consecutive to blood stasis initiates a cascade of events leading to the disorganization of the vessel wall, typical of chronic venous insufficiency. According to this hypothesis, hypoxia determines a decrease in ATP availability which activates endothelial cells to produce the proinflammatory molecule platelet-activating factor and to bind polymorphonuclear neutrophils. These responses lead to

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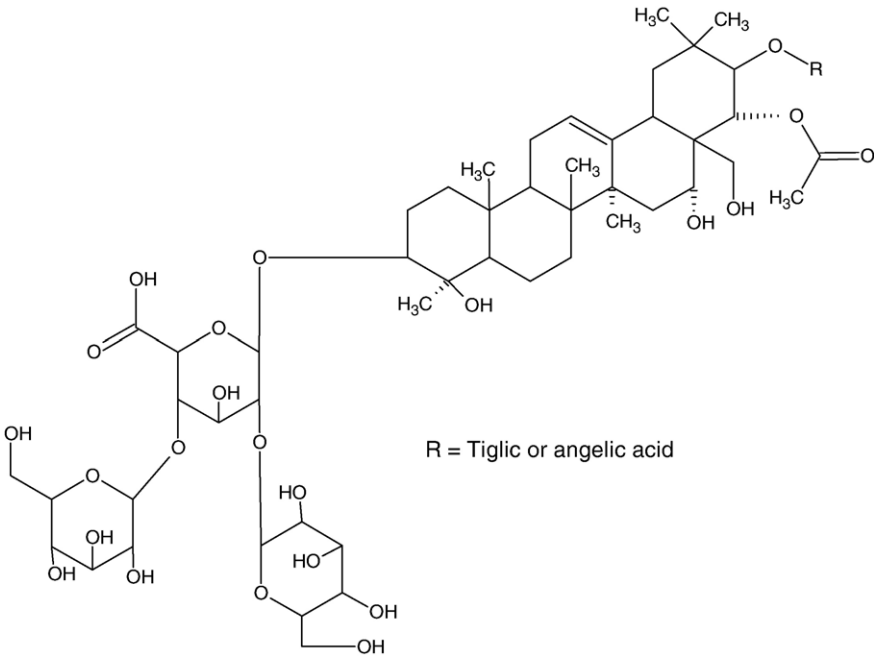


Fig. 1. Structural formula of β-escin.

infiltration of endothelial cells in the media of veins, thus affecting smooth muscle cells and connective tissue. It has been hypothesized that escin, through the pharmacological actions mentioned above, could interfere at several steps with this process (Sirtori, 2001). Accordingly, in human umbilical vein endothelial cells, the drug specifically reduces the hypoxia-mediated decrease in ATP production, as well as the increase in cell adhesiveness to neutrophils (Arnould et al., 1996).

The purported role of the endothelium in venous insufficiency can be considered as a modality of the association of arterial endothelial dysfunction, and the resultant deficiency in nitric oxide production, with cardiovascular disease (Cines et al., 1998; Giles, 2006). Reduced endothelium-dependent vascular relaxation, such as that elicited by acetylcholine, is considered a hallmark of endothelial dysfunction. The finding that a number of cardiovascular drugs, including modulators of the renin–angiotensin system, calcium channel blockers, β-blockers and statins, improve this response, has contributed to identify the endothelium as a pharmacological target for reducing cardiovascular risk factors (Giles, 2006). The present study was therefore carried out in rat aortic rings to determine whether escin behaves as other endothelium-protecting agents in influencing arterial relaxation induced by acetylcholine.

Another aspect of escin action evaluated in the present work was its vascular smooth muscle contracting effect. Venoconstriction has been confirmed in various experimental models (Guillaume and Padioleau, 1994), including human varicose veins (Annoni et al., 1979; Brunner et al., 2001) and attributed to release of prostaglandins (Berti et al., 1977). Experiments were conducted in the abovementioned model to determine whether escin also contracts arterial smooth muscle, a possibility hitherto apparently unexplored.

## 2. Material and methods

### 2.1. Animals

Adult male Wistar rats weighing between 200 and 300 g, raised in the animal facilities of the School of Medicine, Universidad Nacional Autónoma de México, were used in all experiments. Rats were kept in animal rooms illuminated from 07:00 to 19:00 (12-h light/12-h dark cycles) and maintained at 21–23 °C. The animals had free access to food pellets (Purina Chow, St Louis, MO) and tap water. Rats were brought daily to the laboratory for the experiments, which were conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and were approved by the local Ethics Committee.

### 2.2. Relaxation and contraction experiments

Rats were anesthetized with sodium pentobarbital, 20 mg i.p. total dose, and subsequently sacrificed by cervical dislocation. The thoracic aorta was removed and segments 0.5 cm long were obtained and suspended in jacketed 20-mL organ chambers between two nickel–chromium wire hooks. One of the hooks was fastened to the bottom of the chamber and the other was attached to a Grass FT03 force transducer, which was connected in turn to a Grass Model 79 polygraph (Grass Instrument Division, Astro-Med, West Warwick, RI). The baths contained Krebs–Henseleit solution of the following composition: 127 mM NaCl; 4.7 mM KCl; 1.1 mM $MgSO_4$; 1.2 mM $KH_2PO_4$; 2.5 mM $CaCl_2$; 25 mM $NaHCO_3$; 11 mM glucose; and 0.02 mM EDTA. The solution was kept at 37 °C and bubbled with a mixture of 95% $O_2$ and 5% $CO_2$; pH was 7.4. The

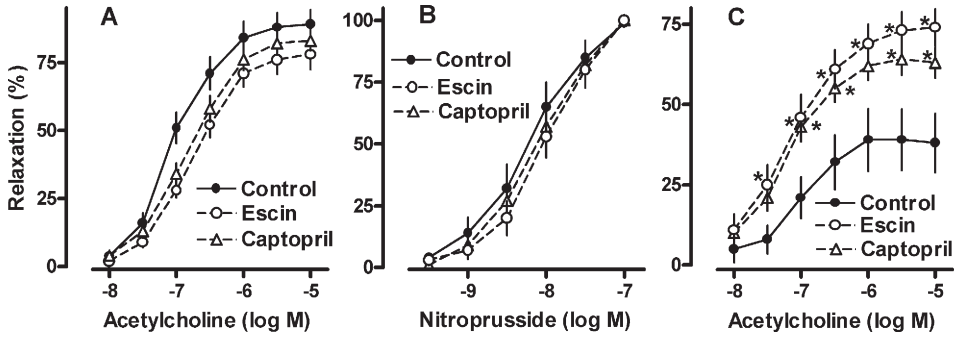


Fig. 2. Concentration–response curves to acetylcholine and nitroprusside obtained in rat aortic rings with endothelium in the absence (Control) and presence of 1 μM escin or 30 μM captopril. Shown are curves in otherwise unpretreated preparations (A and B) and in rings in the presence of 1 μM pyrogallol (C). Vertical lines are standard errors and asterisks denote significant differences from control. Abscissae represent concentrations of the relaxant agents and ordinates, percent relaxation of noradrenaline-induced contractions.

preparations were subjected to a resting tension of 2 g, which was maintained constant throughout the experiments. After a stabilization period of at least 60 min, during which the rings were stimulated several times with 100 nM noradrenaline, integrity of the endothelium was assessed by verifying that the contracted preparations relaxed by at least 50% when challenged with 1 μM acetylcholine. Endothelium was removed in some rings by rubbing intraluminally with a 20-gauge hypodermic needle; in these preparations, absence of the endothelium was confirmed by a less than 10% relaxation upon acetylcholine challenge. All experimental groups consisted of 7 to 9 rings.

In a first series of experiments, the influence of escin on endothelium-dependent and -independent relaxation was assessed in intact rings contracted with 100 nM noradrenaline by constructing cumulative concentration–response curves to acetylcholine, 10 nM to 10 μM, and sodium nitroprusside, 0.3 nM to 100 nM, respectively. These experiments were carried out in unpretreated control rings and in rings preincubated for 30 min with 1 μM escin or 30 μM captopril, which was used as a reference endothelium protectant. In a second series, a superoxide ion donor, 1 μM pyrogallol, was added to the bath just before constructing the curves to acetylcholine. The experiments were repeated preincubating with 30 μM L-NAME or with L-NAME plus escin.

In a third series, the pro-contractile and direct contractile effects of escin were explored in unstimulated endothelium-intact preparations. For the former, curves to noradrenaline, 0.3 nM to 10 μM, were obtained in control rings or in rings preincubated for 30 min with 1 μM escin. For the latter, curves to the saponin, 10 μM to 300 μM, were constructed in rings either unpretreated, preincubated for 30 min with 10 μM indomethacin, or after endothelium removal. The influence of extracellular calcium on escin contractions was explored in experiments carried out with calcium-free Krebs solution. For this purpose, after initial set up, the perfusing solution was changed to one in which $CaCl_2$ was omitted and the concentration of EDTA was increased to 0.2 mM. Over the next 30 min, the preparations were washed three times with this solution and were then incubated undisturbed for another 30 min. Escin was subsequently added at the concentrations noted above.

### 2.3. Nitric oxide metabolites

The influence of escin on the production of nitric oxide by aortic rings was determined by measuring total nitrates as described by Bobadilla et al. (1997). Rings obtained as above were individually incubated at 37 °C in 5 mL of Krebs solution in a shaking water bath. The solution was changed every 15 min until all blood elements were eliminated (30 to 45 min). The tissues were then incubated for an additional 30 min under the conditions noted, in tubes containing 6 mL of Krebs alone (control), 1 μM of escin or 10 μM of the calcium ionophore A23187 ($n=10$ for each group). At the end of this period three 2 mL aliquots of the solution were mixed with 40 μL of a variant of Griess reagent (20 μL 50% trichloroacetic acid, 10 μL 2% procainamide, and 10 μL 1% naphthylethylendiamine) to form a stable azo-dye which was measured in triplicate by spectrophotometry at 548 nM. Nitrite content was determined by interpolating average absorbance values in a standard curve constructed with known concentrations of $KNO_2$. Rings were dried in an oven at 60 °C for 30 min to obtain their dry weight, which was used to express results as μM/mg/30 min of nitrites.

### 2.4. Drugs

Acetylcholine chloride, (±)-noradrenaline hydrochloride, β-escin, captopril, indomethacin, pyrogallol, sodium nitroprusside dihydrate, A23187 calcium magnesium salt and $N_\omega$-nitro-L-arginine methyl ester hydrochloride (L-NAME) were obtained from Sigma-Aldrich, Toluca, Mexico. Noradrenaline was dissolved in 0.1% ascorbic acid, indomethacin in 0.1 N $Na_2CO_3$, subsequently neutralized with 0.1 N HCl, and A23187 in dimethyl sulfoxide. All other drugs were dissolved in Krebs. Stock solutions were prepared daily and appropriate dilutions were made with Krebs.

### 2.5. Data presentation and statistical analyses

Results are presented as means ± S.E.M. Aortic relaxation with acetylcholine and nitroprusside is expressed as percent of the contractile response to 100 nM noradrenaline. Contraction with escin is expressed as percent of the response to 100 nM

noradrenaline obtained previously in each preparation. Responses to individual concentrations of the test drugs in the control and treated groups were compared using either an unpaired t test or a one-way analysis of variance followed by Dunnett's post hoc test, with $P<0.05$ considered significant. Concentration–response curves to noradrenaline were also evaluated by subjecting individual curves to non-linear regression analysis to calculate $pD_2$ values (negative logarithm of $EC_{50}$) for the catecholamine. Statistical evaluation and non-linear regression analyses were carried out with a GraphPad Prism 4.02 package (GraphPad Software, Inc., San Diego, CA).

## 3. Results

In normal rings, neither escin nor captopril affected endothelium-dependent relaxation with acetylcholine (Fig. 2A) or endothelium-independent relaxation with nitroprusside (Fig. 2B). Pyrogallol reduced acetylcholine relaxation by more than one-half, and this effect was prevented by incubation with escin or captopril (Fig. 2C). When rings were preincubated with L-NAME and then challenged with pyrogallol, acetylcholine failed to induce relaxation at any concentration. Exactly the same results were obtained when escin was added to the incubation medium (not shown).

Escin did not influence contractile responses to noradrenaline (Fig. 3A). Analysis of the pharmacodynamic parameters of the catecholamine confirmed such lack of effect, since neither its efficacy, as determined by $E_{max}$, nor its affinity, as determined by $pD_2$, were changed after exposure to escin. Values for $E_{max}$ were 2.14±0.11 and 2.24±0.12 g in control and escin-pretreated rings, respectively. Corresponding values for $pD_2$ were 7.62±0.11 and 7.46±0.11. Escin produced a concentration-related contraction of intact rings, with a maximum effect approximately equivalent to 100% of that elicited by 100 nM noradrenaline (Fig. 3B, C, D). This response was partially diminished by endothelium removal (Fig. 3B) or by indomethacin (Fig. 3C), and was completely prevented by incubation in calcium-free solution (Fig. 3D).

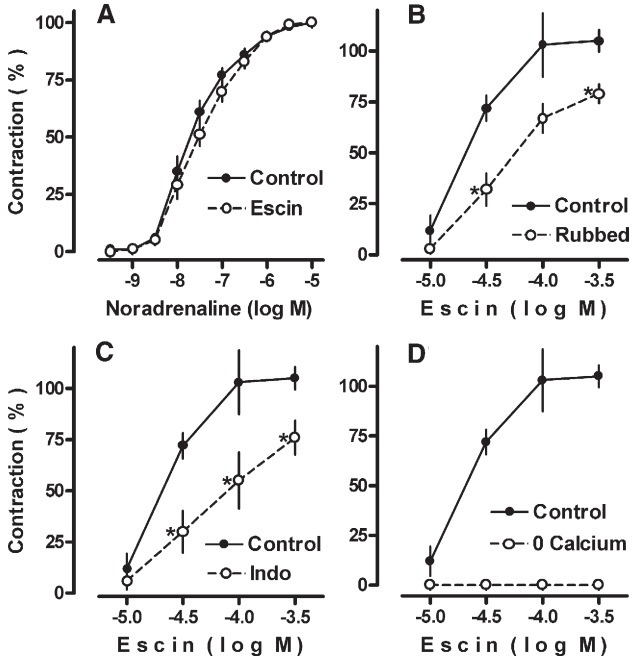


Fig. 3. (A) Concentration–response curves to noradrenaline in rat aortic rings with endothelium in the absence (Control) and presence of 1 μM escin. (B) Curves to escin in rings with (Control) and without (Rubbed) endothelium. (C) Curves to escin in rings with endothelium preincubated with 10 μM indomethacin (Indo). (D) Curves to escin in rings incubated in calcium-free Krebs solution (0 calcium). Vertical lines are standard errors and asterisks denote significant differences from control. Abscissae represent concentrations of the contractile agents and ordinates, contraction as percent of maximum response (A) or as percent of the response to 100 nM noradrenaline (B, C, D).

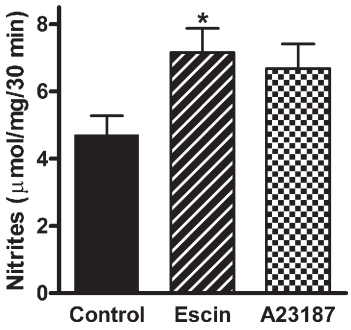


Fig. 4. Production of nitrites by rat aortic rings incubated for 30 min in normal Krebs solution or in Krebs containing 1 μM escin or 10 μM A23187. Bars correspond to means of 10 preparations; vertical lines are standard errors and the asterisk denotes significant difference from control.

Generation of nitric oxide by aortic rings, assessed by determination of its nitrite metabolites, was increased by escin and by the calcium ionophore A23187 (Fig 4). The effect of the latter, although similar in magnitude to that of the saponin, was not statistically significant.

## 4. Discussion

The present results show that in rat aortic rings escin increases the endothelium-dependent relaxation impaired by pyrogallol. In this sense, it resembles the angiotensin-converting enzyme inhibitor captopril, an agent reported to attenuate endothelial dysfunction produced in arteries by homocysteine (Fu et al., 2003), advanced oxidation protein products (Chen et al., 2005), or nicotine (Luo et al., 2006). In the model used in the present work, the endothelial dysfunction induced by pyrogallol can be attributed to generation of superoxide ion, which then inactivates the nitric oxide responsible for acetylcholine relaxation (Moncada et al., 1986).

In the absence of direct measurements of superoxide ion and of nitric oxide, the mechanism of this endothelium-protectant effect of escin cannot be established with certainty by the present study. On one hand, it could be due to scavenging of the superoxide generated by pyrogallol, and thus be a consequence of the antioxidant effect described by Guillaume and Padioleau (1994) and based on inhibition of malondialdehyde formation induced by NADPH in rat liver microsomes. On the other hand, protection could be due to activation of endothelial nitric oxide synthase (eNOS), which would generate increased amounts of

nitric oxide, as suggested by the enhanced basal output of nitrite metabolites by escin-incubated aortic rings observed herein. The finding that in the presence of the non-selective eNOS inhibitor L-NAME, the protectant effect of escin could no longer be detected, is also compatible with an action on the enzyme, since in these experiments participation of eNOS in acetylcholine relaxation is excluded.

Activation of eNOS by escin could be the consequence of an increase in calcium influx into endothelial cells due to enhanced membrane permeability to this ion. The influence of escin on membrane permeability has been known for some time and has determined its use as a tool to obtain "skinned" preparations of vascular (Satoh et al., 1994) and bronchial (Savineau and Marthan, 1994) smooth muscle, as well as of neurons (Fernández et al., 2005). If this occurs in endothelial cells, activation of eNOS is plausible, since this enzyme is calcium-dependent (Alderton et al., 2001). It should be mentioned that the influence of escin on nitrite production in the presence of pyrogallol was also explored, but this agent at the concentration tested (1 μM), either by itself or combined with escin, decreased nitrite output to levels undetectable by the analytical procedure used.

The lack of influence of escin on noradrenaline contractions contrasts with the potentiation of vasoconstriction induced by the catecholamine in dog saphenous veins perfused at constant flow (Guillaume and Padioleau, 1994). Such potentiation is to be expected with an agent like escin which increases membrane permeability to calcium, thus promoting enhanced smooth muscle contraction. Agonists of $\alpha_1$-adrenoceptors such as noradrenaline are known to elicit vascular contractile responses by increasing intracellular levels of this ion (Ruffolo et al., 1991), although the pathways used to achieve this will vary depending on the type of vessel studied (Broadley, 1996). It is possible that in the dog saphenous vein escin-sensitive calcium entry from extracellular sources is important in this response, while in the rat aorta, escin-insensitive calcium mobilization from intracellular deposits predominates.

As has been described in rat and rabbit portal veins (Berti et al., 1977), dog saphenous veins (Guillaume and Padioleau, 1994), and human saphenous veins (Annoni et al., 1979; Brunner et al., 2001), escin also contracts rat aortic rings. Available quantitative estimates of this effect in human saphenous veins indicate an $EC_{50}$ of 10 to 30 μM (Annoni et al., 1979; Brunner et al., 2001), which is in the range of the approximately 20 μM found in rat aorta, extrapolated from Fig. 3B. According to Brunner et al. (2001), the maximum contraction elicited by escin is similar to that produced by phenylephrine, around 70% of that corresponding to a supramaximal concentration of KCl. In the present study, the maximum effect of the saponin was roughly 100% of that of the test concentration of noradrenaline. Thus, the efficacy of escin is similar to that of the reference α-adrenoceptor agonists phenylephrine in veins and noradrenaline in aorta.

Early work on the mechanism of the contractile effect of escin implicated release of the vasoconstrictor prostaglandin $F_2\alpha$, based on blockade by indomethacin and on bioassay of effluent from perfused rat lungs (Berti et al., 1977). The present findings in rat aorta of an indomethacin-sensitive contraction are compatible with this proposition, and further suggest an endothelial source of the constrictor prostanoid. Synthesis and release of prostaglandin $F_2\alpha$ by venous endothelial cells has been reported repeatedly (Michiels et al., 1993b; Camacho et al., 1998); this applies also, although to a lesser extent, to arterial endothelial cells (Revtyak et al., 1987). Prostanoid-mediated contraction of rat aorta by escin constitutes an example of similar effects in this structure by acetylcholine, A23187 and ATP (Yang et al., 2004), as well as of serotonin and vasopressin (Lee et al., 1991) and nitric oxide synthase inhibitors (Pomposiello et al., 1997) in the rat coronary circulation.

The experiments with calcium-free Krebs solution indicate that the major determinant of escin-induced contraction is the availability of extracellular calcium. In contrast to the partial inhibition of contraction observed with endothelium removal or exposure to indomethacin, the response was completely abolished upon incubation in calcium-free medium. Such calcium dependency is compatible with the notion of an effect due to the escin-induced increased membrane permeability mentioned above.

Vascular endothelium, through production of numerous biologically active factors, participates in the regulation of vascular functions such as vasoconstriction and dilation, angiogenesis, coagulation and blood cell trafficking (Cines et al., 1998). Prolonged or exaggerated endothelial activation may lead to its dysfunction, an anomaly that is a prominent component of diverse pathologies, such as atherosclerosis, hypertension, diabetic vasculopathy, transplant rejection and autoimmune diseases. A dysfunction of this type, initiated by hypoxia consecutive to blood stasis, is thought to be a component of chronic venous insufficiency (Michiels et al., 1993a). The endothelium-protectant action of escin found in the present study, if exerted also in veins, could contribute to its beneficial effects in the therapy of varicose veins. On the other hand, the finding that the contractile effects of the saponin are not limited to veins, but extend to arterial smooth muscle, could indicate a limitation to its therapeutic use, particularly in patients with concomitant coronary artery disease. Although the present experiments were carried out in aorta, a conductance vessel, extrapolation of their results to resistance arteries seems justifiable, considering that the postulated calcium influx mechanism of the contractile response is applicable to all vascular smooth muscle cells.

In conclusion, escin, the active component of *A. hippocastanum* seeds, was found to improve endothelial dysfunction in rat aortic rings subjected to oxidative stress, and to induce in these preparations a contraction quantitatively similar to that previously found in veins. These apparently contradicting effects could be due to the well-known ability of escin to enhance cellular permeability to calcium which, on one hand, would increase eNOS activity and nitric oxide production and on the other, would lead to vascular smooth muscle contraction. Endothelial protection could contribute to the therapeutic usefulness of escin, while arterial vasoconstriction could represent a limiting side effect in susceptible patients.

## Acknowledgments

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# Antivasoconstrictor effect of the neuroprotective agent dexrazoxane in rat aorta

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**Abstract**

Dexrazoxane is used clinically to reduce the cardiotoxicity of anthracycline cancer chemotherapeutic agents, acting by an iron-chelating antioxidant mechanism. In a study designed to explore the possible mechanism of the recently described neuroprotective effect of the drug in cerebral ischemia, its influence on vascular reactivity was determined in rat aortic rings. Dexrazoxane was found to be devoid of direct contractile or relaxant activity and to have no influence on responses to acetylcholine or histamine (relaxation), or to angiotensin or serotonin (contraction). In contrast, it decreased contractions to norepinephrine, as evidenced by rightward displacement of the concentration–response curves. The effect was prevented by the removal of the endothelium and by the $\alpha_2$-adrenoceptor antagonist yohimbine; it was partially antagonized by the endothelium-derived depolarizing factor inhibitor clotrimazole, but was not affected by L-NAME or indomethacin, inhibitors of endothelial nitric oxide and prostacyclin production. The anti-contractile effect did not occur in rings stimulated with the $\alpha_1$-adrenoceptor agonist phenylephrine. It was concluded that dexrazoxane opposes norepinephrine vascular contraction by enhancing endothelial $\alpha_2$-adrenoceptor-mediated release of relaxing factor(s). The drug could thus offset the deleterious vasoconstriction elicited by the increased circulating catecholamines present during cerebral ischemia, and by this mechanism produce neuroprotection.


*Keywords:* Dexrazoxane; Endothelium; $\alpha_2$-adrenoceptors; Vasoconstriction; Neuroprotection

## Introduction

Dexrazoxane (Fig. 1) is an iron chelator used clinically to reduce the cardiotoxicity of doxorubicin and other anthracycline cancer chemotherapeutic agents (Cvetkovic and Scott, 2005). The untoward cardiac effects of anthracyclines, characterized by cardiomyocyte necrosis leading to congestive heart failure, are the result of formation of iron-dependent oxygen free radicals by anthracycline iron complexes. In the absence of adequate levels in the heart of oxygen free radical neutralizing systems, damage to myocardial mitochondria occurs by membrane lipid peroxidation (Hasinoff et al., 2003). These effects are prevented by dexrazoxane through chelation of free and anthracycline-bound ferric ions (Hasinoff et al., 1998). Protection is attributed to the ring-opened metabolites of the drug (Hasinoff et al., 2003).

Recently, dexrazoxane was found to exert a neuroprotective effect in mice subjected to global cerebral ischemia (Rodríguez et al., 2003), as evidenced by a reduction of both mortality and neurological deficit. The drug was also able to ameliorate in vitro the impaired contractile response to electrical stimulation produced by ischemia in guinea pig ileum segments (Rodríguez et al., 2006). It is uncertain whether the iron chelating and antioxidant actions of dexrazoxane contribute to neuroprotection (Rodríguez et al., 2003), although it should be noted that the antioxidants ascorbic and dihydrolipoic acids do elicit neuroprotection in the mouse model of cerebral ischemia (Santiago-Mejía et al., 2004). Pertinent to this question is the observation of Hasinoff (2002) that dexrazoxane decreases damage of cultivated rat cardiac myocytes by hypoxia-reoxygenation through iron chelation and prevention of iron-based oxygen radical deleterious effects.

Diverse studies have shown that ischemia induces functional changes in the cerebral vasculature which may be important determinants in the outcome of experimentally-induced stroke.

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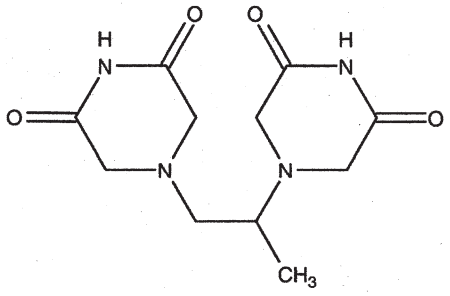

Fig. 1. Chemical structure of dexrazoxane.

Increased contractile responsiveness to angiotensin (Stenman and Edvinsson, 2004) and endothelin (Fadyukova et al., 2004), as well as decreased endothelium-dependent (Volpe et al., 1996) and chemoreflex-induced (Leffler et al., 1989) relaxation, has been reported for cerebral arteries in this condition. Although reactivity to norepinephrine appears to be unchanged after cerebral ischemia (Leffler et al., 1989), increased levels in both brain extracellular (Globus et al., 1989) and circulating (Mizushima et al., 1994) catecholamine do occur. These findings suggest that vasoconstriction could be involved in perpetuating the reduced cerebral blood flow which triggers the so-called neurotoxic cascade in the ischemic penumbra (De Keyser et al., 1999).

It therefore seemed important to determine whether neuroprotection by dexrazoxane involves effects on vascular smooth muscle responses to endogenous vasoactive agents. Results of such investigation, carried out in rat aortic rings, suggest that the drug selectively antagonizes contractile responses to norepinephrine through an endothelium-dependent mechanism.

## Methods

### *Animals*

Experiments were carried out using adult male Wistar rats weighing between 200 and 300 g, raised in the animal facilities of the School of Medicine, Universidad Nacional Autónoma de México. They were kept in animal rooms illuminated from 07:00 to 19:00 h (12-h light 12-h dark cycles) and maintained at 21–23 °C. The animals had free access to food pellets (Purina Chow, St Louis, MO) and tap water. They were brought daily to the laboratory for the experiments, which complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and were approved by the Ethics Committee of the School of Medicine, Universidad Nacional Autónoma de México.

### *Tissue preparation*

Rats were anesthetized with sodium pentobarbital, 20 mg i.p. total dose, and subsequently sacrificed by cervical dislocation. The thoracic aorta was removed and segments 0.5 cm long were obtained and suspended in jacketed 20-ml organ chambers between two nickel–chromium wire hooks. One of the hooks was fastened to the bottom of the chamber and the other was attached to a Grass FT03 force transducer, which was connected in turn to a Grass model 79 polygraph (Grass Instrument Division, Astro-Med, West Warwick, RI). The baths contained Krebs–Henseleit solution of the following composition: 127 mM NaCl; 4.7 mM KCl; 1.1 mM $MgSO_4$; 1.2 mM $KH_2PO_4$; 2.5 mM $CaCl_2$; 25 mM $NAHCO_3$; 11 mM glucose; and 0.02 mM EDTA. The solution was kept at 37 °C and was bubbled with a mixture of 95% $O_2$ and 5% $CO_2$; pH was 7.4. The preparations were subjected to a resting tension of 2 g, which was maintained constant throughout the experiments. After a stabilization period of at least 90 min, during which the rings were stimulated several times with 30 nM norepinephrine, integrity of the endothelium was assessed by verifying that the contracted preparations relaxed by at least 50% when challenged with 1 μM acetylcholine. Endothelium was removed

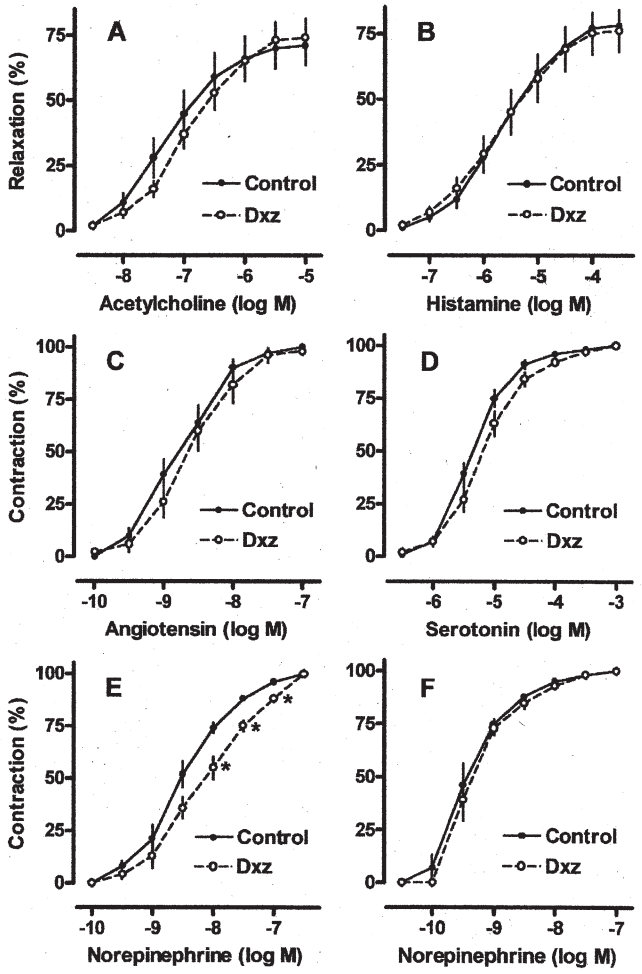


Fig. 2. Concentration–response curves in endothelium-intact control rat aortic rings and in rings preincubated for 30 min with 1 mM dexrazoxane (Dxz). A and B, relaxant effects of acetylcholine and histamine in norepinephrine-contracted rings. C, D and E, contractile effects of angiotensin, serotonin and norepinephrine in unstimulated rings. F, contractile effects of norepinephrine in rings without endothelium. Vertical lines indicate standard errors and asterisks, significant differences from control. Abscissae correspond to log molar concentrations of the test drugs; ordinates, to relaxation as percent of norepinephrine contraction (A and B), and to contraction as percent of maximum response (C, D, E and F).

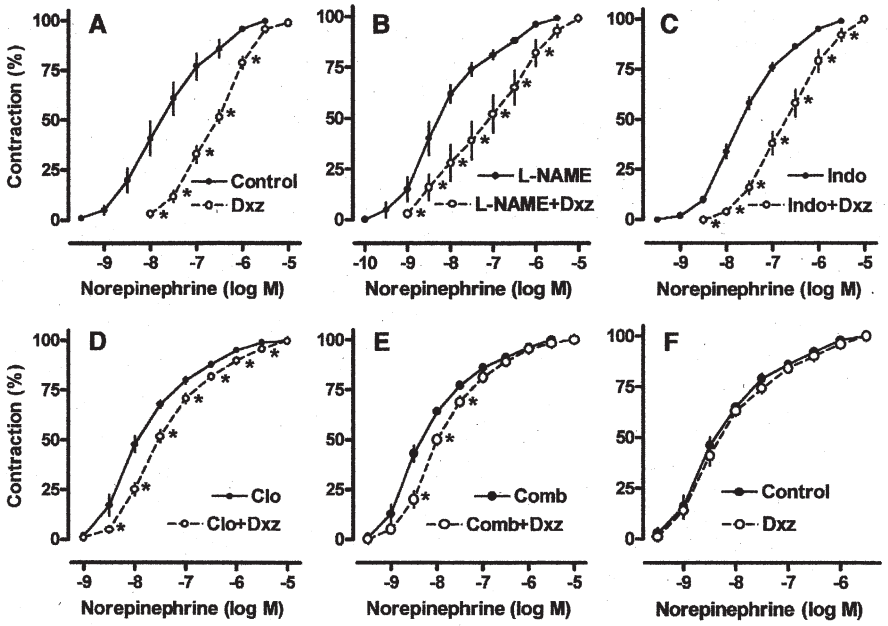


Fig. 3. Concentration–response curves to norepinephrine in endothelium-intact rat aortic rings with or without preincubation for 180 min with 1 mM dexrazoxane (Dxz). A, curves in rings without any additional preincubation. B, C, D and E, rings additionally preincubated for 180 min with 30 μM L-NAME, 10 μM indomethacin, 1 μM clotrimazole, or a combination of the three inhibitors. F, curves in rings without endothelium. Vertical lines indicate standard errors and asterisks, significant differences from rings without dexrazoxane. Abscissae correspond to log molar concentrations of norepinephrine; ordinates, to contraction as percent of maximum response.

in some rings by rubbing intraluminally with a 20-gauge hypodermic needle; in these preparations, absence of the endothelium was confirmed by a less than 10% relaxation upon acetylcholine challenge. All experiments were carried out in groups of 8 or 9 rings.

*Experimental protocol*

In a first series of experiments, the influence of dexrazoxane on the relaxant and contractile effects of several vasoactive endogenous agents was determined. Relaxation was assessed in rings with endothelium contracted with 30 nM norepinephrine by constructing cumulative concentration–response curves to acetylcholine, 3 nM to 10 μM, and histamine, 30 nM to 300 μM. Contraction was explored in unstimulated endothelium-intact preparations by curves to angiotensin, 0.1 nM to 100 nM, serotonin, 300 nM to 1 mM, and norepinephrine, 0.1 nM to 300 nM. These experiments were carried out in unpretreated control rings or in rings previously incubated for 30 min with 1 mM dexrazoxane. Norepinephrine was also tested in preparations without endothelium.

In a second series, the incubation period with dexrazoxane was extended to 180 min and curves to norepinephrine in intact and rubbed rings were constructed as above. In other intact preparations, curves to norepinephrine were obtained after incubation for 180 min with inhibitors of production of diverse endothelium-derived relaxing factors. The inhibitors used were 30 μM L-NAME, 10 μM indomethacin and 1 μM clotrimazole, tested either separately or together. Control rings were exposed to these agents alone and treated rings received in addition 1 mM dexrazoxane, before constructing the concentration–response curves to norepinephrine.

Finally, similar curves were obtained in control and dexrazoxane-incubated intact rings using the selective $\alpha_1$-adrenoceptor agonist phenylephrine, 1 nM to 10 μM, instead of norepinephrine as the contractile agent, and the $\alpha_2$-adrenoceptor antagonist yohimbine, 300 nM, instead of the endothelium inhibitors. In an additional series, the iron chelator deferoxamine, 1 mM, was used instead of dexrazoxane. These experiments were run using 180 min as the preincubation period.

*Drugs*

The hydrochlorides of (±)-norepinephrine, serotonin, L-NAME, (*R*)-(−)-phenylephrine and yohimbine, as well as histamine dihydrochloride, angiotensin II acetate, deferoxamine mesylate,

Table 1
Negative logarithms of the $EC_{50}$ ($pD_2$) and maximum responses in grams ($E_{max}$) to norepinephrine in rat aortic rings subjected to various treatments or maneuvers in the absence (Control) and presence (Dxz) of dexrazoxane

| Treatment | $pD_2$ Control | $pD_2$ Dxz | $E_{max}$ Control | $E_{max}$ Dxz |
|---|---|---|---|---|
| None | 7.72±0.19 | 6.62±0.07[a] | 2.02±0.12 | 1.78±0.30 |
| L-NAME | 8.18±0.15 | 7.16±0.27[a] | 2.71±0.21 | 2.76±0.30 |
| Indomethacin | 7.60±0.06 | 6.68±0.13[a] | 2.72±0.15 | 2.03±0.33[a] |
| Clotrimazole | 7.84±0.06 | 7.44±0.06[a] | 3.08±0.18 | 2.88±0.19 |
| Combination | 8.22±0.07 | 7.88±0.06[a] | 3.47±0.17 | 3.59±0.27 |
| (−) Endothelium | 8.29±0.09 | 8.19±0.07 | 2.47±0.23 | 2.58±0.20 |

Means±S.E.M., $n$=8 or 9.
[a] $P<0.05$ versus control.

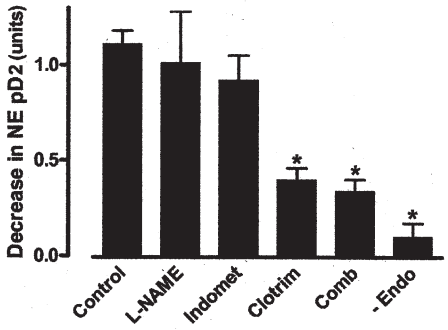


Fig. 4. Dexrazoxane-induced decreases in $pD_2$ values for norepinephrine in rat aortic rings in the absence (Control) and presence of various inhibitors of endothelium-derived relaxing factors, and in rings without endothelium (−Endo). Vertical lines indicate standard errors and asterisks, significant differences from control ($P<0.05$, one-way analysis of variance and Dunnett's test for multiple comparisons with a control). Ordinates correspond to decreases in $pD_2$, calculated from the concentration–response curves of Fig. 3, and expressed as the difference between mean values of rings not exposed to dexrazoxane and individual values of rings treated with the drug.

indomethacin and clotrimazole, were obtained from Sigma-Aldrich, Toluca, Mexico. Dexrazoxane (Cardioxane™, manufactured by Thissen Laboratories, L'Allend, Belgium) was purchased from a local drug distributor (Asofarma de México, S.A. de C.V.). Norepinephrine was dissolved in 0.1% ascorbic acid and indomethacin in 0.1 N $Na_2CO_3$, subsequently neutralized with 0.1 N HCl. All other drugs were dissolved in Krebs–Henseleit solution. Stock solutions were prepared daily and appropriate dilutions were made with Krebs–Henseleit solution.

*Data presentation and statistical analysis*

Results are presented as means±S.E.M., $n=8$ or 9. Aortic ring relaxation is expressed as percent inhibition of the norepinephrine response; contraction, as percent of the maximum effect observed. Individual responses of rings exposed to dexrazoxane or deferoxamine were compared to those of rings not so exposed, by an unpaired Student's $t$-test. In the few cases in which nonhomogeneous variances were detected, a Mann–Whitney nonparametric test was applied. Results of the endothelium inhibitor experiments were additionally evaluated by subjecting individual concentration–response curves to nonlinear regression analysis to calculate the negative logarithm of the $EC_{50}$ for norepinephrine ($pD_2$). Values for dexrazoxane-treated and untreated curves were compared by a $t$-test. In all cases, a probability level of less than 0.05 was accepted as indicating significance. Statistical evaluation and nonlinear regression analyses were carried out with a GraphPad Prism 4.02 package (GraphPad Software, Inc, San Diego, CA).

## Results

In endothelium-intact rings, dexrazoxane in concentrations up to 1 mM lacked contractile or relaxant effects (not shown). Previous incubation with the drug for 30 min did not influence relaxant responses to acetylcholine or histamine (Fig. 2A and B), nor contractile responses to angiotensin or serotonin (Fig. 2C and D). In contrast, this treatment produced a roughly parallel rightward displacement of the concentration–response curve to norepinephrine (Fig. 2E), an effect not observed in preparations without endothelium (Fig. 2F).

Increasing the time of incubation with dexrazoxane to 180 min led to a more marked rightward shift of the norepinephrine curve (Fig. 3A). This was still present after coincubation, also for 180 min, with the inhibitors of the production of the endothelium-derived relaxant factors nitric oxide (L-NAME) and prostacyclin (indomethacin) (Fig. 3B and C). Displacement by dexrazoxane appeared to be reduced after application of the inhibitor of depolarizing factor clotrimazole (Fig. 3D) or of the three drug combination (Fig. 3E), and again was completely prevented by removal of the endothelium (Fig. 3F).

Calculation of the pharmacodynamic parameters $pD_2$ and $E_{max}$ for norepinephrine in the experiments shown in Fig. 3, confirmed that dexrazoxane significantly decreased $pD_2$ values, and that these decreases were still present after exposure to the endothelium inhibitors and only disappeared with endothelium removal (Table 1). In contrast, dexrazoxane did not significantly affect $E_{max}$, except when coincubated with indomethacin. From the table, the extent of the decreases in $pD_2$ in the different treatment groups cannot be clearly appreciated, since the inhibitors by themselves produced changes in this parameter. A more quantitative representation of the dexrazoxane effects is shown in Fig. 4, in which it is apparent that the decreases in $pD_2$

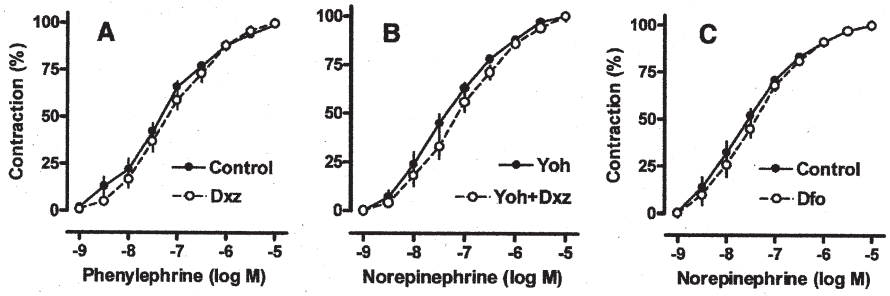


Fig. 5. Concentration–response curves to phenylephrine (A) and norepinephrine (B and C) in endothelium-intact rat aortic rings preincubated for 180 min with 1 mM dexrazoxane (A), 300 nM yohimbine with or without dexrazoxane (B), or 1 mM deferoxamine (C). Vertical lines indicate standard errors. Abscissae correspond to log molar concentrations of phenylephrine or norepinephrine; ordinates, to contractions as percent of maximum response.

produced by the drug alone (control) were not affected by L-NAME or indomethacin, but were significantly reduced by clotrimazole, by the combination of inhibitors, and specially by endothelium removal.

Incubation with dexrazoxane did not modify concentration–response curves to the $\alpha_1$ adrenoceptor-selective agonist phenylephrine (Fig. 5A) or to norepinephrine in the presence of the $\alpha_2$-adrenoceptor antagonist yohimbine (Fig. 5B). Similarly, the iron chelator deferoxamine was unable to affect responses to norepinephrine (Fig. 5C).

## Discussion

The present results in rat aortic rings show that, in the absence of direct relaxant or relaxation-potentiating effects, dexrazoxane produces a decrease in contractile responses to norepinephrine, but not to angiotensin or serotonin. The finding that rightward displacement of the norepinephrine concentration–response curve is absent in rings without endothelium excludes an effect on vascular smooth muscle, such as α-adrenoceptor antagonism, and suggests an action mediated by the endothelium. This structure has long been known to offset norepinephrine-induced contraction of isolated arteries, as shown by comparison of responses in intact and denuded preparations (Cocks and Angus, 1983; Martin et al., 1986; Bullock et al., 1986; Vinet et al., 1991; Tesfamariam et al., 1992; Matsuda et al., 1998). Depression of contraction in the presence of endothelium also occurs with angiotensin (Bullock et al., 1986; Ferrer et al., 1992) and serotonin (Cocks and Angus, 1983; Matsuda et al., 1998), although this phenomenon appears to be quantitatively smaller than that observed with norepinephrine (Dohi et al., 1996).

The anti-contractile influence of the endothelium has been attributed to the release of relaxing factors taking place both spontaneously (Martin et al., 1986) or, in the case of norepinephrine, through activation of endothelial α-adrenoceptors capable of actively inducing such release (Bullock et al., 1986; Tesfamariam et al., 1992; Dohi et al., 1996). Although both $\alpha_1$ and $\alpha_2$ receptors have been implicated in this effect, Miller and Vanhoutte (1985) provided compelling evidence supporting the latter view. More recent studies involving selective antagonists (Figueroa et al., 2001) and specific knockout mice (Shafaroudi et al., 2005) have identified these receptors as corresponding to the $\alpha_{2A}$ subtype. These studies, which confirm the presence of $\alpha_2$ receptors in vascular endothelium, have been carried out in a variety of peripheral arteries, but the fact that such receptors are also found in the endothelium of cerebral arteries (Thorin, 1998), supports the pertinence of the present findings in aorta to the postulation of a vascular mechanism for dexrazoxane neuroprotection.

The anti-contractile effect of dexrazoxane found in the present study appears to be mediated by endothelial $\alpha_2$-adrenoceptors. Evidence for this mechanism can be derived from the following findings: a) the effect occurs with norepinephrine, a non-selective $\alpha_1$ and $\alpha_2$ agonist, but not with angiotensin or serotonin, which do not interact with these receptors, b) it is also absent with the $\alpha_1$ selective agonist phenylephrine, and c) it is blocked by the $\alpha_2$ antagonist yohimbine.

The nature of the interaction between dexrazoxane and the $\alpha_2$-adrenoceptor cannot be ascertained by the present experiments. The possibility of the drug acting as a receptor agonist seems unlikely since it is devoid of the direct relaxant activity reported, for example, for the $\alpha_2$ agonists guanabenz, UK14, 304 and clonidine (Bockman et al., 1996). In addition, the side effects commonly observed during the clinical use of clonidine-like agents (bradycardia, somnolence, dry mouth) have not been reported with dexrazoxane (Hasinoff et al., 1998; Cvetkovic and Scott, 2005), even though this drug is administered at the relatively high dose of 1000 mg/m$^2$. The fact that the anti-contractile effect of dexrazoxane is greater when preincubated for 180 than for 30 min could imply an action at a site inside the endothelial cell, rather than on its surface (the location of the $\alpha_2$ receptor). Dioxopiperazines like dexrazoxane are known to penetrate cells slowly, reaching an equilibrium with the extracellular medium concentration within 2 h (Dawson, 1975). Thus, dexrazoxane could be acting intracellularly on the signaling process subsequent to $\alpha_2$ receptor activation by norepinephrine.

Most recent studies on endothelial $\alpha_2$-receptor-mediated relaxation confirm participation of nitric oxide as the relaxing agent released in this response (Bockman et al., 1996; Figueroa et al., 2001; Shafaroudi et al., 2005). According to Molin and Bendhack (2004), release of prostacyclin or hyperpolarizing factor can be excluded. On the other hand, Thorin et al. (1997) came to the unexpected conclusion that in the rabbit middle cerebral artery oxymetazoline induces endothelium-dependent, $\alpha_2$-mediated relaxation involving decreased endothelin-1 production, rather than release of any of the three known relaxing factors. The present experiments suggest that neither nitric oxide nor prostacyclin appears to participate in the anti-contractile effect of dexrazoxane, as judged from the quantitatively similar decrease in the norepinephrine p$D_2$ value produced by the drug alone and in the presence of L-NAME or indomethacin (Fig. 4). Only endothelium-derived hyperpolarizing factor seems to be involved in this effect. Interpretation of these results is not straightforward, since complete inhibition of production and/or release of each of the relaxing factors by the corresponding drug cannot be expected. This is illustrated by the fact that preincubation with the combination of inhibitors failed to block the effect completely, as occurred with endothelium removal.

Dexrazoxane is known to exert its protective effect against anthracycline-induced cardiotoxicity by previous conversion to its one-ring and two-ring-opened metabolites (Schroeder et al., 2005). This is achieved by the enzymes dihydropyrimidinase and dihydroorotase acting sequentially on the original compound and on the one-ring-opened derivatives, respectively. Since neither of these enzymes is known to be present in blood vessels (Schroeder et al., 2005), metabolism of dexrazoxane is unlikely to occur in the aortic rings used in the present study. The effect observed here can therefore be attributed to the original compound and not to the intermediate or final metabolites, which are the active iron chelators responsible for cardioprotection (Hasinoff et al., 1998). The lack of participation of iron chelation in the effect of dexrazoxane on norepinephrine contractions is supported by the inability of deferoxamine to affect the corresponding concentration–response curves (Fig. 5C).

An entirely different situation could be operating in cardiac myocytes, which dexrazoxane protects against hypoxia-reoxygenation damage (Hasinoff, 2002). These cells do possess the enzymatic machinery necessary for transforming the drug into its ring-opened metabolites (Schroeder et al., 2005), so that protection by an iron-chelating antioxidant mechanism is possible in this model.

It is conceivable that in the setting of cerebral ischemia, in which the levels of both brain and circulating catecholamines are markedly increased (Globus et al., 1989; Mizushima et al., 1994), the anti-contractile effect of dexrazoxane could be decisive in interfering the chain of events leading to neuronal death. The pertinence of the $\alpha_2$-adrenoceptor-mediated effect described in the present study to the previously reported neuroprotective activity of the drug (Rodríguez et al., 2003) is supported by the fact that the anesthetic adjuvant $\alpha_2$ agonist dexmedetomidine improves neurological outcome in rats subjected to cerebral ischemia (Hoffman et al., 1991). Similarly, the antiglaucoma agent brimonidine, also an $\alpha_2$ agonist protects retinal ganglion cells from ischemia (Vidal-Sanz et al., 2001). In the case of dexmedetomidine, neuroprotection has been attributed to normalization of ischemia-induced increased plasma epinephrine and norepinephrine levels (Engelhard et al., 2002). In this study, cerebral norepinephrine, which was also increased by the ischemic insult, was not affected by dexmedetomidine, an unexpected finding, since $\alpha_2$-adrenoceptor agonists are known to decrease brain catecholamines, apparently by an action at prejunctional sites (Meana et al., 1997). The deleterious role of circulating catecholamines in cerebral ischemia is far from being unequivocally established, since in a rat model of this condition involving arterial occlusion and hypotension induced by hemorrhage and ganglionic blockade, exogenously administered epinephrine and norepinephrine both ameliorated (Koide et al., 1986) and exacerbated (Werner et al., 1990) the resulting brain damage. Equally paradoxical is the reported neuroprotection elicited by the $\alpha_2$-adrenoceptor antagonist idazoxan (Gustafson et al., 1989), presumably due to an increase in extracellular norepinephrine levels in the brain (Gustafson et al., 1990). It should be noted, however, that according to Craven and Conway (1997) this effect of idazoxan is due not to an $\alpha_2$ or imidazoline receptor mechanism, but to the production of non-specific hypothermia.

It is recognized that cell death after brain ischemia is due to a complex cascade of biochemical events, including neuronal depolarization, increased glutamate and intracellular $Ca^{2+}$ levels, generation of free radicals, activation of catabolic enzyme systems and induction of inflammation (De Keyser et al., 1999). The present findings allow us to speculate that interference with the initial vasoconstrictive response represents perhaps the single most important variable in neuronal protection, and that this basic action explains the unique neuroprotective properties of dexrazoxane (Rodríguez et al., 2003), which favors rapid restoration of blood flow in the ischemic penumbra, before neurons become irreversibly damaged.

In conclusion, dexrazoxane has been shown to decrease contractile responses of rat aortic rings to norepinephrine, apparently by increasing the release of endothelium-derived hyperzpolarizing factor. This effect is achieved through enhancement of the process of endothelial $\alpha_2$-adrenoceptor activation by the catecholamine and could be involved in the protective action of the drug in models of cerebral and intestinal ischemia.

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